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Integrated immunogenicity risk assessment of therapeutic antibodies: validation and characterization of preclinical assays

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Abbreviations

ADA	*Anti-drug antibody*
ADCC	*Antibody-dependent cellular cytotoxicity*
APC:	*Antigen presenting cells*
ARIA	*Acid-dissociation radioimmunoassay*
BCR	*B cell receptor*
CD	*Cluster differentiation*
CDR	*Complementarity-determining region*
CFSE	*Carboxyfluorescein succinimidyl ester*
CH:	*Constant heavy*
CL	*Constant light*
CLIP	*Class II-associated invariant chain peptide*
CLS	*Clinical lead selection*
CRS	*Cytokine release syndrome*
DAMPs	*Damage-associated molecular patterns*
DC	*Dendritic cells*
ECL	*Electrochemiluminescence*
ECM	*Extracellular matrix*
ELISA	*Enzyme-linked immunosorbent assay*
Fab	*Fragment antigen binding*
Fc	*Fragment crystallizable*
FcγR	*Fragment crystallizable γ receptor*
FDA	*Food and drug administration*
FDC	*Follicular dendritic cell*
GC	*Germinal center*
GM-CSF	*Granulocyte macrophage colony- stimulating factor*
HC	*Heavy chain*

HLA	*Human leukocyte antigen*
iDC	*Immature dendritic cell*
IEDB	*Immune epitope database*
IFN	*Interferon*
Ig	*Immunoglobulin*
IL	*Interleukin*
IND	*Investigational new drug application*
LC	*Light chain*
LN	*Lymph node*
LPS	*Lipopolysaccharide*
MAPPs	*MHC-II associated peptide proteomics*
MHC	*Major histocompatibility complex*
moDC	*Monocyte-derived dendritic cell*
NAb	*Neutralizing antibody*
NK:	*Natural killer*
PAMPs	*Pathogen associated molecular pattern*
PBMCs	*Peripheral blood monocytic cells*
PIA	*pH-shift anti-idiotype antigen binding assay*
pMHC-II	*peptide-MHC-II*
PRRs	*Pattern recognition receptors*
Rh	*Recombinant human*
TCR	*T cell receptor*
Th	*T helper*
TLR	*Toll-like receptors*
TNF-α	*Tumor necrosis factor-α*
Treg	*T regulatory*
TRIA	*Temperature-shift radioimmunoassay*
WBA	*Whole blood assay*

1. Introduction

1.1. Generalities on antibodies

The theory of humoral immunity was proposed for the first time in 1890, when Emil von Behring and Kitasato Shibasaburō observed that the serum could react to an antigen (a term used to describe molecules or parts of molecules recognized by a specific antibody (Ab) or T cell receptor (TCR)). A year later, the term Ab was first used by Paul Ehrlich in 1891, opening the field of humoral immunity research. The interaction between an Ab and its antigen was described in 1897 as a “lock and key” interaction, and their presence as soluble entities was proposed in 1904 (Lindenmann, 1984). In the early 20th century, these theories were confirmed, and B cells were identified as responsible for the generation of Abs. Subsequent work has refined our understanding of the structure and function of Abs.

1.1.1. Structure of natural Abs

Abs or immunoglobulins (Ig) are a product of adaptive immunity in humans and most mammals, more specifically humoral immunity. They are one of the main defense mechanisms against extracellular pathogens and mediate neutralization and subsequent elimination. Abs are Y-shaped proteins composed of four distinct peptide chains: two heavy chains (HC) and two light chains (LC). Each of the chains has a variable domain at their N-terminus responsible for their specificity and binding. Abs come in five main classes, or isotypes - IgG, IgA, IgM, IgD, and IgE depicted in **figure 1** (Duarte, 2016). The Ab isotype is defined by the sequence of the constant region of the heavy chain, which affects its structure and function.

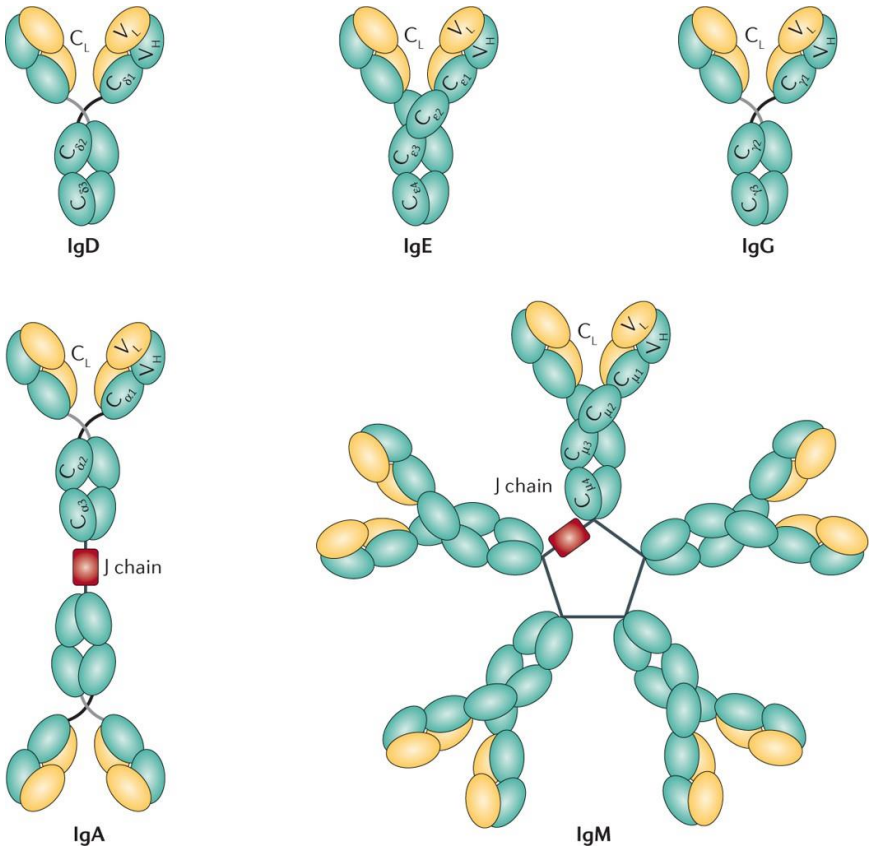

Figure 1. Schematic representation of the different immunoglobulin (Ig) isotypes. IgD, IgE and IgG are monomeric whereas IgA (dimer) and IgM (pentamer) are multimeric (Duarte, 2016).

IgG, IgD and IgE are tetrameric proteins (resulting from the assembly of four chains), but are considered as a monomeric Ig, whereas IgA is a dimer of Igs linked by a joining chain. This characteristic protects against proteases in an adaptation to mucosal immunity, including against microbes in the gut. IgM is a pentamer of Ig and has therefore 10 binding sites, accounting for its increased binding capacity also referred to as increased avidity. Even though monomeric Ig isotypes share the same format, they have specific functions. As an example, IgE are mainly involved in allergy whereas IgG are more polyvalent and the main actors of the humoral response. The Fragment crystallisable (Fc) region of the Ab is critical, in part, because it can bind Fc receptors, which are expressed by most of the immune cells contributing to the protective functions of the immune system. The Ab variable domains or Fragment antigen binding (Fab), formed by the junction between the LC and HC contains three

hypervariable regions called Complementarity Determining Regions (CDR). These domains are the main source of the diversity of Ab binding specificities. From a structural point of view, they constitute the loops facing the outside of the Ab and linking the β-strands of the variable domains. The three CDRs of the HC and the three others from the LC, facing the same direction form the antigen binding site also called idiotype (figure 2).

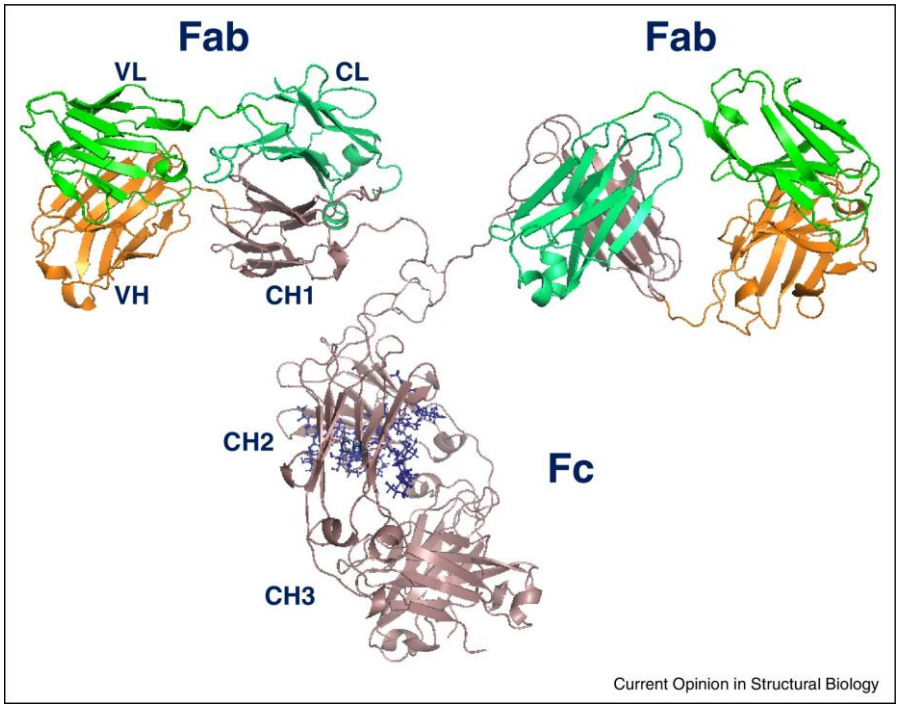

Figure 2. Structural representation of an IgG1 Ab. Variable domains of the LC and HC are respectively colored in orange and green. Together with their constant parts (Constant Light, CL and Constant Heavy, CH1) they form the Fragment Antigen Binding (Fab). The two additional HC constant domains CH2 and CH3 form the Fragment Crystallisable (Fc) portion of the Ab (Chiu & Gilliland, 2016).

1.1.2. Functions of natural of Abs

Abs recognize, as antigens, specific amino acid sequences on many pathogens (such as bacteria, viruses, or toxins), known as epitopes. Such specific Abs can neutralize these pathogens by blocking their interactions with host cells, and by marking the pathogens for Fc-mediated uptake by phagocytes - a process known as opsonization. Viruses are one of the

threats of the immune system, therefore it is also armed to detect and kill infected cells. Indeed, Abs are responsible for sensing and binding to infected cells mostly by detecting the virus spike protein. When bound to an infected cell surface, the Ab mediates the killing of the cell by activating an immune response. There are two types of response to this signal, either by activating the Ab-Dependent Cellular Toxicity (ADCC) mediated by Natural Killer (NK) cells or by activating the complement system.

The last function of Abs is the direct neutralization of viruses and toxins, blocking their normal function. These Ab functions are depicted in the **figure 3** below.

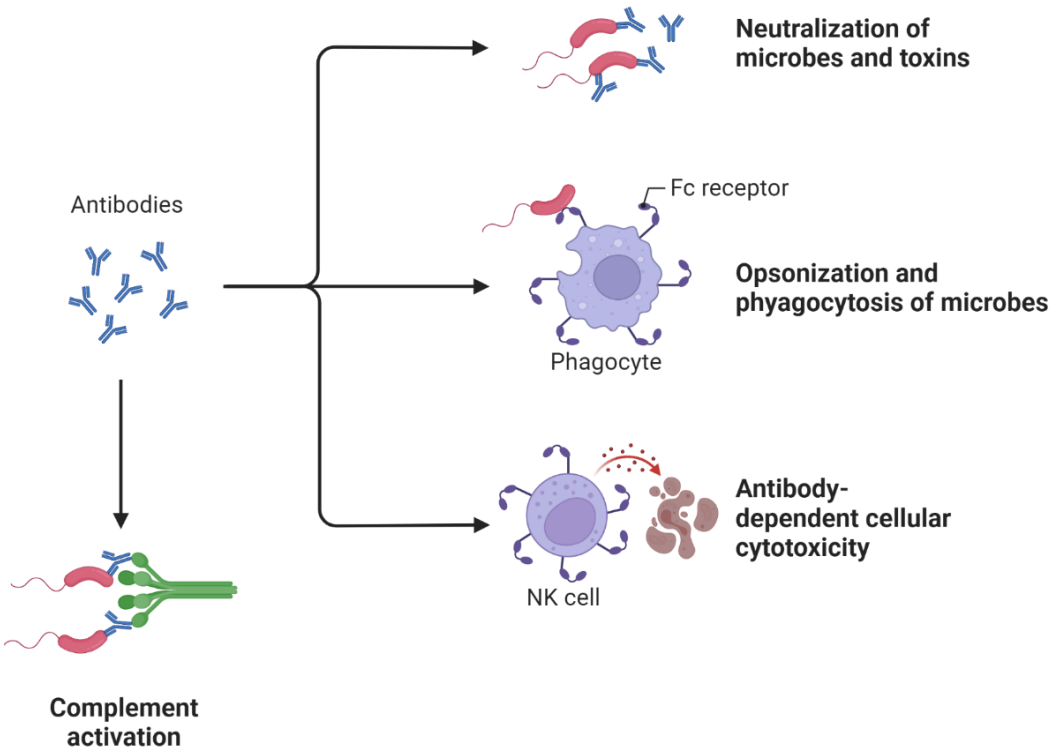

Figure 3. Schematic representation of the Ab functions. Depending on their target antigen, its location (circulation, cell surface bound…) and the Ab isotype the exerted function is different. Created with BioRender.com.

1.1.3. Emergence of the therapeutic Abs

In light of the high specificity and broad diversity of Abs, they have been used in a variety of medical applications. These include research, diagnosis and treatment of disease. Such therapeutic Abs are the focus of this thesis.

The first therapeutic antibody approved by the U.S Food and Drug Administration (FDA) was Muromonab-CD3 in 1986, and the market has only expanded since then, as of today, it encompasses 153 approved therapies (Lyu et al., 2022), with a 186 billion dollar market that is forecast to only grow more over the next ten years. This represents an increase in research and development, leading to a larger variety of targets and Ab formats. The historical IgG format, Ab-drug conjugate, bispecific or Ab fragments are examples of these new formats giving rise to new challenges in terms of production and safety assessment. Approved therapeutic Abs have 91 different targets and are used in as many indications, including many types of cancer and autoimmune/inflammatory conditions, increasing further the complexity of their development. Cancer immunotherapy and ophthalmology have been an historical focus of Roche's portfolio, with 8 and 2 approved Abs respectively. Most of the therapeutic Abs discussed are described in table 1 (Material and Methods section).

1.2. Immunogenicity and its consequences

FDA defines immunogenicity as the propensity of a therapeutic protein product to generate immune responses to itself and to related proteins (www.FDA.gov). As stated, this is a phenomenon observed for therapeutic proteins in general, including therapeutic Abs. The Abs generated by this particular immune response are called Anti-Drug Abs (ADA) and are the main hallmark of immunogenicity. Several factors influence the development of ADA upon treatment with therapeutic Abs. These factors can be categorized into product, patient and treatment-related risks and will be developed in the subsequent section.

1.2.1. Immunogenicity factors in clinical studies

1.2.1.1. Therapeutic Abs properties influencing immunogenicity

It is well accepted that many intrinsic properties of therapeutic Abs can affect their propensity to induce immunogenicity. The amino acid sequence of the therapeutic Abs itself can contain T cell epitopes, which can be presented by APCs and subsequently induce a CD4+ T cell or T helper cell response (these mechanisms will be detailed in the “Epitope presentation and DC-T cell interaction” section), which facilitates a B cell Ab response.

The amino acid sequence of Abs is a crucial property, as it defines its specificity and affinity. With the increased flexibility of the cDNA platform allowing rapid cloning of the Ab variable region of interest and transduction in the producing cell line, it has become possible to design potentially an infinite diversity of Abs, deviating from self-sequences. Lymphocytes are selected to be tolerant to most of naturally occurring amino acid sequences, mainly by central tolerance (detailed in a later section: “The adaptive immune response leading to immunogenicity”). When the sequence deviates and contains a CD4+ T cell epitope, lymphocytes would be thereof armed to respond in consequence. The CD4+ T cell epitope content within the therapeutic Ab sequence is probably the main product related risk factor for immunogenicity. However, with new therapeutic Ab formats, especially the ones modulating T cell activity, new mechanisms arose, breaking the tolerance to previously tolerated sequences (Egli 2022) and creating new challenges in the field of immunogenicity.

Product-related risks also include the critical quality attributes generally associated with the production of Abs (post-translational modifications, aggregates, impurities). Therapeutic Abs have an asparagine consensus sequence for N-glycosylation at the position Asn297 in the heavy chain of the CH2 domain. Alteration of glycan compositions and structures can cause conformational changes of the Fc domain, which could change binding affinity to Fcγ receptors (FcγR), resulting in changes of immune effector functions and potentially recycling properties where FcγR may be involved (Liu, 2015). They are influenced by the culture medium and the

organism used to produce the Ab. Glycosylation of foreign origin (resulting from the Ab production in a foreign system) being different, it can resemble sequences from pathogens and be targeted by the pre-existing ADA. The presence or absence of certain types of oligosaccharides are directly linked to increased ADCC (e.g., fucose) or immunogenicity (Jefferis, 2009).

Impurities is a broad term summarizing the unwanted content of the formulation; Ab fragments, host cell proteins or even high molecular weight species can be considered as such. Impurities often derive from the Abs production process and properties and can be present in the final formulation, thus helping to initiate an immune response. Ab fragments and high molecular weight species are defined as partial or degraded Abs and mispairing of different Ab chains, respectively. Host cell proteins are process-related impurities that are generated by the host organism, which might still be found after Ab purification and considered as foreign by the immune system. This foreign component administered together with the therapeutic Ab can enhance the risk of initiating a response to the Ab. Protein aggregation is mostly a consequence of poor physico-chemical properties of Abs, such as hydrophobicity or charge distribution. Components of the formulation can also have an impact on the probability of aggregates formation hence having an impact on immunogenicity as well (Dingman & Balu-Iyer, 2018).

1.2.1.2. Disease and patient related factors linked to immunogenicity risk

Patients with autoimmune disease such as systemic lupus erythematosus may present a higher risk of immunogenicity, due to a heightened autoreactive immune system (Stebbins et al., 2019). However, this aspect is hardly covered pre-clinically (healthy human donors used as the cell source) which is more relevant to this manuscript, but research is ongoing. An important patient-related characteristic is the Human Leukocyte Antigen (HLA) genotype encoding for a cell-surface protein responsible for the communication between T cells and APCs, the Major Histocompatibility Complex (MHC). In short, there are two classes of MHC, named class-I and class-II, the first being associated with the presentation of intracellular

pathogen derived antigen and expressed on the surface of all nucleated cells. The second is mainly expressed on the surface of APCs and initiates an adaptive immune response following the internalization of an extracellular pathogen. In humans, there are two types of antigens presenting molecules, the MHC-I and MHC-II, encoded by the Human Leukocyte Antigen (HLA) gene complex. Three HLAs correspond to MHC-I (A, B and C) and three for MHC-II (DR, DP, DQ), which are further associated with two numbers giving the genotype of the two chains, which compose the MHC-II.

There is no general correlation between the expressed HLA and immunogenicity, but some studies note an increased risk for immunogenicity linked to HLA expression that is therapeutic Ab specific.

The HLA-DQA1*05 allele, carried by approximately 40% of Europeans, significantly increased the rate of immunogenicity against anti-Tumor Necrosis Factor (TNF) agents, especially adalimumab (Lagassé et al., 2021; McMaster et al., 2021; Sazonovs et al., 2020). This finding is supported by a genome-wide association study of 1240 Crohn's disease patients treated with Infliximab or adalimumab, while identifying the HLA-DRB1*03 allele as well. HLA-DQB1*05, HLA-DRB1*01, and HLA-DRB1*07, however, are associated with a lower risk of developing ADAs to Adalimumab (Lagassé et al., 2021). In rheumatoid arthritis, other alleles have been associated with an increased risk for ADA onset following anti-TNF treatment (Benucci et al., 2018). It highlights the importance of the interplay between the disease status of the patient and their genetic background. The latter study, however, used limited patient cohorts, limiting the significance of such genetic correlations. Other studies are looking at the dependency of the ADA response to the HLA genotype of the patients. DQB1*06:02 and DRB1*15:01 are associated with the development of ADAs against Factor VIII, associated with adverse events (Diego et al., 2020). HLA-DR4, in broader context, is known to be more susceptible to autoimmunity, and could be considered in the case of immunogenicity as well (Quarmby et al., 2018). There are potentially other patient related factors influencing immunogenicity, such as ethnicity (Lagassé et al., 2021).

These associations point toward the need for personalized treatment for therapeutic Abs but also the need to take this factor into consideration while testing healthy donors pre-clinically or in early clinical trials.

1.2.1.3. Clinical regimen and consequences on immunogenicity

The clinical regimen encompasses the duration of the treatment, the number of doses, and the dosage of the administered Ab as well as the route of administration. The dosing is an important aspect of the clinical regimen, but it is difficult to assess its direct effect on immunogenicity. The dose and the schedule of administration is specific to the treatment and is determined in early clinical trials, with the aim to find the efficacious dosing without inducing side effects. There are two main routes of administration for Abs, subcutaneous (under the skin) and intravenous (directly into the bloodstream). The subcutaneous administration is gaining popularity as it decreases the cost and allows self-administration (no hospitalization needed). However, subcutaneous administration of proteins larger than 16 kDa do not readily diffuse into the bloodstream, and therefore rely on lymphatic uptake (Jarvi & Balu-Iyer, 2021). A recent innovation in subcutaneous administration is the co-administration of hyaluronidase to partially degrade the extracellular matrix, greater volume to be administered, therefore more Ab to enter the circulation. This could lead to a danger signal capable of initiating APC maturation and the initiation of an immune response (Crommelin et al., 2019a).

Intravenous administration allows a quicker distribution of the therapeutic Ab. This route is not exempt from adverse events. Intravenous administration can be associated with infusion reactions and be accompanied by symptoms related to hypersensitivity (could also be the case for subcutaneous administration). In the case of therapeutic Abs, it can give rise to a Cytokine Release Syndrome (CRS), which can be caused by the complement but also following an IgE response (Asselin, 2016). The route of administration would affect which cell types the Ab interacts first. An Ab delivered intravenously has a higher chance to be processed by spleen resident cells or macrophages from the marginal zone. However, skin resident cells (Langerhans cells, skin resident APCs) would be the first in contact with the drug and are the

principal initiators of the immunogenic response (Jarvi & Balu-Iyer, 2021) in case of a subcutaneous administration. Although this might increase the risk of immunogenicity, studies are sparse and no firm data exists.

Another confounding factor is co-medication, either included in the use of a therapeutic Ab or as part of standard of care for the condition. Many Abs are co-administered with medications intended to reduce potential side effects, as demonstrated by the use of antihistamines upon administration of rituximab (Pettitt et al., 2012; Richardson et al., 2015). Other examples are described in the literature and are beneficial for the patients (e.g., methotrexate, betamethasone) (Krickaert, 2012; Ragnhammar, 1994). There is no clear link between co-treatment and an increased risk for immunogenicity but it poses challenges when it comes to reporting of immunogenicity related side effects, incorporating biases into the evaluation of its risk.

1.2.2. Consequences of immunogenicity related side effects

Immunogenicity of Abs can affect the efficacy of the treatment and the patient's safety. In addition to a cellular response referred to as CRS (often linked to the therapeutic Ab mode of action), an unrelated humoral response is generating ADA. Both effects will be discussed independently. ADA are classical Abs, most of the time of the IgG subtype, and originate from a standard adaptive immune response against an antigen.

1.2.2.1. Consequences of immunogenicity on the treatment efficacy

One of the functions of an Ab is to neutralize its target and abrogate biological activity (refer to “Functions of natural Abs” section). ADAs, therefore, can block the binding of therapeutic Abs to their target, limiting efficacy. Such Abs are referred to as neutralizing Ab (NAb). Such Abs are referred to as neutralizing Ab (NAb) (Peng et al., 2021). This is an important aspect as many therapies are facing this challenge, which translates into a loss of efficacy for the treatment. The neutralizing function of the ADA due to its idiotype does not prevent it from exerting other Ab functions. When bound to the therapeutic Abs and already blocking its

activity it can also improve its clearance either by phagocytes or other surrounding cells (Groell et al., 2018). The increased clearance of the therapeutic Abs is one of the consequences of immunogenicity and is directly observable by measuring the concentration of circulating therapeutic Abs in the serum. As an example, it has been shown that the presence of ADAs to adalimumab or infliximab, two therapeutic Abs targeting TNF-α, was associated with lower serum drug concentrations and reduced efficacy (Jiang et al., 2021).

ADA are not necessarily directed against the idiotype of therapeutic Abs, and therefore do not directly neutralize their effect. However, as with any antigen bound by Ab, drugs bound by ADA can be internalized by phagocytes and cleared from circulation. This leads to a rapid clearance of the Abs, loss of exposure, and ultimately a loss in efficacy. In some cases the therapeutic Abs itself, by its target or mode of action, can form such immune complexes and favor its own internalization into phagocytes. A comparison between adalimumab/infliximab, that form large complexes with trimeric TNF-α (their target), and etanercept which only target monomers of TNF-α (therefore does not form large complexes) has proven that the large complexes formed by adalimumab or infliximab were more prone to induce an immunogenic response (Kroenke et al., 2021). Bococizumab, an anti-PCSK9 Ab, is a well-known example of clinical trial termination linked to ADA generation in patients and to non-target related clearance. 44 % of the patients treated produced ADA, which resulted in a decrease in efficacy over time (Ridker et al., 2017).

1.2.2.2. Consequences of immunogenicity on patient safety

The induction of an immune response can have consequences on an organism. Although it is a mechanism meant to protect us, an exaggerated immune response or an immune response directed against itself, can have an enormous impact on our health.

Independent of immune complex formation, the binding of IgG or IgM would be recognized by innate immune cells and lead to ADCC, killing the target cell and enhancing the immune response by the release of danger signal (Dingman & Balu-Iyer, 2018). Upon binding of the therapeutic Abs to its target, it can activate bystander cells leading to a cytokine storm, called

CRS, having major clinical consequences. This phenomenon is a major hurdle in the development of T cell engagers for cancer immunotherapies. Indeed, the T cell activation leads to the production of cytokines which are activating surrounding innate immune cells in a large chain reaction (Shimabukuro-Vornhagen et al., 2018). These can go from flu-like illness to life threatening cardiovascular, pulmonary and renal involvement (Shimabukuro-Vornhagen et al., 2018). Several Abs have been withdrawn from early clinical phases and even from the market due to CRS. Campath (alemtuzumab) and TGN-1412 (CD28 superagonist) are probably the most known examples of Abs therapies terminated following CRS findings in patients.

The anti-IL-21R Ab, ATR-107, was intended to suppress lymphoid cell proliferation and B cell differentiation. Despite this, ADAs occur in 76% of tested healthy donors and lead to the withdrawal of ATR-107 from clinical studies (Hua et al., 2014). It is then important to understand the role of other immune cell types to this immunogenic response and the factors influencing such immune response.

Pre-existing Abs can also impact patient safety as it is the case for pre-existing IgE to galactose-α-1,3-galactose which were found to be the cause of hypersensitivity reactions to cetuximab, an EGF receptor targeting Ab (Zeunik et al., 2022). Indeed, a strong immune response can be IgE mediated and leads to type I hypersensitivity reactions and a rapid release of histamine as it would happen in an allergic response. Bound IgEs would then be recognized by mast cells, which are tissue resident cells involved in inflammation.

There are many important considerations around immunogenicity, as described here, the impact on the treatment efficacy but also on the patient safety. Nonetheless, it is important to note that these limitations also affect the immediate cost of Abs treatment. A decrease in efficacy implies an increased dose to reach the desired effect and ultimately increases the cost of the therapy. Patients having immunogenicity related side effects in early clinical trials may stop the development of the therapeutic Abs. However, the development of such

molecules is costly and has, in some way, to be embedded in the cost of Abs reaching the market.

Given the consequences of immunogenicity on both patient's safety and treatment efficacy, it is of utmost importance to understand the mechanisms on which it relies.

1.2.3. Reporting of immunogenicity related side effects

Moreover, a rational risk assessment for immunogenicity is required for the original investigational new drug application (IND) by regulatory agencies. The selected assays have to be developed in parallel with the therapeutic Ab because it is highly dependent on the product. Four examples of immunoassays used for the detection of ADA are represented in **Figure 4** (Suh et al., 2022).

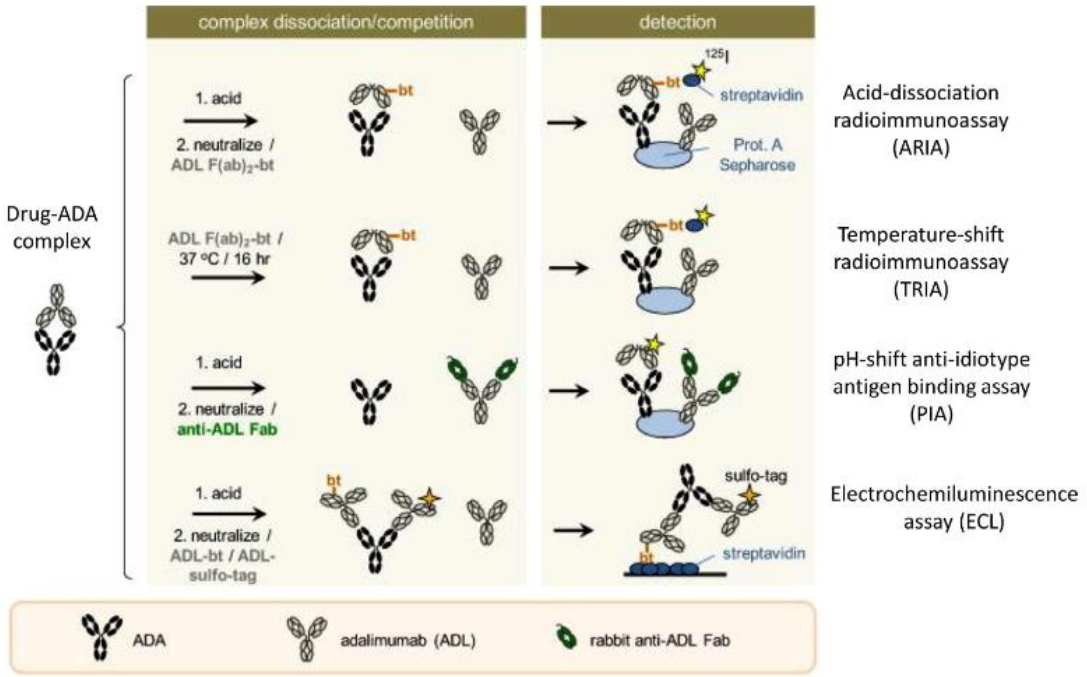

Figure 4. Examples of immunoassays used in the detection of ADA. Adalimumab has been used as an example to illustrate the process. ADL, adalimumab; bt, biotin; prot. A, protein A (Suh et al., 2022).

The first step is the dissociation of the ADA-Abs complex in order to quantify the ADAs. The dissociation is achieved either by a temperature shift (Temperature-shift radioimmunoassay,

TRIA) or by an acidic dissociation (Acid-dissociation radioimmunoassay, ARIA, pH-shift anti-idiotype antigen binding assay, PIA, Electrochemiluminescence, ECL). Subsequently, the interaction is blocked using a biotinylated Fab fragment (ARIA, TRIA), the full Ab (ECL), or by blocking further anti-idiotypic binding on the therapeutic Abs (PIA). Then, both the ADA and the therapeutic Abs are adsorbed on protein A through the Fc fragment. All these methods rely on similar but different readouts and achieve a limit of detection between 5 and 64 ng/mL. With the reduced use of radioactivity and the overall limitation of these assays in terms of sensitivity, they were gradually replaced by Enzyme-Linked ImmunoSorbent Assays (ELISA). Indeed, it can be adapted to the detection and quantification of ADA and has several advantages, including their high sensitivity, low cost, ease-of-use, and relatively high throughput **(Figure 5)**.

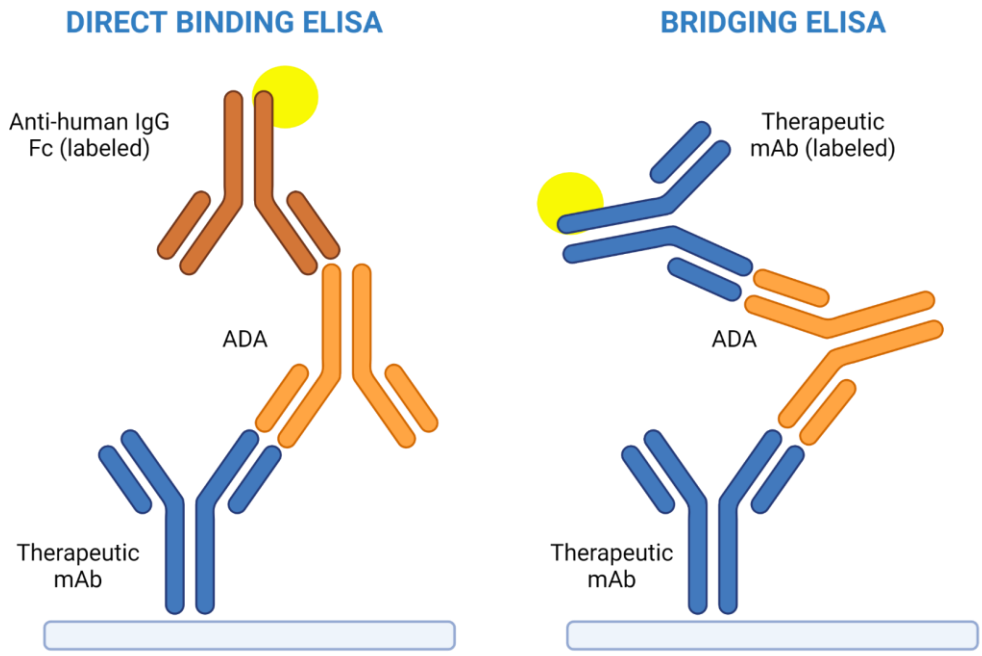

Figure 5. Comparison of two types of ELISA. The bridging ELISA has been optimized for the detection of patient serum ADA, using the labeled therapeutic Ab for detection to increase the specificity of the assay. Created with BioRender.com.

When using the acidic dissociation, the ELISA achieves a 500 ng/mL limit of detection. The ELISA has been optimized for the detection of ADA in patient serum samples and is nowadays

the most common titration method for ADA. This optimized method is called bridging ELISA as it uses twice the therapeutic Ab, for coating and for detection, thus increasing the specificity of the assay and lowering the limit of detection (down to 0.4 ng/mL).

There are additional steps that could be conducted to increase the tolerance to high Ab concentration, starting with the saturation using the Ab therapeutic to form immune complexes followed by an immuno-precipitation with polyethylene glycol before doing the acidic dissociation. Other assays have been used for some particular cases (e.g., therapeutic Ab formats not suited for the aforementioned methods, specific therapeutic protein activity…) like capillary electrophoresis, gene reporter assay, surface plasmon resonance, liquid chromatography-mass spectrometry (Suh et al., 2022).

The source or reference for clinical immunogenicity information (e.g., proportion of ADA positive patients, detection method, clinical symptoms…) is the label of the therapeutic Ab, for which a summary has recently been published (Lagassé et al., 2021). When it comes to the reporting of immunogenicity adverse effects, only 57 % of the Abs approved before 2012 have seen their label being updated when it comes to information related to unwanted immunogenicity (proportion of ADA positive patients, clinical consequences…) with subsequent studies potentially using more sensitive ADA detection methods (Borrega et al., 2019). A direct comparison of immunogenicity rates between therapeutic Abs is generally discouraged. This is because reported incidence of ADA induction may be influenced by many aspects (assay methodology, sample handling, timing of sample collection, concomitant medications, and treated disease) (Gorovits et al., 2020). Additionally, it has been hypothesized that the titer and persistence of ADA better correlate with clinical consequences than incidences (B. A. Cohen & Rivera, 2010). The latter are important to characterize the risk even though only 20.3% of the labels for therapeutic Abs described the association between ADA/NAb incidence and pharmacokinetics/safety/efficacy (Hassanein et al., 2020).

The data currently available for the accurate estimation is limiting and consequently the comparison of the risk of immunogenicity of biotherapeutics in patients. This limitation also affects the potential back translation into pre-clinical development of therapeutic Abs.

Understanding the factors influencing immunogenicity of Ab therapies in light of the consequences of such an immune response is of utmost importance. However, it still corresponds to a normal adaptive immune response, with some critical aspects linked to immunogenicity.

1.3. The adaptive immune response leading to Immunogenicity

The immune system is constantly preventing microbial infections, but also responds when infections do occur. The immune system is divided in two interconnected branches, the innate and the adaptive immunity. The first being an immediate response mostly against bacteria and extracellular pathogens and the second, being delayed and virtually able to respond to any antigen in a very specific manner.

There are two main cell types associated with an adaptive immune response, T and B-lymphocytes also called T and B cells. Both have specific receptors for antigens but the induced signaling is not sufficient for their activation. The immune response is organized in a cascade of events dictated by the co-stimulatory signals needed by the T and B cells respectively. Indeed, T cells need the cooperation of a Dendritic Cell (DC), by means of co-stimulation in addition to the presentation of the antigen on MHC-II. Whereas, the B cells will need the help of a previously activated T cell. Upon activation, lymphocytes proliferate and can execute their functions. Ultimately, activated B cells differentiate into plasma cells, responsible for Ab production. This very simplified view of the adaptive immune response is recapitulated in figure 6 below.

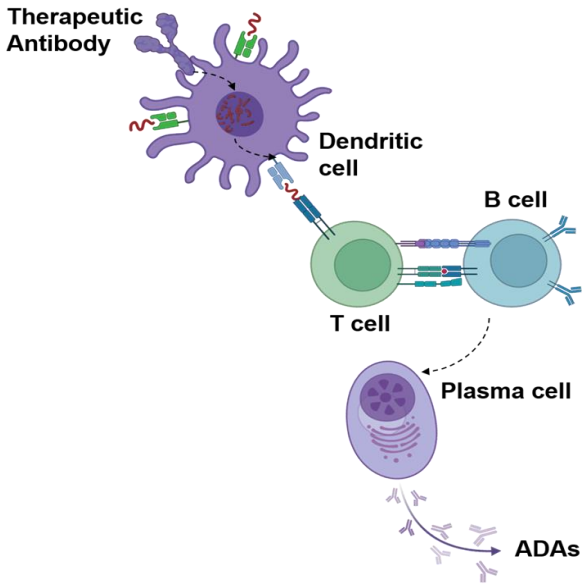

Figure 6. Simplified overview of the adaptive immune response leading to the generation of ADA. The therapeutic Abs is taken up by DCs, subsequently presenting a derived peptide to T cells that will give help to B cells differentiating into ADA producing plasma cells. Figure created using BioRender.com.

There are three critical steps in the generation of an adaptive immune response: (1) the internalization and processing of the antigen, followed by (2) its presentation to the specific T cell together with co-stimulation which will then (3) interact with a B cell inducing its differentiation and the subsequent production of Abs. Important considerations for an immune response leading to the formation of ADA will be detailed along these critical steps in the following sections.

1.3.1. Professional APCs: Dendritic Cells

1.3.1.1. Immature DC: capture and processing of the antigen

DCs are at the frontline of the immune response, they can initiate inflammation but also an adaptive immune response in the tissues as well as in the circulation. As professional APC, DCs can scan their environment for potential threats. All nucleated cells can initiate a CD8+ T cell response via MHC-I peptide presentation following an intracellular infection (e.g., virus), which will not be the focus here. Therapeutic Abs and biotherapeutics in general have to be taken up from the extracellular space. In most cases it is achieved by a nonselective process, namely macropinocytosis, which captures large quantities of extracellular material, including

proteins or bacteria, via plasma membrane ruffling and folding (Blum et al., 2013). In addition to that, caveolae-mediated endocytosis relies on cavities present on the cell surface to capture in a nonspecific manner, proteins of the cell surrounding (Matthaeus & Taraska, 2021). Receptor mediated endocytosis is another mechanism responsible for the internalization of extracellular proteins. Indeed, therapeutic Abs can be targeted towards a specific protein that is expressed on the surface of the DCs, which may not be the targeted cell population. Moreover, DCs express a variety of receptors that bind and internalize extracellular proteins via the clathrin-mediated endocytosis (the name refers to the protein used to build the vesicle). Indeed, C-type lectin receptors, like the mannose receptor or CD209 (DC-SIGN), contribute to the internalization of foreign glycosylation patterns. IgGs are glycosylated molecules, they contain a conserved asparagine residue (Asn297) in the Fc region. Glycan patterns may vary between the different expression systems. Therefore, it is important to monitor the glycosylation patterns of the produced Abs to ensure that there is no influence on the uptake efficiency by DCs (Jambari et al., 2021). However, it is not clear if it increases the risk for immunogenicity in all cases, as no T cell activation following the increased internalization of the glycosylated protein was detected, thus pointing toward the importance of the processing of the protein and the intracellular signaling initiated. Another consideration is that different glycosylation patterns would lead to a potential pre-existing immune response. The Fc domain of Abs is also recognized by FcγR, CD32 or FcγRII being the main FcγR expressed by immature DCs (Wolf et al., 2022a), helping for their internalization upon binding but also enhancing the clathrin mediated endocytosis. The internalized protein can be retained with the cell for several days waiting for an activation signal (Turley et al., 2000). The internalization mechanisms are summarized below (**figure 7**)

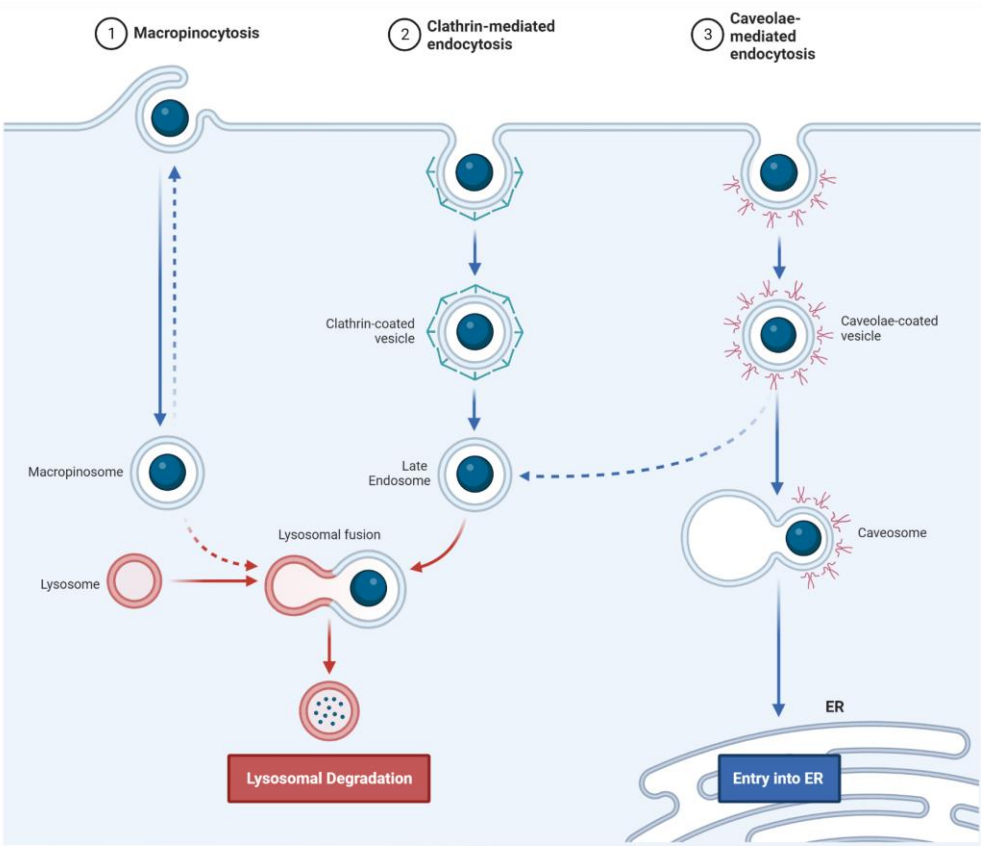

Figure 7. Overview of the main internalization pathways performed by APCs. Macropinocytosis, clathrin and caveolae-mediated endocytosis lead to the formation of vesicles that can later fuse with a lysosome to degrade its content. Adapted from (Behzadi et al., 2017) and created using BioRender.com.

Proteins taken up from the extracellular space are internalized in a membrane bound vesicle, which will fuse with a lysosome. Upon fusion, the protein will be processed into peptides by lysosomal proteases. Cathepsins are the main proteases present in the endosomes or lysosomes. They exert different protease activities defined by their specificity. Cathepsins B, H or S, for example, are some of the 11 cysteine proteases which are playing a particularly important role in endosomal proteolysis. The latter being predominantly expressed in APCs while the majority of the other cathepsins are ubiquitously expressed in human tissues. Cathepsins are tightly regulated to avoid the complete proteolysis of the antigen. The regulation of their activity is linked to the pH,

which is adjusted by an ATP-dependent vacuolar proton pump (Fuchs et al., 1989). This regulation happens early in the cathepsin synthesis, as they are transported as procathepsin from the trans-Golgi network by the mannose-6-phosphate receptor towards the endosomal compartment, where they are dissociated from the receptor and matured in a pH-dependent manner (Kornfeld, S. and Mellman, I, 1989). The regulation of cathepsins and more broadly of extracellular protein processing into peptides is also linked to the redox balance within the phagosome (Ewanchuk et al., 2018). Indeed, internalized proteins are reduced within those compartments facilitating their denaturation and unfolding (Collins et al., 1991). It is a requirement for the processing of proteins containing disulfide bond (important for the secondary and tertiary structure) and this reaction is catalyzed by the γ-interferon-inducible lysosomal thiol reductase (GILT) (Arunachalam et al., 2000). Therefore, the efficiency of disulfide bond reduction influences the repertoire of antigenic peptides, and an increased expression of GILT would universally increase phagosomal peptide diversity and abundance. Moreover, lysosomal cysteine cathepsins require a reduced thiol group in addition to the acidic environment to exert their catalytic function (Turk et al., 2012), which also involves GILT. Its counterpart, the nicotinamide adenine dinucleotide phosphate oxidase 2 (NOX2) complex is responsible for the phagosomal oxidation via the generation of reactive oxygen species (ROS) and has been shown to negatively regulate proteolysis (Savina et al., 2006). While GILT catalyzes cysteine cathepsins activity, NOX2 inactivates them through oxidation by ROS whereas aspartic cathepsins are not sensitive to this regulatory mechanism. NOX2 expression is increased by FcγR or IFN-γ expression but decreased by IL-4 (Balce et al., 2011) and its influence on processing has been shown to be antigen dependent (Allan et al., 2014). Indeed, internalization via FcγR induces an increased NOX2 activity, as opposed to phagocytosis through the mannose receptor (Pfefferkorn et al., 1989). Therefore, the way the protein is internalized influences the redox balance of phagosomes and ultimately the processing efficiency. Overall, the absence of an individual protease has minimal

consequences on the processing of a given antigen (high redundancy of the protease activity). However, the redox balance within the phagosome is heterogeneous and modulated in response to genetic, pharmaceutical, or physiological conditions with implications on the abundance and repertoire of antigenic peptides resulting from the processing of an extracellular protein.

1.3.1.2. Immature DC: Presentation of the derived epitope

The processed peptide has to be loaded on an HLA-II receptor and directed towards the cell surface for a proper presentation to a cognate CD4+ T cell. These receptors are encoded in the MHC locus present on chromosome 6. The corresponding genes are highly polymorphic (Guillemot et al, 1988), meaning that the genetic sequence encoding for them varies among individuals (7183 alleles are deposited in the IMGT database), coding for 27 non-redundant MHC-II haplotypes. This variety of receptors is also due to the association of an alpha and beta chains, needed to form a functional HLA-II receptor. Each individual expresses three types of receptors, HLA-DR, HLA-DP and HLA-DQ. A functional HLA-DR results from the association of the alpha chain with one of the four beta chains (HLA-DRB1, HLA-DRB3, HLA-DRB4, HLA-DRB5) although the HLA-DRB1 chain is expressed at a level five times higher than the others while being present in all individuals (which is not the case for HLA-DRB3, HLA-DRB4 or HLA-DRB5). HLA-DQ has two possible alpha (HLA-DQA1, HLA-DQA2) and beta (HLA-DQB1, HLA-DQB2) chains even though HLA-DQA1 and HLA-DQB1 are largely predominant. Only single alpha and beta chains are expressed and are constitutive of HLA-DP. As HLA-DR is the most represented receptor on the cell surface, in some *in vitro* assays, when healthy donors are selected to match a population, only the HLA-DR haplotype is taken into consideration with a particular focus on HLA-DRB1. In addition, the HLA-DRB1 locus presents the largest allelic diversity (with approximately 2,500 variants) among the other MHC-II loci. However, the alleles are not represented equally in the population which is in favor of focusing on a subset especially when looking at binding affinities *in silico* (methods will be detailed in the “Pre-clinical immunogenicity assessment” section). Indeed, the top 10 DRB1

alleles, for example, are represented in almost 80 % of the European population (http://www.allelefrequencies.net/).

The polymorphic residues of the HLA-II receptors are clustered in the peptide-binding region and are responsible for the different peptide specificities observed for different histocompatibility proteins. In addition, there are allele-specific motifs responsible for additional binding specificities. Hydrogen bonds between atoms of the main-chain along the peptide and HLA-II residues provide a major component to the binding interaction that is independent of peptide sequence (Stern et al., 1994). In the binding site, the pockets accommodate the amino-acid side chains specific to the peptide sequence. Therefore, the overall specificity of peptide binding is dominated by the inability to bind amino-acid side chains with unfavorable electrostatic, hydrophobic or Van der Waals interactions. Additionally, the peptide bound to class II molecules tend to be of variable length (typically between 13 and 25 residues) allowed by the open groove of the binding pocket. The positions 1, 4, 6 and 9 of a T cell epitope are particularly important for the interaction with the MHC-II receptors as they are directed towards the binding groove; they are called anchor residues and are responsible for the specificity of the interaction (Jones et al., 2006).

Because HLA-II heterodimers **(figure 8a)** are unstable in the absence of bound peptides, its invariant chain (CD74) functions as a chaperone to help assemble a stable complex of CD74 and HLA-II heterodimers.

