Clustering: a rational design principle for potentiated antibody therapeutics

Oostindie, S.C.

6

AVIDITY IN ANTIBODY EFFECTOR FUNCTIONS AND BIOTHERAPEUTIC DRUG DESIGN

Simone C. Oostindie[1,2], Greg A. Lazar[3], Janine Schuurman[1] and Paul W.H.I. Parren[2,4,*]

Submitted for publication.

1 Genmab, Utrecht, Netherlands
2 Department of Immunology, Leiden University Medical Center, Leiden, Netherlands
3 Department of Antibody Engineering, Genentech, Inc., South San Francisco, California, USA
4 Lava Therapeutics, Utrecht, Netherlands

* Corresponding author

Correspondence
Paul W.H.I. Parren (p.parren@lavatherapeutics.com)

ABSTRACT

Antibodies, the cardinal effector molecules of the immune system, are being leveraged to enormous success as biotherapeutic drugs. Adaptive immune responses consist of epitope-diverse polyclonal antibody mixtures that are capable of neutralizing their targets via binding interference and by mediating humoral and cellular effector functions. A mechanistic theme fundamental to virtually all aspects of antibody biology, including binding strength, clonal selection and downstream effects, is the utilization of avidity to drive and tune responses beyond a physiological threshold. This principle also underlies the mechanisms of action of many successful antibody drug regimens, and forms an important design principle for the engineering of enhanced and novel properties in next generation biotherapeutics with leapfrog potential. Here we describe the conglomerate of avidity interactions as a central trigger for overall efficacy of functional responses both in natural antibody biology and their therapeutic application. Within this framework, we comprehensively review therapeutic antibody mechanisms of action, with particular emphasis on engineered optimizations and platforms. We describe how affinity- and avidity- tuning of engineered immunoglobulin architectures are enabling a new wave of differentiated antibody drugs with tailored properties and novel functions, marshalling the promise of an ever-more optimal fit for treating a wide variety of diseases.

INTRODUCTION

Antibodies are critical components in the humoral adaptive immune response against disease-causing molecules, viruses and cells by flagging them for destruction. Upon exposure to foreign antigen, the immune system mounts a genetically diverse polyclonal response comprising antibodies that recognize multiple (non-)overlapping antigenic epitopes. While these initial antibodies have low binding affinities, activated antibody-producing B cells undergo a conserved affinity maturation process through somatic hyper mutation and clonal selection, thereby successively generating antibodies with greater affinities[1-3]. Target neutralization and elimination is subsequently triggered via multiple tightly regulated processes including protein- and cellular-mediated effector functions.

The development of the hybridoma technology by Köhler and Milstein enabled the stable generation of monoclonal antibodies of defined specificity, hallmarking a new era of antibody-based drug discovery[4]. Owing to their superb specificity, modular and highly adaptable architecture, favorable pharmacokinetic properties and standardizable drug development, antibodies have since grown into an established and exceptionally versatile class of therapeutic agents for treating a wide spectrum of human diseases. The ascendance and impact of antibody-based therapeutics is evidenced by over 100 granted marketing approvals in Europe and US to date, and more than 600 antibody-based therapeutics at various stages of clinical development[5,6]. Building on this success there is a continued search for next-generation antibody-based therapeutics, fueled by progressive mechanistic insights in both correlates of efficacy as well as emerging limitations of contemporary targeted therapies such as disease heterogeneity, plasticity and refractoriness. Indeed, the rise of diverse innovative antibody formats including bi- and multispecifics, tailored Fc-mediated effector functions, diverse isotypes and fragments, and payload delivery of small molecules or bioactive fusion proteins is steering the antibody landscape towards more tailored drug design[7-11]. Emerging developments in immune-oncology furthermore are demonstrating the revolutionary potential of therapeutic antibodies to transmogrify patients' immune systems and achieve long-term protection against disease. Understanding the multidimensional design principles to generate antibody therapeutics that optimally fit the intended target biology and effectively achieve the envisioned mechanistic objectives remain one of the key challenges in drug discovery.

While many factors contribute to the success of antibody-based treatment, central to their pharmacologic mechanism are the interactions with their targets, and more specifically the affinity and avidity of binding interactions[7,12]. Avidity, defined herein as the accumulated binding strength of multiple affinities derived from individual non-covalent interactions, is fundamental to virtually all aspects of antibody biology. The neutralization of soluble proteins such as toxins or cytokines based on single binding interactions display a limited potency ceiling, which may be surpassed by orders of magnitude in antibody combinations[13,14]. Furthermore, it is generally understood that singular antibody-antigen binding interactions alone are insufficient to achieve successful neutralization or effector function activity for eliminating pathogens or diseased cells[15-18]. Considerable evidence suggests that antibody-based effector functions are triggered by multivalent target binding and clustering of IgG molecules on the cell surface[18-22]. Avidity-driven surface clustering of IgG molecules can be interpreted to serve as a threshold for effector function activation, by providing an "avid docking surface" for the subsequent binding and activation of immune effector molecules. In this review, we describe recent advances in understanding the contribution of avidity interactions to both natural- and therapeutic antibody-based mechanisms of action. The unifying thread is the fundamental role of avidity in determining the quality or "impact" of antibody functional responses. Special emphasis is placed on engineering strategies and platforms that utilize avidity tuning to boost therapeutic activity, including classical effector functions as well as novel engineered mechanisms of action. Within this conceptual framework we also discuss current translational efforts regarding avidity-based antibody concepts in the clinic and provide future perspectives that we believe will aid the development of the next wave of differentiated biotherapeutics that may leapfrog contemporary approaches.

THE ROLE OF AVIDITY IN ANTIBODY BIOLOGY

Antibody functional responses

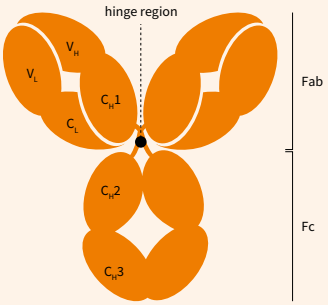
Antibody or immunoglobulin (Ig) function is characterized by three distinct regions of the Ig molecule (Box 1): a fragment antigen-binding (Fab) region defining target specificity and affinity, a fragment crystallizable (Fc) region that interacts with immune effector molecules, and a hinge region linking the Fab and Fc. The iconic Y-shaped IgG structure solved in 1963 provided a first view of the bivalency of the molecule and functional implications for multivalency[23,24]. Two Fab fragments are connected to the Fc by a hinge region, which flexibly links interactions with antigen effector molecules. The strength of a single binding interaction between an antibody Fab fragment and antigen is defined by the term *affinity*. In biology, functional interactions are often not only dependent on affinity, but also on *avidity*. Avidity is defined as the increase in binding strength that results from multivalent interactions, which for antibodies may occur upon binding to antigens on the surface of cells or pathogens, interaction with effector molecules, or cooperation in polyclonal responses[25].

The importance of affinity and avidity to antibody-antigen recognition in both natural immunity and therapeutic application has long been recognized[12,25,26]. The primary mechanism by which antibodies combat infection is through binding and direct neutralization of a target on a pathogen. Antibody Fab arms bind specific pathogen structures or antigens, thereby preventing interactions with host cell receptors and blocking their functionality such as viral entry into a cell. Already in 1937, Burnet and colleagues recognized that a single antibody binding event was likely insufficient to inactivate viruses and argued that neutralization occurs through multivalent antibody binding to a significant proportion of viral epitopes[27]. It is this polyclonal immune complexation, rather than single molecular binding events, that is the central trigger for downstream humoral and cellular responses. Importantly, antibodies are not merely antigen binding molecules, as their Fc domain allows for interactions with multiple immune effector molecules present in human plasma or expressed on immune cells. Classical antibody Fc-mediated effector mechanisms such as complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) are key mechanisms for the elimination of pathogens or diseased cells such as infected or cancerous cells. As with immune complexation, a single binding interaction between antibody and antigen is generally not sufficient to induce an effective Fc-mediated cellular response. Altogether,

Box 1

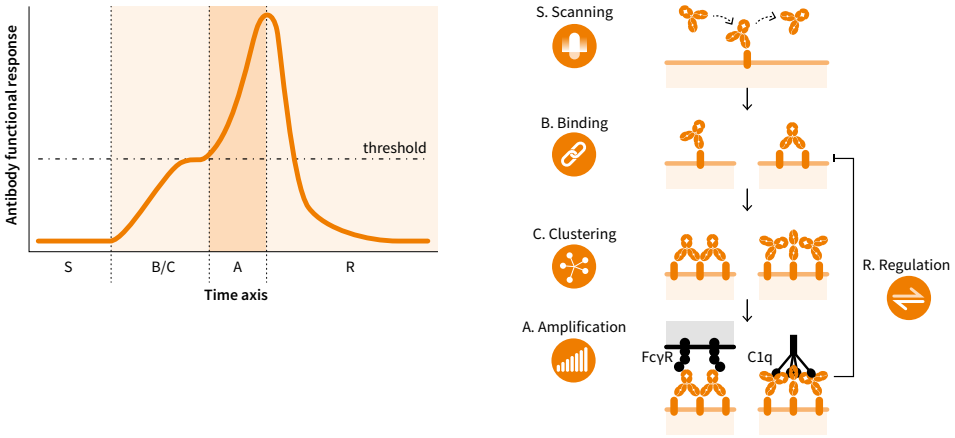
Human immunoglobulin architecture.

Human immunoglobulins (Igs) consist of three distinct regions including an antigen-binding fragment (Fab) region that binds antigen, a crystallizable fragment (Fc) region that interacts with immune effector molecules and a hinge region that links the Fab to the Fc and defines conformational flexibility. A monomeric Ig molecule is Y-shaped and usually composed of four distinct protein chains including two identical heavy- and light chains (HC and LC). The HC is comprised of three or four generic constant domains (C_H), depending on the antibody isotype, while the LC contains a single constant domain (C_L). The constant domains together form the Fc-, hinge- and base of the Fab region. The Fab region comprises the variable domains which determine antigen binding specificity and affinity. Disulfide bonds shape the overall quaternary structure of the molecule, by linking the two HC in the hinge region as well as the HC and LC in the Fab region. In humans, variations in the HC constant domain during an immune response generates five different isotypes; IgM, IgD, IgG, IgA and IgE. IgG and IgA can be further subdivided into subclasses 1-4 and 1-2 respectively. Whilst IgG, IgD and IgE exist as monomers, IgA and IgM may contain an additional polypeptide J-chain that allows the formation of dimers and pentamers respectively. A small fraction of IgM antibodies exist as hexamers in which the J-chain protein is exchanged by an extra monomer subunit. IgA may further contain a secretory component that provides protection against proteolytic degradation. The C_H domains of IgG interact with the neonatal Fc receptor (FcRn) which protects from catabolism and thereby substantially increases IgG plasma half-life. Ig isotypes and subclasses can further be distinguished by variation in the hinge length, the number and location of disulfide bridges and *N*- or *O*-linked glycosylation within the HC. The hinge region serves not only as a connector but also modulates to effector function potency. The hinge region therefore represents a third functional domain; indeed modification of the hinge length and flexibility has allowed for fine tuning of IgG effector activity[157]. Together, changes in antibody valency, hinge length and flexibility and glycosylation status can influence avidity interactions and overall functional response kinetics.



through high avidity binding interactions via both Fab and Fc and with the hinge in a modulatory role, antibodies constitute a remarkably sensitive adaptor system that enables rapid initiation and amplification of different effector mechanisms in combatting human disease.

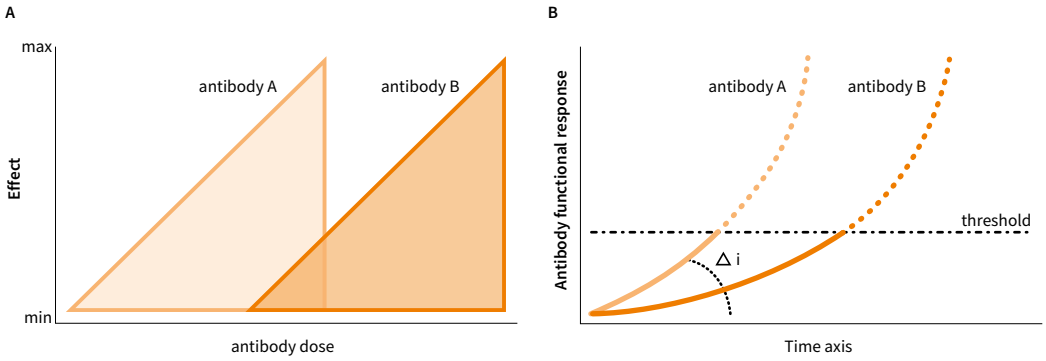
In our view, response kinetics and thresholds governing antibody function can be described in terms that are generally observed in many biological systems that integrate input, output and feedback (Figure 1). We distinguish five distinct activation phases that include a scanning, binding, clustering, amplification and regulation phase. Antibody binding to antigen is a reversible process that follows the law of mass action and is highly dependent on the affinity of antibody for its target. Association and dissociation constants determine the on- and off-rate of binding kinetics at equilibrium (S – scanning phase). Thus, antibody-antigen complex formation is favored when the on-rate is faster than the off-rate (B – binding phase). Subsequently, tethering of multiple Fab arms to antigens on the cell surface enhances the overall strength or avidity of binding (C – clustering phase). The overall avidity sum of these interactions is defined by the intrinsic affinity, valency, flexibility and structural arrangement of proteins in the complex. Antibody-antigen clustering consequently provides an avid docking surface for Fc-mediated binding and clustering of immune effector molecules, thereby triggering potent activation of the functional response (A – amplification phase). However, conditions to reach the threshold before response amplification may vary per effector mechanism. For instance, different types of effector mechanisms might require different antibody concentrations to reach the maximal effect as they operate at distinct target occupancy ratios (Figure 2A)[28]. Similarly, other parameters such as antigen expression and distribution, the presence of systemic effector elements or different Ig subclasses and expression of inhibitory receptors can all impact the overall avidity of antibody interactions and consequent amplification of a functional response (Figure 2B)[29,30]. We propose that all these parameters combine into one overall "impact factor", illustrated by Di, which can be used to describe the quality of an antibody functional response. Finally, negative feedback occurs when the output of a system acts to reduce or dampen the response amplification (R – regulation phase; Figure 1). After amplification of the antibody functional response, regulation can be induced by target-related outputs such as elimination of target cells, target density dropping below the amplification threshold or regulatory molecules expressed on the target cell or recruited from plasma. Similarly, systemic regulation can occur when there is a shortage of effector molecules or cells, or by interfering Ig subclasses[31-33]. Although not the focus of this review, insufficient regulation may conversely cause an antibody functional response to become pathogenic through excessive and/or constitutive activation.



