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# A potent SARS-CoV-2 neutralising nanobody shows therapeutic efficacy in the Syrian golden hamster model of COVID-19

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## Abstract

SARS-CoV-2 remains a global threat to human health particularly as escape mutants emerge. There is an unmet need for effective treatments against COVID-19 for which neutralizing single domain antibodies (nanobodies) have significant potential. Their small size and stability mean that nanobodies are compatible with respiratory administration. We report four nanobodies (C5, H3, C1, F2) engineered as homotrimers with pmolar affinity for the receptor binding domain (RBD) of the SARS-CoV-2 spike protein. Crystal structures show C5 and H3 overlap the ACE2 epitope, whilst C1 and F2 bind to a different epitope. Cryo Electron Microscopy shows C5 binding results

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## Introduction

There are currently seven known coronaviruses that infect humans of which three (SARS-CoV-1, MERS and SARS-CoV-2) have emerged in the last 20 years and caused severe and even fatal respiratory diseases<sup>1</sup>. By far the most serious outbreak has been caused by SARS-CoV-2 which is responsible for the current global pandemic currently associated with 3.94 million deaths worldwide. Although vaccines are now being administered against SARS-CoV-2, building up immunity in the global population will take time. The imperative to treat SARS-CoV-2 infection has led to the search for agents that neutralise the virus for use in passive immunotherapy. Early attention has focused on identifying neutralising monoclonal antibodies from patients who have recovered from COVID-19<sup>2,3,4,5,6</sup>; the therapeutic use of antibodies is widespread and draws on existing knowledge and resources. However, nanobodies or VHHs (Variable Heavy-chain domains of Heavy-chain antibodies) derived from the heavy chain-only subset of camelid immunoglobulins offer an alternative with multiple advantages over conventional antibodies. The small molecular size and stability of nanobodies allows them to be formulated for topical delivery directly to the airways of infected patients through aerosolization. This results in improved bioavailability, simpler therapeutic compliance and easier administration. Secondly, while conventional antibodies that comprise two disulphide-linked polypeptides, heavy and light chain, typically require mammalian cells for production, nanobodies can be manufactured using readily available microbial systems. The potency of nanobodies against SARS-CoV-2<sup>7</sup> infection has been demonstrated in cell-based assays<sup>8,9,10,11,12,13,14,15,16</sup> and most recently in animal studies<sup>17,18</sup>.

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(intraperitoneal route) or via the respiratory tract (intranasal route) with a single dose of the most potent nanobody prevented disease progression in the Syrian hamster model of COVID-19.

## Results

### Isolation and binding characterisation of nanobodies that block ACE2 binding to the Spike protein of SARS-CoV-2

Antibodies to the RBD of SARS-CoV-2 were raised in a llama by primary immunisation with a combination of purified RBD alone and RBD fused to human IgG1, followed by a single boost with purified S (spike) protein mixed with RBD. The S protein sequence was derived from the original Wuhan or Victoria (B) strain of SARS-CoV-2. A phage display VHH library was constructed from the cDNA of peripheral blood mononuclear cells, and RBD binders selected by two rounds of bio-panning. The phage clones with the highest affinity for RBD were identified by an inhibition ELISA and classified by sequencing of complementary determining region 3 (CDR3) (Supplementary Fig. 1). Four VHHs were selected for production and their RBD-binding kinetics measured by surface plasmon resonance (SPR) (Fig. 1a–d). The calculated  $K_D$ s were all in the picomolar range (20–615 pM) with the rank order of affinities  $H3 > F2 > C5 \gg C1$  (Table 1).

**Fig. 1: Nanobody binding kinetics.**

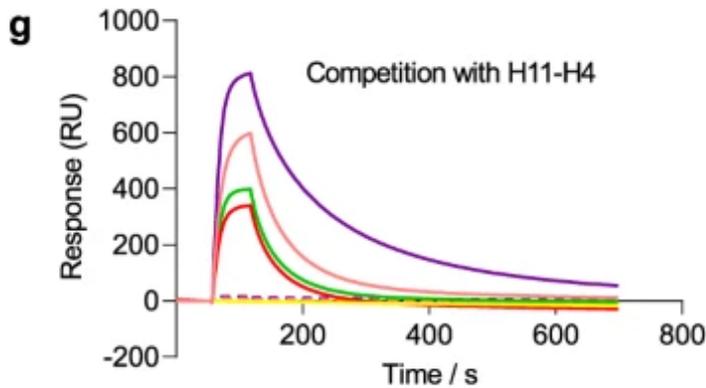
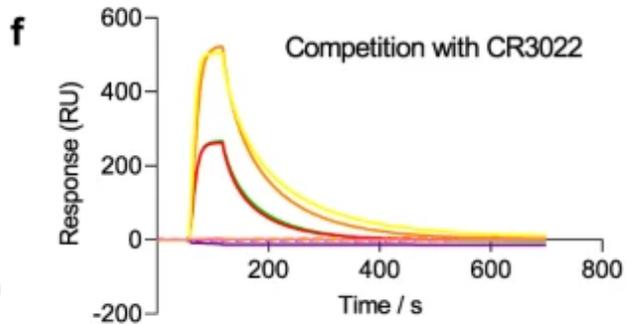
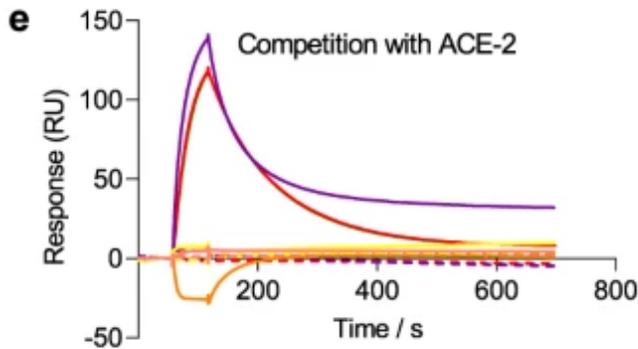
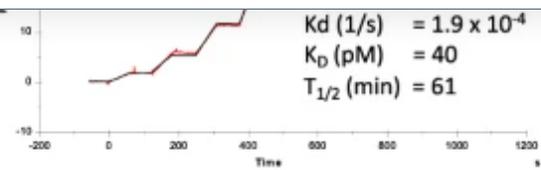
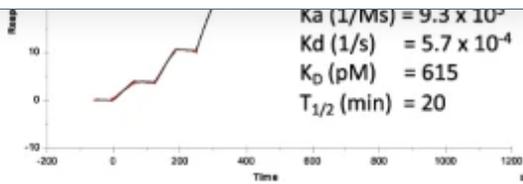


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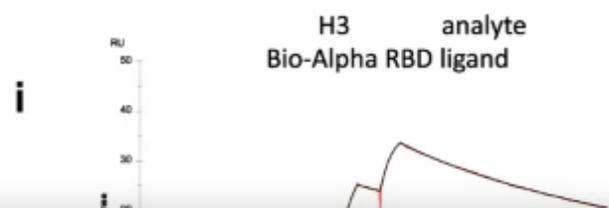
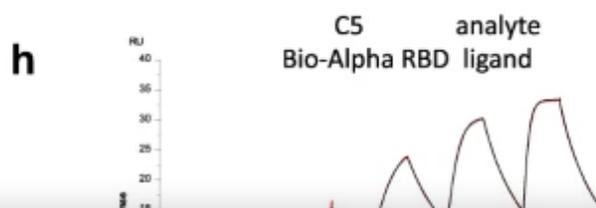
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- C1 + RBD
- C5 + RBD
- H3 + RBD
- F2 + RBD
- C2Nb6 + RBD
- RBD
- - - C1
- - - C5
- - - H3
- - - F2
- - - C2Nb6

