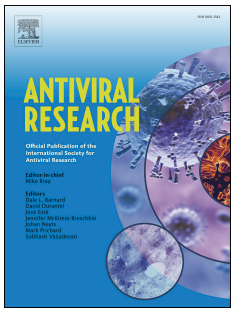

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PII: S0166-3542(24)00181-5

DOI: https://doi.org/10.1016/j.antiviral.2024.105972

Reference: AVR 105972

To appear in: *Antiviral Research*

Received Date: 30 January 2024

Revised Date: 14 July 2024

Accepted Date: 26 July 2024

Please cite this article as: Xie, Z., Protzer, U., Activating adaptive immunity by bispecific, T-cell engager antibodies bridging infected and immune-effector cells is a promising novel therapy for chronic hepatitis B, *Antiviral Research*, https://doi.org/10.1016/j.antiviral.2024.105972.

Activating adaptive immunity by bispecific, T-cell engager antibodies bridging infected and immune-effector cells is a promising novel therapy for chronic hepatitis B

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Abstract

Bispecific antibodies (bsAbs) are engineered immunoglobulins that combine two different antigen-binding sites in one molecule. BsAbs can be divided into two molecular formats: IgG-like and non-IgG-like antibodies. Structural elements of each format have implications for engaging the immune system. T cell engager antibodies (TCEs) are bsAbs designed to engage T cells with target cells. TCEs can be applied not only in cancer but also in infectious disease therapy to activate T-cell responses. In this review, we focus on current literature on the design and use of bsAbs as an innovative strategy to enhance adaptive antiviral immune responses. We summarized the novel T cell-related immunotherapies with a focus on TCEs, that are developed for the treatment of chronic hepatitis B. Chronic infection with the hepatitis B virus (HBV) had a death toll of 1.1 million humans in 2022, mainly due to liver cirrhosis and hepatocellular carcinoma developing in the more than 250 million humans chronically infected. A curative treatment approach for chronic hepatitis B is lacking. Combining antiviral therapy with immune therapies activating T-cell responses is regarded as the most promising therapeutic approach to curing HBV and preventing the sequelae of chronic infection. Attracting functionally intact T cells that are not HBV-specific and, therefore, have not yet been

exposed to regulatory mechanisms and activating those at the target site in the liver is a very interesting therapeutic approach that could be achieved by TCEs. Thus, TCEs redirecting T cells toward HBV-positive cells represent a promising strategy for treating chronic hepatitis B and HBV-associated hepatocellular carcinoma.

Keywords (5-6)

Bispecific antibodies, bispecific T cell engager antibodies, T cell immunity, HBV, immune cell activation

Highlights (separate file)

- Bispecific and T-cell engager antibodies are novel immune therapy approaches for cancer and infectious diseases.
- The format and design of the antibodies largely influence the potency of bispecific antibodies and T-cell engagers.
- There is an urgent need for cure strategies that prevent the deadly sequelae of chronic hepatitis B virus (HBV) infection.
- HBV-infected cells continuously express viral envelop proteins that can serve as targets for antibody and T-cell therapies.
- T-cell engager antibodies can redirect T cells to kill HBV-infected and HBV-associated hepatocellular carcinoma cells.

Introduction

The quest for innovative antiviral strategies has become imperative in the evolutionary arms race between viruses and their hosts. Among the many approaches explored, bispecific antibodies (bsAbs) have emerged as promising candidates with their ability to bridge infected and immune-effector cells, thereby orchestrating a targeted and potent antiviral response. Viruses, with their ability to exploit the host machinery for replication and evade immune surveillance, continue to pose significant threats to global public health. Traditional antiviral strategies often focus on direct inhibition of viral replication or enhancement of the host immune response. While effective, these approaches may encounter challenges such as the emergence of drug-resistant virus strains or insufficient activation of immune effectors. The advent of bispecific antibodies for cancer therapy, designed to simultaneously engage infected cells and immune cells, offers novel opportunities in the battle against viral infections and, in particular, chronic viral infections.

BsAbs are engineered molecules capable of binding to two distinct epitopes, enabling them to simultaneously interact with infected cells and immune effector cells, such as natural killer (NK) or T cells. This unique dual-targeting capability positions bsAbs as potent mediators in redirecting the immune system not only against cancer but also toward virally infected cells. The interaction between bsAbs and infected cells typically involves targeting viral antigens on the cell surface, requiring a timely expression of such antigens by infected cells. An alternative approach provides molecules combining an affinity-enhanced T Cell receptor with an anti-CD3 antibody moiety. Although no classical bsAbs, their mode of action is very similar. The versatility of bsAbs extends beyond their role in direct antiviral activity. Recent studies have highlighted their potential in modulating immune responses, such as activating antigen-presenting cells to enhance antigen presentation and T-cell priming. This immune-modulatory aspect broadens the scope of bsAbs beyond their direct antiviral effects, positioning them as valuable tools in shaping adaptive immune responses against viral pathogens. However, the

optimization of bsAb design, including the selection of target antigens and engineering strategies, remains an ongoing pursuit. Additionally, understanding the kinetics of bsAb-mediated immune responses, as well as potential off-target effects, is critical for ensuring both efficacy and safety in clinical applications.

Harnessing adaptive antiviral responses through bsAbs may represent a paradigm shift in antiviral therapeutics. The unique ability of bsAbs to bridge infected cells with immune effectors, coupled with their multifaceted mechanisms of action, positions them as potent weapons in the fight against viral infections. This review delves into the current literature surrounding the design and utilization of bsAbs as a groundbreaking strategy to harness adaptive antiviral responses, exploring their mechanisms, applications, and the potential they hold for improving antiviral therapeutics, in particular for chronic viral infections, the cure of which requires immune cell activation.

1. Principles of immune-cell engager antibodies

The origin of bsAbs can be traced back to the 1960s when Alfred Nisonoff had the initial idea of substituting one of the two identical antigen-binding arms with an alternative antigen-binding specificity (Nisonoff and Rivers, 1961; Nisonoff et al., 1960). In the 1980s, the initial concept of bispecific antibodies underwent further development to incorporate a second specificity directed against molecules detected on the surface of T cells. Cytotoxic T lymphocytes (CTLs), like all T cells, express variable T-cell receptors (TCRs) in conjunction with invariable CD3 subunits. When this CD3-binding specificity is engineered into antibodies targeting tumor-specific antigens, the CTL response can be redirected towards cancer cells (Perez et al., 1985; Staerz et al., 1985). It was found later that this class of antibodies could activate T cells through CD3 and even non-classical T cells like $\gamma\delta$ T cells NK-T cells (Ferrini et al., 1993; Guo et al., 2020). In the following, bsAbs were also used to target and activate NK cells, monocytes, macrophages, and granulocytes against distinct target cells carrying a specific targeting

molecule on their surface. Thus, using bsAbs, the immune response against tumors could be diversified, addressing the limitations of natural immune reactions in cancer patients.

