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SARS-CoV-2 spike L452R variant evades cellular immunity and increases infectivity

Graphical abstract

Highlights
- L452R and Y453F mutations in the SARS-CoV-2 spike RBM have emerged
- L452R and Y453F mutants escape HLA-A24-restricted cellular immunity
- L452R increases viral infectivity and fusogenicity and promotes viral replication

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In brief
Motozono and G2P-Japan Consortium et al. show that the emerging mutations L452R and Y453F in the SARS-CoV-2 spike receptor-binding motif evade HLA-A24-restricted cellular immunity. L452R increases spike stability, viral infectivity, and viral fusogenicity, thereby promoting viral replication. These data suggest that HLA-restricted cellular immunity potentially affects evolution of viral phenotypes.

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SARS-CoV-2 spike L452R variant evades cellular immunity and increases infectivity

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SUMMARY

Many SARS-CoV-2 variants with naturally acquired mutations have emerged. These mutations can affect viral properties such as infectivity and immune resistance. Although the sensitivity of naturally occurring SARS-CoV-2 variants to humoral immunity has been investigated, sensitivity to human leukocyte antigen (HLA)-restricted cellular immunity remains largely unexplored. Here, we demonstrate that two recently emerging mutations in the receptor-binding domain of the SARS-CoV-2 spike protein, L452R (in B.1.427/429 and B.1.617) and Y453F (in B.1.1.298), confer escape from HLA-A24-restricted cellular immunity. These mutations reinforce affinity toward the host entry receptor ACE2. Notably, the L452R mutation increases spike stability, viral infectivity, viral fusogenicity, and thereby promotes viral replication. These data suggest that HLA-restricted cellular immunity potentially affects the evolution of viral phenotypes and that a further threat of the SARS-CoV-2 pandemic is escape from cellular immunity.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Since an unusual outbreak in Wuhan, Hubei Province, China, in December 2019 (Wu et al., 2020; Zhou et al., 2020), SARS-CoV-2 has rapidly spread worldwide, and as of May 2021, COVID-19 remains an ongoing pandemic:
The emergence of mutated viruses is mainly due to error-prone viral replication, and the spread of emerged variants can be attributed to the escape from immune selective pressures (reviewed in Duffy et al., 2008). In fact, several SARS-CoV-2 mutants may be resistant to the neutralization mediated by antibodies from COVID-19 patients (Baum et al., 2020; Chen et al., 2021; Liu et al., 2021c; McCarthy et al., 2021; Weisblum et al., 2021) as well as those from vaccinated individuals (Liu et al., 2021b). Although the B.1.1.7 variant is sensitive to convalescent and vaccinated sera (Collier et al., 2021; Garcia-Beltran et al., 2021; Shen et al., 2021; Supasa et al., 2021; Wang et al., 2021b), the B.1.351 and P.1 variants are relatively resistant to anti-SARS-CoV-2 humoral immunity (Garcia-Beltran et al., 2021; Hoffmann et al., 2021a; Wang et al., 2021b).

In addition to the humoral immunity mediated by neutralizing antibodies, another protection system against pathogens is the cellular immunity due to cytotoxic T lymphocytes (CTLs) (reviewed in Fryer et al., 2012 and Leslie et al., 2004). CTLs recognize nonself epitopes present on virus-infected cells via human leukocyte antigen (HLA) class I molecules; thus, CTL-mediated antiviral immunity is HLA restricted (reviewed in La Gruta et al., 2018). Recent studies have reported HLA-restricted SARS-CoV-2-derived epitopes that can be recognized by human CTLs (Kared et al., 2021; Kiyotani et al., 2020; Neide et al., 2021; Schulien et al., 2021; Wilson et al., 2021). Furthermore, Le Bert et al. found that the functionality of virus-specific cellular immunity correlates inversely with COVID-19 severity (Le Bert et al., 2021). Therefore, it is conceivable that HLA-restricted CTLs play crucial roles in controlling SARS-CoV-2 infection and COVID-19 disorders, though compared to humoral immune responses, it remains unclear whether SARS-CoV-2 variants can escape cellular immunity.

In this study, we investigated the possibility of the emergence of SARS-CoV-2 mutants that are able to escape HLA-restricted cellular immunity. We demonstrate that at least two naturally occurring substitutions in the receptor-binding motif (RBMon residues 438–506) of the SARS-CoV-2 S protein, L452R and Y453F, which were identified in the two major variants, B.1.427/429 (L452R) and B.1.1.298 (Y453F), can be resistant to cellular immunity in the context of HLA-A24, an allele of HLA-A. More intriguingly, the L452R and Y453F mutants enhance binding affinity for ACE2, and experiments using pseudoviruses show that the L452R substitution increases viral infectivity. Furthermore, we artificially generated SARS-CoV-2 harboring these point mutations by reverse genetics and show that the L452R mutant enhances viral replication capacity.

**RESULTS**

**Evasion from HLA-A24-restricted CTL responses by acquisition of mutations in the RBM of the SARS-CoV-2 S protein**

We set out to address the possibility of the emergence of naturally occurring mutants that can confer resistance to antigen recognition by HLA-restricted cellular immunity. A bioinformatic study has suggested that the 9-mer peptide in the RBM, NYNYLYRFL (we designate this peptide “NF9”), which spans 448–456 of the S protein, is the potential epitope presented by HLA-A24 (Kiyotani et al., 2020), an HLA-A allele widely distributed globally and particularly predominant in East and Southeast Asian areas (Table S1). Additionally, three immunological analyses using COVID-19 convalescent sera have shown that the NF9 peptide is an immunodominant epitope presented by HLA-A*24:02 (Gao et al., 2021; Hu et al., 2020; Kared et al., 2021). To verify these observations, we obtained peripheral blood mononuclear cells (PBMCs) from 16 COVID-19 convalescent donors with HLA-A2*24:02 and stimulated these cells with the NF9 peptide (median 5.2%) was significantly higher than that in its absence (median 0.44%) (Figure 1B; p = 0.0003 by Wilcoxon signed-rank test). In contrast, stimulation with the NF9 peptide did not upregulate CD25 and CD137 on the CD8+ T cells in response to stimulation with NF9. In the 16 samples of the CD8+ T cells from COVID-19 convalescent patients with HLA-A*24:02, the percentage of CD25+ CD137+ cells in the presence of the NF9 peptide (median 5.2%) was significantly higher than that in its absence (median 0.44%) (Figure 1B; p = 0.0003 by Wilcoxon signed-rank test). In contrast, stimulation with the NF9 peptide did not upregulate CD25 and CD137 on the CD8+ T cells in response to stimulation with NF9.
of three seronegative samples with HLA-A*24:02, and the percentage of CD25+CD137+ cells in seronegative samples (median 0.93%) was significantly lower than that in COVID-19 convalescent samples (Figure 1B; p = 0.0003 by Wilcoxon signed-rank test). We also verified that the NF9 peptide did not activate the CD8+ T cells in six COVID-19 convalescent samples with non-HLA-A24 (median 0.43%) (Figure 1B; p = 0.031 by Wilcoxon signed-rank test versus HLA-A24-positive). Consistent with
previous reports (Gao et al., 2021; Hu et al., 2020; Kared et al., 2021; Kiyotani et al., 2020), our data suggest that the NF9 peptide is an immunodominant HLA-A*24:02-restricted epitope recognized by the CD8+ T cells of COVID-19 convalescents in our cohort. In particular, a convalescent sample from donor #13 exhibited a prominent CD8+ T cell-mediated response triggered by the NF9 peptide (Figure 1B; Table S2). Notably, the PBMCs of donor #13 were collected 68 days after PCR positivity, suggesting that NF9-mediated CD8+ T cell immunity can be maintained for a relatively long period and that NF9 is an important epitope.

We then assessed the profile of cytokine production by NF9 stimulation. As depicted in Figure 1C, stimulation with the NF9 peptide induced the production of IFN-γ, TNF-α, and IL-2 by the CD8+ T cells from a COVID-19 convalescent patient. According to analysis using eight COVID-19 convalescent samples, the CD8+ T cells produced multiple cytokines in response to NF9 stimulation (Figure 1D), demonstrating the multifunctional nature of NF9-specific CD8+ T cells of COVID-19 convalescent patients. Moreover, the cytotoxic potential of NF9-specific CD8+ T cells was assessed by staining with surface CD107a, a degranulation marker (Figure 1E). As shown in Figure 1F, the percentage of CD107α+ cells among the CD8+ T cell population treated with the NF9 peptide (median 22.1%) was significantly higher than that among cells not treated with the NF9 peptide (median 0.88%) (p = 0.0078 by Wilcoxon signed-rank test), suggesting the cytotoxic potential of NF9-specific CD8+ T cells.

Next, we analyzed the diversity of SARS-CoV-2 during the current pandemic to assess the presence of naturally occurring variants harboring mutations in this region (residues 448–456 of the S protein). We downloaded 750,243 viral genome sequences from the Global Initiative on Sharing All Influenza Data (GISAID) database (https://www.gisaid.org; as of March 15, 2021). The L452R substitution was most frequent among the sequences analyzed (5,677 sequence), and 1,380 of the sequences reported contain the Y453F substitution (Table 1). Notably, lineages harboring the L452R and Y453F mutations, respectively (Figure 1G; Table S3). We then assessed the possibility that these two mutations affect the binding affinity of NF9 to HLA-A24 molecules. In silico analyses using five tools (Andreatta and Nielsen, 2016; Karosiene et al., 2012; Lundegaard et al., 2008; Nielsen et al., 2003; Reynisson et al., 2020; Zhang et al., 2009) predicted that the L452R mutation decreases the binding affinity of NF9 to HLA-A24, and four out of the five tools predicted that the Y453F mutation decreases the binding affinity (Figure S1A).

To experimentally address the possibility that these naturally occurring mutations, L452R and Y453F in the NF9 region, confer evasion ability from NF9-specific CD8+ T cells in HLA-A24-positive COVID-19 convalescents, two NF9 derivatives containing either substitution (NF9-L452R and NF9-Y453F) were prepared and used for stimulation experiments. As shown in Figure S1B, parental NF9 induced IFN-γ expression in a dose-dependent manner. In contrast, the level of IFN-γ expression induction by the NF9-Y453F derivative was significantly lower than that by parental NF9, and intriguingly, the NF9-L452R derivative did not induce IFN-γ expression, even at the highest concentration tested (10 nM) (Figure S1C). Among the eight HLA-A24-positive COVID-19 convalescent samples tested, parental NF9 peptide significantly induced IFN-γ expression. In contrast, the NF9-L452R derivative did not activate all CD8+ T cells tested, and seven out of the eight HLA-A24-positive COVID-19 convalescent samples failed to be activated by the NF9-Y453F derivative (Figures 1H and 1I). Altogether, these results suggest that the NF9 peptide, which is derived from the SARS-CoV-2 S protein RBD, is an immunodominant epitope of HLA-A24 and that two naturally occurring mutants, L452R and Y453F, are able to evade HLA-A24-restricted cellular immunity.

