Identification of cytotoxic T cells and their T cell receptor sequences targeting COVID-19 using MHC class I-binding peptides

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Since severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, COVID-19) was first reported in China in December 2019, various variants have been identified in different areas of the world such as United Kingdom (alpha), South Africa (beta and omicron), Brazil (gamma), and India (delta). Some of SARS-CoV-2 variants, each of which is characterized by a unique mutation(s) in spike protein, are concerned due to their high infectivity and the capability to escape from neutralizing antibodies elicited by vaccinations. To identify peptide epitopes that are derived from SARS-CoV-2 viral proteins and possibly induce CD8\(^+\) T cell immunity, we investigated SARS-CoV-2-derived peptides that are likely to bind to major histocompatibility complex (MHC) class I molecules. We identified a total of 15 peptides that bind to human leukocyte antigen (HLA)-A*24:02, HLA-A*02:01, or HLA-A*02:06, and possibly induce cytotoxic T lymphocytes (CTLs); thirteen of them corresponded to ORF1ab polyprotein, one peptide to spike protein and the remaining one to membrane glycoprotein. CD8\(^+\) T cells that recognize these peptides were detected in peripheral blood samples in three individuals recovered from COVID-19 as well as non-infected individuals. Since most of these peptides are commonly conserved among other coronaviruses including SARS-CoV and/or MERS-CoV, these might be useful to maintain T cell responses to coronaviruses that are pandemic at present and will become the future threat. We could define pairs of TRA and TRB sequences of nine CTL clones that recognize SARS-CoV-2-derived peptides. We might use these SARS-CoV-2-derived peptide-reactive TCR sequences for investigating the history of SARS-CoV-2 infection.

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INTRODUCTION
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has become world-wide pandemic. Until December 21, 2021, ~273 million people have been confirmed the infection to COVID-19 and more than 5 million died of this disease.

In addition to intensive screening of infected individuals with PCR and/or antigen tests and subsequent isolation of coronavirus-positive individuals, worldwide vaccination is fundamentally essential to overcome the present pandemic situation. Randomized trials demonstrated that Pfizer/BioNTech BNT162b2 and Moderna mRNA-1273 vaccines encoding the SARS-CoV-2 spike protein had ~95% protective ability against COVID-19 [1, 2]. Vaccine-elicited neutralizing antibodies inhibit the spike protein on SARS-CoV-2, which was originally reported in December 2019 in China, to attach to the host cells. While these vaccines against COVID-19 have been used widely, SARS-CoV-2 variants have continuously emerged in different areas of the world. Virus variants, alpha, beta, gamma, delta, and omicron, which were defined by a unique amino acid substitution(s) within the spike protein, were identified in the United Kingdom [3], South Africa [4, 5], Brazil [6], and India [7], respectively. Neutralizing activity against these SARS-CoV-2 variants tends to decrease as compared to that against the original reference strain in individuals who received vaccination [8–12]; therefore, a novel vaccine(s) that is not affected by mutations in the virus genome is required for the fight against SARS-CoV-2.

Although most of the studies have focused on the neutralizing antibodies against the spike protein, we here describe induction of CD8\(^+\) T cells that are expected to play crucial roles in virus clearance through killing of virus-infected cells. In fact, in SARS-CoV-2 infections, the presence of CD8\(^+\) T cells responding to SARS-CoV-2-derived peptides was shown to be associated with better clinical outcomes [13–15]. CD8\(^+\) T cells in COVID-19-recovered individuals could recognize not only spike protein-derived peptides but also other viral proteins including nucleocapsid phosphoprotein, membrane glycoprotein, and ORFs-derived peptides [16–18]. In previous studies, severe acute respiratory syndrome coronavirus (SARS-CoV)-specific memory T cells were detected in blood samples of SARS-recovered individuals after 6–11 years of the initial infection [19, 20]. T cell responses against the nucleocapsid phosphoprotein of SARS-CoV were maintained in individuals with the history of SARS-CoV
infection 17 years after the outbreak of SARS in 2003 and these memory T cells showed cross-reactivity to SARS-CoV-2 [17]. These data indicate that memory T cells may also critically contribute to long-term protection from COVID-19.