▲ **Figure 1**

Response kinetics governing antibody function.

The kinetics of antibody functional response activation are distinguished by five different phases integrating common biological mechanisms of input, output and feedback. (**S**) Scanning phase: association and dissociation constants determine the on- and off-rate of antibody binding kinetics at equilibrium. (**B**) Binding phase: monovalent or bivalent antibody-antigen complex formation is favored when the on-rate is faster than the off-rate. (**C**) Clustering phase: tethering of multiple Fab arms to antigens on the cell surface enhances the overall binding avidity. (**A**) Amplification phase: antibody-antigen clustering provides an avid docking surface for Fc-mediated binding of soluble or cell-bound immune effector molecules, thereby surpassing a threshold and triggering potent activation of the functional response. (**R**) Regulation phase: antibody functional response amplification may be regulated or dampened by for example elimination of target cells, target density dropping below the amplification threshold or regulatory molecules either expressed on the target cell or recruited from plasma.



▲ **Figure 2**

Factors impacting antibody functional response activation

(**A**) Schematic representation of dose-effect relationships for antibodies with different effector mechanisms. Antibody A may require different antibody concentrations to reach the maximal effect compared to antibody B, as both operate at distinct target occupancy ratios. (**B**) Schematic representation of how conditions to reach the threshold before antibody functional response amplification may vary per effector mechanism. Such varying conditions may be defined by different parameters including; (1) antibody dose (A), affinity and valency, (2) antigen expression and distribution, (3) type and presence of systemic effector elements and (4) presence of regulatory molecules expressed on the target cell or recruited from plasma. All these parameters combine into one overall "impact factor", illustrated by Δi, which could be used to describe the quality of an antibody functional response. Note that the example is not intended to imply that antibody B is necessarily 'worse' than antibody A. Depending on the intended use, it may be required to tune a functional response, e.g. to retain activity against target cells while minimizing activity against normal cells expressing the target antigen.

The quality and impact of antibody functional responses is therefore dependent on multidimensional effects anchored in avidity-based interactions between antibodies, antigens and effector molecules. How these interactions orchestrate both natural, autoimmune and therapeutic antibody mechanisms of action is detailed and discussed in the following subsections of this review. Furthermore, we discuss how avidity tuning may serve as a basic engineering principle for optimizing the Δi and thereby the functional response of antibody-based therapeutics.

The humoral adaptive immune response

The humoral adaptive immune response does not consist of a single species of antibody, but instead comprises a complex polyclonal mixture of multiple antibodies directed towards diverse immunogenic epitopes that change and accumulate over time. The huge potential and versatility of this response is illustrated by the projection that humans may produce a quintillion (10^{15}) different antibodies[34]. The role of avidity in humoral immunity is already evident early in the initiation of the immune response, both in the context of triggering antibody production by B cells as well as in their maturation. Upon entry of foreign antigens into lymphoid tissues, naïve B-cells present in lymph node follicles are activated through antigen binding to their B-cell receptors (BCRs, Box 2). BCRs expressed on mature B cells are integral membrane Ig molecules that are activated following antigen-induced aggregation (Figure 1, binding/clustering phase, Figure 3A)[35]. Furthermore, while several different models have been proposed for BCR activation, considerable evidence suggests that BCRs display activation-dependent localization into membrane micro domains, likely facilitating higher order BCR clustering and signal transduction[36,37]. Upon activation, some IgM^+ B cells become plasma cells in which IgM expression through alternative RNA processing switches from being primarily membrane-associated to being abundantly secreted (Box 2)[38]. Secreted IgM antibodies are predominantly pentameric molecules (hexameric molecules comprise <5% of serum IgM[39,40]) that, despite binding with characteristically low intrinsic affinity (K_a 10^4–10^6 $[mol/L]^{-1}$), are able to avidly engage immunogens due to their 10 identical epitope binding sites and high conformational flexibility[41]. As discussed further in a later section, IgM antibodies are especially effective in protection against microbes with highly expressed, closely spaced surface epitopes, a consequence in part of efficient avidity-based interactions between IgM and the complement system. Other activated IgM^+ B cells undergo rapid proliferation aided by additional stimuli provided by T cells, thereby triggering the activation-induced deaminase (AID) enzyme that mediates somatic hypermutation (SHM)[42]. B-cell clones producing higher affinity BCRs are consequently favored in a Darwinian competition for antigens and T-cell help that drive B-cell clonal selection and affinity maturation, resulting in an increase in intrinsic affinity up to 10^{10} $[mol/L]^{-1}$ [43,44]. Addition-

Box 2

Human immunoglobulin functional properties

B-cell receptor

Each of the Ig (sub)classes may be expressed in monomeric form with an integral membrane domain to form the B-cell receptor (BCR) on a B cell's surface. Expression of membrane versus secreted form is regulated by alternative RNA processing. Secreted Ig is identical in sequence to the BCR except for the absence of the transmembrane region and potential post-translational modifications or quaternary structures as discussed below.

IgM: Secretory IgM largely exists as pentamers linked together by disulfide bonds and a polypeptide J-chain, while a small fraction exists as a covalent IgM hexamer in absence of J-chain. Owing to their structural and functional differences, pentameric and hexameric IgM might be viewed as distinct IgM subclasses[64]. Studies have shown that both pentameric and hexameric IgM adopt a hexagonal platform conformation supporting high avidity binding interactions with C1q and efficient complement activation[178,179].

IgG

IgG is most abundant in human serum and accounts for about 10-20% of plasma protein. The four different subclasses in order of decreasing abundance are named IgG1, IgG2, IgG3 and IgG4. Each subclass contains a unique profile with respect to antigen-, complement- and FcγR binding. Structural determinants in the middle or "core" hinge region in particular can influence antibody function. For example, modification of glycan N297 impacts FcγR binding, while increasing hinge length and flexibility enhances binding to antigen and immune complex formation as well as binding to complement component C1q[95,157,180]. The flexibility of the Fab arms impacts the relative binding of subclasses to FcγRs and C1q and is ranked as: IgG3 > IgG1 > IgG4 > IgG2.

IgD

IgD is primarily expressed as a transmembrane antigen receptor on naïve mature B cells. IgD possesses a long hinge region with high flexibility and is thereby capable of acquiring a T-shaped structure. This structural flexibility was suggested to potentially contribute to the regulation of B-cell responsiveness to different types of antigens due to preferential binding of IgD to multimeric rather than monomeric antigens[181,182]. Secreted IgD weakly interacts with complement or FcδRs, but binds basophils, mast cells, monocytes and dendritic cells in an FcδR-independent manner, thereby contributing to mucosal homeostasis through the production of antimicrobial peptides and inflammatory cytokines.

IgA

IgA has two subclasses, IgA1 and IgA2, which are distinguished by a heavily glycosylated T-shaped hinge and a more rigid Y-shaped hinge respectively. IgA exists in monomeric- and dimeric (monomers linked by a polypeptide J-chain and a secretory component) form. Monomeric IgA is abundantly present in serum, while dimeric IgA (secretory IgA) is mainly found in secretions lining the mucosal surfaces. IgA immune complexes efficiently interact with FcαRI on myeloid cells including neutrophils and macrophages[183].

IgE

IgE is the least abundant and fastest clearing (half-life <1 day versus ~3 weeks for IgG) isotype in human plasma. While IgE does not activate complement, it potently binds to the high-affinity FcεRI on mast cells. IgE glycosylation is essential for IgE-FcεRI interactions and this requirement is primarily attributed to one of seven N-linked glycan sites (N394)[184]. IgE can remain bound to FcεRI for weeks to months, thereby contributing to a long tissue half-life[185].

ally, AID facilitates class- or isotype switching from IgM to IgG1-4, IgA1-2 or IgE that results in changes in the antibody Fc domain and associated effector function properties such as complement fixation and Fc receptor (FcR) binding. Through these processes, the adaptive immune system generates a highly diverse pool of antibodies capable of effectively recognizing and eliminating antigens and associated pathogens or diseased cells. Conversely, as typical in any biological system, B-cell activation is subject to physiological control mechanisms via Fc-mediated antibody feedback. Secreted antibodies inhibit B-cell activation by forming antibody-antigen immune complexes that, in the context of interaction with membrane Ig (through antigen), co-engage the inhibitory FcγRIIb (through the antibody's Fc). Simultaneous ligation of both the BCR and FcγRIIb results in inhibition of BCR complex signaling through phosphatases associated with the cytoplasmic tail of FcγRIIb (Figure 1, regulation phase)[35,45,46]. Overall, the BCR diversification that is triggered by antigen exposure is highly conserved over 200 million years of evolution, which despite mechanistic differences in the generation of repertoire diversity (e.g. through combinatorial rearrangements versus gene conversion) follows similar principles in all vertebrates, illustrating the crucial role of affinity and avidity-based antibody interactions in adaptive immunity[1,47,48].

Antibody-mediated effector functions

An antibody-mediated immune response is a culmination of effector functions mediated through both the Fab- and the Fc domain. How antigen binding by the Fab domain translates into Fc-mediated activation of effector functions has been an active area of research for many years. In addition to Fab-me-

diated neutralization of pathogen-host interactions, the Fc domain recruits serum complement component C1q and binds FcRs on innate immune cells to induce cytotoxicity, phagocytosis, and immune cell activation including cytokine release. While the processes of antigen binding by the Fab domain and effector function activation by the Fc domain have long been thought to act independently, multiple studies have challenged this view, and an optimal configuration of immune complexes likely exists for different types of effector functions. The antigen:antibody ratio greatly influences immune complex size, where antibody shortage or excess can reduce FcR-mediated effector potency due to suboptimal clustering or self-competition, also referred to as a hook or prozone effect[49]. In addition to the associative cooperativity observed in effector function activation by IgGs, there is also accumulating evidence for allosteric or intramolecular cooperativity[30]. For example, the optimal immune complex stoichiometry required for complement activation may be facilitated by IgG Fc-Fc interactions and stabilized by the associated Fab domains[50,51]. Fab and Fc function may even be considered interdependent, as efficient effector function activation requires clustering of cell-bound IgGs through both Fab-Fab and Fc-Fc interactions, thereby forming optimal docking sites for recruitment of C1q and crosslinking of FcRs on innate immune cells. Thus, knowledge of how antibody structure impacts function is crucial for understanding different antibody-mediated effector mechanisms, as further discussed individually in the following subsections.

Neutralization

Fab domain-mediated neutralization is the simplest form of pathogen, toxin or self-antigen inactivation. Binding of the Fab domain to specific pathogenic structures prevents interactions with host cells, thereby blocking toxin activity or viral entry into the cell (Figure 3B). While the role of avidity in antibody-mediated neutralization of toxins and viruses has long been recognized, its crucial importance is exemplified by human immunodeficiency virus type 1 (HIV-1) that escapes neutralizing antibodies not only by its rapid mutation rate and shielding of conserved epitopes on the spike trimer, but also by impeding bivalent high avidity antibody binding. In contrast to antibodies targeting viruses such as influenza and respiratory syncytial virus (RSV), which are able to bind viral surface bivalently and thereby take advantage of avidity effects[52,53], antibodies against HIV-1 envelope glycoproteins are thought to predominantly bind the viral surface monovalently[54-56]. Several studies suggest that HIV-1 evades bivalent high avidity antibody binding through its low spike density on the virion surface combined with an unfavorable distribution of epitopes on the spike trimer, thereby limiting both inter- and intra-spike crosslinking[57]. Often, high avidity antibody binding alone is not enough to confer sufficient toxin or pathogen neutralization and instead requires the Fc domain to augment neutralization of toxin activity or pathogen entry through

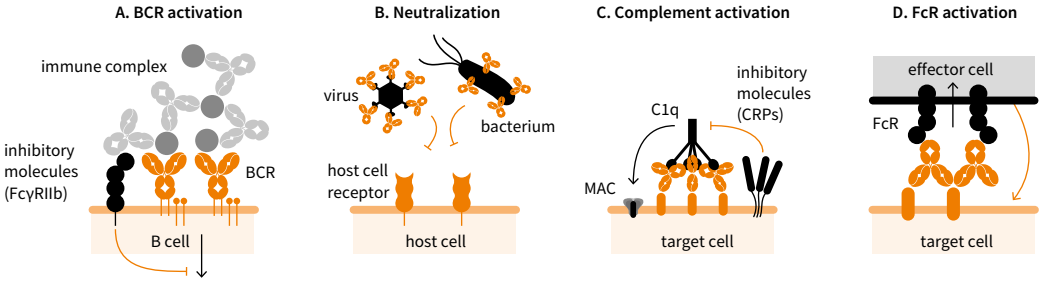
recruitment of complement or binding of FcγRs on immune cells (Figure 1, amplification phase). In the case of HIV-1, the neutralizing capacity of HIV-targeting broadly neutralizing antibodies was compromised upon administration of Fc domain variants that had decreased affinity for FcRs[58,59]. In autoimmune disease, the avidity of autoantibodies against self-antigens impacts on their pathogenicity, such as for example shown by the induction of symptoms of Myasthenia gravis in monkeys treated with bivalent IgG1 antibodies but not monovalent IgG4 antibodies against acetylcholine receptor[60].

One of the most striking illustrations of the selective pressure of repeating microbial epitopes is the VH domain-swapped dimer formed by the anti-HIV-1 antibody 2G12, which enables multivalent interactions with conserved carbohydrate clusters on the HIV-1 envelope glycoprotein subunit gp120[61]. While the germline precursor to this antibody adopts a conventional (non-swapped) structure, a minimum of five somatic mutations permitted a significant fraction of domain exchange[62], suggesting this unique structural rearrangement may be more generally accessible to antibodies. The plot thickened further upon subsequent discovery that domain swap was not limited to intramolecular IgG, but also promoted an intermolecular IgG dimer composed of four Fabs and two Fcs that provided >50-fold neutralization potency compared with monomeric 2G12[63]. This work demonstrates not only the important role that avidity plays for microbial epitope neutralization, but also illustrates how nature truly is the unrivalled antibody engineer, able to couple the extensive immune repertoire with modular immunoglobulin architecture to bring creative avid solutions to the fight against infectious agents.

