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Camelids Nanobodies: Novel Prophylactic and Therapeutic Medication Against Epstein-Barr Virus Infection by Targeting Glycoprotein B

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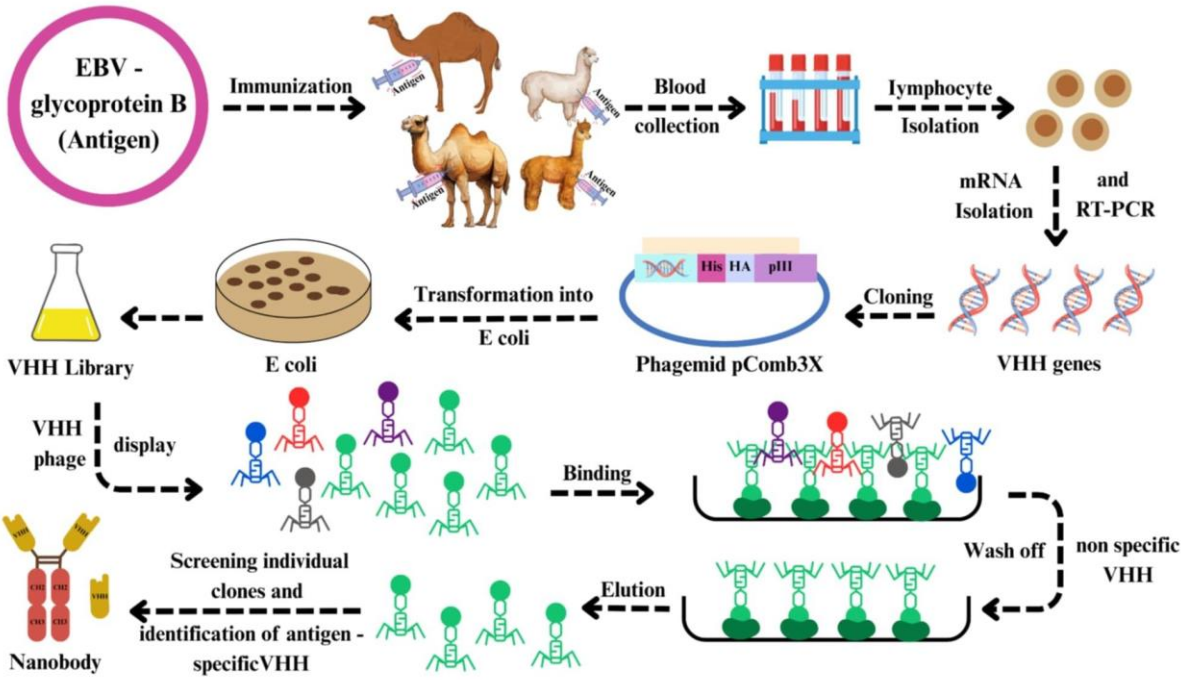

ABSTRACT

The Epstein-Barr virus (EBV) represents a widespread pathogen that infects nearly 98% of adults worldwide, creating persistent latent infections throughout the host's lifetime while contributing to various pathological conditions, encompassing malignancies and autoimmune diseases. EBV's presence within peripheral blood lymphocytes enables transmission through blood transfusion procedures and organ transplantation, creating substantial hazards, especially for immunocompromised patients including transplant recipients who face elevated vulnerability to EBV-associated lymphoproliferative conditions. Presently, neither efficacious vaccination programs nor specific antiviral treatments for EBV are available, highlighting the critical requirement for innovative preventive and treatment approaches. Glycoprotein B (gB) serves as a vital viral fusion protein, performing essential functions in EBV cellular entry, intercellular transmission, viral particle development, and nuclear exodus. Significantly, EBV particles containing elevated gB levels demonstrate superior infectious capacity, with gB establishing direct binding interactions with neuropilin-1 (NRP1) throughout epithelial cell and B lymphocyte invasion processes. Earlier studies demonstrate that EBV-gB successfully stimulates neutralizing antibody responses, establishing its potential as an optimal therapeutic target. This analysis suggests creating camelid-derived single-domain antibody components (VHHs), termed nanobodies (Nbs), directed against EBV-gB. These Nbs, measuring approximately 15 kDa, demonstrate substantially reduced size compared to traditional antibodies, being devoid of light chains and CH1 domains. Their distinctive characteristics encompass simplified production processes, exceptional stability, superior solubility, improved tissue and tumor infiltration capabilities, plus blood-brain barrier crossing potential. Importantly, Nbs demonstrate minimal immunogenic properties, reducing unwanted reactions. Large-volume Nb manufacturing could deliver easily administered prophylaxis for high-risk populations or therapeutic intervention for active infections. Camelid immunization using EBV-gB would simultaneously enable production of powerful neutralizing Nbs while permitting assessment of gB's vaccine potential. The extraordinary properties of Nbs constitute remarkable progress in therapeutic innovation, and we recommend focused initiatives to transform this promise into effective EBV treatments.

Keywords: Epstein-Barr Virus (EBV), Glycoprotein B (gB), Camelids; Nanobodies, VHH, Antiviral Therapy, Prophylaxis.

