Title:

An immunodominance hierarchy exists in CD8⁺ T cell responses to
HLA-A*02:01-restricted epitopes identified from the non-structural polyprotein 1a of
SARS-CoV-2.

Running title:

Dominant hierarchy of CTL epitopes in SARS-CoV-2 pp1a

Authors:

Akira Takagi,¹ and Masanori Matsui^{2*}

Affiliation:

¹School of Medical Technology, Faculty of Health and Medical Care, Saitama Medical
University, 1397-1 Yamane, Hidaka-city, Saitama 350-1241, Japan

²Department of Microbiology, Faculty of Medicine, Saitama Medical University,
38 Morohongo, Moroyama-cho, Iruma-gun, Saitama 350-0495, Japan

*Corresponding author. E-mail address: mmatsui@saitama-med.ac.jp

Tel: +81-49-276-1438; Fax: +81-49-295-9107.

Key words:

SARS-CoV-2, COVID-19, CTL epitope, HLA-A*02:01, pp1a, vaccine,
immunodominance, hierarchy

Abstract

COVID-19 vaccines are being rapidly developed and human trials are underway. Almost all of these vaccines have been designed to induce antibodies targeting spike protein of SARS-CoV-2 in expectation of neutralizing activities. However, non-neutralizing antibodies are at risk of causing antibody-dependent enhancement. Further, the longevity of SARS-CoV-2-specific antibodies is very short. Therefore, in addition to antibody-induced vaccines, novel vaccines on the basis of SARS-CoV-2-specific cytotoxic T lymphocytes (CTLs) should be considered in the vaccine development. Here, we attempted to identify HLA-A*02:01-restricted CTL epitopes derived from the non-structural polyprotein 1a of SARS-CoV-2. Eighty-two peptides were firstly predicted as epitope candidates on bioinformatics. Fifty-four in 82 peptides showed high or medium binding affinities to HLA-A*02:01. HLA-A*02:01 transgenic mice were then immunized with each of the 54 peptides encapsulated into liposomes. The intracellular cytokine staining assay revealed that 18 out of 54 peptides were CTL epitopes because of the induction of IFN- γ -producing CD8⁺ T cells. In the 18 peptides, 10 peptides were chosen for the following analyses because of their high responses. To identify dominant CTL epitopes, mice were immunized with liposomes containing the mixture of the 10 peptides. Some peptides were shown to be statistically predominant over the other peptides. Surprisingly, all mice immunized with the liposomal 10 peptide mixture did not show the same reaction pattern to the 10 peptides. There were three pattern types that varied sequentially, suggesting the existence of an

immunodominance hierarchy, which may provide us more variations in the epitope selection for designing CTL-based COVID-19 vaccines.

Importance

For the development of vaccines based on SARS-CoV-2-specific cytotoxic T lymphocytes (CTLs), we attempted to identify HLA-A*02:01-restricted CTL epitopes derived from the non-structural polyprotein 1a of SARS-CoV-2. Out of 82 peptides predicted on bioinformatics, 54 peptides showed good binding affinities to HLA-A*02:01. Using HLA-A*02:01 transgenic mice, 18 in 54 peptides were found to be CTL epitopes in the intracellular cytokine staining assay. Out of 18 peptides, 10 peptides were chosen for the following analyses because of their high responses. To identify dominant epitopes, mice were immunized with liposomes containing the mixture of the 10 peptides. Some peptides were shown to be statistically predominant. Surprisingly, all immunized mice did not show the same reaction pattern to the 10 peptides. There were three pattern types that varied sequentially, suggesting the existence of an immunodominance hierarchy, which may provide us more variations in the epitope selection for designing CTL-based COVID-19 vaccines.

Introduction

In December 2019, the coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was firstly identified in Wuhan, Hubei province, China. Since then, its subsequent spread of global infection has still continued to gain momentum. As of September 16th, 2020, the COVID-19 pandemic has infected more than 29.4 million people around the world and caused more than 931,000 deaths. Although the clinical symptom is varied from asymptomatic or mild self-limited infection to severe life-threatening respiratory disease, the mechanism of disease outcome remains unclear. Many nations are struggling to find appropriate preventive and control strategies. However, there are no vaccines or antiviral drugs available for the treatment of this infectious disease.

There are seven coronaviruses that infect humans. In addition to SARS-CoV-2, SARS-CoV and middle-east respiratory syndrome coronavirus (MERS-CoV) cause severe pneumonia, whereas the other four human coronaviruses including HCoV-229E, -NL63, -OC43 and -HKU1 cause common cold (1). Like other coronaviruses, SARS-CoV-2 possesses a large single-stranded positive sense RNA genome (2). As shown in Fig. 1, the 5'-terminal two-thirds of the genome are composed of ORF1a and ORF1b. ORF1a encodes the polyprotein 1a (pp1a) containing non-structural proteins (nsp1-11) (Fig. 1). The remaining one-third of the genome encodes the structural proteins involving spike (S), envelope (E), membrane (M), and nucleocapsid (N) as well as accessory proteins (Fig. 1). Coronaviruses depend on S protein for binding to host cells.

The host cell receptor for SARS-CoV-2 is the angiotensin-converting enzyme 2 (ACE2) which is also the receptor for SARS-CoV (3, 4).

It was shown that the increased cell numbers of antibody-secreting cells, follicular helper T cells, activated CD4⁺ T cells and CD8⁺ T cells were observed in a non-severe COVID-19 patient (5), indicating that robust immune responses can be elicited to the newly emerged, SARS-CoV-2. Therefore, the induction of protective immunity against SARS-CoV-2 is considered to help control COVID-19. Vaccines are being rapidly developed in the world, and human trials are underway for several vaccine candidates, ranging from traditional vaccines comprised of inactivated SARS-CoV-2 preparations (6) to innovative vaccines such as subunit (7), RNA (8), DNA (9), and adenoviral (10) vaccines. Almost all of these vaccines have been designed to induce antibodies targeting S protein because some antibodies specific for the receptor binding domain (RBD) of S protein may have neutralizing activities and can interfere with the binding between ACE2 on host cells and virus. In fact, it was reported that DNA vaccine encoding S protein induced neutralizing antibody in rhesus macaques and protected them from the challenge with SARS-CoV-2 (9). However, there are two major issues concerning this vaccine. One issue is that non-neutralizing and sub-neutralizing antibodies to S protein induced by this vaccine are at risk of causing antibody-dependent enhancement (ADE) (11). ADE is the phenomenon in which binding of suboptimal antibodies to viruses enhances viral entry mediated by Fc receptors into immune cells, and promotes inflammatory and tissue injury (12). ADE has been reported in the evaluation of vaccine candidates directed to S protein

for SARS-CoV (13-16). Because of their similarities, these findings enable us to foresee the high risks of ADE in SARS-CoV-2 vaccines directed to S protein. It is worth noting that RBD-specific antibodies with potent neutralizing activity are extremely rare among S-specific antibodies in COVID-19-convalescent individuals (17, 18), suggesting that the development of effective vaccines might require novel strategies to selectively target RBD of SARS-CoV-2. On the other hand, Passive immunization of RBD-specific monoclonal antibodies obtained from convalescent individuals might be safe and effective for the elimination of SARS-CoV-2 (19-21), but much more expensive to produce for worldwide use than vaccines (22). The other issue is the short longevity of neutralizing antibodies to SARS-CoV-2. It was previously reported that the SARS-CoV-specific antibodies are short-lived for at most about 2-3 years (23, 24) in comparison with SARS-CoV-specific memory T cells (25). The longevity of SARS-CoV-2-specific antibodies are likely to be even shorter, as indicated by antibody titers being undetectable or approaching baseline in the majority of SARS-CoV-2-infected individuals after 2-3 months post onset of symptoms (26). Taken together, these data strongly suggest that it does not seem right to rely too much on just the S-specific antibody-induced vaccine to control the COVID-19 pandemic.