In a present study, we demonstrate major histocompatibility complex (MHC) class I-binding epitope peptides derived from SARS-CoV-2 viral proteins to enhance antiviral CD8+ T cell immunity. To characterize cytotoxic T lymphocytes (CTLs) that recognize SARS-CoV-2-derived peptides and play a central role in T cell immunity, we further conduct T cell receptor (TCR) analysis to recognize SARS-CoV-2-derived peptides and play a central role in immunity. To characterize cytotoxic T lymphocytes (CTLs) that were collected before the SARS-CoV-2 pandemic (March 2018–May 2019) were purchased from Cellular Technology Limited. Healthy individuals-derived PBMCs that were collected before the SARS-CoV-2 pandemic (March 2018–May 2019) were purchased from Cellular Technology Limited.

**MATERIALS AND METHODS**

**Samples**

The study protocol was approved by the Institutional Review Board of OncoTherapy Science and the written informed consent was obtained from each of peripheral blood mononuclear cells (PBMCs) donors.

**PBMCs**

For in vitro CTL induction, human leukocyte antigen (HLA)-A*24:02-positive PBMCs were isolated from blood of healthy volunteers by density gradient centrifugation using Ficoll-Paque PLUS (Cytiva) according to an industrial instruction manual. HLA-A*02:01- or HLA-A*02:06-positive PBMCs were purchased from Cellular Technology Limited (Cleveland, OH). For tetramer staining, HLA-A*24:02-positive PBMCs derived from COVID-19-recovered individuals with a prior positive SARS-CoV-2 PCR test were purchased from Precision For Medicine (Bethesda, MD). Healthy individuals-derived PBMCs that were collected before the SARS-CoV-2 pandemic (March 2018–May 2019) were purchased from Cellular Technology Limited.

**Cell lines**

TISI cells (HLA-A*24:02/−, lymphoblastoid cell) were purchased from the IHWG Cell and Gene Bank (Seattle, WA). T2 (HLA-A*02:01/−, lymphoblast), Jiyoye and EB-3 (HLA-A3/Aw32, Burkitt’s lymphoma) cells were purchased from American Type Culture Collection (Manassas, VA). HEV0011 (HLA-A*02:06/−, B lymphocytes) cells were purchased from RIKEN Bioresource Research Center (Tsukuba, Japan). All cells were cultured in RPMI1640 media (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) and 1% antibiotic solution (Wako).

**Peptides**

SARS-CoV-2-derived 9-mer and 10-mer peptides were synthesized by Cosmo Bio Co., Ltd. (Otaru, Japan). The purity (>90%) and the sequences of peptides were confirmed by analytical HPLC and a mass spectrometry analysis. Peptides were dissolved in dimethyl sulfoxide at 20 mg/mL and stored at −80 °C until the use for in vitro CTL induction, an IFN-γ enzyme-linked immunospot (ELISPOT) assay and an IFN-γ enzyme-linked immunosorbent assay (ELISA).

**in vitro CTL induction**

Monocyte-derived dendritic cells (DCs) were generated from PBMCs. Monocytes were isolated from PBMCs by adherence to a plastic tissue culture dish (Becton Dickinson) and cultured in the presence of 1000 IU/mL of granulocyte-macrophage colony-stimulating factor (R&D System) and 1000 IU/mL of interleukin (IL)-4 (R&D System) in AIM-V medium (Invitrogen) supplemented with 2% human AB serum (SIGMA) for 7 days. On day 5, OK-432 (Chugai Pharmaceutical) was added to the culture medium to induce the maturation of DCs (final concentration: 0.1 KE/mL). Autologous CD8+ T cells (3.0 × 105 cells) purified from PBMCs with CD8-Positive Isolation Kit (Dynal) were cultured with DCs (1.5 × 105 cells) with 20 μg/mL of each peptide in AIM-V medium containing 2% human AB serum, 10 ng/mL of IL-7 (R&D System), and 30 ng/mL of IL-21 (Cell Genix). After 3 days of the culture, CD8+ T cells were restimulated with DCs and 20 μg/mL of each peptide. DCs were prepared by the same procedure described above. At day 7 of the culture, CD8+ T cells were further stimulated with 48 IU/mL of IL-2 (Novartis), 5 ng/mL of IL-7 (NovoProtein), and 5 ng/mL of IL-15 (NovoProtein) in AIM-V medium supplemented with 2% human AB serum. At day 11 of the culture, CD8+ T cell responses were examined by an IFN-γ ELISPOT assay.

