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THERAPEUTIC AND DIAGNOSTIC POTENTIAL OF CAMELID NANOBODIES AGAINST COVID-19

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ABSTRACT

The unique structural and functional properties of naturally occurring heavy-chain antibodies (HCAbs) or commercially named Nanobodies (Nbs) are antibody-derived therapeutic proteins. They show excellent robustness, are non-immunogenic in humans, and can easily be engineered and produced in prokaryotic hosts. A variety of methods are currently in use for the fast and efficient generation of target specific Nbs. Such Nbs are produced at low cost and associate with a high affinity to their cognate antigen. The construction of a naive camelid VHHs library from the blood serum of non-immunized camelids with affinity in the sub-nanomolar range and nanobody-based human heavy-chain antibodies as Coronavirus treatment are discussed below.

INTRODUCTION

Antibodies (ab) are glycoprotein, a "Y" shaped molecule which is produced by B lymphocytes in response to antigens. Immunoglobulins or the unique antibody fragments from the Camelidae family interact with antigens under only one single variable domain, referred to as VHH or Nanobody (Nb). Several characteristics make nanobody use superior to conventional antibodies. They are non-immunogenic and show high thermal and chemical stability. They can also recognize unusual antigenic sites such as enzyme active sites and can thus be used as enzyme inhibitors. The greater stability of nanobodies makes them very versatile in terms of potential applications. All these characteristics make them strong candidates as targeting agents for coronavirus treatment. In this review, we discuss the basics of SARS-COV-2 (severe acute respiratory syndrome coronavirus 2), their structure and its binding mechanism; Nanobody, their structure, production, and engineering into multivalent

formats to increase affinity or to produce bispecific antibodies which will help to treat against COVID-19.

Overview of Coronaviruses

Coronaviruses (COV) are a highly diverse family of enveloped positive-sense single-stranded ribonucleic acid (RNA) viruses. They are having largest genomes among all RNA viruses, typically ranges between 27 and 32 kb.[1] Coronavirus virions are spherical particles between 0.06 micron and 0.14 micron in diameter, averaging about 0.125 micron, measured by electron microscope.[2] The spike proteins on their envelope give the virions a crown-like shape ("corona" in Latin means crown) therefore they named as Coronavirus.[3] Their nucleocapsid proteins are basic proteins that encapsulate viral genomic RNA to form part of the virus structure. The nucleocapsid protein of SARS-COV is highly antigenic and associated with several host-cell interactions. They infect humans, other mammalian and avian species and cause respiratory, gastrointestinal, and central nervous system diseases.[4] It is estimated that each infected person carries 10^9-10^{11} virions during peak infection which implies that all SARS-CoV-2 virions currently in the world have a mass of only 0.1-1 kg.[5]

Family of Coronavirus

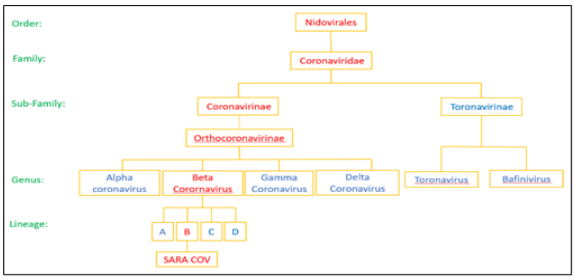

Fig. 01: Coronavirus Family Tree.

Coronaviruses belong to the Coronaviridae family, the Nidovirales order, and the Coronavirus genus. They are categorized into four major genera, α (Alphacoronavirus), β (Betacoronavirus), γ (Gammacoronavirus), and Deltacoronavirus.[6] Here we only discuss Prototypic genus β coronaviruses. This highly pathogenic human pathogen SARS-COV caused the SARS epidemic in 2002 to 2003, with over 8,000 infections and a fatality rate of 10%.[7] The genome of SARS-COV-2 shares about 80% identity with that of SARS-COV and is about 96% identical to the bat coronavirus Bat-COV RaTG13.[8]

Morphology of Coronavirus

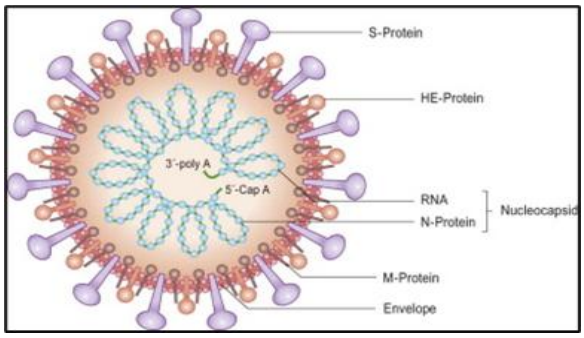

Fig. 02: Structure of Coronavirus.

Coronavirus is a large spherical particle of average diameter 120nm with unique surface projections. Its molecular weight is about 40,000 kDa.[9,10,11] It is enclosed within envelope of lipid bilayer anchored with membrane (M), spike (S) and enveloped (E) protein.[12.13] M protein is the main structural protein which maintain the shape of virus.[14] It is the most abundant protein in viral surface. M protein is also act as central organizer for assembly of coronavirus.[15] S protein is used for viral interaction with cell membrane receptor for fusion and facilitates entry of virus in host cell membrane.[16] E protein is present in minor amount, but it is highly variable in different species and act as surface structural protein. It helps in coronavirus assembling, intramolecular trafficking and budding.[17] M protein, S protein and E protein anchored with lipid bilayer form the outer enveloped of corona virus which protect them from outer environment and help in fusion with cell membrane.[18] N protein is nuclear capsid that coats the RNA genome of coronavirus which play an important role in replication, translation, and transcription. N proteins protect the viral RNA genome and allow the virus to utilize cell enzyme and protein.[19] Viral RNA gnome is comprised of 30,000 nucleotides. Virus also contain hemagglutinin-esterase dimer which is appeared to be important for infection of the host cell sometimes it may be involve in cell fusion but does not have any role in replication.[20] Some non-structural protein like RNA dependent RNA polymerase, papain-like protease and 3-chymotrypsin-like protease helps in replication and infection of the host.[21]

Table 01: Viral protein and their function.

