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Review

Function and mechanism of bispecific antibodies targeting SARS-CoV-2

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ABSTRACT

As the dynamic evolution of SARS-CoV-2 led to reduced efficacy in monoclonal neutralizing antibodies and emergence of immune escape, the role of bispecific antibodies becomes crucial in bolstering antiviral activity and suppressing immune evasion. This review extensively assesses a spectrum of representative bispecific antibodies targeting SARS-CoV-2, delving into their characteristics, design formats, mechanisms of action, and associated advantages and limitations. The analysis encompasses factors influencing the selection of parental antibodies and strategies for incorporating added benefits in bispecific antibody design. Furthermore, how different classes of parental antibodies contribute to augmenting the broad-spectrum neutralization capability within bispecific antibodies is discussed. In summary, this review presents analyses and discussions aimed at offering valuable insights for shaping future strategies in bispecific antibody design to effectively confront the challenges posed by SARS-CoV-2 and propel advancements in antiviral therapeutic development.

1. Background

Despite more than four years having elapsed since the onset of the SARS-CoV-2 outbreak, the persistent global impact and adversity inflicted by this virus endure (Zhu et al., 2020). Intensive efforts in vaccine development have aimed to provide proactive protection to the populations (Dai et al., 2020, 2021; Wang et al., 2020b). Concurrently, the need for specific therapies for individuals infected with SARS-CoV-2 or ineligible for vaccination, such as the elderly and immunocompromised, has become paramount. Among these remedies, monoclonal antibodies, renowned for their high specificity and exceptional targeting effects, emerged as one of the most efficacious specific drugs (Barnes et al., 2020; Cao et al., 2020; Hassan et al., 2020; Joyce et al., 2020; Kreer et al., 2020; Petherick, 2020). Thus, the utilization of monoclonal antibodies for SARS-CoV-2 treatment, evolving from early convalescent plasma to the identification and purification of monoclonal antibodies, unequivocally underscores the feasibility and effectiveness of this treatment approach (Joyner et al., 2021; Libster et al., 2021; Wang et al., 2020c; Writing Committee et al., 2021).

However, the landscape of SARS-CoV-2 remains in flux. Within a short span, a mutation occurring at position N501Y in the spike protein emerged (Brown et al., 2021; Davies et al., 2021; Graham et al., 2021; Socher et al., 2021; Volz et al., 2021), setting off subsequent mutations at various sites, including the variants of concern (VOCs) and variants of interest (VOIs) as designated by the World Health Organization (Aleem et al., 2022; Dejnirattisai et al., 2022; Hoffmann et al., 2021; Zhou et al., 2021a). This progression culminated in the evolution of variants with distinct serotypes (Tan et al., 2023). Consequently, the efficacy of a singular therapeutic drug, including monoclonal antibodies, has become notably inconsistent (Baum et al., 2020a; Dejnirattisai et al., 2022; Shah et al., 2021; Starr et al., 2021). While cocktail (mix of more than two antibodies) therapy appears to offer relatively improved effectiveness against variants compared to monoclonal antibody therapy (Baum et al., 2020a; Baum et al., 2020b; Hansen et al., 2020; Wang et al., 2020a; Yao et al., 2020), this approach presents challenges with higher costs and constraints on production and application due to its complex formulation ratios (Crowe, 2022).

At this pivotal stage, bispecific antibodies (bsAbs) (designing more than two monoclonal antibodies into one molecule through biochemical process) emerge as a highly promising strategy in addressing SARS-CoV-2 and its evolving variants. Originating from the pioneering work by Nisonoff and colleagues, bispecific antibodies have evolved into over a hundred design formats, widely used by scientists and pharmaceutical entities in combating cancer and viral infections (Labrijn et al., 2019;