**IFN-γ ELISPOT assay**

The human IFN-γ ELISPOT kit and AEC substrate set (BD Biosciences) were used to analyze CD8+ T cell responses to SARS-CoV-2-derived peptides. The ELISPOT assay was performed according to the industrial instruction manual. Briefly, TISI, T2, or HEV0011 cells were used as stimulator cells. These cells were cultured with or without 20 μg/mL of peptide overnight at 37 °C. To analyze CD8+ T cell responses, 500 μL of supernatant was removed from each well of culture plates for in vitro CTL induction. Subsequently 100 μL of cell suspensions and stimulator cells (2 × 104 cells) with or without each peptide were co-cultured in 96-well plates coated with anti-IFN-γ antibody. After 16–18 h incubation, IFN-γ spots were detected and counted using ImmunoSpot S6 analyzer (Cellular Technology Limited).

**Limiting dilution**

CD8+ T cells were diluted to 0.5 cell or 10 cells per well in 96-well round-bottom plates (Corning) and cultured with feeder cells in AIM-V medium containing 5% human AB serum, 30 ng/mL of anti-CD3 monoclonal antibody (clone UCHT1; BD Biosciences), and 150 IU/mL of IL-2. Jiyoye and EB-3 cells (1 × 105 cells each) were used as feeder cells after treating with 30 μg/mL of Mitomycin C at 37 °C for 30 min. After 10 days of the culture, IL-2 was added to each well (final concentration: 150 IU/mL). After additional 4 days of the culture, IFN-γ ELISPOT was performed for screening CD8+ T cell responses against each peptide.

**CTL expansion culture**

CTLs that showed positive reactivity in the IFN-γ ELISPOT assay after limiting dilution were expanded. They were cultured with feeder cells in AIM-V medium containing 5% human AB serum and 40 ng/mL of anti-CD3 monoclonal antibody. Jiyoye and EB-3 cells (5 × 106 cells each) were used as feeder cells after treating with 30 μg/mL of Mitomycin C at 37 °C for 30 min. The half volume of culture medium was exchanged with fresh AIM-V containing 5% human AB serum and 72 IU/mL of IL-2 every 3 or 4 days. After 14 days of the culture, IFN-γ secretion from CTLs was measured by ELISA.

**IFN-γ ELISA**

ELISA were performed using IFN-γ ELISA kit (BD Biosciences) according to the industrial instruction manual. Briefly, CTLs (Responders) and TISI, T2, or HEV0011 cells (Stimulators) with or without each peptide were co-cultured in 200 μL of AIM-V medium supplemented with 5% human AB serum at several ratio of Responders to Stimulators (R/S ratio). Responders at 5 × 106, 2.5 × 105, 1.25 × 105, 0.625 × 105, or 0.3125 × 105 cells/well/concentration were plated in 96-well round-bottom plates together with 1 × 104 Stimulators. After 16–18 h of the incubation, supernatants were collected for measurement of IFN-γ. IFN-γ production was calculated as pg/mL using a standard curve.

