

Assay format diversity in pre-clinical immunogenicity risk assessment: Toward a possible harmonization of antigenicity assays

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ABSTRACT

A major impediment to successful use of therapeutic protein drugs is their ability to induce anti-drug antibodies (ADA) that can alter treatment efficacy and safety in a significant number of patients. To this aim, *in silico*, *in vitro*, and *in vivo* tools have been developed to assess sequence and other liabilities contributing to ADA development at different stages of the immune response. However, variability exists between similar assays developed by different investigators due to the complexity of assays, a degree of uncertainty about the underlying science, and their intended use. The impact of protocol variations on the outcome of the assays, i.e., on the immunogenicity risk assigned to a given drug candidate, cannot always be precisely assessed. Here, the Non-Clinical Immunogenicity Risk Assessment working group of the European Immunogenicity Platform (EIP) reviews currently used assays and protocols and discusses feasibility and next steps toward harmonization and standardization.

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Introduction

The concept of “predicting immunogenicity risk” in the pharmaceutical industry is currently understood as the ability to identify a therapeutic protein’s intrinsic and extrinsic factors that may contribute to the mounting of an anti-drug antibody (ADA) response by a patient’s immune system (see Table 1 for a list of terminologies). Key steps in driving this process are: 1) the priming of innate cells such as macrophages and dendritic cells (DCs), followed by 2) the recruitment of T cells to develop a mature immune response, leading to 3) the production of ADAs by plasma cells. Uptake and processing of therapeutic proteins by antigen-presenting cells (APCs) is believed to be one of the initial and critical steps in driving an immune response. APCs are a heterogeneous group of immune cells (including dendritic cells, macrophages, and B cells) that can take up and process antigens that are then presented as linear peptides in the context of class II MHC molecules to naïve T lymphocytes in peripheral lymphoid organs. Priming of naïve T cells requires three distinct signals:¹ 1) activation of the T cell receptor (TCR) by a specific interaction with a distinct Major Histocompatibility Complex (MHC)II:peptide complex, 2) survival and clonal expansion of the activated T cell due to CD28 receptor interaction with CD80/CD86 co-stimulatory molecules, and 3) differentiation into various effector cells depending on the cytokines produced by the APCs.

For example, activated naïve CD4 T cells may differentiate into distinct T effector cell types, such as Th1, Th2, Th9, Th17, T follicular helper cells, T regulatory cells, or into T follicular regulatory cells depending on variations in the third signal (cytokines produced by the DCs).² Activated T cells express numerous surface receptors (e.g., CD25, CD69, CD70, CD71, CD95, HLA-DR-DP-DQ), co-stimulatory molecules (e.g., CD26, CD27, CD28, CD30, CD40L, CD134), chemokine receptors (e.g., CXCR3, CCR5), and adhesion molecules (e.g., ICAM-1, LFA-1)³ that all can modulate and direct further activation of the immune response, in particular the activation of naïve B cells leading to their differentiation to antibody-secreting plasma cells. In the late phase of the immune response, the majority of the antigen-specific T cells die while a pool of memory T cells remain at 10- to 1000-fold higher frequency than the naïve precursors activated during the primary response.^{4,5} In particular, central memory T cells, which are located in secondary lymphoid organs, retain an elevated proliferative capacity and may rapidly re-create a new pool of effector T cells after activation by MHC-II:peptide complexes presented by APCs (including resting B cells), even in the absence of APC co-stimulatory signals.⁶

While a therapeutic protein’s intrinsic and extrinsic properties (Table 1) may initiate the immune response cascade, other factors, such as patient-related factors (Human Leukocyte

Table 1. Terminology of pre-clinical immunogenicity assessment.

Term	Definition
Antigenicity	The ability of a molecule or substance to be specifically recognized by an antigen receptor (T cell receptor – TCR, B cell receptor – BCR or antibody).
Immunogenicity	The ability of a molecule or substance to provoke an integrated systemic adaptive immune response to an antigen, involving recognition by specific antibodies and/or T cells.
Agreptope or MHC binder	Antigen-derived peptide able to bind the major histocompatibility complex molecule (MHC).
Promiscuous agreptope or MHC binder	Antigen-derived peptide able to bind multiple MHC alleles.
T cell epitope	Peptide able to bind MHC class I or II and to trigger CD8 ⁺ cytotoxic T lymphocytes or CD4 ⁺ T helper cells activation, respectively.
Prediction	Forecast of clinical outcome based on <i>in silico</i> , <i>in vitro</i> and/or <i>in vivo</i> findings.
Risk assessment	Process of (1) identifying potential negative clinical outcomes, (2) analyzing and evaluating their likelihood and seriousness, and (3) implementing appropriate risk mitigation and control measures.
Intrinsic properties	Physical-chemical properties of a molecule or substance that may cause or facilitate an interaction with the immune system. In the concept of a therapeutic protein/peptide, these properties might include: <ul style="list-style-type: none"> • Amino acid sequence • Glycosylation profile • Affinity for γFc Receptors • Affinity for FcRn
Extrinsic properties	List of danger signals in therapeutic protein/peptide preparations potentially capable to induce an immune response. There properties might include: <ul style="list-style-type: none"> • Host cell protein contamination • Misfolded proteins • Degradation fragments • Aggregates • Formulation; excipients • Immune complexes formed with DC-shed soluble targets • Target-mediated internalization

Antigen (HLA) haplotype, immune status), therapeutic context, routes of drug delivery, and mode of action (MoA), also contribute to immunogenicity risks.⁷ Thus, the overall contribution of each of these components to the global immunogenic response in the clinic may not become apparent in the absence of large, systematic studies. From a pre-clinical perspective, drug developers have over the years set up a series of assays that recapitulate parts of the immune system, with the aim of capturing critical steps in the immune activation reflecting the *in vivo* situation. However, because pre-clinical assays are reductionist in nature, an immunogenicity risk assessment represents a combination of probabilities and consequences, with the risk level influenced by the degree of confidence in predicting these consequences based on the knowledge and representability of these assays.

The pre-clinical tools and assays discussed here (Figure 1) reflect our current understanding of the immune response as described above and can be broadly summarized in three categories: 1) *in silico* tools, which aim at identifying potential T cell epitopes on the sole basis of predicted HLA affinity; 2) *in vitro* assays, which report the response of a specific population of cell(s) toward a controlled stimulus; and 3) *in vivo* systems, which attempt to replicate a humanized immune response in an animal model. A common challenge and complication met by all pre-clinical immunogenicity assessment tools is MHC-II polymorphism, with currently more than 7300 alleles⁸ known and expressed with different prevalence across the human population. Thus, an important consideration for translation of data to the general population or targeted sub-populations is to ensure that MHC-II diversity is appropriately represented within each assay. The complexity of the immunogenic process and the many factors that contribute to it highlight the need for a standardized approach for methodologies and metrics to attribute commonly accepted risk scores and trigger effective strategies for mitigation.

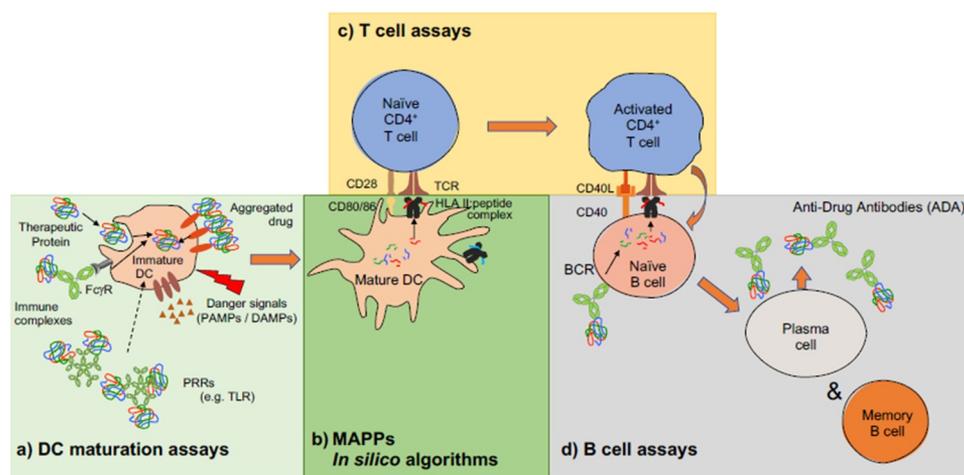


Figure 1. Schematic overview of an immunogenic signal cascade resulting in the production of anti-drug antibodies. Currently available tools and assays cover some of the key areas that drive an anti-drug antibody response to a biotherapeutic. Integrating information from multiple of such tools and assays informs about the intrinsic antigenicity potential of a biotherapeutic. (a) Antigen up-take and processing can be evaluated in dendritic cell uptake and activation assays. (b) *In silico* peptide binding prediction tools and MAPPs help reveal potential T cell epitopes (c) and *in vitro* T cell assays, with multiple different formats available, confirm previously identified potential T cell epitopes and help estimate prevalence of pre-cursor T cell responses in a given population. More recently, B cell assays (d) emerged aiming at identifying and cloning of B cell clones from patients who developed an anti-drug antibody response or to reveal B cell clones which produce cross-reacting antibodies to biotherapeutics, while *in vivo* systems attempt to replicate a humanized immune response in animal models and include all required steps of an anti-drug antibody response.

