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Conserved and novel mouse CD8 T cell epitopes within SARS-CoV-2 spike RBD protein identified following subunit vaccination

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Abstract

The prior existence of human ACE2 protein-expressing mice used to study SARS-CoV and the rapid development of mouse-adapted virus strains, has allowed the study of SARS-CoV-2 in mice, even as we are still learning about its natural pathology in humans. With myriad genetically altered strains on the C57BL/6 background and the abundance of immunological reagents available to interrogate its immune responses, the C57BL/6 mice may provide useful insight into the immunology of SARS-CoV-2 infection and vaccination. In order to conduct more detailed studies on their T cell responses to vaccines and infection, the epitopes eliciting those responses must be characterized in further detail. Here, we mapped CD8 T cell epitopes within the receptor binding domain of the SARS-CoV-2 spike protein in C57BL/6 mice. Our study identified five major CD8 T cell epitopes in immunized C57BL/6 mice, including one, VVLSFELL, presented by H-2K^b and common between SARS-CoV and SARS-CoV-2.

Introduction

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has emerged as one of the most devastating pandemics in a century. The global response to this threat has been swift, leading to the development of multiple safe and efficacious vaccines(1–3).

The primary target for SARS-CoV-2 vaccine studies in humans is the spike (S) protein(4), the surface protein on coronaviruses essential for antibody-mediated neutralization of viral particles. Although the two mRNA-based vaccines now approved for emergency use authorization by the United States FDA elicit strong antibody responses, they also elicit CD8 T cell responses to the S protein(5, 6), as do S-encoding adenoviral vectors of other leading vaccine candidates(7, 8). Future studies may define the protective effect of CD8 T cell responses, especially in the latter. Indeed, in a recent study of COVID-19 patients, CD4 and CD8 T cell responses were independently associated with less severe disease(9).

Despite the availability of multiple mouse models of disease, mechanistic studies into the roles for T cells in vaccine-mediated protection and immunity derived from natural infection

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have been hampered by limited knowledge of the SARS-CoV-2 antigens targeted by CD8 T cells. To define the epitopes contained within the RBD of the S protein, we used a subunit vaccine platform composed of recombinant RBD protein antigen in combination with an adjuvant containing agonistic anti-CD40 antibody and the TLR3 agonist poly(I:C)(10). With peptide stimulation and subsequent cytokine staining, we identified five major and two minor CD8 T cell epitopes in immunized C57BL/6 mice. Furthermore, we defined the MHC class I-restriction as H-2K^b for a peptide epitope that is shared between both SARS-CoV and SARS-CoV-2.

Materials and Methods

Mice and immunizations

All experiments involving mice were conducted following protocols approved by the University of Colorado Institutional Animal Care and Use Committee (IACUC) according to guidelines provided by the Association for Assessment and Accreditation of Laboratory Animal Care. C57BL/6 mice were obtained from the Jackson Laboratory and were subsequently bred in specific-pathogen-free facilities at the University of Colorado Anschutz Medical Campus. Experiments were performed in 6–12-week-old female mice. Mice were immunized via tail-vein injection with 100 μg or 200 μg, of SARS-CoV-2 spike RBD protein plus adjuvant. SARS-CoV-2 RBD protein (Wuhan-Hu-1; GenBank: MT380724.1) was expressed by transfection of Expi293 cells with a His-tagged vector (a gift from F. Krammer, Icahn School of Medicine at Mount Sinai, New York, NY)(11) and subsequently purified from cell culture supernatants by the University of Colorado Cell Technologies Shared Resource. Immunizations were adjuvanted with 40 μg poly(I:C) (Invivogen), and 40 μg anti-CD40 (clone FGK4.5, BioXCell). Vaccines were made immediately prior to immunization and injected in a total volume of 200 μl.

RMA-S MHC class I-stabilization assay

To determine the MHC class I-restriction of SARS-CoV-2 peptide epitopes shown to induce RBD-specific CD8 T cell responses in immunized mice, we employed the murine TAP-deficient RMA-S lymphoma cell line, which is derived from C57BL/6 mice(12, 13). RMA-S cells were cultured overnight at 27°C to stabilize unloaded MHC class I H-2D^b and H-2K^b on the cell surface. RMA-S cells containing peptide-empty H-2D^b and H-2K^b were coincubated with indicated CD8 T cell peptides at 10 μM for 5 h at 37°C. We tested the 15-mer peptides representing the 5 major SARS-CoV-2 RBD epitopes revealed in these studies: $S_{1–14,319}$, $S_{337–351}$, $S_{401–415}$, $S_{477–491}$, $S_{505–519/509–523}$, and the 8-mer $S_{511–518}$. Peptides with known H-2K^b, H-2D^b, and H-2K^b/H-2D^b restriction, respectively, were included as controls: $OVA_{257–264}$, LCMV $NP_{396–404}$, and LCMV $GP_{33–41}$. After 5 h at 37°C, MHC class I stabilization was quantified by flow cytometry using anti-mouse antibodies directed against H-2K^b (clone AF6-14-8) and H-2D^b (clone 28-14-8).