**TCR sequencing**

Total RNAs were extracted from peptide-specific CTL clones using RNeasy mini kit (QIAGEN). cDNAs were synthesized using SMARTScribe Reverse Transcriptase (Clontech). SARS-CoV-2-derived peptides-reactive TCR sequences were identified by Sanger sequencing. TCRs cDNA was amplified by PCR using a common forward primer for adaptor (5’-GGAGGGTGGTCGGCAAGAGATGTTGATATAACGAGACGAGTCTGACATGCTGTTATAGAAGAAGACDBDHICAGGTCAGGGGGTGTTCTGAGATA-3’) and a reverse primer specific for the constant region (5’-TCGTCGCGAGCTGTCAGATGCTGTTATAGAAGAAGACDBDHICAGGTCAGGGGGTGTTCTGAGATA-3’). Sanger sequencing was performed using M13 forward primer (5’-TATTAAAGACGGACGAGCTTCTTG-3’) and M13 reverse primer (5’-CAGGAAACGTTACGATCTG-3’) after TA cloning. TCRβ cDNA was amplified using a common forward primer for adaptor and a reverse primer specific for the constant region (5’-TGCAGGGTGGTCGGCAAGAGATGTTGATATAACGAGACGAGTCTGACATGCTGTTATAGAAGAAGACDBDHICAGGTCAGGGGGTGTTCTGAGATA-3’) and a reverse primer specific for the constant region (5’-TCGTCGCGAGCTGTCAGATGCTGTTATAGAAGAAGACDBDHICAGGTCAGGGGGTGTTCTGAGATA-3’). After 3 days of the culture, CD8+ T cells were further stimulated with 48 IU/mL of IL-2 (Novartis), 5 ng/mL of IL-7 (NovoProtein), and 5 ng/mL of IL-15 (NovoProtein) in AIM-V medium supplemented with
RESULTS
Prediction and selection of MHC class I-binding peptides derived from SARS-CoV-2 viral proteins

The workflow for screening of SARS-CoV-2-derived MHC class I-binding peptides is shown in Fig. 1. Firstly, we predicted MHC class I-binding peptide epitopes derived from SARS-CoV-2 viral proteins including the structural proteins (spike, envelope, membrane glycoprotein, and nucleocapsid phosphoprotein) as well as the non-structural proteins (ORF1ab, ORF3a, ORF6, ORF7a, ORF8, and ORF10) as previously described [24]. Peptide-MHC affinity was calculated using NetMHC 4.0 server [25, 26]. We targeted HLA-A*24:02, HLA-A*02:01, and HLA-A*02:06 because they covered a large proportion (more than 80%) of Japanese populations [27].

We focused peptide epitopes that were conserved in either or both of SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV) to develop common and universal vaccines that can be possibly effective for newly arising coronaviruses in the future.

We firstly selected 14 peptides (P01-P14) that were expected to bind to an HLA-A*24:02 molecule (predicted MHC affinity of <120 nM). Two of the 14 peptides (P06 and P11) were common among coronaviruses including SARS-CoV and MERS-CoV. The remaining 12 peptides were conserved in SARS-CoV. We also selected 15 peptides (P15-P28 and P08) that were expected to bind to both HLA-A*02:01 and HLA-A*02:06 molecules (predicted MHC affinity of <120 nM). They are commonly shared with SARS-CoV. All candidate peptides are shown in Table 1.

Identification of MHC class I-binding peptides derived from SARS-CoV-2 (HLA-A*24:02)
To examined CTL induction by peptides, we first used HLA-A*24:02-positive PBMCs isolated from two healthy donors. Briefly, autologous CD8+ T cells and monocyte-derived DCs were prepared from PBMCs. CD8+ T cells were cultured with DCs pulsed with each peptide. After 11 days of stimulation by each peptide, CD8+ T cells were cultured overnight with HLA-A*24:02-positive TISI cells that were pulsed with or without each peptide. CD8+ T cell responses to each peptide were analyzed by an IFN-γ ELISPOT assay. They were considered as positive when IFN-γ spot counts were 1.5 times or higher, compared with the peptide-negative control. Positive reactivities were detected in 9 of the 14 peptides examined (Fig. 2A).

We subsequently conducted limiting dilution to establish peptide-specific CTL clones from the initial CD8+ T cell populations that showed positive reactivity in the IFN-γ ELISPOT assay. CD8+ T cells were diluted to 10 cells per well in 96-well round-bottom plates. After 14 days of culture, peptide-specific CTLs were screened using an IFN-γ ELISPOT assay. CTL responses to each peptide were examined by an IFN-γ ELISA following the cell expansion. Peptide-specific IFN-γ secretion against eight peptides were observed when TISI cells were used as stimulator cells for presenting individual peptides (Fig. 2B). This indicates that these peptides were likely to be presented on the HLA-A*24:02 molecule and recognized by TCRs on CTLs.

