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Considerations for the Design of Antibody-Based Therapeutics

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Abstract

Antibody-based proteins have become an important class of biologic therapeutics, due in large part to the stability, specificity, and adaptability of the antibody framework. Indeed, antibodies not only have the inherent ability to bind both antigens and endogenous immune receptors; they have also proven extremely amenable to protein engineering. Thus, several derivatives of the monoclonal antibody format, including bispecific antibodies, antibody-drug conjugates, and antibody fragments, have demonstrated efficacy for treating human disease, particularly in the fields of immunology and oncology. Reviewed here are considerations for the design of antibody-based therapeutics, including immunological context, therapeutic mechanisms, and engineering strategies. First, characteristics of antibodies are introduced, with emphasis on structural domains, functionally important receptors, isotypic and allotypic differences, and modifications such as glycosylation. Next, aspects of therapeutic antibody design are discussed, including identification of antigen-specific variable regions, choice of expression system, utility of multispecific formats, and design of antibody derivatives based on fragmentation, oligomerization, or conjugation to other functional moieties. Finally, strategies to enhance antibody function through protein engineering are reviewed while highlighting the impact of fundamental biophysical properties on protein developability.

1. Introduction

The first therapeutic monoclonal antibody, muromonab-CD3 (OKT3), was approved by the Food and Drug Administration (FDA) in 1985 to prevent rejection of kidney, heart, and liver transplants.[1] In a typical mechanism for antibody-based therapeutics, OKT3 binds to and inhibits CD3 on the T cell receptor complex to prevent host T cells from being activated against foreign antigens on the transplanted tissue. Although OKT3 proved effective for preventing host-versus-graft disease, the antibody itself elicits an immune response resulting in its accelerated clearance. The origin of this immune reaction has been traced to non-human sequences on OKT3, a murine antibody. Subsequent generations of therapeutic antibodies have humanized the amino acid sequence of mouse antibodies to chimeric, humanized, and fully human. This humanization of sequence to prevent immunogenicity is

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just one example of how antibody-based therapeutics have been improved through the decades. In fact, each part of the antibody structure has been strategically modified to alter biological effects and improve clinical outcomes.

Antibody therapeutics represent the fastest growing class of drugs on the market, due in large part to naturally favorable attributes such as specificity, potency, and metabolic stability. Knowledge of humoral immunology and advances in protein engineering have further contributed to the development of these important drugs. Currently 76 antibody-based therapeutics are used in the clinic, with nearly as many in late stages of clinical trials.[2] The most fruitful applications of antibodies lie in the fields of oncology (where built-in effector functions help to eliminate tumor cells) and immunology (where inhibition of inflammatory pathways is useful in treating autoimmunity). Over time, increasingly innovative antibody derivatives have replaced the standard monoclonal antibody to address the complex pathobiology of disease and improve upon existing therapies.

When designing antibody-based therapeutics, numerous factors must be considered, with each factor having a direct impact on protein structure and consequent impacts on biological and therapeutic function (Figure 1). For example, the choice of targeted antigen and antibody generation strategy affects the primary and tertiary structure of the antibody variable regions. Differences in this domain of the protein impact the nature of the antibody-antigen interaction, including specificity, affinity, and whether the binding event is activating or inhibitory. These biological properties, in turn, determine clinical properties like potency and therapeutic index. In the same vein, factors like antibody subclass and allotype affect the structure of the constant regions, which in turn influences binding to Fc receptors important for effector function and serum half-life. Thus, several determinants must be considered when creating new antibody-based therapeutics. Although distinct structural features have overlapping functional consequences, antibodies can be designed in a modular fashion to combine all desired features into a single optimized molecule. In this review, various design elements of therapeutic antibodies are discussed, along with their impacts on structure and biological and clinical function. The aim is to cover the wide extent of design strategies and engineering options available, rather than to exhaustively discuss the literature on any given topic. Thus, more focused reviews have been cited for thorough discussion of individual design elements.

2. Antibody structure and function

2.1 Antibody domains

Structurally, each antibody molecule is composed of two identical heavy chains and two identical light chains assembled into three discrete functional domains. While the two antigen-binding fragments (Fabs) are responsible for binding to the specific molecular target with high avidity, the crystallizable fragment (Fc) binds to immune receptors to elicit effector functions. The N-terminal half of the Fab arms contains the variable sequences, which differ between antibodies to confer them distinct specificities. In particular, three complementarity-determining region (CDR) loops on each chain contain hypervariable sequences that are situated at the antigen-binding interface. The remainder of the amino acid sequence contains constant regions that are identical for antibodies of a given subclass.

Within each of the immunoglobulin (Ig) domains of an antibody (of which there are 12 in the IgG class), there is one intrachain disulfide bond. There are also interchain disulfide bonds linking heavy chains to each other (two pairs for IgG1) within the flexible hinge region; and linking each heavy chain to its light chain. Finally, antibodies are modified with glycan molecules that finetune Fc receptor interactions. While the site and number of glycosylation sites varies between antibodies of different classes, the IgG antibodies contain a well-conserved Asn-297 residue for attachment of N-linked glycans.

2.2 Antibody classifications

Antibodies are divided into five structurally and functionally distinct classes (isotypes), each of which may contain additional subclasses (subtypes). This classification is defined by the type of heavy chain so that α, δ, ε, γ, or μ chains create IgA, IgD, IgE, IgG, or IgM antibodies, respectively. In humans, IgA antibodies are further divided into IgA1 and IgA2, while IgG antibodies may be of the IgG1, IgG2, IgG3, or IgG4 subclasses. Structural differences between classes include the number of Ig-like domains (four per α, δ, or γ chain versus five per ε or μ chain), the oligomeric states (IgA dimers and IgM pentamers/hexamers), and diverse patterns of hinge disulfide bonds and glycosylation (Table 1). While each class of antibody uses a defining heavy chain, they share common light chains which may be of the κ or λ class. About two-thirds of endogenous antibodies contain κ light chains, and the majority of clinical antibodies also utilize light chains with the κ framework.[3] The structural differences listed here allow B lymphocytes to generate adaptable immune responses that can be tailored over time via class-switch recombination.

2.3 Antibody functions

The most basic function of antibodies is to neutralize the targeted antigen. By binding to overlapping epitopes or inducing conformational changes, antibodies may inhibit binding of receptors to their coreceptors or growth factors. For endogenously produced antibodies, antigens such as viral entry proteins and bacterial toxins can be neutralized to prevent infection and pathogenesis. Meanwhile, therapeutic antibodies can be generated which bind to human receptors or growth factors, allowing for modulation of targeted pathways. Besides neutralization, other functions of antibodies include cross-linking to induce precipitation of soluble antigens or agglutination of cells.[4] These phenomena result from the bivalent nature of antibodies and the multivalent character of many antigens. Resulting immune complexes form effective opsonins which are efficiently phagocytosed. Thus, the “passive” protective mechanisms of antibodies also potentiate “active” effector functions based on binding to cell-surface Fc receptors and soluble complement factors.

2.4 Fc gamma receptors

Binding to class-specific Fc receptors (FcRs) expressed on leukocytes is one mechanism by which antibodies elicit an adaptive immune response for elimination of infected or malignant cells. FcγRs, which bind to antibodies of the IgG isotype via the lower hinge region, are the best described class of FcRs and the most important for existing antibody therapeutics. In humans, there are six FcγRs (FcγRI/CD64, FcγRIIa/CD32a, FcγRIIb/CD32b, FcγRIIc/CD32c, FcγRIIIa/CD16a, and FcγRIIIb/CD16b) that differ based on the affinity of IgG binding and the downstream response of binding.[5,6] While most FcγRs have

a low affinity for IgG and thus bind only to oligomeric immune complexes, FcγRI binds with high affinity to physiological concentrations of monomeric IgG1, IgG3, and IgG4. Thus, FcγRI is thought to be continuously occupied by monomeric IgG, which is displaced in the event of higher-avidity immune complex formation. FcγRI contains an additional extracellular Ig-like domain compared to the low affinity FcγRs (three instead of two), but the extra domain is not the primary determinant of its tight IgG affinity.[7] FcγRIIb is the only immunoinhibitory FcγR as it contains an intracellular immunotyrosine-based inhibitory motif (ITIM) used for downstream signaling. Conversely, the activating FcγRs generally contain (or are associated with a subunit that contains) an immunotyrosine-based activating motif (ITAM). The exception is FcγRIIIb, which instead signals through a glycosyl-phosphatidylinositol domain.

Expression of each FcγR is restricted to specific subsets of immune cells, which allows the cells to elicit clinically important effector functions. For example, FcγRIIIa is highly expressed on natural killer (NK) cells and is responsible for antibody-dependent cell-mediated cytotoxicity (ADCC).[6] Through this mechanism, NK cells are directed to antibody-coated target cells, which they lyse through delivery of cytotoxic granules. Similarly, myeloid cells like macrophages, monocytes, and dendritic cells express FcγRI and FcγRIIa. These receptors are involved in antibody-dependent cellular phagocytosis (ADCP), another immune mechanism for disposal of tumors or infected cells. In these contexts, activation of effector cells and subsequent cytotoxic mechanisms are dependent on clustering of FcγRs on the cell surface.

Clinically relevant FcγR polymorphisms exist that affect the strength of the IgG-Fc interaction and the potency of the immune response. FcγRIIa H131 (versus R131) and FcγRIIIa V158 (versus F158) have been shown to bind more tightly to certain subtypes of IgG antibodies. Although the differences in IgG1-FcγR affinity are less than two-fold in both cases, cancer patients treated with IgG1 antibodies such as rituximab and trastuzumab (for which ADCC is an important mechanism of tumor killing) show significantly better responses if they express the high affinity FcγR variants.[8–10] While this finding revealed the importance of FcγR-IgG affinity for tumor killing efficacy, the drug industry has focused on engineering the IgG component of the interaction to confer more potent effector function regardless of the patient's receptor genotype.

2.5 Neonatal Fc receptor

The neonatal Fc receptor (FcRn) also binds antibodies exclusively of the IgG subclass, but is structurally similar to major histocompatibility complex (MHC) class I rather than the FcγRs. As the name suggests, FcRn is important for maternal-to-fetal transfer of IgG via the placenta; it is, however, also involved in IgG homeostasis in adults.[11] In fact, the exceptionally long half-lives of IgG and human albumin in the blood are attributed to FcRn-dependent salvage mechanisms. When proteins are endocytosed by endothelial cells en route to lysosomal degradation, IgG and albumin bind to FcRn at the acidic pH (<6.5) of the endosome. The bound proteins then get trafficked back to the cell surface and are released upon reaching physiological pH (7.4) due to the low affinity of the interaction at this pH. Like MHC, the FcRn α chain is associated with the β2-microglobulin protein to create a

transmembrane heterodimer. Both chains contribute contacts to the FcRn-IgG interface, which is located at the Cγ2/Cγ3 domains of the IgG molecule.[12] Expression of FcRn occurs in syncytiotrophoblasts (for fetal transport) and throughout the vascular endothelium (for systemic homeostasis).[11] Additionally, expression in professional antigen-presenting cells allows for trafficking of immune complexes to the lysosome, where antigens can be processed for peptide presentation on MHC.[13]

2.6 Other subclass-specific receptors

There are other classes of FcRs that will become therapeutically important as antibodies of non-IgG classes are explored as drugs. IgA antibodies bind to FcαRI (CD89) on neutrophils and other myeloid cells to elicit either anti- or proinflammatory pathways, depending on the extent of IgA oligomerization.[14,15] Before binding of IgA to FcαRI can occur, the receptor must be primed via “inside-out signaling” originating from inflammatory cytokines. Then, one of two extracellular Ig-like domains of FcαRI becomes capable of binding the Cα2/Cα3 region of IgA to elicit downstream signaling via two intracellular ITAM-containing FcR γ chains.[16] Binding of monomeric or dimeric IgA is anti-inflammatory by preventing immune activation via other effector FcRs. On the other hand, binding of immune complexed IgA activates pro-inflammatory pathways such as phagocytosis, antigen presentation, cytokine release, and ADCC.[14,16]

The receptor for IgM antibodies, FcμR, is the most recently described subclass-specific receptor. It is expressed primarily on B lymphocytes and plays roles in their maturation and differentiation while also preventing survival of autoreactive B cells.[17] Binding of the IgM Fc to the single extracellular Ig-like domain of FcμR results in signal transduction through the intracellular tail and may work in conjunction with B cell receptor signaling.[18] Additionally, the receptor may mediate antimicrobial functions by inducing pro-inflammatory cytokines and supporting the development of inflammatory dendritic cells.

Fcα/μR (CD351) binds to IgA and IgM antibodies and is expressed in both hematopoietic (marginal zone B cells) and non-hematopoietic (follicular dendritic cells) tissues.[19] Endocytosis of IgM immune complexes via Fcα/μR negatively regulates the humoral immune response specifically for T-independent antigens.[19] Structurally, the receptor contains one extracellular Ig-like domain that binds to the Cα3 region of IgA and to the Cμ3/Cμ4 regions of IgM; and a cytoplasmic domain that may be involved in heterodimerization. Fcα/μR may also be important for proinflammatory IL-6 production when challenged with bacterial pathogens by associating with Toll-like receptor (TLR) 4.[20] However, this process does not appear to be Ig dependent. Fcα/μR may also play a role in the endocytosis of IgM-coated pathogens, thus granting IgM antibodies opsonization potential that is independent of the complement pathway.[21]

The polymeric Ig receptor (pIgR) also binds to polymeric forms of both IgA and IgM to concentrate them into mucosal environments.[22] Soluble dimeric IgA binds pIgR on the basolateral side of intestinal epithelial cells to be transcytosed into the intestinal lumen. Proteolytic cleavage on the apical side releases IgA with the secretory component of pIgR bound to the IgA J chain. This secretory component serves to localize secreted IgA to the mucus layer of the intestine and prevent its degradation by digestive proteases.[23]

Structurally, pIgR contains five extracellular Ig-like domains that become the secretory component after cleavage, while its cytoplasmic tail is necessary for intracellular sorting and transcytosis.[22]

FcεRI binds to immune complexes containing IgE antibodies to elicit the degranulation of mast cells and basophils that is characteristic of type I hypersensitivity.[24] The full receptor complex is tetrameric, containing the Cε3-binding α chain and the ITAM-containing $\beta\gamma_2$ chains.[25] IgE binding capacity of FcεRI is confined to the two extracellular Ig-like domains of the α chain, creating a very high-affinity interaction. Although all four chains are present in mast cells and basophils, a trimeric variant lacking the β chain exists in myeloid cells and is involved in immune signaling and antigen presentation. IgE also binds via its Cε3–4 domain to the low affinity FcεRII (CD23), which is actually a C-type lectin that regulates levels of serum IgE.[25] FcεRII exists as two forms that differ in the sequence of the N-terminal intracellular domain. The A form is constitutively expressed on B cells and contributes to endocytosis of IgE complexes for antigen presentation, while the B form is inducibly expressed on myeloid cells and may play a role in the phagocytosis of microbes.[26]

2.7 Complement

Besides cell-surface receptors, IgG and IgM antibodies also bind to bloodborne elements of the classical complement pathway. In the same way that the low-affinity FcγRs do not bind to monomeric IgG, C1q (the first component of the cascade) requires antibody oligomerization to form a stable, high-avidity interaction. In particular, IgG molecules hexamerize via the lower Fc into a C1q-binding conformation when bound to immobilized antigen on the surface of a target cell.[27] C1q itself is a hexamer of trimers, containing six globular head domains that contribute to the strong avidity effect. IgM, which naturally exists as pentamers and hexamers, does not need to further oligomerize to bind C1q; however, deposition onto a surface allows the formation of a more active C1q-binding conformation.[28] After surface-bound antibodies capture C1q, a cascade of chemotactic, inflammatory molecules are generated for recruitment of other immune components. The complement pathway terminates in formation of a membrane attack complex (MAC) in the target cell membrane, causing osmolysis. Thus, the ability to elicit complement-dependent cytotoxicity (CDC) for targeted destruction of specific cells is another attribute of IgG and IgM antibodies that makes them attractive therapeutic options.

2.8 Bacterial Ig-binding proteins

So far, the antibody binding partners discussed have been mainly human proteins that direct immune mechanisms against invading pathogens. However, there is a bacterial family of proteins that have been adapted to evade the humoral response by binding antibodies and inhibiting their interaction with endogenous receptors. The most well-studied of these cell wall-associated molecules are proteins A and G, which are expressed in *Staphylococcus aureus* and *Streptococcus*, respectively. Both proteins bind the antibody Fc region at the C_H2/C_H3 elbow, and thus bind competitively with FcRn.[29] However, they may also bind more weakly to the Fab domains: protein A to the V_H region of human V_H3 family antibodies and protein G to the Cγ1 domain of human IgG antibodies.[30] Subtle differences in the primary Fc binding site cause unique specificities for antibody species and subclasses.

For example, while protein A binds strongly to most mouse and human antibodies, it binds weakly to human IgG3 and mouse IgG1 (both of which are bound strongly by protein G). Another notable antibody-binding bacterial factor is protein L (from *Peptostreptococcus magnus*), which binds light chains of the κ class that are potentially present on antibodies of all subclasses.[29] Recently, protein M from *Mycoplasma genitalium* was found to bind antibodies even more universally with its well-conserved binding site in the V_L region that is present in both λ and κ light chains.[31]

Due to the ability of these proteins to bind a wide array of antibodies with high specificity, they have become convenient tools for affinity-based antibody purification. Recombinant forms of proteins A, G, and L have been developed to further hone their antibody-binding specificities. For example, while native protein G binds to serum albumin in addition to antibodies, the albumin-binding domain was removed from recombinant protein G to prevent binding of this common contaminant.[29] Additionally, the high affinity Fc-binding B-domain of protein A was engineered for increased chemical stability to be used in commercial affinity resins. The properties of distinct bacterial proteins have also been combined into recombinant fusion proteins such as protein A/G. This derivative contains four Fc-binding domains from protein A and two from protein G to allow for capture of antibodies of diverse species and classes.

2.9 Other receptors

While it is tempting to focus on the effector mechanisms that have been directly implicated in therapeutic success, one should also consider the possible effects of therapeutic antibodies binding to less studied receptors. Eight Fc receptor-like (FcRL) proteins have been identified, some of which bind to aggregated Igs. Primarily expressed in B cells and sometimes containing ITAMs and/or ITIMs, FcRLs are thought to be involved in regulation of B cell activation.[32] Tripartite motif (TRIM) 21 is a ubiquitously expressed Fc-binding protein that elicits antibody-mediated proteolysis of intracellular antigens.[33] TRIM21 has been used to target specific intracellular proteins for antibody-mediated degradation via the proteasome. Sialylated IgG antibodies can bind to lectins including some I-type Siglecs (e.g. Siglec-2/CD22) and C-type lectins (e.g. dendritic cell-specific ICAM-3 grabbing non-integrin, DC-SIGN/CD209; dendritic cell immunoreceptor, DCIR). Many of these receptors contain ITIM signaling motifs which may be involved in the favorable immunomodulatory effects of IVIg therapy.[8,34]

3. Antigen specificity

3.1 Mechanisms of action

Perhaps the most impactful decision in therapeutic antibody design is the choice of molecular target, which determines the mechanism by which the drug combats disease (Table 2). When treating cancer with cytotoxic mechanisms such as ADCC and CDC, the ideal antigen would be confined specifically to the tumor for minimization of damage to healthy tissue. For example, cell-differentiating proteins CD19 and CD20 are favorable targets due to their expression almost exclusively on B lymphocytes.[35,36] Of course, this strategy is only appropriate when the malignant tissue expresses a defining antigen, and

when the entire cell population can be safely eliminated. For B cell leukemias and lymphomas in particular, both of these conditions are satisfied due to the abundance of lymphocyte-defining antigens and the ability to deplete B cells without causing severe immunodeficiency or other negative effects. But for many tumors, either cell-specific antigens have not been identified, or the depletion of the entire (healthy and malignant) tissue population would cause loss of essential organ function.

