Avidity in antibody effector functions
and biotherapeutic drug design

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Abstract | Antibodies are the cardinal effector molecules of the immune system and are being leveraged with enormous success as biotherapeutic drugs. A key part of the adaptive immune response is the production of an epitope-diverse, polyclonal antibody mixture that is capable of neutralizing invading pathogens or disease-causing molecules through binding interference and by mediating humoral and cellular effector functions. Avidity — the accumulated binding strength derived from the affinities of multiple individual non-covalent interactions — is fundamental to virtually all aspects of antibody biology, including antibody–antigen binding, clonal selection and effector functions. The manipulation of antibody avidity has since emerged as an important design principle for enhancing or engineering novel properties in antibody biotherapeutics. In this Review, we describe the multiple levels of avidity interactions that trigger the overall efficacy and control of functional responses in both natural antibody biology and their therapeutic applications. Within this framework, we comprehensively review therapeutic antibody mechanisms of action, with particular emphasis on engineered optimizations and platforms. Overall, we describe how affinity and avidity tuning of engineered antibody formats are enabling a new wave of differentiated antibody drugs with tailored properties and novel functions, promising improved treatment options for a wide variety of diseases.

Antibodies are a critical component of the humoral adaptive immune response for the neutralization and destruction of disease-causing molecules, viruses and cells. Upon initial exposure to a foreign antigen, the immune system mounts a diverse polyclonal B cell response, producing a repertoire of antibodies that recognize multiple overlapping and non-overlapping antigenic epitopes. Although these initial antibodies have low binding affinities, activated antibody-producing B cells undergo a conserved affinity maturation process through somatic hypermutation and clonal selection to generate antibodies with successively greater affinities1–3. Target neutralization and elimination is subsequently triggered through multiple tightly regulated processes, including protein-mediated and cell-mediated effector functions.

The discovery of the hybridoma technology by Köhler and Milstein4, followed by the development of standardizable, high-yield recombinant antibody discovery, production and purification platforms, ushered in a new era of antibody-based drugs. Indeed, antibodies have grown into an established and exceptionally versatile class of therapeutic agents for treating a wide spectrum of human diseases, owing to their high specificity, modular and adaptable architecture, predictable bioavailability and pharmacokinetics and availability of standardized manufacturing platforms. In May 2022, an estimated 1,000 therapeutic antibodies were at various stages of clinical development and 119 antibodies had obtained first marketing approval in Europe and the USA (excluding biosimilars; a list of antibody therapeutics approved or in regulatory review in the EU or US is available through the Antibody Society).

Many factors contribute to the success of antibody-based treatments, but central to the pharmacodynamic mechanism is the interaction of antibodies with their targets, and more specifically the affinity and avidity of binding interactions4,5. Avidity therein is defined as the accumulated binding strength of multiple affinities contributed by individual non-covalent interactions in crosslinking events. For example, antibody-based effector functions are triggered by multivalent target binding on the cell surface in which avidity-driven surface clustering of IgG molecules serves as a threshold for effector function activation. Indeed, single antibody–antigen binding interactions may be insufficient for successfully eliminating pathogens or diseased cells1–14 and neutralization of soluble proteins based on single binding interactions display a limited potency ceiling that can be surpassed by orders of magnitude using antibody cocktails15–17. Recent innovative antibody
The role of avidity in antibody biology

The strength of a single binding interaction between an antibody Fab fragment and antigen is defined by the term affinity, which we term zero-order avidity for the purpose of this Review. Avidity arising from different affinity interactions mediated by the antibody Fab and Fc domains and modulated by the hinge region can be grouped into tiers depending on the level of multivalency achieved: first-order avidity, referring to bivalent Fab interactions with antigen; second-order avidity, referring to simultaneous Fab–antigen and Fc–Fab interactions; and third-order avidity, referring to interactions between immune complexes and immune effector molecules. The term avidity is used below as an overarching term to describe the binding strength that results from these multidimensional affinity interactions. It is noted that all tiers of avidity interactions may be increased for polyclonal sera and antibody cocktails binding a multitude of non-overlapping epitopes.

Antibody functional responses

The importance of avidity for antibody–antigen recognition in both natural immunity and antibody therapies has long been recognized. The primary mechanism by which antibodies combat infection is through binding and directly neutralizing a pathogenic target antigen by preventing its interaction with host receptors and blocking its function, such as mediating viral entry into a cell. As early as 1937, Burnet et al. recognized that a single antibody binding event was likely to be insufficient to inactivate viruses and argued that neutralization of viruses by antibodies occurs through multivalent antibody binding to a high proportion of viral epitopes. This polyclonal immune complexation, rather than single molecular binding events, is the central trigger for downstream humoral and cellular responses. Further, antibody Fc domains allow for interactions with multiple immune effector molecules present in human plasma or expressed on immune cells and a single binding interaction between an antigen-bound antibody and an effector molecule is generally insufficient to induce an effective Fc-mediated response. All together, avidity binding interactions through both Fab and Fc — with the hinge in a modulatory role — constitute a remarkably sensitive adaptor system that enables the rapid initiation and amplification of different effector mechanisms to combat disease.

In our view, the response kinetics and thresholds governing functional antibody responses can be defined as the net result of distinct activation phases, including scanning of the antibody over the antigen (zero-order avidity), antigen–antibody binding and complex formation (first-order avidity), clustering of antibody–antigen.

bispecific antibodies owing to space constraints and we refer readers to a recent review by Labrijn et al. for more information. Within our conceptual framework, we discuss current translational efforts regarding avidity-based antibody concepts in the clinic and provide perspectives that we believe will aid the development of the next wave of differentiated biotherapeutics.

Human immunoglobulins consist of three distinct regions: 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 immunoglobulin molecule is Y-shaped and is usually composed of four distinct protein chains, including two identical heavy chains (HCS) and light chains (LCs) (dark and light red, respectively, in the figure). HCs are composed of three or four constant domains (C), depending on the antibody isotype, and LCs contain a single constant domain (C). The constant domains together form the Fc region, the hinge region and the base of the Fab region. The Fab region comprises the variable domains that determine antigen binding specificity and affinity. Disulfide bonds shape the overall quaternary structure of the molecule by linking the two HCs in the hinge region and the HC and LC in the Fab region. In humans, variations in the HC constant domain during an immune response generate five different isotypes: IgM, IgD, IgG, IgA and IgE, with IgG and IgA further subdivided into subclasses 1–4 and 1–2, respectively. IgG, IgD and IgE exist as monomers, whereas 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 exists as hexamers in which the J-chain protein is replaced by an extra monomer subunit. IgA may further contain a secretory component that provides protection against proteolytic degradation. The C2 and C3 domains of IgG interact with the neonatal Fc receptor (FcRn), which protects the antibody from catabolism and thereby substantially increases its plasma half-life. Immunoglobulin isotypes and subclasses can further be distinguished by variation in the hinge length, the number and location of disulfide bridges and N-linked or O-linked glycosylation within the HC.

The hinge region serves as a connector and also modulates effector function potency; indeed, modification of the hinge length and flexibility has allowed for fine tuning of IgG effector activity. Together, changes in antibody valency, hinge length and flexibility, as well as glycosylation status can influence avidity interactions and overall functional response kinetics.

Box 1 | Human immunoglobulin architecture

Antibody cocktails
A mixture of two, three or more antibodies combined into a single formulation.

Multiparatopic
An antibody containing antigen-binding domains recognizing two, three or more epitopes on the same target protein.

Immune complexation
The formation of multivalent antibody–antigen complexes.

formats capable of leveraging antibody avidity, such as bispecific and multispecific antibodies and antibodies with engineered Fc-mediated effector functions, have steered the antibody landscape towards more tailored drug design strategies. Notably, the recent decision of the World Health Organization to revise their generic naming scheme for antibodies and create separate categories for Fc-engineered and multispecific antibodies, in addition to changing nomenclature for unmodified antibodies and antibody fragments, highlights the success and maturity of these novel formats as human therapeutic agents.
complexes on the cell surface, potentially facilitated by the formation of simultaneous intermolecular Fc–Fc and Fab–Fab contacts (second-order avidity), and Fc-mediated binding of immune effector molecules up to a threshold that triggers an amplification of the functional response (third-order avidity). These stages are followed by the regulation and contraction of the response (FIG. 1). Association and dissociation rate constants and local concentration determine the on-rate and off-rate of antibody binding kinetics and complex formation is favoured when the on-rate is faster than the off-rate. The conditions required to reach an activation threshold may vary between effector systems; for example, different types of effector mechanisms require different antibody concentrations because they are triggered at distinct target occupancy levels (FIG. 2). Other factors, including antigen expression and distribution, different immunoglobulin subclasses, and effector elements such as complement factors, effector cell subtypes, Fc receptor subtypes, polymorphisms and expression levels, can also affect the overall avidity of antibody interactions and the consequent amplification of a functional response (refs. 27,28).