Identification of MHC class I-binding peptides derived from SARS-CoV-2 (HLA-A*02:01 and HLA-A*02:06)
We similarly investigated CTL induction for 15 candidate epitopes (P15-P28 and P08 listed in Table 1) using HLA-A*02:01-positive donor-derived PBMCs. CD8+ T cells were cultured overnight with HLA-A*02:01-positive T2 cells pulsed with or without each peptide. Positive reactivity was detected by an IFN-γ ELISPOT assay for 13 of the 15 peptides examined (Fig. 3A). Then, we performed limiting dilution and further confirmed the peptide-specific IFN-γ secretion in expanded T cells stimulated with 8 of the 13 peptides (Fig. 3B). Since HLA-A*02:01 and HLA-A*02:06 have an only one
amino acid substitution (F9Y) in a region of the peptide binding groove [28, 29], it has been indicated that some peptides bind to both HLA-A*02:01 and HLA-A*02:06 molecules [30, 31]. Hence, we investigated the cross-reactivity of these eight peptides to an HLA-A*02:06 molecule using HLA-A*02:06-positive donor-derived PBMCs. As we expected, we found the positive reactivity of seven peptides in an IFN-γ ELISPOT assay (Fig. S1A) as well as the peptide-specific IFN-γ secretions in an ELISA (Fig. S1B).

TCR sequences recognizing SARS-CoV-2-derived peptides

SARS-CoV-2-derived peptide-specific CTL clones were established from CD8+ T cell population that showed positive reactivity to each of the peptides after in vitro CTL induction (Fig. S2A, B). Then DNA sequences of pairs of TCRα and TCRβ were examined using established CTL clones. We show pairs of TRA and TRB sequences that were likely to recognize SARS-CoV-2-derived peptides in Table 2.

Table 1. SARS-CoV-2-derived candidate peptides bind MHC class I (HLA-A*24:02, HLA-A*02:01, or HLA-A*02:06)

| ID | Protein | Sequence | Length (amino acids) | Starting position | Affinity (nM) | HLA | Conserved in |
|----|---------|----------|----------------------|-------------------|---------------|-----|--------------|
| P01 | ORF1ab | FYASALWEI | 9 | 4090 | 18 | A*24:02 | SARS-CoV |
| P02 | S      | PFAMQAMYRF | 10 | 897 | 39 | A*24:02 | SARS-CoV |
| P03 | ORF1ab | YDYLVSTQEF | 10 | 3811 | 41 | A*24:02 | SARS-CoV |
| P04 | ORF1ab | YYSSLMCQPI | 10 | 2560 | 44 | A*24:02 | SARS-CoV |
| P05 | ORF1ab | SYSSLMLPI | 9 | 4628 | 46 | A*24:02 | SARS-CoV |
| P06 | ORF1ab | AYANSVFNI | 9 | 5080 | 56 | A*24:02 | SARS-CoV |
| P07 | ORF1ab | RYKLEGYAF | 9 | 6676 | 62 | A*24:02 | SARS-CoV |
| P08 | ORF1ab | FTYASALWEI | 10 | 4089 | 69 | A*24:02 | SARS-CoV |
| P09 | ORF1ab | SYSLFDMSKF | 10 | 7039 | 85 | A*24:02 | SARS-CoV |
| P10 | ORF1ab | RYFKYWDQTY | 10 | 4677 | 90 | A*24:02 | SARS-CoV |
| P11 | ORF1ab | TAYANSVFNI | 10 | 5079 | 102 | A*24:02 | SARS-CoV |
| P12 | M      | LWLWLVPVTL | 9 | 54 | 108 | A*24:02 | SARS-CoV |
| P13 | ORF1ab | TYASALWEIQ | 10 | 4090 | 111 | A*24:02 | SARS-CoV |
| P14 | ORF1ab | YAYLRKHSFM | 10 | 5138 | 115 | A*24:02 | SARS-CoV |
| P15 | ORF1ab | SLFDMSKFL | 10 | 7041 | 6 | A*02:01 | SARS-CoV |
| P16 | ORF1ab | LMKYKLPWNV | 10 | 6077 | 7 | A*02:01 | SARS-CoV |
| P17 | ORF1ab | FVLWAHGFEL | 10 | 6108 | 8 | A*02:01 | SARS-CoV |
| P18 | ORF1ab | YMPSWVMRI | 10 | 3654 | 12 | A*02:01 | SARS-CoV |
| P19 | ORF1ab | MMGFKMNYQV | 10 | 5982 | 18 | A*02:01 | SARS-CoV |
| P20 | ORF1ab | FELTSMKYFV | 10 | 6115 | 18 | A*02:01 | SARS-CoV |
| P21 | ORF1ab | MMLSDDAV | 9 | 5147 | 35 | A*02:01 | SARS-CoV |
| P22 | N      | FGMSRIGMEV | 10 | 315 | 37 | A*02:01 | SARS-CoV |
| P23 | ORF1ab | TLMIERFVSL | 10 | 5245 | 42 | A*02:01 | SARS-CoV |
| P24 | ORF1ab | YVYNPFMIDV | 10 | 6160 | 51 | A*02:01 | SARS-CoV |
| P25 | ORF1ab | NVLAWLYAAV | 10 | 3466 | 72 | A*02:01 | SARS-CoV |
| P26 | ORF1ab | TLEPEYFNSV | 10 | 5740 | 79 | A*02:01 | SARS-CoV |
| P27 | ORF1ab | YSFLPGVYSV | 10 | 3098 | 81 | A*02:01 | SARS-CoV |
| P28 | ORF1ab | FMIDVQWQGF | 10 | 6165 | 84 | A*02:01 | SARS-CoV |