Augmentation of the binding affinity for ACE2 by the L452R and Y453F mutations

We next sought to determine whether the mutations of interest affect the efficacy of virus infection. Structural analyses have shown that the Y453F and N501 substitutions in the RBM are located at the interface between the SARS-CoV-2 RBM and human ACE2 and directly contribute to human ACE2 binding but that the L452 residue is not located at this interface (Lan et al., 2020; Wang et al., 2020; Zhao et al., 2020) (Figure 2A). To directly assess the effect of these RBM mutations on binding to ACE2, we prepared yeasts expressing the parental SARS-CoV-2 receptor-binding domain (RBD) (residues 336–528) and derivatives (L452R, Y453F, and N501Y) and performed an in vitro binding assay using the yeast surface display of the RBD and soluble ACE2 protein. Consistent with recent studies, including ours (Supasa et al., 2021; Zahradnik et al., 2021b), the N501Y mutation, which is a common mutation in B1.1.7, B1.351, and P.1 variants (reviewed in Plante et al., 2021b), as well as the Y453F mutation (Bayarri-Olmos et al., 2021; Zahradnik et al., 2021b), significantly increased binding affinity for human ACE2 (Figures 2B and 2C).

Table 1. Naturally occurring mutations in residues 448–456 of the SARS-CoV-2 S protein

| Mutation | # sequence | Date first detected | Country first detected | Dominant PANGO lineage | Date first detected in the dominant lineage | Country first detected in the dominant lineage | Nonhuman hosts detected* |
|----------|------------|---------------------|------------------------|------------------------|-------------------------------------------|---------------------------------------------|--------------------------|
| L452R    | 5,677      | March 17, 2020      | Denmark                | B.1.427/429            | July 6, 2020                              | Mexico                                      | gorilla (1)               |
| Y453F    | 1,380      | April 20, 2020      | Denmark                | B.1.1.298              | April 20, 2020                            | Denmark                                    | mink (339) and cat (3)    |
| N450K    | 181        | March 27, 2020      | UK                     | B.1.214.2              | December 5, 2020                         | UK                                         | -                        |
| L452M    | 140        | April 13, 2020      | Japan                  | B.1.22/36              | July 2, 2020                              | Netherlands                               | mink (32)                |
| L452Q    | 111        | March 6, 2020       | Spain                  | B.1.1.374              | October 23, 2020                         | Belarus                                    | -                        |

*The number in parentheses indicates the number of sequences reported.
Figure 2. Increase in the binding affinity to ACE2, viral infectivity, viral fusogenicity, and viral replication capacity by the L452 mutation

(A) Location of the NF9 peptide (residues 448–456) in the cocrystal structure of the SARS-CoV-2 S and human ACE2 proteins (PDB: 6M17) (Yan et al., 2020). An overview (left), an enlarged view of the boxed area in the left panel (middle), and a view of the middle panel rotated 180° on the y axis (right) are shown. Residues 448–456 of SARS-CoV-2 S (corresponding to the NF9 peptide) are shown in black.

(B–D) Binding affinity of SARS-CoV-2 S RBD to ACE2 by yeast surface display. The percentage of the binding of the SARS-CoV-2 S RBD expressed on yeast to soluble ACE2 (B) and the $K_D$ values (C) are shown. Assays were performed in quadruplicate. (D) The level of stable expression of the SARS-CoV-2 RBD on yeast (x axis) and the binding affinity toward ACE2 (y axis) compared to the parental RBD. In (B), the fitting curve of parental RBD is shown as black lines in all panels.

(E and F) Pseudovirus assay. The HIV-1-based reporter virus pseudotyped with the parental SARS-CoV-2 S or its derivatives (E, L452R, Y453F, and N501Y; F, D614G, B.1.429 [S13I/W152C/L452R/D614G], and B.1.1.289 [HV69-70del/Y453F/D614G]) was inoculated into HEK293 cells transiently expressing human ACE2 and TMPRSS2 at four different doses (1, 3, 5, and 10 ng p24 antigens). Percentages of infectivity compared to the virus pseudotyped with parental S (10 ng p24 antigen) are shown.

(legend continued on next page)
In (M), values at respective time points were compared with those at the last time point using a two-tailed, paired Student's t test, and an asterisk denotes statistically significant differences (*p < 0.05) versus parental S (E) and the D614G mutant (F) at the same dose were determined by Student's t test.

In (E and F), statistically significant differences (*p < 0.05) versus parental S (E) and the D614G mutant (F) at the same dose were determined by Student's t test.

In (E and F), statistically significant differences (*p < 0.05) versus parental S (E) and the D614G mutant (F) at the same dose were determined by Student's t test.

Increase in pseudovirus infectivity by the L452R mutation

To directly analyze the effect of these mutations on viral infectivity, we prepared an HIV-1-based reporter virus pseudotyped with the SARS-CoV-2 S protein and its mutants as well as HEK293 cells transiently expressing human ACE2 and TMPRSS2. As shown in Figure 2E, although the N501Y mutation slightly affected viral infectivity in this assay, the L452R mutation significantly increased it compared to the parental S protein. In contrast to the yeast display assay (Figures 2B and 2C), the infectivity of the Y453F mutant was significantly lower than that of the parental S protein (Figure 2E). Similar to the observations using human ACE2 (Figure 2E), the L452R mutant, but not the other mutants including the Y453F mutant, significantly increased infection efficacy using mink ACE2 as the receptor (Figure S1D). Moreover, we demonstrated that the infectivity of the reporter virus pseudotyped with the S protein of the B.1.1.298 variant, which harbors the L452R mutation, was significantly higher than those of parental S as well as the D614G-harboring S, while that of the B.1.1.298 variant, which harbors the Y453F mutation, was not (Figure 2F). Altogether, these findings suggest that the L452R substitution increases the binding affinity of the SARS-CoV-2 RBD toward human ACE2, protein stability, and viral infectivity. Although the L452 residue is not directly located at the binding interface (Figure 2A), structural analysis and in silico mutagenesis suggest that the L452R substitution promotes electrostatic complementarity (Selzer et al., 2000) (Figure 2G). Because residue 452 is located in close proximity to a negatively charged patch of ACE2 residues (E35, E37, and D38), the increase in viral infectivity caused by the L452R substitution can be attributed to an increase in electrostatic interaction with ACE2.

Promotion of SARS-CoV-2 replication in cell cultures by the L452R mutation

To investigate the effect of RBM mutations on viral replication, we artificially generated recombinant SARS-CoV-2 viruses that harbor the mutations of interest as well as parental recombinant virus by a reverse genetics system (Torfi et al., 2021). The nucleotide similarity of SARS-CoV-2 strain WK-521 (GISAID ID: EPI_ISL_408667) (Matsuyama et al., 2020), the backbone of the artificially generated recombinant SARS-CoV-2, to strain Wuhan-Hu-1 (GenBank: NC_045512.2) (Wu et al., 2020) is 99.91% (27 nucleotide difference); the sequences encoding the S protein between these two strains are identical, indicating that strain WK-521 is a SARS-CoV-2 prototype. We verified insertion of the targeted mutations in the viruses generated by direct sequencing (Figure 2H) and performed virus replication assays using these recombinant viruses. As shown in Figure 2I, the cytopathicity of the L452R mutant was higher than that of the other mutants and the parental virus. We also found that the growth rates of the L452R mutant in VeroE6/TMPRSS2 cells (Figure 2J), HEK293 cells stably expressing human ACE2 (HEK293-ACE2; Figure 2K), and A549 cells stably expressing human ACE2 (A549-ACE2; Figure 2L) were significantly higher than those of the parental virus. The predominance of the L452R mutant was observed at different doses (Figure S1E). Moreover, a competition assay using the parental virus and the L452R mutant showed that the L452R mutant expanded more predominantly than the parental virus (Figure 2M). Together with the results of the binding assay (Figures 2B–2D) and the assay using pseudoviruses (Figures 2E, 2F, and S1E), our results suggest that the L452R mutation promotes viral replication.

Increase in viral fusogenicity by the L452R mutation

We next tested how the L452R mutation increases viral infectivity and cytopathicity by performing a SARS-CoV-2 S-based fusion
assay. As shown in Figure S1F, the surface expression levels of parental S and its derivatives on effector cells were comparable. Using the SARS-CoV-2 S-based fusion assay, we demonstrated that the L452R mutation significantly increased fusion efficacy compared to the parental S, as well as the other mutants (Figure 2N). Our data suggest that the L452R mutation promotes viral replication by increasing viral fusogenicity.

Dynamics of the spread of RBM mutants during the current pandemic

We finally assessed the epidemic dynamics of naturally occurring variants containing the L452 and Y453 substitutions. The L452R mutants were mainly found (3,967 sequences) in the B.1.427/B.1.429 lineage, which forms a single clade (Deng et al., 2021) (Figure 3A; Table S4). Although the L452R mutant was first detected in the B.1.39 lineage in Denmark on March 17, 2020 (GISAID ID: EPI_ISL_429311) (Table 1), this variant did not spread. The oldest sequence that contains the L452R mutation in the B.1.427/B.1.429 lineage was isolated in Quintana Roo state, Mexico, on July 6, 2020 (GISAID ID: EPI_ISL_942929) (Figure 3B). The B.1.427/B.1.429 lineage harboring the L452R mutation started expanding in California at the beginning of November 2020 (Figure 3B, top). In 2021, this lineage expanded throughout the USA, and it is currently one of the most predominant lineages in the country (Figure 3B, bottom; Table S5).

Regarding the Y453F mutation, 1,274 of the 1,380 mutated sequences belong to the B.1.1.298 lineage, which has been exclusively detected in Denmark (Table S4). The oldest sequence in the B.1.1.298 lineage that contains the Y453F mutation was first detected in Denmark (April 20, 2020) (Figure 3C), and L452R-harboring mutants were first collected in California, USA, on September 28, 2020 (GISAID ID: EPI_ISL_730092 and EPI_ISL_730345) (Figure 3B). The B.1.427/B.1.429 lineage harboring the L452R mutation started expanding in California at the beginning of November 2020 (Figure 3B, top). In 2021, this lineage expanded throughout the USA, and it is currently one of the most predominant lineages in the country (Figure 3B, bottom; Table S5).