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Nisonoff et al., 1960). The bispecific antibody design strategies have been extensively applied in treating viruses such as HIV, ZIKA, Ebola, H5N1, CMV, with a particularly abundant number of cases targeting HIV (Bournazos et al., 2016; Frei et al., 2016; Huang et al., 2016; Steinhardt et al., 2018; Wang et al., 2017; Zanin et al., 2015). The success of these bispecific antibodies underscored their superior antiviral activity compared to monoclonal antibodies and heightened capability to prevent of immune escape. Compared to cocktail therapy, bispecific antibodies are designed as single-molecule drugs by integrating two or more monoclonal antibodies, streamlining their production formula and reducing the production cost. Moreover, due to their unique action mechanisms, bispecific antibodies often exhibit markedly superior efficacy compared to cocktails. Consequently, in the context of preventing and treating SARS-CoV-2, bispecific antibodies emerge as an evidently superior option over monoclonal antibodies or cocktails (De Gasparo et al., 2021; Hanke et al., 2022; Ku et al., 2022). In this review, we categorize and summarize bispecific antibodies targeting SARS-CoV-2, outlining their design formats, mechanisms of action, and associated advantages and limitations, and aiming to provide insights that steers the development of novel approaches in the future design of bispecific antibodies.

2. Characteristics of representative bispecific antibodies targeting SARS-CoV-2

Bispecific antibodies fuse two or more antibody drug molecules into one through biochemical processes, holding the potential to harness the combined therapeutic benefits of two antibodies concurrently. In the context of combatting SARS-CoV-2, the primary emphasis is on specific antibodies that target the receptor binding domain (RBD) antigen. Notably, antibodies like B38/H4, CB6, and REGN10933/REGN10987 play a crucial role by preventing the interaction between the RBD and angiotensin converting enzyme 2 (ACE2), effectively inhibiting viral infection (Alina Baum et al., 2021; Shi et al., 2020; Wu et al., 2020). In addition, there are also antibodies targeting N-terminal domain (NTD), such as 4A8 (Chi et al., 2020). In response to the need for ensuring antiviral efficacy while minimizing drug dosage and addressing immune escape, researchers have rapidly developed bispecific antibodies, such as the CrossMAb format bispecific antibodies developed by Lei Peng, Raoul De Gasparo and Ping Ren (De Gasparo et al., 2021; Peng et al., 2022; Ren et al., 2023). In the work of De Gasparo and colleagues, two antibodies, *C*121 and C135, targeting different epitopes within the RBD, were employed to develop four bispecific antibodies in scFv format and CrossMAb format. Among them, the CoV-X2 in CrossMAb format, with its Fc region employing the “knob into hole" design to ensure a stable pairing of *C*121-Fc and C135-Fc, exhibited the most robust neutralization efficacy. CoV-X2 demonstrates superior antiviral activity, not only surpassing the neutralization activity of its parent antibodies but also effectively neutralizing both wild-type SARS-CoV-2 and various concerning variants such as B.1 (D614G), B.1.1.7 (N501Y on the RBD), P.1, and B.1.351 (K417N, E484K, and N501Y located on the RBD)). This efficacy is attributed to the complementary function of C121 and C135 against the E484A mutation present on the RBD, wherein C135 can bind to the RBD with the E484A mutation while C121 cannot. Furthermore, CoV-X2 displayed a broader range of binding epitopes compared to the individual parental antibodies, regardless of the spike trimer adopting RBD-3 up, RBD-2 up, RBD-1 up, or RBD-3 down conformation. This versatility enhances its potential to prevent occurrences of immune escape to a considerable extent. However, the CoV-X2 in the CrossMAb format necessitates the transfection of four separate plasmids for expression, and complicates the pairing process, leading to cumbersome purification steps and reduced yield. To streamline the pairing process, an alternative approach involves linking two light chains to their respective heavy chains or linking two different scFvs to the Fc moiety for the construction (Wang et al., 2022a,b; Yuan et al., 2022, 2023).

Li and colleagues incorporated B38 and H4 antibodies targeting different RBD epitopes into DVD-Ig format and IgG-(scFv)2 formats. Interestingly, the IgG-(scFv)2 format bispecific antibody, bsAb15, exhibited superior neutralization activity than B38, H4, and their combination, and could impede immune escape (Li et al., 2022b). DVD-Ig format bispecific antibodies, bsAb13 and bsAb14, could bind to only one epitope on RBD, possibly due to the relative proximity of the binding sites of B38 and H4 (both B38 and H4 fall into class 1 and 2 in classification (Huang et al., 2022)), leading to the spatial hindrance within the DVD-Ig format for these bispecific antibodies. This underscores the importance of selecting parental antibodies with completely non-overlapping binding epitopes, in the meantime considering the distance between these epitopes for effective bispecific antibody design. However, the ideal design doesn't solely rely on maximizing the distance, as the potential for cross-linking two antibodies on the spike also plays a crucial role (Ku et al., 2022). However, as the mechanism of action of bsAb15 remains undisclosed, it may not necessarily possess the characteristic of binding to RBD-down confirmation like CoV-X2 does.