The risk of eliciting innate and adaptive immune responses from next-generation modalities like nucleic acids, viral vectors and cellular therapies is not in scope of this review. Although immunogenicity risk can be assessed to a certain extent with current methods described here, some additional adaptations may be needed to better understand innate responses that would include viral- and nucleic acid-mediated innate receptor-specific assessments, complement activation and adaptive phase responses, such as cytotoxic T lymphocyte (CTL) responses. The transgenes associated with viral gene therapies and chimeric antigen components associated with CAR-T therapies can use existing adaptive response assays, including cellular and humoral response assays. Additional risk assessment considerations and relevant assays would include virology, preexisting serotype reactivity assessments, tissue-specific tropism and associated immune responses. Although concepts discussed in here apply to the field of the new modalities as well, different controls and additional standardization approaches that go beyond the scope of this review are required, and are therefore not discussed here.

This work, conducted by the Non-Clinical Immunogenicity Risk Assessment working group of the European Immunogenicity Platform (EIP), aims to provide an overview of the current approaches and poses the question of whether criteria can be established that can be systematically applied by industry and academic groups that use these risk assessment strategies.

In silico algorithms for potential T cell epitope risk assessment

In silico approaches used for the assessment of immunogenicity risks focus on the identification of potential T cell epitopes based on the requirement for antigen presentation (in the form of linear peptides bound to MHC class II molecules) to trigger an amino acid sequence-based immune response. The primary sequence of the protein drug is scanned in a systematic manner, moving along the sequence, and a binding score is calculated for each 9–15' mer for each HLA molecule included in the analysis. The results of these *in silico* assessments can be used to triage molecules during the discovery phase, as hits are reduced to leads, and during optimization of leads to reduce the number of potential T cell epitopes by the time a clinical candidate is identified. Most drug developers use *in vitro* assays described herein alongside *in silico* algorithms to increase confidence that immunogenicity risk has been reduced through this design process.

Multiple factors contribute to a 9–15' mer amino-acid sequence becoming a functional T cell epitope, including stability of the complexes formed with the HLA molecules, natural processing of the epitope by the APC, and the existence of a specific CD4 T cell repertoire that may recognize the sequence within an MHC-II groove. Therefore, algorithms based solely on HLA affinity tend to be over-predictive and filtering is further required to provide context to the flagged amino acid sequences. For monoclonal antibody (mAb)-based products for instance, framework amino acid sequences found at high frequency across populations are removed from the list of potential epitopes due to presumed immunologic tolerance. Other reasons that HLA binding sequences might not become epitopes include deletion of specific T cell repertoire during negative selection, or because of an avidity that is too low to trigger a specific response. Additional

mechanisms include peripherally induced tolerance, also caused by the presence of so-called Tregitopes, which might induce a T regulatory response.⁹ Likewise, sequences with similarity to previously exposed disease agents as well as microbiomes can further develop a more tolerant T-cell repertoire, leading to lack of response to some of the presented sequences.¹⁰ Clinical experience gained with antibodies derived from common frameworks and isotypes may also provide additional confidence that certain sets of presented epitopes do not elicit long-term immune responses.

Peptide elution-based algorithms of MHC class II epitope prediction have been developed to address factors pertaining to the making of an epitope beyond HLA affinity, incorporating processing and stability data that were experimentally generated using mass spectrometry (MS). Also, much larger input datasets have been used to further improve performance and accuracy. For instance, MixMHC2pred integrates allele-specific motifs, peptide length and binding core offset preferences determined using over 99,000 unique eluted peptides analyzed with MoDec, a novel peptide deep motif deconvolution probabilistic framework.¹¹ Similarly, the multimodal recurrent neural network-based algorithm MARIA includes peptide sequence, cleavage scores based on predictive information from flanking residues, and HLA binding scores, both obtained from new pre-trained neural networks. MARIA also includes gene expression level, which is unique to this approach.¹² Likewise, the Neonmhc2 ligandome predictor was developed and trained on input data obtained from MS analysis generated with a new technology named MAPTAC (mono-allelic purification with tagged allele constructs).¹³ Likewise, another predictor, NNAlign_MAC, showed that the integration of MHC-II associated peptide proteomics (MAPPs) results with conventional affinity data such as collected in the Immune Epitope Database (IEDB; <http://www.iedb.org>) provided context information and HLA binding promiscuity scores, resulting in superior performance in predicting CD4 T cell epitopes in the well-studied infliximab and rituximab molecules.¹⁴ In direct comparison studies, MixMHC2pred, MARIA, neonmhc2, and NNAlign_MAC were found to outperform existing prediction methods.^{11–14} However, these studies are usually limited to very specific scenarios, e.g., prediction of binding to certain HLA-DR alleles, prediction of T cell epitopes identified from certain cell lines, or peptides recovered by MAPPs. It is not surprising that these new methods outperform existing prediction approaches in settings that were used to train and are thus optimized for these novel algorithms, but not for the methods they to which were compared. Therefore, it is essential to fully understand what the *in silico* algorithm was designed for and how it was trained for meaningful use in the context of immunogenicity risk assessment. Applying these methods blindly to “predict immunogenicity” is not recommended. Likewise, prediction of clinical ADA incidence from a pure *in silico* risk assessment is not advised due to the multitude of other contributing factors not included in this assessment.

In vitro assays

Assays described in this section attempt to measure experimentally the ability of an exogenous stimulus to trigger an explicit immune response in an enclosed cellular system correlating directly or indirectly with part(s) of the human immune response cascade. While it is difficult

to fully replicate the tissue environment *in vitro*, the properties of some immune cells have been exploited in the design of assays to evaluate whether therapeutic proteins contain CD4 T cell epitopes capable of driving a memory T cell response. Current *in vitro* assays encompass each step of an immune activation (generation and presentation of an antigen): activation of APCs, presentation of MHC-II peptides, activation of CD4 T cells, activation of B cells, and combination thereof.

Assays developed in the past few decades have been reported with a diversity of setups and conditions reflecting each group's emphasis, core knowledge, and historical experience, which render inter-assay comparison difficult. Also, cell availability and quality (and methods of isolation) are of primary importance, and factors such as genetics, lineage, activation status may contribute to the variability of the final output. For example, T and B cells are either purified in bulk from peripheral blood mononuclear cells (PBMC) or positively isolated using magnetic bead selection, and assay readouts very much depend on the exact lineage composition and activation status. In contrast, while several protocols may be used for the generation of monocyte-derived dendritic cell (moDC), assays based on these immune cells are robust, and outcomes do not seem to depend on which differentiation strategy is used. A common hurdle to all *in vitro* assays is the multiple MHC-II allelic composition, which in turn directs MHC-II peptide restriction. As a consequence, assays must be performed with multiple sample sets, using a wide variety of donors to ensure a coverage representative for a population's HLA composition. This in turn requires assay designs with reasonable throughput and analyses that are performed according to standardized metrics for a statistical evaluation of a protein therapeutics' immunogenicity profile.

Finally, therapeutic proteins' MoA (in particular, those with immunomodulatory functions) and preparation (purity, aggregation state, excipients composition) affect assay designs and readouts. For example, therapeutics containing a CD3-binding domain¹⁵ may not be analyzed in a PBMC-based T cell assay, as

activation of these cells is part of their MoA. Similarly, preparations containing elevated levels of host cell proteins¹⁶ or with degraded, aggregated, or post-translationally-modified forms¹⁷ of the therapeutic protein may elicit false positive responses in certain assays. In conclusion, there is no definite consensus on a minimal set of assays for the evaluation of pre-clinical immunogenicity risk. Rather, each drug developer is encouraged to develop an array of tests informative for each therapeutic in consideration to the intended use and treated population.

DC maturation assay

While the amino acid sequence of a protein directly contributes to the genesis of a T cell epitope, external factors might alert the immune system to the presence of aggravating factors that may activate APCs through cross-linking or via damage/pathogen-associated molecular patterns (DAMPs/PAMPs).¹⁸ DCs, as the paradigm APC (Table 2), play a key role in the induction of an adaptive immune response. Activation of these cells combined with their ability to present antigens is a useful indicator for the potential of biotherapeutics to induce ADAs. Monitoring of DC maturation can also be used as a tool to determine whether external factors, such as formulation or protein aggregation, contribute to the immunogenicity of therapeutics.¹⁹

moDCs may be generated using several protocols. After isolation of PBMCs from human blood, monocytes are isolated with magnetic bead selection or by plastic adherence. Purified monocytes are then differentiated to immature moDCs by culturing the cells in the presence of granulocyte-macrophage colony-stimulating factor and interleukin (IL)-4.²⁰ In the immature state, DCs are challenged with the test biotherapeutics and their maturation status may be monitored from 15 minutes to 48 hours after challenge.²¹ Parameters of interest may include: 1) cell surface-marker expression (Table 3; CD83, CD80, CD86 and CD40 are recommended as minimal panel) measured 4–48 hours after stimulation; 2) cytokine secretion (IL-1 β , IL-6, IL-8, IL-10, IL-12 and tumor necrosis factor (TNF)) measured 24–48 hours after stimulation in cell culture

Table 2. Antigen presenting cell types that have antigen-presenting function *in vitro*.