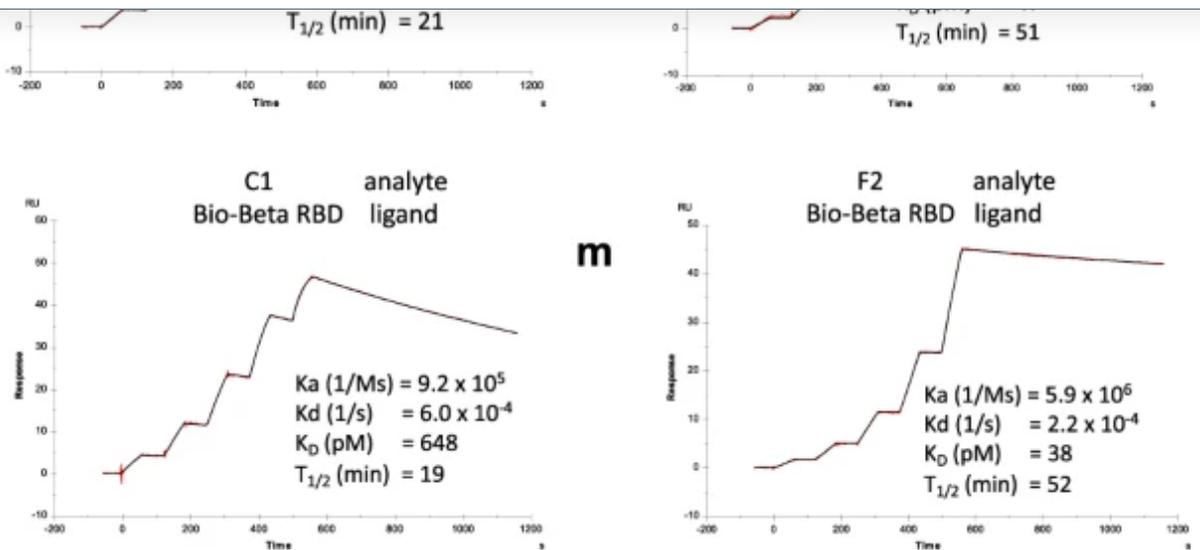


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**a–d** SPR sensorgrams showing binding kinetics of nanobody C5, H3, C1 and F2 for RBD Victoria (immobilised as biotinylated RBD on the chip), **e–g** SPR sensorgrams of competition assays between RBD and C5, H3, C1, F2 for binding to **e** ACE-2, **f** CR3022 and **g** H11-H4, with all ligands immobilised as Fc fusion proteins and C2Nb6 (an anti-Caspr2 nanobody) used as a negative control, **h–k** binding kinetics of nanobody C5, H3, C1 and F2 to Alpha RBD (**l, m**) C1 and F2 binding to Beta RBD (immobilised as biotinylated RBD on the chip).

**Table 1 Summary of nanobody binding kinetics.**

Competition binding experiments were carried out by SPR to investigate whether the VHHs blocked the binding of RBD to ACE2 and the overlap with the epitope recognised by the human

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(cluster 1 antibodies<sup>28</sup>) including CR3022<sup>26</sup> and EY-6A<sup>29</sup> that bind to a region distinct from the ACE2 receptor-binding interface. These two antibodies have been reported to destabilise the trimeric spike protein and by this mechanism prevent receptor engagement<sup>26,29</sup> thereby neutralising the virus.

ITC was used to analyse the binding of C5, F2 and C1 to RBD and spike proteins in solution. However, as the agents bind so tightly conventional ITC has large errors. Therefore a displacement assay was devised using the H11 nanobody previously identified<sup>25</sup> that weakly binds to RBD with a  $K_D$  of 1  $\mu$ M measured by ITC (Supplementary Fig. 2a). Combining the H11 titration with viral proteins (Supplementary Fig. 2a, b), C5 titration with viral proteins (Supplementary Fig. 2c, d) and C5 titration with viral proteins pre-incubated with H11 (displacement assay Supplementary Fig. 1e, f), we determined  $K_D$  for C5 to RBD as  $210 \pm 60$  pM and to Spike as  $350 \text{ pM} \pm 6 \text{ pM}$  (Supplementary Fig. 1g, h). The estimated  $K_D$  confirms sub-nanomolar binding of C5 to the Spike protein in solution and indicates 1:1 stoichiometry. No displacement agent was available for F2 and C1, and therefore the binding  $K_D$  for RBD of  $320 \pm 30$  and  $600 \pm 40$  pM respectively were estimated by direct binding but are subject to considerable uncertainty (Supplementary Fig. 1i, j). Both C1 and F2 when bound to Spike gave complex traces, suggesting that when engaging the Spike other conformational changes occur (Supplementary Fig. 1i, j).

The four nanobodies were also assessed for their binding to RBD from the Alpha (B.1.1.7; N501Y originally identified from the UK) and Beta (B.1.351; N501Y, N417K and E484K, originally identified from South Africa). C5 and H3 bound strongly to the Alpha variant albeit with reduced

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To further define the epitopes recognised by the nanobodies, crystal structures of the C5-RBD (Victoria), H3-C1-RBD (Victoria) and F2-RBD (Victoria) co-complexes were determined to high resolution (Tables 2, 1.5, 1.9 and 2.3 Å, respectively), however, the C1-RBD binary complex failed to give high quality crystals. Examination of the three structures confirmed the results of binding experiments that indeed H3 and C5 occlude the RBD binding site for ACE2 (Fig. 2a). C1 does not occlude the ACE2 epitope but would sterically prevent ACE2 binding to RBD, F2 would not be predicted to interfere with ACE2 binding (Fig. 2a). The C5 epitope has only a small overlap with the H3 epitope or with the H11-H4 epitope that we previously reported<sup>25</sup>. The interface between C5 and RBD is extensive and involves all three CDR loops and the fixed sequence loop (FR2) at A75 of the nanobody (Fig. 2b and Supplementary Fig. 3a).

### Table 2 X-ray crystallography data collection and refinement statistics.

### Fig. 2: Crystal structures of nanobody-RBD complexes.

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**a** The four nanobodies of this study are shown in cartoon and labelled. The figure was generated by superimposing the RBD protein from each crystal structure. only one

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colour scheme as **b** but rotated by 90° to more clearly show the H3 and C5 epitopes. The key molecular interactions between **d** C5, **e** H3, **f** C1, and **g** F2 and RBD are identified and labelled. RBD is in approximately in the same orientation as **a**. In **f** and **g** coloured in magenta and gold respectively is the portion of RBD that is also recognised by both C1 and F2. **h** C1 and F2 bind to RBD in different orientations and overlap at residues 102 and 103. Their spatial relationship can be described as an approximate 40° rotation around the main chain at 102 and 103. **i** In the F2 (blue) RBD (cyan) complex, Y102 of F2 results in a displacement of the helix at Y369 of RBD relative to the C1 (red) and RBD (brown) complex. The orientation of the molecules are the same as shown in Fig. 2a. All structural figures were prepared using PyMOL (<http://www.pymol.org/>).

The epitopes recognised by H3 and H11-H4 as we hypothesised do have a significant overlap (Fig. 3a). H3 however has 100-fold higher affinity than H11-H4. Since H3 and H11-H4 have quite different sequences and this results from many small changes in loops between the structure. This means that the identification of the atomic features that drive the difference in affinity from simple structural analysis is not straightforward. Comparison of the structures reveals several features that may contribute to the increased affinity. The H3-RBD interface buries just under 10% more surface area and satisfies 4 more hydrogen bonds than in H11-H4 RBD. In addition, in H3 the key R52 E484 salt bridge makes additional hydrophobic interactions with W53 and F59 of H3 (Supplementary Fig. 3b), these contacts are absent in H11-H4. In a future study, we suggest these regions should be probed.

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R31 in nanobody H3 and R52 in nanobody C5, respectively. **c** Close-up of the RBD-C5 interfaces for complexes with the Victoria strain of SARS-CoV-2 (N501: left hand side) and Alpha strain (N501Y: right hand side) showing the hydrogen bonding between N501 and Y501 of RBD (coloured green) with N73 of C5 in yellow and wheat respectively. Key residues are shown in stick representations.