Whereas bsAb15 comprises four antibody moieties and might lead to a more intricate cross-linking mechanism, potentially resulting in a substantial spike aggregation compared to CoV-X2. Therefore, the IgG-(scFv)2 format might offer advantages, particularly in cross-linking spikes compared to the CrossMAb format. However, instances such as the case of K202.B, akin to the DVD-Ig format, showing slightly higher affinity for B.1.351-RBD than K202. A in the IgG-(scFv)2 format by approximately 3 folds, indicate nuanced differences, although the neutralization levels was not compared (Kim et al., 2023). This might stem from K202.B having higher epitope coverage on a single RBD molecule. An and colleagues discovered that the IgG-(scFv)2 design (14-H-06) exhibited higher affinity and broader cross-neutralization activity compared to the CrossMAb design (14-crs-06), aligning with findings related to bsAb15. Thus, a comprehensive comparison of affinity and neutralization levels, is warranted to discern the optimal format for specific antibodies.

On the other hand, utilizing NTD neutralizing antibodies to design bispecific antibodies presents a viable option. Joshua Tan's work on the bispecific antibody CV1206_521_GS, targeting RBD and NTD in DVD-Ig format with antibodies CV1206 and CV521 (Cho et al., 2021), demonstrated this approach would be necessary. However, antibodies targeting the NTD supersite are susceptible to variant mutations, posing a concerning factor (Wang et al., 2021), thus resulting in CV1206_521_GS being unable to neutralize Gamma and Delta variants.

Furthermore, nanobodies, characterized by their small molecular weight and strong specificity, are widely applied in mucosal immunity. Li and colleagues developed the inhalable bispecific antibody bn03, employing two single-domain antibodies, n3113v and n3130v, targeting cryptic epitopes. This antibody induces a transition of the Omicron S trimer into an unstable wide-up state, effectively neutralizing VOCs (Li et al., 2022a). This showcases promising application and distinct advantages of single-domain antibodies in the realm of bispecific antibodies (Hanke et al., 2022; Wu et al., 2021). Moreover, heterotrimeric bispecific nanobody Nb_{15}–Nb_H–Nb_{15} was designed by combination variable domain of heavy chain (VHH) and human serum albumin (HSA) to prolong *in vivo* half-life of drug and avoid potential Fc-mediated ADE (Wu et al., 2021).

In summary, these bispecific antibodies demonstrate a shared feature by interacting with two or more distinct epitopes of the antigen. Compared to parental antibodies and cocktails, they exhibit stronger neutralization activity, the ability to suppress immune escape, and complementation in neutralizing specific variants. These cases offer crucial guidance for future bispecific antibody design, highlighting epitope selection is paramount. The imperative lies in selecting two epitopes devoid of overlap, or integrating antibodies with the capacity to broaden breadth or targeting cryptic epitopes for enhanced efficacy against immune escape. Specialized antibodies, such as those inducing S1 shedding, exemplified by antibodies 7D6 and 6D6 (Li et al., 2021), offer unique mechanisms in bispecific antibody design. While reports suggested a correlation between high affinity and activity, evidence remains inconclusive (Dean et al., 2023).

Designing bispecific antibodies from two antibodies with sub-nanomolar affinities may not always confer superiority, emphasizing the need for optimal affinities rather than solely pursuing the highest affinity. Selecting the appropriate design format requires balancing mechanism, complexity and cost implications of later-stage expression, purification, and industrial-scale production. Simplified format designs streamline expression and purification, fostering reproducibility and adoption by others. Moreover, leveraging the mechanisms of tumor-targeting antibodies, presents opportunities for added benefits, such as Fc-mediated antibody-dependent cellular cytotoxicity (ADCC) (Dong et al., 2020) and antibody-dependent cellular phagocytosis (ADCP) effects. However, caution is warranted against inducing antibody-dependent enhancement (ADE) effects, as ADE can promote viral infection (Hohdatsu et al., 1998; Wang et al., 2014; Zhou et al., 2021b). Fusion with receptors like ACE2 or antiviral peptides to block viral entry is an option (Ojha et al., 2022; Weidenbacher et al., 2022), but potential *in vivo* side effects of ACE2 must be carefully considered.