In the cases where no tumor-specific antigens exist, the next best approach is to target antigens that are significantly overexpressed on tumor cells compared to normal tissues. Growth factor receptors such as epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) and adhesion molecules such as epithelial cell adhesion molecule (EpCAM) and carcinoembryonic antigen (CEA) are upregulated in many types of cancer and have been successfully targeted in cancer therapy or diagnosis.[36] Besides inhibiting cell proliferation, antibodies against these targets also work to actively destroy overexpressing cells through signaling-induced cell death, ADCC, and CDC. Although such antibodies have proven efficacious when used as monotherapies, they are susceptible to resistance mechanisms that might be overcome using multispecific antibodies that have additional mechanisms of action.

An alternative strategy in oncology is to inhibit immunomodulatory receptors that tumors use to evade the immune response. As cancer cells grow, they acquire mutations and neoantigens that would normally be recognized by T lymphocytes. In order to prevent immune recognition, many tumors express immunoinhibitory proteins such as programmed death ligand 1 (PDL1/CD274), which binds to programmed cell death protein 1 (PD1/CD279) on T cells to prevent their activation. A therapeutic approach has been to inhibit PD1, PDL1, and other inhibitory receptors like cytotoxic T lymphocyte-associated protein 4 (CTLA4/CD152), which increases the chances of a functional anti-tumor immune response.[36,37] Similarly, OX40 (CD134) is a costimulatory receptor on T cells that has been targeted with agonistic antibodies in the hopes of strengthening the T cell response.[38] Although immunostimulatory antibodies such as anti-PD1 and anti-CTLA4 have been efficacious in the clinic, their autoimmune toxicities are a challenge that remains to be fully addressed.

For systemic inflammatory indications, it is often feasible to target soluble antigens rather than cell-surface receptors. Cytokines such as tumor necrosis factor α (TNFα) and interleukins (IL) 5, 12, 17A, and 23 have all been successfully targeted by inhibitory antibodies for treatment of asthma, rheumatoid arthritis, Crohn's disease, psoriasis, and other inflammatory conditions.[39] Blocking the binding of these proinflammatory cytokines to their receptors helps to dampen systemic inflammation associated with chronic autoimmune disease.

In addition to human proteins, exogenous antigens can be targeted for prevention or treatment of infection. Anti-microbe antibodies work by conferring passive immunity to the patient via pathogen neutralization and Fc-dependent immune mechanisms.[40] For example, antibodies targeting respiratory syncytial virus protein F and *Clostridium difficile* enterotoxin B have been approved for prevention of the corresponding infections. Likewise, antibody binding to antigens on human target cells can also inhibit viral infection, as with

CD4 and human immunodeficiency virus.[40] When designing therapeutic agents for treatment of infection, one should consider passive immunization in addition active vaccination strategies.

3.2 Antibody-antigen affinity

Intuitively, stronger antibody-antigen affinity can translate directly to higher potency and clinical efficacy. This trend has been observed commonly for antagonistic antibodies that bind to pathogenic epitopes, where binding affinity correlates with neutralization efficacy and reduction of infectivity.[41,42] By increasing the strength of the interaction, therapeutic doses can be reduced without sacrificing antagonistic potential. Generation of strongly binding antibody variants can be performed using a diverse array of methods. Besides carrying out affinity maturation in B lymphocytes, several *in vitro* display technologies and even *in silico* mutagenesis approaches have developed for this purpose.[43]

However, it must be emphasized that higher antigen-binding potency does not always create a more efficacious therapeutic. For antibodies targeting solid tumors, it appears that there is an ideal range in antigen affinity that causes the most favorable properties.[44] If the K_D value is too low, the antibody may suffer from poor selectivity of tumor cells versus healthy tissue. Additionally, the slow dissociation rate of tight-binding antibodies may cause them to cluster at the tumor periphery or be internalized before diffusing deeper into the malignant tissue.[45] For antibodies undergoing target-mediated disposition, higher antigen affinity (and especially slower dissociation rate) can lead to accelerated internalization and elimination.[46] Thus, the optimal antigen affinity varies on a case-by-case basis and must be optimized based on factors such as tumor size, antigen concentration, and the kinetics of receptor internalization.

3.3 Epitope

Antibodies targeting the same antigen may elicit different mechanisms of action by binding to distinct molecular features or conformations. For instance, anti-CD20 antibodies can be binned into two groups based on differences in epitope and the types of effector functions induced. While type I antibodies prompt the concentration of CD20 into lipid rafts and cause more efficient deposition of complement, type II antibodies do not re-distribute CD20 in the membrane but cause more potent signaling-induced cell death.[47,48] Trastuzumab and pertuzumab, which target separate epitopes in the extracellular domain of HER2, have distinct modes of action including the inhibition of different types of HER2 heterodimerization.[49] The improved efficacy of trastuzumab/pertuzumab combination therapy indicates that the mechanisms are synergistic.[50]

In extreme cases, antibodies targeting different epitopes on the same antigen will produce the opposite effect. CD28 and CD40 are costimulatory molecules expressed on T cells and antigen presenting cells, respectively. Binding to these receptors in an activating manner enhances the immune response and is useful for cancer applications; meanwhile, inhibitory binding to prevent lymphocyte activation may be useful for treatment of autoimmunity. Interestingly, the epitope on these receptors determines their signaling output based on whether antibody binding mimics the interaction with the native receptor. For CD28,

antibodies that bind near the native B7–1/CD86 or B7–2/CD86 binding site cause a co-stimulatory output that is dependent upon concomitant T cell receptor signaling; but superagonistic antibodies that bind at a distal site of CD28 cause T cell activation even in the absence of T cell receptor ligation.[51] For CD40, the situation is more complicated because activation of antigen presenting cells is dependent upon FcγRIIb-mediated receptor crosslinking. However, it appears that two distinct epitopes defined by CD40L/CD154 competitivity lead to an agonistic or antagonistic response at physiological concentrations of FcγR.[52] Thus, mapping the precise site of antibody-antigen binding can help to define the elicited response.

3.4 Advantages of multispecificity

Combining antibody specificities to simultaneously interact with two distinct antigens allows novel mechanisms to be explored. Bispecific antibodies (bsAbs) targeting two tumor-associated antigens may have increased potency and decreased susceptibility to resistance mechanisms. For example, several bsAbs in clinical trials inhibit combinations of receptor tyrosine kinases including EGFR, HER2, HER3, and insulin-like growth factor 1 receptor (IGFR).[53,54] While monospecific antibodies such as anti-EGFR and anti-IGFR may be prone to tumor resistance mechanisms such as compensatory upregulation of alternate survival pathways, bsAbs targeting both receptors are less likely to be resisted due to the inhibition of orthogonal receptors.[54] Another benefit is that monovalent binding to each antigen may prevent toxicities that result from high-avidity binding or cross-linking.[55] Optimization of the affinity and valency of each antibody-binding domain may allow for increased tumor-targeting selectivity.[56] Thus, bsAbs targeting multiple antigens on the same type of target cell may increase the therapeutic window of anti-cancer drugs by increasing potency and decreasing off-target effects.

BsAbs may also be used for mechanisms that require co-localization of distinct cell types. The most prevalent of these mechanisms is T cell redirection, wherein antibodies targeting both tumor antigens and CD3 on T cells allow for formation of an immunological synapse, activation and proliferation of the T cells, and potent ADCC. Receptors besides CD3 have been used to target other immune cells to the tumor site; for example, FcγRIIIa for recruitment of NK cells.[53,54] Full-length IgG bsAbs may elicit trifunctional effects: in addition to binding tumor and T cells, they engage accessory cells like macrophages via the Fc domain to add phagocytosis to the tumor-killing repertoire, while also eliciting release of inflammatory cytokines.[53] It is thought that memory $CD4^+$ and especially $CD8^+$ lymphocytes dominate the immune response of T cell redirecting bsAbs, since these cells do not require the costimulatory signals that naïve T cells need for activation.[57] Future therapeutics may benefit from optimization of the geometry of the elicited cytolytic synapse, as well as the addition of costimulatory agonists or checkpoint inhibitors for generation of a more robust T cell response.[57]

While traditional monoclonal antibodies are restricted to the circulatory and lymphatic systems, bsAbs can facilitate delivery into otherwise unreachable compartments. A number of neurological targets, such as beta-secretase 1 (BACE1) for Alzheimer’s disease and leucine-rich repeat and immunoglobulin-like domain-containing protein 1 (LINGO1) for

multiple sclerosis, are guarded by the tight junctions of the blood-brain barrier.[58] However, endogenous carrier proteins expressed on the systemic side of the blood-brain barrier can be targeted by one arm of bsAbs for delivery to the brain, while also leaving one arm free to bind the therapeutic target. Using the function of transporters like the transferrin receptor (CD71) and insulin receptor, antibody concentrations within the brain can be significantly increased.[58,59] The antibody's precise affinity and epitope for the transporting receptor are both important parameters that must be optimized. Antibodies with weak transporter affinities may be advantageous, since they allow for efficient release on the brain side of the blood-brain barrier while also minimizing interference of the transporter's native function.[59] Inclusion of the Fc domain is another critical factor, since FcRn mediates antibody export from brain back into systemic circulation.[58] Outside of brain delivery, bsAbs may be useful for localization to other intractable but therapeutically relevant environments such as the intracellular cytosol.[60]

3.5 Delivery of cytotoxic agents

When inhibition and humoral immune mechanisms are insufficient to eliminate tumor cells, conjugation of cytotoxic moieties can be used to potentiate anti-cancer antibodies while retaining their specificity. Although antibody-drug conjugates (ADCs) and other antibody conjugates have been widely used for cancer indications, they have also been investigated for immunosuppression and treatment of infection.[61,62] In each case, the antibody is used to deliver potent drugs or toxins to select cell types while minimizing off-target effects of the unconjugated drug. Since specificity of antigen expression is a favorable attribute of both naked antibodies and ADCs, it is possible for the same antibody to be therapeutically useful with and without conjugation. An example is trastuzumab, which has been approved for treatment of breast cancer both as the naked antibody and as a conjugate to the cytotoxic agent emtansine. However, drug conjugation may potentiate the antitumor activity of a naked antibody, as with brentuximab and brentuxuimab vedotin.[61]

An additional consideration for ADC antigens is their ability to internalize, and thus transfer the antibody and payload into the targeted cell.[63] Ideally, the rate of receptor-mediated internalization (clathrin- or caveolin-mediated) should surpass that of receptor-independent endocytosis, so that ADCs are concentrated into antigen-bearing cells. Rates of internalization of naked and conjugated antibodies are often similar, but in some cases drug attachment alters these kinetics.[63] Affinity of the antibody for the receptor antigen may also affect the rate of ADC internalization, with one study showing faster internalization for ADCs with tighter Ag affinity.[64] While ADC-targeted receptors should internalize efficiently, they should also display minimal shedding from the membrane. Such antigen shedding may lead to accumulation of extracellular ADC-antigen immune complexes and an increase in systemic toxicity.[65] In summary, antibody conjugates offer targeted cytotoxicity, but must be designed to target antigens that are both specifically expressed and favorably localized after binding. Details of payload, linker, and conjugation chemistry are discussed in the section on antibody conjugates.

4. Class, subclass, and allotype

4.1 IgG1

The isotype of an antibody has critical effects on its therapeutic properties, since the structural differences between frameworks determine biological function. Due to factors like long half-life, potent effector function, structural homogeneity, and thorough characterization, IgG1 antibodies dominate the pool of biologics. Human IgG1 antibodies are approximately 146 kDa in size, with 15 amino acids in the hinge region containing two pairs of HC-HC disulfide bonds.[66] In contrast to other IgG subclasses in which the HC-LC disulfide bond occurs at the third cysteine of the heavy chain, IgG1 antibodies contain this linkage at the fifth cysteine of the heavy chain. The paired cysteine occurs as the ultimate (κ) or penultimate (λ) residue of the light chain. IgG1 is not known to have variation in quaternary structure, but is monomeric so that its two Fab arms bind antigen bivalently. Immunologically, an IgG1 response is typically elicited for protein antigens, and IgG1 is a common viral responder.[8] IgG1 is the predominant subtype in the blood, making up roughly half of the total Ig pool (or 67% of IgG antibodies).[15,66]

All the FcγRs are bound by monomeric IgG1. Compared to IgG3, IgG1 binds more tightly to FcγRIIa but less tightly to FcγRIIIa and FcγRIIIb.[8] Thus, IgG1 is capable of mediating clinically useful processes like ADCC and ADCP. Furthermore, IgG1 assembles into hexamers on the surface of target cells, allowing it to fix complement and mediate CDC. An intermediate hinge length and flexibility allow IgG1 to efficiently utilize both Fab arms for bivalent antigen binding and immune complex formation. While IgG1 is less prone to aggregation than IgG2 and IgG4, it is more susceptible to chemical degradation, possibly due to its longer hinge region.[67]

Polymorphisms in Ig constant regions exist for certain subclasses, including IgG1. Of these amino acid variants, those that elicit a serologic response in non-carriers are referred to as allotypes. It is possible for a heavy chain to have amino acid substitutions that are not true allotypes; for example, if the sequence of interest is found in the heavy chain of another subtype. These variants that are unique within a subclass, but redundant in other subclasses, are referred to as isoallotypes. Allotypes are referred to by either alphabetical or numeric labels, coupled with the subclass and the letter m (marker). For example, an allotype within IgG1 is G1m1, which is the same as G1m(a). The heavy chain protein expressed by an individual may contain multiple allotypes, which are often inherited together as a haplotype due to their close genetic proximity and low frequency of crossover. Although allotypes are, by definition, immunogenic, their ability to cause a robust response *in vivo* is less apparent. In fact, it appears that therapeutic antibodies of a given allotype do not elicit more anti-drug antibodies in individuals lacking that allotype.[68,69] Nevertheless, the risk of immunogenicity of biologics may be lowered by using frameworks that lack allelic determinants.

For IgG1, four allotypes have been identified with the designations G1m1 [G1m(a)], G1m2 [G1m(x)], G1m3 [G1m(f)], and G1m17 [G1m(z)].[70] Each allotype involves substitution of one to two amino acids in the C_H1 or C_H3 domains. The four most common combinations of allotypes include the haplotypes G1m3; G1m3,1; G1m17,1; and G1m17,1,2.[70] As for marketed antibodies, the preferred frameworks seem to be G1m3 and G1m17,1.[71]

Interestingly, G1m17, which is not a naturally abundant haplotype, is also found in biologics, where the m1 substitution was removed to reduce immunogenicity.[71] The interplay between the allotypes of patients and therapeutics was highlighted in one study demonstrating that the half-life of infliximab (G1m17,1) is longer in patients with the G1m3 haplotype than those with the G1m17,1 allotype.[72] Apparently, the affinity of G1m17,1 antibodies for FcRn is higher than that of G1m3 antibodies, allowing the administered infliximab to out-compete endogenous G1m3 antibodies for access to endosomal recycling. The same report suggested that G1m3,1 antibodies may have even slower clearance due to their superior FcRn binding.

4.2 IgG2

IgG2 is also about 146 kDa and is the second most prevalent IgG antibody in serum, representing 22% of IgG and 16% of total antibody.[4,15,66] It has a shorter hinge than IgG1, containing 12 amino acids, but actually contains more disulfide bonds in this region for four total HC-HC linkages. An IgG2 response is commonly produced by carbohydrate antigens found on encapsulated bacteria. Since peptide-MHC presentation is impossible for such antigens, the IgG2 response is often T-independent.

Three isomers of IgG2 exist. IgG2A, which is common when the molecule contains λ light chain, has paired hinge disulfides and the typical HC-LC disulfide linkage; IgG2B, common when the molecule contains κ light chain, has shuffled disulfide bonds with the HC-LC disulfide bonds using a set of hinge cysteines; and IgG2A/B contains one of each of these disulfide configurations.[73] The IgG2A isomer is thought to confer more flexibility to the Fab arms relative to IgG2B, which can have functional consequences. For example, an IgG2 antibody was found to interact either agonistically or antagonistically with its antigen depending on whether its hinge disulfides were locked in the A or B configuration.[74] Heterogeneity in the hinge can also cause intermolecular disulfide linkages. Covalent dimers of IgG2 have been observed in recombinant systems as well as the serum, and the antibodies comprising these dimers could feasibly have the same or distinct specificities.[73,75] It has been postulated that antibody dimerization could provide an immunological advantage by increasing avidity for the low-affinity but regularly repeated carbohydrate antigens that are commonly targeted by endogenous IgG2 antibodies.[75] Although the hinge isomers of IgG2 confer it with interesting properties, including the ability to form tetravalent dimers, this heterogeneity could be a problem when creating well-defined therapeutics.

IgG2 has weak effector functions compared to IgG1 and IgG3 due to its weaker affinity for FcγRs. Although monomeric IgG2 displays low micromolar affinity for most of the low-affinity FcγRs, it does not bind significantly to FcγRI or FcγRIIIb.[8] Complement fixation for IgG2 occurs only when the antigen is present on the target cell at high density.[8] Thus, IgG2 is a more immunologically silent isotype that may be useful for therapeutic applications that requires a strict blocking mechanism.

Only one serologically determined allotype of IgG2 has been found, denoted G2m23 [G2m(n)], as opposed to G2m.. which lacks antigenic determinants.[70] The G2m23 allotype contains the V282M substitution in the C_H2 domain, as well as the P189T substitution in the C_H3 domain. Functionally, the G2m23 allotype seems to confer an advantage against

bacterial infections, but the mechanism behind this, and its utility for IgG2 therapeutics, has not been studied.[76]

4.3 IgG3

As the third most abundant IgG subtype in the serum, making up 7% of IgG antibodies and 5% of antibodies overall, IgG3 offers strong effector function at the cost of proteolytic and pharmacokinetic instability.[15,66] Due to the extended hinge containing on average over 60 residues of which 11 are disulfide-forming cysteines, IgG3 antibodies are slightly larger than those of other subtypes at around 170 kDa. Although this long hinge allows for tight binding of Fc binding partners and flexibility of the Fabs, it renders the protein susceptible to cleavage. The IgG3 hinge is also notable for containing threonine residues that can be O-glycosylated.[77] Immunologically, the γ3 chain is the first of the class-switched constant regions, which causes IgG3 antibodies to appear early in the humoral response to viral protein antigens.[8] No prevalent deviations in quaternary structure have been observed for IgG3 antibodies, which exist mostly as monomers.

Compared to antibodies of other IgG subclasses, most IgG3 antibodies have a significantly reduced half-life in serum (one week as opposed to three weeks). This difference has been traced to R435 at the FcRn binding interface, which for all other IgG antibodies is H435. In IgG1, 2, and 4, the titratable histidine at this site is critical for pH-dependent FcRn binding and endosomal recycling. However, the presence of arginine slightly decreases FcRn affinity in the endosome, rendering IgG3 unable to compete with H435-containing antibodies for lysosomal salvage.[78] Interestingly, IgG3 half-life is similar to that of other IgG antibodies for individuals containing allotypes with H435.[8]

Of all IgG subclasses, IgG3 has the strongest effector functions as a result of tight FcγR interactions. Multivalent binding of IgG immune complexes to each of the FcγRs is strongest for IgG3.[79] Furthermore, IgG3 is the most potent of the IgG subclasses in fixing C1q for initiation of the complement pathway.[8] While the potent immune activation of IgG3 may sound promising for treatment of cancer and infectious disease, the complexities of its long hinge region have not been adequately addressed to allow for the formation of stable and homogeneous biologics.