Nowadays, there are over 100 bsAbs in clinical development, most still in the early stages (Brinkmann and Kontermann, 2021). Most bsAbs in development aim to treat cancer. Still, some are focused on chronic inflammatory, autoimmune, and neurodegenerative diseases, vascular (the body's network of blood vessels), ocular (eye-related), and hematologic (blood-related) disorders, and last but not least, target infections. Since 2014, the FDA has approved nine bsAbs marketing applications to treat cancer and hematologic and ocular diseases. These nine bsAbs are summarized in Table 1, their formats are depicted in Figure 1 and explained in Figure 2. Notably, four bsAbs (tebentafusp, faricimab, mosunetuzumab, and teclistamab) targeting combinations of antigens are unique among the currently approved antibody therapeutics and have been approved in 2022 (Kaplon et al., 2023).

1.1. Format

There are two molecular formats of bsAbs: IgG- and non-IgG-like molecules (Figure 2). The structural elements of each format have implications for the engagement and mobilization of the immune system. In this review, we summarize the available antibody platforms currently used for generating IgG-like and non-IgG-like bsAbs and explain which approaches have been used to assemble those bsAbs currently approved for clinical application. More formats under investigation are comprehensively discussed in a review by *Labrijn et al.* (Labrijn et al., 2019).

1.1.1. IgG-like bsAbs

The IgG-like bsAbs incorporate the constant, fragment crystallizable (Fc) region of natural antibodies. The prevalence of IgG-like formats among FDA-approved bsAbs suggests that considerable advantages are associated with this structural configuration. In pharmacokinetics, the Fc region ensures a prolonged *in vivo* half-life by engaging in neonatal Fc receptor (FcRn) that recycles these antibodies and prevents rapid excretion. This

mechanism contributes significantly to the stability and solubility of the antibodies. Moreover, the Fc region serves as a critical determinant in the efficacy of bsAbs to stimulate immune responses. Various biochemical properties of the Fc region of antibodies, including affinity, glycosylation, and antibody isotype, dictate the activation of different immune effector cells (Nimmerjahn and Ravetch, 2007a). The Fc region engages with Fcγ receptors type I (CD64), IIa, b, c (CD32a, b, c), and IIIa, b (CD16a, b) expressed on macrophages, dendritic cells (DC), and NK cells (Nimmerjahn and Ravetch, 2007b). Through these interactions, the Fc region initiates antibody-mediated effector functions such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and antibody-dependent cellular phagocytosis (ADCP) (Demarest and Glaser, 2008). DuoBody® technology is a commonly used approach to combine two stable antibodies with specific mutations in CH3 domains recombining under tailored laboratory conditions, called controlled Fab-arm exchange (Labrijn et al., 2013), resulting in a high-yield bispecific antibody suitable for both discovery-scale and commercial manufacturing applications; Amivantamab (Yanakieva et al., 2022) and Epcoritamab (Thieblemont et al., 2023) are FDA-approved bsAbs which applied the DuoBody technology.

However, the IgG format also presents some intrinsic drawbacks that might interfere with efficiency. The large size of IgG increases the serum half-life but, at the same time, poses challenges in terms of tissue penetration and distribution. Some tissues may be less accessible due to the size of these molecules, limiting their effectiveness in certain physiological contexts. Besides, heavy chain and light chain mispairing pose severe problems for bsAbs made in IgG formats consisting of two distinct polypeptides that may assemble into ineffective antibodies or undesired homodimers. The ‘knobs-into-hole’ strategy mutation promotes the assembly of heavy chains from two different parental antibody chains (one with a 'knob' mutation and the other with 'hole' mutations) into a single new bispecific antibody (Ridgway et al., 1996). This technology was used in the FDA-approved bsAbs Mosunetuzumab (Hosseini et al., 2020).

Although this approach can suppress the formation of Fc heavy chain homodimers, it cannot prevent the mispairing of the light chains associated with the two heavy chains. The light chain mispairing can be overcome by using a single light chain or applying the 'CrossMAb' approach by swapping the CH3 domains between the heavy chains of the two antibody arms (Klein et al., 2016; Schaefer et al., 2011), which were applied in licensed antibodies Faricimab and Glofitmab. Besides, the 'fully orthogonal heavy chain–light chain interface' is a more robust solution to derive the desired heavy chain–light chain specificity (Lewis et al., 2014).

1.1.2. Non-IgG like bsABs

The non-IgG-like bsAb format lacks the Fc region. Blinatumomab is the prime example for a tandem single-chain variable fragment (Fv)-based CD19/CD3 Bispecific T-cell Engager (BiTE) developed by Micromet, a Munich-based biotech company and later on by Amgen for the treatment of acute lymphocytic leukemia (ALL) (Bargou et al., 2008). The non-IgG-like bsAbs, also include formats such as "TandAbs," "Nanobodies," and "Diabody." These bsAbs result in a smaller molecular mass (30-50 kDa) compared to whole IgG (150 kDa), allowing for improved tissue penetration, reducing the unwanted activation of non-specific immune cells, and showing higher tumor specificity due to the smaller size.

However, lacking the Fc region results in a shorter half-life of the construct in the circulation due to rapid renal clearance, a major drawback of non-IgG-like bsAbs. This phenomenon limits the clinical promotion of the application since the short half-life increases the administration doses of therapeutic agents and often requires continuous infusion. Alternative strategies to address this involve fusing or associating the Fab to albumin, other serum proteins, or serum protein-binding modules. The method of connecting two single-chain variable fragments (scFvs) to albumin in the "scFv-HSA-scFv" format and employing Polyethylene glycol (PEGylation) conjugation are viable approaches for extending the circulating half-life (Kontermann, 2011). Additionally, the Dock-and-lock (DNL) (Goldenberg et al., 2008; Rossi et al., 2006) method, which involves heterodimerizing protein domains fused with Fab domains or integral antibodies, allows the creation of multivalent and multifunctional antibody

derivatives, such as trivalent bispecific antibodies, offering a promising platform for constructing antibodies with retained bioactivity.

1.2. Mechanism of action: lessons from cancer therapy

BsAbs can exert their functional roles through various mechanisms. The critical advantage of bsAbs is that they can enable new functions by connecting two different binding moieties in the same molecule. This allows for creative therapeutic ideas, like connecting two types of cells (in-trans binding) or interacting with two molecules on a single cell's membrane (in-cis binding). The process of bridging receptors on cells (in-cis) involves the mandatory crosslinking of specific cell surface receptors, leading to either the inactivation of these cells (Moores et al., 2016) (as a strategy to diminish tumor growth in cancer applications) or their activation (such as conditionally activating a growth factor receptor for diabetes treatment) (Kolumam et al., 2015). BsAbs apply the in-cis binding strategy that bridges cells as their obligate mechanism of action, representing the largest group, with T-cell redirection as the most common denominator, which will mainly be discussed in this section.

1.2.1. Redirecting immune effector cells

1.2.1.1 Activating T cells using T-cell engager bsAb

Most bsAbs undergoing clinical trials comprise CD3-binding, bispecific T cell engagers (TCEs). These TCEs specifically bind to CD3, a constituent of the T cell receptor/CD3 protein complex expressed on the T cell surface, as well as to a tumor-associated antigen (TAA) present on the surface of tumor cells. Upon concurrent engagement of CD3 and TAA, the physical proximity established between the T cell and the tumor cell leads to the formation of an immune synapse. This event triggers T-cell activation and the release of perforin and granzyme, resulting in the T-cell-dependent killing of tumor cells via apoptosis.

