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Bispecific antibodies: potential immunotherapies for HIV treatment.

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Abstract

Bispecific (bs) antibodies (Abs, bsAbs) are engineered immunoglobulins that contain two different antigen-binding sites in one molecule. bsAbs can be divided in two molecular formats; the IgG-like and non-IgG like. The structural elements of each format have implications for engaging the immune system. Elimination of HIV will need sophisticated approaches with immunotherapies being one of the strategies under investigation. Furthermore, HIV genetic variability and functional compromise of the adaptive CTL response complicate the potential usefulness of some immunotherapeutic strategies. Inclusion of novel HIV neutralizing Abs with high potency and breadth as components of bsAbs could represent alternative strategies for virus elimination by harnessing the adaptive immune response *in vivo*.

Keywords

bispecific; HIV; lymph nodes; cure

Introduction

More than 120 years ago von Behring and Kitasato [1] first demonstrated that a serum component present in animals immunized with bacterial toxins protect against bacterial infection by neutralizing the toxin. Antibodies (Abs) were subsequently identified as this serum component. Since then, work on Abs has established them as a major component of the adaptive immune responses against foreign elements and pathogens. Ab-based immunotherapies have now progressed from monoclonal (m)Abs to bispecific (bs)Abs, with most Ab engineering strategies keeping the natural architecture of the Immunoglobulin G (IgG), which is the predominant type of Ab found in blood. IgGs are heterodimeric molecules composed of two heavy (H) and two light (L) chains, with distinct variable (V) domains that bind antigens (Ag) and constant (C) domains that target effector functions such as activation of complement or binding to Fragment crystallizable (Fc) receptors (Figure 1). The fragment variable (Fv) domain represents the smallest fragment of an Ab required for

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Competing interests

The authors declare that no conflict of interest exists

binding and includes VH and VL domains that carry complementarity determining regions (CDRs). Each IgG contains two identical (monospecific) Ag binding sites (Ag-bs) (bivalent) [2] while the introduction of a flexible linker between VH-VL (or VL-VH) gives rise to a single chain Fv (scFv) [2] (Figure 1A).

A plethora of mAbs have been used to treat human diseases [3]. mAb monotherapies targeting a single Ag, either a receptor or a growth factor associated with the tumor, represent early immunotherapy strategies to treat cancer patients. However, resistance to single Ag strategies occurs easily through immune escape. Simultaneous engagement of an Ag present on effector immune T cells and a tumor-associated Ag (TAA) has been used as a strategy to overcome such resistance mechanisms, [4]. Similar to cancer, viruses evolve mechanisms of resistance to counter host immune defenses. Despite their promising performance in preclinical trials to treat infections caused by viruses that have low genetic variability [5-7], only one mAb directed to Respiratory Syncytial Virus (RSV) glycoprotein [8] has been clinically approved to date [9]. Most of the viral infections' symptoms occur after the peak of viremia. Therefore, mAb must be given early, often before clinical symptoms appear. This is certainly true for Human Immunodeficiency Virus (HIV) infection where the virus undergoes rapid viral evolution [10] and the appearance of escape mutations could affect the efficacy of mAb therapy [11].

Bispecific (bs) Abs (bsAbs) bind simultaneously two antigen (Ag) binding sites (Ag-bs). Early studies showed that two pepsin-cleaved IgG molecules give rise to two Ag binding fragments (Fab), which in turn can be artificially combined in one chimeric molecule able to bind two Ag-bs [12] (Figure 1). Since the first reports about the recombinant bsAbs using diabodies [13] or IgG linked to a scFv [14], the rational design of bsAbs has greatly improved and has found application to autoimmune diseases, cancer and infectious diseases [4,15-18].

Molecular formats of bsAbs: structure guides immune responses

There are two molecular formats (IgG- and non-IgG-like) of bsAbs, with the structural elements of each format having implications for engagement and mobilization of the immune system.