Regulation and contraction of the response is induced by target-related outputs such as the elimination of target cells, target densities dropping below the amplification threshold or inhibition by regulatory molecules expressed on the target or effector cell or recruited from plasma. Similarly, systemic regulation can occur when there is a shortage of effector molecules or cells, or by the presence of interfering immunoglobulin subclasses (refs. 29–31). Conversely, insufficient regulation may cause autoimmune through excessive or constitutive activation of the antibody functional response.

**The humoral adaptive immune response**

The role of avidity in humoral immunity is evident early in the initiation of the immune response. Upon entry of foreign antigens into lymphoid tissues, naive B cells present in lymph node follicles are activated through antigen binding to their IgM B cell receptors (BCRs) (Box 2). BCRs expressed on mature B cells are membrane-bound immunoglobulin molecules that are activated following antigen-induced aggregation (refs. 32). Although several different models have been proposed for BCR activation, considerable evidence suggests that BCRs undergo activation-dependent localization to membrane microdomains, probably facilitating higher-order BCR clustering and signal transduction (refs. 33,34). Upon activation, some B cells become plasma cells, which switch from primarily expressing membrane-associated IgM to abnormally secreting it by a mechanism of alternative RNA processing (ref. 35). Secreted IgM antibodies are predominantly pentameric molecules (hexameric molecules comprise <5% of serum IgM) (ref. 36) that, despite binding with characteristically low intrinsic affinity ($K_{D}$ of $10^{-1}$ to $10^{-2}$ mol$^{-1}$), are able to avidly engage immunogens owing to their ten identical antigen binding sites and high conformational flexibility (ref. 16). IgM antibodies are especially effective in protecting against microbes with highly expressed, closely spaced surface epitopes, which is in part a consequence of efficient avidity-based interactions between surface antigen-bound IgM and the complement system.

In an ongoing immune response, B cell clonal selection and affinity maturation can drive the intrinsic affinity of the Fab domain up to $10^{-17}$ mol$^{-1}$ (refs. 39,40). Additionally, class switching from IgM to IgG, IgA,
IgE and their subtypes results in swapping of antibody Fc domains and associated functional properties. Conversely, B cell activation is subject to physiological control mechanisms through Fc-mediated feedback, where secreted antibodies inhibit B cell activation by forming antibody–antigen immune complexes that, in the context of interaction with BCR-bound antigen, co-engage the inhibitory IgG Fc receptor IIb (FcγRIIB). Avidity ligation of both the BCR and FcγRIIB results in inhibitory feedback of BCR complex signalling through phosphatases recruited by the immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic tail of FcγRIIB24,41,42 (Fig. 3a).

**Fc-mediated effector functions**

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 through the activation of complement, NK cells and macrophages.

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**Fig. 2** | **Factors affecting antibody functional response activation.**

- **a.** Schematic representation of dose–response relationships for different Fc-mediated effector mechanisms. Functional responses reach the activation threshold at antibody doses that vary per effector mechanism; here illustrated in order of increasing antibody concentrations required for antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC).
- **b.** Increasing antibody doses result in increasing target occupancy and antibody densities on the target cell surface, thereby favouring distinct tiers of avidity binding; saturation may favour monovalent antibody binding. Fc-mediated effector functions are triggered at different target occupancy levels. IgG Fc receptor (FcγR)-induced ADCC involves the release of cytotoxic granules containing granzymes and perforin (an example of a natural killer (NK) cell is shown); ADCP involves the uptake and lysosomal degradation of target cells (an example of a macrophage is shown); CDC involves the triggering of an amplifiable cascade of complement proteins present in blood, terminating in the generation of a lytic membrane-attack complex. In addition to direct killing, the production of cytokines or bioactive complement fragments may contribute to additional attraction and activation of effector cells. The antibody density required for reaching the activation threshold is defined by different parameters including antibody affinity, valency and concentration; structural constraints related to epitope recognized and antibody isotype, antigen expression and distribution; and the type and presence of effector molecules and regulatory molecules.
or diseased cells such as infected or cancerous cells. Although the processes of antigen binding by the Fab domain and effector function activation by the Fc domain were long thought to act independently, multiple studies have challenged this view and an optimal configuration of immune complexes probably exists for different types of effector functions. In addition to different Fc-density requirements for activation (Fig. 2), there is accumulating evidence for allosteric or intramolecular cooperativity⁴,³⁴; for example, the optimal immune complex stoichiometry required for complement activation may be facilitated by IgG Fc–Fc interactions and modulated by their Fab domains⁴⁵. Fab and Fc function may even be considered interdependent, given that efficient complement activation requires clustering of cell-bound IgGs through concerted antigen–Fab and Fc–Fc interactions, thereby forming optimal docking sites for recruitment of C1q⁴⁶. Furthermore, the binding of IgG–antigen complexes to FcyRIIIA was recently shown to be stabilized through contacts with the IgG light-chain constant region, as well as by Fab and Fc conformational changes⁴⁶. First-order and second-order avidity interactions are therefore a prerequisite for third-order avidity interactions and resulting functional responses.

**Neutralization**

Fab-domain-mediated neutralization is the most direct mechanism by which antigens and pathogens can be inactivated. Indeed, binding affinity is a principal driver of potency for some highly successful therapeutic antibodies that neutralize cytokines, growth factors or complement factors. Binding of the Fab domain to specific pathogenic structures prevents interactions with host cells, thereby blocking toxin activity or viral entry into the cell. Although efficient neutralization fundamentally depends on strong affinity, recent studies provide compelling evidence for additional avidity mechanisms for optimizing pathogen recognition. For example, the repetitive nature of certain *Plasmodium falciparum* circumsporozoite protein (PICSP) epitopes drives the selection of homotypic Fab–Fab interactions between PICSP repeat region-specific antibodies⁴⁷,⁴⁸ (Fig. 5b). These second-order avidity interactions presumably increase B cell receptor clustering and selective clonal expansion of PICSP-reactive B cells.

One of the most striking illustrations of the selective pressure of repeating microbial epitopes involves the heavy chain variable (VH)-domain swapping observed in anti-HIV-1 human antibody 2G12, which enables multivalent interactions with conserved carbohydrate clusters on the HIV-1 envelope glycoprotein subunit gp120 (REF.⁴⁹). A single large and rigid binding domain is formed by the two heavy chains cross-contacted by a polypeptide J-chain but a small fraction exists as a covalent Igm hexamer in absence of a J-chain. Owing to their structural and functional differences, pentameric and hexameric Igm might be viewed as distinct IgM subclasses⁵⁰. 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 upon pentameric and hexameric Igm, which is exported across mucosal surfaces in copious amounts by the polymeric antibody binding²⁰⁴,²⁰⁵. Pentameric IgM can be exported at mucosal surfaces by the high-avidity binding interactions with c1q and efficient complement activation upon pentameric and hexameric Igm. Neutrophils and macrophages²¹¹.

**Box 2 | Human immunoglobulin functional properties**

**BCR**

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

**IgM**

In plasma, IgM largely exists as pentamers linked together by disulfide bonds and a polypeptide J-chain, but a small fraction exists as a covalent IgM hexamer in absence of a J-chain. Owing to their structural and functional differences, pentameric and hexameric IgM might be viewed as distinct IgM subclasses⁵⁰. 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 upon antigen binding²⁰⁴,²⁰⁵. Pentameric IgM can be exported at mucosal surfaces by the polymeric immunoglobulin receptor to form secretory IgM in complex with a fragment derived from the polymeric immunoglobulin receptor, the secretory component²⁰⁵.

**IgG**

IgG is the most abundant immunoglobulin class in human serum and accounts for approximately 10–20% of plasma protein. The four different subclasses, in order of decreasing abundance, are IgG1, IgG2, IgG3 and IgG4. Each subclass contains a unique profile with respect to antigen binding, complement binding and binding to Fc receptors for IgG (FcγRs). Structural determinants in the core hinge region can influence antibody function; for example, increasing hinge length and flexibility enhances binding to antigen for immune complex formation and binding to the complement component C1q²¹²,²⁰⁷. The flexibility of the Fab arms affects the relative binding of subclasses to FcγRs and C1q, with IgG3 having the strongest binding, followed by IgG1, IgG4 and IgG2. Furthermore, modification of glycans attached to amino acid N297 affects FcγR binding. Notably, human polyclonal IgG4 antibodies undergo a process named Fab-arm exchange under natural physiological conditions, in which half-molecules (Hc–lc pairs) recombine with half-molecules from other IgG4 antibodies. This is a continuous and stochastic process resulting in bispecific, functionally monovalent IgG4 antibodies in blood⁵⁰.

