Identification of TCR repertoires in functionally competent cytotoxic T cells cross-reactive to SARS-CoV-2

Kanako Shimizu1, Tomonori Iyoda1, An Sanpei1, Hiroshi Nakazato1, Masahiro Okada1, Shogo Ueda1, Miyuki Kato-Murayama2, Kazutaka Murayama2,3, Mikako Shirouzu2, Naoko Harada4, Michihiro Hidaka4 & Shin-ichiro Fujii1,5

SARS-CoV-2-specific CD8+ T cells are scarce but detectable in unexposed healthy donors (UHDs). It remains unclear whether pre-existing human coronavirus (HCoV)-specific CD8+ T cells are converted to functionally competent T cells cross-reactive to SARS-CoV-2. Here, we identified the HLA-A24-high binding, immunodominant epitopes in SARS-CoV-2 spike region that can be recognized by seasonal coronavirus-specific CD8+ T cells from HLA-A24+ UHDs. Cross-reactive CD8+ T cells were clearly reduced in patients with hematological malignancy, who are usually immunosuppressed, compared to those in UHDs. Furthermore, we showed that CD8+ T cells in response to a selected dominant epitope display multifunctionality and cross-functionality across HCoVs in HLA-A24+ donors. Cross-reactivity of T-cell receptors isolated from them exhibited selective diversity at the single-cell level. Taken together, when stimulated well by immunodominant epitopes, selective pre-existing CD8+ T cells with high functional avidity may be cross-reactive against SARS-CoV-2.
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for causing the COVID-19 pandemic. Recently, several COVID-19 vaccines, such as mRNA-based vaccines or adenovirus-vectored vaccines expressing SARS-CoV-2 spike (S) protein, have now been administered through global vaccination programs[1-3]. Neutralizing antibodies (Abs) need to be generated to protect the body by the SARS-CoV-2 vaccine. Furthermore, to inhibit SARS-CoV-2 more efficiently, CD4+ T cells and CD8+ T cells play important roles in combating SARS-CoV-2.[4]. Recent studies on humoral immunity induced by the vaccines provide evidence regarding the duration of the neutralizing antibody response[5]. Moreover, it is known that CD4+ T cells and CD8+ T cells, in addition to neutralizing Abs, can play an important role in inhibiting SARS-CoV-2.[6]. Anti-viral CD8+ cytotoxic T cells have the potential to eliminate virus-infected cells, even during the latency of viruses, such as HIV, CMV, EBV, and HSV[7-9]. Understanding anti-viral CD8+ T cell immunity may be useful in identifying biomarkers to evaluate the severity of or develop treatment strategies against COVID-19.[10].

Immunodominant epitopes from structural proteins, such as S, membrane, and nucleocapsid proteins, or non-structural proteins, such as NSP7 and NSP13 encoded on ORF1 of SARS-CoV-2, have been predicted and detected in infected patients and convalescent individuals[11-13]. CD8+ T cell responses may be associated with disease severity[14]. A recent report demonstrated that SARS-CoV-2 specific memory CD8+ T cells can be detected over 6 months after the onset of symptoms in 50–70% of the convalescent patients, and their responding epitopes are present on S, membrane, nucleocapsid, and ORF3a[15]. Numerous studies of infected patients or convalescents that traced SARS-CoV-2-specific CD8+ T cells have demonstrated the importance of active effector or memory CD8+ T cells[9-16,18].

Pre-existing CD8+ T cells for seasonal coronaviruses that cross-react with SARS-CoV-2 can be detected in only about 20% of the unexposed healthy donors (UHDs)[11], but may still be biologically relevant[16]. In UHDs, the majority of immunodominant epitopes are derived from non-S regions, ORF1a, and nucleocapsid, but not S protein regions[11,19] because of the low homology of amino acids in S protein. The cross-reactivity of CD8+ T cells for S protein has not been studied sufficiently. The identification of cross-reactive immunodominant epitopes in S protein in UHDs is among the key challenges for CD8+ T cells to combat SARS-CoV-2. Candidate targets for the CD8+ T immunodominant epitope in SARS-CoV-2 spike protein (SARS-CoV-2 S) were predicted using NetMHC 4.0. We selected and synthesized six candidate peptides for these epitopes from the entire S protein sequence and one epitope from nucleocapsid protein as a control (Fig. 1a)[19].

In the current study, we used the PBMCs from healthy volunteers that were collected in early 2020 (from February to April in 2020) and during 2004–2010 (pre-pandemic era) as UHDs (Supplementary Table 1). None of the volunteers suffered from COVID-19, and they were never in close contact with a COVID-19 patient. In addition, all UHDs neither had a history of SARS-CoV-1 nor MERS infections. In addition, we assessed their plasma antibody production twice using two types of commercial kits that were often used in the previous studies[16,19,24]. All UHDs tested negative for antibodies against SARS-CoV-2. For initial screening of the optimal peptide for SARS-CoV-2 CD8+ T cell responses, five HLA-A24+ UHDs were tested. Short-term assays, such as 24-h activation induced marker assays described in the previous reports[11,13,15], are useful for the detection of effector CD8+ T cells, whereas in-vitro expansion assays can be used to detect pre-existing CD8+ T cells against cancer or cross-reactive CD8+ T cells to SARS-CoV-2, even at low levels[14,25-28]. PBMCs were cultured in the presence of each peptide, and the frequencies of epitope-specific IFN-γ-producing CD8+ T cells were assessed on day 21. Although these six peptides from S protein were expected to bind to HLA-A*2402 with high affinity, five of these did not induce a T-cell response, and only Pep3(QYI)-specific CD8+ T cell counts were elevated in cells derived from all UHDs (Fig. 1b and Supplementary Fig. 1a, b). As positive response, we defined a 1.5-fold increase in T-cell response as compared to that in the controls (Supplementary Fig. 1c). Intriguingly, Pep3(QYI) exhibited high sequence homology with other coronaviruses, including four types of seasonal coronaviruses (Fig. 1c). Although all the six peptides of S protein were predicted to exhibit high affinity to HLA-A*2402, five peptides were not homologous to other seasonal coronaviruses (Supplementary Fig. 1d). These results suggest that CD8+ T cells may be cross-reacting, and that pre-existing memory CD8+ T cells may be responsible for the immune response.