S spike protein, M membrane glycoprotein, N nucleocapsid phosphoprotein

SARS-CoV-2-derived peptide-specific CTL clones were established from CD8+ T cell population that showed positive reactivity to each of the peptides after in vitro CTL induction (Fig. S2A, B). Then DNA sequences of pairs of TCRα and TCRβ were examined using established CTL clones. We show pairs of TRA and TRB sequences that were likely to recognize SARS-CoV-2-derived peptides in Table 2.
Detection of SARS-CoV-2-derived peptides-reactive CD8\(^+\) T cells in PBMCs

We analyzed HLA-A*24:02-positive PBMCs from individuals who recovered from COVID-19 (\(n = 3\)) and healthy individuals without any evident SARS-CoV-2 infection (\(n = 3\)) to examine CD8\(^+\) T cells that might recognize MHC class I-binding peptides we identified. After in vitro stimulation, PBMCs were stained with PE-conjugated pMHCI tetramers and analyzed by flow cytometry (Fig. 4A). pMHCI tetramer-binding CD8\(^+\) T cells were detected from three individuals recovered from COVID-19 (Fig. 4B). It is notable that
we found CD8\(^+\) T cells recognizing SARS-CoV-2-derived peptides (P04, P06, and P12), which are conserved in other coronaviruses (Table S1), in one non-infected healthy individual as well (Fig. 4C).

Impact of SARS-CoV-2 omicron variant on MHC class I-binding peptides

We investigated the influence of mutations found in SARS-CoV-2 omicron variant (BA.1), which is a new SARS-CoV-2 variant of concern and has been identified in 89 countries as of 16th of December, 2021, on 15 MHC class I-binding peptides identified in this study. We searched nonsynonymous substitutions and deletions in the GISAID database (https://www.gisaid.org) on 20th of December, 2021. We identified 132 mutations (76 mutations in ORF1ab polyprotein, 51 mutations in spike protein, and 5 mutations in membrane glycoprotein) in 9 or more (>0.1%) of 8993 sequences of omicron variant. Only one mutation at position 5086 (F5086Y) in ORF1ab may affect immunogenicity of P06 and P11, but other peptide sequences are conserved in the

Fig. 3 Identification of HLA-A*02:01-binding peptides derived from SARS-CoV-2. A IFN-γ ELISPOT assay was performed after in vitro CTL induction. CD8\(^+\) T cells and HLA-A*02:01-positive T2 cells pulsed with each peptide were co-cultured overnight. T2 cells without the peptide were used as negative control. Bars represent the number of IFN-γ spots. CD8\(^+\) T cell responses were judged as positive when the number of IFN-γ spots were 1.5 times or higher than the negative control. B To confirm the response of CTLs to SARS-CoV-2-derived peptides, IFN-γ ELISA was performed. CTLs (Responders) were co-cultured overnight with T2 cells (Stimulators) pulsed with or without the peptide at the indicated ratio of Responders to Stimulators (R/S ratio). IFN-γ secretion was measured by ELISA. CTLs that revealed the peptide-specific IFN-γ secretion for eight peptides are shown. Similar results were obtained in independent experiments using three CTLs in the respective peptides.

