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

Kanako Shimizu

RIKEN Center for Integrative Medical Science (IMS)

Tomonori Iyoda

RIKEN Center for Integrative Medical Science (IMS)

An Sanpei

RIKEN Center for Integrative Medical Science (IMS)

Hiroshi Nakazato

RIKEN Center for Integrative Medical Science (IMS)

Masahiro Okada

RIKEN Center for Integrative Medical Science (IMS)

Shogo Ueda

RIKEN Center for Integrative Medical Science (IMS)

Miyuki Kato-Murayama

RIKEN Center for Biosystems Dynamics Research

Kazutaka Murayama

RIKEN Center for Biosystems Dynamics Research

Miyako Shirouzu

RIKEN Center for Biosystems Dynamics Research

Naoko Harada

National Hospital Organization Kumamoto Medical Center

Michihiro Hidaka

National Hospital Organization Kumamoto Medical Center

Shin-ichiro Fujii (✉ shin-ichiro.fujii@riken.jp)

RIKEN Center for Integrative Medical Science (IMS)

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Abstract

SARS-CoV-2-specific CD8⁺ T cells are detectable in infected individuals but are low in unexposed healthy donors (UHD). Little is known about whether pre-existing human coronavirus (HCoV)-specific CD8⁺ T cells are converted to functionally competent T cells cross-reactive to SARS-CoV-2. Induction of cross-reactive immunity requires the recognition of multiple epitopes. Here, we show that SARS-CoV-2-specific T cells elicited in response to a selected dominant epitope are multifunctional and respond to various HCoVs in UHD. TCRαβ chains from each T cell clone were identified; TCRαβ-transduced T cells responded broadly to the relevant epitopes on several HCoVs, thus implying that TCRαβ may exhibit selective diversity at the single-cell level. We further defined four sets of optimal SARS-CoV-2-peptides and demonstrated the response of CD8⁺ T cells even in hematological malignant patients. Together, the proposed epitopes inducing pre-existing CD8⁺ T cells to cross-react with SARS-CoV-2 may be beneficial in vaccine development.

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for causing the COVID-19 pandemic. Recently, mRNA or DNA vaccines have been developed against SARS-CoV-2; however, further studies are required to develop treatment strategies to attenuate the severity of the disease. Studies on humoral immunity against SARS-CoV-1 have provided evidence as to how long the neutralizing antibody response persists¹. The insufficient antibodies (Ab) fail to block the viral entry, aggressive virus-associated lung pathology, recurrence of viral infection, or cytokine storm². Generally, CD8⁺ cytotoxic T cells have the potential to eliminate virus-infected cells, even during the latency of viruses, such as HIV, CMV, EBV, and HSV³⁻⁵. To completely block viruses by vaccination, the vaccine requires CD4⁺ T cells and neutralizing Abs, as well as CD8⁺ T cells⁶.

Immunodominant epitopes from structural protein, such as spike (S), membrane (M) and nucleocapsid (N) protein, or non-structural protein, such as NSP7, NSP13 encoded on ORF1, of SARS-CoV-2 have been predicted *in silico*^{7,8}. SARS-CoV-2-specific CD8⁺ T cells with non-structural and structural proteins can be detected in infected patients and convalescent individuals⁹⁻¹⁴. The CD8⁺ T cell responses may be associated with disease severity^{13,15,16}. A recent report demonstrated that SARS-CoV-2 memory CD8⁺ T cells are detected over 6 months after the onset of the symptoms in 50-70% of the convalescent patients, and their responding epitopes are present on S, M, N and ORF3a^{17,18}.

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 (UHD)⁹, but may still be biologically relevant¹⁴. In UHD, the majority of immunodominant epitopes are derived from the non-spike regions, ORF1a and N, but not the S protein region^{9,19}, because of low homology of the amino acids in the S protein. Thus, the cross-reactivity of the CD8⁺ T cells for the S protein has not been studied sufficiently. The identification of effective epitopes for cross-reactivity from UHD is one of the key challenges for the development of

vaccines that can enhance SARS-CoV-2 cross-reacting CD8⁺ T cells as well as a diagnostic tool. However, the cross-reactivity at the TCR level has not yet been studied.

Patients with hematological malignancies (HM) are vulnerable to infectious diseases, including SARS-CoV-2 infections^{18,20}, and exhibit higher mortality than those with solid tumors. The decrease in the number of CD8⁺ T cells is correlated to high SARS-CoV-2 viral load. Despite an extremely low B cell or Ab production and optimum CD8⁺ T cell frequency, the COVID-19-related mortality is decreased¹⁸. Therefore, developing a CD8⁺ T cell-targeted vaccine is critical for HM-patients. Here, we explored several epitopes and optimized one on the S protein for the generation of SARS-CoV-2 cross-reacting CD8⁺ T cells under the restriction of one of the major HLA, HLA-A24. Using this epitope, we examined the mechanism underlying the cross-reactivity of these proteins to seasonal coronaviruses in a degree dependent manner at the single-cell TCR level. In addition, we characterized SARS-CoV-2 specific CD8⁺ T cells in UHD and HM-patients.