IgG3 contains the most polymorphisms of any Ig subclass in the form of 13 defined allotypes and several hinge variants with differing numbers of repeats. Each of the allotypes occurs as one to three amino acid substitutions in the C_H2 or C_H3 domains.[70] These allotypes are combined into six common alleles, each of which contains four to seven allotypic variants. It is not clear whether any of these alleles might have therapeutic advantages, especially since the IgG3 subclass has been studied relatively little in the biologics field. A more structurally dramatic polymorphism of IgG3 occurs due to heterogeneity in the hinge region. The sequence of the hinge contains one common exon, followed by one to four repeats of a second exon.[71] As a result, the hinge ranges from 27 to 83 residues, which suggests wide variation in conformation, proteolytic stability, and antigen cross-linking.

4.4 IgG4

The least naturally abundant of the IgG subtypes is IgG4, which makes up 4% of IgG antibodies or 3% of antibodies overall.[15,66] IgG4 antibodies are often associated with antigen exposure over an extended period, which may be related to its anti-inflammatory properties and gene locus as the last subtype of γ heavy chain.[8] Like IgG2, IgG4 is around 146 kDa and contains 12 amino acids in the hinge region. However, hinge disulfide bonds of IgG4 resemble those of IgG1, with two covalent linkages between heavy chains. The rigid CPPC amino acid motif in the hinge of IgG1 is replaced with CPSC in IgG4, conferring more flexibility. While IgG4 does not have any antigenically defined allotypes, there are several allelic variants containing isoallotypic substitutions.[70]

The increase in hinge flexibility of IgG4 allows for the formation of intrachain disulfide bonds instead of the normal interchain linkages.[80] In addition, human IgG4 contains R409 instead of K409 that is present in IgG1, 2, and 3. The K409R substitution destabilizes interchain interactions in the C_H3 domain and, combined with the labile hinge of IgG4, allows antibodies to dissociate into half-antibodies and recombine into distinct pairings.[81] This process, termed Fab-arm exchange (FAE), has been observed *in vivo*, resulting in the formation of bispecific molecules that are monovalent for two different antigens.[82] Immunologically, this may be an anti-inflammatory mechanism to decrease antigen cross-linking due to loss of bivalency. Since the exchange of therapeutic half-antibodies with endogenous antibodies has the potential to create poorly defined bsAbs upon human administration, the S228P mutation is now commonly used to prevent this process from occurring for therapeutic IgG4 antibodies.[80] The mutation prevents FAE by stabilizing the IgG4 hinge into a more rigid, IgG1-like state that forms proper interchain disulfide bonds.

IgG4 binds to most FcγRs, except perhaps FcγRIIIb, though generally more weakly than IgG1 or IgG3. Of all the IgG subtypes, IgG4 binds most strongly to the inhibitory FcγRIIb, which may explain its anti-inflammatory effects.[79] It is inefficient at complement fixation, with less C1q binding than even IgG2.[8] As with IgG2, IgG4 may be most applicable for therapeutics that require antigen binding without extensive immune activation.

4.5 IgA

Although IgG antibodies dominate the current pool of biologics, IgA antibodies elicit several effector mechanisms that may be useful therapeutically. Because IgA does not bind to FcRn, it is eliminated more quickly than IgG, with a serum half-life of around one week instead of three weeks.[4,66] Nonetheless, its rate of production is the highest of any other Ig class.[83] IgA1and IgA2 collectively make up ~20% antibodies in the serum, where they are mostly in the monomeric state; however, IgA is the dominant Ig isotype in secretions like saliva and breastmilk, and at mucosal surfaces such as the gastrointestinal and respiratory tracts. At these sites, IgA is processed into polymeric forms (most commonly dimers, but up to tetramers) in which the 15-kDa joining (J) chain covalently links the monomers via disulfide bonds to their 18-residue C-terminal tailpiece domains.[84] In this polymeric form, IgA binds to pIgR and is processed to the lumen of mucosal sites, where it is released with SC as secretory IgA.

IgA1 can be distinguished from IgA2 in terms of structure and distribution. It contains a longer hinge due to sequence duplication, and is more prone to proteolysis. The hinge of IgA1 contains O-glycosylation sites that are absent in the IgA2 hinge.[85] In the serum, IgA is predominantly of the IgA1 subclass, whereas in mucosa IgA2 plays a larger role and even becomes the major subtype in the gut.[84] Both isoforms exist in monomeric and polymeric forms, and both can be processed into secretory IgA by pIgR. While IgA1 responds to protein and polysaccharide antigens, IgA2 seems to specialize in polysaccharide antigens.[86,87]

Despite its lack of FcγR interaction, IgA possesses significant effector functions that occur through binding to FcαRI on various leukocytes. While binding to macrophages and neutrophils elicits phagocytosis, neutrophils and eosinophils release cytotoxic granules in an ADCC mechanism similar to that of IgG-ligated NK cells. Studies with matched IgG and IgA antibodies have demonstrated their complementary mechanisms of action. IgG recruits NK cells for ADCC activity and C1q for initiation of the classical complement pathway. IgA, on the other hand, can cause substantial ADCC in the absence of lymphocytes, and initiates CDC despite not binding to C1q.[84,88] Rather, IgA may recruit complement via the alternative or lectin pathways. It has been postulated that the complement-mediated lysis by IgA is an *in vitro* artifact, and that any observed CDC is independent of IgA.[84] Regardless of complement activity, the cross-linking of FcαRI by IgA clearly elicits potent ADCC and ADCP functions that have not yet been utilized by clinical biologics.

There are two main allotypes of IgA2 (m1 and m2) with notable differences in structure, if not function. A third allotype, IgA2n, has also been observed and may have resulted from recombination of the other alleles.[89] While IgA2m2 and IgA2n contain the normal HC-LC disulfide bonds, IgA2m1 lacks any covalent linkage between paired heavy and light chains.[84] Rather, the two LCs of IgA2m1 form a LC-LC disulfide bond that is unique among all the human Igs. Presumably, this LC-LC linkage could constrict the movement of the Fabs in IgA2m1 while the lack of HC-LC linkage could destabilize the H_2L_2 complex. To address these concerns, the P221R mutation has been incorporated into IgA2m1 to instill it with more IgAm2-like disulfide pairing patterns.[90] In terms of glycosylation, IgA2m1 contains four N-glycosylation sites while IgA2m2 and IgA2n contain one additional site.[85] The effects of these structural changes on the function of each allotype have not yet been thoroughly investigated.

4.6 IgD

IgD is co-expressed with IgM in the membrane of mature naïve B cells, but little is known about its biological function. Whereas IgD has a lengthy hinge region containing multiple O-glycosylation sites, IgM lacks a true hinge but instead contains an extra Ig domain.[91] The distinct structures of IgD and IgM allow for differences in the antigen specificities of B cell receptors. While IgM signaling can be triggered by antigens of low or high valency, IgD signaling appears to occur only for highly polyvalent antigens.[92] Thus, IgD may help to regulate B cell development by distinguishing soluble self-antigens from complexed or polyvalent pathogenic epitopes. Whether this same mechanism plays a role in the outcome of class switch recombination has not been investigated.

Nor is the function of soluble IgD entirely clear. The presence of $IgD^{+}IgM^{-}$ plasmablasts in the upper respiratory mucosa, and the detectable levels of IgD in serum, support an immunological role for antibodies of this Ig subclass.[93] Circulating IgD has been shown to ligate receptors on basophils to elicit production of proinflammatory cytokines (TNFα, IL-1β) and antimicrobial factors (cathelicidin).[93] Thus, while IgD likely plays important immunological roles, these functions are not understood well enough to justify use of this framework in a clinical candidate.

4.7 IgE

IgE is best known for its ability to elicit highly inflammatory responses to allergens, but also to helminths and other pathogens. Its structure contains five Ig domains instead of the more typical four, causing monomeric IgE to be slightly larger than IgG despite lacking a hinge. Seven asparagine residues on the IgE heavy chain can be glycosylated, although one site has been shown to contain exclusively oligomannose sugars rather than complex glycans.[91] IgE in the serum is not only the least prevalent of any Ig class, but also the shortest-lived, having a half-life of just two days.[66] However, molecules of IgE can persist for weeks within tissues by binding to their high-affinity receptor on mast cells and basophils, since the half-life of the IgE-FcεRI complex is on the order of days.[25] This extremely slow dissociation rate also allows IgE to prime immune cells for immediate degranulation upon antigen binding and receptor crosslinking.

A major effector mechanism of IgE is the FcεRI-mediated degranulation of mast cell and basophils, where important contents include histamine, serotonin, proteases, and inflammatory cytokines.[24] However, FcεRI is also expressed on eosinophils, which can similarly degranulate to achieve ADCC via release of cationic proteins, reactive oxygen species, and inflammatory cytokines.[94] Furthermore, expression of CD32 on macrophages and monocytes allows for the phagocytosis of IgE-bound target cells. Thus, IgE antibodies have a variety of clinically relevant effector functions in addition to potentially favorable properties such as long tissue residency, a lack of inhibitory receptors, and interaction with tumor-resident macrophages.[94,95] Despite the lack of human trials, preclinical studies in mice have demonstrated that tumor-targeting IgE and IgG antibodies have complementary modes of action, and that IgE antibodies are in some cases superior.[94] The potential for anaphylactic reactions is a valid concern, but so far IgE has demonstrated no activation of effector cells in the absence of cross-linked antigen.[94] An ongoing clinical study using IgE antibodies targeting folate receptor alpha will help inform whether IgE antibodies are safe agents for the treatment of cancer ().

4.8 IgM

As mentioned previously, IgM is co-expressed with IgD on mature B lymphocytes as a result of differential splicing. Thus, IgM antibodies play a pivotal role in the early immune response, where low antigen affinity, but high avidity and polyreactivity allow for the recognition of pathogens prior to the development of affinity matured antibodies or other adaptive mechanisms.[15] Although IgM is found predominantly in the serum, where it makes up 8% of antibodies and has a half-life of 10 days, it can also be transcytosed to mucosal surfaces via pIgR.[4,15,66] Like the ε heavy chain, the μ chain contains four constant Ig

domains in addition to its variable region and has several conserved N-glycosylation sites.[96] However, IgM forms the largest quaternary structures composed of usually five, but sometimes six, IgM molecules.[15] While the pentameric species can form with or without the J chain, the more potent hexamer form lacks any J chain.[97,98] It appears that J chain is favorable for pIgR binding and transcytosis but inhibitory for complement-mediated lysis.[97,98] IgM heavy chains contain a single disulfide bond (in the Cμ2 domain) linking them to the other heavy chain of the IgM monomer, and two additional cysteine residues (in the Cμ3 and tailpiece domains) allow for formation of inter-monomer disulfide bonds.[96]

The polymeric structure of IgM is perfectly suited to ligation of C1q and potent activation of the classical complement pathway. In addition, IgM has been shown to mediate phagocytosis. While this function was initially attributed to Fcα/μR, it was recently shown that any phagocytosis may result as an extension of the complement cascade (via the opsonin C3 and complement receptor 3 on phagocytes).[21,99] Excluding the IgG class, IgM has the most extensive history of clinical use. One IgM antibody, nebacumab, was approved by several European countries in the early 1990s for treatment of Gram-negative sepsis; but it was subsequently withdrawn due to a variety of factors including high toxicity, high cost, and an inability to diagnose which cases of sepsis would be suitable for nebacumab use.[100] More recently, phase 1 clinical trials with IgM monoclonal antibodies have demonstrated that these therapeutics are generally well-tolerated, with safety concerns including mild skin rash and nosebleed.[101–105] Furthermore, modest but favorable anti-tumor responses have been observed in some of these early trials. Thus, the potent immune-activating function of IgM has shown promise for treatment of infection and cancer.

4.9 κ and λ light chain

antibodies of all subclasses incorporate one of two types of light chains, κ or λ, whose genes are on chromosomes 2 or 22 respectively. Synthesis of a λ light chain only occurs if recombination at both κ alleles is unsuccessful. While the ratio of antibodies containing κ and λ light chains is roughly 2:1 in humans, the ratio is over 9:1 for approved therapeutic antibodies.[2,3] This overwhelming preference for the κ isotype in biologics may be related to the use of hybridoma technology to generate antibody variable regions, since mouse B cells express antibodies with a 20:1 κ:λ ratio.[106] Alternatively, the κ isotype may be purposefully selected based on superiority in stability or other biophysical properties.[107] The third complementarity-determining region (CDR3) of λ light chains is on average longer, more hydrophobic, and more acidic than that of κ light chains.[108,109]

The κ and λ genes contain distinct types of genetic diversity. The κ constant region is encoded by a single gene locus, but this gene has three allotypes (Km1, Km2, Km3).[70] The allele containing Km3 is most common in Caucasoid, Negroid, and Mongoloid populations. Accordingly, most therapeutic antibodies utilize κ light chains of the Km3 allotype. Meanwhile, individuals may have anywhere from 7 to 11 distinct lambda genes due to differences in haplotype.[70] Immunoglobulin constant lambda (IGLC) genes 1, 2, 3, 7, and sometimes 6 code for functional proteins, while the remaining loci are considered pseudogenes. None of the λ genes have serologically defined allotypes.

4.10 Cross-isotypes and cross-subtypes

Using protein engineering, it is possible to combine favorable features of different antibody isotypes into functionally optimized chimeric molecules.[110] For instance, the breadth of antibody effector mechanisms has been increased using an IgG1 framework containing IgA1 sequences in the lower Fc domain.[111] The IgG1/A1 hybrid elicited strong ADCC, ADCP, and CDC by binding to FcαRI in addition to FcγRs and C1q. Conversely, the clinically approved eculizumab combined the IgG2 C_H1 and hinge with the IgG4 C_H2 and C_H3 in order to abrogate both FcγR and C1q binding and serve as an immunologically silent cross-subtype.[112] The use of IgG2 sequences in otherwise IgG1 antibodies has also been used to reduce hinge proteolysis and induce a more agonistic antigen binding response while retaining the favorable properties of the IgG1 subclass.[113,114]

5. Identification of variable regions

5.1 Hybridoma

Multiple strategies can be used to generate and select the antigen-binding variable regions of therapeutic antibodies (Figure 2). The classical method, which is still the most common in successful clinical candidates, uses hybridoma technology.[115] This *in vivo* technique generates antibodies targeting antigens that are immunogenic in mice or other suitable mammalian hosts. Antigens are first injected into the mouse to elicit the expansion of antigen-specific B cells. After a humoral response has been mounted, splenocytes are harvested and the antibody-producing B cells are fused with highly proliferative myeloma cells via strategies such as electroporation and polyethylene glycol treatment. Isolation of fused hybridoma cells is carried out by growing the mixture of cells in hypoxanthine-aminopterin-thymidine (HAT) media, which allows for selective proliferation of cells with properties of both B cells and the myeloma line. Unfused myeloma cells, which are modified to lack the HGPRT gene, cannot grow in HAT media as they are unable to make the nucleotides necessary for DNA replication. Conversely, unfused primary B cells quickly die in culture due to lack of immortalization. Thus, the hybridoma cells are selected and subsequently sorted into new cultures starting from individual cells. These immortalized, antibody-producing cells can then be tested for the desired specificity by conducting binding assays such as ELISA with cell supernatants. In addition to the hybridoma method, B cells can also be immortalized through transformation with Epstein-Barr virus or B cell lymphoma genes.[116,117]

Although the generation of monoclonal antibodies using hybridoma technology was a huge step forward in the development of biologic therapeutics, it later became apparent that use of mouse proteins in humans led to a high incidence of immunogenicity.[118] Since this realization, the chimerization and humanization of mouse antibodies has become commonplace. Chimeric antibodies retain the entire variable region sequences of the hybridoma-generated mouse antibody, but the constant regions are of human origin. Thus, the amino acid sequence of chimeric antibodies is still roughly 1/3 non-human. Humanized antibodies contain a higher percentage of human sequences, as only the mouse CDRs are retained while the rest of the constant and variable framework is human. In some cases, grafting of mouse hypervariable loops onto the human framework leads to decreased antigen

binding, in which case *in vitro* or *in silico* affinity maturation may be performed.[119,120] Fully human antibodies can be generated via mouse hybridoma by using transgenic animals that contain the human Ig genes in place of their mouse counterparts.[121] In addition, isolation of B cells from immunized or infected individuals, or those with cancer, allows for formation of human hybridoma cells from which human antibodies can be isolated.[122] While anti-drug antibody responses in patients correlate with the 'humanness' of the antibody sequence (mouse > chimeric > humanized), even fully human antibodies can be immunogenic depending on the paratope that is formed.[118,123]

Since hybridoma technology functions in the context of an intact immune system, only certain antigens can be effectively targeted using this method.[124] A primary consideration is that antigens must be sufficiently immunogenic to elicit a humoral response in the host animal; thus, antibodies targeting endogenous proteins may be difficult to generate *in vivo*. On the other hand, it is often not feasible or ethical to inject mice with pathogens or toxins that may prove fatal before the generation of a robust B cell response. In summary, while the hybridoma technique is still the standard for monoclonal production and allows for the formation of an immortalized antibody-producing cell line, its dependence on adaptive immunity necessitates antibody generation times of many months and limits the ability to control the precise epitope at which elicited antibodies will bind.

5.2 B cell receptor and immunoglobulin sequencing

Another strategy for identifying monoclonal antibodies of interest is to directly sequence all the V_H and V_L genes isolated from the B cells of immunized animals or humans. B cell receptor sequencing (BCR seq) uses next-generation sequencing to read the variable region sequences of up to several million Ig variants.[124] This method is useful not only for the antibody discovery, but also for detailed characterization of humoral immune responses.[125] While the peripheral blood compartment is the most accessible source of human B cells, the majority of B cells are found in extravascular milieu like the spleen, bone marrow, and lymph nodes, and can only be accessed in lab animals.[126] The localization and time since antigen exposure also affect the functional phenotype of B cells; for example, plasmablasts are abundant in blood one week after antigen exposure, while memory B cells appear one to two weeks later.[124] The B cell subset, in turn, affects properties such as mRNA levels and extent of affinity maturation. Thus, the choice to use mRNA or genomic DNA as the template for BCR seq depends whether information on mRNA expression or B cell clonality is desired.[126]

One drawback of performing BCR seq analysis in bulk is the impossibility of knowing which combinations of V_H and V_L genes came from each B cell. Several strategies have been utilized to deduce the correct pairing of HC and LC sequences, and thus to recreate antibodies with intact paratopes. Pairing sequences based on ranking of clonal frequency is one option that has proven successful.[125] This method is especially powerful following immunization, when Ag-specific B cells make up a significant portion of total B cell pool.[124,126] For antibodies that have undergone significant somatic hypermutation, pairing based on similar levels of mutation accumulation is another approach that has produced viable antibodies.[126]

The most reliable way to ensure physiological pairings of variable sequences, however, is to clone individual BCRs. Single B cells can be isolated by limiting dilution or flow cytometry, which has the added benefit of being able to sort different B cell subsets based on expression of surface markers.[127] Retention of pairing information can be achieved by fusion of V_H and V_L genes into a single amplicon, or by tagging each gene with a barcode unique to its B cell.[126] Selection of Ag-binding BCRs can be performed using Ag-coated beads, flow cytometry with fluorescent antigen, and microarray and microengraving techniques.[127] Alternatively, soluble antibodies secreted by B cells or expressed from cloned genes can be used for binding-based selection. While BCR seq in general is useful for discovery of high-affinity antibodies that have undergone *in vivo* selection, single B cell methods facilitate identification of native gene pairs at the cost of reduced throughput.