Antigen Presenting Cell	Location	Origin	Function	Outputs
Myeloid Dendritic Cells	Under surface epithelium of the skin, mucous membranes of the respiratory tract, genitourinary tract, and the gastrointestinal tract, lymphoid organs and solid tissues	Monocytes	Endogenous and exogenous antigen uptake by MHC Class I and Class II, processing and presentation to CD4+ and CD8 + T cells	Activated dendritic cells express chemokine receptor CCR7 that enables the dendritic cell to migrate toward the chemokine CCL21 expressing lymphoid tissues
Macrophage	Wandering macrophages present all over the body Fixed macrophages are part of reticuloendothelial system Tissue specific cells liver: (Kupffer cells); kidneys (mesangial cells), brain (microglia), bones (osteoclasts), lungs (alveolar macrophages), gastrointestinal tract (peritoneal macrophages)	Blood derived	monocytes	Capture and present antigens to T effector lymphocytes
Produce	chemokines and cytokines like CXCL2, IFN and TNF alpha Receptors that bind and kill infected cells			
B-lymphocyte	Circulate between blood and lymphoid tissues	Bone Marrow	Capture and present peptide epitopes from exogenous peptides to T effector cells through B cell receptors (BCR)	B cells proliferate and differentiate into Antibody secreting Plasma cells

Table 3. DC markers.

Marker	Function	Monocytes	iDCs	mDCs
CD14	LPS-induced Macrophage activation	+++	-/+	-
CD80	Co-stimulation for T cell activation	-	++	++
CD83	Lymphocyte activation	-	-/+	++(+)
CD86	Co-stimulation for T cell activation	-	+	+++
HLA-DR	Antigen Presentation	+	++	+++
CD209	DC-T cell interactions, DC migration	-/+	++	+
CD40	Co-stimulation for T and B cell activation	+	+	+++

iDC: immature dendritic cells; mDC: mature dendritic cells.

supernatant; 3) phosphorylation state of proteins involved in signaling pathways possibly playing a role in DC maturation such as Akt, ERK1/2 and Syk, measured 15 to 30 minutes after immature DC (iDC) challenge with therapeutic,¹⁹ and 4) mRNA quantification (upregulation) of cytokines and/or chemokines at earlier timepoints, measured 6–24 hours after stimulation of DCs with biotherapeutics.¹⁹

Use of relevant assay controls within the assay is essential to assess both the validity of the assay and to allow comparison among different laboratories; we suggest including a negative (medium alone), a positive control (such as keyhole limpet hemocyanin (KLH), a lipopolysaccharide (LPS) titration, or a maturation cocktail) and ideally, one low-immunogenic (e.g., bevacizumab) and one medium-high (e.g., ATR-107) benchmark controls. Evaluation of the results depends on the methodology used and typically involves a statistical analysis over a minimum of 10 donors. When cell surface markers expression is the chosen readout, DC activation is reported as median fluorescence intensity values (% increase per marker for the test condition compared to the unstimulated condition) and % of donors showing an increase of 50% or 100% per marker.²²

MHC-II associated peptide proteomics

In the context of an immunogenicity risk assessment, MAPPs assays have represented the method of choice for the identification of peptides presented by class II MHC molecules on moDCs. The assay generates a repertoire of proteins that have been taken up by the APC, processed, loaded and presented as peptides on MHC-II receptors through the exogenous antigen processing pathway. Thus, MAPPs provide a biologically driven snapshot complementing currently over-predictive T cell epitope bioinformatic platforms. While this assay cannot provide an immunogenicity risk scale for identified MHC-II peptides, it enables the identification of more “authentic” T cell epitopes compared to an overlapping peptide scanning approach.²³ However, as T cell recognition is not tested in this assay, MAPPs will also identify antigen-derived peptides that are not recognized by T cells.

MAPPs assays have been generally performed according to the following protocol using moDCs cell cultures generated as delineated in the previous section. Immature moDC are pulsed with a test compound followed by maturation with LPS or TNF to increase the antigen processing and presentation of MHC-II-peptides complexes on the moDC cell surface. Adherent cells are then pelleted and lysed in the presence of various protease inhibitors. MHC-II-peptide complexes are subsequently immunoprecipitated, typically using anti-MHC-II antibodies (specific for the MHC-II isotype of interest) immobilized to a resin or magnetic nanoparticle beads. After extensive

washing to remove impurities and nonspecific peptides, the MHC-II bound peptides are eluted with acid and separated and sequenced using a liquid chromatography-tandem mass spectrometric approach.^{24,25} Database searching is typically performed against a global protein database containing the amino acid sequence(s) of the biotherapeutics of interest, making sure to omit protease cleavage specificity and including pertinent post-translational modifications (PTMs; for example, methionine sulfoxide or deamidation), as some of the peptides might be modified during the antigen processing or during the MS analysis process. In recent years, the use of increasingly more sensitive (and reliable) mass spectrometers has contributed to the ability of MAPPs assays to accurately characterize MHC-II associated peptides isolated from a number of moDCs compatible with low-throughput screening formats.

There are important contributing factors to the performance and sensitivity of a MAPPs assay, including the use of fresh or frozen cells, the number of cells used per assay, the quantity of test material and the analysis of the mass spectrometric data in terms of threshold and effective false discovery rate settings. Interestingly, while protocols for generating moDCs and performing MAPPs assays remain fairly generic, there is mounting evidence that MAPPs are a fairly robust assay and results compare well across studies.^{26,27} Comparative data from the analysis of infliximab and rituximab using only 15 donors demonstrated a very close alignment with the peptide clusters characterized by Hamze *et al.* who used more than 30 donors for their MAPPs study.²⁶ Moreover, in a separate unpublished study (C.B., Abzena, personal communication) peptide clusters identified by MAPPs and putative T cell epitopes in Factor VII and vatreptacog alfa aligned directly with the point mutation regions shown to associate with MHC-II molecules as published by Lamberth *et al.*²⁸ Due to the relative output of the assay, negative and positive controls are typically included to determine whether the pattern of peptide presentation is consistent and whether the assay has performed as expected. Assuming consistent culture and supplement conditions for the generation of DCs, endogenous reference peptides can be used to evaluate assay performance, such as MHC-II peptides derived from supplements (e.g., from supplemental serum proteins), from tracer proteins added to the assay, or from test proteins such as antibodies that have known germline sequences consistently presented by MHC-II receptors.²⁷ The systematic monitoring of these peptides derived from either media or cell-associated proteins, such as serum albumin, lysosome-associated membrane glycoproteins, and cathepsins, serves as a good measure of MS performance and consistency of the assay with respect to antigen uptake and presentation across conditions and donors.

To date, most of the MHC-II MAPPs studies have been performed to study HLA-DR-presented peptides as the dominant class II genotype, partly because of the availability of well-performing immunoprecipitating pan HLA-DR antibodies, such as the mAb clone L243. Few studies have reported the identification of MHC-II peptides presented on HLA-DP²⁹ or HLA-DQ³⁰ receptors. In particular, HLA-DP is expressed only at low levels (~10 fold) compared to HLA-DR and DQ receptors, and therefore it has been considered less important with respect to immunogenicity risk. Recently, two independent studies using MAPPs on

FVIII and dengue virus, respectively, showed that ~80% of the MHC-II identified peptides were HLA-DR-specific, while ~20% were DQ- and 5–10% DP-specific, indicating the major role of HLA-DR in initiating a CD4 T cell response in these models.^{31,32} With regards to the MAPPs assays' sensitivity, the low expression of HLA-DP, and to a lesser degree HLA-DQ alleles have implications in the purification process and subsequently in the MS identification of peptides binding to HLA-DP and DQ receptors. The necessity and requirement to monitor MHC-II peptides binding to these molecules with respect to immunogenicity risk assessment remain to be demonstrated.

