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Brief Communication

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Patients With Mild COVID-19 Exhibit Low Functional Avidity of SARS-CoV-2 Membrane Protein-Reactive CD4⁺ T Cells

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ABSTRACT

Herein, we found that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-unexposed individuals exhibited an increased frequency of CD4⁺ T cells against SARS-CoV-2 membrane (M) protein, suggesting that SARS-CoV-2 M-reactive cells may be primed by previous infection with common cold coronaviruses (CCCoVs). We confirmed that CCCoV M-reactive CD4⁺ T cells cross-recognize SARS-CoV-2 M in unexposed individuals. Among coronavirus disease 2019 (COVID-19) convalescents and unexposed individuals, SARS-CoV-2 M-reactive CD4⁺ T cells exhibited significantly lower functional avidity than CD4⁺ T cells reactive to other viruses. Importantly, convalescents from mild COVID-19 had SARS-CoV-2 M-reactive CD4⁺ T cells with significantly lower functional avidity than convalescents from severe COVID-19. The current data suggest that pre-existing CCCoV M-specific memory CD4⁺ T cells may contribute to controlling SARS-CoV-2 infection by cross-reactivity, leading to mild disease but leaving memory cells with low functional avidity to SARS-CoV-2 M due to incomplete homology. These data provide indirect evidence that pre-existing cross-reactive CD4⁺ T cells contribute to protection from severe COVID-19.

Keywords: COVID-19; SARS-CoV-2; CD4-positive-T-lymphocytes; Cross-reactivity; Functional avidity

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection causes a broad spectrum of clinical symptoms, from asymptomatic or mild symptoms to severe pneumonia

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Conflict of Interest

The authors declare no potential conflicts of interest.

Abbreviations

BV, Brilliant Violet; CCCoV, common cold coronavirus; COVID-19, coronavirus disease 2019; CTV, CellTrace Violet; EC_{50}, half maximal effective concentration; FP, fusion glycoprotein F0; HCMV, human cytomegalovirus; HCoV, human coronavirus; HD, healthy donor; IAV, influenza A virus; ICS, intracellular cytokine staining; M, membrane; MFI, mean fluorescence intensity; MP1, membrane protein 1; N, nucleocapsid; NIH, National Institutes of Health; ns, not significant; OD, optical density; OLP, overlapping peptide; RSV, respiratory syncytial virus; RT, room temperature; S, spike; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

Author Contributions

Conceptualization: Kim AR, Koh JY, Peck KR, Choi JY, Choi WS, Jeong HW, Shin EC. Data

and acute respiratory distress syndrome, termed coronavirus disease 2019 (COVID-19) (1,2). Although COVID-19 has become an endemic disease, understanding T-cell responses against SARS-CoV-2 is important for preparedness against newly emerging viruses.

T cells contribute to viral control by producing effector cytokines and exerting cytotoxic activity during viral infection, and memory T cells are involved in protective immunity upon viral re-exposure. SARS-CoV-2-specific T cells are detected in patients with COVID-19 (3-6), and SARS-CoV-2-specific CD4+ T cells have been detected in almost 100% of COVID-19 convalescents in *ex vivo* stimulation-based assays (4,7-14).

In several studies, SARS-CoV-2-reactive T cells have also been detected among individuals with no prior SARS-CoV-2 infection or vaccination. SARS-CoV-2-reactive CD4+ T cells are present in 20% to 77% of individuals not exposed to SARS-CoV-2 (4,7-10,14-19). SARS-CoV-2 epitopes recognized by these T cells exhibit considerable levels of homology to endemic common cold coronaviruses (CCCoVs), including OC43, HKU1, 229E, and NL63 (9,10,14-19). These findings suggest that memory T cells primed by previous CCCoV infection are cross-reactive to SARS-CoV-2 proteins to some degree.

Pre-existing cross-reactive T cells have been demonstrated to play a protective role against COVID-19 (20). Pre-existing T cells targeting the highly conserved polymerase protein expand in seronegative individuals after SARS-CoV-2 exposure, leading to abortive infection (18). In addition, T cells specific for conserved coronavirus epitopes correlate with mild COVID-19 (17), and the frequency of baseline cross-reactive T cells correlates with better clinical outcomes following SARS-CoV-2 exposure (21). In an epidemiological study, recent CCCoV infection was associated with a reduced risk of severe COVID-19 (22).

Pre-existing cross-reactive T cells and *de novo* primed T cells during SARS-CoV-2 infection may have different TCR affinity for SARS-CoV-2 proteins. If someone has CCCoV-specific memory T cells that are cross-reactive to SARS-CoV-2 due to considerable levels of protein homology, pre-existing CCCoV-specific memory T cells will rapidly exert antiviral functions upon SARS-CoV-2 infection, leading to mild disease but leaving memory cells with low affinity for SARS-CoV-2 due to incomplete homology between CCCoVs and SARS-CoV-2. However, in the absence of pre-existing CCCoV-specific memory T cells, SARS-CoV-2-reactive T cells may derive from naïve precursor cells that have TCRs specific for SARS-CoV-2, leading to slower effector T-cell responses compared to the rapid responses by pre-existing cross-reactive memory T cells. This scenario may result in severe COVID-19, leaving memory cells with high affinity for SARS-CoV-2. Indeed, a delayed induction of SARS-CoV-2-specific T-cell responses has been found in patients with severe COVID-19 compared to patients with mild COVID-19 (23).

In the present study, we investigated the functional avidity of SARS-CoV-2-reactive CD4+ T cells as a surrogate of TCR affinity and compared it between convalescents from mild and severe COVID-19.