INTRODUCTION

Human herpesvirus 4, commonly referred to as the Epstein-Barr virus (EBV), is classified within the Gammaherpesvirinae subfamily of the Herpesviridae family (Bastawecy et al., 2023). This pathogen demonstrates global distribution, with serological evidence indicating that approximately 98% of the adult population worldwide has experienced infection (Smatti et al., 2017; Smatti et al., 2018). Primary infection typically occurs during early childhood and remains asymptomatic, whereas adolescent infection frequently results in infectious mononucleosis (IM) (Papesch & Watkins, 2001; Reimer et al., 2009). Following successful immune control of the acute phase, EBV establishes persistent latency throughout the host's lifetime, predominantly within B lymphocytes and potentially other sites including parotid gland tissue, thus establishing chronic carriage (Reimer et al., 2009; Wolf et al., 1984).

EBV represents a significant human pathogen of considerable importance. This virus holds the distinction of being the inaugural human virus recognized for its oncogenic properties and receives Group 1 carcinogen classification from the World Health Organization (International Agency for Research on Cancer [IARC], 1997; Wong et al., 2022). Beyond its carcinogenic capabilities, EBV demonstrates association with numerous clinical manifestations, encompassing various autoimmune pathologies (Bastawecy et al., 2023; Hong et al., 2022). Following primary infection, complete viral clearance by host immune mechanisms never occurs, resulting in lifelong persistence (Babcock et al., 1998). Despite its ubiquitous nature and substantial health implications, neither approved vaccination strategies nor targeted antiviral therapies currently exist for EBV management (Sun et al., 2021; Zhao et al., 2024). This therapeutic void presents particular concern for immunocompromised populations, including organ transplant patients, who demonstrate elevated susceptibility to EBV-associated post-transplant lymphoproliferative disorders (PTLD) (Burns & Chaganti, 2021). The viral presence within peripheral blood lymphocytes facilitates transmission via blood transfusion and organ transplantation, emphasizing the critical requirement for effective interventional strategies against this pathogen (Bastawecy et al., 2023; Indari et al., 2024).

Conventional antibodies constitute large protein structures, weighing approximately 150 kDa for IgG molecules, comprising two identical heavy chains paired with two identical light chains (Wahner-Roedler & Kyle, 2005). Heavy chains incorporate three constant regions (CH1, CH2, CH3) plus one variable region (VH), whereas light chains possess one constant region (CL) and one variable region (VL). Heavy chain diseases represent an exception, characterized as uncommon B-cell lymphoproliferative conditions featuring truncated monoclonal immunoglobulin heavy chain production without corresponding light chain association (Wahner-Roedler & Kyle, 2005). In 1989, Hamers-Casterman and research colleagues achieved a revolutionary discovery of novel antibody types in dromedary camels infected with Trypanosoma evansi, which inherently lacked light chain components (Hamers-Casterman et al., 1993). These heavy-chain exclusive antibodies (HcAbs), characteristic of Camelidae species, additionally lack the CH1 region typically required for light chain association. Consequently, HcAbs consist of paired heavy chains, each containing a single variable antigen-recognition domain termed VHH. Despite structural simplification, these HcAbs demonstrate robust binding capacity across diverse antigen targets (Hamers-Casterman et al., 1993). HcAbs, specifically their isolated VHH regions (nanobodies, Nbs), have subsequently garnered substantial attention for diverse diagnostic and therapeutic applications (Jin et al., 2023). Comparable antibody diversification mechanisms exist in additional species; sharks, for instance, generate IgNAR (immunoglobulin new antigen receptor), whose variable regions (VNAR) exhibit similar compact size and stability properties to Nbs (Chen et al., 2023).

The VHH domains can be readily cloned and expressed recombinantly, often in microbial systems like *Escherichia coli*. These ~15 kDa Nbs are typically highly stable, soluble, and retain specific, high-affinity binding to their target antigens (Arbabi-Ghahroudi et al., 1997). Notably, VHHs targeting enzymes often act as potent inhibitors, potentially by accessing active sites or cryptic epitopes less accessible to conventional antibodies (Lauwereys et al., 1998). This unique characteristic, along with their cost-effective production and amenability to engineering (e.g., immunofusions, multimerization), positions Nbs as promising candidates for a wide range of biological applications, including affinity reagents and therapeutics (Muyldermans et al., 2001).

Studies have also shown that camelid HcAbs possess broad antigenic specificities, for instance, against erythrocytes from various species (Sehrawat & Singh, 2006). Furthermore, HcAbs can constitute up to 75% of total serum IgG in camels, and exhibit high neutralizing antibody titers, with affinities comparable to conventional antibodies despite their smaller size (Bastawecy et al., 2014; Rahbarizadeh et al., 2005).

This review aims to: 1) provide an overview of the structure and inherent advantages of Nbs derived from HcAbs; 2) discuss the principles for generating EBV gB-specific neutralizing Nbs in camelids; and 3) highlight the potential of these Nbs as prophylactic and therapeutic agents against EBV infection and associated diseases, focusing on EBV glycoprotein B (gB) as a rational target for Nb-based therapeutics and vaccine design.

VIROLOGICAL ASPECTS OF EPSTEIN-BARR VIRUS

EBV Structure, Transmission, and Pathogenesis

The EBV virion consists of a linear double-stranded DNA genome enclosed within an icosahedral capsid. This capsid is surrounded by a proteinaceous layer known as the tegument, which is, in turn, enveloped by a lipid bilayer embedded with various viral glycoproteins essential for host cell interaction and entry (Figure 1) (Jean-Pierre et al., 2021).