Flow cytometry

Seven days after immunization, single cell suspensions generated from spleens were subjected to ACK red blood cell lysis and counted using a Vi-Cell automated cell counter (Beckman Coulter). For *in vitro* stimulation assays, 1×10^6 cells were incubated with 1

μg/ml peptide and 3 μg/ml brefeldin A for 5 h at 37°C in complete media (RPMI 1640 containing 10% FBS, 10 mM HEPES, 0.1 mM β-ME, 0.1 mM non-essential amino acids, 0.1 mM sodium pyruvate, 2 mM L-glutamine and penicillin-streptomycin). After stimulation, cells were surfaced-stained with CD8α-BV421 (clone 53.67, BioLegend), CD4-FITC (GK1.5, BioLegend), B220-PE-Cy7 (clone RA3-6B2, Tonbo), and a fixable viability dye (Ghost Dye Red 780, Tonbo) for 10 min at room temperature. After staining for surface antigens, cells were fixed and permeabilized with FoxP3 fixation/permeabilization buffers (Tonbo) for 15 min at room temperature. After fixation and permeabilization, cells were washed in perm/wash buffer and stained for intracellular cytokines using IFNγ-APC (XMG1.2, Tonbo) and TNFα-PE (MP6-XT22, BD Biosciences) diluted in perm/wash buffer for 30 min at room temperature. After a final wash, flow cytometry data were acquired on a four-laser (405, 488, 561, 638 nm) CytoFLEX S flow cytometer (Beckman Coulter) and analysis was performed using FlowJo (version 10.7.1; BD Biosciences).

Peptides

Crude preparations of 58 peptides covering the SARS-CoV-2 spike RBD protein (GenBank: MT380724.1), derived from isolate Wuhan-Hu-1, were generated (ChinaPeptides), comprising 15-mer peptides overlapping by 11 amino acids. Highly purified (>96% purity) VVSLFELL peptide was also prepared (ChinaPeptides).

Statistical Analysis

Prism (version 9.01, GraphPad) was used to plot data and perform one-way ANOVA tests with Dunnett's multiple comparisons test to compare all values to stimulation with an irrelevant peptide (HSV glycoprotein $B_{498–504}$).

Results

One week following vaccination via intravenous injection with 100 μg purified, recombinant SARS-CoV-2 RBD protein adjuvanted with poly(I:C) and anti-CD40, splenic CD4 and CD8 T cells from C57BL/6 mice were evaluated by *ex vivo* peptide restimulation and subsequent intracellular cytokine staining for IFNγ and TNFα and flow cytometric analysis. Cells were stimulated using a peptide library of 15-mers, overlapping by 11 amino acids, covering the entire RBD protein (Table I). No CD4 T cell responses to RBD peptides were revealed for C57BL/6 mice by this analysis, however, several major CD8 T cell epitopes were identified. Five peptides were determined to generate statistically significant IFNγ responses in a one-way ANOVA analysis, including $S_{1–14,319}$, $S_{337–351}$, $S_{401–415}$, $S_{477–491}$, and $S_{505–519/509–523}$ (Fig. 1A). The latter sequences, spanning $S_{505–523}$, aligned with a previously identified SARS-CoV CD8 T cell epitope, VVLSFELL(14). Using this same 8-mer sequence, $S_{511–518}$ (511*) was determined to be the minimal epitope for SARS-CoV-2 (Fig. 1A). Two additional minor epitopes were confirmed in an experiment where antigen dose was increased to 200 μg (Fig. 1B). In this experiment, the three strongest epitopes each elicited IFNγ production in roughly 3% of CD8 T cells, each, whereas $S_{529–343}$ and $S_{389–403}$ elicited significant, but relatively modest CD8 T cell responses at about 0.3% of CD8 T cells. Representative flow cytometry plots show most of the CD8 T cells responding to peptide

restimulation stain positive for both IFNγ and TNFα, with negligible background cytokine production in negative control wells (stimulated with $HSVgB_{498–505}$) (Fig. 1C).