MATERIALS AND METHODS

Patients and specimens

A total of 50 convalescents previously diagnosed with SARS-CoV-2 infection were enrolled from Chungbuk National University Hospital, Samsung Medical Center, Severance Hospital,

curation: Kim AR, Koh JY. Formal analysis: Kim AR, Koh JY. Methodology: Kim AR, Koh JY, Rha MS, Jung JH, Ko JH, Choi HK, Jeon JH, Seok H, Park DW, Peck KR, Choi JY, Choi WS, Jeong HW. Resources: Ko JH, Choi HK, Jeon JH, Seok H, Park DW, Peck KR, Choi JY, Choi WS, Jeong HW. Supervision: Park SH, Shin EC. Validation: Kim AR, Koh JY. Writing - original draft: Kim AR, Koh JY, Shin EC. Writing - review & editing: Kim AR, Koh JY, Shin EC.

and Ansan Hospital in 2020 (**Supplementary Table 1**). None of them had been immunized with COVID-19 vaccines. SARS-CoV-2 RNA was detected in specimens from the patients' nasopharyngeal swabs by multiplex real-time RT-PCR using the Allplex 2019-nCoV Assay kit (Seegene, Seoul, Korea) or PowerCheck 2019-nCoV RT PCR kit (KogeneBiotech, Seoul, Korea). In the present study, we categorized patients into asymptomatic and symptomatic (mild to critical illness) as defined by the National Institutes of Health severity of illness categories (24). Peripheral blood was also obtained from 26 healthy donors (HDs), who had never been diagnosed with SARS-CoV-2 infection, before emergence of the COVID-19 pandemic. Informed consent was obtained from all donors and patients.

PBMCs were isolated by density gradient centrifugation using Lymphocyte Separation Medium (Corning, NY, USA). After isolation, the cells were cryopreserved in FBS (Corning) containing 10% DMSO (Sigma-Aldrich, St. Louis, MO, USA) until use.

ELISA

To detect human IgG directed against S proteins from different human coronaviruses (HCoV-OC43, HCoV-HKU1, HCoV-229E, and HCoV-NL63), each recombinant S1 protein was obtained from Sino Biological Inc. (Beijing, China) and in-house ELISAs performed. Briefly, 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA) were coated with 100 μl of 1 μg/ml S1 protein in PBS per well overnight at 4°C. The next day, the plates were washed with PBS containing 0.05% Tween-20 (Junsei Chemical Co.,Ltd., Tokyo, Japan) and blocked with 5% BSA in PBS containing 0.05% Tween-20 for 2 h at room temperature (RT). After blocking and washing, plasma was diluted 1:1,000 with 5% BSA in PBS. Diluted plasma was added and the plates incubated for 2 h at RT. The negative control was 5% BSA in PBS with no plasma. After washing, plates were incubated with 100 μl HRP-conjugated mouse anti-human IgG Ab (1:50,000; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at RT. Reactions were visualized by adding color development (R&D Systems, Minneapolis, MN, USA). The absorbance at 450 nm (optical density, OD) was measured by spectrophotometry (BioTek, Winooski, VT, USA). The detection threshold was determined by the OD value of the negative control.

Peptides

Pools of lyophilized 15-mer peptides with 11-amino-acid overlap, covering the SARS-CoV-2 spike (S), membrane (M), and nucleocapsid (N) proteins were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). The M and N pools included the complete sequences, and the S pool included immunodominant sequence domains. Peptide pools for human cytomegalovirus (HCMV) pp65, influenza A virus (IAV) membrane protein 1 (MP1), and respiratory syncytial virus (RSV) fusion glycoprotein F0 (FP) were purchased from JPT (Berlin, Germany). Pools were resuspended according to the manufacturer's instructions and cells stimulated at a concentration of 1 μg/peptide/ml.

For *in vitro* expansion of CCCoV M-specific T cells and cross-reactivity analysis, lyophilized 15-mer peptides with 10-amino-acid overlap, spanning the entire sequence of the M protein of CCCoVs (HCoV-OC43, HCoV-HKU1, HCoV-229E, and HCoV-NL63) and SARS-CoV-2 were purchased from Mimotopes (Melbourne, Australia). Each peptide was resuspended in distilled water containing 5% DMSO and pooled.

In vitro expansion and re-stimulation of CCCoV M-reactive cells

PBMCs from HDs seropositive for HCoV were labeled with CellTrace Violet (CTV; Invitrogen, Carlsbad, CA, USA) in PBS containing 5% FBS for 20 min at RT and suspended in AIM V

medium (Thermo Fisher Scientific) supplemented with 10 U/ml IL-2 (PeproTech, Cranbury, NJ, USA). Labeled cells (0.5×10^6 cells/well) were cultured in 96-well U-bottom plates and stimulated with the corresponding HCoV M peptide pool at a concentration of 1 μg/peptide/ml at 37°C. After 4 days of culture, the medium was exchanged with fresh medium containing 10 U/ml IL-2 and M pool (1 μg/peptide/ml). After an additional 3 days of culture, the medium was removed and the cells incubated for 24 h in fresh medium containing no cytokine or peptide. After the incubation, the cells were harvested and re-stimulated with the SARS-CoV-2 M peptide pool or the corresponding HCoV M peptide pool at a concentration of 1 μg/peptide/ml. An equimolar volume of DMSO was used as the negative control. After 1 hour of re-stimulation, intracellular cytokine staining (ICS) was performed as described below.