Sr.	Protein Name	Function
1.	Spike Protein Or S protein	It is involved in spike protein receptor interaction, cell fusion and viral entry in host cell.
2.	Membrane Protein or M protein	Act as central organizer for protein assembly and maintains the shape of virus.
3.	Enveloped protein or E protein	It helps in intramolecular trafficking, budding and virus protein assembling.
4.	Nuclear protein or N protein	It is mainly involved in genome packaging and act as protective coat for viral RNA. It generally involved in RNA synthesis, replication, transcription, and translation process.
5.	Chymotrypsin like protease (3CL pro)	It plays a vital role in viral maturation, cleavage of translated polyprotein and in viral replication, etc.
6.	Papain like protease (PL pro)	It is Required for maturation of viral peptide, and it also antagonize the innate cellular antiviral response.
7.	RNA dependent RNA polymerase (RdRP)	It is an enzyme protein required for the synthesis of RNA strand complementary to RNA template.

COVID-19 Outbreaks

The first human cases of COVID-19, the disease caused by the novel coronavirus, subsequently named SARS-CoV-2 were first reported by officials in Wuhan City, China, in December 2019. COVID-19 is highly communicable disease with the lungs as the major pathological target, spread through the of droplet of infected person when they sneeze, speak or cough. After getting infected with SARS COV2 individual shows symptoms like dry cough, fever, shortness of breath, tiredness, chest pain. Older age group, small children with weak immunity and the patients with underlying congenital heart disorders are more vulnerable to get critically ill after they get infected with corona virus. On January 2020 World Health Organization (WHO) declare COVID 19 as public heath outbreak. Due to its high transmission ability at the end of February 2020, COVID 19 cases have been identifies in entire globe and show adverse effect on public health.[22]

Table 02: Symptoms of COVID 19.[23]

Most Common or Less Common Symptoms	Serious Symptoms
• Tiredness • Loss of taste • Sore throat • Headache • Diarrhea • Conjunctivitis • Fever	• Dyspnea • Chest pain • Aphasia • Facial paralysis

On September 2020 first case of COVID19 variant Strain was found in UK (United Kingdom) which is named as 20B501Y.V1/B.1.107. Mutation in new variant strain has been detected in their genome sequence. Researchers found that the mutation is takes place on receptor binding site of spike protein at 501.Y position of amino acid where asparagine (N) has been found to be replace by tyrosine (Y) which results in increase affinity of spike protein towards ACE2 receptor. Another reason of mutation is 69/70 deletion at 69 and 70 amino acid which is likely to change Spike protein conformation. This mutation has been found in many variant strain of SARS COV19. Research shows the new variant strain is 70% more infectious than older one, but it has not been found to be much deadlier and spread rapidly from one person to another. Viral strain has its effect on under 20 age population, which believe so far having less viral complication.[24,25]

The other variant of COVID 19 was first identified at Nelson Mandela Bay in South Africa's east coast in October 2020. this strain is already a dominated viral strain of South Africa and many cases have been found in Japan, Norway, and Austria. This variant strain is name as 20C501YV2/ B.1.3.8. it has found to be different from UK strain and have more mutation variant than Beta variant. According to research, similar to B.1.1.7. this strain show changes on 501Y position of amino acid but does not show 69\70 deletion, which increases its affinity towards ACE2 receptor and can rapidly spread in population. As per the report about 80 to 90% new cases of Africa are related to new strain variant of SARS COV19. Scientists have found that Africa variant might be resistive to current vaccine and might evade the antibody response which show effect on normal SARS COV19 virus. And it again makes the quiz more complicated for the scientists.[24,26]

The another new COVID 19 variant strain firstly detected in India in late of 2020 is named as Delta variant (lineage B. 1.617.2) which is responsible for the mass serge of COVID 19 cases in April and May in India. This variant also has been detected in 80 countries and it is believed to be more contagious than other variants. WHO said that, Delta has the potential "to be more lethal because it's more efficient in the way it transmits between humans," Data suggest that the Delta variant found in India is 60 % more transmissible than Alpha variant and it has been found to be more resistant toward vaccine (as per report particularly when only ne dose of vaccine is given) according to the Health Authorities the variant has been found to have high risk of hospitalization and death.

As of June 25, 2021, globally 180 million cases of COVID 19 are confirm across 191 countries, of these 165 million peoples are recovered. There is total 11.3 million active cases among them 99.3 cases are in mild condition while 0.7% cases are in critical condition and 4.0 million peoples have lose their life.[27]

Spike protein and its function

The coronavirus spike protein (S protein) is a class I fusion protein, an enveloped glycoprotein essential for viral entry into host cells by first binding to a receptor on the host cell surface and then fusing viral and host membranes. The spike protein is composed of a linear chain of 1,273 amino acids, neatly folded into a structure, which is studded with up to 23 sugar molecules. Spike proteins like to stick together, and three separate spike molecules bind to each other to form a functional "trimeric" unit.[28]

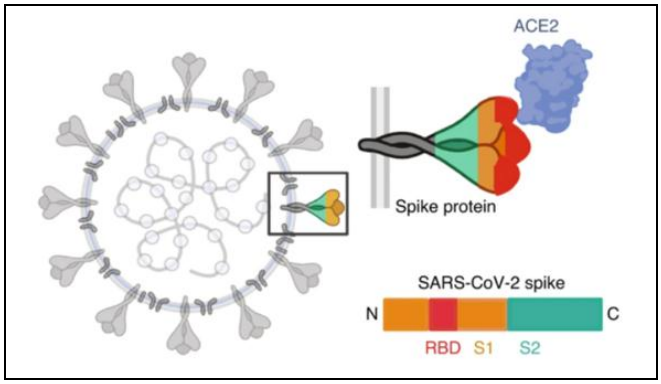

Fig. 03: Structure of spike protein.