Results

Candidates of class I epitopes of SARS-CoV-2 S protein for T cell response *in silico*

To understand the relationship between HLA alleles and COVID-19 in terms of the immune response against SARS-CoV-2, we compared two prominent HLA class I epitopes, HLA-A*02:01 and A*24:02. There was an inverse correlation between the positive cases and death, and the HLA-A*24:02⁺ population in the indicated countries (**Fig. 1a, 1b and 1c**), but not the HLA-A*02:01⁺ population (**Extended Data Fig. 1a, 1b and 1c**). We, therefore, focused on the interaction between HLA-A*24:02 and CD8⁺ T cells cross-reactive for SARS-CoV-2. The candidate targets for the CD8⁺ T immunodominant epitope in SARS-CoV-2 S protein 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 the N protein as a positive control (**Fig. 1d**)⁷.

Screening the optimal peptide and quantitation of SARS-CoV-2-specific CD8⁺ T cell responses from COVID-19 seronegative UHD

For the initial screening of the optimal peptide for the generation of SARS-CoV-2 CD8⁺ T cell responses using an *ex vivo* method, five HLA-A*24:02⁺ UHDs, negative for IgG and IgM Ab specific for SARS-CoV-2, were recruited in this study in 2020 (**Table S1**). To assess the CD8⁺ T cell response, peripheral blood mononuclear cells (PBMCs) were cultured in the presence of each peptide, and the frequencies of epitope specific IFN-γ-producing CD8⁺ T cells were assessed. Although these six peptides are expected to bind to HLA-A*24:02 with high affinity, five of these did not result in a T cell response; only CoV-2 pep#3-specific CD8⁺ T cell counts were elevated in all the patients (**Fig. 1e and 1f**). Intriguingly, CoV-2 pep#3 exhibits high sequence homology with other coronaviruses, including four types of seasonal coronaviruses (**Extended Data Fig. 1d**). These results suggest that the CD8⁺ T cells may be cross-reacting, pre-existing memory CD8⁺ T cells rather than newly generated CoV-2 pep#3-specific CD8⁺ T cells. This further

implies that even high-affinity peptides restricted by HLA-A24 cannot easily elicit the induction of CD8⁺ T cells.

Next, we analyzed the responsiveness of CoV-2 pep#3 for T cells in terms of frequency and polyfunctionality by comparing them to those for EBV and CMV (**Fig. 2a, 2b, 2c and 2d, Extended Data Fig. 2**). To screen the epitope, we established an *ex vivo* T-cell assay using definite peptide-based epitopes namely, Covid2 antigen activation induction method (CovIM), which can detect IFN- γ - and TNF- α -producing T cells after restimulation as multifunctional attributes. We comparatively analyzed the results of the CovIM assays for the robust identification of the antigen specific CD8⁺ T cells. Next, we measured the magnitude of SARS-CoV-2 cross-reactive CD8⁺ T-cell responses, and the responders were separated into two groups, namely, fast and late responders, assessed one or three weeks after the initial culture. Most of the responders, whether fast or late, had IFN- γ -and TNF- α -producing polyfunctional T cells, while some had single positive T cells (**Fig. 2b and 2d**). The late responder group showed a high (>4%) or median (1–4%) frequency of SARS-CoV-2 cross-reactive CD8⁺ T cells. We also compared the frequency of the CoV-2 pep#3-responsive CD8⁺ T cells to that of CMV and EBV in the same participants, using HLA-A24-restricted CMVpp65 or EBV-LPM2 peptides having high affinity. The results demonstrated that cross-reactive T cells for CoV-2 pep#3 were present in 3/10 participants and 8/10 participants, whereas for the CMV peptide in 1/10 participants and 3/10 participants, and EBV peptide in 1/10 participants and 3/10 participants at 1 and 3 weeks, respectively (**Fig. 2b, 2d, and 2e**). These findings indicate that the frequency of cross-reactive CD8⁺ T cells for SARS-CoV-2 was not lower than that for CMV and EBV. Furthermore, we verified that the CoV-2 peptide-specific CD8⁺ T cell lines show cytotoxic activity against CoV-2 pep#3 pulsed A24/CIR, but not the unpulsed peptide (**Fig. 2f**). In conclusion, the frequency and function of SARS-CoV-2-cross-reactive memory CD8⁺ T cells were better than those of CMV and EBV.