A prime example is Teclistamab, a first-in-class BiTE antibody for treating Multiple myeloma (MM). Teclistamab engages in T-cell activation by targeting the CD3 receptor on the surface

of T cells, and BCMA expressed on the surface of MM cells, leading to the release of various proinflammatory cytokines and, ultimately, lysis of BCMA-expressing MM cells (Moreau et al., 2022; Pillarisetti et al., 2020). In the phase1-2 study, Teclistamab resulted in a high rate of deep and durable response in patients with triple-class–exposed, relapsed or refractory MM (Moreau et al., 2022).

Catumaxomab (Removab®) is one of the FDA-approved bsAbs (Seimetz et al., 2010), targeting CD3 and the TAA EpCam (Sebastian et al., 2009), redirects immune cells to the tumor site. The concept of catumaxomab is to activate T-cell mediated lysis, and, by retaining Fcγ RI, RIIa and RIII binding properties (Goere et al., 2013), activate phagocytosis, ADCC, and cytokine-mediated cytotoxicity to destroy tumor cells. However, the tolerated dose is low since it can generally activate T cells, and it was withdrawn from the market in 2017.

Blinatumomab is a BiTE® targeting CD3 and the TAA CD19 (Frankel and Baeuerle, 2013), which has been approved by the FDA since 2014 to treat acute lymphoblastic leukemia (ALL) (Przepiorka et al., 2015). Unlike Catumaxomab, blinatumomab is a monovalent binder of the TCR, T cell activation occurs only if CD19-positive target cells are present (Kufer et al., 2001). Conversely, tumor cell lysis was not observed in the absence of T cells, showing that the cytotoxic effect of blinatumomab is purely T cell-dependent (Wolf et al., 2005).

The affinity for CD3 in TCEs plays a crucial role in their biodistribution. The affinity determines how strongly the TCE binds to CD3 on T cells, influencing the activation and recruitment of T cells to the targeted cells. Although it has been shown that a high CD3 affinity is more efficient in *in vitro* cell culture assays (Leong et al., 2017), a low CD3 affinity seems to have advantages in tumor distribution. In addition, attenuated CD3 affinity endowed potent antitumor activity but limited cytokine release syndrome (CRS) in acute myeloid leukemia (AML) (Lee et al., 2023) as well as in solid tumors (Dang et al., 2021). *Lee* et al. showed that with a limited CD3 binding ability, their bsAb target AML cell lines exhibited more robust T-cell activation and potent tumor cell killing in cell culture and efficient tumor control with less cytokine release in AML mouse models (Lee et al., 2023). Besides, *Dang* et al. showed that low-affinity anti-CD3 is efficacious

while inducing a lower incidence and severity of CRS in patients with prostate cancer compared with TCEs that incorporate high-affinity anti-CD3 domains (Dang et al., 2021). Moreover, a lower affinity for the CD3-binding arm is favored to facilitate the effective distribution of the therapeutic agent within tumors *in vivo*. This preference arises from the desire to avoid rapid plasma clearance of the TCEs and prevent the entrapment of the antibody in tissues rich in T cells, such as the spleen and lymph nodes (Mandikian et al., 2018; Sandker et al., 2023).

1.2.1.2. Activating other immune cells

In addition to T cells, other effector cells or immune cell subsets can also be recruited to tumor cells. For example, bispecific killer engagers (BiKEs) have been developed to target NK cells, potent cytotoxic lymphocytes of the innate immune system. FcγRIII-(CD16A)-mediated recruitment through bsAbs can be realized either through the direct binding of CD16 on the surface of NK cells to the Fc region of the bsAb or through the use of its CD16A-targeting arm in a BiKE. By activating the NK cell receptor CD16A (FcgRIIIA), predominantly expressed on mature NK cells, ADCC may contribute to eradicating tumor cells (Dai et al., 2017).

An example of an NK cell-redirecting antibody is AFM-13, a tandem diabody construct targeting CD16 on NK cells and CD30 on tumor cells (Reusch et al., 2014). In a phase 1 trial in patients with relapsed or refractory Hodgkin's lymphoma, 3 of 26 patients achieved partial remission (11.5%), and 13 patients achieved stable disease (50%), with an overall disease control rate of 61.5% (Rothe et al., 2015). *Kerbauy* et al. described that IL-12/15/18- induced memory-like NK cells from peripheral blood exhibited enhanced killing of CD30+ lymphoma targets directed by AFM-13 (Kerbauy et al., 2021). AFM-13 can be combined with pembrolizumab increasing the response rate to 88% at the highest treatment dose, with an 83% overall response rate. The combination treatment was generally well tolerated, with similar safety profiles compared to the known profiles of each agent alone (Bartlett et al., 2020), and is now applied in a phase 2 clinical trial

In addition to targeting CD16A, a small number of studies have targeted other activation receptors on NK cells, such as natural killer group 2D (Han et al., 2019) and natural cytotoxicity receptor NKp46 (Gauthier et al., 2019). Targeting NK cells offers the advantage of their lower frequency than T cells, as NK cells constitute only 10% of T cell numbers. As a result, the amount of antibodies and the risk of excessive cytokine production is expected to be reduced. Emerging studies consistently suggest that the engagement of NK cells holds promising prospects for future developments in tumor immunotherapy.

1.2.2. Factors determining the targeting by T-cell engager antibodies

TCEs establish a direct connection between T cells and tumor cells, forming an immune synapse that triggers TCR activation. Hereby, the antigen level on the target cells largely determines the activity of TCEs. An *in vitro* study demonstrated that using TCEs targeting the human epidermal growth factor receptor 2 (HER2), the tumor cytotoxicity correlates with the surface HER2 expression in a large panel of human tumor cell lines, irrespective of lineage or tumor type (Lopez-Albaitero et al., 2017). A certain threshold of target expression seems necessary for the cytotoxic activation of T cells by TCEs.

Besides the expression level of antigen, the activity of TCEs also depends on antigen mobility in the membrane; the dimensions of the target molecule play a vital role in the efficiency of synapse formation (Li et al., 2017). *Bluemel* et al. proved that smaller surface target antigens are beneficial for immune synapse formation [38], indicating that antigen size was also an essential determinant for the potency of BiTEs (Khilji et al., 2023). Targeting bivalent tumor antigens is a strategy to enhance the potency of TCEs (Lopez-Albaitero et al., 2017; Slaga et al., 2018). Several tetravalent TCEs were designed and investigated. Cadonilimab is a tetravalent PD-1/CTLA-4 TCE that showed a superior target binding avidity [41], which could lead to preferential accumulation in the tumor microenvironment, thus achieving higher efficacy with less toxicity.

Besides the efficiency and potency, the specificity of the target cells is a significant concern. CD33, CD123, HER2, epithelial cell adhesion molecule, and carcinoembryonic antigen are tumor-associated antigens expressed in normal tissues at low levels. This dual targeting raises concerns about potential side effects and toxicity with TCEs targeting these molecules, especially when considering multiple antigens. It is crucial to note that this concern does not apply to bsAbs or TCEs targeting viral determinants, as these antigens are clearly non-self and only expressed by infected cells.

