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REVIEW ARTICLE

Perspective on therapeutic and diagnostic potential of camel nanobodies for coronavirus disease-19 (COVID-19)

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Abstract

In this paper, we focus on the camelid nanobodies as a revolutionary therapy that can guide efforts to discover new drugs for Coronavirus disease (COVID-19). The small size property makes nanobodies capable of penetrating efficiently into tissues and recognizing cryptic antigens. Strong antigen affinity and stability in the gastrointestinal tract allow them to be used via oral administration. In fact, the use of nanobodies as inhalant can be directly delivered to the target organ, conferring high pulmonary drug concentrations and low systemic drug concentrations and minimal systemic side effects. For that, nanobodies are referred as a class of next-generation antibodies. Nanobodies permit the construction of multivalent formats that may achieve ultra-high neutralization potency and then may prevent mutational escape and can neutralize a wide range of SARS-CoV-2 variants. Due to their distinctive characteristics, nanobodies can be of great use in the development of promising treatment or preventive strategies against SARS-CoV-2 infection. In this review, the state-of-the-art of camel nanobodies design strategies against the virus including SARS-CoV-2 are critically summarized. The application of general nanotechnology was also discussed to mitigate and control emerging SARS-CoV-2 infection.

Keywords Antibodies engineering · Coronavirus disease (COVID-19) · Heavy chain antibodies · Pandemic · Single-domain antibody

Introduction

The coronavirus disease 2019 (COVID-19) is spreading rapidly since its first appearance in Wuhan, China, in December 2019. The occurrence of COVID-19 has been reported from more than 200 countries worldwide. On 16 December 2020, the number of confirmed cases has reached 73,476,721 including 1,635,464 deaths (https://coronavirus.jhu.edu/map.html). Coronavirus was referred to as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and belongs to the beta-CoV genus of the Coronaviridae family (Lu et al. 2020). Phylogenetic analysis showed that SARS-CoV-2 genome has shared the highest nucleotide sequence identity to severe acute respiratory syndrome coronavirus

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(SARS-CoV) and it is an enveloped, single-stranded, positive (+)-sense RNA virus with RNA genome of approximately 30 kilobases in length (Chen et al. 2020b). The emergence of SARS-CoV-2 has been marked as the third major outbreak caused by a new coronavirus in the past 2 decades, following SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV).

The genome of SARS-CoV-2 encodes four important structural proteins named spike (S), envelope (E), membrane (M), and nucleic capsid (N). Similar to other coronaviruses, the SARS-CoV-2 uses its S protein to interact with cellular receptors (Wrapp et al. 2020b; Zhou et al. 2020b). In fact, S protein has been reported as a significant determinant in viral attachment, fusion and entry into target cells (Chen et al. 2020b). The S protein molecule contains two subunits, S1 and S2. The S1 subunit has a receptor-binding domain (RBD) that interacts with its host cell receptor, angiotensin-converting enzyme 2 (ACE2). On the other hand, the S2 subunit mediates fusion of the viral and cellular membranes for releasing viral RNA into the cytoplasm for its survival and replication. A structure model analysis shows that SARS-CoV-2 S protein binds to ACE2 with higher affinity than SARS-CoV S leading to rapid viral replication and then inducing an inflammatory response and provoking an accumulation of a large amounts of pro-inflammatory cytokines (Wrapp et al. 2020b) (Fig. 1; adapted from de la Rica et al. 2020).

Compared to SARS-CoV, transmission route of SARS-CoV-2 among humans seems to be greater (Petersen et al. 2020; Wang et al. 2020b). Several studies suggest that meteorological parameters may be important factors affecting the COVID-19 pandemic. In fact, the association between weather and SARS-CoV-2 spreading deserves special attention to analyze the conditions under which COVID-19 may resurge as a second wave of infections and to define the seasonal characterization of this pandemic. It has been reported that environmental factors, such as a higher temperature and a higher relative humidity, could decrease the virus spreading (Alkhowailed et al. 2020; Qi et al. 2020; Sajadi et al. 2020). Guo et al. (2020) used a distributed lag non-linear model to investigate the association between the COVID-19 incidence and meteorological factors in 415 sites from 190 countries. By comparison with seasonal coronaviruses OC43 and HKU1, Kissler et al. (2020) modelled possible scenarios for COVID-19 up to 2024. The results revealed that SARS-CoV-2 has the highest average of the basic reproductive rate (R_0) in winter, which predicted winter cycles of COVID-19 after the pandemic phase. In many parts of the world, numerous conditions were determined for a second wave of COVID-19. In fact, a recent study proved that a second wave