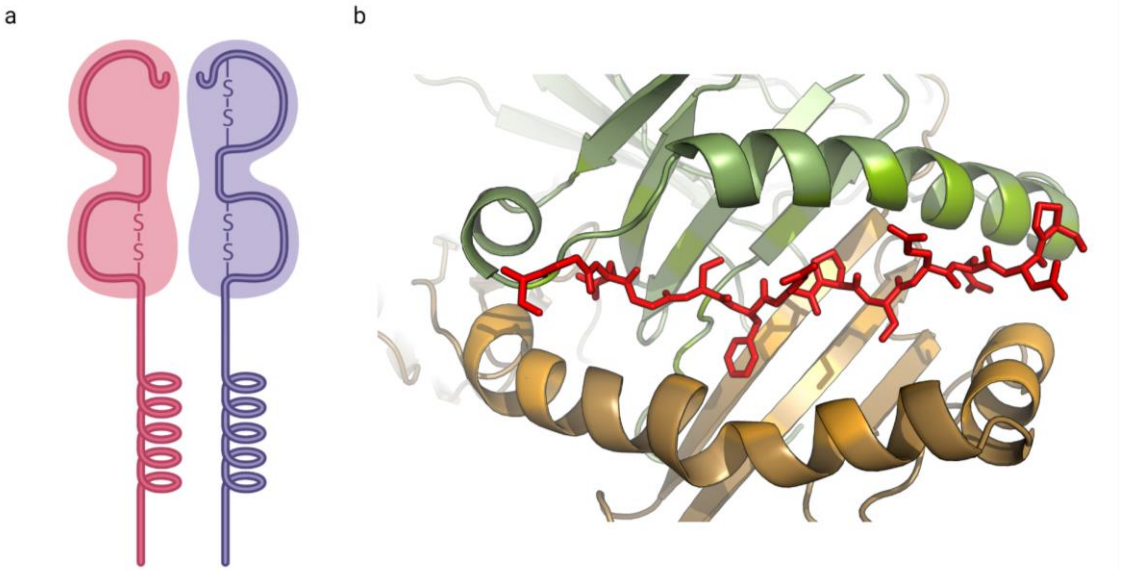

Figure 8. Representations of the HLA-II heterodimer. (a) Schematic representation of the two chains associated to form the HLA-II complex. (b) Structural representation of the peptide-HLA-II complex viewed from the top. Adapted from (Sarri et al., 2018) and created with BioRender.com.

Next, CD74 directs the trafficking of HLA-II complexes to the lysosome or MHC class II compartment, where CD74 is cleaved by proteases such as legumain and cathepsins. CD74 is not entirely cleaved and leaves the Class II-associated invariant chain peptide (CLIP) in the groove, which then competes with the protein-derived peptide. HLA-DM, also present in the lysosome, acts by opening the binding groove of HLA-II to allow for the exchange of the CLIP with high-affinity peptides. If HLA-DM is not present, low affinity peptides cannot replace the CLIP and MHC-II accumulates in the late endosome. HLA-DO regulates the activity of HLA-DM by preventing HLA-DM to induce the dissociation of low-affinity peptides (Blum et al., 2013). This mechanism allows a tight regulation of the peptide loading on the HLA-II receptor and allows the formation of the peptide-HLA-II complex, which is finally transported to the cell surface **(figure 9)**.

2.

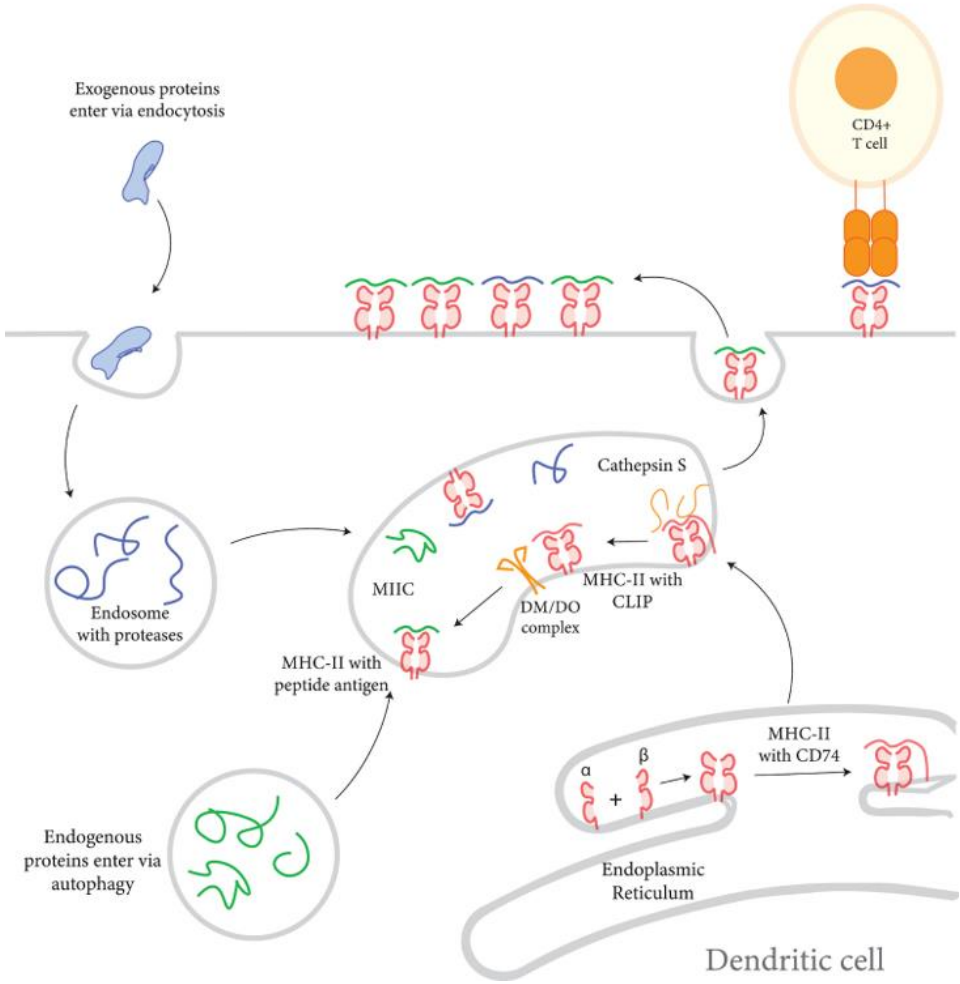

Figure 9. Processing and peptide presentation via MHC-II by a DC (Taylor et al., 2021).

The processing can be impacted by the context (ex. disease) and it becomes clear that the HLA-II antigen presentation pathway plays a critical role in adaptive immune responses. This is demonstrated by the correlation between specific HLA-II allele expression and the occurrence of autoimmune diseases or immunogenicity as described earlier. Antigen presentation has been shown to decline with age and varies according to the individual HLA types indicating the importance of testing a significant cohort of patient or healthy donors before drawing any conclusion (Cornaby et al., 2015b).

1.3.1.3. Mature DC: activation and T cell co-stimulation

An activation signal induces the maturation of DCs and modifies the antigen processing and presentation. Indeed, Toll-like receptor (TLR) ligands can promote a rapid burst of macropinocytosis in DCs (Blum et al., 2013). Moreover, activation of DCs also increases the activity of the ATP-dependent vacuolar proton pump and acidifies antigen-processing compartments (Cornaby et al., 2015a; Roche & Furuta, 2015). The induction of maturation lowers lysosomal pH by ~1 pH unit, from ~pH 5.5 (suboptimal for proteolysis) to pH 4.5 (more adequate pH for proteolysis and peptide loading) (Trombetta & Mellman, 2005).

Upon activation and presentation of a peptide-MHC-II complex (pMHC-II) on its surface, the DC migrates towards a lymph node where it can be recognized by a specific T cell receptor (TCR). While the protein uptake, processing and peptide presentation on MHC-II takes place by APCs at the site of infection, the interaction with specific T cells occurs in the secondary lymphoid organs (e.g., lymph nodes or LN). Upon activation, DCs also express the chemokine receptor CCR7, which binds to its ligands CCL19 and CCL21 expressed by endothelial cells from lymphatic vessels. Therefore, activated DC are attracted towards lymphatic vessels, enter the lymph and subsequently the LN. However, internalization and peptide presentation does not necessarily lead to DC activation, which therefore needs a second signal.

Local inflammation generates host cellular components such as lipids, metabolites, or nucleic acids commonly named Damage-Associated Molecular Patterns (DAMPs). Microbes have conserved molecular motifs, called Pathogen Associated molecular Pattern (PAMPs), from which Lipopolysaccharide (LPS), derived from the cell membrane of Gram-negative bacteria is the most well-known. DAMPs and PAMPs are recognized by Pattern Recognition Receptors (PRRs), such as Toll-like receptors (TLRs) and induce the activation of DCs. Therefore, the second signal is not coming necessarily from the therapeutic Ab sequence itself but potentially from its formulation or co-treatment. Treatment induced cell death, inflammation at the injection site, concomitant infections, are examples of activation signals that can arise upon treatment. DCs, for example, have ATP sensors on their cell surface, which would detect

circulating ATP, indicating neighboring cell death and inducing DC maturation (Clark et al., 2018).

The consequences of DC activation are multiple, starting with the completion of MHC class II biosynthesis to limit the generation of pMHC-II complexes that are irrelevant to the invading pathogen (Hilligan & Ronchese, 2020). The subsequent overexpression of CCR7, helping the migration of DCs to the LN following the CCL21 gradient. Activated or mature DCs express a variety of receptors on their cell surface to convey their message to the T cells, like CD80 or CD86, which bind to CD28 or CTLA-4 on T cells. This interaction highlights the ambivalence of the DCs, where the interaction with CD28 is stimulatory but the one with CTLA-4 is suppressive (Nam et al., 2021). The importance of the interaction between DCs and T cells will be discussed in the next section. It is important to mention that DCs can also be responsible for peripheral tolerance aside their role in the initiation of an immune response. Their ambivalence is driven by the cytokines they produce and the receptors they express. Indeed, they are capable of secreting anti-inflammatory cytokines such as IL-10 to induce tolerance but also pro-inflammatory cytokines like IL-12, IL-1β, IL-6, and TNF-α to initiate an immune response. While being needed and potentially shaping the resulting immune response (Wölfl & Greenberg, 2014): processing, antigen presentation and cytokine production by DCs is not sufficient to initiate an immune response, pointing towards the importance of their interaction with T cells.

1.3.1. Epitope presentation and DC-CD4+ T cell interaction

T cells originate from the bone marrow, like B cells but their maturation and selection takes place in the thymus. T cells express a receptor capable of detecting an antigen in the form of a peptide-MHC complex, which is used for their selection, and ultimately for initiating an immune response: the T cell receptor (TCR).

Once lymphocytes encounter specific antigens, they will get activated and able to exert their function but also expand. Indeed, for antigens the immune system has never encountered,

there are very limited specific circulating lymphocytes (often referred to as precursors) and it is only when they are selected for clonal expansion that mitotic division is highly upregulated and that the specific T or B cells expand. This has major implications for immunogenicity, particularly in the attempt to assess the risk of immunogenicity using in *vitro* assays. It has been demonstrated that the T cell precursors, capable of expanding in response to a therapeutic Ab, are in the order of magnitude of one specific T cell out of a million T cells (Delluc et al., 2011a). This will be discussed further in the dedicated section along with other limitations of *in vitro* assays. Physiologically, this is the reason why antigen presentation by DC to T cells happens in the LN where there is an increased concentration of T cells and a higher probability to initiate this specific interaction. In the context of this interaction and to increase the chance of initiating a specific interaction, a single DC has the capacity of interacting with up to ten T cells (Giese & Marx, 2014). These steps are recapitulated in **figure 10**, along with important parameters in the context of immunogenicity.

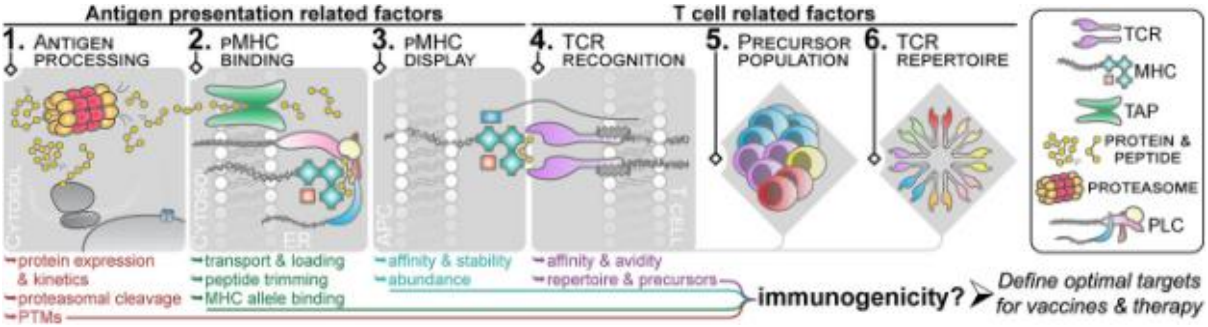

Figure 10. Important steps leading to an immune response. Highlighting important parameters having major consequences for immunogenicity (Croft, 2020).

On **figure 10**, the affinity, the stability and the avidity characterize the pMHC-II-TCR interaction, which could be recapitulated by the duration of the interaction between the two cell types. Indeed, there are different types of interaction that can all lead to a good signaling between DCs and T cells: single long dwell time, or sequential, short, spatially correlated binding events not solely long interactions are responsible for activation (Richard et al., 2021).

The presentation of a peptide via MHC-II by DCs is the first requirement and will be recognized by the cognate CD4+ T cell, leading to downstream signaling and often referred to as “signal one”. Moreover, there is a rapid turnover of pMHC-II in immature DCs due to the ubiquitination of a conserved lysine residue in the MHC-II β-chain by the E3 ubiquitin ligase MARCH1 which does not happen upon maturation where the complex is stable for hundreds of hours (Roche & Furuta, 2015). Although “signal one” is required for T cell activation and stabilized upon APC maturation, it is not sufficient. Indeed, another signal coming from the APC is needed and derived from sensing a potential danger. As previously discussed PAMPs and PRR interaction is one among others that could lead to DC activation. There are different receptors, specific to the PAMPs they bind to, for example TLRs, mostly sense endotoxins and proteins derived from bacteria. The integration of “signal two” leads to the upregulation of co-stimulators, namely B71 (CD80) and B72 (CD86) on the surface of the DCs. Both co-stimulators bind CD28, expressed on the surface of all naive T cells, and transmit the activating signal to the cognate CD4+ T cell. The simultaneous integration of “signal one” and “signal two” by naive CD4+ T cells lead to their activation and expansion.

There are 3 main subsets of CD4+ cells, namely Th1, Th2 and Th17 and they differ in terms of the cytokine they produce. Interferon-γ (IFN-γ) is the hallmark of Th1 cells while Th2 cells produce IL-4, IL-5 and IL-13, which are particularly important to fight against intestinal parasites. Th17 cells secrete mainly IL-17 and help fight against extracellular antigens mainly by promoting the recruitment of neutrophils and monocytes. On the other hand, Th1 cells, via the expression of CD40L and secretion of IFN-γ activate phagocytes and enhance their processing activity, making it the main CD4+ subset involved in immunogenicity.

Over activated or autoreactive T cells can lead to autoimmune disease (e.g., rheumatoid arthritis, multiple sclerosis…). Therefore, there is a need to control the activation of T cells and avoid uncontrolled activation of the immune system. Both PD-1 and CTLA-4 are expressed by activated T cells and the more T cells are activated, the more these receptors interact with

their ligands, dampening the overall immune response. Additionally, T regulatory cells (Tregs), characterized by high expression levels of the high-affinity IL-2 receptor α-chain (CD25) and the transcription factor FoxP3, are known to be of particular importance to maintain immune homeostasis and prevent auto-immunity (Tuzlak et al., 2021). Many FoxP3+ Tregs are of thymic origin. In contrast, FoxP3- but LAG-3 positive CD4+ cells, also referred as T regulatory cells type 1 arise from naive or even memory T cells precursors present in the periphery with major implications in the epitope they recognize. Indeed, they participate in the peripheral tolerance of non-self-antigens for which Tregs originating from the thymus are not specific for. This subset of Tregs maintains tolerance mainly by producing regulatory cytokines (e.g., IL-10) and have been shown to be implicated in the tolerance mechanism of infliximab (Vultaggio et al., 2017). IL-2, which is expressed in a stimulation strength-dependent manner by both CD4+ and CD8+ T cells, results in both autocrine and paracrine signaling through IL-2R (Richard et al., 2021). Activated T cells produce IL-2 but the IL-2 receptor takes 24h upon activation to be expressed on the surface while IL-2 is produced in 6-12h reducing the autocrine pathway and favoring the interaction with surrounding cells. Upon IL-2 sensing, Tregs express CTLA4, which blocks CD80 and CD86 on the surface of the DCs, thus inhibiting the CD28-mediated co-stimulatory pathway (Wong & Germain, 2021).

After going through clonal expansion, controlled by Tregs and checkpoint inhibitors (CTLA-4 and PD-1), the antigen-specific T cells are then able to provide its help to B cells and to eliminate the threat. Subsequently, most of the lymphocytes die but it will remain a larger number than initially forming the pool of memory T cells. This mechanism improves the reactivity of the immune system if the same antigen is encountered again. It could be referred to as pre-existing immunity and lead to a recall response, quicker and more efficient than the initial one. This mechanism also poses challenges to the use of therapeutic Abs in patients, especially if a similar treatment has already been administered in the past. Indeed, the interaction with co-stimulatory signals on APCs is not required for the activation of memory T

cells, allowing a faster response. Ultimately, activated T cells will transduce the signal to B cells in secondary lymphoid organs giving them help for producing large amounts of high affinity Abs.

1.3.2. CD4+ T and B cells interaction leading to the Ab response

B cells originate from the bone marrow but instead of going through a very conservative process like for T cells, their receptor, the BCR, is edited via gene rearrangement making a new light chain until they are no longer self-reactive.

In contrast to T cells, mainly capable of recognizing a peptide bound to the MHC-II receptor on the surface of DCs, B cells can recognize virtually any antigen with their B cell receptor. However, the specific role of B cells in the recognition of the therapeutic lies in the recognition of tri-dimensional epitopes, which should account for 90 % of the cases (Liang & Zhang, 2020), in contrast with the linear epitopes presented by MHC-II and detected by T cells (Rosenberg & Sauna, 2018). A protein that binds to a B cell receptor will be taken up by receptor-mediated endocytosis and processed into peptides that can be displayed on MHC-II molecules also expressed on B cells. B and T cell epitopes, even though being different, are physically linked and are at the origin of the T and B cell collaboration. This interaction happens in the secondary lymphoid organs, which are highly organized into B cell follicles and T cell zones **(figure 11)**.

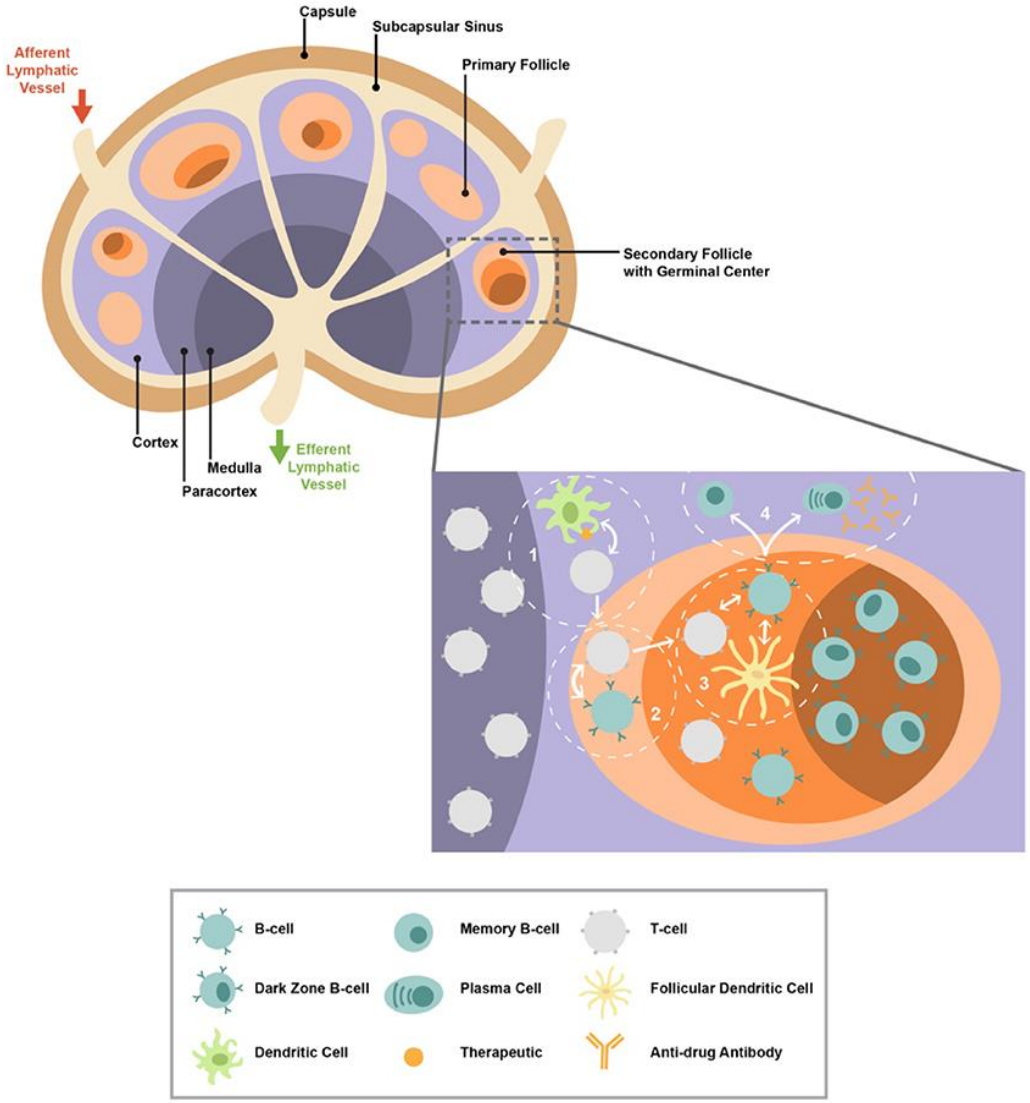

Figure 11. Organization of a lymph node and summary of the main steps. The zoom is done on a follicle to highlight the key stakeholders and steps in a germinal center reaction. (1) dendritic cells that present the antigen interacts with a CD4+ T cells resulting in their activation and differentiation; (2) activated CD4+ T cells begin interacting with B cells, ultimately leading to further differentiation of both cell types and therefore trafficking into follicles or Germinal Centers (GC); (3) within the GC, B cells interact with follicular CD4+ T cells and follicular dendritic cell (FDC); (4) helping B cells promotes their maturation to memory and plasma cells (Fu et al., 2020).

T cells interact with DCs in the paracortex whereas B cells home in the cortex. Activated T cells then migrate to the border of the cortical zone and come into contact with B cells in the

primary follicles to initiate the germinal center reaction. In order to do so, the antigen-specific CD4+ T cells going through clonal expansion also increase their expression of a chemokine receptor, CXCR5, expressed on the B cell surface as well, allowing the encounter of the antigen-specific T cells and the B cells in the B cell zone. Follicular DCs (FDCs) secrete CXCL13 to attract B and T cells to the follicles in a CXCR5-dependent manner (Grasso et al., 2021). At this point, circulating DCs coming to the lymphoid organs are particularly important as they can capture IL-2 via the CD25 receptor on their cell surface. IL-2 being a cytokine responsible for the inhibition of follicular helper T cells, it is an additional mechanism promoting B cell responses, together with the secretion of IL-6 (Hilligan & Ronchese, 2020). Additionally, high endothelial venules or lymphatic endothelial cells are helping the formation of the germinal center by attracting the key stakeholders. At the same time the antigen interacts with the B cell receptor (BCR), is endocytosed, processed into peptides, which are presented via its MHC-II receptor. This also induces the expression of CCR7, normally found on the surface of T cells, which will make these specific B cells migrate toward the T cell zone. This allows the encounter between the specific clonally expanded T cells and the specific B cell presenting the corresponding MHC-II-peptide complex together with CD40. The interaction between CD40 and CD40L present on the surface of the T cells will induce the proliferation of B cells. The expanding B cells will then start to differentiate into short-lived plasma cells, producing low levels of Abs. Subsequently, both cell types migrate to the follicles, where the affinity maturation and isotype switching happens, moving from an early IgM response towards the production of high affinity IgGs. This is referred to as the Germinal Center (GC) reaction. Somatic mutations occur in the gene coding for the variable region of the BCR. These point mutations modify the antigen binding sites of the B cell clones, thus modifying their affinity for the antigen. The B cell clones will then test their newly mutated BCR for affinity to antigens presented by follicular dendritic cells (FDCs). If the B cell clones recognize the antigen, the latter will be internalized and presented to CD4+ follicular helper cells (Tfh), which will provide survival signals to the B cells. During this selection process, the vast majority of the B cell

clones will die by apoptosis. Only the clones that have a high affinity for the antigen will survive. (Giese & Marx, 2014). Subsequently, increased affinity B cells are enriched and produce the corresponding Ab, differentiate into long-lived plasma cells (constantly producing the Ab) or memory B cells (capable of rapidly turning into plasma cells upon re-exposure to the same antigen). However, this is not the only modification that occurs during the GC reaction. Isotype switching is another important modification in the Abs produced by those B cells that will lead to a change in the Ab heavy chain and therefore a change in function. Usually going from an IgM to an IgG thanks to the follicular helper T cell signals, which is providing everything that is necessary for cutting the DNA at the switch regions, allowing the ligation of the chopped DNA and forming the new segment that will code for the new heavy chain. The interactions happening in the secondary lymphoid organs are summarized in **figure 12** below.

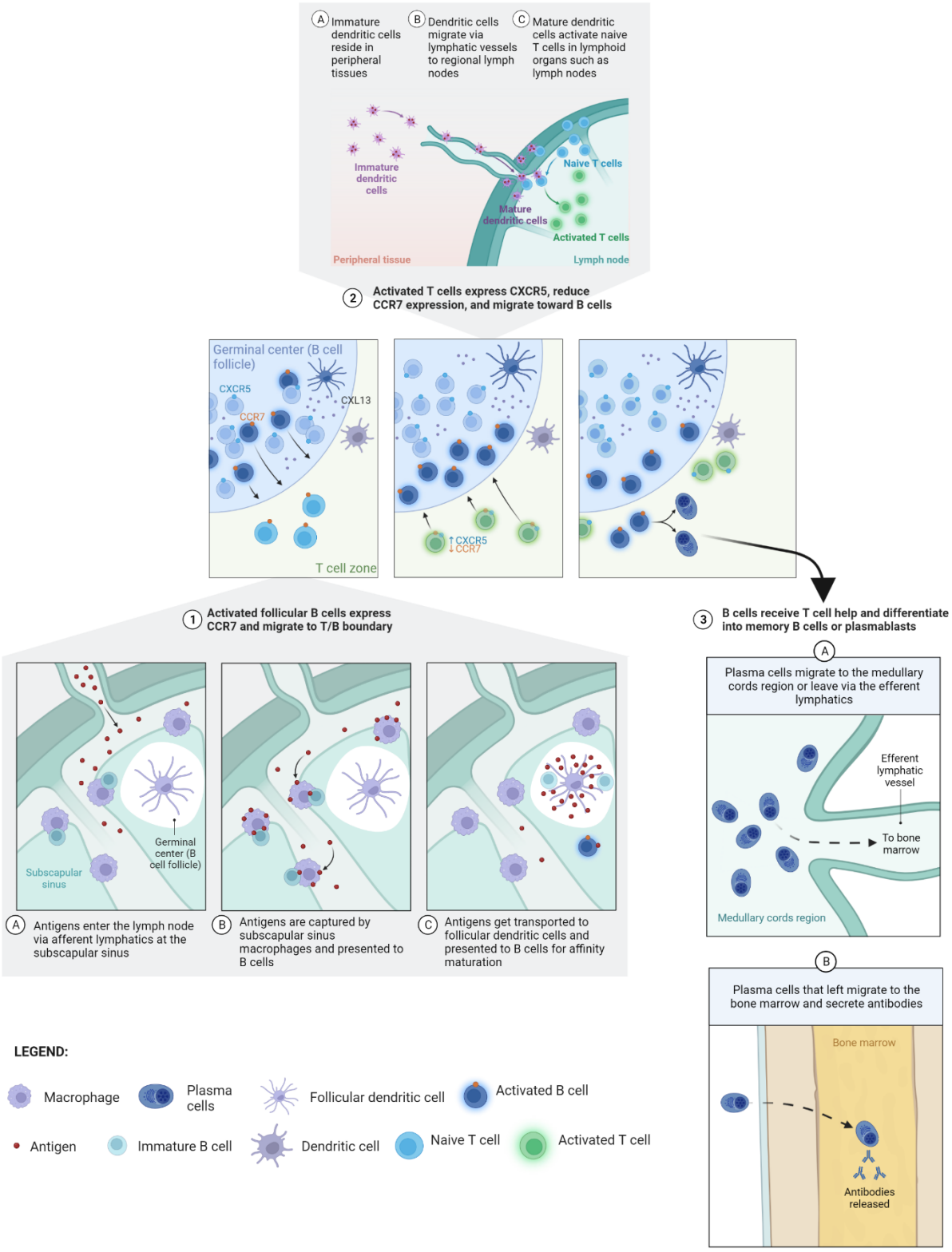

Figure 12. Main interactions leading to the generation of Ab secreting plasma cells. Schematic representation of the main steps, starting with the activation of both B and T cells within the secondary lymphoid organ by DCs. This leads to the expansion of T cells and their

relocalization close to the activated B cell. The interaction between the two lymphocytes will activate further the B cell and prime the germinal center reaction. This reaction leads to a massive proliferation of B cells and a selection for the more specific one, which will ultimately help to make the shift from abundant production of low affinity ABs to highly potent ones. Created with BioRender.Com.

To sum up, the humoral immune response depends on CD4+ T cell activation which follows a three-signal rule for activation (TCR:MHC/peptide interactions, costimulatory interactions such as via CD28, and cytokine production). The selected T cell will then expand and provide the necessary help to B cells in secondary lymphoid organs, to produce high affinity Abs.

1.4. Strategies to reduce immunogenicity

1.4.1. Clinical mitigation strategies

Immunogenicity is a hurdle when using therapeutic Abs and can lead, not only to a decreased treatment efficacy but also to safety concerns as described earlier (see “Consequences of immunogenicity related side effects” section). Therefore, clinical mitigation strategies are implemented to reduce the consequences of immunogenicity.

The first method applied to reduce the risk for adverse events and immunogenicity is the dose escalation. Starting the clinical trial with a lower dose leaves time to adapt in the case adverse events occur. This is the case within a clinical trial but also in the course of a patient treatment. The first administered dose would be lower than the efficacious dose, increasing tolerance to the treatment.

In addition, methotrexate is often used to reduce the proportion of patients developing ADA against adalimumab, as demonstrated in the context of axial spondyloarthritis (Ducourau et al., 2020). Methotrexate is used as a co-treatment with a variety of therapeutic Abs, such as adalimumab. Since methotrexate inhibits the enzyme AICAR transformylase, it leads to the

accumulation of adenosine, which has a broad anti-inflammatory effect (e.g., repression of T-cell activation, down-regulation of B-cells).

Those solutions are in certain cases efficient to reduce the risk for adverse events and immunogenicity but they are very costly. Taken together with the lower chance of success for immunomodulatory Abs to reach the market, the cost of their development exploded and is estimated at $4.5 billion, three times more than the traditional drug or Abs ($1.3 billion) (Vandivort et al., 2020). Therefore, the need for molecules with a low risk of immunogenicity is increasing, which starts with a better understanding of the steps leading to immunogenicity but also a better Ab design strategy to avoid sequence liabilities and unwanted immune reactions. These improvements in Ab design would increase the rate of success for all developed Abs but could also delay entry-into-human. On the other hand, the use of co-treatments would not be needed.

The availability of suitable ADA mitigation strategy (e.g. the induction of immune tolerance (Shakhnovich 2020) co-administration of immunosuppressant (Vermeire 2007), or B cell depletion (Tobinai 2017,Freeman 2018)) can be successful in reducing the impact of ADA on PK/PD and, more generally, in lowering the risks of immunogenicity related adverse events. However, it comes with the need of characterizing this co-administration and its consequences on the treatment efficacy in clinical trials, affecting the time needed to develop the therapy and ultimately its cost.

1.4.2. Production of therapeutic Abs

The production of therapeutic Abs started in 1957, when Georges Köhler and César Milstein established the hybridoma platform (KOHLER, 1975). Since then, various production strategies were developed, such as the use of mouse spleen derived hybridomas, which was used for OKT3, the first approved therapeutic Ab (Wauwe & Goossens, 1980). Abs are specific to a single epitope, which is a prerequisite to avoid off-target consequences and originate from

a hybridoma cell line. However, the use of mouse Abs has faced some challenges, especially the production of human anti-mouse Abs directed against the administered Ab.

Today, the large-scale manufacture and production of therapeutic Abs for clinical application are mainly conducted by recombinant DNA technology using mammalian cell expression systems (Posner et al., 2019). There is now a consensus in the usage of Chinese Hamster Ovary (CHO) cell line to produce therapeutic Abs, as it has the advantage of generating more human-like glycosylation (Clausen et al., 2017). The use of recombinant DNA allows a broader range of Ab specificity, as it does not solely rely on naturally occurring Abs. Indeed, it is possible to screen a large library of Ab idiotypes, derived from the amino acid sequence and design Abs with the desired specificity and even modulate its affinity. The specificity of an Ab lies in the recognition of a three-dimensional epitope by its paratope composed of the CDRs. If there is enough flexibility, allowing a certain degree or rearrangement of the CDRs, the Ab can recognize an additional epitope than the one it was designed to target. Therefore, precise engineering of the CDRs is needed to ensure the specificity of the Ab but not only, as Vernier positions are also important in the formation of the antigen-binding site. The Vernier zone residues refers to residues in framework regions, which have been demonstrated to affect the conformation of CDR loops and affinity of Abs (Makabe 2008). Moreover, this platform makes it possible to produce fully human or humanized Abs less prone to induce an immune response in treated patients.

1.4.3. Therapeutic Abs humanization and de-immunization

The humanization of therapeutic Abs is certainly one of the most critical aspects when it comes to reducing the risk for immunogenicity. Even though it has proven to be a valid strategy, the first humanized Ab, alemtuzumab, achieved by grafting the CDRs on a human framework, was still very immunogenic, with 85 % of the patients developing ADAs (Baker et al., 2020). Sometimes, only grafting the CDRs is not sufficient to keep the same affinity. Therefore, keeping some original mouse residues has proven some efficacy. This process is called back

mutation. Indeed, a simple CDR graft can destabilize and decrease its affinity for the antigen, but a model-guided approach for back mutation can be successful, with significant stabilization and no change in antigen affinity (Goulet et al., 2022). Computer-aided design, phage display and yeast display are used at this step of the humanization process with the goal of being as human as possible while keeping the affinity for the antigen. There is no evidence that the production of ADAs by fully human Abs is less than with humanization. In addition, their administration to patients is by no means free from production of ADAs that may have important adverse effects (Posner et al., 2019). Other risk factors should therefore be taken into consideration. Erythropoietin (a fully human protein) contains a strong HLA binder and can be very immunogenic in rare cases when specific T cells are present. This response has been attributed to product related attributes (impurities...). It is likely that immune modulation enhancing the processing or activation of immune cells could modify the epitopes being presented by DCs or the DC: CD4+ T cells interaction, making even a humanized Ab immunogenic (Jawa et al., 2022). In some cases, it has been shown to be beneficial like with the humanization of a CD52 targeting Ab, alemtuzumab, for which it has been reported to reduce the risk for immunogenicity (Holgate et al., 2015).

Another process used to reduce the risk of immunogenicity would be to remove known T cell epitopes from the Ab sequence. This process is called “de-immunization” and has been used in several studies. For example, Cassota et al. identified T cell epitopes responsible for the onset of ADAs against natalizumab and a “de-immunized” version of the molecule was proposed (Cassotta et al., 2019). This strategy has been applied to IFN-β as well, where one amino acid exchange (I129V) led to complete abolition of the humoral response in BALB/cByJ mice (Yeung et al., 2004). In certain cases, as described above, a single amino acid exchange can have a dramatic effect on immunogenicity.

The modification of an Ab glycosylation can also have a profound effect on its immunogenicity. Indeed, sialylation (addition of sialic acid to the terminal end of glycoproteins) has been associated with anti-inflammatory activity (DC-SIGN or CD22 mediated). Another study showed that a glycosylated IgG was internalized quicker by DCs (Wolf et al., 2022b). In addition, Wolf et al., demonstrated that mannosylation increases uptake by DCs subsequently increasing T cell activation property and enhancing the humoral immune response in BALB/c mice. Fucosylation decreases ADCC by reducing the affinity for the FcγRIII present on NK cells. However, those modifications will not fit for every therapeutic Ab as they had no impact on the DC activation upon TNF-α targeting Abs administration (Wawrzyniak et al., 2021). These studies highlight the need for a more controlled glycosylation to reduce the risk for immunogenicity.

Marketed Abs contain mutations that are also common in human Ab repertoires and not necessarily germline (10^{11} different Ab specificities can be found in a human). The mutation from a serine to an alanine (position 54 of the VH) deviates from germline but increases the stability and decreases immunogenicity for some Abs (Petersen et al., 2021). However, not every modification has been shown to be beneficial. Indeed, a FVIIa variant bearing 99% identity with physiological FVIIa led to the termination of the clinical study because of ADAs (Lamberth et al., 2017). Two of the three mutations that differ from the physiological FVIIa: E296V, and M298Q seemed to have introduced a high affinity T cell epitope. This epitope has been predicted *in silico* but also confirmed *in vitro*.

1.4.4. New Abs format and technologies

It appears critical to have mitigation strategies in place for clinical studies in case of immunogenicity but also develop therapeutic Abs as human-like as possible. However, the Ab must keep its affinity and efficacy even after humanization or co-treatments. Thanks to the increasing knowledge on Abs and immune related processes, new therapeutic Abs engineering strategies arose.

The first consideration is the choice of the Ab isotype as it modifies its function and behavior when used as therapies. For example, the IgG4 isotype was selected for natalizumab and led to a higher number of half Abs making its purification more complex (Ulitzka et al., 2020).

It is also of high importance to characterize the stability of the molecule according to the isotype. Indeed, protein instability can lead to nonspecific interactions up to the formation of aggregates. This is the case for IgG2 Abs, which might be more prone for aggregation than IgG1. However, this property can be reduced by introducing three point mutations (Ulitzka et al., 2020). The IgG3 isotype has a slightly longer hinge, making it more prone to cleavage while bringing the potential advantage of having a more flexible Fab. Hence, the choice of the Ab isotype has consequences on its function but also on other important properties that can enhance the risk for immunogenicity.

As described earlier, Abs are glycosylated proteins and the IgG isotype contains a conserved glycosylation site, which is an asparagine at position 297 in the CH2 domain. However, the attached sugar can differ according to the cell line used for its production (Wolf et al., 2022a). As an example, in the case of rituximab, the change in expressing cells modified the pattern of glycosylation and led to more ADCC. Therefore, a human-like glycosylation pattern is preferred and a change in expressing cells has to be tightly monitored.

The addition of polyethylene glycol polymers has been used as a way to increase the Ab half-life by preventing glomerular filtration or opsonization. Moreover, it has been found to have, in certain cases, a reduced risk for immunogenicity in pre-clinical models, especially reducing their internalization into APCs De Bourayne et al., 2022). Other types of polymers, less hydrophobic than polyethylene glycol, could have an even better safety profile, like polychlorinated biphenyls (Li et al., 2018). DCs, when processing the Ab, may not remove certain glycans, hindering T cell recognition of glycosylated peptides (Trombetta & Mellman, 2005), therefore reducing the risk of initiating an immune response. However, polyethylene glycol motifs were reported to be responsible for fast clearance and injection site reactions (Zeunik et al., 2022) making them not fit for all.

Pharmacologic properties of Abs depend very much on their Fc region; interaction between the Fc domain and molecules of the complement or binding to various Fc receptors on leukocytes can induce ADCC or Ab-dependent cell-mediated phagocytosis. These events can lead to CRS (cytokine release syndrome), one of the adverse effects potentially caused by Ab therapy (e,g., rituximab) (Brennan and Kiessling, 2017). Existing assays aim at anticipating this phenomenon (C1q or FcγR binding assays for example) or more broadly a cytokine release assessment in whole blood upon treatment (Whole Blood Assay, WBA). However, point mutations in the Fc can abrogate these interactions: the N297 point mutation abrogates the binding of Absto the FcRs by limiting the glycosylation of this site (Wang et al., 2021), thus limiting the risk for CRS. In addition, P329G (PG), L234A, L235A (LALA) or commonly referred to as PG-LALA, completely abolished immune effector functions (Schlothauer et al., 2016). LALA mutations reduce binding to the IgG Fc receptors FcγRI, FcγRII and FcγRIII as well as to complement component C1q. Such modifications are useful where binding and activation of Fc receptors is undesirable (in some other cases it can be part of the mode of action). Fc functions, in the case of bispecifics, could lead to off-target toxicities increasing the need for such modifications, as described for intestinal inflammation observed for the CTLA-4/PD-1 bispecific (Wilkinson et al., 2021).

Less stable Abs, which might be a consequence of engineering, have been shown to be processed quicker in lysosomes, thus resulting in less immunogenicity compared to their stable counterparts (Moss et al., 2019). The link between stability and immunogenicity is balanced between highly stable structures that are not degraded by proteolysis and the very unstable ones that are processed too quickly to correctly bind MHC receptors. Without altering the T cell epitope content of an Ab, it is possible to perform amino acid exchange that would make the construct more resistant to certain proteases and therefore reduce antigen processing and subsequently epitope presentation. This method has been used for an enzyme, L-Asparaginase, used in the treatment of acute lymphoblastic leukemia, where an

amino acid exchange made it resistant to cathepsin B and asparagine endopeptidase. Resistance to both proteases increased the serum half-life of the therapeutic enzyme but resistance to cathepsin B alone resulted in less abundant long lived plasma cells but rather plasmablasts, highlighting the dramatic consequences of altered processing by DCs (Rodrigues et al., 2020).

With the constant search of new modes of action, there is an increased interest in developing bispecific or biparatopic Abs. At the moment, four different types of mode of action are explored (1) redirect specific immune effector cells to selectively destroy cancer cells; (2) target more than one cell surface antigen, thus increasing target specificity; (3) deliver drugs or cytokines directly to the tumor; and (4) improve the therapeutic potency and persistence via inhibition of two biological pathways. One advantage of bispecifics over co-medications is that it can bind the two targets on the same cell, as spatial proximity can be important for pharmacology (Zheng et al., 2022). In 2021, more than 110 clinical trials with bispecific Abs were registered (Shi et al., 2021). However, bispecific or biparatopic Abs create new challenges in terms of production, stability and sometimes safety risk assessment.

Specific mutations located in the Fc portion of the Ab allow a good pairing of the two half Abs and reduces the mispairing of LC (S. Cohen, Chung, et al., 2021). This technology is called “Knob-into-Hole”, with one Ab arm containing the “Knob” mutation while the other has the “Hole”. More precisely, a “Knob” is created by one amino acid exchange (T366W) on one HC, and the corresponding “Hole” is made by a triple mutation of T366S, L368A and Y407V on the partner HC (Merchant et al., 1998). This technology allowed a massive increase in the production efficiency of the bispecifics by limiting product-related impurities.

The CrossMab technology has been developed by Roche and allows the control pairing of the different “building blocks” (heavy and corresponding light chain) constitutive of a bispecific Ab. This is achieved by using one Fab with switched VH or CH1 domain(s) with the partner VL or CL domain(s) and by leaving the other Fab untouched (Klein et al., 2016). The correct pairing of the two heavy chains by the “Knob into Hole” leaves only one possible pairing of the VL

portion associated with the CH1, the constant region of the light chain present in one of the heavy chains. These two technologies allowed efficient production of bispecifics and to explore innovative formats as depicted in **figure 13**.

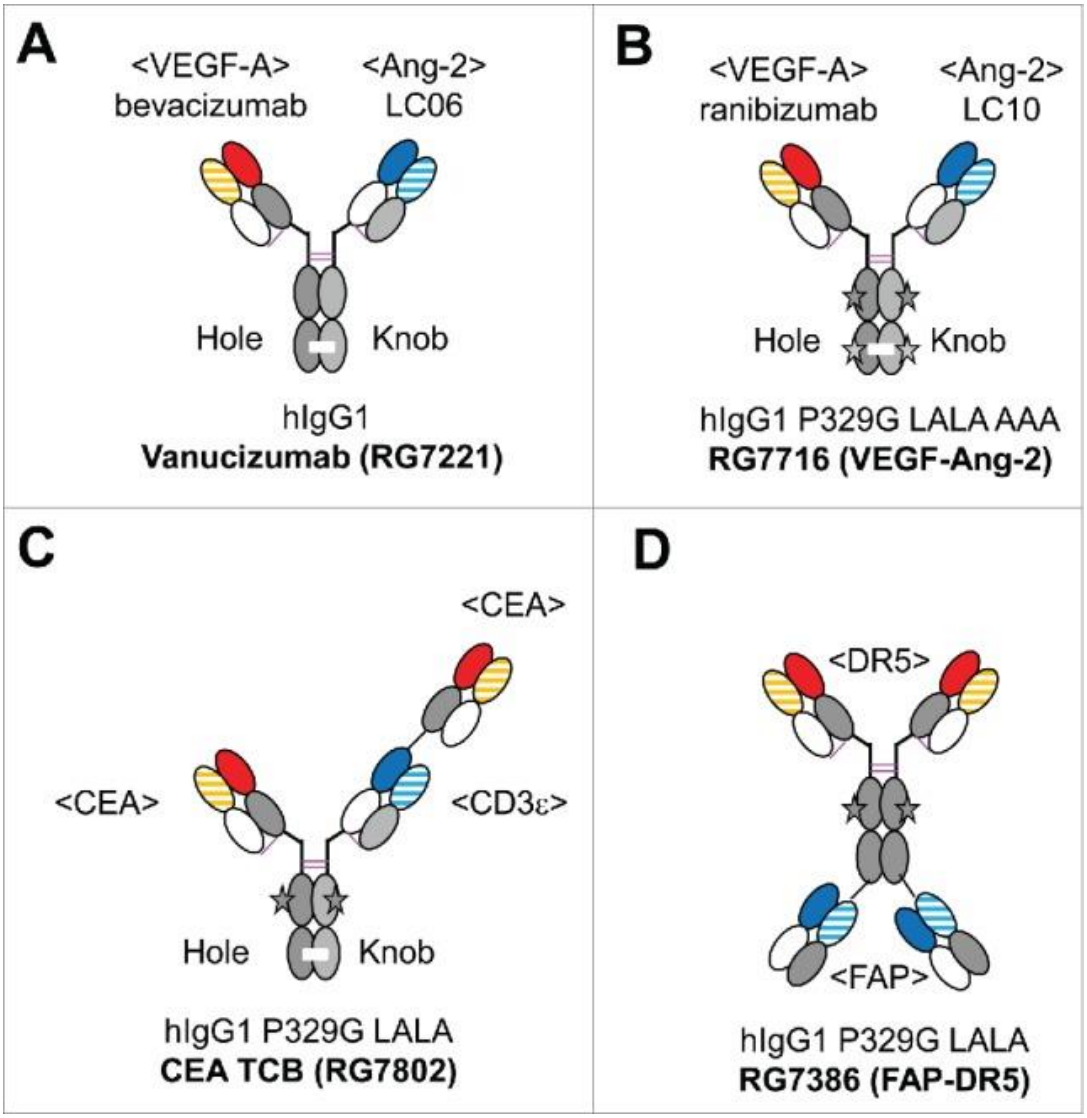

Figure 13. First generation of Abs using the CrossMab and Knob-into-Hole technologies. These technologies allowed efficient production of classical IgG-like bispecifics (A,B) but also to explore more innovative therapeutic Ab formats (C,D). The CrossMab technology is schematically represented on A,B and C, with the CH1 of the ANG2 or CD3 binders (in gray), present on the light chain. The star represents the P329G LALA mutation (Klein, Schaefer, & Regula, 2016).

Stability of these constructs could have been affected but their stability seems comparable to classical IgG1 following various stresses (size variants and post-translational modifications, such as isomerization, deamination, and oxidation) while maintaining activity (Grunert et al., 2022).

Usually, the two binders composing the bispecific have to be assessed for their potential immunogenicity, in certain cases even individually. However, a binder used in a bispecific format may not behave the same way as if it was in a classical IgG format (Spéville & Moreno, 2021). Moreover, the context around the sequence having its importance, the association between a binder A and B will not bear the same risk as binder A alone or A associated with another binder C. It is commonly accepted that the larger the molecule is the bigger the risk for immunogenicity (Vandivort et al., 2020). Recently, the development of a couple of bispecifics has been stopped due to immunogenicity (S. Cohen, Chung, et al., 2021). For example, an IgG4 blocking 2 soluble targets was terminated because of immunogenicity whereas both single targets in an IgG format were tolerated in patients (Schick et al., 2022). In addition, the generated ADA were highly polyclonal making it difficult to identify their specificity.

This phenomenon can be a consequence of epitope spreading, which is a shift from the recognition of immuno-dominant to subdominant epitopes or cryptic epitopes. It can result from altered uptake and processing and might be more likely to happen with larger molecules (Wolf et al., 2022a). This phenomenon can be intra- or intermolecular and initiated by APC or not (2 distinct mechanisms). In the case of an APC-independent epitope spreading, the inflammation is sufficient to activate T cells, which will then give help to the B cells. The APC-dependent mechanism follows the classical MHC-II presentation pathway. This mechanism broadens the ADA specificity leading to the recognition and binding of multiple parts of the therapeutic Ab making the formation of larger immune complexes possible (Gorovits et al., 2020).

Many bispecifics resemble normal IgGs but other formats are currently being explored (Ljungars et al., 2020). While there is an increase in the use of therapeutic mAbs in a broad

range of diseases, there are also many of them that would directly impair or boost the patient's immune system. These mAbs, also called immunomodulators, are more difficult to assess in the current immunogenicity in-vitro assays, thus making the prediction of the risk for immunogenicity burdensome (Joubert et al., 2016).

1.4.5. Pre-clinical immunogenicity assessment

It is only recently that the preclinical immunogenicity assessment appeared in the health authorities recommendations, but with the tragedy observed for some therapeutic Abs in development, it was getting increasing interest, not only by regulators, but also from various research groups (Gorovits et al., 2020; Sauna et al., 2020). There are major implications of the clinical and preclinical immunogenicity strategy especially with the biosimilars hitting the market. It would not be acceptable to introduce a biosimilar in a clinical trial, which would induce an immunogenic reaction because of a different glycosylation pattern or a formulation component (S. Cohen, Myneni, et al., 2021).

Preclinical risk assessment for immunogenicity has already proven its effectiveness as demonstrated for commercially approved anti-PCSK9 Ab candidates (evolocumab, alirocumab) but also anti-IL-17 Abs (secukinumab and ixekizumab), where a good relationship between the prediction outputs to the clinical outcomes measured as clinical immunogenicity was described (Jawa et al., 2022). Therefore, most pharmaceutical companies now have a strategy in place to detect the main liabilities and the potential risk for immunogenicity. However, most of the time, this strategy is applied retrospectively. Most of the immunogenicity experts are conscious of the complexity of this immune reaction and recognize that no single assay or strategy may be used for all products (Ducret et al., 2021a). Thus, further improvements in the individual tools alone are unlikely to resolve important impediments to accurate predictions of immunogenicity in a clinical setting (Rosenberg & Sauna, 2018). However, taking a holistic approach and assessing the different steps leading to the increased risk for immunogenicity could help to develop safer molecules for patients. In light of these

limitations, it is important to consider all the different assays published in the literature, identify their strengths and foremost identify the gaps (validation, limitation of the protocol…).