2. T cell engager antibodies in infectious diseases

In recent years, the experience with BsAbs in cancer therapy has been transferred to the field of antiviral therapy. Virus-specific TCEs were designed to redirect CD8 T cells to viral proteins displayed on the surface of infected cells. These are mainly viral envelop proteins as they are integrated into the host cell membrane to allow the budding of progeny virus particles from infected cells. Such approaches have been described for HIV-1, cytomegalovirus, the coronavirus SARS-CoV2 and the hepatitis B virus (HBV).

Brozy et al. demonstrated through *in vitro* and *ex vivo* experiments that BiTE antibody constructs designed to target HIV gp120 significantly decreased HIV replication (Brozy et al., 2018). They generated BiTE antibody constructs that target the HIV-1 envelope protein gp120 (HIV gp120) using either gp120-specific antibodies or the first two extracellular domains of human CD4 and fused them to an anti-human CD3ε scFv. These engineered human BiTE antibody constructs showed engagement of T cells for redirected lysis of HIV gp120-transfected CHO cells. Furthermore, they substantially inhibited HIV-1 replication in peripheral blood mononuclear cells (PBMCs) and in macrophages cocultured with autologous $CD8^+$ T cells, the most potent being the BiTEs containing the extracellular domains of CD4 that bind the HIV envelop. While the initial CD4 BiTE antibody construct promoted HIV infection of

human $CD4^-/CD8^+$ T cells, the neutralizing antibody BiTE constructs, as well as a BiTE containing CD4 domains fused to an scFv did not (Brozy et al., 2018).

Meng et al. applied the knob-into-hole strategy and constructed a bsAb that can direct non-virus-specific T cells to recognize and exert effector functions against cells infected with the human cytomegalovirus (HCMV) (Meng et al., 2018). Upon HCMV infection, the modified antibody could stimulate the naïve T cells, leading to cytokine production, proliferation, and the expression of distinctive phenotype markers associated with T cell activation.

Early in the SARS-Cov2 pandemic, mAbs targeting the SARS-CoV-2 viral spike protein showed promise as treatments for SARS-CoV-2 infection. However, the amino acid changes of different variants in the epitopes within the spike protein have significantly reduced the activity of the monoclonal antibody therapies (Dean et al., 2023). In response to this challenge, researchers have shifted their focus to developing bsAbs to simultaneously target two epitopes on the virus's spike protein (Yuan et al., 2022). *Li* et al. introduce a bispecific T-cell engager (S-BiTE) strategy that targets the SARS-Cov-2 spike protein to engage T cells to control infection. This method hinders the entry of free viruses into susceptible cells by competing with membrane receptors and eradicates virus-infected cells through potent T-cell-mediated cytotoxicity. Besides, S-BiTE-treated mice showed superior efficiency in viral load control compared to the neutralization-only treated group (Li et al., 2023).

A limitation of targeting HIV, HCMV, and coronaviruses, however, is that these viruses undergo an early-late shift during their gene expression. Early genes express proteins that the viruses require prior *de novo* synthesis of viral particles but allow for alteration of the cells and replication of the viral genome. These early gene products are not displayed on the surface of infected cells and, thus, are no suitable targets for T-cell redirection. The viral envelop proteins that are suitable targets, however, are only expressed late during the infection cycle. In consequence, antibodies binding the envelop proteins can efficiently neutralize the viruses and prevent infection, but infected cells can only be targeted by antibodies for a short time period before they release newly formed viruses or start to undergo cell lysis anyway.

The situation is different for HBV. HBV, unlike most other viruses, does not undergo an early-late shift. HBV expresses its envelop proteins (HBVenv) continuously after productive infection of a hepatocyte and even from most viral genomes randomly integrated during long-term infection. This is clinically detected by a continuous secretion of HBsAg into the patient's blood. HBsAg consists of spheric and filamentous subviral particles, which are empty viral envelopes secreted from infected cells. HBV expresses small (S), medium (M) and large (L) HBVenv proteins that are embedded in the endoplasmic reticulum and plasma membranes and all share the S-domain. The highly abandoned spheric subviral particles consist essentially of the S protein, budd from the endoplasmatic reticulum into Golgi vesicles from where they are secreted and are reposnible for the predominant intracellular staining of S protein. In contrast, the less frequent filamentous subviral particles and virions also contain the L protein and similar to HIV budd into multivesicular bodies. These are vesicles that fuse later their membrane with the plasma membrane to release the virions (summarized in *Patient et al*. (Patient et al., 2009)). By this fusion process, S and L HBVenv proteins can be displayed on the surface of infected cells (Zhao et al., 2021). At the surface of infected cells, they can serve as therapeutic targets, initiating the development of chimeric antigen receptor (CAR)-T cells directed against S and L HBVenv proteins (Bohne et al., 2008; Guo et al., 2023; Wisskirchen et al., 2017; Zhao et al., 2021). How stable the HBVenv proteins are displayed on the cell surface and how they are internalized again and either engulfed into viral particles or degraded remains unknown.

3. Current therapeutic options for treating chronic hepatitis B

There is no finite, curative treatment for the more than 295 million individuals living with persistent HBV infection (Dusheiko et al., 2023) at risk of developing liver cirrhosis or hepatocellular carcinoma (HCC). Chronic hepatitis B (CHB) has become a treatable disease in that antiviral therapy using nucleos(t)ide analogs (NUCs) can suppress replication of the virus to a level below detection in the blood. Nucleoside analogs exert no direct effect on

pgRNA transcription from covalently closed circular DNA (cccDNA) and do not directly affect HBsAg expression from integrated genomes. Thus, hepatitis B e antigen (HBeAg) loss, indicating a reduction of the risk of developing liver cirrhosis, is only 20-30%, and HBsAg concentrations show only limited declines: an annual rate of 0.22% (0.17-0.28) and a 10-year cumulative incidence of 2.11% (1.54%–2.88%) has been reported (Hsu et al., 2021) but HBsAg loss. HBsAg loss, if it occurs, may be associated with a favorable outcome. Although nucleoside analogs improve clinical outcomes, they do not eliminate the risk of cirrhosis and HCC (Dusheiko et al., 2023), and treatment indication is limited to those who have developed inflammatory liver disease and are at high risk of developing HCC. In addition, patients continue to face the stigma of chronic infection and the economic and personal burdens of long-term treatment.