**IgD**

IgD is primarily expressed as a transmembrane antigen receptor on naive mature B cells. IgD possesses a long hinge region with high flexibility and is capable of acquiring a T-shaped structure. This structural flexibility potentially contributes to the regulation of B cell responsiveness to different types of antigens owing to preferential binding of IgD to multimeric antigens over monomeric antigens²⁵,²⁵⁰. Secreted IgD weakly interacts with complement or FcαRs. It binds basophils, mast cells, monocytes and dendritic cells in an FcγR-independent manner, contributing to mucosal homeostasis through the production of antimicrobial peptides and inflammatory cytokines.

**IgA**

IgA has two subclasses — IgA1 and IgA2 — that contain a heavily glycosylated t-shaped heavy chain and a more rigid Y-shaped hinge, respectively. IgA in serum exists as a monomer and a functional structure, five somatic mutations were sufficient for antibody function; for example, increasing hinge length and flexibility enhances binding to antigen for immune complex formation and binding to the complement component C1q²¹²,²⁰⁷. The flexibility of the Fab arms affects the relative binding of subclasses to FcγRs and C1q, with IgG3 having the strongest binding, followed by IgG1, IgG4 and IgG2. Furthermore, modification of glycans attached to amino acid N297 affects FcγR binding. Notably, human polyclonal IgG4 antibodies undergo a process named Fab-arm exchange under natural physiological conditions, in which half-molecules (Hc–lc pairs) recombine with half-molecules from other IgG4 antibodies. This is a continuous and stochastic process resulting in bispecific, functionally monovalent IgG4 antibodies in blood⁵⁰.

**IgE**

IgE is the least abundant and fastest-clearing isotype in human plasma (its half-life is less than a day, compared to about 3 weeks for IgG). IgE binds to the high-affinity FcεRI on mast cells and the low-affinity CD23 on haematopoietic cell types²⁵¹. IgE glycosylation is essential for IgE–FcεRI interactions and this requirement is primarily attributed to one of seven N-linked glycan sites (N394)²¹³. IgE can remain bound to FcεRI for weeks to months, thereby contributing to long tissue persistence²¹⁴.

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**Fig. 2** | Human immunoglobulin functional properties.

**Fig. 3b** | Human immunoglobulin functional properties.
The crucial importance of avidity in antibody-mediated neutralization of toxins and viruses is further exemplified by HIV-1 itself, which escapes neutralizing antibodies by impeding bivalent high-avidity antibody binding, in addition to other mechanisms such as its rapid mutation rate and shielding of conserved epitopes on the trimeric spike protein. As a result, antibodies against HIV-1 envelope glycoproteins are thought to predominantly bind the viral surface monovalently53–55. Several studies suggest that evasion of bivalent antibody binding occurs owing to the low spike protein density on the virion surface and an unfavourable distribution of epitopes on the viral spike protein trimer, thereby limiting interspike and intraspike crosslinking, respectively56 (Fig. 3b, right). By contrast, antibodies targeting viruses such as influenza and respiratory syncytial virus can bind bivalently and thereby take advantage of second-order avidity effects57,58.

Often, Fc-domain-mediated third-order avidity interactions are required to augment toxin or pathogen inactivation through recruitment of complement or FcγRs on immune cells (Fig. 2). In the case of HIV-1, in vivo protection by broadly neutralizing antibodies (bNAbs) was shown to be augmented for antibodies with Fc domains capable of engaging activating FcγRs 59,60. Similarly, in an elegant Fc engineering approach, Gunn and colleagues recently used a library of Fc variants with identical Fab domains to demonstrate the critical
Antibody-dependent enhancement
A phenomenon in which non-neutralizing antibodies (or neutralizing antibodies at a suboptimal concentration) bind to pathogens without blocking or clearing infection and instead exacerbate disease by leading to increased infection or enhanced immune activation.

importance of FcγR-mediated effector functions and complement activation in antibody-mediated protection against Ebola virus infection.20

The potentially deleterious effects of effector functions, such as antibody-dependent enhancement of viral infection, represent an important design consideration. Interactions between virus-bound antibodies and FcγRs or complement receptors may enhance viral entry and replication in phagocytic cells, as has been demonstrated for flaviviruses such as dengue virus.19 A cocktail of antibodies targeting distinct epitopes on dengue virus envelope glycoprotein E and engineered to reduce FcγR binding (L234A–L235A mutations) induced efficient dengue virus neutralization without precipitating antibody-mediated complement activation19. This indicates that potentiating first-order and second-order avidity binding in a polyclonal antibody mixture while minimizing third-order avidity may be crucial for obtaining optimal protection in certain cases.

Autoimmunity
The avidity of autoantibodies against self-antigens can increase their pathogenicity, as demonstrated by the induction of symptoms of myasthenia gravis in monkeys by bivalent IgG1 antibodies against the acetylcholine receptor when infused alone, but not when combined with IgG4 antibodies. This can be explained by the finding that IgG4 antibodies in plasma cannot engage in first-order Fab-mediated avidity interactions as they become functionally monovalent in vivo by the dynamic process of Fab-arm exchange between IgG4 antibodies of unrelated specificity.21 Notably, interspecies exchange between human and macaque IgG4 antibodies occurs with higher efficiency than intraspecies exchange because of the increased stability of the human/macaque IgG4 heterodimer22,23, which may have contributed to the observed effects. Paradoxically, avidity binding may also ameliorate autoimmunity, as was demonstrated in the case of myasthenia gravis caused by autoantibodies against muscle-specific kinase (MuSK). Signalling through MuSK has an essential role in maintaining neuromuscular junctions and a recent study demonstrated that monospecific, functionally bivalent anti-MuSK antibodies act as partial agonists and are therefore less pathogenic than bispecific, functionally monovalent IgG4 anti-MuSK antibodies, which abolished MuSK signalling. These findings suggest that a lack of first-order avidity of IgG4 molecules could be a pathogenic mechanism in other IgG4-mediated autoimmune diseases.24

Complement activation
The complement system is a potent innate immune defence mechanism composed of an amplifiable enzymatic cascade that kills pathogens and attracts immune effector cells. Complement is activated through three distinct pathways: the classical pathway, the lectin pathway and the alternative pathway, which depend on the binding of C1q, mannann-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, chemotactants and the formation of the membrane attack complex, which breaches the target cell membrane to kill the cell.25

The classical pathway is triggered upon binding of C1q to the Fc region of cell-bound IgG or IgM. C1q consists of six collagen-like triple-helical stalks connected to globular headpieces that resemble the shape of a bunch of tulips.26 The binding affinity of monomeric IgG Fc for C1q is weak (Kₐ ≈ 10⁻⁸ M), resulting in highly transient interactions. Consequently, functional C1q binding and activation requires second-order and third-order avidity interactions, leading to an increase in Kₐ by four orders of magnitude.27 IgM antibodies naturally exist in a multimeric form, which allows them to interact with C1q to activate complement following a conformational change.28,29,30 By contrast, IgGs require assembly into ordered antigen-bound hexamers to form an optimal structure for binding and activating C1 (REF.1). Mass spectrometry, cryo-electron tomography and mutational studies have given compelling evidence for the requirement of higher-order IgG oligomers for optimal complement activation, demonstrating that IgG monomers, dimers and trimers do not contribute much to CDC and that engagement of least four C1q binding sites is required for C1 activation.31,32,33,34,35 The assembly of IgG hexamers on antigenic surfaces results from an intricate process in which incoming IgGs are predominantly recruited from solution through second-order avidity events in which Fc–Fc interactions facilitate antigen binding (FIG. 3c). At low antigen densities or high IgG concentrations, monovalently bound IgGs may contribute to oligomerization mediated through lateral interactions between FcγRs on adjacent B cells.36