The spike protein consists of three segments—an ectodomain, a single-pass transmembrane anchor, and a short intracellular tail. The ectodomain compound a receptor binding S1 subunit and a membrane fusion S2 subunit. The S1 contains two independent subdomains, an N-terminal domain (NTD) and a C-terminal domain (CTD), both can function as receptor binding domains (RBDs) and bind a variety of proteins and sugars. The S2 is responsible for fusion, contains the putative fusion peptide (FP), a transmembrane domain, and two heptad repeat (HR) regions, HR1 and HR2. The S2 domain is the most conserved region of the protein, whereas the S1 domain diverges in sequence even among species of a single coronavirus.

The S protein plays a crucial role in the induction of neutralizing-antibody and T-cell responses, as well as protective immunity. The pivotal functions for the spike protein are as follows:

1. Mediate receptor binding and membrane fusion, efines the range of the hosts and the specificity of the virus.
2. The main component to bind with the neutralizing antibody.
3. Key target for vaccine design.
4. Can be transmitted between different hosts through gene recombination or mutation of the receptor-binding domain (RBD), leading to a higher mortality rate.[29,30,31]

What is ACE2 receptor?

Angiotensin-converting enzyme 2 (ACE-2) is a type I transmembrane zinc-dependent carboxypeptidase with homology to ACE, which is an important regulator of the renin-angiotensin system (RAS), which plays a vital role in maintaining fluid salt balance and blood pressure homeostasis in mammals. In Acute respiratory distress syndrome (ARDS), the RAS appears crucial in maintaining oxygenation, possibly as widespread lung injury would otherwise result in a complete pulmonary shutdown.[32]

The predicted human ACE-2 protein sequence consists of 805 amino acids, including an N-terminal signal peptide, a single catalytic domain, a C-terminal membrane anchor, and a short cytoplasmic tail. ACE-2 helps to cleave angiotensin I and II as a carboxypeptidase. ACE2 is present in many cell types and tissues, including the lungs, heart, blood vessels, liver, kidneys, and gastrointestinal tract. In addition, it is also present in epithelial cells, which line certain tissues and create protective barriers.[33]

Because of its location and specificity, ACE plays auxiliary roles in immunity, reproduction, and neuropeptide regulation. ACE exists in two isoforms. Somatic ACE consists of two highly similar protease domains while germinal ACE consists of a single protease domain. ACE-2 comprises a single protease domain and is an essential regulator of lung and heart function. ACE2 protein also emerges to be the entry point receptor for the severe acute respiratory syndrome (SARS) coronavirus.[34]

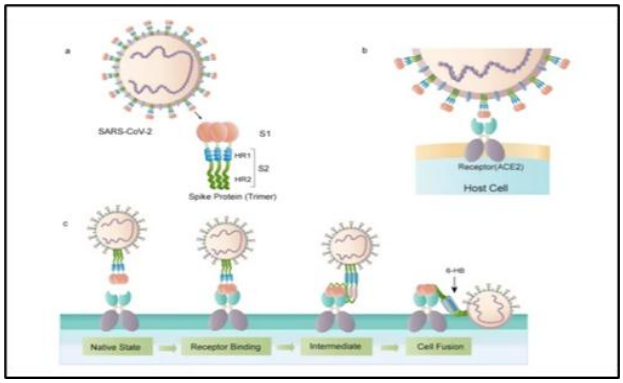

Fig. 04: Binding of viral protein spike with ACE2 receptor.

Receptor recognition by viruses is the first and essential step of viral infections of host cells. It is an important determinant of viral host range and cross-species infection and a primary target for antiviral intervention. Coronaviruses recognize a variety of host receptors, infect many hosts, and are health threats to humans and animals. The receptor-binding S1 subunit of coronavirus spike proteins contains two distinctive domains, the N terminal domain (S1-NTD) and the C-terminal domain (S1-CTD), both of which can function as receptor-binding domains (RBDs). S1-NTDs and S1 CTDs from three major coronavirus genera recognize at least four protein receptors and three sugar receptors and demonstrate a complex receptor recognition pattern. For example, highly similar coronavirus S1-CTDs within the same genus can recognize different receptors, whereas very different coronavirus S1-CTDs from different genera can recognize the same receptor. Moreover, coronavirus S1-NTDs can recognize either protein or sugar receptors. Structural studies in the past decade have elucidated many of the puzzles associated with coronavirus-receptor interactions.

Overall, coronaviruses have evolved a complex receptor recognition pattern: (I) coronaviruses use one or both S1 domains as RBDs; (ii) highly similar coronavirus S1- CTDs within the same genus can recognize different protein receptors, whereas very different coronavirus S1-CTDs from different genera can recognize the same protein receptor and (iii) coronavirus S1-NTDs can recognize either protein or sugar receptors. Understanding the receptor recognition mechanisms of coronaviruses can provide critical insight into the origin, evolution, and receptor selection of coronaviruses. The SARS-CoV belong to the β-genus and their S1-CTDs recognize receptor is angiotensin-converting enzyme 2 (ACE2).

The spike (S) glycoprotein of SARS-CoV-2 binds angiotensin-converting enzyme 2 (ACE2) on host cells. S is a trimeric class I viral fusion protein that is proteolytically processed into

S1 and S2 subunits that remain noncovalently associated in a prefusion state. Upon engagement of ACE2 by a receptor binding domain (RBD) in S1, conformational rearrangements occur that cause S1 shedding, cleavage of S2 by host proteases, and exposure of a fusion peptide adjacent to the S2' proteolysis site. Folding of S to a post fusion conformation is coupled to host cell–virus membrane fusion and cytosolic release of viral RNA. Atomic contacts with the RBD are restricted to the extracellular protease domain of ACE2. Soluble ACE2 in which the transmembrane domain has been removed is sufficient for binding S and neutralizing infection.