Only a fraction of peripheral B cells will go on to secrete soluble antibodies that are the basis of the humoral immune response. Therefore, a proteomics approach must be applied to discern which BCR sequences are found on circulating antibodies. Ig seq uses LC-MS/MS to determine the amino acid sequence of antigen-specific antibodies isolated from serum.[128] While BCR seq allows for description of the entire BCR repertoire, protein Ig seq enriches antibodies that are found to bind the antigen of interest. Together, BCR and Ig seq provide complementary methods for identification of variable regions and in-depth analysis of the adaptive response to antigens of interest.

5.3 Display

The essential aspects of *in vivo* immunological selection have been emulated *in vitro* to yield display technologies that allow for identification of functional antibody variable regions in the absence of an animal host.[119,125] Thus, antibodies to almost any antigen, even those that are toxic or weakly immunogenic, can be discovered in a fraction of the time. The first step for display technologies is to select a library of heavy and light chain genes that can be cloned into the system of interest. The library is either derived from immunized, infected, or otherwise immune-challenged individuals; or from universal donors that have not undergone specific immune activation. While the former libraries are more focused and potentially affinity-matured, naïve libraries have to potential to select antibodies against virtually any antigen. After cloning the library into the desired expression system, combinations of heavy and light chains, often in the scFv or Fab format, are displayed on the particle surface and selected by their ability to bind the antigen of interest. Non-binders are removed via wash steps to leave the best antibody candidates, which can be further refined through additional panning steps using the same or different binding conditions. Since the genes of interest are immediately available, the soluble protein can be expressed and used to quantify antibody-antigen affinity via methods such as ELISA. While hybridoma-derived antibodies still dominate the pool of therapeutics, six display-derived antibodies have been approved, and new *in vitro* methods for antibody selection continue to be discovered.[129]

The first display technology developed, and still the most widely used, uses bacteriophage for surface expression of antibody variable domains and selection of antigen binders.[130] Phage display, which uses viruses such as M13 phage, works by fusing the antibody scFv sequence with that of phage surface molecules like coat protein pIII.[131] The DNA sequence

within the plasmid codes for the corresponding surface protein, allowing for phenotypic selection and subsequent genotypic identification. Phage particles that bind antigen strongly are amplified via infection of bacterial hosts such as *E. coli*. This selective expansion of tightly binding phage particles allows for further rounds of panning or DNA sequencing of the associated scFvs. Several antibodies in clinical use were derived from phage display technology, with the first being adalimumab in 2002.[129] Mammalian-derived antibodies tend to have more favorable biophysical properties than phage-derived antibodies, which might be related to *in vivo* selection of antibodies with stable variable regions.[132]

In addition to phage display, various cell-based display platforms have also been developed. Bacterial, yeast, and mammalian display have all been used to select for antibodies with the desired antigen binding properties.[133–135] The main advantage of cell display is the ability to quickly isolate the cells expressing the most active antibody domains using fluorescence-activated cell sorting. Cells containing extracellular scFv or Fab are bound to fluorescently labeled antigen and then sorted by brightness. The level of binding can be normalized for antibody expression on the surface using additional fluorescence channels. Cell-based display methods tend to have smaller library sizes due to low efficiency of transformation. However, the shift from a prokaryotic to eukaryotic, and ultimately mammalian system, has the advantage that the antibodies selected will be expressed in cells with similar folding pathways and post-translational modifications.

Finally, ribosome and mRNA display are cell-free technologies that reduce the problem of antibody display to one of protein translation.[136] In ribosome display, mRNA sequences are *in vitro* translated, and the resulting polypeptide is left tethered to the ribosome due to lack of mRNA stop codon. After selection of antigen-binding proteins, the antibody-ribosome-mRNA complex is disassembled for sequencing of the selected mRNA. For mRNA display, puromycin serves as an adaptor molecule linking the translated polypeptide to its mRNA precursor. Since transformations into cells are not required in either case, these two methods have larger libraries and allow for more diversity than other display techniques. The presence of a single antigen binding domain per complex prevents the avidity effects that sometimes complicate cell-based display. In addition to using high fidelity PCR for sequencing of the mRNA candidates, PCR using an error-prone polymerase can also be used for affinity maturation via random mutagenesis if necessary.

6. Expression system

6.1 Mammalian expression

The vast majority of approved therapeutic antibodies are produced in mammalian cells due to their ability to express, fold, post-translationally modify, and secrete proteins in an analogous manner to endogenous human proteins, and thus to avoid unwanted immunogenicity.[137] Additionally, the ability of mammalian cells to properly glycosylate the conserved Asn-297 residue of IgG antibodies is vital for solubility, stability, and effector function, while glycan composition also impacts antibody pharmacokinetics.[138–141]

Human cell lines such as human embryonic kidney 293 (HEK293) and its derivatives are commonly used for batch expressions in the discovery phase due to the favorable

transfection efficiency of these cells.[142] Antibody production with HEK cells is facilitated by their growth in chemically defined, serum-free media in suspension.[137] A significant advantage of HEK cells, and other human cells like PER.C6 embryonic retinoblasts and HT-1080 fibrosarcoma cells, is their production of proteins with fully human glycosylation patterns. Because human cell lines and human patients are of the same species, there is a risk of pathogen contamination and transmission; however, this ability to propagate virus also makes HEK cells useful in vaccine development.[142] Although no therapeutic antibodies are currently expressed in human cells, other types of approved biologics have been produced in HEK293 and HT-1080 cells.[142]

Non-human mammalian cells exemplified by the Chinese hamster ovary (CHO) line are used to produce most antibody therapeutics due to thorough characterization, high protein yield, and ability to be stably transfected.[143] While these cells can produce proteins with complex N-glycans, glycosylation patterns are not identical to those of human cells. CHO cells do not express all the human glycosylating enzymes and have been shown to sialylate proteins to a greater extent than HEK cells.[142,144] Some murine cell lines, such as NS0 and Sp2/0, express an α-galactosylating enzyme not present in humans, which introduces a potentially immunogenic epitope on glycoproteins produced in these systems.[142] Nonetheless, plasma-, CHO-K1-, and HEK293-derived IgG have core-fucosylated structures containing similar levels of galactose and sialic acid. Cell engineering has created non-human cell lines with more human-like glycosylation patterns, as well as cell lines with targeted changes to antibody glycosylation and function.[142] For example, the Lec13 variant of CHO cells reduces synthesis of the fucosylation substrate to generate low-fucose antibodies with increased ADCC via enhanced FcγRIIIa binding.[145] Thus, human and non-human mammalians cells form antibodies with optimal folding and post-translational modifications, but genetic manipulation is relatively difficult and maintenance costly.

6.2 Other expression systems

Other cell-based expression systems can be used when cost and convenience are the primary considerations. While bacterial systems such as *Escherichia coli* may have difficulty producing complex full-length proteins, they have shown utility in generating antibody fragments such as Fab and Fv that do not require glycosylation to fulfill their intended functions.[137] In contrast to gram negative bacteria, which generally transport antibody products to the periplasm, gram positive bacteria have the advantage of secreting proteins directly into the media. Eukaryotic cells such as the yeast *Pichia Pastoris* and insect cells retain some of the advantages of prokaryotic systems while also being able to post-translationally modify expressed proteins.[137,143,146] Although glycosylation in these cells is not identical to that of human cells, cell engineering of glycosylating enzymes allows for production of antibodies with appropriate effector function.[137,143] Other genetic manipulation strategies include the co-expression of folding chaperones to facilitate assembly of complex antibody structures.[137] Besides cell-based expression, it is also possible to use prokaryotic and eukaryotic cell lysates in combination with template DNA and additional supplements to synthesize antibody fragments *in vitro*. These cell-free techniques eliminate the need for transformation of expression plasmids while enabling the facile incorporation of non-natural or isotopically labeled amino acids.[147]

For production of large batches of antibody-based proteins, whole-organism approaches have also been explored. antibody genes can be transformed into plants via the transfer DNA of *Agrobacterium tumefaciens*, which allows for purification of protein from tobacco leaves or rice seeds.[137,148] Alternatively, antibodies can be harvested from mouse or goat milk or chicken eggs using transgenic animals that have human Ig genes in place of the native Ig loci.[137] While these latter systems are expected to produce proteins with human-like glycosylation, plant systems have been engineered to prevent the attachment of potentially immunogenic sugars like xylose.[137] It seems unlikely that antibody expression in transgenic plants or animals will soon replace current mammalian cell production considering the time required to establish and maintain such protein-producing organisms.

7. Post-translational modifications

7.1 Glycosylation

Clearly, glycosylation of antibodies is a vital determinant of their biological and therapeutic activity. Although antibodies of various classes may have several N- and O-glycosylation sites, the best characterized glycans are those attached to Asn297 in the C_H2 region of IgG antibodies (Figure 3). These complex N-glycans contain a core heptasaccharide motif with four N-acetyl glucosamine (GlcNAc) residues and three mannose residues in a biantennary arrangement.[8,149] Fucose may be added to the protein-proximal GlcNAc residue, bisecting GlcNAc may be added to the central mannose residue, galactose may be added to each terminal mannose residue, and sialic acid (N-acetylneuraminic acid in mammals or N-glycolylneuraminic acid in some non-human mammals) may be additionally added to these galactose residues. All these possibilities for individual glycans, combined with the potential for differential glycosylation on each heavy chain, allow for significant heterogeneity in otherwise similar molecules of antibody. Studies from serum samples have demonstrated that >90% of endogenous IgG is core fucosylated, while only ~15% contains bisecting GlcNAc.[150] Glycoforms lacking galactose or containing a single galactose residue are more common than doubly galactosylated glycoforms.[151] Similarly, sialic acid is incorporated in <10% of structures, with disialylation even less common.[152] While hybrid and high-mannose type glycans are rare in endogenous IgG antibodies (2% and <0.1%, respectively), they are important to study in the context of therapeutics since non-human recombinant systems like NS0 and CHO cells can produce ~29% and ~3.5% high-mannose glycans.[142,153] It should also be noted that ~20% of IgG Fabs contain saccharides as a result of N-glycosylation motifs in the variable regions.[154] Due to increased accessibility for glycosylating enzymes, Fab glycans tend to have higher levels of bisecting GlcNAc, galactose, and sialic aid compared to their Fc counterparts.[8] Interestingly, Fab glycans are less likely to contain fucose, which may be explained by the inhibitory effect of bisection on core fucosylation.[150]

There is substantial evidence that glycosylation of endogenous IgG antibodies is tailored to dampen or potentiate the elicited immune response. On the one hand, the frequency of different glycoforms is altered as a function of physiologic states like age, pregnancy, and inflammatory status.[8,34,155] Low inflammation is associated with increases in both galactosylation and sialylation, with rheumatoid arthritis and lupus patients having higher

levels of agalactosylated antibodies.[155–157] On the other hand, glycan composition has direct effects on immune receptor binding and antibody function.[158] Addition of sialic acid to terminal galactose residues causes decreased binding to inflammatory FcγRs and increased binding to anti-inflammatory lectins like DC-SIGN.[34] Fucose is a well-established immunoregulator, with afucosylated IgG antibodies having more potent ADCC through tighter FcγRIIIa binding.[145] Likewise, the presence of bisecting GlcNAc inhibits core fucosylation, and therefore increases ADCC.[150,155] Complete lack of glycosylation all but eliminates binding to FcγRs and complement, and therefore ablates IgG effector functions.

Fc glycosylation affects the thermodynamic and serum stability of IgG antibodies.[141,159] Structurally, the effect of IgG deglycosylation is increased flexibility of the C_H2 domain, which causes tighter packing in crystal structures but a larger radius of gyration in solution.[160,161] Deglycosylation decreases the thermal stability of the C_H2 domain and may cause increased aggregation relative to normally glycosylated IgG.[139] While complete loss of glycans does not significantly alter FcRn binding or pharmacokinetics, the presence of terminal mannose or galactose causes shorter half-life via lectin-mediated clearance. Antibodies with terminal mannose glycans can be cleared through the mannose receptor on endothelial and immune cells, while those lacking terminal sialic acid can be eliminated by the hepatic asialoglycoprotein receptor.[141,159] Thus, complex glycan-bearing antibodies have longer half-lives than those containing hybrid or high-mannose glycans.[162] Similarly, IgG that has been degalactosylated, and therefore contains terminal GlcNAc, was shown to have extended pharmacokinetics compared to unmodified antibodies.[163] Increased sialylation has been associated with longer half-life of IgG antibodies as well as reduced clearance of Fc-receptor fusion proteins.[46,164] Given the important pharmacokinetic and pharmacodynamic effects of Fc glycosylation, an ongoing challenge is the glycan heterogeneity that occurs using current antibody expression systems.

While IgG heavy chains contain a single N-glycosylation site that has been extensively characterized, antibodies of other classes contain up to seven N-glycans per heavy chain. These glycans may be of the complex type, equivalent to the trimannosyl chitobiose structure found in IgG, or classified as oligomannose glycans. IgA1 contains two complex-type glycans, whereas IgA2 has two (IgA2m1) or three (IgA2m2) additional glycosylation sites.[84,165] IgD contains two complex and one oligomannose, IgE contains six complex and one oligomannose, and IgM contains three complex and two oligomannose sites.[91,165,166] Functionally, the oligomannose glycans (as well as GlcNAc-terminating complex glycans) may be involved in binding lectins such as mannose-binding lectin, which initiates the lectin complement pathway.[91,165] Furthermore, the oligomannose glycan proximal to the IgD inter-heavy chain disulfide bond was found to be essential for secretion of functional protein.[167]

Likewise, antibodies of the IgA1, IgD, and IgG3 subclasses have been found to contain O-glycosylation sites in the hinge region. While the number of O-linked glycosylation sites is suggested to be nine for IgA1, five for IgD, and up to three for IgG3, not all sites are fully occupied.[77,165,166,168] Antibody O-glycans are of the Core I type, containing the GalNAc-Gal disaccharide attached to serine or threonine hydroxyl groups.[91,165] Both the GalNAc and galactose residues may be sialylated. Functionally, these modifications have been

implicated in hinge conformation and resistance to proteolysis.[169,170] Abnormalities in O-glycosylation have been linked to IgA nephropathy, wherein agalactosylated IgA1 (with O-glycans terminating in GalNAc or sialic acid) acts as an autoantigen and results in formation of IgG-IgA immune complexes.[165] Given the substantial effect of antibody glycosylation on function and stability, numerous glycoengineering strategies have been developed to create next generation antibody therapeutics with more controlled glycan composition. One of the most explored methods to increase IgG effector potency is to generate low fucose glycoforms that bind more tightly to FcγRIIIa. Common strategies to modify glycosylation in cell culture include the addition of glycan precursors to increase saccharide incorporation or glycosyltransferase inhibitors to decrease incorporation.[149,159] Additionally, the genes for glycosylating enzymes within host cells may be knocked out to reduce fucosylation and non-human glycosylation, or knocked in to provide a more human-like glycosylation profile. Clinical trials such as one with the FDA-approved obinutuzumab have demonstrated that low fucose glycovariants from engineered cell lines may lower the risk of disease progression, but increase adverse events, compared to non-glycoengineered antibodies.[171] Completely aglycosylated antibodies have been extensively studied in contexts where strong immune recruitment is not necessary, since they can be expressed in prokaryotic hosts and lack the heterogeneity of glycosylated antibodies.[172] Protein engineering strategies have identified Fc mutations that restore FcγR-mediated function to aglycosylated antibodies, thus paving the way for antibody therapeutics that are both homogeneous and immune competent.

7.2 Amino acid modifications

Chemical alteration of amino acids is common both endogenously and in recombinant antibody preparations (Figure 3).[173,174] Some types of modifications add or remove charges, which can interfere with antigen or receptor binding depending on the site of the altered residue.[159,175,176] Formation of pyroglutamate from N-terminal glutamine or glutamate removes the N-terminal positive charge, and also the negative charge of glutamate. Likewise, C-terminal amidation replaces a negatively charged carboxylate with an uncharged primary amide. Deamidation of interior asparagine or glutamine residues to aspartate or glutamate leads to introduction of negative charge, while isomerization of aspartate to isoaspartate can change protein conformation through alteration of the polypeptide backbone. Glycation of lysine residues removes a positive charge while introducing new functionality.

Notably for IgG antibodies, whose heavy chains terminate in a lysine residue, cleavage of this C-terminal lysine by carboxypeptidase B has important functional consequences.[177] While most endogenous antibodies lack the terminal lysine and are thus better able to hexamerize for complement fixation, recombinant antibodies can have higher levels of uncleaved heavy chain. Once recombinant antibodies are localized to the serum, remaining lysine residues are efficiently cleaved with a half-life of approximately one hour.[178] However, it may be important to characterize C-terminal composition of purified antibodies being compared in CDC assays, as those antibodies with two C-terminal lysines elicit CDC with significantly weaker potency compared to unfractionated antibodies from hybridoma cells.[177]

Oxidation of exposed amino acid side chains, especially those of methionine, tryptophan, and histidine, is commonly observed in antibodies and other proteins, and has potential impacts on protein partner binding.[175,176] Methionine residues 252 and 428 located at the $C_\gamma2$-$C_\gamma3$ interface are particularly prone to oxidation, which can lead to weaker FcRn binding and in extreme cases, reduced half-life.[179,180] Oxidation of tryptophan residues within the hydrophobic antigen binding pocket can lead to significant decreases in antigen affinity.[181] Thus, it is important to optimize formulation conditions, or even replace liable residues, to reduce these sources of heterogeneity.

Conversely, reduction of cystines can also introduce unwanted complexity into antibody preparations.[175] In the native structure of an antibody, all framework cysteine residues are paired into either intra- or inter-chain disulfide bonds that serve to stabilize antibody tertiary and quaternary structure. The presence of free thiols represents a deviation from the proper antibody structure, and has been associated with decreased thermal stability and the ability to form covalent antibody aggregates.[182,183] Thus, it is common to ensure integrity of recombinant antibodies by incorporating free sulfhydryl quantification into quality control assessments. Additional heterogeneity associated with cysteine residues includes formation of trisulfides, thioethers, and racemized D-cysteine, although impacts of these modifications on function are less apparent.[175]

8. Fragmentation

8.1 Antigen-binding fragments

While whole antibody frameworks such as IgG are well-established as therapeutics, smaller frameworks of individual antibody domains may confer distinct biological advantages. The major difference between full antibodies and antibody fragments is molecular size, with the 150-kDa IgG being reduced to 50 kDa in the case of Fabs, and even smaller sizes for other antibody fragments (Figure 4). One favorable result of small size is increased rate of diffusion, allowing for more efficient penetration into tissues and tumors that are being targeted.[184] Bypassing of the lymphatic system can increase absorption and bioavailability of fragmented frameworks when administered extravascularly.[185] Furthermore, small, non-glycosylated antibody fragments can be expressed efficiently in prokaryotic cells, reducing the time and cost of protein production. On the other hand, proteins <60 kDa are preferentially cleared via renal filtration, meaning 50-kDa Fabs and especially smaller antibody fragments are eliminated more quickly than 150-kDa IgG antibodies.[184,186] Valency of binding is another consideration, since bivalent antibodies have tighter avidity for antigen compared to monovalent antibody fragments.