diagram of antibody biology. a | B cell receptors (BCRs) constitute antibodies expressed with a transmembrane region on the B cell surface. First-order avidity interactions induce BCR crosslinking and phosphorylation of the BCR-associated transducer molecules that recruit SYK and LYN tyrosine kinases, resulting in protein kinase C (PKC) activation, mitogen-activated protein kinase (MAPK) activation and calcium release. Second-order and third-order avidity binding of antibody–antigen immune complexes recruit FcγRIIB, which comprises an immunoreceptor tyrosine-based inhibitory motif (ITIM motif) that activates phosphatases and reduces BCR signalling. b | Fab–domain-mediated neutralization of pathogen structures, preventing interactions with host cells and blocking pathogen entry into the cell. Binding of a protective antibody against the repetitive Pfalciparum circumsporozoite protein (PfCSP) epitope consisting of repeats of the amino acids NPNA is facilitated by second-order avidity Fab–Fab interactions between Fabs from neighbouring IgGs (bound in pairs). Neighbouring Fabs each bind a two NPNA repeat and are shifted 77° along the NPNA spiral, with five IgGs completing a full circle. The Fabs of the IgGs are oriented non-symmetrically in a repeating light chain-bottom/heavy chain-top orientation, facilitating Fab–Fab contacts (left). HIV-1 virions escape second-order avidity binding by sparse surface expression of HIV-1 envelope glycoprotein spikes. The neutralizing antibody ZG12 evolved a domain-swapped structure in which each heavy chain contacts both light chains, thereby creating a large rigid surface that facilitates avid binding of conserved carbohydrate clusters on the HIV-1 envelope spike (right). c | Clustering of immune complexes is initiated by IgG molecules that assemble into ordered hexameric structures through non-covalent Fc–Fc interactions, which facilitate third-order avidity binding and activation of C1 or intracellular signalling through recruitment of signalling molecules such as cIAP1 and TRAF2. IgG hexamer formation proceeds through recruitment of additional IgG molecules through second-order avidity interactions mediated by the Fc domains until ring closure. d | Antibodies that predominantly bind monovalently (such as those with moderate affinity or those engineered for monovalent binding) may elicit stronger effector functions because higher cell surface densities of Fc domains can be achieved. Part a adapted from REF.41, Springer Nature Limited.
diffusion. Interestingly, functionally monovalent antibodies, such as bispecific antibodies comprising only a single Fab arm that is capable of antigen binding on a target cell, were shown to induce CDC more potently than the corresponding bivalent (parent) antibodies, potentially owing to optimal geometric positioning and a superior ability to form hexamers and engage C1 [REFS 14,46,72]. The high amplification potential of the IgG hexamer–complement system is counterbalanced by strong regulation through numerous soluble factors — for example, C1 inhibitor, C4Bp, and complement factors I and H — and cell-surface-expressed proteins such as CD46, CD55 and CD59, which block the complement cascade from progressing at multiple checkpoints.

As discussed earlier, an ideal immune complex stoichiometry probably exists for efficient activation and amplification of Fc-mediated effector functions such as the complement cascade. Aside from optimal immunoglobulin 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 positioning of IgG hexamers on the cell surface, including antigen−dependent constraints such as their size and distribution, and epitope geometry and orientation. Antigen−dependent and epitope−dependent constraints are most clearly exemplified by the enigmatic existence of two functional types of antibodies against the established therapeutic target CD20 in B cell malignancies and inflammatory diseases. Type I CD20 antibodies induce CD20 translocation into lipid rafts and induce cell killing through complement activation and ADCC, whereas type II CD20 antibodies do not cluster CD20 and instead kill through apoptosis and ADCC. Recent studies employing structural and thermodynamic analyses have shed light on the mechanistic basis of this class distinction, showing that CD20 forms a dimer that provides two binding sites for type I CD20 antibodies such as rituximab and ofatumumab, facilitating avidity interactions and complement activation. These interactions include Fab–Fab and Fc–Fc second-order avidity interactions enabled by the two binding sites on the CD20 dimer. By contrast, each antigen binding site of the type II CD20 antibody obinutuzumab can bind only a single CD20 dimer, therefore precluding second-order avidity interactions that facilitate complement activation. These insights suggest that besides Fc–Fc interactions, Fab–Fab homotypic interactions can also be exploited to avidity−engineer therapeutic antibody function.

Combinations of IgG antibodies simultaneously targeting CD20 and CD37 antigens can induce synergistic CDC of tumour B cells by forming mixed hetero-hexameric complexes on the cell surface, even where the monoclonal parent antibodies are unable to activate complement. This synergy between antibodies targeting two different antigens is probably caused by a favourable distribution of target epitopes on the cell surface that facilitates avidity binding. Conversely, structural constraints such as hinge length and glycan heterogeneity influence IgG oligomerization and complement activation. The composition of polyclonal mixtures of IgG subclasses or including other antibody classes such as IgA and IgE may therefore affect complement activation through competition for antigen binding and differences in flexibility, conformation and valency.

**Box 3 | IgG Fc receptors**

The Fc domain of antibodies interacts with a family of FcRs expressed on various immune cells to mediate effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP). IgG binds to FcRs (Box 3), which are broadly classified as either activating or inhibitory, depending on whether their intracellular signalling domains contain an immunoreceptor tyrosine activating motif (ITAM) or an immunoreceptor tyrosine inhibitory motif (ITIM), respectively. Activating FcRs in humans include FcγRI (CD64), FcγRIIA (CD32a) and FcγRIIB (CD16a) and the less well characterized FcγRIC (CD16c). By contrast, FcγRIIB (CD16b) represents the sole inhibitory FcγR, which regulates the function of activating FcRs, controls antibody production by B cells and serves as the main scavenging receptor in the liver. Finally, FcγRIIB (CD16b) uniquely contains no intracellular signalling domain and is instead anchored to the membrane by glycosylphosphatidylinositol. FcγRs are differentially expressed on lymphoid-derived and myeloid-derived effector cells, although the receptor distribution is unique to each cell type. FcγRIIA 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 signalling molecules after engaging IgG-opsonized target cells. Phagocytes such as macrophages and dendritic cells express a combination of different FcγRs, including FcγRI, FcγRIIA, FcγRIIB, FcγRIID and FcγRIIF, that mediate ADCP for innate destruction of IgG-opsonized targets cells and also promote cross-presentation to T cells to elicit immunological memory. Studies have shown that 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 dendritic cells. Although the link between FcγRs and cellular immunity has historically been viewed as indirect through antigen presentation by cells, it was recently demonstrated that FcγRIIA is capable of directly promoting activation of CD4+ T cells by IgG immune complexes.

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**Afucosylated**

Describes an IgG antibody that lacks one or both fucose residues in the N-linked branched glycan moiety of the Fc domain, leading to strongly enhanced affinity for FcγRIIA.

**Fc-receptor-mediated cellular effector functions**

FcγRs are bound by the different human IgG isotypes with varying affinities (Box 3). Only FcγRI can bind monomeric IgG with high (nanomolar range) affinity and as a consequence may hamper avidity interactions within immune complexes in vivo, given that the receptor is probably nearly saturated by the high IgG content in serum. Cytokine stimulation during inflammation may induce FcγRI clustering in membrane microdomains, thereby facilitating avidity binding of immune complexes over monomeric IgG. All other FcγRs exhibit low, micromolar affinities for monomeric IgG, and as a consequence require third-order avidity interactions for IgG binding 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 over a threshold that triggers receptor signalling through phosphorylation of immunoreceptor tyrosine activating motif (ITAM) domains, which in turn leads to elimination of target cells through ADCC or ADCP. Notably, FcγRIIB — which is responsible for mediating ADCC by natural killer (NK) cells — binds afucosylated IgG, with 40-fold greater affinity than fucosylated IgG. The relevance of this for immune regulation was demonstrated by the observation that afucosylated IgG is selectively produced against membrane antigens on enveloped viruses — triggered by an as-yet-unknown mechanism — and the degree of afucosylation correlates with disease severity in COVID-19 and dengue
Polymorphic
Indicates that the protein occurs as two or more variants or alleles in the population with functional polymorphisms of Fc receptors referring to isomers with different immunoglobulin-binding properties.

Avidity engineering of antibody therapeutics
Antibodies have become widely established as therapies for numerous diseases, including cancer, infectious diseases, inflammatory diseases and autoimmunity. The design of therapeutics against hard-to-hit targets that provide meaningful improvements for patients compared to contemporary drug regimens is shifting antibody drug development from canonical IgG antibodies towards formats with novel functionalities. The search for approaches to enhance antibody function is not surprising considering the limitations imposed by monospecificity; indeed, the use of monoclonal antibodies as a single agent for therapy seems contradictory in light of the polyclonal, avidity-tuned nature of antibodies generated during the natural immune response.

The avidity engineering toolbox
A broad spectrum of novel antibody engineering strategies and formats that enhance or tune avidity are emerging. These strategies include those that optimize physiological immune pathways, such as effector function enhancement, and those that enable non-native mechanisms of action, for example effector cell redirection or receptor agonism.