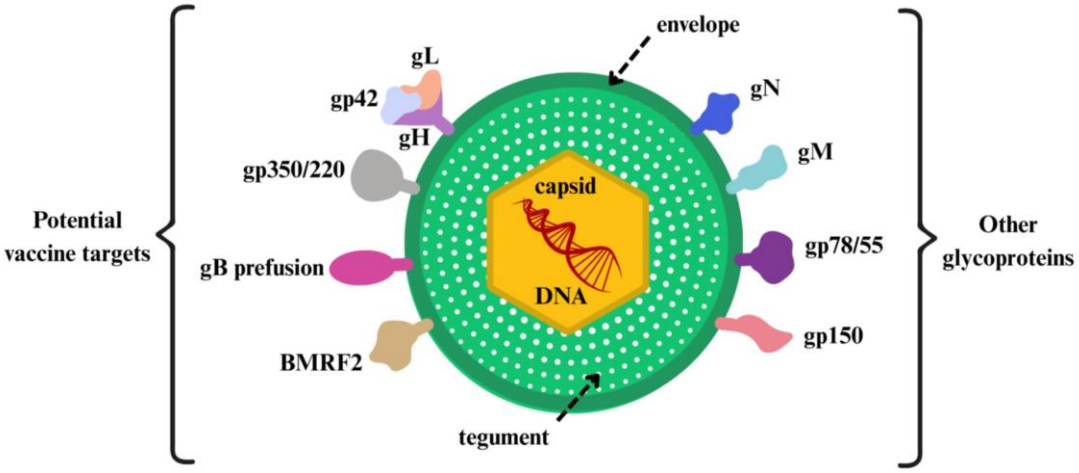

Figure 1: Schematic of an Epstein-Barr virus (EBV) particle, illustrating the core double-stranded DNA, icosahedral capsid, tegument layer, and outer envelope containing key glycoproteins, including gB.

Primary EBV transmission predominantly occurs via oropharyngeal secretions through close personal contact. Upon exposure, the virus infects epithelial cells of the oropharynx and subsequently B lymphocytes, initiating a lytic infection phase (Cohen, 2000; Indari et al., 2024). Alternative transmission routes include transplacental transfer, sexual intercourse (EBV is detectable in semen and cervical secretions), and fecal-oral routes (EBV detected in stool samples) (Bastawecy et al., 2023). Iatrogenic transmission through organ transplantation and blood transfusion also contributes to viral spread (Rickinson & Kieff, 2007).

EBV's capacity to infect diverse organ systems underpins its association with a broad spectrum of diseases and inflammatory conditions (Figure 2) (Bastawecy et al., 2023). These include, but

are not limited to: infectious mononucleosis (IM); various autoimmune diseases (e.g., systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, type 1 diabetes, inflammatory bowel disease); vasculitis; organ-specific inflammation (e.g., hepatitis, pancreatitis, myocarditis, encephalitis); and malignancies such as Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma, and gastric cancer. Other manifestations include hairy leukoplakia, post-transplant lymphoproliferative disease (PTLD), chronic active EBV infection (CAEBV), hemophagocytic lymphohistiocytosis, and potentially contributions to chronic fatigue syndrome and Alzheimer's disease (Bastawecy et al., 2023).

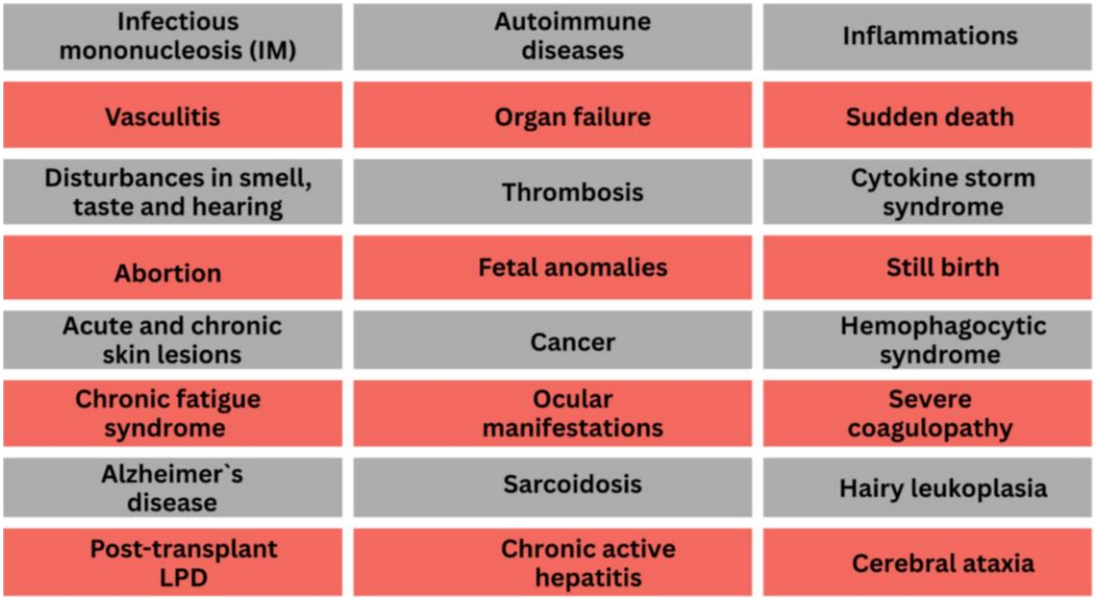

Figure 2: Overview of EBV-associated pathologies, categorized into infectious (e.g., IM), oncogenic (e.g., lymphomas, carcinomas), autoimmune, and other inflammatory conditions