See also Figure S2 and Tables S4 and S5.
isolated from a human in Denmark on April 20, 2020 (GISAID ID: EPI_ISL_714253) (Figure 3C). Intriguingly, the B.1.1.298 variants containing either Y453 or F453 were detected not only in humans but also in minks (Figure 3D). Phylogenetic analysis of the entire genome sequences of the B.1.1.298 lineage suggested multiple SARS-CoV-2 transmissions between humans and minks (Figure S2). Additionally, the three sequences that contain the Y453F mutation were isolated from cats in Denmark: two identical sequences (GISAID ID: EPI_ISL_683164 and EPI_ISL_683166) and another sequence (GISAID ID: EPI_ISL_683165) of a distinct origin (Figure S2). These results suggest that this SARS-CoV-2 variant has transmitted from humans to cats multiple times and that some of them may have spread among the Danish cat population. Nevertheless, the epidemic of a fraction of the B.1.1.298 lineage containing the Y453F mutation in Denmark peaked from October to November 2020 and then gradually declined (Figure 3D). The variant containing the Y453F mutation was last collected in Denmark on January 18, 2021 (GISAID ID: EPI_ISL_925998), and it has not been reported worldwide since (Figure 3D).

**DISCUSSION**

In the present study, we demonstrate that at least two naturally occurring mutations in the SARS-CoV-2 RBM, L452R and Y453F, escape HLA-restricted cellular immunity and further promote affinity toward the viral receptor ACE2. We also demonstrate that the L452R mutation increases the stability of the S protein and viral infectivity and thereby enhances viral replication. Our data suggest that the L452R mutant escapes HLA-A24-restricted cellular immunity and further strengthens its infectivity. Consistent with our findings, Deng et al. performed a pseudovirus assay and showed that the L452R mutation increases viral infectivity (Deng et al., 2021). However, the mechanism of action was not revealed. Here, we demonstrated that the L452R mutation increases viral fusogenicity.

Lines of recent evidence show the emergence of SARS-CoV-2 variants that evade anti-SARS-CoV-2 neutralizing humoral immunity (Baum et al., 2020; Chen et al., 2021; Garcia-Beltran et al., 2021; Hoffmann et al., 2021a; Liu et al., 2021b, 2021c; McCarthy et al., 2021; Wang et al., 2021a; Weissblum et al., 2020), forewarning the risk of the spread of immune escape variants. In addition to humoral immunity, Zuo et al. reported that functional SARS-CoV-2-specific cellular immune responses are retained for at least 6 months after infection (Zuo et al., 2021). Le Bert et al. also found that functional cellular immune responses can contribute to controlling the progression of COVID-19 (Le Bert et al., 2021). These observations suggest the importance of cellular immunity that elicits efficient antiviral effects. However, the possibility of the emergence of SARS-CoV-2 variants that are able to evade cellular immunity has not yet been addressed. Here, we demonstrate that the L452R and Y453F mutations contribute to escape from HLA-restricted cellular immunity. In addition to our findings, recent papers have documented that the L452R (Deng et al., 2021; Li et al., 2020) and Y453F (Baum et al., 2020; Hoffmann et al., 2021b) substitutions may confer resistance to neutralizing antibodies, suggesting that these mutants can evade both humoral and HLA-restricted cellular immunity. Furthermore, we demonstrate that the L452R mutation significantly improves viral replication capacity by increasing binding affinity to human ACE2 as well as S protein stability. Altogether, our findings suggest that the emergence of variants that can escape HLA-restricted cellular immunity and further enhance viral replication capacity is a potential risk for deterioration of the situation regarding the COVID-19 pandemic.

Previous papers have reported that the L452R (Garcia-Beltran et al., 2021; Liu et al., 2021c) and Y453F mutations (Baum et al., 2020; Garcia-Beltran et al., 2021) had less effect on the sensitivity to neutralizing antibodies (e.g., convalescent/vaccinated sera and monoclonal antibodies). On the other hand, a recent study has shown that the B.1.427/429 variant, which harbors the L452R mutation, is 2–6.7-fold more resistant to neutralizing antibodies than the non-L452R prototypic virus (Deng et al., 2021). Although the effects of L452R and Y453F mutations on sensitivity to neutralizing antibodies are controversial, our data demonstrate that these mutations evade HLA-A24-mediated cellular immunity. Therefore, the L452R/Y453F mutations would more crucially impact HLA-A24-mediated cellular immunity than sensitivity to neutralizing antibodies.

As suggested in previous reports (Koopmans, 2021; Oude Munnink et al., 2021; WHO, 2020b), our data show that the B.1.1.298 variant possessing the Y453F substitution is closely associated with the outbreak among minks in Denmark. Although it remains unclear whether the emergence of the Y453F mutant may be associated with evasion from acquired immunity in minks, we demonstrate here that this mutation does confer resistance to HLA-A24-restricted human cellular immunity. Because the Y453F mutation did not increase infection efficacy when using mink ACE2, our results suggest that the emergence of this mutation is not due to enhanced viral fitness in minks. Nevertheless, the host range of SARS-CoV-2 in terms of the use of ACE2 molecules as receptors mediating infection is broad in a variety of mammals (Liu et al., 2021a; OIE, 2021). More importantly, although murine ACE2 cannot be used for infection of prototype SARS-CoV-2, recent studies have revealed that some SARS-CoV-2 variants, including B.1.351 and P.1, have gained the ability to utilize murine ACE2 for infection, expanding their host range to mice (Li et al., 2021; Montagutelli et al., 2021). In addition to evasion from acquired immunity in humans, zoonotic and zooanthroponotic SARS-CoV-2 transmission can contribute to the accumulation of mutations in spreading viruses and further impact viral phenotypes, including infectivity, replication efficacy, pathogenicity, transmissibility, and even host range. Therefore, surveillance of novel variant emergence, even in nonhuman mammals, and assessment of the potential of these viruses to adapt to nonhuman ACE2 for infection receptors will be critical.

In contrast to the B.1.1.298 variant, the B.1.427/429 variant harboring the L452R substitution appears to have emerged during spread in the human population. Because the L452R mutation reinforces binding to human ACE2 and further enhances viral replication capacity, this variant might have emerged to improve viral fitness in humans. Another possibility is that the L452R mutant developed to evade HLA-A24-restricted cellular immunity: HLA-A24 is relatively predominant in East Asian individuals (Gonzalez-Galarza et al., 2020), and the proportion of Asian Americans in the USA is highest in California (CDC, 2019). For instance, the proportion of HLA-A24-positive individuals is
~20% in San Diego (Moore et al., 2018), California, where approximately 280,000 SARS-CoV-2 infection cases have been reported thus far (https://coronavirus.jhu.edu; as of May 22, 2021). Because the B.1.427/429 variant harboring L452R mutation has been predominately spreading in California, it may be assumed that the emergence of the L452R mutant (or the B.1.427/429 lineage) was driven by HLA-A24-mediated cellular immunity.

In addition to escape from antiviral acquired immunity, recent studies have shown that variants emerging during the current pandemic, particularly the B.1.1.7 variant, can even increase SARS-CoV-2 pathogenicity and the mortality of COVID-19 (Challen et al., 2021; Davies et al., 2021; Grint et al., 2021). Importantly, HLA-A24 individuals are relatively highly frequent in East and Southeast Asian countries such as Japan (Japanese, allele frequency = 0.364, n = 1,550) and Malaysia (Malaysian, allele frequency = 0.361, n = 1,974) (Gonzalez-Galarza et al., 2020) (Table S1), where both the number of confirmed cases and COVID-19 mortality are relatively lower than those in European countries and the USA (https://coronavirus.jhu.edu; as of May 22, 2021).

There are some limitations in this study. First, we hypothesized that the emergence of the L452R and Y453F mutations might be attributed to the immune pressure triggered by HLA-A24-mediated cellular immunity. However, since the frequency of HLA-A24-positive individuals in California and Denmark is inaccessible, the magnitude of HLA-A24-mediated immunity against SARS-CoV-2 in these states and countries remains unclear. Second, the magnitude of NF9-mediated HLA-A24-restricted antiviral cellular immunity was not revealed. To clarify this, further investigations with larger cohorts of COVID-19 patients are essential. Third, the effects of the L452 and Y453 substitutions on viral pathogenicity, disease severity, mortality, and transmissibility remain unaddressed. To fully characterize the virological features of these mutants, further investigations using animal models and epidemiological data will be required. Nevertheless, we provide direct evidence suggesting that mutations in the RBM, including L452R (in the B.1.427/429 lineage) and Y453F (in the B.1.298 lineage), may confer escape from HLA-A24-restricted cellular immunity, and furthermore, that the L452R mutant has increased replication capacity. Accordingly, variants, particularly those possessing the L452R mutation, such as the B.1.427/429 lineage, are a potential threat in countries and regions with a predominance of HLA-A24 individuals, and deep surveillance and tracing of the epidemic related to these variants are urgently required.

Finally, in May 2021, a novel variant of concern, the B.1.617 lineage, emerged that was closely associated with a massive COVID-19 surge in India. Notably, in addition to the B.1.427/429 lineage, the L452R mutation is a hallmark of the B.1.617 lineage. Therefore, the L452R mutation would be a mutation of concern and should be targeted by deep surveillance.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**QUANTIFICATION AND STATISTICAL ANALYSIS**

**SUPPLEMENTAL INFORMATION**

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**CONSORTIA**

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AUTHOR CONTRIBUTIONS

C.M., M.T., J.Z., A.S., H.N., T.S.T., I.N., I.K., K.U., Y.Y., R.S., T.I., and K.S. performed the experiments. S.T., T.F., G.S., and Y.M. prepared experimental materials. J.Z. and Y.K. performed structural analysis. S.N. performed molecular phylogenetic analysis. A.Y., N. Shimoto, Y.N., R.M., T.T., and N. Sekiya performed clinical analysis and collected clinical samples. C.M., M.T., J.Z., A.S., J.I., T.I., and K.S. designed the experiments and interpreted the results. K.S. wrote the original manuscript. C.M., J.Z., A.S., T.I., S.N., and T.U. modified the manuscript. All authors reviewed and proofread the manuscript. The Genotype to Phenotype Japan (G2P-Japan) Consortium contributed to the project administration.