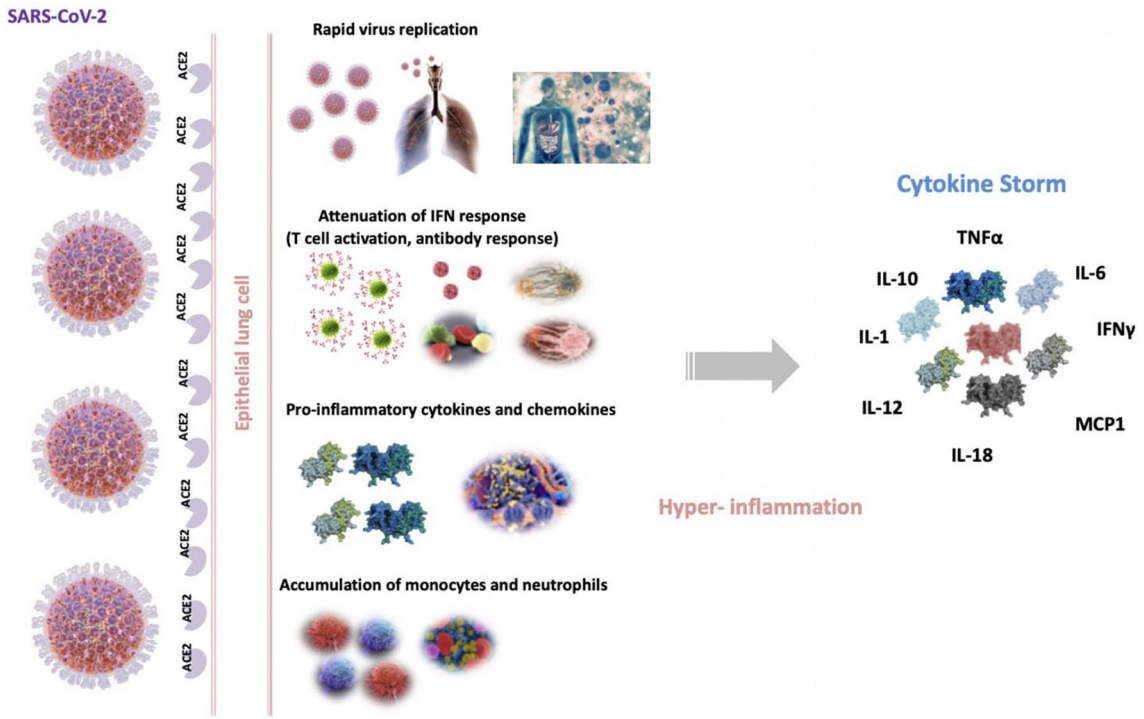

Fig. 1 Schematic representation of the origin of COVID-19 cytokine storm. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) enters cells via binding to angiotensin-converting enzyme 2 receptor (ACE2). The rapid viral replication in the first infection stage leads to an inflammatory response and provokes an accumulation of large amounts of pro-inflammatory cytokines referred as cytokine storm (adapted from de la Rica et al. 2020)

can occur across a broad range of plausible model input parameters governing epidemiological and social conditions, on the account of instabilities generated by behavior–disease interactions (Pedro et al. 2020). The same study found that the second wave tends to have a higher peak than the first although the efficacy of restrictions is greater than 40% and when the basic reproduction number R_0 is less than 2.4. The letter estimated value has been proposed for SARS-CoV-2 similarly to that of SARS-CoV, the 1918 influenza pandemic and higher than that of MERS-CoV (R_0 0.9) and the 2009 H1N1 influenza pandemic (R_0 1.5) (Petersen et al. 2020). Similarly, more recent studies showed that the second wave seems to be significant especially when non-pharmaceutical measures are relaxed (Bontempi 2020; Cacciapaglia et al. 2020; Glass 2020; Renardy et al. 2020). In presence of novel pharmaceutical interventions, such as vaccines, a substantial third wave can be reversed. However, the present knowledge on chronology and durability of the immunity after natural infection with SARS-CoV-2 is incomplete (Prévost et al. 2020; Seow et al. 2020). The results of several studies showed that pre-existing immunity to common coronaviruses does not confer cross-protection against SARS-CoV-2 in vivo (Miyara et al. 2020). Taken together, the combination of several factors, such as the durability of protective immunity, the degree of cross-immunity between SARS-CoV-2 and other coronavirus and the relaxation of effective mitigation measures, defines the dynamics of SARS-CoV-2. Population protective immunity has the potential to block the relentless spread of this pandemic. The percentage of the population that needs to be immunized to achieve this goal has been estimated to be ~67% (Vashishtha and Kumar 2020). Based on these observations, we expect that the long-term dynamics of COVID-19 pandemic tends to come in waves over the next five years until the herd immunity builds-up naturally or through vaccination.

Clinically, symptoms of SARS-CoV-2 caused by COVID-19 range from complete absence of symptoms (asymptomatic) to severe symptoms including fever, dry cough, shortness of breath, pneumonia and death (Mishra et al. 2020). As a matter of fact, diagnosis of COVID-19 can be achieved by several methods. These include CT radiography, a real-time reverse transcription-polymerase chain reaction (RT-PCR) and serological assays like point-of-care blood test (POCT) of IgM/IgG or enzyme-linked immunosorbent assay (ELISA kits) for SARS-CoV-2 (Li et al. 2020b). Intensified efforts have been made to develop preventive and therapeutic interventions strategies to combat SARS-CoV-2. Importantly, both early diagnosis and therapeutic options are key requirements for managing the current pandemic. At the moment, more than 30 vaccine candidates have entered clinical trials to prevent COVID-19 infection in humans (Dong et al. 2020c). Indeed Sputnik vaccine based on recombinant adenoviral vectors rAd26-S and rAd5-S showed that the vaccine is safe, well tolerated, and induces strong humoral and cellular immune responses in 100% of healthy participants. Results showed that volunteers who received the heterologous rAd26 and rAd5 vaccine elicited the same titre of SARS-CoV-2 neutralizing antibodies as did people who had recovered from COVID-19 (Logunov et al. 2020). Currently, another procedure makes available a vaccine using mRNA constructed for the first time with a large scale by Pfizer Company. This vaccine solicits the activation of both the humoral and the cellular immune responses. Pfizer's vaccine is found to be 90% effective in preventing COVID-19. It appears that in human organism, many cell types are able to internalize these mRNAs which are then translated into proteins. This process mimics what happens in a natural infection, the cell infected by the antigens (from the mRNA) presents them via its Major Histocompatibility Complex (MHC) to the immune cells. This vaccine appears to stimulate both cellular and humoral immune responses (Jackson et al. 2020). However, vaccination is only useful in a preventive environment and cannot be applied for immunodeficient patients or pregnant women.

Among the different strategies for treatment and prevention of COVID-19, passive immunotherapy using single-domain antibodies (sdAbs), namely nanobodies (Nbs), may be of great benefit. Nbs derived from heavy-chain-only antibodies (HcAbs) are only found in camelids and some cartilaginous fish (Hamers-Casterman et al. 1993; Stanfield et al. 2004). Camelid-derived sdAbs, known as VHHs, exhibit unique characteristics in comparison with conventional antibodies and their recombinant fragments. The specific properties of VHHs, like high affinity, small size (only 15 kDa) and easy manipulation, make them promise tools for a wide range of applications including virus neutralization. In the present review, we summarize recent findings on Nbs and how they can be used as a diagnostic and therapeutic reagent that control and mitigate the emergence of COVID-19.