EBV Life Cycle: Lytic and Latent Phases

The EBV lifecycle demonstrates alternation between two separate phases: a dormant stage and a productive (or active replication) stage (Indari et al., 2024). Throughout the dormant period, the pathogen maintains relative inactivity, producing a restricted set of proteins (including EBNAs, LMPs) that primarily function to preserve the viral genetic material as an autonomous circular DNA element within the host cell's nuclear compartment while facilitating host cell viability and growth. This limited protein production reduces immune system detection, enabling persistent viral maintenance throughout the host's lifetime (Münz, 2019; Murata & Tsurumi, 2014). The autonomous viral DNA duplicates concurrently with host cellular division processes and distributes to offspring cells throughout cell division (Murata & Tsurumi, 2014). The lytic phase encompasses the systematic activation of approximately 100 viral genes, resulting in new viral particle generation and consequent host cell destruction (Münz, 2019). Active replication contributes to disease development through immediate cell-damaging mechanisms and by stimulating the release of viral or cellular cytokines and growth mediators that drive inflammatory responses and blood vessel formation (Ye et al., 2021; Yokoe et al., 2022). Transition from the dormant state to the active replication cycle can be initiated by diverse triggers, encompassing cellular damage, immune system suppression, and simultaneous infection with additional pathogens (Indari et al., 2024).

Glycoprotein B (gB) as a Therapeutic Target

Within the spectrum of EBV glycoproteins, glycoprotein B (gB), produced by the BALF4 open reading frame, holds critical importance (Hong et al., 2022). This protein functions as a type I transmembrane component and class III viral fusion mediator, proving indispensable for merging viral envelopes with host cellular membranes throughout invasion of epithelial cells and B lymphocytes (Figure 3) (Hong et al., 2022; Reimer et al., 2009). Throughout active replication, gB primarily accumulates at the perinuclear membrane and endoplasmic reticulum locations, with reduced quantities appearing at the cellular surface (Reimer et al., 2009). The gB genetic sequence ranks among the most highly preserved across herpesvirus species, with its sequence frequently utilized for evolutionary analysis within the *Herpesviridae* classification (Bastawecy et al., 2023). Apart from cellular invasion, gB proves crucial for viral particle development, nuclear departure, and intercellular transmission (Bastawecy et al., 2023; Hong et al., 2022). Therefore, EBV variants missing functional gB genetic material cannot generate infectious particles, while viral particles containing elevated gB concentrations demonstrate superior infectious potential (Hong et al., 2022; Neuhierl et al., 2002; Reimer et al., 2009).

EBV-gB comprises an 857-amino acid sequence, incorporating a removable N-terminal signal peptide and nine possible N-linked glycosylation locations within its extracellular region (Pellett et al., 1985). Mutagenesis experiments have demonstrated the significance of its C-terminal intracellular tail region (CTD) for appropriate subcellular positioning, protein expression, and membrane fusion function (Garcia et al., 2013).

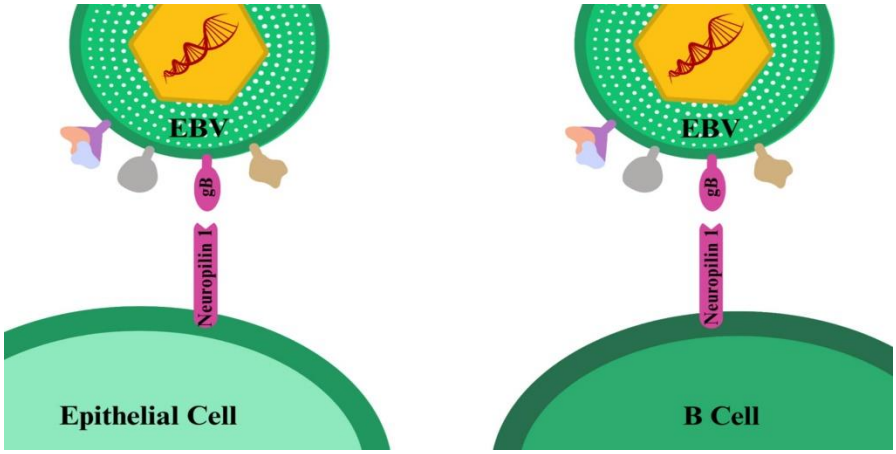

Figure 3: Schematic depicting the role of EBV glycoprotein B (gB) in viral entry and infection of host cells (epithelial cells and B cells) by attaching to their neuropilin 1 receptors.

Current findings reveal that EBV-gB establishes direct binding with neuropilin-1 (NRP1) serving as an entry receptor on nasopharyngeal epithelial tissues and B lymphocytes (Escalante et al., 2022; Wang et al., 2015).

Significantly, gB represents a primary target for host antibody-mediated immune responses, with gB-specific antibodies capable of inhibiting EBV infection through in vitro neutralization (Hong et al., 2022; Zhu et al., 2020). Vaccination strategies utilizing gB from alternative herpesvirus species have shown protective effectiveness in both laboratory and human clinical trials (Plotkin et al., 2020; Scarpini et al., 2021; Schleiss et al., 2017). Regarding EBV, vaccination

using trimeric gB configurations has proven capable of generating elevated neutralizing antibody levels and delivering passive immunological defense against fatal EBV exposure in humanized mouse systems, achieving protective effectiveness equal to or exceeding that of gH/gL protein complexes (Bu et al., 2019; Cui et al., 2021; Escalante et al., 2020; Hong et al., 2022). These features—including its fundamental necessity, evolutionary preservation, immune-stimulating properties, and vital function across various viral lifecycle stages—establish EBV-gB as an exceptionally promising candidate for developing neutralizing Nbs.