The IgG-like bsAbs include the Fc region which facilitates its purification. In terms of pharmacokinetics, the Fc region ensures long *in vivo* half-life due to neonatal Fc receptor (FcRn) mediated-recycling [19], contributing to Ab stability and solubility. Furthermore, the Fc region is an important determinant for efficacy of the bsAb to stimulate immune responses. Biochemical properties of the Fc region of Ab (affinity, glycosylation and isotype) are responsible to trigger different immune effector cells [20]. The Fc region interacts with type I (CD64), IIa, b, c (CD32a, b, c) and IIIa, b (CD16a,b) Fc γ receptors which are expressed on macrophages, dendritic cells (DC) and natural killer (NK) cells [21]. By interacting with these receptors, the Fc region triggers Ab-mediated effector functions such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and antibody-dependent cellular phagocytosis (ADCP) [22].

The IgG-like bsAbs format presents some intrinsic drawbacks that may account for their reduced specificity. To overcome random assembly of different chains, one approach is to fuse a second binding moiety like a single-chain Fv (scFv) to CL, to the Fc region or to VH and VL domains, generating bsAb-scFv [23] (Figure 1). A second approach is to generate an asymmetric IgG-like bsAb formed via the use of the knobs-into-hole technology (KiH) by introducing mutations in CH3 that force heterodimerization between different HCs [24]. KiH-IgG, however, can undergo random pairing of LCs even if the HCs heterodimerize. CrossMab technology overcomes this aspect through a correct pairing of LC by exchanging CH1 domain of one HC with the constant domain of the LC (Figure 1) [25].

The non-IgG-like bsAb format lacks the Fc region and as a consequence, the molecular mass (30-50kDa) is smaller than that of a whole IgG (150kDa). The small size facilitates penetration into tissues but results in a shorter half-life in serum due to rapid renal clearance. Because the structure of a non-IgG-like format includes only VH and VL domains or the Fab fragment, such bsAb format depends only on Ag binding capacity for its therapeutic function. The fusion of two scFv produces a tandem scFv molecule, an example of which is the Bispecific T cell engager (BiTE®) in which the two binding moieties are connected with a flexible linker while keeping independent folding of the two scFvs [26,27]. The diabody (Db) format contains VH and VL for two antibodies (A and B; VHA-VLB; VHB-VLA) with a short peptide as linker to force heterodimerization. A variant of this bsAb category, the “Dual-affinity retargeting molecule” (DART) [28] has been used for killing of HIV infected cells [29,30] (Figure 1). A major limitation of the non-IgG-like bsAbs is their short-half life. Bi-scFvs have serum half-life less than two hrs, thereby requiring an infusion pump for continuous delivery [27]. Alternative strategies that have been used to increase the half-life of the Fab include the fusion to albumin, to other proteins, or to serum protein-binding modules [31].

BsAbs: lessons from cancer therapy

Cancer is among the leading causes of death worldwide (<https://www.cancer.gov/aboutcancer/understanding/statistics>) and the development of novel therapeutics is of great importance. Currently, more than 30 bsAbs are in clinical development phase [4,17,32] and two have already been approved. BsAbs can simultaneously recruit immune effector cells using one Ab arm directed to receptor CD3 or to Fc and a second Ab arm targeting the cell surface TAA. Catumaxomab (Removab®), one of the approved bsAbs [33], is targeting CD3 and the TAA EpCam [33-36], and by retaining Fcγ RI, RIIa and RIII [37] redirects immune cells to the tumor site. However, the tolerated dose is low due to a general activation of T cells [38]. On the other hand, Blinatumomab is a BiTE® targeting CD3 and the TAA CD19 [39] used for the treatment of acute lymphoblastic leukemia (ALL). However, its ability to redirect T cell cytotoxicity at picomolar range is counterbalanced by the short half-life that requires continuous infusion of the product [16].