The different capabilities of SARSCoV-2 RBD proteins to enhance ACE2 enzymatic activity suggested that the stronger interaction between RBD and ACE2. These results highlighted the potential for SARS-CoV-2 infection to enhance ACE2 activity, which may be relevant to the cardiovascular symptoms associated with COVID19. The viral spike protein-mediated cleavage enhancement is specific to carboxypeptidase activity of ACE2 rather than potential contaminating protease present in the spike preparation as the enzymatic enhancement was abolished in the presence of a potent ACE2 specific inhibitor MLN-4760. Surprisingly, SARS-CoV-2 spike enhanced ACE2 proteolytic activity on both caspase-1 substrate and bradykinin-analogue, and the enzymatic enhancement was mediated by the spike RBD domain binding. The ability of SARS-CoV2 spike protein to alter ACE2 enzymatic activity may result in dysregulation of RAS and.[29,30,31,35]

Structure of nanobody

The conventional antibodies or the most abundant type of antibody in circulation, immunoglobulin G (IgG) comprise two identical heavy (H) and two identical light (L) chains held together by interchain disulfide bonds. The antigen is recognized through an interplay between the variable N-terminal domains of the heavy (VH) and the light (VL) chain and six CDRs. Binders derived from IgGs can be classified as antigen-binding fragments (Fab, ca. 50 kDa), single-chain variable fragments (scFv, ca. 25 kDa,) and heavy- or light-chain single domains (VH or VL, ca. 12.5 kDa). Fab and scFv binders consist of both the VH and VL domain of the parental IgG and retain the size and affinity of the area binding the antigen.

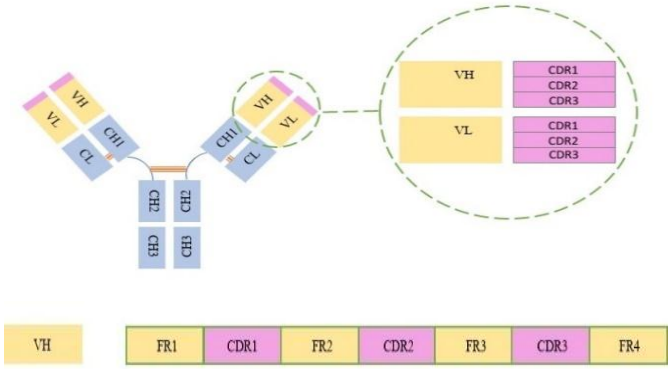

Fig. 05: IgG schematic representation with heavy chain (domain VH, CH1, CH2 and CH3) and light chain (domain VL and CL).

A nanobody is typically composed of three complementarity-determining regions (CDRs), alternated with four framework regions (FRs). The HCAbs of Camelidae have a unique structure. They are devoid of light chain polypeptide and composed of three instead of four globular domains as they lack the first constant domain (CH1) which is present in the genome, but is spliced out during mRNA processing. The absence of the CH1 domain leads to their absence of the light chain in the HcAbs, at this domain is the anchoring place for the constant domain of the light chain. As a result the functional antigen-binding unit reduced to a single variable domain (VHH). These properties allow the generation of potent, recombinant VHHs by isolating and engineering the corresponding single domain region of Camelidae B cells after immunization. Based on their small size of around 13–14 kDa, recombinant VHHs are often referred to as 'Nanobodies'. The hinge region and two constant domains (CH2 and CH3) are highly homologous to the Fc domains (CH2-CH3) of classical antibodies.

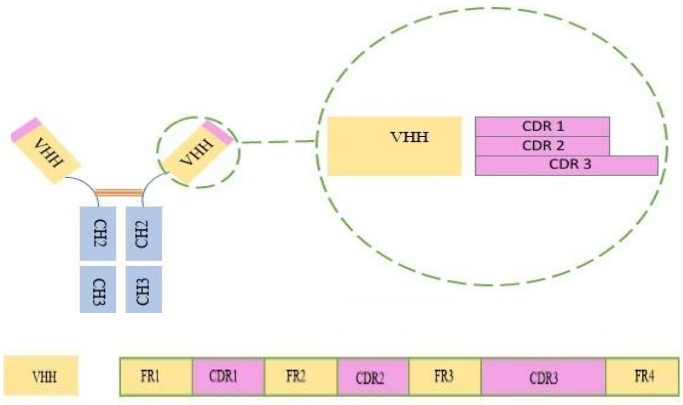

Fig. 06: Heavy chain only antibody (HCAbs), schematic representation with domain VHH, CH2, CH3.

2D structure of nanobody

The VHH domain is comprised of a folded βsheet with three loops in the regions homologous to the CDRs of the IgG VH domains. As nanobodies lack the variable domain of the light chain, they only contain three instead of six CDRs. The length of CDR3 loop in the VHH can exceed 20 amino acid residues, compared to the typical length of nine (mouse) or twelve (human) in the conventional antibodies. In order to provide an adequate antigen-interacting surface of 600–800 Å2, nanobodies have longer CDR1 and CDR3 loops than VHs (variable domain of the heavy chain) of conventional antibodies, resulting in similar binding affinities. In dromedary nanobodies, these long loops are often connected by a disulfide bond that restricts their flexibility and consequently, favors antigen binding. A longer CDR3, the convex shape of the antigen binding site and the small size allow nanobodies to access epitopes that may be cryptic and non-antigenic for conventional antibodies. In fact, unlike the conventional antibodies, which more of ten detect linear or planar epitopes, many nanobodies bind to concave and discontinuous epitopes that only form in the folded protein. Nanobodies that bind discontinuous epitopes can be selected from the initial panel by competition with linear peptides or with an unfolded protein, or by using the masked selection technique. These CDRs are organized in three loops, separated by more conserved framework regions (FRs) and cluster at the N-terminal side of the nanobody.

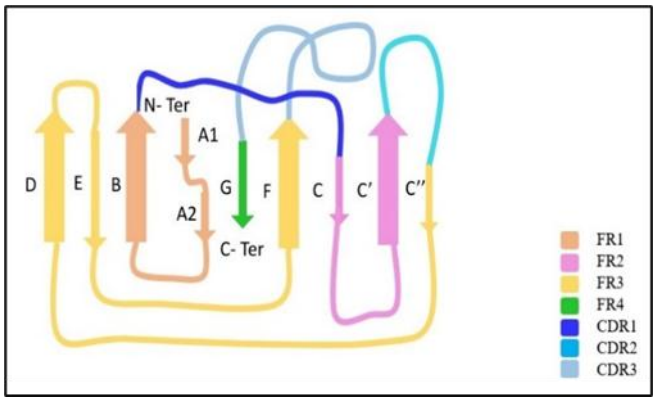

Fig. 07: 2D representation of immunoglobin fold of VHH domain with remarketed framework region (FRs) and Complementary Determining Region (CDRs) loop length are approximated for representation purpose FR1 is composed of β-stands A1, A2 and B, FR2 is composed of β-strand Cand C' FR3 of 4β-strand C'', D, E and F FR4 end the VHH sequence by last β-strand, G.