Current treatment strategies of SARS-CoV-2

Antiviral medications

As reported previously, numerous antiviral drugs may have great potential against SARS-CoV-2 and some of them are in phase III trials for COVID-19.

Among these antiviral drugs, Remdesivir, an adenosine analogue, that can block viral RNA replication, might have some clinical efficacy against SARS-CoV-2, similar to prior studies using them against SARS (Sheahan et al. 2017; Agostini et al. 2018). Chloroquine, a 9-aminoquinoline, used in therapy of malaria can be effective in the treatment of patients with COVID-19. Therefore, recent studies reported its strong activity against SARS-CoV-2 through different

mechanims of action (Gao et al. 2020; Gautret et al. 2020). Other therapeutic agents, including Lopinavir/Ritonavir, Favipiravir (T-705) and Arbidol could also be the choices for the management of COVID-19/SARS-CoV-2 infection (Tse et al. 2020; Wang et al. 2020c; Lu 2020).

Plasma therapy and neutralizing antibodies

Passive immunisation by administration of antibodies (immunoglobulins: IgG) has been known for more than one hundred years as a very efficient tool and affordable solution for immediate treatment against infectious diseases. Thus, the role of plasma therapy in protection from SARS-CoV-2 infection has been thoroughly reviewed elsewhere (Klasse and Moore 2020; Wang et al. 2020c). Because of the high morbidity and mortality associated with COVID-19, the plasma of some convalescent SARS-CoV-2-infected patients represents an easily applied for treatment of COVID-19 infection clinically.

Patients with resolved COVID-19 infection will develop a specific immune defense against the SARS-CoV-2. Plasma from convalescent donors provides an immediate immunity to passively immunized persons that can last from weeks to months. Blood plasma from recovered patients could boost the immune state, limit the virus reproduction and then reduce the damage in patients with SARS-CoV-2 infection. However, functional antibody titers may be the determining factor (e.g., 50% plaque-reduction neutralization titer: PRNT50) to obtain effective therapeutic results as reported previously with MERS-CoV infection (Ko et al. 2018). Also, a donor selection criteria need to be strictly enforced to ensure the safety of plasma IgG specific to SARS-CoV-2. Therapeutic trials based on the administration of plasma with high antibody titers may be more beneficial than low-titer plasma in non-incubated patients, particularly when administered within 72 h of COVID-19 diagnosis. However, the use of convalescent plasma did not always lead to the recovery (Gharbharan et al. 2020).

Several human monoclonal antibodies (mAbs) have been isolated and characterized (Ju et al. 2020; Wang et al. 2020a; Chen et al. 2020a). Developed mAbs showed promising neutralization activity against SARS-CoV-2. In a recent review paper, Renn et al. (2020) summarized current development of neutralizing antibodies. Some of them have entered clinical trials or are in preclinical stages. Despite the large number of neutralizing antibodies identified until now, there are still many challenges. In fact, potential problems related to somatic mutations and antibody-dependent enhancement (ADE) may reduce the efficacy of a single neutralizing antibodies. To fight these problems, antibody cocktails can be used. Some of them have entered clinical evaluation. On the other hand, multiple research studies are focused on antibody fragments that can be used in combination to balance neutralization and ADE side effects (Zhou et al. 2020a; Huo et al. 2020).

Nbs as new approach for the prevention and treatment of COVID-19

Camel heavy chain antibodies and Nbs

In addition to conventional antibodies (heterotetrameric structure with two heavy and two light chains) produced by all mammals, camelidae members family including Old World species (camels and dromedaries) and New World species (llamas, alpacas, and vicuñas) are able to produce non-conventional antibodies (Hamers-Casterman et al. 1993). Structures of these IgG are devoid of the complete light chain and the first constant heavy domain CH1 and

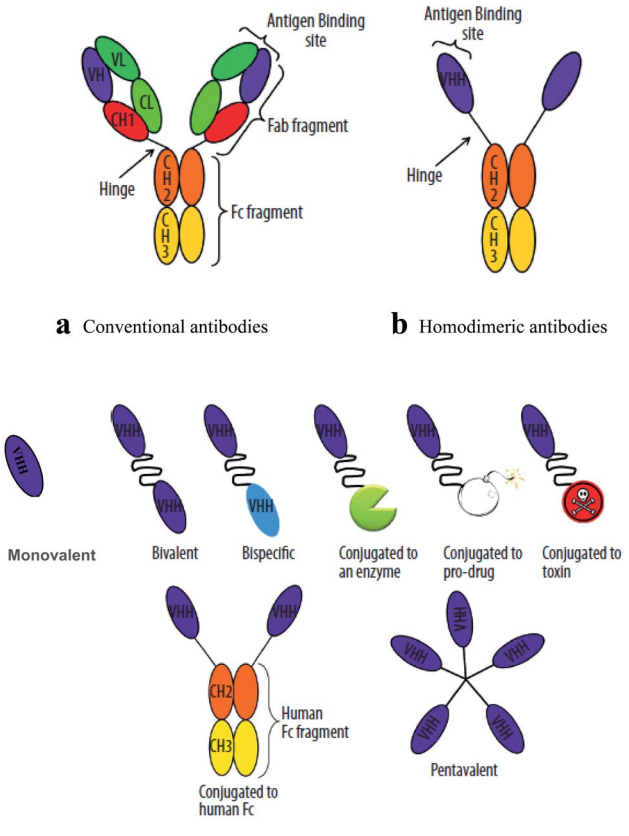

Fig. 2 Schematic diagram of camelid antibodies (**a**, **b**) and different Single-domain antibody fragment (VHH) constructions (**c**). **a** The common structure of conventional antibodies: The antigen-binding fragment (Fab) consisting of Variable Light (VL), Variable Heavy (VH), Constant Light (CL) and Constant Heavy 1 (CH1) domains. **b** The structure of homodimeric camelid antibody: The antigen-binding fragment lack the VL, CL and CH1 domains and named Single-domain antibody fragment (VHH). **c** Different VHH constructions (adapted from Smolarek et al. 2012)

then named HcAbs. The account of HcAbs in the sera varies between camel species. It can reach 50–80% in *Camelus bactrianus* and *Camelus dromedarius*; however, it does not exceed 25% in the serum of the South American camelids (alpacas and llamas) (De Simone et al. 2008; Blanc et al. 2009).