ICS and flow cytometry

PBMCs or *in vitro* expanded T cells were resuspended in complete medium (RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin) and 1×10^6 cells cultured per well in 96-well U-bottom plates (Corning) with the relevant peptide pool (each at 1 μg/ml). After 1 h of stimulation, brefeldin A (1 μl/ml; BD Biosciences, Franklin Lakes, NJ, USA) was added. After incubating for 5 h at 37°C, cells were washed in PBS supplemented with 2% FBS and 2 μM EDTA (FACS buffer) and stained for 20 min at 4°C with fluorochrome-conjugated Abs for specific surface markers. Dead cells were stained using LIVE/DEAD red fluorescent reactive dye (Invitrogen). The cells were then washed with FACS buffer and fixed/permeabilized using the FoxP3/Transcription Factor Staining Buffer Set (Invitrogen). Intracellular cytokine proteins were detected by the addition of fluorochrome-conjugated Abs for 20 min at RT. Multi-color flow cytometry was performed using an LSR II instrument (BD Bioscience) and the data analyzed by FlowJo software (FlowJo, LLC, Ashland, OR, USA).

Abs

The following fluorochrome-conjugated mAbs were used for multicolor flow cytometry: anti-CD3-Brilliant Violet (BV) 786 or -PE-Cy7 (565491 or 563423; clone UCHT1), anti-CD4-APC-Cy7 or -BV650 (566319 or 563875; clone SK3), and anti-CD8-BV510 (563919; clone SK1; all from BD Bioscience). For intracellular staining, we used the following Abs: anti-IFN-γ-APC or BV711 (554702 or 564039; clone B27; BD Bioscience), anti-TNF-FITC or PE-Cy7 (11-7349-82 or 25-7349-82; clone Mab11), and anti-IL-2-PE or PerCP-eFluor710 (12-7029-42 or 46-7029-42; clone MQ1-17H12; all from eBioscience, San Diego, CA, USA).

Measurement of functional avidity

To measure the functional avidity, PBMCs were stimulated with 4-fold serially diluted peptide concentrations (1, 0.25, 0.0625, 0.0156, 0.0039, 0.0009 μg/peptide/ml) and analyzed by ICS. The peptide concentrations required for a half-maximal response (EC_{50} values) were calculated from the dose-normalized response curves using GraphPad Prism (GraphPad Software, San Diego, CA, USA). The functional avidity values were determined as $Log(EC_{50})$.

Statistical analysis

Statistical analyses were performed using GraphPad Prism. Significance was set at $p<0.05$. The Wilcoxon signed-rank test was used to compare data between 2 paired groups and the Mann-Whitney U test to compare data between 2 unpaired groups. The analytical methods are described in the corresponding figure legends.

RESULTS AND DISCUSSION

SARS-CoV-2 M protein-reactive CD4⁺ T cells are frequently observed in unexposed individuals

First, we performed ICS assays for IFN-γ, TNF, and IL-2 after *ex vivo* stimulation of PBMCs from COVID-19 convalescents with overlapping peptide (OLP) pools covering SARS-CoV-2 S, M, and N proteins and analyzed the CD4⁺ T-cell responses (**Supplementary Fig. 1**). As expected, the frequencies of IFN-γ⁺ cells in S, M, or N-stimulated cells were significantly higher than the frequencies in non-stimulated controls (**Fig. 1A and B**). Similar results were obtained when TNF⁺ or IL-2⁺ cells were evaluated. In CD8⁺ T cells, the frequencies of IFN-γ⁺ cells in S, M, or N-stimulated cells were also significantly higher than the frequencies in non-stimulated controls (**Supplementary Fig. 2A**).

We also examined SARS-CoV-2-reactive CD4⁺ T-cell responses using prepandemic PBMCs banked before the emergence of COVID-19. The frequency of IFN-γ⁺ or TNF⁺ cells in M-stimulated cells was significantly higher than the frequency in non-stimulated controls, though the frequency of IL-2⁺ cells was not significantly different (**Fig. 1C and D**). However, S or N stimulation did not result in higher frequencies of IFN-γ⁺, TNF⁺, or IL-2⁺ cells compared to non-stimulation controls. In CD8⁺ T cells, the frequencies of IFN-γ⁺ cells in