Limitations of directly acting antiviral therapies under development, such as capsid assembly modifiers or siRNA, include the fact that they act at late steps in HBV replication. The viral persistence form, cccDNA, remains untouched in the nucleus of infected cells (Lucifora and Protzer, 2016). Therefore, HBsAg secretion persists, and integration of HBV-DNA may continue. This necessitates long-term treatment. When antiviral therapy is withdrawn, most patients experience a rebound in HBV DNA, with some patients developing liver inflammation and potentially life-threatening flares (Berg et al., 2017). A finite treatment that allows long-term control and, ultimately, a cure for HBV infection is therefore desired. This will likely require coordinated therapies targeting the virus and boosting immunity to clear the infected reservoir of hepatocytes (Gehring and Protzer, 2019). In attempts to cure HBV infection, it is important to remember that HBV does not only persist via cccDNA - it may also integrate into the host genome. This integrated DNA does not replicate HBV but can still express HBV antigens, including HBsAg. In addition, cccDNA persists decades after the resolution of HBV infection (Rehermann et al., 1996) and can account for the reactivation of HBV infection when patients with resolved infection are immunosuppressed. A functional cure will most likely only be possible if directly acting antivirals are combined with immunotherapies (Lim et al., 2023).

The efficiency of the HBV-specific immune response becomes evident during the resolution of acute, self-limiting HBV infection. Patients who resolve acute HBV infection have a robust, HBV-specific T cell response with both CD4+ and CD8+ T cells essentially participating. These cells produce antiviral cytokines and provide co-stimulation to B cells. B cells produce anti-HBs, which clear antigens and viruses from the circulation and prevent or limit re-infection. The dichotomy between patients who resolve acute infections and those with chronic HBV infection is apparent in the magnitude and function of their immune responses (Gehring and Protzer, 2019). HBV-specific T cells are detected at significantly lower frequencies in patients with chronic versus acute infections. In the few patients who achieve a functional cure for a chronic HBV infection, an activation of CD4+ T cell responses seems decisive (Hoogeveen et al., 2022). A high frequency of activated, intrahepatic HBV-specific CD8+ T cells, combined with peripheral cytotoxic T cells that produce cytokines and expand, correlates with HBV control (Gehring and Protzer, 2019).

In a cross-sectional study investigating the magnitude of T-cell responses, patients who controlled HBV after long-term NUC therapy had T-cell frequencies similar to those of patients who resolved an acute infection (Boni et al., 2012). Lack of expansion and arming of effector T cells, lack of co-stimulation by hepatocytes, apoptosis, and NK-cell mediated depletion of activated T cells contribute to the low frequency of T cells observed in patients with chronic HBV infection (summarized in: (Gehring and Protzer, 2019)). In the HBV-specific T cells that remain, proliferation and antiviral function are restricted. In patients with chronic infection, persistent antigen exposure results in T-cell exhaustion (inactivation) and increased expression of inhibitory receptors PD-1, CTLA4, and TIM3. Amino acid restriction by myeloid-derived suppressor cells and mitochondrial dysfunction of HBV-specific T cells are examples of metabolic defects that reduce T-cell function (Fisicaro et al., 2017; Pallett et al., 2015). The lack of sufficient effector cells and functional immune tolerance is why T cells no longer recognize or respond to HBV antigens. Attracting functionally intact T cells that are not HBV-specific and, therefore, have not yet been exposed to these regulatory mechanisms and

activating those at the target site in the liver seems a very interesting therapeutic approach that could be achieved by T-cell engager antibodies.

4. T-cell therapy for chronic hepatitis B

The adoptive transfer of functional effector T cells redirected against HBV antigens is an interesting approach to overcoming the immune tolerance in chronic HBV infection. The first observation that immune cell therapy may cure HBV infection has been made in the setting of stem cell transplantation. Immune reconstitution through transplantation of bone marrow cells from donors with immunity to HBV into patients with chronic infection cleared HBV from the infected liver without severe side effects (Lau et al., 1997; Lau et al., 2002). As allogeneic stem cell transplantation is limited by severe side effects, such as graft versus host disease and high mortality, reconstituting immunity using autologous T cells genetically engineered to express HBV-specific receptors was then developed to treat CHB, prevent HBV-related complications, or treat HBV-related HCC (reviewed in Schreiber and Tan (Tan and Schreiber, 2020)).

As the first attempt to re-target T cells against HBV-infected cells that has been described worldwide, *Bohne et al.* generated CARs, recognizing S and L HBVenv proteins on the surface of replicating cells and showed that S-CAR grafted T cells recognize HBV-infected cells (Bohne et al., 2008) (Figure 3 A). These first-generation CARs were composed of an N-terminal leader sequence, heavy and light chain variable regions of an scFv recognizing the HBVenv proteins, an Fc spacer domain of human IgG1, transmembrane and intracellular CD3z signaling domains, in addition to the co-stimulatory signaling domain of CD28. Optimal scFv were selected by comparing a series of scFv-binding HBV surface proteins, demonstrating that an scFv that recognizes a conformational epitope in the external loop of the S domain of all HBVenv proteins of a broad range of HBV genotypes (Zhao et al., 2021) was best suited; it was designated as S-CAR. Patient T cells grafted with the S-CAR could clear HBV from infected, autologous primary hepatocyte cultures (Bohne et al., 2008).

As an alternative approach, T cell receptors were cloned from HBV-specific T cells isolated from donors that had cured an HBV infection (Gehring et al., 2011; Wisskirchen et al., 2017). Applying T cells grafted with S-specific TCRs could control the infection in immune-deficient, liver-humanized mice infected with HBV (Kah et al., 2017) (Figure 3 C). *In vivo* applicability of the S-CAR for adoptive T cell therapy was first assessed in an HBV-transgenic mouse model, and later in AAV-HBV infected mice serving as an HBV carrier model (Krebs et al., 2013; Wisskirchen et al., 2017), demonstrating that S-CAR grafted T cells relocated to the liver. $CD8^+$ S-CAR-grafted T cells targeting the HBV envelop proteins on the surface of infected cells effectively controlled the virus and caused only transient liver damage with no other obvious side effects.

Subsequently, *Guo et al*. used the affinity-matured antibody 2H5-A14, recognizing the pre-S1 domain of the L HBVenv protein to generate a second-generation CAR. A14 CAR T cells were capable of killing HBV-infected hepatocytes with high specificity. Adoptive transfer of A14 CAR-T cells to HBV-infected humanized immunodeficient mice resulted in reductions of all serum and intrahepatic virological markers to levels below the detection limit, demonstrating the curative potential of the approach *in vivo* (Guo et al., 2023).

However, TCR-T cells and CAR-T cells require handling the patient's cells ex vivo in a good-manufacturing production-compliant laboratory to graft them with the respective TCR using retro- or lentiviral vectors. This procedure is expensive, time-consuming, and bears certain risks. bsAbs that can be injected and engage and redirect the patient's T cells to HBV-infected cells in the liver would be an interesting alternative, easier to produce, and more convenient to apply. bsAb would thus provide a more broadly applicable therapeutic alternative. In addition, potential side effects may be easier to handle due to the limited half-life of a recombinant antibody compared to a dividing and long-term persisting cell product.

5. T-cell engager antibodies to target HBV

The first bsAb target HBV was described by Park *et al.* in 2000 as a tetravalent antibody that showed both anti-S and anti-pre-S2 binding activities (Park et al., 2000). In this study, the bsAb only acted as a neutralizing antibody without immune cell engagement. Kruse *et al.* showed that combining HBVenv-targeting antibodies with a CD3-binding domain improves their antiviral efficacy when the antibodies were expressed in the liver after hydrodynamic tail vein injection of plasmids encoding the constructs (Kruse et al., 2017). They demonstrated that these in vivo expressed bsAb can reduce HBV gene expression in a mouse model of acute HBV infection. However,the value of this study was limited because all mice, including the control-treated mice, cleared HBV. In addition, the delivery relied on hydrodynamic injection, which results in efficient co-delivery of plasmids to the same cells (Sebestyen et al., 2006) that cannot be applied in humans.