HcAbs were reffered to as IgG2 (IgG2a and IgG2b) and IgG3 IgG-subclasses to distinguish them from conventional antibodies (IgG1). Despite their particular structure, HcAbs antibodies are fully functional and still able to bind antigens with a high affinity through their antigen-binding site known as VHH (Fig. 2; adapted from Smolarek et al. 2012).

Structure and peculiar characteristics of VHHs

Despite it shares general structural features with human variable heavy domain (VH), the camelid heavy chain variable named as VHH, with a molecular weight of 15 kDa, presents an important difference with VH. In fact, there are four major amino acid substitutions observed in the framework region 2 (FR2) that substitute the hydrophobic residues (involved in the VH/VL interaction in conventional antibodies) by more hydrophilic amino acids (Nguyen et al. 2001). These substitutions compensate the lack of the variable light domain (VL) and confer the higher solubility of VHHs when compared to other single-domain antibodies. Additionally, complementarity determining regions (CDRs) exhibit a long CDR3 loop that increases the antigen- binding loop size in VHHs and enables theme to bind concave epitopes that cannot be recognized by traditional antibodies ((Muyldermans et al. 1994; Vu et al. 1997). The stability of extended CDR3 loops is maintained by a disulfide bond between CDR1 and CDR3 or between FR2 and CDR3. These particular features increase the stability and the solubility of VHH even under denaturing conditions or high temperatures (Van der Linden et al. 1999; Dumoulin et al. 2002; Conrath et al. 2005; Kunz et al. 2018). Moreover, VHHs are easily engineered with high yields and low-cost production using various expression systems (Liu and Huang 2018; De Marco 2020).

The most important characteristics of VHH fragments include negligible immunogenicity in the human body, rapid penetration into the tissue, a nanomolar affinity for their target and flexible formatting (multimerization). More importantly, the high stability of VHHs under harsh conditions (in the presence of proteases, chaotropic agents as well as at extreme pHs) facilitate their administration by inhaled delivery for the treatment of respiratory diseases. These distinctive properties provide VHHs numerous advantages compared to conventional antibodies and their recombinant fragments and make them a powerful tool for immunotherapy as well as immunodiagnostics immunoassay development.

Production process of VHHs

Over three decades ago, the phage display technology has been shown to be an effective and efficient platform to develop and produce therapeutic antibodies. Numerous recombinant antibodies with desired functional properties are selected from immune, naïve, or synthetic libraries via phage display technology. This fast methodology enables the selection of Nbs with a reasonable specificity and affinity by successive rounds of bio-panning (Silacci et al. 2005).

Nbs are successfully expressed in a variety of expression systems including prokaryotic, eukaryotic and plant hosts. Nbs are characterized by large-scale production, solubility and stability compared to conventional antibody fragments (antigen-binding fragments (Fab) or single-chain variable fragments (scFv)) that can aggregate due to their low solubility (Van der Linden et al. 1999).

Other strategies, such as ribosome display and yeast surface display can be used for the selection of specific VHHs. The mono-domain format of VHH offers significant advantages in cost of production and engineering compared to conventional antibodies.

Camel nanobodies: promising therapeutic tools to combat the emerging pandemic virus

Neutralizing VHH against viral zoonosis

Due to their peculiar properties, rapid progress has been made regarding the production of VHH domains for therapeutic and diagnostic applications (Wesolowski et al. 2009; Khodabakhsh et al. 2018; Lafaye and Li 2018; Sanaei et al. 2019; Chames and Rothbauer 2020). It has been demonstrated that these Nbs can be easily engineered without loss of functionality. Currently, several Nbs produced by Ablynx (now Sanofi), are in different clinical trials and with Caplacizumab, the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) approved the first sdAb-based medicine for adults in November 2018 and in February 2019, respectively (Morrison 2019; Jovčevska and Muyldermans 2020). Viral neutralizing sdAb has been described as valuable biomolecules, with high potentials, able to perturb different steps in the viral life cycle with many examples of viral neutralizing Nbs (De Vlieger et al. 2019; Sroga et al. 2020). These Nbs have been generated from an immune or synthetic phage display library against a wide range of target classes. Table 1 shows Nbs selected for the prevention and treatment of infectious viral diseases including that of SARS-CoV-2.

For example, sdAb specific for influenza viruses has been successfully isolated through selections against the nucleoprotein and M2 ion channel protein of Influenza A, the neuraminidase and trimeric spike protein and hemagglutinin of

Table 1 Nanobodies-based therapeutic tools for viral infection diseases including emerging corona viruses