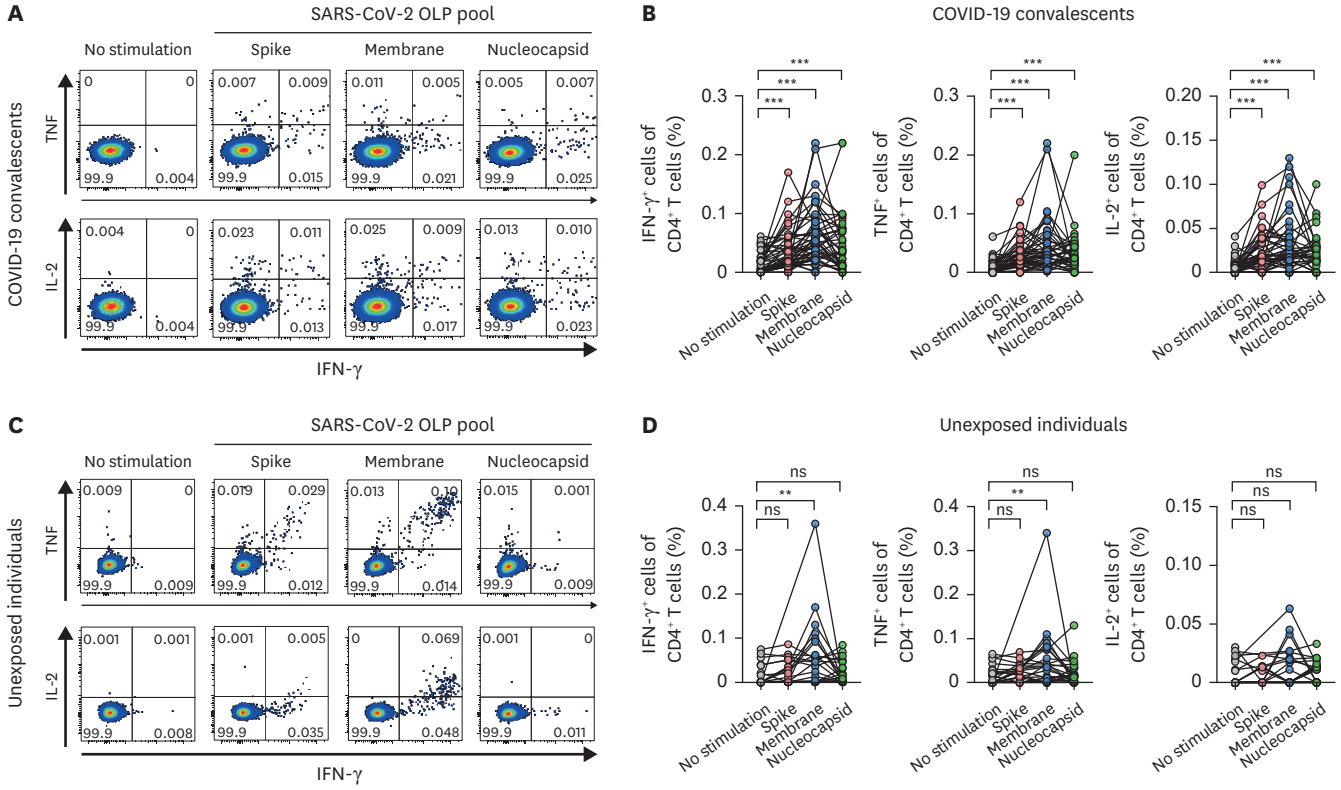

Figure 1. Cytokine production by SARS-CoV-2-reactive CD4⁺ T cells in COVID-19 convalescents and unexposed donors. PBMCs from COVID-19 convalescents (A, B) and unexposed donors (C, D) were stimulated with SARS-CoV-2 protein peptide pools (spike, membrane, or nucleocapsid). Cytokine-producing T cells were detected by intracellular cytokine staining after stimulation. (A) Representative flow cytometry plots and (B) summary data of the frequencies of IFN-γ⁺, TNF⁺, or IL-2⁺ cells among CD4⁺ T cells against spike (n=46), membrane (n=50), and nucleocapsid (n=40) pools from COVID-19 convalescents. (C) Representative flow cytometry plots and (D) summary data of the frequencies of IFN-γ⁺, TNF⁺, or IL-2⁺ cells among CD4⁺ T cells against spike (n=23), membrane (n=26), and nucleocapsid (n=26) pools from unexposed individuals. Data in (B, D) are presented with lines connecting data from the same individuals.
ns, not significant.
**p<0.01, ***p<0.001, Wilcoxon signed-rank test.

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S, M, or N-stimulated cells were comparable to the frequencies in non-stimulated controls (**Supplementary Fig. 2B**). Collectively, these data indicate that SARS-CoV-2 M-reactive CD4⁺ cells exist in individuals without prior infection with or vaccination for SARS-CoV-2.

CCCoV M-specific CD4⁺ T cells are cross-reactive to SARS-CoV-2 M in SARS-CoV-2-unexposed individuals

We wondered whether prior infection with CCCoVs can explain the presence of SARS-CoV-2 M-reactive CD4⁺ T cells in SARS-CoV-2-unexposed individuals. To this end, we examined serum IgG specific to CCCoVs, such as OC43, HKU1, 229E, and NL63, among SARS-CoV-2-unexposed HDs and found that all of the HDs had high IgG titers over the cut-off against all 4 CCCoVs (**Fig. 2A**), indicating that they commonly had prior infections with CCCoVs. Next, we directly examined whether CCCoV M-specific CD4⁺ T cells are cross-reactive to SARS-CoV-2 M protein. We generated CCCoV M-specific short-term (1-wk) T cell lines by stimulating CTV-labelled PBMCs from SARS-CoV-2-unexposed HDs with CCCoV M OLPs. We selected a species of CCCoVs for the OLPs according to the species with the highest IgG titer in each HD. We performed IFN-γ ICS assays by stimulating 1-week T cell lines with either the CCCoV M OLPs that were used for the generation of a T cell line or SARS-CoV-2 M OLPs. We detected IFN-γ⁺ cells among CTV^{lo}CD4⁺ T cells following CCCoV M OLP stimulation, and they were detected at