3. Mechanistic analysis of bispecific antibodies targeting SARS-CoV-2

Bispecific antibodies targeting SARS-CoV-2 have demonstrated superior antiviral activity compared to parental antibodies and cocktails. This review endeavors to consolidate findings from prior studies to unveil the intricate mechanisms of bispecific antibodies, aiming to offer valuable insights that can inform their practical design and optimization.

3.1. Disruption of RBD-ACE2 interaction and multivalent antigen binding

The SARS-CoV-2 monoclonal antibodies predominantly focus on targeting the RBD. Studies have classified these RBD-targeting antibodies into 7 classes based on their distinct binding epitopes (Hastie et al., 2021). Bispecific antibodies derived from these antibodies leverage the antiviral mechanisms by targeting two distinct RBD epitopes. In comparison to individual antibody molecules, this approach serves to achieve

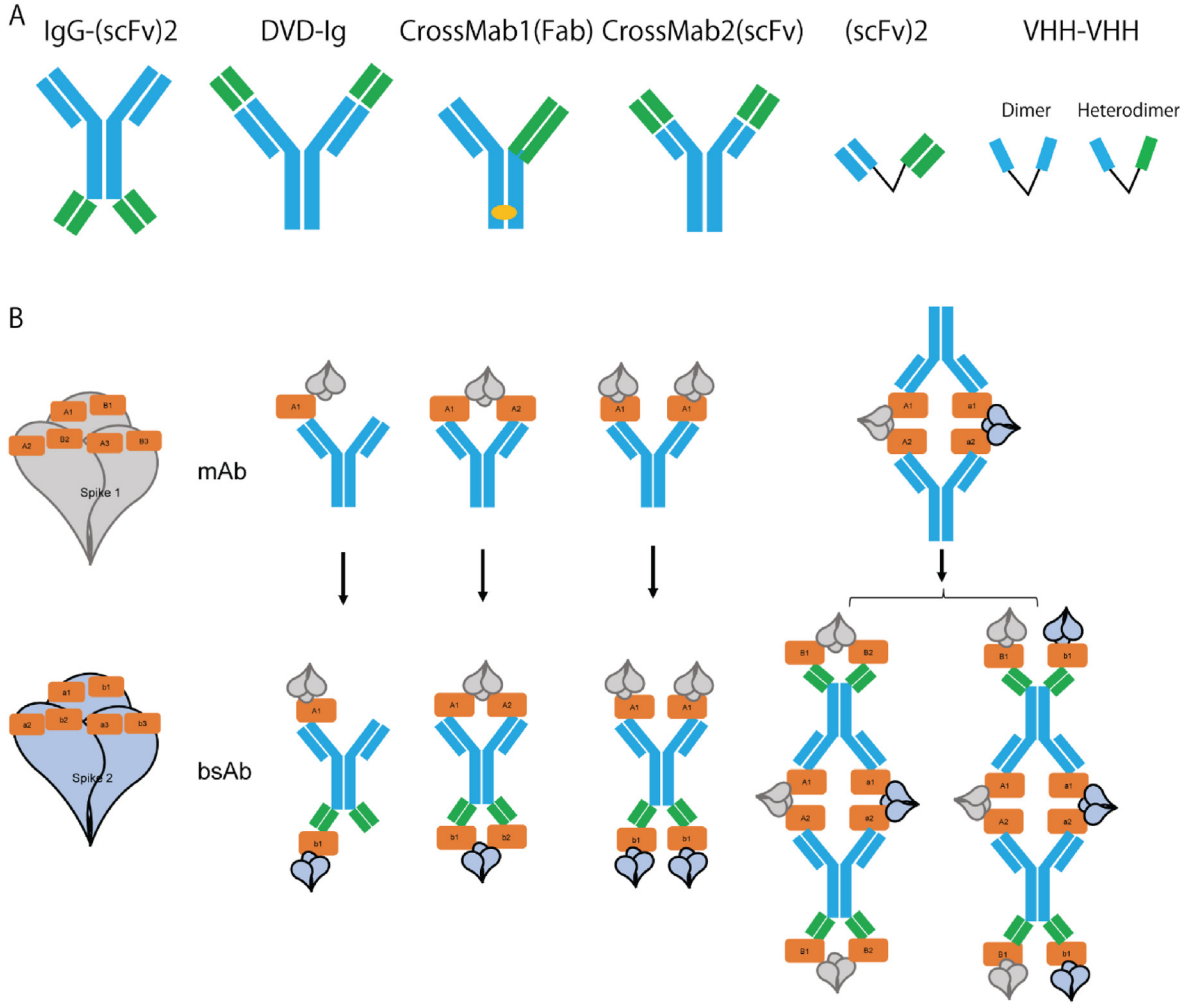

Fig. 1. Schematic of Classical Bispecific Antibody Formats and Crosslinking Mechanism. (A) Schematic of several representative formats of bispecific antibodies. In the IgG-(scFv)2 format, the single-chain variable fragment (scFv) is linked to the C-terminus of the antibody's Fc region through a linker. The DVD-Ig (Dual Variable Domain) format connects the variable regions of one antibody's heavy and light chains to the N-terminus of another antibody's heavy and light chains via a linker. The CrossMab1 (Fab) format replaces one side of Fab with that of another antibody, requiring knobs-into-holes mutations in the CH3 region of the two different heavy chains to ensure proper pairing. The CrossMab2 (scFv) format connects the variable regions of two distinct antibodies through a linker, omitting the CH1 and CL regions. The (scFv)2 format links the scFvs of two different antibodies via a linker. The VHH or nanobody derived from llama was designed to dimer and heterodimer bispecific antibody in VHH-VHH format. The blue and green cylinders represent two distinct antibodies, while the brown ellipses denote knobs-into-holes mutations. Fab is composed of CH1-VH and CL-VL, and scFv is composed of VH and VL. (B) Schematic of crosslinking of bispecific antibody in IgG-(scFv)2 format. The gray and light blue trimeric spheres represent two spike trimers, with mAb indicating a monoclonal antibody and bsAb representing a bispecific antibody. A1, A2, A3, or a1, a2, a3 represent epitopes recognized by the blue monoclonal antibody on the spike trimer, while B1, B2, B3, or b1, b2, b3 represent epitopes targeted by the green monoclonal antibody on the spike trimer. Using the IgG-(scFv)2 format as an example, the number of spike crosslinking by bispecific antibody is shown to be higher than that by monoclonal antibody, whether in case of intra-spike or inter-spike, often doubling or tripling the latter's crosslinking capacity.