1.4.5.3. *In silico* approaches for Ab properties and T cell epitope content

In silico, tools are databases that can be used to get a first level of information on the potential for immunogenicity. Immune Epitope Database (IEDB), is the main source of information on potential peptides that could be presented as pMHC-II complexes. About 16,000 MHC-II epitopes are annotated as positive (capable of binding an MHC-II receptor) which represent half of the total database. Repiotpe (https://github.com/masato-ogishi/Repitope) make use of this database to focus on the propensity of an epitope to induce a T cell response, by predicting the pMHC-II-TCR interaction according to the peptide sequence (Ogishi & Yotsuyanagi, 2019a). They use the binding affinity of a pMHC-II complex to a small portion of the CDR3 loop and are refined by integrating the additional interactions. However, they demonstrated that none of the 27 epitope phys-chem properties tested could be linked to an increased risk for immunogenicity (Ogishi & Yotsuyanagi, 2019b).

In silico prediction tools have been available for several years now, but they are gaining a lot of interest lately as they continue to get more predictive. The immunogenicity assessment should be done as early as possible during drug development. Indeed, the earliest the safety liabilities are detected, the easiest it is to select the appropriate candidate or to remove the potential T cell epitopes. In this context, where hundreds of molecules are still envisaged, *in silico* tools are very valuable to make a first selection of the candidates with the least sequence liabilities. One of the most advanced algorithms is called NetMHCIIPan (Reynisson et al., 2020a) and analyzes 15-mer peptides along the protein sequence for their binding affinity to all possible HLA alleles. This information gives a first indication of the presence of potential T cell epitopes (with the reserve that it can be presented on the surface as a pMHC-II complex and active the cognate T cells) contained in the protein sequence. This algorithm is now, in its version 4, informed by *in vitro* data, namely from peptide-MHC-II binding experiments but also MHC-II Associated Peptide Proteomics (MAPPs, which will be detailed later), thus improving

its reliability. Other companies have been developing such algorithms as well, with the aim of providing them as a service to drug developers.

B cell epitopes are believed to be most probably three dimensional, but the majority of the reported and deposited epitopes in databases are linear and it has been postulated that they likely result from antigens degraded proteolytically by various immune cells and subsequently exocytosed (Hebditch & Warwicker, 2019a). Additionally, marginal zone B cells are capable of hijacking the pMHC-II complex presented on circulation DCs, via the process of trogocytosis (Schriek et al., 2022). This process is also in favor of prioritizing linear epitopes over the three dimensional ones for which few data and only limited *in silico* prediction are available.

Lonza's AggreSolveTM and other *in silico* tools can also be used to detect Ab regions prone to induce aggregation (Chae et al., 2021a). Even though this algorithm does not predict directly a risk for immunogenicity, aggregation increases the probability of mounting an immune response (Ratanji et al., 2014).

Other biophysical properties of Abs can be analyzed as for other proteins (stability, charge patches, hydrophobicity…). Even if these properties are not directly correlated with immunogenicity, unfavorable properties are seen as increased risk factors when developing therapeutic Abs.

1.4.5.4. Assess the DC internalization propensity of Abs

In most of the assay looking at the contribution of DCs to an immune response, monocyte derived DCs (moDC) are used as surrogate. Indeed, DCs are rare in the circulation and hence difficult to isolate from blood. However, monocytes make up to 25 % of Peripheral Blood Mononuclear Cells (PBMCs) and can serve as progenitors for DCs after differentiation with IL-4 and GM-CSF (Granulocyte macrophage colony-stimulating factor). They are thought to behave similarly to circulating DCs, which is making them very attractive to study. The scheme of IL-4 administration has major implications for the moDC phenotype (Sander et al., 2017). The control of their differentiation is then of utmost importance in in-vitro assays to ensure consistency.

Different mechanisms are contributing to internalization into moDCs. The main driver in assays using immature moDCs is macropinocytosis, as described in Kraft et al., 2019a. Other mechanisms playing a role in DC internalization and already mentioned in the “Professional APCs: Dendritic Cells” section are the clathrin-dependent, receptor-mediated, and caveolae-mediated endocytosis (Trombetta & Mellman, 2005).

One way of assessing the internalization of a protein into DCs is to compare the fluorescence of a labeled protein between incubations at 4°C and at 37°C (Jambari et al., 2021). At 4°C, the cellular processes are stopped and the residual fluorescence detected is solely due to protein binding (an Ab to a cell surface receptor for example). On the other hand, an increase in fluorescence at 37°C is the result of protein internalization. Other DC internalization assay formats were already described to measure internalization into APCs (Melendez et al., 2022); (Kovalova et al., 2020), (Wen et al., 2020); (Xue et al., 2016b) using different readouts (e.g., targeting the human Ig Fcγ fragment with a labeled F(ab’)2, Fluorescence Resonance Energy Transfer based where the fluorophore and its quencher are separated upon lysosomal degradation, microscopy).

However, rapid internalization into DCs will not necessarily result in their activation (S. Cohen & Chung, 2021a) and do not strictly correlate with the onset and/or the intensity of the elicited immune response.

1.4.5.5. Assess the risk of DC activation

One of the main readouts for the activation of immune cells are cell surface receptors that can be followed by flow cytometry allowing a precise characterization of the population at a certain time point. This is one of the limitations of flow cytometry and requires the optimization of the timing of the readout. In general, CD11c+ cells are considered the closest to circulating DC and CD209 is considered as a specific marker for in vitro differentiated DCs (Clark et al., 2018). These two markers, along with the downregulation of CD14, ensure that the monocytes have followed an appropriate differentiation into moDCs.

The assessment of the DC activation status has been mostly studied in the context of protein aggregates. In this context, *in vitro ex vivo* models have been proposed to look at moDCs activation markers (Ahmadi et al., 2015; Groell et al., 2018; Morgan et al., 2019). CD40, CD80, CD83, CD86, CD209, HLA-DR, are the main moDCs surface markers that have been assessed to estimate the activation status of moDCs. In certain cases, even with protein aggregates, activation of moDCs is hardly detectable *in vitro*, making the development of such assay for non-stressed mAbs a challenge. Recently, an assay looking at DC activation upon treatment with non-modified Abs was published together with a thorough validation (Wickramarachchi et al., 2020). Cell density was an important aspect of this assay format over others used in the past. Here, the cells were differentiated in a 96 well plate at 1.9 million cells/cm². Nonetheless, the authors demonstrated that ATR-107 could induce the increased surface expression of CD86, CD40 and CD274 on moDCs as well as inducing the secretion of TNF-α and IL-13 (Xue et al., 2016a). Other activation markers showed great promises, like CCL22, which showed a strong correlation with the inflammatory status of DCs (Sander et al., 2017). Multiplex cytokine release assays have also been used to assess the activation status of moDCs but detected low treatment-induced variation, looking at sarilumab, elotuzumab, trastuzumab and ustekinumab after modifications (e.g., deamination, isomerization) (Zeunik et al., 2022).

1.4.5.6. Assess the T cell epitope presentation

The first assays looking at peptide presentation were focused on the binding affinity of a peptide (usually 20-mer) to MHC-II receptors (Perry et al., 2008). These were also the first datasets used to inform *in silico* algorithms. However, some of the limitations of these methods were the coverage of the MHC-II receptor diversity and more importantly the lack of information on the processing leading to these peptides. Indeed, there is no certainty that all 20-mer peptides could be naturally derived from a molecule and presented as pMHC-II.

To circumvent this, the MHC-II Associated Peptide Proteomics (MAPPs) assay has been developed by Roche and patented (HARALD 2000). This is probably the more widely used

assay to inform immunogenicity risk assessment across industries with a relative consensus on the protocol details. In short, moDCs are differentiated from blood monocytes, challenged with the treatment of interest and lysed. An immunoprecipitation is then performed on the lysate to purify the pMHC-II complexes that will be dissociated and the peptide analyzed by mass spectrometry.

In this assay moDC activation with LPS is used to stabilize and increase the number of MHC-II receptors expressed on the surface of the moDCs and potentially pMHC-II derived from the treatment of interest. The overexpression of MHC-II is only transient but is stabilized upon TLR-4 engagement (Cella et al., 1997). LPS is preferentially used over TNF-α to provide the activation signal to the moDCs as, unlike TNF-α, LPS is not presented via MHC-II receptors and therefore interfere with peptide presentation (Quarmby et al., 2018). To increase even more the sensitivity of this assay, sepharose beads historically used were replaced by magnetic beads and in certain cases the immuno-precipitation could be entirely automated. These optimizations allowed the use of fewer cells for the assessment of one treatment, down to 1.4 million moDCs, leaving room to test different treatments within the same donor for better comparison (Sekiguchi et al., 2018).

MAPPs cannot be used alone to rank molecules because detected epitopes (also referred to as clusters) could induce tolerance by stimulating Tregs that suppress the immune response toward the biotherapeutic (S. Cohen & Chung, 2021a) or be devoid of specific T cells. Indeed, it is hypothesized that certain sequences in the constant domains of Abs (Fab, Fc) could exert a central role in immune tolerance. The proposed mechanism of action is that these epitopes when being presented would induce the production of regulatory cytokines (Matucci et al., 2019) and immune tolerance to other co-administered antigen (Su et al., 2013). Additionally, the stability of the peptide, or epitope, within the MHC-II binding groove is most of the time unknown (Gorovits et al., 2020) and might not bind strongly enough to interact with a specific CD4+ T cell.

Competitive binding assays could be used to assess the capacity of DCs to present peptides on their surface. In short, the interaction of this TCR with its specific epitope is measured in the presence of the test article; a decrease in the DC:T interaction would imply that the DCs present more of the test article derived epitopes (Tasker et al., 2021). However, there is more interest in having a qualitative assessment of the peptide presentation (which regions are prone to be presented) than a quantitative assessment (how well does it compete with another epitope).

1.4.5.7. Assess the consequences of the DC: CD4+ T cell interaction

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 biotherapeutic-derived epitopes. However, the complexity of the immune response and the variety of interactions involved led to the development of several assay formats probing various effector functions and outcomes. In addition, the nature of the evaluated biotherapeutic and mode of action may also dictate, which T cell activation assay, must be used. In a PBMC-based assay, the surface expression of CD134 and CD137 on $CD4^+$ T cells was detected upon treatment with marketed biotherapeutics with high clinical immunogenicity (S. Cohen, Myneni, et al., 2021), efficiently evaluating the risk for immunogenicity. There are other assays looking at T cell activation in PBMCs but finding a good readout for such assays is challenging. Surface receptor expression by flow cytometry has been one the main readouts used, allowing the focus on a specific T cell population (CD45, CD3, CD4 positive cells for example to focus the analysis on helper T cells). CD69 has been described as the most stable activation marker for T cells, even if the cytokines, TNF-α and IFN-γ are good alternatives (O'Donoghue et al., 2021). CD40L, CD25, and CD46 have been proposed as good alternative indicators of T cell activation *in vitro* (Groell et al., 2018), for which, both the proportion of positive cells and intensity (abundance of the receptor) are important (Richard et al., 2021). It has been shown that $CD8^+$ T cells dampen the $CD4^+$ T cells response *in vitro*, therefore, the addition of a $CD8^+$ depletion step would ameliorate the sensitivity of PBMC-based T cell assays (Walsh et al.,

2020). However, PBMCs also lack the functional mature and professional APCs matching T cell subsets with different effector functions.

Therefore, more complex assay systems, where moDCs and CD4+ T cells are generated side-by-side and put together, controlling for the ratio between the two cell types were developed. This assay format ensures that the CD4+ T cells are not directly in contact with the test article and would see only the epitopes presented by the moDCs. Additionally, irradiated APCs can be used as good initiators of a T cell response, as they would not proliferate and influence the readout of the assay (S. Cohen & Chung, 2021a; Schultz et al., 2017). A ratio of 1:10 (moDC:CD4+ T cell) is usually described as a co-culture ratio between the two cell types (Gorovits et al., 2020), which reflects the capability of a DC to interact with multiple T cells for a more efficient scanning of the presented peptides. Moreover, it increases the odds of a specific interaction to happen, as the specific T cells are probably rare, as discussed earlier. During the co-culture, it is possible to follow the antigen dependent proliferation of the T cells by carboxyfluorescein succinimidyl ester (CFSE)-labeled incorporation (Jambari et al., 2021). However, various readouts can be associated with this type of assay, like thymidine incorporation (Ito et al., 2019; Jankowski et al., 2019; Joubert et al., 2016), ELISpot (Chalasani et al., 2019; Joubert et al., 2016) or fluoroSpot assessed after a second stimulation round to amplify the T cell signal. This principle is used in the currently applied DC: CD4+ T cell restimulation assay platform (Siegel et al., 2022). There are few examples showing the uses of in vitro assays focusing on CD4+ T cell activation. Infliximab, rituximab, adalimumab and natalizumab have been tested in such assay format and their increased risk for immunogenicity could be detected (Schultz et al., 2017). In another study, the combination of in silico prediction of potential T cell epitopes, using EpiVax with a DC:T cell assay format could demonstrate that 19 amino-acid exchanges were needed to reduce FVIII-induced T cell proliferation (Winterling et al., 2021).

Currently, *in vitro* T cell activation assessment is widely used, however, no *in vitro* assay looking at B cell activation upon treatment with therapeutic Abs. However, it is believed that

co-localization of the therapeutic Ab with the B cell surface should be enough to characterize the risk for B cell activation (Greenfield et al., 2021b).

In vivo models for a mechanistic approach to immunogenicity

Only a few in vivo models were described as valuable tools for immunogenicity prediction. Indeed, immunogenicity is considered as a breakage of immune tolerance, and therefore, when testing mAbs in animal models, they are by nature foreign and will elicit an immune response. To circumvent that issue, Bessa et al. developed an hIgG1 transgenic mouse model. The murine Ab repertoire has been modified to express the heavy chain (IgH-gamma 1) and light chain (Ig-kappa and Ig-lambda) transgenes. As a consequence, these mice express human circulating IgG1 making them tolerant to most of the Abs of the same isotype (Bessa et al., 2015). This model can give mechanistic insight for immunogenicity but also a readout for B cell activity through ADA quantification and analysis of their specificity by ELISA. However, species cross-reactivity and the T cell repertoire are still major limitations of those models for immunogenicity studies. Other humanized mouse models were described but are rarely used for immunogenicity testing (PBMCs transferred, bone marrow, liver and thymus of human origin transferred into a mouse, BLT model…) (Morillon et al., 2020; Yan et al., 2019). The human HLA transgenic mice were successfully used in the study of autoimmune diseases as reviewed elsewhere (Gregersen et al., 2004). This model overcomes the limitations around the T cell repertoire mentioned above, as they arise from the same epitopes as in humans. However, it does not reflect the polymorphism of the human HLA and the mice are not tolerized against human proteins, limiting their value for immunogenicity study of human or humanized therapeutic Abs.

1.4.6. Outlook

Despite their widespread use, a thorough validation of the assays used to evaluate the risk for immunogenicity of therapeutic mAbs is lacking. (Paul et al., 2020). There is also the need for

“fit for purpose” validation of the assays. Along with the assay, a robust data reporting has to be proposed, thresholds to identify positive hits have to be statistically validated based on background stimulation variability, positive and negative assay controls, but also donor variability when the assay is using primary cells (Wickramarachchi et al., 2020).

Improvement of the preclinical immunogenicity risk assessment will certainly help to decrease the drug development costs and time by bringing safer medicine to the patients and terminating early enough the ones that bear a high risk for adverse effects. This strategy will not only accelerate drug development, but also enable another horizon for personalized medicine, where informed decisions on treatment would be taken based on the predicted immunogenicity, efficacy and safety of a given product in a given patient (Tourdot & Hickling, 2019).

2. Material and Methods

2.1. Compounds

Stock solutions of keyhole limpet hemocyanin (KLH-Imject Maleimide-Activated mcKLH, Thermo Fisher Scientific, #77600) were reconstituted and stored at -80°C in single-use aliquots according to the manufacturer's recommendations under sterile conditions. CD44 antibody variants (CD44var20, CD44var104, CD44var1, CD44var27, CD44var112) bearing different charge patches were produced internally (by collaborators from Roche Innovation Center Munich, Penzberg). The initial variants were further engineered with the addition of the main CD4+ T cell epitope derived from ovalbumin (ISQAVHAAHAEINEAGR) into the CH1 domain (Rasmussen 2001). The latter were used to assess the consequences of altered internalization on CD4+ T cell activation with a common epitope.

All biotherapeutics (see Table 1); were bought from Runge Pharma GmbH & Co in their respective formulation and stored according to the manufacturer's recommendations. Peptides were synthesized by Cambridge Research Biochemicals and reconstituted in sterile ultra-pure water (Invitrogen, #10977015) and 50 % Acetonitrile (≥99.95%, VWR, #83639.320).

Table 1. Overview of the test items and their respective clinical ADA rates. Therefore, considerations should be taken when looking at the reported ADA rates. The information was extracted from FDA labels (https://labels.fda.gov/; accessed on 24 October 2022). If several clinical ADA rates were reported, studies mentioning a co-treatment were excluded and mean value for the remaining study outcomes was taken.

Antibody name	**Trade name**	**Format**	**Target**	**Main target patient population**	**Clinical ADA rate**
Adalimumab	Humira	Human IgG1	TNF-α	Rheumatoid Arthritis	23
Alemtuzumab	Lemtrada	Humanized IgG1	CD-52	Multiple Sclerosis	35
Alirocumab	Praluent	Human IgG1	PCSK9	Cardiovascular disease	5
Atezolizumab	Tecentriq	Human IgG1 no-Glyco	PD-L1	Non-Small-Cell Lung Carcinoma (NSCLC)	44
Avelumab	Bavencio	Human IgG1	PD-L1	Urothelial Carcinoma	17
Benralizumab	Fasenra	Humanized IgG1	CD-125	Asthma	13
Bevacizumab	Avastin	Humanized IgG1	VEGF	Solid Tumor	0.6
Brentuximab	Adcetris	Chimeric IgG1-ADC	CD-30	Classical Hodgkin Lymphoma (late stage)	30
Bococizumab	NA	Chimeric-IgG2	PCSK9	Cholesterol	48
Briakinumab	NA	Human IgG1	IL-12/IL-23	Rheumatoid arthritis	NA
Certolizumab	Cimzia	FabPEG	TNF-α	Crohn Disease and Rheumatoid Arthritis	8
Cetuximab	Erbitux	Chimeric IgG1	EGFR	Head, Colorectal and Neck Cancer	5
Daratumumab	Darzalex	Human IgG1	CD-38	Multiple myeloma	0
Durvalumab	Imfinzi	Human IgG1	PD-L1	Locally advanced or Metastatic Urothelial Carcinoma, NSCLC	3
Elotuzumab	Empliciti	Human IgG1	SLAMF7	Multiple Myeloma	27
Evolocumab	Repatha	Human IgG2	PCSK9	Cardiovascular Disease	0.3
Galcanezumab	Emgality	Humanized IgG4	Calcitonin	Migraine	5
Infliximab	Remicade	Chimeric IgG1	TNF-α	Psoriatic Arthritis	27
Ipilimumab	Yervoy	Human IgG1	CTLA-4	Metastatic melanoma, advanced renal cell carcinoma, metastatic colorectal cancer	8
Ixekizumab	Taltz	Humanized IgG4	IL-17a	Plaque Psoriasis	8.4
Necitumumab	Portrazza	Human IgG1	EGFR	NSCLC	4
Nivolumab	Opdivo	Human IgG4-CPPC	PD-1	NSCLC	11
Pembrolizumab	Keytruda	Humanized IgG4-CPPC	PD-1	Cancer	2
Sarilumab	Kevzara	IgG1	IL-6R	Rheumatoid Arthritis	9
Secukinumab	Cosentyx	Human IgG1	IL-17a	Psoriasis	1
Tocilizumab	Actemra	Humanized IgG1	IL-6R	Rheumatoid Arthritis	2

Trastuzumab	Herceptin	Humanized IgG1	HER2	Breast Cancer	1
Ustekinumab	Stelara	Human IgG1	IL-12/IL-23	Plaque Psoriasis	6
Utomilumab	NA	Human IgG2	4-1BB	Solid tumor	NA
Vedolizumab	Entyvio	Humanized IgG1	Integrin α4β7	Ulcerative colitis and Crohn's disease	6

For the DC internalization assay, antibodies were labeled using the SiteClick Antibody Azido Modification Kit (Thermo Fisher, #S20026) according to the manufacturer's instructions. Briefly, N-linked galactose residues of the Fc region were removed by β-galactosidase and replaced by an azide-containing galactose via the β-1,4-galactosyltransferase. This azide modification enables a copper-free conjugation of sDIBO-modified dyes. The pH-sensitive amine-reactive dye was coupled to a sulfo-DBCO PEG4 amine. Antibodies were labeled with a molar dye excess of 3.5. Excess dye was removed using the Amicon Ultra-2 Centrifugal Filter (Merck, #UFC205024) with a cutoff at 50 kD and antibodies were re-buffered in 20 mM histidine 140 mM NaCl buffer (pH 5.5). The absorbances of the labeled molecules at 280 nm and 532 nm were determined using a Nanodrop spectrometer and the concentration [1] as well as the dye-to-antibody ratio (DAR) [2] was calculated as follows.

$$c(AB) = [A280nm - [A280nm * CF(Dye)]] / \varepsilon(AB) \quad [1]$$

$$DAR = [A532nm * MW(AB)] / [c(AB) * \varepsilon(Dye)] \quad [2]$$

(A = absorbance; AB = antibody; c = concentration; DAR = dye to antibody ratio; ε (dye) = extinction coefficient dye = 47225; CF = correction factor = 0.36)

To confirm the efficient removal of unbound dye and to exclude possible antibody aggregates or fragments, a size exclusion chromatography of the labeled antibodies and their unlabeled counterparts was performed. Samples were separated using a BioSuite Diol (OH) column (Waters, 186002165) with a potassium dihydrogen phosphate buffer (pH 6.2) as the mobile phase at a flow rate of 0.5 ml/min. Detectors at 280 nm and 532 nm were used to quantify and analyze the labeled antibodies.

2.2. Epibase® DC: CD4+ re-stimulation assay (outsourced at LONZA)

Healthy donors were recruited at Phase I clinical trial units in the UK. All samples were collected under an ethical protocol approved by a local Research Ethics Committee (reference number: 21/LO/0474), and written informed consent was obtained from each donor prior to sample donation. All samples were stored according to the terms of Lonza's Human Tissue Authority license for the use of samples in research. Peripheral blood mononuclear cells (PBMC) from healthy donors were prepared from whole blood or leukopaks using Lymphorep density gradient medium (Cedarlane, # CL5120) within six hours of blood withdrawal. PBMC were controlled-rate frozen and stored in vapor-phase nitrogen at -196°C until used in the assays. The quality and functionality of each PBMC preparation were analyzed after seven days of activation, with positive controls such as KLH to assess naïve T cell responses. For each screen, the donor cohorts consisted of typically 30 donors selected to represent the world population in terms of their HLA-DRB1 allele frequency distribution. Monocytes were isolated from frozen PBMC samples by magnetic bead selection using CD14 microbeads (Miltenyi Biotec # 130-050-201 on an AutoMACS Pro system) and differentiated into immature DC (iDC) using 1000 IU/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF) and 1000 IU/mL of IL-4 in a serum-free medium (Cell-Genix # 20805-0500, supplemented with 0.05 mg/mL Gentamicin Lonza # 17-518L) for 5 days at 37 C, 5% CO2. iDC were then harvested, washed and loaded with each test protein/peptide individually for 4 h at 37 C, 5% CO2. A DC maturation cocktail containing TNF-α (800 IU/mL) and IL-1β (100 IU/mL) was then added for a further 40–42 h to activate/mature the DC (mDC). The expression of key DC surface markers (CD11c-3.9, CD14-63D3, CD40-5C3, CD80-2D10, CD83-HB15E, CD86-BU63, CD209-9E9A8 and HLA-DR-L243) at both the immature and mature stage were assessed by flow cytometry (Bio-Rad ZE5 Cell Analyzer) to ensure the DC were activated prior to T cell interaction. After a thorough washing procedure, 100,000 mDCs were then co-cultured with 1

million autologous CD4+ T cells (isolated by magnetic bead selection, Miltenyi Biotec # 130-045-101 on an AutoMACS Pro system) in a deep-well plate (final volume of 1.2 mL, Greiner # 780271). The DC:CD4+ T cells ratio is 1:10 and the co-culture is incubated for 6 days at 37 C, 5% CO2 in a humidified atmosphere. On day 6, autologous monocytes were isolated from PBMC using magnetic bead selection (Miltenyi Biotec # 130-050-201 on an AutoMACS Pro system) and loaded with the selected protein or peptide that were initially used to load the DC. After incubation at 37 C, 5% CO2 in a humidified atmosphere for 4 h, the monocytes were washed and then added to anti-IFN-γ pre-coated FluoroSpot plates (Mabtech # FSP-0108-10) along with the corresponding DC:CD4 co-culture in quadruplicate (25,000 monocytes: 250,000 CD4+ T cells in a final volume of 200 L). The FluoroSpot plates were incubated for 40–42 h at 37 C, 5% CO2 in a humidified atmosphere. After incubation, the FluoroSpot plates were developed according to the manufacturer's procedure (IRIS FluoroSpot reader, Mabtech) and the number of spot-forming cells (SFC) per well were assessed for each test condition in an automated and unbiased manner. Data management and statistical analysis were performed in the R programming language (https://www.R-project.org/, accessed on 28 October 2022, versions 3.6.1 up to 4.1.2), including essential packages for handling generalized linear models ("nlme", "emmeans") and carrying out variance component analyses (VCA, version 1.4.3). The calculation of Stimulation Indices (SI) was performed as follows. Spot forming cells (SFC) from the FluoroSpot assay were transformed to a log2 scale, and a generalized linear model (GLM) was applied to estimate the SI (i.e., the ratio between a treatment condition and the donor-matched blank on a linear signal scale) and associated confidence intervals. Quadruplicate SFC measurements were implicitly aggregated by the GLM to yield one SI value for each combination of a specific test compound, donor, and screen. The screens were analyzed sequentially and independently from each other, with the linear model considering a specific cytokine readout of an entire screen as input. The processing workflow was tailored to address a few peculiarities of the given data. Specifically, we used an exponential type of heteroscedasticity adjustment in the GLM to achieve scale-

invariance of residuals and injected some Gaussian noise at the low end of the SFC scale to support model convergence with the frequent presence of ties of discrete values around zero. (The standard deviation of this normally distributed, zero-centered noise was chosen to correspond to the replicate variability inferred by the GLM in the limit of zero SFC counts at the low end of the SFC scale and drops down exponentially by a factor of exp (-2) = 0.14 for every unit increase of the log2 SFC). Furthermore, we observed a consistent trend in the data to the effect that higher blank values of a donor corresponded to systematically lower SI values for that donor. The relation between 'pre-stimulation' of the blank and observed stimulation indices could be well captured by linear regressions performed for each treatment within a screen. We corrected the raw SI values then for every donor treatment pair with the respective linear model, basically extrapolating to the value which would have been observed with a common blank value of 0. Standard quality control plots were generated for every data set, including the visualization of DC differentiation markers, the reproducibility of reference compound data across studies, and (if possible) the variability of repeated compound testing with the same donor. We also looked at the individual stimulation profile of each donor within a study, as the overall inducibility of T-cell response could vary from person to person; simultaneously, this enabled us to rule out the presence of generally inert sample material. A donor response was recorded as "positive" if a SI fold-change of 2 or above (compared to its blank control) was measured at a statistical significance of $p < 0.05$ (using non-adjusted p-values from the GLM). The fraction of positive donor responses (within a cohort of typically 30 healthy donors per screen) provided the response rate for the treatment in a specific screen.

2.3. DC Internalization Assay (DCIA)

Buffy coats from healthy human volunteers were supplied by the Blood Donation Centre (SRK Aargau-Solothurn, Switzerland) according to current ethical practices. Subsequently, human peripheral blood mononuclear cells (PBMC) were isolated by gradient density centrifugation

using Ficoll-Paque PLUS (GE Healthcare Europe GmbH, Glattbrugg, Switzerland,GE Healthcare #17-1440-03) according to the manufacturer's instructions.

For further enrichment of monocytes, magnetic activated cell sorting was performed using anti-huCD14 beads (Miltenyi, #130-050-201) and LS columns (Miltenyi, #130-042-401) according to the manufacturer's instructions. Briefly, monocytes and beads were incubated in MACS Buffer for 15 min on ice and separated using a magnet. The isolated monocytes were suspended in pre-warmed serum-free Cellgro (CellGenix #20901-0500) medium containing 1% GlutaMAX (GIBCO #35050-061), 1% penicillin/streptomycin (GIBCO #15140-122), 1% MEM Non-Essential Amino Acids Solution (GIBCO #11140-035), 1 mM Sodium Pyruvate (GIBCO #11360-039), 5 ng/mL recombinant human IL-4 (rhIL-4, R&D systems, #204-IL) and 50 ng/mL rhGM-CSF (R&D system, #215GM-500) on ultra-low attachment culture dishes ($0.3x10^6$ cells/ml, Corning, #354407) differentiated into moDCs at 37 °C with 5% CO2 for 5 days.

On the day of the experiment, cells were detached from the ultra-low attachment culture dishes by pipetting and plated into ultra-low attachment 96-well plates at a density of 8x104 cells/well (50µl/well). Antibody solutions were prepared at a concentration of 400 nM in Cellgro (CellGenix #20901-0500) medium and 50 µl were applied to the cells for a final concentration of 200 nM. Cells were incubated for two and four hours at 37°C and 5% CO2. Here, the labeled antibodies with the pH-sensitive dye, are used in order to detect the increased fluorescence linked to the internalization of the Abs into more acidic cell compartments (e.g., lysosomes). Cells were transferred into U-bottom 96-well plates for sedimentation (300 g, 5 min), the pellet was washed with 200 µl ice cold PBS, centrifuged and resuspended in 200 µl FACS buffer containing 50 ng/mL DAPI. The mean fluorescent intensity (MFI) of the internalized antibodies was acquired using a Fortessa X20 flow cytometer (BD) equipped with a 532 nm-emitting laser. Signals were collected at 572 nm ± 35 nm. The exact same conditions, gains, and gates were used for all time points. Data extraction was performed using the FlowJo-V10.8.1 software (BD Life Sciences). Cells were gated for singlets, morphology and viability. Values

of the negative control were subtracted from all geo-mean values followed by normalization to the Dye to Antibody Ratio (DAR). The normalized geo-mean values from each antibody were plotted as a linear regression curve using R Statistical Software (v4.1.2; R Core Team 2021) to extract the slope (Geo Mean MFI/min for 120 and 240 min). Statistical significance of differences in internalization rates were calculated by one-sided paired t-test. Statistical analysis was performed using R. Significance level: $p < 0.0001$= ****; $p < 0.001$= ***; $p < 0.01$=**; $p < 0.05$= *; not significant= ns.

2.4. DC Activation Assay

Monocytes were isolated as described in the above section (“DC Internalisation Assay (DCIA)”) and suspended in pre-warmed serum-free Cellgro (CellGenix #20901-0500) medium containing 1% GlutaMAX (GIBCO #35050-061), 1% penicillin/streptomycin (GIBCO #15140-122), 1% MEM Non-Essential Amino Acids Solution (GIBCO #11140-035), 1 mM Sodium Pyruvate (GIBCO #11360-039), 10 ng/mL rhIL4 (R&D systems, #204-IL) and 100 ng/mL rhGM-CSF (R&D system, #215GM-500) on ultra-low attachment 96-well culture plates (200 µL, $3x10^6$ cells/ml, Corning, #3262) differentiated into moDCs at 37 °C with 5% CO2 for 5 days. On day 5, the cells were seeded and half of the medium was replaced by the treatment of interest containing medium (100 µL at 600 nM for a final concentration of 300 nM) and incubated for 48 hours at 37°C and 5% CO_2.

The cells were then centrifuged (300 g, 5 min) and resuspended in 200 µL PBS containing a Fixable Viability Stain BV510 (BD, #564406) and a FcR blocking agent (Miltenyi, #130-059-901) for 15 minutes at room temperature. The medium was changed for the Ab master mix composed of CD80 BUV 737 (clone L307, BD, #741865), HLA-DR FITC (clone G46-6, BD, #555811), CD40 BV786 (clone 5C3, BD, #740985), CD209 BV421 (clone DCN46, BD, #564127), CD11c BUV395 (clone B-ly6, BD, #563787), CD14 PerCP (clone M5E2, BioLegends, #301848), CD83 APC (clone HB15E, BD, #551073), CD86 PE (clone 2331, BD, #555658) diluted in brilliant stain buffer (BD, #566349) - PBS solution and incubated 30

minutes at 4°C. Cells were finally washed twice in FACS buffer and the fluorescence was acquired using the Fortessa X20 (BD). Data extraction was performed using the FlowJo-V10.8.1 software (BD Life Sciences). In short, cells were gated for singlets, morphology and viability. MFI were extracted for the different activation markers (CD80, HLA-DR, CD86, CD83, CD209 and CD40) on CD11c+ CD14- viable cells. Values of the non-treated control were used to calculate the Stimulation Index (SI) specific to each activation marker and individual. The SI were plotted for each treatment to compare for their moDCs activation capacity. An arbitrary threshold at SI = 1.4 was used to classify the individual as responder for the particular treatment and activation marker. Keyhole Limpet Hemocyanin (KLH, Sigma, #SRP6195) response was used as an inclusion criteria for an individual. For an individual to be considered in further treatment comparison, KLH response has to be positive (SI ≥ 1.4) for at least three of the tested activation markers. While for the treatment, a response with SI ≥ 1.4 for any 1 of the 6 populations was considered a positive response, as described for a similar protocol (Wickramarachchi et al., 2020). Statistical significance of differences in SI were calculated by a one-sided paired t-test. Statistical analysis was performed using R. Significance level: $p < 0.0001$= ****; $p < 0.001$= ***; $p < 0.01$=**; $p < 0.05$= *; not significant= ns.

2.5. Mass Spectrometry Associated Peptide Proteomics (MAPPs)

Monocytes were isolated as described in the above section (“DC Internalisation Assay (DCIA)”) and suspended in pre-warmed serum-free Cellgro (CellGenix #20901-0500) medium containing 1% GlutaMAX (GIBCO #35050-061), 1% penicillin/streptomycin (GIBCO #15140-122), 1% MEM Non-Essential Amino Acids Solution (GIBCO #11140-035), 1 mM Sodium Pyruvate (GIBCO #11360-039), 5 ng/mL rhIL4 (R&D systems, #204-IL) and 50 ng/mL rhGM-CSF (R&D system, #215GM-500) on ultra-low attachment culture dishes ($0.3x10^6$ cells/ml, Corning, #354407) differentiated into moDCs at 37 °C with 5% CO2 for 5 days. The day of the experiment, MAPPs assay was performed according to the standard protocol and analyzed

according to Steiner et al. ((Steiner et al., 2020)). In short, moDCs cells were challenged with the test protein at 300 nM in the presence of 1 µg/mL of lipopolysaccharide (LPS) from Salmonella abortus equi (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) for 24 h. Mature moDCs were harvested, washed with PBS and the cell pellets were frozen at −80 °C. Frozen cell pellets were lysed in 20 mM Tris-buffer solution pH 7.8 containing 1% (v/v) Digitonin and protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) for 1 h at 4 °C on a ThermoMixer at 1100 rpm. The HLA-DR immune complexes were isolated by immunoprecipitation using the biotin-conjugated anti-human HLA-DR monoclonal antibodies (clone L243, BioLegends). Lysates were incubated with the antibody on a rotator overnight at 4 °C. Samples were washed five times with a buffer containing 20 mM N-(2-hydroxyethyl)piperazine-N′-ethanesulfonic acid-NaOH (pH 7.9), 150 mM KCl, 1 mM MgCl2, 0.2 mM CaCl2, 0.2 mM ethylenediaminetetraacetate, 10% (v/v) glycerol, and 0.1% (v/v) Digitonin and five times with purified water. MHC-II peptides were eluted twice from HLA-DR molecules by adding 18 µL of 0.1% trifluoroacetic acid. The eluates were collected and analyzed by tandem mass spectrometry. MHC-II peptide preparations were separated on a nanocapillary liquid chromatography system (UltiMate 3000 RSLC, Thermo Scientific, CA, USA) using self-packed fused-silica C18 reversed phase column (75 µm i.d. × 170 mm, ReproSil-Pur C18-AQ, 3 µm, Dr. Maisch GmbH) connected to a Q-Exactive Plus/HF/HFX Orbitrap mass spectrometers (Thermo Scientific) via electrospray ionization (LC-ESI-MS/MS). Samples (10–20 µL volume dissolved in 0.5% (v/v) formic acid in 2% (v/v) acetonitrile/water) were loaded for 2–3 min at 10 µL/min onto an Acclaim PepMap C18 trap column (100 µm i.d. × 20 mm, Thermo Scientific) using a Vented Tee design. Peptides were then eluted at a flow rate of 250 nL/min using a nonlinear 39 min gradient of 2–45% B, followed by an 11 min column wash, and re-equilibration for 10 min [buffer A: 0.1% (v/v) formic acid in 2% (v/v) acetonitrile/water; buffer B: 0.1% (v/v) formic acid in acetonitrile]. MHC-II peptides were analyzed by tandem MS using standard operating parameters. Survey scans (scanning range m/z 400–1650) were recorded in the Orbitrap mass analyzer at a resolution of 60,000, with

the lock mass option enabled. Data-dependent MS/MS spectra of the 12 most abundant ions from the survey scan were recorded in the Orbitrap cell at a resolution of 15,000. Target ions selected for MS/MS were dynamically excluded for 15 s.

Peptides were identified using the most updated PEAKS Studio version available at the time (version 8.5, Bioinformatics Solutions Inc., ON, Canada). The raw MS data were searched against the human protein database UniProtKB (http://www.uniprot.org, release 2015_10, approx. 88,500 TrEMBL and SwissProt entries containing the amino acid sequences of the test therapeutic proteins) with a mass tolerance of ±10 ppm for precursor ions and ±0.025 Da for fragment ions. Met-sulfoxide, Asn/Gln deamidation, and N-terminal pyroglutamylation were considered as differential modifications. Data were searched without enzyme specificity, and peptide results were reported at 1% specFDR cutoff, if not reported differently otherwise. Label-free quantification was performed using the corresponding PEAKS module. All LC–MS/MS runs of a given donor were processed batchwise, and the areas under the curve of identified features (2 min retention time shift tolerance; features reporting option: all) were exported in a tab-delimited table without further normalization. One of the features of dataMAPPs pipeline is to record the peptides' associated peak area for enabling advanced QC and normalization procedures. The peak area is transformed to the log2 scale prior to processing.

Detected peptides were grouped into clusters and represented along the sequence of the corresponding antibody using Geneious Prime 2022.1.1 (https://www.geneious.com). A numerical estimation of the MAPPs assay outcome was calculated using the number of epitopes detected and their signal intensities like follows:

$n_{epitopes}$/ $total_{epitopes}$ x $mean_{signal}$ intensity

2.6. Estimation of the T cell precursor frequency

This assay has been performed according to the published protocol proposed by Delluc 2011. In short, moDCs were obtained according to the procedure detailed in the section above

(“Mass Spectrometry Associated Peptide Proteomics (MAPPs)”) and loaded either with KLH, CD44var1 or CD44var112 (300 nM) and matured over night with 1 ug/mL of LPS (Sigma-Aldrich #L5886). T cells were isolated from PBMCs by negative selection (Myltenyi, #130-096-533) as recommended by the manufacturer and co-cultured with previously loaded autologous moDCs. 10,000 loaded moDCs were co-cultured with 100,000 T cells (n=20 wells per condition) in a total volume of 200 uL of Iscove's Modified Dulbecco's Medium (IMDM, GIBCO #21980-032) supplemented with 10 % human serum (Sigma-Aldrich #H3667-100ml), 1000 U/mL rh-IL-6 (R&D Systems #7270-IL-025/CF), 10 ng/mL rh-IL-12 (R&D Systems #10018-IL-020) and incubated at 37°C and 5% CO2 ambient on ultra-low attachment 96-well plates (Corning #3474). The CD4+ T cells were re-stimulated on day 7 and 14 with fresh autologous moDCs loaded with one of the antibody variants or KLH, 10 U/mL of IL-2 (R&D Systems #202-IL-010/CF) and 5 ng/mL of IL-7 (R&D Systems #207-IL-010/CF). At day 21, the specificity of the CD4+ T cells was assessed by IFN-γ enzyme-linked immunospot (ELISpot) following the recommended procedure (Mabtech, #3420-4APT-10). To assess the T cell response against KLH, 3300 moDCs (either naïve or loaded with KLH 4 hours prior co-culture without LPS) were added to 10,000 CD4+ T cells (n=20) expanded for KLH. The T cell response directed against the two CD44 antibody variants was measured in relation to a single epitope (the one presented by the OVA peptide): 3300 moDCs (either naïve or loaded with ovalbumin (Fisher Scientific #A/1280/48,) 4 hours prior co-culture without LPS) were added to 10,000 CD4+ T cells (n=20) expanded for either CD44var1 or CD44var112 containing the main ovalbumin CD4+ T cell epitope as introduced earlier. Each of these co-cultures were evaluated in duplicate. For the estimation of the T cell precursor frequency, spots were counted in a computer-assisted video image analyzer (AID, Strassber, Germany). A response was considered positive when the spot count was increased by 2-fold compared to the well where non-loaded moDCs were added. The frequency of CD4+ T cell precursors was calculated as proposed in Delluc 2011, using the Poisson distribution:

frequency = -ln(negative wells/total wells tested)/(CD4 T cells/well)

Statistical significance was calculated using a one-sided t-test. Statistical analysis was performed using R.

2.7. In silico T cell epitope prediction

NetMHCIIpan-4.0 (Reynisson et al., 2020b) was used to predict potential T cell epitope content for the five antibody variants. The algorithm has been run for 13 DRB1 alleles (DRB1-0101, DRB1-0301, DRB1-0401, DRB1-0701, DRB1-0801, DRB1-0901, DRB1-1001, DRB1-1101, DRB1-1201, DRB1-1301, DRB1-1401, DRB1-1501, DRB1-1601) screening for binding affinities of 15-mer derived from the antibody sequences. The top 2 % of the hits were kept as strong binders and further analyzed. Detected peptides were grouped into clusters and represented along the sequence of the corresponding antibody using Geneious Prime 2022.1.1 (https://www.geneious.com).

3. Objective of the thesis

The number of patients suffering from immunogenicity related adverse events remains high and can lead to consequences on both patient safety and treatment efficacy, sometimes leading to the termination of clinical trials. Furthermore, the increased complexity of the therapeutic Abs format and their derived properties, has made the risk evaluation of immunogenicity even more challenging. Taken together, these elements highlight the need for the development of biotherapeutics with a better immunogenicity profile to reduce those adverse events and the overall development cost for these therapies. Currently available techniques to assess the risk of biotherapeutics' immunogenicity include *in-silico* algorithms, *in-vitro* assays and *in-vivo* experiments, which are already applied in practice to guide the selection of molecules with presumably better immunogenic profiles. However, these methods are presently not fully validated and therefore an integrated approach is considered to evaluate the potential immunogenicity risk (Davda et al., 2019). Indeed, the evaluation of the risk for immunogenicity will not be achieved by a single assay but could be helped by a better characterization of the existing ones and by the development of new assays providing a better understanding of the mechanisms leading to this immune reaction. Integrating the different assay readouts as early as possible in the development process would make it possible to optimize the developed biotherapeutic (e.g., CD4+ T cell epitope removal, improved properties). The main objectives of my thesis were therefore to better understand Abs properties that could lead to an increased risk for immunogenicity while improving the current preclinical evaluation applied to the biotherapeutics in development.

4. Publication 1

Enhanced immunogenic potential of cancer immunotherapy antibodies in human IgG1 transgenic mice

Jerome Egli, Stefan Heiler, Felix Weber, Guido Steiner, Timo Schwandt, Katharine Bray-French, Christian Klein, Sebastian Fenn, Gregor P. Lotz, Eugenia Opolka-Hoffmann, Thomas E. Kraft, Laetitia Petersen, Rebecca Moser, Jonathan DeGeer, ***Michel Siegel****, Daniela Finke, Juliana Bessa & Antonio Iglesias*

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The enhanced ADA production against the idiotype often displayed by cancer immunotherapy antibodies can lead to exposure loss and subsequently affect anti-tumor efficacy and cause undesired effects on safety. Most conventional therapeutic antibodies are now of human origin or humanized, and hence immunologically tolerated in most patients. In contrast, the contribution of additional factors, other than the protein sequence, to the clinical ADA rates- of some CitAbs, especially T cell engagers, remains poorly understood. Here, we used human immunoglobulin gamma 1 (IgG1) transgenic mice), which are immunologically tolerant to human IgG1, to study the immunogenicity of 13 conventional antibodies and 2 CitAbs.

We found that tolerance to non-germline encoded idiotypes is maintained in part by the function of neonatal Fc-receptor (FcRn). Additionally, the incorporation of T cell-engaging moieties was sufficient to revert tolerance and trigger ADA production directed to the idiotypes of these compounds. A potential mechanism has been proposed.

My main contribution was to perform the experiments related to the internalization of these constructs into mouse-derived DCs. I also participated in the project discussions and review of the manuscript.

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REPORT

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Enhanced immunogenic potential of cancer immunotherapy antibodies in human IgG1 transgenic mice

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ABSTRACT

Clinical anti-drug-antibody (ADA) responses represent a substantial obstacle to the development of efficacious therapeutic antibodies. The enhanced ADA production against the idiotype (Id) often displayed by cancer immunotherapy antibodies (CitAbs) can lead to exposure loss and subsequently affect anti-tumor efficacy and cause undesired effects on safety. Thus, ADA responses contribute to prolonged clinical development and high attrition rates. Most conventional therapeutic antibodies are now of human origin or humanized proteins, and are hence immunologically tolerized in most patients. In contrast, the contribution of additional factors, other than the protein sequence, to the higher rates of clinical ADA to certain CitAbs, remains poorly understood. Here, we used human immunoglobulin *gamma* 1 (IgG1) transgenic mice (named "hIgG1 transgenic mice" or "TG"), which are immunologically tolerant to human IgG1, to study the immunogenicity of 13 conventional antibodies and 2 CitAbs. We found that tolerance to non-germline encoded Ids is maintained in part by the function of neonatal Fc-receptor (FcRn). Additionally, the incorporation of T cell-engaging moieties like an interleukin 2 (IL-2)-based immunocytokine or a CD3ε-specific antigen-binding fragment (Fab) was sufficient to revert tolerance and trigger ADA production directed to the Id of these compounds. We postulate that T cell receptor or IL-2 receptor activation may result in activation of unresponsive T cells specific for the crystallizable fragment (Fc) that typically inactivate Id-specific B cells and mediate "linked-antigen tolerance". Reversal of this unresponsiveness by the action of CitAbs on T cells may be the cause of undesired ADA responses.

Abbreviations: ADA Anti-Drug Antibodies; BCR B Cell Receptor; BId Idiotype-specific B Cell; BiTE Bispecific T cell Engager; BMC Bone Marrow Chimeric Mice; BSA Bovine Serum Albumin; CDR Complementary Determining Region; CEA Carcinoembryonic Antigen; CIT Cancer Immunotherapy; CitAbs Cancer Immunotherapy Antibodies; DC Dendritic Cell; ELISA Enzyme-Linked Immunosorbent Assay; FcRn Neonatal Fc Receptor; FcγR Fc gamma Receptor; GM-CSF Granulocyte-Macrophage Colony Stimulating Factor; gMFI Geometric Mean Fluorescence Intensity; H Heavy Chain; IC Immune Complex; Id Idiotype; IgA Immunoglobulin alpha; IgG1 Immunoglobulin gamma 1; IL-2 Interleukin 2; IL-2R Interleukin 2 Receptor; IL2v Interleukin 2 Variant; IVIG1 Intravenous Immunoglobulin 1; KLH Keyhole Limpet Hemocyanin; L Light Chain; MAPPs MHC-associated Peptide Proteomics; MHC Major Histocompatibility Complex; PBMC Peripheral Blood Mononuclear Cells; PBS Phosphate Buffered Saline; SHM Somatic Hypermutation; scFv Single-chain Variable Fragment; TCR T cell Receptor; TFc Fc-specific T cell; TId Id-specific T cell; UV Ultraviolet; V Variable.

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Introduction

The broad clinical use of therapeutic antibodies has revealed that in some cases this treatment results in unwanted immune responses and the production of anti-drug antibodies (ADA). In some cases, ADA can cause substantial exposure loss that affects efficacy, with the potential to provoke undesired adverse events.[1] Presently, it is unknown which factors, in addition to foreign immunogenic protein sequences, contribute to the onset of ADA production by some therapeutic antibodies and not by others. In a recent survey, the collected ADA data from 60 clinical antibodies revealed that the majority (76%) of these antibodies display an immunogenicity rate of less than 10% and the minority (10%) of the antibodies tested cause an ADA rate higher than 20%.[2] Thus, most human or humanized conventional therapeutic antibodies are weakly immunogenic in humans. In contrast, bispecific, immunomodulatory antibodies frequently used in cancer immunotherapy (CIT) – herein referred to as CitAbs – appear to cause ADA with a higher incidence than expected, given their

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generally human/humanized origin.[1,3] Therefore, it is crucial to better understand the mechanisms involved in breaking tolerance toward CitAbs, leading to ADA responses.

CitAbs are engineered bispecific antibodies bearing binding arms directed at multiple targets.[4] They can cross-link agonistic receptors like the T cell receptor (TCR) or interleukin 2 receptor (IL-2R) on immune cells and a tumor antigen or checkpoint inhibitor on the respective target cancer cells in order to induce tumor cell killing by the immune cell.[5] Here, we aimed to study the immunogenic properties of CitAbs by making use of a transgenic system that provides immune tolerance to a broad range of human antibodies of the immunoglobulin *gamma* 1 (IgG1) isotype, the hIgG1 transgenic mouse.[6] As this transgenic system has previously been shown to sense immunogenic modifications of an otherwise tolerated human therapeutic antibody,[7] we reasoned that it would also help with the study of the immunogenicity of CitAbs preclinically. To this end, we first conducted a study to validate this transgenic mouse system using 13 commercially available conventional therapeutic antibodies with known clinical immunogenicity rates. The study revealed that the immunogenicity in hIgG1 transgenic mice is substantially reduced as compared to control mice. Then, we tested the immunogenic potential of CEA-IL2v (cergutuzumab amunaleukin) and CEA-TCB (cibitasamab), two prototypical CitAbs,[8,9] and investigated possible mechanisms responsible for the enhanced ADA responses they elicit in the hIgG1 transgenic mice.