The FRs regions are commonly considered as 'constant' regions both in terms of sequence and structure while the CDRs are variable/ hypervariable.[36,37,38,39,40]

Production mechanism of Nanobodies[41]

In early 1990 microorganisms had been used as source for the production of conventional antibodies, now a days nanobodies are the versatile tool of immunization due to its simple structure, high specificity and high affinity. Some species of camelids have become a popular source for the generation of nanobodies, as they can produce both conventional antibodies as well as heavy chain antibodies which allow the production of single domain antibodies. Immunization of specific antigen to camelids and the blood withdrawal is the basic principle of nanobody production. It also involves RNA recombination, PCR technique, library construction, M13 phage display for the production, isolation, and selection of single domain antibody.

It involves following main steps

- Generation of antigen specific alpaca antibody
- Lymphocyte purification for library construction.
- Antibody panning.

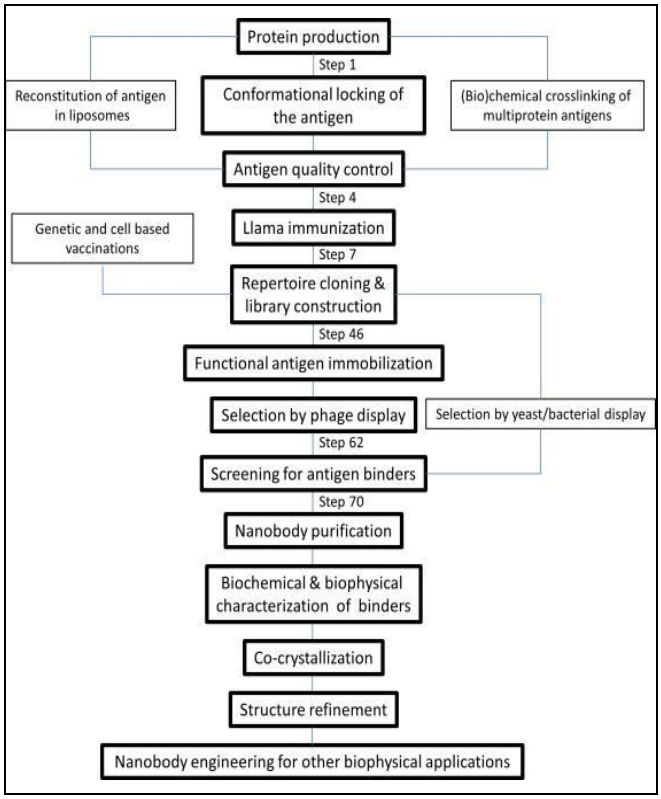

Fig. 08: Steps for nanobody production.

A) Generation of antigen specific alpaca antibodies

It consists of following steps

1. Alpaca handling

Obtain adult alpacas, 6 years aged with a body condition score at the minimum of 3.0 (scale 1-5). As they are herd animals acquire a minimum of two, but preferably three animals together.

Note: Males are recommended because, habitually, they have a more even temperament and are easier to work with.

Check the health status of animals through a veterinary examination, including the review of diet, housing, vaccination, and parasite control programs and ensure that the animals are up to date with vaccinations appropriate to the local geographic region based on professional Due to alpacas' typically thicker coats, alpacas require close examination to reveal potential ailments and injuries that you may not notice through a cursory observation.

Acclimatize to halter training and exposure to restraint for at least one week.

Collect a 4 ml blood sample to acquire serum for use as a pre-immunization control. Freeze the sera and store it at -20 °C in 0.5 ml aliquots.

Note: Promptly, additional blood can be taken for a complete blood cell (CBC) analysis to monitor the animals' initial health status.

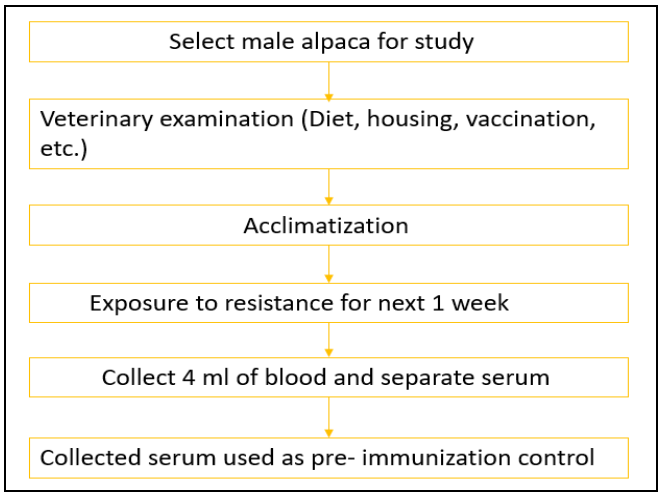

Fig. 09: Alpaca handling flow chart.

2. Antigen preparation

Formulate purified protein antigens in phosphate Suffered saline (PBS) or HEPES-buffered saline (HBS) and concentrate to ≥1 mg/ml. Approximately 3 mg of protein is necessitated for the complete protocol, coupled with immunization, panning, and confirmation of clones.

Note: Camelid antibodies exhibit high specificity and selectivity against protein antigens but are habitually not well adapted for unstructured proteins or peptide antigens.

- An analytical method such as DS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) helps to establish > 90% desired antigen purity.
- Prepare frozen aliquots of antigen that contain a total of 100 µg of antigen. 50 µl of a 2 mg/ml preparation is ideal. 100 µL of 1 mg/ml is the most dilute protein solution suitable for immunization. Alternatively, if the antigen cannot undergo freeze/thaw and is known to be stable in the solution long term, it can be stored at 4 °C.