DECLARATION OF INTERESTS

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script. The Genotype to Phenotype Japan (G2P-Japan) Consortium contributed to the results. K.S. wrote the original manuscript. C.M., J.Z., A.S., T.I., S.N, and T.U. designed the experiments and interpreted the results. K.S. wrote the original manuscript. C.M., M.T., J.Z., A.S., T.I., and K.S. performed the experiments. S.T., T.F., G.S., and Y.M. prepared experimental materials. J.Z. and Y.K. performed structural analysis. S.N. performed molecular phylogenetic analysis. A.Y., N. Shimoto, Y.N., R.M., T.T., and N. Sekiya performed clinical analysis and collected clinical samples. C.M., M.T., J.Z., A.S., J.I., T.I., S.N., and T.U. designed the experiments and interpreted the results. K.S. wrote the original manuscript. C.M., J.Z., A.S., T.I., S.N., and T.U. modified the manuscript. All authors reviewed and proofread the manuscript. The Genotype to Phenotype Japan (G2P-Japan) Consortium contributed to the project administration.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| FITC-conjugated anti-human CD3 antibody | Biolegend | Cat# 300440; RRID: AB_2562046 |
| BV510-conjugated anti-human CD3 antibody | Biolegend | Cat# 317331; RRID: AB_2561376 |
| APC-Cy7-conjugated anti-human CD8 antibody | Biolegend | Cat# 301016; RRID: AB_314134 |
| PerCP-Cy5.5-conjugated anti-human CD14 antibody | Biolegend | Cat# 325622; RRID: AB_893250 |
| PerCP-Cy5.5-conjugated anti-human CD19 antibody | Biolegend | Cat# 320220; RRID: AB_2073119 |
| PE-Cy7-conjugated anti-human CD25 antibody | Biolegend | Cat# 356107; RRID: AB_2561974 |
| APC-conjugated anti-human CD137 antibody | Biolegend | Cat# 309809; RRID: AB_830671 |
| BV421-conjugated anti-human CD107a antibody | Biolegend | Cat# 326825; RRID: AB_10899581 |
| PE-conjugated anti-human IFN-γ antibody | BD Biosciences | Cat# 554552; RRID: AB_395474 |
| PE-Cy7-conjugated anti-human TNF-α antibody | Biolegend | Cat# 502930; RRID: AB_2204079 |
| APC-conjugated anti-human IL-2 antibody | Biolegend | Cat# 500310; RRID: AB_315097 |
| Anti-ACE2 antibody | R&D Systems | Cat# AF933; RRID: AB_355722 |
| APC-conjugated anti-goat IgG | R&D Systems | Cat# F0108; RRID: AB_573124 |
| Goat normal IgG | R&D Systems | Cat# AB-108-C; RRID: AB_354267 |
| Anti-SARS-CoV-2 S antibody | GeneTex | Cat# GTX635654; RRID: AB_2888548 |
| APC-conjugated anti-rabbit IgG | Jackson ImmunoResearch | Cat# 111-136-144; RRID: AB_2337987 |
| Rabbit normal IgG | SouthernBiotech | Cat# 0111-01; RRID: AB_2732899 |

### Bacterial and virus strains

| SARS-CoV-2 (strain WK-521) | Matsuyama et al., 2020 | GISAID ID: EPI_ISL_408667 |

### Biological samples

| Human PBMCs | This study | N/A |

### Chemicals, peptides, and recombinant proteins

| Ficoll-Paque Plus | GE Healthcare Life Sciences | Cat# 17-1440-03 |
| RPMI1640 medium | Thermo Fisher Scientific | Cat# 11875101 |
| Fetal calf serum (FCS) | Sigma-Aldrich | Cat# 172012-500ML |
| Penicillin streptomycin (PS) | Sigma-Aldrich | Cat# P4333-100ML |
| Dulbecco’s modified Eagle’s medium (high glucose) | Wako | Cat# 44-29765 |
| Eagle’s minimum essential medium (with L-glutamine and phenol red) | Wako | Cat# 051-07615 |
| Dulbecco’s modified Eagle’s medium (low glucose) | Wako | Cat# 041-29775 |
| G418 | Nacalai Tesque | Cat# G8168-10ML |
| Dulbecco’s modified Eagle’s medium (high glucose) | Sigma-Aldrich | Cat# R8758-500ML |
| Blasticidin | InvivoGen | Cat# ant-bl-1 |
| Ham’s F-12K medium | Wako | Cat# 080-08565 |
| Expi293 expression medium | Thermo Fisher Scientific | Cat# A1435101 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| SARS-CoV-2 PepTivator peptide pools | Miltenyi Biotec | Cat# 130-126-700 |
| Recombinant human IL-2 | Peprotec | Cat# 200-02 |
| NF9 peptide (NYNYLYRLF, residues 448-456 of the SARS-CoV-2 S protein) | This study | N/A |
| L452R peptide (NYNYRYRLF, L5R in NF9) | This study | N/A |
| Y453F peptide (NYNYLFRLF, Y6F in NF9) | This study | N/A |
| 7-aminoactinomycin D | Biolegend | Cat# 420404 |
| Paraformaldehyde | Nacalai Tesque | Cat# 09154-85 |
| Brefeldin A | Sigma-Aldrich | Cat# B7651 |
| Monensin | Biolegend | Cat# 420701 |
| PrimeSTAR GXL DNA polymerase | Takara | Cat# R050A |
| Kpnl | New England Biolab | Cat# R0142S |
| NotI | New England Biolab | Cat# R1089S |
| Mlu-HF | New England Biolab | Cat# R3198L |
| Smal | New England Biolab | Cat# R0141L |
| Hpal | New England Biolab | Cat# R0105L |
| Xhol | New England Biolab | Cat# R0146S |
| Bilirubin | Sigma-Aldrich | Cat# 14370-1G |
| CF640R succinimidyl ester | Biotium | Cat# 92108 |
| Lipofectamine 3000 | Thermo Fisher Scientific | Cat# L3000015 |
| Lipofectamine 2000 | Thermo Fisher Scientific | Cat# 11668019 |
| Dnase I | Sigma-Aldrich | Cat# 11284932001 |
| TransIT-LT1 | Takara | Cat# MiR2300 |
| Puromycin | InvivoGen | Cat# ant-pr-1 |
| Doxycycline | Takara | Cat# 1311N |
| TURBO DNase | Thermo Fisher Scientific | Cat# AM2238 |
| Minimum essential medium (2 x ), no phenol red | Thermo Fisher Scientific | Cat# 11935046 |
| Nonessential amino acid | Thermo Fisher Scientific | Cat# 11140-050 |
| Carboxymethyl cellulose | Sigma-Aldrich | Cat# C9481-500G |
| Formaldehyde | Nacalai Tesque | Cat# 37152-51 |
| Crystal violet | Nacalai Tesque | Cat# 09804-52 |
| Methylene blue | Nacalai Tesque | Cat# 22412-14 |
| Recombinant RNase inhibitor | Takara | Cat# 2313B |
| Transcriptor reverse transcriptase | Roche | Cat# 0353137001 |

Critical commercial assays

| Cytofix/Cytoperm Fixation/Permeabilization solution kit | BD Biosciences | Cat# 554714 |
| QI Amp RNA blood mini kit | QIAGEN | Cat# 52304 |
| SuperScript III reverse transcriptase | Thermo Fisher Scientific | Cat# 18080085 |
| ExpIfectamine 293 transfection kit | Thermo Fisher Scientific | Cat# A14525 |
| KAPA HiFi HotStart ReadyMix kit | Roche | Cat# KK2601 |
| One-Glo luciferase assay system | Promega | Cat# E5130 |
| GENE ART site-directed mutagenesis system | Thermo Fisher Scientific | Cat# A13312 |
| QI Amp viral RNA mini kit | QIAGEN | Cat# 52906 |
| One Step TB Green PrimeScript PLUS RT-PCR kit | Takara | Cat# RR096A |
| SARS-CoV-2 direct detection RT-qPCR kit | Takara | Cat# RC300A |
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| GeneJET gel extraction kit | Thermo Fisher Scientific | Cat# K0692 |
| EnduRen live cell substrate | Promega | Cat# E6481 |

**Experimental models: Cell lines**

| Human: C1R-A2402 cells | Karaki et al., 1993 | N/A |
| Human: HEK293 cells | ATCC | CRL-1573 |
| Human: HEK293T cells | ATCC | CRL-3216 |
| African green monkey (*Chlorocebus sabaeus*): Vero cells | JCRB | JCRB0111 |
| African green monkey (*Chlorocebus sabaeus*): VeroE6/TMPRSS2 cells | JCRB | JCRB1819 |
| Mink (*Neovison vison*): Mv.1.Lu cells | ATCC | CCL-64 |
| Human: HEK293-C34 cells | Torii et al., 2021 | N/A |
| Human: HEK293-ACE2 cells | This study | N/A |
| Human: A549-ACE2 cells | This study | N/A |
| Human: Expi293F cells | Thermo Fisher Scientific | Cat# A14527 |
| Yeast (*Saccharomyces cerevisiae*): strain EBY100 | ATCC | MYA-4941 |