Absence of Fc-mediated function is another major difference between full-length antibodies and antibody fragments. Since the Fc domain is required for FcRn binding and endosomal recycling, antibody fragments lacking this domain are quickly cleared by lysosomal degradation in addition to renal filtration.[186] Thus, higher or more frequent dosing is usually required for small antibody-based therapeutics. Lack of the Fc domain also eliminates FcR- and complement-mediated effector functions. This loss of function may be advantageous for therapeutic mechanisms that require antigen binding without immune activation, but detrimental for indications like oncology, where ADCC, ADCP, and CDC are important

tumor-killing functions. Finally, lack of an Fc domain may eliminate binding to protein A- and G-based resins, necessitating the use of alternative purification strategies based on, for example, protein L. It should be noted that the fast clearance of antibody fragments, combined with their lack of FcR binding, makes them well-suited to diagnostic applications.[184]

In the laboratory, various Fab-containing fragments can be generated via enzymatic proteolysis. Traditionally, papain has been used to cleave above the hinge disulfide bonds to yield two monovalent Fabs and one Fc per molecule of IgG.[187] Pepsin cleaves below the hinge disulfides, producing one bivalent $F(ab')^2$ per IgG that can be further split into two F(ab') molecules in the presence of reducing agent. The apostrophe in these names denotes the presence of hinge/Fc sequences including oxidizable cysteines. Pepsin tends to cleave multiple sites in the Fc, preventing the purification of functional Fc using this enzyme. Following IgG cleavage, protein A or other affinity techniques can be used to purify the species of interest. Besides papain and pepsin, other enzymes like IdeS are used for site-specific IgG proteolysis.[188] While these enzymatic methods are useful for preparing small samples of Fab, recombinant expression of antibody fragments from custom genes may be more feasible for production of large, homogeneous preparations.

The Fab framework has the longest clinical history of all antibody fragments, with four molecules achieving approved status.[189] While ranibizumab and certolizumab pegol are expressed in *E. coli* as Fab or Fab' fragments, respectively, abciximab is produced as a full-length IgG in mammalian cells before papain digestion to Fab. Generally, Fabs have been successfully employed to treat acute indications where fast clearance is not a major concern. However, certolizumab pegol is used to treat chronic inflammatory conditions like rheumatoid arthritis and Crohn's disease. For this reason, the free cysteine near the heavy chain C-terminus is used for site-specific conjugation to ~40-kDa polyethylene glycol (PEG), which increases its serum half-life.[190] Thus, Fab fragments are attractive options when the Fc region is unnecessary or detrimental, and modifications such as PEGylation or albumin-binding functionality can be used to mitigate drawbacks like fast clearance.

8.2 Single-chain variable fragments

The next most explored types of antibody fragment are the variable fragment (Fv), disulfide-stabilized variable fragment (dsFv), and most commonly, single-chain variable fragment (scFv).[191] These frameworks are composed of the V_H and V_L domains, which for the dsFv are stabilized by an engineered interchain disulfide bond, and for the scFv are covalently linked with a hydrophilic 10–25 amino acid linker. At roughly 28 kDa, the scFv is the smallest antibody-based protein that retains the native variable regions of a human antibody. Similar to Fabs, scFvs have the potential for low-cost prokaryotic expression and increased tissue penetration, at the cost of fast clearance and lack of effector function.

The modular nature of the scFv facilitates its multimerization into homo-oligomers with increased antigen-binding valency or hetero-oligomers with multiple functionalities.[191] For example, use of progressively shorter linkers allows for formation of scFv dimer, trimer, and tetramer (diabody, triabody, tetrabody) since the V_H and V_L domains cannot properly pair when connected by a short peptide. It is possible to create bispecific scFvs by incorporating

the V_H and V_L sequences for two separate antibodies into a single 55-kDa polypeptide. This and other bsAb platforms are discussed in the section on multispecificity.

8.3 Single domain antibodies

Even smaller than scFvs are single domain antibodies (sdAbs), which are 15-kDa V_L, V_H, or V_{HH} domains.[192] The most popular of these are V_{HH} sdAbs, or nanobodies, which are derived from heavy chain antibodies that are produced by camelids. These 80-kDa heavy chain antibodies (so named because they lack light chains) contain a single antigen-binding domain, V_{HH}, directly N-terminal of the hinge. Since the V_{HH} domain is not complexed with other antibody domains, its surface tends to be much more hydrophilic than that of V_H and V_L domains, which are hydrophobic at their pairing interface. Therefore, camelid-derived V_{HH} nanobodies generally have favorable biophysical characteristics like high solubility and low aggregation compared to human sdAbs.[193] In addition, the V_{HH} CDR3 is often longer than the V_H CDR3, potentially allowing it to form more favorable contacts with its binding epitope.[194] Besides camelids, cartilaginous fishes produce a distinct type of heavy chain antibody containing a single variable region, V_{NAR}, which could also serve as a therapeutic sdAb.[195]

Like scFvs, sdAbs are amenable to tandem multimerization. Fusion of the same nanobody allows for increased valency and decreased antigen dissociation rate, while fusion to distinct nanobodies allows for bispecific mechanisms to be explored. A common strategy is to pair antigen-binding and albumin-binding specificities into the same molecule, allowing it to fulfill its intended function while circulating longer in serum.[192] Additional engineering efforts have focused on reducing the immunogenicity of V_{HH} domains, which may not be crucial given their sequence similarity to human V_H3 domains and their size comparability to the non-human domains in full-length chimeric antibodies.[192] While humanization of V_{HH} molecules may decrease their antigenicity, it can also confer unfavorable V_H properties like low solubility. Thus, the complementary strengths of human and non-human sdAbs should be considered when designing therapeutics with these frameworks.

8.4 Crystallizable fragments

While most therapeutic antibody fragments retain Ag-binding domains, the free Fc domain can also be used to antagonize FcRs. This format is ideal when FcRs should be occupied without co-localizing a specific Ag. As one example, the IgG1 Fc has been engineered to bind with high affinity and less pH dependence to FcRn.[196] Upon administration, the IgG mutant binds tightly to endosomal FcRn, preventing FcRn-mediated salvage of endogenous IgG antibodies and accelerating their degradation. The utility of these antibody-degrading Fc molecules (Abdegs) has been explored for treatment of autoimmune diseases mediated by pathogenic IgG.[197] Since the Fab domains are unnecessary for the Abdeg mechanism, the Fc domain alone can be used therapeutically. A clinical example is ARGX-113, which has shown both depletion of endogenous IgG and efficacy treating myasthenia gravis.[197]

9. Multimerization

9.1 Antibody multimerization

Intuitively, antibody oligomers can have enhanced binding to antigen and FcRs, largely through multivalency and a decreased dissociation rate. The affinity of naturally low-abundant recombinant IgG1 dimers for low-affinity FcγRs is hundreds of times higher than that of the corresponding monomers.[198] Similarly, aggregates of thermally stressed IgG1 have enhanced affinity for FcRn, while immune complexed and hexamerized IgG1 have enhanced FcRn-mediated transepithelial transport.[199,200] For CD40 antibodies, agonism is generally dependent on FcγRIIb-mediated crosslinking. However, covalent multimers of mouse IgG2a were shown to activate CD40 in a FcγR-independent manner, allowing for increased survival in a mouse lymphoma model.[201] Thus, multimerized antibody frameworks (Figure 4) can provide enhanced FcR- and Ag-mediated functions, but may introduce heterogeneity or potential for immunogenicity depending on the oligomerization strategy.

IgG hexamerization, which occurs on the surface of Ag-coated cells, can be augmented for improvement of complement-mediated effector function.[27] By mutating residues at the IgG complexation interface, hexamerization on the cell surface has been increased, allowing for improved C1q recruitment and CDC.[202] The requirement of antigen-expressing cells for enhancement of effector function distinguishes this approach from other Fc engineering strategies, which tend to increase immune activation independent of target binding. Attachment of the IgM tailpiece to the IgG C-terminus is another hexamerization approach that allows for enhanced binding to Fc-binding proteins.[200,203] Depending on structure and context, these multimerizing antibodies may be useful for both potentiating and inhibiting the immune functions of complement and FcRs.

9.2 Domain multimerization

Duplication of Ag-binding domains is possible through tandem fusion of gene sequences, and may be used to maximize potency. As discussed previously, multimerization of scFvs and nanobodies allows for higher-avidity antigen binding, and introduces the opportunity for bispecific and multispecific mechanisms to be explored.[191,192] These Ag-binding domains may be used alone or as Fc fusions to improve pharmacokinetics and impart effector function.

The Fc domain can be analogously duplicated to generate antibody variants that are multivalent for both Ag and FcRs. Several studies have demonstrated the utility of IgG1 molecules that contain one or two extra Fc domains tandemly linked to the IgG C-terminus.[204–207] Whether the additional Fc domains are linked by the IgG2 hinge or a flexible linker, they elicit significantly stronger ADCC and ADCP than the wild-type IgG. Intriguingly, the Fc domains can also be of distinct classes. For example, fusion of the IgA2 Fc to the C-terminus of a normal IgG1 antibody led to enhanced ADCC through FcαRI binding.[208] Alternatively, Fc multimers that lack Ag binding display marked FcR antagonism, which could be useful for treatment of autoimmunity.[209] A potential drawback of these frameworks is faster clearance due to differences in FcRn binding avidity.[207] Regardless, Fc duplication

is a creative engineering strategy for generating antibodies with stronger effector functions. In contrast to the standard approach of introducing framework mutations that alter FcR binding, this strategy uses the avidity effect to enhance binding. Use of the proper linker may allow for potent IgG derivatives that retain high stability and low immunogenicity through conservation of native antibody sequences.

10. Conjugation

10.1 Payload

Antibodies can be conjugated to cytotoxic drugs to create ADCs or fused to other proteins to add novel functionality (Figure 4). A primary consideration for the design of ADCs is the type of cytotoxic agent that will be conjugated to the tumor-targeting antibody. Due to the limited number of antibody-targeting receptors on the cell surface, and the limited capacity of drugs per antibody, the delivery of traditional chemotherapeutic agents is often not sufficient to eliminate malignant cells.[61] Instead, ADCs have made use of increasingly potent drugs that are cytotoxic in the picomolar range, and therefore not therapeutically feasible without conjugation to antibodies to increase their specificity.

While potent antitumor activity is essential for ADC payloads, other factors should also be considered. The physicochemical properties of the drug are important, as excessively hydrophobic agents can lead to low aqueous solubility, increased ADC aggregation, immunogenicity, or accelerated clearance.[61,210] For example, the hydrophobicity of some drugs limits the number of molecules that can be attached to the antibody before aggregation occurs. The warhead should also be amenable to linker attachment, and retain activity and stability after conjugation to the antibody. Finally, ideal conjugated drugs should not be substrates for efflux transporters like multidrug resistance protein 1 (MDR1), as this would increase their systemic toxicity and introduce the opportunity for resistance mechanisms.

Several classes of warheads have been conjugated to approved ADCs, and many more are conjugated to ADCs that have been in clinical trials. Auristatins and maytansinoids, potent tubulin inhibitors, are derivatives of compounds produced by *Dolabella auricularia* sea slugs and *Maytenus ovatus* plants, respectively.[61] Monomethyl auristatins E and F (MMAE and MMAF), are two examples of auristatins that were selected for favorable potency and stability. MMAE is the cytotoxic component of the approved ADC brentuximab vedotin. Examples of stable and soluble maytansinoids include DM1 and DM4, with DM1 being the warhead for trastuzumab emtansine. The calicheamicins are a third class of payload found on approved ADCs, derived from a compound produced by the bacterium *Micromonospora echinospora*. Both gemtuzumab ozogamicin and inotuzumab ozogamicin utilize the DNA-cleaving N-acetyl-γ-calicheamicin to kill malignant cells. Notably, the distinct cytotoxic mechanism makes calicheamicins efficient at lysing all cells, in contrast to auristatins and maytansinoids, which preferentially target rapidly dividing cells.[211] While several other classes of cytotoxic compounds have been conjugated to antibodies, they generally induce cell death via DNA, RNA, or tubulin interference and so far have not been used in approved ADCs.[61]

Antibody-radionuclide conjugates (ARCs) can be used diagnostically, but also therapeutically due to the damaging effects of radiation on DNA, membranes, and mitochondria.[212] Attachment of radionuclides to antibodies is commonly achieved through antibody conjugation to metal chelators such as diethylenetriaminepentaacetic acid (DTPA) or 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), or through iodination of tyrosine residues in the case of iodine isotopes.[213] Selection of the radionuclide is critical for delivery of optimal radiation energy at an appropriate pathlength. Most commonly used are β emitters, such as ^{90}Y and ^{131}I, which exert long-range (1–10 mm) effects but have low linear energy transfer.[213] Examples include the approved ^{90}Y-ibritumomab (ibritumomab tiuxetan) and the approved but withdrawn ^{131}I-tositumomab. Although both of these ARCs target CD20 for treatment of non-Hodgkin's lymphoma, it is thought that β emitters may be suitable for elimination of high-volume solid tumors, due to the large crossfire effect that damages cells distant to the radionuclide.[212,213] Conversely, α emitters (e.g. ^{211}At, ^{213}Bi) and Auger emitters (e.g. ^{111}In, ^{125}I), which have a higher linear energy transfer but a lower range, may be more apt for treatment of small tumors or metastasized clusters of cells. For all types of emitters, another important consideration is the physical half-life of the radionuclide, which should ideally be similar to the biological half-life of the ARC.[214] An ongoing challenge for ARCs is the cost and expertise required for administration, which often involves complex dose calculations and the need for radioprotection and waste management.[213,214] Indeed, the declining sales and resulting withdrawal of ^{131}I-tositumomab was partly attributed to the complexity of administering the drug.[210] Overall, radionuclides serve as potent warheads when conjugated to antibodies and provide beneficial crossfire effects for targeting heterogeneous tumors, but a deeper understanding of the underlying radiobiology would help clarify dose requirements and radio-resistance mechanisms.

In addition to ADCs and ARCs, other types of antibody conjugates have been explored. Moxetumomab pasudotox, which was recently approved for treatment of hairy cell leukemia, is composed of an anti-CD22 dsFv linked to a truncated form of *Pseudomonas* endotoxin A.[215] Thus, protein toxins are viable alternatives to small molecule drugs and natural products, with sufficient activity at the low concentrations achieved by antibody conjugates. A concern for use of exogenous toxins is the possibility of immunogenicity and increased clearance, which is more problematic for larger toxins containing more potential epitopes. Antibodies can also be conjugated to more specific cytotoxic agents, like antibacterials, for treatment of intracellular infections.[216] In each of these cases, the naked antibody, free conjugate, and intact ADC should be regarded as three fundamentally distinct species with their own distribution, metabolism, and toxicity profiles. A common goal of ADCs is to capitalize on the favorable properties of the conjugated species while minimizing systemic toxicity that occurs upon release of free payload.

10.2 Conjugation strategy

Early ADCs used the intrinsic reactivity of native amino acids to couple drugs to antibodies. The primary amine of lysine sidechains readily reacts with activated esters on linker moieties, allowing for lysine-specific conjugation. Unfortunately, IgG molecules contain approximately 80 lysines which are largely surface exposed. This excess of reactive sites leads to significant heterogeneity both in drug-antibody ratio (DAR) and the site of

conjugation, with possibility for reduced Ag binding or serum half-life.[217] Most approved ADCs (gemtuzumab ozogamicin, trastuzumab emtansine, and inotuzumab ozogamicin) have used this lysine conjugation strategy, which can result in some antibodies being left unconjugated while others are loaded with a DAR of up to 8.[61] Another straightforward conjugation strategy, employed by brentuximab vedotin, is reactivity of reduced interchain cysteines with the linker maleimide group. Although there are only 8 interchain cysteines per IgG, there is still significant heterogeneity associated with this method.[217] The loss of stabilizing interchain disulfide bonds can be rectified by using re-bridging groups that covalently link cysteines while also adding a handle for conjugation.[61,218] Overall, conjugation via native amino acids has produced several efficacious ADCs, but also left room for improvement of product homogeneity.

Molecular biology paved the way for site-specific ADCs via conjugation to mutated amino acids. At the forefront are THIOMABs, which contain engineered cysteine residues at ideal locations for drug attachment.[219] Since the introduced cysteines are unpaired, they react with Michael acceptors more readily than disulfide-bonded cysteine residues. Advantages of THIOMABs, and other engineered cysteine technologies, include homogeneity of structure and DAR, increased therapeutic index, and improved pharmacokinetics.[219] The site of the engineered cysteine has a large impact on the properties of the ADC. Introduced cysteines should not be too solvent accessible, for fear of thiol exchange with endogenous proteins; and they can be positioned at patches containing positive charge to improve linkage stability via succinimide hydrolysis.[220] In addition to engineered cysteines, incorporation of peptide tags into the antibody sequence has allowed for enzymatic conversion to site-specific conjugation handles.[61,217] For example, presence of the CXPXR amino acid motif allows for introduction of an electrophilic formylglycine residue via formylglycine-generating enzyme, while the LLQGA motif allows for direct coupling of an amine-containing substrate to the internal glutamine residue via transglutaminase.

Incorporation of non-canonical or unnatural amino acids outside the standard 20 is another approach for generating site-specific ADCs.[221] Since selenocysteine has a lower pKa than cysteine and lysine, it is more reactive than other nucleophilic residues at low pH. This amino acid can be introduced at the antibody C-terminus using the UGA codon paired with the selenocysteine insertion sequence.[222] Unnatural amino acids like para-acetylphenylalanine and para-azidomethylphenylalanine can likewise be introduced using the amber codon (UAG) along with the proper charged tRNA.[223,224] These amino acids integrate orthogonal ketone and azide groups, respectively, into the antibody for conjugation to alkoxyamines and dibenzocyclooctynes. While use of unnatural residues requires a substantial initial investment and may affect protein yields, it ultimately allows for stable and well-defined conjugation.

Glycans attached to IgG Asn297 represent an additional target for site-specific conjugation.[61,217] Metabolic engineering with media supplementation has allowed for incorporation of 6-thiofucose in place of the normal fucose residue.[225] As with THIOMABs, the unpaired thiol group of 6-thiofucose is more prone to reduction and maleimide conjugation. Similarly, post-expression glycan engineering allows for introduction of galactose and sialic acid residues that contain azide or ketone functionality.[61,226] This is achieved using glycosylating

enzymes that naturally have, or are mutated to have, expanded substrate specificity. Finally, chemical oxidation of vicinal diols within glycan residues such as sialic acid introduces electrophilic aldehyde groups.[61,217] While periodate oxidation is a relatively straightforward method to incorporate a chemical handle for conjugation, it can cause off-target oxidation of prone methionine residues.[227]

Conjugation strategy directly determines the possible range of DAR values, as well as the chemical properties of the antibody-linker bond. While conjugation to native lysines or cysteines leads to heterogeneity in DAR and potential for excessive drug loading, introduction of engineered cysteines or unnatural amino acids allows for well-defined DAR values. Generally one or two sites are mutated (on each HC or LC), allowing for DAR values of 2 or 4.[61] This reduction in maximum DAR has benefits such as reduced aggregation propensity and improved pharmacokinetics.[217] On the other hand, high DAR values have been linked to increased clearance, decreased exposure, and increased volume of distribution due to toxic accumulation in the liver.[228] Meanwhile, the conjugation chemistry also impacts ADC stability, with labile linkages allowing for drug loss and resulting off-target toxicity. Thiol-maleimide coupling is often used to load native and engineered cysteines due to its speed and selectivity; however, the resulting thioether is susceptible to thiol exchange with endogenous thiols.[217] By adding groups with positive charge adjacent to the maleimide, succinimide hydrolysis is favored for prevention of retro-Michael reactions.[229] Oxime, and to a greater extent hydrazone, linkages undergo acid-catalyzed hydrolysis, so the possible instability of these groups should also be considered.[217] As the number of site-specific conjugation strategies continues to increase, it will be interesting to see which sites and functionalities produce the safest and most efficacious ADCs.