We then focused on Pep3(QYI)-specific CD8+ T cells from UHDs. In this assay, we isolated CD8+ T cells from PBMCs and cocultured them with irradiated autologous PBMCs pulsed with Pep3(QYI), and the frequencies of epitope-specific IFN-γ-producing CD8+ T cells were assessed on day 14 (Fig. 1d). We found that antigen-specific CD8+ T cells were present in 15 out of 18 UHD participants (83.3%) (Fig. 1e, f, g). The response rate of Pep3(QYI)-specific T cells in HLA-A*2402+ UHDs was higher than that in 20% of the UHDs in a previous study[11], even if the frequency was low, most of the responders possessed IFN-γ- and TNF-α-producing T cells (Fig. 1e, f).

Assessment of the polyfunctionality of Pep3(QYI)-specific CD8+ T cells from UHDs. We assessed the polyfunctionality of Pep3(QYI)-specific CD8+ T cells by cytokine production capacity, cytotoxic activity, and degranulation markers. First, we found that half the T-cell populations produced more than two cytokines (IFN-γ, TNF-α, and IL-2) (Fig. 2a). In contrast, we could not detect IL-10 (Supplementary Fig. 2). Next, the cytotoxicity of Pep3(QYI)-specific CD8+ T cell lines was assessed.
peptide mix contained the other immunodominant epitopes in this region around the Pep#3(QYI) epitope, to examine the other immunodominant epitopes predicted in silico under HLA-A*24:02 restriction. However, other peptides may bind to other HLAs.

**Fig. 1 SARS-CoV-2-specific CD8+ T cell response in HLA-A24-positive unexposed healthy donors (UHDs).** a Selected peptides for CD8+ T cells. Peptides derived from Spike (S) protein of SARS-CoV-2, shown in the list, were selected based on high-affinity binding (% rank) to HLA-A*24:02 via in silico prediction using NetMHC4.0. Pep#7 for nucleocapsid protein was selected as the control. b The data of IFN-γ+ response of antigen-specific CD8+ T cells from one representative donor (UHD2). PBMCs were stimulated with seven types of peptides individually, cultured for 3 weeks in the presence of IL-2, and then assessed for IFN-γ production following recall with their cognate peptide by intracellular cytokine analysis (ICS). c Homology of the reported epitope in the Pep#3 region of SARS-CoV-2 compared with that in other human coronaviruses. Identical residues are same color-coded. d Culture protocol of Pep#3(QYI)-specific CD8+ T cell response. The sorted CD8+ T cells from PBMCs of HLA-A24+ UHDs were stimulated with irradiated autologous PBMCs pulsed with Pep#3(QYI). The Pep#3(QYI)-specific CD8+ T cells were examined by the frequency of IFN-γ+TNF-α+ CD8+ T cells by ICS analysis on day 14. e Flow cytometry data are shown for representative donors (UHDs, 6, 13, 14, 18, and 19) (Fig. 2b) and the frequency of IFN-γ+TNF-α+ CD8+ T cells is summarized (n = 18) (f). ※ responder (>1.5 fold change (restimulation+ /restimulation−)) g Pie chart representing the proportions of responders or non-responders against Pep#3(QYI) in 18 HLA-A24+ UHDs.

(Fig. 2b). We detected cytotoxicity against Pep#3(QYI)-pulsed A24/CIR, but not the unpulsed A24/CIR (Fig. 2b). Finally, we performed the CD107a degranulation assay, which is used to measure the potential of cells to secrete cytotoxic molecules, i.e., perforin and granzyme. We found that approximately 80% of IFN-γ+ CD8+ T cells expressed CD107a (Fig. 2c).

Detection of the high affinity region around the Pep#3(QYI). Next, we synthesized four types of 11-mer-overlapped 15-mer peptides based on the S2 region (1200-1226) of S protein around the Pep#3(QYI) epitope, to examine the other immunodominant epitopes in this region (Fig. 3a). Since the relevant epitopes are relatively conserved across HCoVs and completely conserved across the SARS-CoV-2 variant strains, such as VOC-202012/01, 501Y.V2, and 501Y.V3, the peptide set is available for SARS-CoV-2 variant strains (Supplementary Fig. 3). We cultured PBMCs from UHDs in the presence of a 15-mer peptide mix and evaluated the response of antigen-specific CD8+ T cells at day 14 (Fig. 3b). Surprisingly, we detected SARS-CoV-2-specific CD8+ T cells in all the UHDs exposed to the peptides (Fig. 3c). Moreover, CD8+ T cells responded primarily to 15-mer pep#2 and #3, but in some cases, they also responded to 15-mer pep#1 and #4 (Fig. 3d–f). These findings indicated that the 15-mer peptide mix contained the other immunodominant epitopes in addition to Pep#3(QYI) for HLA-A24+ individuals and would be more useful for detecting or expanding pre-existing CD8+ T cells in HLA-A24+ individuals.

Multiple epitopes in SARS-CoV-2 S are highly immunogenic in HLA-A24+UHDs. Next, to verify the parts of the epitopes of four types of the 15-mer peptide mix responsible for CD8+ T cell activation, a consecutive 9-mer peptide library was synthesized using a S protein sequence (Fig. 4a). We assessed peptide-specific T-cell response by restimulation with each 9-mer-peptide after priming with the 15-mer peptide mix (Fig. 4b). We found that the 9-mer peptides#1204 to #1218, particularly #1208 (Pep#3(QYI)), induced CD8+ T cell-specific responses comparable to 15-mer pep#2 to #3 (Fig. 4c, d). Accordingly, we conducted an in silico screening of this region in SARS-CoV-2 and seasonal coronaviruses using IEBD and NetMHC4.0 (Fig. 4e) to examine which 9-mer peptides were recognized by these CD8+ T cells under the HLA-A24 restriction. We found six and eight types of 9-mer-epitopes with high IEBD and NetMHC scores expressed in-silico for all the HLA-A24 individuals. However, other peptides may bind to other HLAs.
Analysis of SARS-CoV-2-specific CD8⁺ T cells in patients with HM. We examined whether Pep#3(QYI) can induce epitope-specific CD8⁺ T cells in HLA-A24+ patients with HM (Supplementary Table 2), who represent a high-risk group that may be susceptible to COVID-19 due to immune-cell dysfunction related to disease progression or chemotherapy. We confirmed that all patients with HM recruited in this study were negative for IgG specific for SARS-CoV-2 (Supplementary Table 2). Pep#3(QYI)-specific CD8⁺ T cells were observed in 14.8% (4 out of 27) of the patients with HM, and the induction ratio of Pep#3(QYI)-specific CD8⁺ T cells was significantly lower in patients with HM than in UHDs (p < 0.01; Fisher’s/Chi-square tests) (Fig. 5a–c). This indicated that the levels of SARS-CoV-2 cross-reacting CD8⁺ T cells against seasonal coronaviruses were lower in patients with HM than in UHDs.