**Oligonucleotides**

| Oligonucleotide | Source | Identifier |
|-----------------|--------|------------|
| Forward primer for pC-SARS2-S: TGTCGACC ATG TTT TTC CTG GTG CTG | Ozono et al., 2021 | N/A |
| Reverse primer for pC-SARS2-S: GTG GCGGCCGC TCT AGA TTC AGG TGT AGT GCA GTT T | Ozono et al., 2021 | N/A |
| Forward primer for S L452R: GTG GGA GGC AAC TAC AAC TAC CGT TAC | This study | N/A |
| Forward primer for S Y453F: GTG GGA GGC AAG TAC AAC TAC CTC TCC AGA | This study | N/A |
| Reverse primer for S L452R/L453F: GGT GTA GTT GCC TCC CAC CTT | This study | N/A |
| Forward primer for S N501Y: TCC TAT GGC TTC CAA CCA CAT TAT GGA | This study | N/A |
| Reverse primer for S N501Y: TGG TTG GAA GCC ATA GGA TTG | This study | N/A |
| Forward primer for S S13I: TGC CAC TGG TGT CCA TCC AGT GTG TGA ACC T | This study | N/A |
| Reverse primer for S S13I: AGG TTC ACA CAC TGG ATG GAC ACC AGT GGC A | This study | N/A |
| Forward primer for S W152C: GAA CAA CAA GTC TGT TAT GGA GTC TGA GTT C | This study | N/A |
| Reverse primer for S W152C: GAA CTC AGA CTC CAT ACA GGA CCT GTT GTT C | This study | N/A |
| Forward primer for S D614G: CTG TGC TCT ACC AGG GTG TGA ACT GTA CTG A | This study | N/A |
| Reverse primer for S D614G: TCA GTA CAG TTC ACA CCC TGG TAG AGC ACA G | This study | N/A |
| Forward primer for S HV69-70del: GGT TCC ATG CCA TCT CTG GCA CCA AGT GCA | This study | N/A |
| Reverse primer for S HV69-70del: TGC CAT TGG CAG AGA TGG CAT GGA ACC | This study | N/A |
| Mink ACE2 forward primer for cloning: ATG TTA GGC TCT TCC TGG CTC CTT | This study | N/A |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mink ACE2 reverse primer for cloning: CTA AAA TGA CGT CTG AAC ATC ATC GAC | This study | N/A |
| Mink ACE2 forward primer for the preparation of mink ACE2 expression: TTG GGT ACC ATG TTA GGC TCT TCC TGG CTC C | This study | N/A |
| Mink ACE2 reverse primer for the preparation of mink ACE2 expression: AAA GAT CTC GAG CTA AAA TGA CGT CTG AAC ATC | This study | N/A |
| Primer for the preparation of RBD L452R expression plasmid (RBD L452R):GGA CAG CAA GTG GGG AGG CAA CTA CAA CTA CAG ATA CAG ACT GTT CAG GAA GAG CAA C | This study | N/A |
| Primer for the preparation of RBD Y453F expression plasmid (RBD Y453F): CTC AAA TGG TTG CTT GCT CTC CAT GAG GTC GAA GAG GTA GTT GTA GTT GCC TCC C | This study | N/A |
| Primer for the preparation of RBD N501Y expression plasmid (RBD N501Y): GGA CAG CAA GTG GGG AGG CAA CTA CAA CTA CAG ATA CAG ACT GTT CAG GAA GAG CAA C | This study | N/A |
| Primer for the preparation of RBD expression plasmid CAT GGT AAA ACA TGT TGT TTA CGG AG | This study | N/A |
| Forward primer for the preparation of Fragment 8, S L452R: CTA AGG TTG GTG GTA ATT ATA ATT ACC GGT ATA GAT TGT TTA GGA AGT CTA ATC | This study | N/A |
| Reverse primer for the preparation of Fragment 8, S L452R: GAT TAG ACT TCC TAA ACA ATC TAT ACC GGT AAT TAT AAT TAC CAC CAA CCT TAG | This study | N/A |
| Forward primer for the preparation of Fragment 8, S Y453F: GGT TGG TGG TAA TTA TTA CCT GCT TGT TAG ATT TAG GAA GTC TAA TCT C | This study | N/A |
| Reverse primer for the preparation of Fragment 8, S Y453F: GAG ATT AGA CTT CCT AAA CAA CCT AAA CAG GTA ATTATA ATT ACC ACC AAC C | This study | N/A |
| Forward primer for the preparation of Fragment 8, S N501Y: GGA CAG CAA TAT GTG TTA CAA CCC ACT TAT GTG GTT GGT TAC CAA CCA TAC AG | This study | N/A |
| Reverse primer for the preparation of Fragment 8, S N501Y: CTG TAT GTG TGG TAA CCA ACA CCA TAA GTG GGT TG GAAA CCA TAT GAT TG | This study | N/A |
| SARS-CoV-2 S forward primer for the mutation verification (WK-521, 22607-22630 forward): GCA TCT GTT TAT GCT TG GAG CAA AGG | Torii et al., 2021 | N/A |
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| SARS-CoV-2 S reverse primer for the mutation verification (WK-521, 23342-23367 reverse): CCT GGT GTT ATA ACA CTG ACA CCA CC | Torii et al., 2021 | N/A |
| SARS-CoV-2 N forward primer for real-time RT-PCR: AGC CTC TTC TCG TTC CTC ATC AC | This study | N/A |
| SARS-CoV-2 N reverse primer for real-time RT-PCR: CCG CCA TTG CCA GCC ATT C | This study | N/A |
| Forward primer for competition assay: CAG GGC AAA CTG GAA AGA TT | This study | N/A |
| Reverse primer for competition assay: GCA ATG TCT CTG CCA AA | This study | N/A |
| Sequencing primer for competition assay: CAG GGC AAA CTG GAA AGA TT | This study | N/A |
| Random hexamer | Sigma-Aldrich | N/A |

**Recombinant DNA**

| Plasmid: pc-SARS2-S | Ozono et al., 2021 | N/A |
| Plasmid: pCAGGS | Niwa et al., 1991 | N/A |
| Plasmid: pc-SARS2-S-L452R | This study | N/A |
| Plasmid: pc-SARS2-S-Y453F | This study | N/A |
| Plasmid: pc-SARS2-S-N501Y | This study | N/A |
| Plasmid: pc-SARS2-S-D614G | Ozono et al., 2021 | N/A |
| Plasmid: pc-SARS2-S-B.1.1.298 [S13I/W152C/L452R/D614G] | This study | N/A |
| Plasmid: pc-SARS2-S-B.1.1.298 [HV69-70del/Y453F/D614G] | This study | N/A |
| Plasmid: pTarget-human ACE2 | Fukushi et al., 2007 | N/A |
| Plasmid: pLV-EF1a-IRES-Puro | Addgene | Cat# 85132 |
| Plasmid: pLV-EF1a-human ACE2-IRES-Puro | This study | N/A |
| Plasmid: pc-ACE2 | Ozono et al., 2021 | N/A |
| Plasmid: pc-mink ACE2 | This study | N/A |
| Plasmid: pHL-sec | Addgene | Cat# 99845 |
| Plasmid: pHL-sec-human ACE2 (residues 18-740) | Zahradnik et al., 2021a | N/A |
| Plasmid: pJYDC1 | Addgene | Cat# 162458 |
| Plasmid: pJYDC1-SARS-CoV-2 S RBD (residues 336-528) | Zahradnik et al., 2021a | N/A |
| Plasmid: pJYDC1-SARS-CoV-2 S RBD L452R | This study | N/A |
| Plasmid: pJYDC1-SARS-CoV-2 S RBD Y453F | This study | N/A |
| Plasmid: pJYDC1-SARS-CoV-2 S RBD N501Y | This study | N/A |
| Plasmid: psPAX2-IN/HIBIT | Ozono et al., 2020 | N/A |
| Plasmid: pWP1-Luc2 | Ozono et al., 2020 | N/A |
| Plasmid: pc-TMPRSS2 | Ozono et al., 2021 | N/A |
| Plasmid: pDA-NRF | Naldini et al., 1996 | N/A |
| Plasmid: pVSV-G | Addgene | Cat# 138479 |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Kei Sato (KeiSato@g.ecc.u-tokyo.ac.jp).

Materials availability
All unique reagents generated in this study are listed in the Key Resources Table and available from the Lead Contact with a completed Materials Transfer Agreement.

Data and code availability
N/A

REAGENT or RESOURCE SOURCE IDENTIFIER

Plasmid: template plasmids for the preparation of the partial SARS-CoV-2 genome fragments for CPER, see Table S6
Torii et al., 2021 N/A

Plasmid: pDSP1-7
Kondo et al., 2011 N/A

Plasmid: pDSP8-11
Kondo et al., 2011 N/A

Software and algorithms

NetMHCpan (v 4.1)
Reynisson et al., 2020; Katoh and Standley, 2013 http://www.cbs.dtu.dk/services/NetMHCpan/

NetMHC (v 4.0)
Andreatta and Nielsen, 2016; Lundegaard et al., 2008; Nielsen et al., 2003 http://www.cbs.dtu.dk/services/NetMHC/

SMM tool
Andreatta and Nielsen, 2016 http://tools.iedb.org/mhci/help/

PickPocket (v 1.1)
Zhang et al., 2009 http://www.cbs.dtu.dk/services/PickPocket/

MAGFT suite (v7.467)
Katoh and Standley, 2013 https://mafft.cbrc.jp/alignment/software/

ModelTest-NG (v0.1.5)
Darriba et al., 2020 https://github.com/ddarriba/modeltest

RAxML-NG (v1.0.0)
(Kozlov et al., 2019) https://github.com/amkozlov/raxml-ng

FtigTree (v1.4.4)
Andrew Rambaut http://tree.bio.ed.ac.uk/software/figtree

Python (v3.7)
Python Software Foundation https://www.python.org

Sequencer (v5.1)
Gene Codes Corporation N/A

PyMOL (v2.3.0)
Schrödinger https://pymol.org/2/

UCSF Chimera (v1.13)
Pettersen et al., 2004 N/A

Prism
GraphPad Software https://www.graphpad.com/scientific-software/prism/

Tracy
Rausch et al., 2020 https://www.gear-genomics.com/teal/

EditR
Kluesner et al., 2018 http://baseeditr.com

GISAID
Freunde von GISAID e.V. https://www.gisaid.org

Pangolin
Rambaut et al., 2020 https://cov-lineages.org/pangolin.html

0.45-μm pore size filter
Thermo Fisher Scientific Cat# 09-740-114

40-μm cell strainer
SPL Life Sciences Cat# 93040

HisTrap Fast Flow column
Cytiva Cat# 17-525S-01

Ultracel-3 regenerated cellulose membrane
Merck Cat# UFC900324

0.45-μm pore size filter
Merck Cat# SLGVR33PB

96-well black plate
PerkinElmer Cat# 6005225
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethics statement
All protocols involving human subjects recruited at Kyushu University Hospital, Japan, National Hospital Organization Kyushu Medical Center, Japan, and Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital, Japan, were reviewed and approved by the Ethics Committee for Epidemiological and General Research at the Faculty of Life Science, Kumamoto University (approval numbers 2066 and 461). All human subjects provided written informed consent.