The key binding interaction between C5 and H3 nanobodies and RBD is a combined salt bridge  $\pi$ -cation interaction involving an arginine from the nanobody (R31 in C5, R52 in H3) with E484 and F490 of RBD. This arrangement of the positively charged guanidine group, phenyl ring and glutamate was previously highlighted in the H11-H4 study<sup>25</sup>. In C5, R31 is located in CDR1 and as result the side chain of R31 enters the salt bridge  $\pi$ -cation interaction from the opposite side to R52 but preserves the interaction (Fig. 3b). The E484K mutation found in the recently emergent South African and Brazilian strains will disrupt this interface in both C5 and H3 (as well as H11-H4). The formation of a salt bridge with E484 is a feature of many antibodies isolated from the B cells of COVID-19 convalescent and vaccinated individuals and escape mutants at this position are obviously a major concern for the efficacy of current vaccines<sup>30,31</sup>.

In addition to R31, residues T28 to G30 from CDR1 of C5 are also in contact with residues Y453, L455, Q493 and S494 of RBD (Fig. 2b and Supplementary Fig. 3a). The aromatic ring of Y449 of the RBD makes extensive hydrophobic contacts with the main chain residues, T53 to G56 from CDR2 of C5. From C5 FR2 the main chain of S72, the side chains of N73 and N74 make hydrogen bonds with the side chains of Q498, N501 and the main chain of S494 respectively. The

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F490) (Fig. 2c and supplementary Fig. 3a). From CDR3, I101 to Y106 make either (or both) hydrogen bonds and van der Waals contacts with RBD (Y449, L455, F456, E484, Y489, F490, L492-S494). Compared to the H11-H4 interaction, H3 has pivoted around V102 resulting in a shift of 2 Å at R52. It is this pivot that brings FR2 of H3 into contact with RBD (Fig. 2b and Supplementary Fig. 3a).

Based on the structure, the H3 interaction would not be expected to be sensitive to the mutation (N501Y) (Fig. 2c). The observation of the lower affinity of H3 for Alpha RBD is therefore surprising. In order to investigate this further the crystal structures of both H3 and C5 in complex with the Alpha RBD were determined. In neither the H3-RBD or H3-Alpha RBD complex is there any direct contact with residue 501. The crystal structures of these complexes do not reveal any differences in the nanobody RBD interface that result from the mutation. Molecular dynamics studies have identified that this mutation alters the dynamics of RBD and leads to an increase in affinity for ACE2<sup>32</sup>. It may be that altered dynamics are responsible for modifying the binding of H3. In the C5-Alpha RBD complex, N73 still makes a hydrogen bond interaction with Y501 but the arrangement is less geometrically ideal than with N501, consistent with the lower binding affinity observed (Fig. 3c).

The RBD epitopes recognised by C1 and F2 substantially overlap (Y369-A372, F374-T385 in common) but are not identical (Fig. 2a, f, g and Supplementary Fig. 3c, d). The C1 and F2 nanobodies are oriented differently, the relationship can be described as an approximate 40° rotation around residues 102 and 103 of CD3 (Fig. 2h). Interestingly this is very similar pivot point as we observed between H3 and H11-H4 (Fig. 3a). C1 buries more surface area and engages

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Residues T376- T385 of RBD also form part of the binding site of the VH domain of CR3022<sup>26</sup>. Koenig et al.<sup>11</sup> very recently reported two anti-RBD nanobodies (VHH\_V and VHH\_U) that bind in a similar location to C1 (and F2) and target this epitope (residues Y369-K378). On repeated passage of SARS-CoV-2 escape mutations were observed at these interface residues (Y369H, S371P, F377L and K378Q/N)<sup>11</sup>, however actual variants incorporating these changes have yet to be identified<sup>33</sup>.

In the context of the whole virus and from ultrastructural analysis of purified Spike by cryo-EM, RBD exists in an equilibrium of up and down conformations. Interaction between the spike protein and cell-surface ACE2 requires at least one RBD in the up or open conformation<sup>34,35</sup>. The cryo-EM structure of the C5 bound to the spike protein (stabilised in the prefusion state<sup>34</sup>) was determined by single particle cryo-EM (Table 3 and Supplementary Figs. 4 and 5). C5 nanobodies were observed bound to the 3 down (inactive)<sup>36</sup> form of the spike trimer (Fig. 4a). Simple modelling shows that C5 (unlike H11-H4) is unlikely to bind to the 1 up 2 down active form due to steric clashes (Fig. 4b). We conclude that although C5 can only bind to the all down of the Spike, dynamic equilibrium between Spike conformers, results in the conversion to the all down complex. Other nanobody bound spike complexes have shown binding to either both up and down RBDs<sup>12</sup> or only up conformations<sup>11</sup>. Incubation of C1 or F2 with the trimeric spike protein led to ill-defined aggregates on EM grids, indicating they destabilise the trimer, which would disrupt ACE2 engagement (Supplementary Fig. 4). Similar findings were reported for CR3022<sup>26</sup> and EY-6A<sup>29</sup> that recognise this epitope and are consistent with the complex ITC traces observed for binding of C1 and F2 to the spike protein in solution (Supplementary Fig. 2) This was attributed to the epitope being in the middle of the molecule and binding of a protein to this epitope is

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and purple wheat and throughout and show that all three RDBs are in the down conformation. **b** Superimposition of C5 onto the spike protein in the two down one up conformation shows that there would be significant clashes that would prevent this interaction.

## Potent neutralisation of SARS-CoV-2 in vitro by trimeric nanobodies

Linking more than one nanobody together to create bivalent and trivalent assemblies significantly increases antigen-binding due to avidity<sup>11,13,23,37,38,39</sup>. Therefore, trivalent versions of the four nanobodies were constructed by joining the VHH domains with a glycine-serine flexible linker, (GS)<sub>6</sub>. The nanobody homo-trimers (C5, C1 and H3) were produced by transient expression in expi293 cells and purified by metal chelate affinity chromatography and size exclusion. Although the F2 trimer was expressed it proved to be unstable on purification and was not pursued further. Binding of the trimeric nanobodies to the RBD was measured by SPR, and an approximate 10 to 100-fold enhancement in  $K_D$  was observed compared to the monomers (Table 1 and Supplementary Fig. 6). Notably, the H3 trimer was shown to have a sub-picomolar  $K_D$  for the RBD-Victoria with an off rate of ~6 h. Binding of C5 trimer to RBD-Kent was shown to be only two-fold weaker than to RBD-Victoria, whilst binding of C5 monomer was ~25-fold weaker (Table 1, Fig. 1 and Supplementary Fig. 6).

Microneutralisation assays were carried out to test the effectiveness of the three nanobody trimers to block infection of Vero E6 cells by either Victoria, Alpha or Beta strains of the virus. All

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Neutralisation curves of the anti-RBD nanobody trimers for **a** Victoria (BVIC01), **b** Alpha, (B1.1.7) and **c** Beta, (B1.351) strains of SARS-CoV-2 measured in a microneutralisation assay. Data are shown as the mean ( $n = 4$ )  $\pm$  95% CI.

The neutralisation potency of the C5 trimer was confirmed in the Gold Standard Plaque Reduction Neutralization Test (PRNT) against the Victoria strain which gave an ND50 of 3 pM (Supplementary Fig. 7). This corresponds to one of the most potent neutralising nanobodies that

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are similar to those seen in infected humans. Since an anti-MERS-CoV nanobody fused to immunoglobulin Fc fragment has previously shown to extend the half-life of the protein in vivo and ameliorate disease in a mouse challenge model<sup>45</sup> we first tested C5 as a huIgG1 Fc fusion protein. The RBD-binding affinity ( $K_D$  37 pM) and virus neutralisation potency (ND50 of 2 pM; 180 pg/ml) of C5-Fc was similar to the trivalent C5 protein, confirming the importance of multivalency for effective neutralisation (Table 1 and Supplementary Figs. 6 and 7). Efficacy of a human IgG1 antibody has also been demonstrated in the Syrian hamster model with the isotype matched control showing no therapeutic effect<sup>6</sup>.