BsAbs in cancer therapy could provide insight into the mode of action of each bsAbs arm. Most of them engage T cells *via* the co-receptor CD3 expressed on one arm of the bsAb. As a consequence, all cells expressing CD3, including T cell subsets with immunoregulatory and immunosuppressive capacity and CD4 and CD8 T cell subsets expressing different

levels of exhaustion or anergic status, could be redirected to the tumor site. Therefore, the final outcome of tumor lysis is affected by the level of bsAb- induced activation as well as the abundance and relative distribution of relevant T cell subsets within the tumor microenvironment. The ultimate goal is to balance the potency of activated T cells and/or to counter-select subsets of cells that enable immunosuppression and tumor growth. Anti-CD3 bsAbs are expected to cause a generalized T cell activation that triggers a cytokine storm [40]. Administering low doses of bsAb could avoid cytokine-release-related symptoms [41] but it does not ensure that specific effector subsets can be engaged. Furthermore, an early recruitment of effector CD8⁺T cells and a late recruitment of regulatory T cells (Tregs, CD4⁺CD25⁺Foxp3⁺) is desirable to increase the efficacy of a bsAb treatment. Supporting this is the observed poor survival for cancer patients who have high levels of Tregs [42-44].

Combination immunotherapy studies have revealed the importance of recruiting activated rather than resting cells into the tumor site [45-47]. Expression of “activation receptors” on cells in the tumor microenvironment could be targets for the manipulation of the local milieu and facilitation of the bsAbs function. Crosslinking NK group 2 domain (NKG2D) or DNAX accessory molecule-1 (DNAM-1) on T $\gamma\delta$ cells [48] could recruit/activate them as scavengers of malignant tumor cells by eliciting cytokines and chemokines to regulate immune cells and present Ag for bsAb-mediated T cell priming [49,50]. Activating invariant NK cells (iNKT) by crosslinking of glycolipid presented from CD1d, could further activate effectors (NK and CTL) in an IFN- γ dependent fashion [51]. In combination with bsAbs where the Fc is engineered to improve affinity for Fc γ RIIIA, they could significantly enhance the *in vivo* NK-mediated ADCC activity. Alternatively, engineering Fc region to increase affinity to DCs is also a critical aspect to improve the current design. In fact, DCs are able to induce long-lasting immunity against tumors by processing and presenting tumor cells and materials derived from the tumor [52].

Regarding the TAA-based strategies, a major concern is the “specificity” of the targeted cells. CD33, CD123, human epidermal growth factor receptor 2 (HER2), EpCAM, and carcino-embryonic Ag (CEA) represent cell-surface targets for TAAs that are also expressed in normal tissues although at low levels. This could be a confounding factor for the specific action of bsAbs targeting these molecules and lead to possible side effects due to toxicity. This is of particular concern when multiple TAAs that could lead to recruitment/activation of more effector cells and higher efficacy of the treatment are under consideration. It is important to emphasize, however, that this is not a concern in case of bsAbs targeting viral determinants where the antigen-targets are mainly expressed by infected cells.

BsAbs in HIV treatment

HIV causes 40,000 new infections every year in the United States and 2 million cases worldwide (HIV Global statistics, URL: <https://www.hiv.gov/hiv-basics/overview/data-and-trends/global-statistics>). Recent studies have led to the discovery of several antibodies referred to as broadly neutralizing antibodies (bNAbs) which have broad and potent HIV neutralization activity [53], and have the potential for immunotherapeutic use [54,55]. The cellular and molecular mechanisms regulating the generation of such antibodies [56,57] are not well understood. An association between the quality of Env- specific follicular CD4⁺ T

cells (Tfh) cells, the degree of hypermutation in Env-specific germinal center B cells, the breadth of neutralization and the magnitude of Env sequence diversity was recently described in non-human primates infected with SHIV [58]. These findings suggest that co-evolution of virus and relevant follicular immune cells is an important biological factor underlying the development of broadly neutralizing antibodies in HIV. Furthermore, structural studies have revealed the modes by which Ab recognizes surface Env and guided the efforts for a structure-based design of HIV vaccines. Env is present as a functional trimer on the viral membrane and based on its conserved vulnerable sites, bNAbs have been classified as 1) CD4bs (CD4 receptor-binding site) group (VRC01, VRC07, 3BNC117, 8ANC131, CH103 and N6, the most potent antibody in the family [59]; 2) V3-glycan group that recognize the base of the variable loop V3 near to the N322 glycan (PTG121, PGT128, PGT135, and 10-1074); 3) V1V2-glycan group, recognizing the variable loop V2 and its neighboring glycans (PG9/16, PGT145, and PGDM1400) 4) MPER group, recognizing the membrane proximal external region of gp41 (10E8) and 5) the group targeting the gp120-gp41 interface including the fusion peptide (PGT151, 3BC176, 8ANC195 and VRC34.01).