Complement activation

The complement system is a potent innate immune defense mechanism composed of an amplifiable enzymatic cascade that kills pathogens and attracts immune effector cells. Complement is activated through three distinct pathways; the classical-, lectin- and alternative pathway, which depend on binding of C1q, mannan-binding lectin or spontaneously activated complement to pathogenic surfaces respectively. All pathways ultimately converge in the generation of C3 and C5 convertases that cooperate in the production of opsonins, anaphylatoxins, chemoattractants and the formation of the membrane attack complex (MAC) that breaches the target cell membrane to kill the cell[64].

The classical pathway is triggered upon binding of C1q to the Fc region of cell-bound IgG or IgM antibodies. C1q consists of six collagen-like triple-helical stalks connected to a globular headpiece that closely resembles the shape of a bunch of tulips[65]. The binding affinity of monomeric IgG Fc for C1q is weak (K_d ~ 10^4 M) resulting in highly transient interactions (Figure 1 – scanning phase). Consequently, functional C1q binding and activation requires an



▲ **Figure 3**

Avidity in antibody biology

(**A**) B-cell receptor (BCR) activation and FcγRIIb inhibition following antigen-induced aggregation during clonal selection. (**B**) Fab domain-mediated neutralization of pathogen structures to prevent interactions with host cells and blocking pathogen entry into the cell. (**C**) Complement activation initiated by IgGs that assemble into ordered hexameric structures through non-covalent Fc-Fc interactions. Ordered IgG Fc domains serve as an optimal docking structure for C1q binding, which trigger a series of proteolytic events leading to lysis of the cell via formation of the membrane attack complex (MAC). Complement activation is regulated by membrane-bound complement regulatory proteins (CRPs). (**D**) FcR-mediated (innate and adaptive) immune effector function activation through multimerization of cell-bound IgG. Activation of FcRs may induce a number of antibody-dependent cellular effector functions including ADCC and ADCP.

increase in apparent binding affinity (K_d ~ 10^8 M) through multivalent antibody interactions[66] IgM antibodies naturally exist in multimerized form, either organized into a pentameric (>95%) or hexameric arrangement (<5%), thereby providing them with a superior ability to interact with C1q to activate complement[39,40,67]. Diebolder et al. reported that IgGs assemble into ordered hexameric structures after target engagement on the cell surface through non-covalent interactions between neighboring IgG Fc domains (Figure 1 – binding/clustering phase)[19]. These ordered IgG Fc domains form an optimal docking structure, effectively serving as a danger signal for C1q binding, enabling subsequent C1 activation and amplification of the complement cascade (Figure 1 – amplification phase, Figure 3C). The high amplification potential of the complement system is counterbalanced by strong regulation through numerous soluble (e.g. C1 inhibitor, C4bp, factors I and H) and cell surface expressed proteins, (e.g. CD46, CD55 and CD59; Figure 1 – regulation phase)[68]. Compelling evidence for the requirement of higher order IgG oligomers for optimal complement activation was provided by mass-spectrometry-, cryo-EM tomography- and mutational studies. These studies demonstrated that IgG monomers, dimers and trimers do not significantly contribute to CDC and illustrated the requirement of at least four C1q binding sites to achieve sufficient CDC potency[17,50,51]. Additional studies focusing on the dynamics and kinetics of IgG-C1q interactions showed that at high antigen density, oligomerization may occur through IgG recruitment from solution to predominantly bivalently-attached IgGs that then serve as nucleation sites. However at low antigen density or high IgG concentrations, predominantly monovalently-bound IgGs have been reported to additionally be capable of oligomerization via lateral

diffusion[51]. Interestingly, functionally monovalent antibodies (e.g. bispecific antibodies comprising only a single Fab arm for a target cell), were shown to induce CDC more potently, potentially due to a superior ability to form hexamers and engage C1[17,19,51,69].

As discussed earlier, an ideal immune complex stoichiometry likely exists for efficient activation and amplification of Fc-mediated effector functions such as the complement cascade. Apart from optimal Ig oligomerization, a number of additional factors can influence triggering of complement activity. For example, there are several structural prerequisites that affect IgG Fc-Fc interactions and optimal placement of the hexameric C1q docking platform relative to the cell surface, including antigen-dependent constraints such as size, distribution, epitope geometry and orientation. These antigen- and epitope-dependent constraints are perhaps most evidently exemplified by recent studies that solve the enigma of the existence of two functional types of antibodies against the well-known therapeutic target CD20 in B-cell malignancies and inflammatory diseases. Type I CD20 antibodies are known to induce CD20 translocation into lipid rafts and kill via complement and ADCC, whereas type II's do not cluster CD20 and instead mediate cell killing via apoptosis and ADCC[70,71]. In these studies, the authors used structural and thermodynamic analyses to illustrate that CD20 forms a dimer that provides two binding sites for type I CD20 antibodies such as rituximab and ofatumumab, thereby facilitating efficient antibody clustering and complement activation. By contrast, each antigen binding site (i.e. Fab arm) of the type II CD20 antibody obinutuzumab can only bind a single CD20 dimer, thereby precluding higher order avidity interactions[21,72]. In another study, combinations of IgG antibodies simultaneously targeting CD20 and CD37 antigens were shown to induce synergistic CDC of tumor B cells by forming mixed hetero-hexameric complexes on the cell surface, while the individual antibodies were unable to activate complement[73]. This cooperativity between antibodies targeting two different antigens is likely caused by an optimal (re)distribution of antigen targets on the cell surface, thereby enabling colocalization and co-clustering. Additionally, antibody-dependent structural constraints such as hinge length and glycan heterogeneity also influence Ig oligomerization and complement activation[74-77]. These structural elements also account for the differences in complement activation observed between IgG subclasses (Box 2). Similarly, the presence of different IgG subclasses (e.g. certain IgG4 forms that are functionally monovalent[60]) and other classes like IgA and IgE could potentially dampen or regulate IgG-mediated complement activation, resulting from differences in flexibility, conformation, valency and steric interference with Fc-Fc interactions required for efficient oligomerization[60,78-81]. Thus, efficient activation and amplification of the complement system is dependent on a complex interplay between both Ig Fab- and Fc-mediated avidity interactions (Figure 1).

FcR-mediated cellular effector functions

The Fc domain of antibodies can interact with different FcRs expressed on various immune cells to mediate effector functions such as ADCC and ADCP. IgG binds to FcγRs (Box 2), which are broadly classified as either activating or inhibitory, depending on whether their intracellular signaling domain contains an immunoreceptor tyrosine activating motif (ITAM) or immunoreceptor tyrosine inhibitory motif (ITIM) respectively. Activating FcγRs in humans include FcγRI (CD64), FcγRIIa (CD32a) and FcγRIIIa (CD16a), in addition to the less well characterized FcγRIIc (CD16c). By contrast, FcγRIIb (CD32b) represents the sole inhibitory FcγR, regulating the function of activating FcγRs, as well as serving as the main scavenging receptor in the liver, thereby further controlling antibody production by B lymphocytes. Finally FcγRIIIb (CD16b) uniquely contains no intracellular signaling domain and is instead glycosylphosphatidylinositol (GPI)-anchored to the membrane. FcγRs are differentially expressed on lymphoid- and myeloid-derived effector cells, although the receptor distribution is unique to each cell type[82]. FcγRIIIa is expressed on monocytes/macrophages and natural killer (NK) cells, and is the main receptor for mediating ADCC, promoting release of cytotoxic granules and pro-apoptotic signaling molecules after engaging IgG-opsonized target cells. Phagocytes such as macrophages and dendritic cells (DCs) express a combination of different FcγRs including FcγRI, FcγRIIa, FcγRIIIa and FcγRIIb that mediate not only ADCP for innate destruction of IgG-opsonized targets cells, but can also promote cross-presentation to T cells to mediate adaptive cellular immunity for a vaccinal effect[83-85]. Studies have shown that FcγRIIa and in particular the balance of signals between activating FcγRIIa and inhibitory FcγRIIb are important determinants of the ability of antibody:antigen immune complexes to activate monocytes and DCs[83,86-88]. While the link between FcγRs and cellular immunity has historically been indirect through antigen presentation by cells, in a recent twist it was demonstrated that FcγRIIa is capable of directly promoting activation of CD4+ T cells by IgG immune complexes[89].

FcγRs are bound by the different human IgG isotypes with varying affinities. Only FcγRI can bind monomeric IgG with high, nM range, affinity. All other FcγRs exhibit low, µM affinities for monomeric IgG, and as a consequence monomeric binding is transient under physiological conditions (Figure 1 – scanning phase)[90]. In the extreme, low affinity FcγRs including FcγRIIa and FcγRIIIa only interact with multimerized IgG on opsonized cells or multimeric IgG immune complexes under physiological conditions, thereby preventing inappropriate effector cell activation in the absence of a pathogenic trigger. Multimerization of cell-bound IgG strengthens FcγR binding interactions, thereby generating sufficient avidity (Figure 1 – binding/clustering phase) to trigger receptor signaling through phosphorylation of ITAM domains

(Figure 1 – amplification phase), which in turn leads to elimination of target cells via ADCC or ADCP and other immune cellular effects (Figure 3D).

Besides IgG binding to FcγRs, other Ig subclasses bind to corresponding FcRs including FcαRI (IgA), FcεRI (IgE), FcμR (IgM) and IgD (IgδR) and Fcα/μR (IgA/IgM). IgA plays a key role in mucosal areas by regulating the tolerance to- and protection from exposure to antigens, food and (commensal) microorganisms, while IgE is well known for its role in allergic reactions through binding the high affinity to FcεRI expressed on mast cells (Box 2)[91,92]. IgA immune complexes avidly bind IgαRI expressed on myeloid cells including neutrophils, eosinophils, monocytes and macrophages. IgA antibodies have been reported to recruit neutrophils by crosslinking FcαR1 and tumor cells in the context of a bispecific molecule and additionally induced efficient neutrophil-mediated killing of various tumor cell types via ADCC or ADCP[92-94].

A multitude of factors influence the overall avidity and degree of antibody-FcR crosslinking, including antibody and FcR binding affinity, location of the antigenic epitope, and cell surface rigidity[22]. Several studies have demonstrated that different FcγR allotype variants display a wide range of binding affinities for different IgG subclasses, which may impact FcγR crosslinking and activation[90,95]. More recently, Mazor et al., showed that at saturating concentrations, high affinity antibody variants targeting EGFR and HER2 elicited a weaker ADCC response compared to low affinity antibody variants[49]. The observed difference was attributed to increased cell surface opsonization by low affinity antibodies, which display faster off rates and are thus expected to predominantly engage their target via monovalent binding interactions, leading to a higher local density of Fc domains. In contrast, high affinity antibodies were hypothesized to engage target bivalently, thereby occupying more antigen sites and consequently displaying lower local Fc domain densities. We additionally suggest that monovalent (low affinity) binding interactions may allow greater mobility and flexibility in crosslinking FcγRs. One could also hypothesize that high affinity interactions and a slow off-rate may thwart the assembly of antibodies into higher order avidity structures. This view is supported by the earlier mentioned studies demonstrating that while bivalent (high affinity) binding can lead to stagnation, monovalent binding can trigger association and enhanced complement activation via lateral diffusion across cell surfaces[51,96]. In a related study, phagocytosis of emulsion droplets was shown to be more efficient at lower IgG concentrations compared to solid particles[97]. The authors speculated that lateral diffusion of IgGs attached to the surface observed in emulsion droplets was otherwise prevented in solid particles displaying higher cell surface rigidity. Altogether these examples illustrate the interplay between affinity and avidity interactions in tuning of antibody effector biology for responsiveness to infectious immune complexes.[90]

AVIDITY ENGINEERING OF ANTIBODY THERAPEUTICS

Antibodies have become widely established as therapies for numerous diseases, including cancer, infectious diseases, inflammatory diseases and autoimmunity[16]. The challenges of designing differentiating therapeutics, next to disease heterogeneity, therapy resistance and escape have shifted therapeutic antibody development from canonical IgG antibodies towards formats with novel functionalities, increased potency and breadth. The search for approaches to enhance antibody function is not surprising considering the limitations imposed by monospecificity. Indeed, the single-agent use of monoclonal antibodies for therapy seems contradictory in light of the avidity-tuned polyclonal nature of antibody responses generated during natural immune responses.

The avidity engineering toolbox

A broad spectrum of novel antibody engineering strategies and format concepts are emerging that enhance or tune avidity to boost 'classical' effector functions and enable novel therapeutic mechanisms. Some approaches may be biologically relevant optimizations (e.g. effector function enhancement), while others design elements for *de novo* yet non-native mechanisms of action (e.g. effector cell redirection or receptor agonism).

We describe an 'avidity engineering toolbox', and discuss the impact of different strategies on the different phases of antibody functional response activation as defined in Figure 1. Strategies include (i) multi-targeting approaches directed towards multiple epitopes, (ii) multi-targeting approaches directed to multiple surface receptors expressed on the same- or on different target cells, (iii) increasing antibody valency and (iv) optimizing binding towards complement or FcγRs. Many of these approaches have advanced into clinical development, and as of March 2021, the commercial clinical pipeline included 26 programs leveraging avidity to increase antibody function (Table 1). This overview table excludes clinical programs investigating obligate or combinatorial bispecific antibodies for which we refer to a recent review by Labrijn et al[10]. In the subsections below we highlight some of these key engineering strategies exploiting avidity to boost antibody functional responses.

Multispecific targeting

In recent years, a growing number of antibody format technologies were introduced that enable increased antibody binding by targeting more than one epitope or target. While the initial design goal may be focused on improv-

ing tissue selectivity through targeting multiple disease-related targets or signaling pathways, multi-targeting concepts may simultaneously benefit from enhanced functional activity by already promoting avidity interactions in early phases of antibody functional response activation. Multi-targeting concepts include combinations of two or more antibody molecules with different defined specificities; e.g. antibody mixtures or designer polyclonals, and antibody architectures and formats combining two or more specificities in a single antibody molecule; e.g. bispecifics or multispecifics.