Cell culture
Human PBMCs were obtained from three HLA-A*24:02-positive seronegative donors (average age: 44, range: 36-56, 100% male), sixteen HLA-A*24:02-positive COVID-19 convalescent donors (average age: 53, range: 27-83, 81% male, median sampling day post PCR positivity: 20, day range: 3-173), and six HLA-A24-negative COVID-19 convalescent donors (average age: 44, range: 24-67, 67% male, median sampling day post PCR positivity: 15, day range: 2-171) (Table S2). The PBMCs were purified by a density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare Life Sciences, cat# 17-1440-03) and stored in liquid nitrogen until further use. C1R cells expressing HLA-A*24:02 (C1R-A2402) (Karaki et al., 1993) were maintained in RPMI 1640 medium (Thermo Fisher Scientific, cat# 11875101) containing 10% fetal calf serum (FCS) and 1% antibiotics (penicillin and streptomycin; PS). HEK293 cells (a human embryonic kidney cell line; ATCC CRL-1573) and HEK293T cells (a human embryonic kidney cell line; ATCC CRL-3216) were maintained in Dulbecco’s modified Eagle’s medium (high glucose) (Wako, cat# 044-29765) containing 10% FCS and 1% PS. Vero cells (an African green monkey (Chlorocebus sabaeus) kidney cell line; JCRB0111) were maintained in Eagle’s minimum essential medium (Wako, cat# 051-07615) containing 10% FCS and 1% PS. VeroE6/TMPRSS2 cells [an African green monkey (Chlorocebus sabaeus) kidney cell line; JCRB1819] (Matsuyama et al., 2020) and Mv.1.Lu cells [a mink (Neovison vison) lung epithelial cell line; ATCC CCL-64] were maintained in Dulbecco’s modified Eagle’s medium (low glucose) (Wako, cat# 041-29775) containing 10% FCS, G418 (1 mg/mL; Nacalai Tesque, cat# G8168-10ML) and 1% PS. HEK293-C34 cells, the IFNAR1 KO HEK293 cells expressing human ACE2 and TMPRSS2 by doxyycycline treatment (Torii et al., 2021), were maintained in Dulbecco’s modified Eagle’s medium (high glucose) (Sigma-Aldrich, cat# R8758-500ML) containing 10% FCS, blasticidin (10 µg/mL; InvivoGen, cat# ant-bl-1) and 1% PS. A549 cells (a human lung epithelial cell line; ATCC CCL-185) were maintained in Ham’s F-12K medium (Wako, cat# 080-08565) containing 10% FCS and 1% PS. Expi293F cells (Thermo Fisher Scientific, cat# A14527) were maintained in Exp293 expression medium (Thermo Fisher Scientific, cat# A1435101).

METHOD DETAILS

Viral genomes and phylogenetic analyses
All viral genome sequences and annotation information used in this study were downloaded from GISAID (https://www.gisaid.org) as of March 15, 2021 (750,243 sequences). We used viral nucleotide sequences that did not contain any undetermined nucleotides in the region coding the S protein for our analysis (581,367 sequences). SARS-CoV-2 variants containing the L452R or Y453F mutation were sorted from the verified 581,367 sequences (Tables S3, S4, and S5). To infer the phylogeny of the B.1.1.298 lineage (Figure S2), we collected 657 sequences belonging to the B.1.1.298 lineage that do not contain any undetermined nucleotides. For the phylogenetic analysis, we used 345 representative SARS-CoV-2 sequences by removing identical ones. We aligned entire genome sequences by using the FFT-NS-2 program in MAFFT suite v7.467 (Katoh and Standley, 2013) and removed gapped regions using trimAl v1.4.rev22 with the gappyout option (Capella-Gutierrez et al., 2009). We selected GTR+1 as the best-fit nucleotide substitution model using ModelTest-NG v0.1.5 (Darriba et al., 2020). Based on this model, we generated a maximum-likelihood-based phylogenetic tree using RAxML-NG v1.0.0 (Kozlov et al., 2019) with a bootstrap test (n = 100).

Activation induced marker assay
Expansion of antigen-specific human CD8+ T cells and the analysis of the surface expression levels of the activation markers CD25 and CD137 were performed as previously described (Wolf et al., 2007). Briefly, human PBMCs were pulsed with 1 µg/mL SARS-CoV-2 PepTivator peptide pools (“S overlap peptides”) (Miltenyi Biotec, cat# 130-126-700) and maintained in RPMI 1640 medium (Thermo Fisher Scientific, cat# 11875101) containing 10% FCS and 30 U/mL recombinant human IL-2 (Peprotec, cat# 200-02) for 10-14 days. The in-vitro expanded CD8+ T cells (i.e., CTL lines) were stimulated with or without the NF9 peptide (NYNYLYRLF, residues 448-456 of the SARS-CoV-2 S protein; synthesized by Scrum Inc.). After incubation at 37°C for 1 h, the cells were washed, and surface proteins (CD3, CD8, CD14, CD19, CD25 and CD137) were stained with the antibodies listed in Key Resources Table. Dead cells were stained with 7-aminoactinomycin D (Biolegend, cat# 420404). After incubation for 20 min on ice, the cells were fixed with 1% paraformaldehyde (Nacalai Tesque, cat# 09154-85), and the levels of protein surface expression were analyzed by flow cytometry using a FACS Canto II (BD Biosciences). The data obtained by flow cytometry were analyzed with FlowJo software (Tree Star).

Analysis of the multifunctionality and cytotoxic potential of CD8+ T cells
C1R-A2402 cells were pulsed with or without the NF9 peptide or its derivatives [the NF9-L452R peptide (NYNYLYRRLF, LSR in NF9) and the NF9-Y453F peptide (NYNYLFRLF, Y6F in NF9); synthesized by Scrum Inc.] at concentrations from 0.1 to 10 nM at 37°C for 1 h. The cells were washed twice with PBS, mixed with the CTL lines generated from COVID-19 convalescent samples (see above) and...
incubated with RPMI 1640 medium (Thermo Fisher Scientific, cat# 11875101) containing 10% FCS, 5 μg/mL brefeldin A (Sigma-Aldrich, cat# B7651), 2 μM monensin (Biologend, cat# 420701) and a BV421-anti-CD107a antibody (Biologend, cat# 420404) in a 96-well U plate at 37°C for 5 h. The cells were washed, and surface proteins (CD3, CD8, CD14 and CD19) were stained with the antibodies listed in Key Resources Table. Dead cells were stained with 7-aminoactinomycin D (Biologend, cat# 420404). After incubation at 37°C for 30 min, the cells were fixed and permeabilized with a Cytofix/Cytoperm Fixation/Permeabilization solution kit (BD Biosciences, cat# 554714); intracellular proteins (IFN-γ, TNF-α and IL-2) were stained with the antibodies listed in Key Resources Table. After incubation at room temperature for 30 min, the cells were washed, and levels of protein expression were analyzed by flow cytometry using a FACS Canto II (BD Biosciences) followed by analysis using FlowJo software (Tree Star).

In silico HLA binding assay
The binding affinity of NF9 and its derivatives to HLA-A24 was predicted using the following five tools: NetMHCpan (v 4.1) (Reynisson et al., 2020), NetMHIC (v 4.0) (Andreatta and Nielsen, 2016; Lundegaard et al., 2008; Nielsen et al., 2003), SMM tool (Andreatta and Nielsen, 2016), PickPocket (v 1.1) (Zhang et al., 2009), and NetMHCcons (v 1.1) (Karosiene et al., 2012).

Plasmid construction
Plasmids expressing the SARS-CoV-2 S protein mutants (pC-S-L452R, pC-SARS2-S-Y453F, pC-SARS2-S-N501Y, pC-SARS2-S-B.1.429 [S13I/W152C/L452R/D614G] and pC-SARS2-S-B.1.1.298 [HV69-70del/Y453F/D614G]) were generated by site-directed mutagenesis PCR using pC-SARS2-S (kindly provided by Kenzo Tokunaga) (Ozono et al., 2021) as the template and the following primers: S forward, 5’-TTG GGT ACC ATG TTT GTG TTC CTG GTG CTG-3’; S reverse, 5’-GGT GCG GCC CGC TCT AGA TCT AGG TGT AGA TTT GTG TTC CTG GTG CTG-3’; S N501Y forward, 5’-TTC TAT GGC TTC CAA CCA ACC TAT GGA-3’; S N501Y reverse, 5’-TGG TGA GAA GCC ATG TGA TGG-3’; S S13I forward, 5’-TGC CAC TGG TGT CCA TCC ACC TAT GGA TGG-3’; S S13I reverse, 5’-AGG TCT ACA CAC TGG ATG ACC AGG ATG AAC TAC AAT GAC-3’; S W152C forward, 5’-GAA CAA CAA CAA CTA CTG TGT GAA GTC TGA GTT C-3’; S W152C reverse, 5’-GAA CTC AGA CTA CAC GAA CTT GTT GGT C-3’; S D614G forward, 5’-CTG TGC TCT ACC AGG ATG TGA ACT GTA CTG A-3’; S D614G reverse, 5’-CTG ATC ATA GCC TGT GGA GTT CTT GGA-3’; S D614G reverse, 5’-TCC GAT CAC TGG TGT CCA TCT CAC TGG TCA ACC TAT GGA TGG-3’; S HV69-70del forward, 5’-GTC TAT ATG TTC CCA CCT GCA AGA TGA ACC-3’; and S HV69-70del reverse, 5’-TGC CAT TGG TGG CAG AGA TGG CAT GGA ACC-3’. The resulting PCR fragment was digested with KpnI and NotI and inserted into the KpnI-NotI site of the pCAGGS vector (Niwa et al., 1991).

To construct the expression plasmid for human ACE2 (GenBank: NM_021804.3) (pLV-ER1a-human ACE2-IRE5-Puro), the MluI-Smal fragment pTargeT-human ACE2 (kindly provided by Shuetsu Fukushi) (Fukushi et al., 2007) was inserted into the MluI-Hpal site of pLV-ER1a-IRE5-Puro (Addgene #85132).

To construct the expression plasmid for mink ACE2 (GenBank: MW269526.1) (pC-mink ACE2), mink cDNA was used as the template. To prepare mink cDNA, cellular mRNA was extracted from Mv. 1. Lu cells using a QiAamp RNA blood mini kit (QIAGEN, cat# 52304), and mink mRNA was reversed transcribed using SuperScript III reverse transcriptase (Thermo Fisher Scientific, cat# 18080085) according to the manufacturers’ protocols. The DNA fragment including the mink ACE2 open reading frame was obtained by RT-PCR using PrimStar GXL DNA polymerase (Takara, cat# R050A) and the following primers: Mink ACE2 forward, 5’-ATGG TTA GGC TCT TCC TGG CTC CTT-3’; and Mink ACE2 reverse, 5’-CTA AAA TGA CGT CTG AAC ATC ATC ACC TAT GAC-3’. To add the linker sequence, additional PCR was performed using PrimStar GXL DNA polymerase (Takara, cat# R050A). The DNA fragment including the mink ACE2 open reading frame was used as the template, and two primers, KpnI mink ACE2 forward, 5’-TTG GGT ACC ATG TGA ACT GTA CTG A-3’; and XhoI mink ACE2 reverse: 5’-AAA GAT CTC GAG TTA GGA GGC AAC TAC AAC TAC CTC TTC AGA-3’; and XhoI mink ACE2 reverse: 5’-AAA GAT CTC GAG TTA GGA GGC AAC TAC AAC TAC CTC TTC AGA-3’, were used. The resultant DNA fragment was digested with KpnI and NotI and inserted into the KpnI-NotI site of pCACE2 (a human ACE2 expression plasmid kindly provided by Kenzo Tokunaga) (Ozono et al., 2021). Nucleotide sequences were determined by a DNA sequencing service (Fasmac), and the sequence data were analyzed by Sequencher v5.1 software (Gene Codes Corporation).