In the viral infection, CD8⁺ cytotoxic T lymphocytes (CTLs) play an important role for the clearance of virus as well as neutralizing antibodies. CTLs recognize virus-derived peptides in association with major histocompatibility complex class I (MHC-I) molecules on the surface of antigen presenting cells and kill virus-infected target cells. It was

reported that CD4⁺ T cells and CD8⁺ T cells are decreased in proportion to the disease severity and are exhausted in severe COVID-19 patients (27, 28), suggesting the significance of CD8⁺ CTLs in the clearance of SARS-CoV-2. Furthermore, SARS-CoV-specific memory T cells persisted up to 11 years (25), predicting the long life of SARS-CoV-2-specific memory T cells. Therefore, in addition to antibody-induced vaccines, novel vaccines based on SARS-CoV-2-specific CTLs should also be considered in the future vaccine development for the prevention and disease control of COVID-19.

For the development of CTL-based COVID-19 vaccine, we here attempted to identify HLA-A*02:01-restricted, dominant CTL epitopes derived from pp1a of SARS-CoV-2. Pp1a is a largest protein composed of 4,401 amino acids among SARS-CoV-2 proteins, and therefore, it seems highly possible to find dominant epitopes in this protein. Furthermore, pp1a is produced first in SARS-CoV-2-infected cells, suggesting pp1a-specific CTLs could kill infected cells before the formation of mature virions. In addition, this protein is composed of 11 non-structural regulatory proteins that are highly conserved among many different coronaviruses (29). To identify CTL epitopes, we used computational algorithms, HLA-A*02:01 transgenic mice and the peptide-encapsulated liposomes. In a similar way, we previously identified CTL epitopes of SARS-CoV pp1a (30).

Results

Prediction of HLA-A*02:01-restricted CTL epitopes derived from SARS-CoV-2

pp1a.

We firstly attempted to predict HLA-A*02:01-restricted CTL epitopes derived from SARS-CoV-2 pp1a using four computer-based programs, SYFPEITHI (31), nHLAPred (32), ProPred-I (33), and IEDB (34). Eighty-two epitopes with high scores for all four programs were selected and synthesized into 9-mer peptides (Table 1). These peptides were then evaluated for their binding affinities to HLA-A*02:01 molecule using TAP2-deficient RMA-S-HHD cells. As the half-maximal binding level (BL₅₀) values of the influenza A virus matrix protein 1-derived peptide, FMP₅₈₋₆₆ (35) as a high binder control and the HIV reverse transcriptase-derived peptide, HIV-pol₄₇₆₋₄₈₄ (36) as a medium binder control were 2.3 μM and 80.6 μM, respectively, we defined a high binder with a BL₅₀ value below 10 μM, a medium binder with a BL₅₀ value ranging from 10 to 100 μM, and a low binder with a BL₅₀ value above 100 μM. As shown in Table 1, 20 peptides were high binders, whereas 34 peptides were medium binders, suggesting that the bioinformatics prediction was mostly successful. In contrast, the remaining 28 peptides displayed low affinities or no binding to HLA-A*02:01 molecules (Table 1). In the following experiments, 54 peptides involving high binders and medium binders were chosen to further investigate their abilities of peptide-specific CTL induction.

Detection of SARS-CoV-2 pp1a-specific CD8⁺ T cell responses in mice immunized with peptide-encapsulated liposomes.

Each of 54 peptides selected were encapsulated into liposomes as described in the materials and methods. HLA-A*02:01 transgenic (HHD) mice (37) were then subcutaneously (s.c.) immunized twice at a one-week interval with each of peptide-encapsulated liposomes together with CpG adjuvant. One week later, spleen cells of immunized mice were prepared, stimulated *in vitro* with a relevant peptide for 5 hours, and stained for their expression of cell-surface CD8 and intracellular interferon-gamma (IFN- γ). As shown in Fig. 2, the intracellular cytokine staining (ICS) assay showed that significant numbers of IFN- γ -producing CD8⁺ T cells were elicited in mice immunized with 18 liposomal peptides including pp1a-38, -52, -84, -103, -445, -597, -641, -1675, -2785, -2884, -3083, -3403, -3467, -3583, -3662, -3710, -3732, and -3886, revealing that these 18 peptides are HLA-A*02:01-restricted CTL epitopes derived from SARS-CoV-2 pp1a. As indicated in Table 2, multiple epitopes are located in small proteins such as nsp1 (180 aa) and nsp6 (290 aa), whereas only one epitope is seen in the large nsp3 composed of 1945 amino acids. On the other hand, the remaining 36 peptides out of 54 peptides in liposomes were not able to stimulate peptide-specific CTLs in mice (data not shown), demonstrating the necessity to generate data through wet-lab experiments. Interestingly, four epitopes including pp1a-103, -2884, -3403, and -3467 are located in the amino acid sequence of SARS-CoV pp1a as well (Table 2). pp1a-3467 was previously reported by us in the identification of SARS-CoV-derived CTL epitopes (30). However, any of 18

epitopes are not found in the amino acid sequence of either MERS-CoV or the four common cold human coronaviruses involving HCoV-OC43, HCoV-229E, HCoV-NL63, and HCoV-HKU1.

In the 18 positive peptides, 10 peptides including pp1a-38, -84, -641, -1675, -2884, -3467, -3583, -3662, -3710, and -3732 were selected for the following analyses because of the high ratios of IFN- γ ⁺ cells in CD8⁺ T cells (Fig. 2).

Identification of dominant CTL epitopes.

To confirm that the 10 peptides are effective epitopes for peptide-specific CTL responses, we examined whether peptide-specific killing activities were elicited in mice with each of 10 peptides in liposomes. HHD mice were immunized s.c. twice with each of peptide-encapsulated liposomes and CpG adjuvant. One week later, equal numbers of peptide-pulsed and -unpulsed target cells were transferred into immunized mice via i.v. injection, and peptide-specific lysis was analyzed by flow cytometry. In support of the data of ICS (Fig. 2), peptide-specific killing was observed in mice immunized with any of 10 liposomal peptides (Fig. 3A).

We next attempted to identify dominant CTL epitopes among the 10 CTL epitopes. The same amounts of the 10 peptide solutions at an equal concentration were mixed together and encapsulated into liposomes. Seventeen mice were immunized once with the liposomes containing the mixture of 10 peptides. One week later, spleen cells were incubated with each of the 10 peptides for 5 hours, and the ICS assay was performed. As

shown in Fig. 3B & C, pp1a-38 and -84 were statistically predominant over almost all other peptides in the induction of peptide-specific IFN- γ ⁺ CD8⁺ T cells. Furthermore, pp1a-641 and pp1a-3732 were significantly superior to pp1a-1675/-3583 and pp1a-1675/-3583/-3662 in the stimulation of IFN- γ -producing CD8⁺ T cells, respectively (Fig. 3B).

We also examined the peptide-specific induction of CD107a⁺ CD8⁺ T cells and CD69⁺ CD8⁺ T cells. CD107a and CD69 are markers of degranulation and early activation on CD8⁺ T lymphocytes, respectively. Nine mice were immunized once with the liposomes encapsulating the mixture of the 10 peptides. After one week, spleen cells were stimulated with each peptide, and stained for their expression of CD107a or CD69 of CD8⁺ T cells. At first glance, the graphs of CD107a (Fig. 4A) and CD69 (Fig. 4B) expression were similar to that of IFN- γ expression of CD8⁺ T cells (Fig. 3C). As shown in Figs. 4A & 4C, both pp1a-38 and pp1a-84 were superior to almost all other peptides for the CD107a expression of CD8⁺ T cells. Moreover, pp1a-641 and pp1a-3732 elicited CD107a-positive T cells better than pp1a-1675/-2884/-3467 and pp1a-1675/-2884/-3467/-3583, respectively. In the case of CD69 expression (Figs. 4B & 4D), pp1a-38 and pp1a-3732 were more dominant than pp1a-1675/-2884/-3467/-3583 and pp1a-1675/-2884/-3467/-3583/-3662, respectively. Further, pp1a-84 was superior to pp1a-1675/-2884.

Taken together, 10 peptides differed significantly in their ability to induce SARS-CoV-2 pp1a-specific CTLs when mice were immunized with the mixture of 10

peptides in liposomes. Thus, some peptides were found to be dominant CTL epitopes although each peptide alone of the 10 peptides has the capability to efficiently activate peptide-specific CTL (Figs. 2 & 3A).

Existence of an immunodominance hierarchy.