Note: To ensure the antigen can undergo a thaw cycle, test a 20 µL aliquot of the antigen should be dispensed into a thin-walled PCR tube and snap-frozen in liquid nitrogen. Thaw the tube, perform high-speed centrifugation, and verify the quantitative recovery by comparing UV/VIS absorption before and after freezing or by running an SDS-PAGE gel. If feasible, the antigen should also be tested for the retention of biological activity. If the freeze-thaw cycle is successful, the remaining antigen is snap-frozen in 100 µL aliquots and stored at -80 °C. If there are issues with the freeze/thaw cycle, the protocol can be repeated with the addition of up to 20% glycerol.

3. Immunization

- Trim alpaca's hair and clear the site of immunization (trim the hair mostly on neck region of alpaca.)
- Mix five antigen each of about ≈ 200µg in a glass test tube and dilute the solution up to ≈300 to 1000µl using water.

Note: Use glycine, MOPS (3-(N-morpholino) propane sulfonic acid), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) as buffers if necessary.

PH of between 5 and 6 has often prove to be more favorable than neutral PH. Avoid contact of polyvalent ion like phosphate ion, citrate ion, etc. as they can cause coagulation.

- Inject the antigens in alpaca using 22G needle.

- Monitor the animal at least for 20 minutes, monitor for signs of anaphylaxis, monitor the inflammation at the site of injection.
- Ensure that excessive blood leaking is not occur from site of injection.
- Give five injections weekly containing 100µg of each antigen in the mixture.

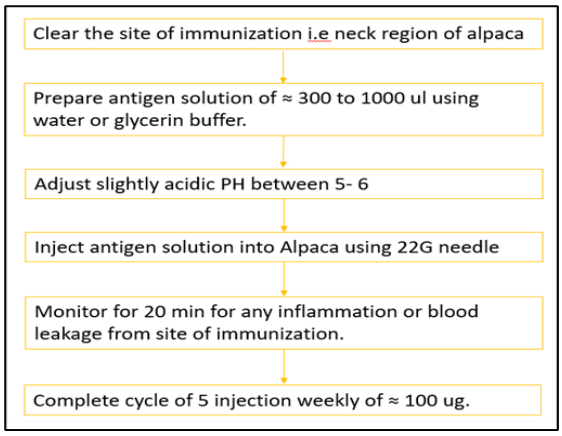

Fig. 10: Immunization flow chart.

4. Blood withdrawal from alpaca

- Disinfect the site of blood collection using ethanol swab and properly hold the neck of alpaca.
- Apply the pressure on medial of vein for proper visualization and palpitation. (As jugular vein is present in neck region, due to thick skin and neck musculature of male alpaca it is difficult to visualize jugular vein easily.)
- Inject the injection needle (most probably at 45°) to the jugular vein, in such a way that projection should be towards neck.
- Inject 4 to 10 ml of alpaca's blood for analytical purpose and collect it in glass test tube containing EDTA.

5. Immune monitoring and Alpaca monitoring

- After blood withdrawal take blood sample test tube and to obtained serum. Take additional blood sample to monitor the health of animals.
- Determine the presence of antigen specific antibodies by performing ELISA (enzyme-linked immunosorbent assay).
- Prepare antigen affinity plates using surface treated well plate.

- Dilute 0.1 µg of antigen in 100 mm of sodium carbonate, maintain alkaline PH (PH= 9.0) and add 100 µL of solution in each well and keep the plates for incubation over night at 4°c.
- Prepare antigen affinity plates using 96- well plate. Dilute 0.1 µg of antigen in mm of sodium carbonate, adjust the PH at 9.
- Add 100 µL of solution in each well and keep plates for overnight incubation at 4 °C. Ten dilution of all well are tested.
- After overnight incubation remove the solution of wells by inverting the plates and wash the plates with 0.1 ml of PBS.
- Add 0.1 ml of BSA in wells and incubate the plates for 1 h at room temperature. after 1 h wash the plates with PBS and remove the content by inversion of plates.
- Prepare dilution of pre immune and post immune serum each of 0.3 ml in a ratio of 1\100.
- Add 100 µL of each dilution to the wells and incubate the plates for 2 h. After incubation remove the content from each well and wash the plates three times using PBS.
- Add 0.1 mL of HRP-conjugated goat anti-llama IgG antibody in each well and incubate the plates for 1h and then remove the solution and wash the plates with PBS.

Note: Here we can record immune response including both conventional as well as heavy chain antibodies.

- Add 100 µL of chromogenic peroxidase substrate to the well and incubate at room temperature until the intensity of the two highest concentration points have become saturated. After getting higher concentrated point add 100 µL of 0.1 M sulfuric acid to each well to stop the reaction.
- By using plate reader measure absorbance rate of each well and plot the graph of absorbance.
- Monitor alpacas throughout the process.

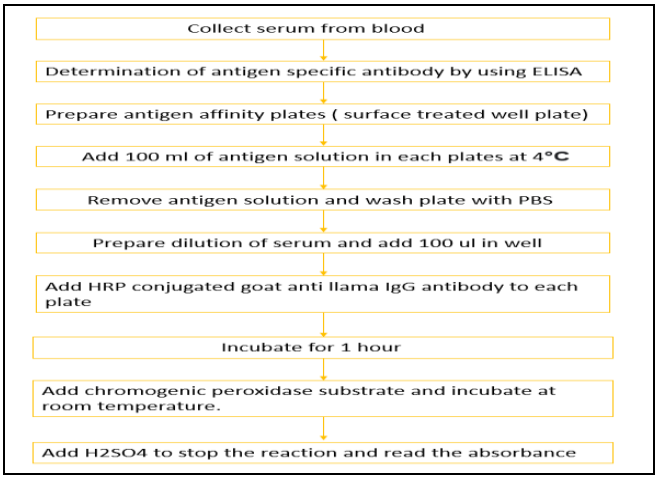

Fig. 11: Immune monitoring flow chart].