Preparation of soluble human ACE2
To prepare soluble human ACE2, the expression plasmid for the extracellular domain of human ACE2 (residues 18-740) based on pHl-sec (Addgene, cat# 99845) (Zahradnik et al., 2021a) was transfected into ExpI293F cells using an ExpIectamine 293 transfection kit (Thermo Fisher Scientific, cat# A1452S) according to the manufacturer’s protocol. At three days posttransfection, the culture medium was harvested, centrifuged, and filtered through a 0.45-μm pore size filter (Thermo Fisher Scientific, cat# 09-740-114). The filtered medium was applied to a 5 mL HisTrap Fast Flow column (Cytiva, cat# 17-5255-01) equilibrated with phosphate-buffered saline (PBS) using a ÄKTA pure chromatography system (Cytiva). The column was washed with PBS, and the pure human ACE2 protein (residues 18-740) was eluted using PBS supplemented with 300 mM imidazole (pH 7.4). The buffer was exchanged with PBS by using an Ultragel-3 regenerated cellulose membrane (Merck, cat# UFC900324), and the purified protein was concentrated. The purity of the prepared protein was assessed using a Tycho NT.6 system (NanoTemper).

Preparation of a yeast-based SARS-CoV-2 RBD expression system
The labeling-free yeast surface display plasmid pJYDC1 (Addgene, cat# 162458) (Zahradnik et al., 2021b) encoding the SARS-CoV-2 S RBD (residues 336-528) (pJYDC1-RBD) (Zahradnik et al., 2021a) was modified by the restriction enzyme-free cloning procedure
To prepare the plasmids with the mutated RBD, megaprimers were amplified by PCR using the KAPA HiFi HotStart ReadyMix kit (Roche, cat# KK2601) with the following primers according to the manufacturer’s protocol: RBD L452R forward: 5’-GGA CAG CAA GGTT GGG AGC CAA CTA CAA CTA CAG ATG CTG GAA GAA GAA C-3’; RBD Y453F reverse: 5’-CTG AAT TGG TTT CAG GGT GCT CTT CCA GAA CAG TGT GAA GAG GTA GAA GTC TTC GTC GCC CAC CTA CAC ATG CTG GAA GAA GAA C-3’; RBD N501Y reverse: 5’-GTA GAG TGT GTA GCC CAC CAC TTA CTA CTA CAG ATG CTG GAA GAA GAA C-3’. pCT_seq reverse: 5’-CAT GGG AAA ACA TGT TGT TTA CGG AG-3’; and pCTCON_seq forward: 5’-GCA GCC CCA TAA ACA CAC AGT AT-3’. The PCR products were integrated into pYDJC1 by integration PCR as previously described (Peleg and Unger, 2014).

Analysis of the binding affinity of the SARS-CoV-2 S RBD variants for human ACE2 by yeast surface display

The pYDJC1-based yeast display plasmids expressing SARS-CoV-2 RBD and its mutants were transformed into yeast (Saccharomyces cerevisiae; strain EBY100, ATCC MYA-4941) and selected by growth on SD-W plates (Peleg and Unger, 2014). Single colonies were grown in 1 mL liquid SD-CAA medium (Zahradník et al., 2021a) overnight at 30 °C (220 rpm) and used to inoculate 1/9 medium (Zahradník et al., 2021a) with 1 nM bilirubin (Sigma-Aldrich, cat# 14370-1G) for expression cultures. The cells were washed with PBS-B buffer [PBS supplemented with bovine serum albumin (1 g/l)] and aliquoted to solutions consisting of PBS-B buffer with 14 different concentrations (ranging from 100 nM to 1 pM) of the human ACE2 protein (residues 18-740) labeled with CF640R succinimidyl ester (Biotium, cat# 92108). The volume of the analysis solution was adjusted (1-100 µl) to reduce the effect of ligand depletion (Zahradník et al., 2021b). Yeasts expressing the SARS-CoV-2 S RBDs were incubated with the analysis solution overnight to allow for equilibrium. Subsequently, the yeast cells were washed with PBS-B buffer and passed through a 40-µm cell strainer (SPL Life Sciences, cat# 93040), after which binding affinity to the CF640R-labeled human ACE2 protein (residues 18-740) was examined using an Accuri C6 flow cytometer (BD Biosciences). The fluorescent signal was processed as previously described (Zahradník et al., 2021b), and the standard noncooperative Hill equation was fitted by nonlinear least-squares regression using Python v3.7 (https://www.python.org).

Pseudovirus assay

To prepare pseudoviruses, lentivirus (HIV-1)-based, luciferase-expressing reporter viruses pseudotyped with the SARS-CoV-2 S protein and its derivatives, HEK293T cells (1 × 10⁶ cells) were cotransfected with 1 µg of psPAX2-IN/HiBiT (Ozono et al., 2020), 1 µg of pWPI-Luc2 (Ozono et al., 2020), and 500 ng of plasmids expressing parental S or its derivatives (L452R, Y453F, N501Y, D614G, B.1.429 and B.1.1.298) using Lipofectamine 3000 (Thermo Fisher Scientific, cat# L3000015) according to the manufacturer’s protocol. At two days posttransfection, the culture supernatants were harvested, centrifuged, and treated with 37.5 U/mL DNase I (Sigma-Aldrich, cat# 11284932001) at 37 °C for 30 min. The amount of pseudoviruses prepared was quantified using the HiBiT assay, and the measured value was normalized to the level of HIV-1 p24 antigen as previously described (Ozono et al., 2021; Ozono et al., 2020). The pseudoviruses prepared were stored at −80 °C until use.

To prepare target cells for pseudovirus infection, HEK293T cells (1 × 10⁶ cells) were cotransfected with 250 ng of pc-TMPRSS2 (Ozono et al., 2021) and 500 ng of either pc-ACE2 or pc-mink ACE2 using Lipofectamine 2000 (Thermo Fisher Scientific, cat# 11668019) according to the manufacturer’s protocol. At two days posttransfection, the cells (22,000 cells/100 µl) were seeded in 96-well plates and infected with 100 µl of the pseudoviruses prepared at 4 different doses (1, 3, 5 and 10 ng of p24 antigen). At two days postinfection, the infected cells were lysed with a One-Glo luciferase assay system (Promega, cat# E6130), and the luminescent signal was measured using a CentroXS3 plate reader (Berthold Technologies).

Lentiviral transduction

Lentiviral transduction was performed as described previously (Anderson et al., 2018; Ikeda et al., 2019). Briefly, the VSV-G-pseudotyped lentivirus vector expressing human ACE2 was generated by transfecting 2.5 µg of plLV-EF1a-human ACE2-IRE5-Puro plasmid with 1.67 µg of pA-NRF (expressing HIV-1 gag, pol, rev, and tat genes) (Naldini et al., 1996) and 0.83 µg of pVSV-G (expressing VSV-G; Addgene, cat# 138479) into HEK293T cells (3 × 10⁶ cells) using TransIT-LT1 (Takara, cat# MIR2300) according to the manufacturer’s protocol. At two days posttransfection, the culture supernatants were harvested and centrifuged, and the supernatants were filtered with a 0.45-µm pore size filter (Millipore, cat# SLGVR33RB) and collected as the lentiviral vector. The lentivirus vectors were concentrated by centrifugation (at 22,000 × g for 2 h at 4 °C), and the concentrated vectors were inoculated into target cells (HEK293 cells and A549 cells) and incubated at 37 °C. At two days posttransduction, the cells were selected using culture medium containing 1 µg/ml puromycin (InvivoGen, cat# ant-pr-1). Puromycin-selected cells with relatively higher ACE2 expression were sorted by a FACS Aria II (BD Biosciences) and expanded. After expansion, the expression level of surface ACE2 was verified by a FACS Canto II (BD Biosciences). A goat anti-ACE2 polyclonal antibody (R&D Systems, cat# AF933) and an APC-conjugated donkey anti-goat IgG (R&D Systems, cat# F0108) were used for surface ACE2 staining. Normal goat IgG (R&D Systems, cat# AB-108-C) was used as the negative control for this assay.

Protein structure

PyMOL v2.3 (https://pymol.org/2/) was used for 3D visualization of the SARS-CoV-2 S and human ACE2 proteins (Figures 2A and 2G) with the cocrystal structure of the SARS-CoV-2 S and human ACE2 proteins (PDB: 6M17) (Yan et al., 2020). The L452R substitution (Figure 2G) was prepared using UCSF Chimera v1.13 (Petterson et al., 2004).
SARS-CoV-2 reverse genetics
Recombinant SARS-CoV-2 was generated by circular polymerase extension reaction (CPER) as previously described (Torii et al., 2021). In brief, 9 DNA fragments encoding the partial genome of SARS-CoV-2 (strain WK-521; GISAID ID: EPI_ISL_408667) (Matsuyama et al., 2020) were prepared by PCR using PrimeSTAR GXL DNA polymerase (Takara, cat# R050A). A linker fragment encoding hepatitis delta virus ribozyme (HDVr), bovine growth hormone (BGH) polyA signal and cytomegalovirus (CMV) promoter was also prepared by PCR. The corresponding SARS-CoV-2 genomic region and the templates and primers of this PCR are summarized in Table S6. The 10 obtained DNA fragments were mixed and used for CPER (Torii et al., 2021).

