The alpha/B.1.1.7 SARS-CoV-2 variant exhibits significantly higher affinity for ACE-2 and requires lower inoculation doses to cause disease in K18-hACE2 mice.

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Running head: Functional characterization of the alpha/B.1.1.7 SARS-CoV-2 variant

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Abstract (currently 166 words)

The alpha/B.1.1.7 SARS-CoV-2 lineage emerged in autumn 2020 in the United Kingdom and transmitted rapidly until winter 2021 when it was responsible for most new COVID-19 cases in many European countries. The incidence domination was likely due to a fitness advantage that could be driven by the RBD residue change (N501Y), which also emerged independently in other Variants of Concern such as the beta/B.1.351 and gamma/P.1 strains. Here we present a functional characterization of the alpha/B.1.1.7 variant and show an eight-fold affinity increase towards human ACE-2. In accordance with this, transgenic hACE-2 mice showed a faster disease progression and severity after infection with a low dose of B.1.1.7, compared to an early 2020 SARS-CoV-2 isolate. When challenged with sera from convalescent individuals or anti-RBD monoclonal antibodies, the N501Y variant showed a minor, but significant elevated evasion potential of ACE-2/RBD antibody neutralization. The data suggest that the single asparagine to tyrosine substitution remarkable rise in affinity may be responsible for the higher transmission rate and severity of the B.1.1.7 variant.

Introduction

Since the global expansion of SARS-CoV-2, there has been a world-wide surveillance effort to identify new emerging variants empowered by the unprecedented generation of full-genome sequences of circulating strains shared under the GISAID umbrella (www.gisaid.org). Many genetically drifted SARS-CoV-2 variants have been reported since the early spring of 2020. Some of these that affect the S (spike) gene are non-synonymous mutations. These mutations have
received much attention since the spike protein binds directly to the angiotensin-converting enzyme-2 receptor (ACE-2) responsible for viral entry into the human host cells\textsuperscript{1-3}. Furthermore, almost all current vaccine formulations, except for the few vaccines that rely on the inactivated SARS-CoV-2, focus on raising immunity specifically towards the spike protein or parts of it, mostly the receptor-binding domain (RBD)\textsuperscript{4}. The possible humoral or cellular immunity evasion due to critical residue changes in B- and T-lymphocyte epitopes of the spike protein is a major concern. In fact, several of the non-synonymous mutations identified to date are located in the RBD of the spike protein and may improve viral fitness by raising the affinity of the virus towards ACE-2 or by hindering antibody-mediated viral neutralization\textsuperscript{5}. Some of these residue substitutions, including the N501Y mutation, seem to have arisen independently by convergent evolution and selection and are found in the B.1.1.7 (alpha), the South African B.1.351 (beta), and the Brazilian P.1 (gamma) variants of concern (VOC)\textsuperscript{6-8}; as well as the variant of interest (VOI) B.1.621 (mu), first identified in Colombia\textsuperscript{9}, and the former VOI P.3 (theta) from The Philippines\textsuperscript{10}. All the above VOC/VOI have been for the most part replaced by the B.1.617.2 (Delta), which emerged in India in December 2020\textsuperscript{11,12} and has become the dominant variant globally (\texttt{www.gisaid.org}, accessed October 2021). A not-yet-peer reviewed publication on vaccine breakthrough, found that the N501Y mutation was associated with increased number of breakthrough infections\textsuperscript{13}. Recent studies suggest that the B.1.1.7, B.1.351, P.1, and B.1.617.2 VOC have increased transmissibility\textsuperscript{7,8,14-16} and appear to cause a more severe disease\textsuperscript{17-20}, albeit most rely primarily on epidemiological and prediction data. Moreover, other reports indicate that especially the B.1.351, P.1, and B.1.617.2 have a reduced potential of antibody-dependent virus neutralization from convalescent individuals and
might pose a challenge to current vaccines\textsuperscript{21–26}. The same immune evasion capacity has not been reported for the B.1.1.7 variant, although some evasion cannot be excluded\textsuperscript{27}.