These data suggest a promiscuity of the peptide VVLSFELL ($S_{511–518}$) for MHC of multiple haplotypes, here eliciting responses in C57BL/6 mice and in another recent publication, $S_{511–525}$ elicited responses in BALB/c mice immunized with a DNA-based vaccine encoding the S protein(15). Using the MHC-I peptide binding prediction tool NetH2pan(16), the only 8–14-mer peptides predicted to bind H-$2K^d$ or H-$2D^d$ within $S_{511–525}$ are VVLSFELL and VVVLSFELL ($S_{510–518}$), which are both predicted to strongly bind H-$2D^d$. Interestingly, the 9-mer VVVLSFELL is also predicted to bind to H-$2D^b$. To determine whether this epitope was restricted to H-$2K^b$ and/or H-$2D^b$, we performed a cell-based MHC-I stabilization assay. RMA-S cells were interrogated with the 15-mers $S_{1–14,319}$, $S_{337–351}$, $S_{401–415}$, $S_{477–491}$, $S_{505–519}$, and $S_{509–523}$, as well as the minimal 8-mer $S_{511–518}$. RMA-S cells are deficient in the expression of the TAP peptide transporter, critical for stabilizing MHC-I through peptide loading in the endoplasmic reticulum. This results in little to no MHC-I expression on the cell surface at 37°C(12). However, when RMA-S cells are cultured at 27°C, empty H-$2D^b$ and H-$2K^b$ MHC-I molecules accumulate on the cell surface. The addition of peptides able to bind to either K^b or D^b, followed by shifting the cells to 37°C, permits identification of the MHC-I molecules (i.e., K^b, D^b, or both) stabilized on the cell surface. Staining with antibodies specific for H-$2K^b$ and H-$2D^b$ indicated that the 8-mer VVLSFELL ($S_{511–518}$) was clearly restricted to H-$2K^b$ (Fig. 2). In contrast, $S_{505–519}$, and $S_{509–523}$, which contain the $S_{511–518}$ 8-mer as well as the 9-mer VVVLSFELL, appeared to stabilize H-$2D^b$, as predicted, with $S_{509–523}$ stabilizing both K^b and D^b. Results for the 15-mer peptides covering the remaining major epitopes were less clear, with the exception of $S_{477–491}$, which also stabilized H-$2D^b$. It is not surprising that the RMA-S assay was unable to define the restriction for every 15-mer, as it is likely a less sensitive measure of peptide binding as the cytokine staining of activated T cells, known to react to picomolar quantities of peptide-bound MHC(17). However, use of the MHC-I peptide binding prediction tool NetH2pan(16) indicated a likely VFLVLLPL epitope binding H-$2K^b$ within $S_{1–14,319}$, a NATRFASV epitope binding H-$2K^b$ in $S_{337–351}$, and a STPCNGVEGF epitope binding H-$2D^b$ in $S_{477–491}$.

Discussion

The relative durability of the antibody responses to SARS-CoV-2 infection has been controversial, with initial studies reporting a dramatic early decline in titers that may leave patients susceptible to reinfection(18). More recent, much larger studies, however, indicate that neutralizing antibody titers persist for at least 5 months after infection(19). In line with these data, preliminary studies suggest the risk of reinfection remains very low, and is associated with asymptomatic disease(20). Yet, whether or not antibody responses ultimately demonstrate long-term durability, cellular immune responses are likely an important determinant of prolonged protection.

COVID-19 patients show T cell reactivity toward multiple proteins, including membrane (M), nucleocapsid (N) and non-structural proteins (NSPs)(21); in fact, one recent study identified an epitope within the nucleocapsid, $N_{219–227}$, shared by both mouse (H-$2D^b$) and