Several studies have shown that *in vivo* administration of bNAbs can confer protection against infection [60-63]. All known bNAbs have related viral escape mutations, due to HIV reverse transcriptase function or immunological pressure induced mutations, challenging the therapeutic efficacy of bNAb monotherapies to prevent and treat HIV infection. Administration of bNAb cocktails has shown better efficacy for control of the virus *in vivo* [54,64-67]. Still, resistant viral isolates that exist prior or after infusion of bNAbs represent a major challenge for this therapeutic strategy [68]. Over the past years, an increasing number of bsAbs against HIV has been designed and tested for the ability to neutralize different HIV strains [69-71] (Table 1). Still, Env diversity, bNAb escape mutations and access to anatomical sites like follicular areas in lymph nodes or central nervous system, where the virus can establish reservoirs, are major challenges for bsAbs based HIV treatment. Overcoming these obstacles, bsAbs in the CrossMab format targeting more than one Env element have been tested (Figure 1). bNAb combinations like VRC07, targeting CD4bs and the glycan dependent PG9-16 bNAb have shown increased breadth and potency [69]. Swapping the hinge domain of IgG1 with that of IgG3, which is known to offer more flexibility based on its length and amino acid composition, facilitates better Env binding [70]. The engineered IgG3C- CrossMAB with two Fab arms targeting the CD4bs and gp120 V3-glycan epitope showed synergistic effects leading to increased neutralization potency and breadth (Figure 1) [70]. The hypothesis that targeting multiple epitopes with a single molecule improves potency and breadth has been demonstrated by Xu et al, [71]. Three bNAbs directed to CD4bs, MPER, and the V1V2 glycan site were linked in an alternative IgG configuration (Cross-Over Dual Variable IgG, CODV-IgG [72]) that inverts the mode by which V regions are arrayed in tandem, with each V domain able to interact with its own target. This tri-specific (Tri-Ab) fully protected NHP against a mucosal challenge with a mixture of SHIVs showing increased breadth with respect to the parental bNAbs [71]. By developing a structure-based approach considering the topology of the complex bNAb- Env trimer and the importance of the inter-protomer distance, bsAbs and tri-Abs that were able to cross-link adjacent protomers within the same Env trimer were generated [73]. An

alternative strategy is the generation of bsAbs that target host proteins, like CD4 or CCR5, and viral epitopes aiming to block virus entry and prevent virus from infecting cells [74-76].

Strategies to improve the *in vivo* “availability” of bsAbs, under controlled inflammatory conditions, could further improve their efficacy. Taking advantage of the *in-vitro* delivery transcribed platforms (IVT), mRNA-based therapeutics have been recently tested [77-79]. One advantage of the mRNA-based production of bsAbs is the capability of avoiding systemic release of proinflammatory cytokines [79] while repeated administrations of mRNA have been proven safe [78].

bsAbs for HIV cure: engage and kill

HIV persistence in latent reservoirs, even in combination antiretroviral therapy (cART) treated individuals, is a major obstacle for HIV cure. Several strategies have been undertaken to eliminate the latent HIV reservoir, including the use of latency-reversing agent whose function is to reactivate provirus for viral expression, followed by elimination of latently infected cells either by cytolytic effectors or via endogenous immune responses [80]. Drugs like histone deacetylase inhibitors modestly reduce the reservoir, whereas drugs such as protein kinase C activator and disulfiram activate latent HIV expression [81,82]. However, the shock and kill strategy is complicated by the fact that virus is reactivated in CD4⁺ T cells located in the sanctuary sites such as the lymph node B cell follicles where the viral clearance is limited by a paucity of virus-specific CD8⁺ cytotoxic cells (CTLs) [83-85].