More in-depth knowledge of the functional biophysical characteristics of circulating and emerging variants is needed. A large yeast two-hybrid study characterizing the mutational landscape of SARS-CoV-2 RBD indicated that the N501Y residue-change results in increased affinity towards ACE-2\textsuperscript{28}. Two recent studies, one still non-peer reviewed, have focused on the N501Y mutation showing a variable impact on affinity (from 0.5 nM to sub pM)\textsuperscript{29,30}.

In the present study we have performed detailed biophysical characterization of the RBD variants N439K (the most prevalent RBD mutation to date) and N501Y (shared between the B.1.1.7, B.1.351, and P.1), showing that the 1:1 interaction affinity to human ACE-2 is two- and eight-fold increased, respectively, compared to the original Wuhan RBD.

Using a transgenic humanized ACE-2 mouse model we found that a lower infection dose was required to establish infection when challenged with the alpha/B.1.1.7 variant compared to an early 2020 isolate (differing at the N501Y position) and that the alpha variant resulted in increased disease severity. Additionally, we present data suggesting a partial decrease in the antibody capacity to neutralize N501Y:ACE-2 interaction \textit{in vitro} using sera from convalescent individuals (\(n = 140\)). However, no significant difference was seen when we tested a small cohort of convalescent individuals (\(n = 10\)) in a plaque reduction neutralization test (PRNT) and a group of anti-RBD monoclonal antibodies (\(n = 8\)).
Results

Biophysical characterization

We expressed recombinant SARS-CoV-2 RBD wild-type (wt) (Wuhan-hu-1), RBD N439K, and RBD N501Y in Expi293 HEK cells and performed thermal stability and binding kinetic analyses to determine the biophysical relevance of the RBD variants (Fig. 1). Protein purity and homogeneity was evaluated by SDS-PAGE (Fig. 1A) and size-exclusion HPLC (Fig. 1B). We monitored the thermal unfolding using the intrinsic fluorescence ratio at 350 and 330 nm and observed a ~2.5 °C reduction in the inflection temperature (Ti) for the N439K variant (Fig. 1C). This suggests that the N439K, but not the N501Y, has a moderately deleterious effect on the RBD stability. Next, we measured their binding kinetics towards the human ACE-2 by biolayer interferometry (BLI) to determine their functional importance. The N439K variant bound with an approx. two-fold higher affinity than the wt (8.6 nM vs 17 nM) (Fig. 1D, E), while the N501Y variant did so with an eight-fold higher affinity (2.23 nM vs 17 nM) (Fig. 1F). Analyses of the binding response curves indicate that the variants bound to ACE-2 faster (KaN439K = 4.15x10^5 M⁻¹s⁻¹, KaN501Y = 4.76x10^5 M⁻¹s⁻¹, Kawt = 3.34x10^5 M⁻¹s⁻¹) and mostly the N501Y had noticeable slower dissociation rates (KdisN439K = 3.57x10⁻³ s⁻¹, KdisN501Y = 1.06x10⁻³ s⁻¹, Kdiswt = 5.9 x10⁻³ s⁻¹).
**Figure 1. Biophysical characterization of recombinant RBD variants.**

(A) SDS-PAGE total protein stain of RBD variants and ACE-2.

(B) SEC profiles of the purified proteins run in a BioSep-SEC-S3000 column. Purity was determined by peak integration with the Empower software.

(C) Thermal denaturation curves of the RBD wt, N439K, and N501Y variants. Data are represented as the first derivative of the intrinsic fluorescence ratio 350nm:330nm of the mean of 3 replicates. Vertical dashed lines represent the inflection temperatures (Ti).

(D, E, F) BLI sensorgrams of RBD wt (D), N439K (E), and N501Y (F) binding to ACE-2-Fc.
immobilized in anti-human Fc capture (AHC) sensors. ACE-2-immobilized sensors were dipped into 7- to 11-point dilution series of RBD for 500 s, followed by dissociation for another 500 s.