either a broader blockade of the RBD responsible for ACE2 binding, thereby impeding the virus from utilizing ACE2 to infect cells, or confers multivalent binding attributes to the bispecific antibody. For instance, CoV-X2 exhibits a bivalent binding mechanism to engage with the spike trimer in RBD-3 up, RBD-2 up, RBD-1 up, or RBD-3 down conformations. In contrast, individual parental antibodies predominantly engage with the S trimer in a monovalent manner (De Gasparo et al., 2021). This bivalency inherent in bispecific antibodies significantly augments their ability to neutralize viral particles by enhancing their binding and blocking capacity.

3.2. Crosslinking

While monoclonal antibodies possess direct ACE2 competition binding (where one fab of one antibody molecule binds to one RBD site on a single spike protein), they also exhibit bivalent intra-spike binding (where two fabs of one antibody molecule simultaneously bind to two identical RBD epitopes on a single spike protein) and inter-spike crosslinking characteristics (where two fabs of one antibody molecule simultaneously bind to two identical RBD epitopes on two different spike proteins, leading to the crosslinking of these proteins) (Hastie et al., 2021) (Fig. 1B). However, the crosslinking mechanism of bispecific antibodies appears to be notably more intricate (Fig. 1). For instance, Ku et al. illustrated that the tetravalent bispecific antibody 14-H-06 in the IgG-scFv format has the capacity to crosslink up to four spikes, surpassing the bivalent CrossMab format bispecific antibody 14-crs-06 and parental antibody interacting with two spikes (Ku et al., 2022). This attribute of 14-H-06 allows for increased crosslinking interactions among spikes present on virus particles, effectively impeding the interaction between spikes and the ACE2 receptor. Even though the number of crosslinked spikes is merely twice that of parental antibodies, the impact of increased crosslinking via bispecific antibodies is anticipated to be magnitudes higher than what's observed with parental antibodies. This is particularly noteworthy when considering the virus's replication and proliferation dynamics following cell infection.