10.3 Linker

The first step in linker design is choosing two functionalities that will allow for covalent bond formation between the antibody on one end and the payload on the other. Thus, the chemistry of the linker must be compatible with whichever conjugation site and drug are selected.

An important property of these two reactive ends is the extent to which their conjugated products are cleavable by lysosomal proteases, acidic pH, and reducing conditions. Ideally, both antibody-linker-payload linkages would be completely stable in the blood, and only efficiently cleaved after delivery to the target cell. One strategy to release the warhead within the cell is to use dipeptide linkers such as valine-citrulline, which are C-terminally cleaved by cathepsin B in the lysosome.[230,231] Brentuximab vedotin is an ADC in clinical use with such a linker. A second strategy is to incorporate pH-sensitive linkages that allow for drug release specifically in the lysosome; for example, the hydrazone group which was mentioned previously for its acid lability.[230] Although ADCs like gemtuzumab ozogamicin and inotuzumab ozogamicin have shown efficacy using such a linker, it is now appreciated that hydrazone groups may release the payload prematurely to increase off-target toxicity.[61,230] Thirdly, disulfide linkers are cleaved much more efficiently in the reducing environment within cells, and therefore also allow for targeted drug release. Sterically masking the disulfide bond may help to decrease reduction by low concentrations of reductant in the

serum.[232] In contrast to cleavable linkers, linkers containing thioethers and other non-cleavable groups may be used to decrease the chances of unintended systemic drug exposure.[230] For trastuzumab emtansine, drug release from the antibody does not occur until the antibody has been proteolytically degraded. Because the metabolic product contains the payload linked to an antibody-derived amino acid, it is vital that the payload retain activity even with the added bulk and charge. In one comparison of linker types, it was found that ADCs containing a non-cleavable linker had similar potency, but a higher therapeutic window, than the corresponding ADCs containing a protease-cleavable linker.[233]

In addition to chemical reactivity, the physicochemical properties of the linker as a whole should also be considered. Since ADCs contain more hydrophobic surface area than naked antibodies, they are generally more prone to aggregation.[230] Additionally, hydrophobic drugs are more likely to be substrates for the transporter MDR1.[61] Therefore, polar linkers that contain solubilizing groups like sulfonate and PEG not only reduce ADC aggregation propensity, but also potentiate elimination of MDR1-expressing cells (assuming the polar functionality is retained in the final warhead).[234,235] As a result, properties like potency, pharmacokinetics, and therapeutic index can all be improved by using charged or polar linkers.[236] Presence of charge in the final drug moiety also largely determines its ability to elicit the bystander effect, as only uncharged molecules efficiently cross lipid membranes to reach neighboring cells. Whereas S-methyl metabolites originating from disulfide linkers are uncharged and therefore enable bystander killing, amino acid-conjugated payloads with non-cleavable linkers are charged and less likely to elicit these effects.[237,238] It should be noted that toxicity to neighboring cells can be favorable (in the case of targeting tumors with heterogenous antigen expression) or unfavorable (when the primary casualties are healthy cells).

10.4 Fusion proteins

Fusion proteins are another class of antibody conjugate that combine the favorable features of antibodies with therapeutic properties of other biomolecules. Molecular biology approaches can be used to genetically fuse an antibody or antibody domain to the molecule of interest, allowing for recombinant expression of multifunctional proteins.

Some fusion proteins utilize the Ag-binding specificity of antibodies for localization into targeted environments. For example, antibody-cytokine conjugates have been used to increase tumor accumulation of pro-inflammatory interleukins (e.g. IL-2, IL-12) for activation of cytotoxic T cells and NK cells.[239] For these immunomodulatory agents, the affinity and valency of the cytokine and antibody components should be optimized to maximize tumor localization and minimize off-target cytokine effects. Similarly, antibody-directed enzyme prodrug therapy (ADEPT) uses antibody specificity to deliver enzymes to the site of malignancy, where they can activate low-toxicity prodrugs into the active cytotoxic agent.[240] Although immunogenicity is a concern, engineering of non-human enzymes, or use of human enzymes not present in blood (e.g. β-glucuronidase), can be used to mitigate this risk factor. A widely applicable strategy for half-life extension of biologics is incorporation of an albumin-binding domain. Nanobodies targeting albumin (AlbudAbs)

take advantage of FcRn-mediated endosomal recycling to prolong the pharmacokinetics of other proteins while only adding a small ~12-kDa domain.[241]

Likewise, the Fc domain of an antibody can be conjugated to other proteins to impart physical and biological properties. Biomolecules like receptors, cytokines, enzymes, and peptides can be genetically fused to antibody Fc domains, and several such molecules are approved for clinical use or are in clinical development for indications like cancer, autoimmunity, and blood disorders.[242] An important advantage of IgG Fc fusion is the ability to bind FcRn and increase half-life, though Fc-fusion proteins generally have weaker FcRn binding and shorter half-lives (~2 weeks vs. ~3 weeks) than IgG molecules.[242,243] Depending on the groups attached, Fc-fusion may retain FcγR-mediated effector functions but generally lose the ability to fix complement.[243,244] Other potential benefits include increased stability and solubility, as well as facile purification using protein A- and G-based resins.[242] From a design standpoint, it is possible to fuse proteins to either end of the Fc. In practice, it is more common to attach functionality to the N-terminus of the Fc to more closely mimic the native IgG structure. Since the Fc domain is a dimer of heavy chains, the folded product will be a dimer of the fusion partner as well. While domain dimerization may be advantageous, in some cases it is preferable to generate molecules that are monomeric for the non-antibody component. For example, co-transfection of the protein-Fc fusion with a standard Fc domain allows for a mixture of products from which the desired species (monomeric protein, dimeric Fc) can be purified.[245] Such monomeric Fc-fusions may have improved pharmacokinetics, but presumably a lower yield. Regardless of which design strategy is used, the Fc-fusion format has proven useful for instilling diverse biotherapeutics with beneficial properties of the antibody framework.

10.5 Conjugation to hydrophilic polymers

As mentioned previously, certolizumab pegol is a Fab' fragment that is site-specifically PEGylated to partially compensate for lack of FcRn-mediated recycling.[246] In fact, addition of PEG and other hydrophilic groups is a general strategy to increase solubility and hydrodynamic radius of therapeutic proteins. The increase in apparent size prevents renal filtration of small proteins that would otherwise be quickly eliminated.[247] Conjugation can be achieved using the same amino acid-targeting chemistries discussed for ADCs, where modification of site-specific C-terminal Fab' thiols may be preferential to non-specific amine conjugation that can impede Ag binding. Favorable effects of PEG include increased stability, bioavailability, and half-life; and decreased immunogenicity and proteolysis.[246,247] On the other hand, anti-PEG antibodies have been detected in patients before and after administration of PEGylated proteins and may lead to faster clearance of PEGylated proteins.[248] Several groups besides PEG, including sugars like polysialic acid and dextran, can be chemically conjugated to antibody fragments to similarly alter their biophysical characteristics.[247] Furthermore, proteins can be recombinantly modified to include hydrophilic peptide repeats or glycosylation motifs. These strategies reinforce the notion that covalent conjugation of antibodies to drugs, polymers, and other proteins allows the properties of antibodies to be tailored to their specific therapeutic purpose.

11. Multispecificity

11.1 Asymmetric IgG-like frameworks

As mentioned in the section on antigen selection, some mechanisms of action require multiple functionalities to be combined into one molecule. With deeper understanding of genetic and protein engineering, it has become increasingly practical to design bispecific and multispecific antibodies with a diverse array of architectures (Figure 5).[249,250] While it is outside the scope of this review to describe all the multispecific technologies that have been developed, it is worthwhile to summarize the main strategies used and to highlight demonstrative examples of their implementation. The first family of frameworks uses the whole IgG structure to generate bivalent, bsAbs that retain Fc-mediated properties like pharmacokinetic stability and immune activity.

Classically, IgG bsAbs could be generated by fusing two hybridomas into a quadroma that expresses two distinct Ig HCs (HC_A, HC_B) and two distinct LCs (LC_A-LC_B) targeting antigens A and B.[251] Similarly, expression hosts can be transfected with DNA sequences corresponding to the four polypeptide chains of interest. When the chain pairing possibilities are considered, it is easy to understand why these methods are inefficient at producing bsAb with the desired [LC_A-HC_A]-[HC_B-LC_B] composition. When left to chance, the HCs may homodimerize (HC_A-HC_A or HC_B-HC_B) instead of heterodimerizing (HC_A-HC_B), and each HC may associate with the wrong LC (e.g., HC_A-LC_B instead of HC_A-LC_A). These opportunities for chain mispairing have been termed the HC problem and LC problem, respectively. Assuming equal efficiency of transfection/expression of each chain and unbiased chain pairing, only 1/8 of the tetrameric protein product will have the desired composition. While it is possible to purify the bsAb fraction from the mixture, the low yield and additional steps are significant drawbacks. Use of antibody chains from different species (e.g. mouse and rat) simplifies the matter somewhat by minimizing the LC problem and allowing for pH step elution of the three possible HC dimers.[252] An example of such a Triomab is catumaxomab (α-CD3 x α-EPCAM), which was the first bsAb approved for clinical use in 2009 but later withdrawn for commercial reasons.[39]

To facilitate proper chain assembly without resorting to non-human frameworks, genetic engineering strategies have been employed with much success. The HC problem can be remedied by engineering a preference for HC heterodimerization over homodimerization, increasing yield of bsAb from 1/8 to 1/4 of tetrameric products in the absence of additional HC-LC pairing strategies. A pioneering technology in this field was the “Knobs-into-Holes” set of mutations that creates a “knob” in the C_H3 domain of one HC (T366Y) that fits into a “hole” of the other HC (Y407T).[253] Subsequent generations of Knobs-into-Holes technology incorporated additional mutations at the C_H3 interface, including introduction of an engineered disulfide bond to stabilize the HC heterodimer.[254,255] In addition to steric complementarity, electrostatic steering is another way to create a preference for the HC heterodimer. By making one C_H3 domain more negatively charged (K392D/K409D) and the other more positively charged (E356K/D399K), the HCs of the same types are repelled while opposite HCs attract.[256] Charge-based HC heterodimerization methods, combined with a common LC, were used to create the recently approved bsAb emicizumab (α-factor

IXa x α-factor X).[257] A third strategy is to use IgA/IgG chimeric strand-exchange engineered domain (SEED) C_H3 domains where the heterodimer contains more class-matched area at the C_H3 interface.[258] An advantage of each of these methods is the ability to drive heterodimerization in cells that would otherwise assemble HCs randomly.

Combination of HC-HC pairing and HC-LC pairing strategies generates IgG bsAbs with high purity. One way to circumvent the LC problem is to combine the HC and LC into a single chain, as for scFv-Fc or scFab-Fc fusions.[249] Alternatively, the correct HC-LC pairings can be favored by incorporating mutations into each chain that generate orthogonal Fab interfaces.[259] Moving the site of the HC-LC disulfide bond on only one of the Fab domains (as with DuetMab) is another way to drive correct HC-LC assembly.[260] A particularly elegant solution to the LC problem is to swap the C_H1 and C_L domains on only one Fab arm.[261] This CrossMAb approach reliably generates the desired HC-LC pairings without the use of potentially destabilizing mutations and has inspired a whole family of multispecific Ig frameworks.[262] In cases where it is possible to generate functional Fvs against two distinct antigens using a common LC or common HC, the LC and HC problems can be avoided and these chain pairing strategies become unnecessary.

The bsAb technologies covered so far rely on co-transfection of Ig chains directed toward distinct antigens into the same batch of antibody-expressing cells. It is also possible to separately express and purify parental antibodies before recombining them *in vitro* to the corresponding bsAb. For example, parental antibodies containing “knob” or “hole” mutations can be separately purified and then incubated together in the presence of reducing agent to allow for hinge reduction and half-antibody exchange driven by the energetic preference for HC heterodimer.[263] Mutation of the hinge to lack disulfide bonds facilitates this process by obviating the need for reducing agent, but also eliminates stabilizing disulfide bonds in the product.[264] A similar method takes advantage of the Fab-arm exchange process that takes place naturally for IgG4 due to the weaker C_H3 interactions and hinge lability of this subtype. For controlled Fab-arm exchange, parental antibodies containing the F405L or K409R mutations are combined under reducing conditions to allow formation of >90% pure bsAb.[265] Since the correct HC-LC pairing is not disrupted, even in the presence of reductant, these methods sidestep the LC problem and only require mutations for HC heterodimerization. Another benefit of the post-expression recombination approach is the ability to generate panels of parental antibodies containing complementary mutations, which can be recombined to evaluate the synergy of each combination.

Rather than driving proper HC-HC and HC-LC pairings, strategies have also been devised to facilitate purification of bsAb from mixtures of other chain combinations. For instances where two Fv fragments are discovered or engineered to use the same V_H sequence (i.e. common HC) and each V_L is from a different class (κ and λ), κ/λ bodies can be easily purified.[266] After transfecting cells with the single HC, Ag A-specific κ LC, and Ag B-specific λ LC, the desired product will contain two HCs and one of each LC. Thus, successive KappaSelect and LambdaSelect chromatography steps allow for purification of bsAbs of the desired composition. For any two antibodies containing a common LC, bsAb purification can be expedited by incorporating mutations that ablate protein A binding into one of the parental HCs.[267] After co-expression of the two HCs and common LC, bsAb can

be purified from the mixture using protein A resin combined with incremental decreases in buffer pH. The species containing two mutated HCs flows through the column, while the heterodimer elutes at intermediate pH and the wild-type HC homodimer elutes only at low pH due to strong protein A avidity. If recombination of half-antibodies is performed using purified parental antibodies or culture supernatants (rather than co-expression), the process can be used to purify bsAb without the requirement for a common LC.[268]

In summary, several strategies exist for the preferential formation and purification of IgG-like bsAbs. Although bsAbs with the whole IgG framework necessitate methods for proper HC-HC and HC-LC pairing, they inherit many of the favorable structural and functional features of the well-characterized IgG framework. Selection of which bsAb technology to employ may be guided by considerations including ease of implementation and minimization of mutational load, while patent rights are a valid concern for commercial projects.

11.2 Fusion of Ag-binding domains

To avoid the HC and LC pairing problems, or to alter properties like size, valency, and geometry, multispecific antibodies lacking the Fc domain can be formed via fusion of Ag-binding domains. Small size in particular may be beneficial for tissue penetration, while the resulting decrease in half-life can be overcome through incorporation of albumin-binding capacity, for example.[184,187] Lack of the Fc domain eliminates FcR- and C1q-mediated functions, but specific immune activity can be achieved by domains that bind receptors of interest (e.g. α-CD3). Without the constraints of the IgG framework, factors like flexibility and inter-Ag distance can be optimized using different binding domains and linkers. Although chemical conjugation to generate, for example, bispecific $F(ab')_2$ is possible, most work has focused on genetic engineering approaches to create bsAbs from Ag-binding fragments.[269,270]

The scFv format is well-suited for bsAb applications, as scFv domains with different specificities can be easily combined using single or multiple polypeptide chains. For cancer indications, tandem linkage of scFv domains targeting a target-associated antigen and a T cell or NK cell antigen (α-CD3 or α-FcγRIIIa) are used to create bispecific T cell engagers (BiTEs) or bispecific killer cell engagers (BiKEs).[271,272] For example, the BiTE blinatumomab (α-CD3 x α-CD19) was approved in 2014 for treatment of acute lymphoblastic leukemia, and is thought to function by stimulating cytotoxic T cells to act on co-localized tumor cells. In addition to $scFv_2$ formats, $scFv_3$ proteins can be engineered to bind three separate antigens, or to bind one antigen bivalently and a second antigen monovalently.[249] The scFv-based Diabody framework is also amenable to multispecificity. Since Diabody chains may undergo unwanted homodimerization, derivatives like dual-affinity retargeting (DART) proteins and single-chain Diabodies have been engineered to drive heterodimerization through incorporation of a stabilizing disulfide bond and consolidation into a single polypeptide chain, respectively.[273,274] Shortening the linker of the single-chain Diabody allows for generation of TandAbs, which bind bivalently to each of two Ags.[275]

Like scFvs, distinct Fab fragments can be combined into bifunctional agents. For example, HCs can be fused in tandem and co-expressed with both LCs.[276] The correct HC-LC pairing is driven by strategies such as the orthogonal Fab interface mutations that resolve the LC problem. sdAbs are particularly amenable to genetic fusion, since their binding capacity resides in a single domain that does not require chain pairing. Tandem fusion of sdAbs allows for generation of multispecific and multivalent proteins that retain low molecular weights.[249]

In summary, sdAbs, scFvs, and Fabs of distinct specificities can be combined into compact and multifunctional agents. These Fc-less bsAbs are not limited to sdAb-sdAb, scFv-scFv, or Fab-Fab fusions; indeed, combination of different frameworks may be beneficial. For instance, Fab-scFv and Fab-sdAb fusions do not have the chain-pairing problem of Fab-Fab fusions, and the increased spacing creates geometry distinct from that of scFv-scFv and sdAb-sdAb fusions. bsAb properties like Ag affinity, expression yield, and aggregation propensity can vary between bsAb formats.[277] Thus, careful consideration and experimentation are likely required to determine the optimal domain configurations for a given application.

11.3 Fusion of Ag-binding domains to IgG

Finally, Ag-binding fragments can be fused to the IgG or Fc framework to create multispecific antibodies that are often multivalent for each antigen. A straightforward strategy is to append Ag-binding domains to the native IgG framework. Domains such as scFv, and sdAb can be fused either N- or C-terminally to the HC or LC, allowing many symmetrical constructs to be explored that may differ in their geometry and ability to co-engage with two antigens.[249] Because bispecificity results from the native IgG and appended domain targeting different antigens, it is not necessary to address the HC and LC problems. An example of this approach is the dual-variable-domain antibody (DVD-Ig), in which a distinct V_H and V_L are fused N-terminally to the standard IgG HC and LC, respectively.[278] If the size of IgG fusions is deemed too large, the same strategies can be applied to make bispecific molecules based on Fc or C_H3 homodimerizing domains. Combinations of different domain types, linkers, and appendage sites allow for almost limitless possibilities to be explored.

At the same time, domains can also be appended to heterodimerizing Fc domains to generate asymmetric architectures with opportunities for multispecificity. For example, the trispecific scFab-Fc-scFv contains heterodimerizing HCs attached to different scFabs at the N-terminus and a distinct scFv at one or both of the C-termini.[279] Applying the DVD-Ig format together with Knobs-into-Holes mutations to drive HC heterodimerization and CrossMAb domain swapping to drive correct HC-LC pairing allow for creation of tetraspecific antibodies that are monovalent for each antigen.[280] It has become increasingly clear that combinations of protein engineering strategies can be used to generate multifunctional frameworks that are custom-tailored to the application at hand.

12. Protein engineering

12.1 Mutations that alter effector function

Early work in the IgG engineering field revealed residues important for FcγR binding via alanine scanning, and also showed that mutation of selected residues could enhance FcγR affinity and FcγR-mediated effector functions.[281] Since then, numerous studies have reported sets of 1–5 amino acid mutations that significantly improve both FcγR binding and ADCC/ADCP.[282–284] While all of the FcγRs share a binding site comprising the lower hinge and upper C_H2, slight differences in binding modes means that a given set of mutations may differentially impact affinity to each of the FcγRs. Improved binding to FcγRIIIa for more potent ADCC is a common goal that can be achieved through amino acid mutation in addition to the glycan engineering methods mentioned previously. Similarly, variants with tighter FcγRIIa binding have enhanced macrophage-mediated ADCP. Although it may be important in some contexts to maximize the relative affinity for activating and inhibitory FcγRs, it has been shown that FcγRIIa affinity is more important than the FcγRIIa/FcγRIIb affinity ratio for ADCP function.[285,286] Because the IgG HCs bind FcγRs asymmetrically, each HC can be made to contain a distinct set of mutations that synergistically enhance FcγR binding.[287] As all of these mutation strategies incorporate framework changes and potentially novel epitopes, they may have altered properties, such as stability and immunogenicity, compared to a native IgG.