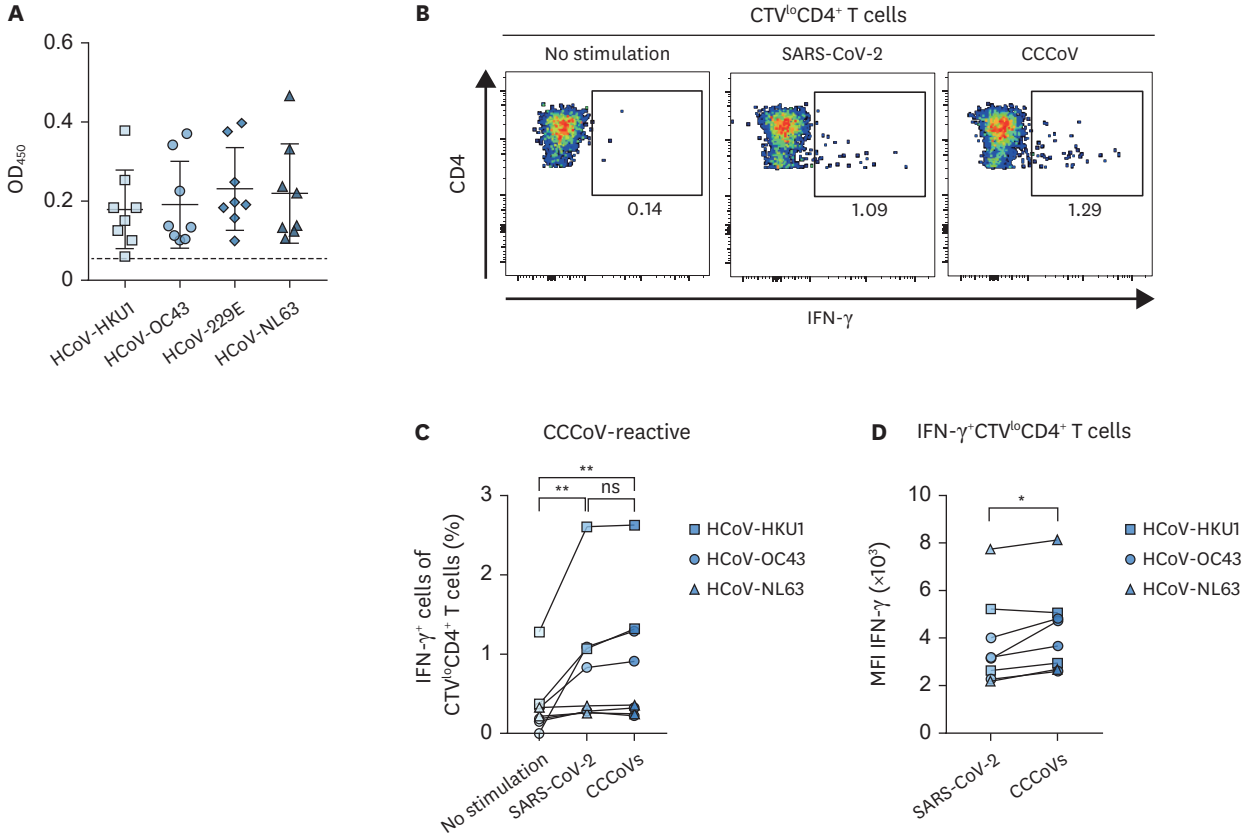

Figure 2. Cross-reactivity of CCCoV-reactive CD4⁺ T cells to SARS-CoV-2 in unexposed donors. (A) Plasma titers of anti-spike IgG towards CCCoVs (HCoV-HKU1, HCoV-OC43, HCoV-229E, and HCoV-NL63) in unexposed donors (n=8). (B-D) CTV-labeled PBMCs from unexposed donors (n=8) were expanded *in vitro* with the CCCoV membrane peptide pool for 7 days and re-stimulated with the SARS-Co-V-2 membrane peptide pool or corresponding CCCoV membrane pool for 6 h. After the stimulation, ICS was performed and IFN-γ⁺ cells detected among CTV^{lo}CD4⁺ T cells. (B) Representative flow cytometry plots and (C) summary data of the frequency of IFN-γ⁺ cells among CTV^{lo}CD4⁺ T cells. (D) Comparison of mean fluorescence intensities of IFN-γ among IFN-γ⁺CTV^{lo}CD4⁺ T cells against SARS-CoV-2 and CCCoV. Data in (A) are presented as mean and SD, and data in (C, D) are presented with lines connecting data from the same individuals.
ns, not significant.
*p<0.05, **p<0.01, Wilcoxon signed-rank test.

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a comparable frequency following SARS-CoV-2 M OLP stimulation (**Fig. 2B and C**), demonstrating that CCCoV M-specific CD4+ T cells are cross-reactive to SARS-CoV-2 M in SARS-CoV-2-unexposed individuals. However, SARS-CoV-2 M OLP stimulation resulted in a significantly lower mean fluorescence intensity (MFI) of IFN-γ among IFN-γ+CTVloCD4+ T cells than CCCoV M OLP stimulation (**Fig. 2D**). Given previous reports that high avidity T cells exhibit enhanced effector functions, including high IFN-γ MFI (25,26), we hypothesized that CCCoV M-specific CD4+ T cells may have low functional avidity to SARS-CoV-2 M, probably due to incomplete homology between CCCoV and SARS-CoV-2 M proteins.

SARS-CoV-2 M-reactive CD4+ T cells exhibit low functional avidity in COVID-19 convalescents and SARS-CoV-2-unexposed individuals

We directly assessed the functional avidity of SARS-CoV-2 M-reactive CD4+ T cells by performing IFN-γ ICS assays with 4-fold serial dilution of SARS-CoV-2 M OLPs. In COVID-19 convalescents, the frequency of IFN-γ+CD4+ T cells reactive to SARS-CoV-2 M or IAV MP1 abruptly decreased when the concentration of the OLP pools was diluted from 1 μg/ml to 0.25 μg/ml, whereas the frequency of IFN-γ+CD4+ T cells reactive to HCMV pp65 or RSV FP was maintained (**Fig. 3A and B**). The functional avidity was determined by the EC_{50}. The functional avidity of SARS-CoV-2 M-reactive CD4+ T cells was comparable to that of IAV MP1-reactive CD4+ T cells but significantly lower than that of HCMV pp65- or RSV FP-reactive CD4+ T cells (**Fig. 3C**). The low functional avidity of SARS-CoV-2 M-reactive CD4+ T cells might be attributed to partial cross-reactivity between CCCoV and SARS-CoV-2 M proteins. Such low functional avidity was also anticipated in T cell responses against IAV because an individual tends to have a history of repeated infections with various strains of IAV, and low functional avidity of IAV MP1-reactive CD4+ T cells was observed indeed. In the analysis according to the age, there was no significant difference in the functional avidity of SARS-CoV-2 M-reactive CD4+ T cells (**Fig. 3D**) and in IFN-γ MFI (**Supplementary Fig. 3A**) between different age groups although the frequency of IFN-γ+ cells was significantly higher in convalescents over 60 years old than those under 40 years old (**Supplementary Fig. 3B**).