In current models of immune tolerance, early expression of germline-encoded self-antigens in the bone marrow and the thymus predisposes B and T lymphocytes, respectively, for a lifelong state of unresponsiveness toward most self-proteins. The case of antibodies is peculiar because, although germline-encoded, the amino acid composition of the antigen-binding site can be further altered during the process of gene rearrangement (to assemble the variable (V) region) and of somatic hypermutation (SHM), which increases the affinity to antigen during maturation of antibody responses. Thus, the resulting array of complementary-determining regions (CDR) of mature, high-affinity antibodies that bind defined antigens can diverge substantially from corresponding germline-encoded sequences. Nonetheless, the variant amino acid segments of secreted antibodies during immune responses normally do not provoke antibody responses to the newly formed CDR combinations or idiotypes (Id), even if they deviate from the tolerated V regions. Similarly, the iterative clinical infusion of large quantities of antibody-based therapeutics does not generally result in immunogenic ADA responses. In a simplified experimental system, we made use of the mouse immune system, transgenically rendered tolerant to human antibodies, and asked the two basic questions: 1) How is tolerance to unforeseen V region epitopes (idiotypes) maintained? and 2) What causes the enhanced immunogenic properties of CitAbs, as compared to conventional antibodies?

The results of our study revealed two regulatory pathways associated with the Fc region of antibodies that downmodulate anti-Id ADA responses. We propose a model to explain the predominant tolerance to non-germline Id determinants and its breakdown by CitAbs and discuss mitigation strategies derived thereof.

Results

Immunogenicity of conventional antibodies

As described previously, hIgG1 transgenic mice are immunologically tolerant to a broad range of human IgG1 antibodies.[6] Here, we interrogated if this transgenic system could reproduce experimentally the clinical ADA incidence of 13 therapeutic antibodies of the IgG1 isotype surveyed by Dingman and Balu-Iyer.[2] We selected 5 antibodies with an immunogenicity rate higher than 10% and 8 antibodies with an immunogenicity rate lower than 10% (Table 1). According to regulatory documents and patents, cross-reactivity to the target is absent or only low in mice. Hence, ADA data in transgenic mice refer mostly to the intrinsic antigenicity, and not to the function-related immunogenic properties, of the antibodies analyzed.

Table 1 Comparison of clinical ADA rates and experimental in vivo immunogenicity of 13 selected human IgG1 clinical mAbs. Table 1 lists the 13 clinical mAbs with reported clinical ADA rates and experimental in vivo immunogenicity (Incidence; IGF) observed in hIgG1 transgenic (TG) and wild-type (WT) mice. In addition, the target, format, and highest V gene identity to the closest VH and VL gene of the transgene or germline is indicated in percent.

				IGF (2)		Incidence [%] (3)		Identity TG [%] (4)		Identity WT [%] (4)	
Compound	Target	Format	Clin. ADA Incidence [%] (1)	TG	WT	TG	WT	VH	VL	VH	VL
Alemtuzumab	CD52	Humanized	1.9 - 8.3	0	4.2	0	90	73	81	61	77
Alirocumab	PCSK-9	Human	1.2 - 4.8	0	3	0	60	89	66	75	81
Avelumab	PD-L1	Human	4.1	0.2	8	10	90	91	99*	77	54*
Benralizumab	CD125	Humanized	13.0	0.7	2.7	10	60	74	81	70	76
Bevacizumab	VEGFA	Humanized	0.6	0	3.2	0	80	74	84	67	80
Brentuximab	CD30	Chimeric	7.0 - 30.0	0.8	3.4	20	60	86	64	93	97
Cetuximab	EGFR	Chimeric	5.0	0.3	2.1	10	60	56	61	91	95
Daratumumab	CD38	Human	0.0	0	0.6	0	20	95	89	74	74
Elotuzumab	SLAMF7	Human	18.5	0	1.6	0	40	76	77	87	81
Infliximab	TNF-a	Chimeric	10.0 - 51.0	0.3	2.4	10	60	66	n.f.	87	n.f.
Ipilimumab	CTLA-4	Human	1.1 - 4.9	0	1.8	0	40	56	98	78	65
Necitumumab	EGFR	Human	4.1	0	3.7	0	60	92	90	73	65
Vedolizumab	Integrin α4β7	Humanized	13.0	0	5.2	0	80	78	62	80	84

(1) Dingman et al., 2019
(2) Immunogenicity Factor
(3) In vivo incidence
(4) Sequence identity to transgenic V-gene (TG) or closest germline V-gene (WT)
* Ig lambda light chain
n.f.: Not found

Clinical-grade preparations of the selected antibodies were tested in groups of 10 hIgG1 transgenic and 10 littermate wild-type mice except for avelumab, where only 9 wild-type mice were used. The mice were immunized following the established immunization protocol with seven consecutive subcutaneous applications of 10 µg IgG antibody without adjuvant over a three-week period.[6] Supplementary Figure 1 gives an overview on the general study outline of all in vivo immunogenicity experiments in this work. The results of the individual immunization experiments are shown in Supplementary Figure 2 and are summarized in Table 1. As indicated in Table 1, the ADA incidence in wild-type mice varies from 20% to 90% of treated mice. These differences probably reflect intrinsic immunogenic differences of the V region sequences used, since all compounds tested bear the same human constant region. Two of the three chimeric antibodies bear murine V regions with a high degree (over 90%) of sequence identity with endogenous mouse germline V gene sequences (brentuximab, cetuximab) for both the heavy (H) and the light (L) chain. The finding that two (infliximab, brentuximab) of the three antibodies displaying higher clinical ADA scores are chimeric human/mouse antibodies might suggest that the murine V sequences increase their immunogenic potential in humans.

In contrast, hIgG1 transgenic mice display a low ADA incidence that is generally reminiscent of that observed in humans with these compounds. The clinical ADA incidence reported for eight antibodies analyzed range between 0 and 10%, but is very variable for two of the remaining five antibodies studied (brentuximab and infliximab), ranging between 7% and 51% (Table 1). Compared with the large case numbers contributing to the clinical ADA rates of the compounds listed in Table 1, the ADA frequency in hIgG1 transgenic mice stems from experimental groups of 10 mice. This explains the better granularity of the clinical ADA rates, especially for the small numbers, as ADA incidence values between 0% and 10% effectively cannot be reproduced in hIgG1 transgenic mice (less than one mouse). In fact, all compounds with a clinical ADA incidence up to 13% cause an ADA rate between 0% and 10% in hIgG1 transgenic mice (Table 1). Thus, given the group size limitation, the hIgG1 transgenic mice consistently reproduce the range of clinical immunogenicity reported for the compounds tested here.

Tolerance to human antibodies does not depend on V sequence similarity to resident V gene repertoire

Given the observed fluctuation of the measured ADA titer values within immunization groups (Supplementary Figure 2), and in order to provide a tool reflecting both the incidence and the intensity of individual immune responses, we introduced the concept of the "Immunogenicity Factor" (IGF in Table 1). This value integrates the frequency of

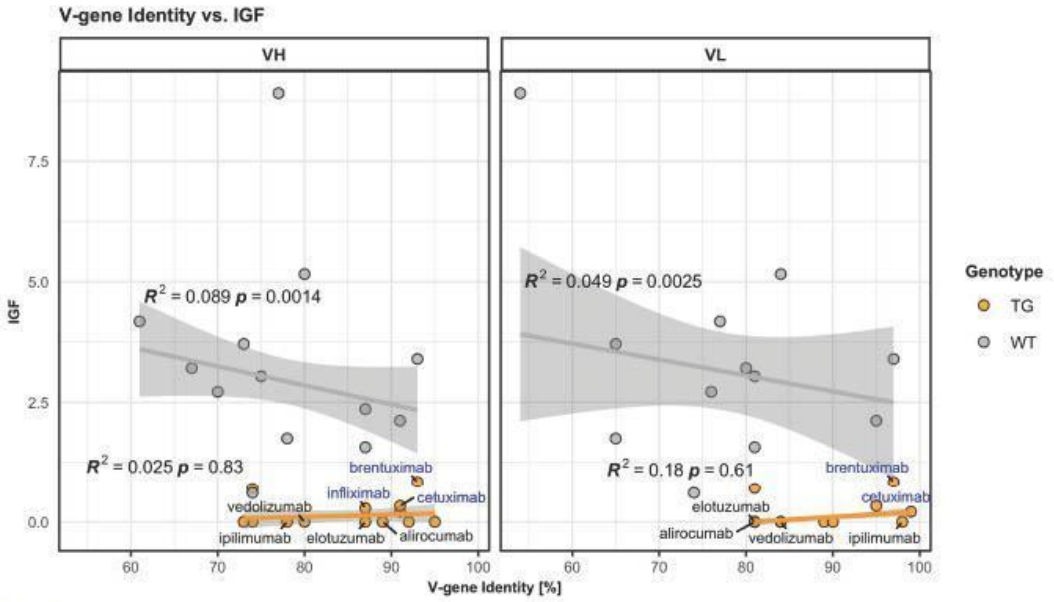


Figure 1. Low identity of antibody V genes to endogenous V genes does not correlate with increased immunogenicity. V gene identity is given as percent amino acid sequence identity of the variable (V) domain of each antibody listed with the best matching hIgG1 transgenic (TG, orange circles) or endogenous murine wild-type (WT, gray circles) V heavy (VH, left panel) and V light (VL, right panel) gene elements. The Immunogenicity Factor (IGF) as a degree of immunogenicity is correlated with the V gene identity for hIgG1 transgenic (orange lines) or wild-type mice (gray lines) and R^2 values, as well as p-values are displayed in the plot. A robust linear model was used, based on Huber's M-estimator, which downweighs the influence of single outlying data points, as well as deviations from non-normality. R^2 coefficients of determination were calculated using modified residual and total sums of squares, i.e., weighing the squared deviations with the respective weights assigned by the robust estimator. Compounds showing higher identity to endogenous V genes compared to transgenic V genes are labelled and in addition, chimeric compounds are labelled in blue.

responders and the respective titer values as described in the materials and methods section.

We determined the level of identity between the V sequence of the 13 tested antibodies and the endogenous murine or the five transgenic human V gene sequences for the H and the L chain in each case. These values are given in Table 1 for the H and the L chain V gene of each antibody as sequence identity (in %) to the closest resident transgenic or endogenous murine V genes. For example, for alirocumab, a maximum of amino acid identity of 89% with one of the five transgenic VH genes and of 66% with one of the two transgenic V *kappa* (Vκ) genes is indicated. Figure 1 displays the IGF of the responses to each compound as a function of the sequence identity of its V gene. Interestingly, this comparison in wild-type mice denotes a trend toward increased tolerance when V sequence identity increases (Figure 1, gray symbols). However, this systematic effect only accounts for a few percent of the overall variability in the data, based on R2 values, therefore not constituting a pivotal factor determining the IGF. In hIgG1 transgenic mice, V sequence identity does not correlate at all with observed immunogenicity (Figure 1, orange symbols). The data suggest an "equalizing principle" in hIgG1 transgenic mice causing a general flattening of IGF irrespective of their V sequence identity to resident transgenic or endogenous V sequences. In summary, we conclude that tolerance to human antibodies has a broader coverage than expected by the five transgenic V genes expressed in hIgG1 transgenic mice. Therefore, we next asked if the common Fc domain has an influence in maintaining unresponsiveness to V sequences.

Fab fragments display enhanced ADA responses

We analyzed the immunogenic properties of purified antigen-binding fragments (Fab) lacking the crystallizable fragment (Fc) of three representative IgG1 antibodies: the therapeutic antibody bevacizumab, the scaffold antibody CEA-IgG containing the same sequence as the CitAbs CEA-IL2v and CEA-TCB, and the experimental antibody DP47-IgG bearing VH and Vκ regions encoded by the transgenic human VH3-23 and Vκ3-20 genes, respectively. The data in Figure 2A illustrate that the immunogenic potential of these antibodies observed in wild-type mice is strongly reduced or abolished in hIgG1 transgenic mice. In contrast, the Fab preparations of bevacizumab (beva.-Fab) and CEA-IgG (CEA-Fab), but not of DP47-

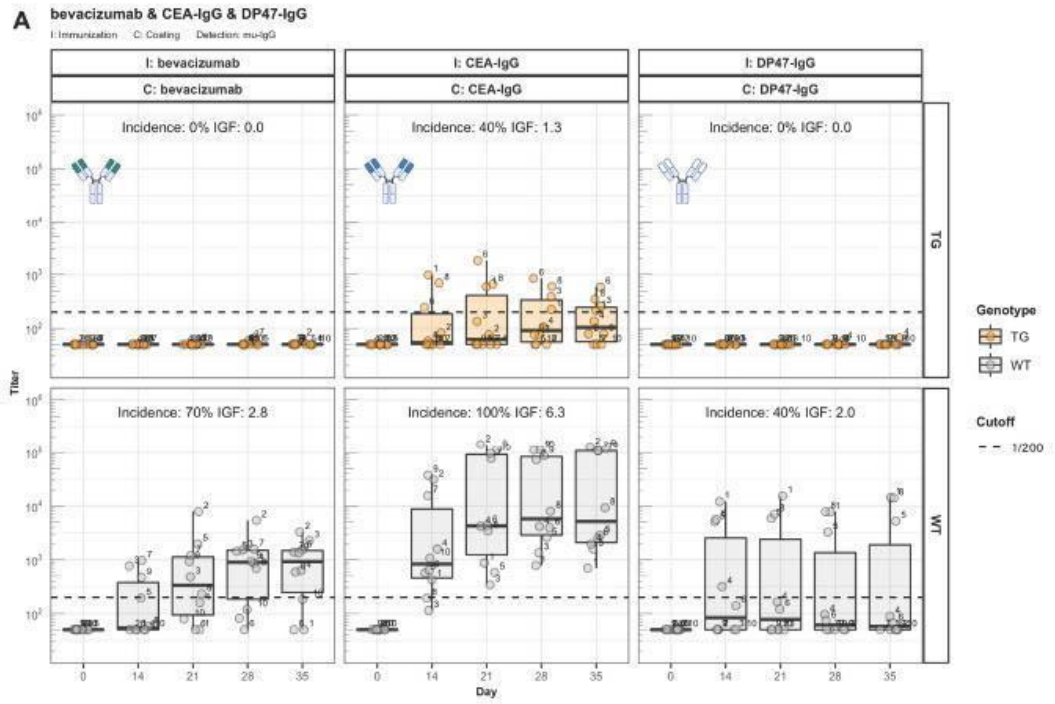


Figure 2. Fc contributes to the unresponsiveness towards conventional mAbs. Murine IgG anti-drug antibody (ADA) titers in hIgG1 transgenic (orange, upper panels) and wild-type (gray, lower panels) are measured over time before (day 0) and after the first immunization. Groups of 10 hIgG1 transgenic and 10 wildtype mice were immunized with the full antibody and the respective Fab preparation in the same experiment. The immunization compound (I:) and the coating compound for the ELISA (C:) are displayed above each plot. ADA titers above the arbitrary threshold of 200 are considered as positive and hence used to calculate the ADA incidence displayed in percent and the Immunogenicity Factor (IGF). Panel A) displays ADA titers against the full IgG1 antibodies bevacizumab, CEA-IgG, DP47-IgG and panel B) shows ADA titers against the respective Fab fragments beva.-Fab, CEA-Fab, and DP47-Fab. Panel C) shows ADA titers against the Intravenous Immunoglobulin subclass 1 Fab fragment (IVIG1-Fab) induced by immunization with the full IVIG1 or IVIG1-Fab preparation.

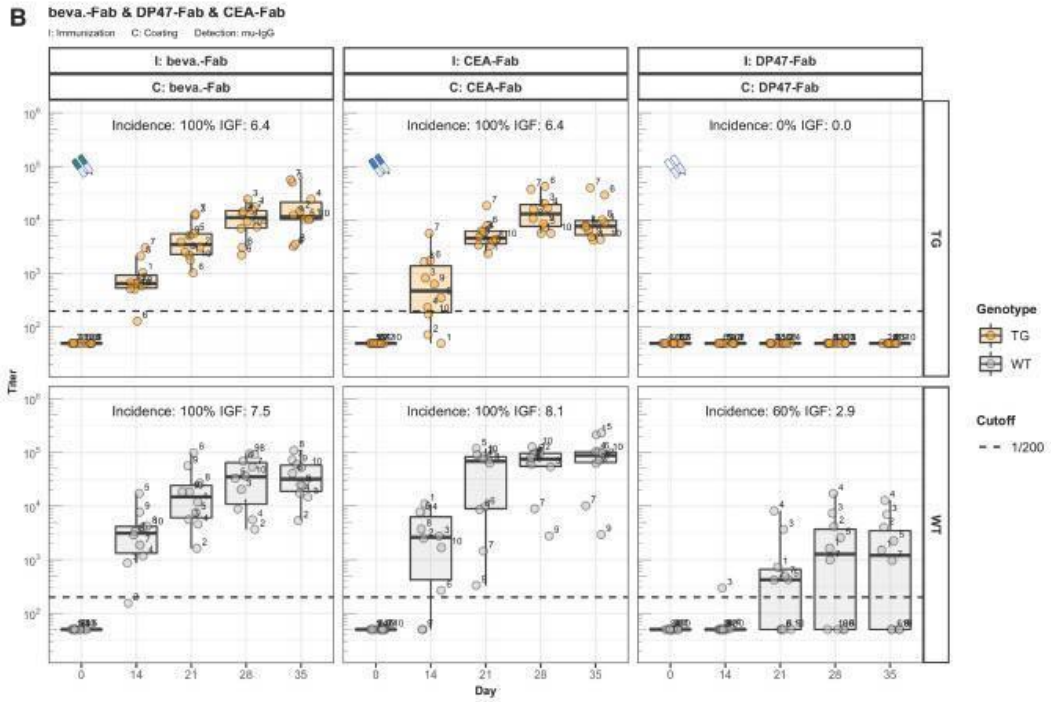


Figure 2. (Continued).

IgG (DP47-Fab), display enhanced immunogenicity both in hIgG1 transgenic and wild-type mice (Figure 2B). The results imply that Fab-specific B cells exist in hIgG1 transgenic mice that are silenced upon immunization with full antibody, but are unleashed when triggered with Fab preparations. Interestingly, the Fab of the antibody DP47-IgG fails to elicit this "reactivation" response (Figure 2B). Given that the same human VH3-23, joining (J)H4, Vκ3-20, and Jκ1 genes of DP47 are also expressed in hIgG1 transgenic mice,[6] we interpret the absence of a corresponding antibody response to DP47-Fab as resulting from central tolerance-induced deletion of DP47-specific lymphocytes. Immunization with IgG1 fraction of intravenous immunoglobulin (IVIG1) and Fab preparations thereof (IVIG1-Fab) extend the validity of this assumption over the two cases with beva.-Fab and CEA-Fab. As shown in Figure 2C, the incidence and the intensity of the ADA response to IVIG1-Fab is enhanced when compared with IVIG1, both in hIgG1 transgenic and in wild-type mice.

Reitan and Hannestad[10] discovered that the immunogenicity of Id depends on the isotype of the antibody. To assess the influence of the constant region on immunogenicity of the CEA-binder, we generated antibody constructs composed of this particular human V region associated with the murine IgA constant region (CEA-mIgA). Immunization of hIgG1 transgenic and wild-type littermate mice demonstrates that the V region associated with the mIgA isotype elicits a similar ADA response as the corresponding CEA-Fab preparation (Figure 3A). Further, the results in Figure 3A demonstrate that the observed ADA response to Fab is not caused by cryptic epitopes exposed in the Fab, but results from the absent tolerogenic effect of IgG-Fc.

IgG binding to FcRn is required for immune tolerance

One major difference between IgG and IgA is the binding capacity to the neonatal Fc receptor (FcRn).[11] To investigate the contribution of this receptor, we generated a variant of CEA-IgG bearing a murine IgG1 constant region (CEA-mIgG) and an AAA variant thereof (CEA-mIgG-AAA) containing a triple mutation (I253A, H310A, H435A) that abolishes binding to the FcRn.[12] The elevated ADA response of CEA-mIgG-AAA clearly links FcRn to the Fc-mediated unresponsiveness with CEA-mIgG (Figure 3B).

Elevated ADA response in mice deficient for FcRn

Immunization experiments in mice deficient for the FcRn receptor (FcRn-KO) further corroborated this assumption. Because this mouse strain does not express human IgG1 as self-protein, it requires immunizations with the mouse surrogate CEA-mIgG antibody. In analogy to the enhanced ADA response found with

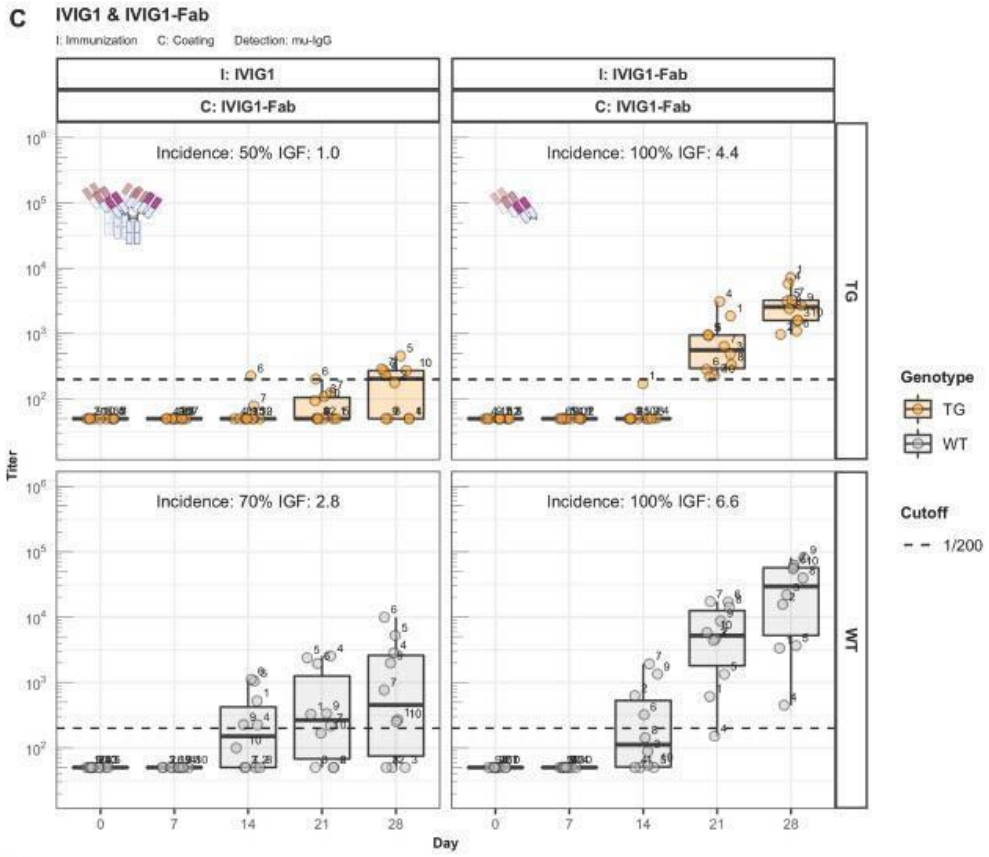


Figure 2. (Continued).

CEA-mIgG-AAA, the CEA-mIgG antibody elicits a stronger ADA response in FcRn-KO mice as compared to wild-type mice (Figure 3C), despite the expected strongly reduced exposure of the injected antibody (Supplementary Figure 3A) and the overall reduced endogenous IgG levels (Supplementary Figure 3B) in mutant mice due to the lack of FcRn-mediated recycling. The control immunization with the T cell-dependent antigen keyhole limpet hemocyanin (KLH) in this experiment demonstrates that FcRn-KO mice are capable to mount ADA responses, but the titers are generally reduced in comparison to wild-type mice (Figure 3C). Taken together, we conclude that tolerance to idiotype components of antibodies is mediated by the Fc domain of IgG (but not by IgA isotype) through interaction with FcRn.

IL-2 immunocytokine and T-cell bispecific antibody display enhanced immunogenicity

The identification of Fc-associated mechanisms preserving unresponsiveness to conventional antibodies prompted us to address the widely observed strong immunogenic potential of bispecific therapeutic antibodies.[1] Thus, we extended our studies to two forms of CitAbs, based on the same CEA-specific scaffold antibody. The structure of CEA-based CitAbs used is shown in Figure 4A. The T cell bispecific (TCB) cibisatamab (CEA-hTCB) is composed of two Fab arms specific for CEA and one arm specific for the CD3ε chain of the TCR protein complex.[8,] In our experiments, we also used a variant with a V domain of the hamster-anti mouse CD3ε clone 145-2C11 (CEA-mTCB), in the similar, though not identical, 2:1 format of clinical CEA-hTCB. The Fc-distal position of the CD3ε binder in CEA-mTCB, as compared to its Fc-proximal position in CEA-hTCB, results from protein expressibility and activity limitations of the murine version. The immunocytokine cergutuzumab amunaleukin (CEA-IL2v) is composed of the same CEA-binding scaffold antibody fused to a modified human IL-2 molecule at the carboxy terminal of one of the Fc moieties by a linker.[9] This IL-2 variant (IL2v) used in this immunocytokine contains mutations that abolish binding to the high-affinity IL-2Rα chain (CD25), but preserve binding to the intermediate affinity IL-2Rβγ heterodimer receptor.[9] Therefore, it preferentially stimulates $CD25^{dull}$ effector T cells and NK cells, while

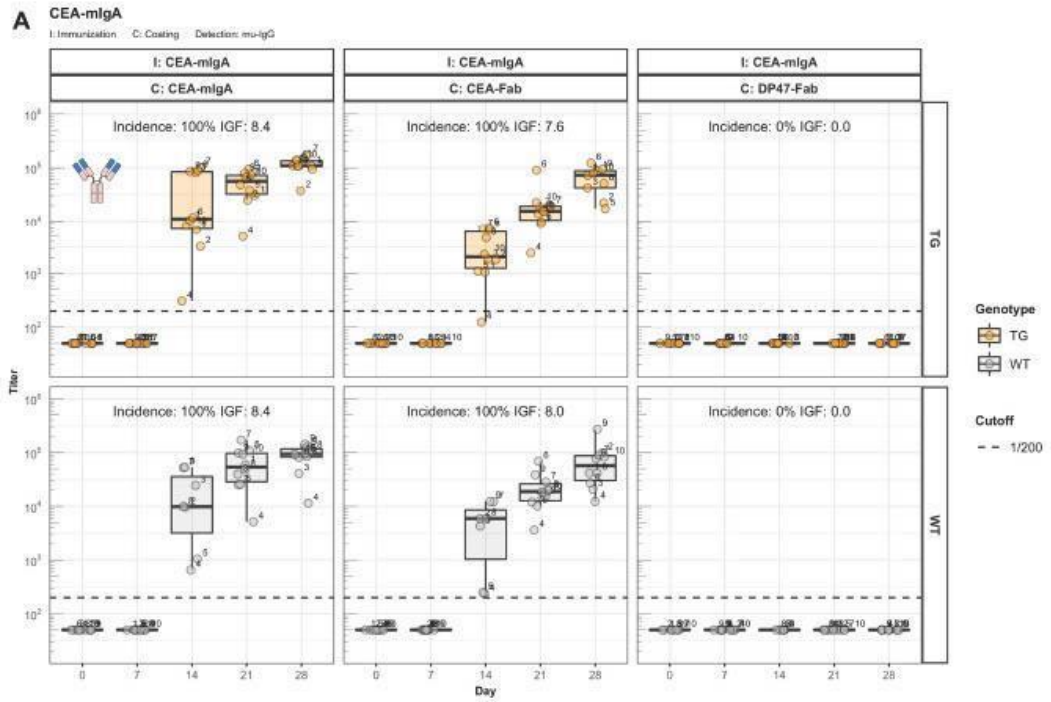


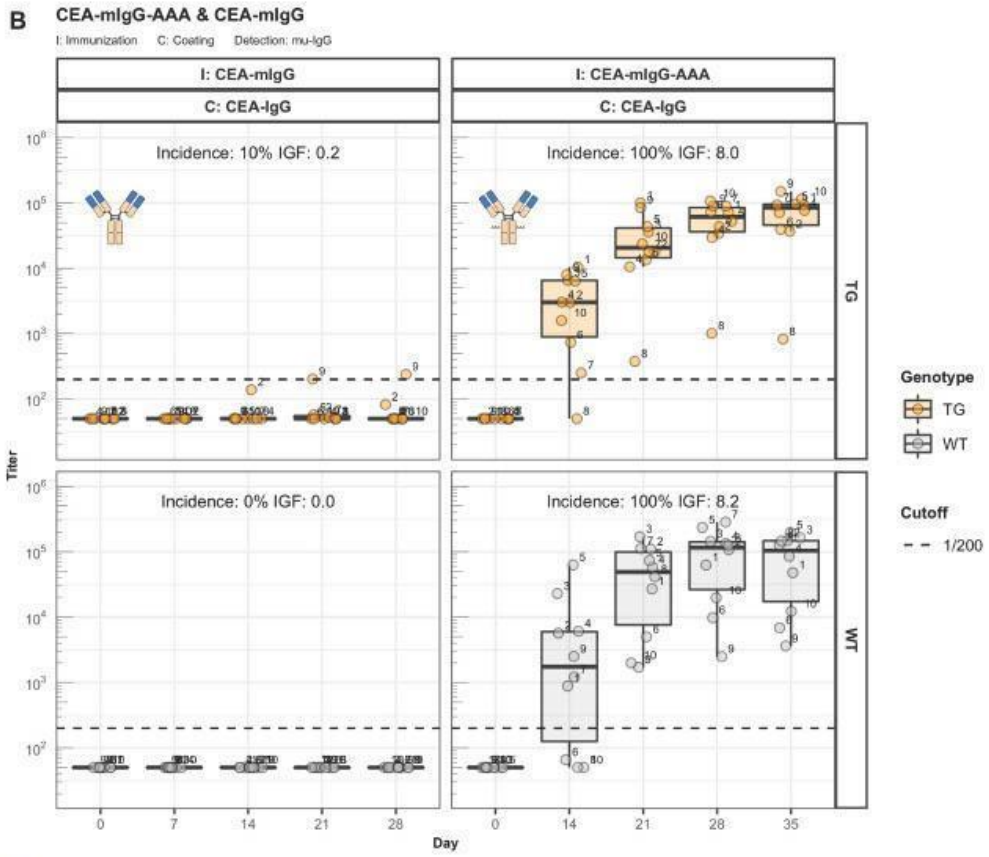
Figure 3. The role of FcRn in immunogenicity. Murine IgG anti-drug antibody (ADA) titers in hIgG1 transgenic (orange), wild-type (WT, gray) and FcRn-KO (blue) mice are plotted over time before (day 0) and after the first immunization. For each immunization, groups of 10 mice were used. The immunization compound (I:) and the coating compound for the ELISA (C:) are displayed above each plot. ADA titers above the arbitrary threshold of 200 are considered as positive and hence used to calculate the ADA incidence displayed in percent and the Immunogenicity Factor (IGF). Panel A) shows the ADA response of 10 hIgG1 transgenic and 10 wild-type mice immunized with a CEA-mIgA against the full molecule (C: CEA-mIgA), the variable domain (C: CEA-Fab) or the constant region (C: DP47-Fab). Panel B) shows ADA responses against the CEA-binder in 10 hIgG1 transgenic and 10 wild-type mice immunized with CEA-mIgG and CEA-mIgG-AAA. In panel C), FcRn-KO and wild-type mice were immunized with CEA-mIgG and KLH. The immunization with CEA-mIgG was repeated to confirm the result leading to the duplicated sample size (n=20 each).

avoiding activation of $CD25^{high}$ regulatory T cells.[9] In addition, the CEA-IgG backbone bears the PGLALA modification within the Fc region that eliminates the Fc-*gamma* receptor (FcγR) and complement C1q binding, while retaining FcRn binding.[13] In the experiments presented here, the CEA-IgG scaffold antibody served as control of the baseline immunogenic potential. As shown in Figure 4A, CEA-IgG and CEA-hTCB are poorly immunogenic, whereas CEA-mTCB and CEA-IL2v elicit immune responses with high incidence and titer in hIgG1 transgenic mice. It is particularly significant that the fusion with the human CD3ε-specific Fab in CEA-hTCB does not raise the immunogenic potential of CEA-IgG, whereas addition of the mouse CD3ε-reactive Fab in CEA-mTCB and of the human IL2v protein in CEA-IL2v results in increased immunogenicity. As both CEA-mTCB and CEA-IL2v can engage the corresponding receptors in murine T cells (CD3ε and IL-2R), the data support a role for this interaction in reverting the pre-existing unresponsiveness to CEA-IgG. Furthermore, Figure 4B shows that immunization with CEA-IL2v induces ADA responses directed against CEA-Fab, indicating the involvement of Fab-specific B cell clones. In contrast to wild-type, hIgG1 transgenic mice usually do not produce ADA against the IgG1 Fc portion, suggesting an elimination of Fc-specific B cells mediated by the expression of the transgene.

When similarly modified variants of the germline antibody DP47-IgG are tested for their immunogenicity, a different picture emerges. It is evident that no ADA against DP47 are elicited with DP47-IL2v (Figure 4C) and DP47-mTCB (Figure 4D) despite the addition of IL2v or mouse CD3ε binder, respectively. This demonstrates that the absence of anti-DP47 ADA reflects the lack of germline-specific B cell clones. Interestingly, the detection of mTCB-specific ADA in mice immunized with DP47-mTCB (Figure 4D, lower panels) suggests the presence of T and B cell epitopes in the hamster-anti mouse CD3ε binder.

Enhanced Immunogenicity of CitAbs does not correlate with dendritic cell uptake

It is known that pre-existing ADA, even below the detection limit, can contribute to the formation of immune complexes (ICs) with infused antibodies, thus increasing their immunogenicity.[14] The underlying mechanism could be enhanced uptake, processing, and presentation by dendritic cells (DC).[15,16] Concerns were raised that structural



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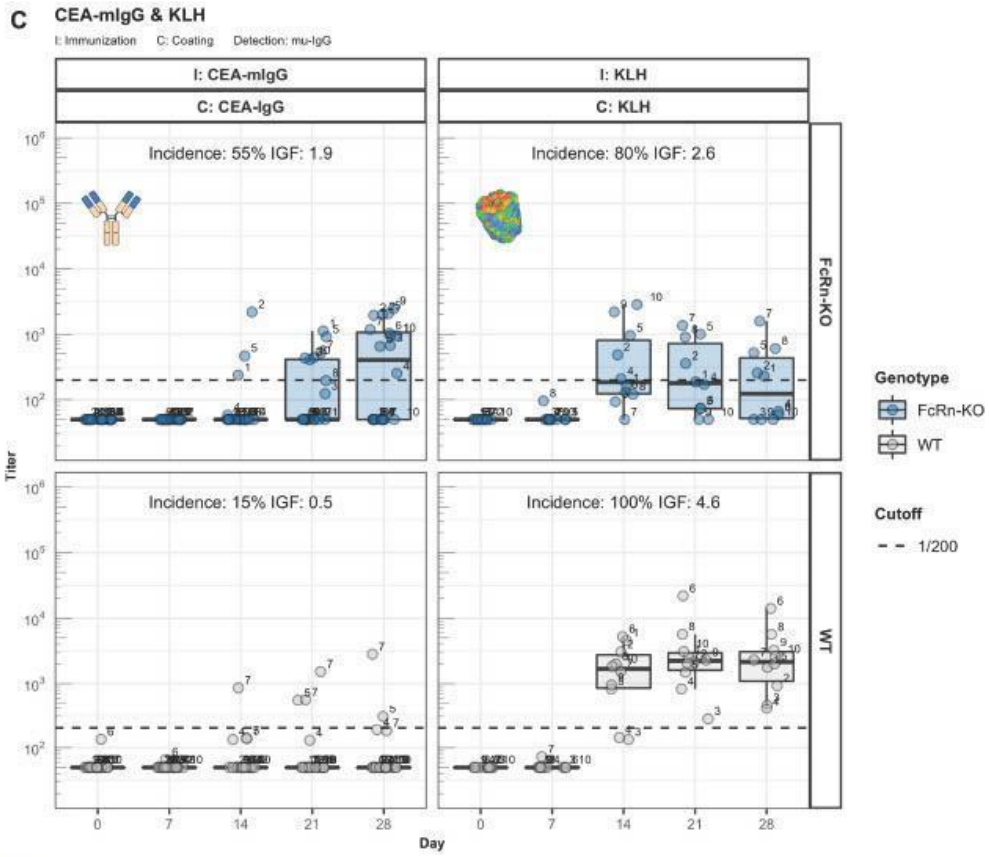
modifications of CEA-IgG, such as the fusion of IL2v or TCB and the knob-into-hole amino acid changes for engineering of bispecific antibodies,[17] could promote binding of pre-existing ADA, and hence increase immunogenicity or cause stimulation of DCs via other mechanisms. Thus, we generated large ICs formed by CEA-IgG and mouse IgG2a antibodies specific to the idiotype of CEA-IgG (CEA-IgG-IC) to investigate this hypothesis (Supplementary Figure 4A). Immunizing hIgG1 transgenic and wild-type mice with CEA-IgG-IC leads to a distribution of intact ICs in the serum (Supplementary Figure 4B) but causes only a slight increase of the ADA response against CEA-IgG (Figure 5A). This slight increase caused us to question the contribution of DC internalization to the immunogenicity of CitAbs. To that end, we differentiated DCs from the bone marrow of three hIgG1 transgenic and three wild-type mice using recombinant murine granulocyte-macrophage colony stimulating factor (GM-CSF). Subsequently, we measured by flow cytometry the internalization rate of the compounds labeled with a pH sensitive fluorescent dye, which is brighter in the acidic environment of the lysosome. As expected, DCs internalized CEA-IgG-IC more efficiently than monomeric CEA-IgG (Figure 5B), which could contribute to the slightly enhanced immunogenicity. The internalization rate of monomeric CEA-IL2v and CEA-mTCB is also in the range of CEA-IgG-IC, even though these compounds induce a stronger ADA response in hIgG1 transgenic mice (Figure 4A). Interestingly, CEA-mIgG displays the highest DC internalization despite its low immunogenicity in hIgG1 transgenic and wild-type mice (Figure 3B). Similarly, CEA-hTCB is not immunogenic in hIgG1 transgenic mice (Figure 4A), but shows an intermediate DC internalization rate. From the combination of these data, we conclude that there is no simple correlation between the rate of uptake and internalization and the immunogenic attributes of the analyzed compounds. Thus, other explanations are needed to explain the enhanced immunogenicity of CitAbs.

The assumption that ADA responses to CEA-mTCB and CEA-IL2v are stimulated by activation of T cells targeted by their mTCB or the IL2v moieties is further supported by immunization experiments with mixtures of scaffold CEA-IgG with purified IL2v or a one-arm variant of the mCD3ε-specific antibody (mCD3-OA). These immunization studies



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show an incremental difference in immunogenicity comparable to that found with the corresponding T cell engager antibodies (Supplementary Figure 5). From these results, we conclude that idiotype-specific B cell clones exist that are indirectly reactivated by the action of IL2v or CD3ε binder molecules. Given the T cell-activating nature of the IL2v and anti-CD3ε Fab, the question arises whether this reactivation is an antigen-cognate process, or the sole result of bystander responses mediated by nonspecific T cells.

ADA responses elicited by CitAbs require cognate T-B cell interaction

To address the question of cognate T-B cell activation, we studied the role of antigen-presentation by B cells in the formation of ADA. To do so, we used an experimental system of lethally irradiated mice reconstituted with a mix of bone marrow cells. The mix is composed of 80% bone marrow cells from mice homozygous for the Igh-J^{tm1Cgn} targeted mutation ($JhT^{-/-}$), and thus lacking functional B cells, and of 20% bone marrow cells derived from MHC-II deficient mice ($MHC\text{-}II^{-/-}$) in order to create a system where only B cells are deficient for MHC-II expression. A mix containing 20% bone marrow cells from wild-type mice serves as a control. The generation of the bone marrow chimeric mice (BMC) is described in Figure 6A and in the materials and methods section (Mice and Immunization). The B cells in such reconstituted mice are $MHC\text{-}II^{-/-}$, while all other immune cells bear the $JhT^{-/-}$ mutation but express MHC-II (Figure 6, B and C). The system is apt to test if the CitAbs CEA-IL2v and CEA-mTCB were able to induce antigen presentation-independent, non-cognate T cell activation, and hence MHC-II-independent ADA production. As shown in Figure 6D, the experiment evinced that only mice reconstituted with the mix containing wild-type bone marrow cells (WT BMC) produced ADA to CEA-IL2v and CEA-mTCB upon immunization. In contrast, chimeric mice containing MHC-II-deficient B cells (MHC-II KO BMC) were unable to respond to the immunization with CEA-IL2v and CEA-mTCB. Since the bone marrow reconstitution generated similar numbers of lymphocytes in the two groups of chimeras (Figure 6C), we totally exclude a non-cognate type of T cell activation and ADA production as explanation for the immunogenic potential of CEA-

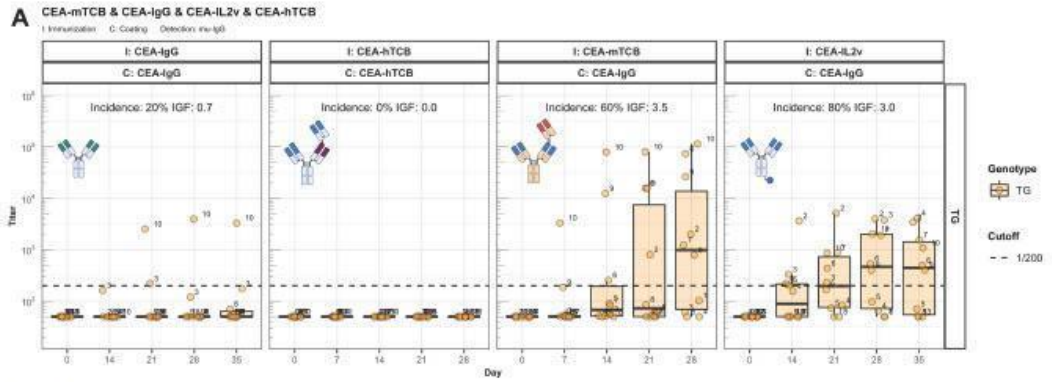
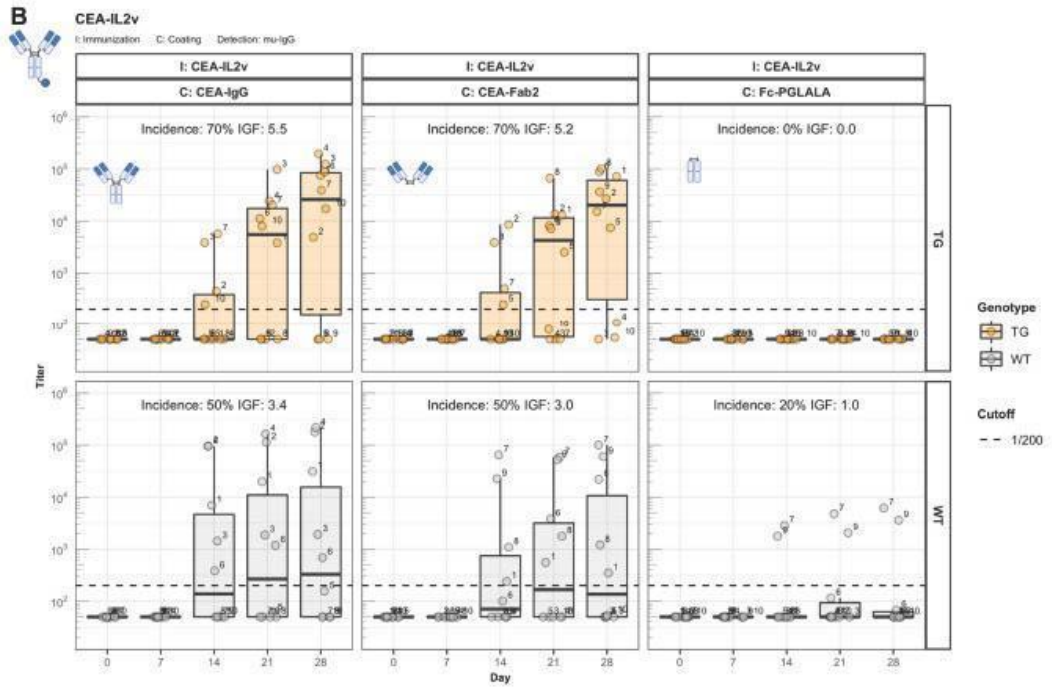


Figure 4. The enhanced immunogenicity of CitAbs. Panel A) ADA response of hIgG1 transgenic mice (TG, orange, n=10) immunized with either CEA-IgG, CEA-hTCB, CEA-mTCB or CEA-IL2v against the scaffold antibody (CEA-IgG) or the injected compound (CEA-hTCB) is shown. Panel B) shows the specific ADA response of hIgG1 transgenic mice (orange, n=10) immunized with either CEA-IL2v directed against the scaffold antibody (CEA-IgG), the F(ab')2 fraction (CEA-Fab2) and the Fc domain (Fc-PGLALA). Panel C) depicts the specific ADA response of hIgG1 transgenic mice (orange, n=10) immunized with either CEA-IL2v (as in A)) or DP47-IL2v directed against the scaffold antibody, CEA-IgG or DP47-IgG, respectively. Panel D) shows the specific ADA response of hIgG1 transgenic mice immunized with CEA-mTCB or DP47-mTCB specific for the parental molecule or the murine CD3 binding moiety (mCD3-IgG).



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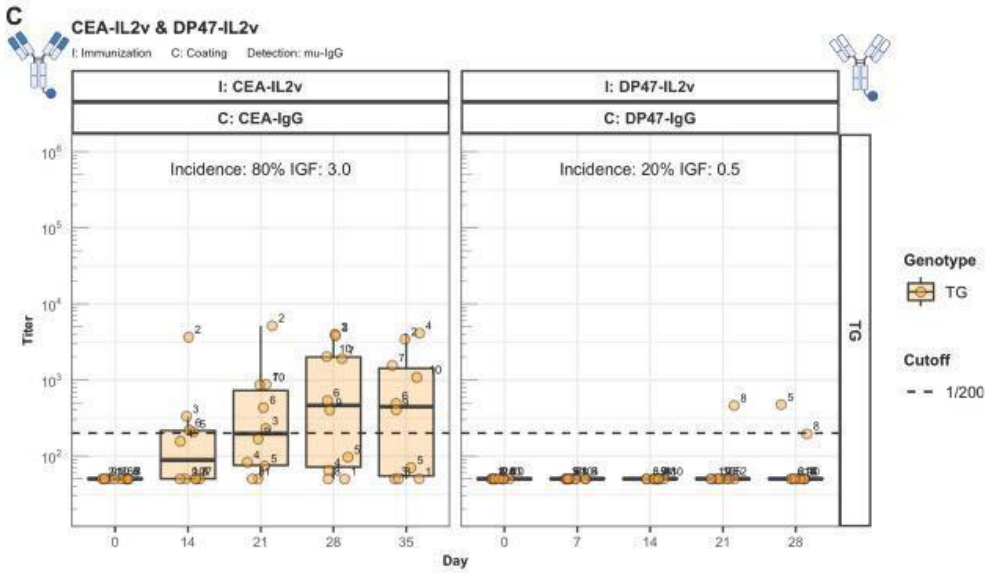

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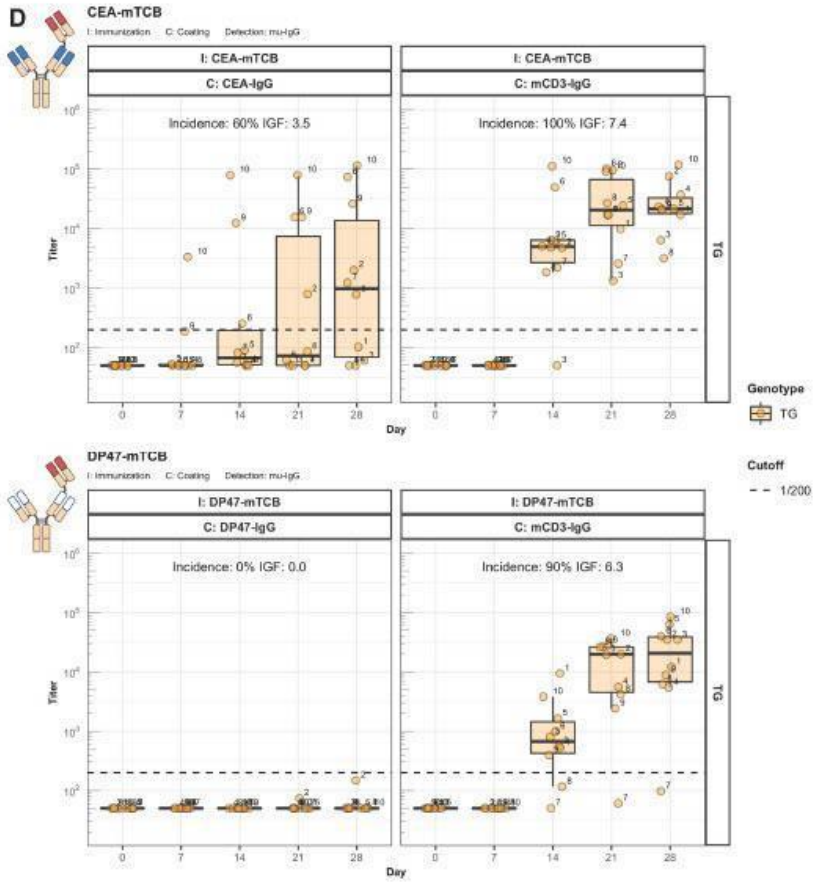
IL2v and CEA-mTCB. Although MHC-II KO BMC displays reduced endogenous IgG titer (Supplementary Figure 6A), both groups can be considered equally immunocompetent. This was also evident from the comparable antibody response elicited by the T cell-independent antigen NP-Ficoll in both groups of chimeric mice (Supplementary Figure 6B). Therefore, reversal of unresponsiveness to CEA-IgG by the bispecific versions CEA-IL2v and CEA-mTCB requires both extracellular binding of IL-2 or TCB to their receptors on the T cell surface and intracellular processing and presentation by MHC-II.

Discussion

Despite intensive efforts in the development of bispecific antibody therapeutics specifically designed for cancer immunotherapy, few have reached the market.[1] Doubtless, antibody therapy based on CitAbs and related biotherapeutics has the potential to be highly efficacious in cancer treatment.[5,18] Given that immunogenicity represents one of the major liabilities of this class of anti-tumor drugs, the preclinical assessment of their immunogenic properties is the objective of intensive research efforts.[19–22] Methods are in place aiming to identify potential antigenic epitopes of defined biotherapeutic proteins in silico (e.g., EpiVax, netMHC-pan), in vitro (MAPPs), ex vivo (e.g., PBMC and DC-TC assays) or in vivo with transgenic mouse systems.[23,24] Yet, the basic question of the mechanism(s) causing break of tolerance to antibody proteins in general, and more specifically to CitAbs in particular, was often raised but insufficiently addressed experimentally.[1] Immunogenicity of antibodies is centered around anti-Id responses.[25–27] Anti-Id ADA responses have been studied in detail in experimental murine systems based on the transgenic expression of Id-specific B and T cell receptors.[28–30] These studies demonstrated that, upon administration of Id-bearing antibodies, Id-specific naive B and T cells collaborate in vivo to the formation of germinal centers and plasma cells, resulting in isotype-switched anti-Id ADA. This process occurs in the absence of adjuvants and does not require antigen presentation by DCs.[31] These mechanisms differ significantly from conventional antibody responses to foreign antigens and could also apply to Id-specific ADA responses to human antibodies in clinical trials. Using these transgenic tools, it became evident that self-reactive B and T cell clones specific for germline-encoded Id are deleted centrally.[28,30] However, lymphocytes with specificity for non-germline Ids can mature and persist in the periphery and could respond to challenge by idiotypic determinants arising during SHM of antibodies or appearing upon infusion of exogenous therapeutic antibodies.