3.3. Induction of spike conformational changes or disassembly/aggregation

Given the dynamic nature of the spike protein during receptor binding, developing antibodies that can target various functionalities across various stages of spike conformational changes becomes critical. This underscores the significance of designing bispecific antibodies by combining antibodies with distinct functionalities, allowing the resulting bispecific antibody to encompass the characteristics of both parental antibodies simultaneously. For instance, Cheng Li et al. revealed that the bispecific antibody bn03 induced the dissociation of Omicron S trimer into monomers, destabilizing the RBD and impeding virus particle infection (Li et al., 2022a). Yingdan Wang et al. reported that a bispecific antibody, designed from non-Omicron RBD antibodies, showed neutralizing activity against Omicron by inducing the transition of RBD-down to 3 RBD-up states and subsequently promoting spike trimer dimerization, leading to virus particle aggregation (Wang et al., 2022a). Similar findings were corroborated by Hanke et al. (2022), Walter et al. (2022). Dong et al. further unveiled that the induction of RBD-up resulted in increased affinity of the trispecific antibody 3F–1B-2A-Fc, indicating enhanced neutralization potency (Dong et al., 2020). These studies underscore the adaptability and versatility of bispecific antibodies in inducing spike conformational instability or driving virus particle aggregation, even when many monoclonal antibodies induce the transition of RBD-down to RBD-up states.

4. Cross-neutralization and immune escape inhibition of bispecific antibodies targeting SARS-CoV-2

Mutations in the SARS-CoV-2 RBD have rendered many monoclonal antibodies ineffective. Consequently, expanding the breadth of activity and monitoring mutations within bispecific antibodies becomes crucial. The selection of parental antibodies necessitates adjustments, considering their susceptibility to variants (Huang et al., 2022).

Though 1 and 2-class RBD antibodies are more prevalent, they tend to induce mutation escape. For instance, bsAb15, composed of the 1-class antibody B38 and the 2-class antibody H4, encountered challenges combating Beta variants carrying both K417 and E484 mutations (Huang et al., 2022; Li et al., 2022b). In contrast, bispecific antibodies like G9, crafted from the 7 class GW01 and 1-class REGN10989 antibodies, exhibit potent neutralization against immune escape variants (Wang et al., 2022b). Similarly, bn03, designed using the 4-class n3113v and 7-class n3130v antibodies, demonstrated efficacy against VOCs (Li et al., 2022a). CoV-X2, designed by combining 2-class *C*121 and 5-class C135 antibodies, exhibited neutralization capabilities against Alpha, Beta, and Gamma variants (De Gasparo et al., 2021). Moreover, 14-H-06, a bispecific antibody designed using the 5-class CoV2-06 and 1-class CoV2-14 antibodies, displayed neutralization potency against Alpha, Beta, Gamma, and Delta variants (Ku et al., 2022) (Table 1). These findings align with the evolutionary characteristics of SARS-CoV-2, where RBD-exposed binding epitopes more readily induce neutralizing antibodies. While 1 and 2-class antibodies are more numerous and effective in neutralization, they are more prone to mutations, resulting in loss of neutralizing activity. In contrast, 4, 5, 6, and 7-class antibodies, though exhibiting slightly lower neutralization activity, demonstrate cross-neutralization and immune escape prevention. Notably, 4 and 5-class antibodies can also bind to RBD-down conformations (Hastie et al., 2021; Huang et al., 2022). Therefore, designing bispecific antibodies by combining ACE2-blocking and broadly neutralizing antibodies or antibodies targeting cryptic epitopes enhances both blocking effects and cross-neutralization characteristics (Bianchini et al., 2023). However, ACE2-blocking antibodies might reduce neutralization activity due to mutations. Learning from Huan Ma's approach of introducing the Y29G mutation to bsAb nanobodies for neutralizing the BA.2.75 variant offers a potential solution (Ma et al., 2022). Another viable strategy involves isolating cross-neutralizing antibodies from patients infected with variants and using them to design bispecific antibodies, further amplifying neutralization potency and broadening the breadth (Guerra et al., 2023).