B. Lymphocyte purification for library construction.

- After three to five days of last injection collect 50 ml of blood.
- Dilute 15 ml of blood with 5 ml of PBS in lymphocyte separation tube and spin it for 20min to isolate blood lymphocyte by centrifugation method.
- Isolate the total RNA.
- By combining 10 µg of isolated RNA with a mixture 0.1 µg of Oligo(dT)12-18 primers and reverse transcriptase enzyme synthesize cDNA.
- Using specific vector and cloning technique generate gene library.

C. Antibody panning

- Prepare antigen affinity plates using 96- well plate. Dilute 0.1 µg of antigen in mm of sodium carbonate, adjust the PH at 9.
- Add 100 µL of solution in each well and keep plates for overnight incubation at 4 °C. Ten dilution of all well are tested.
- After overnight incubation remove the solution of wells by inverting the plates and wash the plates with 0.1 ml of PBS.
- Treat the phage solution with 100 µL of 40 mg/mL BSA in PBS on vibrating machine for 30 minutes.
- Add phage solution to each antigen coated well and incubate the plates for 2 h.
- Remove unbound phage solution by the inversion of plates and wash the plates with PBS.
- Remove the absorb unwanted substance from phage bounded region by adding 0.1 mL of 0.25 mg/mL trypsin in PBS and incubate the plates for 30 min on vibrating plate form

- Then add content to small glass bottle containing 5 µL of 4 mg/mL 4-benzenesulfonyl fluoride hydrochloride (AEBSF) solution to balance the activity of trypsin.
- Grow TG1 phage display competent cells at 37 °C with shaking, add 50 µL of eluted phage to 3 mL of the TG1 cells and incubate for 30 min.
- Add 7 ml of Lysogeny broth media containing 100 µg/mL ampicillin, 2% glucose and keep it for overnight incubation.
- After overnight incubation, plate the bacteria on newly prepared LB media and keep the plate for incubation at 37°C.
- Pick the colonies and inoculate it in sterile 96- well plates100 µL of 2XYT with 100 µg/mL ampicillin, 2% glucose, 10% v/v glycerol per well and keep the plates for overnight incubation.
- Take 0.2 µL of media in PCR tube. Perform PCR reaction using appropriate primer for getting positive clones.
- Inoculate positive clones into fresh new tubes and keep the tubes for overnight incubation at 37°C.
- Obtained plasmid DNA and standard sequence of positive clones using pEX-Rev primer.
- Perform computational analysis to get single domain antibody sequence and ensure that no stop codons are present in the sequence.
- Express the single domain antibody using a BL21-derived expression strain of E. coil.
- Purify the single domain antibody using immobilized metal affinity chromatography (IMAC) technique.
- Perform ELISA technique to determine accuracy of single domain antibody production.

Neutralization of SARS-CoV-2 spike against ultrapotent synthetic nanobody

1. Researchers created a pool of DNA sequences, where each strand of DNA codes for a unique nanobody.
2. They screened through yeast surface-display library to narrow the pool of 2 billion nanobody sequences that would likely bind to SARS-CoV-2 spike ectodomain and within three weeks, they identified 800 potential candidates.
3. Researchers then put those DNA sequences into individual yeast cells which has a unique nanobody tethered to its surface. So, in a test tube full of more than 2 billion of yeasts, each individual yeast will have a different nanobody on its surface.

4. Then mixed these individual nanobody-displaying yeast cultures with fluorescently labeled mutant form of SARS-CoV-2 Spike (Spike S2P) and excess human ACE2 protein.
5. Researchers then added a magnetic particle to each spike and eventually they turned on a magnetic field and appeared like "fishing lures" rise to the surface with the nanobodies, bound most effectively to the spikes attached. Some nanobodies held loosely to the spike proteins; others were bound more closely.
6. After a two to three-week whittling process, 21 distinct nanobodies showed competition with the ACE2 protein that each appeared to optimize three competing factors. But in the presence of a dimeric construct of the ACE2 extracellular domain (ACE2-Fc), they showed decreased binding.
7. These nanobodies are later classified into two types. Class I nanobodies, namely Nb6 and Nb11, binds the RBD and competes directly with ACE2-Fc for the same binding site on the spike and displays binding to RBD alone. On the other hand, Class II nanobodies such as Nb3 targets a different domain but displays no binding to RBD alone. In the presence of excess ACE2-Fc, binding of class I nanobodies is blocked entirely, whereas binding of class II nanobodies is moderately decreased. These results suggest that class I nanobodies target the RBD to block ACE2 binding, whereas class II nanobodies target other epitopes. As both classes revealed decreased binding activity in the presence of increased ACE2, this classification would allow the team to identify the most potent candidate and engineer an ultrapotent version of itself.
8. The ideal nanobody would need to bind quickly and tightly to the spike, remain robust in the presence of heat and other degrading real-world factors, and be as "human-like" as possible.
9. Ultimately, they narrowed the nanobody field down to the best of the entire lot; what they called Nb6. Using cryo-EM, they studied Nb6 at the atomic scale as it clamped on to the COVID-19 spike protein. After some adjustment they enhanced Nb6's binding affinity to the spike by another 500 times.
10. The researchers turned their modified nanobody (mNb6) into a trifold nanobody specifically engineered to fit the precise contours of the novel coronavirus's trimer spike protein, namely mNb6-tri. The structure of Nb6 bound to closed Spike enabled us to engineer bivalent and trivalent nanobodies predicted to lock all RBDs in the down state.
11. Affinity, maturation, and structure-guided design of multivalency yielded a trivalent nanobody, mNb6-tri, with femtomolar affinity for SARS-CoV-2 Spike and picomolar

neutralization of SARS-CoV-2 infection. mNb6-tri retains stability and function after aerosolization, lyophilization, and heat treatment. These properties may enable aerosol-mediated delivery of this potent neutralizer directly to the airway epithelia, promising to yield a widely deployable, patient-friendly prophylactic and early infection therapeutic agent to stem the worst pandemic in a century.[42,43,44]