Nonetheless, the accumulated clinical data of numerous marketed therapeutic antibodies suggest a low to moderate immunogenicity potential for most human therapeutic antibodies clinically tested so far (Table 1, ref. 2). In contrast, CitAbs tend to elicit ADA more frequently, often causing exposure and efficacy loss and even adverse events, which contribute to high attrition rates.[1]

Here, we addressed the apparent broad tolerance to Ids encoded by non-germline V genes of exogenous conventional therapeutic antibodies and the comparably pronounced immunogenic attributes of CitAbs. We used the hIgG1 transgenic mouse previously shown to display immunological tolerance to a broad range of human IgG1 antibodies and to be sensitive to immunogenic modifications thereof.[6,7]



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Of course, this model cannot evaluate patient-related factors contributing to ADA occurrence in clinical trials, including cross-reacting pre-existing antibodies, dosing regimens, premedication, administration route, biodistribution, and density of the tumor target antigen(s). While all these factors, along with genetic factors, can influence the ADA outcome to a defined treatment with CitAbs, our study addresses fundamental immunological mechanisms governing immune tolerance and its reversal, that are shared by the human and mouse immune systems. In this study, we have not addressed other questions potentially adding to the immunogenic attributes, such as the affinity of CitAbs to the T cell target antigens or the different formats of CitAbs. However, given the tightly coordinated interaction between T and B cells, anti-CD3ε with higher affinities could in turn lead to stronger B cell help and thus be more immunogenic.

Our results confirm the previously reported broad range of tolerance to human IgG1 antibodies in the hIgG1 transgenic mice.[6] Nonetheless, this is somehow surprising considering that only five human V sequences are included in the germline of hIgG1 transgenic mice, in comparison to the marked diversity of V gene sequences of the human antibodies tested (Table 1 and Figure 1). The lack of correlation between immunogenicity and sequence identity to resident V sequences in hIgG1 transgenic mice suggests a mechanism ensuring tolerance to V sequences not encoded in the germline. We hypothesized that the IgG1 constant region plays this tolerizing role. Indeed, we found that Fc-devoid preparations of two prototypical antibodies (bevacizumab and CEA-IgG) and of IVIG1 elicit strong ADA responses in hIgG1 transgenic mice (Figure 2), indicating that Id-specific B and T cells exist in these mice, but are kept silent toward Fc-containing antibodies. Taken together, these results support the general tolerogenic role of the Fc moiety of IgG1 antibodies, but not of antibodies of other isotypes like IgA (Figure 3A) in hIgG1 transgenic mice. Even considering that Fab are unnatural, artificial

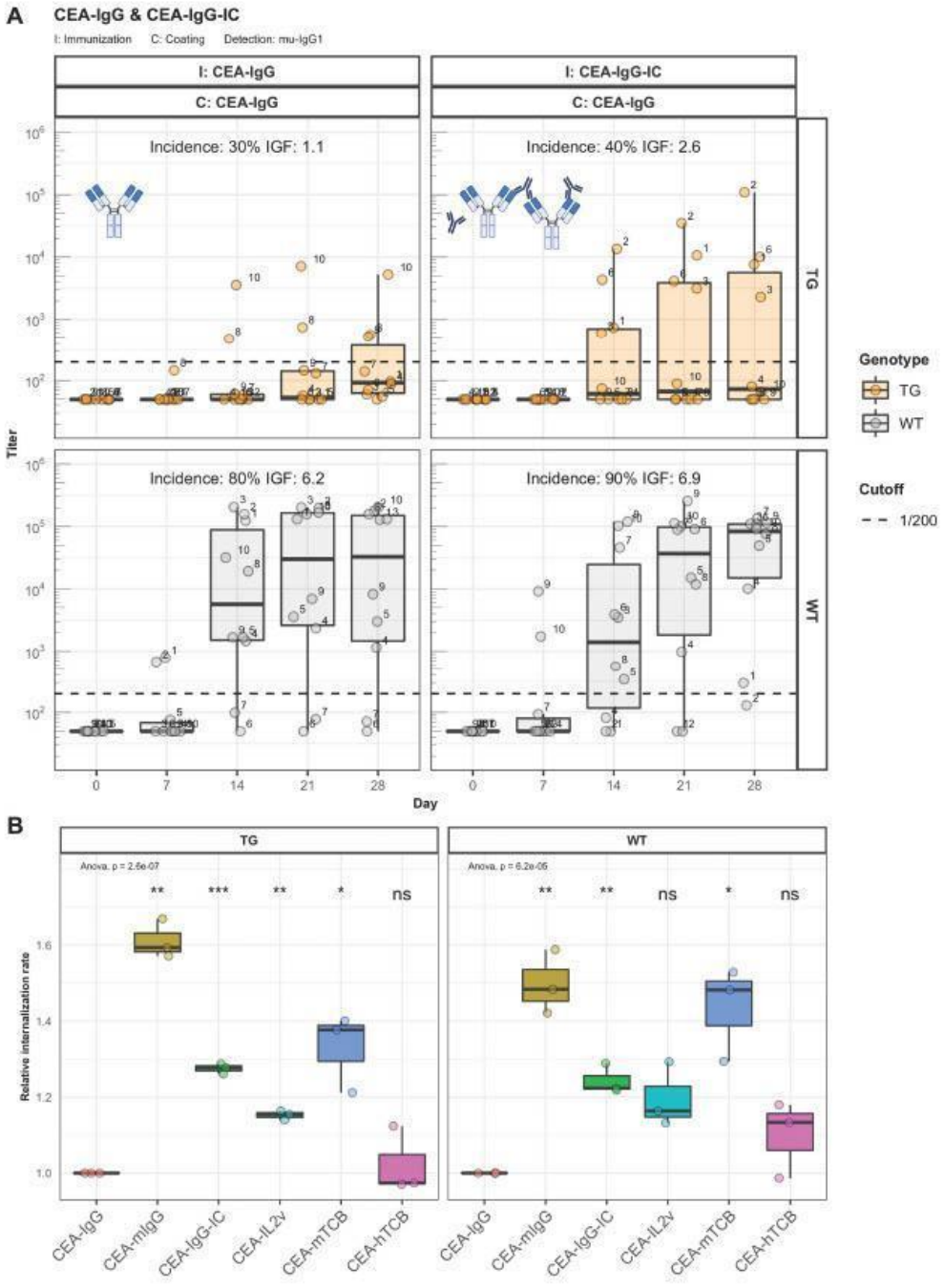


Figure 5. IC formation leads to slightly enhanced immunogenicity. Panel A) shows murine IgG ADA titers against the scaffold antibody (CEA-IgG) of hIgG1 transgenic (TG, orange, n=10) and wild-type (WT, gray, n=10) mice immunized with either CEA-IgG or CEA-IgG in immune complex with a mouse IgG2a anti-idiotype (CEA-IgG-IC). Panel B) displays the internalization rate of compounds with a pH sensitive labelling into the acidic lysosome of $CD11c^+/CD11b^+$ DCs cultivated from bone marrow of three hIgG1 transgenic and three wild-type mice.

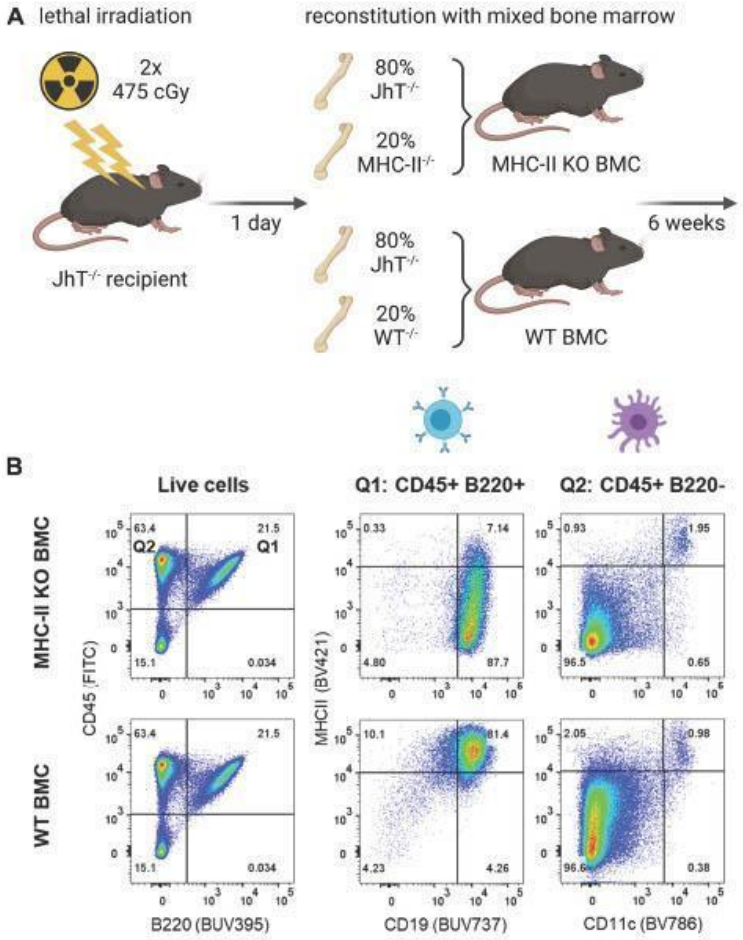
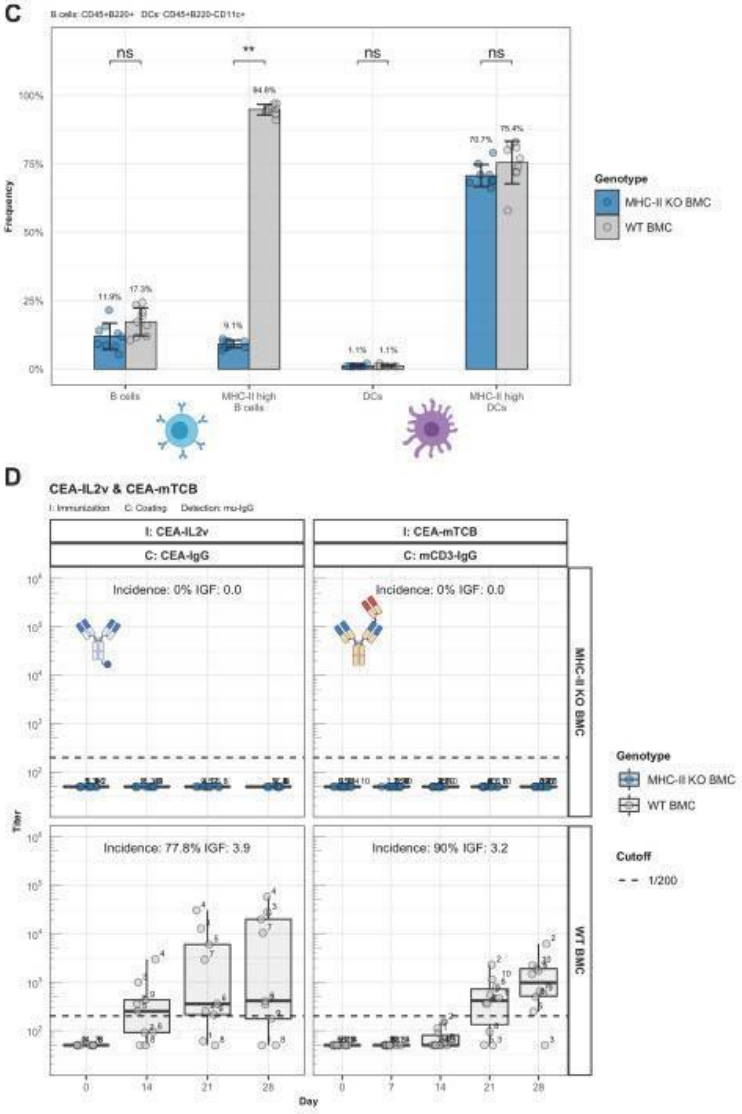


Figure 6. ADA responses to CitAbs require MHC-II dependent T/B collaboration. Panel A) Graphical abstract of the generation of bone marrow chimeric mice using JhT$^{-/-}$, MHC-II$^{-/-}$ and WT mice. Panel B) Representative FACS plots showing the MHC-II expression on B cells and DCs in spleens of WT BMC and MHC-II KO BMC on day 28. Panel C) Quantification of key populations as shown in Panel B). Mean frequencies for each population are given above the bar. Panel D) ADA response induced by CEA-IL2v and CEA-mTCB in WT BMC (gray) and MHC-II KO BMC (blue). The ADA response against the parental CEA-IgG molecule and the ADA response against the T cell binding moiety is shown for CEA-IL2 and CEA-mTCB, respectively. I: Immunization; C: Coating; D: Detection

substances used here for experimental purposes, the results clearly suggest that the Fc has a role in modulating immune responses toward Id determinants of antibodies. Interestingly, the few published immunogenicity studies on bispecific T cell engagers (BiTEs) have revealed the immunogenic character of this class of small therapeutic antibodies composed of two different single-chain variable fragment (scFv) binding one tumor antigen and an activating receptor on T cells.[32,33] According to our findings, it should be expected that appendage of an Fc moiety would abrogate or mitigate the immunogenic properties of BiTEs in humans.

Using FcRn-deficient mice we demonstrated that the tolerogenic role of Fc is related to the function of FcRn (Figure 3). Based on these data, we propose that FcRn-directed recycling diverts internalized antibodies away from the proteasomal degradation pathway to peptide presentation and immune activation.[12]

Similar observations have been made for murine anti-Id ADA responses. Indeed, certain murine Id-bearing antibodies proved to be very immunogenic and efficient in inducing anti-Id ADA responses even when administered adjuvant-free in syngeneic mice.[34] In that study, Reitan et al. showed that the immunogenicity of these mouse idiotypes depends on the associated isotype. Indeed, the same idiotype is tolerized or can elicit vigorous anti-Id responses when borne by IgG isotypes or by IgM, IgE and IgA isotypes, respectively. Furthermore, this IgG-associated tolerogenic Id becomes immunogenic when administered Fc-free, as Fab fragments.[10]



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Here, using hIgG1 transgenic mice, we demonstrate that germline encoded V regions of human antibodies promote central elimination of corresponding Id-specific lymphocytes, as exemplified by the antibody DP47-IgG, while unresponsiveness to non-germline human antibodies (bevacizumab, CEA-IgG) is aided by a recycling pathway mediated by FcRn. Assuming efficient binding of bevacizumab and CEA-IgG to the murine FcRn[35] and the lack of binding to murine FcγRs of the CEA-Fc with the PGLALA mutations,[13] we conclude that FcRn, but not FcγR, substantially contributes to the observed unresponsiveness to infused antibodies. Upon B cell receptor (BCR) ligation, antigen-specific B cells are also able to present BCR-derived Id determinants and receive help from Id-specific T cells.[29] However, naive B cells initially bind antigen with low affinity via non-mutated germline BCR. These B cells are not likely to encounter T cells specific for such germline-encoded Id, as they are clonally deleted in the thymus.[28] In contrast, whenever Id-bearing antibodies are generated through SHM (or are exogenously added) and capable of ligating a corresponding anti-Id BCR, ADA can result[29] from direct Id-

specific B-T cell interaction.[31] Here, we describe that this process is down-modulated by the action of FcRn (Figure 3) and by an additional suppressive and unresponsive state conveyed by putative Fc-specific, suppressor, or anergic T cells.

In contrast to the poorly immunogenic scaffold antibody CEA-IgG, the derivatives CEA-IL2v and CEA-mTCB elicit vigorous anti-Id ADA responses in hIgG1 transgenic mice (Figure 4A). This fundamental finding identifies the IL2v and TCB moieties as responsible for the immunogenicity increment. This assumption is further supported by the finding that the fusion of a human CD3ε-binding Fab, which does not cross-react with mouse CD3ε, does not cause an increase in immunogenicity (Figure 4A). The fact that germline V-based DP47-IL2v was immunologically inert (Figure 4C) and DP47-mTCB elicited exclusively an anti-mTCB ADA response (Figure 4D) further substantiates the deletion of DP47-Id B cell clones during central tolerance. The absence of ADA against DP47 excludes the formation of new epitopes in CEA-IL2v and CEA-mTCB as the primary reason for the onset of immunogenicity.

The possible formation of IC composed of CEA-IL2v or CEA-mTCB and pre-existing low levels of ADA was simulated with antibodies directed against the Id of CEA-IgG, yet the immunogenicity rate of this preparation failed to explain the typical enhancement of immunogenicity of the CitAbs. The measurement of antibody internalization in antigen-presenting DCs has been proposed as a tool for immunogenicity risk assessment because a higher degree of internalization generally correlates with higher clinical immunogenicity.[36] However, in a similar assay, the internalization rate of these compounds in murine DC revealed no simple correlation with their corresponding immunogenic potential in hIgG1 transgenic mice. Finally, the artificial mixture of CEA-IgG with soluble IL2v or a one-armed antibody specific for mCD3ε confirmed T cell activation as the mechanism causing the ADA responses of CEA-IL2v and CEA-mTCB, respectively (Figure 4 and Figure 5 and Supplementary Figure 5).

Taken together, these results add another level of control of anti-Id responses in addition to FcRn that is sensitive to T cell engagement. Binding of IL-2R or TCR-CD3 by CEA-IL2v and CEA-TCB, respectively, seemingly causes the reversal of a state of unresponsiveness not compensable by the recycling pathway driven through FcRn. This unresponsiveness has features reminiscent of T cell anergy, as it is sensitive to T cell stimulation by activating molecules like IL-2 or TCR-binding moieties of certain CitAbs (Figure 4). Indeed,[37–39] FcRn relates to a salvage pathway of internalized antibody while reversal of unresponsiveness by CitAbs like CEA-IL2v and CEA-TCB occurs through the extracellular triggering of T cell activation surface receptors like IL-2R and TCR-CD3. The immunomodulatory property of the Fc region discussed before seems to be overruled by direct engagement of surface T cell activators. Thus, the questions arise, if the reversal of the unresponsive state requires extracellular ligation of the affected T cell surface receptors alone, or if cognate antigen presentation by the Id-specific B cell is also needed. We answered these questions with bone marrow chimeric mice designed to lack expression of MHC-II in B cells, but not in other antigen-presenting cells like[40] DCs (Figure 6A-C). These animals are apt to discern the need for antigen presentation by B cells in the process of breaking tolerance to CEA-IgG by immunization with CEA-IL2v and CEA-mTCB. The experiment clearly demonstrated the need for MHC-II-dependent antigen presentation by B cells (Figure 6D).

In summary, we conclude that tolerance to antibodies, in addition to clonal deletion, is also the result of a peripheral state of T cell unresponsiveness that can be broken if the CitAb includes additional T cell-activating entities like the IL2v or TCR-CD3-binding moieties. This could lead to the activation of hitherto inactive Id-specific B cells, and to ADA production. We postulate that immunomodulatory T cells specific for common "public" epitope(s) of the Fc region (T_{Fc} cells), do maintain this unresponsive state on B cells of different "private" Id specificities. Hence, reversal of the unresponsive status of T_{Fc} cells with CitAbs will result in activation of all possible Id-specific B cell clones (B_{Id}) ligated by the corresponding CitAb. This type of linked-antigen presentation would then lead to unresponsiveness rather than to activation, and hence it is termed *"linked-antigen tolerance"*. Interestingly, the immunogenicity study of commercial antibodies shown in Table 1 and Figure 1 additionally supports the idea that expression of the human Fc in hIgG1 transgenic mice creates an immunological resort ensuring tolerance to associated V sequences independent of their degree of identity to (immune tolerized) resident V sequences.

Figure 7 summarizes schematically the features of a T-B cell cooperation set in motion by CitAbs that would break the postulated status of linked-antigen tolerance toward IgG antibodies and provoke the observed ADA production to the involved CitAbs. This model invokes a status of broad immune tolerance to non-germline encoded V regions as achieved by unresponsive T_{Fc} cells specific for the common Fc region. The linked-antigen tolerance model postulates a frequency of the unresponsive (anergic, suppressive) T_{Fc} cells emerging from the thymus being theoretically higher than that of functional, rare Id-specific T cells (T_{Id}). Upon binding of the CitAb (Figure 7, Panel 1), B_{Id} cells would process and present Id epitopes on MHC-II, possibly under the influence of FcRn-mediated recycling (Figure 7, Panel 2). Of course, rare T_{Id} cells can eventually interact with, and activate, B_{Id} cells to produce anti-Id ADA. Yet, more abundant Fc-specific, unresponsive T_{Fc} cells are more probable to interact with B_{Id} cells and maintain their unresponsive state. Therefore, T_{Fc} cells mediating unresponsiveness and silencing of B_{Id} cells is the predominant result (Figure7, Panel 3). Such a mechanism of linked-antigen tolerance would explain the extended low immunogenicity of conventional therapeutic antibodies in humans and hIgG1 transgenic mice (Table 1). In contrast, the ligation of the BCR of B_{Id} cells by CitAbs is accompanied by the triggering of activating surface receptors (IL-2R, TCR-CD3) on T_{Fc} cells (Figure 7, Panel 4). The ensuing reversal of unresponsiveness and re-activation of T_{Fc} cells would set in motion the program of helping the cognate B_{Id} cells for anti-Id ADA production. This model could explain the high rates of ADA observed with many of the CitAbs.[1] If confirmed, the hypothesis proposed here of breakage of linked-antigen tolerance to CitAbs represents an intrinsic hurdle asking for specific mitigation strategies. Confirmation of the linked-antigen tolerance hypothesis

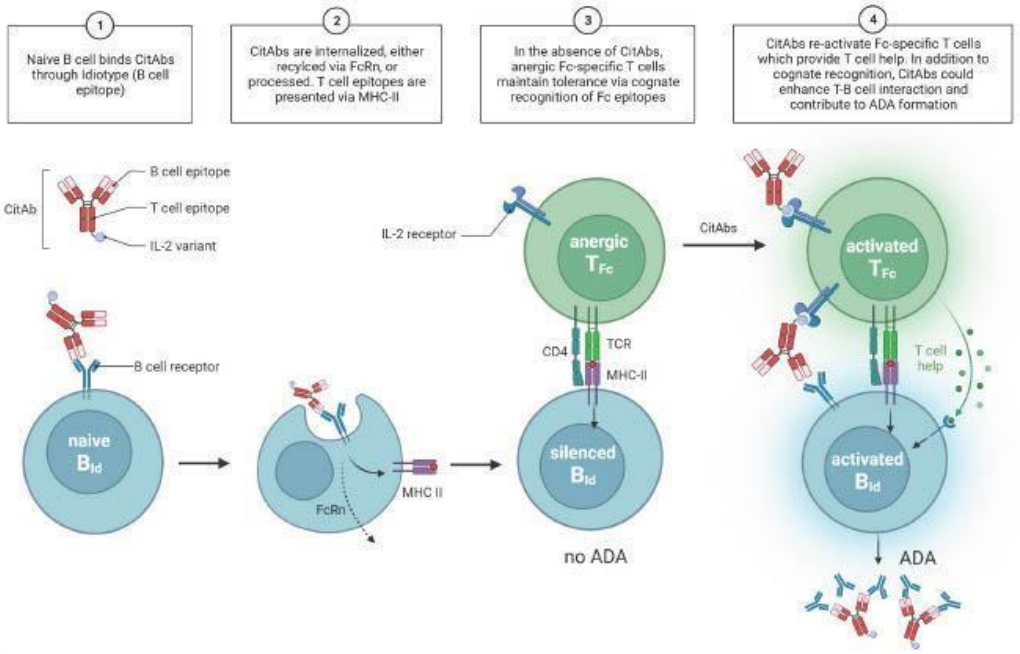

Figure 7. Mechanistic model. A graphical summary of how CitAbs can lead to the break of antigen-linked tolerance and formation of ADA response. Adapted from the figure template "B Cells Internalize and Process Antigens Only if Their BCR is Specific to the Antigen", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates

requires identification of the putative T_{Fc} cells and of the predicted Fc epitopes involved in unresponsiveness. If verified, the model would open a door for mitigation strategies through elimination of the putative Fc epitopes recognized by the unresponsive T_{Fc} cells, such as to cancel their cognate reactivation by CitAbs. This Fc amino acid modification(s) would result in mitigation of all immunogenic CitAbs. In essence, this Fc mutation would restrict anti-Id ADA production to that resulting from interaction of rare B_{Id} with rare T_{Id} cells, to an extent possibly comparable to other, low immunogenic antibodies (Table 1), thus reducing their high attrition rate.

Materials and methods

Antibodies and compounds

All marketed therapeutic antibodies (Table 1) are clinical-grade products purchased from pharmacies. All other therapeutic antibodies (overview in Supplementary Table 1) were transiently expressed in HEK293 cells or produced in stable CHO clones and subsequently purified using Protein A affinity chromatography and size exclusion chromatography. All Fabs were prepared by enzymatic digestion from IgG fractions and subsequently purified using Protein A affinity chromatography or Kappa select capture resin, followed by size exclusion chromatography. The IVIG1 and enzymatically digested IVIG1-Fab preparations were obtained from Athens Research & Technology. Fab preparations of bevacizumab were generated at Genscript by papain digestion, Protein A/G Fc removal, and subsequent endotoxin removal. All compounds were assessed for purity and endotoxin after manufacturing.

NP-Ficoll (Biosearch Technologies; Cat: F-1420) and keyhole limpet hemocyanin (KLH) subunits (Thermo Scientific; Cat: 77649) served as T cell independent and T cell dependent immunogens, respectively.

Immune complexes were prepared and characterized as described previously.[41] In short, a mixture containing 2 mg/ml CEA-IgG and 3 mg/ml monoclonal mIgG2a anti-idiotype antibody was prepared in histidine buffer (20 mM histidine, 140 mM NaCl, pH 6.0) and incubated for 1 h at room temperature on a shaker at 500 rpm. The ICs were characterized as previously described.[41] In brief, a Waters XBridge Protein BEH SEC Guard Column, 450 Å, 3.5 µm, 7.8 mm × 30 mm and a XBridge Protein BEH SEC Column, 450 Å, 3.5 µm, 7.8 mm × 300 mm were used in a Dionax UltiMate 3000 system from Thermo Fisher Scientific GmbH (ultraviolet (UV) detector MWD-3000, auto sampler, automated fraction collector). 20 µl of centrifuged dosing solutions were injected for analysis. Phosphate-buffered saline (PBS) with 5% ethanol (v/v) was used as running buffer with a flow rate of 0.5 ml/min. Online UV detection was performed at 280 nm.

Antibodies for the DC internalization assay were labeled using the SiteClick Antibody Azido Modification Kit (Invitrogen; Cat: S20026) according to manufacturer's instructions. Briefly, antibodies were labeled with a molar dye excess of 3.5, which was subsequently removed using the Amicon Ultra-2 Centrifugal Filter with a MWCO of 50 kD and re-buffered in 20 mM histidine 140 mM NaCl (pH 5.5). The dye-to-antibody ratio is calculated from the absorbance at 280 nm and 532 nm, the extraction coefficient of the dye and a correction factor of 0.36. ICs for DC internalization were generated as described above using labeled CEA-IgG and unlabeled anti-idiotype antibody.

Mice and immunization

Animal experiments were conducted in AAALAC certified facilities of F. Hoffmann-La Roche in Basel or at the Department for Biomedicine of the University of Basel (Switzerland) in accordance with local rules and regulations of the veterinary authorities under cantonal licence number 2634 and 3125. Throughout the studies, mice were kept under specific-pathogen-free conditions with continuous health monitoring.

The hIgG1 transgenic mouse model (C57BL/6-Tg(hIgg1,k,l) ait) breeds heterozygous at Taconic in Denmark and the resulting wild-type C57BL/6J littermates serve as controls. The FcRn-KO mice (B6.129X1-Fcgrttm1Dcr/DcrJ; Strain #003982), and wild-type C57BL/6J control mice (Strain #000664) originate from the Jackson Laboratory (USA).

To generate B cell-specific MHC-II deficient bone marrow chimeras, lethally irradiated $JhT^{-/-}$ mice (B6.129P2-Igh-Jtm1Cgn/J) were reconstituted with 80% bone marrow of $JhT^{-/-}$ mice and 20% bone marrow of MHC-$II^{-/-}$mice (B6.129S2-H2dlAb1-Ea/J). Control mice were reconstituted with 20% bone marrow of wild-type C57BL/6 mice instead of MHC-II KO mice. One day prior to reconstitution, recipient mice were irradiated twice in an interval of 4 h with 475 cGy using a Gammacell 40 137Cs irradiator. The bone marrow of donor mice was extracted by crushing femur and tibia, depleted from red blood cells using Lysing Buffer (BD Biosciences; Cat: 555899), depleted from T cells using CD90.2 beads on LD columns (Miltenyi; Cat: 130-121-278) and mixed in the previously mentioned ratio prior to intravenous injection of 10×10^6 cells per recipient. All mice used for studies employing bone marrow chimeric mice were bred and maintained in an animal facility at the Department of Biomedicine, University of Basel.

Unless otherwise mentioned, five female and five male mice between 6 and 15 weeks of age were carefully assigned to treatment groups in order to balance relevant factors such as litter, sex, body weight and exact age at the time of the experiment, reducing the potential danger of confounding the treatment effect with these factors. Mice were immunized two times per week with a total of seven subcutaneous injections into the abdominal region, alternating between the left and right sides. A dose of 10 µg full IgG molecule or the equimolar equivalent in the case of antibody fragments or conjugates was used per injection. The dose of ICs was adjusted to 10 µg CEA-IgG per mouse and the KLH subunits (Thermo Scientific; Cat: 77649) were injected twice on day 0 and 7 at a dose of 125 µg/mouse. The test items were freshly diluted in a total volume of 100 µl DPBS (Gibco; Cat: 12559069) or histidine buffer (20 mM histidine, 140 mM NaCl, pH 6.0) at dosing dates prior to administration.

Blood was collected prior to dosing on day 0 (naive), and weekly on day 7, 14, 21, and 28 by tail vein sampling into serum gel micro-sample tubes (Sarstedt; Cat: 41.1500.005). After clotting, the samples were centrifuged as indicated and stored at -20°C until analysis by enzyme-linked immunosorbent assay (ELISA). In case drug exposure was assessed, additional blood was sampled 7 and 24 h after the first injection.

Sample analysis

ADA was detected by ELISA as previously described.[6] In short, 96-well Nunc MaxiSorp™ plates (Thermo Scientific; Cat: 44-2404-21) were coated overnight with 5 µg/ml of the indicated antigens in 0.1 M sodium bicarbonate buffer, pH 8.5 (Alfa Aesar; Cat: J60408.AP). Subsequently, the plates were washed three times with DPBS + 0.05% Tween (Sigma; Cat: P9416-100ML), blocked for 2 h with DPBS + 2% bovine serum albumin (BSA; Thermo Scientific; Cat: 37520), and washed again. Serially diluted serum samples (1:50, then 1:3 for a total of eight dilutions) were incubated for 2 h at room temperature followed by further washing. Binding ADA was detected using alkaline phosphatase-conjugated goat anti-mIgG-Fcy (Jackson ImmunoResearch; Cat: 115-055-071) for 1 h and OD 450 nm values were measured after washing and 10 min incubation with p-Nitrophenyl Phosphate (Merck Millipore; Cat: P7998).

The drug exposure and endogenous IgG concentrations were measured similarly by coating with anti-Id antibody (in house production) or goat anti-mIgG light chain specific (Jackson ImmunoResearch; Cat: 115-005-174) antibody, and detection with alkaline phosphatase-conjugated goat anti-mIgG Fcy fragment-specific antibody.

The baseline for titer determination was calculated as the mean OD value at 1:50 dilution of all naive samples plus six times the standard deviation of those values (6 sigma cutoff). OD values greater than the upper quartile plus 1.5×IQR (interquartile range) were classified as outliers and excluded from baseline calculation. For a few cases, due to high background signals, cut-off values were adjusted manually.

The titration curve was fitted using the 5^{th} degree polynomial and intersected with the baseline to obtain titer values. If no intersection occurred within the range of applied dilutions and the titration curve stayed consistently below baseline, we fixed the titer at the value corresponding to an intersection at the lowest dilution (1:50). If the intersection was not reached until the highest dilution, we allowed extrapolation of the titration curve up to a dilution of 1:328050, i.e., three times as high as the actual highest one used, applying a more stable 2^{nd} degree polynomial fit. In any case, the titer was bounded by a maximum value corresponding to a 1:328050 dilution, and a flag was retained to indicate whether the result was obtained by proper interpolation or whether extrapolation/bounding was applied instead. Titers above the arbitrary threshold of 200 are considered ADA positive results.

The Immunogenicity Factor (IGF) integrates the response frequency and the intensity using the following equation:

$$IGF = 10 * \frac{Responder}{GroupSize} * \frac{MeanPosMaxTiterInc}{(MaxTiter - MinTiter)}$$

Responder: Mice with titer > 200 at any time point after first immunization

Group Size: Number of mice per group

MeanPosMaxTiterInc: Increase of the average of highest titer of all responses

MaxTiter: Maximum possible titer (50×3^8= 328050 (extrapolated))

MinTiter: Lowest possible titer (50×3^0= 50)

Sequence analysis

The V gene identity displays the amino acid sequence identity of the variable domains of the therapeutic antibodies with the best matching transgenic or endogenous murine variable heavy- or light-chain genes. Sequence identity was obtained by pairwise alignment (EMBOSS water) of antibody V sequences with the five transgenic VH and two VL sequences or by blasting against mouse genomic sequences in the PRO ensmusp-Ensembl database, release 99.[42]

DC internalization assay

DCs for the use in the internalization assay were cultured from isolated bone marrow of hIgG1 transgenic mice and wild-type littermates as previously described.[43] In short, the cells were seeded into 100 mm Ultra-Low Attachment Culture Dishes (Corning; Cat: 4615) at a concentration of 2×10^5 cells/ml in a total volume of 10 ml complete RPMI-1640 medium (Gibco; Cat: 61870-010) with 100 mM sodium pyruvate (Gibco; Cat: 11360-070), 50 mM 2-mercaptorethanol (Gibco; Cat: 31350-010), 10'000 U/ml Penicillin/Streptomycin (Gibco; Cat: 15140-122), 10% FBS (Seradigm; Cat: 97068-085), and containing 20 ng/ml recombinant mouse GM-CSF (Gibco; Cat: PMC2016). The medium was replenished on day 3 and 6 and the cultures were harvested on day 9 by pipetting. This mouse DC internalization assay is designed according to a protocol developed in-house for the validated human DC internalization assay. The GM-CSF differentiated mouse DCs were seeded in ultra-low attachment 96-well plates at a concentration of 1×10^5 cells/well and incubated with a final concentration of 200 nM of the labeled antibodies for 120 min and 240 min in complete medium. Subsequently, FcγRs were blocked using TruStain FcX Plus (Biolegend; Cat: 156604) and the cells were stained using antibodies against CD11c (N418, BUV737) from BD Biosciences, and B220 (RA3-6B2, BV421), CD11b (M1-70, PE-Cy7), CD8a (53-6.7, APC), CD86 (GL-1, PE), and I-A/I-E (M5/114.15.2, BV786) from Biolegend. After dead cell staining with Zombie NIR (Biolegend; Cat: 423106), the events were acquired on BD LSR Fortessa. Cells were gated based on their morphology, aggregation, viability, and expression of $CD11c^+$, $CD11b^+$. The geometric mean fluorescence intensity (gMFI) in the FITC channel (excitation at 532 nm, filter at 572/35 nm) is recorded as a measure of the internalization of the dye into the acidic lysosome. gMFI values of a medium-stimulated control were subtracted, followed by normalization to the dye-to-antibody ratio. The normalized gMFI from each antibody is plotted as a linear regression curve to extract the slope (gMFI/min for 120 min and 240 min).

Characterization of bone marrow chimeras

The expression of MHC-II on B cells and DCs in bone marrow chimeric mice was assessed by flow cytometry. For this, the spleens of the bone marrow chimeras were sampled and splenocytes were extracted by mechanical dissociation in RPMI-1640 medium (Gibco; Cat: 61870-010) using the gentleMACS Octo Dissociator (Miltenyi). Red blood cells were depleted using Lysing Buffer (BD Biosciences; Cat: 349202), Fc block (Biolegend; Cat: 101320) was applied and extracellular markers were stained using antibodies against CD45 (30-F11, FITC), B220 (RA3-6B2, BV421), IgD (11-26c.2a, PerCP-Cy5.5), TCR-β (H57-597, BV605), CD4 (GK1.4, PE-Cy7), CD8 (53-6.7, APC), MHC-II (M5/114.15.2, BV786), CD25 (PK136, PE) from Biolegend and CD11c (N418, BUV737) and IgM (II/41, BUV395) from BD Biosciences. Subsequently, dead cells were stained using Zombie Aqua (Biolegend; Cat: 423102) and events were acquired on BD LSR Fortessa and analyzed using FlowJo software.

Statistical analysis

Statistical significance of differences in titers between groups in exposure and endogenous IgG assessments were calculated by Wilcoxon test (non-parametric) followed by p-value adjustment (Holm method). For DC internalization assay, statistically significant differences were calculated with ANOVA and paired t-test. Statistical analysis was performed using R.[44] Significance level: $p < 0.0001$= ****; $p < 0.001$= ***; $p < 0.01$= **; $p< 0.05$= *; not significant= ns.

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Disclosure statement

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5. Publication 2

Validation of a dendritic cell and CD4+ T cell restimulation assay contributing to the immunogenicity risk evaluation of biotherapeutics

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Immunogenicity is still difficult to evaluate pre-clinically but could lead to serious adverse events in patients. Consequently, evaluation of the risk of immunogenicity early in the development of biotherapeutics is of critical importance for defining their efficacy and safety profiles. Here, we describe and validate a fit-for-purpose FluoroSpot-based in vitro assay for the evaluation of drug-specific T cell responses. A panel of 24 biotherapeutics with a wide range of clinical anti-drug antibody response rates were tested in this assay. We demonstrated that using suitable cutoffs and donor cohort sizes, this assay could identify most of the compounds with high clinical immunogenicity rates (71% and 78% for sensitivity and specificity, respectively) while we characterized the main sources of assay variability.
Overall, we demonstrated that the assay gives good indications on the risk for immunogenicity, especially when comparing different drug candidates. However, part of the variability remains unexplained and would not be decreased by simply increasing the sampling. The value of the assay lies in an integrated approach for the evaluation of the risk of immunogenicity.

All the experiments have been performed at the contract research organization (LONZA). My contributions were in the design and planning of the studies and statistical analysis of the experiments. I wrote the original version of the manuscript and participated in the discussions and revisions.

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Article

Validation of a Dendritic Cell and CD4+ T Cell Restimulation Assay Contributing to the Immunogenicity Risk Evaluation of Biotherapeutics

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Abstract: Immunogenicity, defined as the ability to provoke an immune response, can be either wanted (i.e., vaccines) or unwanted. The latter refers to an immune response to protein or peptide therapeutics, characterized by the production of anti-drug antibodies, which may affect the efficacy and/or the safety profiles of these drugs. Consequently, evaluation of the risk of immunogenicity early in the development of biotherapeutics is of critical importance for defining their efficacy and safety profiles. Here, we describe and validate a fit-for-purpose FluoroSpot-based in vitro assay for the evaluation of drug-specific T cell responses. A panel of 24 biotherapeutics with a wide range of clinical anti-drug antibody response rates were tested in this assay. We demonstrated that using suitable cutoffs and donor cohort sizes, this assay could identify most of the compounds with high clinical immunogenicity rates (71% and 78% for sensitivity and specificity, respectively) while we characterized the main sources of assay variability. Overall, these data indicate that the dendritic cell and CD4+ T cell restimulation assay published herein could be a valuable tool to assess the risk of drug-specific T cell responses and contribute to the selection of clinical candidates in early development.

Keywords: immunogenicity; immunomodulation; biotherapeutics; in vitro T cell assay; assay validation

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1. Introduction

Despite success in the clinic, a substantial number of biotherapeutics elicit unwanted immune or immunogenic responses—termed immunogenicity. One of the hallmarks of immunogenicity is the onset of anti-drug antibodies (ADAs). Due to ADAs exhibiting major consequences for both patient's safety and treatment efficacy, it is of utmost importance to assess this risk as early as possible during drug development [1,2].

Partially or fully humanized biotherapeutics (i.e., antibodies with minimal non-germline amino acid sequences) are usually at lesser risk of an unwanted immunogenicity response; however, this measure may not completely abrogate ADA formation. It is now established that a compound immunogenicity risk assessment must include multiple complex factors ranging from product-related risks, such as protein structure, formulation, or impurities [3]; patient and disease-related factors, including genetic factors, age, concomitant treatment; and route of administration [4]. In the case of immunomodulatory drugs, adverse events may also be caused by target binding in healthy tissues, or enhanced pharmacology attenuating the activity of target molecules on cells.

Consequently, an integrated preclinical risk assessment should be considered as a key element in biotherapeutics development. Regulatory bodies, such as the European

Medicines Agency (EMA) and the Food and Drug Administration (FDA), are now encouraging drug developers to consider risk factors related to the product and to the patient, mentioned above, as early as possible in the development process. An integrated approach relies on the use of specific tools and methods to identify relevant immunogenicity factors and to develop corresponding risk mitigation strategies [5]. Currently, these tools include in silico screening algorithms to scan for sequence liabilities, in vitro cell-based assays to measure various readouts from the immune response (dendritic cell internalization, activation and presentation, T cell activation), and the use of transgenic animal models designed to study the intimate mechanisms of an immune response from a mechanistic viewpoint [6]. However, most of these tools have not undergone a formal qualification process, and factors contributing to assay variability are not always understood. For example, T cell-dependent responses are the major drivers for immunogenicity, and in vitro T cell assays are frequently used to identify and measure CD4+ T cell responses to biotherapeutics. These assays have been derived in different formats and reviewed elsewhere [6–8]. However, the sensitivity of these assays is usually quite low, as the size of the pre-existing CD4+ T cell repertoire reactive to the drug is very small, ranging from 1 to 10 cells out of 10^8 T cells [9].

Here, we describe and characterize a dendritic cell and CD4+ T cell restimulation assay and discuss the potential of such an assay to assess a CD4+ T cell-driven immunogenicity risk. This assay consists of a co-culture between monocyte-derived dendritic cells (moDCs) and autologous CD4+ T cells, including a re-stimulation step to increase assay sensitivity. The main goals of this study were to establish an assay threshold to distinguish between positive and negative responses, to determine the optimal cohort size for the assay, and to identify factors affecting assay variability. We are currently using this assay as part of an integrated approach to rank candidate biotherapeutics during the initial selection process, enabling the selection of lower-risk clinical leads for subsequent large-scale production and clinical trials.

2. Materials and Methods

2.1. Compounds

Stock solutions of keyhole limpet hemocyanin (KLH-Imject Maleimide-Activated mcKLH, Thermo Fisher Scientific, Basel, Switzerland, #77600) were reconstituted and stored at −80 °C in single-use aliquots according to the manufacturer's recommendations under sterile conditions. All biotherapeutics were bought from Runge Pharma GmbH & Co (Lörrach, Germany) in their respective formulation and stored according to the manufacturer's recommendations. Peptides were synthesized by Cambridge Research Biochemicals and reconstituted in sterile ultra-pure water (Invitrogen, Basel, Switzerland, #10977015) and 50% Acetonitrile (≥99.95%, VWR, #83639.320). Biotherapeutics were used at a final concentration of 0.3 µM (peptides were used at a final concentration of 10 µg/mL) for both the DC stimulation stage and re-stimulation stage.

2.2. Healthy Donor Cohort

Healthy donors were recruited at Phase I clinical trial units in the UK. All samples were collected under an ethical protocol approved by a local Research Ethics Committee (reference number: 21/LO/0474), and written informed consent was obtained from each donor prior to sample donation. All samples were stored according to the terms of Lonza's Human Tissue Authority license for the use of samples in research. Peripheral blood mononuclear cells (PBMC) from healthy donors were prepared from whole blood or leukopaks using Lymphorep density gradient medium (Cedarlane, # CL5120) within six hours of blood withdrawal. PBMC were controlled-rate frozen and stored in vapor-phase nitrogen at −196 °C until used in the assays. The quality and functionality of each PBMC preparation were analyzed after seven days of activation, with positive controls such as KLH to assess naïve T cell responses. For each screen, the donor cohorts consisted of typically 30 donors selected to represent the world population in terms of their HLA-DRB1 allele frequency distribution [5] (Supplementary Figure S1).

2.3. DC:CD4+ Re-Stimulation Assay (Epibase®IV, Lonza)

Monocytes were isolated from frozen PBMC samples by magnetic bead selection using CD14 microbeads (Miltenyi Biotec # 130-050-201 on an AutoMACS Pro system) and differentiated into immature DC (iDC) using 1000 IU/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF) and 1000 IU/mL of IL-4 in a serum-free mediun (CellGenix # 20805-0500, supplemented with 0.05 mg/mL Gentamicin Lonza # 17-518L) for 5 days at 37 °C, 5% CO_2. iDC were then harvested, washed and loaded with each test protein/peptide individually for 4 h at 37 °C, 5% CO_2. A DC maturation cocktail containing TNFα (800 IU/mL) and IL-1β (100 IU/mL) was then added for a further 40–42 h to activate/mature the DC (mDC). The expression of key DC surface markers (CD11c-3.9, CD14-63D3, CD40-5C3, CD80-2D10, CD83-HB15E, CD86-BU63, CD209-9E9A8 and HLA-DR-L243) at both the immature and mature stage were assessed by flow cytometry (Bio-Rad ZE5 Cell Analyzer) to ensure the DC were activated prior to T cell interaction. After a thorough washing procedure, 100,000 mDCs were then co-cultured with 1 million autologous CD4+ T cells (isolated by magnetic bead selection, Miltenyi Biotec # 130-045-101 on an AutoMACS Pro system) in a deep-well plate (final volume of 1.2 mL, Greiner # 780271). The DC:CD4+ T cells ratio is 1:10 and the co-culture is incubated for 6 days at 37 °C, 5% CO_2 in a humidified atmosphere. On day 6, autologous monocytes were isolated from PBMC using magnetic bead selection (Miltenyi Biotec # 130-050-201 on an AutoMACS Pro system) and loaded with the selected protein or peptide that were initially used to load the DC. After incubation at 37 °C, 5% CO_2 in a humidified atmosphere for 4 h, the monocytes were washed and then added to anti-IFN-γ/anti-IL-5 pre-coated FluoroSpot plates (Mabtech # FSP-0108-10) along with the corresponding DC:CD4 co-culture in quadruplicate (25,000 monocytes: 250,000 CD4+ T cells in a final volume of 200 µL). The FluoroSpot plates were incubated for 40–42 h at 37 °C, 5% CO_2 in a humidified atmosphere. After incubation, the FluoroSpot plates were developed according to the manufacturer's procedure (IRIS FluoroSpot reader, Mabtech) and the number of spot-forming cells (SFC) per well were assessed for each test condition in an automated and unbiased manner.

2.4. Data Analysis

Data management and statistical analysis were performed in the R programming language (https://www.R-project.org/, accessed on 28 October 2022, versions 3.6.1 up to 4.1.2), including essential packages for handling generalized linear models (nlme, emmeans) and carrying out variance component analyses (VCA, version 1.4.3).

The calculation of Stimulation Indices (SI) was performed as follows. Spot forming cells (SFC) from the FluoroSpot assay were transformed to a log2 scale, and a generalized linear model (GLM) was applied to estimate the SI (i.e., the ratio between a treatment condition and the donor-matched blank on a linear signal scale) and associated confidence intervals. Quadruplicate SFC measurements were implicitly aggregated by the GLM to yield one SI value for each combination of a specific test compound, donor, and screen. The screens were analyzed sequentially and independently from each other, with the linear model considering a specific cytokine readout of an entire screen as input. The processing workflow was tailored to address a few peculiarities of the given data. Specifically, we used an exponential type of heteroscedasticity adjustment in the GLM to achieve scale-invariance of residuals and injected some Gaussian noise at the low end of the SFC scale to support model convergence with the frequent presence of ties of discrete values around zero. (The standard deviation of this normally distributed, zero-centered noise was chosen to correspond to the replicate variability inferred by the GLM in the limit of zero SFC counts at the low end of the SFC scale and drops down exponentially by a factor of exp (−2) = 0.14 for every unit increase of the log2 SFC). Furthermore, we observed a consistent trend in the data to the effect that higher blank values of a donor corresponded to systematically lower SI values for that donor. The relation between 'pre-stimulation' of the blank and observed stimulation indices could be well captured by linear regressions performed for each treatment within a screen. We corrected the raw SI values then for every donor-

treatment pair with the respective linear model, basically extrapolating to the value which would have been observed with a common blank value of 0.

Standard quality control plots were generated for every data set, including the visualization of DC differentiation markers, the reproducibility of reference compound data across studies, and (if possible) the variability of repeated compound testing with the same donor. We also looked at the individual stimulation profile of each donor within a study, as the overall inducibility of T-cell response could vary from person to person; simultaneously, this enabled us to rule out the presence of generally inert sample material. A donor response was recorded as "positive" if a SI fold-change of 2 or above (compared to its blank control) was measured at a statistical significance of $p < 0.05$ (using non-adjusted p-values from the GLM). The fraction of positive donor responses (within a cohort of typically 30 healthy donors per screen) provided the response rate for the treatment in a specific screen.

3. Results

3.1. DC:CD4+ T Cell Restimulation Assay Workflow

The general workflow of the assay is illustrated on Figure 1a. Test items were investigated in independent screens of the DC:CD4+ T cell restimulation assay over a time span of several years. Therefore, various controls were employed to ensure a consistent and comprehensive analysis of the data.