Similar to cancer bsAb-based immunotherapies, HIV specific bsAb could target Env and simultaneously engage CD3 on CTLs. Work in the late 90s set the precedent for a strategy to generate T cell engaging HIV bsAbs where anti-CD3 is present to re-target host effector immune cells to eliminate HIV infected cells and a second arm that target viral elements [86]. Recent work based on Env-specific DARTs showed that many bsAbs (PTG121, PGT145, A32, 7B) can induce a CTL-mediated killing of resting HIV-infected CD4⁺ T cells *in vitro* and reduce HIV protein expression *ex-vivo* [30,87]. Interestingly, combination of DARTs that allow recognition of diverse Env antigen revealed that PGT121xCD3 paired with A32xCD3 or 7B2xCD3 did not show synergistic nor additive effects [87] possibly due to the high potency of each DART. In the BiTE format, the combination of domain 1 and 2 with a neutralizing scFv against gp120 and an anti-CD3 scFv demonstrated potent activity in targeting latently HIV-infected cells [88]. bsAbs containing the Fab region of bNAbs VRC07 linked via C-terminus to the humanized scFv of anti-CD3 has been used for the clearance of infected cells too [89]. T-cell lysis can be ascribed to both arms of the bsAb where CD3-mediated activation of latently infected T cells is followed by proviral gene expression and bsAb can cross-link the newly exposed Env molecules to effector CD8 T cells leading to lysis of previously latently-infected cells [89]. Several studies have shown that B cell follicles and particularly germinal centers (GCs) are major sites for HIV reservoir establishment [90]. Tfh cells hold HIV even after initiation of antiretroviral treatment [91]. Therefore, the efficacy of bsAb immunotherapies relying on the local stimulation of effector cells depends on the recruitment of such cells in these immunologically privileged areas like GCs. Previous reports have shown the limited access of HIV/SIV specific CD8⁺ T cells into these areas [83]. More recently, the phenotype, functionality, molecular profile and

localization of bulk CD8⁺ T cells in the follicular areas (fCD8s) was described in HIV infected subjects [92] and SIV infected non-human primates [93,94]. Generalized immune activation is at least one of the driving forces for the accumulation of fCD8 cells in chronic HIV/SIV [93], a profile found even after initiation of cART [92,93]. *In vitro* bsAb-mediated redirected killing assays showed that fCD8 cells have a potent cytotoxic capacity [92,93]. These observations provide an immunological basis for the investigation of treatments combining bsAbs with reagents like IL-15, which increases the trafficking of CD8⁺ T cells in the lymph node follicles [95], aiming to increase the likelihood of *in situ* activated fCD8 to attack and clear Tfh cells harboring activated virus. Many aspects related to bsAbs (i.e. *in vivo* pharmacokinetics, distribution in the follicular areas, modifications to minimize generalized immune activation) and the response of the host to such treatment (i.e. immune activation, changes in the follicular dynamics, trafficking of effector T cells into tissues) remain to be solved. The SHIV NHP model can be used to assess the *in vivo* efficacy of these therapies since the virus expresses the native HIV envelope which are targeted by these bsAbs and therefore will provide key insight towards the development of bsAb dependent treatment of HIV infection.