We also assessed the functional avidity of CD4+ T cells among SARS-CoV-2-unexposed HDs. The functional avidity of SARS-CoV-2 M-reactive CD4+ T cells was comparable to that of IAV MP1-reactive CD4+ T cells and significantly lower than that of HCMV pp65-reactive cells (**Fig. 3E**). When COVID-19 convalescents and unexposed individuals were compared, the functional avidity of SARS-CoV-2 M-reactive CD4+ T cells was significantly lower in the unexposed group than the convalescent group (**Fig. 3F**). The functional avidity of IAV MP- or HCMV pp65-reactive CD4+ T cells was not different between 2 groups.

Our findings indicate that the functional avidity of SARS-CoV-2 M-reactive CD4+ T cells is low in both COVID-19 convalescents and SARS-CoV-2-unexposed individuals. We hypothesized that SARS-CoV-2 infection activates CCCoV M-specific memory CD4+ T cells that are partially cross-reactive to SARS-CoV-2 M, resulting in the low functional avidity of SARS-CoV-2 M-reactive CD4+ T cells among COVID-19 convalescents.

Convalescents from mild COVID-19 have SARS-CoV-2 M-reactive CD4+ T cells with lower functional avidity than convalescents from severe COVID-19

Finally, we compared SARS-CoV-2 M-reactive CD4+ T cell responses between COVID-19 convalescents who recovered from mild (non-hospitalized) and severe (hospitalized) disease. Convalescents from mild COVID-19 exhibited significantly lower frequencies of IFN-γ+, TNF+, or IL-2+ cells than convalescents from severe disease although there was no significant