The data in Fig. 5 indicate reactivity to the 10 peptides in each of 15 mice immunized with the mixture of the 10 peptides in liposomes. Each graph represents reactivity of an individual mouse (Fig. 5). Unexpectedly, all mice did not show the same reaction pattern against the 10 peptides. It looks like there were roughly three types that varied sequentially in terms of the reaction pattern to the 10 peptides. Type A is a group of mice in which pp1a-38, -84, -641-specific IFN- γ^+ CD8 $^+$ T cells were predominantly elicited, whereas the remaining 7 peptides were not able to activate peptide-specific IFN- γ^+ CD8 $^+$ T cells. In the case of type B, pp1a-3732 stimulated peptide-specific IFN- γ^+ CD8 $^+$ T cells as well as pp1a-38, and -84. In addition to these three peptides, several other peptides also induced IFN- γ^+ CD8 $^+$ T cells in Type C. These data suggest that there seems to be an immunodominance hierarchy composed of three stages in CD8 $^+$ T cell responses to the 10 peptides. The immunodominance hierarchy may provide us more variations for designing CTL-based COVID-19 vaccines.

Discussion

After the epidemics of SARS and MERS, scientists have not succeeded yet in developing preventive or therapeutic vaccines available for re-emergence of them. In the SARS and MERS vaccine development, the full-length S protein or its S1 subunit have frequently been used as an antigen to produce anti-RBD neutralizing antibodies. However, these vaccine candidates provided partial protection against virus challenge in animal models accompanied by safety concerns such as ADE (1). Furthermore, antibody responses to coronaviruses rapidly wane following infection or immunization (23, 24, 26). Considering the above, it should be necessary to consider CTL-based vaccine against SARS-CoV-2 to provide robust long-lived T cell memory although neutralizing antibody responses are a primary vaccine target.

In the current study, we aimed to find HLA-A*02:01-restricted CTL dominant epitopes derived from SARS-CoV-2. Dominant epitopes induce strong immune response to eliminate a certain pathogen fast and effectively, and also contribute to make the memory T cell pool. We focused on pp1a of SARS-CoV-2 to find out CTL epitopes because pp1a is a largest and conserved polyprotein among the constituent proteins. Further, pp1a is produced earlier than structural proteins, suggesting that pp1a-specific CTLs can eliminate infected cells before the formation of mature virions. To predict CTL epitopes, we utilized bioinformatics to select 82 peptides with high scores in four kinds of computer-based programs (Table 1). In the evaluation of peptide binding, 54 peptides showed high or medium binding affinities to HLA-A*02:01 molecules, whereas the

remaining 28 peptides displayed low binding affinities or no binding (Table 1). Out of them, only eighteen peptides were found to be CTL epitopes. Hence, we have to keep in mind that currently available algorithms have a limited ability to accurately predict CTL epitopes although the bioinformatics approach is very useful to quickly predict a number of epitopes in a large protein (38, 39).

Among 18 epitopes which we have identified in the current study, 4 epitopes including pp1a-103, -2884, -3403, and -3467 are present in the amino acid sequence of SARS-CoV pp1a (100% identity) (Table 2). Therefore, CTLs induced by these four epitopes could work fine for the clearance of SARS-CoV as well. In support of this data, Le Bert et al., reported that long lasting memory T cells in SARS-recovered individuals cross-reacted to N protein of SARS-CoV-2 (40). Recently, several studies found that SARS-CoV-2-reactive CD4⁺ and CD8⁺ T cells were detected in a substantial proportion of healthy donors who have never infected with SARS-CoV-2 or SARS-CoV (40-44). It is most likely that these individuals were previously infected with one of the four human coronaviruses (HCoV-229E, -NL63, -OC43 and -HKU1) that cause seasonal common cold. Nelde et al. demonstrated evidence that the amino acid sequences of several SARS-CoV-2 T cell epitopes recognized by unexposed individuals are similar to some amino acid sequences in the four seasonal common cold human coronaviruses with identities ranging from 10% to 89% (not 100% identity) (44). However, anyone has not shown evidence that people with this cross-reactivity are less susceptible to COVID-19. It may be also possible to assume that pre-existing T cell immunity might be detrimental

through mechanisms such as original antigenic sin or ADE (45). In the current data, any of the 18 epitopes was not found in the amino acid sequences of the four human coronaviruses, suggesting that effective common CTL epitopes derived from pp1a, if any, might be very few.

Here, we focused on CTL epitopes restricted by HLA-A*02:01 which is the most common HLA class I allele in the world, and used highly reactive HLA-A*02:01 transgenic mice, termed HHD mice (37). Although we can use lymphocytes of SARS-CoV-2-infected individuals to identify CTL epitopes, there are mainly two advantages to using HHD mice. First, a large amount of blood of patients is required for examine many candidates of CTL epitopes, but any number of mice can be prepared for this purpose. Second, when using patients' lymphocytes, we are only testing whether the peptide candidates are recognized by memory CTLs. When using naïve mice, however, we can find whether the epitope candidates are able to prime peptide-specific CTLs, which may be a better criterion to judge them as vaccine antigens. It is supposed that the efficient epitope for CTL recognition is not always efficient for CTL priming. However, we should take into account that the immunogenic variation in HLA class I transgenic mice may not be identical to that in humans because the antigen processing and presentation differ between them.

In the previous studies, we used peptide-linked liposomes as an immunogen (30). The surface-linked liposomal peptides were effective for peptide-specific CTL induction in mice. However, attaching peptides to the surface of liposomes followed by purifying

them through the column is a fairly complicated process and time-consuming. In the current study, peptide-encapsulated liposomes were used as an immunogen. In contrast to the peptide-linked liposomes, the peptide-encapsulated liposomes are prepared by just mixing liposomes and the peptide. In addition, the peptide-encapsulated liposomes are able to prime peptide-specific CTLs in mice as efficiently as the peptide-linked liposomes. Liposome itself consisting of lipid bilayers is a very safe material for humans. Therefore, the peptide-encapsulated liposomes are considered to be promising as a CTL-based vaccine candidate.

Understanding the mechanism of immunodominance is obviously important for the development of effective vaccines. When mice were immunized with liposomes containing the mixture of 10 peptides, it was found that some peptides induced peptide-specific CTLs stronger than other peptides (Figs. 3 & 4). As shown in Figs. 3 & 4, pp1a-38 and -84 are considered to be relatively dominant in comparison with other peptides. In general, dominant epitope-specific CTLs are activated sooner and proliferate faster than subdominant epitope-specific CTLs. This immunodominance may be associated with the affinity of peptide to MHC-I molecules and the affinity of T-cell receptor (TCR) to the peptide-MHC-I complex. As shown in Table I, the peptide affinity of pp1a-84 to HLA-A*02:01 is very high ($BL_{50} = 6.8 \mu M$), while pp1a-38 is a medium binder ($BL_{50} = 76.8 \mu M$). Interestingly, the peptide affinity of pp1a-38 is lowest among the 10 peptides selected (Table I). These data suggest that the affinity of TCR to the peptide-MHC-I complex is critical for CTL immunodominance. In the selection of

antigenic epitopes for the CTL-based vaccine against SARS-CoV-2, dominant epitopes such as pp1a-38 and -84 should be chosen because they produce strong CTL response to eliminate virus-infected cells effectively. However, the immunological pressure exerted by dominant epitopes may allow the epitope sequences of SARS-CoV-2 to be mutated, and therefore, a vaccine containing multiple antigenic epitopes should be recommended for a successful COVID-19 vaccine.

It was surprising that all of the genetically identical mice did not show the same reactive pattern against the 10 peptides when they were immunized with liposomes containing the mixture of 10 peptides (Fig. 5). There were roughly three pattern types, A-C, that varied sequentially, suggesting the existence of an immunodominance hierarchy composed of three stages in CD8⁺ T cell responses to the 10 peptides (Fig. 5). The differences among the three types might be explained by the timing of CTL expansion. In the type A, dominant peptides, pp1a-38, -84, and -641 presumably activated T cells more efficiently than the other peptides, and hence, dominant peptide-specific CTLs proliferate faster and curtail the expansion of CTLs specific for the other peptides. In the type B, it is considered that the expansion of dominant CTLs specific for pp1a-38, and -84 was delayed for some reason compared to that in type A, and thereby subdominant CTLs specific for pp1a-3732 could afford to expand. It is also thought that even non-dominant CTLs proliferated because the expansion of both dominant CTLs and subdominant CTLs in the type C was later than that in the type B. Although it is difficult to explain what caused difference in the timing of CTL expansion

among three reaction types, it should be important to find out what it is for the development of CTL-based peptide vaccine.