Targeting multiple epitopes

Multi-targeting antibody technologies that come closest to mimicking high avidity interactions of natural polyclonal antibodies are those directed towards non-overlapping epitopes on the same target (Figure 4A). Such technologies often build on increasing target occupancy and local Fc domain density to enhance antibody clustering and overall functional activity, while some may even potentiate additional novel effector functions (Figure 1, binding/clustering phase). For example, while complement activity by monoclonal antibodies has rarely been shown in solid tumor models, complement activation was reportedly triggered by combining two antibodies targeting non-overlapping epitopes on EGFR[98,99]. Similarly, biparatopic bispecific heavy chain antibody constructs targeting non-overlapping epitopes on CD38 were reported to potently enhance CDC activity in multiple myeloma and Burkitt's lymphoma cell lines[100]. Potentiation of CDC was additionally reported for simultaneously targeting non-overlapping epitopes on a number of other cell surface targets including CD37, HLA, Factor H-binding protein and folate receptor[101-104]. Targeting multiple non-overlapping epitopes may overcome complement defense mechanisms through increased binding and crosslinking of the same target, thereby exploiting the natural capacity of antibodies to form hexameric complexes and induce CDC.

Targeting non-overlapping epitopes using multi-specific antibody technologies is being explored in viral diseases to reduce resistance and lower the risk of viral escape. Trispecific antibody formats combining binding domains of broadly neutralizing antibodies (bNAbs) directed against different functional HIV-1 epitopes on the envelope spike exhibited higher neutralization potency and breadth compared to (combinations of) the parental bNAbs both in vitro and in vivo[105,106].

Formats that enhance antibody binding by targeting multiple non-overlapping epitopes on a single antigen represent the largest group of clinical programs leveraging avidity tuning (Table 1). Such formats range from mixtures of two or more non-competing antibodies to bi- or multi-specific antibody architectures combining different epitope binding specificities in a single molecule. A num-

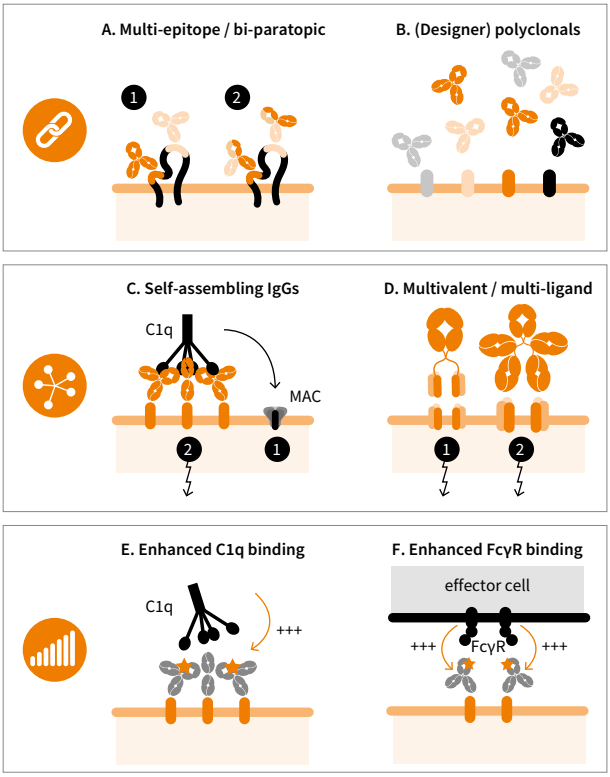


Figure 4

Antibody avidity engineering strategies
(**A**) Enhancing antibody binding; (1) dual epitope targeting, (2) biparatopic bispecific targeting. (**B**) Enhancing antibody binding; (designer) polyclonals. (**C**) Enhancing antibody (-mediated) clustering; (1) self-assembling IgGs inducing complement activation or (2) self-assembling IgGs inducing cell surface receptor clustering and agonism. (**D**) Enhancing antibody-mediated clustering; (1) Hera-ligand technology or (2) multivalent antibody architectures inducing TNFRSF receptor clustering and agonism. (**E-F**) Enhancing antibody functional response amplification; increasing C1q- (**E**) and FcγR (**F**) binding affinity.

ber of clinical programs are investigating the efficacy of therapeutics targeting multiple epitopes on one of the EGFR family members that are overexpressed in many solid tumor indications[107-109]. There are substantial clinical data illustrating that patients who initially respond to EGFR-specific antibodies eventually become resistant. Targeting multiple receptor epitopes may overcome resistance, as was demonstrated by a proof of principle study with the anti-EGFR antibody cocktail Sym004 showing clinical activity in a metastatic colorectal cancer (mCRC) patient with acquired, EGFR mutation-mediated resistance to cetuximab[110]. Similarly, multi-epitope-targeting antibody formats also hold promise for treating patients with highly variant RNA viruses, who are likely to culminate escape variants after treatment with a single mAb. Indeed, a dual-epitope-targeting antibody cocktail is currently in phase III clinical development for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and has recently received an emergency use authorization from the United States Food and Drug Administration[111-113]. *In vitro* studies with this antibody cocktail prevented the selection of escape mutants in cultures growing a pseudo virus expressing the SARS-COV-2 spike, in contrast to pseudo virus grown in the presence of the individual antibodies[114]. In addition to dual-epitope targeting, multi-epitope targeting is also being explored in a phase I clinical trial

in the form of a triparatopic antibody containing binding domains from three broadly neutralizing antibodies (bnAbs; VRC01-LS, PGDM1400 and 10E8v4) directed towards the HIV-1 envelope spike[106]. Furthermore, a cocktail of three non-competing IgG1 antibodies (atoltivimab, maftivimab and odesivimab) was recently approved for treatment of Zaire ebolavirus (ZEBOV)[115].

Targeting multiple cell surface receptors

Antibody cocktails targeting two or more cell surface receptors represent an archetypical example of avidity engineering through enhanced binding (Figure 4B). While such cocktails generate increased target occupancy, they may simultaneously benefit from additional avidity effects through enhanced antibody clustering (Figure 1, binding/clustering phase). We recently described a mixture of two antibodies targeting CD20 and CD37 antigens on tumor B cells that co-engaged in hetero-hexameric complexes, thereby potentiating complement activity[61]. Likewise, Jacobsen and colleagues reported on a mixture of six antibodies which, in synergistic pairs, target each of the HER family members EGFR, HER2 and HER3[116]. This PAN-HER mixture (Sym013) was shown to down modulate all three targets and overcome acquired resistance due to compensatory receptor upregulation in HER family-expressing tumors.

Other approaches to target multiple cell surface receptors include combining multiple binding specificities into a single molecule in the form of bi- or multispecifics which, either in-*cis* or in-*trans*, bridge different receptors on the same cell or on different cell types respectively. The strength of bi- or multispecifics lies within their capacity to activate novel or 'designed' effector functions that could not otherwise be achieved by antibody mixtures, which are also referred to as 'obligate' mechanisms of action in the case of bispecifics[10]. Furthermore, their therapeutic index may be increased through localized tethering of receptors, thereby restricting therapeutic activity to preferred cells or tissues and limiting on- and off-target toxicity[117]. The most widely studied 'obligate' bispecific antibodies are those focusing on redirecting the cytotoxic activity of T cells or other effector cell types to eliminate tumor cells[118]. These bispecific antibodies are composed of effector cell-binding domain(s) physically linked to tumor cell-binding domain(s) and represent an inherent example of avidity tuning, as effector cells are brought in close contact with tumor cells. Such in-*trans* binding antibody concepts increase avidity interactions already in early phases of antibody functional response activation through enhanced binding and recruitment of effector cells and enhanced clustering via formation of the immunological synapse, while simultaneously boosting functional response amplification by effector-cell mediated killing of the target cell (Figure 1, binding/clustering/amplification phase). T-cell redirection has proven particularly useful in mediating sufficient avidity when targeting low-den-

sity cell surface receptors. In a clever use of avidity to improve therapeutic index, engineering two low affinity anti-Her2 Fab arms into an anti-HER2/CD3 bispecific enabled selective targeting of HER2-overexpressing tumor cells with high potency while sparing cells that express low HER2 found in normal tissue[119].

An interesting example of how in-*cis* bridging can unlock obligate mechanisms of action is represented by amivantamab (Table 1), a bispecific antibody targeting EGFR and MET. Crosslinking of MET using bivalent antibodies causes undesirable tumor cell activation, which can be circumvented by combining a single (non-crosslinking) MET-binding arm with an EGFR-binding arm in a bispecific configuration that blocks both MET and EGFR signaling[120]. This example is also noteworthy in terms of avidity tuning, as it illustrates the importance of understanding both antibody- and target biology in the design of effective antibody-based therapeutics.

The targeting of multiple receptors surprisingly can also be achieved in a single binding domain. Lameris et al. recently described a single-domain antibody (VHH) 1D12 targeting the glycolipid-presenting major histocompatibility complex (MHC) class I-like molecule CD1d that can simultaneously interact with CD1d and the type I natural killer T (NKT) cell receptor. Cross-linking by VHH 1D12 stabilizes this interaction and induces anti-tumor activity by type I NKT cell through intrinsic bispecificity while retaining the T-cell receptor's (TCR) specificity for presented antigen[121]. VHH 1D12 is linked to a VHH targeting the Vg9Vd2 TCR in LAVA-051, which through its unique trispecific properties recruits both type I NKT cells and Vg9Vd2 T cells for tumor cells killing. LAVA-051 is a next-gen T-cell engager in development for the treatment of hematological cancers including CLL, MM and AML (Table 1).

Combined *cis*- and t*rans*-bridging of receptors can also be achieved by engineering additional antibody fragments onto a classical bispecific antibody format, thereby creating tri- or multi-specific antibodies that enable simultaneous targeting of multiple cell types and same-cell receptors[122,123]. For instance, Shivange and colleagues recently reported on the generation of a Bispecific-Anchored Cytotoxicity-Activator (BaCa) antibody that simultaneously targets DR5 and folate receptor-1 (FOLR-1) both expressed on ovarian cancer cells. This Baca antibody was reported to bind FOLR-1 and crosslink DR5 both in-*cis* and in-*trans*, by which FOLR-1 served as tumor-specific anchor and primary clustering point for agonist DR5 signaling[124].

Enhancing / optimizing valency

Over the past few years multiple engineering strategies have focused on enhancing antibody clustering to improve antibody function (Figure 1, clustering phase). Such engineering strategies are often aimed at increasing antibody valency by transferring IgG variable regions to IgM-like formats or through multiplication of (parts of) Fab or Fc domains in (non-) IgG-like structures or fragments. Several studies reported on the design of antibody architectures containing multiple Fc domains to enhance FcγR crosslinking and ADCC[125-128]. In a more recent study, Miller and colleagues adapted the tetramerization domain of p53 and fused it to different antigen binding domains to create octavalent monospecific and bispecific (Quads) antibody variants with increased functional activity[129]. Alternatively, CDC by IgG1 antibodies can be enhanced through the covalent association of up to six IgG1 monomers using the µ-tailpiece of IgM, thereby generating IgM-like structures that efficiently bind and activate complement[130]. In contrast to pre-assembled antibody multimers, the ability of IgGs to self-assemble into ordered oligomers on antigenic surfaces via Fc-Fc interactions may be exploited in therapeutic applications, with notion that the use of IgG antibodies with standard architecture would simplify design and development. De Jong and colleagues were first to report on a novel HexaBody® technology platform that uses specific single-point mutations in the Fc domain to enhance self-assembly of IgG molecules into hexameric complexes after target binding on a cell surface (Figure 4C)[131]. In a mutational screening approach, they identified two Fc residues, E345 and E430 which, when substituted with any other amino acid, enhanced IgG hexamer formation and complement activation for a wide range of cell surface targets. Furthermore, by systematically depleting individual complement components, Taylor et al, elegantly showed that antibodies containing such hexamer-enhancing mutations required a reduced presence of MAC-forming complement components to promote CDC[132]. Thus, cell surface oligomerization of hexamer-enhanced antibodies is likely to be highly efficient, resulting in a lower complement activation threshold compared to regular IgGs.

Besides improving antagonistic antibody function, enhancement of antibody clustering may also have novel applications in amplifying 'designed' antibody functions such as agonistic receptor signaling. Some receptors, including those with multimeric cognate ligands or those that interact at cell-cell synapses, require higher order clustering before activation of downstream signaling. The most widely studied receptors of this class are members of the Tumor Necrosis Factor Receptor Super Family (TNFRSF), which are of growing interest to drug developers owing to their important function in regulating cell survival and inflammatory signaling. Regular binding of agonistic immunomodulatory antibodies targeting these receptors is generally not sufficient to engage higher order TNFRSF clustering and activation. Many require additional extrinsic

crosslinking via FcγRs, in particular FcγRIIb, on immune effector cells[133-135]. Pioneering work by Ashkenazi, Presta, and colleagues demonstrated that engineered tandem Fab repeats can enhance the forward signaling activity of antibodies targeting DR5 and CD20[136]. Alternatively, introducing hexamer-enhancing mutations in antibodies targeting TNFRSF members also proved an effective strategy to enhance agonistic signaling and tumor cell death. Recently, Overdijk et al. described the generation of a novel antibody mixture of two non-competing DR5-targeting (TRAIL-R2) antibodies containing an E430G hexamer-enhancing mutation that improves DR5 agonistic signaling and tumor cell death independent of FcγR crosslinking in a wide range of tumor types[137]. Intermolecular Fc-Fc interactions between noncompeting hexamer-enhanced DR5 IgG molecules were shown to be essential for DR5 agonistic activity and binding of complement component C1q reportedly contributed to the potency observed *in vitro* and *in vivo*, potentially resulting from increased clustering after C1 binding (Figure 1, clustering phase).

Besides targeting TNFRSF members such as TRAIL on tumor cells, immune effector cells also express TNFRSF activating receptors including OX40, CD27, CD40, 4-1BB and GITR. Much effort has been directed towards the use of their respective ligands or agonist antibodies to stimulate T-cell proliferation and cytotoxic activity. Enhanced hexamerization using an E435R Fc mutation was reported to increase the agonistic activity of OX40-targeting antibodies[138,139]. Alternative approaches to enhance TNFR activation independent of FcγR crosslinking include Fc fusions with six (hexavalent) ligand receptor binding domains, IgM class switching, covalently trimerized fusion proteins and tetravalent bi-epitopic targeting concepts (Figure 4D)[140-143]. The hexavalent receptor agonist (HERA) technology for example, makes use of single chain trimeric TNFR ligand binding domains fused to a silenced IgG1-derived Fc domain to enhance clustering of CD40, GITR and CD27 and boost antigen-specific T-cell responses[144-146]. Likewise, combined multivalent and multi-epitopic engagement of DR5 and OX40 using various tetravalent bi-epitopic antibody formats was shown to induce agonistic signaling without the need for extrinsic crosslinking[143].