**Alpha/B.1.1.7 establishes disease-causing infection at lower inoculation doses than original SARS-CoV-2 isolate**

In order to examine whether the increased affinity of the N501Y variant for ACE-2 was associated with a more efficient establishment of infection and development of disease, we challenged transgenic ACE-2 humanized K18-hACE2 mice with the early 2020 SARS-CoV2 B.1 (Freiburg isolate, FR-424831) and the B1.1.7 (alpha) strains. The model has been reported to reflect many aspects of COVID-19, including viral replication and histopathological changes in the lungs32. Upon infection with two different doses of the B.1 strain, we observed no weight loss as an indication of disease development (**Fig. 2A**). However, infection with B.1.1.7 at the same doses led to severe disease development in the mice. Mann-Whitney pair-wise comparisons (B.1 vs B.1.1.7) showed a significant difference in weight at low viral doses at days 6 and 7 (multiple comparisons corrected q values 0.009743 and 0.000291, respectively, n = 9 per group). The same tendency, albeit not statistically significant, was observed for the high viral doses (n = 5 per group). We also observed that the virus replicates to higher levels in the lungs at day 2 post-infection with B.1.1.7 compared to B.1 when infected with low viral doses (**Fig. 2B**).
**Figure 2. Development of COVID-19-like disease in mice.** (A) Weight evolution of K18-hACE2 mice challenged intranasally with $2.5 \times 10^3$ (low, $n = 9$ per group) or $2.5 \times 10^4$ p.f.u. (high, $n = 5$ per group) SARS-CoV-2 B.1 (Freiburg isolate, FR-4286) or B.1.1.7. Global differences between the groups were analysed with the Kruskal-Wallis test. (B) Viral load measured in the lungs on day 2 post-infection. The mice were infected with the same doses as described in (A). The expression of Sars-CoV-2 RNA was analysed by real-time qPCR. Values normalized to 18S-rRNA are presented as mean ± SEM.

**Determination of the evasion capacity of the N439K and N501Y variants in naturally-induced antibody-mediated immunity**

Next, we sought to clarify whether the residue changes could affect the folding and binding properties of the RBD and viral fitness by providing immune evasion. To do so, we first determined the antibody-mediated inhibition potency, measured as the inhibition of the ACE-2/RBD interaction, in sera of recovered COVID-19 patients ($n = 140$) using a validated *in vitro* antibody inhibition assay$^{33}$ (Fig. 3A, B, C). There was a statistically significant reduction in the inhibition of the N439K and N501Y RBD compared to the wt ($p < 0.0001$
for both) (Fig. 3A). The inhibition potencies towards the wt and the variants had a highly significant correlation ($\rho = 0.9774$ and $\rho = 0.9581$ for N439K and N501Y respectively, $p < 0.0001$), with the best-fit X-intercept ranging from 12.38 to 15.89 for the N439K and N501Y respectively (Fig. 3B, C). However, analyses of convalescent sera ($n = 10$) using a PRNT virus neutralization platform showed no significant difference in the neutralization potency towards the B.1 and B.1.1.7 strains (Fig. 3D).

**Figure 3. Antibody-mediated inhibition potency of recovered COVID-19 patient sera.** (A) Inhibition of wt, N439K, and N501Y RBD towards ACE-2 in serum from convalescent COVID-19 individuals ($n = 140$). Friedman test with Dunn’s multiple comparisons. Orange lines represent medians. ****, $p < 0.0001$. (B, C) Linear regression and Spearman correlation
analyses for N439K vs wt (B) and N501Y vs wt (C). Trend line represents linear regression.

(D) Neutralization of serum from convalescent COVID-19 individuals (*n* = 10) against B.1 and B.1.1.7 calculated by the PRNT and analysed using Wilcoxon matched-pairs signed ranked tests with multiple comparisons corrections. NS, no serum.