In summary, we have identified 18 kinds of HLA-A*02:01-restricted CTL epitopes derived from pp1a of SARS-CoV-2 using computational algorithms, HLA-A*02:01 transgenic mice and the peptide-encapsulated liposomes. Out of 18 epitopes, we have also found some dominant CTL epitopes such as pp1a-38 and -84. In the process of finding dominant epitopes, we showed the existence of an immunodominance hierarchy in CD8⁺ T cell responses to these epitopes. The immunodominance hierarchy composed of multiple stages may offer us more variations in the epitope selection for designing CTL-based COVID-19 vaccines. These data may provide important information for further studies of T cell immunity in COVID-19 and the development of preventive and/or therapeutic CTL-based vaccines against SARS-CoV-2.

Materials and Methods

Prediction of CTL epitopes.

Four computer-based programs including SYFPEITHI (31), nHLAPred (32), ProPred-I (33), and IEDB (34) were used to predict HLA-A*02:01-restricted CTL epitopes derived from pp1a of SARS-CoV-2 (GenBank accession numbers: LC528232.1 & LC528233.1). As shown in Table 1, 82 peptides with superior scores were selected and were synthesized by Eurofins Genomics (Tokyo, Japan). Two control peptides, FMP₅₈₋₆₆ (sequence: GILFGVFTL) (35) and HIV-pol₄₇₆₋₄₈₄ (sequence: ILKEPVHGV) (36), were synthesized as well.

Mice.

We used HLA-A*02:01 transgenic mice (37) that express a transgenic HLA-A*02:01 monochain, designated as HHD, in which human β 2-microglobulin is covalently linked to a chimeric heavy chain composed of HLA-A*02:01 (α 1 and α 2 domains) and H-2D^b (α 3, transmembrane, and cytoplasmic domains). Six- to ten-week-old mice were used for all experiments. Mice were housed in appropriate animal care facilities at Saitama Medical University, and were handled according to the international guideline for experiments with animals. This study was approved by the Animal Research Committee of Saitama Medical University.

Cell lines.

RMA-S-HHD is a TAP2-deficient mouse lymphoma cell line, RMA-S (H-2^b) transfected with the HHD gene (37). RMA-S-HHD was cultured in RPMI-1640 medium (Nacalai Tesque Inc., Kyoto, Japan) with 10% FCS (Biowest, Nuaille, France) and 500 µg/ml G418 (Nacalai Tesque Inc.)

Peptide binding assay.

Binding of peptides to the HLA-A*02:01 molecule was measured using RMA-S-HHD cells, as described (46). Briefly, RMA-S-HHD cells were pre-cultured overnight at 26°C in a CO₂ incubator, and then pulsed with each peptide at various concentrations ranging from 0.01 µM to 100 µM for 1 hour at 26°C. After 3 hours' incubation at 37°C, peptide-pulsed cells were stained with anti-HLA-A2 monoclonal antibody, BB7.2 (47), followed by FITC-labeled goat anti-mouse IgG antibody (Sigma-Aldrich, St. Louis, MO). Mean fluorescence intensity (MFI) of HLA-A2 expression on the surface of RMA-S-HHD cells was measured by flow cytometry (FACSCanto™ II, BD Biosciences, Franklin Lakes, NJ), and standardized as the percent cell surface expression by the following formula: % relative binding = [(MFI of cells pulsed with each peptide) – (MFI of cells incubated at 37°C without a peptide)] / [(MFI of cells incubated at 26°C without a peptide) – (MFI of cells incubated at 37°C without a peptide)] × 100. The concentration of each peptide that yields the 50% relative binding was calculated as the half-maximal binding level (BL₅₀). FMP₅₈₋₆₆ and HIV-pol₄₇₆₋₄₈₄ were used as control peptides.

Peptide-encapsulated liposomes.

Peptide-encapsulated liposomes were prepared using Lipocapsulater FD-U PL (Hygieia BioScience, Osaka, Japan) according to the manufacturer's instructions with a slight modification. In brief, each of 54 synthetic peptides was dissolved in DMSO at a final concentration of 10 mM. For the first screening, the same amounts of 4 to 5 kinds of 10 mM peptides were mixed to make a total 100 μ l, which was then diluted with 1.9 ml of H₂O. For the second screening, 20 μ l of each peptide at 10 mM was diluted to 2 ml with H₂O. For the identification of dominant epitopes among the 10 peptides selected, 20 μ l of each of the 10 peptide solutions at a concentration of 10 mM was mixed together, and the total volume was increased to 2 ml by adding 1.8 ml of H₂O. The peptide solution was added into a vial of Lipocapsulater containing 10 mg of dried liposomes, and incubated for 15 min at room temperature. The resultant solution contains peptide-encapsulated liposomes.

Immunization.

Mice were immunized s.c. once or twice at a one-week interval with peptide-encapsulated liposomes (100 μ l/mouse) together with CpG-ODN (5002: 5'-TCCATGACGTTCTTGATGTT-3', Hokkaido System Science, Sapporo, Japan) (5 μ g/mouse) in the footpad.

Intracellular cytokine staining (ICS).

ICS was performed as described (30). In brief, after 1 wk following immunization, spleen cells were incubated with 50 μ M of each peptide for 5 hours at 37°C in the presence of brefeldin A (GolgiPlugTM, BD Biosciences), and were stained with FITC-conjugated anti-mouse CD8 monoclonal antibody (mAb) (BioLegend, San Diego, CA). Cells were then fixed, permeabilized, and stained with phycoerythrin (PE)-conjugated rat anti-mouse IFN- γ mAb (BD Biosciences). After washing the cells, flow cytometric analyses were performed using flow cytometry (FACSCantoTM II, BD Biosciences).

Detection of CD107a and CD69 molecules on CD8⁺ T cells.

For the detection of CD107a, spleen cells of immunized mice were incubated with 50 μ M of each peptide for 6 hours at 37°C in the presence of monensin (GolgiStopTM, BD Biosciences) and 0.8 μ g of FITC-conjugated anti-mouse CD107a mAb (BioLegend). Cells were then stained with PE-Cy5-conjugated anti-mouse CD8 mAb (BioLegend). For the examination of CD69 marker, spleen cells of immunized mice were stimulated with 50 μ M of each peptide for 4 hours at 37°C. Cells were then stained with PE-conjugated anti-mouse CD69 mAb (BioLegend) and FITC-conjugated anti-mouse CD8 mAb. Stained cells were analyzed by flow cytometry (FACSCantoTM II, BD Biosciences).

***In vivo* CTL assay.**

In vivo CTL assay was carried out as described (46). In brief, spleen cells from naive HHD mice were equally split into two populations. One population was pulsed with 50 μ M of a relevant peptide and labeled with a high concentration (2.5 μ M) of carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR). The other population was unpulsed and labeled with a lower concentration (0.25 μ M) of CFSE. An equal number (1×10^7) of cells from each population was mixed together and adoptively transferred i.v. into mice that had been immunized once with a liposomal peptide. Sixteen hours later, spleen cells were prepared and analyzed by flow cytometry. To calculate specific lysis, the following formula was used: % specific lysis = $[1 - \{(\text{number of CFSE}^{\text{low}}$ cells in normal mice)/(\text{number of CFSE}^{\text{high}} cells in normal mice)}/\{(\text{number of CFSE}^{\text{low}} cells in immunized mice)/(\text{number of CFSE}^{\text{high}} cells in immunized mice)}] \times 100.

Statistical analyses.

One-way ANOVA followed by post-hoc tests was performed for statistical analyses among multiple groups using Graphpad Prism 5 software (GraphPad software, San Diego, CA). A value of $P < 0.05$ was considered statistically significant.

ACKNOWLEDGMENTS

The authors are grateful to Professor François A. Lemonnier (Pasteur Institute, Paris, France) for providing HHD mice and RMA-S-HHD cells. This work was supported by a Grant-in-Aid for Scientific Research (C) (JSPS KAKENHI Grant Number: JP18K06631) to M. M., a Grant-in-Aid for Early-Career Scientists (JSPS KAKENHI Grant Number: JP18K15430) to A. T. from Japan Society for the Promotion of Science, and a Grant from Ochiai memorial award 2018 to A. T. The authors have no conflicting financial interests.