The study comprised an experimental and a control group each of six animals. All animals in both groups were challenged intranasally (IN) with SARS-CoV-2 Victoria ( $5 \times 10^4$  pfu). The experimental group was treated 24 h later with a single dose of C5-Fc (4 mg/kg) administered intraperitoneally (IP) whilst the control group were left untreated (Fig. 6a). As a measure of disease progression, the animals were weighed each day over 7 days and nasal washes and oropharyngeal swabs were taken every other day (Fig. 6a). On day 7 the animals were culled and viral load in lung, trachea and duodenum measured by subgenomic (sg)-RT-qPCR. Vital organs were formalin-fixed for histopathology (H&E staining) and ISH RNAScope staining with SARS-CoV-2 S-gene probe to detect presence of virus RNA. SARS-CoV-2 infected animals exhibited progressive mean body weight loss (up to 17%) from day 1 to day 7 post challenge (pc) (Fig. 6b). In contrast, by day 7 post challenge (pc), animals in the nanobody treated group had lost significantly ( $P < 0.005$ , Mann–Whitney) less weight (7%). High levels of nasal shedding of live virus ( $10^4$ – $10^5$  FFU/ml) were detected in 6/6 untreated animals (100%) on day 2 pc, whereas only 3/6 (50%) animals in the nanobody treated group shed virus (Fig. 6c). Some live viral shedding

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**Fig. 6: C5-Fc neutralisation of SARS-CoV-2 in the Syrian hamster model.**

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**a** Golden Syrian hamsters ( $n = 6$  biologically independent animals per group) were challenged with SARS-CoV-2 (B Victoria  $5 \times 10^4$  pfu) at day 0 and then treated with either C5-Fc (IP 4 mg/kg) or PBS, delivered by the intraperitoneal route 24 h post-challenge and Throat Swab (TS) and Nasal Wash (NS) samples collected on days 2, 4, 6 and 7 post virus challenge. **b** Body weight was recorded daily and the mean percentage weight change from baseline was plotted (mean  $\pm$  1 SE). Filled in square represents data from control animals (virus only) and filled in circles represents data from nanobody treated. Nasal washes (i–iii) and oropharyngeal swabs (iv–vi) were collected at days –2 to 2, 4, 6 and 7 pc for all virus challenged groups. Tissue samples (lung, trachea and duodenum) were collected at post-mortem (day 7 pc) (vii & viii). Open square represents data from control animals (virus only) and open circle represents data from nanobody-treated hamsters. Symbols show values for individual animals, columns represent the calculated group geometric means. **c** Quantitation of live virus in the nasal wash and oropharyngeal swabs using a micro-

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Histopathology and RNAScope ISH techniques were used to compare the pathological changes and the presence of viral RNA in tissues from nanobody-treated and untreated control hamsters. A semiquantitative scoring system was combined with digital image analysis to calculate the area of lung with pneumonia and the quantity of virus. Viral RNA and lesions consistent with infection with SARS-CoV-2 were observed only in the nasal cavity (Supplementary Fig. 8) and lungs (Supplementary Fig. 9). No lesions were observed in any other organ studied. The lung lesions consisted of a bronchointerstitial pneumonia showing areas of parenchymal consolidation and were characterised by infiltration of macrophages and neutrophils, but also some lymphocytes and plasma cells (Supplementary Fig. 8c). The lesions in the nasal cavity consisted in necrosis of the respiratory and olfactory mucosa and presence of inflammatory exudates and cell debris within the nasal cavity lumen. The area with pneumonia was significantly lower in the nanobody-treated hamsters together with a marked reduction of histopathology scores in the nasal cavity (Supplementary Fig. 9a). Statistically significant differences were also found for the presence of virus RNA in the lung or the nasal cavity (Supplementary Fig. 8b and 9b). Together, these results showed that a single therapeutic dose of C5-Fc administered IP reached the site of action in the lungs and nasal cavity and reduced viral load and associated pathological changes. Therefore, based on these promising results we undertook a larger study to evaluate the C5 trimer in the Syrian hamster model.

## Trimeric C5 nanobody shows efficacy when administered via the respiratory route

The smaller molecular size of the C5-trimer (40 kDa) compared to the C5-Fc (80 kDa plus 2N-linked glycans) renders the nanobody suitable for respiratory administration directly to the

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tested. As in the first study, animals in the untreated group showed a significant and progressive weight loss (20% by day 7), whereas all animals treated therapeutically, 24 h after viral challenge, showed only a small weight loss and from day 2 had recovered to pre-challenged weights (Fig. 7b). The animals pre-treated 2 h before IN virus inoculation with 4 mg/kg C5 via the intranasal route showed no change in weight. The weight loss in all C5-treated groups was significantly different from the control group given PBS alone ( $p < 0.01$ ; repeated measures two-way ANOVA). Analysis of viral load in the post-mortem lungs at day 7 by qPCR for Nucleoprotein (NP) RNA showed a decrease in the median value in treated compared to the untreated control animals. (Fig. 7c). This decrease was significantly different in the IP treated group. While there was a clear trend in the other groups, there were two outliers with higher RNA load in each of the groups treated via the intranasal route. No live virus was detected by plaque assay in day 7 samples of lung homogenates consistent with what was observed in the first animal study (Fig. 6c).

**Fig. 7: Therapeutic efficacy of C5 Trimer in Syrian hamster model.**

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0.0001, PBS vs. 4 mg/kg 24 hpi i/n; \*\*\* $P=0.0005$ , PBS vs. 4 mg/kg 24 hpi i/p; \*\*\* $P=0.0002$ , PBS vs. 0.4 mg/kg 24 hpi i/n; \*\*\* $P=0.0003$ . **c** RNA extracted from lungs was analysed for SARS-CoV-2 viral load using qRT-PCR for the N gene levels by qRT-PCR. Assays were normalised relative to levels of 18S RNA. Data for individual animals are shown with the median value represented by a horizontal line. Data are mean value ( $n=6$ )  $\pm$ SEM and were analysed using a Kruskal–Wallis one-way ANOVA with Dunn's multiple comparisons test; PBS vs. 4 mg/kg 2 h pre-inf i/n;  $P=0.1682$  (ns), PBS vs. 4 mg/kg 24 hpi i/n;  $P>0.9999$  (ns), PBS vs. 4 mg/kg 24 hpi i/p; \* $P=0.0287$ , PBS vs. 0.4 mg/kg 24 hpi i/n;  $P=0.4044$  (ns). **d** Morphometric analysis of HE-stained sections scanned and analysed using the software programme Visiopharm to quantify the area of non-aerated parenchyma and aerated parenchyma in relation to the total area. Results are expressed as the mean free airspace in lung sections. Data are mean value ( $n=6$ )  $\pm$ SEM and were analysed using a one-way ANOVA with Dunnett's multiple comparisons test; PBS vs. 4 mg/kg 2 h pre-inf i/n; \* $P=0.0109$ , PBS vs. 4 mg/kg 24 hpi i/n; \* $P=0.0406$ , PBS vs. 4 mg/kg 24 hpi i/p; \* $P=0.0270$ , PBS vs. 0.4 mg/kg 24 hpi i/n; \* $P=0.0110$ . **e** Lung sections of hamsters, infected intranasally with  $10^4$  PFU/100  $\mu$ l SARS-CoV-2 and euthanized at day 7 post infection. Animals had been untreated prior to infection (PBS) or treated with 4 mg/kg C5 IN 2 h prae infection (h prae inf) or 24 h post infection (h post inf) or IP at 24 h post inf, or had received 0.4 mg/kg C5 IN at 24 h post inf. In the untreated animal (PBS) the lung parenchyma exhibits a large consolidated area (arrow) and multifocal patches with extensive viral antigen expression in particular by pneumocytes. In treated animals there are only a few small areas of consolidation (arrows). The animal treated with 4 mg/kg C5

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The histological and immunohistological examination showed multifocal extensive consolidation of the lung parenchyma in the untreated group, with multifocal patches of cells that expressed viral antigen (mainly type I and II pneumocytes, some cells morphologically consistent with macrophages) (Fig. 7d). The consolidated areas contained aggregates of macrophages and some neutrophils and were otherwise comprised of activated type II pneumocytes with occasional syncytial cell formation, and hyperplastic bronchiolar epithelial cells (Supplementary Fig. 10). In all treated groups, the extent of parenchymal consolidation was substantially reduced as quantified by automated morphometric analysis which resulted in a statistically significantly larger area of ventilated lung parenchyma (Fig. 7d). The lungs of treated animals showed very limited viral antigen expression and only in occasional individual macrophages within small infiltrates or in pneumocytes in individual alveoli (Fig. 7e).