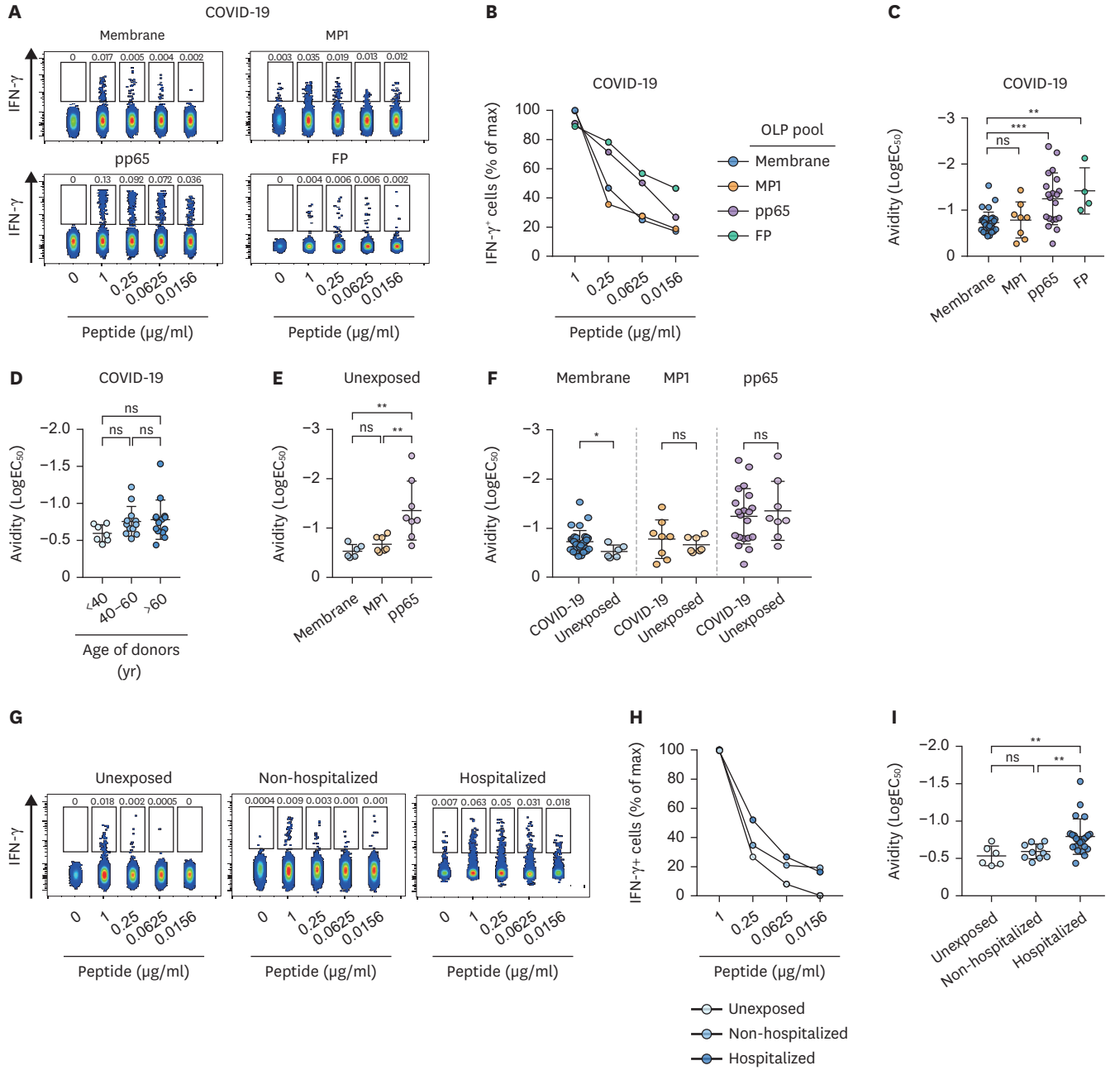

Figure 3. *Ex vivo* functional avidity among virus Ag-reactive CD4⁺ T cells in COVID-19 convalescents and unexposed individuals. PBMCs from COVID-19 convalescents (A-D, F-I) and unexposed donors (E-I) were stimulated with 4-fold diluted peptide pools. EC_{50} values were calculated as the peptide concentration yielding a half-maximal response in the IFN-γ intracellular cytokine staining assay, and avidities are represented as Log(EC_{50} values). (A) Concatenated flow cytometry plots of the frequencies of IFN-γ⁺ cells among CD4⁺ T cells. (B) Mean values of the normalized IFN-γ⁺ cells among CD4⁺ T cells against SARS-CoV-2 membrane (n=33), IAV MP1 (n=8), HCMV pp65 (n=20), and RSV FP (n=4) peptide pools in COVID-19 convalescents for the indicated concentration per peptide. (C) Comparison of avidity against SARS-CoV-2 membrane (n=33), IAV MP1 (n=8), HCMV pp65 (n=20), and RSV FP (n=4) peptide pools in COVID-19 convalescents. (D) Comparison of avidity against SARS-CoV-2 membrane peptide pool among different age groups of COVID-19 convalescents (<40 years, n=7; 40-60 years, n=12; >60 years, n=14). (E) Comparison of avidity against SARS-CoV-2 membrane (n=6), IAV MP1 (n=7), and HCMV pp65 (n=8) peptide pools in unexposed donors. (F) Summary data showing the avidities against SARS-CoV-2 membrane, IAV MP1, and HCMV pp65 peptide pools among COVID-19 convalescents and unexposed donors. (G-I) The COVID-19 convalescents were grouped by COVID-19 severity level according to NIH criteria into non-hospitalized (asymptomatic and mild symptoms in the acute phase) and hospitalized (moderate, severe, and critical symptoms in the acute phase) groups. (G) Concatenated flow cytometry plots of the frequencies of IFN-γ⁺ cells among CD4⁺ T cells. (H) Mean values of the normalized IFN-γ⁺ cells among CD4⁺ T cells against the SARS-CoV-2 membrane peptide pool in unexposed donors (n=6) and non-hospitalized (n=10) or hospitalized (n=23) COVID-19 convalescents for the indicated concentration per peptide. (I) Comparison of avidity against the SARS-CoV-2 membrane peptide pool in unexposed donors (n=6) and non-hospitalized (n=10) or hospitalized (n=23) COVID-19 convalescents. Data in (C-F, I) are presented as mean and SD. ns, not significant.
*p<0.05, **p<0.01, ***p<0.001, Mann-Whitney test.

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difference in IFN-γ MFI (**Supplementary Fig. 4A and B**). Next, we compared the functional avidity of SARS-CoV-2 M-reactive CD4⁺ T cells. Convalescents from mild COVID-19 had SARS-CoV-2 M-reactive CD4⁺ T cells with lower functional avidity than convalescents from severe disease, but the functional avidity was comparable to SARS-CoV-2 M-reactive CD4⁺ T cells in SARS-CoV-2-unexposed HDs (**Fig. 3G-I**).

These data support our hypothesis that individuals with high levels of pre-existing CCCoV M-specific memory CD4⁺ T cells that are partially cross-reactive to SARS CoV-2 M may have mild disease upon SARS-CoV-2 infection due to rapid T-cell responses although their functional avidity for SARS-CoV-2 M might be relatively low (**Fig. 4A**). In those individuals, SARS-CoV-2-cross-reactive memory CD4⁺ T cells may outgrow SARS-CoV-2-specific naïve precursor CD4⁺ T cells during SARS-CoV-2 infection. In this scenario, SARS-CoV-2 M-specific naïve precursor CD4⁺ T cells might not have a chance to be fully activated due to early disappearance of the cognate Ag. After convalescence, these individuals may have SARS-CoV-2 M-reactive memory CD4⁺ T cells with relatively low functional avidity. In contrast, individuals without pre-existing CCCoV M-specific memory CD4⁺ T cells may have severe disease upon SARS-CoV-2 infection due to slow primary T-cell responses starting from SARS-CoV-2 M-specific naïve precursor CD4⁺ T cells (**Fig. 4B**). These individuals may have SARS-CoV-2 M-reactive memory CD4⁺ T cells with high functional avidity after convalescence because their memory cells carry TCRs that are more highly specific to SARS-CoV-2 M.

Bacher et al. (27) previously reported that SARS-CoV-2-reactive CD4⁺ T cells exhibited low functional avidity in severe cases, contrasting with our current findings. This discrepancy can be potentially explained by Ag specificity and patient status. They primarily examined S-reactive T cells whereas we focused on M-reactive T cells. In addition, they included