References

1. Sariol A, Perlman S. 2020. Lessons for COVID-19 immunity from other coronavirus infections. *Immunity* 53:248-263.
2. Srinivasan S, Cui H, Gao Z, Liu M, Lu S, Mkandawire W, Narykov O, Sun M, Korkin D. 2020. Structural genomics of SARS-CoV-2 indicates evolutionary conserved functional regions of viral proteins. *Viruses* 12:360. <https://doi.org/10.3390/v12040360>.
3. Walls AC, Park YJ, Tortorici MA, Wall A, McGuire AT, Velesler D. 2020. Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein *Cell* 181:281-292.
4. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. 2020. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell* 181:271-280.
5. Thevarajan I, Nguyen THO, Koutsakos M, Druce J, Caly L, van de Sandt CE, Jia X, Nicholson S, Catton M, Cowie B, Tong SYC, Lewin SR, Kedzierska K. 2020. Breadth of concomitant immune responses prior to patient recovery: a case report of non-severe COVID-19. *Nat Med* 26:453-455.
6. Gao, Q, Bao L, Mao H, Wang L, Xu K, Yang M, Li Y, Zhu L, Wang N, Lv Z, Gao H, Ge X, Kan B, Hu Y, Jiangning Liu J, Cai F, Jiang D, Yin Y, Qin C, Li J, Gong X, Lou X, Shi W, Wu D, Zhang H, Zhu L, Deng W, Li Y, Lu J, Li C, Wang X, Yin W,

499 Zhang Y, Qin C. 2020. Development of an inactivated vaccine candidate for
500 SARS-CoV-2. *Science* 369:77-81.

501 7. Guebre-Xabier M, Patel N, Tian J-H, Zhou B, Maciejewski S, Lam K, Portnoff AD,
502 Massare MJ, Frieman MB, Piedra PA, Ellingsworth L, Glenn G, Gale Smith G.
503 2020. NVX-CoV2373 vaccine protects cynomolgus macaque upper and lower
504 airways against SARS-CoV-2 challenge. *bioRxiv*
505 <https://doi.org/10.1101/2020.08.18.256578>.

506 8. Walsh EE, Frenck R, Falsey AR, Kitchin N, Absalon J, Gurtman A, Lockhart S,
507 Neuzil K, Mulligan MJ, Bailey R, Swanson KA, Li P, Koury K, Kalina W, Cooper
508 D, Fontes-Garfias C, Shi P-Y, Türeci Z, Tompkins KR, Lyke KE, Raabe V,
509 Dormitzer PR, Jansen KU, Şahin U, Gruber WC. 2020. RNA-based COVID-19
510 vaccine BNT162b2 selected for a pivotal efficacy study. *medRxiv*
511 <https://doi.org/10.1101/2020.08.17.20176651>.

512 9. Yu J, Tostanoski LH, Peter L, Mercado NB, McMahan K, Mahrokhian SH, Nkolola
513 JP, Liu J, Li Z, Chandrashekar A, Martinez DR, Loos C, Atyeo C, Fischinger S,
514 Burke JS, Slein MD, Chen Y, Zuiani A, Lelis FJN, Travers M, Habibi S, Pessaint L,
515 Ry AV, Blade K, Brown R, Cook A, Finneyfrock B, Dodson A, Teow E, Velasco J,
516 Zahn R, Wegmann F, Bondzie EA, Dagotto G, Gebre MS, He X, Jacob-Dolan C,
517 Kirilova M, Kordana N, Lin Z, Maxfield LF, Nampanya F, Nityanandam R, Ventura
518 JD, Wan H, Cai Y, Chen B, Schmidt AG, Wesemann DR, Baric RS, Alter G,

519 Andersen H, Lewis MG, Barouch DH. 2020. DNA vaccine protection against
520 SARS-CoV-2 in rhesus macaques. *Science* 369:806-811.

521 10. Hassan AO, Kafai NM, Dmitriev IP, Fox JM, Smith BK, Harvey IB, Chen RE,
522 Winkler ES, Wessel AW, Case JB, Kashentseva E, McCune BT, Bailey AL, Zhao
523 H, VanBlargan LA, Dai Y-N, Ma M, Adams LJ, Shrihari S, Danis JE, Gralinski LE,
524 Hou YJ, Schäfer A, Kim AS, Keeler SP, Weiskopf D, Baric RS, Holtzman MJ,
525 Fremont DH, Curiel DT, Diamond MS. 14 August 2020. A single-dose intranasal
526 ChAd vaccine protects upper and lower respiratory tracts against SARS-CoV-2.
527 *Cell* <https://doi.org/10.1016/j.cell.2020.08.026>

528 11. Iwasaki A, Yang Y. 2020. The potential danger of suboptimal antibody responses in
529 COVID-19. *Nat Rev Immunol* 20:339-341.

530 12. Arvin AM, Fink K, Schmid MA, Cathcart A, Spreafico R, Havenar-Daughton C,
531 Lanzavecchia A, Corti D, Virgin HW. 2020. A perspective on potential
532 antibody-dependent enhancement of SARS-CoV-2. *Nature* 584:353-363.

533 13. Weingart H, Czub M, Czub S, Neufeld J, Marszal P, Gren J, Smith G, Jones S,
534 Proulx R, Deschambault Y, Grudeski E, Andonov A, He R, Li Y, Copps J, Grolla A,
535 Dick D, Berry J, Ganske S, Manning L, Cao J. 2004. Immunization with modified
536 vaccinia virus Ankara-based recombinant vaccine against severe acute respiratory
537 syndrome is associated with enhanced hepatitis in ferrets. *J Virol* 78:12672-12676.

538 14. Tseng CT, Sbrana E, Iwata-Yoshikawa N, Newman PC, Garron T, Atmar RL, Peters
539 CJ, Couch RB. 2012. Immunization with SARS coronavirus vaccines leads to

540 pulmonary immunopathology on challenge with the SARS virus. PLoS One
541 7:e35421.

542 15. Wang SF, Tseng SP, Yen CH, Yang JY, Tsao CH, Shen CW, Chen KH, Liu FT, Liu
543 WT, Chen YM, Huang JC. 2014. Antibody-dependent SARS coronavirus infection
544 is mediated by antibodies against spike proteins. Biochem Biophys Res Commun
545 451:208-214.

546 16. Liu L, Wei Q, Lin Q, Fang J, Wang H, Kwok H, Tang H, Nishiura K, Peng J, Tan Z,
547 Wu T, Cheung K-W, Chan K-H, Alvarez X, Qin C, Lackner A, Perlman S, Yuen
548 K-Y, Chen Z. 2019. Anti-spike IgG causes severe acute lung injury by skewing
549 macrophage responses during acute SARS-CoV infection. JCI Insight 4:e123158.

550 17. Robbiani DF, Gaebler C, Muecksch F, Lorenzi JCC, Wang Z, Cho A, Agudelo M,
551 Barnes CO, Gazumyan A, Finkin S, Hägglöf T, Oliveira TY, Viant C, Hurley A,
552 Hoffmann HH, Millard KG, Kost RG, Cipolla M, Gordon K, Bianchini F, Chen ST,
553 Ramos V, Patel R, Dizon J, Shimeliovich I, Mendoza P, Hartweger H, Nogueira L,
554 Pack M, Horowitz J, Schmidt F, Weisblum Y, Michailidis E, Ashbrook AW, Waltari
555 E, Pak JE, Huey-Tubman KE, Koranda N, Hoffman PR, West AP Jr, Rice CM,
556 Hatziioannou T, Bjorkman PJ, Bieniasz PD, Caskey M, Nussenzweig MC. 2020.
557 Convergent antibody responses to SARS-CoV-2 in convalescent individuals. Nature
558 584:437-442.

559 18. Juno JA, Tan H-X, Lee WS, Reynaldi A, Kelly HG, Wragg K, Esterbauer R, Kent
560 HE, Batten J, Mordant FL, Gherardin NA, Pymm P, Dietrich MH, Scott NE, Tham

W-H, Godfrey DI, Subbarao K, Davenport MP, Kent SJ, Wheatley AK. 13 July
2020. Humoral and circulating follicular helper T cell responses in recovered
patients with COVID-19. <https://doi.org/10.1038/s41591-020-0995-0>.