More detailed assessment of the consolidated areas in untreated animals confirmed that at day 7 post SARS-CoV-2 infection, the pathological processes in the lungs are dominated by regenerative attempts, as shown by type II pneumocyte and bronchiolar epithelial hyperplasia, in combination with macrophage dominated inflammatory infiltration (Supplementary Fig. 10). Animals that had received either C5-trimer (4 mg/kg) 2 h pre-infection or the lower dose (0.4 mg/kg) at 4 h post infection, resulted in substantially less regenerative processes; the observed small, consolidated areas were dominated by infiltrating macrophages (Supplementary Fig. 10). These findings at the late, i.e., regenerative stage of SARS-CoV-2 infection in hamsters<sup>42</sup> indirectly confirm that the C5-trimer treatment significantly reduced pulmonary infection and induced a strong macrophage response, likely leading to phagocytosis and thereby sequestration of the virus. Double immunofluorescence for viral N protein and the macrophage marker Iba1

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animal) of the C5-trimer demonstrating the high potency of this biological agent. A further dose ranging study will be required to establish the minimum amount of the nanobody required to be therapeutically effective in the hamster disease model.

## Discussion

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The RBD of SARS-CoV-2 is the immuno-dominant region of the virus spike protein and the target for neutralising antibodies generated either by vaccination or infection. Following immunisation of a llama with a combination of the RBD and stabilised spike trimer<sup>34</sup> based on the Victoria strain sequence, we obtained nanobodies designated C5, F2, H3 and C1 that bound one of two orthogonal sites on the RBD. The site recognised by C5 and H3 overlapped with the ACE2 binding site on the top surface of the domain, whilst the second recognised by C1 and F2 corresponded to a location on the side of the RBD originally identified by the SARS-CoV antibody CR3022<sup>24,26,47</sup> and nanobody VHH72<sup>48</sup>. Consistent with other recent reports<sup>10,17,39</sup> nanobodies that bound to both sites showed very potent neutralisation activity when configured as multivalent trimers, with the C5 trimer demonstrating complete inhibition of infection of Vero cells at < 100 pM in a PRNT assay. This activity was translated into a marked disease-modifying effect in the Syrian golden hamster model of COVID-19 with treated animals showing minimal weight loss and very limited pulmonary infection and associated changes following a single dose of C5 trimer 24 h post virus challenge. Most importantly, administration of the nanobody agent either directly by nasal administration or systemically (IP) was effective at 4.0 mg/kg. Nasal administration appeared to promote faster recovery than IP perhaps reflecting increased levels of

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The independent emergence of SARS-CoV-2 variants which appear to be more transmissible is now a major concern. Although in this study, animals were challenged with the Victoria and Liverpool (lineage B) strains, the in vitro neutralisation data strongly indicates the C5 trimer will be equally effective against the lineage B.1.1.7 or Alpha variant in this COVID-19 disease model. Although, the Alpha variant dominated infections in the UK in early 2021, the new Delta virus (B.1.671.2) that first originated in India has become the most recent variant of concern. The epitope recognised by C5 does not include the two residues that are mutated in the RBD of the Delta virus, L452R and T478K. However, F54 in Framework 3 of C5 does make a Van der Waal interaction with L452 that may be disrupted by mutation to R452 (Supplementary Fig. 3). The B.1.351 (Beta variant) and P.1 (Gamma variant) lineages are characterised by three mutations (K417N, E484K and N501Y) in the RBD, which, although less prevalent, are a serious concern as they are associated with immune evasion<sup>30</sup>. Structural analysis of the C5-RBD and H3-RBD complexes showed the central importance of E484 in RBD to the interaction and unsurprisingly these nanobodies failed to neutralise the Beta virus. The C1 nanobody is significantly less potent than C5 against the Victoria strain, NT50 of C1 trimer is 4.9 nM compared to 18 pM and binds to a different epitope. However, C1 was equally effective against all three strains of the virus tested for neutralisation in vitro, thus it has the potential to be a broadly neutralising agent.

The relative size and stability of nanobody based bio-therapeutics has fuelled interest in their use as inhaled drugs for the treatment of respiratory diseases<sup>49</sup>, including for COVID-19<sup>50</sup>.

Furthermore, since some of their formulations, for example the trimeric molecule discussed here, do not require mammalian cell culture, they are relatively inexpensive to produce. In laboratory tests, anti-SARS-CoV-2 nanobody trimers, similar to the ones we report here, have already been

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The two epitopes correspond to those targeted by human antibodies recovered from convalescent patients pointing to their cross species immunodominance. We show that SARS-CoV-2 infection in a hamster model can be treated with a single dose of the most potent trimeric nanobody delivered either systemically or intranasally. Combinations of nanobodies that target different epitopes may improve resilience in combating new variants of the virus.

## Methods

---

### Immunisation and construction of VHH library

The SARS-CoV-2 receptor-binding domain (amino acids 330-532), SARS-CoV-2 receptor-binding domain fused to hIgG1 Fc (RBD-Fc) and trimeric spike protein (amino acids 1–1208) were produced as described by Huo et al.<sup>25</sup>. Antibodies were raised in a llama by intramuscular immunisation with 200 µg of recombinant RBD and 200 µg of RBD-Fc on day 0, and then 200 µg RBD and 200 µg S protein on day 28. The adjuvant used was Gerbu LQ#3000. Blood (150 ml) was collected on day 38. Immunisations and handling of the llama were performed under the authority of the project license PA1FB163A. Peripheral blood mononuclear cells were prepared using Ficoll-Paque PLUS according to the manufacturer's protocol; total RNA was extracted using TRIzol™; reverse transcription and PCR was carried out with SuperScript IV Reverse Transcriptase using primer CALL\_GSP. The pool of VHH encoding sequences were amplified by two rounds of PCR using CALL\_001 and CALL\_02 (round 1), VHH\_For and VHH\_Rev\_IgG2 plus VHH\_Rev\_IgG3 (round 2). Following purification by agarose gel electrophoresis, the VHH

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Phages displaying VHHs specific for the RBD of SARS-CoV-2 were enriched after two rounds of bio-panning on 50 nM and 2 nM of biotinylated RBD respectively, through capturing with Dynabeads™ M-280 (Thermo Fisher Scientific). Enrichment after each round of panning was determined by plating the cell culture with 10-fold serial dilutions. After the second round of panning, 93 individual phagemid clones were picked, VHH displaying phages were recovered by infection with M13K07 helper phage and tested for binding to RBD by a combination of competition and inhibition ELISAs. In these assays, RBD was immobilised on a 96-well plate and binding of phage clones was measured in the presence of excess soluble RBD (inhibition ELISA) or the RBD-binding H11-H4-Fc<sup>25</sup> (competition ELISA). Bound phage were detected with an HRP-coupled anti-M13 antibody (1:5000; Cytiva 27-9421-01).

Phage binders were ranked according to the inhibition assay and then classified as either competitive with H11-H4 (i.e., sharing the same epitope) or non-competitive (i.e. binding to a different epitope on RBD). Clones were sequenced and grouped according to CDR3 sequence identity.