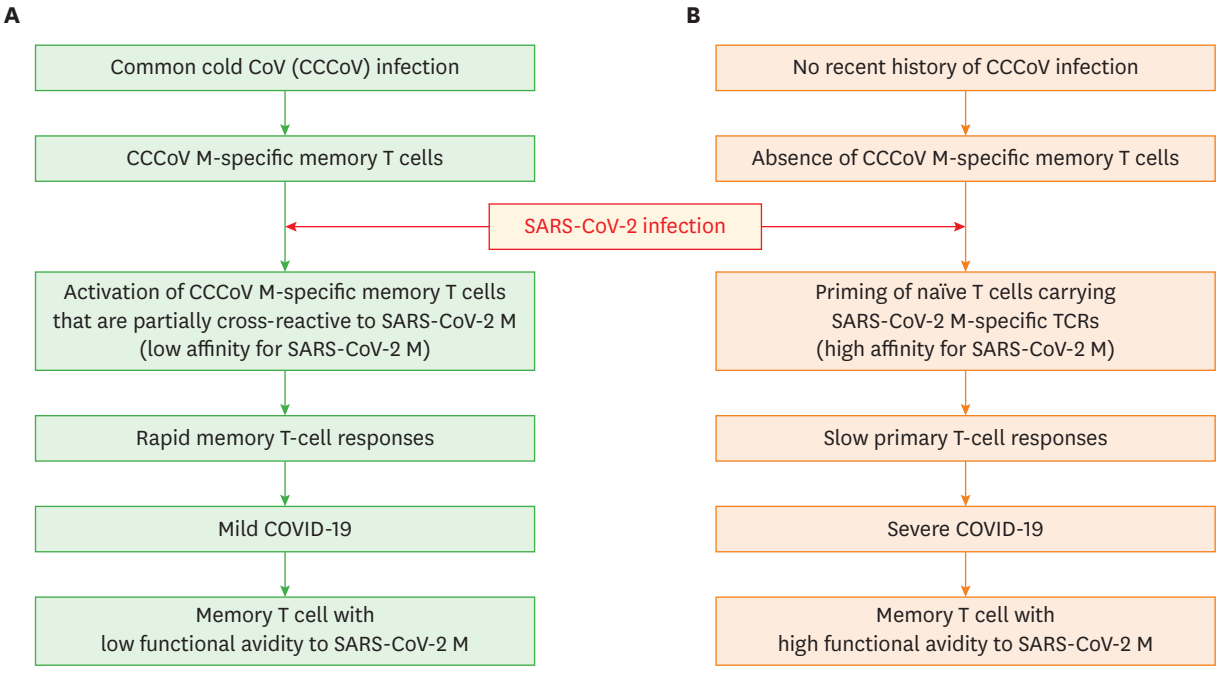

Figure 4. Differential T-cell responses to SARS-CoV-2 based on prior CCCoV infection history. The diagram illustrates how prior exposure to CCCoVs can influence the severity of COVID-19 through the activation of pre-existing CCCoV M-specific memory T cells with partial cross-reactivity to SARS-CoV-2 M (A, green) versus the priming of SARS-CoV-2 M-specific naïve precursor T cells (B, orange).

hospitalized patients with active COVID-19 in their study whereas we only recruited convalescents who already recovered from COVID-19 in our study.

Study limitations

There are limitations in our study. First, we examined cross-reactivity at the protein level not at the epitope level. Therefore, we do not know epitope peptides that are responsible for the cross-reactivity. Second, we do not know a species of CCCoVs that elicited cross-reactive CD4+ T cells in each individual. Third, we did not examine HLA allotypes of enrolled subjects.

Conclusions

The current data suggest that pre-existing CCCoV M-specific memory CD4+ T cells contribute to controlling SARS-CoV-2 infection by partial cross-reactivity, leading to mild disease but leaving memory cells with low functional avidity to SARS-CoV-2 M. These data provide indirect evidence that pre-existing cross-reactive CD4+ T cells contribute to protection from severe COVID-19. Our study not only provides insights into SARS-CoV-2 immunity, but also may aid in preparing for newly emerging viruses.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1

Clinical information of the COVID-19 convalescents

Supplementary Figure 1

Representative flow cytometry plots of SARS-CoV-2-reactive CD4+ T cells from COVID-19 convalescents. The representative flow cytometry plots show the expression of IFN-γ, TNF, or IL-2 among CD4+ T cells after stimulation of PBMCs with SARS-CoV-2 protein peptide pools (spike, membrane, or nucleocapsid). Numbers indicate the percentages of IFN-γ+, TNF+, and IL-2+ cells among CD4+ T cells. No stimulation was used as a negative control. αCD3 and αCD28 was used as a positive control.

Supplementary Figure 2

Cytokine production by SARS-CoV-2-reactive CD8+ T cells. PBMCs from COVID-19 convalescents (A) and unexposed donors (B) were stimulated with SARS-CoV-2 protein peptide pools (spike, membrane, or nucleocapsid). Cytokine-producing T cells were detected by intracellular cytokine staining after stimulation. (A) Summary data of the frequencies of IFN-γ+, TNF+, or IL-2+ cells among CD8+ T cells against spike (n=46), membrane (n=50), and nucleocapsid (n=40) pools from COVID-19 convalescents. (B) Summary data of the frequencies of IFN-γ+, TNF+, or IL-2+ cells among CD8+ T cells against spike (n=23), membrane (n=26), and nucleocapsid (n=26) pools from unexposed individuals. Data are presented with lines connecting data from the same individuals.

Supplementary Figure 3

IFN-γ responses of SARS-CoV-2-reactive CD4+ T cells in different age groups. Mean fluorescence intensity of IFN-γ among IFN-γ+CD4+ T cells (A) and the frequency of IFN-γ+ cells among CD4+ T cells (B) in response to stimulation with SARS-CoV-2 membrane peptide pool were compared in different age groups of COVID-19 convalescents: <40 years, n=7; 40–60 years, n=12; >60 years, n=14 in (A) and <40 years, n=15; 40–60 years, n=15; >60 years, n=20 in (B). Data are presented as mean and SD.

Supplementary Figure 4

IFN-γ responses of SARS-CoV-2-reactive CD4+ T cells in COVID-19 convalescents according to clinical severity in the acute phase. The COVID-19 convalescents were grouped by COVID-19 severity level according to NIH criteria into non-hospitalized (asymptomatic and mild symptoms in the acute phase) and hospitalized (moderate, severe, and critical symptoms in the acute phase) groups. PBMCs from COVID-19 convalescents were stimulated with SARS-CoV-2 membrane peptide pool. Cytokine-producing CD4+ T cells were detected by intracellular cytokine staining. (A) Comparison of the frequencies of IFN-γ+, TNF+, or IL-2+ cells among CD4+ T cells in non-hospitalized (n=18) and hospitalized (n=32) convalescents. (B) Comparison of mean fluorescence intensity of IFN-γ among IFN-γ+CD4+ T cells in non-hospitalized (n=10) and hospitalized (n=23) convalescents. Data are presented as mean and SD.

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