19. Pinto D, Park Y-J, Beltramello M, Walls AC, Tortorici A, Bianchi S, Jaconi S,
Culap K, Zatta F, De Marco, A, Peter A, Guarino B, Spreafico R, Cameroni E, Case
JB, Chen RE, Havenar-Daughton C, Snell G, Telenti A, Virgin HW, Lanzavecchia
A, Diamond MS, Fink K, Veisler D, Corti D. 2020. Cross-neutralization of
SARS-CoV-2 by a human monoclonal SARS-CoV antibody. *Nature* 583:290-295.

20. Liu L, Wang P, Nair MS, Yu J, Rapp M, Wang Q, Luo Y, Chan JF-W, Sahi V,
Figuerola A, Guo XV, Cerutti G, Bimela J, Gorman J, Zhou T, Chen Z, Yuen K-W,
Kwong PD, Sodroski JG, Yin MT, Sheng Z, Huang Y, Shapiro L, Ho DD. 2020.
Potent neutralizing antibodies against multiple epitopes on SARS-CoV-2 spike.
Nature 584: 450-456.

21. Zost SJ, Gilchuk P, Case JB, Binshtein E, Chen RE, Nkolola JP, Schäfer A, Reidy
JX, Trivette A, Nargi RS, Sutton RE, Suryadevara N, Martinez DR, Williamson LE,
Chen EC, Jones T, Day S, Myers L, Hassan AO, Kafai NM, Winkler ES, Fox JM,
Shrihari S, Mueller BK, Meiler J, Chandrashekar A, Mercado NB, Steinhardt JJ,
Ren K, Loo Y-M, Kallewaard NL, McCune BT, Keeler SP, Holtzman MJ, Barouch
DH, Gralinski LE, Baric RS, Thackray LB, Diamond MS, Carnahan RH, Crowe Jr
JE. 2020. Potently neutralizing and protective human antibodies against
SARS-CoV-2. *Nature* 584: 443-449.

- 582 22. Ledford H. 2020. Antibody therapies could be a bridge to a coronavirus vaccine-but
583 will the world benefit? *Nature* 584:333-334.
- 584 23. Cao W-C, Liu W, Zhang P-H, Zhang F, Richardus JH. 2007. Disappearance of
585 antibodies to SARS-associated coronavirus after recovery. *N Engl J Med*
586 357:1162-1163.
- 587 24. Mo H, Zeng G, Ren X, Li H, Ke C, Tan Y, Cai C, Lai K, Chen R, Chan-Yeung M,
588 Zhong N. 2006. Longitudinal profile of antibodies against SARS-coronavirus in
589 SARS patients and their clinical significance. *Respirology* 11:49-53.
- 590 25. Ng OW, Chia A, Tan AT, Jadi RS, Leong HN, Bertoletti A, Tan YJ. 2016. Memory
591 T cell responses targeting the SARS coronavirus persist up to 11 years
592 post-infection. *Vaccine* 34:2008-2014.
- 593 26. Seow J, Graham C, Merrick B, Acors S, Steel KJA, Hemmings O, O'Bryne A,
594 Kouphou N, Pickering S, Galao RP, Betancor G, Wilson HD, Signell AW, Winstone
595 H, Kerridge C, Temperton N, Snell L, Bisnauthsing K, Moore A, Green A, Martinez
596 L, 6 , Stokes B, Honey J, Izquierdo-Barras A, Arbane G, Patel A, O'Connell L,
597 O'Hara G, MacMahon E, Douthwaite S, Nebbia G, Batra R, Martinez-Nunez R,
598 Edgeworth JD, Neil SJD, Malim MH, Doores KJ. 2020. Logitudinal evaluation and
599 decline of antibody responses in SARS-CoV-2 infection. *medRxiv*
600 <https://doi.org/10.1101/2020.07.09.20148429>.
- 601 27. Diao B, Wang C, Tan Y, Chen X, Liu Y, Ning L, Chen L, Li M, Liu Y, Wang G,
602 Yuan Z, Feng Z, Zhang Y, Wu Y, Chen Y. 2020. Reduction and functional

- exhaustion of T cells in patients with coronavirus disease 2019 (COVID-19). *Front immunol* 11:1729. <https://doi.org/10.3389/fimmu.2020.01729>.
28. Lucas C, Wong P, Klein J, Castro TBR, Silva J, Sundaram M, Ellingson MK, Mao T, Oh JE, Israelow B, Takahashi T, Tokuyama M, Lu P, Venkataraman A, Park A, Mohanty S, Wang H, Wyllie AL, Vogels CBF, Earnest R, Lapidus S, Ott IM, Moore AJ, Muenker MC, Fournier JB, Campbell M, Odio CD, Casanovas-Massana A; Yale IMPACT Team, Herbst R, Shaw AC, Medzhitov R, Schulz WL, Grubaugh ND, Dela Cruz C, Farhadian S, Ko AI, Omer SB, Iwasaki A. 2020. Longitudinal analyses reveal immunological misfiring in severe COVID-19. *Nature* 584: 463-469.
29. Cui J, Li F, Shi ZL. Origin and evolution of pathogenic coronaviruses. 2019. *Nat Rev Microbiol* 17:181-192.
30. Kohyama S, Ohno S, Tatsuya Suda T, Taneichi M, Yokoyama S, Mori M, Kobayashi A, Hayashi H, Tetsuya Uchida T, Matsui M. 2009. Efficient induction of cytotoxic T lymphocytes specific for severe acute respiratory syndrome (SARS)-associated coronavirus by immunization with surface-linked liposomal peptides derived from a non-structural polyprotein 1a. *Antiviral Res* 84:168-177.
31. Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanović S. 1999. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* 50:213-219.
32. Bhasin M, Raghava GPS. 2006. A hybrid approach for predicting promiscuous MHC class I restricted T cell epitopes. *J Biosci* 32:31-42.

33. Singh H, Raghava GPS. 2003. ProPred1: Prediction of promiscuous MHC class-I binding sites. *Bioinformatics* 19:1009-1014.
34. Vita R, Mahajan S, Overton JA, Dhanda SK, Martini S, Cantrell JR, Wheeler DK, Sette A, Peters B. 2019. The immune epitope database (IEDB): 2018 update. *Nucleic Acids Res* 47(D1):D339-D343.
<https://doi.org/10.1093/nar/gky1006>.
35. Gotch F, Rothbard J, Howland K, Townsend A, McMichael A. 1987. Cytotoxic T lymphocytes recognize a fragment of influenza virus matrix protein in association with HLA-A2. *Nature* 326:881-882.
36. Tsomides TJ, Walker BD, Eisen HN. 1991. An optimal viral peptide recognized by CD8⁺ T cells binds very tightly to the restricting class I major histocompatibility complex protein on intact cells but not to the purified class I protein. *Proc Natl Acad Sci U S A* 88:11276-11280.
37. Pascolo S, Bervas N, Ure JM, Smith AG, Lemonnier FA, Pérarnau B. 1997. HLA-A2.1-restricted education and cytolytic activity of CD8⁺ T lymphocytes from beta2 microglobulin (beta2m) HLA-A2.1 monochain transgenic H-2D^b beta2m double knockout mice. *J Exp Med* 185:2043-2051.
38. Grifoni A, Sidney J, Zhang Y, Scheuermann RH, Peters B, Sette A. 2020. A sequence homology and bioinformatics approach can predict candidate targets for immune responses to SARS-CoV-2. *Cell Host & Microbe* 27:671-680, 2020.