While more complex and challenging than IgGs with respect to production, glycosylation, and pharmacokinetics, naturally multivalent immunoglobulin isotypes such as IgM and IgA are being clinically translated, with a steady increase in engineering for their optimization[41,147,148]. In a unique ocular application, Agard and colleagues leveraged not only the multivalent nature of IgM to effectively agonize Tie2, but also capitalized on its large size to reduce vitreal clearance[149].

Avidity-engineered formats are advancing clinically particularly in the context of antibody-mediated targeting of TNFRSF members (Table 1). Previous efforts to develop TNFRSF-targeting antibodies have yielded limited clinical success, partly due to lack of efficacy due possibly to insufficient (FcγR-mediated) crosslinking, and in some cases due to adverse events or toxicity[150,151]. Interest has revived upon development of antibody formats with increased valency that allow for superior TNFR crosslinking and signaling independent of Fc interactions. At present, four valency-increased antibodies targeting TNFRSF members have entered early-stage clinical trials, including a pentameric IgM-based antibody construct targeting DR5 (IGM-8444), and tetravalent (INBRX-109) or hexavalent (INBRX-106, ABBV-621) formats containing multiple (ligand) binding domains fused to an Fc domain targeting DR5, TRAIL and OX40 respectively. Moreover, at present, two clinical programs leverage avidity through a combination of enhanced binding and clustering, both in the context of antibodies targeting two non-overlapping epitopes that contain an Fc domain mutation driving self-assembly of IgGs in hexameric complexes. Combining dual epitope targeting with enhanced hexamerization is being explored as an antibody mixture targeting DR5 on solid tumors (GEN1029) and as a bispecific (biparatopic) molecule targeting CD37 in hematologic malignancies (GEN3009)[102,137].

Optimizing binding to Fc effector receptors

Canonical therapeutic antibodies rely on their Fc domain to effectively initiate immune effector functions (Figure 1, amplification phase). Therefore, much effort has been made to engineer the Fc domain and enhance binding affinity towards immune effector molecules and cells. As the various IgG subclasses differ in their complement activating potency (IgG3 > IgG1 >> IgG2 ≈ IgG4), reshuffling of segments from different IgG subclasses has been a common engineering strategy for optimization [152-155]. In human IgG1, multiple amino acid residues were identified to be critical for binding of C1q including D270, K322, P329 and P331 in the second heavy chain constant domain (C_H2)[156]. Several groups have enhanced C1q binding affinity and CDC by mutating amino acid positions in the Fc region (Figure 4E)[156-160]. Other positions in the IgG Fc region differentially affect FcγR binding and can likewise be modified to enhance ADCC or ADCP activity (Figure 4F)[88,161,162]. For example, S239D/I332E amino acid substitutions in the Fc domain of antibodies targeting EGFR, CD52 and CD20 were shown to enhance FcγRIIIa/FcγRIIb affinity and increase ADCC and ADCP activity[161]. Another successful engineering approach for enhancing antibody effector function is glycoengineering of the Fc region for reduced fucose content, which selectively improves binding to FcγRIIIa. Enhancement of FcγR binding interactions may not only improve classical FcγR-mediated effector functions, but can also be employed to increase the agonistic activity of immunomodulatory antibodies. For instance, FcγR affinity enhancement

using S267E/L328F or E233D/G237D/H268D/P271G/A330R Fc mutations was reported to increase the anti-tumor activity of DR5 and OX40 TNFRSF members[133,139,163-165]. Additionally, isoelectric point engineering or selective enhancement to FcγRIIb in pH-dependent antibodies, which bind to antigen at neutral pH in plasma and dissociate at acidic pH in endosomes, was also reported to improve soluble antigen clearance from circulation[166,167].

At present, seven active clinical programs apply Fc engineering to boost (amplify) FcγR-mediated effector functions (Table 1). A combination of two Fc domain single point mutations (S239D/I332E) enhances ADCC and ADCP functional activity of antibodies targeting the envelope spike on HIV-1 and CD19 on B tumor cells, of which the latter (tafasitamab) was recently approved for treatment of relapsed/refractory diffuse large B-cell lymphoma (DLBCL) in combination with lenalidomide. Recently margetuximab, an anti-HER2 antibody Fc engineered with the variant L235V/F243L/R292P/Y300L/P396L, was approved for HER2+ breast cancer[168,169]. Glycoengineering for effector function enhancement has met broad clinical success, with three approved antibodies with reduced Fc fucose content (mogamulizumab)[170,171], obinutuzumab[172], benralizumab[173,174] and one under regulatory review (amivantamab)[120,175].

As described above, FcγRs not only mediate cytotoxic and phagocytic effector functions, but can also play a role in activation of DCs by antibody:antigen immune complexes to mediate adaptive cellular immunity for a vaccinal effect. Recently, Ravetch and colleagues have demonstrated that antibodies Fc engineered for selective enhancement to FcγRIIa[88] can increase DC maturation and induce protective $CD8^+$ T cell anti-viral response[176].

CONCLUSIONS AND FUTURE PERSPECTIVES

Antibody function relies on complex interactions between antibodies and their target, as well as their ability to associate with effector molecules and cells of the immune system. There is considerable evidence demonstrating the crucial role of avidity in natural antibody biology, from antibody affinity maturation early in the immune response, to effector function activation after target engagement. Antibody effector mechanisms including CDC, ADCC and ADCP all require a certain level of ordered clustering prior to activation either via self-assembling Ig Fc domains or crosslinking of FcRs on immune effector cells. Although the threshold by which antibody avidity interactions translate in an effective functional response may vary per effector mechanism, antibody clustering may be viewed as the checkpoint before response amplification and thus in large part determines the efficacy or impact of functional response (Figure 1). Understanding the key determinants that shape antibody functional response kinetics, including avidity interactions and response regulators, is therefore crucial in the design of novel and 'impactful' antibody-based therapeutics. In that light, we take lessons from the natural polyclonal antibody response, which is highly efficient at establishing avidity interactions through the generation of antibodies with different affinities, valencies and binding specificities. Upon repeat exposure, somatic mutation and selection, such responses mature to high affinity responses which are more oligoclonal in nature while retaining multi-epitope targeting as a principal feature. In essence, the historical single-agent use of monoclonal antibodies for therapy seems contradictory to antibody function as established in natural biology. In traditional drug development, single agent safety and activity is required for a drug to be registered. Drug combinations are typically sought thereafter, often using empirical approaches. This process however, is in conflict with the biologic function of immunity, in which the body matures a polyclonal immune response that is combinatorial from the start. Awareness on this paradox has steadily grown as antibody-based therapeutics have evolved from classical, non-modified monoclonal antibodies towards more complex antibody architectures and formats in an effort to enhance functional activity.

Novel formats are emerging that leverage antibody avidity interactions to boost classical as well as novel or 'designed' effector functions. Many of these formats feature some of the strengths offered by polyclonals including increased affinity, valency or binding specificities, either as antibody cocktails or encompassed within a single antibody molecule. Using this concept, distinct avidity engineering approaches may be combined for incremental avidity

effects. Thus for example, multi-epitope or multi-valent formats may be combined with (self-) assembling technologies that may be further enhanced by effector molecules (Figure 4 and see Table 1 for examples). We believe that multi-agent approaches are a major part of the future of antibody therapy, either in the form of bi- or multispecifics with obligate functions or designer polyclonals. The ever-growing list of investigational therapeutic antibodies entering or undergoing clinical development argues for greater effort towards rational design of oligoclonal antibody cocktails. For example, with five programmed cell death protein 1 (PD-1) antibodies approved for clinical use and over ten in clinical development, combination trials with multiple existing antibodies targeting non-overlapping epitopes on PD-1 may offer advantages over (yet another) single-agent approach[177]. Notably, forward-looking antibody discovery approaches that perform unbiased screening of antibody libraries in their final format will be critical to identification of the most optimal multispecific candidates or antibody combinations. Collectively, recent novel insights into antibody effector biology together with current antibody design efforts that extend our capabilities beyond the classic monoclonal antibody format are paving the way for novel transformative biotherapeutics that positively impact patient lives.

Table 1

Avidity-based therapeutic antibody concepts in the clinic and engineering platforms that optimize them

Data available as of 1 March 2021. ADC, antibody-drug conjugate; ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; AML, acute myeloid leukemia; bnAb, broadly neutralizing antibody; BLA, Biologics License Application; B-NHL, B-cell non-Hodgkin lymphoma; CCR4, C-C Motif Chemokine Receptor 4; CD, cluster of differentiation; CLL, chronic lymphocytic leukemia; CODV-Ig, cross-over dual variable Ig-like protein; COVID-19, coronavirus disease 2019; CRIB, charge repulsion induced bispecificity; DLBCL, diffuse large B-cell lymphoma; DR5, death receptor 5; EGFR, epidermal growth factor receptor; Fc, fragment crystallizable; FL, follicular lymphoma; HER2, human epidermal growth factor receptor 2; HIV-1, human immunodeficiency virus 1; IL, interleukin; mCRC, metastatic colorectal cancer; MET, tyrosine-protein kinase Met; MM, multiple myeloma; MPER, membrane-proximal external region; NKT, natural killer T cell; OX40, tumor necrosis factor receptor superfamily member 4; NSCLC, non-small cell lung cancer; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; sdAb, single-domain antibody; TCR, T-cell receptor; TNFRSF, tumor necrosis factor receptor super family; TRAIL, TNF-related apoptosis-inducing ligand; VHH, single-domain antibodies; ZEBOV, Zaire ebolavirus. Engineering data provided in the fourth column were obtained from public documents (scientific literature, abstracts, posters and patent publications).

Antibody name(s) (Company)	Target	Optimizing[1]	Format (Technology platform)	Indication (Selected)	Development status (Selected trials)
Targeting multiple epitopes					
Sym015 (Symphogen)	cMET		Mixture of two IgG1 antibodies binding non-overlapping epitopes on the SEMA-a domain of MET	Solid tumors (NSCLC)	Phase IIa (NCT02648724)
REGN5093 (Regeneron)	cMET		Biparatopic bispecific IgG1 antibody targeting two non-overlapping epitopes on MET (Veloci-Bi® bispecific technology)	Solid tumors (NSCLC)	Phase I/II (NCT04077099)
Sym004 (Symphogen)	EGFR		Mixture of two IgG1 antibodies (futuximab, modotuximab) binding non-overlapping epitopes on EGFR	Solid tumors (mCRC)	Phase III ready (NCT02083653)
KN026 (Alphamab)	HER2		Biparatopic bispecific IgG1 antibody targeting two non-overlapping epitopes on HER2 (CRIB technology)	Solid tumors (breast and gastric cancer)	Phase I (NCT03619681 and NCT03847168)
MBS301 (Beijing Mabworks Biotech)	HER2		Afucosylated biparatopic bispecific IgG1 antibody targeting two non-overlapping epitopes on HER2 (knobs-into-holes bispecific technology)	Solid tumors ($HER2^+$)	Phase I (NCT03842085)
Zanidatamab, ZW25 (Zymeworks)	HER2		Biparatopic bispecific IgG1 antibody targeting two non-overlapping epitopes on HER2 (Azymetric™ bispecific technology)	Solid tumors ($HER2^+$)	Phase II (NCT04466891, NCT03929666 and NCT04224272)

Binding Clustering Amplification

1 See Figure 1 for additional information about different phases (binding, clustering and amplification) of antibody functional response activation.

Antibody name(s) (Company)	Target	Optimizing[1]	Format (Technology platform)	Indication (Selected)	Development status (Selected trials)
ZW49 (Zymeworks)	HER2		Biparatopic bispecific IgG1 antibody targeting two non-overlapping epitopes on HER2 (Azymetric bispecific technology) conjugated to auristatin (Zymelink™ ADC technology)	Solid tumors (HER2+)	Phase I (NCT03821233)
Casirivimab, imdevimab, REGN-COV2 (Regeneron)	SARS-CoV-2 spike		Mixture of two human IgG1 antibodies binding non-overlapping epitopes on the SARS-CoV-2 viral spike	COVID-19	Emergency approval, Phase III (NCT04452318 and NCT04381936)
ADM03820 (Ology Bioservices)	SARS-CoV-2 spike		Mixture of two human IgG1 antibodies binding non-overlapping epitopes on the SARS-CoV-2 viral spike	COVID-19	Phase I (NCT04592549)
SAR441236 (Sanofi)	HIV-1 envelope		Triparatopic IgG1 bnAb (derived from VRC01-LS, PGDM1400 and 10E8v4) targeting the CD4bs, gp41 MPER and V1/V2 glycan-directed binding sites on HIV-1 (CODV-Ig technology)	HIV-1	Phase I (NCT03705169)
Inmazeb, REGN-EB3 (Regeneron)	ZEBOV glyco-protein		A mixture of three IgG1 antibodies (atoltivimab, maftivimab and odesivimab) that each bind to different, non-overlapping epitopes on the Zaire ebolavirus glycoprotein	ZEBOV	Approved
Targeting multiple cell surface receptors					
LAVA-051 (Lava Therapeutics)	CD1d, Vd2 TCR		CD1d bispecific gamma-delta T-cell engager: VHH targeting CD1d that simultaneously binds CD1d and the type I NKT TCR, thereby stabilizing this interaction through intrinsic bispecificity linked to a VHH targeting Vg9Vd2 TCR	Hematologic malignancies (CLL, MM, AML)	Phase I (pending)
Enhancing / optimizing valency					
IGM-8444 (IgM Biosciences)	DR5		Pentameric IgM antibody with 10 binding sites specific for DR5	Solid tumors	Phase I (NCT04553692)

 Binding  Clustering  Amplification

1 See Figure 1 for additional information about different phases (binding, clustering and amplification) of antibody functional response activation.