Assessment of SARS-CoV-2-specific memory CD8⁺ T cell frequency in patients with HM

As the UHD may be asymptomatic carriers with non-apparent infection or latent infection, we examined the PBMCs from 11 completely unexposed healthy controls (collected between 2004-2010, the SARS-CoV-2 unexposed era) Next, we focused on 11 completely unexposed healthy controls (collected between 2004-2010, the SARS-CoV-2 unexposed era) (**Table S1**). To raise the bottom of the immune response, CD8⁺ T cells were isolated and cultured with CoV-2 pep#3-pulse irradiated autologous PBMCs for one week and again restimulated with this feeder for another week. We detected high epitope-specific CD8⁺ T cell responses in 8 out of 11 (72.7%) UHDs on day 14 (**Fig. 3a, 3b, 3c, and 3d**). Next, we examined whether CoV-2 pep#3 can induce epitope-specific CD8⁺ T cells in HM-patients (**Table S2**), who represent a high-risk group that may be susceptible to COVID-19 due to immune-cell dysfunction related to disease progression or chemotherapy. Epitope-specific CD8⁺ T cells were observed in 14.8% (4 out of 27) of the HM-patients and the induction ratio of CoV-2 pep#3-specific CD8 T cells was significantly lower in HM-patients compared to that in the UHDs ($p < 0.01$) (**Fig. 3e, 3f, 3g, and 3h**). This indicates that the levels of SARS-CoV-2 cross-reacting memory CD8⁺ T cells against seasonal coronaviruses are lower in HM-patients than in UHD.

Cross-reactivity of SARS-CoV-2-specific CD8⁺ T cells to other coronaviruses

To study the cross-reactivity for SARS-CoV-2-specific CD8⁺ T cells, we established CD8⁺ T cell lines. Furthermore, to determine whether the CD8⁺ T cells generated by CoV-2 pep#3 from UHD cross-reacted with other coronavirus families and quantify SARS-CoV-2-specific CD8⁺ T cells, we used CD8⁺ T cell lines from 10 UHD and two HM-patients (**Fig. 4a**). The polyfunctionality of the CD8⁺ T cell line was assessed by the production of IFN- γ and TNF- α in response to the relevant peptide of each virus as the mode of cross-reactivity of the CD8⁺ T cells for the coronavirus families (**Fig. 4b**). The CD8⁺ T cell line responded to betacoronaviruses (SARS-CoV-1, MERS, HKU-1 and OC43) with 83–100% responders and to alphacoronaviruses (NL63, 229E) with 58.3–66.7% responders (**Fig. 4c**). Six out of 12 donors showed a certain degree of response to all coronaviruses, whereas two showed a response against betacoronaviruses but not alphacoronaviruses. The cross-reactivity of the CD8⁺ T cell line for the HCoV family showed different patterns (**Fig. 4d**).

Next, we monitored the cross-reactivity of CD8⁺ T cells to coronaviruses in UHD by inducing the cells using two approaches, namely, by priming with CoV-2 peptide and other coronavirus-specific peptide mixtures (**Fig. 4a and 4e**). The CD8⁺ T cells elicited peptides from seasonal coronaviruses responded to SARS-CoV-2, predominantly with high reactivity to seasonal betacoronaviruses (**Fig. 4b and 4f**), suggesting that UHD1 probably may have been previously infected with one or more seasonal betacoronaviruses. In contrast, CD8⁺ T cells elicited by peptides from seasonal coronaviruses had high reactivity to seasonal alphacoronaviruses (**Fig. 4b and 4f**), thus indicating that UHD8 was previously infected in seasonal alphacoronaviruses, but not betacoronaviruses. Evaluation of T cell responses for HKU1 and 229E showed that the extent of T cell response to seasonal coronaviruses differed irrespective of their similarity to SARS-CoV-2. Those infected with some types of seasonal coronaviruses had some pre-existing clones. The differences between UHD1 and 8 indicate that the distinction between these different T cell clones may rely on either the plural recognition of TCR repertoires of CD8⁺ T cells or the existence of multiple clones in the CD8⁺ T cell line, probably due to repetitive seasonal corona infections.