human (HLA-A2) T cells(22). However, in serum isolated from PCR-confirmed SARS-CoV-2 positive patients, the primary target for neutralizing antibody is the S protein, with epitope specificity of neutralization directed against both the S protein RBD, and the S protein N-terminal domain (NTD)(23). As such, the S protein may experience greater pressure to mutate from one virus strain to another, and, thus, the T cell epitopes identified within S, are more likely to be unique to SARS-CoV-2 than those from other structural proteins. Indeed, the sequence identity between SARS-CoV and SARS-CoV-2 is 91% for both the membrane (M), nucleocapsid (N) proteins, whereas it is only 76% for S, and 73% for the RBD. In spite of this, we identified one epitope shared by the two viruses within the RBD, $S_{511–518}$. Two of the five major epitopes ($S_{337–351}$ and $S_{401–415}$) had high sequence homology but were not known to the authors to be previously described epitopes for SARS-CoV. In addition, we identified two unique CD8 T cell epitopes – the sequence homology at $S_{1–14,319}$ and $S_{477–491}$ is only 50% and 40%, respectively, between SARS-CoV and SARS-CoV-2. Although the minimal epitope within $S_{1–14,319}$, could comprise a hybrid peptide between the signal peptide and the RBD, not seen in natural infection, this is unlikely, given that NetH2pan predictions only predict MHCI binding for $S_{1–8}$, and $S_{3–10}$.

The combination of both conserved and unique epitopes within the RBD of the S protein may foster future investigations into serial infections using SARS-CoV and SARS-CoV-2 in either mouse-adapted coronavirus strains, or hACE2-expressing C57BL/6 mice. During infection, CD8 T cell responses to additional structural and non-structural proteins will undoubtedly also arise, as recently reported for the nucleocapsid protein(22), and each may contribute to viral control. Moreover, infection may elicit CD8 T cell responses to these epitopes to varying degrees compared to what we have reported here for vaccination, especially as CD8 T cells responding to immunogenic epitopes within other proteins compete for immunodominance. Nonetheless, we expect one or more of these epitopes to be involved in the infectious response, and we hope the data reported here will be a useful resource, reducing the financial and practical threshold for new studies of SARS-CoV-2 infection or vaccination in mice.

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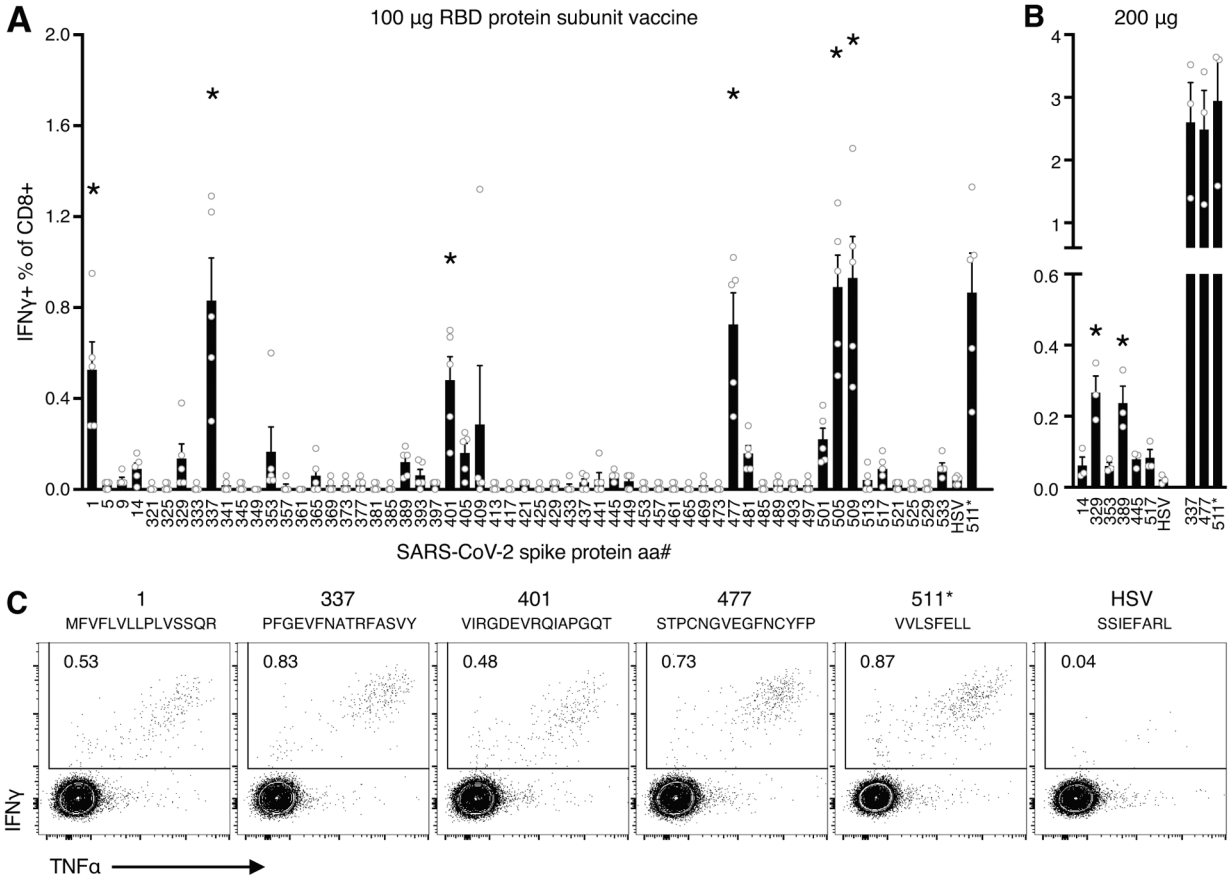