Virus	Target	Nanobodies	Source	References
MERS-CoV	RBD	Monomeric VHH: NbMS10	Llama immune VHH library (phage display)	Zhao et al. (2018)
MERS-CoV	RBD	VHH and camel/human chimeric (HcAbs:HCAb-83)	Dromedary camel immune VHH library (phage display)	Raj et al. (2018)
MERS-CoV	RBD	Mono-Nb, dimeric Nb (Di-Nb) and Trimeric Nb (Tri-Nb)	Llama immune VHH library (phage display)	He et al. (2019)
SARS-CoV-2	RBD	Monomeric VHH: NIH-CoVnb-112	Llama immune VHH library (phage display)	Esparza and Brody (2020)
SARS-CoV-2	RBD	Multivalent Nb: $Nb21_3$ and $Nb20_3$	Llama immune VHH library (phage display)	Xiang et al. (2020)
SARS-CoV-2	RBD	Monomeric VHH	Alpaca immune VHH library (phage display)	Nieto et al. (2020)
SARS-CoV-2	RBD	Monomeric VHH: NM1226, NM1228 and NM1230	Alpaca immune VHH library (phage display)	Wagner et al. (2020)
SARS-CoV-2	RBD	Trimeric Nb: mNb6-tri	Llama synthetic VHH library (yeast display)	Schoof et al. (2020)
SARS-CoV-2	RBD	Monomeric VHH: H11-D4 and H11-H4; chimeric fusions:H11-H4-Fc and H11-D4-Fc	Naïve llama VHH library	Huo et al. (2020)
SARS-CoV-2	RBD	Monomeric VHH: Ty1	Alpaca immune VHH library (phage display)	Hanke et al. (2020)
SARS-CoV-2	RBD	Monovalent Nb: Nb11-59	Camel immune VHH library (phage display)	Gai et al. (2020)
SARS-CoV-2	RBD	Monomeric VHH: SR31	Synthetic sdAb phage display library	Yao et al. (2020)
SARS-CoV-2	RBD	Monovalent VHH: 2F2, 3F11 and 5F8 Fc-fused sdAbs	Synthetic sdAb phage display library	Chi et al. (2020)
SARS-CoV-2	RBD	Sybody Sb23	Three sybody libraries (concave, loop and convex)	Custódio et al. (2020)
SARS-CoV-2	RBD	Sybody MR3	Three sybody libraries (concave, loop and convex)	Li et al. (2020a)
SARS-CoV-2	RBD	Sybodies	Three large combinatorial libraries, using ribosome and phage display	Walter et al. (2020)
SARS-CoV-2	Recombinant SARS-CoV-2 S protein	Bispecific VHH-Fc antibody, Tri-specifc VHH-Fc antibody	Naïve and synthetic llama VHH library	Dong et al. (2020a, b, c)
SARS-CoV-2	RBD, S1 protein	Human single-domain antibodies n3130	Naïve antibody libraries	Wu et al. (2020)
SARS-CoV-2, MERS-CoV, SARS-CoV-1	prefusion-stabilized coronavirus spikes	Bivalent VHH:VHH-72 VHH-55, VHH-72-Fc	Llama immune VHH library (phage display)	Wrapp et al. (2020a)
Influenza A and B viruses	Hemaglutinins	Multivalent VHH: MD3606	Llama immune VHH library	Laursen et al. (2018)
H1N1	Hemaglutinin	bivalent VHH: R1a-B6	Alpaca immune VHH library (phage display)	Hufton et al. (2014)
Respiratory syncytial virus	Fusion (F) protein	Trivalent nanobody: ALX-0171	Llama immune VHH library (phage display)	Van Heeke et al. (2016), Detalle et al. (2015), Wilken et al. (2017)

Table 1 (continued)

Virus	Target	Nanobodies	Source	References
Hepatitis B virus	Capsid protein: HBcAg	VHH intrabodies	Llama immune VHH library (phage display)	Serruys et al. (2010)
HIV	gp120	Monovalent VHH: A12, C8, and D17,	Llama immune VHH library (phage display)	Forsman et al. (2008)
HIV	gp140	Monovalent VHH: 2E7	Llama immune VHH library (phage display)	Strokappe et al. (2012)
Influenza A virus	Nucleoprotein (NP)	Monovalent VHH:NP-VHHs	Alpaca immune VHH library (phage display)	Ashour et al. (2015)
Influenza A virus	Native M2 ion channel protein	Monovalent VHH: M2-7A	Synthetic Camel single-domain antibody (VHH) libraries	Wei et al. (2011)
H5N1	Hemaglutinin	Trivalent VHH	Llama immune VHH library (phage display)	Hultberg et al. (2011)
H5N1	Influenza virus neuraminidase (NA) Neuraminidase	Bivalent VHH: N1-VHHb, N1-VHH-Fc	Alpaca immune VHH library (phage display)	Cardoso et al. (2014)
Poliovirus type 1	Capsid	Monovalent VHH: PVSS21E	Dromedary immune VHH library (phage display)	Strauss et al. (2016)
Norovirus	VLPs	Monomerci: Nano-26 and Nano-85	Alpaca immune VHH library (phage display)	Koromyslova et al. (2017)
Rotavirus	VP6 inner capsid protein	Monovalent VHH	Llama immune VHH library (phage display)	Van der Vaart et al. (2006)
Chikungunya virus (CHIKV)	CHIKV virus-like particles contained the capsid, E1 and E2 proteins	CC3 VHH	Llama immune VHH library (phage display)	Liu et al. (2019)
Ebola virus	Recombinant EBOV GP and EBOV VLPs	sdAbs	Llama immune VHH library (phage display)	Liu et al. (2017)

H5N1 and H1N1 (Wei et al. 2011; Hultberg et al. 2011; Cardoso et al. 2014; Hufton et al. 2014; Ashour et al. 2015; Laursen et al. 2018). An sdAb-specific viral protein (PV1) of poliovirus (PV) was identified from the immunised phage display library. The mechanism by which these VHHs reduce viral loads is by blocking the ligand–receptor interactions (Strauss et al. 2016). Human norovirus is classified among the leading cause of gastroenteritis worldwide. Several Nbs against virus-like particles (VLPs) of norovirus have shown promise for disease treatment (Koromyslova and Hansman 2017).