Fergusson *et al.* (Fergusson et al., 2020) chose an alternative approach to classical bsAb. They designed a series of immune mobilizing monoclonal T-cell receptors against virus (ImmTAV) molecules that combine an affinity-enhanced T-cell receptor with an anti-CD3 T-cell-activating moiety. They utilized TCRs recognizing the HLA-A*02:01-restricted epitopes from HBV envelope (Env), polymerase, and core antigens as targets, optimized their affinity using directed molecular evolution and phage display selection and generated ImmTAV molecules by fusing the affinity-enhanced HBV-specific TCRs to a scFv anti-CD3 via a flexible linker (Fergusson et al., 2020). They demonstrated that picomolar concentrations of ImmTAV-Env can redirect T cells from healthy and HBV-infected donors toward hepatocellular carcinoma cells containing integrated HBV DNA and induce a cytokine release and cytolysis of HBV-positive HCC cells and cells infected with HBV *in vitro.* These TCR-like bsAbs that recognize a processed peptide in the context of a given MHC-I molecule on the surface of HBV-positive cells may be a more sensitive trigger compared to conventional bsAbs, which recognize the native protein. However, their applicability is limited to a proportion of patients that have the proper HLA-type, in that case, HLA-A*0201. The dependence on a certain, human HLA-type also limits the *in vivo* testing of ImmTAVs, since this requires the use of a

double humanized mouse model reconstituted not only with human HLA-A*02:01 HBV-infected hepatocytes but also with HLA-matched T cells to avoid alloreactivity between human hepatocytes and T cells. Therefore, an S-peptide-directed ImmTAV directly entered a first-in-human, interventional clinical trial for hepatitis B and HBV-associated hepatocellular carcinoma in 2020 (NCT05867056) with results expected end of 2024.

Based on the promising data using S-CAR T cells to target and kill HBV-infected cells in preclinical models *in vivo* (Bohne et al., 2008), *Quitt, Luo et al.* developed immune-cell engager antibodies that target the native HBs antigen and are not restricted by a certain HLA haplotype. This was further supported by the finding that S-CAR T cells can control HBV and deplete HBV cccDNA from infected cells in a non-cytolytic fashion by activating the secretion of the antiviral cytokines interferon-gamma, tumor necrosis factor, and lymphotoxin (Koh et al., 2018; Xia et al., 2016).

First, they tested different TCE formats in terms of their expression level, stability and functionality. Two suitable TCE formats, designated as Bi-Mab (TetravalentMab) and Fab-Mab (scFv-Fab) (Figure 2), were selected (Quitt et al., 2021). BiMAb contained, and Fab-MAb contained the Fab-fragment of a murine monoclonal antibody as HBVenv binding domains. BiMAb represents a ~170 kD homodimer of two copies of the same, HBVenv-specific scFv as the S-CAR connected via a $(G_4S)_3$ linker to the N-termini of a modified IgG1 Fc domain. CD3-specific scFv generated from variable domains of OKT3 (Kung et al., 1979), or a CD28-specific scFv derived from antibody 9.3 (Baroja et al., 1989) was linked C-terminally. FabMAb is a ~78 kD monovalent binder composed of the human IgG-CH1 and scFv from antibody 5F9 (Golsaz-Shirazi et al., 2016) connected to the CD3- or CD28-specific scFvs by a glycine-serine $(G_4S)_3$ linker.

The TCEs were added to co-cultures of peripheral blood lymphocytes and HBV-infected target cells. Activation of CD4+ and CD8+ T-cells was demonstrated by the secretion of pro-inflammatory cytokines and activation of cytotoxic effector function. The two CD3- and CD28-binding TCEs synergistically activated T cells of healthy donors and of chronic hepatitis B

patients to become polyfunctional effectors upon binding of recombinant HBsAg or HBVenv on HBV-infected cells (Figure 3 B). The activated T cells showed a polyfunctional phenotype and elicited potent antiviral effects by specifically killing HBV-infected cells in a dose-dependent fashion (Quitt et al., 2021).

In addition, the T-cells activated by Bi-MAb and Fab-MAb could control HBV via non-cytolytic, cytokine-mediated antiviral mechanisms. A significant reduction of HBeAg, intracellular HBV-DNA and cccDNA in HBV-infected HepaRG cells without direct contact to activated T cells demonstrated a strong cytokine-mediated antiviral effect. The antiviral effect correlated directly with the levels of IFNγ, IL-2 and TNFα secreted by the activated cells (Quitt et al., 2021). Further experiments demonstrated that the T-cell engager antibodies license both CD4+ and CD8+ T cells to kill target cells in the presence of antigen. When using lymphocytes from CHB patients, the killing kinetics were comparable to that observed with lymphocytes of healthy donors (Quitt et al., 2021).

For *in vivo* analysis, BiMAb's were employed due to their more favorable pharmacodynamics with a longer serum half-life. To study in vivo efficacy, immune-deficient mice were transplanted with HBVenv-positive and -negative hepatoma cells on either flank and treated by intravenous injection BiMAb after injection of human lymphocytes (Quitt et al., 2021). *In vivo* in the mice, the antibodies attracted T cells specifically to the tumors expressing HBVenv, resulting in T-cell activation, tumor infiltration, and reduction of tumor burden.

Recently, the TCEs were analyzed in HBV-infected, liver-humanized mice by *Volmari et al*. (https://doi.org/10.1016/S0168-8278(23)03102-1). The immunodeficient mice received two injections of human PBMC 5 weeks after HBV infection and were treated with a combination of CD3- and CD28-targeting BiMabs. The treatment induced efficient recruitment of the human T cells to the liver and allowed for recognition of infected human hepatocytes as well as induction of cytolytic and cytokine-mediated HBV-specific T cell immunity. Notably, this effect was observed despite the presence of high levels of circulating HBsAg.

The high levels of soluble HBsAg in patients' blood certainly is a concern for clinical application as it may either capture and inactivate the TCEs or even activate T cells in the blood circulation and contribute to a cytokine release syndrome. In the *in vivo* models, however, high levels of HBsAg in the circulation did neither prevent targeting of HBVenv on the hepatocyte membrane nor over-activated T cells nor induced a systemic cytokine release syndrome.

Taken together, these studies demonstrated that the co-administration of HBVenv-targeting T-cell engager antibodies activating CD3 and CD28 facilitated a robust redirection of T-cell toward HBV-positive target cells providing an interesting approach for the treatment of chronic viral hepatitis and HBV-associated HCC.