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Table 2. SARS-CoV-2-derived peptide-reactive TCRs

| Peptide ID | Peptide-reactive TCRs | Chain | V region | J region | CDR3 | HLA |
|------------|------------------------|-------|----------|----------|------|------|
| P04        | Alpha TRAV10           | TRAV10 | TRAJ31   | CVVSA LNARLMF | A*24:02 |
| Beta TRBV20-1 | TRBV20-1 | TRBU2-1 | CSARDYTSSYNEQFF | A*24:02 |
| P06        | Alpha TRAV1-2          | TRAV1-2 | TRAJ32   | CAVRGSGGGATKNIIF | A*24:02 |
| Beta TRBV9 | TRBV9                | TRBU2-1 | CASSPSGNYEQYF | A*24:02 |
| P07        | Alpha TRAV20           | TRAV20 | TRAJ11   | CAVHHFPGYSTLF | A*24:02 |
| Beta TRBV30 | TRBV30        | TRBU2-7 | CAGAGRGYEQYF | A*24:02 |
| P08        | Alpha TRAV26-1         | TRAV26-1 | TRAJ5    | CIVSDMGRRALTF | A*24:02 |
| Beta TRBV4-1 | TRBV4-1 | TRBU2-1 | CASSFTGSGSLGEOFF | A*24:02 |
| P11        | Alpha TRAV21           | TRAV21 | TRAJ20   | CAVFNDYKLSF  | A*24:02 |
| Beta TRBV7-9 | TRBV7-9 | TRBU2-7 | CASSSRTSGRYSYEQYF | A*24:02 |
| P08        | Alpha TRAV21           | TRAV21 | TRAJ48   | CAVRRTYFGNEKLF | A*02:01 |
| Beta TRBV20-1 | TRBV20-1 | TRBU2-7 | CSARGSDSYEQYF | A*02:01 |
| P23        | Alpha TRAV8-6          | TRAV8-6 | TRAJ36   | CAITGANNLFF  | A*02:01 |
| Beta TRBV20-1 | TRBV20-1 | TRBU1-5 | CSAEDRGSNSNPQHF | A*02:01 |
| P25        | Alpha TRAV1-2          | TRAV1-2 | TRAJ20   | CAVRDPRNDYKLSF | A*02:01 |
| Beta TRBV27 | TRBV27            | TRBU1-1 | CATQVNEAFF  | A*02:01 |
| 2          | Alpha TRAV24           | TRAV24 | TRAJ29   | CAFLSGNTPLVF  | A*02:01 |
| Beta TRBV27 | TRBV27            | TRBU1-6 | CASSPAAYSPHLF  | A*02:01 |

The omicron variant (Table S2). This indicates that the peptides identified in this study may be used as a sort of universal peptides to induce CTLs against COVID-19 variants.

**DISCUSSION**

We screened 29 SARS-CoV-2-derived epitope peptides (14 peptides for HLA-A*24:02 and 15 peptides for HLA-A*02:01) that were predicted to bind to MHC class I molecules for activation of CD8\(^+\) T cells and found that eight peptides each for HLA-A*24:02 and HLA-A*02:01 could activate CD8\(^+\) T cells isolated from PBMCs. We subsequently established CTL clones and confirmed their immune reactivities against these peptides presented on the expected HLA molecules. Among the eight peptides that activated CD8\(^+\) T cells derived from HLA-A*02:01 donors, seven peptides also activated CD8\(^+\) T cells derived from HLA-A*02:01:02-positive individuals recovered from COVID-19, indicating that CD8\(^+\) T cells were activated in vivo by coronavirus-derived epitope peptides (possibly same as peptides identified in this study) presented on MHC class I molecules on antigen-presenting cells including DCs in their body. It is notable that we found CD8\(^+\) T cells that recognized SARS-CoV-2-derived peptides (P04, P06, and P12) in one SARS-CoV-2-non-infected healthy individual. Since these peptides are conserved among other human coronaviruses, the presence of CD8\(^+\) T cells reacting to these peptides may reflect previous infections with these viruses, or this individual might have experienced non-symptomatic COVID-19. Understanding the effect of pre-existing SARS-CoV-2-derived peptide-reactive CD8\(^+\) T cells on non-symptomatic patients or prevention of COVID-19 should be important to consider the strategy for controlling pandemic or the future threat to newly arising coronaviruses.