CAMELID HEAVY-CHAIN ANTIBODIES AND NANOBODIES

Unique Structural Features of HcAbs and Nbs

The Camelidae classification includes Old World camels (dromedaries – *Camelus dromedarius*; Bactrian camels – *Camelus bactrianus*) and New World camelids (llamas – *Lama glama*; alpacas – *Vicugna pacos*; vicuñas – *Vicugna vicugna*; guanacos – *Lama guanicoe*) (Han, 2010). Camelid serum IgG demonstrates distinctive organization into three primary isotypes: IgG1, representing traditional antibodies containing paired heavy and light chains; plus two HcAb variants, IgG2 (extended hinge region) and IgG3 (compact hinge region), which lack light chains and the CH1 heavy chain domain (Arbabi-Ghahroudi, 2017). These HcAbs may represent a substantial fraction, reaching 75%, of total circulating IgG (Arbabi-Ghahroudi, 2017). As noted earlier, HcAbs comprise an Fc segment analogous to conventional antibodies, connected directly to an antigen-recognition region containing a single VHH domain (Figure 4) (Jin et al., 2023). The elimination of CH1 domains and light chains produces a decreased molecular weight of roughly 90 kDa for HcAbs versus ~150 kDa for traditional IgGs (Muyldermans, 2013). The individual VHH domain, possessing a molecular mass of merely ~15 kDa and approximate dimensions of 2.5 nm x 4.0 nm, is designated a nanobody (Nb) (Jin et al., 2023). Nbs can be easily isolated and recombinantly produced as single-unit proteins that maintain complete antigen-binding functionality of the original HcAb (Jin et al., 2023).

Both Nbs and VH domains from traditional antibodies possess a shared structural foundation, containing four preserved framework regions (FRs) and three variable complementarity-determining regions (CDRs) – CDR1, CDR2, and CDR3 – which constitute the paratope (antigen-recognition site) (Jin et al., 2023). Nevertheless, Nbs exhibit multiple distinct structural characteristics that provide unique attributes (Figure 4) (Jin et al., 2023):

Hydrophilic FR2:

Unlike conventional VH domains, which have hydrophobic amino acid residues in FR2 (e.g., V42, G49, L50, W52 in human VH) that interact with the VL domain, Nbs typically feature hydrophilic substitutions at these positions (e.g., F/Y42, E/Q49, R/C50, G/L/C52) (Muyldermans, 2013; Muyldermans et al., 2001). These hydrophilic residues enhance the solubility and stability of Nbs as single domains, preventing aggregation.

Extended CDR3 Loop:

Nbs often possess a significantly longer CDR3 loop compared to conventional VH domains. This extended CDR3 can fold over the former VL-binding site, contributing to paratope diversity and often forming unique convex or protruding structures that can access epitopes (e.g., enzyme active sites, receptor clefts) inaccessible to the typically flatter or concave paratopes of conventional antibodies (De Genst et al., 2006; Vu et al., 1997).

Modified CDR1:
The CDR1 loop in Nbs can also be extended, further increasing the paratope surface area and contributing to antigen recognition diversity, partially compensating for the absence of the VL domain (Mitchell & Colwell, 2018a; Mitchell & Colwell, 2018b).

Inter-loop Disulfide Bonds:
An additional disulfide bond frequently connects the CDR1 or CDR2 with the CDR3 loop, particularly in dromedary Nbs, which can stabilize the CDR3 conformation (Muyldermans et al., 2001).

Amino Acid Substitutions:
A common substitution in FR1, L11S, is more prevalent in dromedary VHH sequences compared to llama VHHs (Vu et al., 1997). While CDR3 is the primary contributor to Nb antigen interaction, CDR1 and CDR2 also play roles, though perhaps less dominant than in conventional antibodies where all six CDRs (three from VH, three from VL) contribute more equally (De Genst et al., 2006; Wrapp et al., 2020).

Sequence and structural examinations demonstrate that the varied antigen-recognition capacity of Nbs originates not mainly from enhanced sequence variability in CDR1 and CDR2, but instead from conformational differences in CDR1 and the extended length plus sequence heterogeneity of CDR3 (Mitchell & Colwell, 2018b; Muyldermans, 2021a).

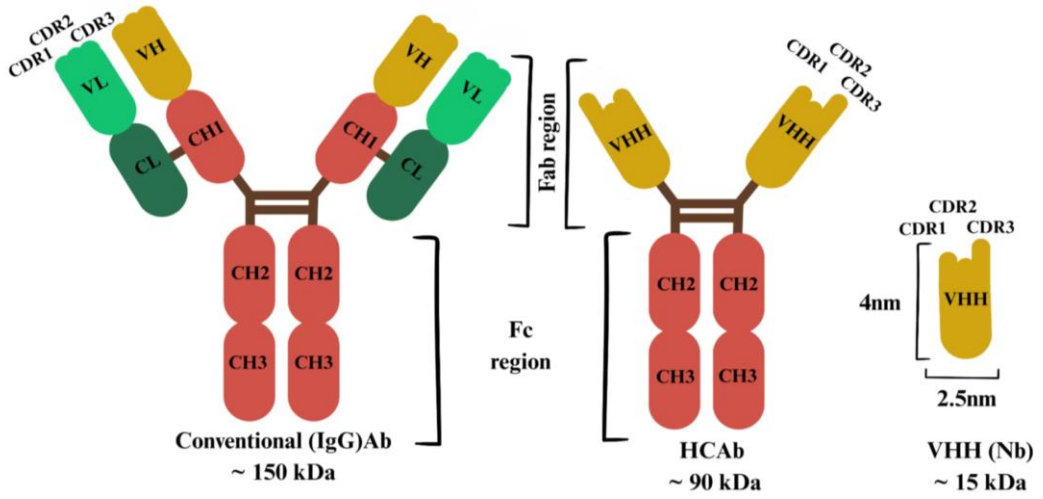

Figure 4: Diagrammatic representation of conventional IgG, camelid HcAb and the derived VHH.