Below, we describe an ‘avidity engineering toolbox’ and discuss the impact of different strategies on the different phases of antibody functional response activation as defined above and in Fig. 1. Avidity engineering strategies include targeting multiple epitopes; targeting multiple cell surface receptors in cis; tuning antibody valency; and tuning binding strength for complement or FcγRs. Antibody therapies employing these engineering strategies have advanced into clinical development and as of May 2022, the commercial clinical pipeline includes 35 programmes leveraging avidity to increase antibody function (Table 1). 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 have been introduced that enable increased antibody binding by targeting more than one epitope or target. Although these antibodies were initially developed to improve selectivity by targeting multiple disease-related antigens or signalling pathways, multi-targeting concepts may simultaneously enhance functional activity by promoting avidity interactions in early phases of the antibody functional response. Multi-targeting concepts include combinations of two or more antibody molecules with different defined specificities — for example, antibody mixtures or designer polyclonals — and antibody architectures and formats combining two or more specificities in a single antibody molecule such as bispecific or multispecific antibodies.

Targeting multiple epitopes. Multi-targeting antibody technologies directed towards non-overlapping epitopes on the same target come closest to exploiting the potentiation of first-order and second-order avidity interactions as they occur in natural polyclonal antibody responses (Fig. 4a). These technologies can increase target
**Table 1 | Avidity-based therapeutic antibody concepts in the clinic and engineering platforms**

| Agent (company) | Target | Format | Indication (selected) | Development status (selected trials) |
|-----------------|--------|--------|-----------------------|-------------------------------------|
| **Targeting multiple epitopes** |
| Sym015 (Symphogen) | MET | Mixture of two IgG1 antibodies binding non-overlapping epitopes on the Semaphorin domain of MET | Solid tumours (NSCLC) | Phase Ila completed (NCT02648724) |
| REGN5093 (Regeneron) | MET | Biparatopic, bispecific IgG4 antibody targeting two non-overlapping epitopes on MET (Veloci-Bi bispecific technology) | Solid tumours (NSCLC) | Phase I/II (NCT04077099) |
| REGN5093-M114 (Regeneron) | MET | Biparatopic, bispecific IgG4 antibody targeting two non-overlapping epitopes on MET (Veloci-Bi bispecific technology) conjugated to a maytansinoid | Solid tumours (NSCLC) | Phase I/II (NCT04077099) |
| Sym004 (Symphogen) | EGFR | Mixture of two IgG1 antibodies (futuximab, modotuximab) binding non-overlapping epitopes on EGFR | Solid tumours (mCRC) | Phase III completed (NCT02083653) |
| KN026 (Alphamab) | HER2 | Biparatopic, bispecific IgG1 antibody targeting two non-overlapping epitopes on HER2 (CRIB technology) | Solid tumours (breast and gastric cancer) | Phase I (NCT03619681, NCT03847168) |
| MBS301 (Beijing Mabworks Biotech) | HER2 | Afucosylated, biparatopic, bispecific IgG1 antibody targeting two non-overlapping epitopes on HER2 (knobs-into-holes bispecific technology) | Solid tumours (HER2) | Phase I (NCT03842085) |
| Zanidatamab, ZW25 (Zymeworks) | HER2 | Biparatopic, bispecific IgG1 antibody targeting two non-overlapping epitopes on HER2 (Asymmetric bispecific technology) | Solid tumours (HER2) | Phase II (NCT04466891, NCT03929666, NCT04224272) |
| ZW49 (Zymeworks) | HER2 | Biparatopic, bispecific IgG1 antibody targeting two non-overlapping epitopes on HER2 (Asymmetric bispecific technology) conjugated to a maytansinoid (Zymelink antibody-drug condensate technology) | Solid tumours (HER2) | Phase I (NCT03821233) |
| REGN-COV, casirivimab/imdevimab (Regeneron) | SARS-CoV-2 spike | Mixture of two human IgG1 antibodies, binding non-overlapping epitopes on the SARS-CoV-2 viral spike | COVID-19 | EUA, Phase III completed (NCT04381936) |
| Evusheld, tixagevimab/cilgavimab, (AstraZeneca, Vanderbilt) | SARS-CoV-2 spike | Mixture of two human IgG1 antibodies with mutations that extend half-life (M252Y,S254T,T256E) and reduce Fc receptor and C1q interactions (L234F, L235E, P331S), binding non-overlapping epitopes on the SARS-CoV-2 viral spike | COVID-19 | EUA, Phase III (NCT04625725) |
| BMS-986414/BMS-986413 (Bristol Myers Squibb/ Rockefeller University) | SARS-CoV-2 spike | Mixture of two human IgG1 antibodies with mutations that extend half-life (M428L/N434S), binding non-overlapping epitopes on the SARS-CoV-2 viral spike | COVID-19 | Phase II/III (NCT04518410) |
| 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 from the VRC01-LS, PGDM1400 and 10E8v4 therapeutic antibodies, targeting the CD4bs, gp41 MPER and V1/V2 glycan-directed binding sites on HIV-1 (CVDig-1g technology) | HIV-1 | Phase I (NCT03705169) |
| Inmazeb, atoltivimab/maftivimab/odesivimab (Regeneron) | Zaire ebolavirus (ZEBOV) glycoprotein | A mixture of three afucosylated IgG1 antibodies that each bind to different, non-overlapping epitopes on the ZEBOV glycoprotein | ZEBOV | Approved (NCT03576690) |
| **Targeting multiple cell surface receptors** |
| LAVA-051 (Lava Therapeutics) | CD1d, V82 TCR | A trispecific 6T cell engager comprised of a single-domain antibody (VHH) that binds both CD1d and the INKT TCR, and a VHH targeting the Vy9V62 TCR, which triggers iNKT and Vy9V62 T cell activation | Haematologic malignancies (CLL, MM, AML) | Phase I/II (NCT04887259) |
| Sym013 (Symphogen) | EGFR, HER2, HER3 | A mixture of six humanized monoclonal IgG1 antibodies (three pairs) that bind to non-overlapping epitopes on EGFR, HER2 and HER3 | Advanced epithelial malignancies | Phase I/II terminated (NCT02906670) |
| **Tuning antibody valency** |
| IGM-8444 (IgM Biosciences) | DR5 | Pentameric IgM antibody with ten binding sites specific for DR5 | Solid tumours | Phase I (NCT04553692) |
| INBRX-109 (Inhibrix) | DR5 | Four single-domain antibodies (tetravalent) fused to an Fc domain (sdAb technology; binding units derived from heavy-chain-only antibodies) | Solid tumours | Phase I (NCT03715933, NCT04950075) |
| INBRX-106 (Inhibrix) | OX40 | Six single-domain antibodies (hexavalent) fused to an Fc domain (sdAb technology; binding units derived from heavy-chain-only antibodies) | Solid tumours | Phase I (NCT04198766) |
| ABBV-621, APG350, Efotuzanerin alpha (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) | Multiple myeloma | Phase IIb (NCT04570631) |
Table 1 (cont.) | Avidity-based therapeutic antibody concepts in the clinic and engineering platforms