Antibody name(s) (Company)	Target	Optimizing[1]	Format (Technology platform)	Indication (Selected)	Development status (Selected trials)
INBRX-109 (INHIBRX)	DR5		Four single domain antibodies (tetravalent) fused to an Fc domain (sdAb technology – binding units derived from heavy chain only antibodies)	Solid tumors	Phase I (NCT03715933)
INBRX-106 (INHIBRX)	OX40		Six single domain antibodies (hexavalent) fused to an Fc domain (sdAb technology – binding units derived from heavy chain only antibodies)	Solid tumors	Phase I (NCT04198766)
ABBV-621, APG350, HERA-TRAIL (Abbvie, Apogenix)	TRAIL		Hexavalent TNFRSF agonist comprising a fusion protein composed of three receptor binding domains in a single chain arrangement, linked to a silenced human IgG1 Fc-domain (HERA-ligand technology)	Solid tumors, hematologic malignancies	Phase I (NCT03082209)
GL-2045 (Gliknik)	C1q		Recombinant human IgG1-based Fc multimer; fusion of the complete IgG1 hinge-CH2-CH3 coding region to the IgG2 hinge region (Stradomer technology™)	Autoimmune diseases	Phase I (NCT03275740)
Targeting multiple epitopes & enhancing / optimizing valency					
GEN3009, DuoHexaBody-CD37 (Genmab, AbbVie)	CD37		Biparatopic bispecific IgG1 antibody targeting two non-overlapping epitopes on CD37 containing an E430G hexamerization-enhancing Fc mutation ((DuoHexaBody® technology)	Hematologic malignancies (B-NHL, CLL)	Phase I (NCT04358458)
GEN1029; HexaBody DR5/ DR5 (Genmab)	DR5		Mixture of two IgG1 antibodies targeting non-overlapping epitopes on DR5, both containing an E430G hexamerization-enhancing Fc mutation (HexaBody® technology)	Solid tumors	Phase I (NCT03576131)
Optimizing binding to Fc effector receptors					
Monjuvi, tafasitamab-cxix (MorphoSys/ Incyte)	CD19		IgG1 antibody containing S239D/I332E Fc mutations for enhanced ADCC and ADCP (high cytotoxicity XmAb® Fc domain technology)	Hematologic malignancies (DLBCL)	Approved

 Binding  Clustering  Amplification

1 See Figure 1 for additional information about different phases (binding, clustering and amplification) of antibody functional response activation.

Antibody name(s) (Company)	Target	Optimizing[1]	Format (Technology platform)	Indication (Selected)	Development status (Selected trials)
Elipovimab, GS-9722 (Gilead Sciences)	HIV-1 envelope		IgG1 bnAb (derived from PGT121) containing S239D/I332E and M428L/N434S Fc mutations for lower immunogenicity, improved PK, enhanced ADCC and ADCP (high cytotoxicity XmAb & Xtend™ Fc domain technology)	HIV-1	GS-US-420-3902 Adisinsight entry (not listed in clinicaltrials.gov)
Margenza, margetuximab (Macrogenics)	HER2		IgG1 antibody containing L235V/F243L/R292P/Y300L/P396L Fc mutations for enhanced ADCC	Solid tumors ($HER2^+$ breast cancer)	Approved
Poteligeo, Mogamulizumab (Kyowa Hakko Kirin)	CCR4		IgG1 antibody containing reduced fucose in the Fc region for enhanced ADCC (Potelligent® Technology)	Adult T-cell leukemia or lymphoma	Approved
Gazyva, obinutuzumab (Roche)	CD20		IgG1 antibody containing reduced fucose in the Fc region for enhanced ADCC (GlycoMab® Technology)	Hematologic malignancies (CLL, FL)	Approved
Fasenra, benralizumab (Astra Zeneca, Kyoa Hakko Kirin)	IL-5Ra		IgG1 antibody containing reduced fucose in the Fc region for enhanced ADCC (Potelligent Technology)	Asthma	Approved
Targeting multiple cell surface receptors & optimizing binding to Fc effector receptors					
Amivantamab (Janssen/JNJ)	HER2, cMET		IgG1 bispecific antibody containing reduced fucose in the Fc region for enhanced ADCC (DuoBody® technology)	Solid tumors (Metastatic NSCLC with EGFR exon 20 insertion mutations)	BLA

 Binding  Clustering  Amplification

1 See Figure 1 for additional information about different phases (binding, clustering and amplification) of antibody functional response activation.

REFERENCES

1. Eisen HN. Affinity Enhancement of Antibodies: How Low-Affinity Antibodies Produced Early in Immune Responses Are Followed by High-Affinity Antibodies Later and in Memory B-Cell Responses. *Cancer Immunol Res* 2014; **2**(5): 381.

2. Rajewsky K. Clonal selection and learning in the antibody system. *Nature* 1996; **381**(6585): 751-8.

3. Tonegawa S. Somatic generation of antibody diversity. *Nature* 1983; **302**(5909): 575-81.

4. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 1975; **256**(5517): 495-7.

5. Kaplon H, Reichert JM. Antibodies to watch in 2021. *mAbs* 2021; **13**(1): 1860476.

6. https://www.antibodysociety.org/resources/approved-antibodies/.

7. Chiu ML, Goulet DR, Teplyakov A, Gilliland GL. Antibody Structure and Function: The Basis for Engineering Therapeutics. *Antibodies* 2019; **8**(4): 55.

8. Goulet DR, Atkins WM. Considerations for the Design of Antibody-Based Therapeutics. *J Pharm Sci* 2020; **109**(1): 74-103.

9. Wang Y, Yang S. Multispecific drugs: the fourth wave of biopharmaceutical innovation. *Signal Transduct Target Ther* 2020; **5**(1): 86.

10. Labrijn AF, Janmaat ML, Reichert JM, Parren P. Bispecific antibodies: a mechanistic review of the pipeline. *Nat Rev Drug Discov* 2019; **18**(8): 585-608.

11. Schuurman J, Parren PW. Editorial overview: Special section: New concepts in antibody therapeutics: What's in store for antibody therapy? *Curr Opin Immunol* 2016; **40**: vii-xiii.

12. Rudnick SI, Adams GP. Affinity and avidity in antibody-based tumor targeting. *Cancer Biother Radiopharm* 2009; **24**(2): 155-61.

13. Marks JD. Deciphering antibody properties that lead to potent botulinum neurotoxin neutralization. *Mov Disord* 2004; **19 Suppl 8**: S101-8.

14. Wang XZ, Coljee VW, Maynard JA. Back to the future: recombinant polyclonal antibody therapeutics. *Curr Opin Chem Eng* 2013; **2**(4): 405-15.

15. Parren PW, Burton DR. The antiviral activity of antibodies in vitro and in vivo. *Adv Immunol* 2001; **77**: 195-262.

16. Carter PJ, Lazar GA. Next generation antibody drugs: pursuit of the 'high-hanging fruit'. *Nat Rev Drug Discov* 2018; **17**(3): 197-223.

17. Ugurlar D, Howes SC, de Kreuk B-J, et al. Structures of C1-IgG1 provide insights into how danger pattern recognition activates complement. *Science* 2018; **359**(6377): 794-7.

18. Sondermann P, Szymkowski DE. Harnessing Fc receptor biology in the design of therapeutic antibodies. *Curr Opin Immunol* 2016; **40**: 78-87.

19. Diebolder CA, Beurskens FJ, de Jong RN, et al. Complement Is Activated by IgG Hexamers Assembled at the Cell Surface. *Science* 2014; **343**(6176): 1260-3.

20. Hiramoto E, Tsutsumi A, Suzuki R, et al. The IgM pentamer is an asymmetric pentagon with an open groove that binds the AIM protein. *Sci Adv* 2018; **4**(10): eaau1199.

21. Rougé L, Chiang N, Steffek M, et al. Structure of CD20 in complex with the therapeutic monoclonal antibody rituximab. *Science* 2020; **367**(6483): 1224-30.

22. Patel KR, Roberts JT, Barb AW. Multiple Variables at the Leukocyte Cell Surface Impact Fc γ Receptor-Dependent Mechanisms. *Front Immunol* 2019; **10**(223).

23. Fleischman JB, Porter RR, Press EM. The arrangement of the peptide chains in gamma-gobulin. *Biochem J* 1963; **88**(2): 220-8.

24. Porter RR. The hydrolysis of rabbit y-globulin and antibodies with crystalline papain. *Biochem J* 1959; **73**: 119-26.

25. Karush F. Multivalent binding and functional affinity. *Contemp Top Mol Immunol* 1976; **5**: 217-28.

26. Vorup-Jensen T. On the roles of polyvalent binding in immune recognition: Perspectives in the nanoscience of immunology and the immune response to nanomedicines. *Adv Drug Deliv Rev* 2012; **64**(15): 1759-81.

27. Burnet FM, Keogh EV, Lush D. The Immunologieal Reactions of the Filterable Viruses. *Aust J Exp Biol Med Sci* 1937; **15**(Pt. 3): 227-368.

28. Bleeker WK, Lammerts van Bueren JJ, van Ojik HH, et al. Dual mode of action of a human anti-epidermal growth factor receptor monoclonal antibody for cancer therapy. *J Immunol* 2004; **173**(7): 4699-707.

29. Rhoden JJ, Dyas GL, Wroblewski VJ. A Modeling and Experimental Investigation of the Effects of Antigen Density, Binding Affinity, and Antigen Expression Ratio on Bispecific Antibody Binding to Cell Surface Targets. *J Biol Chem* 2016; **291**(21): 11337-47.

30. Yang D, Kroe-Barrett R, Singh S, Roberts CJ, Laue TM. IgG cooperativity – Is there allostery? Implications for antibody functions and therapeutic antibody development. *mAbs* 2017; **9**(8): 1231-52.

31. Beurskens FJ, Lindorfer MA, Farooqui M, et al. Exhaustion of cytotoxic effector systems may limit monoclonal antibody-based immunotherapy in cancer patients. *J Immunol* 2012; **188**(7): 3532-41.

32. Lilienthal G-M, Rahmöller J, Petry J, Bartsch YC, Leliavski A, Ehlers M. Potential of Murine IgG1 and Human IgG4 to Inhibit the Classical Complement and Fcγ Receptor Activation Pathways. *Front Immunol* 2018; **9**(958).

33. Prodjinotho UF, Hoerauf A, Adjobimey T. IgG4 antibodies from patients with asymptomatic bancroftian filariasis inhibit the binding of IgG1 and IgG2 to C1q in a Fc-Fc-dependent mechanism. *Parasitol Res* 2019; **118**(10): 2957-68.

34. Briney B, Inderbitzin A, Joyce C, Burton DR. Commonality despite exceptional diversity in the baseline human antibody repertoire. *Nature* 2019; **566**(7744): 393-7.

35. Dal Porto JM, Gauld SB, Merrell KT, Mills D, Pugh-Bernard AE, Cambier J. B cell antigen receptor signaling 101. *Mol Immunol* 2004; **41**(6-7): 599-613.

36. Feng Y, Wang Y, Zhang S, Haneef K, Liu W. Structural and immunogenomic insights into B-cell receptor activation. *J Genet Genomics* 2020; **47**(1): 27-35.

37. Treanor B. B-cell receptor: from resting state to activate. *Immunology* 2012; **136**(1): 21-7.

38. Peterson ML. Mechanisms controlling production of membrane and secreted immunoglobulin during B cell development. *Immunol Res* 2007; **37**(1): 33-46.

39. Cattaneo A, Neuberger MS. Polymeric immunoglobulin M is secreted by transfectants of non-lymphoid cells in the absence of immunoglobulin J chain. *EMBO J* 1987; **6**(9): 2753-8.

40. Hughey CT, Brewer JW, Colosia AD, Rosse WF, Corley RB. Production of IgM Hexamers by Normal and Autoimmune B Cells: Implications for the Physiologic Role of Hexameric IgM. *J Immunol* 1998; **161**(8): 4091.

41. Keyt BA, Baliga R, Sinclair AM, Carroll SF, Peterson MS. Structure, Function, and Therapeutic Use of IgM Antibodies. *Antibodies* 2020; **9**(4).

42. Noia JMD, Neuberger MS. Molecular Mechanisms of Antibody Somatic Hypermutation. *Annu Rev Biochem* 2007; **76**(1): 1-22.

43. Batista FD, Neuberger MS. Affinity dependence of the B cell response to antigen: a threshold, a ceiling, and the importance of off-rate. *Immunity* 1998; **8**(6): 751-9.

44. Foote J, Eisen HN. Kinetic and affinity limits on antibodies produced during immune responses. *Proc Natl Acad Sci U S A* 1995; **92**(5): 1254-6.

45. Daëron M. Fc receptors as adaptive immunoreceptors. *Curr Top Microbiol Immunol* 2014; **382**: 131-64.

46. Nimmerjahn F, Ravetch JV. FcγRs in health and disease. *Curr Top Microbiol Immunol* 2011; **350**: 105-25.

47. Maizels N. Immunoglobulin gene diversification. *Annu Rev Genet* 2005; **39**: 23-46.

48. Arakawa H, Buerstedde JM. Immunoglobulin gene conversion: insights from bursal B cells and the DT40 cell line. *Dev Dyn* 2004; **229**(3): 458-64.

49. Mazor Y, Yang C, Borrok MJ, et al. Enhancement of Immune Effector Functions by Modulating IgG's Intrinsic Affinity for Target Antigen. *PLoS One* 2016; **11**(6): e0157788-e.

50. Wang, de Jong, van den Bremer, et al. Molecular Basis of Assembly and Activation of Complement Component C1 in Complex with Immunoglobulin G1 and Antigen. *Mol Cell* 2016; **63**(1): 135-45.

51. Strasser J, de Jong RN, Beurskens FJ, et al. Unraveling the Macromolecular Pathways of IgG Oligomerization and Complement Activation on Antigenic Surfaces. *Nano Lett* 2019; **19**(7): 4787-96.

52. Schofield DJ, Stephenson JR, Dimmock NJ. Variations in the neutralizing and haemagglutination-inhibiting activities of five influenza A virus-specific IgGs and their antibody fragments. *J Gen Virol* 1997; **78**(10): 2431-9.

53. Wu H, Pfarr DS, Tang Y, et al. Ultra-potent Antibodies Against Respiratory Syncytial Virus: Effects of Binding Kinetics and Binding Valence on Viral Neutralization. *J Mol Biol* 2005; **350**(1): 126-44.

54. Klein JS, Gnanapragasam PN, Galimidi RP, Foglesong CP, West AP, Jr., Bjorkman PJ. Examination of the contributions of size and avidity to the neutralization mechanisms of the anti-HIV antibodies b12 and 4E10. *Proc Natl Acad Sci U S A* 2009; **106**(18): 7385-90.

55. Ofek G, Tang M, Sambor A, et al. Structure and mechanistic analysis of the anti-human immunodeficiency virus type 1 antibody 2F5 in complex with its gp41 epitope. *J Virol* 2004; **78**(19): 10724-37.