Ebola virus (EBOV) is extremely virulent and causes fatal hemorrhagic fever in ~50% of the cases. The interaction between viral envelope glycoprotein GP and host cell receptors plays a critical role in pathogenicity of this virus. An immune sdAb phage display library derived from a llama immunized with killed EBOV and recombinant GP was constructed and SdAbs specific for Ebola GP were selected and evaluated for their affinity and thermal stability (Liu et al. 2017). Also, monovalent, bivalent and trivalent VHHs targeting the glycoproteins, gp120 and gp41 of VIH have been generated to disrupt virus entry into cluster of differentiation 4 $(CD4)^+$ T cells (Forsman et al. 2008; Strokappe et al. 2012; Weiss and Verrips 2019). Chikungunya virus (CHIKV) is a member of Togaviridae family that causes severe joint pain which is associated with fever, rash and headache. Five anti-CHIKV sdAbs have been generated in immunized llamas. These viral neutralizing sdAbs have been successfully isolated through selections against the CHIKV VLPs (Liu et al. 2019).

Nbs developed against the core antigen of hepatitis B (HBcAg) have an effect on the viral life cycle in HBV-transfected hepatocytes (Serruys et al. 2010). Moreover, VHHs directed against rotavirus serotype G3 reduce morbidity of rotavirus-induced diarrhea in vivo (Van der Vaart et al. 2006). In another example, it has been shown by Terryn et al. (2016) that multi-merization of VHH domains significantly improves protection of mice from lethal rabies infection.

Nbs have been described as inhaled bio-therapeutics for lung diseases. Human respiratory syncytial virus (RSV) belongs to the Pneumoviridae family and represents the most important cause of lower respiratory tract infections in young infants. Successful phase I/IIa clinical trials were completed for an anti-RSV VHH domain called ALX-0171 (Detalle et al. 2015; Van Heeke et al. 2017; Wilken and McPherson 2018). Neutralizing activities of ALX-0171 were compared to Palivizumab, a marketed neutralizing

monoclonal antibody, and it was demonstrated that ALX-0171 inhibits virus replication in 87% of viruses tested versus 18% observed with Palivizumab administration. In addition, to develop lung-targeting drugs, Nbs targeting pulmonary surfactant protein A (SPA) have been isolated. The authors showed fast accumulation of selected Nbs in the lungs (Wang et al. 2015).

Nbs against different coronavirus species have been isolated and characterized to block interaction between virus and host cell. As example, Abs targeting RBD of MERS-CoV were identified and selected from the immunized dromedary and it was shown that camel/human chimeric HcAbs bind to their target with picomolar affinity (Raj et al. 2018). In addition, Zhao and colleagues panned an immune VHH library derived from llama immunized with a recombinant MERS-CoV RBD protein, and isolated monomeric VHH (Mono-Nb, NbMS10) (Zhao et al. 2018). He et al. (2019) generated two oligomeric Nbs, dimeric and trimeric Nbs, from an immunized library directed against the RBD of MERS-CoV. According to a multiple recent studies, VHHs exhibited high neutralization potency SARS-CoV-2. Humanized sdAbs-binding SARS-CoV-2 RBD proteins have been discovered in a synthetic sdAb phage display library (Chi et al. 2020). The inhibition efficiency on SARS-CoV-2 pp and affinity kinetics was tested in vitro. These Nbs, named 2F2, 3F11 and 5F8 could be very advantageous to find new specific drugs to prevent SARS-CoV-2 infection by inhibiting membrane fusion between RBDs of the viral Spike and their host cell receptors and then blocks the entry of SARS-CoV-2 into cells (Fig. 3; adapted from Esparza et al. 2020). Another illustration of a VHH against SARS-CoV-2 is VHHs against prefusion-stabilized MERS-CoV and SARS-CoV-1 spikes of Betacoronaviruses. These neutralizing sdAbs were isolated from immunized llamas and it was demonstrated that engineered bivalent Nbs exhibits cross-reactivity against SARS-CoV-1 RBD and SARS-CoV-2 RBD and able to neutralize SARS-CoV-2 S pseudoviruses with high affinity (Wrapp et al. 2020a). Using a combiation of two llama VHH libraries, humanized VHH has been constructed (Dong et al. 2020a, b). In their studies, Dong and collaborators demonstrated that multi-specific antibodies showed better affinity and avidity than individual monoclonal VHH-Fcs. More importantly, these multi-specific antibodies showed more potent neutralization activity than a combination of monoclonal antibodies. Hanke et al. (2020) reported the isolation of a monomeric Nb (Ty1) from immunised alpaca. The highly specificity and high-affinity binding of Ty1 to the RBD have been confirmed. Hanke and colleagues, suggest that the generation of homodimeric or trimeric formats is likely to further increase its neutralization activity. More recently, Schoof et al. (2020) have successfully isolated a panel of Nbs, from synthetic Nbs library, that bind to multiple epitopes on Spike. These Nbs were divided into two classes. Class I bound directly to the RBD and competed with the ACE2 receptor from the surface of human cells. While class II recognized another binding region leading to modification of structural conformation of the RBD so that it cannot recognize ACE2 receptor. Structural analyses have clearly shown that the binding domain of class II Nbs occurred on a protected area on the spike protein well away from the RBD. Two class I Nbs designated Nb6 and Nb11 bound to both open and closed conformations of Spike. To enhance the reactivity, Nb6 has been considered to make dimers and trimers. The novel structures inhibited more strongly the trimeric spike S protein by binding to more than one RBD on its surface. To assess the binding efficacy the measurement of IC50 revealed that Nb6 had an IC50 of 2 micromolar, while the Nb6-trimeric form (mNb6-tri) was 1.2 nM. In this assay, the trimeric form showed two-thousand-fold improvement in efficacy. This result was confirmed in a test of Vero cell infection with real SARS-CoV-2 coronavirus where mNb6-tri was able to prevent viral attack with an IC50 of 160 picomolar, which is truly impressive. Moreover, a mutation generate on mNb6-tri reached femtomolar affinity for SARS-CoV-2. Even if at the prophylactic level, Sputnik vaccine and mRNA vaccine have been shown to be effective. The therapeutic mNb6-tri is very interesting with its high interaction with RBD as well as its stability after aerosolization, lyophilization and heat treatment (Schroof el al. 2020). It is easy and not expensive to obtain this Nb in large number by cultivation on yeast. The study of its efficacy on patient's infected with SARS-CoV-2