| Agent (company) | Target | Format | Indication (selected) | Development status (selected trials) |
|-----------------|--------|--------|-----------------------|--------------------------------------|
| **Tuning antibody valency (cont.)** | | | | |
| PF-06755347, GL-2045 (Pfizer/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) |
| **Tuning antibody oligomerization** | | | | |
| GEN3014, HexaBody-CD38 (Genmab) | CD38 | IgG1 antibody containing an E430G hexametization-enhancing Fc mutation (HexaBody technology) | Haematologic malignancies (MM) | Phase I/II (NCT04824794) |
| **Targeting multiple epitopes and tuning antibody oligomerization** | | | | |
| GEN3009, DuoHexaBody-CD37 (Genmab/AbbVie) | CD37 | Biparatopic, bispecific IgG1 antibody targeting two non-overlapping epitopes on CD37 containing an E430G hexametization-enhancing Fc mutation (DuoHexaBody technology) | Haematologic malignancies (B cell NHL, CLL) | Phase I/II (NCT04358458) |
| GEN1029, HexaBody-DR5/DR5 (Genmab) | DR5 | Mixture of two IgG1 antibodies targeting non-overlapping epitopes on DR5, both containing an E430G hexametization-enhancing Fc mutation (HexaBody technology) | Solid tumours | Phase I (terminated) (NCT03576131) |
| **Tuning binding to Fc effector receptors** | | | | |
| GS-1811, JXT-1811 (Gilead Sciences/Jounce Therapeutics) | CCR8 | IgG1 antibody afucosylated for enhanced ADCC | Solid tumours | Phase I (NCT05007782) |
| BMS-986340 (Bristol Myers Squibb) | CCR8 | IgG1 antibody afucosylated for enhanced ADCC | Solid tumours | Phase I (NCT04895709) |
| SEA-CD70 (Seattle Genetics) | CD70 | IgG1 containing afucosylated for enhanced ADCC (SEA technology) | Myeloid malignancies (MDS, AML) | Phase I (NCT04227847) |
| Elipovimab, GS-9722 (Gilead Sciences) | HIV-1 envelope | IgG1 bNAb (derived from PGT121) containing S239D/I332E and M428L/N434S Fc mutations for improved PK, enhanced ADCC and ADCP (high cytotoxicity XmAb and Xtend Fc domain technology) | HIV-1 | Phase Ib (GS-US-420-3902) |
| Monjuvi, tafasitamab (MorphoSys/Incyte) | CD19 | IgG1 antibody containing S239D/I332E Fc mutations for enhanced ADCC and ADCP (high-cytotoxicity XmAb Fc domain technology) | Haematologic malignancies (DLBCL) | Approved |
| Margenza, margetuximab (Macrogenics) | HER2 | IgG1 antibody containing L235V/F243L/R292P/Y300L/P339L+ insertion Fc mutations for enhanced ADCC | Solid tumours (HER2+ breast cancer) | Approved |
| Poteligeo, mogamulizumab (Kyowa Hakko Kirin) | CCR4 | IgG1 antibody afucosylated for enhanced ADCC (Potelligent Technology) | Adult T cell leukaemia or lymphoma | Approved |
| Gazvya, obinutuzumab (Roche) | CD20 | IgG1 antibody afucosylated for enhanced ADCC (GlycoMab Technology) | Haematologic malignancies (CLL, FL) | Approved |
| Fasenra, benralizumab (Astra Zeneca/Kyoo Hakko Kirin) | IL-5Ra | IgG1 antibody afucosylated for enhanced ADCC (Potelligent Technology) | Asthma | Approved |
| **Targeting multiple cell surface receptors and tuning binding to Fc effector receptors** | | | | |
| Rybrevant, amivantamab (Janssen/JNJ) | EGFR, MET | IgG1 bispecific antibody afucosylated for enhanced ADCC (Duobody technology) | Solid tumours (metastatic NSCLC with EGFR exon 20 insertion mutations) | Approved |
| MCLA-129 (Merus/Betta Pharmaceuticals) | EGFR, MET | IgG1 bispecific antibody afucosylated for enhanced ADCC (Bicitonics and GlymavaX technology) | Solid tumours (NSCLC) | Phase I/II (NCT04868877) |

Data available as of 1 May 2022. Engineering data were obtained from public documents (scientific literature, abstracts, posters and patent publications). This overview table excludes clinical programmes investigating bispecific antibodies, for which we refer to Labrijn et al. 20. ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; AML, acute myeloid leukaemia; bNAb, broadly neutralizing antibody; CCR, CC-chemokine receptor; CLL, chronic lymphocytic leukaemia; CODV-ig, crossover 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; EUA, emergency use authorization; Fc, crystalizable fragment; FL, follicular lymphoma; HER2, human epidermal growth factor receptor 2; iNKT cell, type I natural killer T cell; mCRC, metastatic colorectal cancer; MDS, myelodysplastic syndrome; MET, tyrosine-protein kinase MET; MM, multiple myeloma; MPER, membrane–proximal external region; NHL, non-Hodgkin lymphoma; NSCLC, non-small-cell lung cancer; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; sdAb, single-domain antibody; TCR, T cell receptor; TNFRSF, tumour necrosis factor receptor superfamily; TRAIL, tumour necrosis factor-related apoptosis-inducing ligand; VHH, variable domain of a heavy chain-only antibody. *Adinsight entry, trial not listed in ClinicalTrials.gov.
occupancy and local Fc-domain density to enhance antibody clustering and overall functional activity, and some may even lead to novel functions. For example, in separate studies targeting the oncogenic drivers EGFR and MET, combinations of two antibodies against non-overlapping epitopes on the same cell surface receptor showed enhanced antitumour activity over individual antibodies in preclinical and clinical studies. Both EGFR and MET antibody combinations promoted receptor internalization and degradation, which for EGFR could be attributed to enhanced receptor crosslinking and clustering within detergent-insoluble, plasma-membrane-associated tubules. Increased receptor internalization can also be achieved using multispecific antibody formats. Biparatopic antibodies individually targeting HER2 and MET are currently being developed to induce increased degradation of target receptors or alternatively, as biparatopic antibody–drug conjugates.
The activation threshold for effector functions may be increased through multi-epitope targeting.

Classical Fc-mediated effector functions may be enhanced through multi-epitope targeting. Antibody combinations targeting multiple epitopes of either MET or EGFR were reported to more potently trigger complement activation than monoclonal antibodies targeting a single epitope of either protein.\(^{105,106,108}\) Potentiation of CDC through multi-epitope targeting has been reported for a number of other cell surface targets including CD37, human leukocyte antigen (HLA), the Neisseria meningitides factor H-binding protein, folate receptor alpha (FRα)\(^{105-108}\), and against multiple myeloma and Burkitt lymphoma cell lines using engineered biparatopic heavy-chain-only antibody constructs targeting CD38 (REF.\(^{109}\)). We speculate that avidity engineering of multispecific antibodies may effectively overcome complement defence mechanisms through increased second-order avidity interactions, exploiting the natural capability of antibodies to hexamerize and induce CDC.

Targeting non-overlapping epitopes using multispecific antibody technologies has been explored to reduce virus resistance and lower the risk of viral escape. Trispecific antibody formats combining Fab-derived binding domains of broadly neutralizing antibodies (bNAbs) directed against different functional HIV-1 epitopes on the envelope spike exhibited greater neutralization potency and activity against a larger set of diverse HIV-1 isolates compared to combinations of the parental bNAbs both in vitro and in vivo.\(^{106,107}\)

Formats that enhance antibody binding by targeting multiple, non-overlapping epitopes on a single antigen represent the largest group of clinical programmes leveraging avidity tuning (TABLE 1). Such formats range from mixtures of two or more non-competing antibodies to bispecific or multispecific antibody architectures that combine different epitope binding specificities into a single molecule. For example, multiple clinical programs are currently investigating therapeutics that target multiple epitopes on EGFR family members overexpressed in many solid tumour indications.\(^{100,122,123}\) Clinical data show that patients who initially respond to EGFR-specific antibodies eventually become resistant. A proof-of-concept study using the anti-EGFR antibody cocktail Sym004 consisting of the antibodies futuzumab and modotuzumab (Symphogen) showed clinical activity in a patient with metastatic colorectal cancer with acquired EGFR mutation-mediated resistance to cetuximab.\(^{114}\) Unselected patients treated with Sym004 did not show an increase in overall survival in a phase II study, although a retrospective analysis indicated a survival benefit for a patient subgroup negative for RAS/BRAF/EGFR extracellular domain mutations (NCT02083653).\(^{19}\)

Multi-epitope-targeting antibody cocktails are being explored for prophylaxis or treatment of infection with the highly variant RNA virus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). A combination of casirivimab and imdevimab (Regeneron, NCT04381936) targeting non-overlapping epitopes on the SARS-CoV-2 S glycoprotein was shown to prevent in vitro selection of escape mutants of a pseudovirus expressing the SARS-CoV-2 S glycoprotein, whereas neutralization escape quickly developed in the presence of the individual antibodies or combinations of antibodies against overlapping epitopes.\(^{108-110}\) The cocktail was found to be inactive against the omicron BA.1 variant of SARS-COV-2 although it did neutralize the emerging omicron BA.2 variant, albeit with a reduction in potency of two orders of magnitude.\(^{129,130}\) A recent study using in vitro and in vivo models of SARS-CoV-2 infection demonstrated synergism between two potently neutralizing non-competing antibodies.\(^{131}\) These antibodies were optimized for extended half-life and reduced FcγR binding and are being developed as tixagevimab and cilgavimab (AstraZeneca, NCT04625725). It should be noted that Fc-mutated antibodies with a reduced potential for third-order avidity interactions would be unable to leverage the contribution of Fc-mediated effector functions to in vivo therapy,\(^{122-124}\) although these mutations would decrease the potential risk for Fc-mediated enhancement of disease.\(^{22}\)