To produce recombinant SARS-CoV-2, the CPER products were transfected into HEK293-C34 cells using TransIT-LT1 (Takara, cat# MIR2300) according to the manufacturer’s protocol. At one day posttransfection, the culture medium was replaced with Dulbecco’s modified Eagle’s medium (high glucose) (Sigma-Aldrich, cat# R8758-500ML) containing 2% FCS, 1% PS and doxycycline (1 μg/mL; Takara, cat# 1311N). At six days posttransfection, the culture medium was harvested and centrifuged, and the supernatants were collected as the seed virus. To remove the CPER products (i.e., SARS-CoV-2-related DNA), 1 mL of the seed virus was treated with 2 μL TURBO DNase (Thermo Fisher Scientific, cat# AM2238) and incubated at 37°C for 1 h. Complete removal of the CPER products (i.e., SARS-CoV-2-related DNA) from the seed virus was verified by PCR. To prepare the working virus of recombinant SARS-CoV-2 for virological experiments (Figures 2H–2L), 100 μL of the seed virus was inoculated into VeroE6/TMPRSS2 cells (5,000,000 cells in a T-75 flask). At one h after infection, the culture medium was replaced with Dulbecco’s modified Eagle’s medium (low glucose) (Wako, cat# 041-29775) containing 2% FCS and 1% PS; at two days postinfection, the culture medium was harvested and centrifuged, and the supernatants were collected as the working virus.

To generate recombinant SARS-CoV-2 mutants, insertions were inserted in fragment 8 (Table S6) using the GENEART site-directed mutagenesis system (Thermo Fisher Scientific, cat# A13312) according to the manufacturer’s protocol with the following primers: Fragment 8_S L452R forward, 5’-CTA AGG TTG GTA ATT ATA ACC ACC GTG GTA ATT ATA CTA GCA -3’; Fragment 8_S L452R reverse, 5’-GAT ACT TCC TAA ACA ATC ACC GAT ATT AAT TAC CCA CCA CCT CAG TAG TG-3’; Fragment 8_S Y453F forward, 5’-GAT TGG TGG TAA TTA TAA TTA CCT GTT TAG ATT GAA TGA GTC TAA TCT C-3’; Fragment 8_S Y453F reverse, 5’-GAA AGT AGA CTT CCT AAA CAA TCT AAA CAG GTA ATT ATA ATT ACC ACC AAC C-3’; Fragment 8_S N501Y forward, 5’-CAA TCA TAT GGT TTC CAA CCC ACT TAT GGT GTT GGT TAC CAA CCA TCA CAC AG-3’; and Fragment 8_S N501Y reverse, 5’-CTG TAT GTG TTA CCA ACA AAA CCA TAA GTG TGG TGG AAA CCA TAT GAT TG-3’, according to the manufacturer’s protocol. Nucleotide sequences were determined by a DNA sequencing service (Fasmac), and the sequence data were analyzed by Sequencher version 5.1 software (Gene Codes Corporation). The CPER for the preparation of SARS-CoV-2 mutants was performed using mutated fragment 8 instead of parental fragment 8. Subsequent experimental procedures correspond to the procedure for parental SARS-CoV-2 preparation (described above). To verify insertion of the mutation in the working viruses, viral RNA was extracted using a QIAamp viral RNA mini kit (QIAGEN, cat# 25906) and reverse transcribed using SuperScript III reverse transcriptase (Thermo Fisher Scientific, cat# 18080085) according to the manufacturers’ protocols. DNA fragments including the mutations inserted were obtained by RT-PCR using PrimeSTAR GXL DNA polymerase (Takara, cat# R050A) and the following primers: WK-521 22607-22630 forward, 5’-GCA TCT GTT TAT ACC ACC AAC C-3’; and Fragment 8_S N501Y reverse, 5’-CTG TAT GTG TTA CCA ACA AAA CCA TAA GTG TGG TGG AAA CCA TAT GAT TG-3’, according to the manufacturer’s protocol. Nucleotide sequences were determined as described above, and sequence chromatograms (Figure 2H) were visualized using the web application Tracy (https://www.gear-genomics.com/teal/) (Rausch et al., 2020).

Plaque assay
One day prior to infection, 200,000 Vero cells were seeded into a 12-well plate. The virus was diluted with serum-free virus dilution buffer [1 × minimum essential medium (Temin’s modification) (Thermo Fisher Scientific, cat# 11935046) with 20 mM HEPES, nonessential amino acids (Thermo Fisher Scientific, cat# 11140-050) and antibiotics]. After removing the culture medium, the cells were infected with 500 μl of diluted virus at 37°C. At two h postinfection, 1 mL of mounting solution [1 × minimum essential medium containing 3% FCS and 1.5% carboxymethyl cellulose (Sigma, cat# C9481-500G)] was overlaid, followed by incubation at 37°C. At three days postinfection, the culture medium was removed, and the cells were washed with PBS three times and fixed with 10% formaldehyde (Nacalai Tesque, cat# 37152-51) or 4% paraformaldehyde (Nacalai Tesque, cat# 09154-85). The fixed cells were washed with tap water, dried, and stained with staining solution [2% crystal violet (Nacalai Tesque, cat# 09804-52) or 0.1% methylene blue (Nacalai Tesque, cat# 22412-14) in water]. The stained cells were washed with city water and dried, and the number of plaques was counted to calculate PFU.

SARS-CoV-2 infection
One day prior to infection, 10,000 HEK293-ACE2, A549-ACE2 and VeroE6/TMPRSS2 cells were seeded into a 96-well plate. Recombinant SARS-CoV-2 (10, 100 or 1,000 PFU) was inoculated and incubated at 37°C for 1 h. The infected cells were washed, and 180 μl of culture medium was added. The culture supernatant (10 μl) was harvested at indicated time points and used for real-time RT-PCR to quantify the viral RNA copy number.

Real-time RT-PCR
The amount of viral RNA in culture supernatant was quantified by real-time RT-PCR as previously described (Shema Mugisha et al., 2020), with some modifications. In brief, 5 μl of culture supernatant was mixed with 5 μl of 2 × RNA lysis buffer [2% Triton X-100, 50 mM KCl, 100 mM Tris-HCl (pH 7.4), 40% glycerol, 0.8 U/μl recombinant RNase inhibitor (Takara, cat# 2313B)] and incubated at
room temperature for 10 min. RNase-free water (90 μl) was added, and the diluted sample (2.5 μl) was used as the template for real-time RT-PCR performed according to the manufacturer’s protocol using the One Step TB Green PrimeScript PLUS RT-PCR kit (Takara, cat# RR096A) and the following primers: Forward N, 5’-AGC CTC TTC TCG TTC ATC AC-3’; and Reverse N, 5’-CCG CCA TTG CCA GCC ATT C-3’. The copy number of viral RNA was standardized with a SARS-CoV-2 direct detection RT-qPCR kit (Takara, cat# RC300A). The fluorescent signal was acquired using a QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific), a CFX Connect Real-Time PCR Detection system (Bio-Rad) or a 7500 Real Time PCR System (Applied Biosystems).

**Competition assay**

One day prior to infection, 100,000 HEK293-ACE2 cells were seeded into 12-well plates. Parental virus and the L452R mutant were mixed at a 1:1 ratio based on the PFU. The virus mixture was inoculated at a final concentration of 1,000 PFU (i.e., 500 PFU for each virus) and incubated at 37˚C for 1 h. The infected cells were washed, and 1 mL of culture medium was added. The culture supernatant (140 μl) was harvested at the indicated time points, and 140 μl culture medium was supplied. Viral RNAs were isolated from the harvested culture supernatant using a QIAamp viral RNA mini kit (QIAGEN, cat# 52906), and cDNA was synthesized using reverse transcriptase (Roche, cat# 03531317001) with a random hexamer (Sigma-Aldrich). The resultant cDNAs were amplified with a primer set (forward, 5’-CAG GGC AAA CTG GAA AGA TT-3’ and reverse, 5’-TCA GCA ATG TCT CTG CCA AA-3’). The PCR products were purified using a GeneJET gel extraction kit (Thermo Fisher Scientific, cat# K0692) and subjected to Sanger sequencing with the following primer: 5’-CAG GGC AAA CTG GAA AGA TT-3’. The nucleotide variation at position 22,917 (i.e., T for parental virus, G for the L452R mutant) was quantified by using EditR (Kluesner et al., 2018).

**SARS-CoV-2 S-based fusion assay**

The SARS-CoV-2 S-based fusion assay was performed according to the HIV-1 envelope protein-based fusion assay (Ikeda et al., 2018; Kondo et al., 2011) with some modifications. This assay utilizes a dual split protein (DSP) encoding Renilla luciferase (RL) and GFP genes, and the respective split proteins, DSP1-7 and DSP8-11, are expressed in effector and target cells by transfection (Ikeda et al., 2018; Kondo et al., 2011).

On day 1, effector cells (i.e., S-expressing cells) and target cells (i.e., ACE2-expressing cells) were prepared. To prepare effector cells, HEK293 cells were cotransfected with the expression plasmids for parental S or its derivatives (L452R, Y453F and N501Y) (400 ng) and pDSP1-7 (400 ng) using TransIT-LT1 (Takara, cat# MIR2300). To prepare target cells, HEK293 cells were cotransfected with pC-ACE2 (1,000 ng) and pDSP8-11 (400 ng). On day 2 (24 h posttransfection), 16,000 effector cells were detached and reseeded into 96-well black plates (PerkinElmer, cat# 6005225), and target cells were reseeded at a density of 1,000,000 cells/2 mL/well in 6-well plates. On day 4 (72 h posttransfection), target cells were incubated with EnduRen live cell substrate (Promega, cat# E6481) for three h and then detached, and 32,000 target cells were applied to a 96-well plate with effector cells. RL activity was measured at the indicated time points by using a Centro XS3 LB960 (Berthold Technologies). The S proteins expressed on the surface of effector cells were stained with a rabbit anti-SARS-CoV-2 monoclonal antibody (GeneTex, cat# GTX635654) and an APC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, cat# 111-136-144). Normal rabbit IgG (SouthernBiotech, cat# 0111-01) was used as a negative control. Expression levels of surface S proteins were analyzed using a FACS Canto II (BD Biosciences). RL activity was normalized to the mean fluorescence intensity (MFI) of surface S proteins, and the normalized values are shown as fusion activity.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Data analyses were performed using Prism 7 (GraphPad Software). In Figures 1B and 1F, data are presented as median with SD, and n represents the number of PBMC donor. In the other figure panels, data are presented as average with SD, and n represents the number of technical replicate. In Figures 1B and 1F, statistically significant differences are determined by the Wilcoxon signed-rank test. In Figure 1I, statistically significant differences are determined by ANOVA, with multiple comparisons by Bonferroni correction. In Figure 2C, statistically significant difference are determined by the Mann-Whitney U test. In Figures 2E, 2F, and 2J–2N, statistically significant difference are determined by Student’s t test.