Next, we analyzed these patients by four types of 11-mer-overlapped 15-mer peptides (Fig. 5d–f). Surprisingly, we detected SARS-CoV-2-specific CD8⁺ T cells in 65.4% of patients with HM (Fig. 5e, f). T-cell response in the presence of a mix of four peptides was more efficient than that induced by the 9-mer peptide, particularly in patients with HM. Thus, there were fewer cross-reactive CD8⁺ T cells in patients with HM than in UHDs (65.4% vs 14.8%, p < 0.001; Fisher’s/Chi-square tests), which indicated that seasonal coronavirus-specific memory T cells may be reduced in response to disease progression or therapy-related toxicity. However, CD8⁺ T cells that have the potential to respond to the 15-mer mix, mainly to 15-mer pep#2 and #3, can be activated by rechallenging with the 15-mer peptide mix. Furthermore, in some cases, they also responded to 15-mer pep#1 and #4 (Supplementary Fig. 4).

Fig. 2 Function of SARS-CoV-2-specific CD8⁺ T cells from UHDs. a Polyfunctionality of SARS-CoV-2 Pep#3(QYI)-specific CD8⁺ T cells. Pep#3(QYI)-specific CD8⁺ T cell lines were established from UHDs and re-stimulated individually with Pep#3(QYI) in an ICS assay (n = 15). The frequency of CD8⁺ T cells with different cytokine production profiles (IFN-γ, TNF-α, and IL-2) was determined. (i) The outer ring of the double ring pie shows the cytokine profile in different colors, and the inner ring represents the number of cytokines produced, with gray scale. (ii) The mean of the frequency of number of cytokines produced in UHDs (early 2020) and UHDs (pre-pandemic 2004–2010) and all was summarized. b Peptide-specific cytotoxic activity of Pep#3(QYI)-specific CD8⁺ T cells from representative donors. The cytotoxicity assay was performed in duplicate. Data represent the mean of duplicate. c Frequency of the degranulation marker CD107a positive in Pep#3(QYI)-specific IFN-γ⁺ CD8⁺ T cells (n = 12).

CD8⁺ T cells exhibit cross-reactivity between SARS-CoV-2- and HCoV-derived S peptide. We evaluated whether the Pep#3(QYI)-specific CD8⁺ T cells generated from ten HLA-A24+ UHDs and two patients with HM cross-reacted with other coronavirus families, and quantified SARS-CoV-2-specific CD8⁺ T cells (Fig. 6a). The Pep#3(QYI)-specific CD8⁺ T cell lines were assessed based on the production of IFN-γ and TNF-α in response to the relevant peptide of each virus (Fig. 6b). The Pep#3(QYI)-specific CD8⁺ T cell lines from UHDs exhibited cross-reactivity across the HCoVs. In these two patients with HM, cross-reactivity of CD8⁺ T cells was also found to be preserved. The CD8⁺ T cell lines responded to betacoronaviruses (SARS-CoV-1, MERS, HKU-1, and OC43), with 83–100% responders, and to alphacoronaviruses (NL63 and 229E), with 58.3–66.7% responders (Fig. 6c–e). Next, we assessed the level of the cross-reactivity of Pep#3(QYI)-specific CD8⁺ T cells to coronaviruses in UHDs. We also evaluated the functional avidities of these CD8⁺ T cells by IFN-γ production. Peptide titration experiments defined a dose range of 0 to 10,000 ng/mL for SARS-CoV-2 and seasonal coronavirus with the highest affinity (e.g., HCoV-HKU1) (Fig. 6f). EC₅₀ is calculated as the peptide concentration required to reach one-half maximal response. EC₅₀ for SARS-CoV-2 S was comparable to that for seasonal coronaviruses (Fig. 6f). These data demonstrated similar functional avidity of the SARS-CoV-2 cross-reactive CD8⁺ T cell response relative to the CD8⁺ T cell response for seasonal coronaviruses. Furthermore, we assessed the cytotoxic activity across different coronaviruses as another measure of cross functionality (Fig. 6g, h). The cytotoxicity against A24/CIR pulsed with the target peptide from SARS-CoV-2 was compared to that...
from seasonal coronaviruses with the highest affinity for Pep#3(QYI)-specific CD8^+ T cells in each UHD cell line (Fig. 6g). However, no statistical difference was observed between them (Fig. 6h). These findings indicated that although some pre-existing memory CD8^+ T cells in HLA-A24^+ UHDs had the potential to recognize SARS-CoV-2, they can be sufficiently skewed toward SARS-CoV-2 under optimal conditions.