Multi-epitope targeting is being explored in a phase I clinical trial of a triparatopic antibody that contains binding domains from three bNAbs (VRG011S, PGDM1400 and 10E8v4 (Sanofi)) against the HIV-1 envelope spike (NCT03705169).\(^{131}\) Further, a cocktail of the three non-competing IgG1 antibodies atoltivimab, maftivimab and odesivimab (REGN-EB3, Regeneron) was recently approved for treatment of Zaire Ebola virus (ZEOBV) (NCT03576690).\(^{108}\) The antibodies were selected for their ability to bind the ZEOBV glycoprotein simultaneously and were produced as afucosylated IgGs owing to the role of Fc-mediated effector functions in protecting against Ebola virus infection.\(^{125-127}\)
Targeting multiple cell surface receptors in cis. Antibody cocktails targeting two or more cell surface receptors represent an archetypal example of avidity engineering by employing the learnings of natural polyclonal antibody responses. Targeting multiple cell surface receptors may increase both antibody occupancy [Fig. 5d] and antigen clustering through crosslinking and Fc–Fc interactions, resulting in enhanced third-order avidity interactions. We recently described a mixture of two antibodies targeting CD20 and CD37 antigens on tumour B cells that co-engaged in hetero-hexameric complexes, thereby potentiating complement activation19 (Fig. 4a, right). Jacobsen and colleagues reported a mixture of six antibodies which, in synergistic pairs, target each of the HER family members EGFR, HER2 and HER3 (Ref.190). This mixture, known as Pan-HER (Sym013, Symphogen) was shown to downregulate the expression of all three targets and overcome acquired resistance in HER family-expressing tumours caused by compensatory receptor upregulation; the cocktail entered clinical development, but was terminated after phase I (NCT02906670).

Other approaches to targeting multiple cell surface receptors include combining multiple binding specificities into a single molecule in the form of bispecific or multispecific antibodies that bridge different receptors on the same cell (in cis) or on different cell types (in trans). One of the strengths of bispecific or multispecific antibodies is their capacity to activate novel ‘designed’ functions that cannot be achieved by antibody mixtures (for bispecific antibodies, these are referred to as ‘obligate’ mechanisms of action)191. Further, their therapeutic index can be increased through the localized tethering of receptors, which can focus therapeutic activity to preferential cells or tissues and limit on-target and off-tumour toxicity192. This concept makes use of a particular aspect of avidity binding that has been referred to as cross-arm binding, in which the first binding event restricts the second binding arm to a small volume, thereby increasing its effective concentration and probability of binding193,194. Notably, the increase in avidity achievable by cross-arm binding is highly dependent on the epitope, target expression levels, antibody format and valency.

The most widely studied ‘obligate’ bispecific antibodies employ in trans bridging to redirect the cytotoxic activity of T cells or other effector cell types to eliminate tumour cells. We refer the reader to recent reviews for a deeper discussion of this important therapeutic antibody class195,196.

An interesting example of how in cis bridging can unlock obligate mechanisms of action is represented by amivantamab (TABLE 1), a recently FDA-approved and EMA-approved bispecific antibody targeting EGFR and MET (Janssen). Crosslinking of MET using bivalent antibodies causes undesired tumour cell activation and amivantamab circumvents this by combining a single, non-crosslinking MET-binding arm with an EGFR-binding arm in a bispecific configuration that effectively blocks both MET and EGFR signalling197,198. The potential to engage in third-order avidity interactions was increased by engineering the bispecific antibody for low fucosylation. This example is 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.

Avidity binding to a protein complex can be achieved by a single binding domain. Lameris et al. recently described a single-domain antibody, VHH1D12, against the antigen-presenting glycoprotein CD1d, a glycolipid-presenting molecule closely related to the HLA class I proteins. VHH1D12 can simultaneously interact with CD1d and the type I natural killer T cell receptor (TCR), which triggers antitumour activity by type I natural killer T (NK) cells owing to CD1d–TCR crosslinking199. The ability of a single antibody binding site to interact with multiple proteins or structures has previously been described for antibodies against HIV-1, for which polyreactivity increased the observed affinity for the virion200. Specifically, bNAb against the HIV envelope glycoprotein membrane-proximal region (MPER) were shown to interact with both the gp41 subunit and the viral lipid membrane201.

Combined in cis and in trans bridging of receptors can also be achieved by engineering additional antibody fragments onto bispecific antibodies, thereby creating tri-specific or multi-specific antibodies that enable simultaneous targeting of multiple cell types and same-cell receptors202,203. For example, the trispecific antibody LAVA-051 (Lava therapeutics) — based on VHH1D12 linked to an additional single-domain antibody targeting the Vγ9Vδ2 TCR of γδ T cells — recruits both type I NK T cells and Vγ9Vδ2 T cells for tumour cell killing. LAVA-051 is currently being investigated as a next-generation T cell engager for the treatment of haematological cancers (NCT04887259) (TABLE 1).

In an additional example, Shivange and colleagues recently reported on the generation of a single-agent, bispecific-anchored cytotoxicity-activator (BaCa), an antibody that co-engages death receptor 5 (DR5, also known as TRAIL-R2) and FRα, which are expressed together on ovarian cancer cells. The BaCa antibody was reported to bind FRα and crosslink DR5 both in cis and in trans, by which FRα served as a tumour-specific anchor and primary clustering point for DR5 agonist signalling. Consistent with the potential for an avidity gain resulting from cross-arm binding, in cis killing required lower antibody concentrations than killing in trans204.

Tuning avidity through antibody valency

Over the past few years, engineering strategies for enhancing antibody clustering to improve antibody function have primarily aimed at increasing antibody valency by replicating antibody binding or Fc domains in fragments and IgG-like or IgM-like structures. Indeed, several studies have reported on the design of antibody formats containing multiple Fc domains to enhance FcγR crosslinking and ADCC205–207. In a recent study, Miller and colleagues adapted the tetramerization domain of the tumour suppressor p53 and fused it to different antigen binding domains to create octavalent monospecific and bispecific antibody variants with increased functional activity, known as quads208. Further, CDC can be enhanced for all four human IgG isotypes through covalent hexamerization, which is achieved...
by genetically fusing the 18-amino-acid μ-tailpiece of IgM to their carboxyl termini, thus generating multivalent structures that efficiently bind and activate complement149.

Some receptors, including those with multimeric cognate ligands or those that interact at cell–cell synapses, require higher-order oligomerization of adjacent complexes for activation of downstream signalling. The most widely studied receptors of this class are members of the tumour necrosis factor receptor superfamily (TNFRSF), which are of growing interest to drug developers owing to their function in regulating cell survival and inflammatory signalling. Regular binding of immunomodulatory antibodies targeting these receptors generally induces insufficient TNFRSF clustering and many require additional third-order avidity binding through FcRs, in particular FcyRIIB, on immune effector cells to engage in agonist activity149–151. Pioneering work by Ashkenazi, Presta and colleagues demonstrated that avidity engineering by increasing valency using tandem Fab repeats can enhance the forward signalling activity of antibodies targeting the TNFRSF DR5 (REF 152).

Previous efforts to develop TNFRSF-targeting antibodies have yielded limited clinical success, possibly owing to insufficient FcR-mediated crosslinking and in some cases adverse events or toxicity153,154. Interest in targeting TNFRSF has revived building upon the role of pre-existing anti-drug antibodies potentially mediating hypercrosslinking151. All together, these studies demonstrate the importance of careful design and rigorous preclinical investigation for avidity-engineered biotherapeutic agonists.

There has been a steady increase of studies detailing the engineering and clinical translation of naturally multivalent immunoglobulin isotypes such as IgM and IgA, despite being more challenging to develop than IgGs owing to their more complex multichain architectures, more extensive and heterogeneous glycosylation patterns and shorter in vivo half-lives156,157,158. In a unique ocular application, Agard and colleagues used the multivalent nature of IgM to effectively agonize the tyrosine-protein kinase receptor TIE2; this application capitalized on the large size of IgM to reduce vitreal clearance, which was shown to be inversely proportional to the molecule’s hydrodynamic radius159.

**Tuning avidity through oligomerization**

The ability of IgGs to self-assemble into ordered oligomers on antigenic surfaces through Fc–Fc interactions can be exploited in therapeutic applications and the design and development of IgG antibodies with standard architecture is more straightforward than that of pre-assembled antibody multimers. De Jong and colleagues reported a novel HexaBody technology platform that uses specific single-point mutations in the Fc domain to enhance target-dependent self-assembly of IgG molecules into hexameric complexes on cell surfaces160 (FIG. 4b, right). In a mutational screening approach, they identified two conserved glutamic acid residues in the Fc region of IgG — E345 and E430 — for which the substitution of any other amino acid enhanced IgG hexamer formation and complement activation for a wide range of cell surface targets. Sopp et al. demonstrated that self-assembly dependent on target binding can also be facilitated by extending IgG1 antibodies with the μ-tailpiece mentioned above, but in which the carboxyl-terminal cysteine is mutated to a serine to prevent covalent hexamerization161. By systematically depleting individual complement components, Taylor et al.
showed that antibodies containing hexamer-enhancing mutations require a reduced presence of membrane attack complex-forming complement components to promote CDC compared to the non-mutated parent antibodies. As discussed previously, on-target self-assembly of hexamerization-enhanced antibodies on antigenic surfaces generates an increased density of IgG hexamers, which therefore probably results in a lower complement activation threshold than regular IgGs.