**Determination of the evasion capacity of the RBD variants in vaccine-induced immunity**

Next, we analysed the variants’ evasion capacity using a previously established vaccine mouse model\(^{20}\). Briefly, mice were immunized with wt RBD (*n* = 3) or wt prefusion-stabilized spike protein ectodomain (*n* = 3). Polyclonal sera were collected after 3 rounds of immunizations, and monoclonal antibodies (mAbs) were developed and characterized from cloned hybridomas. As shown previously\(^{33,35}\), polyclonal sera from RBD immunized mice was approx. 4-fold more effective than spike-immunized mice sera, with IC\(_{50}\) values of 2.4–2.6x10\(^4\) (RBD) and 6.6–8.2x10\(^3\) (spike), respectively (**Fig. 4A, B**). Sera from RBD-immunized mice showed no difference in the inhibitory potency against the wt and the variants. However, best-fit IC\(_{50}\) values from sera from mice immunized with spike differed slightly between strains, as 11% and 19% higher serum concentration was required for the N439K and N501Y variants, respectively, to achieve the same inhibition levels as for the wt RBD. We also evaluated the inhibition potency of mouse mAbs raised against wt RBD and wt spike (*n* = 18) (**Fig. 4C, D**). The mAbs were screened for high affinity towards the SARS-CoV-2 RBD wt, and their epitopes mapped via competition assays\(^{33}\). Linear regression and Spearman correlation analyses of the mAbs with best-fit IC50s within the range of concentration tested (*n* = 8) showed that the N439K and N501Y mutations had very minor
effects on the inhibition potency of the mAbs ($N439K \ R^2 = 0.9777, \ p = 1, \ p < 0.0001; \ N501Y \ R^2 = 0.9832, \ p = 0.9762, \ p < 0.0001$).

Figure 4. Antibody-mediated inhibition potency of polyclonal sera and mAbs isolated from mice immunized against wt RBD or spike protein. IC$_{50}$ comparison of the inhibition of polyclonal mouse sera from an animal vaccine model based on RBD (A) or spike (B) challenged with RBD wt, N439K, and N501Y. Connecting lines represent non-linear fits using the equation [inhibitor] vs normalized response with variable slope. Vertical dashed lines delimit the IC$_{50}$ values, where they intersect the horizontal 50% inhibition dashed line. Data are presented as mean ± SEM. Comparison of the inhibition potency (log[IC$_{50}$]) of mouse mAbs ($n = 8$) calculated from three independent experiments against RBD wt and RBD N439K (C) or RBD N501Y (D), analysed by linear regression and Spearman correlation. The trend represents a linear regression. Dashed line signals equidistance between axis (i.e. slope 1). Neutralization fold changes over 1.5 are highlighted. Only mAbs with IC$_{50}$ values within the
range of concentrations tested were included in the statistical analyses (i.e. 8 out of 18 tested mAbs).

Discussion

Emerging clusters of genetically drifted SARS-CoV-2 variants have received much attention due to concerns of enhanced adaptive fitness and viral escape of neutralizing antibodies or T-cell mediated responses. A specific focus has been on the spike protein changes that interact with the ACE-2 receptor and mediate host cell entry. This is also the target for the vast majority of the vaccine strategies. A worldwide effort to sequence viral strains has revealed many emerging variants. However, although many spike variants have been reported so far, there seems to be a limitation in terms of the “freedom” possibilities to which and how many non-synonymous mutations are emerging in the S gene and particularly in the RBD coding region\textsuperscript{28}. One of these RBD residue changes is N501Y, which has appeared by convergent evolution in three of the so-called VOC: B.1.351 (beta), P.1 (gamma), and the B.1.1.7 (alpha) variant, but interestingly not in the rapidly spreading B.1.617.2 (delta) variant, where other mutations appear to be of importance. Nevertheless, from an epidemiological viewpoint, all these variants seem to have a higher transmission rate and have outcompeted the original Wuhan strain in the regions they have arisen or been introduced to. In Europe, the B.1.1.7 (alpha) variant was, at the end of March 2021, dominating many countries only few months after its emergence with severe consequences on the health care system, while now just a few months later in the end of August 2021, the B.1.617.2 (delta) variant has taken over (www.gisaid.com, accessed August 2021).
We aimed to characterize the functional properties of the B.1.1.7 (alpha) variant and address any potential evasion capacity of antibody-mediated neutralization. We also did this for the prevalent RBD mutation N439K and we found a two-fold affinity increase to ACE-2 and a partial evasion of antibody-mediated neutralization, lending support to a recently published paper\(^5\).

When we did BLI measurements of the 1:1 interaction of N501Y RBD on ACE-2 immobilized sensors, we found a remarkable eight-fold affinity increase of the variant (2.2 nM) compared to wild-type Wuhan RBD (17 nM). This might be a central explanation for the observed higher transmission rate and the fitness advantage of the B.1.1.7 variant. Furthermore, the 1:1 molecular affinity determination might even underestimate the true in vivo interaction potential covering multivalent avidity interactions between the trimeric spike scaffolds on the viral surface and the ACE-2-covered host membrane. In agreement with this, we found that transgenic ACE-2 humanized K18-hACE2 mice developed severe disease following a low inoculation dose of B.1.1.7, which did not cause disease for the early B.1 strain. Another report has shown that the B.1.1.7 variant might also infect WT mice though not as efficient as the P.1 and B.1.351\(^29\).