CoV-2 pep#3-specific TCR-expressing CD8⁺ T cell clones recognize different epitopes of other coronavirus families at the single-cell level

To understand why CD8⁺ T cells exhibit multiple responses to various coronaviruses and their underlying mechanisms, we studied the cross-reactivity of CD8⁺ T cells at the single TCR level. After stimulation with CoV-2 pep#3, we isolated the CD8⁺ T cells by gating the CD107a⁺CD8⁺ cells and performed single-cell analysis of the TCR repertoires. A total of 227 T cells were screened, and 44 TCR clonotypes were identified from five UHD and one HM-patient (**Extended Data Fig. 3a**). Furthermore, we focused on the dominant types of TCR from among the clonotypes and identified four types of TCR α and TCR β pairs from four donors (three UHD and one HM). To assess the specificity and functions of the TCR α and TCR β pairs, we transduced the TCR α and β genes into the SKW3-CD8AB (human T-ALL) cell line and demonstrated their peptide-specific response (**Fig. 5a**). Intriguingly, the four types of TCR repertoires from

UHD significantly varied from each other (**Fig. 5b**). TCR-T (TCR-1) cells from UHD2 responded well to all the epitopes derived from coronaviruses, whereas the TCR-T (TCR-2) cells from UHD8 responded only to CoV-1 and CoV-2. Furthermore, the TCR-T (TCR-3) cells from UHD6 responded to CoV-2, Cov1, NL63, and 229E while those (TCR-4) from HM16 responded to CoV-2, CoV-1, MERS, HKU1, and OC43, but not to NL63 and 229E (**Fig. 5c**). Thus, despite 1–3 amino acid substitutions at P1(Q), P3(I), P8(Y), and P9(I), the TCR-1 pair exhibited cross-reactivity. However, if each peptide was shifted back and forth in the array, these TCR pairs could not react with any peptides (**Extended Data Fig. 3b**). Thus, TCR $\alpha\beta$ chains from each T cell clone were identified in both UHD and HM. Some types of single TCR $\alpha\beta$ -transduced T cells responded broadly to the relevant epitopes on HCoV-229E, whereas others responded only to two or four peptides. These findings on the SARS-CoV-2-epitope-responding T cell repertoires imply that the potential TCR $\alpha\beta$ may be selective at the single-cell level.

Crystal structures of CD8⁺ T cell binding to HLA-A*24 :02 and TCR complex

The crystal structures of HLA-A*24:02 and three peptides, namely, CoV2, 229E, and HKU1, were determined (**Fig. 6a, Table S3**). These three complex structures were almost identical to each other and their rmsd values were 0.15 Å between HLA-A*24:02•CoV2 and HLA-A*24:02•229E, 0.41 Å between HLA-A*24:02•CoV2 and HLA-A*24:02•HKU1, and 0.41 Å between HLA-A*24:02•229E and HLA-A*24:02•HKU1. 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 on the equivalent position of the HLA-A*24:02 structure (**Fig. 6b**). Although the electron densities of the peptide backbones were observed, those of some side chains were partially poor, especially, 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 of P2, P3, P6 and P9 positions were involved in HLA-A*24:02•peptide interactions at the bottom of the binding pocket. In contrast, the side chains of the P1, P4, P5, P7 and P8 positions were directed to the solvent region and oriented differently in the complex structures (**Fig. 6c**). 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. The peptide lengths are in the range of 8-mer to 11-mer. Of these, two complex structures are with a 9-mer peptide²¹. Comparison between these 9-mer peptides and the peptides in this study revealed that all the peptides anchor to the binding pocket at P2 and P9 positions, and conformational variations are observed at the P4-P7 positions (**Fig. 6d**). This region forms a bulge structure and corresponds to the putative interaction site with TCR. Furthermore, we investigated the influence of the peptide sequence on the interface and the intrinsic plasticity of the TCR/pMHC trimolecular and pMHC biomolecular complexes by structural modeling performed by replacing the HLA moiety of the HLA•peptide•TCR complex structure²² (PDB code 3VXM) with the structure mentioned in our study.

Analysis of the high affinity CoV-2-epitopes binding to HLA-A*24:02 eliciting multiple SARS-CoV-2-specific T cells in patients with HM

Although most of the UHD responded to the CoV-2 pep#3 epitope, HM-patients did not (**Fig. 3**). Thus, we searched for other hot spots surrounding the epitope region, including candidates for the restriction of HLA-A*24:02 by *in silico* screening using NetMHC and IEBD (**Fig. 7a, 7b**), and found four and six types of 9-mer-epitopes with high scores by NetMHC and IEBD expressed on SARS-CoV-2 as high-affinity functional hot spots. From the aspect of the sequence of seasonal coronaviruses, we detected 7 and 7 epitopes for HKU1, 6 and 4 epitopes for OC43, 5 and 3 epitopes for NL63, and 5 and 6 epitopes for 229E, by NetMHC and IEBD, respectively (**Fig. 7b**). To utilize all of these regions, which are available for many people, we synthesized four types of 11 mer-overlapped 15-mer peptides (**Fig. 7b**). Since the relevant epitopes are conserved across the SARS-CoV-2 variant strains, such as VOC-202012/01, 501Y.V2, 501Y.V3, the peptide set is available for SARS-CoV-2 variant strains. Surprisingly, we detected CoV-2-specific CD8⁺ T cells in UHDs and HM-patients (100% vs 65.4%, $p < 0.01$) (**Fig. 7c and 7d**). The T-cell response in the presence of a mixture of four peptides was more efficient than that of the 9-mer peptide especially in HM-patients (65.4% vs 14.8%, $p < 0.001$). When CD8⁺ T cells were rechallenged with each 15-mer peptide to test the epitopes, CD8⁺ T cells responded mainly to 15-mer pep#2 and #3, but in some cases, they also responded to 15-mer pep#1 and #4 (**Fig. 7e, 7f, and 7g**).