## Construction of trivalent VHHs

To generate the trimeric VHHs, the C1, C5, H3 and F2 gene fragments were used as templates to amplify three fragments by PCR with the following pairs of primers: TriNb\_Neo\_F1 and TriNb\_R1; TriNb\_F2 and TriNb\_R2; TriNb\_F3 and TriNb\_Neo\_R1; the three fragments were then joined together with a PCR reaction using primers TriNb\_Neo\_F2 and TriNb\_Neo\_R2. The trimeric gene product was then inserted into the pOPINTTGneo vector by Infusion® cloning.

pOPINTTG contains a myc phosphatase leader sequence and C-terminal His6 tag.<sup>51</sup>

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The Beta RBD gene was generated using the Alpha RBD as a template, in two steps. Firstly, two fragments were amplified with pairs of primers of (1) TTGneo\_RBD\_F and E484K\_R and (2) TTGneo\_RBD\_R and E484K\_F. The two fragments were then joined together with a PCR reaction using primer TTGneo\_RBD\_F and TTGneo\_RBD\_R and cloned into the pOPINTTNeo vector by Infusion® cloning. Secondly, using this plasmid as a template, the two fragments were amplified with pairs of primers of (1) TTGneo\_RBD\_F and K417V\_R and (2) TTGneo\_RBD\_R and K417V\_F. The fragments were then joined together with a PCR reaction using primer TTGneo\_RBD\_F and TTGneo\_RBD\_R and the resulting gene product was then cloned into the pOPINTTNeo vector by Infusion® cloning to produce the Beta-RBD expression vector.

To generate the huIgG1 Fc-fusion versions of RBDs, the RBD genes from the pOPINTTNeo vector were amplified by a pair of primers TTGneo\_RBD\_F and RBD\_Fc\_R, followed by being cloned into the pOPINTTNeo-Fc vector by Infusion® cloning. The pOPINTTNeo-Fc contains a mu-phosphatase leader sequence, a huIgG1 Fc and C-terminal His6 tag<sup>51</sup>.

## Protein production

In general, the monovalent VHs were cloned into the vector pOPINO<sup>52</sup> containing an OmpA leader sequence and C-terminal His6 tag. The C5 and H3 VHH constructs used for the crystallisation of C5-Kent RBD and H3-Kent RBD complexes, respectively, were generated through amplification with a pair of primers PelB\_F and PelB\_R, followed by being cloned into the phagemid vector pADL-23c by Infusion® cloning. pADL-23c contains a PelB leader sequence and C-terminal His6 tag. The plasmids were transformed into the WK6 *E. coli* strain and protein expression induced by 1 mM IPTG grown overnight at 20 °C. Periplasmic extracts were

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IMAC (GE, Capto S 1 mL column) to lower endotoxin levels which were further reduced to <0.1 EU/ml by passing in the final purified product through two Proteus NoEndo™ clean-up columns (Generon, Slough, UK). Endotoxin levels were quantified using the Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (ThermoFisher Scientific). Protein was concentrated to 4 mg/ml and flash frozen for storage at −80 °C. The biotinylated and non-biotinylated RBDs, ACE2-Fc and CR3022-Fc were produced by transient expression in expi293® cells<sup>25</sup>. Briefly, Proteins were purified from culture supernatants 72 h post-transfection by immobilised metal affinity using an automated protocol implemented on an ÄKTExpress (GE Healthcare, UK), followed by a Hiload 16/60 Superdex 75 or a Superdex 200 10/300GL column, using phosphate-buffered saline (PBS) pH 7.4 buffer.

All PCR primers used in this work are listed in Supplementary Table 1 and nanobody sequences are provided in the Supplementary Table 2. The pOPINO vectors for producing nanobodies C1, C5, F2 and H3 have been deposited with Addgene ([www.addgene.org](http://www.addgene.org)) with IDs 171924, 171925, 171926 and 171927.

## Surface plasmon resonance & ITC

The surface plasmon resonance experiments were performed using a Biacore T200 (GE Healthcare). All assays were performed with a running buffer of PBS pH 7.4 supplemented with 0.005% vol/vol surfactant P20 (GE Healthcare) at 25 °C.

The competition assay was performed with a Sensor Chip Protein A (Cytiva). CR3022-Fc, ACE2-Fc or H11-H4-Fc was used as the ligand. ~1000 RU of CR3022-Fc, ACE2-Fc or H11-H4-Fc was

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the two flow cells at a range of five concentrations prepared by serial twofold dilutions, at a flow rate of  $30 \mu\text{l min}^{-1}$  using a single-cycle kinetics programme. Running buffer was also injected using the same programme for background subtraction. All data were fitted to a 1:1 binding model using Biacore T200 Evaluation Software 3.1.

To determine the binding kinetics between the SARS-CoV-2 RBD-WT and C5-Fc, a Biotin CAPture Kit (Cytiva) was used. Biotinylated RBD was immobilised onto the sample flow cell of the sensor chip. The reference flow cell was left blank. C5-Fc was injected over the two flow cells at a single concentration of 10 nM, at a flow rate of  $30 \mu\text{l min}^{-1}$ . Running buffer was also injected using the same programme for background subtraction. All data were fitted to a 1:1 binding model using Biacore T200 Evaluation Software 3.1.

To determine the binding kinetics between the SARS-CoV-2 RBD and the trimeric nanobodies C1/C5/H3, a Sensor Chip Protein A (Cytiva) was used. The huIgG1 Fc-fusion versions of RBDs were immobilised onto the sample flow cell of the sensor chip. The reference flow cell was left blank. Trimeric nanobody was injected over the two flow cells at a single concentration of 25 nM for C1 trimer, 10 nM for C5 trimer and 10 nM (RBD-Kent interaction) or 2.5 nM (RBD-WT interaction) for H3 trimer, at a flow rate of  $30 \mu\text{l min}^{-1}$ . Running buffer was also injected using the same programme for background subtraction. All data were fitted to a 1:1 binding model using Biacore T200 Evaluation Software 3.1.

Isothermal titration calorimetry (ITC) measurements were carried out using an iTC200 and PEAQ-ITC MicroCalorimeter (GE Healthcare) at 25 °C. RBD and all nanobodies were dialysed

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AFFINImeter for global fitting of the displacement assay. For the fitting of C5 and H11 into spike, the monomeric concentration of spike and a single binding mode have been used. Data analysis was performed by generating a binding isotherm and best fit using the following parameters:  $N$  (number of sites),  $\Delta H$  ( $\text{kJmol}^{-1}$ ),  $\Delta S$  ( $\text{JK}^{-1}\text{mol}^{-1}$ ) and  $K$  (binding constant in  $\text{molar}^{-1}$ ). Following data analysis,  $K$  was converted to the dissociation constant ( $K_d$ ).

## Determination of the structure of VHH- RBD complexes by X-ray crystallography

Purified VHHs were mixed with de-glycosylated RBD at a molar ratio of 1.2:1, and the complex purified by size exclusion chromatography as described<sup>8</sup>. The optimal conditions for crystallisation of each complex were F2-RBD 0.1 M Succinic Acid, Sodium Dihydrogen Phosphate and Glycine (SPG), pH 8, 25% Polyethylene glycol (PEG) 1500, H3-C1-RBD and H3-C1-Alpha RBD 1.0 M Lithium chloride, 0.1 M Citric acid pH 4, 20% PEG 6000 and C5-RBD 0.2 M Sodium Acetate, 0.1 M Sodium Cacodylate pH 6.5, 30% w/v PEG 8000 and the C5-Alpha RBD 0.2 M Ammonium fluoride and 20% PEG 3350. The protein concentrations for all complexes were 18 mg/ml except for F2-RBD, where 34 mg/ml was used. Crystals were grown at 20 °C by sitting drop vapour diffusion method by mixing 0.1  $\mu\text{l}$  of protein complex (C5-RBD) with 0.1  $\mu\text{l}$  of reservoir; mixing 0.2  $\mu\text{l}$  of protein complex (F2-RBD; H3-C1-RBD) with 0.1  $\mu\text{l}$  of reservoir or 0.1  $\mu\text{l}$  of protein complex (C5-Alpha RBD; H3-C1-Alpha RBD) and 0.2  $\mu\text{l}$  of reservoir as stated above. Crystals were cryoprotected with 30% glycerol, cryocooled in liquid nitrogen, diffraction data collected and processed at the beamlines I03, I04 and I24 of Diamond Light Source, UK. The structures were solved by molecular replacement with PHASER<sup>53,54</sup>, as implemented in CCP4<sup>55</sup> using the individual components of the complex between H11-H4-RBD<sup>8</sup>