Figure 1. Epitope mapping of CD8 T cell responses to SARS-CoV-2 RBD protein in C57BL/6 mice.

Five mice were immunized with RBD protein plus adjuvant and their spleens harvested one week later. **A**) The percentage of CD8 T cells staining for IFNγ after a 5 h incubation with individual 15-mer peptides spanning SARS-CoV-2 RBD. Responses that were significantly greater than those induced by an irrelevant peptide ($HSVgB_{498–504}$), as determined by Dunnett's multiple comparisons test (where $p<0.01$), were indicated by an asterisk. **B**) The percentage of CD8 T cells staining for IFNγ for the six potential minor epitopes and three of the major epitopes identified in A) in mice immunized 200 µg of RBD plus adjuvant. **C**) Representative intracellular IFNγ and TNFα staining. Cells were pre-gated on lymphocytes, singlets, live cells, and $CD8^{+}CD4^{-}B220^{-}$.

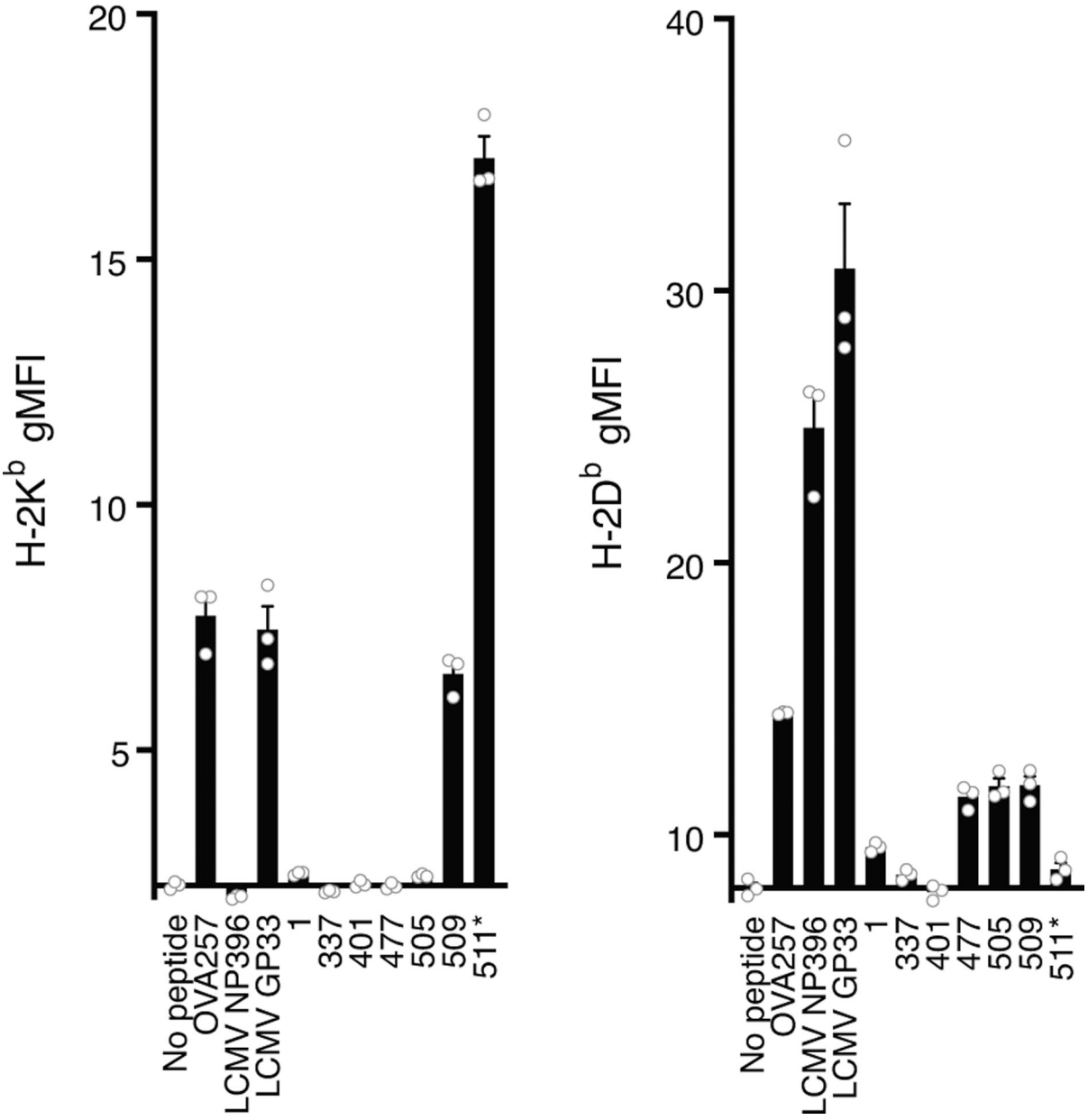

Figure 2. Determination of peptide MHC class I-restriction.

H-2K^b or H-2K^b staining of RMA-S cells 5 h after incubation with the indicated peptide. For both graphs, the x-axis intersects the y-axis at the average gMFI value for controls without peptide.

Table I.

Amino acid sequences for peptides used in in vitro cytokine stimulation assays.

Spike protein aa#	Sequence
1–14, 319	MFVFLVLLPLVSSQR
5–14, 319–323	LVLLPLVSSQRVQPT
9–14, 319–327	PLVSSQRVQPTESIV