TCR avidity for recognition of SARS-CoV-2 and HCoVs at the single-cell level. To understand why CD8^+ T cells exhibit multiple responses to various coronaviruses and the underlying mechanisms, we studied the cross-reactivity of CD8^+ T cells at the single TCR level. After restimulation with Pep#3(QYI), we isolated CD8^+ T cells from UHDs and performed single-cell analysis of TCR repertoires. To analyze the clonalities of TCR repertoires, we transduced T cells by upregulation of CD69 expression (MFI). These findings (Fig. 6g) showed that the TCR repertoires varied from each other in their epitope recognition (Fig. 7b). TCR-T (TCR-T-1) cells from UHD2 responded well to all the epitopes derived from coronaviruses, whereas the TCR-T (TCR-T-2) cells from UHD8 responded only to SARS-CoV-1 and SARS-CoV-2. These data are interesting because UHD8 had not been infected with either of them. Dykema et al. also reported the same phenomenon in a previous study, wherein SARS-CoV-2-specific, monoreactive CD4^+ T clonotypes were detected in the UHDs and indicated the possibility of cross-reactive response to unknown coronaviruses or other pathogens. The TCR-T data, however, may also be a chance finding. Furthermore, the TCR-T (TCR-T-3) cells from UHD6 responded to SARS-CoV-2, SARS-CoV-1, NL63, and 229E, while those (TCR-T-4) from HM16 responded to SARS-CoV-2, SARS-CoV-1, MERS, HKU1, and OC43, but not to NL63 and 229E (Fig. 7b, c). Some types of single TCRαβ-transduced T cells responded broadly to the relevant epitopes on HCoVs, whereas others responded only to two or four peptides. These findings on the SARS-CoV-2-epitope-responding T cell repertoires imply that TCRαβ may be selective at the single-cell level.

We next evaluated the functional avidities of these TCR-transduced T cells by upregulation of CD69 expression (MFI). Peptide titration experiments defined a dose range of 10 fg/mL to 10 μg/mL for SARS-CoV-2 and each seasonal coronavirus (Fig. 7d). TCR-T-1 exhibited the highest avidity for OC43 compared to that for SARS-CoV-2 and others. However, other TCR-T cells (TCR-T-2 to TCR-T-4) from UHDs exhibited the highest avidity for SARS-CoV-2 (Fig. 7e). Comparing TCR-T-2
(monoreactive) to other cross-reactive TCRs (TCR-T-1, -3, and -4), we could not find large differences in their functional avidities (Fig. 7e). Irrespective of the previous infection status, Pep#3(-QYI)-specific CD8+ T cells from UHDs displayed sufficient cross-functionality at the single cell level (Fig. 7e). From these data, we concluded that Pep#3(QYI)-specific CD8+T cells from UHDs did not exhibit low avidity.

Crystal structures of CD8+ T cells binding to HLA-A*24:02 and TCR complex. The crystal structures of HLA-A*24:02 and three peptides, namely, SARS-CoV-2, 229E, and HKU1, were determined (Fig. 8a, Table 1). These three complex structures were almost identical to each other and their rmsd values were 0.15 Å, 0.41 Å, and 0.41 Å between HLA-A*24:02-CoV2 and HLA-A*24:02-229E, HLA-A*24:02-CoV2 and HLA-A*24:02-HKU1, and HLA-A*24:02-229E and HLA- A*24:02-HKU1, respectively. In the complex structures, crystal packing was not involved in the interactions between HLA- A*24:02 and each peptide.

The 9-mer peptides were identified at the equivalent position of the HLA-A*24:02 structure (Fig. 8b). Although electron densities of the peptide backbones were observed, those of some side chains were poor, particularly, Lys4 for three peptides, Trp5 and 7 for CoV2 and 229E, and Pro6 for HKU1. The conformation of these peptide backbones overlapped well. Of the nine positions, the side chains at positions P2, P3, P6, and P9 were involved in the HLA-A*24:02-peptide interactions at the bottom of the binding pocket. In contrast, the side chains at positions P1, P4, P5, P7, and P8 were directed to the solvent region and oriented differently in the complex structures (Fig. 8c). Thus, these side chains are expected to directly interact with TCR.

In the Protein Data Bank, 31 structures of the HLA-A*24:02 peptide complex have been deposited so far. Peptide sequences on the interface and the intrinsic plasticity of the TCR/HLA-A24 complex structures are with the 9-mer peptide29. A comparison between these 9-mer peptides and the peptides in this study revealed that all the peptides anchored to the binding pocket at positions P2 and P9, and conformational variations were observed at positions P4–P7 (Fig. 8d). This region forms a bulge structure and corresponds to the putative interaction site with TCR. Furthermore, we investigated the influence of peptide sequence on the interface and the intrinsic plasticity of the TCR/PMHC trimeric and PMHC biomolecular complexes with structural modeling performed by replacing the HLA moiety of the HLA-peptide-TCR complex structure30 (PDB code 3VXM) with the structure mentioned in the present study.

Discussion

Elucidating the role of pre-existing, cross-reactive T cells in COVID-19 is important for understanding the development and severity of COVID-19 and determining the optimal T-cell engagement strategies for development of diagnostic tools and vaccines. An expansion of cross-reactive T cells with low avidity may lead to exacerbation of infection. Therefore, it is critical not only to identify pre-existing T cells but also to find an approach to expand functionally competent, cross-reactive T cells with high avidity. In the current study, we focused on an amino acid sequence conserved between SARS-CoV-2 and seasonal coronaviruses in the S2 region, and selected 9-mer peptides. One of the selected peptides, Pep#3(QYI), efficiently induced SARS-CoV-2 cross-reactive CD8+ T cells in HLA-A24+ UHDs. These Pep#3(QYI)-specific CD8+ T cells from UHDs exhibited polyfunctionality and similar cytotoxicity against seasonal coronaviruses. Furthermore, we identified immunodominant epitopes, including Pep#3 (QYI), which were covered by three 15-mer overlapping peptides. Moreover, to elucidate the mechanism, we assessed the cross-reactivity and functional avidity of Pep#3 (QYI)-specific TCR at the single TCR level.
Although cross-reactivity with pre-existing T cells has been reported\textsuperscript{10,14,19,31}, whether SARS-CoV-2 cross-reactive T cells are protective is still an issue of debate. SARS-CoV-2 cross-reactive CD4\textsuperscript{+} T cells have been reported to exhibit low avidity, and some studies have demonstrated that they are non-protective\textsuperscript{24,27}. However, there are three studies that address the cross-reactivity of pre-existing CD8\textsuperscript{+} T cells in UHDs. Schulien et al. performed an analysis of pre-existing and induced CD8\textsuperscript{+} T cells in three patients pre- and post-SARS-CoV-2 infection and showed the importance of anti-viral effector CD8\textsuperscript{+} T cells\textsuperscript{16}. T cell cross-reactivity can be defined to recognize more than one distinct peptide-MHC structures by single TCR\textsuperscript{32}. Mallajosyula et al. demonstrated that T cells that recognize peptides conserved among coronaviruses are abundant in UHDs\textsuperscript{33}. Moreover, they verified that these are more abundant in COVID-19 patients by spheromer technology. They also detected shared TCR motifs of SARS-CoV-2 specific CD8\textsuperscript{+} T cells between UHDs and mild COVID-19 patients\textsuperscript{33}, suggesting a protective role of pre-existing CD8\textsuperscript{+} T cells in COVID-19. A recent study reported immunodominant, HLA-B7-restricted SARS-CoV-2 nucleocapsid protein epitope cross-reactive CD8\textsuperscript{+} T cells\textsuperscript{28}. The authors found the presence of a shared CDR3\textbeta motif in epitope-specific CD8\textsuperscript{+} T clonotypes in exposed and unexposed donors, which suggested that pre-existing immunity in HLA-B7\textsuperscript{+} individuals favored clonal expansion. In the current study, we demonstrated the pattern of Pep\#3 (QYI)-specific CD8\textsuperscript{+} T cell TCR cross-reactivity across HCoVs under HLA-A24\textsuperscript{+} restriction at the single-cell level, which exhibited substantial functional avidity. Nevertheless, further molecular studies that examine TCR characteristics of HLA-A24\textsuperscript{+} UHDs and infected patients are required.