In a follow-up study, *Debelec-Butener, Quitt, et al.* generated a tri-specific TCE binding the S domain of HBVenv proteins and activating CD3 and CD28 using a single molecule (Debelec-Butuner et al., 2022). The publication compares the activation of distinct antiviral T-cell immunity employing bi- and trispecific T-cell engager antibodies, as well as the S-CAR targeting HBV envelope proteins using the same binder. The study evaluated the effectiveness of these approaches in inducing antiviral T-cell responses. The findings contribute to understanding the potential of different T-cell-engaging strategies for combating HBV infection and enhancing antiviral immune responses.

Cytotoxic and non-cytolytic antiviral activities of these bi- and trispecific T-cell engager antibodies were assessed in co-cultures of human PBMC with HBV-positive hepatoma cells, and compared to that of S-CAR-grafted T cells (Debelec-Butuner et al., 2022). Activation of T cells *via* the S-CAR or a combination of the CD3- and CD28-targeting bispecific antibodies or the trispecific antibody allowed for specific elimination of HBV-positive target cells. While S-CAR-grafted effector T cells displayed faster killing kinetics, combinatory treatment with the bispecific antibodies or single treatment with the trispecific antibody was associated with a more pronounced cytokine release. Clearance of viral antigens and elimination of the HBV persistence form, the cccDNA, through cytolytic as well as cytokine-mediated activity was

demonstrated in all three settings with the combination of bispecific antibodies showing the strongest non-cytolytic, cytokine-mediated antiviral effect (Debelec-Butuner et al., 2022).

Taken together, these studies demonstrate that bi- and trispecific TCE can serve as a potent, off-the-shelf alternative therapy to cure HBV that is broadly applicable because no restriction by HLA-type of HBV genotype applies. Whether the binding of circulating HBsAg or the limited amount of HBenv proteins displayed on the surface of infected hepatocytes will limit clinical application, however, needs to be determined in clinical trials.

6. Concluding remarks

Therapeutic bsAbs, particularly those designed to engage immune cells, represent a rapidly growing category of diverse molecules. The current emphasis is notably on cancer applications, but bsAbs also hold promise for non-cancer applications. BsAbs exhibit various formats that impact manufacturing, valency, Fc-mediated effector functions, and *in vivo* half-life. The selection of the appropriate bsAb format is significantly influenced by the desired target product profile and clinical indication. Beyond the realm of cancer, there is also a burgeoning interest in applying bsAbs or TCEs to autoimmune and infectious diseases. Numerous studies propose that TCEs hold promise as a strategy for eliminating HBV. Administering TCEs targeting HBVenv effectively enables the redirection of T cells toward HBV-positive target cells, presenting a viable and promising approach for treating chronic hepatitis B and HBV-associated hepatocellular carcinoma. We believe that the progress in the platforms and ideas outlined in this review will influence not only cancer therapy but also the therapy of infectious diseases, particularly CHB.

Funding

This work was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) SFB-TRR 179/2 2020–272983813 and SFB-TRR 338/1 2021–452881907.

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Tables

Table 1. A summary of FDA-approved bispecific antibodies.

Trade Name	Active Ingredient	Target	Platform	Year Approved	Indication	Reference
Blincyto	Blinatumomab	CD3×CD19	BiTE	2014	To treat Philadelphia chromosome-negative relapsed or refractory B cell precursor acute lymphoblastic leukemia	(Sanford, 2015)
Hemlibra	Emicizumab-kxwh	FIX×FX	Duobody	2017	To prevent or reduce the frequency of bleeding episodes in hemophilia A with factor VIII inhibitors	(Scott and Kim, 2018)
Rybrevant	Amivantamab-vmjw	EGFR×MET	Duobody	2021	To treat locally advanced or metastatic non-small cell lung cancer with certain mutations	(Syed, 2021)
Kimmtrak	Tebentafusp-tebn	CD3×IMCgp100	ImmTAC	2022	To treat a form of unresectable or metastatic uveal melanoma	(Dhillon, 2022)
Vabysmo	Faricimab-svoa	VEGFA×Ang-2	CrossMab	2022	To treat neovascular (wet) age-related macular degenerated and diabetic macular edema	(Shirley, 2022)
Tecvayli	Teclistamab-cqyv	CD3×BCMA	Duobody	2022	To treat relapsed or refractory multiple myeloma	(Kang, 2022b)
Lunsumio	Mosunetuzumab-axgb	CD3×CD20	Knobs-into-holes	2022	To treat relapsed or refractory follicular lymphoma	(Kang, 2022a)
Epkinly	Epcoritamab-bysp	CD3×CD20	Duobody	2023	To treat relapsed or refractory diffuse large B-cell lymphoma	(Frampton , 2023)
Columvi	Glofitamab-gxbm	CD3×CD20	Knobs-into-holes CrossMab	2023	To treat relapsed or refractory diffuse large B-cell lymphoma or large B-cell lymphoma	(Shirley, 2023)

FIX, factor IX; FX, factor X; EGFR, epidermal growth factor receptor; MET, mesenchymal–epithelial transition; BCMA, B-cell maturation antigen; ANG2, angiopoietin 2; VEGFA, vascular endothelial growth factor A; BCMA: B-cell mutation antigen.

Figures

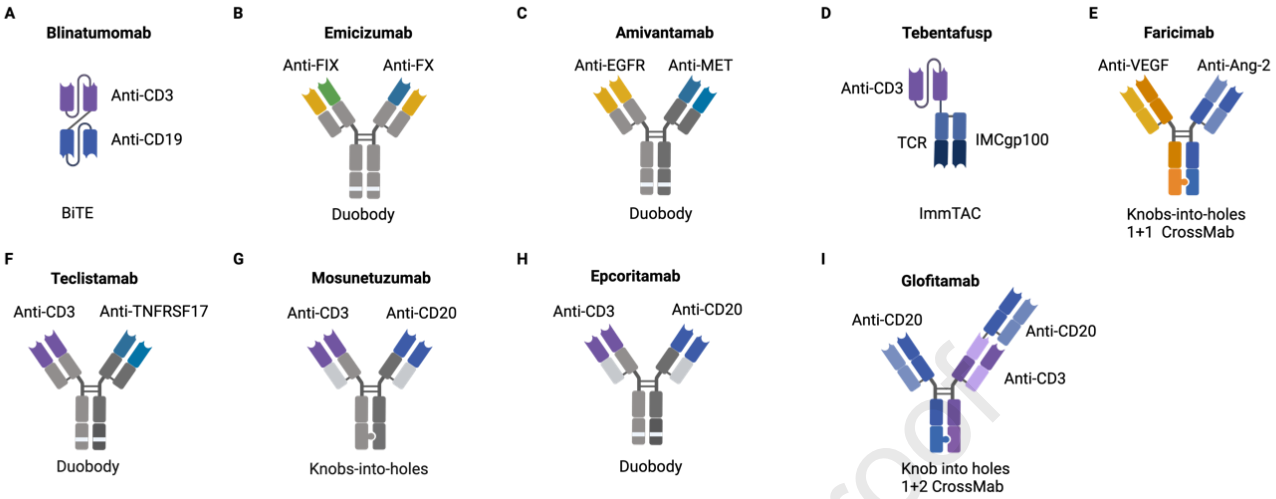

Figure 1: FDA-approved bispecific antibodies.