Enhancement of antibody clustering by promoting self-assembly may also have particular applications in amplifying ‘designed’ antibody functions such as agonistic receptor signalling. The introduction of hexamerization-enhancing mutations in antibodies targeting DR5 has proved to be an effective agonist strategy for inducing tumour cell death; recently, a novel antibody mixture of two non-competing, DR5-targeting antibodies containing an E430G hexamer-enhancing mutation was shown to improve DR5 signalling and tumour cell death independently of FcγR crosslinking in a wide range of tumour types. Intermolecular Fc–Fc interactions between the two IgG molecules were essential for DR5 agonistic activity and binding of complement component C1q reportedly contributed to the crucial role of avidity in natural antibody biology, as well as to promote CDC compared to the non-mutated parent antibodies. As discussed previously, on-target occupancy at lower antibody densities or antibody immune complex concentrations, or by extending interaction times (owing to a decreased off-rate). The Fc regions of IgG subclasses differ in their affinity for C1q and their potency to activate complement component C1q, and ADCP of antibodies targeting the envelope spike on HIV-1 and CD19 on B tumour cells. Tafasitamab, an antibody targeting the latter, was recently approved for treatment of relapsed/refractory diffuse large B cell lymphoma (DLBCL) in combination with lenalidomide. Margetuximab, an anti-HER2 antibodyFc engineered with the mutations L235V/F243L/R292P/Y300L/I332E amino acid substitutions in the Fc domain of antibodies targeting EGFR, CD52 and CD20 were shown to enhance FcγRIIA/FcγRIIB affinity and increase ADCC activity of DR5 and OX40 antibodies. Further, selective enhancement of antibody binding to FcγRIIB has been leveraged to inhibit BCR-mediated B cell activation and suppress humoral immunity, as well as to enhance the clearance of immune complexes.

At present, seven active clinical programs apply Fc engineering to amplify FcγR-mediated effector functions (Table 1). The combination of two Fc-domain single-point mutations (S239D/I332E) enhances ADCC and ADCP of antibodies targeting the envelope spike on HIV-1 and CD19 on B tumour cells. Tafasitamab, an antibody targeting the latter, was recently approved for treatment of relapsed/refractory diffuse large B cell lymphoma (DLBCL) in combination with lenalidomide. Margetuximab, an anti-HER2 antibody Fc engineered with the mutations L235V/F243L/R292P/Y300L/I332E, was recently approved for HER2-positive breast cancer. Glycoengineering for effector function enhancement has met with clinical success in five approved afucosylated antibody products, including mogamulizumab, an antibody targeting the latter, was recently approved for treatment of relapsed/refractory diffuse large B cell lymphoma (DLBCL) in combination with lenalidomide. Margetuximab, an anti-HER2 antibody Fc engineered with the mutations L235V/F243L/R292P/Y300L/I332E, was recently approved for HER2-positive breast cancer. Glycoengineering for effector function enhancement has met with clinical success in five approved afucosylated antibody products, including mogamulizumab, an antibody targeting the latter, was recently approved for treatment of relapsed/refractory diffuse large B cell lymphoma (DLBCL) in combination with lenalidomide. Margetuximab, an anti-HER2 antibody Fc engineered with the mutations L235V/F243L/R292P/Y300L/I332E, was recently approved for HER2-positive breast cancer.

**Conclusions and future perspectives**

Antibody function relies on complex interactions between antibodies, their targets and their association 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 system. Recent advances in understanding the role of avidity in natural antibody biology, from antibody affinity maturation early in the immune system. Recent advances in understanding the role of avidity in natural antibody biology, from antibody affinity maturation early in the immune system. Recent advances in understanding the role of avidity in natural antibody biology, from antibody affinity maturation early in the immune system. Recent advances in understanding the role of avidity in natural antibody biology, from antibody affinity maturation early in the immune system. Recent advances in understanding the role of avidity in natural antibody biology, from antibody affinity maturation early in the immune system. Recent advances in understanding the role of avidity in natural antibody biology, from antibody affinity maturation early in the immune system. Recent advances in understanding the role of avidity in natural antibody biology, from antibody affinity maturation early in the immune system. Recent advances in understanding the role of avidity in natural antibody biology, from antibody affinity maturation early in the immune system.
response to effector function activation after target engagement. Specific interactions between antibody and antigen, which we have termed zero-order avidity binding, can vary from highly transient (fast off-rate) to long lasting (slow off-rate). Next, avidity interactions critically contribute to antibody biology by increasing the strength of binding until a threshold is reached that triggers functional activity. We distinguish first-order avidity (bivalent Fab–antigen binding), second-order avidity (simultaneous Fab–antigen and Fab–Fab or Fc–Fc interactions) and third-order avidity (interactions between the immune complex and effector molecule). All levels of avidity binding can be potentiated by the polyclonality of antibody responses targeting multiple epitopes. Although the threshold at which antibody avidity interactions translate into an effective functional response varies between effector mechanisms, avidity binding can be viewed as a checkpoint before response amplification that in large part determines the efficacy of the functional response. Understanding the key determinants that shape antibody functional response kinetics, including the balance of multiple levels of antibody avidity interactions and response regulators, is therefore crucial in mastering antibody mechanisms of action and designing more effective antibody-based therapeutics.

The single-agent use of monoclonal antibodies for therapy is in many ways contrary to antibody function as established in natural biology. In traditional drug development, single-agent safety and activity is usually required for a drug to be registered and drug combinations are typically only sought following first registration, often using empirical approaches. However, in the body, a combinatorial, polyclonal response is the starting point of the immune response to an antigen. An appreciation of this paradox has steadily grown, and antibody-based therapeutics have evolved from canonical monoclonal antibodies towards more complex antibody architectures and formats in an effort to enhance functional activity and cocktails are being rationally designed and assessed in combination in clinical trials.

Novel formats are emerging that leverage antibody avidity interactions to boost classical effector functions and novel or ‘designed’ mechanisms. These formats feature avidity improvements, including multiplicities of binding specificities, increased valency, enhanced Fab–Fab or Fc–Fc interactions or enhanced interactions between Fc and the effector molecule. Using this concept, distinct avidity engineering approaches may be combined to achieve incremental avidity effects. For example, multi-epitope or multivalent formats can be combined with self-assembling technologies, which may be further enhanced by effector molecules. We believe that multi-agent approaches will be a major player in the future of antibody therapy, either in the form of bispecific/multispecific antibodies with obligate functions or designer polyclonal antibodies. There is a strong argument for greater efforts towards the rational design of antibody cocktails in response to the growing list of therapeutic antibodies entering or undergoing clinical development. Notably, forward-looking antibody discovery approaches that perform unbiased screening of antibody libraries in their final format will be critical to identifying optimal multispecific antibodies or antibody combinations. Similarly, thoughtful antibody design, together with advances in engineering capabilities and improvements in preclinical characterization, will enable antibody-optimized molecular formats that better achieve tuned potency to balance maximal functional activity with minimizing the risks for adverse events. Collectively, recent novel insights into antibody effector biology together with current antibody design efforts that extend our capabilities beyond the classic monoclonal format are paving the way for novel transformative biotherapeutics that can positively affect patient lives.

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Author contributions
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Competing interests
J.S. and S.C.O. are employees of Genmab (a biotechnology company developing therapeutic antibodies including bispecific and avidity-engineered molecules), own Genmab warrants and/or stock and are inventors on patent applications relating to bispecific and avidity-engineered molecules assigned to Genmab. J.S. is a board member of the Antibody Society. G.A.L. is an employee of Genentech, a member of the Roche Group that develops and commercializes therapeutics including antibody-based drugs. P.W.H.I.P. is an employee of Lava Therapeutics and Leiden University Medical Center and a former employee of Genmab, owns options and/or stock of Lava Therapeutics and is an investor on applications relating to bispecific and avidity-engineered molecules assigned to Lava Therapeutics and Genmab. He is a board member of The Antibody Society and provides consulting services on behalf of Sparring Bioconsult and served as an expert witness in a case of Roche versus Takeda. He is also an operational partner at Gilde Healthcare.

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