Next, to verify the parts of epitopes from four types of 15-mer peptide mixtures responsible for CD8⁺ T cell activation, a consecutive 9-mer peptide library was synthesized using the S protein sequence. We assessed the peptide-specific T cell response by restimulation with a 9-mer-peptide library after priming with 15-mer peptides (**Fig. 8a**). We detected 9-mer-peptide specific CD8T cell responses from #1204 to #1218 peptides comparable to 15-mer pep#2 to #3, which are included in CD8⁺ T cells using 15-mer libraries (**Fig. 8b and 8c**).

Discussion

The S protein region in SARS-CoV-2 is immunodominant in memory CD8⁺ T cells in infected or convalescent patients⁷⁻¹⁴. Nevertheless, it exhibits a low degree of homology with the S protein of seasonal coronaviruses, and therefore, epitopes outside of the S protein region have been shown to be involved in inducing the pre-existing CD8⁺ T cell response^{9, 10}. An analysis of the pre-existing and induced CD8⁺ T cells in three patients pre- and post-SARS-Cov-2 infection indicated that the generation of not only induced memory T cells post-infection but also cross-reactive memory T cells from pre-existing T cells is important for protection against COVID-19¹⁴. However, the cross-reactivity in the pre-existing CD8⁺ T cells has not yet been demonstrated at the TCR level. In the current study, we focused on an amino acid sequence conserved between SARS-CoV-2 and seasonal coronaviruses in the S2 region, selected 9-mer peptides, and demonstrated high levels of memory CD8⁺ T cells that exhibit cross-reactivity toward seasonal coronaviruses in UHD. Furthermore, we demonstrated the pattern of TCR cross-reactivity at the single-cell level and the difference in activity between α and β types of HCoVs due to the structure of HLA-

A*24:02 and 9-mer peptide. However, most HM-patients, failed to generate CD8⁺ T cells in response to this peptide. In silico analysis revealed that the area surrounding 9-mer peptide region has a high concentration of peptide candidates that elicit the production of CD8⁺ T cells in an HLA-A*24:02 restriction; thus, we extended and established 15-mer mixed peptides. There was a concentration of the identical immunodominant regions, including high-affinity epitopes of seasonal coronaviruses under the HLA-A*24:02 restriction. We then examined the CD8⁺ T-cell response using 15-mer peptide mixtures and successfully induced the generation of CD8⁺ T cells against SARS-CoV-2 in many HM-patients. This may imply that if the cross-reactive memory CD8⁺ T cells with high affinity were expanded in immunocompromised hosts, the immune defense against COVID-19 may be strengthened. It also indicates the importance of HLA in COVID-19 pathogenesis and immune monitoring of patients. Thus, the 15-mer mixtures may act as CD8⁺ T-cell-induced type vaccines and might help develop herd immunity in specific populations in several countries.

The forms of T cell cross-reactivity were first proposed as the basis of “molecular mimicry” in which microbial peptides share structural features with host peptides that stimulate T cells. This leads to cross-reactivity with self-proteins, thereby initiating and/or perpetuating autoimmune disease²³. In a previous study, the cross-reactive CD8⁺ T cell population was observed in animal models by sequential heterologous viral infections with two distantly related arenaviruses, LCMV and Pichinde²⁴. However, this study did not involve the assessment of the CD8⁺ T cell clones or single-cell levels. In the current study, to characterize the cross-reactive CD8⁺ T cells and further elucidate the mechanism underlying the CD8⁺ T cell response against COVID-19 by cross-reacting with seasonal coronaviruses, we examined TCR at the single-cell clone level and the structure of HLA-A*24:02. Even at the single-cell level, cloned TCR α/β -T cells cross-reacted to several or all types of coronaviruses simultaneously, thus demonstrating the different patterns in individuals.