56. Zhang MY, Xiao X, Sidorov IA, et al. Identification and characterization of a new cross-reactive human immunodeficiency virus type 1-neutralizing human monoclonal antibody. *J Virol* 2004; **78**(17): 9233-42.

57. Klein JS, Bjorkman PJ. Few and Far Between: How HIV May Be Evading Antibody Avidity. *PLOS Pathog* 2010; **6**(5): e1000908.

58. Bournazos S, Klein F, Pietzsch J, Seaman MS, Nussenzweig MC, Ravetch JV. Broadly neutralizing anti-HIV-1 antibodies require Fc effector functions for in vivo activity. *Cell* 2014; **158**(6): 1243-53.

59. Hessell AJ, Hangartner L, Hunter M, et al. Fc receptor but not complement binding is important in antibody protection against HIV. *Nature* 2007; **449**(7158): 101-4.

60. van der Neut Kolfschoten M, Schuurman J, Losen M, et al. Anti-Inflammatory Activity of Human IgG4 Antibodies by Dynamic Fab Arm Exchange. *Science* 2007; **317**(5844): 1554.

61. Calarese DA, Scanlan CN, Zwick MB, et al. Antibody domain exchange is an immunological solution to carbohydrate cluster recognition. *Science* 2003; **300**(5628): 2065-71.

62. Huber M, Le KM, Doores KJ, et al. Very few substitutions in a germ line antibody are required to initiate significant domain exchange. *J Virol* 2010; **84**(20): 10700-7.

63. Wu Y, West AP, Jr., Kim HJ, Thornton ME, Ward AB, Bjorkman PJ. Structural basis for enhanced HIV-1 neutralization by a dimeric immunoglobulin G form of the glycan-recognizing antibody 2G12. *Cell Rep* 2013; **5**(5): 1443-55.

64. Melis JP, Strumane K, Ruuls SR, Beurskens FJ, Schuurman J, Parren PW. Complement in therapy and disease: Regulating the complement system with antibody-based therapeutics. *Mol Immunol* 2015; **67**(2 Pt A): 117-30.

65. Reid KBM, Porter RR. Subunit composition and structure of subcomponent C1q of the first component of human complement. *Biochem J* 1976; **155**(1): 19-23.

66. Burton DR. Antibody: the flexible adaptor molecule. *Trends Biochem Sci* 1990; **15**(2): 64-9.

67. Collins C, Tsui FWL, Shulman MJ. Differential activation of human and guinea pig complement by pentameric and hexameric IgM. *Eur J Immunol* 2002; **32**(6): 1802-10.

68. Meyer S, Leusen JHW, Boross P. Regulation of complement and modulation of its activity in monoclonal antibody therapy of cancer. *mAbs* 2014; **6**(5): 1133-44.

69. Strasser J, de Jong RN, Beurskens FJ, et al. Weak Fragment Crystallizable (Fc) Domain Interactions Drive the Dynamic Assembly of IgG Oligomers upon Antigen Recognition. *ACS Nano* 2020; **14**(3): 2739-50.

70. Alduaij W, Ivanov A, Honeychurch J, et al. Novel type II anti-CD20 monoclonal antibody (GA101) evokes homotypic adhesion and actin-dependent, lysosome-mediated cell death in B-cell malignancies. *Blood* 2011; **117**(17): 4519-29.

71. Beers SA, Chan CH, French RR, Cragg MS, Glennie MJ. CD20 as a Target for Therapeutic Type I and II Monoclonal Antibodies. *Semin Hematol* 2010; **47**(2): 107-14.

72. Kumar A, Planchais C, Fronzes R, Mouquet H, Reyes N. Binding mechanisms of therapeutic antibodies to human CD20. *Science* 2020; **369**(6505): 793.

73. Oostindie SC, van der Horst HJ, Lindorfer MA, et al. CD20 and CD37 antibodies synergize to activate complement by Fc-mediated clustering. *Haematologica* 2019; **104**(9): 1841-52.

74. Dangl JL, Wensel TG, Morrison SL, Stryer L, Herzenberg LA, Oi VT. Segmental flexibility and complement fixation of genetically engineered chimeric human, rabbit and mouse antibodies. *EMBO J* 1988; **7**(7): 1989-94.

75. Tan LK, Shopes RJ, Oi VT, Morrison SL. Influence of the hinge region on complement activation, C1q binding, and segmental flexibility in chimeric human immunoglobulins. *Proc Natl Acad Sci U S A* 1990; **87**(1): 162-6.

76. Coloma MJ, Trinh KR, Wims LA, Morrison SL. The hinge as a spacer contributes to covalent assembly and is required for function of IgG. *J Immunol* 1997; **158**(2): 733.

77. Raju TS. Terminal sugars of Fc glycans influence antibody effector functions of IgGs. *Curr Opin Immunol* 2008; **20**(4): 471-8.

78. Kerr MA. The structure and function of human IgA. *Biochem J* 1990; **271**(2): 285-96.

79. Dechant M, Valerius T. IgA antibodies for cancer therapy. *Crit Rev Oncol Hematol* 2001; **39**(1): 69-77.

80. Labrijn AF, Aalberse RC, Schuurman J. When binding is enough: nonactivating antibody formats. *Curr Opin Immunol* 2008; **20**(4): 479-85.

81. Davies AM, Jefferis R, Sutton BJ. Crystal structure of deglycosylated human IgG4-Fc. *Mol Immunol* 2014; **62**(1): 46-53.

82. Bruhns P, Jönsson F. Mouse and human FcR effector functions. *Immunol Rev* 2015; **268**(1): 25-51.

83. Dhodapkar KM, Dhodapkar MV. Recruiting dendritic cells to improve antibody therapy of cancer. *Proc Natl Acad Sci U S A* 2005; **102**(18): 6243.

84. Dhodapkar MV, Geller MD, Chang DH, et al. A Reversible Defect in Natural Killer T Cell Function Characterizes the Progression of Premalignant to Malignant Multiple Myeloma. *J Exp Med* 2003; **197**(12): 1667-76.

85. Groh V, Li YQ, Cioca D, et al. Efficient cross-priming of tumor antigen-specific T cells by dendritic cells sensitized with diverse anti-MICA opsonized tumor cells. *Proc Natl Acad Sci U S A* 2005; **102**(18): 6461.

86. Boruchov AM, Heller G, Veri MC, Bonvini E, Ravetch JV, Young JW. Activating and inhibitory IgG Fc receptors on human DCs mediate opposing functions. *J Clin Invest* 2005; **115**(10): 2914-23.

87. DiLillo DJ, Ravetch JV. Differential Fc-Receptor Engagement Drives an Anti-tumor Vaccinal Effect. *Cell* 2015; **161**(5): 1035-45.

88. Richards JO, Karki S, Lazar GA, Chen H, Dang W, Desjarlais JR. Optimization of antibody binding to FcγRIIa enhances macrophage phagocytosis of tumor cells. *Mol Cancer Ther* 2008; **7**(8): 2517.

89. Holgado MP, Sananez I, Raiden S, Geffner JR, Arruvito L. CD32 Ligation Promotes the Activation of CD4+ T Cells. *Front Immunol* 2018; **9**(2814).

90. Bruhns P, Iannascoli B, England P, et al. Specificity and affinity of human Fcgamma receptors and their polymorphic variants for human IgG subclasses. *Blood* 2009; **113**(16): 3716-25.

91. Sutton BJ, Davies AM, Bax HJ, Karagiannis SN. IgE Antibodies: From Structure to Function and Clinical Translation. *Antibodies* 2019; **8**(1): 19.

92. Breedveld A, van Egmond M. IgA and FcαRI: Pathological Roles and Therapeutic Opportunities. *Front Immunol* 2019; **10**: 553-.

93. Heemskerk N, van Egmond M. Monoclonal antibody-mediated killing of tumour cells by neutrophils. *Eur J Clin Invest* 2018; **48 Suppl 2**(Suppl Suppl 2): e12962.

94. van Egmond M, Bakema JE. Neutrophils as effector cells for antibody-based immunotherapy of cancer. *Semin Cancer Biol* 2013; **23**(3): 190-9.

95. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol* 2014; **5**: 520-.

96. Preiner J, Kodera N, Tang J, et al. IgGs are made for walking on bacterial and viral surfaces. *Nat Commun* 2014; **5**(1): 4394.

97. Ben M'Barek K, Molino D, Quignard S, et al. Phagocytosis of immunoglobulin-coated emulsion droplets. *Biomaterials* 2015; **51**: 270-7.

98. Dechant M, Weisner W, Berger S, et al. Complement-Dependent Tumor Cell Lysis Triggered by Combinations of Epidermal Growth Factor Receptor Antibodies. *Cancer Res* 2008; **68**(13): 4998-5003.

99. Klausz K, Berger S, Lammerts van Bueren JJ, et al. Complement-mediated tumor-specific cell lysis by antibody combinations targeting epidermal growth factor receptor (EGFR) and its variant III (EGFRvIII). *Cancer Sci* 2011; **102**(10): 1761-8.

100. Schütze K, Petry K, Hambach J, et al. CD38-Specific Biparatopic Heavy Chain Antibodies Display Potent Complement-Dependent Cytotoxicity Against Multiple Myeloma Cells. *Front Immunol* 2018; **9**: 2553.

101. Macor P, Mezzanzanica D, Cossetti C, et al. Complement Activated by Chimeric Anti-Folate Receptor Antibodies Is an Efficient Effector System to Control Ovarian Carcinoma. *Cancer Res* 2006; **66**(7): 3876-83.

102. Oostindie SC, van der Horst HJ, Kil LP, et al. DuoHexaBody-CD37®, a novel biparatopic CD37 antibody with enhanced Fc-mediated hexamerization as a potential therapy for B-cell malignancies. *Blood Cancer J* 2020; **10**(3): 30.

103. Peschiera Ilaria, Giuliani Maria, Giusti Fabiola, et al. Structural basis for cooperativity of human monoclonal antibodies to meningococcal factor H-binding protein. *Commun Biol* 2019; **2**(1): 241-.

104. Rijkers M, Schmidt D, Lu N, et al. Anti-HLA antibodies with complementary and synergistic interaction geometries promote classical complement activation on platelets. *Haematologica* 2019; **104**(2): 403-16.

105. Steinhardt JJ, Guenaga J, Turner HL, et al. Rational design of a trispecific antibody targeting the HIV-1 Env with elevated anti-viral activity. *Nature commun* 2018; **9**(1): 877-.

106. Xu L, Pegu A, Rao E, et al. Trispecific broadly neutralizing HIV antibodies mediate potent SHIV protection in macaques. *Science* 2017; **358**(6359): 85-90.

107. Huang S, Li F, Liu H, et al. Structural and functional characterization of MBS301, an afucosylated bispecific anti-HER2 antibody. *mAbs* 2018; **10**(6): 864-75.

108. Koefoed K, Steinaa L, Søderberg JN, et al. Rational identification of an optimal antibody mixture for targeting the epidermal growth factor receptor. *mAbs* 2011; **3**(6): 584-95.

109. Wei H, Cai H, Jin Y, et al. Structural basis of a novel heterodimeric Fc for bispecific antibody production. *Oncotarget* 2017; **8**(31): 51037-49.

110. Sánchez-Martín FJ, Bellosillo B, Gelabert-Baldrich M, et al. The First-in-class Anti-EGFR Antibody Mixture Sym004 Overcomes Cetuximab Resistance Mediated by EGFR Extracellular Domain Mutations in Colorectal Cancer. *Clin Cancer Res* 2016; **22**(13): 3260-7.

111. Baum A, Ajithdoss D, Copin R, et al. REGN-COV2 antibodies prevent and treat SARS-CoV-2 infection in rhesus macaques and hamsters. *Science* 2020: eabe2402.

112. Hansen J, Baum A, Pascal KE, et al. Studies in humanized mice and convalescent humans yield a SARS-CoV-2 antibody cocktail. *Science* 2020; **369**(6506): 1010.

113. Cohen MS. Monoclonal Antibodies to Disrupt Progression of Early Covid-19 Infection. *N Engl J Med* 2021; **384**(3): 289-91.

114. Baum A, Fulton BO, Wloga E, et al. Antibody cocktail to SARS-CoV-2 spike protein prevents rapid mutational escape seen with individual antibodies. *Science* 2020; **369**(6506): 1014.

115. Mulangu S, Dodd LE, Davey RT, et al. A Randomized, Controlled Trial of Ebola Virus Disease Therapeutics. *N Engl J Med* 2019; **381**(24): 2293-303.

116. Jacobsen HJ, Poulsen TT, Dahlman A, et al. Pan-HER, an Antibody Mixture Simultaneously Targeting EGFR, HER2, and HER3, Effectively Overcomes Tumor Heterogeneity and Plasticity. *Clin Cancer Res* 2015; **21**(18): 4110-22.

117. Deshaies RJ. Multispecific drugs herald a new era of biopharmaceutical innovation. *Nature* 2020; **580**(7803): 329-38.

118. Clynes RA, Desjarlais JR. Redirected T Cell Cytotoxicity in Cancer Therapy. *Annu Rev Med* 2019; **70**(1): 437-50.

119. Slaga D, Ellerman D, Lombana TN, et al. Avidity-based binding to HER2 results in selective killing of HER2-overexpressing cells by anti-HER2/CD3. *Sci Transl Med* 2018; **10**(463): eaat5775.

120. Grugan KD, Dorn K, Jarantow SW, et al. Fc-mediated activity of EGFR x c-Met bispecific antibody JNJ-61186372 enhanced killing of lung cancer cells. *mAbs* 2017; **9**(1): 114-26.

121. Lameris R, Shahine A, Pellicci DG, et al. A single-domain bispecific antibody targeting CD1d and the NKT T-cell receptor induces a potent antitumor response. *Nat Cancer* 2020; **1**(11): 1054-65.

122. Kügler M, Stein C, Kellner C, et al. A recombinant trispecific single-chain Fv derivative directed against CD123 and CD33 mediates effective elimination of acute myeloid leukaemia cells by dual targeting. *Br J Haematol* 2010; **150**(5): 574-86.

123. Wu L, Seung E, Xu L, et al. Trispecific antibodies enhance the therapeutic efficacy of tumor-directed T cells through T cell receptor co-stimulation. *Nature Cancer* 2020; **1**(1): 86-98.