In conclusion, the intrinsic genomic instability of RNA viruses makes immune escape an inevitable scientific challenge. When designing bispecific antibodies, it is imperative to leverage their enhanced neutralizing capacities while ensuring a delicate balance with cross-neutralization characteristics. This requires a comprehensive understanding of the mechanisms that govern bispecific antibody action, along with the judicious selection of neutralizing antibodies. Despite the challenges, the development and application of bispecific antibodies stand as pivotal endeavors, as they offer superior advantages in bolstering neutralization potency, diversifying mechanisms of function, fortifying broad-breadth enhancement, and inhibiting immune escape. This is particularly vital for effectively managing and preventing of SARS-CoV-2.

5. Discussion

Drawing inspiration from the design principles of SARS-CoV-2 bispecific antibodies holds significant value for coronavirus drug development. In the development of bispecific antibodies, factors such as binding epitopes, potential mechanisms of action, and suitable formats must be carefully considered. The choice of format can significantly impact the functionality of bispecific antibodies, as well as the complexity involved in their expression and purification. To streamline production without compromising efficacy, simpler bsAb formats are favored over more intricate designs with multiple chains. Additionally, the incorporation of additional effects like ADCP and ADCC proves advantageous (Dong et al., 2020), but careful vigilance is essential to avoid the potential risks associated with ADE.

Table 1
Representative of bispecific antibodies against SARS-CoV-2.

Bispecific Antibody	BsAb Format	Parent Antibodies	Neutralization Activity	Mechanism	Classification and Structure		Clinical Phase	References
G9	DVD-Ig	GW01, REGN10989	• Wildtype •Escape variants of parent antibodies •Omicron	The simultaneous targeting of the binding epitopes of GW01 and REGN10989 by G9 ensures a broad-spectrum neutralizing activity	 7-GW01 (PDB:7EPX)	 1-REGN10989 (PDB:7M42)	Null	Wang et al. (2022a,b)
bn03	(scFv)2	n3130v, n3113v	•Wildtype •VOCs	The two arms of the bn03 antibody can concurrently bind and synergistically act on the RBD of the spike trimer, inducing the Omicron S trimer into an unstable wide-up conformation	 4-n3113v (PDB:7VNB)	 7-n3130v (PDB:7WHI, 7WHJ)	Preclinical China Mar 09, 2022	Li et al. (2022a)
CoV-X2	CrossMAb (knobs-into-holes)	C121, C135	•Wildtype •Alpha •Beta •Gamma	The CoV-X2 exerts its inhibitory effect by simultaneously engaging two distinct sites on the RBD, thereby impeding the binding of ACE2	 2-C121 (PDB:7K8X)	 5-C135 (PDB:7K8Z)	Null	De Gasparo et al. (2021)
14-H-06, 14-crs-06	IgG-(scFv)2: 14-H-06 CrossMAb: 14-crs-06	CoV2-06, CoV2-14	•Wildtype •VOCs	14-H-06 demonstrates enhanced inter-spike crosslinking potential	 5-CoV2-06 (PDB:7WPH)	 1-CoV2-14 (PDB:7XXL)	Preclinical US 27 Dec 2023	Ku et al. (2022)
bsAb15	IgG-(scFv)2	B38, H4	•Wildtype •Escape variants of parent antibodies •Alpha •Delta	The bsab15 antibody exhibits the capability to simultaneously engage two distinct epitopes on the RBD	 1-B38 (PDB:7BZ5)	2-H4 (no PDB data)	Preclinical China 28 Feb 2022	Li et al. (2022b)

Yellow color indicates the epitope of antibody binding, number before antibody name is the antibody class.