Cancer patients have an increased risk of severe illness from COVID-19, with a high mortality rate\textsuperscript{20,34,35}. Particularly, adult patients with HM having COVID-19 were found to have a 34% risk of death. Patients aged >60 years had a significantly higher risk of death\textsuperscript{32}. Furthermore, patients with impaired B-cell function, such as myeloma, lymphoma, or CML, may not respond to standard vaccines and may require special attention\textsuperscript{36,37}. Using a 15-mer peptide mix, SARS-CoV-2 cross-reactive CD8\textsuperscript{+} T cells were successfully detected at 75%, 50%, and 56% higher rates in patients with HM. Since these findings indicate the importance of HLA in COVID-19 pathogenesis, other HLA and peptide interactions should also be studied.

The restricted formula for linking HLA-peptide and TCR confers heterotypic cross-reactivity across SARS-CoV-2. Furthermore, to elucidate the molecular basis of cross-reactive TCR/\beta recognition between SARS-CoV-2 and seasonal coronaviruses, we examined the structure of SARS-CoV-2 peptides and the relevant peptides of seasonal coronaviruses bound to the HLA-A24:02 molecule (Fig. 8). The obtained data on the HLA-A24:02-peptide complex structures imply that the side chains at positions P1, P4, P5, P7, and P8 interact with the TCR. The superimposed model (where the HLA moiety is replaced by the structure presented in our study) indicates that the peptide...
positions mentioned above are close to the contact surface of the TCR. The side chains at positions P1/P4/P5 and P7/P8 can interact with the loop moieties of TCRα and TCRβ, respectively. In the sequence alignment among TCR1-4, insertions/deletions were found in these loop regions, suggesting variety of affinity in the HLA-A*24:02 peptide complex and TCRs. Furthermore, our data suggest that the selective cross-reactivity of T cells in SARS-CoV-2 epitopes depends on combinations of the structure mode of the peptide on the HLA-A*24:02 complex and the sequences/loop region of the TCR conformation. Further studies are required to broadly underpin the cross-reactivity by analyzing the structure of the cross-reactive TCR complex with HLA- A*24:02+SARS-CoV-2-S2 and HLA- A*24:02+seasonal coronavirus epitopes.

Overall, we found that the potential of cross-reactivity may depend on the interaction between TCR and the structure of specific HLA, i.e., HLA- A*24:02. Moreover, TCRα/β from Pep3(QYI)-specific CD8+ T cells exhibited varying patterns of cross-reactivity against HCoVs. Lastly, we determined the optimal 15 mer-mixed peptides that may stimulate SARS-CoV-2-specific CD8+ T cells, even for most patients with HM in addition to all UHDs; the results indicated the possibility of stimulatory epitopes. We speculated that some CD8+ T cells that act against seasonal coronaviruses might persist as long-term memory cells in UHDs. If these cross-reactive T cells were stimulated by a type of vaccine, including immunodominant epitopes, these could be skewed toward SARS-CoV-2. Development of a vaccine for modulating cross-reactive CD8+ T cells may help reduce the rates of the disease.

**Methods**

**Human samples and preparation.** Peripheral blood samples were obtained from healthy blood donors at our Institute (early 2020) or from the buffy coats of healthy blood donors (2004–2010) (Tokyo Red Cross, Tokyo, Japan). Peripheral blood samples from 28 patients with hematological malignancies (HM) were obtained from the National Hospital Organization Kumamoto Medical Center (Kumamoto, Japan). None of the UHDs or unexposed patients with HM suffered from COVID-19, and they did not have any close contact with a COVID-19 patient. In addition, none of the UHDs had a history of SARS-CoV-1 or MERS infections. Plasma samples from healthy donors and patients with HM were assessed by COVID-19 Human IgM IgG Rapid tests (Abnova) and the anti-SARS-CoV-2 IgG ELISA kit (EUROIMMUN). The sensitivity of the Euroimmun kit in samples after day 10 is 94.5%, according to the manufacturer. None of the patients tested positive for either antibody. These donors are referred to as unexposed donors (UHDs) in this manuscript. PBMCs were separated via Ficoll–Paque PLUS (GE Healthcare, Uppsala, Sweden) density centrifugation and washed twice with phosphate-buffered saline (PBS) and stored in liquid nitrogen until further use. Information on healthy blood donors and the characteristics of patients with HM are listed in Supplementary Tables 1 and 2, respectively. This study was approved by the Institutional Review Board for Human Research at RIKEN IMS.