This restricted formula for linking HLA-peptide and TCR confers heterotypic cross-reactivity across SARS-CoV-2. Further, to elucidate the molecular basis of cross-reactive TCR α/β recognition between SARS-CoV-2 and seasonal coronaviruses, we showed the structure of the SARS-CoV-2 peptide and the relevant peptides of seasonal coronaviruses bound to the HLA-A*24:02 molecule (**Fig. 6**). These data on the HLA-A*24:02-peptide complex structures imply that the side chains of the P1, P4, P5, P7 and P8 positions interact with the TCR. The superimposed model (where the HLA moiety is replaced by the structure of 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 TCR α and TCR β , respectively. In the sequence alignment among TCR1-4, insertions/deletions were found in these loop regions, suggesting variety of affinities between HLA-A*24:02-peptide complex and TCRs. Furthermore, our data suggest that selective cross-reactivity of T cells in SARS-CoV-2 epitopes depends on combination of the structure mode of the peptide on the HLA-A*24:02 complex and the sequences/loop region the conformations of TCRs. 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-S2 and HLA-

A*24:02•seasonal coronavirus epitopes. It is also important to note that epitope conformation may be altered by TCR engagement.

Understanding the antigenic origin of the broadly cross-reactive epitopes is crucial for the development of SARS-CoV-2-specific CD8⁺ T cell vaccines. A strategy for selecting universal peptides for optimizing TCR with high affinity for antigen and selection of TCR would be required for CoV-2. There is a 40–48% homology in the region of the 15-mer mixture between SARS-CoV-2 and seasonal coronaviruses, which is much higher than that observed in the S1 region (12.3–16.4%), RBD (13.1–19.2%), and total S2 region (31.2–40.2%) (**Extended Data Fig. 4**). Furthermore, several high-affinity 9-mer epitopes of seasonal coronaviruses are concentrated in this region, indicating that it may be useful as a cross-reactive universal vaccine (**Fig. 7b**). The cross-reactivity of T cells for different viral species enables heterologous immunity to a pathogen after exposure to a nonidentical pathogen. Such heterologous immunity may mediate either beneficial or unbeneficial events. In the closely related viruses, such as the influenza families, CD8⁺ T-cell responses elicited by universally cross-reactive influenza vaccines show an anti-viral response for influenza A, B, and C viruses²⁵. Thus, we speculated that some CD8⁺ T cells against seasonal coronaviruses may persist as long-term memory in UHD. When optimal memory T cells were selected in vivo by administering a 15-mer-peptide mixture, the counts of memory T cells could be boosted by peptide therapy. The protective function of cross-reactive CD8⁺T cells can be evaluated by analysis using CoVIM.

Interestingly, when 15-mer peptides were used, CD8⁺ T cell induction against SARS-CoV-2 in patients with AML and CML was comparable to that against CMV and EBV even when patients with AML and CML were treated with chemotherapy. However, half of the patients with myeloma failed to generate CD8⁺ T cells possibly due to steroid therapy²⁶. Moreover, diseases including the impaired function of B cells or antibody production, for example, myeloma or lymphoma, deserve special attention. The duration after therapy, steroid therapy, and residual tumor burden should be cautioned for COVID-19. A recent study reported that when a patient with malignant B-cell lymphoma was diagnosed with COVID-19 and treated with an infusion of neutralizing antibody, variants of SARS-CoV-2 emerged²⁷. We speculate that if CoV-2 viruses persist in immunocompromised hosts for a long period, viruses cannot be eliminated, and new variants may appear. Progression or chemotherapy in HM affects the severity of symptoms and prognosis of COVID-19. After vaccine or antibody treatment, CD8⁺ T-cell targeting therapy would be useful in healthy people as well as in high-risk groups.

In summary, we found that the potential of cross-reactivity may depend on the interaction between TCR and the structure of specific HLA, for example, HLA- A*24:02. Moreover, some TCR α/β from SARS-CoV-2 reactive T cells may recognize different peptide epitopes, while others cannot. Lastly, the determination of optimal and long peptides as universal vaccines is required to broadly generate CD8⁺ T cells. These data may have implications in designing universal SARS-CoV-2 CD8⁺ T cell vaccines that elicit broadly cross-reactive CD8⁺ T cells. The vaccines for stably producing neutralizing antibodies as well as CD8⁺ T-cell

inductive vaccines against CoV-2 would help reduce the annual rates of SARS-CoV-2-induced morbidity and mortality globally.

Declarations

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Author contributions: SF and KS conceptualized the work and strategy, planned and analyzed human experiments and wrote the paper. NH and MH conducted patient data and sample collection as well as medical evaluation of patients with hematological malignancies. AS, US, TI and KS performed isolation of PBMCs and generation of CTL, and cellular analysis. MO prepared SKW-3-CD8AB line and provided the information of TCR cloning and HN performed these experiments. AS and KS established TCR-transduced SKW-3-CD8AB and performed their functional assay. MKM, KM and MS performed crystal analysis of HLA-peptide complex and interpretation of interaction of TCR and HLA-peptide complex.