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Preparation of cryo-EM grids, data collection and processing were carried out as previously described<sup>8</sup>. Briefly, purified spike protein in 10 mM Hepes, pH 8, 150 mM NaCl, at 1 mg/ml was incubated with nanobody C5, purified in PBS, at a molar ratio of 1:1.2 (Spike monomer:nanobody) at 16 °C overnight. SPT Labtech prototype 300 mesh 1.2/2.0 nanowire grids were glow-discharged on low for 4 min (Plasma Cleaner PDC-002-CE, Harrick Plasma) and used in a Chameleon EP system (SPT Labtech) at 80% relative humidity, ambient temperature. Frozen grids were screened, and data collected using Titan Krios G2 (Thermo Fisher Scientific) equipped with a Bioquantum-K3 detector (Gatan, UK) operated at 300 kV. Data collection statistics are given in Supplementary Table 3. The RELION\_IT.py processing pipeline as implemented in eBIC was used for automatic data processing up to 2D classification. The data were first processed as C1 but as the complex showed C3 symmetry, this was later changed to C3. The best 3D class was selected for further refinement, CTF refinement and particle polishing within Relion. An initial model based on PDB ID 6VXX was created and the RBD-C5 crystal structure placed into density. The final model with correlation coefficient 0.76 was generated by multiple cycles of manual intervention in coot<sup>56</sup> followed by jelly body refinement using RefMac5 via CCP-EM GUI<sup>53,54</sup>. Model validation was carried out in PHENIX<sup>62,63,64</sup>. Data processing and refinement statistics are given in Table 3.

## Microneutralisation assay

VHH trimers were serially diluted into Dulbecco's Modified Eagles Medium (DMEM) containing 1% (w/v) foetal bovine serum (FBS) in a 96-well plate. SARS-CoV-2 strains (B VIC01, B1.17 and B1.351) passage 4 (Vero 76 clone e6 [ECACC 85020206])  $9 \times 10^4$  pfu/ml diluted 1:5 in

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reader. The neutralisation titre was defined as the titre of VHH trimer that reduced the Foci forming unit (FFU) by 50% compared to the control wells.

## PRNT assay

Plaque reduction neutralization tests (PRNT) were carried out at Public Health England using SARS-CoV-2 (hCoV-19/Australia/VIC01/2020) (GISAID accession number EPI\_ISL\_406844)<sup>65</sup> generously provided by The Doherty Institute, Melbourne, Australia at P1 and passaged twice in Vero/hSLAM cells [ECACC 04091501]. Virus was diluted to a concentration of 933 p.f.u. ml<sup>-1</sup> (70 p.f.u./75 µl) and mixed 50:50 in minimal essential medium (MEM; Life Technologies) containing 1% FBS (Life Technologies) and 25 mM HEPES buffer (Sigma) with doubling antibody dilutions in a 96-well V-bottomed plate. The plate was incubated at 37 °C in a humidified box for 1 h to allow neutralisation to take place. Afterwards, the virus-antibody mixture was transferred into the wells of a twice Dulbecco's PBS-washed 24-well plate containing confluent monolayers of Vero E6 cells (ECACC 85020206, PHE) that had been cultured in MEM containing 10% (v/v) FBS. Virus was allowed to adsorb onto cells at 37 °C for a further hour in a humidified box, then the cells were overlaid with MEM containing 1.5% carboxymethyl cellulose (Sigma), 4% (v/v) FBS and 25 mM HEPES buffer. After 5 days incubation at 37 °C in a humidified box, the plates were fixed overnight with 20% formalin/PBS (v/v), washed with tap water and then stained with 0.2% crystal violet solution (Sigma) and plaques were counted. A mid-point probit analysis (written in R programming language for statistical computing and graphics) was used to determine the dilution of antibody required to reduce SARS-CoV-2 viral plaques by 50% (ND50) compared with the virus-only control ( $n = 5$ ). The script used in R was

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Golden Syrian hamsters (*Mesocricetus auratus*) (males and females) aged between 7 and 9 weeks old, weighing 110–140 g, were obtained from Envigo, London, UK. Hamsters were assigned randomly and housed in individual cages with access to food and water ad libitum. All experimental work was conducted under the authority of a UK Home Office approved project license that had been subject to local ethical review at PHE Porton Down by the Animal Welfare and Ethical Review Body (AWERB) as required by the Home Office Animals (Scientific Procedures) Act 1986.

Twelve hamsters were briefly anaesthetised with 5% isoflurane (Zoetis, Leatherhead, UK) and 4 L/m O<sub>2</sub> and inoculated by the intranasal route with  $5 \times 10^4$  p.f.u/animal of SARS-CoV-2 (hCoV-19/Australia/VIC01/2020) delivered in 100  $\mu$ l per nostril (200  $\mu$ l in total). At day 1 post-challenge (pc) 6 hamsters were treated with 4 mg/kg of C5 Nanobody via the intraperitoneal route. Control hamsters ( $n = 6$ ) received no treatment. Temperature (taken using a microchip reader and implanted temperature/ID chip) and clinical signs were monitored twice daily, weight once daily. Clinical signs were scored as follows; healthy = 0, behavioural changes = 1, ruffled fur = 2, wet tail = 2, dehydrated = 2, eyes shut = 3, arched back = 3, wasp waisted = 3 and laboured breathing = 5. Clinical samples of nasal washes in Dulbecco's PBS (DPBS, Gibco) (200  $\mu$ l) as well as oropharyngeal (throat) swabs (MWE, Corsham, UK) were obtained prior to infection (day -2) and on days 2, 4, 6 and 7 pc; animals were briefly anaesthetised for the collection of these samples. On day 7 all the hamsters were euthanized by an overdose of anaesthetic (sodium pentobarbitone [Dolelethal, Vetquinol UK Ltd]) via the intraperitoneal route. At necropsy nasal washes and oropharyngeal swabs and tissue samples (lung, trachea and duodenum) were collected in PBS and stored frozen at  $-80$  °C for viral RNA measurement and viral culture. Tissue samples for

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viscous overlay (1% carboxymethylcellulose, Sigma) was added. The plates were then incubated for 24 hr at 37 °C. The cells were then fixed using 8% formalin for >8 h and an immunostaining protocol was performed on the fixed cells (Bewley et al.). Stained foci [foci forming units (FFU)] were counted using an ELISpot counter (Cellular Technology Limited, USA). The counted foci data was then plotted using Graph Pad version 9. A SARS-CoV-2 positive control at  $1 \times 10^5$  PFU/ml was run alongside the animal samples, on each assay plate, with uninfected assay diluent as negative control.

RNA was isolated from nasal washes, oropharyngeal swabs and tissue samples (lung, trachea and duodenum). Weighed tissue samples were homogenised and inactivated in RLT (Qiagen) supplemented with 1% (v/v) beta-mercaptoethanol. Tissue homogenate was then centrifuged through a QIAshredder homogenizer (Qiagen) and supplemented with ethanol as per manufacturer's instructions. Downstream extraction was then performed using the BioSprint™96 One-For-All vet kit (Indical Bioscience) and Kingfisher Flex platform as per manufacturer's instructions. Non-tissue samples were inactivated in AVL (Qiagen) and ethanol, with final extraction using the BioSprint™96 One-For-All vet kit (Indical Bioscience) and Kingfisher Flex platform as per manufacturer's instructions. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed using TaqPath™ 1-Step RT-qPCR Master Mix, CG (Applied Biosystems™), 2019-nCoV CDC RUO Kit (Integrated DNA Technologies) and QuantStudio™ 7 Flex Real-Time PCR System. Sequences of the N1 primers and probe were: 2019-nCoV\_N1-forward, 5' GACCCCAAATCAGCGAAAT 3'; 2019-nCoV\_N1-reverse, 5' TCTGGTACTGCCAGTTGAATCTG 3'; 2019-nCoV\_N1-probe, 5' FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1 3'. The cycling conditions were 25 °C for 2 min,

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LLOQ of  $1.31 \times 10^4$  copies/g and LLOD of  $5.71 \times 10^4$  copies/g. Samples detected between LLOQ and LLOD were assigned  $2.86 \times 10^4$  copies/g.