**Cell lines.** CIR cells expressing HLA-A*24:02 (A24/CIR) were kindly provided by Dr. Masafumi Takiguchi (Kumamoto University). The SKW-3 (T-ALL) line was
Epitope prediction and peptide selection. The S protein CD8+ T epitopes of SARS-CoV-2 and other HCoV for HLA-A*24:02 were predicted using the Immune Epitope Database and Analysis Resource (IEDB) (https://www.iedb.org/)98 and NetMHC 4.0 (https://www.cbs.dtu.dk/services/NetMHC/).40,41 The corresponding protein accession identification numbers are: SARS-CoV-2 isolate, Wuhan-Hu-1 NCBI: YP_009472404.1, HCoV(HKU1) NCBI: YP_173238.1, HCoV(OCA) NCBI: YP_00955241.1, HCoV(NL63) NCBI: YP_003767.1, HCoV(229E) NCBI: NP_073551.1. NFKDQVILL in the nucleocapsid phosphoprotein has been previously reported as a SARS-CoV-2 dominant T cell epitope.

In-vitro expansion of SARS-CoV-2-specific T cells. For the peptide screening test, PBMCs (3–5 × 10^6 cells) were stimulated with the indicated peptide (each peptide 10 µg/mL) (summarized in Supplementary Table 3) in culture medium in the presence of IL-2 (100 U/mL) (Shionogi CO., LTD) and restimulated with 30 Gy-irradiated autologous PBMCs pulsed with each peptide weekly. Then, these cells were analyzed on day 21. For determining CD8+ T cell response against Pep3(QYI), CD8+ T cells (0.5–1 × 10^6 cells) were isolated from PBMCs using CD8+ MACS beads (Miltenyi Biotec), and the isolated cells were stimulated with irradiated Pep3(QYI)-pulsed autologous PBMCs (1:1 ratio) in the presence of IL-2 (100 U/mL) and further restimulated with irradiated autologous PBMCs pulsed with Pep3(QYI) on day 7. Then, the cells were analyzed on day 14. For determining CD8+ T cell response using 15mer peptides, PBMCs (3–5 × 10^6 cells) were stimulated with the 15mer peptide mixture (each peptide 10 µg/mL) in culture medium in the presence of IL-2 (100 U/mL) and further restimulated with irradiated autologous PBMCs pulsed with peptide mixture on day 7. Then, these cells were analyzed at day 14. Cultures were supplemented with R10 media (RPMI-1640 medium supplemented with 10% FBS, 55 mM 2-ME, and 1% penicillin/streptomycin) containing IL-2 twice or thrice weekly. The harvested cells were washed out with R10, restimulated with or without the indicated peptide (10 µg/mL) in the presence of brefeldin A (50 µg/mL) (SIGMA) and monensin (750 ng/mL) (SIGMA) for 16 h, and analyzed using flow cytometry. To determine the threshold, we prepared PBMCs or sorted CD8+ T cells from UHDs and cultured them without peptide in the presence of IL-2. Two weeks later, the cells were restimulated with Pep3 (control cells were not restimulated), and IFN-γ production was analyzed by intracellular cytokine staining. Then, we calculated the fold change of the % of IFN-γ+ CD8+ T cells in the restimulated group compared to that in the control group. Since the fold change in these experiments did not exceed 1.5 (mean ± 2SD: 0.94 ± 0.4) (Supplementary Fig. 1c), we set the threshold at 1.5.

Flow cytometry. Antibodies were purchased from BD Bioscience, Biolegend, or eBioscience (summarized in Supplementary Table 4). Following stimulation, the cells were incubated with Human TruStain FcX (BioLegend) in FACs buffer (PBS with 2% heat-inactivated FBS) for Fc-Blocking and then stained with surface antibodies, CD3-PE/Cy7, CD4-PerCP/Cy5, and CD8-BUV737 or FITC and Live/Dead Fixable Aqua or Violet Stain (Life Technologies) for 30 min. For intracellular cytokine staining, the cells were washed twice with 2%FBS in PBS, fixed and permeabilized using BD Cytofix/Cytoperm solution and then, intracellularly stained with anti-IFN-γ-APC, IL-2-BUV786, TNF-α-PE or IL-10-PE for further 30 min. For degranulation assay, we added anti-CD107a-Alexa488 in the culture during restimulation for 6 h. Then, after Fc-Blocking, the cells were stained with surface antibodies, CD3-PE/Cy7, CD4-PerCP/Cy5 and CD8-BUV737 and Live/Dead Fixable Aqua or Violet Stain (Life Technologies) for 30 min and fixed and permeabilized using BD Cytofix/Cytoperm solution and then intracellularly stained with anti-IFN-γ-APC for further 30 min. The LSR Fortessa X-20 instrument and FACSDiva (v 8.0.1) (BD Biosciences) or FlowJo software (v10.3B2) were used for data analysis.

Analysis of cross-reactivity. The established Pep3(QYI)-specific CD8+ T cell lines were restimulated with Pep3(QYI) or the relevant peptide of HCoV (each
peptide 10 μg/mL) and analyzed for cytokine production via intracellular cytokine staining. We used a graded dose of the peptide, ranging from 0.1 μg/mL to 10 μg/mL, to assess TCR avidity for each stimulating peptide. TCR avidity was assessed by %IFN-γ in CD8+ T cells. Furthermore, EC50 was estimated by calculating the peptide concentration required to reach one-half maximum %IFN-γ reached in our assay.

Single-cell TCR sequencing and construction of retroviral TCR vector. Single-cell TCR sequencing was performed as below. All primers used are listed in Supplementary Table 5. CoV-2 pep#3-specific CD8+ T cell lines were restimulated with anti-human CD107a-BV421 in the presence or absence of 10 μM CoV-2 pep#3 for 6 h and then stained with anti-human CD8-PE and Aqua. The peptide-specific CD8+ CD107a+ cells were sorted as single cells into 5 μL of RT-PCR mix in a 96-well plate, using FACS Aria III (BD Biosciences). The RT-PCR mix comprised 1 μL of 5× Prime STAR GXL Buffer, 0.45 μL RT-PCR primer mix, 0.4 μL of 2.5 mM dNTP, 0.05 μL of 40 U/μL RNase Inhibitor, 200 U/μL PrimeScript II Reverse Transcriptase (Takara Bio Inc,), and 2.95 μL nuclease-free water. The program used for the one-step RT-PCR was as follows: 45 °C for 40 min, 98 °C for 1 min, and 35 cycles of 98 °C for 10 s, 55 °C for 15 s, and 68 °C for 1 min. The resultant PCR products were diluted 10-fold with nuclease-free water and used as templates for the 2nd-PCR. The 2nd PCR was performed using PrimeSTAR GXL with the pMXs-BamHI-InFusion primer and CA-rev2 primer for TCRα or CB-rev2 primer for TCRβ in a 10 μL reaction volume. The program for the 2nd-PCR was as follows: 98 °C for 1 min and 44 cycles of 98 °C for 10 s, 55 °C for 15 s, and 68 °C for 1 min. The 2nd-PCR products were treated with ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific K.K.) and sequenced using the CA-rev3 primer for TCRα or CB-rev3 primer for TCRβ. Sequencing results were analyzed using the V-QUEST tool of the IMGT database (http://www.imgt.org/IMGT_vquest/input)42. For cloning TCR, VDJ and C regions were amplified from the 2nd-PCR products and PBMC-derived cDNAs of the donors, respectively. PrimeSTAR Max was used for amplification with the pMXs-BamHI-InFusion and CA-rev or CB-rev3 primer. Each fragment was cloned into BamHI-digested linearized pMXs-IRES-GFP for TCRα or -TdTomato for TCRβ by the InFusion HD reaction.

Establishment of SKW-3-CD8AB-expressing cells. SKW-3 cells were maintained in R10 media. hCD8A-IRES-Puro and hCD8B-IRES-Puro VSV-G retroviruses were transduced into SKW-3 cells in the presence of 5 μg/mL polybrene (Millipore) via centrifugation at 2300 rpm for 90 min at 35 °C. CD8AB-expressing cells were stained with PE-CD8A (RPA-T8) and BV421-CD8B (2ST8.5H7) and sorted twice using FACS Aria III. SKW-3-CD8AB-expressing cells were maintained with 3 μg/mL puromycin (InvivoGen).

TCR clonotyping and αβ TCR cell line generation. The concentrated TCR Va and TCR Vβ viruses were used to infect 2 × 10⁶ SKW-3-CD8AB cells with 5 μg/mL polybrene (Millipore) via centrifugation for 1 h at 15000 × g at 35 °C. The virus was removed, and the medium for cell culture was replaced with fresh medium. TCR-transduced SKW-3-CD8AB cells were enriched by sorting the GFP+ tdTOMATO+ cells.

Fig. 8 Structure of the peptide-binding site. a Crystal structures of three HLA-A*24:02:peptide complexes. HLA-A24 structures are drawn as ribbon models in gray/light purple, and peptide structures are colored in green (CoV2), magenta (229E), and cyan (HKU1). b Electron densities of the peptides. The electron density for each peptide is contoured at 1.0σ in the 2Fo-Fc omit map. Peptide structures are shown in green (CoV2, upper panel), magenta (229E, middle panel), and cyan (HKU1, bottom panel). c Superimposition of the three peptide structures. Peptide structures are depicted by the same colors as in (b). d Superposition of the 9-mer peptide structures in the HLA-A*24:02:peptide complexes. Peptides are shown in green (CoV2, this study), light gray (PDB code: 2BCK), and dark gray (PDB code: 3I6L).
Epitope identification and avidity analysis of TCR-transduced SKW-3-CD8AB cells. The transduced SKW-3-CD8AB cells were co-cultured with A24/CIR pulsed with the indicated peptide in a 1:1 ratio for 16 h. CD69 was measured to detect early T cell activation by flow cytometry. Graded titrations of the peptide (from 10 μg/mL to 10 fg/mL) were used to assess TCR avidity. TCR avidity was assessed using the molecular replacement method (MR) with Phaser53. The coordinates of the HLA-A*24:02 complex with the newly identified peptide (PDB ID: 4F7M) were used as the search model. The model was corrected and further refined using Phenix24 and Coot55. The Ramachandran plot indicated that 97.4%/2.6% for the CoV2 complex, 97.4%/2.6% for the 229E complex, and 97.7%, 2.3% for HKU1 complex are in favored and allowed regions. Data collection and refinement statistics are summarized in Table 1. ribbon models in the figures are depicted using PyMOL software (http://www.pymol.org).

Statistics and reproducibility. Statistical analysis was performed using StatMate V (Nihon 3B Scientific Inc.). Fisher’s/Chi-square tests were used to compare ratio between two groups. Data were analyzed using Mann–Whitney U-test for two independent groups and Turkey or Neuman–Keuls tests for non-parametric multiple comparisons. p < 0.05 was considered statistically significant.

The number of biological replicates or sample size are given in figure legend.

Table 1 Data collection and refinement statistics.

| Data collection          | HLA-A*24:02+CoV2 | HLA-A*24:02+229E | HLA-A*24:02+HKU1 |
|--------------------------|-----------------|-----------------|-----------------|
| Space group              | P2,2,2,1        | P2,2,2,1        | P2,2,2,1        |
| Cell dimensions          |                 |                 |                 |
| a, b, c (Å)              | 67.72, 85.47, 91.37 | 67.95, 85.59, 91.09 | 66.70, 77.72, 87.95 |
| α, β, γ (°)              | 90.00, 90.00, 90.00 | 90.00, 90.00, 90.00 | 90.00, 90.00, 90.00 |
| Resolution (Å)           | 1.89 (1.96-1.89) | 1.71 (1.77-1.71) | 2.10 (2.18-2.10) |
| Rmerge (%)               | 0.220 (2.797)   | 0.096 (2.250)   | 0.136 (2.179)   |
| Completeness (%)         | 98.73 (97.72)   | 99.99 (99.97)   | 99.96 (99.90)   |
| Redundancy (Å)           | 7.7 (7.8)       | 7.7 (7.6)       | 7.1 (7.3)       |
| Reflections              |                 |                 |                 |
| No. reflections          | 41,912          | 57,838          | 26,856          |
| Rwork/Rfree              | 0.200/0.255     | 0.192/0.221     | 0.203/0.264     |
| No. atoms                |                 |                 |                 |
| Protein                  | 3071            | 3071            | 3087            |
| Ligand/ion               | 94              | 93              | 91              |
| Water                    | 378             | 491             | 136             |
| B-factors                |                 |                 |                 |
| Protein                  | 32.1            | 30.3            | 52.9            |
| Ligand/ion               | 49.6            | 44.3            | 70.2            |
| Water                    | 37.9            | 37.9            | 50.3            |
| R.m.s. deviations        |                 |                 |                 |
| Bond lengths (Å)         | 0.007           | 0.007           | 0.008           |
| Bond angles (°)          | 0.86            | 0.83            | 0.93            |

*Values in parentheses are for highest-resolution shell.

Epitope expression and purification. HLA-A24 a chain and β-2-microglobulin with an N-terminal histidine tag and a TEV protease cleavage site were co-expressed in a cell-free expression system43,44. The cell-free reaction mixture was with an N-terminal histidine tag and a TEV protease cleavage site were co-expressed in a cell-free expression system43,44. The cell-free reaction mixture was 10 μg/mL to 10 fg/mL) were used to assess TCR avidity. TCR avidity was measured to detect early T cell activation by flow cytometry. Graded titrations of the peptide were calculated by estimating the peptide concentration required to reach one-half maximum of CD69 MFI reached in our assay.

Cytotoxicity assays. CD8+ T-cell lines were cultured for three weeks and then harvested as effector cells. T-cell cytotoxicity assays were performed using the following methodology: CFSE (Molecular Probe)-labeled A24-CIR cells were pulsed with the indicated peptide or DMSO for 2 h and washed twice. Effector cells were cultured with 1 x 10⁷ target cells, at effector/target cell ratios of 12.5, 25, and 50, for 6 h and then stained with TO-PRO3 (Molecular Probe) immediately prior to their analysis to identify dead cells. Spontaneous target cell death (SD) was determined by labeling the target cells that were cultured alone. As a positive control for total cytotoxicity (TD), the labeled target cells were permeabilized with BD Cytofix/Cytoperm reagent (BD Pharmingen). Specific lysis was calculated using the following formula: (Sample – SD/TD – SD) x 100. The cells were analyzed using flow cytometry.

Protein expression and purification. HLA-A24 a chain and β-2-microglobulin with an N-terminal histidine tag and a TEV protease cleavage site were co-expressed in a cell-free expression system43,44. The cell-free reaction mixture was prepared with 0.15 mg/mL of each peptide (CoV2, 229E, and HKU1). The HLA-A*24:02-peptide complexes were purified via chromatography using a HisTrap column (Cytiva) and subjected to TEV protease digestion. Protein solutions were then applied to the HisTrap column to remove the histidine-tag. The through fractions were subsequently purified using Hitrap Q and Superdex200 gel filtration chromatography (Cytiva). The HLA-A*24:02-peptide complexes were concentrated in 20 mM Tris-HCl buffer (pH 8.0) containing 50 mM NaCl.

Crystallization and data collection. Crystallization was performed using the sitting-drop vapor-diffusion method at 293 K. The HLA-A*24:02-CoV2 peptide complex crystals were grown in 1.0 M Na/K phosphate (pH 8.2) at a concentration of 6.6 mg/mL. The HLA-A*24:02-229E peptide complex crystals were grown in 0.2 M sodium citrate and 20% PEG3350 at a concentration of 6.9 mg/mL. The HLA-A*24:02-HKU1 peptide complex crystals were grown in 0.1 M Tris-HCl (pH 8.5) and 25% PEG3350 at a concentration of 2.4 mg/mL. Data collection was carried out at 100 K with 10% glycerol as a cryoprotectant. Data were collected at 1.000 Å. The diffraction data were processed and scaled using the ZOOGAMO: Automated Data Processing System for Microcrystals50,51 and the CCP4 software suite52.

Data availability. The structures of HLA-A*24:02-peptide (CoV2, 229E, and HKU1) complexes were deposited in the RCSB Protein DataBank (PDB) under accession codes 7EJL, 7EJM, and 7EIN respectively. The TCR sequence data were deposited in the BioProject database (PRJNA779816). All source data underlying the graphs presented in the main figures are available in Supplementary Data 1.

Received: 27 April 2021; Accepted: 12 November 2021; Published online: 02 December 2021

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Acknowledgements
We are deeply grateful to all the patients and healthy volunteers who contributed to this research. We would like to thank Drs. Yoshiko Inoue and Toshio Kawakita (National Hospital Organization Kumamoto Medical Center) for sample collection as well as medical evaluation, and Masami Kawamura (RIKEN) for technical support in human in-vitro assays. We would like to thank Mio Inoue, Kazuharu Hanada, Mayumi Yonemochi, and Kazuhiko Katsumura for help with plasmid preparation and protein expression studies. We acknowledge the technical support of the Biomedical Research Core of the Tohoku University Graduate School of Medicine. We also thank Toshiki Hosaka, Kentaro Ibara, and the beamline staffs at SPring-8 (BL26B2 and BL32XU) for their assistance during data collection and processing.

Author contributions
S.F. and K.S. conceptualized the work and strategy, planned and analyzed human experiments, and wrote the paper. N.H. and M.H. collected patient data and samples and conducted medical evaluation of patients with hematological malignancies. A.S., U.T. and K.S. performed the isolation of PBMCs and generation of CTL and cellular analysis. M.O. prepared the SKW-3-CD8AB line and provided information about TCR
cloning, and H.N. performed these experiments. A.S. and K.S. established TCR-transduced SKW-3-CD8AB and performed the related functional assays. M.K.M., K.M., and M.S. performed structure analysis of the HLA-peptide complex and interpretation of the interaction of the TCR and HLA-peptide complex.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s42003-021-02885-6.

Correspondence and requests for materials should be addressed to Shin-ichiro Fujii.

Peer review information Communications Biology thanks the anonymous reviewers for their contribution to the peer review of this work. Primary Handling Editors: Joanna Hester and George Inglis.

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