14, 323–331	SQRVQPTESIVRFPN
321–335	QPTESIVRFPNITNL
325–339	SIVRFPNITNLCPFG
329–343	FPNITNLCPFGEVFN
333–347	TNLCPFGEVFNATRF
337–351	PFGEVFNATRFASVY
341–355	VFNATRFASVYAWNR
345–359	TRFASVYAWNRKRIS
349–363	SVYAWNRKRISNCVA
353–367	WNRKRISNCVADYSV
357–371	RISNCVADYSVLYNS
361–375	CVADYSVLYNSASFS
365–379	YSVLYNSASFSTFKC
369–383	YNSASFSTFKCYGVS
373–387	SFSTFKCYGVSPTKL
377–391	FKCYGVSPTKLNDLC
381–395	GVSPTKLNDLCFTNV
385–399	TKLNDLCFTNVYADS
389–403	DLCFTNVYADSFVIR
393–407	TNVYADSFVIRGDEV
397–411	ADSFVIRGDEVRQIA
401–415	VIRGDEVRQIAPGQT
405–419	DEVRQIAPGQTGKIA
409–423	QIAPGQTGKIADYNY
413–427	GQTGKIADYNYKLPD
417–431	KIADYNYKLPDDFTG
421–435	YNYKLPDDFTGCVIA
425–439	LPDDFTGCVIAWNSN
429–443	FTGCVIAWNSNNLDS
433–447	VIAWNSNNLDSKVGG
437–451	NSNNLDSKVGGNYNY
441–455	LDSKVGGNYNYLYRL
445–459	VGGNYNYLYRLFRKS
449–463	YNYLYRLFRKSNLKP
453–467	YRLFRKSNLKPFERD
457–471	RKSNLKPFERDISTE

Spike protein aa#	Sequence
461–475	LKPFERDISTEIYQA
465–479	ERDISTEIYQAGSTP
469–483	STEIYQAGSTPCNGV
473–487	YQAGSTPCNGVEGFN
477–491	STPCNGVEGFNCYFP
481–495	NGVEGFNCYFPLQSY
485–499	GFNCYFPLQSYGFQP
489–503	YFPLQSYGFQPTNGV
493–507	QSYGFQPTNGVGYQP
497–511	FQPTNGVGYQPYRVV
501–515	NGVGYQPYRVVVLSF
505–519	YQPYRVVVLSFELLH
509–523	RVVVLSFELLHAPAT
513–527	LSFELLHAPATVCGP
517–531	LLHAPATVCGPKKST
521–535	PATVCGPKKSTNLVK
525–539	CGPKKSTNLVKNKCV
529–541, 2xH	KSTNLVKNKCVNFHH
533–541, 6xH	LVKNKCVNFHHHHHH
HSVgB 498–505	SSIEFARL
511*–518	VVLSFELL