Subgenomic RT-qPCR was performed on the QuantStudio™ 7 Flex Real-Time PCR System using TaqMan™ Fast Virus 1-Step Master Mix (Thermo Fisher Scientific) and oligonucleotides as specified by Wolfel et al.<sup>67</sup>, with forward primer, probe and reverse primer at a final concentration of 250 nM, 125 nM and 500 nM respectively. Sequences of the sgE primers and probe were: 2019-nCoV\_sgE-forward, 5' CGATCTCTTGATAGATCTGTTCTC 3'; 2019-nCoV\_sgE-reverse, 5' ATATTGCAGCAGTACGCACACA 3' and 2019-nCoV\_sgE-probe, 5' FAM-ACACTAGCCATCCTTACTGCGCTTCG-BHQ1 3'. Cycling conditions were 50 °C for 10 min, 95 °C for 2 min, followed by 45 cycles of 95 °C for 10 s and 60 °C for 30 s. RT-qPCR amplicons were quantified against an in vitro transcribed RNA standard of the full-length SARS-CoV-2 E ORF (accession number NC\_045512.2) preceded by the UTR leader sequence and putative E gene transcription regulatory sequence described by Wolfel et al. in 202049. Positive samples detected below the lower limit of quantification (LLOQ) were assigned the value of 5 copies/μl, whilst undetected samples were assigned the value of  $\leq 0.9$  copies/μl, equivalent to the lower limit of detection of the assay (LLOD). For nasal washes and oropharyngeal swabs extracted samples this equated to an LLOQ of  $1.29 \times 10^4$  copies/mL and LLOD of  $1.16 \times 10^3$  copies/mL. For tissue samples this equates to an LLOQ of  $5.71 \times 10^4$  copies/g and LLOD of  $5.14 \times 10^3$  copies/g.

The lung, nasal cavity including olfactory and respiratory mucosa, heart, liver, spleen, pancreas, trachea/larynx brain and small intestine (duodenum) were taken from each animal and were fixed in 10% neutral-buffered formalin, processed, embedded in paraffin wax and 4 μm thick sections

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RNAscope (an in-situ hybridisation method used on formalin-fixed, paraffin-embedded tissues) was used to identify the SARS-CoV-2 virus in all tissues. Briefly, tissues were pre-treated with hydrogen peroxide for 10 mins at room temperature (RT) target retrieval for 15 mins (98–101 °C) and protease plus for 30 mins (40 °C) (all Advanced Cell Diagnostics). A V-nCoV2019-S probe (Advanced Cell Diagnostics) targeting the S-protein gene was incubated on the tissues for 2 h at 40 °C. Amplification of the signal was carried out following the RNAscope protocol (RNAscope 2.5 HD Detection Reagent – Red) using the RNAscope 2.5 HD red kit (Advanced Cell Diagnostics). Appropriate controls were included in each ISH run. Digital image analysis was carried out with the Nikon NIS-Ar software package in order to calculate the total area of the tissue section positive for viral RNA. The images were scanned digitally using a Hamamatsu NanoZoomer S360 digital slide scanner and examined using Ndp.view2 v2.9.22 software. Nikon NIS-Ar software was used to perform digital image analysis in order to quantify the presence of viral RNA in lung sections. Graph and statistical analysis were performed with Graphpad Prism 9 and Minitab version 16.

## **Evaluation of C5 trimer therapeutic efficacy in the Syrian hamster model (University of Liverpool)**

Animal work was approved by the local University of Liverpool Animal Welfare and Ethical Review Body and performed under UK Home Office Project Licence PP4715265. Male golden Syrian hamsters (8–10 weeks old) were purchased from Janvier Labs (France). Animals were maintained under SPF barrier conditions in individually ventilated cages. For virus infection the Liverpool strain was used, a PANGO lineage B strain of SARS-CoV-2 (hCoV-2/human/Liverpool/PEMPO0001/2020)<sup>68</sup>. Animals were randomly assigned into multiple cohorts

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wax embedded. Consecutive sections (3–5  $\mu\text{m}$ ) were prepared and stained with HE for histological examination or subjected to immunohistological staining. Immunohistology was performed to detect SARS-CoV-2 antigen, macrophages (Iba1+), type II pneumocytes (SP-C+) and epithelial cells (pan-cytokeratin+), using the horseradish peroxidase (HRP) method and the following primary antibodies: rabbit anti-SARS-CoV nucleocapsid protein (1:6000, Rockland, 200-402-A50), rabbit anti-human Iba1/AIF1 (1:1000, Wako, 019-19741), rabbit anti-human prosurfactant protein-C (1:4000, SP-C; Abcam, ab40879) and mouse anti-human pan-cytokeratin (1:10000, clone PCK-26; Novus Biologicals, NB120-6401). Briefly, after de-paraffination, sections underwent antigen retrieval in citrate buffer (pH 6.0; Agilent) (anti-SARS-CoV-2, -Iba1) or Tris-EDTA buffer (pH 9.0) (anti-SP-C, -pan-cytokeratin) for 20 min at 98 °C and for 20 min at 37 °C respectively, followed by incubation with the primary antibody overnight at 4 °C (anti-SARS-CoV, SP-C) or 60 min at RT (anti-Iba1, -pan-cytokeratin). This was followed by blocking of endogenous peroxidase (peroxidase block, Agilent) for 10 min at room temperature (RT) and incubation with the secondary antibody, EnVision+/HRP, Rabbit and Mouse respectively (undiluted ready-to-use reagent, Agilent K406311-2) for 30 min at RT, followed by EnVision FLEX DAB + Chromogen in Substrate buffer (Agilent) for 10 min at RT, all in an autostainer (Dako). Sections were subsequently counterstained with haematoxylin. The anti-Iba1, -SP-C and -pan-cytokeratin antibodies were tested for their cross reactivity in hamster tissues, using the lung of an uninfected control hamster as positive control.

For double immunofluorescence, sections underwent antigen retrieval in citrate buffer (pH 6.0) and were then incubated with the first primary antibody (rabbit anti-SARS-CoV), overnight at 4 °C, followed by blocking of the endogenous peroxidase (see above) and 1 h incubation with the

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For morphometric analysis, the HE-stained sections were scanned (NanoZoomer-XR C12000; Hamamatsu, Hamamatsu City, Japan) and analysed using the software programme Visiopharm (Visiopharm 2020.08.1.8403; Visiopharm, Hoersholm, Denmark) to quantify the area of non-aerated parenchyma and aerated parenchyma in relation to the total area (= area occupied by lung parenchyma on two sections prepared from the left lung lobes) in the sections. This was used to compare the amount of air space (as an equivalent for the gas exchange surface) in the lungs between untreated and treated animals. A first app was applied that outlined the entire lung tissue as Region Of Interest (ROI, total area). For this a Decision forest method was used and the software was trained to detect the lung tissue section (total area). Once the lung section was outlined as ROI the large bronchi and vessels were manually excluded from the ROI. Subsequently, a second app with Decision forest method was trained to detect dense parenchyma (non-ventilated) and alveolar spaces (clear spaces; ventilated area) within the ROI.

## Reporting summary

Further information on research design is available in the [Nature Research Reporting Summary](#) linked to this article.

## Data availability

The coordinates and structure factors were deposited in the wwPDB with accession nos. C5 – RBD [7OAO](#), H3- RBD-C1 [7OAP](#), F2–RBD [7OAY](#), C5-Alpha-RBD [7OAU](#) and H3-Alpha RBD-

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## Contributions

J.H. isolated the nanobodies, designed trimers and carried out SPR analyses. M.W. and D.K.C. performed the EM studies. H.M., L.M., A.L.B., J.H. and J.H.N. performed the crystallography and ITC experiments. J.H., A.L.B., J.D., C.N., P.J.H., P.N.W. and M.D. produced proteins for the

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## Ethics declarations

## Competing interests

The Rosalind Franklin Institute has filed a patent that includes the four nanobodies described here, R.J.O., J.H. and J.H.N. are named as inventors. The other authors declare no competing interests.

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