Concluding remarks, future directions

Several studies suggest that bsAbs could be a promising strategy for the *in vivo* elimination of HIV. In the context of a shock-and-kill strategy, special effort should be also given to complementary interventions for the efficient recruitment of effector killers in the site of viral reactivation. The presence of viral escape mutations, the incomplete recruitment into the follicular and germinal center areas, and the expression of exhaustion molecules on CTLs are all biological factors compromising the ability of infection- induced virus-specific CTLs to clear infected cells. Targeting bulk CD8 T cells by bsAbs represents a strategy that overcomes these limitations. However, the expression of co- inhibitory receptors like PD-1 can affect the survival of bulk CD8⁺ T cells too [96]. This would be particularly important for the follicular CD8⁺ T cells that co-express several co- inhibitory receptors even after cART initiation [92]. Therefore, engineering bsAbs to include also specificities against such receptors could increase their efficacy. Further development of bsAbs could include the testing of anti-CD3 specificities with different CD3 affinities aiming to avoid unrestrained T cell activation or the targeting of more than one Env domains increasing the specificity and efficacy of the bsAbs activity. Rigorous, pre-clinical assessments of such treatments are warranted. Active ongoing research is focused on understanding local, tissue effector immune cell populations and the molecular signature of HIV infected cells. These studies could further inform the design of novel bsAbs with higher specificity and efficacy as well as the identification of molecular targets for bsAb-based combination immunotherapies against HIV.

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Fig. 1.

bs- and Tri-specific Abs for HIV. (A) Schematic of IgG structure in two mAbs. Variable regions (ovals): V_H, green or purple; V_L, orange or pink; Constant domains: C_L, light grey/dark grey; C_H1-C_H2-C_H3, light black/dark black. IgG elements: single chain Fragment variable (scFv) carrying a flexible linker between V_HV_L; Fragment antigen binding (Fab) including V_H-V_L, C_H1-C_L. Left: example of IgG like-bsAb with the combination of two Fabs from two mAbs. Antigen (Ag) (pentagon) is red, or black. (B-D) Examples of bsAb for HIV. The two different Ags are shown in red and black (B) CrossMab with Knob-in-hole

(KiH) technology; CH3 dimerization white circle). Note CH1-CL domain-exchanged (75); (C) Hinge-domain-engineered IgG3 enhances Fab domain flexibility (76); (D) tandem scFv joined with DNA linkers (G4S)_n (79). Increased binding moiety in two examples of Tri-specific for HIV by combining CrossMab KiH with (E) CODV-Ig (78) format or with (F) tandem scFv joined with DNA linkers. Three different Ags are shown in red, grey and black. Example of non-Ig- like bsAb. (G) bispecific T cell engager (BiTE) composed two Fv, one targeting the virus (solid fill color) and another targeting CD3 (grey checker board pattern); (H) Dual-Affinity re-Targeting molecule (DART), in which VH of one mAb is linked to VL of another mAb to form a ‘diabody’ structure linked tightly by a disulphide bond (H shape). The arm targeting the host is shown as grey checker board pattern.

Table 1:

List of bsAbs and tri-Abs for HIV.

Format	Arm 1	Arm 2	References
IgG like bsAbs			
CrossMab (CH1-CL)	VRC07	PG9-16	[73]
(G ₄ S) flexible linker-Fc	VRC01	PGT121	[76]
mAb-scFv (BibNAb)	PG9	iMab (αCD4)	[77]
CrossMab (CH1-CL)	10E8v2.0	iMab (αCD4)	[78]
IgG3C-hinge variant	3BNC117	PGT135	[74]
Tandem bs-bnAb	PGT128	Hu5A8 (αCD4)	[79]
Non-IgG like bsAbs			
Fab-scFv	VRC07	αCD3	[93]
BiTE: (scFv) ₂	CD4d1d2 +17b	αCD3	[92]
DART: (scFv) ₂	A32	αCD3	[30, 91]
DART-Fc	MP3-A32	αCD3	[91]
Tri-Abs			
CODV-Ig based	VRC01	PGDM1400-10E8v4	[75]
(G ₄ S) and Knob-in-hole	10E8-VRC01	PGT151	[76]

¹. Bispecific antibodies (BsAbs) are engineered immunoglobulins with the capacity of recognizing two different binding sites.

². The molecular format is a major determinant for the immune responses elicited by BsAbs.

³. Cancer studies have revealed the potential for combinatorial immunotherapies including BsAbs.

⁴. BsAbs is a promising immunotherapy for HIV elimination in the context of a “shock and kill” strategy.

⁵. BsAbs activity is not affected by viral mutations or impaired endogenous, infection-induced immune responses.