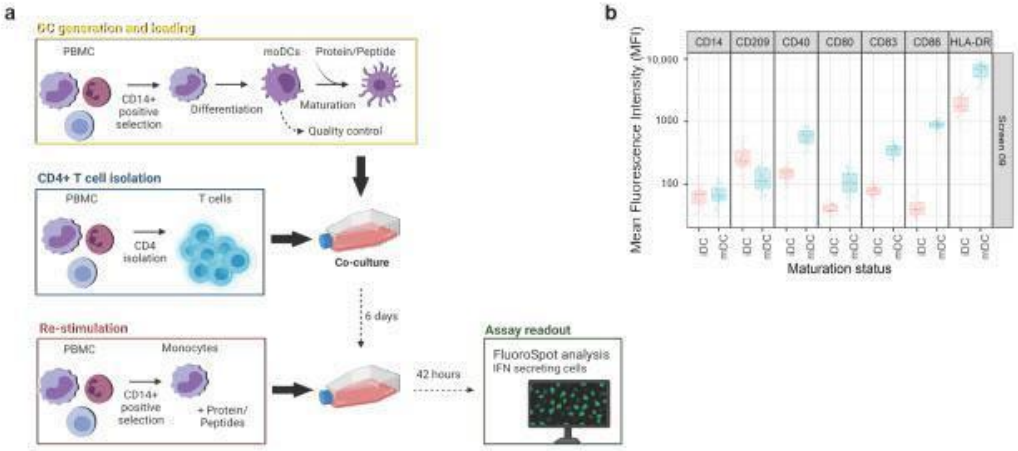

Figure 1. The DC:CD4+ T cell restimulation assay. (**a**) Experimental setup of the DC:CD4+ restimulation assay. The assay starts with the isolation of monocytes from healthy donor PBMCs, followed by the loading of the protein of interest and maturation of the monocyte derived Dendritic Cells (moDC). Autologous CD4+ T cells are isolated and co-cultured with the loaded moDCs. After an incubation of 6 days, freshly isolated monocytes are challenged with the same protein and added to the co-culture for an additional 42 h before analyzing the production of IFN-γ by FluoroSpot. (**b**) DC were characterized by the expression of the following cell surface markers: CD11c, CD14, CD80, CD83, CD86, CD209, and HLA-DR before and after DC activation by addition of TNF-α and IL-1β to ensure good cell fitness. Created with BioRender.com, accessed on 27 November 2022.

For each screen, 30 healthy donors were selected based on their HLA-DRB1 alleles to reflect the world population [5] (Supplementary Figure S1). In addition, a characterization of the dendritic cells (DCs) was included in every screen to assess the phenotype of these cells before and after maturation by flow cytometry. Activation of the DCs was determined by upregulation of key maturation markers on the cell surface that are known to be

correlated with T-cell priming capacity: CD40, CD80, CD83, CD86, and HLA-DR [10]. Moreover, CD209, a pathogen-recognition receptor expressed on the surface of immature DCs, is internalized together with other markers, thus resulting in efficient presentation [11]. Accordingly, the downregulation of CD209 is the consequence of a shift from an immature to a mature DC phenotype. A representative distribution of cell surface marker expression at both the immature (iDC) and mature stage (mDC) is shown in Figure 1b. The addition of the DC maturation cocktail, composed of TNF-α and IL-1β, led to a slightly higher expression of CD40, CD80, CD83, and HLA-DR, but also a substantial increase in CD86 expression, resulting in a more than ten-fold increase in the average MFI for this surface marker. In addition, we also observed a moderate decrease in CD209 expression. Altogether, this analysis confirmed that DCs from all donors of the cohort have the potential to be activated prior to their interaction with autologous CD4+ T cells. Moreover, the assay is qualified for a given immunomodulatory protein by treating the DCs together with KLH to assess what impact the protein has on the KLH-induced T cell response. This enables us to highlight proteins that may influence the DC-induced activation of T cells.

3.2. DC:CD4+ T Cell Restimulation Assay Precision Assessment and Comparators

We investigated first the repeatability of the assay by testing the IFN-γ response of donors to KLH and Avastin (same production batch) in multiple assay screens. To this aim, we plotted the SI for KLH and Avastin for all donors, grouped by batches. All the donors analyzed over 24 screens consistently showed high SIs (with a geometric mean of 225 across all screens) upon treatment with KLH (a widely accepted positive control), while SIs obtained with treatment with bevacizumab were distributed around 1 (Figure 2a), suggesting that there was no substantial change in IFN-γ release compared to the blank. Moreover, very few donors (40/607, 6.6%) in this treatment group showed a two-fold SI change or above (our criterion for calling a positive response, see Section 2). Based on these findings, we recommend the use of bevacizumab as a negative comparator in this assay. We used KLH as the technical positive control in our analyses, as highly immunogenic biopharmaceuticals tend not to reach marketing authorization [12].

We used the DC:CD4+ T-cell restimulation assay to investigate 24 biotherapeutics developed by a range of pharmaceutical companies, comprising a broad range of drug formats and targets. Details about the molecules were extracted from the corresponding FDA label [13] and are summarized in Table 1.

However, for most of the labels, important data about the trial were missing, ultimately limiting the interpretability of the reported ADA rates. Moreover, for a number of trials, drugs were administered in combination with radiotherapy, which is known to impact the immune system and the subsequent production of ADA [14]. In other cases, biopharmaceuticals were administered with corticoid pre-treatment to dampen the immune response, which also influences the production of ADA. In this manuscript, data from combination trials were omitted, except for Alemtuzumab, Cetuximab, Daratumumab, Elotuzumab, Sarilumab, and Tocilizumab, which are always co-administered with other drugs.

Results are summarized in Figure 2b,c. Most of the tested biopharmaceuticals elicited low levels of IFN-γ release (alirocumab, avelumab, benralizumab, bevacizumab, brentuximab, certolizumab, cetuximab, durvalumab, evolocumab, galcanezumab, necitumumab, nivolumab, sarilumab, tocilizumab, ustekinumab, vedolizumab). However, we saw stronger T cell responses with alemtuzumab, elotuzumab, pembrolizumab, infliximab, and daratumumab, for which more than 10% of the donors showed a SI statistically significant above 2. Interestingly, antibodies with identical modes of action (i.e., infliximab, adalimumab, and certolizumab all target TNF-α) triggered different T cell responses with regards to IFN-γ production. In addition, when compounds were tested several times in different screens, we observed that SIs and the derived response rates showed a significant variability (Figure 2c). We observed that for adalimumab, for which screens 02 and 06 resulted in 23.3% and 26.7% of positive donors, respectively, whereas it dropped to 0% in screen 07. These discrepancies are seen for pembrolizumab, atezolizumab, and

elotuzumab, as well. One explanation for this observation could be a compound batch effect, as illustrated for adalimumab in Figure 2d.

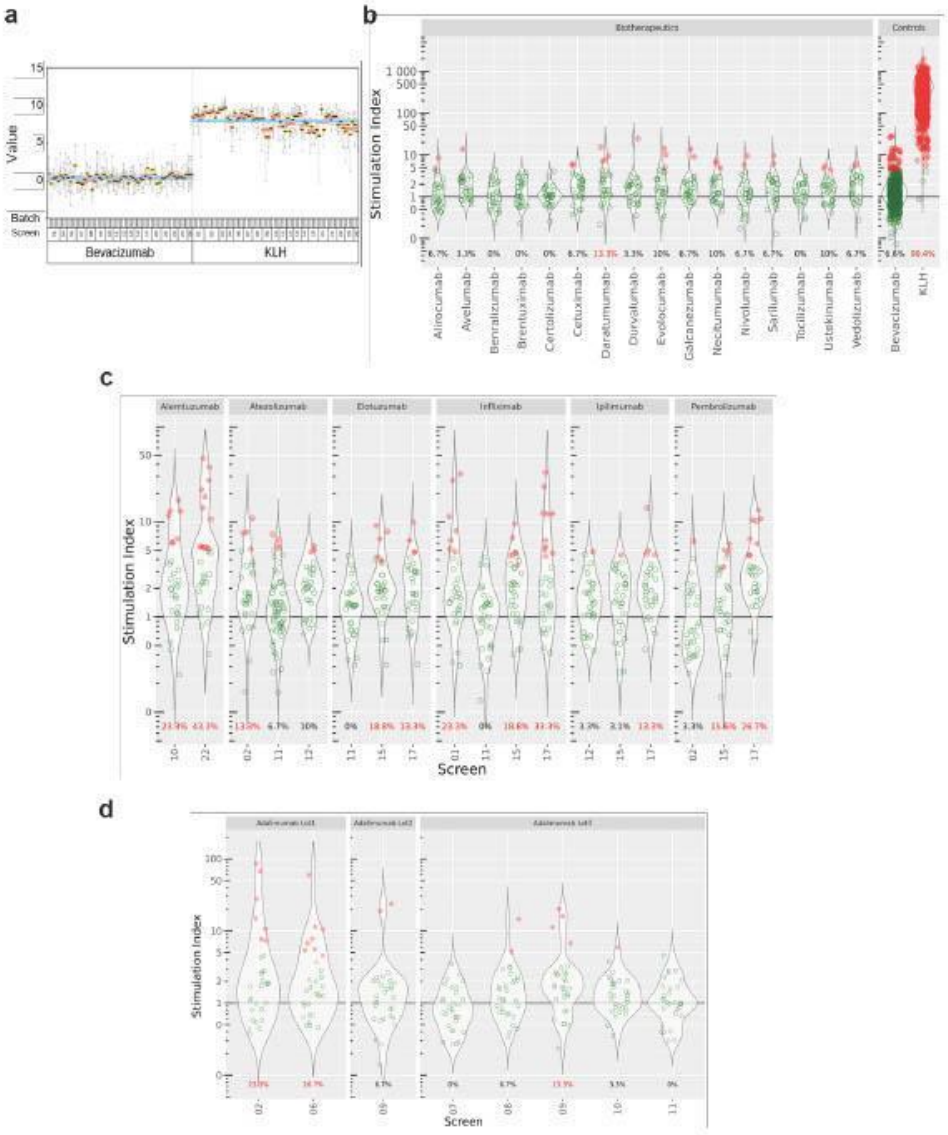

Figure 2. Overview of the stimulation indexes (SI) obtained in the DC:CD4+ T cell restimulation assay. (a) Stability of the controls over different assay screens and donor batches. Data were generated for the set of benchmark compounds in single (b) or multiple screens (c,d). SI represents the number of IFN-γ positive cells over baseline. If a datapoint is significantly above the SI threshold of 2, the donor is considered as positive for this condition and appears in red.

Table 1. Overview of the test items and their respective clinical ADA rates. Alemtuzumab, cetuximab, daratumumab, elotuzumab, sarilumab and tocilizumab are part of a co-treatment. Therefore, consideration should be taken when looking at the reported ADA rates. The information was extracted from FDA labels [13]. If several clinical ADA rates were reported, studies mentioning a co-treatment were excluded and the mean value for the remaining study outcomes was taken. In many cases, larger deviations may be due to systematic differences in the treated patient populations, as well as different analytical methods.

Antibody Name	Trade Name	Format	Target	Main Target Patient Population	Clinical ADA Rate	Screens
Adalimumab	Humira	Human IgG1	TNF-α	Rheumatoid Arthritis	23	02; 06; 07; 08; 09; 10; 11
Alemtuzumab	Lemtrada	Humanized IgG1	CD-52	Multiple Sclerosis	35	10; 22
Alirocumab	Praluent	Human IgG1	PCSK9	Cardiovascular disease	5	10
Atezolizumab	Tecentriq	Human IgG1 no-Glyco	PD-L1	Non-Small-Cell Lung Carcinoma (NSCLC)	44	02; 11; 12
Avelumab	Bavencio	Human IgG1	PD-L1	Urothelial Carcinoma	17	12
Benralizumab	Fasenra	Humanized IgG1	CD-125	Asthma	13	11
Bevacizumab	Avastin	Humanized IgG1	VEGF	Solid Tumor	0.6	ALL
Brentuximab	Adcetris	Chimeric IgG1-ADC	CD-30	Classical Hodgkin Lymphoma (late stage)	30	11
Certolizumab	Cimzia	FabPEG	TNF-α	Crohn Disease and Rheumatoid Arthritis	8	10
Cetuximab	Erbitux	Chimeric IgG1	EGFR	Head, Colorectal and Neck Cancer	5	12
Daratumumab	Darzalex	Human IgG1	CD-38	Multiple myeloma	0	12
Durvalumab	Imfinzi	Human IgG1	PD-L1	Locally advanced or Metastatic Urothelial Carcinoma, NSCLC	3	10
Elotuzumab	Empliciti	Human IgG1	SLAMF7	Multiple Myeloma	27	11; 15; 17
Evolocumab	Repatha	Human IgG2	PCSK9	Cardiovascular Disease	0.3	10
Galcanezumab	Emgality	Humanized IgG4	Calcitonin	Migraine	5	10
Infliximab	Remicade	Chimeric IgG1	TNF-α	Psoriatic Arthritis	27	01; 11; 15; 17
Ipilimumab	Yervoy	Human IgG1	CTLA-4	Metastatic melanoma, advanced renal cell carcinoma, metastatic colorectal cancer	8	12; 15; 17
Necitumumab	Portrazza	Human IgG1	EGFR	NSCLC	4	12
Nivolumab	Opdivo	Human IgG4-CPPC	PD-1	NSCLC	11	02
Pembrolizumab	Keytruda	Humanized IgG4-CPPC	PD-1	Cancer	2	02; 15; 17
Sarilumab	Kevzara	IgG1	IL-6R	Rheumatoid Arthritis	9	10
Tocilizumab	Actemra	Humanized IgG1	IL-6R	Rheumatoid Arthritis	2	10
Ustekinumab	Stelara	Human IgG1	IL-12/IL-23	Plaque Psoriasis	6	10
Vedolizumab	Entyvio	Humanized IgG1	Integrin α4β7	Ulcerative colitis and Crohn's disease	6	11

3.3. Statistical Characterization of the Assay

We investigated the stability of the assay across independent screens and the potential influence of confounding experimental factors using a variance component analysis on the full data set, which included the controls as well as the marketed compounds.

The data reported in this study were recorded in 24 different screens over several years. Hence, data replication occurred on various levels (i.e., bevacizumab and KLH were measured in all screens, several compounds were repeatedly measured in some

screens, while subsets of compounds were tested on all donors within each screen), we could estimate the variance contributions of the treatments, the donor, the treatment-donor interaction, and the screen. As a typical screen is done in 3–4 batches, running a given subset of donors on all compounds in each batch, we can also assess the batch effect that is nested in the screen. During the course of the study, healthy donors that had given their blood could visit the blood donation center again, and the derived cells were used in two (or more) screens (i.e., same donor, same treatment but different screens). The results of this analysis are summarized in Figure 3a. Treatment-related effects (the expected effect from a compound in the assay, here driven primarily by the large number of strong KLH responses) accounted for 54% of the total variance; in contrast, the contribution of purely experimental factors was quite small (screen-to-screen variability: 0.5%, batch-to-batch variability within a screen: 2.0%). The donor factor (i.e., a factor accounting for a generally higher or lower donor-specific IFN-γ release independently of the treatment) accounted for 6.9% of the total variability; a similar proportion of the variance (5.4%) was attributed to the donor-treatment interaction (i.e., a factor taking account of a subject-specific response to a given treatment). A relatively high proportion of the total variance (23.6%) could not be readily accounted for by the known experimental factors. This could be due, for example, to the unavoidable technical variability in the protocol used to carry out the assay, or to heterogeneities unaccounted for when collecting sample material from a given donor at different times. In general, it would be very difficult to single out these technical and biological sources of variance and to investigate their relative impact on the assay reproducibility without some very cumbersome additional quality control processes.

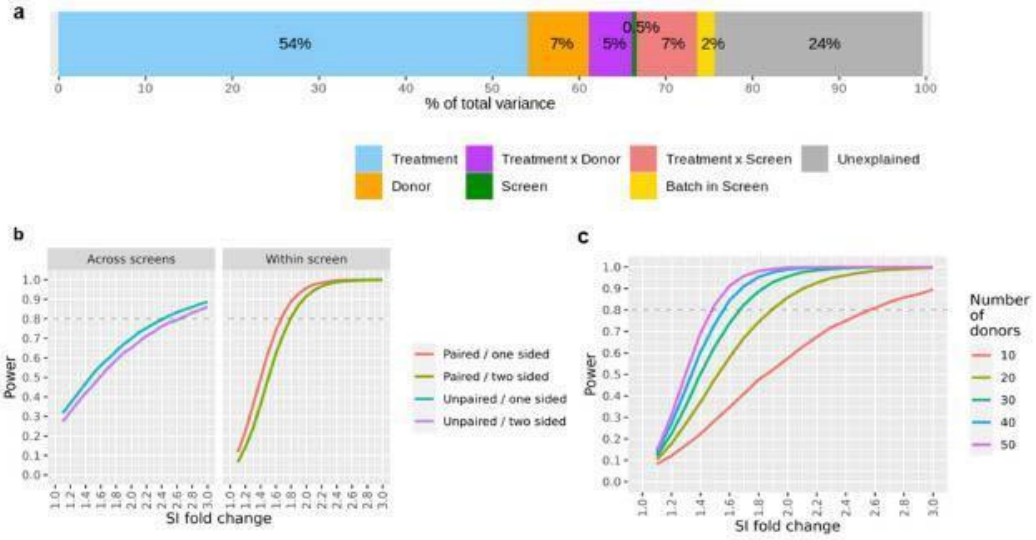

Figure 3. Variance and assay power. (a) Main factors contributing to assay variability estimated by a variance component analysis. The fitted model is the following: log2 (SI) ~ (Compound × DonorID) + Screen/Batch. There is a relatively low relative impact of assay batching variables (Screen, Batch within a screen) in comparison to the compound component. (b) DC:CD4+ T cell restimulation assay power curves for compound comparison. (c) Assay power curves showing the statistical power to detect a treatment effect by comparing a compound with a comparator treatment. A one-sided paired test within-study ($\alpha = 0.05$) according to various donor cohort sizes has been used.

The breakdown of the SI readouts into individual variance components enables us to simulate data sets with specified effect sizes for hypothetical treatments. Hence, we can estimate the statistical power (i.e., the probability of detecting a true compound effect) in a wide range of conditions. For example, Figure 3b shows the resulting statistical power when comparing a compound SI fold-change response with the one of a reference or comparator treatment; here, we differentiate the case where both compounds of interest were assessed in the same screen, in contrast to a comparison that was conducted across different screens. A major advantage of a within-screen comparison is that one could apply paired testing (i.e., using 'donor' as a covariate) to yield higher statistical power because the donor-to-donor variability would be partially accounted for in this approach. This is, in our opinion, the recommended setting for a compound ranking study. Moreover, depending on the hypothesis of interest, some additional statistical power may be gained by using a one-sided testing approach. This is legitimate when only a higher (or lower) compound response is of interest as compared to a reference treatment, which, in fact, could be the most relevant scenario. As a rule of thumb, we expect that SI differences of about 75% on a linear scale (i.e., a SI fold-change of 1.75 or 0.8 log2 units) can be detected with a statistical power of 80%, assuming one-sided testing within the same screen, alpha = 0.05, and n = 30 donors.

Statistical power is also a function of the sample size (here, number of donors per screen); we next examined this dependency and the impact of this variable in the interpretation of our assay results (Figure 3c). We observe a considerable gain in statistical power for studies including up to 30 donors per study. Increasing the number of donors beyond this point leads to noticeably smaller gains in statistical power at the cost of a considerable increase in effort and expenses, which is associated with larger experiments. In our experience, a standard study size of 30 donors per screen strikes the right balance, both for the experimental and statistical angles. In the case that enhanced statistical power is desired, we believe that a reduction of the residual assay variance by experimental protocol refinements could be a more promising approach than merely increasing the donor count.

3.4. Qualification of the Assay Threshold

An essential aspect of the study was to investigate the assay's ability to predict the potential for unwanted immune responses in line with FDA labels [13]. Accordingly, we characterized our assay in terms of accuracy (overall rate of correct predictions on compound level), sensitivity (probability to detect an immunogenic treatment), specificity (probability of correctly identifying a non-immunogenic treatment), and Positive/Negative Predictive Value (confidence in assigning either label correctly). To this end, we tested the aforementioned 24 molecules for which clinical data were available; however, since ADA responses in a limited number of patients would not necessarily be considered a relevant risk, we divided the tested molecules into two categories: high risk (≥20% reported ADA rate) and low risk (<20% reported ADA rate) for immunogenicity according to the reported data upon treatment. This classification was correlated to the proportion of donors for which a given biopharmaceutical triggered a CD4+ T cell-driven IFN-γ production in the assay: a positive assay readout was set to generate a SI statistically significant above 2 compared to the blank control, while a negative assay readout would not.

Using 10% as an optimal threshold (>3/30 positive donors according to our criteria), the assay reported 4 true positives (TP) and 16 true negatives (TN) for a total of 24 tested biopharmaceuticals (6 categorized as high risk, 18 labeled as low risk). It categorized 2 antibodies (daratumumab and pembrolizumab) at high risk of immunogenicity, even though their clinical ADA rates were below 20% (false positives, FP), while brentuximab and atezolizumab are categorized as low risk of immunogenicity, even though their clinical ADA rate were above 20% (false negatives, FN). The accuracy is the sum of true positives and true negatives over the total of tested compounds, yielding an estimated assay accuracy of 83% (20/24). The sensitivity, TP/(TP + FN), and specificity, TN/(TN + FP), are two additional important estimators, which represent the two types of possible errors. At this threshold,

the DC:CD4+ T cell restimulation assay provides a 67% sensitivity at 89% specificity, with a 67% (4/6) and 89% (16/18) Positive and Negative Predictive Value, respectively.

3.5. Case Studies in Pre-Clinical Research

An important motivation of running a DC:CD4+ T cell restimulation assay in a pre-clinical setting is to derive information on whether compounds in development might be at risk of inducing an immunogenic response in treated patients. In this context, it is important to reduce false positive compound categorization, even at the expense of a higher false negative rate (i.e., over-classifying new molecules in the high immunogenicity risk category). As part of an integrated immunogenicity risk assessment, other risk factors (e.g., peptide presentation, mode of action, etc.) should also be taken into consideration. Our analysis demonstrates that a direct comparison of the responder rates in the DC:CD4+ T cell restimulation assay with the proportion of ADA-positive patients for a given treatment may not provide the best context of use for this assay. Our proposed strategy is to apply a given threshold to interpret results, essentially reducing the assay output to a binary outcome for biotherapeutics immunogenicity hazard identification. This enables us to retain the essential information on compound risk categorization, while minimizing the impact of noise in the data. Our data suggest that a selected threshold of 10% positive responders to classify a molecule as bearing a higher potential for immunogenicity is the optimal cutoff to flag compounds with high immunogenic potentials, while limiting the number of false negatives at an early stage of preclinical development.

To illustrate the strategy delineated above, we provide here a case study derived from one of our internal programs where seven potential clinical candidates from the same project, which differ from their primary sequence, have been tested in the assay (Figure 4a). The results showed that compounds A, B, D, and G were above the threshold, whereas variants C, E, and F were below the threshold and, therefore, associated with a lower risk of immunogenicity.

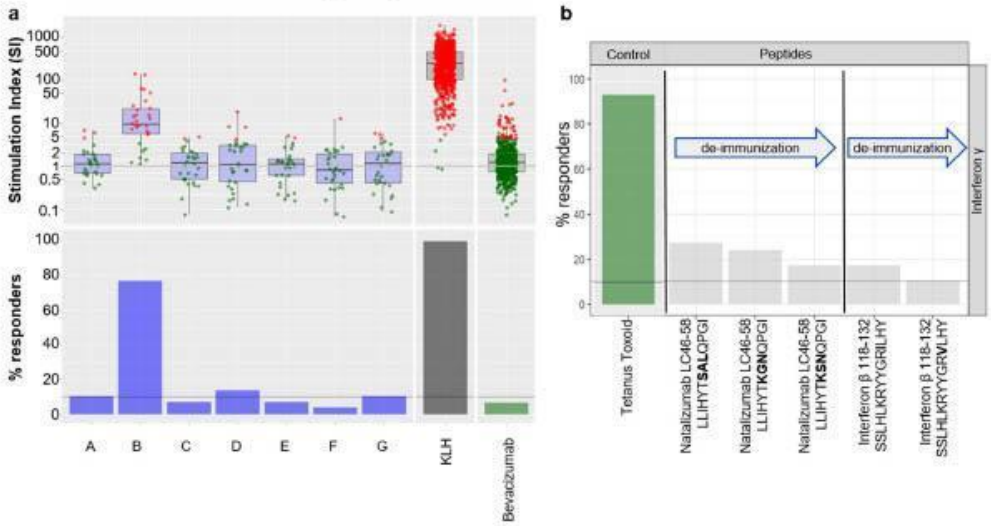

Figure 4. Case studies in pre-clinical research. (a) Stimulation Index (SI) obtained for 7 different candidate compounds of the same project (named A to G). The change in color, from green to red, depicts the positivity of the donor within the screen. The lower panel represents the proportion of positive donors. The threshold derived from the validation study is set at 10% positive donors. (b) DC:CD4+ re-stimulation results obtained for a selection of known T cell epitopes derived from biotherapeutics and their "de-immunized" counterparts.

Furthermore, we demonstrated that this assay was also suitable for testing whether peptides could trigger a CD4+ T cell response. Hence, we tested known T cell epitopes from Natalizumab and Interferon β, as well as potential deimmunized versions [15,16] (Figure 4b). Peptides were tested at 2 ug/mL and followed the same experimental procedure as described in the Material and Methods section. Results from the assay demonstrate that minor changes in the amino acid sequence of the T cell epitopes could reduce the onset of a CD4+ T cell response, thus confirming the published findings, but also that this assay can accommodate peptides (e.g., peptide based biotherapeutics or T cell epitopes).

4. Discussion

The multifactorial nature of immunogenicity requires that an integrated preclinical risk assessment should be a key element of biotherapeutics development. As T cell-dependent responses are major drivers of immunogenicity, in vitro T cell assays are frequently used as tools to identify and measure CD4+ T cell-dependent responses to biotherapeutics. The DC:CD4+ T cell restimulation assay described here assesses the propensity of a biotherapeutic to trigger a CD4+ T cell response that may result in B cell activation and ADA production. This assay plays a key role in our integrated approach to therapeutic protein immunogenicity risk estimation, which could accelerate drug development.

While a number of assays probing T cell activation in the context of immunogenicity have been published in recent years [6], we believe that the DC:CD4+ T cell restimulation assay described here provides a more comprehensive insight into the role of dendritic cells (taken here as the archetypal APC) in the context of their activation of T cells [17]. The immune response follows a three-signal rule for activation (TCR:MHC/peptide interactions, costimulatory interactions such as via CD28, and cytokine production); the assay published herein captures the interplay of all three signals [18]. In addition, the number of preexisting T cells specific to biotherapeutics is very low, ranging between 1 out of 10^8 (e.g., trastuzumab and etanercept) and 1 out of 10^7 T cells (e.g., rituximab) [9], but the assay format of the DC:CD4+ T cell restimulation assay allows screening of more CD4+ T cells than in a classical PBMC-based assay. We also believe that the re-stimulation step increases the likelihood of capturing a sustained T cell response [19].

A key part of validating the DC:CD4+ T cell restimulation assay was assessing repeatability and reliability, and its potential to categorize biotherapeutics according to their risk of inducing an unwanted immune response in the clinic. To this end, we carried out a detailed analysis of 24 biotherapeutics with various levels of clinically-detected ADA rates as a proxy for immunogenicity risk. Our robust assessment comprised several levels of repetitions, including repeated assessment of some compounds in multiple assay screens, to provide insights into both the potential and inevitable limitations of the assay. Variance component analysis showed that the primary factors governing the experimental setup, i.e., the screen and the batched donor processing within each screen, did not have a major systematic impact on the readouts. Notable variability arose, however, when compounds were re-tested in another screen, presumably related to subtle variation in compound preparation or the used production batches. Nonetheless, the compound batch effect is not specific to this assay. It is likely that the handling and storage of the sample plays a role here, influencing post-translational modifications and aggregation. Additionally, non-product related factors (e.g., DNA and host cell protein contaminations) have an impact on the risk of immunogenicity and could also influence the assay readout. General donor specific inducibility and the donor specific response to individual compounds also explained parts of the signal variability. However, there was a rather substantial residual unexplained variance, which should caution the user with regard to overinterpretation of individual readouts. Notably, quantification of SI changes in a strict sense were not directly informative, as even clinically-tested compounds with low immunogenic risks (for example, bevacizumab) resulted in a few positive readouts. We presume that additional insights might be gained by fundamental and costly changes of the lab protocol, i.e., performing replicated measurements in different experimental batches for every condition. In our

experience, donor cohorts of 30 individuals per screen offer a reasonable tradeoff between the cost, timelines, and statistical power of the assay. While not specifically discussed in the manuscript, we found it important to test all compounds using a panel of donors that showed an HLA-DRB1 allele frequency that broadly reflects the world population. It has been demonstrated that certain HLA alleles were associated with an increased immunogenic response towards certain biopharmaceuticals [20–25]. Nevertheless, in the context of use of this preclinical assay carried out in 30 donors, we primarily investigated whether compounds may be at risk of inducing an enhanced immunogenic response in a general population. As each screen usually comprised different sets of donors, an arbitrary selection of pre-typed donors with respect to their allelic HLA-DRB1 composition enabled a higher comparability of the data in the long term.

While tempting, it is problematic to compare SI values measured in a DC:CD4+ T cell restimulation assay with actual ADA rates in the clinic, although it is one of the few available measures directly related to clinical immunogenicity. Assays used to measure ADA in clinics are based on different methodologies sensitive to sample handling, the timing of sample collection, concomitant medications utilized in the study, and the underlying nature of the treated disease [26]. Furthermore, while we believe we have used the most recent information available on the FDA database, most labels may not be updated on a regular basis: in a recent review, Borrega et al. showed that 57% (39/69) of the biological drugs authorized before 2012 did not have updated summaries of product characteristics, especially in the immunogenicity section [27]. In our study, we collected the ADA rates of the 24 assessed marketed compounds as a starting point to build a database to benchmark newly developed immunogenicity estimation methods and to have a retrospective and comprehensive overview of the immunogenicity of marketed antibodies. We used the available data to create two categories of compounds, at high (≥20% reported ADA rate) and low (<20% ADA rate) risk for immunogenicity, on which we calibrated the assay's linear mixed model. Thus, this binary high/low risk paradigm is the most reasonable for implementation in preclinical risk evaluation for therapeutics. To facilitate this process, we found it essential to add in the panel a few standard compounds (at minimum, a negative control, such as bevacizumab, and a positive control, such as KLH; any additional comparators also provide useful comparisons) to help set precise boundaries of low and high risk of immunogenicity while mitigating intrinsic donor variability. Accordingly, in this context of use, one of the most useful applications of the DC: CD4+ T cell restimulation assay is to provide a relative ranking for compounds with similar amino acid sequences and mode of action, or compounds that have different formulation or have been produced in different batches.

While assays measuring T cell activation in response to novel biopharmaceuticals are not yet required by regulatory agencies, there is added value in presenting the results of such assays as part of the risk assessment submitted in the Integrated Summary of Immunogenicity [28]. A current challenge is that none of the published assays is considered to be fully validated. We here propose a new assay format that captures the interaction between DCs and CD4+ T cells by monitoring the production of IFN-γ by CD4+ T cells in response to biotherapeutics processed by DCs. We tested the predictive power of this assay vs. clinical ADA rate by assessing 24 marketed antibodies, which resulted in 83% accuracy. Predicting the actual rate of ADA-positive patients in a clinical setting with a single in vitro assay is unlikely to be possible, given the myriad contributing factors. However, the DC:CD4+ T cell restimulation assay can help flag potentially immunogenic biopharmaceuticals in preclinical drug development, allowing for selection or de-immunization before a clinical trial starts, improving both patient safety and the cost of pharmaceuticals. Implementation of this assay as part of a comprehensive risk assessment has the potential to provide a more robust and informative immunogenicity risk assessment in early drug development.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pharmaceutics14122672/s1, Figure S1: Heatmap showing the relative distribution of the HLA-DRB1 supertype frequencies among the donor cohorts per screen of the DC:CD4+ T cell restimulation assay.

Author Contributions: Conceptualization, M.S., G.S., L.C.F., K.B.-F., T.P.H., N.S. and C.M.-D.; Formal analysis, M.S., G.S., L.C.F., A.D. and C.M.-D.; Investigation, M.S., G.S., L.C.F., F.C., J.H., K.H., C.M.L., N.S. and C.M.-D.; Writing—original draft, M.S., G.S., L.C.F., A.D. and C.M.-D.; Writing—review & editing, F.C., J.H., K.H., C.M.L., K.B.-F., O.R., T.P.H. and N.S. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are contained within the article or Supplementary Materials.

Conflicts of Interest: M.S., G.S., L.C.F., K.H., C.M.L., A.D., K.B.F., T.P.H. and C.M.D. are employees of F. Hoffmann. La Roche (Switzerland); F.C., J.H. and N.S. are employees of Lonza Biologics (United Kingdom). The company had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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6. Publication 3

Development and validation of dendritic cell assays contributing to the immunogenicity risk evaluation of biotherapeutics

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Manuscript in preparation

Dendritic cells (DCs) are the first actors of an immune response; their contributions are from the internalization of extracellular antigens, to their processing and subsequent presentation of T cell epitopes on MHC-II receptors. These mechanisms constitute the first steps of the immunogenicity cascade, putting DCs in the spotlight to help with immunogenicity prediction. To better understand the mechanisms of DC uptake and activation and their relationship with immunogenicity, we developed and validated a flow cytometry-based uptake and activation assay that will contribute to the overall risk assessment of immunogenicity of our molecules.

We describe in the manuscript herein two additional assays looking at the contribution of DCs to an immunogenic response. We evaluated both assays with a set of marketed biotherapeutics, identifying for some, a potential influence on DC, which might contribute to their observed immunogenicity in clinical studies.

I contributed to the discussions and organization of the DC internalization benchmarking study. I designed the DC activation assay, optimized it and performed all the experiments related to this assay (except the experiment represented in fig. 2.c). I created the figures, wrote the original version, participated in the discussions and revisions of the manuscript.

Development and validation of dendritic cell assays contributing to the immunogenicity risk evaluation of biotherapeutics

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KEY WORDS

Immunogenicity, immunomodulation, biotherapeutics

Introduction

Immunogenicity, defined as the ability to provoke an immune response, can be either wanted or unwanted. Wanted immunogenicity is typically induced by vaccines, which elicit an immune response against pathogens. On the other hand, unwanted immunogenicity refers to an immune response after the administration of biotherapeutics, leading to the production of anti-drug antibodies (ADA), which often negatively affects the efficacy by reducing exposure and/or the safety profile of these drugs. Consequently, evaluation of the risk of immunogenicity during lead identification and optimization phase in the development of biotherapeutics is of critical importance. Most of the experts in the field are cognizant of the complexities and agree that no single assay or strategy may be used for immunogenicity assessment of all therapeutic protein products. Thus, further improvements in the existing individual preclinical tools alone are unlikely to resolve important impediments to accurate predictions of immunogenicity in a clinical setting (Rosenberg & Sauna, 2018). However, taking an holistic approach and assessing the combination of differential relevant steps leading to an immunogenic response could lead to a better prediction of clinical immunogenicity and help to develop safer and more effective medicines for patients.

ADA production is the last step in a series of immunological events that starts with the internalization of antigens (Ag) by dendritic cells (DCs). The internalized Ag is processed in the lysosome into peptide fragments which are subsequently presented as peptide-MHC-II (pMHC-II) complexes at the surface of the dendritic cells. Subsequently, specific T cells recognize these complexes and in presence of a co-stimulatory signal are able to provide the necessary help to B cells, which will differentiate into plasma cells and produce ADAs. As DCs play a crucial role in the initial steps of immunogenicity, assays that measure their ability to present Ag-derived peptides on MHC-II receptors, such as MAPPs (MHC-II Associated Peptide Proteomics) are widely used in preclinical immunogenicity assessment (Steiner et al., 2020) whereas other biomarkers of their activity (e.g, internalization, activation) have been

often overlooked. Nevertheless, a few studies have highlighted the critical role of the biology of the DCs on the onset of ADAs. . For example, Xue et al. demonstrated that DC internalization and activation contribute to the development of the immunogenic response against ATR-107 (Xue et al., 2016a). In addition, the contribution of the DCs in the initiation of an immunogenic response has been studied primarily in the context of protein aggregates. Indeed, sub-visible antibody aggregates are more prone to be internalized by APCs, to activate them and to elicit the presentation of Ag-derived peptides on MHC-II receptors (Gallais et al., 2017; Rombach-Riegraf et al., 2014). Different methods were already described to measure internalization into APCs (Melendez et al., 2022); (Kovalova et al., 2020), (Wen et al., 2020); (Xue et al., 2016b) using different readouts (e.g., targeting the human Ig Fcγ fragment with a labeled F(ab')2, FRET based, microscopy).

In addition to measuring the internalization rate of therapeutic antibodies into DCs, determining their impact on the activation of DCs is also important to better evaluate their risk for immunogenicity. Indeed, the immune response follows a three-signal rule for activation (TCR:MHC/peptide interactions, costimulatory interactions, and cytokine production) (Goral, 2011); the assay published herein captures the contribution of DCs in providing the costimulatory signal to T cells

In this context *in vitro* models looking at DC activation markers have been proposed. CD40, B7 (CD80, CD86), CD83, DC-SIGN (CD209) and HLA-DR are the most established surface markers to estimate the activation status of DCs (Ahmadi et al., 2015; Groell et al., 2018; Morgan et al., 2019). Despite the use of protein aggregates, moDCs activation is hardly detectable *in vitro*, making the development of such an assay for non-stressed, monomeric antibodies challenging. Recently, an assay investigating moDC activation upon treatment with native antibodies was published (Wickramarachchi et al., 2020). In light of the increasing importance of understanding the role of DCs in immunogenicity, we describe a new method to evaluate antibody internalization into moDCs. In addition, an adapted version of the DC activation assay described by (Wickramarachchi et al., 2020) was added to the workflow.

Evaluating the performance of immunogenicity assays to evaluate the risk of immunogenicity is of great importance (Paul et al., 2020). Therefore, a validation of these methods has been performed and the role of DCs in the initiation of an immunogenic response was highlighted.

Results

Validation of the DC internalization assay (DCIA)

A set of commercially available therapeutic antibodies was tested to evaluate the performance of the DCIA and to better understand properties that could influence internalization of therapeutic antibodies. The workflow of the DCIA depicted on Figure 1.

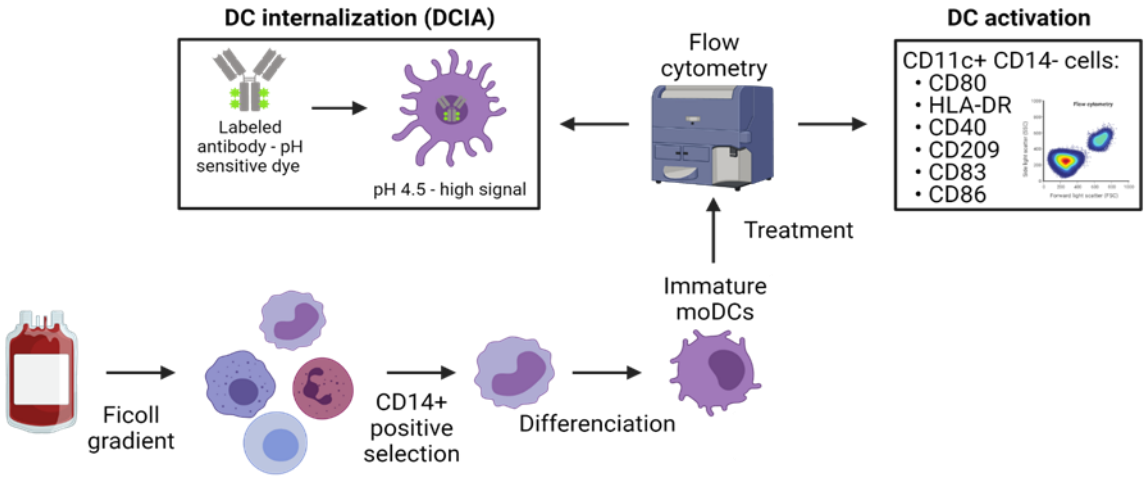

Figure 1. Overview of the experimental procedure to assess moDCs contribution to immunogenicity. The common starting point of the assays is the PBMC isolation according to standard protocols. CD14+ cells isolation and differentiation into immature moDCs are also shared between the procedures. Immature moDCs are challenged with the treatment according to the Material and Methods for the internalization and activation assays

The relative internalization rates, scaled to our internal control (untargeted IgG1), for a large set of antibodies (trastuzumab, ustekinumab, ixekizumab, alirocumab, evolocumab, utomilumab, bevacizumab, briakinumab, adalimumab, bococizumab, ATR-107 and secukinumab) (fig.2).

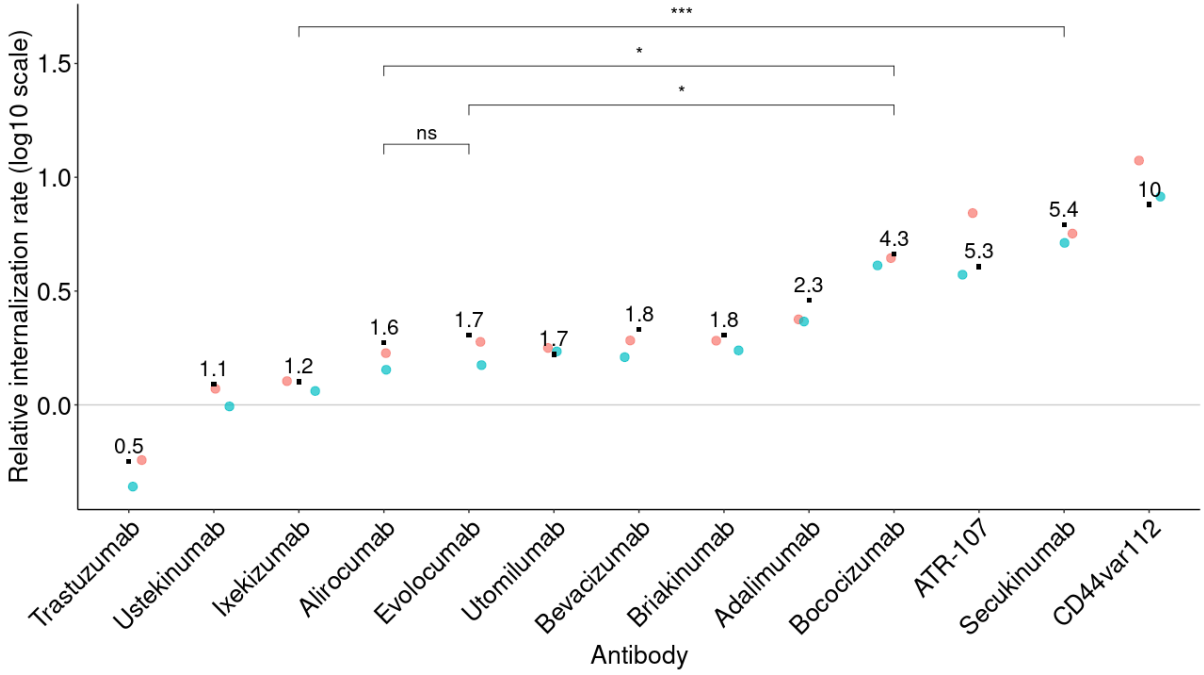

*Figure 2. Internalization rates of monoclonal antibodies. The plot displays the internalization rate of compounds with a pH sensitive labeling into the acidic lysosome of CD11c+ moDCs from 2 individual healthy blood donors. The relative internalization rate represents the internalization efficiency as calculated by the slope of mean fluorescence (MFI) values between the 120 min and 240 min time points, normalized with the medium control and scaled to our internal control (according to the Material and Methods section). A one-sided paired t-test was applied for the comparison of antibodies sharing the same target (p < 0.0001= ****; p < 0.001= ***; p < 0.01= **; p< 0.05= *; not significant= ns).*

ATR-107, bococizumab and Secukinumab (Adalimumab to a lesser extent) showed an important increase in DC internalization compared to our internal IgG1 control. The other benchmark molecules (trastuzumab, ustekinumab, ixekizumab, alirocumab, evolocumab, utomilumab, bevacizumab, briakinumab) showed to be internalized by DCs at a similar rate compared to the internal control (around 1 relative internalization rate). We observed that the donor inter-variability was quite low in this assay. As a consequence, a limited cohort of donors is sufficient to maintain the statistical power when looking at differences between

treatments. This comparison is especially important for molecules sharing the same target, as in some cases target engagement could influence the outcome of the assay. As an example, we observed that secukinumab was accumulating quicker into lysosomes than ixekizumab, both targeting IL-17. The same comparison can be done for alirocumab, evolocumab and bococizumab, all targeting PCSK9 with the latter showing a dramatic increase in DC internalization rate.

Comparison of different DC activation assay setups

Many protocols used to differentiate monocytes into monocyte-derived DCs in-vitro are described in the literature (Sander et al., 2017a). Based on past experience and the use of moDCs in the MAPPs assay internally (Steiner et al., 2020), we used five days of differentiation as a starting point for generating the cells. As previously reported, shorter differentiation periods were used in the case of such assay development (Wickramarachchi et al., 2020). Therefore, we compared the two timepoints to ensure that we have the best moDCs phenotype on the day of the challenge with the Ag of interest. This was assessed by flow cytometry as described in the Material and Methods section. Briefly, cells were gated for singlets, morphology and viability. Mean Fluorescence Intensities (MFI) were extracted for the different activation markers (CD80, HLA-DR, CD86, CD83, CD209 and CD40) on CD11c+ CD14- viable cells (fig. 3.a).

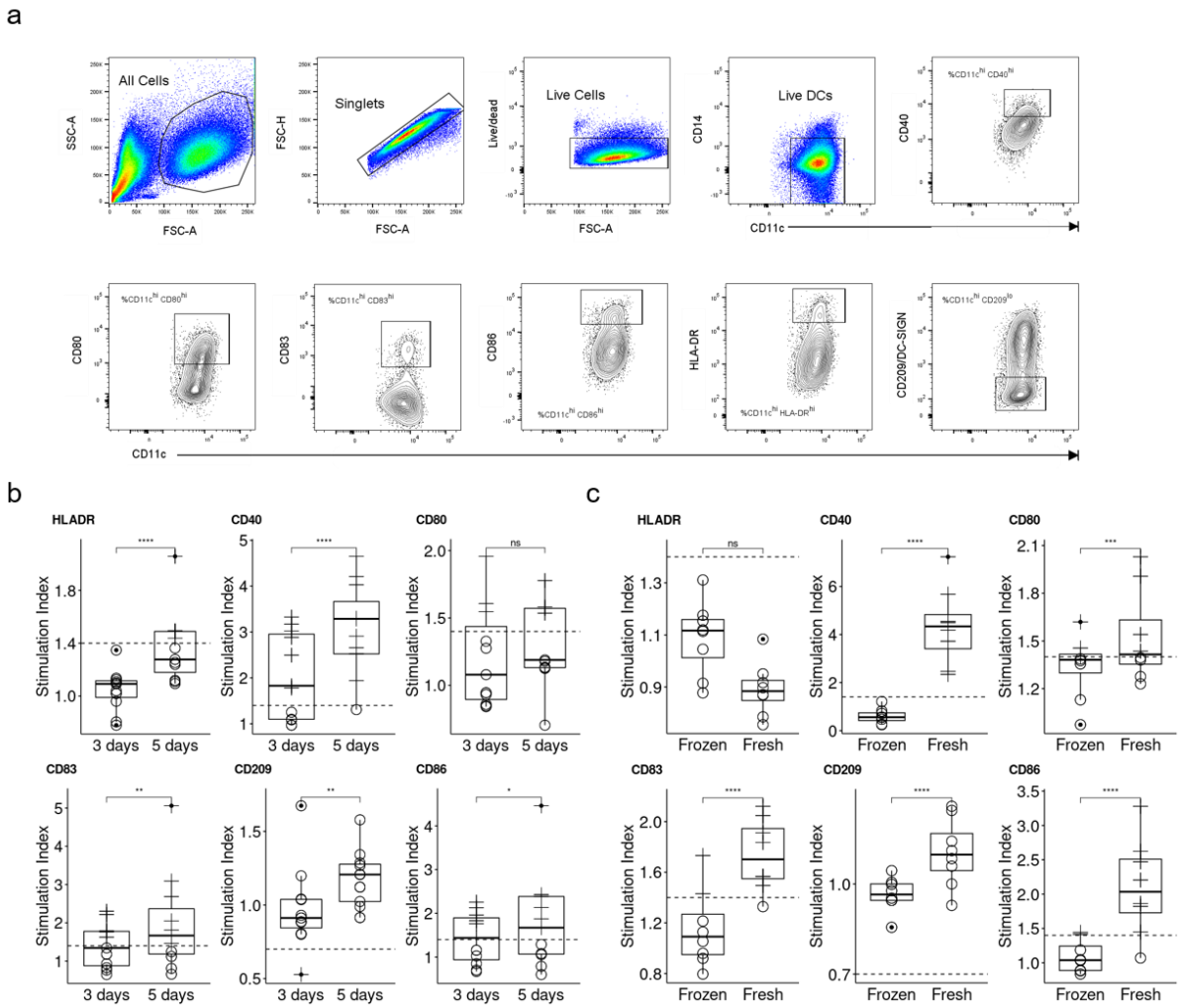

*Figure 3. Comparison of two moDCs differentiation durations and cell source using the response to KLH. (a) Representation of the flow cytometric gating strategy applied for the assessment of DC activation. (b) Three days of differentiation was compared to an extended differentiation of five days by assessing the moDCs response to KLH. Individual moDCs SI were calculated and a plot per activation marker generated. (c) Freshly isolated and frozen PBMCs have been compared in their ability to respond to KLH. A one-sided paired t-test has been performed ($p < 0.0001$= ****; $p < 0.001$= ***; $p < 0.01$= **; $p < 0.05$= *; not significant= ns).*

The assay system was tested first for responsiveness after LPS treatment, which is known to activate moDCs via the TLR-4 pathway (Supp. fig 1.a). The set of activation markers tested (HLA-DR, CD40, CD86, CD83, CD209 and CD80) all behave similarly in both differentiation

protocols. Indeed, all of them were upregulated in a dose dependent manner, except for CD209 which was downregulated as expected (Suppl. Fig 1.b). However, as LPS leads to receptor mediated moDCs activation, we also tested KLH, an Ag which does not interact with specific surface receptors expressed on moDCs, as it would be the case for most of the biopharmaceuticals (fig. 3.b). As the moDCs response towards KLH was weaker compared to the one against LPS, we could better distinguish the differences in the dynamic of the response, Indeed, in our hands the moDCs were more responsive to KLH treatment after a longer differentiation period (except for CD209 downregulation). Another important consideration was the source of the starting material. Indeed, we could show that there were significant differences in moDCs responsiveness to KLH when comparing freshly isolated with frozen PBMCs (fig 3.c). We would therefore recommend the use of freshly isolated PBMCs and the capacity of the cells to respond to KLH as an inclusion criterion to ensure cell fitness. Hence, only moDCs that responded to KLH with at least three activation surface markers significantly overexpressed (SI > 1.4) were included in the subsequent benchmarking study.

Benchmarking of the DC activation assay

The differentiation period of five days and the inclusion criterion determined above were used to benchmark this assay against a set of commercially available biopharmaceuticals (fig.4).