We confirmed the presence of CD8\(^+\) T cells that recognize SARS-CoV-2-derived peptides (P01, P02, P07, P08, and P12) in HLA-A*24:02-positive individuals recovered from COVID-19, indicating that CD8\(^+\) T cells were activated in vivo by coronavirus-derived peptides (possibly same as peptides identified in this study) presented on MHC class I molecules on antigen-presenting cells including DCs in their body. It is notable that we found CD8\(^+\) T cells that recognized SARS-CoV-2-derived peptides (P04, P06, and P12) in one SARS-CoV-2-non-infected healthy individual. Since these peptides are conserved among other human coronaviruses, the presence of CD8\(^+\) T cells reacting to these peptides may reflect previous infections with these viruses, or this individual might have experienced non-symptomatic COVID-19. Understanding the effect of pre-existing SARS-CoV-2-derived peptide-reactive CD8\(^+\) T cells on non-symptomatic patients or prevention of COVID-19 should be important to consider the strategy for controlling pandemic or the future threat to newly arising coronaviruses.

Recently, one report indicated that a spike protein-derived polypeptide with adjuvant on nanoparticle, which was administrated by intramuscular route, successfully induced neutralizing antibodies and CD4\(^+\) T cell responses in rhesus macaques [32]. This report also supports the concept that SARS-CoV-2-derived epitope peptides might be a promising COVID-19 vaccine that would induce and maintain the T cell immunity against SARS-CoV-2 that will continuously cause new mutations. In addition, we found no significant homology with peptides possibly derived from human proteins by the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi), suggesting there is little possibility that our peptides might cause unintended immune reactions, which are unfavorable to our health condition.

Furthermore, T-Detect COVID Test (Adaptive Biotechnologies), a next-generation TCR sequencing-based test, was approved by FDA for identifying the recent or prior SARS-CoV-2 infection. Our peptide-reactive TCR sequences might be useful to investigate SARS-CoV-2 infection history or monitor the effectiveness in vaccinated individuals.
In this study, we have reported identification of 15 candidate vaccine peptides against COVID-19. Since T cell memories seem to be maintained in a longer period (few to several years) than antibodies and the peptides reported here are likely to avoid the influence of additional mutations that may occur in SARS-CoV-2 in the future, our peptides may enable us to provide the useful information to design the novel vaccine(s).

Fig. 4 Detection of CD8+ T cells that recognized SARS-CoV-2-derived MHC class I-binding peptides in PBMCs. A Gating strategy for detecting SARS-CoV-2-derived peptides-reactive CD8+ T cells. Firstly, lymphocytes population was separated by a forward scatter (FSC-A) and back scatter (BSC-A) gates. Doublets were excluded on forward scatter height (FSC-H) and forward scatter width (FSC-W). Living CD3+CD4- cells were selected by staining with DAPI, and expression of CD3 and CD4. pMHC1 tetramer-binding CD8+ T cells were defined by gating based on HIV control tetramer staining. B HLA-A*24:02-positive PBMCs from three COVID-19-recovered individuals were stained using pMHCI tetramers after in vitro stimulation. CD8+ T cells that recognize SARS-CoV-2-derived peptides were detected from every individual. C HLA-A*24:02-positive PBMCs from three healthy individuals without any history of SARS-CoV-2 infection were stained using pMHCI tetramers after in vitro stimulation. Pre-existing CD8+ T cells that recognize SARS-CoV-2-derived peptides were detected from one of the three non-infected individuals.
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COMPETING INTERESTS

TH, M. Sakamoto, MH, M. Saito, YY, and KT are employees of OncoTherapy Science, Inc. YN is a stockholder and scientific advisor of OncoTherapy Science, Inc. TH and YN are listed as the inventors on pending patent application that is related to findings in this study.

ADDITIONAL INFORMATION

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