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Figures

Figure 1

SARS-CoV-2 specific CD8+ T cell response in HLA-A*24:02 positive uninfected healthy donors a. Data on the HLA-A*24:02 positive population in the indicated countries. b, c. Correlation between HLA- A*24:02 and COVID-19 cases- cumulative total per 105 population (b) and COVID-19 deaths- cumulative total per 105 population (c) Pearson's correlation coefficients (Pearson's r) are shown. d. Selected peptides for CD8+ T cells. The peptides derived from the Spike (S) protein of SARS-CoV-2, shown in the list, were selected based on high-affinity binding (% rank) to HLA-A*24:02 by in silico prediction using NetMHC4.0. A previously reported peptide for nucleocapsid protein was selected as a positive control. e. Antigen-specific IFN-γ+ CD8+ T cells using CoVIM. PBMCs from UHD were cultured in the presence of seven types of peptides separately. The cultured cells were harvested and restimulated with DMSO or the same peptide three weeks later for intracellular IFN-γ-APC analysis by flow cytometry. Data shown are representative flow cytometric analyses (UHD2) gating on CD8+ T cells from five donors in independent experiments. f. Same as e but the comparison of peptide-specific CD8+ T cells was shown by the frequency of IFN-γ+ CD8+ T cell response to each peptide in the representative donor (left) and summarized in the five UHDs (right). The data are calculated by subtracting the frequency of the non-stimulated control (-pep). The black bar and other symbols represent the mean of the five UHDs and each data, respectively. *p<0.05 (Tukey's test)

Figure 2

Memory T cell response for SARS-CoV-2 (pep#3) versus CMV or EBV in HLA-A*24:02 positive UHD a-d. PBMCs from HLA-A*24:02 positive UHDs were cultured with the peptides of CoV-2 pep#3, CMVpp65, or EBV-LMP2, having high affinity to HLA- A*24:02 individually, and restimulated with the same peptides weekly. The polyfunctionality of peptide-specific CD8+ T cells was determined by the frequency of IFN-

γ +TNF- α + CD8+ T cells by intracellular cytokine staining on day 7 (a, b) and 21 (c, d). *Generation of peptide-specific CD8+ T cells was determined by a 1.5-fold increase in +pep vs. -pep as a responder. a, c. Data are representative of IFN- γ -APC and TNF- α -PE on CD8T cells in response to CoV-2 pep#3, CMV, or EBV on day 7 (A) and 21 (C). b, d. Generation of CoV-2 pep#3, CMV, and EBV-peptide specific CD8+ T cells at day 7 (B) and 21 (D) from three and seven UHDs, respectively, are summarized. e. Pie charts representing the proportions of responders or non-responders to CoV-2 pep#3, CMV, and EBV peptide. f. Peptide-specific cytotoxic activity of CoV-2 pep#3-specific CD8+ T cell lines.

Figure 3

Induction of SARS-CoV-2-specific CD8+ T cells from PBMCs of UHDs before the COVID-19 pandemic and patients with hematological malignancies (HM) a-h. CD8+ T cells were isolated from PBMCs of 11 UHDs who provided samples between 2004 and 2010 or 27 SARS-CoV-2-uninfected patients with HM. These were cultured with CoV-2 pep#3-pulsed irradiated autologous PBMCs and restimulated on day 7. Two weeks later, the CD8+ T cells were analyzed using intracellular IFN- γ and TNF- α staining after stimulating with or without the same peptide. *Generation of peptide-specific CD8+ T cells was determined by the 1.5-fold increase of +pep vs -pep as a responder. a, e. Flow cytometry data of IFN- γ -APC and TNF- α -PE in CD8+ T cell line from representative UHD (a) and patients (e). b, f. Generation of polyfunctional CoV-2 pep#3-specific CD8+ T cell in 11 UHDs (b) and 27 patients (f). c, g. Cytokine production pattern (IFN- γ +TNF- α +, IFN- γ -TNF- α +, IFN- γ +TNF- α -) of peptide-specific CD8+ T cells in responders of UHDs (c) and patients (g). d, h. Pie charts representing the proportions of responder or non-responder against CoV-2 pep#3 in UHD (d) and HM-patients (h).