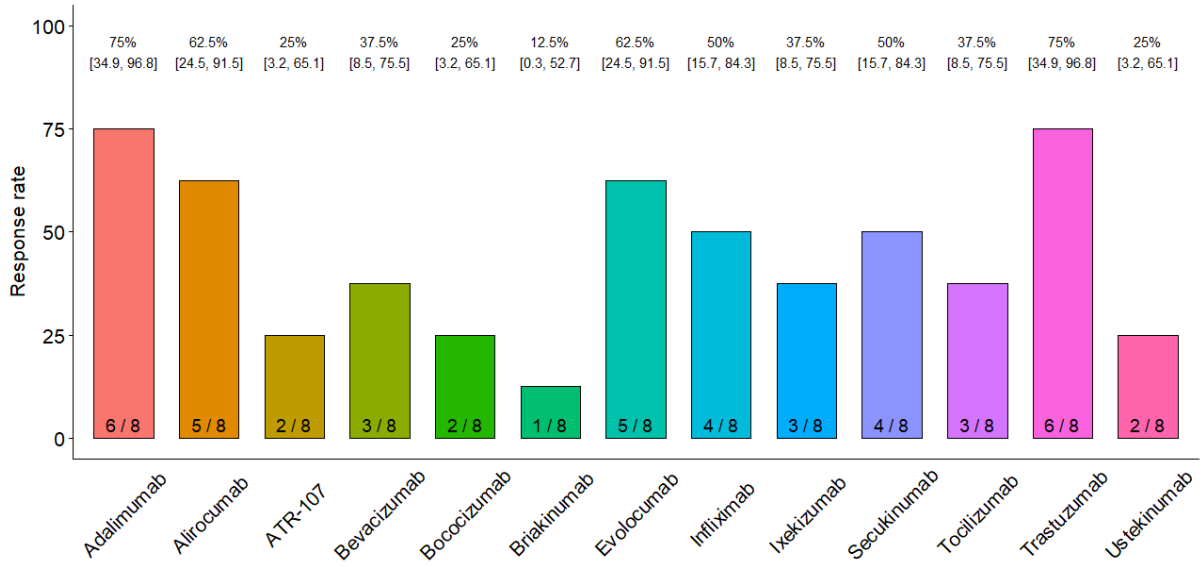

Figure 4. MoDCs activation potential of a set of therapeutic antibodies. Individual donors were included into the dataset based on their KLH response (as described in Material and Methods). The barplot represents the proportion of donors responding (SI ≥ 1.4 for at least one of the activation markers, see Suppl. Fig. 2) to a treatment. The proportion together with the confidence interval are displayed on top of the barplot.

In the comparison of those test articles we found that the significant expression increase of one out of the six surface markers was sufficient to distinguish between the treatments. Using this rule, the response rate of the tested healthy donors could be calculated and allowed the comparison of the moDCs activation propensity for this set of biopharmaceuticals. Adalimumab and trastuzumab being the two antibodies with the greatest moDCs activation propensity compared to briakinumab or even ustekinumab showing a lower response rate in our DC activation assay.

Discussion

Several cell culture aspects are of importance when using CD14+ derived cells which applies to both assays. MoDCs differentiate in a time and IL-4 dependent manner with major implications for the moDC phenotype (Sander et al., 2017b). Therefore the comparison of the differentiation protocols was an important step in the assays' development. Additionally, serum free media are especially important while developing moDC-based assays as the serum composition is highly variable. Additionally, serum contains proteins that are susceptible to be taken up by the DCs and would interfere with the assay (Sauter et al., 2019). The surface used to culture the cells also has consequences, here we used ultra-low binding surfaces based on past experience and studies showing that it has no influence on their T cell activation capacity (Sauter et al., 2019). In the assays described herein, LPS is not added as co-treatment because DC maturation might inhibit macropinocytosis (Roche & Furuta, 2015). There are multiple properties that could enhance an antibody internalization into APCs or their subsequent activation. As an example, the improved immunogenicity profile of ixekizumab compared to secukinumab, both targeting IL-17, could be partially driven by favorable properties and decreased internalization into APCs as demonstrated here.

It has been shown that tissue uptake and clearance from the circulation are enhanced by higher isoelectric point values because of the tendency for these more basic antibodies to adhere to anionic sites of cell surfaces (Boswell et al., 2010). With the hypothesis that this might be a conserved mechanism, it could also lead to increased internalization in our moDCs model and might be an explanation for the increased internalization of bococizumab, having an higher isoelectric point compared to other PCSK9 targeting antibodies (alirocumab, evolocumab), which could contribute to increasing the risk for immunogenicity. Charge interaction might also increase FcRn binding, like described for briakinumab (Schoch et al., 2015). Binding at low endosomal pH and immediate release/non-binding at serum pH would result in good FcRn recycling properties (Kraft et al., 2019) which could explain the reduced lysosomal accumulation for briakinumab. However, slower dissociation with FcRn at a lower

pH in the lysosome could lead to the degradation instead of the recycling of the antibody and modify the derived epitopes. It is therefore difficult to evaluate the implications of this mechanism on immunogenicity as briakinumab has been withdrawn from its clinical trials and no information about immunogenicity has been published.

Another possible mechanism of action for increased antibody uptake could be mediated by target binding. Indeed, Tumor Necrosis Factor (TNF) is a transmembrane protein, upon engagement with the antibody, the complex would be internalized, processed and might lead to the activation of DCs with major implication for immunogenicity (Deora et al., 2017; Kroenke et al., 2021). In our assays, the TNF targeting antibody, adalimumab, did show an increased propensity for internalization, as observed elsewhere (Kovalova et al., 2020). Another consideration is that different glycosylation patterns, which can be batch-dependent, would lead to a difference in uptake efficiency by DCs (Jambari et al., 2021a, 2021b; Wolf et al., 2022). Indeed, C-type lectin receptors, like the mannose receptor or DC-SIGN (CD209), would add to the nonspecific uptake already performed by professional APCs. However, it is not clear if it increases the risk for T cell activation, pointing toward the importance of the processing of the protein and the intracellular signaling initiated.

Last, components of the formulation can also have an impact on the probability of aggregates formation hence having an impact on immunogenicity as well (Dingman & Balu-Iyer, 2018). Aggregates can have a profound impact on antibody internalization and moDCs activation which could also be recapitulated in our assays. In fact, DC activation assays have been historically used to describe immunogenicity of antibody aggregates.

The internalization rate used as the readout of the DCIA, described herein, summarizes all these properties without potentially being affected by the addition of a large payload. The assessment of moDC activation can in some cases help to better understand the DCIA findings. It has been demonstrated that ATR-107 is more present in the late endosome than a comparator molecule. Its internalization into moDCs also led to their activation, assessed by the expression of CD86, CD40 and CD274 (Xue et al., 2016b). ATR-107 targets IL-21R

expressed on the cell surface of DCs, similarly to TNF targeting antibodies could lead to its enhanced internalization. We also demonstrated that this antibody internalizes very quickly in our DCIA but failed to detect moDCs activation. In the above mentioned study, another DC targeting antibody with the same internalization behavior as ATR-107 was tested but did not show DC activation. It is possible that a component of its formulation, a contaminant or a Post Translational Modification (PTM) specific to the tested batch could be responsible for these observations. Understanding why differences between biotherapeutics' immunogenicity profile, sharing the same target arises is an important matter and the DCIA might not always be sufficient.

CD80 and CD86 act as co-stimulator for T cells binding CD28 and CD152 respectively, making them good predictors of moDCs activation. CD83 is an early marker of activation stabilizing the surface expression of MHC-II and CD86 receptors. Overall, having this holistic view on moDCs surface receptor expression might help to understand DCIA findings.

Individual *in vitro* models as described herein have limitations in the mechanisms they can recapitulate. The link between stability and immunogenicity is balanced between highly stable structures that are not degraded by proteolysis and the very unstable ones that are processed too quickly to correctly bind MHC receptors (Moss et al., 2019). These differences would not be accounted for in the DCIA as accumulation in the lysosome would stay constant. In addition, interactions with certain cell types or components, not recapitulated in our system, have consequences for immunogenicity. This can be target-mediated but also non specific, for example, binding to red blood cells increases the immunogenicity risk for weak antigens (Greenfield et al., 2021a), especially by increasing their uptake rate (Greenfield et al., 2021b). When a therapeutic antibody is administered subcutaneously, it most of the time comes with Hyaluronidase in the formulation to partially degrade the ExtraCellular Matrix (ECM) allowing the highly concentrated antibody to enter circulation (Crommelin et al., 2019). This could lead to a danger signal capable of initiating a DC or other APC maturation and the initiation of an immune response (Jawa et al., 2020). Having the DCs cultured in the context of this ECM

could further improve the model predictive power. Understanding the role of DCs in the initiation of an immunogenic response and predicting it may have major implications for therapeutic antibody development. T lymphocytes may form better synapses with DCs that present abundant peptides as epitopes on their surface (Osugi et al., 2002), which is certainly exemplified with an increased uptake of the presented protein. In the context of biosimilars hitting the market, there are major challenges for the clinical and preclinical immunogenicity strategy. Precise understanding of biosimilar immunogenicity is needed to avoid inducing an immunogenic reaction because of a different glycosylation pattern or a formulation component (Cohen et al., 2021). The herein discussed assays will certainly be instrumental to this constantly evolving immunogenicity strategy.

Materials and Methods

Antibody Labeling

For the DC internalization assay, antibodies were labeled using the SiteClick Antibody Azido Modification Kit (Thermo Fisher, #S20026) according to the manufacturer's instructions. Briefly, N-linked galactose residues of the Fc region were removed by β-galactosidase and replaced by an azide-containing galactose via the β-1,4-galactosyltransferase. This azide modification enables a copper-free conjugation of sDIBO-modified dyes. The pH-sensitive amine-reactive dye was coupled to a sulfo-DBCO PEG4 amine. Antibodies were labeled with a molar dye excess of 3.5. Excess dye was removed using the Amicon Ultra-2 Centrifugal Filter (Merck, #UFC205024) with a MWCO of 50 kD and antibodies were re-buffered in 20 mM histidine 140 mM NaCl buffer (pH 5.5). The absorbances of the labeled molecules at 280 nm and 532 nm were determined using a Nanodrop spectrometer and the concentration [1] as well as the dye-to-antibody ratio (DAR) [2] was calculated as follows.

$$c(AB) = [A280nm - [A280nm * CF(Dye)]] / \varepsilon(AB) \quad [1]$$

$$DAR = [A532nm * MW(AB)] / [c(AB) * \varepsilon(Dye)] \quad [2]$$

(A = absorbance; AB = antibody; c = concentration; DAR = dye to antibody ratio; ε (dye) = extinction coefficient dye = 47225; CF = correction factor = 0.36)

Quality control of the labeled antibodies

To confirm the efficient removal of unbound dye and to exclude possible antibody aggregates or fragments, a size exclusion chromatography of the labeled antibodies and their unlabeled counterparts was performed. Samples were separated using a BioSuite Diol (OH) column (Waters, 186002165) with a potassium dihydrogen phosphate buffer (pH 6.2) as the mobile phase at a flow rate of 0.5 ml/min. Detectors at 280 nm and 532 nm were used to quantify and analyze the labeled antibodies.

Cell culture and maintenance

Human peripheral blood mononuclear cells were isolated by Pancoll density gradient centrifugation from whole blood according to the manufacturer's instructions. Therefore, EDTA-whole blood donations from healthy volunteers were diluted 1:2 with PBS. For each experiment, different donors were used. For further enrichment of monocytes, magnetic activated cell sorting was performed using anti-huCD14 beads (Miltenyi, #130-050-201) and LS columns (Miltenyi, #130-042-401) according to the manufacturer's instructions. Briefly, monocytes and beads were incubated in MACS Buffer for 15 min on ice and separated by a magnet. The isolated monocytes were suspended in a pre-warmed medium.

Internalisation Assay

CD14+ monocytes were differentiated into monocyte derived DCs (moDCs), by culturing within a DC medium (sterile filtered CellGenix GMP DC medium, with GlutaMAX, non-essential amino acids, sodium pyruvate and Penicillin-Streptomycin) supplemented with 5 ng/mL rhIL4 (R&D systems, #204-IL) and 50 ng/mL rhGM-CSF (R&D system, #215GM-500) for 5 days at 37°C and 5% CO2 ambient on ultra-low attachment culture dishes (0.3x106 cells/ml, Corning, #354407). On the day of the experiment, cells were detached from the ultra-low attachment culture dishes by pipetting and plated into ultra-low attachment 96-well plates at a density of 8x104 cells/well (50µl/well). Antibody solutions were prepared at a concentration of 400 nM in DC medium and 50 µl were applied to the cells for a final concentration of 200 nM. Cells were incubated for two and four hours at 37°C and 5% CO2. Cells were transferred into U-bottom 96-well plates for sedimentation (300 g, 5 min), the pellet was washed with 200 µl ice cold PBS, centrifuged and resuspended in 200 µl FACS buffer containing 50 ng/mL DAPI.

Activation Assay

CD14+ monocytes were differentiated into dendritic cells (DCs), by culturing within medium supplemented with 10 ng/mL rhIL4 (R&D systems, #204-IL) and 100 ng/mL rhGM-CSF (R&D system, #215GM-500) for 5 days at 37°C and 5% CO_2 ambient on ultra-low attachment 96-well culture plates (200 µL, $3x10^6$ cells/ml, Corning, #3262). At day 5 the cells are seeded (300 g, 5 min) and half of the medium was changed for the treatment of interest containing medium (100 µL at 600 nMol/L or 100 µg/mL for a final concentration of 300 nMol/L or 50 µg/mL) and incubated for 48 hours at 37°C and 5% CO_2 ambient.

The cells were then spin down (300 g, 5 min) and resuspended in 200 µL PBS containing a Fixable Viability Stain BV510 (BD, #564406) and a FcR blocking agent (Miltenyi, #130-059-901) for 15 minutes at room temperature. The medium was changed for the antibody mastermix composed of CD80 BUV 737 (clone L307, BD, #741865), HLA-DR FITC (clone G46-6, BD, #555811), CD40 BV786 (clone 5C3, BD, #740985), CD209 BV421 (clone DCN46, BD, #564127), CD11c BUV395 (clone B-ly6, BD, #563787), CD14 PerCP (clone M5E2, BioLegends, #301848), CD83 APC (clone HB15E, BD, #551073), CD86 PE (clone 2331, BD, #555658) in a brilliant stain buffer (BD, #566349) - PBS solution and incubated 30 minutes at 4°C. Cells were finally washed twice in FACS buffer and the fluorescence was acquired using the Fortessa X20 (BD).

Data analysis

The mean fluorescent intensity (MFI) of the internalized antibodies was acquired using a Fortessa X20 flow cytometer (BD) equipped with a 532 nm-emitting laser. Signals were collected at 572 nm ± 35 nm. The exact same conditions, gains, and gates were used for all time points. Data extraction was performed using the FlowJo-V10.8.1 software (BD Life Sciences). Cells were gated for singlets, morphology and viability. Values of the negative control were subtracted from all geo-mean values followed by normalization to the Dye to Antibody Ratio (DAR) and to our internal untargeted IgG1 control. The normalized geo-mean

values from each antibody were plotted as a linear regression curve using R Statistical Software (v4.1.2; R Core Team 2021) to extract the slope (Geo Mean MFI/min for 120 and 240 min).

Concerning the activation assay, data extraction was performed using the FloJo_V10 software as well. Cells were gated for singlets, morphology and viability. MFI were extracted for the different activation markers (CD80, HLA-DR, CD86, CD83, CD209 and CD40) on $CD11c^{+}$ $CD14^{-}$ viable cells. Values of the non-treated control were used to calculate the Stimulation Index (SI) specific to each activation marker and individual. The SI were plotted for each treatment to compare for their moDCs activation capacity. An arbitrary threshold at SI = 1.4 was used to classify the individual as responder for the particular treatment and activation marker. Keyhole Limpet Hemocyanin (KLH, Sigma, #SRP6195) response was used as an inclusion criteria for an individual. For an individual to be considered in further treatment comparison, KLH response has to be positive ($SI \geq 1.4$) for at least three of the tested activation markers. While for the treatment, a response with $SI \geq 1.4$ for any 1 of the 6 populations was considered a positive response, as described for a similar protocol (Wickramarachchi et al., 2020). Statistical significance of differences in internalization rates and SI were calculated by a paired t-test. Statistical analysis was performed using R. Significance level: $p < 0.0001$= ****; $p < 0.001$= ***; $p < 0.01$=**; $p < 0.05$= *; not significant= ns.

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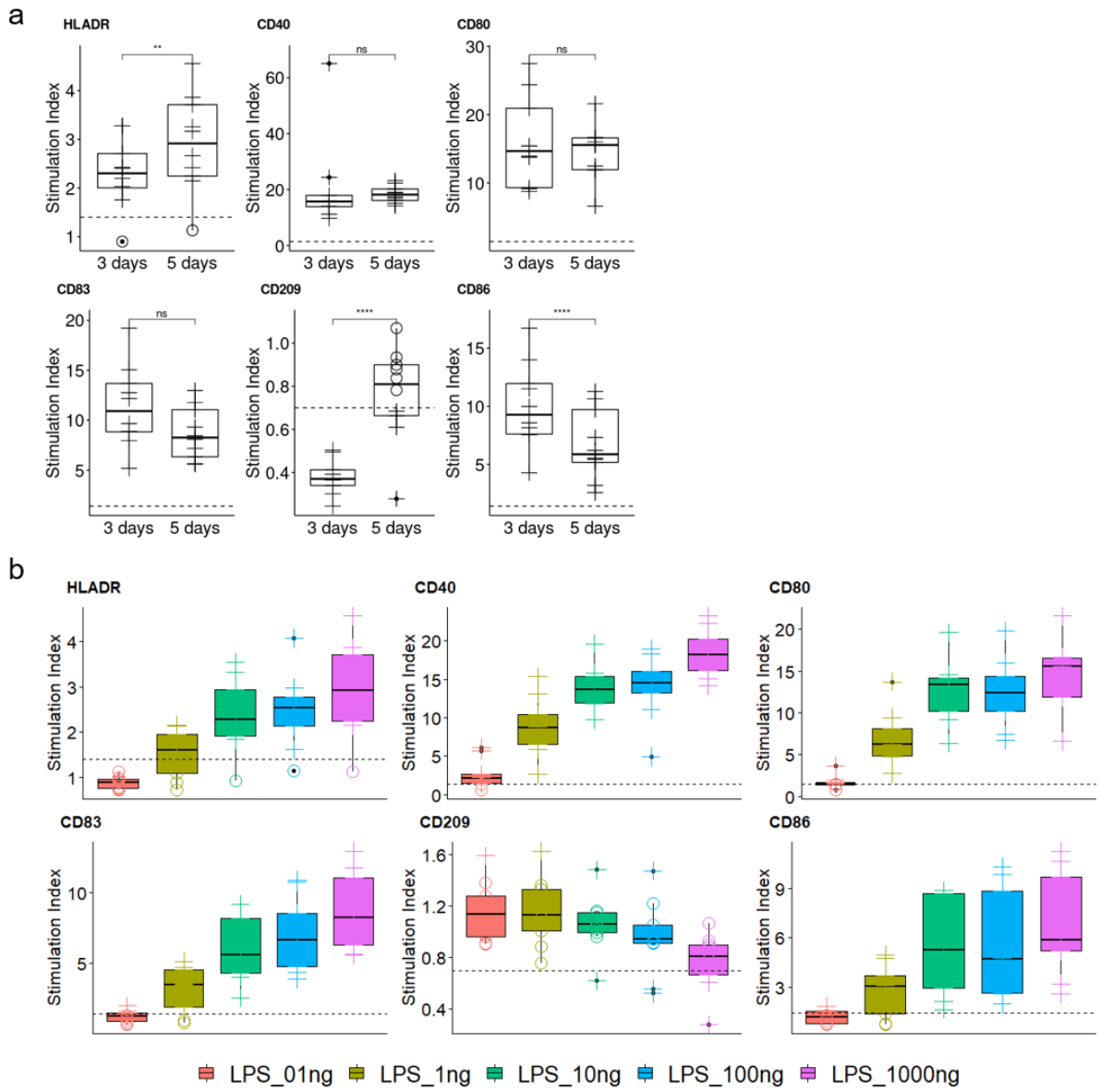
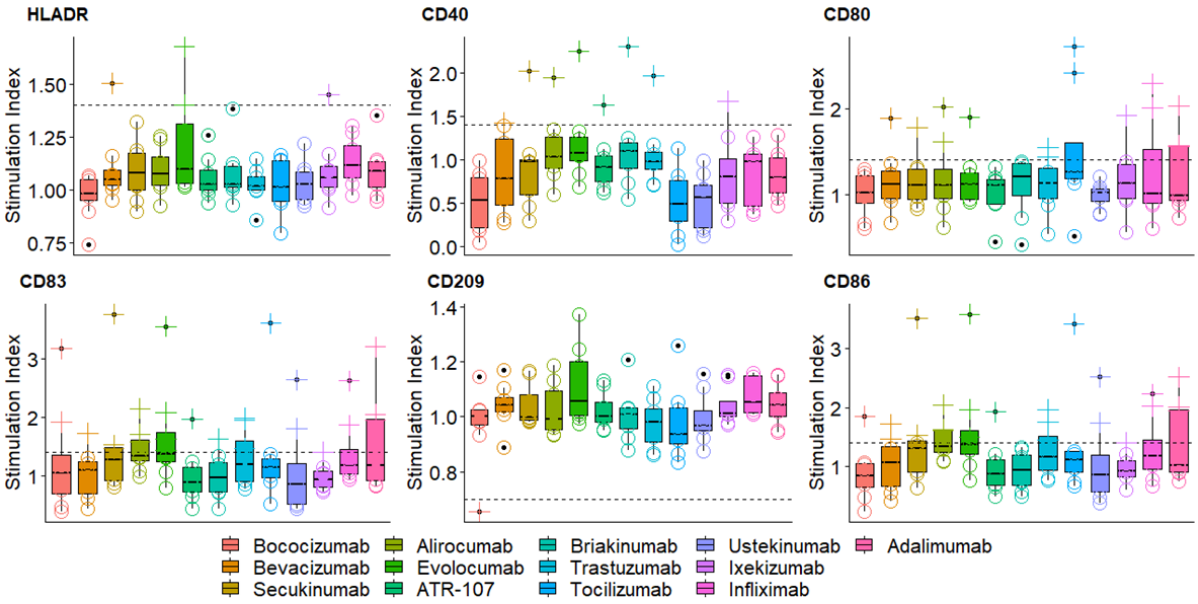


Supplementary figure 1. Comparison of two moDCs differentiation durations using LPS. (a) Three days of differentiation was compared to an extended differentiation of five days by assessing the moDCs response to 1 ug/mL of LPS. (b) A dose response to increasing LPS concentrations using the 5 days differentiation period. Individual moDCs SI for the different LPS concentrations were calculated and a plot per activation marker generated.

Supplementary figure 2.



Supplementary figure 2. The SI for the tested antibodies are plotted for each treatment according to the activation marker. The dashed line represents the threshold (SI = 1.4).

7. Publication 4

Internalization into Dendritic cells as a risk factor for immunogenicity

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Manuscript in preparation

DC internalization can be influenced by the physico-chemical properties of therapeutic antibodies. Here, we investigated the link between charge patches and internalization in DCs. A set of five tool antibodies have been engineered to display large positive or negative charge patches. In short, we demonstrated the influence of positive charge patches on the uptake by DC. Additionally, we showed that an altered internalization could echo on the subsequent immune response by looking at MHC-II epitope presentation and CD4+ T cell activation.

I performed the MAPPs assay with help on the mass spectrometric analysis and did the *in silico* analysis using NetMHCIIpan-4.0. I optimized the assay looking at CD4+ T cell response to fit our needs and tested our tool molecules. I created the figures, wrote the original version, participated in the discussions and revisions of the manuscript.

Internalization into Dendritic cells as a risk factor for immunogenicity

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KEY WORDS

Immunogenicity, immunomodulation, biotherapeutics

Introduction

There are three critical steps in the generation of an adaptive immune response: (1) the internalization and processing of the antigen, followed by (2) its presentation to the specific CD4+ T cell together with co-stimulation which will then (3) interact with a B cell inducing its differentiation and the subsequent production of antibodies (Abs). This scheme holds true for immunogenicity, referring to an immune response following the administration of a biotherapeutic. Immunogenicity leads to the production of anti-drug antibodies (ADA), which often negatively affects the efficacy by reducing exposure and/or the safety profile of these molecules. It is a multifactorial phenomenon and risk factors influencing each and every step of the immune response may have major consequences. Previously, it has been observed that biophysical properties like positive charge patches affect the biodistribution of Abs, pharmacokinetic properties like clearance and alter their uptake into endothelial cells (Boswell et al., 2010; Liu et al., 2021; Stüber et al., 2022; Yadav et al., 2015). We hypothesized that the same properties affecting pharmacokinetic properties could also affect internalization into dendritic cells (DCs) and thus the risk for immunogenicity. In order to test this hypothesis we devised experiments to measure the effects of 5 well characterized tool compounds with different biophysical properties (e.g. charge patches) but high sequence homology on several key steps in the immunogenicity response cascade.

Here we explore the influence of internalization rates and the subsequent cellular accumulation of Abs on the initiation of an immunogenic response in DCs. The main drivers for cellular accumulation *in vitro* are macropinocytosis and recycling via the neonatal Fc receptor (FcRn) (Kraft et al., 2019). However, clathrin-dependent, receptor-mediated, and caveolae-mediated endocytosis also play a role in this process (Trombetta & Mellman, 2005). These mechanisms are recapitulated in our DC internalization assay where we measure the rate of accumulation of a test compound in the lysosomes of human monocyte-derived DCs. We then determined the presentation of peptides on MHC-II complexes via the MAPPs assay and subsequently characterized the CD4+ T cell epitope presentation pattern and assessed

the risk for CD4+ T cell activation. It has been demonstrated that the T cell precursors, capable of expanding in response to a biotherapeutic, are in the order of magnitude of one specific T cell out of 1 to 10 million T cells (Delluc et al., 2011). We then applied a fit-for-purpose strategy, inserting the main CD4+ T cell epitope of ovalbumin (Rasmussen et al., 2001) into our test Abs, increasing the odds of a specific T cell response directed towards a strong and shared epitope.

Given the multifactorial nature of immunogenicity and the variety of interactions involved, looking at the outcome of different assays individually does not seem to be an appropriate strategy. Here, we propose an integrated approach looking at the consequences of charge patches and altered internalization into monocyte derived DCs (moDCs) on the subsequent steps of the immune response.

Results

Positive charge patches enhance the internalization into moDCs.

All antibody variants described here were derived from a common human IgG1 Ab that was previously engineered to abolish binding to its original target, human and murine CD44. Surface-exposed residues were manually identified in the modeled three dimensional structure of the variable domain and replaced with selected amino acids carrying specific properties like positive, negative or neutral net charge. Specifically, groups of amino acid residues were selected to create molecules with high sequence similarity but distinct biophysical properties like charge patches and charge distribution (Fig. 1). Best candidates (located both in CDRs and framework regions) were then produced and subsequently characterized. The parent and the four generated CD44 variants show strong differences with respect to their calculated isoelectric points (from 4.8 for CD44var20 up to 9.8 for CD44var112) correlating with the markedly difference in their charge distribution in the Fab domain.

The presence of positive charge patches on an Ab was described to mediate rapid and transient tissue uptake (Stüber et al., 2022). In order to determine whether this would translate to other cell types, we investigated the cellular accumulation rate of the five CD44 variants into moDCs (**fig. 1**). Using a DC internalization assay, we show that the two antibodies engineered with positive charge patches (CD44var27 and CD44var112) showed a significantly higher cellular accumulation rate into moDCs compared to the parental molecule (CD44var1) and the one engineered with negative charge patch (CD44var20) or the one with even charge distribution (CD44var104). This observation points toward a conserved mechanism and a potential increased risk for the initiation of an immunogenic response.

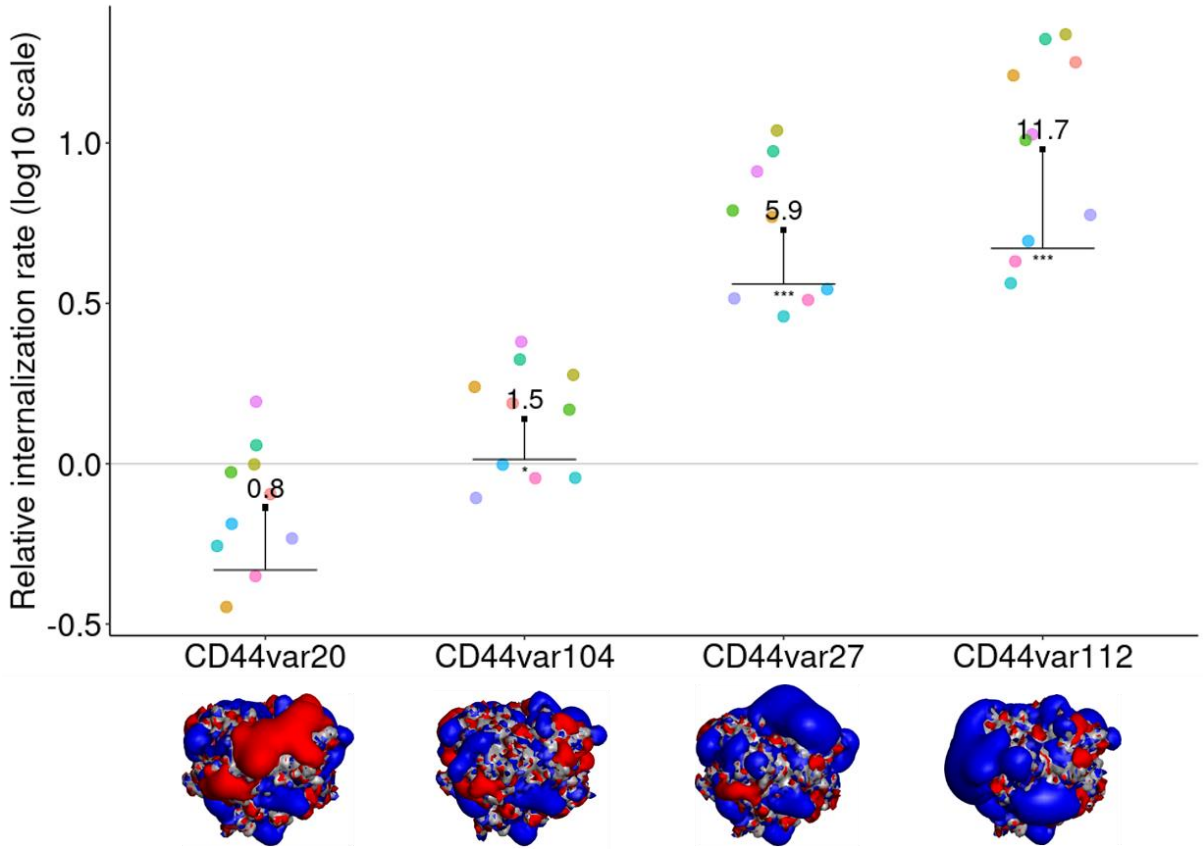

*Figure 1. The modification of biophysical properties like the insertion of charge patches or the modification of charge distribution alters the internalization rate of untargeted antibody variants. The dot plot represents the relative internalization rate of each variant normalized to CD44var1 (each color representing a donor, n=9). The internalization efficiency corresponds to the slope between the 120 min and 240 min time points looking at the mean fluorescence (MFI) values and normalized with the medium control. The average fold change is displayed for each group. The paratopes' isopotential surfaces (viewed from the top of the Fab) of the antibody variants are represented (blue: positive charges; red: negative charges). Isocontour renderings shown, were generated using PyMOL (Schrödinger, LLC, Stüber et al., 2022). A one-sided paired t-test has been performed (p < 0.001= ***; p < 0.01= **; p< 0.05= *).*

Increased internalization enhances T cell epitope presentation by moDCs

Internalization and processing of antigens by professional antigen presenting cells mainly result in CD4+ T cell epitope presentation via MHC-II receptors. However, the relationship

between altered internalization rates into APCs and CD4+ T cell epitope presentation is presently unclear. In order to explore further this hypothesis, T cell epitope presentation was assessed for the five Ab variants by MAPPs using cells isolated from the same nine donors investigated in the DCIA (**fig. 2a).**

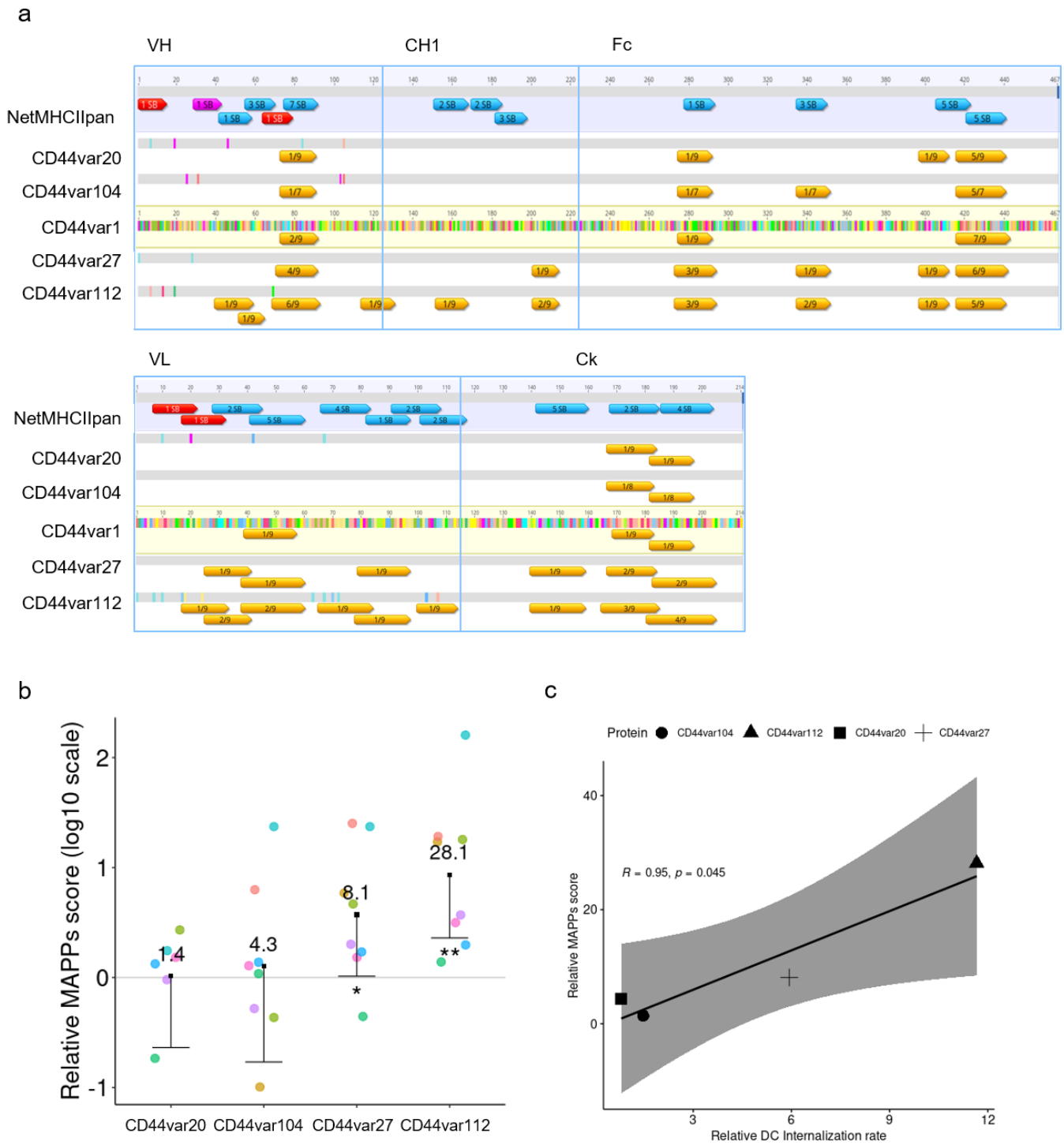

Figure 2. An increased internalization rate leads to increased peptide presentation in MHC-II

*Peptide Associated Proteomics (MAPPs). (a) Analysis of the T cell epitope content for the five antibody variants. The differences in amino acids to CD44var1 are highlighted (color corresponding to the amino acid). Epitopes detected by MAPPs (in orange, annotated with the proportion of donors presenting the T cell epitope) are represented together with T cell epitope prediction (annotated with the number of strong binders, SB) along the amino acid sequence. T cell epitope predictions in blue are common to all variants whereas the ones in pink and red are respectively specific for CD44var104 and CD44var112. (b) Comparison of the MAPPs score for the different antibody variants. The MAPPs score summarizes the number of epitopes detected and their signal intensities ($n_{epitopes}$/ $total_{epitopes}$ x $mean_{signal}$) normalized to CD44var1 (each color representing a donor, n=9). (c) Correlation plot of the MAPPs score according to the DC internalization rate. Relative MAPPs score and DC internalization rate were obtained by taking the mean of all the tested donors (n=9). A one-sided paired t-test has been performed ($p < 0.01$= **; $p< 0.05$= *).*

All MAPPs-detected clusters were predicted as strong binders using NetMHCII-pan4.0 with the exception for two clusters located in the CH1 domain. Most interestingly, we observed a sharp increase in the number and abundance of detected peptides in the MAPPs assay performed with the Ab variants bearing positive charge patches (CD44var27 and CD44var112). In contrast, analysis using NetMHCII-pan4.0 indicates that the residue exchange required to generate the charge patches resulted in no (variant 27 and 20), 1 (variant 104), or 5 (variant 112) neo-epitopes in comparison to CD44var1. In particular, the amino acid sequence of the two aforementioned clusters were present in all tested Ab variants but they were only detected in the MAPPs assay using the two Ab variants with positive patches. To explore this further, we converted the MAPPs findings into a score considering, for each Ab variant, the number and intensity of the detected epitopes in relation to the sum of overall detected clusters. The “MAPPs score” shows a clear positive trend for CD44var 27 and CD44var112 (**fig. 2b**) that correlates tightly with the relative internalization rate as

measured by the DCIA (**fig. 2c**). In summary, these results suggest that Ab with positive charge patches will accumulate in the lysosome of moDCs at a higher rate resulting in a generally enhanced CD4+ T cell epitope presentation on MHC-II complexes; in contrast, Abs with neg. charge patches or even charge distribution show low cellular accumulation rates and a moderate presentation of CD4+ T cell epitopes on MHC-II complexes.

Insertion of an ovalbumin CD4+ T cell epitope within the antibody variant sequences had no influence on the studied properties.

The next step in the immunological response ultimately leading to ADAs is the recognition of MHC-II presented peptides by specific CD4+ T cells leading to their activation and their subsequent expansion. In order to assess whether an increased cellular accumulation and presentation by DCs would also lead to an increase in the specific CD4+ T cell response, while avoiding a bias from sequence differences of the tested Abs, we inserted a strong T cell epitope into the sequence of all test Abs and determined the effect on specific CD4+ T cell proliferation. In the first step, we repeated the DCIA, the MAPPs assay, and the T cell epitope prediction for these new Ab variants to better understand the consequence(s) of inserting the peptide sequence corresponding to the epitope in the molecules (**fig.3**).

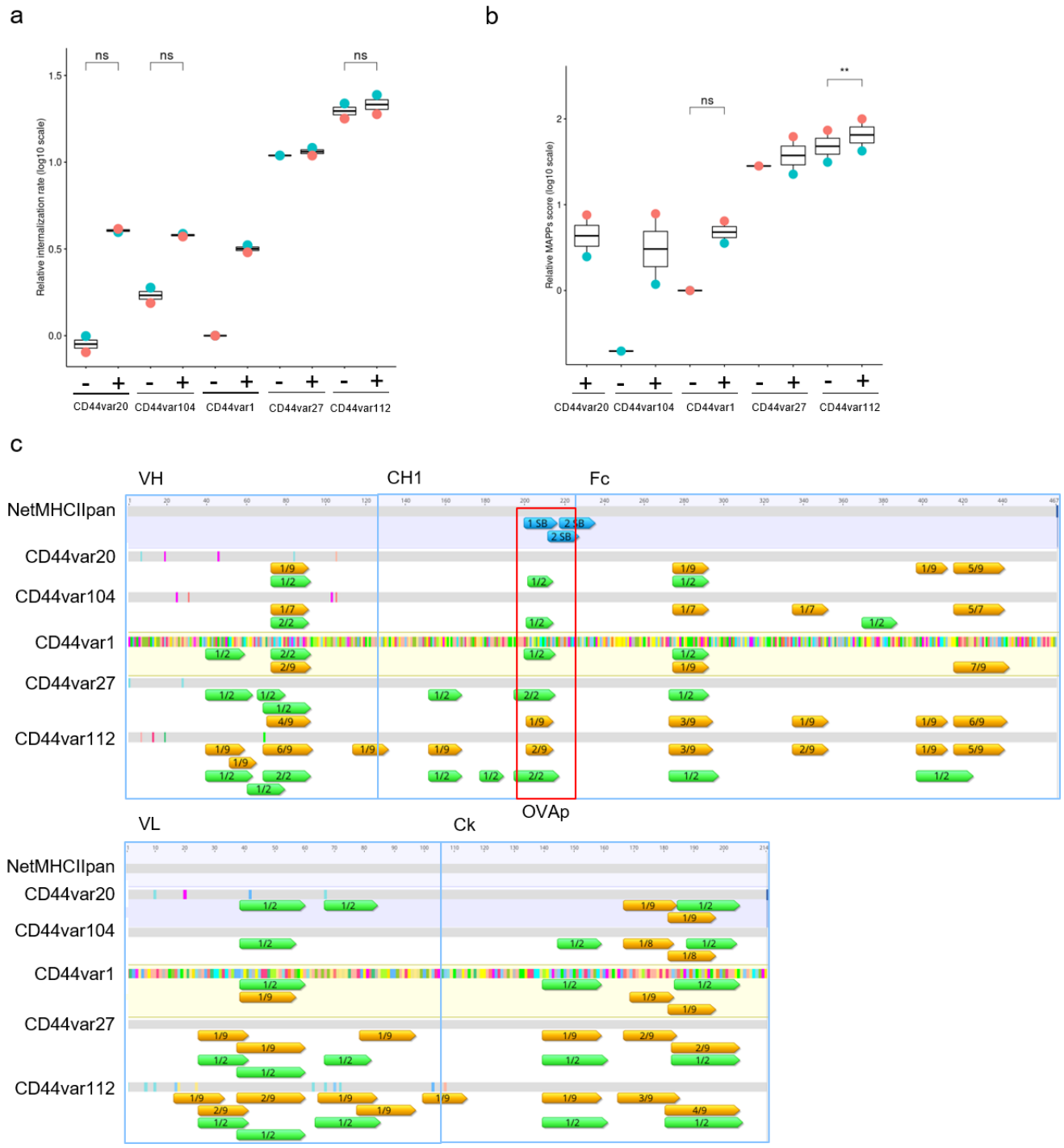

Figure 3. Generation of ovalbumin CD4+ T cell epitope (OVAp) containing variants without altering their properties. (a) Box plot representing the relative internalization rates before (-) and after (+) the addition of the OVAp (each color representing a donor, n=2). (b) Box plot representing the relative MAPPs score before (-) and after (+) the addition of the OVAp (each color representing a donor, n=2). (c) Analysis of the T cell epitope content for the five antibody variants. The differences in amino acids to CD44var1 are highlighted (color corresponding to

*the amino acid). Epitopes detected by MAPPs (annotated with the proportion of donors presenting the T cell epitope) of the initial variants (in orange) are represented together with the epitopes detected by MAPPs for the OVAp variants (in green) along the amino acid sequence. Only the predicted T cell epitopes induced by the insertion of the OVAp are represented (in blue, common to all variants). A one-sided paired t-test has been performed (p < 0.01= **; p< 0.05= *; not significant= ns).*

While the addition of the OVA CD4+ T cell epitope (OVAp) led overall to a slight increase in moDCs internalization rates (**fig 3a**), differences between Ab variants, especially when comparing CD44var1 to CD44var112, were mainly driven by the positive charge patches; in particular, the increase in internalization rate was observed for the entire series of compounds allowing the qualitative comparison within the molecule series. The same trend was observed for the “MAPPs score” (**fig 3b**) and for the clusters detected in MAPPs (**fig 3c**): they mostly overlapped for CD44variants with or without the OVAp insertion. Overall, the data confirms that the link between an increased internalization rate (driven by the charge patches) and an enhanced CD4+ T cell epitope presentation via MHC-II remains valid for all CD44 variants. In addition, we show that the OVAp insertion is predicted to generate a strong immunogenic sequence using NetMHCII-pan4.0 (**fig. 3c** in blue); the sequence was also presented on MHC-II receptors in the MAPPs assay using the OVAp-containing CD44 variants (**fig 3c**, annotated in green).

Increased internalization lead to higher risk of T cell activation

It has been demonstrated that the T cell precursors, capable of expanding in response to a therapeutic Ab, are in the order of magnitude of one specific T cell out of 1 million T cells (Delluc et al., 2011). Therefore, the number of T cells in an *in vitro* assay is a limiting factor which could be circumvented by additional rounds of stimulation, selectively expanding specific T cells. This approach has been shown successful elsewhere (Castelli et al., 2007;

Hamze et al., 2017) and coupled to the OVAp containing Ab variants is a fit for purpose strategy to explore the link between altered internalization, epitope presentation by APC and CD4+ T cell activation, ultimately leading to immunogenicity. Moreover, the assay format and the insertion of a common strong CD4+ T cell epitope within the sequence of the five Ab variants allowed us to focus on this specificity and rule out other confounding factors. The data comparing the CD4+ T cell activation propensity of CD44var1 to CD44var112 are summarized below (**fig.4**).

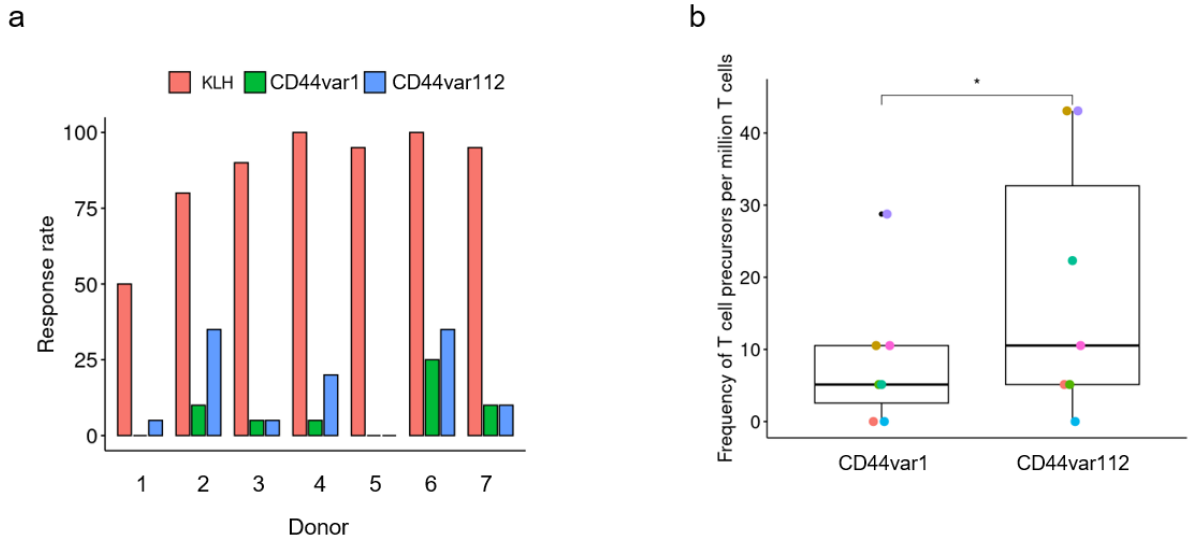

*Figure 4. Increased CD4+ T cell activation following increased internalization and peptide presentation directed towards a common epitope. (a) Representation of the assay response rate (n= 20) from 7 individual donors according to the treatment. In red, T cells have been expanded using autologous KLH-loaded moDCs and their response to autologous KLH-loaded moDCs has been assessed at week 4 by IFNy ELISPOT. For the antibody variants, T cells have been expanded using autologous CD44var1-loaded moDCs (green) or CD44var112-loaded moDCs (blue) and their response to autologous OVA-loaded moDCs has been assessed at week 4 by IFNy ELISPOT (Delluc et al., 2011). (b) Estimation of the number of T cell precursors for the two tested variants. The calculation has been done according to ref. In short the Frequency = -Ln (negative wells/total wells tested)/(CD4 T cells/well)). A one-sided paired t-test has been performed (p < 0.0001= ****; p < 0.001= ***; p < 0.01= **; p< 0.05= *; not significant= ns).*

The proportions represented on **fig.4a**, represents the number of wells considered as positive out of the 20 DC:CD4+ T cell co-culture initiated (see Material and Methods section). KLH was used as a positive control for its well documented capacity to induce a strong CD4+ T cell response. The two other conditions, namely CD44var1 and CD44var112, were tested for reactivity following co-culture with ovalbumin loaded moDCs and the proportion of positive wells represented in green and blue respectively. It seems that out of the 20 co-culture run over 21 days with weekly re-stimulation by moDCs loaded either with CD44var1 (green) and CD44var112 (blue), the ones challenged with CD44var112 (containing the positive charge patches) were more prone to respond to ovalbumin loaded moDCs. This has been confirmed by the estimation of the CD4+ T cell precursors based on the Poisson distribution (**fig4.b**). Indeed, there is a significant difference in the estimation of the number of CD4+ T cell precursors responsive to ovalbumin following weekly challenges with the Ab variants containing the OVAp. Pointing towards the increased likelihood of finding a responsive CD4+ T cell to the common epitope after exposure to CD44var112. This conclusion goes in the same direction as the previous observations and links the increased accumulation into the lysosomes to the increased CD4+ T cell epitope presentation via MHC-II and to the increased likelihood to generate an effective CD4+ T cell response. Ultimately leading to the conclusion that increased internalization rates into moDCs, here mediated by positive charge patches, is an important risk factor for immunogenicity.

Discussion

The complexity of therapeutic antibody formats continues to increase, and these molecules may substantially differ in their pharmacokinetic and biodistribution properties based on molecular size and geometry, altered nonspecific or target-mediated clearance, or FcRn interactions (Carter & Lazar, 2018; Thompson et al., 2012a). Each monoclonal Ab has unique biophysical properties, mainly due to differences in the CDR residues and framework scaffolds (Chae et al., 2021). In a meta-data analysis of phase I clinical trials, positive charge patches in those regions showed lower success rates (Raybould & Deane, 2021). Indeed, it has already been observed that increased positive charge in the CDRs, correlates with an increased risk of self-association, potentially leading to aggregation, viscosity and poor specificity (Hebditch & Warwicker, 2019; Pérez et al., 2021). However, we showed herein that positive charge patches, independent of aggregation, have a dramatic effect on DC internalization.