The format of nine FDA-approved bispecific antibodies is shown. Binding sites and applied technology platforms are indicated in the graph. For a detailed explanation of the different bsAb formats, see Figure 2.

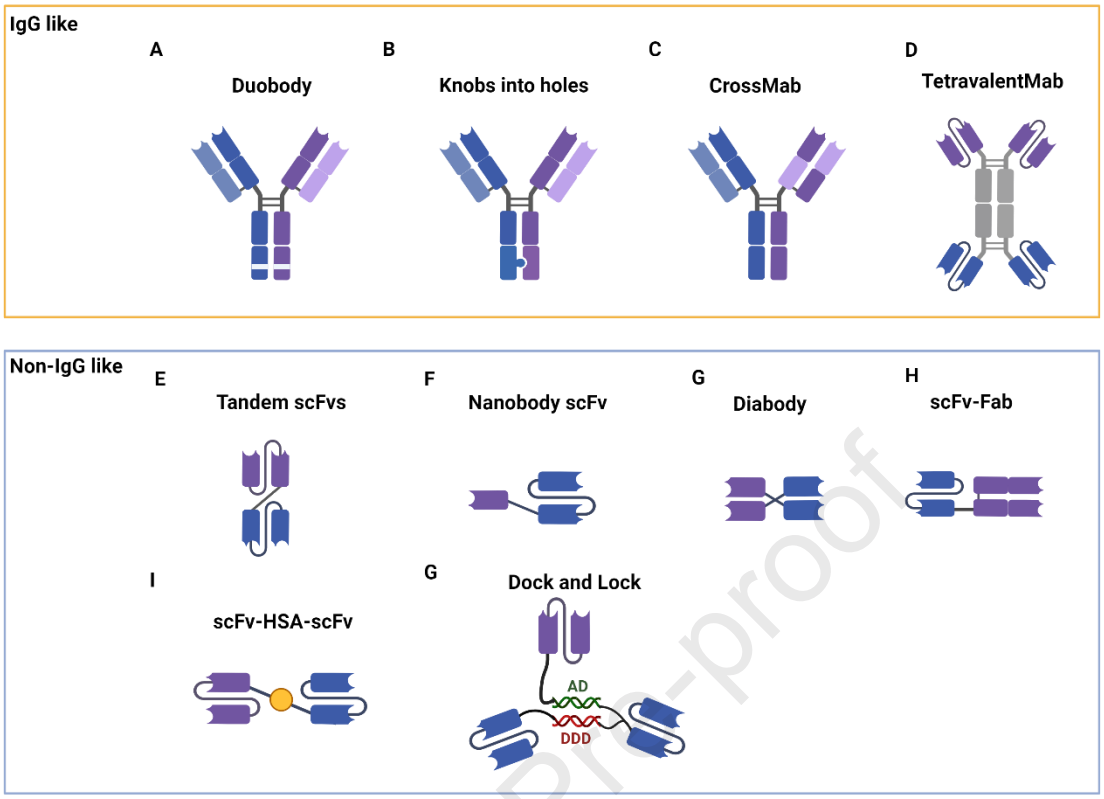

Figure 2: A selection of some common bispecific antibodies.

IgG-like (A-D) and non-IgG-like (E-J) bispecific antibody formats are depicted. (A) Duobody: its Fc region was suppressed by inserting mutations, which circumvents the Fc-mediated cytotoxicity. (B) Fc-modified IgG format: built with the knob into holes (KIH) technology to heterodimerize two different heavy chains. (C) CrossMab: antibody domain crossover enables the proper connection of generic light chains. (D) TetravalentMab. (E) Tandem scFv (taFv): the minimum bsAb. (F) Nanobody. (G) Diabody (DB): a short protein linker joins the heavy chain variable (VH) and light chain variable (VL) domains of an scFv segment to form a noncovalent heterodimer. (H) ScFv-Fab. (I) ScFv-HSA-scFv strategy: two different scFv molecules are fused by human serum albumin (HSA) to increase stability. (J) Dock and lock (DNL)strategy: the basic strategy of DNL involves generating two types of modules, one containing the dimerization and docking domain (DDD) of cAMP-dependent protein kinase A and the other containing the anchoring domain (AD) of an interactive A-kinase anchoring protein.

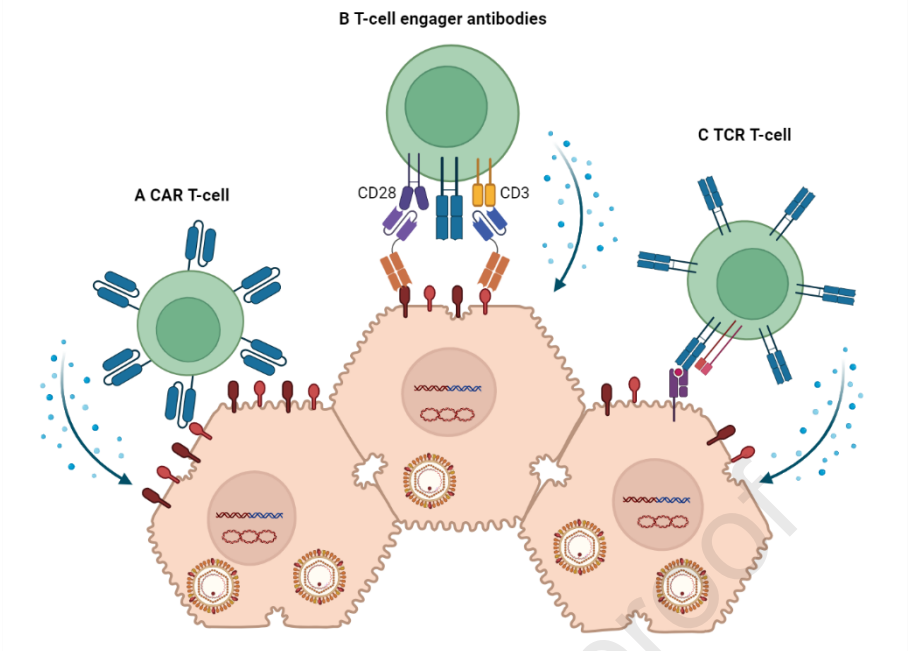

Figure 3: Target recognition in chronic HBV infection. HBV-infected cells contain the so-called cccDNA, the persistence form of HBV DNA in the nucleus. Over time, HBV DNA frequently integrates into the genome. Episomal cccDNA, as well as integrated HBV-DNA, express HBV envelop proteins and secrete the hepatitis B surface antigen (HBsAg). HBV-specific chimeric antigen receptors (CAR) (A), T-cell engager antibodies (B) or T-cell receptors (TCR) (C) can be used to redirect T cells toward HBV-positive cells. The redirected T cells are activated through target recognition on the cell surface (A,B) or by HBV-peptide recognition on MHC molecules (C), respectively. TCEs (B) bind envelope proteins on the surface of the HBV-positive cell and CD3 or CD28 on T cells. They can induce a potent antiviral and cytotoxic T-cell response that leads to cytokine secretion and the elimination of HBV-positive cells.

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: