Development of multi-specific humanized llama antibodies blocking SARS-CoV-2/ACE2 interaction with high affinity and avidity

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Coronaviruses are enveloped, positive-sense single-strand RNA viruses with mammalian and avian hosts. Previous coronaviruses are known to infect humans include 229E, NL63, OC43, HKU1, SARS-CoV, and MERS-CoV, which cause a range of mild seasonal illnesses to severe diseases outbreaks. Notably, the past outbreaks of severe acute respiratory syndrome (SARS) (2003) and the Middle East respiratory syndrome (MERS) (2012) were caused by the coronaviruses SARS-CoV and MERS-CoV, respectively [1]. SARS-CoV-2, that emerged in December 2019, is the seventh known coronavirus to infect humans, and block the S/ACE2 interaction were identified. Furthermore, pairwise combination of VHHs showed synergistic blocking. Multi-specific antibodies with enhanced affinity and avidity, and improved S/ACE2 blocking are currently being developed using an in-silico approach that also fuses VHHs to Fc domains. Importantly, our current bi-specific antibody shows potent S/ACE2 blocking (KD \sim 0.25 \text{nM}, IC100 \sim 36.7 \text{nM}, IC95 \sim 12.2 \text{nM}, IC50 \sim 1 \text{nM}) which is significantly better than individual monoclonal VHH-Fcs. Overall, this design would equip the VHH-Fcs multiple mechanisms of actions against SARS-CoV-2. Thus, we aim to contribute to the battle against COVID-19 by developing therapeutic antibodies as well as diagnostics.

Coronaviruses cause severe human viral diseases including SARS, MERS and COVID-19. Most recently SARS-CoV-2 virus (causing COVID-19) has led to a pandemic with no successful therapeutics. The SARS-CoV-2 infection relies on trimeric spike (S) proteins to facilitate virus entry into host cells by binding to ACE2 receptor on host cell membranes. Therefore, blocking this interaction with antibodies are promising agents against SARS-CoV-2. Here we describe using humanized llama antibody VHVs against SARS-CoV-2 that would overcome the limitations associated with polyclonal and monoclonal combination therapies. From two llama VHH libraries, unique humanized VHHs that bind to S protein and block the S/ACE2 interaction were identified. Furthermore, pairwise combination of VHHs showed synergistic blocking. Multi-specific antibodies with enhanced affinity and avidity, and improved S/ACE2 blocking are currently being developed using an in-silico approach that also fuses VHHs to Fc domains. Importantly, our current bi-specific antibody shows potent S/ACE2 blocking (KD \sim 0.25 \text{nM}, IC100 \sim 36.7 \text{nM}, IC95 \sim 12.2 \text{nM}, IC50 \sim 1 \text{nM}) which is significantly better than individual monoclonal VHH-Fcs. Overall, this design would equip the VHH-Fcs multiple mechanisms of actions against SARS-CoV-2. Thus, we aim to contribute to the battle against COVID-19 by developing therapeutic antibodies as well as diagnostics.
targeting multiple epitopes have better viral neutralizing ability than single monoclonal antibodies [7]. However, immunoglobulin isolation from COVID-19 survivors is limited by the lack of availability of plasma from donors, and combinatorial treatment with several monoclonal antibodies is also limited due to high cost of production and potential toxicity. In order to overcome these existing limitations, we employed a novel approach of using humanized llama antibodies that blocks the interaction of SARS-CoV-2 S protein and ACE2, with the goal of rapidly developing high affinity and avidity bi- or tri-specific therapeutic antibodies that neutralize SARS-CoV-2 before it infects cells. In addition, previous reports have shown that if viruses are bound by low titre therapeutic antibodies with low affinity and avidity, the Fc-FcR interaction might trigger antibody-dependent enhancement (ADE) of virus entry into host cells [8]. Therefore, we also aimed to circumvent ADE by developing high titre neutralizing antibodies.

We used one naïve and one designed synthetic llama VHH library for this approach. The naïve library was constructed with PBMCs from 65 llamas, and the synthetic library was constructed from the naïve VHH library, where the VHH framework was partially humanized and the CDR1, 2, and 3 of VHH were shuffled to generate enhanced diversity and keep low immunogenicity. We panned the two llama VHH libraries against recombinant SARS-CoV-2 S protein. After panning, we obtained 91 high-affinity VHH hits for SARS-CoV-2 S protein binding, among which, 69 were unique sequences. We also assessed the ability of VHVs to block the S/ACE2 interaction in-vitro and discovered that 15 out of 69 unique S protein binders had S/ACE2 blocking function, top 9 of them are listed in Figure 1. Follow-up studies revealed that pairwise combination of some of the 9 VHH blockers led to synergistic blocking efficacy of S/ACE2 interaction. Notably, a combination of VHH1 with any other VHH did not affect its blocking function, making it the possible base unit in multi-specific antibody design (Figure 1).

By selecting and fusing two or three different humanized VHH sequences that target different but adjacent S protein RBD epitopes with high affinity and avidity into a single multi-specific antibody, while avoiding...
binding competition among the VHHs to S protein, we aim to improve overall S protein binding affinity and avidity and increase the S/ACE2 blocking function of our therapeutic antibodies. This design is aided by analysing different VHH combinations in-silico with modelled structures via our signature computer-aided antibody design (CAAD). We also use CAAD to further improve the VHH sequences so that the VHHs have low immunogenicity in humans, and high developability/manufacturability. The designed candidates are fused to human IgG Fc domains, and analysed in-vitro by binding and blocking assays to determine their S protein binding and S/ACE2 blocking capabilities, respectively. As expected, the bispecific VHH-Fc antibody we have currently generated shows significantly better S protein binding and S/ACE2 blocking functions than each monoclonal VHH-Fc at therapeutically relevant concentrations, that is consistent in three independent attempts. It shows potent S/ACE2 blocking, with ~100% blocking at 36.7 nM, and ~95% blocking at 12.2 nM that will be important in successful elimination of the SARS-CoV-2 infection (Figure 1). We will test these multi-specific VHH-Fc antibodies and study their SARS-CoV-2 neutralizing capabilities with our collaborators. These molecules would potentially protect against SARS-CoV-2 by blocking S/ACE2 interaction and subsequent virus internalization, promoting virus aggregation, and inducing Fc-dependent antiviral functions, thereby possessing multiple mechanisms of action (Figure 1). In addition, these multi-specific antibodies would be easier to manufacture than polyclonal antibodies due to the production needs of only one molecule.

We will also assess the remaining 60 S protein high-affinity binders without S/ACE2 blocking function with our collaborators to probe whether they could be used for diagnostic applications to detect serum S protein and/or SARS-CoV-2 virions, either as single VHHs or in combination (Figure 1). Thus, we look forward to contributing in the fight against COVID-19 by developing antibody-based therapeutics and diagnostics.

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No potential conflict of interest was reported by the author(s).

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