Advantages of Nanobodies over Conventional Monoclonal Antibodies
The unique structural attributes of Nbs translate into several advantages over conventional mAbs and their fragments (e.g., Fab, scFv) (Table 1) (Jin et al., 2023; Jovčevska & Muyldermans, 2020):

Epitope Recognition:
Their small size and often convex paratope, particularly due to the extended CDR3, enable Nbs to bind unique epitopes, including cryptic sites or clefts on antigens, that are inaccessible to larger conventional mAbs (Jovčevska & Muyldermans, 2020; Khodabakhsh et al., 2018).

High Affinity and Specificity:
Despite their smaller paratope (three CDRs vs. six in conventional antibodies), Nbs can exhibit high binding affinities, often in the nanomolar to picomolar range, comparable to those of conventional mAbs (Muyldermans, 2013; Sockolosky et al., 2016).

Enhanced Tissue Penetration:
The small size of Nbs facilitates improved penetration into tissues and tumors, which is often a limitation for larger antibody formats. This is advantageous for diagnostic imaging and targeted drug delivery (Richards, 2018; Yang & Shah, 2020).

Blood-Brain Barrier (BBB) Permeability:
Nbs have demonstrated an ability to cross the BBB, either naturally to some extent or via engineered mechanisms, opening avenues for treating central nervous system (CNS) pathologies, including EBV-associated CNS diseases (Bastawecy et al., 2023; Ruiz-de-Angulo et al., 2021).

Low Immunogenicity:
Nbs generally exhibit low immunogenicity in humans, partly due to their high sequence homology to human VH domains. The risk of adverse immune reactions can be further mitigated through humanization techniques (mutating camelid-specific residues to human counterparts) or by selecting Nbs from pre-humanized synthetic libraries (Jin et al., 2023; Vincke et al., 2009).

Biophysical Stability:
Nbs are renowned for their robust stability under harsh conditions, including high temperatures (often refolding correctly after denaturation) and extreme pH values. They are also resistant to proteases (Jin et al., 2023).

Solubility and Resistance to Aggregation:
The hydrophilic FR2 residues contribute to high solubility and prevent aggregation, allowing for high-concentration formulations (Jin et al., 2023).

Ease of Production and Engineering:
Nbs are single-chain proteins that can be efficiently produced in microbial systems (e.g., *E. coli*, yeast), which is generally more cost-effective and scalable than mammalian cell culture required for full-length mAbs. Their simple structure also facilitates genetic engineering, such as multimerization (to increase avidity or create bispecific/multispecific constructs) or fusion to other moieties (e.g., toxins, enzymes, fluorescent proteins, Fc domains for effector functions or half-life extension) (Bobkov et al., 2018; de Beer & Giepmans, 2020; Jin et al., 2023).

Simplified Purification:
Nbs undergo straightforward purification using conventional chromatographic methods, frequently utilizing a C-terminal hexahistidine-tag for immobilized metal affinity chromatography (IMAC) or exploiting their structural similarity to human VH domains for Protein A affinity chromatography (Crauwels et al., 2020; Muyldermans et al., 2001).

Table 1: Key Advantages of Nanobodies Compared to Conventional Monoclonal Antibody Formats (Adapted from Jin et al., 2023).

Feature	Nanobodies (Nbs)	Conventional mAbs (e.g., IgG)	scFv/Fab Fragments
Size	~15 kDa; smallest functional antigen-binding unit.	~150 kDa; large heterotetrameric structure.	Fab: ~50 kDa; scFv: ~25-30 kDa. Intermediate size.
Production	Efficient, high-yield production in microbial systems (e.g., *E. coli*, yeast); cost-effective.	Requires complex mammalian cell culture systems; generally expensive.	Can be produced in microbial systems; yield/stability can be variable.
Stability	Robust thermostability, pH resistance, protease resistance; often refold correctly after denaturation.	Generally, less stable to harsh conditions; prone to aggregation.	Stability is variable; often less stable than full mAbs or Nbs without engineering.
Epitope Access	Unique ability to access cryptic, concave, or hidden epitopes due to small size and extended CDR3.	Typically bind to more accessible, often flatter or convex, surface epitopes.	Improved access compared to mAbs but may not reach all epitopes accessible to Nbs.
Immunogenicity	Generally low immunogenicity; high homology to human VH; amenable to humanization with minimal loss of function.	Can be immunogenic (especially non-human); humanization/fully human formats reduce but may not eliminate immunogenicity.	Can be immunogenic; humanization is often required for therapeutic use.
Tissue Penetration	Enhanced penetration into tissues, tumors, and across biological barriers (e.g., BBB to some extent).	Limited tissue and tumor penetration due to large size.	Better penetration than mAbs, but generally less than Nbs.
Engineering	Simple to engineer; ideal for multimerization (bispecific/multispecific constructs), fusions (e.g., toxins, Fc).	More complex to engineer into novel formats.	Amenable to engineering (e.g., bispecifics, fusions), similar flexibility to Nbs.
Cost	Comparatively economical production, especially in microbial systems.	High production costs.	Moderate production costs; generally lower than mAbs but potentially higher than Nbs depending on scale and purity.
Solubility	High solubility due to hydrophilic FR2; resistant to aggregation.	Solubility can be a concern, may require formulation optimization.	Solubility can vary; sometimes prone to aggregation if not optimized.
Half-life (native)	Short (hours) due to renal clearance; requires	Long (days to weeks) due to FcRn recycling.	Short (hours); also requires

	engineering for extension (e.g., Fc fusion, PEGylation).		engineering for half-life extension.