39. Ong E, Wong MU, Huffman A, He Y. COVID-19 coronavirus vaccine design using reverse vaccinology and machine learning. *Front Immunol* 11:1581. <https://doi.org/10.3389/fimmu.2020.01581>.
40. Le Bert N, Tan AT, Kunasegaran K, Tham CYL, Hafezi M, Chia A, Chng MHY, Lin M, Tan N, Linster M, Chia WN, Chen MI, Wang LF, Ooi EE, Kalimuddin S, Tambyah PA, Low JG, Tan YJ, Bertoletti A. 2020. SARS-CoV-2-specific T cell immunity in cases of COVID-19 and SARS, and uninfected controls. *Nature* 584:457-462.
41. Mateus J, Grifoni A, Tarke A, John Sidney J, Ramirez SI, Dan JM, Burger ZC, Rawlings SA, Smith DM, Phillips E, Mallal S, Lammers M, Rubiro P, Quiambao L, Sutherland A, Yu ED, Antunes RS, Greenbaum J, Frazier A, Markmann AJ, Premkumar L, de Silva A, Peters B, Crotty S, Sette A, Weiskopf D. 2020. Selective and cross-reactive SARS-CoV-2 T cell epitopes in unexposed humans. *Science* 4 August 2020. eabd3871. <https://doi.org/10.1126/science.abd3871>.
42. Grifoni A, Weiskopf D, Ramirez SI, Mateus J, Dan JM, Moderbacher CR, Rawlings SA, Sutherland A, Premkumar L, Jadi RS, Marrama D, de Silva AM, Frazier A, Carlin AF, Greenbaum JA, Peters B, Krammer F, Smith DM, Crotty S, 1,2,5, Sette A. 2020. Targets of T cell responses to SARS-CoV-2 coronavirus in humans with COVID-19 disease and unexposed individuals. *Cell* 181:1489-1501.
43. Braun J, Loyal L, Frentsch M, Wendisch D, Georg P, Kurth F, Hippenstiel S, Dingeldey M, Kruse B, Fauchere F, Baysal E, Mangold M, Henze L, Lauster R,

665 Mall MA, Beyer K, Röhm J, Voigt S, Schmitz J, Miltenyi S, Demuth I, Müller
666 MA, Hocke A, Witznath M, Suttrop N, Kern F, Reimer U, Wenschuh H, Drosten
667 C, Corman VM, Giesecke-Thiel C, Sander LE, Thiel A. 2020.
668 SARS-CoV-2-reactive T cells in healthy donors and patients with COVID-19.
669 Nature <https://doi.org/10.1038/s41586-020-2598-9>.

670 44. Nelde A, Bilich T, Heitmann JS, Maringer Y, Salih HR, Roerden M, Lübke M,
671 Bauer J, Jonas Rieth J, Wacker M, Peter A, Hörber S, Traenkle B, Kaiser PD,
672 othbauer U, Becker M, Junker D, Krause G, Strengert M, Schneiderhan-Marra N,
673 Templin MF, Joos TO, Kowalewski DJ, Stos-Zweifel V, Fehr M, Graf M, Gruber
674 L-C, Rachfalski D, Preuß B, Hagelstein I, Märklin M, Bakchoul T, Gouttefangeas
675 C, Kohlbacher O, Klein R, Stevanović S, Rammensee H-G, Walz JS. 2020.
676 SARS-CoV-2-epitopes define heterologous and COVID-19-induced T-cell
677 recognition. Research Square <http://doi.org/10.21203/rs.3.rs-35331/v1>.

678 45. Sette A, Crotty, S. 2020. Pre-existing immunity to SARS-CoV-2: the knowns and
679 unknowns. Nat Rev Immunol 20:457-458.

680 46. Matsui M, Kawano M, Matsushita S, Akatsuka T. 2014. Introduction of a point
681 mutation into an HLA class I single-chain trimer induces enhancement of CTL
682 priming and antitumor immunity. Mol Ther Methods Clin Dev 1:14027.
683 <https://doi.org/10.1038/mtm.2014.27>.

684 47. Parham, P and Brodsky, FM. 1981. Partial purification and some properties of
685 BB7.2. A cytotoxic monoclonal antibody with specificity for HLA-A2 and a variant
686 of HLA-A28. Hum Immunol 3: 277-299.
687

Figure legends

Fig. 1. The linear diagrams of the SARS-CoV-2 genome and the protein subunits of ORF1a. The SARS-CoV-2 genome consists of ORF1a, ORF1b, Spike (S), ORF3a, Envelope (E), Membrane (M), ORF6, ORF7a, ORF7b, ORF8, Nucleocapsid (N), and ORF10. The ORF1a polyprotein is composed of eleven non-structural proteins, nsp1-nsp11.

Fig. 2. Intracellular IFN- γ staining of CD8⁺ T cells specific for peptides derived from SARS-CoV-2 pp1a. HHD mice were immunized twice with each of predicted peptides of SARS-CoV-2 pp1a in liposomes together with CpG. After one week, spleen cells were prepared and stimulated with (+) or without (-) a relevant peptide for 5 hours. Cells were then stained for their surface expression of CD8 (x axis) and their intracellular expression of IFN- γ (y axis). The numbers shown indicate the percentages of intracellular IFN- γ ⁺ cells within CD8⁺ T cells. Three to five mice per group were used in each experiment, and the spleen cells of the mice per group were pooled.

Fig. 3. (A) *In vivo* killing activities specific for the 10 peptides. HHD mice were immunized twice with either each of the 10 liposomal peptides (pp1a-38, -84, -641, -1675, -2884, -3467, -3583, -3662, 3710, and -3732) or liposomes alone together with CpG. One week later, *in vivo* peptide-specific killing activities were measured. Three to five mice per group were used, and the data of % specific lysis are shown as the mean $\pm$

SD. (B & C) Comparison of the 10 peptides in the induction of IFN- γ^+ CD8 $^+$ T cells. Seventeen HHD mice were immunized once with the mixture of 10 peptides involving pp1a-38, -84, -641, -1675, -2884, -3467, -3583, -3662, -3710, and -3732 in liposomes with CpG. After one week, spleen cells were stimulated with or without each of the 10 peptides, and intracellular IFN- γ in CD8 $^+$ T cells was stained. (C) Y-axis indicates the relative percentages of IFN- γ^+ cells in CD8 $^+$ T cells which were calculated by subtracting the % of IFN- γ^+ cells in CD8 $^+$ T cells without a peptide from the % of IFN- γ^+ cells in CD8 $^+$ T cells with a relevant peptide. Each blue circle represents an individual mouse. Data are shown as the mean (horizontal bars) $\pm$ SD. (B) Statistical comparisons of the relative % values of IFN- γ^+ CD8 $^+$ T cells among the 10 peptides in Fig. 3C were made by one-way ANOVA followed by post-hoc tests. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

Fig. 4. Comparison of the 10 peptides in the induction of CD107a $^+$ CD8 $^+$ T cells (A) and CD69 $^+$ CD8 $^+$ T cells (B). Nine HHD mice were immunized once with the mixture of 10 peptides involving pp1a-38, -84, -641, -1675, -2884, -3467, -3583, -3662, -3710, and -3732 in liposomes with CpG. After one week, spleen cells were stimulated with or without each of the 10 peptides, and the expression of CD107a (A) or CD69 (B) on CD8 $^+$ T cells was analyzed. Data indicates the relative percentages of CD107a $^+$ (A) and CD69 $^+$ (B) cells in CD8 $^+$ T cells which were obtained by subtracting the % of CD107a $^+$ and CD69 $^+$ cells in CD8 $^+$ T cells without a peptide from the % of CD107a $^+$ and CD69 $^+$ cells

in CD8⁺ T cells with a peptide, respectively. Each red (A) or green (B) circle represents an individual mouse. Data are shown as the mean (horizontal bars) $\pm$ SD. Statistical analyses of the data among the 10 peptides in Fig. 4A and Fig. 4B were performed by one-way ANOVA followed by post-hoc tests in Fig. 4C and Fig 4D, respectively. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

Fig. 5. Three types of reactivity in mice immunized with the mixture of the 10 peptides. Fifteen mice were immunized once with the mixture of 10 peptides including pp1a-38, -84, -641, -1675, -2884, -3467, -3583, -3662, -3710, and -3732 in liposomes with CpG. After one week, spleen cells were stimulated with or without each of the 10 peptides, and intracellular IFN- γ in CD8⁺ T cells was stained. Based on the reactivity pattern to the 10 peptides, fifteen mice were divided into three types, A-C. Each graph represents reactivity of an individual mouse. Y-axis indicates the relative percentage of IFN- γ ⁺ cells in CD8⁺ T cells which was calculated by subtracting the % of IFN- γ ⁺ cells in CD8⁺ T cells without a peptide from the % of IFN- γ ⁺ cells in CD8⁺ T cells with a relevant peptide. Statistical analyses of the relative % values to 10 peptides in each type were performed by one-way ANOVA followed by post-hoc tests. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