Presently, the descriptive nature of the MAPPs assay (the outcome is a list of presented MHC-II peptides that may contain potential T cell epitopes) has limited its use as an *in vitro* immunogenicity risk assessment tool. So far, most published studies have been conducted retrospectively on known immunogenic therapeutics. Also, it remains to be shown that the MHC-II peptide repertoire presented by moDC is the same as that of *in vivo* DCs. However, emerging data show that MHC-II peptides presented by moDCs from healthy donors are similar to those presented by moDCs isolated from patients; moreover, some of these peptides overlapped with reactive T cell epitopes identified in both healthy donors and patients.^{32,33}

The rich nature of the data it generates added to increasing indications of its robustness are contributing to the growing popularity of the MAPPs platform to support immunogenicity profiling. For example, MAPPs are an optimal tool for the deimmunization of therapeutic peptides and proteins. Efforts to reduce the binding and presentation of peptides on APCs can easily be assessed by analyzing parent molecule and deimmunized variants hereof. There are, however, a number of important aspects of the assay to standardize. A minimum of 10 donors should be used for early sequence assessment, while a minimum of 15–30 genotyped donors ought to be probed to account for MHC-II diversity across the global population. Currently, there are no anti-HLA-DP or -DQ antibodies with appropriate performance available for the MAPPs assay. Therefore, the antibody used for MHC-II-peptide complex purification should be a standard antibody specific for HLA-DR, for example, clone L243, until satisfactory HLA-DR, DP, and DQ antibodies, which could be used in a mixture, or pan MHC-II antibodies may become available and allow a comprehensive analysis to be performed. The total number of identified mammalian endogenous peptides and proteins should be in the range of 5,000 and 1,000, respectively, as evidence for a process with good MHC-II peptide purification and high sensitivity MS detection. Lastly, clear information on identified target molecule peptide clusters and associated common sequence for each cluster (including the MHC-II binding core sequence) should be provided to enable harmonization between laboratories.²⁷ Standardization will also benefit from routine reporting of MAPPs datasets on qualified and easily accessible control protein(s), such as the well-studied infliximab and rituximab.

T cell functional analysis

In vitro T cell activation assays attempt to evaluate the immunogenic risk of therapeutic proteins and peptides by measuring the activation of CD4 T cells in response to drug-derived

epitope(s) (Figure 1). However, the presence of and/or requirement for many different immune players led to the development of several assay formats to probe various effector functions and outcomes (Table 4). In addition, the nature of the evaluated biologics and MoA, such as immune-modulatory vs metabolic nonimmune modulatory target, may also dictate which T cell activation assay must be used.^{34–38} Each assay format presents discrete advantages and disadvantages with regards to sample preparation, ease of use, and interpretation of activation signal(s). For example, whole blood-based assays require less sample manipulation and ensure a rapid turnaround, but need to be conducted within 4–6 hours of draw since the immune cells function in blood samples cannot be preserved over long periods of time. Also, activation signals detected from such assays lack specificity since immune cells have not been previously sorted. Similarly, for whole PBMC-based assays, activation signals cannot be attributed to a particular subset of cells and usually cannot be used to assess immune modulatory drugs. PBMCs also lack the functional mature and professional APCs matching T cell subsets with different effector functions. Therefore, the use of peptides instead of intact proteins as challenge antigens can lead to over-representation of the HLA-binding peptides presented by the APCs.³⁹ Some approaches to address these limitations include depleting PBMCs of CD8 T cells to reduce interference and to assess more specifically the CD4 T effector response. DC-CD4 T cell or DC-PBMC co-culture-based assays may also prove advantageous to evaluate therapeutic proteins with immune modulatory MoA, in particular those containing

Table 4. Overview of effector cell activation assays.

Application	Assay Setup and Components	Readout
Assessment of immediate immune response	Whole blood WBC, RBC, granulocytes, macrophages	Cytokine secretion
Assessment of T cell response toward biotherapeutic drug candidates	PBMC Immature Dendritic cells, monocytes, B cells, CD4 ⁺ T cells, CD8 ⁺ T cells CD8-depleted PBMC Immature Dendritic cells, monocytes, B cells, CD4 + T cells DC-T cell /DC-PBMC assay Dendritic cells (DCs), CD4 ⁺ /CD8 ⁺ T cells, B cells, Monocytes	Read-out of T cell proliferation assays <ul style="list-style-type: none"> • by flow cytometry: • a cell division marker (CFSE), • a DNA proliferation marker (BrdU or EdU) • a protein proliferation marker (Ki67) radioactive techniques (³H-thymidine incorporation); • cytokine secretion by Elispot/Fluorospot or Luminex
Assessment of T cell response toward peptides of biotherapeutic drug candidates	Enriched PBMC peptide assay T cell lines/ rechallenge DC:T cell peptide assay	Read-out of T cell proliferation assays <ul style="list-style-type: none"> • by flow cytometry: • a cell division marker (CFSE), • a DNA proliferation marker (BrdU or EdU) • a protein proliferation marker (Ki67) radioactive techniques (³H-thymidine incorporation); • cytokine secretion by Elispot/Fluorospot or Luminex

T cell binding domains. Staggered cultures, wherein the intact biologic is only administered to DCs prior to co-culture, or co-culture with adjusted T cells to DCs ratios, may so avoid interference with the assay's readout.⁴⁰ A further expansion of PBMC-based assays may lead to the generation of enriched T cell clones with antigen specificity. Such assays were originally intended to be high throughput, but require multiple rounds of antigen stimulation and cytokine challenge. Alternatively, individual peptides or peptide pools can also be used to elicit antigen-specific T cell clones while considering that not all peptides will be naturally presented by APCs.⁴¹

T-cell assays are typically composed of four distinct components and sequential steps: 1) antigen challenge, 2) cell culture components and stimulus, 3) assay readout, and 4) data analysis; some essentials of these steps are discussed below and summarized in (Table 5). The amount of antigen needed for activation depends on the assay format, on the nature of the cells being challenged and of the target, on the time and frequency of such challenges, and whether the assay's primary aim is to elicit a naïve vs. a memory response. Similarly, the setup of cell cultures and the required nutrients and growth factors may vary depending on whether the investigated cells have an effector or an antigen-presenting role. Accordingly, T cell activation assay outputs may range from an antigen-specific modulation of a cytokine, as reported by an enzyme-linked immune absorbent spot (ELISPOT) assay, to complex readouts, such as the profiling of secreted proteins using MS. It is therefore of importance to carefully design and execute such experiments following a formal analytical plan to enable a statistical interpretation of the data, since activation is usually based on an arbitrary activation threshold, as discussed below.

Data analysis

A positive T cell activation response to a protein therapeutic is determined by statistical analysis of the data based on an empirical threshold determined within the experiment. Several models are being used to carry out this analysis, such as an unpaired Student's t-test to compare the means of two independent groups (usually the T cell response for a test compound against that of the medium blank and/or a known non-immunogenic control),^{48,52,53} The Distribution Free Resampling (DFR) method and its derivative, the DFR(2x) method,⁵⁴ have also been used in this context, especially for the analysis of ELISPOT-based assays. The *p* value is usually set at a significance of 0.05 (*p* < .05) below which a T cell activation signal will be considered as true. Overall, an empirical T cell stimulation index (SI) >2 has been a widely accepted threshold to report a positive response based on data acquired from known immunogenic test compounds,⁵⁵ while some authors have used lower SI, such as SI>1.9,⁵³ to increase sensitivity of the assay. T cell activation response based on proliferation-based assays is interpolated from dose-response curves at concentrations of KLH inducing 50% of maximum proliferation (EC₅₀). For these specific experiments, a response is considered positive when an antigen induces a (mean+2 standard deviation) proliferation rate greater than the response obtained in the absence of antigen or a non-immunogenic control.

Table 5. Phases of T-cell assays and key factors for consideration.

Antigen Challenge	<p>Antigen dose/concentration: optimized amount or concentrations that would not lead to cellular toxicity; naïve T cells- DCs and macrophage interaction and subsequent activation is antigen dose dependent.^{42,43} Variability (100-fold or more) across donors in the EC₅₀ of antigen required for inducing 50% of maximum proliferation.⁵</p> <p>Time/Rounds of stimulation:</p> <ul style="list-style-type: none"> • For memory/recall response: single challenge^{6,44} is adequate; readouts at 24–72 h post challenge for cytokine readouts, at 7 days for a proliferation readout. • For naïve response: multiple sequential challenges to generate antigen-specific T cell lines;^{33,45,46} incubation times may vary between 7 and 28 days.^{6,33,40,44–46} <p>Antigen mode of action: Based on antigenicity and engagement of target, cells and assay format will change.</p>
Cell culture components	<p>Medium:^{40,47} RPMI, DMEM, Earle's modified</p> <p>Serum: pooled human AB serum, autologous serum or serum-free</p> <p>Stimulating antigens only⁴⁰ /cytokines added to the culture: amount, time-point and duration of addition of IL-2,^{5,6} IL-6, IL-7, IL-12,^{45,46} IL-15, IL-17, IL-21.⁴⁴</p> <ul style="list-style-type: none"> • Cytokines used for DC maturation: IL-4, GM-CSF and LPS, decrease bystander T cell response and reducing background using IFN-α.⁴⁷ • Exogenous stimuli: LPS, TNF-α, IFN-α, CD40L and maturation cocktails for protein loaded immature DCs.
Outputs/Readouts	<p>ELISPOT and Fluorospot: (multiplexed and capable of identifying Th subtypes based on cytokine profiles);^{45,48,49} ideal for short-term memory T-cell assays, identifying low T-cell precursor frequencies of memory T-cells.</p> <p>T cell proliferation: markers of T-cell activation; fluorescent marker (e.g. CFSE); DNA proliferation marker and intracellular staining of Ki67; best suitable for extended stimulation times to generate a naïve T cell response.⁴⁸ measured for example using</p> <ul style="list-style-type: none"> • Flow cytometry-based phenotypic cell surface markers like CD154 indicate antigen-specific CD4 T cell activation⁵⁰ or intracellular cytokine staining^{40,51} as markers for T cell activation • Multiplexed bead-based secreted cytokine assessments with platforms like Luminex[®] and MSD.