Disadvantages and Mitigation Strategies for Nanobodies

Despite their numerous advantages, Nbs also present certain limitations:

Monovalency:

Native Nbs are monovalent, which may result in lower avidity compared to bivalent conventional antibodies. This can be addressed by engineering multivalent Nb constructs (e.g., bivalent or trivalent homodimers/trimers) (Jin et al., 2023).

Short Serum Half-Life:

Their small size (<60-70 kDa renal filtration threshold) leads to rapid renal clearance and a short in vivo half-life (typically a few hours), which can be disadvantageous for therapeutic applications requiring sustained exposure (Zavrtanik et al., 2018). Strategies to extend half-life include fusion to albumin, albumin-binding Nbs, PEGylation, or fusion to an Fc domain (Hu et al., 2022; Jin et al., 2023; Salvador et al., 2019).

Lack of Effector Functions:

Nbs lack an Fc region and therefore do not naturally mediate effector functions like antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). This can be overcome by fusing Nbs to an Fc domain (Bobkov et al., 2018).

Generation Process:

The conventional method of Nb generation involves immunizing camelids, which requires specialized animal facilities and ethical considerations. While transgenic mice producing HcAbs or fully synthetic/naïve libraries can bypass this, humanization may still be needed to minimize immunogenicity, potentially affecting affinity or stability (Moutel et al., 2016; Vincke et al., 2009).

Epitope Range:

While Nbs excel at recognizing conformational or concave epitopes, they may be less effective at binding flat or linear epitopes compared to conventional antibodies, potentially limiting the range of targetable epitopes (Zavrtanik et al., 2018).

NANOBODIES AS PROPHYLACTIC AND THERAPEUTIC AGENTS FOR VIRAL INFECTIONS

Nbs have emerged as highly promising antiviral agents due to their unique structural and functional properties. Their small size allows them to penetrate virion structures and access epitopes that may be sterically hindered for larger antibodies, such as those within receptor-binding sites or fusion machinery components on viral glycoproteins (Mostafa & Mohamed, 2023). This enables Nbs to act as potent neutralization agents by directly interfering with viral attachment, entry, or uncoating (Mostafa & Mohamed, 2023). Furthermore, Nbs can be engineered to target viral enzymes like polymerases or proteases, thereby inhibiting viral replication and reducing viral load (Mostafa & Mohamed, 2023).

The cost-effectiveness and scalability of Nb production in microbial systems are significant advantages over conventional mAbs, which often require expensive and complex mammalian cell culture systems (Arbabi-Ghahroudi, 2022). Large quantities of Nbs can be produced relatively inexpensively, facilitating their potential use as broad-spectrum prophylactics in high-risk populations or as therapeutics for established infections (Mostafa & Mohamed, 2023). While mAbs are used therapeutically, their long-term application can be limited by production costs, immunogenicity (even for humanized versions), and suboptimal tissue penetration (Zambrano et al., 2022). For example, Nbs developed to bind the SARS-CoV-2 spike protein's receptor-binding domain (RBD) successfully prevented viral entry in vitro by blocking its interaction with the ACE2 receptor (He et al., 2023). Biparatopic Nbs targeting SARS-CoV-2 have shown ultra-potent neutralization across variants (Chi et al., 2022).

Nanobodies Targeting EBV Glycoprotein B

As established, EBV gB is an ideal candidate for Nb-mediated neutralization due to its essential and conserved role in viral fusion and entry (Hong et al., 2022; Neuhierl et al., 2002). The strategy involves isolating or constructing the EBV gB antigen (or immunogenic fragments thereof), potentially as a trimeric form which has shown efficacy in eliciting neutralizing antibodies (Hong et al., 2022), and using it to immunize camelids. Following immunization, typically involving multiple boosts with adjuvants (e.g., Freund's adjuvant) over several weeks, a robust HcAb response is elicited (Hong et al., 2022). This review posits that harnessing this approach will yield potent neutralizing Nbs against EBV-gB.

The advantages of Nbs are particularly pertinent for targeting EBV:

- **Neutralizing Latent and Lytic Infection:** Nbs could neutralize cell-free virions produced during lytic reactivation, preventing new infections or spread to new cell types.
- **Penetration into Sanctuary Sites:** EBV can persist in various anatomical sites. The superior tissue penetration of Nbs might allow them to reach virions or infected cells in these reservoirs more effectively than conventional antibodies.
- **Prophylaxis in High-Risk Groups:** For immunocompromised patients (e.g., transplant recipients), Nbs could be administered prophylactically to prevent primary infection or reactivation leading to PTLD.
- **Vaccine Candidate Evaluation:** The immunization of camelids with EBV-gB not only serves to generate Nbs but also provides a platform to further evaluate the immunogenicity and protective potential of gB as a vaccine antigen.