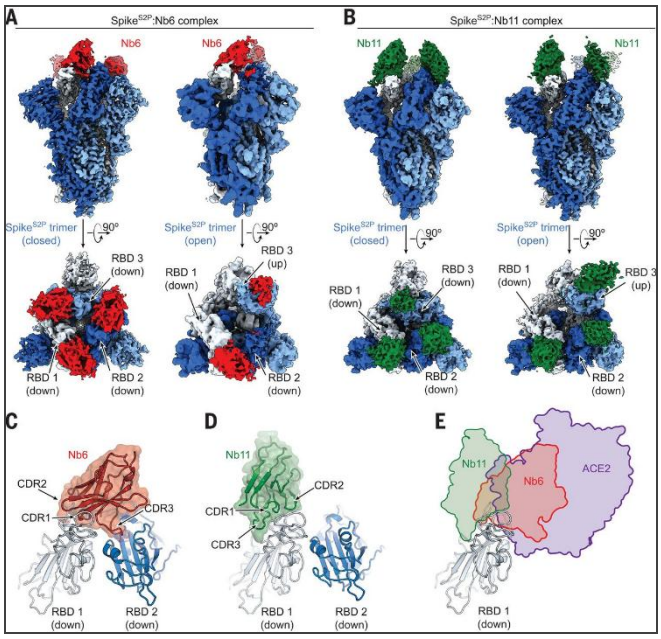

Fig. 12: Cryo-EM maps of the Spike S2P -Nb6 complex in either closed (left) or open (right)Spike S2P conformation. (B) Cryo-EM maps of the Spike S2P -Nb11 complex in either closed (left) or open (right) Spike S2P conformation. The top views show RBD up or down states. (C) Nb6 straddles the interface of two down-state RBDs, with CDR3 reaching over to an adjacent RBD. (D) Nb11 binds a single RBD in the down state (displayed) or similarly in the up state. No cross-RBD contacts are made by Nb11 in either RBD up or down state. (E) Comparison of RBD epitopes engaged by ACE2 (purple), Nb6 (red), or Nb11 (green). Both Nb11 and Nb6 directly compete with ACE2 binding.[42]

To define the binding sites of class I nanobodies, first determined their cryo-EM structures bound to Spike (Fig. A and B). Both the Nb6 and Nb11 recognize RBD epitopes that overlap the ACE2 binding site (Fig. E). Nb6 bound to closed Spike straddles the interface between two adjacent RBDs. Most of the contacting surfaces mostly contain CDR1 and CDR2 of Nb6 (Fig. C). CDR3 contacts the adjacent RBD positioned counterclockwise when viewed from the top (Fig. C). The binding of one Nb6 therefore stabilizes two adjacent RBDs in the down

state and likely preorganized the binding site for a second and third Nb6 molecule to stabilize the closed Spike conformation. By contrast, Nb11 bound to down-state RBDs only contacts a single RBD (Fig. D).

AeroNabs[45,46,47,48]

A research team at the University of California, San Francisco (UCSF) engineered a more potent version of the nanobodies, which resulted in the creation of a completely synthetic, production-ready molecule; "AeroNabs" that straitjackets the crucial SARS-CoV-2 machinery. In an aerosol formulation, these molecules could self-administer with a nasal spray or inhaler. While it is not a traditional vaccine, used once a day, AeroNabs could provide powerful, reliable protection against SARS-CoV-2 and has promising, immediate therapeutic treatment.

Nanobodies can be both an order of magnitude lighter than COVID and relatively easy to characterize and potentially even engineer on an atom-by-atom basis. Through a lock-and-key-like interaction between an ACE2 receptor and a spike RBD, the virus enters the cell but most potent nanobodies act as a potential neutralizer, blocked spike-ACE2 interactions by strongly attaching themselves directly to the spike RBDs. Cryogenic Electron Microscopy (Cryo-EM) revealed that one exceptionally stable nanobody, Nb6, function a bit like a sheath that covers the RBD "key" and prevents it from being inserted into an ACE2 "lock", an inaccessible down-state which is incapable of binding ACE2. Due to inherent stability of nanobodies, there is no loss of antiviral potency in the aerosolized form which suggests AeroNabs are a potent SARS-CoV-2 antiviral that could be practical to administer via a shelf-stable inhaler or nasal spray.

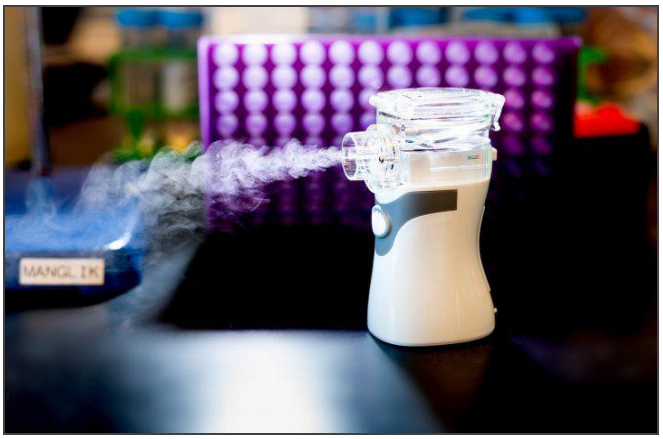

Fig. 13: AeroNabs.

Advantages of AeroNabs

Several advantages of AeroNabs over traditional therapeutic antibodies.

- These molecules are inexpensive to mass produce in bacteria or yeast, and stable to transport in the form of powders.
- AeroNabs can be easily aerosolized and self-administered with a nasal spray or inhaler.
- Vaccination process is critical, and it may take years before the whole world can get vaccinated. This llama inspired AeroNabs could be used as a sort of personal protective equipment, a "stopgap" until the vaccines become available for all.

As the dynamics of the virus change over the coming year, especially that there is a new potential pandemic continues to emerge, one can image there is a version of virus resistant to vaccine effort and there is a new version of vaccine that only provides 50 percent effectiveness. So, in that situation options like nanobody treatment become far more important.

CONCLUSION

Stability, resistivity, and specificity of nanobody make them boon for health services. Its similarity with the sequence of conventional antibody and its potential to give strong and specific immune response against any antigen may reduce the concern about the immunological reaction that clear its way to enter clinical practices in future. Its high specific binding potential with the spike protein of an antigen may make nanobodies a powerful medication against the sever diseases which are not cured by the conventional antibody.

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