Harmonization of T cell assays

The large diversity of existing T cell assays to accommodate different purposes is likely to prevent the adoption of a universal format, which raises the question whether results and data can be effectively compared across approaches. However, and possibly more importantly, there is also a growing need to establish commonly accepted quality controls (QC) and to define the minimal set of data to report from T cell assays.

The first harmonization we propose here is to set QC criterion for the PBMCs used in the assays. The QC should include post-thaw viability and recovery assessment when using frozen PMBCs. To confirm that T cells are functional and not pre-activated, the QC should include assessment of baseline or background responses as well as the cells capability of responding to polyclonal and neo- or recall antigens. Acceptable QC criteria for a PBMC preparation include a viability >85% and an SI for polyclonal activation ≥ 2 . Baseline responses may differ between donors and sometimes even between samples taken at different time points from the same donor due to biological variability. Ideally, a baseline response should be recorded for each PBMC preparation to establish sample-specific cut-points. It is important to stress that calculations to set SI thresholds are highly dependent on assay types and on the statistical method used to evaluate the results, in

particular, on how measurement replicates are taking into account to report significance; it is therefore desirable that reports on T cell assays provide a comprehensive summary of the experimental procedure and measurements and describe the statistical models used to report results for better inter-laboratory comparability.

A second harmonization we suggest is to define a minimal number of donors to include in a study. This number might depend on the stage of development of the drug candidate, on the type of T cell assay that is performed, but also on prior experience and data collected in past experiments. At early discovery stages, where assays are typically run in an investigational manner, we consider that studies including 10 to 25 donors representative for HLA allele frequencies of a target population may be appropriate to report on PBMC-based assays results. This number might be lower for a DC-T cell assay due to the time and labor required to obtain results. A pool of non-self-peptides may be designed to flag sequences potentially binding to prevalent MHC-II allele supertypes. Similarly, pools spanning multiple epitopes found in high-risk regions of a biologic amino acid sequence would enable identification of a potentially highly immunogenic molecule at an early stage of development. Still, such relatively small donor panels might not be sufficiently representative of a genetically diverse population. At a later stage, we recommend confirming early findings using a larger pool (50) of HLA-characterized donors to fit the HLA allele distribution of the intended target population.

A further harmonization we suggest is to add a panel of control molecules/drugs with well-characterized naïve as well as memory T cell responses to calibrate and monitor the assay performance over time. The frequency of antigen-specific T cell responses for control molecules should be sufficient at the cellular and population levels to be run with a standard average number of cells in tested individuals. One such universally accepted assay control is KLH because of its high neo-antigenic content, which elicits a positive response in >90% of the tested cells. However, assay consistency and performance require a careful examination of the origin and batch-to-batch quality of the KLH protein; it is therefore recommended to titrate every new batch of KLH and to avoid changes during a screening assay. Similar considerations apply for other essential reagents and materials to keep the experimental variability within one assay as low as possible.

Although T cell assays are less robust in their performance compared to DC activation and MAPPs assays, and their outcome depends on the setup and the MoA of the tested biologics, relative ranking is possible using control molecules with known immunogenic outcomes in each of the assay format. For example, bococizumab is known to elicit a positive response in 50–70% of the donors tested in a T cell assay, whereas it is lower for ATR-107 (40–60%) and minimal (<5%) for bevacizumab. Assays investigating peptides as challenge antigens should include relevant positive controls such as a CD3/CD28 cross-linker, which elicits a positive response in >90% of the donors and positive peptide controls such as PADRE for CD4 T cell peptide assays⁵⁶ and a CEF/CEFA/CEFT pool for MHC Class I and Class II -restricted T cell epitope assays.^{57,58}

To achieve full harmonization, we propose that any laboratory using such assays and willing to participate should establish jointly a common QC protocol for *in vitro* T cell assays immunogenicity

risk assessment. A central lab would provide PBMCs from a central bank of donors and relevant controls to participating laboratories. These would run T cell assays using previously agreed parameters around assay setups, cell numbers and viability, time of incubation, and readouts. Raw data would be sent anonymously to designated biostatisticians who could provide feedback to all participating labs and identify sources of variability that affect consistency across assays and laboratories. Results may lead to the drafting of a guidance document stating general considerations for performing T cell assays, including QC acceptance criteria for cells used in each type of assay, number of donors to be included at different stages of drug development, the coverage of the MHC-II allele supertypes distribution in a target population, and a selection of relevant controls for innate vs adaptive immune response assays. Such an initiative could also set more standardized guidelines on statistical methods to be used for the data analysis from T cell assays, accounting for the nature of the data (for example, to take into consideration the high variability of results when working with primary cells and/or the low activation or proliferation signals due to the rare T cell receptor frequency), and for the calculation of robust activation thresholds.

B cell assays

Commonly used immunogenicity risk assessment tools focus mainly on the potential of a given biologic to activate CD4 T cells, due to the pivotal role they play in activating B cells and leading to their differentiation to ADA-producing plasma cells. Biologics also need to be directly recognized and internalized by specific populations of B cells for subsequent peptide presentation to T cells. This interaction is required for the activation of B cells, which leads to B cell differentiation into memory and plasma B cells, which are ultimately responsible for the production of high-affinity IgG (ADA) against the biologic. Hence, the B cell epitope content and the number of B cells producing IgG that are specific for a given biologic might serve as an important ranking criterion for immunogenicity risk. However, the B cell component of the immunogenicity risk assessment is currently greatly overlooked, due in part to technical limitations to assess the B cell response *in vitro*.

Predictive *in silico* methods have been developed, such as SEPIa, an algorithm combining 13 different sequence-derived features, including amino acid composition, hydrophilicity, solvent accessibility, and backbone flexibility. SEPIa was found to outperform both sequence-based and structure-based preexisting methods, but still exhibited limited performance compared to T cell epitope predictors.⁵⁹

To date, only one ELISpot/FluoroSpot-based assay has been reported to assess cellular B cell response to a biotherapeutic *in vitro*.⁶⁰ It is performed with PBMCs and reports the number of B cells secreting antibodies specific to the target antigen. The assay is initiated by treating B cells with a polyclonal activation cocktail (typically containing at least a Toll-like receptor agonist and IL-2), which differentiates memory B cells into plasma cells. These plasma cells secrete large amounts of antibody that can be captured on a polyvinylidene fluoride (PVDF) membrane. The antibody capture step can be carried out in two different ways: 1) The target antigen can be directly immobilized onto the PVDF membrane and is available to bind to the secreted antibodies, which can

be detected and quantified using an anti-Fc antibody labeled for colorimetric or fluorescent detection; or 2) an anti-Fc antibody is used to capture all antibodies secreted by the B-cell derived plasma cells; detection and quantification is performed using the directly labeled antigen by colorimetric or fluorescent detection. The latter method provides the advantage that the total number of antibody-secreting cells can be determined alongside the number of antigen-specific secreting cells and enables the calculation of the frequency or % of antigen-secreting cells in the memory B cell population. The assay procedure can also be modified to detect various antibody isotypes (IgA, IgE, IgG1-4 and IgM) while the use of a FluoroSpot-based instrumentation enables the multiplexed detection of multiple antigens or different antibody isotypes (e.g., IgM and IgG) in the same well. In this modality, when run on healthy donor PBMCs, the assay will primarily detect memory B cells specific for an unrelated antigen that cross-react with the biologic of interest. In a recent study, Liao and colleagues demonstrated that the B cell response generated *in vitro* against a proprietary compound originated solely from memory B cells, with antibody-secreting cells detectable in treatment naïve subjects with preexisting ADA. When the assay was performed with peripheral blood from ADA⁺ subjects, ADA titers and the number of ADA-secreting B cells correlated, further demonstrating the specificity of the response detected by the assay.⁶¹

Although currently limited to assessing the memory B cell response, the B cell ELISpot/FluoroSpot assay could become a tool to assess and compare the risk of preexisting cross-reactive antibodies present in drug-naïve healthy donors during the early stages of biotherapeutic development. In these settings, an optimal positive control would recall a B cell response in a high percentage of the donor panel and could take the form, for example, of an influenza-derived protein antigen mix representative of the main serotypes used in flu vaccines over the past decades. Harmonization of the assay would involve the use of a common protocol in terms of starting cells and readouts, and the use of a standard positive control for which a range of B cell memory response frequency would have been established on an extended panel of healthy donor PBMCs.

There is currently no broadly available *in vitro* strategy shown to induce a robust IgG-based response in naïve B cells. This is due to the complex processes involved in class switching, wherein B cells switch from low-affinity IgM production to high-affinity IgG antibodies associated with ADA responses in the clinic. Potential *in vitro* strategies to assess the naïve B cell repertoire rather than the cross-reactive memory repertoire have been evaluated and include measurement of B cell activation and proliferation in response to the biologic and B cell receptor-specific binding and internalization. However, these assays are yet to be optimized and validated.