Mutations in the C_H2 domain can also alter affinity for C1q binding, and thus modulate CDC activity. Since FcγRs and C1q bind at proximal sites in the IgG Fc domain, improvement of CDC may come at the cost of decreased ADCC.[283,284] For example, one study found that a set of three mutations caused a 7-fold increase in CDC, but a 20-fold decrease in ADCC.[288] Addition of two additional mutations not only increased CDC further, but also caused a modest increase in ADCC relative to the wild-type IgG. Another approach designed an IgG1/3 chimera by fusing the IgG1 C_H1 and hinge with the IgG3 C_H2 and C_H3 domains.[289] By combining the properties of each subclass, CDC was increased relative to IgG1 while ADCC was increased relative to IgG3. As mentioned previously, mutations in the lower Fc domain can also be used to enhance CDC by increasing IgG hexamerization.[27]

Conversely, Fc engineering can be used to generate silent antagonists that have minimal effector function. Previously mentioned strategies to reduce FcγR and C1q binding include deglycosylation and hybridization of weakly activating subclasses like IgG2 and IgG4.[112,154] In addition, the C_H2 domain can be mutated to decrease engagement with activating receptors and the resulting release of inflammatory cytokines.[284,290] Different sets of 2–6 amino acid mutations in the IgG1, IgG2, and IgG4 framework in some cases caused complete elimination of ADCC, ADCP, and CDC functions, depending on the antibody/host species.[291] For example, while D265A/N297G (DANG) and L234A/L235A/P329G (LALA-PG) ablated effector function in primates, the DANG variant retained complement activation as murine IgG2a, making LALA-PG the better surrogate in mouse studies. Meanwhile, properties like stability, immunogenicity, and biological half-life appeared to be minimally affected by the mutations. Rather than reducing binding to all FcγRs, it may be desirable to increase affinity for the inhibitory FcγRIIb in some immunosuppressive applications.[290]

Overall, the introduction of even a few amino acid mutations can have powerful and specific effects on the biological and biophysical properties of antibodies which enables effector functions to be fine-tuned based on indication.

12.2 Mutations that alter pharmacokinetics

Structural and mutational studies have identified residues at the Cγ2-Cγ3 elbow that are responsible for FcRn binding.[284] This knowledge, in combination with display technologies, has allowed for the discovery of IgG Fc point mutations that strengthen the interaction with FcRn at endosomal pH.[282,284,290,292] Since FcRn is vital for endosomal salvage of IgG, it was thought that tight-binding IgG mutants might have an extended PK profile. Indeed, one set of mutations (M252Y/S254T/T256E, YTE) not only increased affinity for FcRn at pH 6.0 by ~10-fold, but also prolonged serum half-life in humans by 2- to 4-fold.[293,294] Functionally, the long-lived LS mutants (M428L/N434S) have been shown to elicit stronger anti-tumor activity due to maintenance of higher serum concentrations.[295] Other studies have stressed the importance of retaining pH sensitivity, showing that an increased affinity for FcRn at physiological pH may actually accelerate clearance.[282,292] It seems that strong FcRn affinity at pH 6.0 generally extends half-life, but that some threshold for pH 7.4 affinity exists which, if surpassed, counteracts this effect.[296] Although FcRn and FcγRs bind at distal sites, potential allosteric effects have been suggested based on the decreases in effector function observed for mutants with enhanced FcRn binding.[297] FcRn and protein A, on the other hand, bind to the same Cγ2/Cγ3 region; thus, care should be taken not to interfere with protein A binding if it is required for purification. Due to advantages such as lower dose requirements, less frequent dosing, and lower cost, PK-enhancing mutations will likely be incorporated into more novel antibodies and biosuperiors in the future.

By contrast, IgG interaction with FcRn can also be modulated to increase clearance of fast-acting therapeutics or diagnostics..[46] IgG mutants that bind FcRn with high affinity at acidic and neutral pH can be used to accelerate degradation of endogenous antibodies or antigens. The systemic load of potentially autoreactive, pathogenic antibodies can be depleted by antagonizing FcRn and preventing its salvage of endogenous IgG.[292] Because this FcRn-blocking strategy is not dependent on Fab activity, the Abdeg format uses Fc fragments containing mutations that enhance FcRn binding at a broad pH range.[196] For antibodies that antagonize soluble antigens, the long IgG half-life may be conferred to the antigen unwantedly.[292] To accelerate clearance of pathogenic antigens, sweeping antibodies can be used that have enhanced FcRn affinity at neutral pH.[298] This improved FcRn binding allows for receptor-mediated cellular uptake and lysosomal degradation. Concomitant engineering of the variable regions can be used to release antigen in the endosome and prevent it from recycling with IgG.[292,298] It is important to note that Abdegs and sweeping antibodies have shorter half-lives than native IgGs, which may necessitate more frequent dosing. Nevertheless, it is apparent that alteration of pH-dependent FcRn binding is a powerful tool for half-life modulation of IgGs as well as their targeted antigens.

13. Biophysical properties

13.1 Stability and aggregation

The conformational stability of an antibody is defined by the relative free energies of the native and unfolded states (Figure 6). These energies are based on fundamental forces like hydrogen bonds, hydrophobic and van der Waals interactions, and electrostatic attraction and repulsion, both between protein atoms and between protein and solvent. Aggregation is closely coupled to stability.[173,174,299,300] While all proteins undergo continuous conformational sampling, less stable proteins are more likely to partially unfold and reveal hydrophobic residues that are buried in the native state. Transient exposure of hydrophobic, uncharged patches allows for intermolecular association of these regions. Because aggregation of this sort locks proteins in non-native conformations, it is often considered to be irreversible.[300] Some regions of antibodies are more likely than others to initiate aggregation. The intra- and interdomain contacts, such as those between V_H and V_L domains, are especially prone to aggregation due to their hydrophobic character. For this reason, sdAbs (V_H, V_L) are often engineered to reduce hydrophobicity at the normal domain interface, and scFvs may be modified to minimize transient opening that can lead to aggregation.[300–302] IgG binding sites (CDRs for antigen binding, lower hinge and upper Cγ2 for FcγR and C1q binding, and the Cγ2/Cγ3 elbow for FcRn binding) also tend to have hydrophobic residues that contribute to the energy of binding.[300] For ADCs and other conjugates, hydrophobic linkers or payloads have the potential to increase aggregation.[300] Although IgG molecules are considered especially stable proteins, efforts to improve developability have focused on protein engineering and formulation strategies to further reduce the incidence of aggregation.[303–305]

Clinical IgGs are routinely concentrated to >100 mg/mL in order to deliver sufficient quantities of drug via small-volume injection.[306] Because aggregation is more likely at higher concentrations, there is a clear need to quantify aggregation and understand its effects. Indeed, antibodies and other therapeutic proteins must be thoroughly characterized to ensure that no more than a few percent of the drug consists of non-monomeric species. This homogeneity is essential because pre-clinical data is usually available only for the species of interest. Oligomers and large aggregates do not necessarily share the same biological properties as the monomer, and in many cases aggregates have less desirable characteristics.[299,300] For example, the repeated epitopes or misfolded regions on protein multimers may make them more immunogenic.[307] The generation of an immune response to aggregates not only compromises patient safety, but may also lead to immune recognition of the active, monomeric species. Thus, aggregation-induced immunogenicity can increase clearance of a drug (often via anti-drug antibodies), reducing exposure and efficacy.[308]

The negative aspects of aggregation may be minimized by protein engineering, either through rational alteration of problematic sequences or through evolutionary screening approaches.[300] In both cases, a common goal is to decrease the free energy of the folded protein in order to disfavor the unfolded states that are more likely to initiate aggregation. An example of rational design is the inclusion of a novel intradomain disulfide bond into sdAbs, which increases thermal stability and decreases aggregation.[309] Certain HCs and LCs

may be selected to generate antibodies with more favorable biophysical properties. For example, the sub-family V_H3 has superior thermodynamic stability compared to other V_H domains, and V_κ is generally more stable than V_λ.[107] The pairings of these domains are also important to consider, as certain V_H and V_L combinations are more stable and more common *in vivo*.[107,310] Engineering to decrease intermolecular encounters is another option for decreasing aggregation. For instance, addition of charge (especially acidification) via mutagenesis may be used to induce molecular repulsion, while addition of hydrophilic residues into otherwise hydrophobic stretches decreases hydrophobic nucleation.[300] When engineering for increased stability, it is important to consider functional sites that could be impacted by proximal or even distant mutations. There is a complex interplay between properties like affinity, specificity, and stability that must be co-optimized to generate molecules with the desired characteristics.[311] In contrast to rational mutagenesis, evolutionary approaches generate libraries of variants and isolate those with enhanced biophysical properties by screening under destabilizing conditions.[300]

Several methods exist for assessing the conformational stability and aggregation propensity of drug candidates, as well as related properties like colloidal stability and self-association.[132,312,313] Stress tests attempt to quickly predict the long-term stability of proteins by exposing them to conditions that allow for exploration of non-native conformations. In principle, aggregation can be accelerated by increasing the free energy of the native state, decreasing the free energy of the unfolded state, or adding energy to the system to overcome kinetic barriers between states. Temperature ramping methods like differential scanning calorimetry are frequently used to compare protein stability based on the thermal unfolding of each domain. Alternatively, the percent of monomer can be tracked using size-based analyses such as size-exclusion chromatography (SEC).[314] Note that these techniques measure fundamentally different biophysical properties. While thermal shift assays like differential scanning calorimetry/fluorimetry quantify conformational stability by detecting the temperature of melting transitions, size-based methods like SEC and dynamic light scattering measure aggregation end products including dimers and other oligomers. SEC may be used to rank stability after long-term incubation at formulation conditions, but is more frequently used following short-term incubation under stress conditions including extreme salt or pH.For example, stability at low pH is frequently tested since elution from affinity resin and viral inactivation are performed in low pH buffers. Recently, the ability of thermal unfolding assays to predict physical stability has been questioned, with properties like self-association, colloidal stability, and chemical denaturation stability being proposed as superior predictive parameters than melting temperature.[313,315] Tryptophan fluorescence is another property potentially correlated to total monomer loss (fragmentation plus aggregation) rather than aggregation alone.[313] The presence of aggregation-prone regions may also be predicted using sequence-based computational modeling approaches.[316] But while *in silico* methods may be helpful in guiding lead selection, they are unlikely to replace *in vitro* stability tests that experimentally evaluate the presence of problematic aggregation pathways.

13.2 Immunogenicity

It is logical that exogenous proteins have the potential to elicit an immune response when administered to animals or patients. On the one hand, some anti-drug antibody (ADA) responses lead to non-neutralizing antibodies that minimally impact the therapeutic mechanism but may accelerate clearance.[308] On the other hand, ADAs developed against important drug epitopes can reduce both potency and half-life. Severe immune reactions may inactivate the therapeutic agent while also causing potentially fatal infusion reactions and anaphylaxis.[307] Immunogenicity depends not only on extrinsic factors like dose, frequency, route of administration, formulation, and patient background; but also on intrinsic biophysical properties of the therapeutic agent.[308] Thus, it is important to understand which molecular features are likely to be immunogenic in order to develop safer and more effective biologics.

As previously mentioned, aggregated antibodies and other proteins are often more immunogenic than their monomers.[307,308] This may be due in part to the presence of regularly repeated epitopes that mimic pathogenic patterns of surface expression. Repeated structures present on other antibody-based drugs can also be cause for concern. For example, conjugation of hydrophobic drugs to antibodies can not only increase immunogenicity through aggregation, but also through the repeated linker and payload motifs present on a monomeric ADC.[317] Formation of ADC immune complexes and subsequent uptake into phagocytes can also lead to off-target toxicities and loss of efficacy.

The main source of immunogenicity is novel structures not present in endogenous antibodies. Thus, mouse antibodies elicit a stronger ADA response than chimeric antibodies, which in turn elicit a stronger response than humanized antibodies.[118] Antibody fusions may be immunogenic due to the presence of non-human proteins or unnatural peptide linkers.[317] Small molecules and other cytotoxic payloads can also serve as antigenic haptens when present on ADCs, as can the linkers connecting them to the protein. Even minimally disruptive amino acid mutations and oxidative or chemical modifications have been suggested as sources of immunogenicity.[176,318] Strategies to avoid the development of ADAs include the introduction of masking groups like PEG and glycans, which can limit exposure to neoepitopes.[318]

Although it is difficult to replicate the complexity of the human immune systems, a number of preclinical models exist for the prediction of immunogenicity in humans.[319] Since immunity is species-specific, it is preferable to do *in vivo* studies in non-human primates which have high homology with humans. While rodents may develop an immune response to human proteins and could thus overestimate immunogenicity, they may be useful predictors of relative immunogenicity.[308,319] A more efficient approach is to use transgenic mice that express the human antibody genes and/or human MHC. Although this should generate immune tolerance for the administered antibody and allow for identification of neoepitopes, ongoing challenges include lack of genetic diversity in these models and incomplete understanding of mechanisms that induce human immunogenicity.[319] In addition to whole organisms, immunogenicity can be predicted *in vitro* by incubating cells with the antibody and monitoring surface expression of receptors on antigen-presenting cells, T cell proliferation, or cytokine release.[319] Identification of T cell epitopes *in silico* may also be

useful for predicting antigenicity of novel sequences.[319] In summary, immunogenicity can be avoided by rationally minimizing novel and repeated epitopes, and by masking and formulation strategies. While several methods exist for preclinical prediction of immunogenicity, it remains challenging to fully replicate the complexity of the immune response in humans.

13.3 Charge and isoelectric point

An important biophysical property of antibodies is their surface charge, both in terms of net charge and distribution. Patches of uncharged, hydrophobic amino acids can serve as hot spots for antibody aggregation.[300] Thus, incorporation of acidic or basic residues into these regions can help to prevent intermolecular association. However, positively charged patches can also increase non-specific tissue uptake and reduce exposure of antibodies. Studies have demonstrated that engineering variable regions to reduce patches of positive charge can decrease the clearance of antibodies.[320,321] In these instances, the increased serum stability might also be related to small decreases in the isoelectric point (pI) of the proteins.

The overall charge of a protein at physiological pH is determined by its pI, which in turn is related to the number of titratable side chains it contains. It is well-established that antibodies with more basic pI values tend to have increased tissue uptake and faster clearance.[46,322] This phenomenon is likely related to the propensity of positively charged residues to interact with negatively charged cell membranes. Reducing the pI of an antibody, e.g. by engineering the variable domains, allows for improvement of several PK parameters. Acidification is thought to decrease interactions at cell surfaces, decrease non-specific tissue uptake, decrease clearance, and increase bioavailability.[322] On the other hand, increasing pI tends to increases clearance and volume of distribution, but could possibly be used to favor penetration of the blood-brain barrier. Significant changes in PK properties have been proposed to occur only once the pI has been changed by >1 pH unit.[46] Engineering to modulate charge and pI is therefore a valid option to control aggregation and PK properties of antibodies.

13.4 Formulation

The conformational stability of a protein is a function of intramolecular interactions, but also of interactions between the protein and its surrounding solvent. Thus, formulation is a powerful tool to stabilize antibodies and prevent them from aggregating or degrading over the normal shelf life of several years.[174,306] One variable to optimize is pH, where intermediate pH formulations tend to have undesirably high viscosity, but extreme pH formulations may accelerate degradation pathways like isomerization and deamidation. In cases where protein self-association is controlled by electrostatic interactions, ionic strength may be modulated to prevent self-association and high viscosity.

Addition of excipients to formulation buffers is broadly used to improve long-term stability.[306,323] Surfactants like polysorbates 20 and 80 may be added to mitigate aggregation that occurs at air-liquid interfaces. Similarly, amino acids like arginine and histidine and non-reducing sugars like sucrose and trehalose are commonly used to prevent aggregation at high protein concentrations. These same components may also have stabilizing effects in the

context of protein lyophilization and freeze-thaws.[306] Since each antibody is structurally distinct and may contain unique instabilities, panels of buffers conditions are often tested to determine the ideal formulation on a case-by-case basis. Type of storage vessel is another important consideration, as different materials are known to leach potentially destabilizing components into the antibody formulation.[306] Despite these formulation strategies, intrinsic batch-to-batch microheterogeneity in glycan profile, disulfide oxidation, and other subtle but important structural elements remains a serious challenge for the assurance of consistent product quality. While intravenous and subcutaneous administration routes remain the most widely used, several novel delivery strategies have been proposed to expand the options available to patients and physicians.[324]

13.5 Behavior in serum

Although formulations are optimized to maximize long-term antibody stability, these therapeutics are ultimately delivered to complex biological compartments where their behavior may differ from that in simple buffers. The primary environment for most therapeutic antibodies is the blood, where they are delivered directly via infusion or indirectly via injection and the lymphatic system.[46] Plasma is a crowded matrix that contains high concentrations of endogenous proteins and other components.[325] The excluded volume effect can increase the apparent concentration of antibodies while the abundance of other proteins introduces opportunities for non-specific interactions.[326] Thus, it is important to determine whether administered antibodies interact with serum components in a way that alters their functional properties (Figure 6).

The presence of high concentrations of endogenous proteins complicates the analysis of antibody behavior in serum. Due to the high background and heterogeneity of serum samples, it is difficult to monitor individual proteins using traditional (e.g., absorbance-detected) methods. However, strategies such as fluorescent labeling have allowed for comparison of antibody behavior in buffers and in serum. Intriguingly, differences in antibody-antigen affinity, stoichiometry, and complex size have been noted based on serum matrix effects.[327,328] Thus, measures of binding affinity in dilute buffers may not accurately represent the binding that occurs *in vivo*. Furthermore, mixing of formulated antibodies with serum can result in aggregation of antibodies with serum proteins in an antibody- and excipient-dependent manner.[329,330] Administration of certain antibody/formulation combinations could therefore result in aggregation-induced infusion reactions or loss of effective drug. Differences in the number and size of aggregates has also been reported based on environment.[331] Whereas heat-stressed antibodies aggregated similarly in buffer and serum, pH-stressed antibodies formed smaller but more numerous aggregates in serum. Because serum allows for both self-association or aggregation with serum components, nanoparticle-based techniques have been developed to distinguish between these mechanisms.[332] Clearly, antibody functionality can vary significantly between formulated buffers and complex biological matrices. In the future, it will be important to characterize antibody functions in biologically relevant environments in order to increase understanding of therapeutic mechanisms as they occur *in vivo*.

14. Conclusions

The first generation of antibody therapeutics focused primarily on specific binding of molecular targets to elicit simple inhibitory mechanisms. While these early molecules established large molecules as a valid class of drugs, they did not fully capitalize on all aspects of the antibody platform. More recently, therapeutic mechanisms have been customized not only based on type of antigen, but by antigen affinity, valency, and epitope. Use of different antibody subclasses allows for fine-tuning of pharmacokinetics and effector function due to differential binding to endogenous FcRs and complement proteins. Meanwhile, small antigen-binding domains can be used to eliminate effector function, multimerizing antibodies increase complement fixation, and antibody fusions instill other proteins with favorable properties of the antibody framework. Conjugation of cytotoxic agents to antibodies allows for specific delivery of payloads to tumors, while multispecific antibodies grant novel mechanisms that increase specificity and facilitate delivery to historically intractable compartments. In parallel with these framework innovations, antibody engineering allows for incorporation of amino acid and glycan changes that selectively alter biological and physical properties.