PRINCIPLES FOR NANOBODY GENERATION AND HALF-LIFE EXTENSION

Nanobody Generation Strategies

Antigen-specific Nbs are typically selected from one of three types of libraries: immune, naïve, or synthetic (Muyldermans, 2021a; Salvador et al., 2019).

Immune Libraries:

These are generated from B-lymphocytes of camelids vaccinated with the target antigen (e.g., EBV-gB). Peripheral blood mononuclear cells (PBMCs) undergo isolation, followed by mRNA extraction and cDNA synthesis. VHH-coding sequences receive amplification through PCR,

commonly focusing on the FR1 to FR4 region. These amplified products are subsequently inserted into phagemid vectors for phage display library construction (Liu & Yang, 2022). The resulting library, displaying Nbs on phage surfaces, experiences multiple biopanning cycles against immobilized target antigens. Non-binding elements are removed through washing, while bound phages undergo elution, amplification in *E. coli*, and utilization in additional panning cycles to concentrate high-affinity binding candidates. Individual clones then receive selection, sequencing, and characterization for binding strength and neutralizing capabilities (Mei et al., 2022).

Naïve Libraries:

These are generated from unimmunized camelids, theoretically containing Nbs against a vast repertoire of antigens. The process is similar to immune library construction but without prior antigen exposure (Muyldermans, 2021a).

Synthetic Libraries:

These are created by introducing diversity (e.g., by randomizing CDR sequences) into one or more pre-defined humanized or camelid Nb scaffolds. This approach avoids animal immunization and can yield Nbs with tailored properties (Liu & Yang, 2022; Valdés-Tresanco et al., 2020). An alternative "two-step" approach involves transplanting CDRs from existing conventional antibodies with known specificity onto an Nb framework. While initial grafts may have suboptimal affinity, these can serve as templates for targeted synthetic phage libraries to affinity-mature the Nbs (Wagner et al., 2018). This method leverages the vast data on existing antibody CDRs and can expedite Nb development (Mostafa & Mohamed, 2023; Wagner et al., 2018). Regardless of the library source, rigorous in vitro and in vivo validation of neutralizing activity and efficacy is essential.

Strategies for Enhancing Nanobody Half-Life

The short circulating half-life of Nbs due to rapid renal clearance is a significant hurdle for many therapeutic applications (Arbabi-Ghahroudi, 2017). Several strategies are employed to extend their persistence in vivo:

Fc Fusion:

Fusing an Nb to the Fc region of human IgG1 is a common and effective method. The Fc domain binds to the neonatal Fc receptor (FcRn), which rescues the fusion protein from lysosomal degradation and recycles it back into circulation, significantly prolonging half-life. Fc fusion can also confer effector functions (Bobkov et al., 2018; Chi et al., 2022).

PEGylation:

Covalent attachment of polyethylene glycol (PEG) chains increases the hydrodynamic volume of the Nb, reducing renal filtration and extending half-life (Salvador et al., 2019).

Albumin Binding/Fusion:

Nbs can be fused to albumin or to an albumin-binding Nb. Albumin itself has a long half-life (around 19 days in humans) due to FcRn-mediated recycling, and this property can be conferred to the Nb conjugate (Hu et al., 2022; Jin et al., 2023; Salvador et al., 2019).

Site-Specific Conjugation and Glycosylation:
More advanced chemical biology approaches can be used for precise modification, although these are generally more complex (Arbabi-Ghahroudi, 2017).

Among these, Fc fusion has proven highly effective for antiviral Nbs, enhancing both serum persistence and, in some cases, effector functions leading to improved efficacy (Brentville et al., 2021; Chi et al., 2022).

CONCLUSION AND FUTURE OUTLOOK

Since their discovery, Nbs have transitioned from a scientific curiosity to a versatile platform with immense therapeutic and diagnostic potential. For Epstein-Barr virus, a pathogen associated with significant global morbidity for which no specific therapies or vaccines exist, Nbs targeting the essential gB glycoprotein offer a compelling new avenue. The unique attributes of Nbs—small size, high stability, deep tissue penetration, low immunogenicity, and ease of production and engineering—make them highly suitable for combating EBV. Immunizing camelids with EBV-gB presents a dual opportunity: the generation of potent neutralizing Nbs and the concomitant evaluation of gB as a vaccine antigen.

The development pipeline for Nbs, from library construction to lead optimization and half-life extension strategies, is well-established. We anticipate that compact and stable Nbs will increasingly complement or replace conventional mAbs and their fragments in various biomedical applications. While challenges such as optimizing in vivo half-life and ensuring efficient delivery to target sites remain, ongoing research is actively addressing these issues.

The potential of Nbs to improve human health is substantial. Given EBV's ubiquity and its role in a multitude of diseases, which may be exacerbated by increasing global stressors affecting immune competence, the development of effective anti-EBV Nbs is a critical unmet need. The low immunogenicity of Nbs is a key advantage for long-term prophylactic or therapeutic use. Ultimately, for Nbs to realize their full therapeutic promise against EBV, they must demonstrate robust efficacy, achieve adequate concentrations at target sites, and exert their intended biological activity. The compelling preclinical data for Nbs against other viral pathogens, coupled with the critical role of gB in EBV's life cycle, strongly supports prioritizing the development and clinical translation of Nbs targeting EBV-gB. This endeavor warrants collaborative efforts from academia and industry to expedite the availability of these novel agents for patients in need.

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