In vivo models

The complexity and the challenges to capture relevant parts of the immune system through *in silico* and *in vitro* systems have led researchers to investigate *in vivo* models that consider the entire immune system, as it is arguably better suited to trigger ADA generation when tolerance is breached. Standard animal models are not appropriate because any human therapeutic protein might be recognized by the animal as foreign.

Therefore, numerous non-clinical animal models have been generated in an effort to recapitulate the entire ADA response. Here, we review both transgenic models expressing human transgenes such as HLA and immunoglobulins,⁶² and models in which the human immune system has been incorporated into a mouse to diverse extents, with varying levels of success in generating the expected response to a known antigen.

Human HLA transgenic mice express a human MHC transgene and consequently can have a T cell repertoire that reflects more closely the human counterpart. These models are largely used to study autoimmune conditions with known risk alleles,⁶³ but they show limited value for immunogenicity prediction since they do not reflect the diversity of MHC alleles in the human population and the TCR repertoire remains entirely murine. Transgenic models expressing large fractions of the human germline V gene repertoire of the immunoglobulin family are more widely used to study immunogenicity because their expression provides immune tolerance to a broad range of human antibodies.⁶⁴ Mice made tolerant to certain subclasses of human immunoglobulin, such as the Xeno-het mouse, have been used to evaluate the IgG2 mAbs attribute-related immunogenicity risk. Aggregates made by mechanical stirring were able to induce an ADA response, although weak and transient.⁶⁵ Despite expression of a large repertoire of human IgG antibodies, the membrane form of human antibodies lead to allelic exclusion of murine antibody repertoire, thus forcing a laborious crossing of the transgenic line with knock-out Ig alleles and one wild-type allele. In contrast, other groups have generated transgenic mice bearing only the five most used human V_H and V_L (kappa and lambda) genes. These mice rearrange the human VDJ genes and, importantly, bear an intact murine repertoire as the transgenic C_γ1 constant region encodes only the secretory form of human antibodies.⁶⁶ These transgenic mice have been shown to tolerate antibody aggregates⁶⁶ and sub-visible particles⁶⁷ devoid of new T cell epitopes, but they mount an immune response against antibody preparations that contained chemical modifications of primary amino acid residues.

As the aforementioned transgenic animals bear T and B cell repertoires as well as murine MHC alleles, they cannot be used for the identification of potential immunogenic human T cell epitopes; only mice carrying a human MHC II allele may be used for the determination of an immunodominant CD4 T cell epitope^{68,69} (see also below). However, protein sequence is only one factor contributing to ADA formation. Other product-related factors such as MoA and compound format can also influence the immunogenicity of biologics. Thus, infliximab, a chimeric anti-TNF antibody, elicits similar or even lower ADA rates in human than adalimumab, a fully human anti-TNF.⁷⁰ Also, work performed by Reipert's group has shown that the route of administration and degree of PEGylation influenced the immune response against Factor VIII in immunetolerant mice.^{68,69} Thus, these transgenic mouse models are useful tools to study product-related factors causing a break of immune tolerance against human antibodies and other proteins.

Currently, the human immune response is best modeled in humanized mice (i.e., Human Immune System "HIS"), created by myeloablation of immunodeficient mice and reconstitution with human hematopoietic stem cells obtained either from fetal liver or (more commonly) from umbilical cord blood. Three

models have shown potential in this respect: 1) the bone marrow, liver, thymus (BLT) mouse, 2) the Balb/c Rag2^{-/-}IL2Rγ^{-/-}Sirpa^{NOD} TSLP^{Tg} (BRGSF/T) mouse, and 3) the NOD.SCID.IL2Rγ^{-/-} (NSG) mouse.^{71,72} Since their introduction, these models have consistently achieved high rates of reconstitution, averaging 60–90% HIS (as calculated by the % of hCD45⁺ (total mCD45⁺ + hCD45⁺)). These humanized mice have historically been tested in the context of infectious disease; in recent years, however, providers of HIS mice have focused their efforts on generating models for the burgeoning immuno-oncology field. Table 6 provides an overview of the three different HIS models and their potential for immunogenicity testing.

To date, the use of humanized mice has not entered the pre-clinical immunogenicity risk assessment toolkit as a standard go-to platform when evaluating biotherapeutics. Commitment to such an approach is non-trivial due to costs and time to reach an appropriate decision point, and therefore the use of such *in vivo* models will be focused on the study/assessment of biotherapeutics deemed to have high immunogenicity risk. In the absence of other pre-clinical models replicating all steps required for ADA formation (i.e., lymph node on a chip), transgenic immune tolerant and humanized animals represent the only available tool to understand better the likelihood of biologics to trigger ADA formation in the clinic. Future studies using next-generation sequencing to interrogate deeply the T and B cell repertoires combined with single cell RNA sequencing in *in vivo* models, as well as the systematic study of ADA-positive patients, will be critical to allow researchers to identify immunogenicity biomarkers and provide a better understanding of translatability of individual models.

Immunogenicity modeling

Mechanistic modeling is used across drug development to support our understanding of complex systems and to provide simulations in predicting outcomes in experiments and in clinical trials. The integration of data from assays that investigate key mechanisms of the immune response in mathematical models is expected to result in better prediction of immunogenicity outcomes in clinical trials.⁸⁷ Data that can be used for such modeling may be obtained from DC activation, protein uptake by DCs, T cell epitopes (*in silico* predicted and/or identified using MAPPs), and T cell proliferation assays. Model sensitivity analysis identifies inputs on DC activation, number of naïve T cells, and the affinity of HLA-peptide interactions as being most influential for ADA incidence simulations, indicating the high value of generating these data. Additional variables that are currently investigated (but which will require additional assays to refine modeling outputs) include B cell activation, B cell epitopes determination, B cell proliferation, and rate of ADA production. Also, extrinsic variables derived from ADA determination and PK/PD assays or from patient-centric parameters, such as individualized T and B cell epitopes and HLA genotyping, may also be included in modeling.

At present, immune response modeling has been shown to predict appropriately the clinical outcome of drugs such as adalimumab.⁸⁸ Simulations were able to describe the overall ADA generation in a population, its impact on pharmacokinetics, and the likelihood that patients will lose efficacy due to reduced exposure. Additionally, sub-models can be used to interrogate the assays and specific biological components in order to improve our

Table 6. Overview of transgenic animal models.

Models	Advantages	Limitations	Utility	References
<ul style="list-style-type: none"> • BLT (bone marrow hematopoietic stem cells, human fetal liver and thymus tissue) 	<ul style="list-style-type: none"> • Near complete and fully functional human immune system; in particular, the HLA-restricted T cell repertoire is more representative of the human's as it is educated on some human thymic epithelial antigens • can accurately replicate the human mucosal system 	<ul style="list-style-type: none"> • Its generation is very laborious requiring fetal tissue and surgical expertise • Risk of GVHD induction by certain HLA Class I alleles, thus masking compound's immunogenic signals 	<ul style="list-style-type: none"> • used to evaluate prophylactics and therapeutic response against viral infections, including HIV and Dengue 	48,73–80
<ul style="list-style-type: none"> • BRGS(F/T) • Balb/c Rag2^{-/-}IL2Rγ^{-/-}Sirpa^{NOD} • F = Flk2^{-/-} • T = TSLP^{Tg} 	<ul style="list-style-type: none"> • possess most of human hematopoietic cell subsets, including B, T, T regulatory, NK and the myeloid compartment including DCs and pDCs and monocytes/macrophages. • CD47/SIRPα interaction avoiding mouse phagocytosis of human xenograft • BRGST develops lymphoid tissue with compartmentalized B and T cell areas, increased IL-21 secreting T follicular helper cells leading to strong antigen-specific responses 	<ul style="list-style-type: none"> • BRGST is not commercially available • BRGSF develops poor IgG response to classical protein antigen such as KLH 	<ul style="list-style-type: none"> • largely used in immuno-oncology, infectious disease and vaccine field • BRGST used in HIV settings 	81,82
<ul style="list-style-type: none"> • NSG (NOD.SCID.IL2Rγ^{-/-}) 	<ul style="list-style-type: none"> • HIS model with best characterized immune response • Recapitulates well human T cell responses, in particular cytotoxic T cell responses 	<ul style="list-style-type: none"> • poor myeloid compartment • suboptimal humoral response, in particular mature IgG responses relying on germinal center formation • T cell repertoire educated on mouse thymic epithelial antigens 	<ul style="list-style-type: none"> • Good model for studying anti-tumor immunity and others diseases, including infectious and autoimmune disease • Most advanced HIS model for <i>in vivo</i> immunogenicity assessment. However model can be improved by (1) boosting the myeloid compartment for ADA initiation, (2) supporting secondary lymphoid structure and germinal center formation leading to Ig class switching and affinity maturation. 	71,72,83–86

understanding of how an individual assay⁸⁹ or individual risk factors¹⁸ contribute to immunogenicity prediction. The model contains multiple components that are targets of, or would-be biomarkers for, immunomodulatory therapeutics. Therefore, the immunomodulatory pharmacology of a therapeutic can be modeled directly or indirectly, for example by reducing the number of B cells for B cell depleting therapies or enhancing T cell activation for cancer immunotherapies. Future developments for immunogenicity modeling will include the ability to distinguish the effects of neutralizing ADA from clearing ADA, which could be achieved using modeling concepts already in use. For example, ADAs can be modeled as if there were *bona fide* compounds, with clearing and neutralizing functions with respect to the investigative drug as the target. A longer-term aim would be to include mechanisms of hypersensitivity to investigate whether safety endpoints (in particular cytokine release syndromes) could be simulated.

Conclusions and future developments

The aim of pre-clinical immunogenicity risk assessment is to estimate the likelihood of a biotherapeutic to induce an ADA response in treated patients. In this review, we gathered pre-clinical methods, platforms and strategies that we believe are currently used to interrogate the potential immunogenic liabilities of protein drug candidates. While the data generated by these assays and platform is becoming increasingly informative and valuable, the interpretation and translation into an overall immunogenicity risk of a given therapeutic protein remains challenging due to lack of assay standardization and harmonization. As of today, *in vitro*-based pre-clinical risk assessments do not (yet) enable accurate prediction of clinical immunogenicity. This is in part because most methods and assays focus on sequence-related risk factors, leaving out additional risk factors, such as MoA of the drug, route of administration, dosing regimen, and patient-related risk factors (e.g., a possible interaction with concomitant medication). The addition of approaches such as *in silico* modeling and *in vivo* models, which recapitulate most of the multiple factors pertaining to immunogenicity development, might increase the predictive power of pre-clinical risk assessments. However, validation of such outcomes through post-hoc analyses is challenging due to the use of disparate, non-standardized bioanalytical methods to report ADA occurrence.⁹⁰ While incidence is typically reported, kinetics, magnitude of response, the presence of neutralizing species and cross-reactivity to endogenous analyte or other therapeutic drug(s) should also be stated to provide a more complete picture of the immunogenic response. Recent regulatory guidance for therapeutic proteins development (FDA 2019 <https://www.fda.gov/media/119788/download>; EMA 2017 https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-immunogenicity-assessment-therapeutic-proteins-revision-1_en.pdf) and peptides (FDA 2017 <https://www.fda.gov/files/drugs/published/ANDAs-for-Certain-Highly-Purified-Synthetic-Peptide-Drug-Products-That-Refer-to-Listed-Drugs-of-rDNA-Origin-Guidance-for-Industry.pdf>) recommend that intrinsic and extrinsic risks identified during pre-clinical and clinical development

should guide the development of a robust bioanalytical and clinical strategy. An example of roadmaps for conducting and summarizing such risk-based strategies has been recently published for fusion proteins, pegylated proteins and multi-domain therapeutics.⁹¹

Selecting the most appropriate pre-clinical immunogenicity risk assessment tool(s) among the many available might be a delicate exercise, as no universal strategy currently exists (see (Table 7) for an overview of the assays discussed in this review). It may help, however, to consider first the following points to select an initial approach: 1) in which development stage is the drug candidate? and 2) what is the pharmacology of the drug candidate?

In silico approaches are the most high-throughput and cost-effective way of filtering out candidates with high sequence-associated risks, and can provide results even before the availability of the biologics of interest. They are therefore most successful during discovery and early drug design stages. Cell-based assays are amenable to relatively large sample sets (several tens of donors). However, they should be conducted with control molecules to allow suitable benchmarking and sample ranking. In cases where biotherapeutics have an immunomodulatory component, assay formats should be selected with caution so as not to generate false positive or negative results. T cell activation assays (using isolated DCs or PBMCs) in the presence of whole antigens are usually the most biologically relevant approach to assess overall immunogenicity risk. Depending on the drug development stage, the test molecule will also have different characteristics (e.g., expression cell line, formulation buffer, contaminants, post-translational modifications) and may not fully recapitulate the final drug product. Finally, due to costs and sample throughput, *in vivo* models may only be used in the selected cases where the recapitulation of the entire immune system is required for assessing immunogenicity risks in a comprehensive manner.

Further, selection of an assay (or a set of assays) should be guided by the nature of the data that will be generated by the study and its intended use in the evaluation of a potential immunogenicity risk, for example: 1) To inform a clinical safety management plan, e.g., if data suggests a moderate immunogenicity risk, then dosing and ADA monitoring strategy may be adjusted; 2) To compare or rank against similar product candidates or biosimilar molecules to select the best candidate to take forward for clinical development; and 3) To identify sequence liabilities and remove them using protein engineering techniques.

Different assays will provide different types of data that may be better suited to one objective compared to another. For example, the growing utilization of peptide proteomics (MHC-II MAPPs assays) has generated a wealth of data enabling the interrogation of peptides that are physically presented in the context of MHC class II molecules. While these are not necessarily T cell epitopes, it is a highly sensitive and robust method to investigate potential liabilities within a protein sequence or to compare antigen presentation profiles between very similar biologics, as exemplified in the investigation of therapeutic Factor VIII proteins for the treatment of hemophilia A.^{32,92} Therefore, it is reasonable to expect the MAPPs platform could also be used to profile biosimilars or to assess the effect of formulation on the immunogenic profile of a lead biotherapeutic.

Table 7. Assay overview.

Assay	Pros	Cons	Utility	Stage
<i>In silico</i> algorithms	<ul style="list-style-type: none"> High throughput Low cost 	<ul style="list-style-type: none"> Solely based on primary sequence Mainly relies on prediction of MHC binding do not account for key antigen processing, presentation and T cell recognition processes most recently developed tools typically include additional selection criterion 	<ul style="list-style-type: none"> Filter-out high-risk variants from large panels of sequences Rank clones of high homology Support humanization/deimmunization strategies 	Early – Discovery
DC maturation assays	<ul style="list-style-type: none"> Consistent responses across donors 	<ul style="list-style-type: none"> Not an assessment of antigenicity or immunogenicity 	<ul style="list-style-type: none"> Assess the effect of external factors on antigen presentation (e.g. aggregation, contaminants) and potentially on immunogenicity Assess potential interference of target-mediated biology with preclinical immunogenicity assays (e.g. anti-TNFs) 	Late – Formulation
MAPPs	<ul style="list-style-type: none"> Fairly robust assay Relies on natural antigen processing by DCs Data rich 	<ul style="list-style-type: none"> Not all MHC class II binders will be recognized by T cells or trigger an immune response Currently only well established for HLA-DR 	<ul style="list-style-type: none"> Identify ‘hot spots’ for humanization/deimmunization Assess the effect of non-sequence related changes on antigen presentation (e.g. biosimilars) 	Early – Lead optimization Intermediate – Lead selection
T cell activation assays <i>Peptide-based assays</i>	<ul style="list-style-type: none"> No protein required Assessment of T cell responses 	<ul style="list-style-type: none"> Linear peptides generated chemically (not all peptides may be generated by the cellular machinery) Varied readouts and assay set-ups used in the industry 	<ul style="list-style-type: none"> Identify ‘hot spots’ for humanization/deimmunization 	Early – Lead optimization
<i>Whole protein, PBMC assays</i>	<ul style="list-style-type: none"> Relies on natural antigen processing by DCs Assessment of T cell responses 	<ul style="list-style-type: none"> Immuno-modulating therapeutics may interfere with the readout Varied readouts and assay set-ups used in the industry 	<ul style="list-style-type: none"> Assessment of frequency of specific TCR in T cell repertoire that recognizes presented peptides of the biotherapeutic across different donors representing a targeted HLA-distribution 	Intermediate – Lead selection
<i>Whole protein, DC:T cell assays</i>	<ul style="list-style-type: none"> Relies on natural antigen processing by DCs Assessment of T cell responses 	<ul style="list-style-type: none"> Immuno-modulating therapeutics may interfere with the readout Varied readouts and assay set-ups used in the industry 	<ul style="list-style-type: none"> Assessment of the uptake, processing, and presentation of the biotherapeutic, followed by assessment of frequency of specific TCR in T cell repertoire that recognizes presented peptides of the biotherapeutic across different donors representing a targeted HLA-distribution 	Intermediate – Lead selection
B cell assays	<ul style="list-style-type: none"> Only assay that detects individual antibody-secreting B cells 	<ul style="list-style-type: none"> Currently limited to memory B cell responses Immuno-modulating therapeutics may interfere with the readout Controls need to recall a response across the donor panel 	<ul style="list-style-type: none"> Assessment of preexisting/cross-reactive antibodies to the biotherapeutic Identification of B clones in patients producing ADA 	Intermediate – Lead selection Post-hoc analysis
<i>In vivo models</i>	<ul style="list-style-type: none"> To date, only assay involving all steps of immune response required to culminate on ADA formation 	<ul style="list-style-type: none"> Generation of HIS mice is generally costly and laborious, preventing its broad application Transgenic systems generally are restricted to a specific protein, including single human HLA It does not inform on immunogenic human T (and B) cell epitopes 	<ul style="list-style-type: none"> Assessment of product-related factors, including post-translation modifications, formulation and mode-of-action of biotherapeutics 	Intermediate – Lead selection Late – Formulation

Colors of each section matches the scheme used in [Figure 1](#).