Two recent studies, one yet-to-be peer reviewed, have focused on the biophysics of the N501Y variant, reporting affinities ranging from 0.5 nM\(^29\) to undefined sub pM\(^30\). Even though they numerically deviate from our findings, they conceptually observe increased affinity of the N501Y variant. However, in both studies, immobilized RBD was incubated with dimeric ACE-2 in the soluble phase. Therefore, avidity interactions might have contributed to the determined \(K_D\) values using the 1:1 fitting model employed in the studies. Of note, our affinity findings are in good agreement with those of a recent study using surface plasmon resonance spectrometry to evaluate the binding between
immobilized ACE-2 and soluble RBD \( (K_D \ 2.4 \pm 0.4) \), published during the revision process for the current manuscript\(^{36}\).

Whether the alpha variant escapes immunity is still a matter of debate, but it has been shown that alpha might confer some reduction in virus neutralization potential compared with the wild-type both in naturally infected and vaccinated individuals\(^{27}\). However, the decrease in response varies highly between individuals and whether the variation has clinical relevance requires further studies.

When we tested the antibody-mediated inhibition of the wild-type, N439K, and N501Y RBD interaction with human ACE-2, we found a minor, but significant reduction of the neutralization potency of convalescent sera \( (n = 140) \) compatible with the data observed by Supasa et al\(^ {27}\). The relative neutralization evasion level from these experiments was WT RBD\(\text{<}\)N439K\(\text{<}\)N501Y. Conversely, hyperimmune sera and high affinity mAbs from mice that received several immunizations with recombinant wild-type spike or RBD did not show this significant neutralization difference, indicating that a fully established vaccine response will overcome the evasion potential of the N501Y. As shown by others\(^ {37}\) and us\(^ {33}\), focused immunization with RBD leads to higher antibody titers and higher neutralization potency than immunization with the full spike ectodomain. At sufficiently high antibody titers, the N439K and N501Y variants' potential evasion advantage might of less immunological importance.

When we addressed the antibody neutralization in sera from a group of convalescent individuals using the PRNT assay, we found no statistical difference between the early 2020 Freiburg isolate and the alpha variant; an observation also made by others performing live virus PRNT
assays, which is not in full agreement with the study from Supasa et al. The discrepancy between the findings can be attributed to several reasons: First, the viral neutralization assays are challenging to perform and might be hampered by intrinsic high biological variation compared with the ACE-2/RBD ELISA inhibition assay that can be performed on a large scale and is optimized to display little assay variability. Another critical issue is that the neutralization assays address the whole virus and contain multiple elements involved in the interaction and infection, i.e. membrane clusters of trimeric spike and the intracellular structural proteins that determine viral fitness, which might lead to differences. Since the different neutralization studies involve relatively few investigated individuals, the risk of type 1 and type 2 errors should be taken into account. Interestingly, one study proposes that the N501Y variant might pose challenges for the MHC class II presentation and the CD4+ T cell response. In contrast to this, another study has demonstrated a negligible impact of SARS-CoV-2 variants on CD4+ and CD8+ T cell reactivity.

In this study, we present comprehensive biophysical data showing that the single N501Y residue change in the RBD region results in an eight-fold increase in the affinity to human ACE-2. This affinity adaptation is likely also to be found in the two other VOC, the B.1.351 and P.1. In line with this, we find that the alpha variant induces a more severe disease than an early 2020 SARS-CoV-2 isolate in K18-hACE-2 mice, indicating a more efficient establishment of infection in vivo. Moreover, our data show a minor but significant immune evasion effect of the N501Y substitution.

Materials and methods
Production of recombinant SARS-CoV-2 RBD variants and human ACE-2 ectodomain

The nucleotide sequence corresponding to the SARS-CoV-2 RBD (QIC53204.1, aa R319–S593) with either an N439K or N501Y substitution and a C-terminal 10xHis-AviTag were synthesized and subcloned into pcDNA3.4-TOPO expression vectors by GeneArt (Thermo Fisher Scientific, Massachusetts, USA). The sequences were optimized towards higher codon adaptation indexes, 5’ mRNA folding energies, removal of cryptic splice sites, and tandem repeats as described elsewhere. The production and purification of recombinant human ACE-2 ectodomain and ACE-2-Fc; and production, purification, and biotinylation of the RBD variants was performed as described elsewhere. Protein purity and quality were determined by 4–12% Bis-Tris SDS-PAGE and Coomassie staining and size-exclusion HPLC. The recombinantly produced RBDs were identified as having the expected mass by intact mass LC-MS.

Protein stability determination

The impact of the N439K and N501Y substitutions on the thermal stability of the RBD was analysed in triplicates on a Tycho NT.6 (NanoTemper Technologies GmbH, Munich, Germany) using a thermal ramp of 30 °C/min in PBS. Protein unfolding was assessed from the ratio of the intrinsic fluorescence recorded at 350 and 330 nm. Inflection temperatures (Ti), representing a discrete unfolding transition or change in the structural integrity of the proteins, were calculated by the Tycho software.

ACE-2/RBD affinity determination by biolayer interferometry
Binding kinetics experiments were performed on an Octet RED383 system (ForteBio, California, USA) as described elsewhere with minor modifications. Briefly, 13 µg/ml ACE-2-Fc was loaded on anti-human Fc capture (AHC) sensors (Pall Life Sciences, California, USA) for 500 s, followed by baseline for 60 s, association to 12-point 1.5-fold serial dilutions starting at 150 nM for RBD wt, N439K, and N501Y for 500 s, and finally dissociation for another 500 s. For each 16-channel column sensor, four sensors loaded with ACE-2-Fc were assigned as a reference and dipped into buffer during the association and dissociation phases. Final sensorgrams were reference subtracted column-wise and globally fitted to a 1:1 binding model.

**ACE-2/RBD antibody inhibition assay**

The antibody neutralization potency, calculated from the degree of inhibition of the ACE-2/RBD interaction, was measured in serum samples from recovered individuals with a past PCR-confirmed COVID-19 infection, mice immunized with wt RBD or spike, and anti-RBD mouse monoclonal antibodies (mAbs) using an ELISA-based ACE-2/RBD antibody inhibition test described elsewhere. Briefly, ACE-2 ectodomain (1 µg/ml) was coated onto Nunc Maxisorp microtiter plates (Thermo Fisher Scientific) overnight at 4 °C in PBS. Samples were incubated with a solution of biotinylated RBD (4 ng/ml) and Pierce high sensitivity streptavidin (Thermo Fisher Scientific) (1:16,000) as follows: convalescent sera in a 10% dilution, immunized mice sera in an 8-point 4-fold dilution starting at a 0.625% dilution, and mAbs in a 6-point 4-fold dilution starting at 20 µg/ml. After 1 h incubation in low-binding round-bottom plates (Thermo Fisher Scientific), the samples/RBD mixes were transferred to ACE-2-coated wells for 15 min. The plates were developed with TMB One (KemEnTec Diagnostics, Taastrup, Denmark) for 20 min, the reaction stopped with 0.3 M H2SO4,
and the optical density (OD) recorded at 450 nm. Coating buffer (PBS, 10.1 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, 2.7 mM KCl, 137 mM NaCl), sample and washing buffer (PBS, 0.05% Tween-20). The wells were washed three times with washing buffer between steps, and all incubations—unless otherwise stated—were done at room temperature in an orbital shaker.

**K18-hACE2 mouse COVID-19 model**

K18-hACE C57BL/6J mice (strain: 2B6.Cg-Tg(K18-ACE2)2Prlmn/J) were obtained from The Jackson Laboratory (Stock number: 034860). Age-matched male and female mice, randomized in groups, were fed standard chow diet and housed in a pathogen-free facility. Animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) and administered either 2.5x10$^3$ or 2.5x10$^4$ plaque-forming units (p.f.u.) SARS-CoV-2 via intranasal administration. Mice were weighed every day at the same time of the day until day 7 post-infection.

**RNA isolation and real-time PCR (qPCR).**

Lungs were homogenized with steel beads in a Tissuelyser (II) (both from Qiagen, Hilden, Germany) in PBS and immediately used for RNA isolation. RNA was isolated using the High Pure RNA Isolation Kit (Roche, Basel, Switzerland) and an equal amount of RNA was used for standard One-Step RT-PCR (TaqMan® RNA-to-Ct™ 1-Step Kit, Applied Biosystems, Massachusetts, USA). For the SARS-CoV-2 $N$ gene, qPCR primers AAATTGGGGACCAGGAAC and TGGCACCTGTGTAGGTCAAC and probe FAM-ATGTCGCGCATGGCATGGA-BHQ were used. For the 18S-rRNA qPCR the Hs99999901_s1 18S rRNA Taqman Gene Expression Assays were used (Applied Biosystems). RNA levels of SARS-CoV-2 $N$ gene were normalized to the mouse housekeeping gene.
18S-rRNA using the formula $2^{\Delta Ct(18S-rRNA)-Ct(Sars-CoV-2 \ RNA)}$. The resulting normalized ratio is presented directly in the figures.

**SARS-CoV-2**

The early 2020 B.1 strain, Freiburg isolate FR-4286, was kindly provided by Professor Georg Kochs, University of Freiburg, Germany and B.1.1.7 SARS-CoV2 (Kent, UK, isolate) was provided under MTA by Professor Arvind Patel, University of Glasgow. The viruses were propagated in VeroE6 cells expressing human TMPRSS2 (VeroE6-hTMPRSS2) (kindly provided by Professor Stefan Pöhlmann, University of Göttingen). We have confirmed the expression of hTMPRSS2 in the Green monkey VeroE6 cell line with PCR analysis and the cells were tested negative for mycoplasma contamination. Briefly, VeroE6-hTMPRSS2 cells were infected with a multiplicity of infection (MOI) of 0.05, in DMEM (Gibco) + 2% FCS (Sigma-aldrich) + 1% Pen/Strep (Gibco) + L-Glutamine (Sigma-Aldrich) (From here, complete medium). The supernatant containing new virus progeny was harvested 72h post infection, and concentrated on 100 kDa Amicon ultrafiltration columns (Merck, New Jersey, USA) by centrifugation at 4000 x g for 30 min. Virus titer was determined by TCID$_{50}$ assay and calculated by the Reed-Muench method.

Virus isolates were prepared for whole-genome sequencing using the EasySeq™ RC-PCR SARS CoV-2 Whole Genome Sequencing kit (Nimagen, Nijmegen, Netherlands) and sequenced using Illumina sequencing (Illumina, California, USA). Adapter and primer sequences were removed, and reads were mapped to the NC_045512.2 reference using Minimap and iVar (PMID: 29750242, PMID: 30621750). Variants and pangolin lineages
(PMID: 32669681) were determined based on iVar consensus sequences with minimum 80% base frequency.

The Freiburg isolate FR-4286 is the Wuhan-like early European B.1 lineage containing the S:D614G, and ORF1b:P314L, as well as E:L37R. The B.1.1.7 differs from the B.1 in the spike protein on positions S:N501Y, S:A570D, S:T716I, S:P681H, S:S982A, S:D1118H, S:del69/70, S:del144/145. Both the B.1.1.7 and the B.1 strains contains the S:D614G and ORF1b:P314L mutations. The viruses used are clinical isolates. The B.1.1.7 variant is in the database as MZ314997. The Freiburg isolate sequence has been uploaded and has the accession ID no: EPI_ISL_852748.

**Plaque reduction neutralization test (PRNT)**

Neutralizing capacity of convalescent serum against B.1 SARS-CoV2 (FR-4286) or B.1.1.7 was assessed in a neutralization assay (PRNT), performed as previously described. In short, convalescent serum from human COVID-19 patients was heat-inactivated (30 minutes at 56 °C) and prepared in a 9 point 2-fold serum dilution starting at 1:20, in DMEM (Gibco) + 2% FCS (Sigma-Aldrich) + 1% Pen/Strep (Gibco) + L-Glutamine (Sigma-Aldrich). Sera were mixed with SARS-CoV-2 to a final titer of 100 TCID50/well, and incubated at 4 °C overnight. A “no serum” and a “no virus” (uninfected) control samples were included. TCID50 control plates (in triplicates) of each of the viruses were included to control for actual virus titer of B.1 and B.1.1.7, respectively. The following day, virus:serum mixtures were added in octuplicates to 2 x 10^4 VeroE6-hTMPRSS2 cells seeded in flat-bottomed 96-well plates (Thermo Fisher), and incubated 72 h in a humidified CO2 incubator at 37 °C, 5% CO2. Cytopathic effect (CPE) was scored after fixing with 5% formalin (Sigma-Aldrich) and crystal violet stain (Sigma-Aldrich), using a light microscope (Leica DMi1).
Blood samples

The antibody neutralization potency was assessed in 140 randomly-selected convalescent serum samples (the patient cohort has been described elsewhere\(^{42}\)) with IC\(_{50}\) values for the ACE-2/RBD wt interaction ranging from low to high (estimated from 6-point 4-fold dilution series done as part of a previous study\(^{33}\)). A serum pool from healthy individuals was used as a negative control.

Ethics

The collection and use of blood samples have been approved by the Regional Ethical Committee of the Capital Region of Denmark (H-20028627) and (H-20079890). The human studies were conducted in agreement with the Helsinki declaration with. We have received informed consent to do the experiments included in this study. The Danish Animal Experiments Inspectorate has approved the experimental animal procedures and were carried out in accordance with the Danish Animal Welfare Act for the Care and Use of Animals for Scientific Purposes. (License ID 2019-15-0201-00090 and 2020-15-0201-00726). All procedures followed the recommendations of the Animal Facilities at the Universities of Copenhagen and Aarhus.

Biosafety

All aspects of this study were approved by the office of the Danish Working Environment Authority, Landskronagade 33, 2100 Copenhagen Ø, before the initiation of this study. Work with SARS-CoV-2 was performed in a biosafety level 2+ laboratory by personnel equipped with powered air-purifying respirators.
Statistics

Statistical analyses were performed with GraphPad Prism 9 (GraphPad Software, California, USA). Global differences in the weight of K18-hACE2 mice exposed to high and low doses of SARS-CoV-2 were analyzed with Kruskal-Wallis. Pair-wise comparisons of the effect of SARS-CoV-2 variants in weight loss were performed with multiple Mann-Whitney tests (ranks computed for each day) and false discovery rate approach using the two-stage step-up method of Benjamini, Krieger, and Yekutieli. Comparison of the neutralization potency of serum samples from COVID-19 recovered, vaccinated individuals, and mouse monoclonal antibodies was performed by Friedman test comparing the mean of each of the variants with the wt, as well as pair-wise (variant vs wt) Spearman rank correlation tests (two-tailed, reported as $\rho$ and a significance value $p$) and linear regression analyses (reported as $R^2$). Neutralization indexes of convalescent sera for the B.1 and B.1.1.7 strains obtained from the PRNT were analyzed by Wilcoxon matched-pairs signed ranked tests corrected for multiple comparisons using the Holm-Šídák method. The extra-sum-of-squares F test was used to compare best-fit IC$_{50}$ values, interpolated with the equation [inhibitor] vs normalized response with a variable slope as described elsewhere, of mice sera and mouse mAbs. P values < 0.05 were considered statistically significant.

Source data files

Figure 1 Source Data File 1, Accompanying Figure 1

Figure 1 Source Data File 2, Accompanying Figure 1

Figure 1 Source Data File 3, Accompanying Figure 1

Figure 2 Source Data File, Accompanying Figure 2
Figure 3 Source Data File, Accompanying Figure 3

Figure 4 Source Data File, Accompanying Figure 4
References


doi:10.1093/cid/ciab411


doi:10.1101/2021.10.18.21264623


doi:10.1093/infdis/jiab082


32. Winkler, E. S. et al. SARS-CoV-2 infection of human ACE2-transgenic mice causes severe lung


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Authors' contributions

RB-O, PG and MOS conceived and designed the study; RB-O, CH and JRB enabled recombinant protein production; RB-O, MI, AR, LBJ, CH, JRB, LR, CBH and MOS performed experiments; RB-O, MI, LBJ, SRP and MOS analysed the data; RB-O, PG and MOS wrote the paper with inputs from all co-authors. All authors approved the final version of the manuscript.

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Conflict of interest

The authors declare that they have no conflicts of interest to disclose.