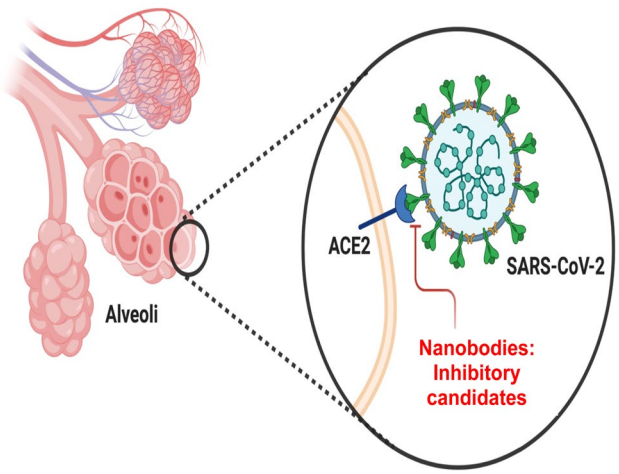

Fig. 3 The potential mechanisms of SARS-CoV-2 neutralization by nanobodies. The major therapeutic goal is to develop inhibitory agents that disrupt the interaction between the receptor-binding domain of SARS-CoV-2 (green color) with its host cell receptor (angiotensin-converting enzyme 2: ACE2). Nanobodies bound directly to the receptor-binding domain (RBD) and competed with the ACE2 receptor from the surface of human cells (adapted from Esparza et al. 2020)

needs to be monitored. In a similar study, the identification and characterization of two other high-affinity Nbs (H11-D4 and H11-H4) have been reported (Huo et al. 2020). Both H11-D4 and H11-H4 Nbs blocked RBD binding to ACE2. Furthermore, in the same study, a bivalent Human Fc-Nb fusion (homodimeric chimeric protein) showed neutralizing activity against SARS-CoV-2 and additive neutralization with the SARS-CoV-1/2 antibody CR3022. In fact, it has been demonstrated that the CR3022 and these Nbs recognized non-overlapping epitopes on RBD. Interestingly, such additive combinations are a well-known strategy to reduce mutational escape.

In a recent study, three potent Nbs (Nb21, Nb20 and Nb89) with picomolar affinity have been isolated from a Llama immune VHH library (Xiang et al. 2020). Multivalent Nbs have been constructed to enhance the antiviral activities. Results showed that up to ~ 30-fold improvement of inhibitory activity was observed with an IC50 of 1.3 picomolar and 4.1 picomolar for the homotrimeric constructs of $Nb21_3$ and $Nb20_3$, respectively. Importantly, multivalent constructs keep excellent physicochemical properties after lyophilization and aerosolization make them suitable for inhalation administration. Nieto et al. (2020) reported a rapid selection of monomeric Nb with sub-nanomolar affinity. Nevertheless, the neutralization activity of this Nb against SARS-CoV-2 pseudo-virus needs to be achieved. In another study, Nbs targeting different spike antigens (RBD, S1 domain or homo-trimeric spike) with high neutralizing potency have been successfully isolated from immunised alpaca VHH library (Wagner et al. 2020). Using these Nbs (NM1226, NM1228 and NM1230), a competitive multiplex binding assay called "NeutrobodyPlex" has been developed. Authors demonstrated that NeutrobodyPlex approach using RBD-specific Nbs was more efficacious than conventional antibodies which showed cross-reactive signals. Furthermore, the test has been validated by analyzing serum samples collected from 18 patients and 4 healthy donors in comparison to standard assays. It can determine whether the examined people carry neutralizing antibodies preventing re-infection. Interestingly, this novel diagnostic test opens the door to surveil the emergence of neutralizing antibodies in infected patients. Thus, it might be useful during vaccination campaigns in the future.

Nbs library derived from immunised camel has been constructed (Gai et al. 2020). It has been shown that seven Nbs represented good binding capacity to RBD including eight SARS-CoV2-RBD mutants. Among these candidates, Nb11-59 exhibited the best neutralizing activities with a good stability. According to these results, Nb11-59 might be novel therapeutic molecule, as an inhaled drug, for COVID-19 treatment. In a study, 63 sybodies, against the SARS-CoV-2 RBD, were generated from three large combinatorial libraries, using ribosome and phage display. Described flycode technology provides new opportunities for passive immunization to protect people against SARS-CoV-2 escape mutants (Walter et al. 2020). In addition, other studies reported the rapid isolation and characterization of potent synthetic Nbs which neutralize SARS-CoV-2 pseudo-viruses with high affinity (sybodies MR3 and Sb23) (Custodio et al. 2020; Li et al. 2020a). The last one (Sb23) can also bind the RBD in both "up" and "down" conformation (Custodio et al. 2020). Also, Yao et al. (2020) identified a new synthetic Nb. Selected sybody (named SR31) displayed poor neutralization activity against SARS-CoV-2 pseudo-virus. However, when fusioned with two other sybodies, increased binding affinities and neutralizing activities were observed. According to this result, SR31 cannot be used alone. Nevertheless, it may be combined with monoclonal antibodies or other antibody fragments to improve affinity and potency.

In another study, several Nbs that bind to the SARS-CoV-2 RBD have been isolated (Esparza and Brody 2020). Among those, the lead therapeutic candidate named NIH-CoVnb-112 showed high affinity in monomeric form and blocked interaction between ACE2 and several variant forms of the spike protein. Furthermore, Wu et al. (2020) selected fully human single-domain antibodies against five types of epitopes on SARS-CoV-2 RBD, using phage-displayed VHH library by grafting naïve CDRs into FR regions. The use of these Nbs may represent a novel approach to battle against COVID-19 given that human showed immunogenicity towards other antibody fragments (Wu et al. 2020).