Figure 4

Cross-reactivity of SARS-CoV-2-specific CD8⁺ T cells to other coronaviruses a. The cross-reactivity of CoV-2 pep#3-specific CD8⁺ T cell line from UHD or HM-patients to the relevant peptides of other coronaviruses (SARS-CoV-1, MERS, HKU1, OC43, NL63, and 229E). CD8⁺ T cell lines from UHD or HM-patients were co-cultured with the indicated peptide-pulsed A24/CIR for 16 h and analyzed using IFN-γ-APC and TNF-α-PE by intracellular staining. Representative data from UHDs and patient are shown. b, c. Frequency of responding donors for each peptide of the coronaviruses. d. Summary of data on cross-reactivity. The peptide-specific CD8⁺ T cell frequency was calculated by subtracting the frequency of the CD8⁺ T cells (–pep; DMSO). The ratio of each coronavirus-derived peptide-specific IFN-γ+TNF-α+ CD8⁺ T cell frequency to that of CoV-2 pep#3 is depicted. e, f. CD8⁺ T cell lines were established by culturing with a mixture of peptides, including HKU1, OC43, NL63, and 229E. The cross-reactivity was then assessed by challenging with each coronavirus peptide. The CD8⁺ T cell lines were co-cultured with the indicated peptide-pulsed A24/CIR for 16 h and analyzed using IFN-γ-APC and TNF-α-PE by flow cytometry (e). Representative data from two UHD are shown (f).

Figure 5

Cross-reactivity of TCR among (across) the coronaviruses a. To obtain functional antigen-specific TCR molecules from T cell repertoires of CoV-2 pep#3-specific CD8⁺ T cell line, TCR- α/β chains were cloned from CoV-2 pep#3-specific CD8⁺ T cells, followed by functional validation of the completed TCRs. The TCR-transduced SKW3-CD8AB cells were cocultured with the indicated peptide-pulsed A24/CIR for 16 h, and the upregulation of CD69 expression was measured by flow cytometry. b. CD69 expression (upper) and MFI (lower) of TCR-transduced SKW3-CD8AB. **p<0.01, ***p<0.001 (Newman-Keuls test) c. Summary of TCR-T responses in b are depicted.

Figure 6

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 the peptide structures are colored in green (CoV2), magenta (229E) and cyan (HKU1), respectively. b. Electron densities of the peptides. The electron density for each peptide is contoured at 1.0 σ in the 2Fo-Fc omit map. The peptide structures are shown in green (CoV2, upper panel), magenta (229E, middle panel), and cyan (HKU1, bottom panel),

respectively. c. Superimposition of the three peptide structures. The peptide structures are depicted by the same colors in the figure 6b. d. Superposition of the 9-mer peptide structures in the HLA-A*24:02•peptide complexes. Each peptide is shown in green (CoV2, this study), light gray (PDB code: 2BCK) and dark gray (PDB code: 3I6L), respectively.

Figure 7

Design of the broadly responding 15-mer peptide mixtures a. Global alignment of the Spike protein between SARS-CoV-2 and other coronaviruses and design of 15-mer peptide library including CoV-2 pep#3 epitopes in the S2 region of SARS-CoV-2. b. 9-mer peptide library selected from the region of 15-mer peptide mixture. In silico analysis revealed that the two lists indicate the high affinity of SARS-CoV-2 and the relevant peptide epitopes of seasonal coronaviruses to HLA-A*24:02, predicted by IEBD (upper) and NetMHC4.0 (lower). c-g. PBMCs from 20 UHD and 23 patients with hematological malignancies were cultured with 1-mer-mixture peptides and stimulated on day 7. Two weeks later, the cultured cells were restimulated with or without the same peptide mixtures, each 15-mer peptide for 16 h in the presence of monensin and brefeldin. The frequency of IFN-γ+TNF-α+ CD8+ T cells was measured after gating the CD8+ T cells by intracellular staining. c. Data show the frequency of IFN-γ+TNF-α+ CD8+ T cell response to 15-mer peptide mixture in UHD (upper) and patients with hematological malignancies (lower). *Generation of peptide-specific CD8+ T cells was determined by a 1.5-fold increase in +pep vs. –pep as a responder. d. Pie charts representing the proportions of responders or non-responders against 15-mer peptide mixture in UHD (left) and patients with hematological malignancies (right). e. Flow cytometry data from representative donors. f. Summary of the 15-mer peptide-specific CD8+ T cells. The peptide-specific CD8+ T cell frequency was calculated by subtracting the frequency of –pep (DMSO). Data indicate the ratio of the frequency of each 15mer peptide-responding IFN-γ+TNF-α+ CD8+ T cells to that of 15mer peptide-mixtures responding IFN-γ+TNF-α+ CD8+ T cells. g. Frequency of responding donors for each 15-mer peptide.

Figure 8

Epitope mapping in spanning 15-mer peptide mixture a. Design of 9-mer peptide library in the Spike protein 1200-1226. b. 15-mer mixture-specific CD8⁺ T cell lines were isolated and examined by epitope mapping using 9-mer peptide library. Flow cytometry data from representative donors is shown. c. Frequency of responding donors for each peptide of the 9-mer peptide library. d. The response to 9-mer peptide-specific CD8⁺ T cells selected from the 15-mer peptide mixture. Data indicate that the ratio of +pep vs -pep was 1.5 times greater (orange) and 3 times greater (red) in the 9-mer peptide, respectively. Gray indicates "not done".

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