In this context, even the blocking of macropinocytosis could not abrogate internalization completely, indicating that receptors mediated uptake (e.g., sRAGE, galectin-3, CD36) might play a role (Deng et al., 2020). Moreover, internalization of charged proteins has been shown to induce accumulation of transferrin and endosomal maturation (Thompson et al., 2012b) or modify secreted cytokines (Deng et al., 2020). This points toward an altered processing which may have consequences on the generation of epitopes that will be subsequently presented via MHC-II. We demonstrated that following the increased internalization of Abs, the peptide presentation pattern was modified and overall more epitopes were loaded on MHC-II receptors. This is a major risk factor as in most cases, immunogenicity is dependent on the presentation of a CD4+ T cell epitope via MHC-II (Vultaggio et al., 2016). This may even have consequences on marginal zone B cells which are capable of hijacking DC's peptide-MHC-II complexes via trogocytosis, which is enhanced by the increased availability of these complexes (Schriek et al., 2022). Moreover, CD4+ T cells may form better synapses with DCs that present abundant peptides as epitopes on their surface (Osugi et al., 2002). However, the

pMHC-II-TCR interaction is multiparametric (defined by the affinity, the stability and the avidity) (Croft, 2020) and different types of interaction can lead to a good signaling between DCs and CD4+ T cells (e.g., short, spatially correlated binding events)(Richard et al., 2021). Not all peptides detected by MAPPs or predicted by NetMHCIIpan-4.0 will bind strongly enough to interact with a specific CD4+ T cell (Gorovits et al., 2020) and that a specific CD4+ T cell would be present. Therefore, it has been important to look at the specific response toward a strong and shared epitope, like OVAp, to correlate the increased lysosomal accumulation with an increased risk for CD4+ T cell activation. Importantly, free peptides to MHC-II may have a different conformation and specificity for T cell stimulation than the same pMHC formed by the same peptide extracted from the intact protein by intracellular processing (Trombetta & Mellman, 2005). We therefore made the choice of loading the moDCs with ovalbumin instead of the epitope as a peptide. We therefore could determine that the Ab variant with the positive charge patches increased the likelihood of finding a specific CD4+ T cell when compared to its non-charged counterpart.

Rapid internalization into the DCs will not always result in their activation (Cohen & Chung, 2021). We demonstrated that this is a risk factor for immunogenicity but in absence of an appropriate CD4+ T cell epitope, might not echo on the subsequent response which needs further evaluation. This highlights the need for a precise characterization of the risk for immunogenicity pre-clinically by means of an integrated approach.

Materials and Methods

Proteins

Stock solutions of keyhole limpet hemocyanin (KLH-Imject Maleimide-Activated mcKLH, Thermo Fisher Scientific, #77600) were reconstituted and stored at -80°C in single-use aliquots according to the manufacturer's recommendations under sterile conditions. CD44 antibody variants (CD44var20, CD44var104, CD44var1, CD44var27, CD44var112) bearing different charge patches were produced internally. The initial variants were further engineered with the addition of the main CD4+ T cell epitope derived from ovalbumin (ISQAVHAAHAEINEAGR) into the CH1 domain (Rasmussen et al., 2001). The latter were used to assess the consequences of altered internalization on CD4+ T cell activation with a common epitope.

Antibody Labeling

For the DC internalization assay, antibodies were labeled using the SiteClick Antibody Azido Modification Kit (Thermo Fisher, #S20026) according to the manufacturer's instructions. Briefly, N-linked galactose residues of the Fc region were removed by β-galactosidase and replaced by an azide-containing galactose via the β-1,4-galactosyltransferase. This azide modification enables a copper-free conjugation of sDIBO-modified dyes. The pH-sensitive amine-reactive dye was coupled to a sulfo-DBCO PEG4 amine. Antibodies were labeled with a molar dye excess of 3.5. Excess dye was removed using the Amicon Ultra-2 Centrifugal Filter (Merck, #UFC205024) with a MWCO of 50 kD and antibodies were re-buffered in 20 mM histidine 140 mM NaCl buffer (pH 5.5). The absorbances of the labeled molecules at 280 nm and 532 nm were determined using a Nanodrop spectrometer and the concentration [1] as well as the dye-to-antibody ratio (DAR) [2] was calculated as follows.

$$c(AB) = [A280nm - [A280nm * CF(Dye)]] / \varepsilon(AB) \quad [1]$$

$$DAR = [A532nm * MW(AB)] / [c(AB) * \varepsilon(Dye)] \quad [2]$$

(A = absorbance; AB = antibody; c = concentration; DAR = dye to antibody ratio; ε (dye) = extinction coefficient dye = 47225; CF = correction factor = 0.36)

Quality control of the labeled antibodies

To confirm the efficient removal of unbound dye and to exclude possible antibody aggregates or fragments, a size exclusion chromatography of the labeled antibodies and their unlabeled counterparts was performed. Samples were separated using a BioSuite Diol (OH) column (Waters, 186002165) with a potassium dihydrogen phosphate buffer (pH 6.2) as the mobile phase at a flow rate of 0.5 ml/min. Detectors at 280 nm and 532 nm were used to quantify and analyze the labeled antibodies.

Cell culture and maintenance

Human peripheral blood mononuclear cells were isolated by Ficoll density gradient centrifugation from buffy coats according to the manufacturer's instructions (GE Healthcare #17-1440-03). For further enrichment of monocytes, magnetic activated cell sorting was performed using anti-huCD14 beads (Miltenyi, #130-050-201) and LS columns (Miltenyi, #130-042-401) according to the manufacturer's instructions. Briefly, monocytes and beads were incubated in MACS Buffer for 15 min on ice and separated by a magnet. The isolated monocytes were suspended in a pre-warmed medium. CD14+ monocytes were differentiated into monocyte derived DCs (moDCs), by culturing within a DC medium (sterile filtered CellGenix GMP DC medium, with GlutaMAX, non-essential amino acids, sodium pyruvate and Penicillin-Streptomycin) supplemented with 5 ng/mL rhIL4 (R&D systems, #204-IL) and 50 ng/mL rhGM-CSF (R&D system, #215GM-500) for 5 days at 37°C and 5% CO_2 ambient on ultra-low attachment culture dishes ($0.3x10^6$ cells/ml, Corning, #354407).

Internalization Assay

On the day of the experiment, cells were detached from the ultra-low attachment culture dishes by pipetting and plated into ultra-low attachment 96-well plates at a density of $8x10^4$ cells/well (50µl/well). Antibody solutions were prepared at a concentration of 400 nM in DC medium and 50 µl were applied to the cells for a final concentration of 200 nM. Cells were incubated for two and four hours at 37°C and 5% CO_2. Cells were transferred into U-bottom 96-well plates for sedimentation (300 g, 5 min), the pellet was washed with 200 µl ice cold PBS, centrifuged and resuspended in 200 µl FACS buffer containing 50 ng/mL DAPI.

MHC-II associated Peptide Proteomics (MAPPs)

MAPPs assay was performed according to the standard protocol and analyzed according to (Steiner et al., 2020). In short, moDCs cells were challenged with the test protein at 300 nM in the presence of 1 µg/mL of lipopolysaccharide (LPS) from Salmonella abortus equi (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) for 24 h. Mature moDCs were harvested, washed with PBS and the cell pellets were frozen at −80 °C. Frozen cell pellets were lysed in 20 mM Tris-buffer solution pH 7.8 containing 1% (v/v) Digitonin and protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) for 1 h at 4 °C on a ThermoMixer at 1100 rpm. The HLA-DR immune complexes were isolated by immunoprecipitation using the biotin-conjugated anti-human HLA-DR monoclonal antibodies (clone L243, BioLegends). Lysates were incubated with the antibody on a rotator overnight at 4 °C. Samples were washed five times with a buffer containing 20 mM N-(2-hydroxyethyl)piperazine-N′-ethanesulfonic acid-NaOH (pH 7.9), 150 mM KCl, 1 mM MgCl2, 0.2 mM CaCl2, 0.2 mM ethylenediaminetetraacetate, 10% (v/v) glycerol, and 0.1% (v/v) Digitonin and five times with purified water. MHC-II peptides were eluted twice from HLA-DR molecules by adding 18 µL of 0.1% trifluoroacetic acid. The eluates were collected and analyzed by tandem mass spectrometry. Detected peptides were grouped into clusters and represented along the sequence of the corresponding antibody using Geneious Prime 2022.1.1 (https://www.geneious.com).

In silico T cell epitope prediction

NetMHCIIpan-4.0 (Reynisson et al., 2020) was used to predict potential T cell epitope content for the five antibody variants. The algorithm has been run for 13 DRB1 alleles (DRB1-0101, DRB1-0301, DRB1-0401, DRB1-0701, DRB1-0801, DRB1-0901, DRB1-1001, DRB1-1101, DRB1-1201, DRB1-1301, DRB1-1401, DRB1-1501, DRB1-1601) screening for binding affinities of 15-mer derived from the antibody sequences. The top 2 % of the hits were kept as strong binders and further analyzed. Detected peptides were grouped into clusters and represented along the sequence of the corresponding antibody using Geneious Prime 2022.1.1 (https://www.geneious.com).

Estimation of the T cell precursor frequency

This assay has been performed according to the published protocol proposed by Delluc et al (Delluc et al., 2011). In short, moDCs were loaded either with KLH, CD44var1 or CD44var112 (300 nM) and matured over night with 1 ug/mL of LPS (Sigma-Aldrich #L5886). T cells were isolated from PBMCs by negative selection (Myltenyi, #130-096-533) as recommended by the manufacturer and co-cultured with previously loaded autologous moDCs. 10,000 loaded moDCs were co-cultured with 100,000 T cells (n=20 wells per condition) in a total volume of 200 uL of Iscove's Modified Dulbecco's Medium (IMDM, GIBCO #21980-032) supplemented with 10 % human serum (Sigma-Aldrich #H3667-100ml), 1000 U/mL rh-IL-6 (R&D Systems #7270-IL-025/CF), 10 ng/mL rh-IL-12 (R&D Systems #10018-IL-020) and incubated at 37°C and 5% CO_2 ambient on ultra-low attachment 96-well plates (Corning #3474). The CD4+ T cells were re-stimulated on day 7 and 14 with fresh autologous moDCs loaded with one of the antibody variants or KLH, 10 U/mL of IL-2 (R&D Systems #202-IL-010/CF) and 5 ng/mL of IL-7 (R&D Systems #207-IL-010/CF). At day 21, the specificity of the CD4+ T cells was assessed by IFN-γ enzyme-linked immunospot (ELISpot) following the recommended procedure (Mabtech, #3420-4APT-10). To assess the T cell response against KLH, 3300 moDCs (either

naïve or loaded with KLH 4 hours prior co-culture without LPS) were added to 10,000 CD4+ T cells (n=20) expanded for KLH. The T cell response directed against the two CD44 antibody variants was measured in relation to a single epitope (the one presented by the OVA peptide): 3300 moDCs (either naïve or loaded with ovalbumin (Fisher Scientific #A/1280/48) 4 hours prior co-culture without LPS) were added to 10,000 CD4+ T cells (n=20) expanded for either CD44var1 or CD44var112 containing the main ovalbumin CD4+ T cell epitope as introduced earlier. Each of these co-cultures were evaluated in duplicate.

Data analysis

The mean fluorescent intensity (MFI) of the internalized antibodies was acquired using a Fortessa X20 flow cytometer (BD) equipped with a 532 nm-emitting laser. Signals were collected at 572 nm ± 35 nm. The exact same conditions, gains, and gates were used for all time points. Data extraction was performed using the FlowJo-V10.8.1 software (BD Life Sciences). Cells were gated for singlets, morphology and viability. Values of the negative control were subtracted from all geo-mean values followed by normalization to the Dye to Antibody Ratio (DAR). The normalized geo-mean values from each antibody were plotted as a linear regression curve using R Statistical Software (v4.1.2; R Core Team 2021) to extract the slope (Geo Mean MFI/min for 120 and 240 min).

A numerical estimation of the MAPPs assay outcome was calculated using the number of epitopes detected and their signal intensities like follows:

$n_{epitopes}$/ $total_{epitopes}$ x $mean_{signal\ intensity}$

For the estimation of the T cell precursor frequency, spots were counted in a computer-assisted video image analyzer (AID, Strassber, Germany). A response was considered positive when the spot count was increased by 2-fold compared to the well where non-loaded moDCs were added. The frequency of CD4+ T cell precursors was calculated as proposed in (Delluc et al., 2011), using the Poisson distribution:

frequency = -ln(negative wells/total wells tested)/(CD4 T cells/well)

Statistical significance was calculated using a one-sided t-test. Statistical analysis was performed using R.

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8. Discussion and future prospective

8.1. Risk evaluation of immunogenicity: towards an integrated immunogenicity risk assessment

Immunogenicity is a major impediment to the successful use of therapeutic Abs. Induced anti-drug antibodies (ADA) can alter treatment efficacy and safety in a significant number of patients (Ducret et al., 2021a). With the aim of reducing this phenomenon, *in silico* (e.g., EpiVax, netMHCIIpan), *in vitro* (e.g., MAPPs, PBMC and DC-T cell assays) and *in vivo* (e.g., humanized mouse model) tools have been developed to assess compound liabilities (e.g., antigenic epitopes) contributing to ADA development. As T cell-dependent immune response is a major driver of immunogenicity, *in vitro* T cell assays are frequently used as tools to identify and measure CD4+ T cell-dependent responses to biotherapeutics. The DC:CD4+ T cell restimulation assay described in publication 2 (Siegel et al., 2022) assesses the propensity of a biotherapeutic to trigger a IFN-γ release by CD4+ T cell that may help the generation of ADA. The main limitation of many assays looking at CD4+ T cell activation lies in the low number of T cells sampled. Hence, the number of preexisting T cells specific to a biotherapeutic ranges between 1 out of 10^8 (e.g., trastuzumab and etanercept) and 1 out of 10^7 T cells (e.g., rituximab) (Delluc et al., 2011a). However, our assay format of the DC:CD4+ T cell restimulation assay allows screening of more CD4+ T cells than most of currently used T cell assay (Ducret 2021) and includes a re-stimulation step increasing the likelihood of capturing a sustained T cell response (Pennock et al., 2013). As a starting point towards the validation of this assay, we collected the clinical ADA rates for 24 marketed biotherapeutics. This exercise allowed us to build a database to benchmark newly developed immunogenicity assays and to have a retrospective and comprehensive overview of the immunogenicity of these molecules. We then split these compounds into two categories, of high (≥20% reported ADA rate) and low (<20% ADA rate) risk for immunogenicity. This database was used to

calibrate the assay's linear mixed model, which ultimately helped to understand the main sources of variability in this assay. We believe that a binary high/low risk paradigm is the most reasonable for implementation into a preclinical risk evaluation of immunogenicity for therapeutics. However, the most useful applications of the DC:CD4+ T cell restimulation assay is still to provide a relative ranking for candidate compounds approaching the clinical lead selection (CLS) milestone

Predicting clinical immunogenicity with a single assay would certainly not be accurate enough and is a concept that is now accepted by the immunogenicity community. Hence, the multifactorial nature of immunogenicity indicates that an integrated preclinical risk assessment of immunogenicity should be a key element of biotherapeutics development. The DC: CD4+ T cell restimulation assay, even while playing a key role in our integrated approach to biotherapeutics immunogenicity risk estimation, would not be sufficient to select candidate leads. However, the complexity of therapeutic antibody formats continues to increase, and these molecules may substantially differ in their pharmacokinetic and biodistribution properties based on molecular size and structure, altered non-specific or target-mediated clearance, or FcRn interactions (Carter & Lazar, 2018; Thompson et al., 2012). Even a classical IgG1 format has unique biophysical properties, mainly due to differences in the CDR residues and framework scaffolds (Chae et al., 2021b). Therefore, the implementation of this assay as part of a comprehensive risk assessment has the potential to provide a more robust and informative immunogenicity risk assessment early in development.

Other assays would then complement the DC: CD4+ T cell restimulation assay, as for example the DC internalization and activation assays. Indeed, favorable properties as well as safety concerns could be attributed to the contribution of DC in the initiation of an immunogenic response. As an example, the improved immunogenicity profile of ixekizumab compared to secukinumab, both targeting IL-17, could be partially driven by favorable physicochemical properties (i.e., charge patches, stability, and hydrophobicity) and decreased internalization into APCs as demonstrated in publication 3. These assays could also help to better

understand the consequences of target expression on the surface of DCs as hypothesized for TNF-α targeting antibodies. In fact, in our assay, the TNF-α targeting antibody, adalimumab, did show an increased propensity for internalization, as observed elsewhere (Kovalova et al., 2020a).

The assessment of moDC activation can also in some cases help to better understand the findings of the DCIA and their consequences on DCs. Overall, understanding the role of DCs in the initiation of an immunogenic response has major implications for therapeutic antibody development. The widely used MAPPs assay would then be used, eventually coupled with *in silico* predictions, to identify where sequence liabilities may lie. Their identification would point to specific positions in the amino acid sequence and therefore by means of amino acid exchange allow the reduction of potential CD4+ T cell epitope content.

8.2. Positive charge patches alter internalization into DC constitute a risk factor for immunogenicity

Positive charge patches were already described as poor properties for therapeutic Abs (Hebditch & Warwicker, 2019b; Pérez et al., 2021; Raybould & Deane, 2021). However, we showed in publication 4 that positive charge patches, independently of aggregation, have a dramatic effect on DC internalization hence potentially on immunogenicity. This has been demonstrated using the DCIA even if the direct link with immunogenicity is not clear, solely relying on this readout. Moreover, internalization of charged proteins has been shown to induce accumulation of transferrin and endosomal maturation (Thompson et al., 2012) or modify secreted cytokines (Deng et al., 2020). This points toward an altered processing which may have consequences on the generation of epitopes that will be subsequently presented via MHC-II. We demonstrated that following the increased internalization of Abs, the peptide presentation pattern was modified and overall more epitopes were loaded on MHC-II receptors. Furthermore, we could show that in this context the two processes were tightly correlated. In this regard, the integrated analysis of the DCIA and MAPPs already pointed

toward a link between increased internalization into DCs and subsequent peptide presentation via MHC-II. Therefore, classifying high internalization in the DCIA as a risk factor for immunogenicity. Nevertheless, it is still unclear if this would always be the case as charge interaction might also increase FcRn binding, like for briakinumab (Schoch et al., 2015), resulting in favorable FcRn recycling properties (Kraft et al., 2019b) and which could explain its reduced lysosomal accumulation (see publication 3). However, slower dissociation with FcRn at a lower pH in the lysosome, following an increased charge interaction, could lead to the degradation instead of the recycling of the Ab and modify the derived epitopes. It is therefore not advisable, even if the two assay correlated in publication 4, to use one over the other. More importantly, it highlights the importance of both readouts to better understand where the liabilities of the therapeutic Ab are and act on them when possible. Additionally, we demonstrated that these properties also had consequences for the subsequent CD4+ T cell activation. This was an important confirmation of high internalization into DCs as a risk factor for immunogenicity as increased internalization into DCs was not always correlated to their activation (S. Cohen & Chung, 2021b). We did not explore the consequences on the DC phenotype, which would be the confirmation of their major role in this process. We investigated the CD4+ T cell activation propensity using a strong epitope (derived from ovalbumin), linking increased internalization into DCs to an increased risk for CD4+ T cell activation. However, in the absence of an appropriate CD4+ T cell epitope, altered internalization into DCs might not correlate to the subsequent immune response, which would need further evaluation. This study highlights the strengths of an integrated risk evaluation of immunogenicity in exploring properties that could act as risk enhancing factors, which could be applied to most of the biotherapeutics in development.

8.3. New modalities and T cell engager

The current risk evaluation of immunogenicity has proven efficacious in studying IgG1-like therapeutic Abs but format, modalities and target are constantly evolving and so are the

challenges to evaluate the risk for immunogenicity. Indeed, recent Ab formats make use of multiple binders which, in terms of immunogenicity risk evaluation, does not correspond to a combination of IgG1 using those binders. It has been shown that processing and presentation are affected by several factors, including the physical form of the antigen at time of delivery (Trombetta & Mellman, 2005), which can also relate to a difference in size or conformation induced by those formats. Despite intensive efforts in the development of bispecific Ab therapeutics, few have reached the market (Zhou et al., 2022). Given that immunogenicity represents one of the major liabilities of this class of Ab, the preclinical assessment of their immunogenic properties is the objective of intensive research efforts (Bi et al., 2013; Filipe et al., 2012; Harding et al., 2010; Jawa et al., 2020).

We used the hIgG1 transgenic mouse previously shown to display immunological tolerance to a broad range of human IgG1 antibodies and to be sensitive to immunogenic modifications thereof (Bessa et al., 2015). We showed that Fc-devoid Abs elicit strong ADA responses in our model, indicating that idiotypic-specific B and T cells exist in these mice, but are kept silent toward Fc-containing Abs (as full constructs did not elicit ADA). Additionally, we demonstrated that this phenomenon might be mainly mediated by FcRn-directed recycling, which diverts internalized antibodies away from the proteasomal degradation pathway to peptide presentation and immune activation (Qiao et al., 2008). Mechanism already observed for Abs having an increased affinity for FcRn mentioned before. Interestingly, small therapeutic antibodies composed of two different single-chain variable fragments (bispecific T cell engagers or BiTEs) were shown immunogenic, which goes in line with our observations (Hummel et al., 2021; You et al., 2021).

In contrast to the low levels of ADA detected for CEA-IgG in our hIgG1 mouse model, the derivatives CEA-IL2v and CEA-mTCB elicit strong anti-idiotypic ADA responses. This fundamental finding identifies the IL2v and CD3 binding moieties as responsible for the immunogenicity increment. The absence of ADA against DP47, an untargeted IgG1 Ab, even fused to a CD3 binding moiety, excludes the formation of new epitopes in CEA-IL2v and CEA-

mTCB as the primary reason for the onset of immunogenicity. Artificially induced immune-complexes composed of CEA-IL2v or CEA-mTCB and pre-existing low levels of ADA failed to explain the typical enhancement of immunogenicity. Additionally, the internalization rate of these compounds in murine DC revealed no simple correlation with their corresponding immunogenic potential in hIgG1 transgenic mice. Highlighting that correlations or observations made for classical IgG1 may not hold true for these T cell engaging Abs. The immunomodulatory property of the Fc region discussed above seems to be overruled by direct engagement of surface T cell activators. Upon binding of the T cell engaging Ab, B cells would process and present derived epitopes on MHC-II, rare idiotype-specific T cells can eventually interact with, and provide help to the B cell to produce ADA. Yet, potentially more abundant Fc-specific, unresponsive T cells are more prone to interact with B cells and maintain their unresponsive state. In contrast, the binding of the BCR of idiotype-specific B cells by T cell engaging Ab is accompanied by the triggering of activating surface receptors (IL-2R, TCR-CD3) on the hypothetical Fc-specific T cells. The ensuing reversal of unresponsiveness and re-activation of those cells would initiate the help to the cognate B cell to produce ADA. Confirmation of this hypothesis would require the identification of the putative Fc-specific T cell and the epitopes involved in unresponsiveness. Moreover, this phenomenon might be difficult to assess using the currently available assays for the risk evaluation of immunogenicity pointing to potential limitations of the strategy.

8.4. Limitation of the current risk evaluation strategy and further improvements

There are limitations that would be difficult to overcome for both *in vitro* and *in vivo* models, like patient-related factors contributing to ADA occurrence in clinical trials (e.g., cross-reacting pre-existing antibodies, dosing regimens). While these factors can influence the ADA outcome to a defined treatment, our current integrated risk evaluation of immunogenicity can address fundamental immunological mechanisms governing immune tolerance and its reversal. Even

the well established *in vivo* model, C57BL/6 mice has a different leukocyte subsets distribution (e.g., reduced Treg compartment), different TLR compared to humans and respond differently to pro and anti-inflammatory cytokines than Balb/c mice for example (Dingman & Balu-Iyer, 2018)(Wong & Germain, 2021)(Dingman & Balu-Iyer, 2018). These considerations together with the goal of reducing animal use at their minimum are in favor of *in silico* and *in vitro* methods which are not devoid of limitations.

Hence, even being informed by MAPPs data, NetMHCIIpan would not address the antigen processing and polymorphism of the cathepsin cleavage sites across individuals that can influence the peptides that would be presented in the context of MHC-II (Jawa et al., 2022). Additionally, the processing would also be affected by the therapeutic Ab conformation and stability, which is not taken into account by solely considering the amino acid sequence.

MHC-II polymorphism is a complication for all preclinical immunogenicity assessment tools in contrast to *in silico* predictions, which currently contain a database of more than 7300 alleles (Ducret et al., 2021b). Studies suggest that a subset of around 100 HLA-II heterodimers will be sufficient to cover at least one HLA-II heterodimer in greater than 90% of the global population (Taylor et al., 2021) which is currently not achievable. In addition, *in vitro* differentiated moDCs are often used as surrogates for DCs in research, as the availability of monocytes account for up to 20 % of the PBMC compared to circulating DCs (Osugi et al., 2002b). However, very few studies have focused on their comparison, especially when looking at the epitope being presented on their cell surface or their activation (Quarmby et al., 2018).

In the context of a pre-clinical immunogenicity assessment, the assays are mainly performed using healthy blood donors. However, it has been demonstrated that only patients treated with infliximab and tested ADA positive could mount an immune response against the treatment in T cell assay (recall response) (Karle et al., 2016; Vultaggio et al., 2016). This highlights again the limitations of these assays due to the low number of antigen specific T cells. This limitation has been partially circumvented by the assay format we used in publication 4, looking at IFN-

γ release by CD4+ T cells after four rounds of stimulation with autologous moDCs loaded with the compound of interest (Delluc et al., 2011b).

Similar T cell assays have been optimized for testing peptide pools while providing the co-stimulatory signal artificially (Bozkus et al., 2021). This assay format moves even further away from the physiological conditions, because peptides shorter than 80 amino acids would not go through the endosomal pathway (Tasker et al., 2021). Moreover, free peptides may have a different conformation and specificity for CD4+ T cell than endogenous peptides processed by DCs (Trombetta & Mellman, 2005). These observations are in line with the strategy described in publication 4, where moDCs are challenged with ovalbumin to assess the responsiveness of pOVA specific CD4+ T cells. When considering the use of peptides, recombinant ones would often be a better choice if they were folded properly (Greenfield et al., 2021b).

Considerable limitations of the currently used *in vitro* assays are their limited characterization and validation. We therefore validated three immunogenicity assays that we are currently using to evaluate the risk for immunogenicity of our projects, namely the DC: CD4+ restimulation assay, the DCIA and the DC activation assay. For the DC:CD4+ restimulation assay, we defined the main sources of variability and found that notable variability arose when compounds were re-tested in another screen, presumably related to different production batches. In addition, it is likely that the handling and storage of the sample plays a role, influencing post-translational modifications and aggregation (Joubert et al., 2016). Additionally, non-product related factors (e.g., DNA and host cell protein contaminations) have an impact on immunogenicity and could influence the assay readouts (Thacker et al., 2022). This emphasizes the need for an assay capable of detecting low amounts of contaminants or little changes in the therapeutic Ab structure, most likely looking at innate immune cell activation.

In addition, most of the immunogenicity assays lack complexity and interaction between cell types is mostly overlooked. Hence, non-specific (e.g., binding to red blood cells) or on-target (target expressed on DCs) binding can increase the risk for immunogenicity (Greenfield et al.,

2021a). Such interaction could for example increase their uptake into DCs (Greenfield et al., 2021b), interaction which is not recapitulated in the DCIA internalization assay. Furthermore, the interaction between therapeutic antibodies and the extracellular matrix (ECM) is currently not often included into immunogenicity risk assessments. However, formulation may affect the ECM directly and affect surrounding cells (Crommelin et al., 2019b). This could, for example, lead to a danger signals capable of initiating APC maturation and thus the initiation of an immune response (Jawa et al., 2020).

Importantly, the integrated risk evaluation of immunogenicity is often lacking the B cell contribution, which is often deduced as a consequence ofCD4+ T cell activation. Currently, antigen-specific B cell activation is difficult to assess *in vitro*.

Two strategies, certainly complementary, could be evaluated to improve the current immunogenicity risk assessment. The first one would be the increased use of APC cell lines and consequently assay automatization. However, the cell lines have to be carefully selected, characterized and suitable to a specific assay. Monocytic cell lines (e.g., THP-1) were compared to monocytes and moDCs for internalization and activation(Wen et al., 2021) and showed a very similar profile (Wen et al., 2021). However, the THP-1 cells were overall less sensitive to the tested conditions. More sensitive readouts (e.g., RNA sequencing) could also be used in PBMC-based assays or cell lines to assess their influence on innate immune cells, for example in the context of testing non-product related impurities (Thacker et al., 2022). Similarly, cell lines could also be developed for looking at MHC-II peptide presentation (e.g., overexpression of specific HLA alleles). The second strategy would be to implement complex *in vitro* systems into the strategy for the risk evaluation of immunogenicity. As a very first step, having DCs cultured with ECM could further improve the predictive value of the assay.

Finally, access to clinical samples from early trials might help to improve the clinical value of the preclinical assays. Moreover, the clonal dominance at a single time point is probably less informative regarding antigen-responsiveness compared to the changes in clonal frequency between the pre- and post-stimulation (Pollastro et al., 2021). For example, the monoclonality

or the specificity of the TCR of the T cells that expanded the most after a long period of exposure to the therapeutic Ab would not imply that the immunogenic response was initiated toward the same epitope. Such analysis would be made possible by an efficient use of patient samples in the most relevant immunogenicity assay.

9. Conclusion

The number of patients suffering from immunogenicity related adverse events remains high and can lead to severe clinical consequences on both patient safety and treatment efficacy, sometimes provoking the termination of clinical trials. Furthermore, the increased complexity of therapeutic Abs formats and their derived physicochemical properties has made the risk evaluation of immunogenicity even more challenging. We therefore characterized extensively the immunogenicity assays we judged relevant for an integrated immunogenicity risk assessment (e.g. DC: CD4+ T cell restimulation assay, hIgG1 mouse model) and adapted or developed new ones (e.g., DCIA, DC activation assay). The use of such an integrated approach allowed the identification of potential risk factors enhancing immunogenicity, such as positive charge patches. We demonstrated that positive charge patches on Abs induce high internalization into DCs, which could be the trigger for an immune response. However, this strategy still faces difficulties in identifying the liabilities of T cell engagers while still providing important insights into the mechanism underlying their immunogenicity. Overall, a fit-for-purpose validation of the immunogenicity assays used in the risk assessment and continuous improvements on the integrated evaluation of immunogenicity (e.g., new assays, automatization) will improve the identification of potential liabilities and increase our understanding of immunogenicity. If identified early enough in the molecule development, these liabilities could be removed, thus improving the success rate in early clinical trials and ultimately benefit the patients.

10. References

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Résumé en français

Introduction

La théorie de la réponse immunitaire humorale a été proposée pour la première fois en 1890 quand Emil von Behring et Kitasato Shibasaburō ont observé que le sérum pouvait réagir en réponse à un pathogène. Cette réactivité peut être généralisée à n'importe quel antigène, terme utilisé pour décrire une molécule ou au moins une partie pouvant être reconnue par un anticorps spécifique. Le terme d'anticorps a été décrit l'année suivante par Paul Ehrlich, puis vint la description de l'interaction avec un antigène, similaire à une interaction clé-serrure. Les anticorps ont ensuite été étudiés et sont considérés comme le mécanisme de défense principal contre les pathogènes extracellulaires (en opposition aux virus ou autres pathogènes intracellulaires). Les anticorps sont impliqués dans la neutralisation et l'élimination de ces pathogènes extracellulaires, des fonctions qui sont intimement liées à leur structure. Les anticorps sont des protéines composées de quatre chaînes peptidiques : deux chaînes lourdes et deux légères. Chacune de ces chaînes ayant un domaine variable à leur extrémité N-terminale, responsable de leur spécificité à un antigène et de sa fixation. La fixation de l'antigène par un anticorps pouvant entraîner la neutralisation de la protéine ciblée, bloquant toute autres interactions ou encore rendant cette protéine visible par le reste du système immunitaire. Ces interactions ont été pour une large variété d'utilisations dans le domaine médical (recherche, diagnostique ou encore traitement de pathologies). Le premier anticorps à usage thérapeutique approuvé par les autorités américaines (US Food and Drug Administration, FDA) a été Muromonab-CD3 en 1986 et depuis 153 nouvelles thérapies ont été approuvées, composant un marché de plus de 186 milliards de dollars. Ces anticorps ont été développés pour cibler 91 cibles thérapeutiques différentes et sont utilisés dans encore plus d'indications, particulièrement pour le traitement de maladies auto-immunes, inflammatoires ou encore différents cancers.

Cependant, une contrainte majeure à l'utilisation d'anticorps thérapeutique est leur capacité à induire la production d'anticorps dirigé à leur égard (anticorps anti-thérapie, AAT), pouvant réduire l'efficacité du traitement. Cette réaction est communément appelée immunogénicité. Cette réponse, qui se conclut par la production d'AAT suit le schéma d'une réponse immunitaire adaptative. En résumé, elle démarre par l'internalisation de l'antigène, ici l'anticorps thérapeutique, par les cellules dendritiques ou bien les macrophages (considérés comme des cellules spécialisées dans la présentation d'antigène). Dès la prise en charge de l'anticorps thérapeutique par ces cellules, il est dégradé dans les lysosomes et dirigé vers la surface de la cellule sous forme d'un peptide lié au récepteur du complex majeur d'histocompatibilité de classe II (CMH-II). Ce récepteur et cette voie cellulaire sont dédiés à la présentation d'antigènes extra-cellulaires, tel qu'il est le cas pour l'anticorps thérapeutique. La présentation du complexe peptide-CMH-II est le résultat d'une succession d'interactions et de processus qui commencent à la surface de la cellule présentatrice de l'antigène. En effet, plusieurs mécanismes peuvent être responsables de l'internalisation de l'antigène dans la cellule présentatrice, certains pouvant être considérés comme spécifiques (lié à l'interaction avec un récepteur) ou non (induit par des intéractions de charge par exemple). Ensuite le processus permettant la formation du complexe peptide-CMH-II peut éventuellement être influencé par l'antigène en lui-même (stabilité, présence de séquences considérées comme étrangères). C'est en effet seulement, les peptides générés par la dégradation de l'antigène dans les lysosomes, ayant la plus grande affinité pour le récepteur du CMH-II qui vont être présentés et considérés comme des épitopes. La présentation de l'épitope via le CMH-II n'est pas la seule contribution des cellules dendritiques mais elles doivent aussi être activées préalablement à leur interaction avec in lymphocyte T. En effet, l'activation d'un lymphocyte T nécessaire à la suite de la réponse immunitaire, n'est possible que suite à la présence de récepteurs de costimulation (CD80 ou CD86) aux côtés de la présentation de l'épitope à la surface de la cellule dendritique. L'activation d'une cellule dendritique, elle aussi peut dépendre de plusieurs facteurs, avec un objectif qui est de transmettre l'information, qu'un

danger est présent. Ce danger peut donc être détecté de plusieurs façons, la première étant la reconnaissance spécifique de certains motifs présents à la surface de pathogènes, appelé motif moléculaire associé aux pathogènes, reconnus par des récepteurs spécifiques présents à la surface des cellules dendritiques. D'autres récepteurs ont pour fonction de détecter une inflammation locale, la mortalité de cellules environnantes ou encore d'autres motifs étant considérés comme étrangers et donc donnant lieu à un signal de danger. Une fois les informations récoltées sur le lieu de l'infection (présentation d'un épitope dérivé de l'antigène via le CMH-II et incorporation d'un signal de danger donnant lieu à l'expression de récepteurs de costimulation), la cellule dendritique va migrer vers les organes lymphoid secondaire (par exemple un ganglion lymphatique) afin de rencontrer un lymphocyte T spécifique à l'antigène et transmettre le signal. Cette interaction s'effectue dans un organe lymphoïde secondaire afin de pallier la rareté de ces lymphocytes T. En effet, il existe une énorme variété de lymphocytes T de par la spécificité de leur récepteur lié à la reconnaissance d'un peptide-CMH-II donné. Les organes lymphoïdes secondaires étant un lieu de forte concentration en lymphocyte T, cela augmente les probabilités d'une interaction spécifique avec la cellule dendritique. Cette limitation dans le nombre de lymphocytes T spécifiques est vraie dans le cas d'une première exposition à l'antigène, ce qui est principalement le cas lors d'un traitement avec un anticorps thérapeutique. Ce lymphocyte T spécifique va donc interagir avec la cellule dendritique décrite précédemment et intégrer le signal donné par le complexe peptide-CMH-II de par son récepteur de cellules T mais aussi le signal de costimulation via un autre récepteur (CD28). Cette interaction va entraîner l'activation du lymphocyte T, s'exprimant par la production de cytokines (petites protéines transmettant un signal à plus longue distance), l'apparition sur sa surface de la protéine ligand du récepteur CD40 ainsi que par une rapide prolifération. En parallèle, un lymphocyte B résidant dans ce même organe lymphoïde secondaire aura rencontré et reconnu le même antigène qu'il va présenter de la même manière que la cellule dendritique, tout en exprimant le récepteur CD40 et en migrant à la rencontre du lymphocyte T décrit précédemment. Cette interaction, régi par la fixation de CD40, exprimé par le

lymphocyte B spécifique et le ligand exprimé par le lymphocyte T mais aussi de part la détection du même complexe peptide-CMH-II par ce dernier va entraîner la sélection et l'activation du lymphocyte B. Une prolifération cellulaire massive va s'en suivre et seulement les lymphocytes B ayant une affinité accrue pour l'antigène vont être sélectionnés pour ensuite produire des anticorps et constituer une réserve plus importante de lymphocytes prête à être mobilisée dans le cas d'une nouvelle rencontre avec ce même antigène. Cette étape de la réaction immunitaire humorale se nomme la réaction du centre germinatif. Au cours de cette réaction, les anticorps produits sont d'abord de faible affinité mais au fur et à mesure de la prolifération des lymphocytes B et avec l'aide du signal fourni par les lymphocytes T celle-ci va drastiquement augmenter.

Il est important de comprendre les principaux mécanismes liés à la réaction immunitaire humorale afin de pouvoir agir et diminuer cette réaction quand elle cible l'anticorps thérapeutique et dans certains cas essayer de prévoir une telle réaction. Même si des propriétés directement liées à l'anticorps thérapeutique peuvent être à l'initiative d'une réponse immunitaire humorale, ou plus simplement immunogénique, d'autres facteurs tels que ceux liés au patient (génotype, statut du système immunitaire…), à la voie d'administration de la thérapie ou encore son mode d'action (cible thérapeutique et possible conséquences) contribuent aussi au risque d'initier une telle réaction. Il y'a donc différentes stratégies en place afin de limiter ces réponses immunogéniques soit directement en réponse à celle-ci dans le cas d'un essai clinique (ou même lorsqu'un patient est sous thérapies approuvée) soit de manière préventive en sélectionnant voire en modifiant l'anticorps thérapeutique qui présentera moins de risque d'initier cette réaction.

Dans le but de détecter les propriétés qui contribuent au développement des AAT à différents niveaux de la réponse immunitaire, des modèles in silico, in vitro et in vivo ont été développés. Ces méthodes sont utilisées afin de déceler les facteurs intrinsèques et extrinsèques à l'anticorps thérapeutique en développement qui contribue à la réponse immunitaire chez le patient sous thérapie. Les méthodes actuellement en place peuvent être regroupées en trois

catégories: 1) les méthodes *in silico* avec l'objectif d'identifier les potentiels epitopes pouvant être présent dans la séquence de l'anticorps thérapeutique; 2) les méthodes *in vitro* ayant pour principal objectif de retranscrire la réponse d'une population spécifique de cellules immunitaires (cellule dendritique, lymphocyte T principalement) à la suite d'un stimulus; 3) les modèles *in vivo* tentant de reproduire le système immunitaire humain principalement chez les murins. Différents formats de ces méthodes sont décrits dans la littérature mais ceux discutés dans ce manuscript relatent principalement de la prédiction *in silico* d'épitope (NetMHCIIpan) ainsi que de leur validation in vitro (MHC-II Associated Peptide Proteomics, MAPPs). Mais aussi de la contribution des cellules dendritiques (internalisation et activation) ainsi que leur interaction avec les lymphocytes T dans différentes méthodes. Malgré leur usage répandu, ces méthodes manquent toujours de validation, il est donc nécessaire d'affiner leurs caractérisations et de définir rigoureusement leurs usages. Cependant, l'amélioration et la caractérisation de ces méthodes, bien que utiles, ne seront pas suffisantes pour rendre l'évaluation du risque pour l'immunogénicité efficace. Il est important d'apporter d'avantages de méthodes et d'intégrer les différents résultats provenant de différentes méthodes avant de tirer des conclusions. C'est seulement de cette façon que l'évaluation pré-clinique du risque pour l'immunogénicité pourra réduire le coût de développement de ces thérapies tout en apportant des traitements plus sûrs pour les patients.

Objectif et résultats

Le nombre de patients souffrant d'événements indésirables liés à l'immunogénicité reste élevé et peut avoir des conséquences à la fois sur leur sécurité et sur l'efficacité du traitement, conduisant parfois à l'arrêt d'essais cliniques. De plus, la complexité accrue anticorps thérapeutiques et de leurs propriétés a rendu l'évaluation du risque d'immunogénicité encore plus difficile. Ces éléments soulignent la nécessité de développer des thérapies avec un meilleur profil d'immunogénicité afin de réduire ces événements indésirables et le coût global de développement de ces thérapies. Les techniques actuellement disponibles pour évaluer le

risque d'immunogénicité ne sont actuellement pas entièrement validées et rarement appliquées dans une approche intégrée pour évaluer le risque d'immunogénicité. En effet, l'évaluation du risque d'immunogénicité ne sera pas réalisée par un test unique mais pourrait être aidée par une meilleure caractérisation des méthodes existantes et par le développement de nouvelles méthodes permettant de mieux comprendre les mécanismes conduisant à cette réaction immunitaire. L'intégration des différentes données issues ces dernières, le plus tôt possible dans le processus de développement permettrait d'optimiser la molécule développée (par exemple, élimination des épitopes, propriétés améliorées). Les principaux objectifs de ma thèse étaient donc de mieux comprendre les propriétés des anticorps thérapeutiques qui pourraient conduire à un risque accru d'immunogénicité tout en améliorant l'évaluation pré-clinique actuelle appliquée aux molécules en développement.

Dans un premier temps une nouvelle méthode décrivant l'interaction entre les cellules dendritiques et les lymphocytes T a été exposée. Cette méthode *in vitro* utilisant le FluoroSpot pour l'évaluation de la réponse des lymphocytes T spécifiques à anticorps thérapeutique a aussi été analysée statistiquement. Pour cela, un panel de 24 anticorps thérapeutiques avec des risques pour l'immunogénicité différents ont été testés. Nous avons démontré qu'en utilisant un seuil de positivité et une cohorte suffisante de donneurs sains, cette méthode pouvait identifier la plupart des composés avec des taux d'immunogénicité élevés chez les patients (71 % et 78 % pour la sensibilité et la spécificité, respectivement). Les principales sources de variabilité de la méthode ont aussi été caractérisées. Dans l'ensemble, nous avons démontré que cette méthode donne de bonnes indications sur le risque d'immunogénicité, en particulier lors de la comparaison de différents candidats. Cependant, une partie de la variabilité reste inexpliquée et ne serait pas diminuée par une simple augmentation de l'échantillonnage. L'intérêt du test réside dans une approche intégrée d'évaluation du risque d'immunogénicité induisant donc le développement de méthodes complémentaires.

Etant donné que les cellules dendritiques sont les premiers acteurs de la réponse immunitaire, et leurs contributions allant de l'internalisation des antigènes extracellulaires, à leur traitement

et à la présentation ultérieure des épitopes aux cellules T, il nous a semblé important d'explorer dans les détails la contribution de ce type cellulaire. Ces mécanismes constituent les premières étapes de la cascade d'immunogénicité, mettant les DC à l'honneur pour aider à la prédiction de l'immunogénicité. Pour mieux comprendre les mécanismes d'internalisation et d'activation des DC et leur relation avec l'immunogénicité, nous avons développé et validé un test d'internalisation et d'activation basé sur la cytométrie en flux qui contribuera à l'évaluation globale du risque d'immunogénicité de nos molécules. Nous avons évalué les deux tests avec un ensemble d'anticorps thérapeutiques commercialisés, identifiant pour certains une influence potentielle sur les DC qui pourrait contribuer à leur immunogénicité observée dans les études cliniques.

La quatrième publication peut être considérée comme une étude de cas, mettant en pratique l'approche intégrée d'évaluation du risque d'immunogénicité. En effet, l'internalisation des cellules dendritiques peut être influencée par les propriétés physico-chimiques des anticorps thérapeutiques. Ici, nous avons étudié le lien entre les patchs de charge et l'internalisation dans ces dernières. Un ensemble de 5 anticorps a été conçu pour afficher de grands patchs de charge positifs ou négatifs. Nous avons démontré l'influence des patchs de charge positifs sur l'internalisation par les cellules dendritiques. De plus, nous avons montré qu'une internalisation altérée pouvait se répercuter sur la réponse immunitaire en examinant la présentation d'épitopes via le CMH-II et l'activation des lymphocytes T. Cette étude met en évidence les points forts d'une évaluation intégrée des risques d'immunogénicité dans l'exploration des propriétés qui pourraient agir comme facteurs aggravant et qui pourrait être appliquée à la plupart des anticorps thérapeutique en développement.

La stratégie actuelle pour l'évaluation du risque d'immunogénicité s'est avérée efficace dans l'étude des anticorps thérapeutiques de type IgG1 mais le format, les modalités et les cibles évoluent constamment et constituent un défi dans l'évaluation du risque d'immunogénicité. En effet, les formats récents anticorps thérapeutiques utilisent la possibilité de fixer de multiple cibles (récepteurs…) simultanément, ce qui, en termes d'évaluation du risque

d'immunogénicité, ne correspond pas seulement à une combinaison d'IgG1 ciblant ces récepteurs. Il a été démontré que le traitement et la présentation sont affectés par plusieurs facteurs, notamment la forme physique de l'antigène, qui peut également être liée à une différence de taille ou de conformation induite par ces formats. Malgré des efforts intensifs dans le développement de ce type de thérapies, peu ont atteint le marché. Étant donné que l'immunogénicité représente l'un des principaux obstacles, l'évaluation préclinique de leurs propriétés immunogènes est l'objectif d'efforts de recherche intensifs.

Nous avons donc exploré la contribution de facteurs supplémentaires, autres que la séquence protéique, à l'immunogénicité de ces anticorps thérapeutiques, en particulier ceux ciblant de lymphocytes T. Ici, nous avons utilisé des souris transgéniques à immunoglobuline humaine gamma 1 (IgG1), qui sont immunologiquement tolérantes à l'IgG1 humaine, pour étudier l'immunogénicité de 13 anticorps conventionnels et de 2 autres ciblant de lymphocytes T. Nous avons constaté que la tolérance aux idiotypes est maintenue en partie par la fonction du récepteur Fc néonatal (FcRn). De plus, l'incorporation de fragments engageant les lymphocytes T était suffisante pour inverser la tolérance et déclencher la production d'AAT dirigée contre les idiotypes de ces composés. Enfin, ce phénomène pourrait être difficile à évaluer à l'aide des méthodes actuellement disponibles pour l'évaluation des risques d'immunogénicité, ce qui indique les limites potentielles de la stratégie et indiquant de futures améliorations discutées dans ce manuscrit.

Conclusion

Le nombre de patients souffrant d'événements indésirables liés à l'immunogénicité reste élevé et peut avoir des conséquences à la fois sur la sécurité des patients et sur l'efficacité des traitements, provoquant parfois l'arrêt d'essais cliniques. Nous avons donc caractérisé de manière approfondie les méthodes existantes (par exemple, “DC:CD4+ T cell restimulation assay”, le modèle de souris transgénique hIgG1) et en avons développé de nouveaux (par exemple, internalisation dans les cellules dendritiques, ou leur activation) pour améliorer

l'évaluation actuelle du risque d'immunogénicité effectuée en pré-clinique. L'utilisation d'une telle approche intégrée pour l'évaluation des risques d'immunogénicité a permis d'identifier les facteurs potentiels d'augmentation des risques. Nous avons démontré que les patchs de charge positifs induisent une forte internalisation dans les cellules dendritiques qui se répercutent sur la suite de la réponse immunitaire jusqu'à l'activation des lymphocytes T. Cependant, cette stratégie rencontre encore des difficultés pour identifier les risques pour les anticorps thérapeutiques ciblant directement l'activation des lymphocytes T. Bien qu'utile, en fournissant des informations importantes sur le mécanisme sous-jacent à leur immunogénicité. Dans l'ensemble, des validations adaptées et une amélioration continue de l'évaluation intégrée de l'immunogénicité (par exemple, de nouvelles méthodes, automatisation) amélioreront encore la compréhension de l'immunogénicité. Si les propriétés induisant l'immunogénicité sont identifiées suffisamment tôt dans le développement de la molécule, des modifications pourraient être apportées, améliorant le taux de réussite dans les premiers essais cliniques et, en fin de compte, le bénéfice pour le patient.

Michel Siegel, 2023

Titre : Evaluation intégrée des risques pour l'immunogénicité : validation et caractérisation des méthodes précliniques

Mots clés : Anticorps thérapeutiques, immunogénicité, patches de charges, recherche préclinique, validation de méthodes.

Résumé : La capacité des anticorps thérapeutiques à induire une réponse immunitaire indésirable est un phénomène multifactoriel appelé immunogénicité. Il est important de concevoir de nouvelles molécules à faible potentiel d'immunogénicité car cela a des conséquences pour les patients mais aussi éventuellement sur le développement de la molécule. Les méthodes actuellement disponibles pour évaluer le risque d'immunogénicité des anticorps thérapeutiques comprennent des algorithmes *in silico*, des tests *in vitro* et des expérimentations animales, qui sont déjà appliquées dans la pratique pour guider la sélection de molécules avec des profils immunogènes vraisemblablement meilleurs. Cependant, ces méthodes ne sont actuellement pas entièrement validées et, par conséquent, une approche intégrée est envisagée pour évaluer le risque potentiel d'immunogénicité. Par conséquent, nous avons validé trois de nos méthodes, élargissant le panel de test d'immunogénicité disponible, afin de mieux comprendre les propriétés et les mécanismes conduisant à l'immunogénicité des anticorps thérapeutiques. En appliquant cette stratégie nous avons démontré que les patches de charges positifs sur les anticorps et une internalisation élevée dans les cellules dendritiques sont d'importants facteurs de risque pour l'immunogénicité.

Title: Integrated immunogenicity risk assessment of therapeutic antibodies: validation and characterization of preclinical assays

Keywords: Assay validation, charge patches, immunogenicity, preclinical research, therapeutic antibodies.

Abstract: The ability of therapeutic antibodies to induce an unwanted immune response is a multifactorial phenomenon called immunogenicity. There is a growing need to design new molecules with a low potential for immunogenicity as it has consequences on patient safety and eventually on the molecule development. Currently available techniques to assess the risk of drug immunogenicity include *in-silico* algorithms, *in-vitro* assays and *in-vivo* experiments, which are already applied in practice to guide the selection of molecules with presumably better immunogenic profiles. However, these methods are presently not fully validated and therefore an integrated approach is considered to evaluate the potential immunogenicity risk. Therefore, we validated three of our assays, expanding the immunogenicity assay toolbox available; to better understand the properties and mechanisms leading to immunogenicity of therapeutic antibodies. We demonstrated, applying this integrated approach, that positive charge patches on antibodies and high internalization into dendritic cells, are important risk factors for immunogenicity.

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