Table 1. Predicted CTL epitopes for the SARS-CoV-2 non-structural polyprotein 1a

High binders					
Name	Sequence	BL ₅₀	Name	Sequence	BL ₅₀
pp1a-84	VMVELVAEL	6.8 ± 0.3	pp1a-103	TLGVLVPHV	1.3 ± 0.2
pp1a-214	TLSEQLDFI	2.0 ± 0.2	pp1a-445	GLNDNLLEI	6.0 ± 0.2
pp1a-597	VMAYITGGV	9.5 ± 0.9	pp1a-619	TVYEKLKPV	5.6 ± 0.7
pp1a-641	FLRDGWEIV	0.9 ± 0.1	pp1a-1675	YLATALTL	8.4 ± 0.2
pp1a-2270	YLNSTNVTI	8.9 ± 0.3	pp1a-2569	ILLDQALV	9.6 ± 1.5
pp1a-2785	AIFYLITPV	5.1 ± 1.6	pp1a-2968	YLEGSVRVV	7.5 ± 0.1
pp1a-3013	SLPGVFCGV	3.1 ± 0.2	pp1a-3115	YLTNDVSFL	4.8 ± 0.3
pp1a-3122	FLAHIQWMV	6.9 ± 0.4	pp1a-3128	WMVMFTPLV	9.8 ± 1.1
pp1a-3587	ILTSLLVLV	8.3 ± 0.9	pp1a-3710	TLMNVLTIV	2.0 ± 0.4
pp1a-3732	SMWALIISV	8.9 ± 0.4	pp1a-4094	ALWEIQQVV	6.4 ± 0.4
Medium binders					
Name	Sequence	BL ₅₀	Name	Sequence	BL ₅₀
pp1a-15	QLSLPVLQV	79.4 ± 4.4	pp1a-38	VLSEARQHL	76.8 ± 6.8
pp1a-52	GLVEVEKGV	14.5 ± 4.9	pp1a-106	VLVPHVGEI	76.0 ± 2.0
pp1a-468	KLNEEIAII	56.2 ± 4.8	pp1a-600	YITGGVVQL	48.5 ± 7.9
pp1a-685	FLALCADSI	10.2 ± 0.5	pp1a-702	ALNLGETFV	40.7 ± 2.8
pp1a-1109	NLAKHCLHV	38.5 ± 8.2	pp1a-1114	CLHVVGPV	62.9 ± 2.3
pp1a-1161	SLRVCVDTV	85.7 ± 1.9	pp1a-1312	MLAKALRKV	95.2 ± 0.7
pp1a-2168	YMPYFFTL	99.0 ± 2.4	pp1a-2332	ILFTRFFV	96.5 ± 14.0
pp1a-2563	QLMCQPILL	11.1 ± 1.8	pp1a-2884	FLPRVFSV	29.3 ± 6.5
pp1a-3047	IVAGGIVAI	50.5 ± 2.5	pp1a-3083	LLFLMSFTV	39.4 ± 0.9
pp1a-3183	FLLNKEMY	12.9 ± 6.8	pp1a-3403	FLNGSCGSV	18.7 ± 3.2
pp1a-3467	VLAWLAAV	45.0 ± 10.8	pp1a-3475	VINGDRWFL	85.0 ± 5.4
pp1a-3482	FLNRFTTL	79.0 ± 9.6	pp1a-3583	LLLTILSL	63.8 ± 3.1
pp1a-3639	FLLPSLATV	15.0 ± 4.4	pp1a-3662	RIMTWLDMV	76.5 ± 7.1
pp1a-3753	FLARGIVFM	71.2 ± 12.9	pp1a-3798	CLLNRYFRL	81.6 ± 20.2
pp1a-3807	TLGVYDYL	61.7 ± 8.7	pp1a-3871	VLLSVLQQL	44.9 ± 6.0
pp1a-3886	KLWAQCVQL	27.2 ± 9.4	pp1a-4183	ALLSDLQDL	42.4 ± 6.1
pp1a-4266	VLSFCAFAV	20.1 ± 6.1	pp1a-4283	YLASGGQPI	39.8 ± 6.2
Low binders					
Name	Sequence	BL ₅₀	Name	Sequence	BL ₅₀
pp1a-572	GISQYSLRL	ND	pp1a-589	DLATNNLVV	ND
pp1a-692	SIHGGAKL	ND	pp1a-881	KTLQPVSEL	161.7 ± 28.3
pp1a-1143	VLLAPLLSA	ND	pp1a-1148	LLSAGIFGA	ND
pp1a-1214	FITESKPSV	122.1 ± 31.7	pp1a-1367	ILGTVSWNL	123.2 ± 11.3
pp1a-1387	KLMPVCVET	174.2 ± 33.5	pp1a-1433	SLINTLNDL	123.4 ± 11.0
pp1a-1642	FLGRYMSAL	124.1 ± 1.9	pp1a-1962	DLNGDVVAI	ND
pp1a-2076	ILKPANNSL	ND	pp1a-2226	LINIIWFL	ND
pp1a-2228	NIIWFLLL	ND	pp1a-2230	IIWFLLLSV	176.7 ± 6.3
pp1a-2235	LLSVCLGSL	ND	pp1a-2242	SLIYSTAAL	134.0 ± 7.6
pp1a-2249	ALGVLMNSL	ND	pp1a-2348	QLFFSYFAV	146.4 ± 19.7
pp1a-2363	WLMWLIINL	102.7 ± 6.4	pp1a-2364	LMWLIINLV	159.0 ± 42.8
pp1a-2901	KLIEYTDFA	126.0 ± 2.5	pp1a-3344	SMQNCVLKL	104.5 ± 2.2
pp1a-3827	GLLPPKNSI	ND	pp1a-3839	KLNIKLLGV	213.5 ± 85.7
pp1a-3917	VLLSMQGAV	112.3 ± 7.2	pp1a-4032	MLFTMLRKL	165.8 ± 21.7

Data of peptide binding assays are shown as BL₅₀, indicating a concentration (μM) of each peptide that yields the 50% relative binding as shown in the materials and methods. Experiments were performed in triplicate and repeated twice with similar results. Data are given as mean values ± SD. High binders: BL₅₀ < 10 μM; Medium binders: 10 μM ≤ BL₅₀ < 100 μM; Low binders: BL₅₀ ≥ 100 μM or ND (not detected).

Table 2

Comparison of amino acid sequences of SARS-CoV-2 pp1a CTL epitopes with the amino acid sequence of SARS-CoV

SARS-CoV-2				SARS-CoV	
Name	Start-End	Sequence	Protein	Sequence	Identity (%)
pp1a-38	38-46	VLSEARQHL	nsp1	ALSEAREHL	89
pp1a-52	52-60	GLVEVEKGV	nsp1	GLVELEKGV	89
pp1a-84	84-92	VMVELVAEL	nsp1	KVVELVAEM	67
pp1a-103	103-11	TLGVLVPHV	nsp1	TLGVLVPHV	100
pp1a-445	445-53	GLNDNLLEI	nsp2	TLNEDLLEI	67
pp1a-597	597-605	VMAYITGGV	nsp2	IMAYVTGGL	67
pp1a-641	641-9	FLRDGWEIV	nsp2	FLKDAWEIL	67
pp1a-1675	1675-83	YLATALLT	nsp3		
pp1a-2785	2785-93	AIFYLITPV	nsp4		
pp1a-2884	2884-92	FLPRVFSAV	nsp4	FLPRVFSAV	100
pp1a-3083	3083-91	LLFLMSFTV	nsp4	LLFLMSFTI	89
pp1a-3403	3403-11	FLNGSCGSV	nsp5	FLNGSCGSV	100
pp1a-3467	3467-75	VLAWLYAAV	nsp5	VLAWLYAAV	100
pp1a-3583	3583-91	LLLILTSL	nsp6		
pp1a-3662	3662-70	RIMTWLDMV	nsp6	RIMTWLELA	67
pp1a-3710	3710-8	TLMNVLTIV	nsp6	TLMNVITLV	89
pp1a-3732	3732-40	SMWALIISV	nsp6	SMWALVISV	89
pp1a-3886	3886-94	KLWAQCVQL	nsp7