Reported ADA data from clinical studies represent an important source of information in understanding the potential for pre-clinical assessment to predict immunogenicity risk of biologics. Reverse translational analysis of clinical data enables a more detailed analysis of a biotherapeutic’s features informing whether an ADA response might be expected and how it might occur. For example, a number of studies have validated the use of MAPPs assays as a method to identify native T cell epitopes correlating

with a positive CD4 T cell response in ADA positive patients.³³ A systematic comparative analysis may lead to the identification of reactive epitopes that can be subsequently modified to dampen the initial T cell induction, therefore reducing the overall immunogenicity risk.³² However, despite the many putative T cell epitopes that can be characterized in a MAPPs assay, recognition by T cells remains highly patient-dependent and may hinder the identification of the one dominant epitope inducing ADA production.⁹³

Table 8. Recommendations for harmonization or standardization of human *in vitro* cellular assays^a.

Assay	Topic	Recommendations	References
DC maturation	Donors	Minimum of 10	
	Controls	<ul style="list-style-type: none"> Negative control: medium positive control: KLH, LPS titration, or maturation cocktail Benchmark therapeutics: Bevacizumab (Low), ATR-107 (medium-high) 	22
QC	QC	SI ^b ≥ 2 for one or more endpoints using a positive control	
	Readouts	One or more of the following: <ul style="list-style-type: none"> Cell surface markers (4–48 h after stimulation): CD83, CD80, CD86 and CD40 Cytokines (24–48 h after stimulation measured in cell culture supernatant): IL-1β, IL-6, IL-8, IL-10, IL-12 and TNF-α Signaling pathway/phosphorylation (15–30 min after stimulation): pAkt, pERK1/2 and pSyk mRNA upregulation (6–24 h after stimulation): cytokines, chemokines All readouts (except surface marker) should report the % of donors showing a SI ≥ 1.5 and ≥ 2.0; for cell surface markers, activation is typically reported as MFI values (% increase per marker for the test condition compared to the negative control) and % of donors showing an increase of 50 or 100% per marker.	19–21
DC MAPPs	Donors	Minimum of 10 donors for early sequence assessment to 15–30 genotyped donors for risk assessment to account for HLA diversity.	
	Controls	<ul style="list-style-type: none"> negative control: medium positive control: KLH, tracer proteins Benchmark therapeutics: Infliximab, Rituximab 	27
	QC	<ul style="list-style-type: none"> Quality control (MS performance): detection of 1000 different endogenous proteins represented by 5000 different MHC-II peptides per sample for HLA-DR or pan Class II pull-down cell-associated proteins (reference value for 5 × 10⁶ moDCs/sample); Quality control (sample quality): detection of consistent sets of MHC-II peptides for selected proteins (e.g. serum albumin, lysosome-associated membrane glycoproteins, cathepsins, KLH, test protein, etc.) per donor. 	27
	Readouts	<ul style="list-style-type: none"> Aggregated amino acid sequences of the observed peptides, with the core HLA binding sequence highlighted for the relevant HLA type (if known). Number of peptides (and possibly relative abundance) for the observed cluster 	26,27
T cell functional analyses	Donors	10–25 donors for early sequence selection to 50 donors for risk assessment to account for HLA diversity. HLA typing (4 digits) is highly recommended to facilitate population level representation	
	Controls	<ul style="list-style-type: none"> Negative control: medium positive control: KLH Benchmark therapeutics: Bevacizumab (Low), Bococizumab/ATR-107 (High) Benchmark peptides: PADRE or CEF/CEFA/CEFT pool 	
	QC	<ul style="list-style-type: none"> Post-thaw viability (PBMC preparations) ≥ 85% polyclonal and/or antigen-specific T cell activation SI ≥ 2 baseline measurement of each preparations to set sample-specific cut-point 	56–58
	Readouts	Please see Table 5 for a detailed list of possible readouts depending on assay type <ul style="list-style-type: none"> T cell proliferation assay: fluorescent markers (e.g. CFSE-based assay); DNA proliferation markers; Ki67 staining T cell activation assay: ELISPOT or Fluorospot-based multiplex assays to profile cytokines (Th subtypes-specific); T cell activation markers (e.g. CD154) Due to the large variety of assays and possible outputs, please provide a comprehensive summary of the experimental procedure and measurements and describe the statistical models used to report results for better inter-laboratory comparability	40,45,48–51
B cell	Donors	A minimum of 30–50 donors should be used to cover a reasonable population and can be selected for broad HLA Class II allele coverage.	
	Controls	<ul style="list-style-type: none"> negative control: Human protein (e.g. albumin) or other naïve proteins positive control: Antigen from a common immunization program such as tetanus toxoid or common viral antigens that the general population are frequently exposed to (e.g. Influenza-derived protein antigens) 	60
	QC	Not defined at this point of time; in this paper, we suggest <ul style="list-style-type: none"> Cell number and viability after polyclonal activation Use an anti-Fc antibody to capture the total number IgG-secreting B cells per million PBMC Measure the number of antigen-specific B cells per million PBMC and the % of antigen-specific B cells in the total IgG-secreting B cell repertoire (frequency of donor responses) 	
	Readouts	B cell ELISpot/FluoroSpot	60,61

^athe present table summarizes at high level the main characteristics and outputs that can be expected from the listed *in vitro* assays. By design, this table cannot be exhaustive, and researchers are expected to derive their own list of parameters and QC conditions depending on the context and the experimental readouts.

^bSI, Stimulation Index; a stimulation index (SI) is calculated relative to the concurrent vehicle control. By definition the SI of the control (vehicle) group is set to 1; an SI of 2 means a doubling of the values measured for the controls.

Efforts are ongoing to streamline the analytical process and to identify parameters predicting high-risk sequences with better confidence.^{94,95} In another example, Walsh *et al.* reported a post-hoc analysis of three clinically immunogenic antibodies that differ by the mechanism of T cell activation, and demonstrated that a biotherapeutics' intrinsic properties influence immunogenicity risk.⁹⁶ Interestingly, the authors mention that a T cell proliferation assay performed with PBMCs devoid of CD8 T cells provided the most predictive immunogenicity risk assessment if the results were correlated to the data collected in the Phase 1 clinical trial, although the assay data in isolation was not sufficient to explain all the immunogenic events.

The inherent complexity of some of the assays described in this review makes it challenging to harmonize and standardize; in Table 8, we attempt to summarize, for all *in vitro* assays, what we believe to be a minimal set of conditions and controls to better understand the nature of the generated data and to facilitate its replication if needed. For example, T cell activation assays have been developed and used by many different laboratories for over 20 years without common directives on how to perform such assays and report data. In light of an increasing reliance on these assays, we believe that standardization and definition of common standardized platforms that enable cross-validation and data comparability would increase our confidence in conducting pre-

clinical risk assessments. Concomitantly, minimal quality requirements for cells, samples, and standards need to be established. The ability of T cells to be activated and DC to mature in the presence of known stimuli should be monitored systematically for every donor where practical, and when changes are applied to the process (e.g., when using a new source of blood cells). Universal controls and benchmarks from centralized sources should ideally be used by all laboratories, with stringent acceptance criteria for purity. Controls and benchmarks should also be routinely batch-tested, bridging studies conducted, and trend analysis performed to ensure consistent assay performance. Most laboratories use bevacizumab as a low immunogenicity benchmark in T cell-based assays, which has the advantage of being commercially available in a Good Manufacturing Practices (GMP)-quality grade. In contrast, a collaborative effort is most likely needed to identify a suitable universal 'high immunogenicity' benchmark for T cell activation assays. With the support of drug developers, a set of biologics with no direct immunomodulatory activity but known clinical immunogenicity, either marketed, under development, or withdrawn from development could be identified, manufactured to agreed specifications, and tested in multiple assay formats at various locations. This may lead to the election of an approved benchmark, which can then be manufactured to GMP grade and made commercially available to all for general use.

In summary, we foresee that significant improvement and refinement of pre-clinical immunogenicity assessment tools will come through common agreements across drug makers on assay QC acceptance criteria (despite possible hurdles such as confidentiality, legal, and commercial considerations), as well as from learnings from reverse translation of data collected on compounds found to be immunogenic in the clinic.

Abbreviations

Ab	antibody
ADA	anti-drug antibody
APC	antigen presenting cells
DAMP/PAMP	damage/pathogen-associated molecular patterns
DC	dendritic cell
ELISPOT	enzyme-linked immune absorbent spot
GMP	Good Manufacturing Practices
HIS	human immune system
HLA	human leukocyte antigen
Ig	immunoglobulin
KLH	keyhole limpet hemocyanin
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAPPs	MHC-II associated peptide proteomics
MHC	major histocompatibility complex
MoA	mode of action
moDC	monocyte-derived dendritic cell
PBMC	peripheral blood mononuclear cell
PVDF	polyvinylidene fluoride
QC	quality control
TCR	T cell receptor

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