Nanobodies as modulators of inflammation

Immune responses play a key role during SARS-CoV-2 virus infection. The latest reports suggest that acute respiratory distress syndrome (ARDS) is the common immunopathological event for this infectious disease (Shi et al. 2020; Wen et al. 2020). One of the main mechanisms for ARDS is the uncontrolled systemic inflammation, named as cytokine storm, resulting from the release of large amounts of pro-inflammatory cytokines (interferons: IFN-α and IFN-γ; interleukins: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-9, IL-10, IL-12, IL-18 and IL-33; tumour necrosis factor: TNF-α; transforming growth factor β: TGFβ; etc.) and chemokines (CCL2, CCL3, CCL5, CXCL8, CXCL9, CXCL10, etc.) by immune effector cells upon viral infection (Fig. 1) (Ong et al. 2020; Li et al. 2020a, b; de la Rica et al. 2020). Given the pivotal role of these proteins during inflammation, specific inhibitors of their activities might be useful as new tools to modulate immune functions in COVID-19 patients (Shi et al. 2020).

The generation of Nbs directed against chemokines, cytokines, and ecto-enzymes can be tailored to modulate inflammation responses and then beneficial for the recovery

of COVID-19 patients. Nbs that modulate immune function have been successfully generated in immunized camelids. Several reports have been published in raising sdAbs directed against cytokine by phage display technology (Nosenko et al. 2017). For example, TNFα is an important cytokine implicated in a number of chronic inflammatory disorders. TNFα-blocking sdAbs have been successfully isolated from a llama immunized with human and mouse TNFα which are more effective at neutralizing TNFα than the conventional TNFα-blocking antibodies Infliximab and Adalimumab (Coppieters et al. 2006). Another Nb that binds to human IL-6-R and IL23 was generated for treatment of rheumatoid arthritis (Tillib et al. 2015; Desmyter et al. 2017).

Koch-Nolte et al. (2007) selected a novel Nb from an immunized phage display library directed against the T cell ecto-enzyme, ART2.2, which plays a key role in inflammatory settings and induces T cell death. Researchers demonstrate that these Nbs block effectively the enzymatic activity of ART2.2 in vivo.

Another study presented an interesting example of generating an ion-channel blocking Nb. The P2X7 ion channel is expressed by both monocytes and T cells. This ion channel responsible for the release of IL-1β during inflammation represents a potential therapeutic target in inflammatory diseases. It has been shown that sdAbs recognizing P2X7 were isolated from immunized llama effectively blocked ATP-induced the release of IL-1β with sub-nanomolar affinity (Danquah et al. 2016).

CXCL10 expression level increases in several diseases including SARS-CoV-2 infection. The group of Sadeghian-Rizi et al. (2019) reported the isolation of anti-CXCL10 polyclonal HcAbs for the development of a specific Nbs that specifically target CXCL10 for in vivo therapeutic applications (Sadeghian-Rizi et al. 2019). Several VHHs targeting other chemokine receptors including CXCR4, CXCR7 and ChemR23 have been described (Jahnichen et al. 2010; Maussang et al. 2013; Peyrassol et al. 2016). Similarly, VHHs Blockade CCL2, CCL5, CXCL11 and CXCL12 have been selected by Blanchetot et al. (2013). The selected Nbs showed preventing chemokine receptor activation-induced immune cells migration in vitro.

The adaptive immune response in SARS-CoV had been extensively investigated. It was reported that CD4 + T cells promoted the proliferation of neutralizing antibodies, whereas CD8 + T cells were responsible for the destruction of viral-infected cells. Increasing evidences have been reported that insufficient T cell responses could play a decisive role in clearance of SARS-CoV (Rajaei and Dabbagh 2020; Vellingiri et al. 2020). In this context, targeted delivery of antigens to antigen presenting cells (APCs) improve immune responses by enhancing antibody production, activation of $CD4^+$ T cells and elicitation of $CD8^+$ T cell responses. MHC-II products, integrins (CD11b) and scavenger receptors (CD36) are abundantly expressed on APCs. Duarte et al. (2016) showed that VHHs specific for these molecules enhanced immune responses in distinct dendritic cells (DCs) populations. So, VHH can be used to deliver proteins or peptides to APCs to trigger humoral immunity and to track inflammation to treat or prevent SARS-CoV-2 infection.

Conclusion

The pandemic outbreak of COVID-19 is a potentially fatal and highly contagious disease. Apart from conventional antibodies produced by mammals, camelidae family members produce functional Heavy chain antibodies. These antibodies present a very small binding domain, named VHH or Nb. The burst of VHH application in the last decades is highly apparent. Different VHH formats were developed for their use in therapeutic and/or diagnostic applications. Since there were peculiar characteristics like small size, low immunogenicity and high affinity and stability, combined with their easy expression as recombinant proteins, Nbs can be delivered to the infection site via inhalation. Neutralizing Nbs against the SARS-CoV-2 RBD have been successfully isolated. Some of them showed ultra-high affinity, excellent physicochemical properties after aerosolization, lyophilization and heat treatment which are desirable characteristics for a large-scale manufacturing. Selected Nbs represent an interesting approach to combat the COVID-19 re-emergence in the future. Thereby, VHHs may be of great benefit and could be an appropriate replacement for other therapeutics particularly for vulnerable population. In addition, they may find application in a cocktail of laboratory-synthesized neutralizing antibodies to enhance their neutralizing activities to reduce mutational escape, to treat severely ill COVID-19 patients and to mitigate future SARS-related CoV infections. Also, isolation of Nbs against chemokines, cytokines and ecto-enzymes indicates their potential utility as anti-inflammatory and immune modulating agents to prevent and treat COVID-19 infection. So that, it can be given to front-line health care workers or immunocompromised patients to provide them long-term passive immune protection during COVID-19 pandemic.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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