124. Shivange, Urbanek, Przanowski, et al. A Single-Agent Dual-Specificity Targeting of FOLR1 and DR5 as an Effective Strategy for Ovarian Cancer. *Cancer Cell* 2018; **34**(2): 331-45.e11.

125. Goulet DR, Zwolak A, Williams JA, Chiu ML, Atkins WM. Design and characterization of novel dual Fc antibody with enhanced avidity for Fc receptors. *Proteins* 2019.

126. Jain A, Poonia B, So EC, et al. Tumour antigen targeted monoclonal antibodies incorporating a novel multimerisation domain significantly enhance antibody dependent cellular cytotoxicity against colon cancer. *Eur J Cancer* 2013; **49**(15): 3344-52.

127. Rowley Tania, Peters Shirley, Aylott Mike, et al. Engineered hexavalent Fc proteins with enhanced Fc-gamma receptor avidity provide insights into immune-complex interactions. *Proc Natl Acad Sci U S A* 2018; **1**(1): 146-.

128. Zhang X, Olsen HS, Chen S, et al. Anti-CD20 Antibody with Multimerized Fc Domains: A Novel Strategy To Deplete B Cells and Augment Treatment of Autoimmune Disease. *J Immunol* 2016; **196**(3): 1165-76.

129. Miller A, Carr S, Rabbitts T, Ali H. Multimeric antibodies with increased valency surpassing functional affinity and potency thresholds using novel formats. *mAbs* 2020; **12**(1): 1752529.

130. Smith RI, Coloma MJ, Morrison SL. Addition of a mu-tailpiece to IgG results in polymeric antibodies with enhanced effector functions including complement-mediated cytolysis by IgG4. *J Immunol* 1995; **154**(5): 2226-36.

131. de Jong RN, Beurskens FJ, Verploegen S, et al. A Novel Platform for the Potentiation of Therapeutic Antibodies Based on Antigen-Dependent Formation of IgG Hexamers at the Cell Surface. *Plos Biol* 2016; **14**(1): e1002344.

132. Cook EM, Lindorfer MA, van der Horst H, et al. Antibodies That Efficiently Form Hexamers upon Antigen Binding Can Induce Complement-Dependent Cytotoxicity under Complement-Limiting Conditions. *J Immunol* 2016; **197**(5): 1762-75.

133. Li F, Ravetch JV. Apoptotic and antitumor activity of death receptor antibodies require inhibitory Fcγ receptor engagement. *Proc Natl Acad Sci U S A* 2012; **109**(27): 10966-71.

134. White AL, Chan HT, Roghanian A, et al. Interaction with FcγRIIB is critical for the agonistic activity of anti-CD40 monoclonal antibody. *J Immunol* 2011; **187**(4): 1754-63.

135. Wilson NS, Yang B, Yang A, et al. An Fcγ receptor-dependent mechanism drives antibody-mediated target-receptor signaling in cancer cells. *Cancer Cell* 2011; **19**(1): 101-13.

136. Miller K, Meng G, Liu J, et al. Design, Construction, and In Vitro Analyses of Multivalent Antibodies. *J Immunol* 2003; **170**(9): 4854-61.

137. Overdijk MB, Strumane K, Beurskens FJ, et al. Dual Epitope Targeting and Enhanced Hexamerization by DR5 Antibodies as a Novel Approach to Induce Potent Antitumor Activity Through DR5 Agonism. *Mol Cancer Ther* 2020; **19**(10): 2126.

138. Zhang D, Armstrong AA, Tam SH, et al. Functional optimization of agonistic antibodies to OX40 receptor with novel Fc mutations to promote antibody multimerization. *mAbs* 2017; **9**(7): 1129-42.

139. Zhang D, Goldberg MV, Chiu ML. Fc Engineering Approaches to Enhance the Agonism and Effector Functions of an Anti-OX40 Antibody. *J Biol Chem* 2016; **291**(53): 27134-46.

140. Gieffers C, Kluge M, Merz C, et al. APG350 induces superior clustering of TRAIL receptors and shows therapeutic antitumor efficacy independent of cross-linking via Fcγ receptors. *Mol Cancer Ther* 2013; **12**(12): 2735-47.

141. Liu H, Su D, Zhang J, et al. Improvement of Pharmacokinetic Profile of TRAIL via Trimer-Tag Enhances its Antitumor Activity in vivo. *Sci Rep* 2017; **7**(1): 8953.

142. Piao X, Ozawa T, Hamana H, et al. TRAIL-receptor 1 IgM antibodies strongly induce apoptosis in human cancer cells in vitro and in vivo. *Oncoimmunology* 2016; **5**(5): e1131380.

143. Yang Y, Yeh SH, Madireddi S, et al. Tetravalent biepitopic targeting enables intrinsic antibody agonism of tumor necrosis factor receptor superfamily members. *mAbs* 2019; **11**(6): 996-1011.

144. Merz C, Sykora J, Marschall V, et al. The Hexavalent CD40 Agonist HERA-CD40L Induces T-Cell-mediated Antitumor Immune Response Through Activation of Antigen-presenting Cells. *J Immunother* 2018; **41**(9): 385-98.

145. Richards DM, Marschall V, Billian-Frey K, et al. HERA-GITRL activates T cells and promotes anti-tumor efficacy independent of FcγR-binding functionality. *J Immunother Cancer* 2019; **7**(1): 191-.

146. Thiemann M, Richards DM, Heinonen K, et al. A Single-Chain-Based Hexavalent CD27 Agonist Enhances T Cell Activation and Induces Anti-Tumor Immunity. *Front Oncol* 2018; **8**: 387.

147. Leusen JH. IgA as therapeutic antibody. *Mol Immunol* 2015; **68**(1): 35-9.

148. van Tetering G, Evers M, Chan C, Stip M, Leusen J. Fc Engineering Strategies to Advance IgA Antibodies as Therapeutic Agents. *Antibodies* 2020; **9**(4).

149. Chen Y, Paluch M, Zorn JA, et al. Targeted IgMs agonize ocular targets with extended vitreal exposure. *mAbs* 2020; **12**(1): 1818436.

150. de Miguel D, Lemke J, Anel A, Walczak H, Martinez-Lostao L. Onto better TRAILs for cancer treatment. *Cell Death Differ* 2016; **23**(5): 733-47.

151. von Karstedt S, Montinaro A, Walczak H. Exploring the TRAILs less travelled: TRAIL in cancer biology and therapy. *Nat Rev Cancer* 2017; **17**(6): 352-66.

152. Natsume A, In M, Takamura H, et al. Engineered antibodies of IgG1/IgG3 mixed isotype with enhanced cytotoxic activities. *Cancer Res* 2008; **68**(10): 3863-72.

153. Natsume A, Shimizu-Yokoyama Y, Satoh M, Shitara K, Niwa R. Engineered anti-CD20 antibodies with enhanced complement-activating capacity mediate potent anti-lymphoma activity. *Cancer Sci* 2009; **100**(12): 2411-8.

154. Sensel MG, Kane LM, Morrison SL. Amino acid differences in the N-terminus of C(H)2 influence the relative abilities of IgG2 and IgG3 to activate complement. *Mol Immunol* 1997; **34**(14): 1019-29.

155. Xu Y, Oomen R, Klein MH. Residue at position 331 in the IgG1 and IgG4 CH2 domains contributes to their differential ability to bind and activate complement. *J Biol Chem* 1994; **269**(5): 3469-74.

156. Idusogie EE, Presta LG, Gazzano-Santoro H, et al. Mapping of the C1q binding site on rituxan, a chimeric antibody with a human IgG1 Fc. *J Immunol* 2000; **164**(8): 4178-84.

157. Dall'Acqua WF, Cook KE, Damschroder MM, Woods RM, Wu H. Modulation of the Effector Functions of a Human IgG1 through Engineering of Its Hinge Region. *J Immunol* 2006; **177**(2): 1129.

158. Idusogie EE, Wong PY, Presta LG, et al. Engineered antibodies with increased activity to recruit complement. *J Immunol* 2001; **166**(4): 2571-5.

159. Michaelsen TE, Sandlie I, Bratlie DB, Sandin RH, Ihle O. Structural difference in the complement activation site of human IgG1 and IgG3. *Scand J Immunol* 2009; **70**(6): 553-64.

160. Moore GL, Chen H, Karki S, Lazar GA. Engineered Fc variant antibodies with enhanced ability to recruit complement and mediate effector functions. *mAbs* 2010; **2**(2): 181-9.

161. Lazar GA, Dang W, Karki S, et al. Engineered antibody Fc variants with enhanced effector function. *Proc Natl Acad Sci U S A* 2006; **103**(11): 4005-10.

162. Shields RL, Namenuk AK, Hong K, et al. High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R. *J Biol Chem* 2001; **276**(9): 6591-604.

163. Chu SY, Vostiar I, Karki S, et al. Inhibition of B cell receptor-mediated activation of primary human B cells by coengagement of CD19 and FcγRIIb with Fc-engineered antibodies. *Mol Immunol* 2008; **45**(15): 3926-33.

164. Mimoto F, Igawa T, Kuramochi T, et al. Novel asymmetrically engineered antibody Fc variant with superior FcγR binding affinity and specificity compared with afucosylated Fc variant. *mAbs* 2013; **5**(2): 229-36.

165. Mimoto F, Katada H, Kadono S, et al. Engineered antibody Fc variant with selectively enhanced FcγRIIb binding over both FcγRIIa(R131) and FcγRIIa(H131). *Protein Eng Des Sel* 2013; **26**(10): 589-98.

166. Iwayanagi Y, Igawa T, Maeda A, et al. Inhibitory FcγRIIb-Mediated Soluble Antigen Clearance from Plasma by a pH-Dependent Antigen-Binding Antibody and Its Enhancement by Fc Engineering. *J Immunol* 2015; **195**(7): 3198-205.

167. Sampei Z, Haraya K, Tachibana T, et al. Antibody engineering to generate SKY59, a long-acting anti-C5 recycling antibody. *PLoS One* 2018; **13**(12): e0209509.

168. Nordstrom JL, Gorlatov S, Zhang W, et al. Anti-tumor activity and toxicokinetics analysis of MGAH22, an anti-HER2 monoclonal antibody with enhanced Fcγ receptor binding properties. *Breast Cancer Res* 2011; **13**(6): R123.

169. Rugo HS, Im SA, Cardoso F, et al. Efficacy of Margetuximab vs Trastuzumab in Patients With Pretreated ERBB2-Positive Advanced Breast Cancer: A Phase 3 Randomized Clinical Trial. *JAMA Oncol* 2021.

170. Niwa R, Shoji-Hosaka E, Sakurada M, et al. Defucosylated chimeric anti-CC chemokine receptor 4 IgG1 with enhanced antibody-dependent cellular cytotoxicity shows potent therapeutic activity to T-cell leukemia and lymphoma. *Cancer Res* 2004; **64**(6): 2127-33.

171. Ollila TA, Sahin I, Olszewski AJ. Mogamulizumab: a new tool for management of cutaneous T-cell lymphoma. *Onco Targets Ther* 2019; **12**: 1085-94.

172. Tobinai K, Klein C, Oya N, Fingerle-Rowson G. A Review of Obinutuzumab (GA101), a Novel Type II Anti-CD20 Monoclonal Antibody, for the Treatment of Patients with B-Cell Malignancies. *Adv Ther* 2017; **34**(2): 324-56.

173. Ghazi A, Trikha A, Calhoun WJ. Benralizumab--a humanized mAb to IL-5Rα with enhanced antibody-dependent cell-mediated cytotoxicity--a novel approach for the treatment of asthma. *Expert Opin Biol Ther* 2012; **12**(1): 113-8.

174. Kolbeck R, Kozhich A, Koike M, et al. MEDI-563, a humanized anti-IL-5 receptor alpha mAb with enhanced antibody-dependent cell-mediated cytotoxicity function. *J Allergy Clin Immunol* 2010; **125**(6): 1344-53.e2.

175. Vijayaraghavan S, Lipfert L, Chevalier K, et al. Amivantamab (JNJ-61186372), an Fc Enhanced EGFR/cMet Bispecific Antibody, Induces Receptor Downmodulation and Antitumor Activity by Monocyte/Macrophage Trogocytosis. *Mol Cancer Ther* 2020; **19**(10): 2044-56.

176. Bournazos S, Corti D, Virgin HW, Ravetch JV. Fc-optimized antibodies elicit CD8 immunity to viral respiratory infection. *Nature* 2020; **588**(7838): 485-90.

177. Guo L, Wei R, Lin Y, Kwok HF. Clinical and Recent Patents Applications of PD-1/PD-L1 Targeting Immunotherapy in Cancer Treatment-Current Progress, Strategy, and Future Perspective. *Front Immunol* 2020; **11**: 1508-.

178. Müller R, Gräwert MA, Kern T, et al. High-resolution structures of the IgM Fc domains reveal principles of its hexamer formation. *Proc Natl Acad Sci U S A* 2013; **110**(25): 10183.

179. Sharp TH, Boyle AL, Diebolder CA, Kros A, Koster AJ, Gros P. Insights into IgM-mediated complement activation based on in situ structures of IgM-C1-C4b. *Proc Natl Acad Sci U S A* 2019; **116**(24): 11900.

180. Redpath S, Michaelsen TE, Sandlie I, Clark MR. The influence of the hinge region length in binding of human IgG to human Fcgamma receptors. *Hum Immunol* 1998; **59**(11): 720-7.

181. Hobeika E, Maity PC, Jumaa H. Control of B Cell Responsiveness by Isotype and Structural Elements of the Antigen Receptor. *Trends Immunol* 2016; **37**(5): 310-20.

182. Übelhart R, Hug E, Bach MP, et al. Responsiveness of B cells is regulated by the hinge region of IgD. *Nat Immunol* 2015; **16**(5): 534-43.

183. Breedveld A, van Egmond M. IgA and FcαRI: Pathological Roles and Therapeutic Opportunities. *Front Immunol* 2019; **10**(553).

184. Shade KT, Platzer B, Washburn N, et al. A single glycan on IgE is indispensable for initiation of anaphylaxis. *J Exp Med* 2015; **212**(4): 457-67.

185. Kubo S, Nakayama T, Matsuoka K, Yonekawa H, Karasuyama H. Long term maintenance of IgE-mediated memory in mast cells in the absence of detectable serum IgE. *J Immunol* 2003; **170**(2): 775-80.