The strategic selection of parental antibodies significantly influences the mechanism, neutralization efficacy, and cross-neutralization activity of bispecific antibodies. Incorporating antibodies with distinctive mechanisms, such as those inducing S1 protein shedding (Li et al., 2021) or non-neutralizing antibodies with potential alternative effects (Weidenbacher et al., 2022), alongside antibodies targeting cryptic epitopes, can amplify the overall effectiveness of bispecific antibodies. For instance, Yuan et al. designed the bsAb Bi-Nab35B5-47D10 targeting RBD and S2, showcasing neutralization against diverse variants, spanning Alpha, Beta, Kappa, Delta, Omicron BA.1, and Omicron BA.2 (Yuan et al., 2022). The integration of antibodies that induce RBD-down to RBD-up transition into bsAb design holds promise in further enhancing neutralization activity and broadening the breadth efficacy (Peng et al., 2022; Wang et al., 2022a). While certain bispecific antibodies exhibit increased antigen-binding affinities (Dong et al., 2020; Ku et al., 2022), not all follow this patter. For instance, bsAb15, designed by B38 and H4, maintains a similar affinity to its parent antibodies (Li et al., 2022b). However, recognizing that increased affinity may correlate with the ability to induce RBD-up states, selecting bispecific antibody designs that enhance affinity could potentially signify improved neutralization activity.

Moreover, bispecific antibodies in scFv format offer the advantage of smaller molecular size for improved tissue penetration, but their shorter half-life may pose limitations in sustaining their efficacy. For instance, the CrossMAb format of CoV-X2 tends to outperform the scFv1-scFv2 format (De Gasparo et al., 2021), possibly due to larger antibody molecules creating substantial steric hindrance on the virus particle surface and the fusion Fc region contributing to an extended half-life (Li et al., 2022b). Furthermore, leveraging Fc-mediated effects, such as ADCC and ADCP, widely applied in cancer treatment, could significantly enhance viral clearance by engaging immune cells in the process (Dong et al., 2020). Therefore, bispecific antibodies not only target viral surface antigens, capitalizing on their unique advantages, but also guide immune cells to eliminate virus particles, significantly amplifying virus clearance.

On the other hand, the majority of bispecific antibodies containing the Fc moiety typically exhibit an extended serum half-life *in vivo*. However, they may experience a reduction in tissue penetration ability due to their larger molecule size compared to monoclonal antibodies. In addition, the polymer formed by bispecific antibody pairing could increase the potential risk of immunogenicity (Rossi et al., 2013). While the bispecific antibodies designed using VHH or nanobody enhance tissue penetration ability but they face challenges in sustaining *in vivo* (Chames et al., 2009; Chen et al., 2017). As a result, these antibodies could find utility in mucosal immunotherapy.

Based on the currently available data, IgG-(scFv)2 emerges as a highly promising candidate among the formats, considering the complexity of expression and purification, the number of antigen binding moiety and crosslinking potential. The simplicity of pairing of bispecific antibody is evident when expressed with two plasmids than three or more plasmids likes the CrossMab1(Fab) format (Fig. 1A), ensuring a reliable yield of bispecific antibodies. However, in an effort to minimize heavy chain and light chain mismatches, the CrossMab format was adopted in the design of bispecific antibodies (Klein et al., 2016). The enhanced binding affinity of bispecific antibody maybe contributed to the transition of RBD-down to RBD-up state induced by parent antibody (Dong et al., 2020; Ku et al., 2022), rather than the specific format employed. Additionally, compared to DVD-Ig and CrossMab2(scFv) formats, the IgG-(scFv)2 format features four antigen binding moieties in a theoretically non-interfering pattern, thereby contributing to increased numbers of antigen binding and diverse crosslinking potential (Fig. 1B). Despite DVD-Ig and CrossMab2(scFv) formats sharing the same antigen binding moieties as the IgG-(scFv)2 format, steric hindrance among two different antibody moieties could lead to an inevitable concern (Fig. 1A).

In conclusion, bispecific antibody plays a vital role in treatment against SARS-CoV-2. The parent antibodies selection, format and functional mechanism are three key factors in the design of bispecific antibodies. Moreover, the breadth and cross-neutralizing activity of bispecific antibodies targeting SARS-CoV-2 are intricately dictated by the distinct epitopes of the parent antibodies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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