In conclusion, the humoral immune response creates astoundingly complex antibody molecules with the ability to bind both antigens and elements of the adaptive immune system. This antibody format has proven to be extremely amenable to protein engineering, which allows for modular design of structural domains that best integrate the desired therapeutic functions. With increased understanding of immunobiology and the continued development of molecular biological methods, the possibilities for antibody-based therapeutics are bounded only by the scope of human ingenuity.

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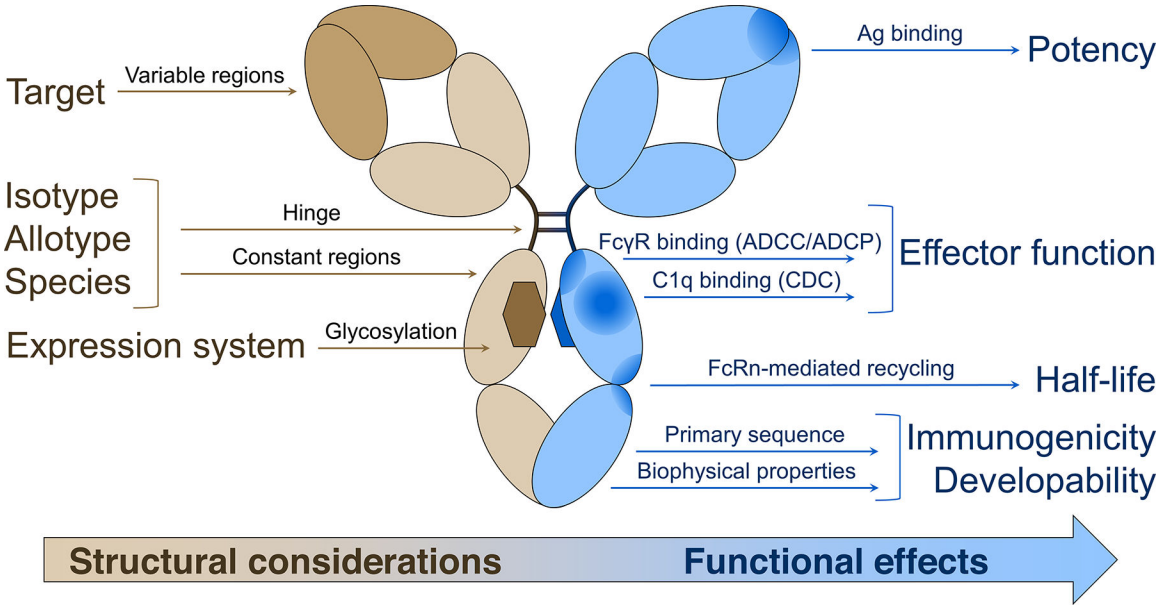

Figure 1:
Structural considerations for the design of IgG-based therapeutics and their effects on biological and clinical function.

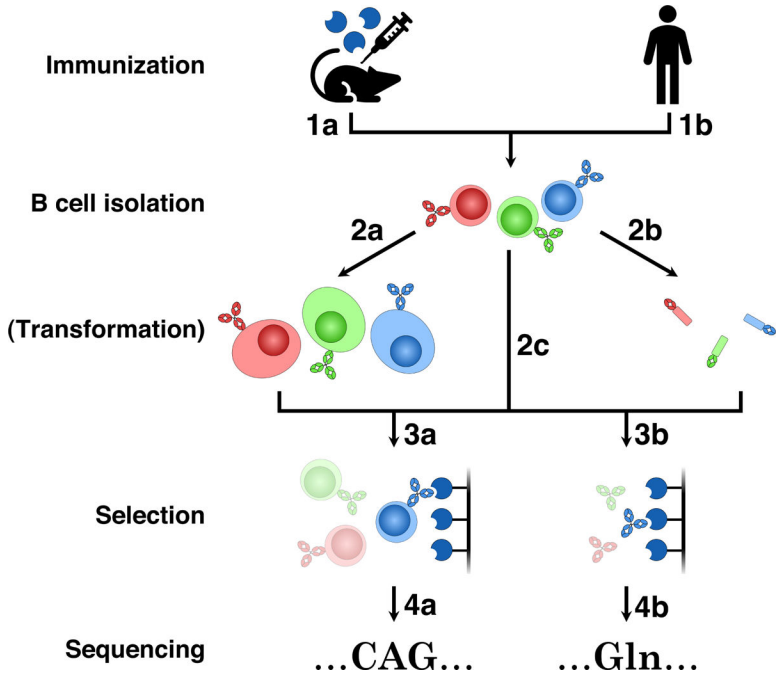

Figure 2:

Strategies for identification of antibody variable regions. Rearranged V(D)J genes may be sourced from the spleens of immunized animals (1a), or from the blood of naïve, vaccinated, or chronically ill patients (1b). Synthetic DNA libraries have also been created with an emphasis on diversity within complementarity determining regions. After isolating B cells, the variable regions of their B cell receptors are used to generate surface-expressed antibody domains that may be used in functional screens. One approach is to immortalize the B cells via fusion with myeloma cells, producing highly proliferative antibody-producing hybridomas (2a). Alternatively, mRNA can be isolated from B cells, converted to cDNA, and used to construct libraries of phage, bacteria, yeast, or mammalian cells with surface display of Fab or scFv fragments (2b). The repertoire of B cells may also be directly sequenced via Ig seq or sorted/diluted into single B cell populations for sequencing (2c). Whether using hybridoma, surface display, or (single) B cells, screening steps are used to select for functional antigen binders. This selection may occur at the cell level using FACS (3a) or at the protein level using ELISA of cell supernatants (3b). After enrichment of functional cells or proteins, successful candidates may be identified at the DNA (4a) or protein (4b) levels. While cloning, selection, and sequencing are required steps for identification of functional Ig genes, they may be performed in orders other than those represented here.

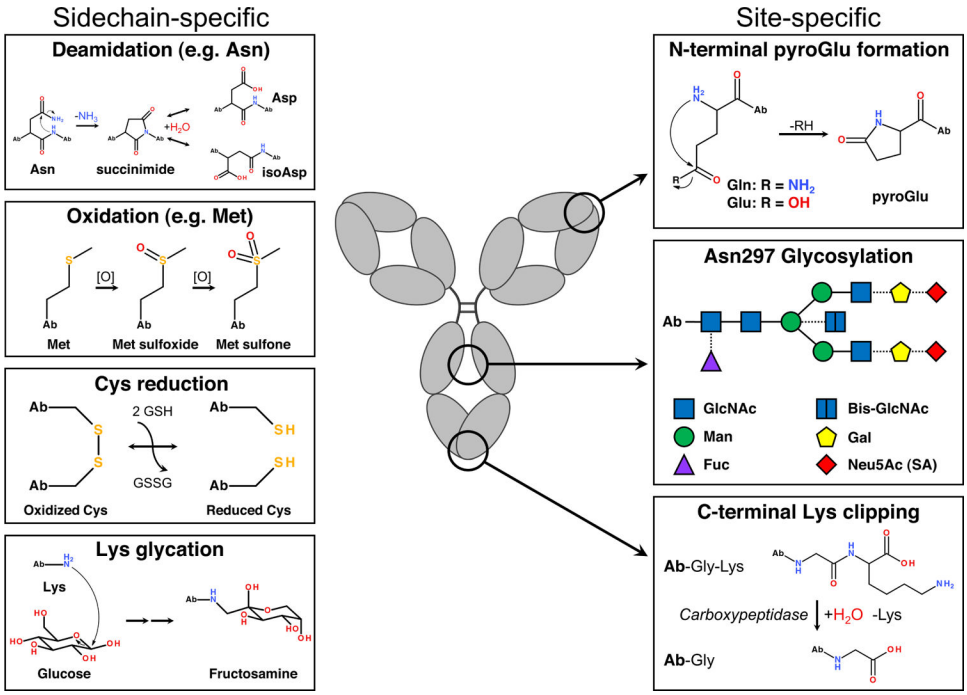

Figure 3:

Common post-translational modifications of IgG antibodies. Shown on the left are amino acid modifications that occur in a side-chain dependent but site-independent manner. These chemical alterations may negatively affect properties like antigen or receptor binding. Shown on the right are amino acid modifications that occur at specific sites. While N-terminal formation of pyroglutamate occurs only for chains that begin with glutamine or glutamate, C-terminal lysine clipping occurs for all IgG antibodies, whose heavy chains terminate with a glycine-lysine motif. Glycosylation at asparagine 297 leads to a core glycan (solid lines) to which additional sugars may be added (dotted lines). These differences in glycan composition have significant effects on binding to Fc receptors. Abbreviations: Bis-GlcNAc (bisecting N-acetylglucosamine), Fuc (fucose), Gal (galactose), Man (mannose), Neu5Ac (N-acetylneuraminic acid), SA (sialic acid)

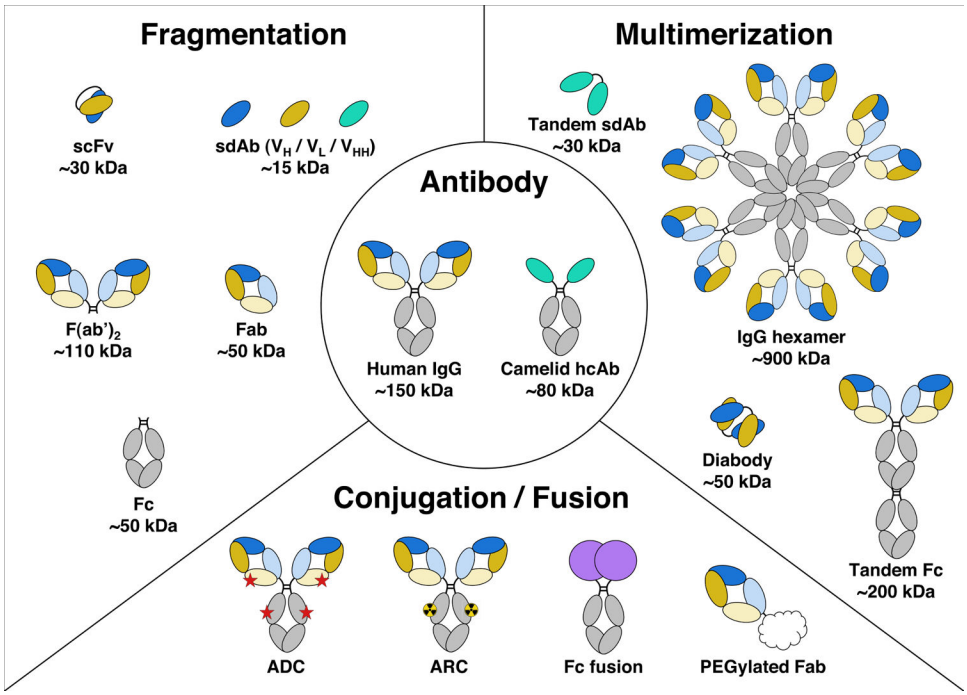

Figure 4:

Therapeutic frameworks based on fragmentation, multimerization, conjugation, and fusion of human or non-human antibody domains. Abbreviations: ADC (antibody-drug conjugate), ARC (antibody-radionuclide conjugate), Fab (antigen-binding fragment), Fc (crystallizable fragment), hcAb (heavy chain antibody), IgG (immunoglobulin G), PEG (polyethylene glycol), scFv (single-chain variable fragment), sdAb (single domain antibody)

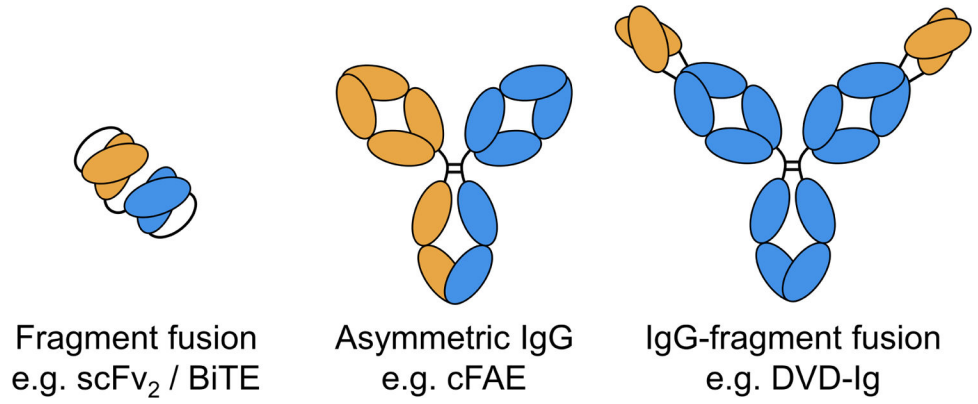

Figure 5:

Types of bispecific antibody frameworks. Fragment fusions are small proteins created by genetic fusion of antibody domains (Fab, scFv, sdAb). Their lack of Fc domain confers high diffusion and tissue penetration, but fast clearance and lack of effector function. Asymmetric IgG frameworks retain many properties of native IgG but bind distinct antigens via each Fab arm. Fusion of antibody fragments to the IgG framework creates large, generally symmetric molecules that are often multivalent.

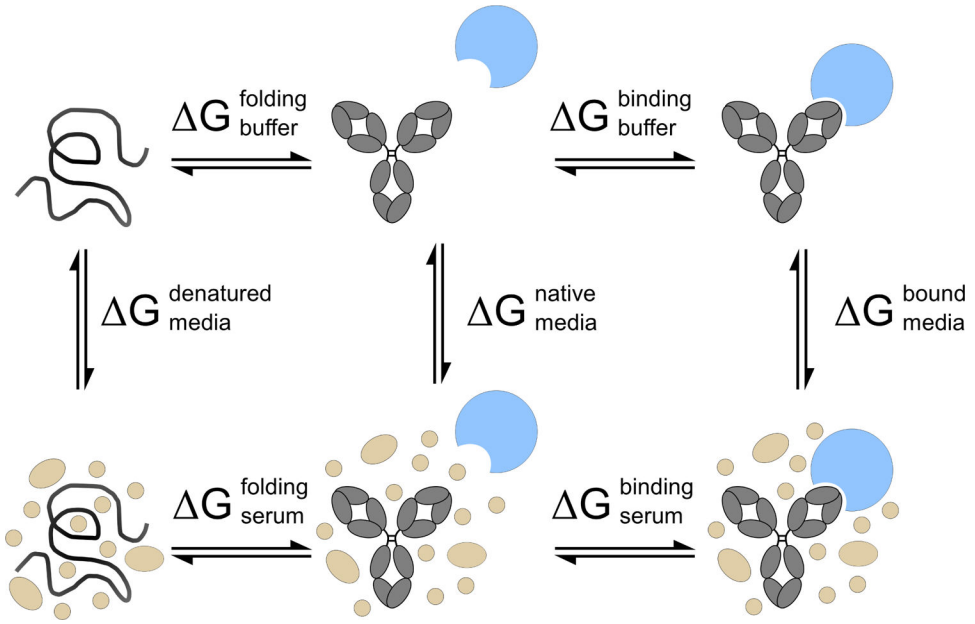

Figure 6:

Thermodynamics of antibody folding and binding in dilute buffer and in complex matrices like the serum. Because the free energy of unfolded, native, and bound antibodies may differ significantly in buffer and in serum, the stability ($\Delta G_{folding}$) and binding affinity ($\Delta G_{binding}$) may also differ in these two types of media. Thus, it is important to characterize and understand the thermodynamic properties of antibodies in complex but biologically relevant environments.

Table 1:

Properties of antibody subclasses

	IgA1	IgA2	IgD	IgE	IgG1	IgG2	IgG3	IgG4	IgM
Structure									
Heavy chain	α_1	α_2	δ	ε	γ_1	γ_2	γ_3	γ_4	μ
Ig domains per HC	4	4	4	5	4	4	4	4	5
Monomer size (kDa)	160	160	184	188	146	146	165	146	194
Oligomeric forms	1–4	1–4	1	1	1	1–2	1	0.5–1	5–6
Hinge amino acids	23	10	64	0	15	12	62[i]	12	0
HC-HC disulfide bonds	3	3	1	2	2	4	11[i]	2	3[ii]
N-glycan sites per HC	2	4–5[iii]	3	7	1	1	1	1	5
O-glycan sites per HC	9[iv]	0	5	0	0	0	3[i]	0	0
Biology									
Serum level (g/L)	3	0.5	0.03	0.00005	9	3	1	0.5	1.5
Serum half-life (d)	6	6	3	2	21	20	7	21	10
Predominant antigen[v]	p	c	-	a	p	c	p	a	c
Allotypes	0	3	0	1	4	1	13	0	0
Distribution									
Mucosal transcytosis[vi]	+++	+++	-	-	-	-	-	-	+
Placental transfer	-	-	-	-	+++	+	++	+	+
Extravascular diffusion[vii]	++	++	-	+	+++	+++	+++	+++	-
Effector function									
Cytotoxicity (ADCC)[viii]	++	++	-	++	+++	+	+++	+	-
Phagocytosis (ADCP)	++	++	-	-	+++	-	+++	+	+
Complement (CDC)[ix]	+	+	-	-	++	+	+++	-	+++

[i] Due to differences in allotype and number of exon repeats, there is considerable variation in the IgG3 hinge region.

[ii] Polymeric IgM contains 1 HC-HC disulfide bond within each monomer, and an additional 2 disulfide bonds linking each HC to HCs of other monomers

[iii] IgA2m1 contains 4 N-glycosylation sites while IgA2m2 and IgA2n contain 5

[iv] While IgA1 has 9 potential Ser/Thr glycosylation sites in the hinge, <6 are occupied

[v] p=protein; c=carbohydrate; a=allergen

[vi] Only polymeric IgA (predominantly dimer) is transported to secretions

[vii] Only monomeric IgA has significant diffusion to extravascular sites

[viii] ADCC is mediated primarily by NK cells (IgG) and myeloid cells (IgA, IgE)

[ix] Complement activation of IgA occurs through the alternative or lectin, rather than classical, pathway

Table 2:

Examples of antigens targeted by therapeutic antibodies

Indication	Antigen type	Example antigen	Approved antibodies
Cancer	Lineage-defining	CD19	Blinatumomab (CD19 x CD3)
		CD20	Ibritumomab tiuxetan I-131 Tositumomab * Obinutuzumab Ofatumumab Rituximab
		CD22	Inotuzumab ozogamicin
		CD30	Brentuximab vedotin
		CD33	Gemtuzumab ozogamicin
		CD38	Daratumumab
		SLAMF7	Elotuzumab
	Overexpressed	EpCAM	Catumaxomab * (EpCAM x CD3) Edrecolomab *
		EGFR	Cetuximab Necitumumab Panitumumab
		HER2	Pertuzumab Trastuzumab (emtansine)
		PDGRFα	Olaratumab
	Immunomodulatory	CTLA4	Ipilimumab
		PD1	Cemiplimab Nivolumab Pembrolizumab
		PDL1	Atezolizumab Avelumab Durvalumab
Inflammatory disease	Soluble cytokine	BAFF	Belimumab
		IL-1β	Canakinumab
		IL-5	Mepolizumab Reslizumab
		IL-12	Ustekinumab (IL-12/23)
		IL-17A	Ixekizumab Secukinumab
		IL-23	Guselkumab T ildrakizumab
		TNFα	Adalimumab Certolizumab pegol Golimumab Infliximab
Infection	Pathogen	*B. anthracis*	Obiltoxaximab Raxibacumab
		C. difficile enterotoxin B	Bezlotoxumab
		RSV protein F	Palivizumab

* Withdrawn