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Production of a functionally active recombinant SARS-CoV-2 (COVID-19) 3C-like protease and a soluble inactive 3C-like protease-RBD chimeric in a prokaryotic expression system

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Abstract

During the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) intracellular life-cycle, two large polyproteins, pp1a and pp1ab, are produced. Processing of these by viral cysteine proteases, the papain-like protease (PLpro) and the chymotrypsin-like 3C-like protease (3CL-pro) release non-structural proteins necessary for the establishment of the viral replication and transcription complex (RTC), crucial for viral replication. Hence, these proteases are considered prime targets against which anti-coronavirus disease 2019 (COVID-19) drugs could be developed. Here, we describe the expression of a highly soluble and functionally active recombinant 3CL-pro using *Escherichia coli* BL21 cells. We show that the enzyme functions in a dimeric form and exhibits an unexpected inhibitory profile because its activity is potently blocked by serine rather than cysteine protease inhibitors. In addition, we assessed the ability of our 3CL-pro to function as a carrier for the receptor binding domain (RBD) of the Spike protein. The co-expressed chimeric protein, 3CLpro-RBD, did not exhibit 3CL-pro activity, but its enhanced solubility made purification easier and improved RBD antigenicity when tested against serum from vaccinated individuals in ELISAs. Chimeric proteins containing the 3CL-pro could represent an innovative approach to developing new COVID-19 vaccines.

Introduction

The severe acute respiratory syndrome coronavirus (SARS-CoV-2) was first identified in Wuhan, China, in December 2019 and subsequently reported throughout the world [1–3]. Person-to-person transmission of the virus resulted in rapid distribution of SARS-CoV-2, leading to the unprecedented pandemic of coronavirus disease 2019 (COVID-19), which up to now has claimed >6 million lives [4]. The impact of this pandemic on the global health and economy prompted the rapid action on the development, testing and approval of prophylactic COVID-19 vaccines, followed by mass immunisation programs [5].

SARS-CoV-2 is an enveloped virus that contains a single-strand of positive-sense RNA. Infection begins when the virus attaches to cells via the angiotensin-converting enzyme 2 (ACE2) receptor, mediated by the receptor-binding domain (RBD) of the major glycoprotein expressed on the virus surface, the Spike protein [6, 7]. Fusion of the viral membrane with the lumen of the endosomal membrane leads to endocytosis, facilitating infection via entry of the viral RNA into the cytosol. Applying new approaches and technologies, a multitude of vaccines have been developed, four of which were licensed by the regulatory agencies [4] and have been administrated across the world, representing a relief and a unique opportunity to prevent the deaths of millions of people and control the pandemic. In general, each of these four vaccines induces antibodies against the Spike protein and bind to the RBD to block its interaction with ACE2 [8, 9].

During the intracellular viral life cycle, two large polyproteins, pp1a and pp1ab, are translated. Sixteen non-structural proteins (nsp) are co-translationally and post-translationally released from pp1a and pp1ab upon proteolytic activity of two virus cysteine proteases, the papain-like protease (PLpro) and the chymotrypsin-like 3C-like protease (3CL-pro), also known as the main protease. These proteases allow the establishment of the viral replication and transcription complex (RTC), which is crucial for virus replication inside the cells [10]. The 3CL-pro plays a prominent role on viral gene expression and replication [11, 12]. Moreover, recent studies comparing the protease from other coronaviruses, SARS-CoV and Middle East Respiratory Syndrome CoV (MERS-CoV), or from picornaviruses, show that they are all highly conserved in terms of proteolytic activity and structure [13–15] and revealed important immunomodulatory properties for this enzyme. Amongst other mechanisms, during viral infection 3CL-pro contributes to the delay of host anti-viral innate immune response by cleaving or inactivating key elements of the Retinoic acid-inducible gene I (RIG-I) like receptors (RLRs)-mediated Type I interferon (INF-I) signalling pathway, which allows effective viral infection and contribute for disease progression and severity [15–17]. Thus, the 3CL-pro could be considered an attractive target for the development of future anti-COVID-19 treatments.

Here we describe the production of a recombinant 3CL-pro in a prokaryotic expression system and its purification as a highly soluble and functionally active protease. We also generated a 3C-like protease-RBD gene construct that enabled the production of a chimeric protein, named 3CLpro-RBD. This strategy proved useful to enhance the solubility and antigenicity of the RBD, albeit the recombinant chimeric protein did not exhibit proteolytic activity, understandable since the functional 3CL-pro functions as a dimer.

Methods

Ethical statement

Human experimental work was conducted according to Human Research Ethics Committees. Sera samples from individuals double-vaccinated with Pfizer/BioNTech (BNT162b2) vaccine were obtained from healthy volunteers following ethical approval by the National University of Ireland Galway, Ireland, research ethics committee (R20.Jun.06). The samples were pooled and immediately stored at −80 °C. All participants provided written informed consent prior to the study. Negative control samples obtained from the Irish Blood Transfusion Service. These blood samples were previously characterised by De Marco Verissimo *et al.* [18].

Recombinant protein production in *Escherichia coli* cells and purification

Sequences encoding the 3CL-pro and RBD proteins were codon optimised for expression in *Escherichia coli* and cloned into the pET-28a(+) vector (Genscript Biotech). The chimeric protein 3CLpro-RBD was produced by generating a gene construct that linked the 3CL-pro and RBD genes by a bridge sequence that encoded for glycine-proline triple repeat (GPGPGP) (see Fig. 1). The recombinantly produced proteins contain a thrombin cleavage site followed by a C-terminal His-tag. The synthesised vectors were transformed into BL21 competent *E. coli* cells (ThermoFisher Scientific) following the manufacturer's instructions and stored in Luria Bertani (LB) broth (Sigma-Aldrich) supplemented with 25% glycerol at −80 °C. LB broth supplemented with 50 µg/ml kanamycin was inoculated from the glycerol stock and incubated shaking (200 rpm) at 37 °C overnight. The culture was then diluted in fresh LB broth supplemented with kanamycin, incubated at 37 °C to OD_{600} 0.6 and protein expression induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG; ThermoFisher Scientific) for 4 h at 30 °C (3CL-pro and RBD); 18 h at 16 °C (3CLpro-RBD chimer). Following centrifugation at 10 000 × ***g*** for 10 min at 4 °C, the bacterial pellets were re-suspended in 10 mL ST buffer (10 mM Tris, 150 mM NaCl, pH 8.0).

The bacteria pellets were treated with lysozyme (10 µg/ml), sonicated on ice (6 × 10 s, 40% amplitude) and centrifuged 15 000 × ***g*** at 4 °C for 30 min. The soluble recombinant protein within the supernatant was purified and dialysed using the Profinia Affinity Chromatography Protein Purification System (Bio-Rad), with the mini profinity IMAC and mini Bio-Gel P-6 desalting cartridges (Bio-Rad). The protein concentration and purity were verified by Bradford Protein Assay (Bio-Rad) and by 4–20% SDS-PAGE gels (Bio-Rad) stained with Biosafe Coomassie (Bio-Rad), respectively. The gels were visualised using a G:BOX Chemi XRQ imager (Syngene).

As RBD protein was found within the inclusion bodies, processing of the pellets, protein purification and dialysis were performed as described by Schlager *et al.* [19] and employed by us previously to extract recombinant SARS-CoV-2 proteins previously [18]. Briefly, 1% (*w/v*) SDS buffer (8 mM Na_2HPO_4, 286 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, 1% (*w/v*) SDS, pH 7.4) containing 0.1 mM DTT was added to the cell pellet to solubilise the inclusion bodies. After sonication, the samples were centrifuged 15 000 × ***g*** at 4 °C for 30 min and the resulting supernatant containing the target protein was filtered and purified using a pre-equilibrated Ni-NTA beads column (Qiagen). The recombinant protein was eluted using 4 mL of elution buffer (8 mM Na_2HPO_4, 286 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, 0.1% Sarkosyl (*w/v*), 250 mM imidazole, pH 7.4) and buffer-exchanged into 1x PBS containing 0.05% sarkosyl, pH 7.4.

Size exclusion chromatography

The purified recombinant 3CL-pro purified was additionally subjected to size-exclusion (gel filtration) chromatography to resolve its dimerisation state. The purification was performed using a high-performance Superdex 7510/300 GL (Tricorn) column, with a flow rate of 400 µl/min and eluted into 1x PBS. Three known proteins of different molecular sizes were resolved in the column as standards, namely conalbumin (75 kDa), carbonic anhydrase (29 kDa) and aprotinin (6.5 kDa) (Sup Fig. S1). Once the retention parameters were determined, the r3CL-pro, at 1 mg/ml in PBS, was added to the column for purification. Aliquots of 200 µl of the sample were collected and stored at 4 °C for further analysis using an activity assay (see below).

Fluorogenic assay to assess the enzymatic activity of the recombinant 3CL-pro

The enzymatic activity of the recombinant r3CL-pro purified by affinity chromatography and of the different fractions produced by gel filtration was verified using a fluorogenic assay using the substrate LGSAVLQ-rhodamine 110-dp (BostonBiochem). Unless highlighted, all the screening assays were performed at 37 °C, in a 100 µl reaction volume Hepes buffer (20 mM Hepes, 2 mM EDTA, pH 7.4). Initially, the reaction buffer was mixed with either of the recombinant proteins, r3CL-pro (500 nM), rRBD (500 nM) or r3CLpro-RBD chimer (500 nM), and incubated for 5 min at room temperature. The fluorogenic substrate (20 µM) was added to the wells and the proteolytic activity was measured at 37 °C, over 1 h, as relative fluorescent units (RFU) in a PolarStar Omega Spectrophotometer (BMG LabTech). All

A

3C-like protease

SGFRKMAFPSGKVEGCMVQVTCGTTTLNGLWLDDVVYCPRHVICTSEDMLNPNYEDLLIRKSNHNFLVQA
GNVQLRVIGHSMQNCVLKLKVDTANPKTPKYKFVRIQPGQTFSVLACYNGSPSGVYQCAMRPNFTIKGSF
LNGSCGSVGFNIDYDCVSFCYMHHMELPTGVHAGTDLEGNFYGPFVDRQTAQAAGTDTTITVNVLAWLYA
AVINGDRWFLNRFTTTLNDFNLVAMKYNYEPLTQDHVDILGPLSAQTGIAVLDMCASLKELLQNGMNGRT
ILGSALLEDEFTPFDVVRQCSGVTFQ

B

Receptor Binding Domain of the Spike protein (RBD)

RVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRKRISNCVADYSVLYNSASFSTFKCYGVSPTKLNDLC
FTNVYADSFVIRGDEVRQIAPGQTGKIADYNYKLPDDFTGCVIAWNSNNLDSKVGGNYNYLYRLFRKSNLK
PFERDISTEIYQAGSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQPYRVVVLSFELLHAPATVCGPKKSTNLV
KNKCVNFN

C

Fig. 1. Primary sequence of the SARS-CoV-2 proteins and schematic representation of the 3CLpro-RBD chimeric protein structure. a: The amino acid sequence of the SARS-CoV-2 3C-like protease (3CL-pro) used for recombinant expression in *Escherichia coli*. b: The amino acid sequence of the receptor-binding domain (RBD) (residues 319–542 of the full SARS-CoV-2 Spike protein). c: Schematic representation of the 3CLpro-RBD chimeric protein structure showing the unique GP linker. SARS-CoV-2 proteins, 3CL-pro and the RBD are linked by a GP triplet (Glycine, G, and Proline, P), allowing their expression as a stable chimeric protein. Thr: Thrombin cleavage site; 6His: Histidine tag added to the protein C-terminal.

assays were carried out in triplicate. Commercial broad-spectrum protease inhibitors, namely serine protease inhibitors AEBSF (5 mM; Sigma-Aldrich) and Futhan-175 (FUT-175, 200 µM; BD-Pharmingen-Bioscience), and the cysteine protease inhibitor E-64 (200 µM; Sigma-Aldrich), were added to the reaction, individually, for further characterisation of the proteolytic activity of the recombinant r3CL-pro.

Assessment of the immunogenicity of the r3CL-pro, the rRBD and the chimeric r3CLpro-RBD

Seven weeks-old male and female CD1 outbred mice were used to assess the immunogenicity of the recombinantly-produced proteins according to the schedule shown in Figure 2. All animal experimental procedures were carried out by Eurogentec, BE, as follows: Group 1, adjuvant control group (Montanide ISA 206VG, Seppic) ($n = 9$); Group 2, r3CLpro-RBD chimer (15 µg) formulated in the Montanide adjuvant (1:1 *v*/*v*) ($n = 10$); Group 3, r3CL-pro (15 µg) formulated in the Montanide adjuvant (1:1 *v*/*v*) ($n = 7$); Group 4, rRBD (15 µg) formulated in the Montanide adjuvant (1:1 *v*/*v*) ($n = 7$); Group 5, r3CL-pro + rRBD (7.5 µg of each) formulated in the Montanide adjuvant (1:1 *v*/*v*) ($n = 7$).

ELISA to assess antibodies against the recombinant SARS-CoV-2 proteins in serum of vaccinated humans

Flat-bottom 96 well microtitre plates (Nunc MaxiSorp, Biolegend) were coated with r3CL-pro, r3CLpro-RBD chimer, rRBD or cmRBD as described above. After incubation in blocking buffer (2% BSA in PBS 0.05% Tween-20 (*v*/v), pH 7.4, PBST) and washing steps, pooled serum samples from (a) 10 vaccinated individuals (collected at least 10 days after the second dose Pfizer/BioNTech (BNT162b2) vaccine), or from 10 negative controls individuals (samples from the Irish Blood Transfusion Service obtained before COVID-19 pandemic) were diluted 1:100 in blocking buffer and added to the plate. After 1 h incubation at room temperature (RT), and washing five times with PBST, the secondary antibody HRP anti-Human IgG (Fc specific) (Sigma-Aldrich) was added (1: 15 000), and the plates incubated for 1 h at RT. After washing five times, TMB (3,3′,5,5′-Tetramethylbenzidine Liquid Substrate Supersensitive, Sigma-Aldrich) substrate was added to each well. Following a three-minute incubation the reaction was stopped with 2 N sulphuric acid and plates read at 450 nm in a PolarStar Omega Spectrophotometer. All samples were analysed in triplicate.

Analysis of the immune response of mice to the recombinant 3CL-pro and r3CLpro-RBD chimer by ELISA

The antibody response of individual mouse serum at day 0 and day 35 (Fig. 2) was assessed by ELISA using r3CL-pro and r3CLpro-RBD chimer as antigens. Flat-bottom 96 well microtitre plates (Nunc MaxiSorp, Biolegend) were coated overnight at 4 °C with either r3CL-pro (2 µg/ml) or r3CLpro-RBD chimer (2 µg/ml) diluted in carbonate buffer (pH 9.6). After incubation in blocking buffer (2% BSA in PBS-0.05% Tween-20 (*v*/*v*), pH 7.4; PBST) and washing steps, mice serum diluted 1:100 in blocking buffer was added to the antigen-coated wells and incubated for 1 h at RT. After washing five times with PBST, the secondary antibody HRP goat to mouse-anti-IgG (ThermoFisher Scientific) was added (1: 10 000), and the plates incubated for 1 h at RT. After washing five times, TMB substrate was added to each well. Following a three-minute incubation the reaction was stopped with 2 N sulphuric acid and plates read at 450 nm in a PolarStar Omega Spectrophotometer. All samples were analysed in triplicates.

Results

Production of SARS-CoV-2 recombinant proteins in E. coli

The 3C-like protease (r3CL-pro) was readily produced as a recombinant protein in *E. coli;* analysis of bacterial lysate showed

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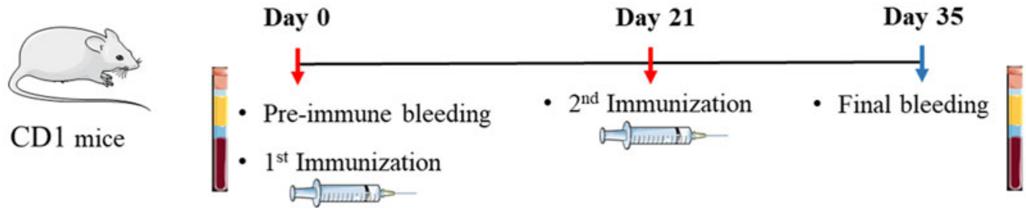

Fig. 2. Graphical schematic showing the schedule for the immunisation of CD1 outbred mice using the recombinant SARS-CoV-2 proteins. Red arrows indicate when the mice were immunised with adjuvant alone or with the recombinant 3CL-pro and 3CLpro-RBD chimer. Blue arrow indicates the end of the experiment and when the final bleeding was taken.

that it was a prominent protein that separated into the soluble fraction making it easy to isolate by affinity chromatography. The purified protein resolved at the expected molecular size of ~34 kDa, as a highly soluble protein, and our purification yielded 5.3 mg enzyme per litre of bacterial culture (Fig. 3a).

By marked contrast, we found that rRBD did not extract with the solubilisation buffers used but remained in the insoluble pellet, presumably in inclusion bodies. Accordingly, we employed an alternative means of solubilisation that included the chaotropic detergent sodium dodecyl sulphate (SDS) in the buffer, which proved successful in extracting the protein from the pellet [18, 19]. After this extraction procedure, the recombinant RBD could be isolated by NTA-affinity chromatography (Fig. 3b). The purified ~29 kDa protein remained soluble after dialysis against PBS containing 0,05% sarkosyl to remove the SDS detergent. This yielded ~1.5 mg of protein per litre of bacterial culture.

By expressing the 3CL-pro and RBD proteins as a chimer (Fig. 1), 3CLpro-RBD (~60 kDa), we found that the recombinant protein exhibits intermediate solubility to the protein expressed alone and, therefore, we were able to purify the chimer using the same automated protocol adopted with the r3CL-pro. It provided a yield of ~1.2 mg per litre of bacterial culture (Fig. 3b).

Further confirmation that we had purified the targeted proteins was obtained by western blot analysis, where we probed the purified proteins with antibody to the His-tag present on all three recombinant proteins (Fig. 3c).

Recombinant 3CL-pro of SARS-CoV-2 is functionally active in dimeric form, while the chimeric protein does not exhibit activity

Once we successfully produced a soluble recombinant 3CL-pro, we proceeded to check its proteolytic activity using an appropriate substrate. Since the cleavage site of 3CL-pro is highly unique the commercially available LGSAVLQ-rhodamine 110-dp substrate could be utilised to specifically assay the activity of the recombinant enzyme. The assay revealed that our recombinant enzyme is a functionally active protease at 37 °C in neutral pH (Fig. 4). Unexpectedly, however, despite 3CL-pro being described as a cysteine protease, we found that the enzyme was not inhibited by the cysteine proteases inhibitors such as E-64, but was susceptible to two broad-spectrum serine protease inhibitors, AEBSF and Futhan-175; the enzyme was completely inhibited by these latter compounds at concentrations of 5 and 200 µM, respectively (Fig. 4).

Using gel filtration to further purify the functionally active recombinant r3CL-pro we were able to determine the presence of a mixture of dimers and oligomers within the product purified by affinity chromatography (Peak 1 and 2, respectively; Fig. 5). The importance of the dimerisation for proteolytic activity of the r3CL-pro was determined by assaying the individual fractions within the two main protein peaks detected during purification. Our data revealed that the r3CL-pro is only functionally active when in its dimeric form, which represents the predominant peak in the chromatogram obtained (Fig. 5).

In order to examine if the expression of the 3CL-pro in a chimeric format with the RBD, 3CLpro-RBD chimer, had an effect on its proteolytic activity, we also assayed the activity of the recombinant chimeric protein and rRBD produced. Neither protein exhibited enzymatic activity when assayed in the same conditions used with the r3CL-pro (Fig. 4).

Antibodies in serum of naturally infected and vaccinated individuals recognise r3CLpro-RBD chimeric protein

In order to determine if our recombinant SARS-CoV-2 proteins had common epitopes with those present in the virus or with the viral proteins expressed upon vaccination, we performed ELISA tests with sera from vaccinated humans using our recombinant proteins r3CL-pro, r3CLpro-RBD chimer and rRBD as target antigens. In parallel, we used the commercial RBD recombinantly produced in mammalian cells (cmRBD), which is commonly used in immunological and functional assays [20]. Our results show that, when compared to negative control samples, sera from vaccinated individuals contain antibodies that recognise the r3CLpro-RBD chimer. These individuals also recognised the rRBD, albeit with lower intensity (Fig. 6). Surprisingly, a discrete antibody response against the r3CL-pro was also observed with the vaccinated group (Fig. 6). In addition, comparing the antibody response of individuals naturally infected using nucleocapsidic (Npro) and S2Frag proteins as target antigens in parallel, we verified that 50% of the individuals analysed mounted a significant antibody response to 3CL-pro (Supplementary Fig. S2).

r3CL-pro and r3CLpro-RBD chimer induce antibody response in vaccinated mice

In order to assess and compare the immunogenicity of the r3CL-pro and r3CLpro-RBD chimer, we immunised outbred CD1 mice with each protein in a regime similar to that initially recommended for the available COVID-19 vaccines (i.e., initially the recommendation for Pfizer and Oxford vaccines was 2 doses administered 3 weeks a part, Fig. 2) [8, 9]. Groups of CD1 mice were immunised with each of the proteins (15 µg) or with adjuvant Montanide ISA 206VG alone, as base-line controls, which was used to formulate the preparations with the recombinant proteins. Pre-immune (Day 0) and immune sera (Day 35) of each animal was assessed for antibodies using ELISA tests.

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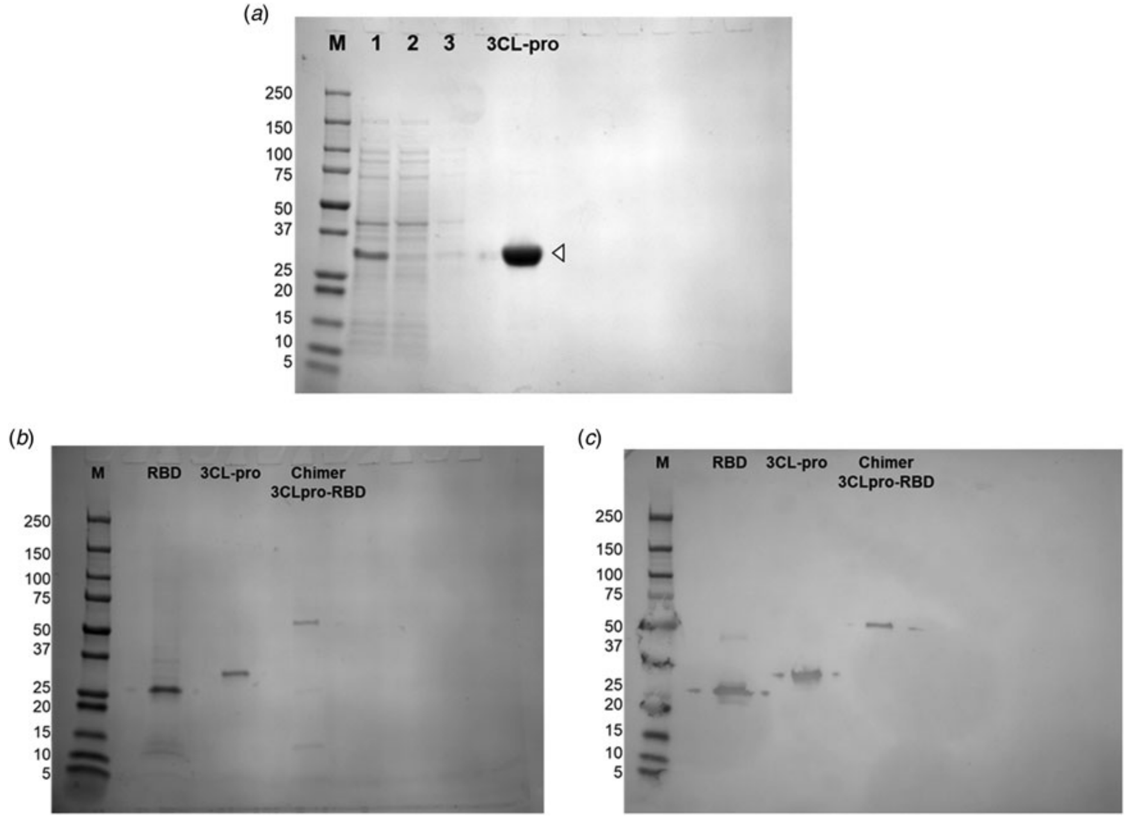

Fig. 3. Recombinant expression of the SARS-CoV-2 proteins, 3C-like protease, receptor binding domain (RBD), and 3CLpro-RBD chimer. a: Purification of recombinant 3C-like protease. The supernatant after bacterial pellet digestion (1); proteins that did not bind to the column in the run through (2); proteins in the wash (3); purified and dialysed recombinant protein (3CL-pro). b: The proteins were recombinantly expressed in the prokaryotic expression system, *E. coli,* purified and resolved in SDS-PAGE at the expected respective molecular size: RBD, ~29 kDa; 3CL-pro, ~34 kDa; 3Cpro-RBD chimer, ~60 kDa. c: Western blot of the recombinant proteins probed with the monoclonal anti-6Histidine tag antibody. M: Molecular weight in kilodaltons.

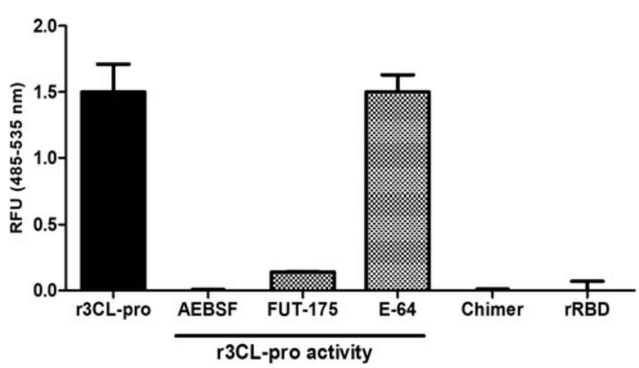

Fig. 4. Enzymatic activity of the SARS-CoV-2 recombinant proteins. The enzymatic activity of the r3C-Like protease (r3CL-pro, 500 nM) was tested with or without various broad-spectrum protease inhibitor, namely the serine protease inhibitors AEBSF (5 mM) and Futhan-175 (FUT-175, 200 µM), and the cysteine protease inhibitor E-64 (200 µM). The activity of the r3CLpro-RBD chimer (500 nM) and of the receptor biding domain (rRBD; 500 µM) was assessed in parallel using the same substrate, LGSAVLQ-Rh110 (20 µM). Enzymatic activity presented as relative fluorescence units (RFU) at 485–535 nm. Error bars indicate standard deviation of three separate experiments.

Our ELISA results show that those animals immunised with adjuvant alone did not react to any recombinant protein (Fig. 7). Mice immunised with r3CL-pro responded with high levels of antibodies against the r3CL-pro but their antibodies did not bind well to the r3CLpro-RBD chimeric (Fig. 7a and b). Conversely, mice immunised with the r3CLpro-RBD chimeric produced with antibodies that bound to the chimeric protein in ELISA, but elicited a low level response to the r3CL-pro (Fig. 7a).

Discussion

The 3C-like protease is regarded as a prime target for therapeutic drug treatment of COVID-19 due to its unique specificity for cleaving peptide bonds that are absent in human proteins [14, 21, 22]. This protease plays a central role during viral replication, being responsible for the cleavage of 11 sites within the polyprotein 1ab, ultimately releasing 13 non-structural proteins involved in SARS-CoV-2 replication inside the host's cells [23, 24]. Such activity is associated with the ability of this protease to recognise and cleave the unique peptide sequence Leu/Phe/Met-Gln ↓ Gly/Ser/Ala (↓ denotes the cleavage site). We were able to demonstrate that the r3CL-pro produced in the present study using bacterial expression systems has the same requirement for proteolytic activity, including for a glutamine (Gln) at the P1 position, as the recombinant enzyme was able to cleave specifically the LGSAVLQ-Rh110 substrate. Since 3CL-pro is reported to be functionally active as a dimer [25, 26], we further investigated its molecular state using size-exclusion chromatography and determined that the purified product was dominated by r3CL-pro dimers. Together with the results of the activity assays, our data indicate that the production of this enzyme in the *E. coli* system employed here allowed for protein fold and dimerisation

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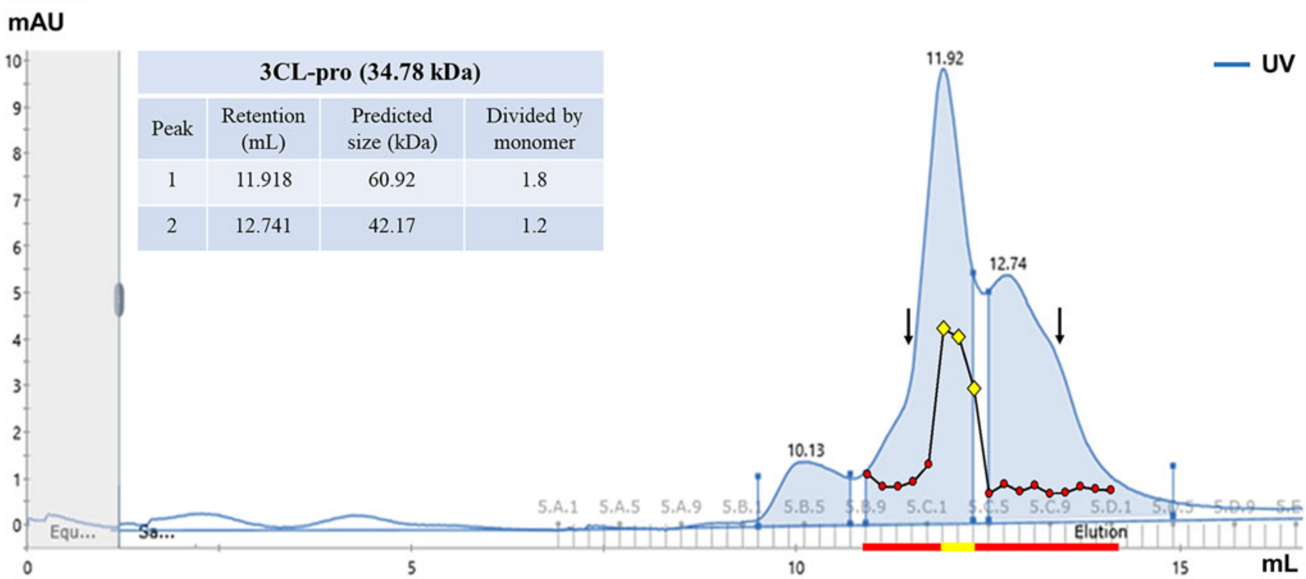

Fig. 5. Gel filtration chromatography of the recombinant 3CL-pro. The chromatogram of the purification of the r3CL-pro by gel filtration. Peak 1 (light blue), appeared at 11.918 ml was calculated to represent a protein of ~ 60.9 kDa, while the Peak 2 (light blue), at 12.74 ml, represents a protein of ~ 42 kDa, which indicates the presence of 3CL-pro as a dimer and a oligomer, respectively (for the protein standards data see Supplementary Fig. S1). The enzymatic activity of each fraction within the peaks (*n* = 17, in red) was determined in relation to the activity detected with the r3CL-pro purified only by affinity chromatography. In yellow, the three fractions where enzymatic activity was detected. Black arrows indicate the retention (ml) for the standards conalbumin (11.37) and carbonic anhydrase (13.50). The complete chromatogram for the standards is presented in the Supplementary Fig. S2.

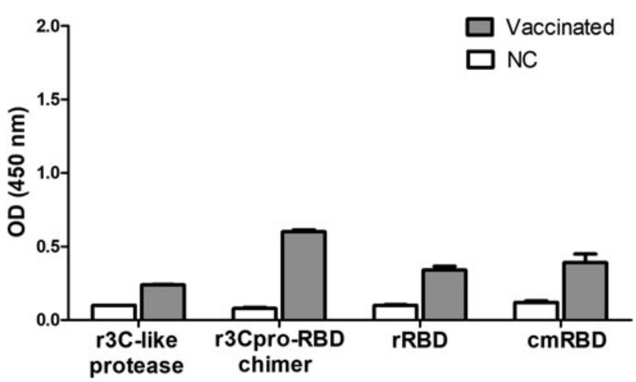

Fig. 6. Immune recognition of the recombinant SARS-CoV-2 proteins by antibodies in sera from COVID-19 fully-vaccinated individuals. ELISA tests were performed to assess the presence antibodies in serum of negative control individuals (NC) or COVID-19-vaccinated individuals that bind r3CL-pro, r3CLpro-RBD chimer, rRBD or commercial RBD (cmRBD). Results presented as the mean and standard deviation of OD 450 nm values of all the individuals of the group.

similar to that of the native form. The unexpected inhibition of this protease by broad-spectrum serine protease inhibitors, but not by cysteine protease inhibitors, does not support the claim that this protease is a cysteine peptidase, but is more in keeping with the reported chymotrypsin-like protease activity of 3CL-pro [10, 27]. Perhaps this observation could be exploited in anti-3CL-pro novel drug designing.

In our previous study aimed at improving COVID-19 diagnostics [18] by using the Npro and S2Frag proteins as parallel target antigens on ELISA tests, we also evaluated the antibody response of individuals naturally infected with SARS-CoV-2 against the r3CL-pro. Our data showed that, although higher antibodies titres are generated against Npro and S2frag, 50% of the individuals analysed had significant levels of antibodies against 3CL-pro, indicating that this protease is naturally immunogenic during COVID-19 infection (Supplementary Fig. S2). As expected, individuals vaccinated against COVID-19 with the currently available vaccines do not raise a significant antibody response against 3CLpro (Fig. 6). Nonetheless, in the present study, we demonstrated that the recombinant version of this protein is immunogenic and induces a strong and specific antibody response in mice. Together, this data indicates the potential application of the 3CL-pro as a vaccine target against COVID-19. Inducing antibodies to the 3CL-pro could provide broader antibody and cellular immunity to individuals and thus induce a stronger protection against SARS-CoV-2 infection [15, 16].

Given the difficulty in producing recombinant soluble SARS-CoV-2 proteins in prokaryotic systems [18, 28, 29], we considered that the highly soluble 3CL-pro could serve as a carrier protein to produce and deliver RBD. Thus, we designed a construct to generate a recombinant chimeric protein, 3CLpro-RBD. The two proteins were linked using a GP-linker that allows separation and flexibility between them, so that each molecule is stable and can function separately. This approach improved the solubility and production of the RBD. However, we observed that the functional activity of the r3CL-pro chimeric was lost, likely due to incorrect protease folding or the inability of the 3CL-pro to form dimers when linked to the RBD.

Surprisingly, our ELISA tests revealed that CD1 mice immunised with the chimeric protein developed antibodies against the 3CL-pro portion of the antigen, but not to the RBD part. Nonetheless, antibodies in serum from individuals fully vaccinated against COVID-19 recognised the chimeric protein more efficiently than the rRBD alone, indicating that the presentation of the RBD is improved when produced in the chimeric format. Furthermore, the ability of vaccinated individuals to recognise the commercial RBD produced in mammalian cells (cmRBD), but not our rRBD produced in *E. coli* cells, suggests that

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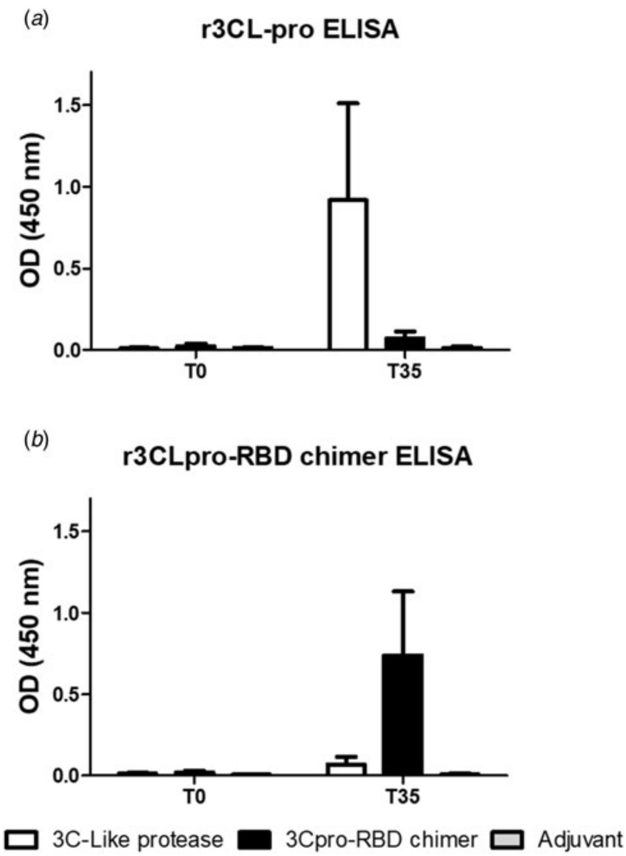

Fig. 7. Evaluation of the antibody response induced by the recombinant 3CL-pro and 3CLpro-RBD chimer proteins in CD1 outbred mice. Groups of CD1 outbred mice were immunised with either r3CL-pro, r3CLpro-RBD chimer or Adjuvant only and evaluated for specific antibodies using ELISA tests. a: ELISA using r3CL-pro as target antigen; b: ELISA using r3CLpro-RBD chimer as target antigen to assess specificity immune response stimulated. Results presented as the mean and standard deviation of OD 450 nm values of all the animals of the group.

glycosylated epitopes are important in antibody recognition. Based on current data, antibodies that bind to the RBD can neutralise the SARS-CoV-2 virus by preventing it binding to ACE2 receptors and consequent host cells invasion and infection [22, 30–32]. A chimeric protein carrying the RBD certainly could help to circumvent the low cellular immunogenicity problem faced with vaccines, and the combination of RBD with more immunogenic molecules has been demonstrated to be a useful strategy to create alternative vaccine targets to fight COVID-19 [28, 29, 33]. This is the first time that another SARS-CoV-2 molecule, and specifically the 3CL-pro, has been considered as an option to fuse with the antigenic RBD, which could represent a future strategy to develop COVID-19 vaccine targets, as the 3CL-pro could have a superior ability to induce long-term neutralising antibody responses, as well as potent cellular immunity, and also overcome the problems with the SARS-CoV-2 variants that are mainly associated high plasticity of the Spike protein [34–36]. The concept of chimerisation of SARS-CoV-2 molecules that we introduced in this study might open new avenues on the discovery of drug and vaccine targets to fight COVID-19.

Conclusions

Here we present a straightforward, efficient and cheap method to express and purify a highly soluble and functional 3C-like protease, which is regarded as a main drug target at which to develop therapies against SARS-CoV-2 infection. The enzyme could be useful in the development of high-throughput assays for the screening and isolation of new anti-COVID-19 compounds. In addition, given its solubility and potential for triggering a cellular immune response to fight infection, we introduced the idea of using the 3CL-pro as a carrier to other SARS-CoV-2 proteins, in this case RBD, to improve their expression and delivery as potential vaccines. Co-expressing 3CL-pro and RBD in a chimeric format resulted in loss of the 3CL-pro activity (likely due to the inability of the enzyme domain to dimerise), but enhanced the solubility of the RBD expressed alone, and improved its antigenic properties. Chimeric proteins containing the 3CL-pro could represent a new approach to engender next generation protein-subunit COVID-19 vaccine candidates.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0950268822001078

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Conflict of interest. None.

Data availability. The data that support the findings of this study are all included in the publication.

References

1. **Sun B *et al.*** (2020) Kinetics of SARS-CoV-2 specific IgM and IgG responses in COVID-19 patients. *Emerging Microbes & Infections* **9**, 940–948.
2. **Meo SA *et al.*** (2021) Omicron SARS-CoV-2 new variant: global prevalence and biological and clinical characteristics. *European Review for Medical and Pharmacological Sciences* **25**, 8012–8018.
3. **Aleem A, Akbar Samad AB and Slenker AK** (2022) Emerging Variants of SARS-CoV-2 And Novel Therapeutics Against Coronavirus (COVID-19). In: *StatPearls*. Treasure Island (FL): StatPearls Publishing Copyright © 2022, StatPearls Publishing LLC.
4. **World Health Organization** (2022) Coronavirus disease (COVID-19) pandemic. Available at https://covid19.who.int/ (Accessed 20th May 2022).
5. **Coronavirus (COVID-19) Vaccinations: Our World in Data** (2022) Available at https://ourworldindata.org/covid-vaccinations (Accessed 22nd May 2022).
6. **Letko M, Marzi A and Munster V** (2020) Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. *Nature Microbiology* **5**, 562–569.
7. **Holmes KV** (2003) SARS-associated coronavirus. *New England Journal of Medicine* **348**, 1948–1951.
8. **Polack FP *et al.*** (2020) Safety and efficacy of the BNT162b2 mRNA COVID-19 vaccine. *New England Journal of Medicine* **383**, 2603–2615.
9. **Voysey M *et al.*** (2021) Safety and efficacy of the ChAdOx1 nCoV-19 vaccine (AZD1222) against SARS-CoV-2: an interim analysis of four randomised controlled trials in Brazil, South Africa, and the UK. *Lancet (London, England)* **397**, 99–111.
10. **V'Kovski P *et al.*** (2021) Coronavirus biology and replication: implications for SARS-CoV-2. *Nature Reviews Microbiology* **19**, 155–170.
11. **Gordon CJ *et al.*** (2020) The antiviral compound remdesivir potently inhibits RNA-dependent RNA polymerase from Middle East respiratory syndrome coronavirus. *Journal of Biological Chemistry* **295**, 4773–4779.
12. **Dai W *et al.*** (2020) Structure-based design of antiviral drug candidates targeting the SARS-CoV-2 main protease. *Science (New York, N.Y.)* **368**, 1331–1335.
13. **Anand K *et al.*** (2003) Coronavirus main proteinase (3CLpro) structure: basis for design of anti-SARS drugs. *Science (New York, N.Y.)* **300**, 1763–1767.

14. **Zhang CH *et al.*** (2021) Potent noncovalent inhibitors of the main protease of SARS-CoV-2 from molecular sculpting of the drug perampanel guided by free energy perturbation calculations. *ACS Central Science* **7**, 467–475.
15. **Ng CS, Stobart CC and Luo H** (2021) Innate immune evasion mediated by picornaviral 3C protease: possible lessons for coronaviral 3C-like protease? *Reviews in Medical Virology* **31**, 1–22.
16. **Blanco-Melo D *et al.*** (2020) SARS-CoV-2 launches a unique transcriptional signature from in vitro, ex vivo, and in vivo systems. bioRxiv. 2020:004655.
17. **Lokugamage KG *et al.*** (2020) Type I interferon susceptibility distinguishes SARS-CoV-2 from SARS-CoV. *Journal of Virology* **94**, e01410–20.
18. **De Marco Verissimo C *et al.*** (2021) Improved diagnosis of SARS-CoV-2 by using nucleoprotein and spike protein fragment 2 in quantitative dual ELISA tests. *Epidemiology and Infection* **149**, e140.
19. **Schlager B, Straessle A and Hafen E** (2012) Use of anionic denaturing detergents to purify insoluble proteins after overexpression. *BMC Biotechnology* **12**, 95.
20. **Huang WC *et al.*** (2020) SARS-CoV-2 RBD neutralizing antibody induction is enhanced by particulate vaccination. *Advanced Materials* **32**, e2005637.
21. **Jukič M *et al.*** (2021) Prioritisation of compounds for 3CL(pro) inhibitor development on SARS-CoV-2 variants. *Molecules (Basel, Switzerland)* **26**, 3003.
22. **Chen YW, Yiu C-PB and Wong K-Y** (2020) Prediction of the SARS-CoV-2 (2019-nCoV) 3C-like protease (3CL pro) structure: virtual screening reveals velpatasvir, ledipasvir, and other drug repurposing candidates. *F1000Research* **9**, 129.
23. **Froggatt HM, Heaton BE and Heaton NS** (2020) Development of a fluorescence-based, high-throughput SARS-CoV-2 3CL(pro) reporter assay. *Journal of Virology* **94**, e01265-20.
24. **Jaskolski M *et al.*** (2021) Crystallographic models of SARS-CoV-2 3CL (pro): in-depth assessment of structure quality and validation. *IUCrJ International Union of Crystallography* **8**, 238–256.
25. **Chen S *et al.*** (2008) Residues on the dimer interface of SARS coronavirus 3C-like protease: dimer stability characterization and enzyme catalytic activity analysis. *Journal of Biochemistry* **143**, 525–536.
26. **Goyal B and Goyal D** (2020) Targeting the dimerization of the main protease of coronaviruses: a potential broad-spectrum therapeutic strategy. *ACS Combinatorial Science* **22**, 297–305.
27. **Molavi Z *et al.*** (2021) Identification of FDA approved drugs against SARS-CoV-2 RNA dependent RNA polymerase (RdRp) and 3-chymotrypsin-like protease (3CLpro), drug repurposing approach. *Biomedicine and Pharmacotherapy* **138**, 111544.
28. **Bellone ML *et al.*** (2021) Production in Escherichia coli of recombinant COVID-19 spike protein fragments fused to CRM197. *Biochemical and Biophysical Research Communications* **558**, 79–85.
29. **Tan TK *et al.*** (2021) A COVID-19 vaccine candidate using SpyCatcher multimerization of the SARS-CoV-2 spike protein receptor-binding domain induces potent neutralising antibody responses. *Nature Communications* **12**, 542–542.
30. **Weinreich DM *et al.*** (2020) REGN-COV2, a neutralizing antibody cocktail, in outpatients with COVID-19. *New England Journal of Medicine* **384**, 238–251.
31. **Hoffmann H-H *et al.*** (2021) Functional interrogation of a SARS-CoV-2 host protein interactome identifies unique and shared coronavirus host factors. *Cell Host & Microbe* **29**, 267–280, e5.
32. **Chen P *et al.*** (2020) SARS-CoV-2 neutralizing antibody LY-CoV555 in outpatients with COVID-19. *New England Journal of Medicine* **384**, 229–237.
33. **Hong SH *et al.*** (2021) Immunization with RBD-P2 and N protects against SARS-CoV-2 in nonhuman primates. *Science Advances* **7**, eabg7156.
34. **Gómez CE, Perdiguero B and Esteban M** (2021) Emerging SARS-CoV-2 variants and impact in global vaccination programs against SARS-CoV-2/COVID-19. *Vaccines (Basel)* **9**, 243.
35. **Plante JA *et al.*** (2021) Spike mutation D614G alters SARS-CoV-2 fitness. *Nature* **592**, 116–121.
36. **Oude Munnink BB *et al.*** (2021) The next phase of SARS-CoV-2 surveillance: real-time molecular epidemiology. *Nature Medicine* **27**, 1518–1524.