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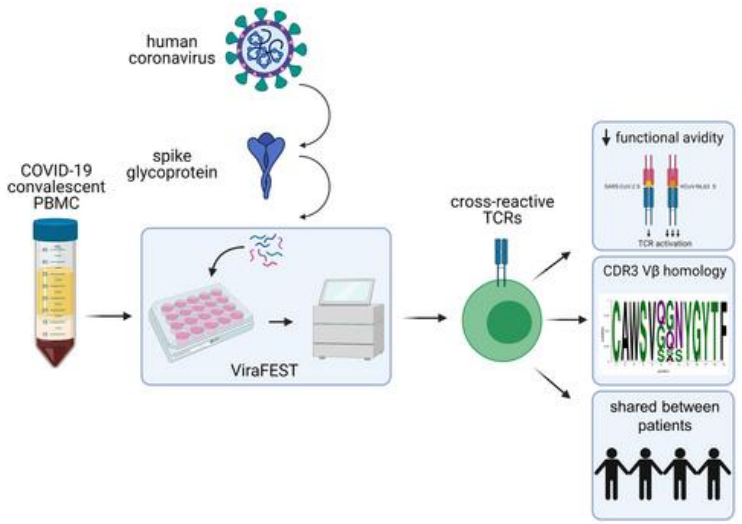
Graphical abstract



Functional characterization of CD4+ T-cell receptors cross-reactive for SARS-CoV-2 and endemic coronaviruses

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CONFLICTS OF INTEREST

DMP and KNS have filed for patent protection on a subset of the technologies described herein (US provisional patent application no. 62/407,820). AD, ALC, FD, DMP, JB, and KNS have filed for patent protection on a subset of the technologies described herein (US provisional patent application no. 63/135,534). SZ is a founder of, holds equity in, and serves as a consultant to Personal Genome Diagnostics. SZ holds equity in Thrive Earlier Detection and has a research agreement with BioMed Valley Discoveries Inc. DMP reports stock and ownership interests in Aduro Biotech, DNAtrix, Dracen Pharmaceuticals, Dragonfly Therapeutics, Ervaxx, Five Prime Therapeutics, Potenza Therapeutics, RAPT, Tizona Therapeutics, Trieza Therapeutics, and WindMIL; a consulting or advisory role in Amgen, DNAtrix, Dragonfly Therapeutics, Ervaxx, Five Prime Therapeutics, Immunocore, Immunomic Therapeutics, Janssen Pharmaceuticals, MedImmune/AstraZeneca, Merck, RAPT, and WindMIL; research grants from Compugen; patent royalties, and/or other intellectual property through their institution with Aduro Biotech, Arbor Pharmaceuticals, Bristol-Myers Squibb, Immunomic Therapeutics, NexImmune, and WindMIL; and travel, accommodations, and expenses from Bristol-Myers Squibb and Five prime Therapeutics. KNS receives commercial research funding from Bristol-Myers Squibb, Astra Zeneca, and Enara Bio and has received travel support/honoraria from Illumina, Inc. KNS, DMP, and SZ own founder's equity in ManaT Bio. These arrangements have been reviewed and approved by the Johns Hopkins University in accordance with its conflict-of-interest policies.

ABSTRACT

Background. Recent studies have reported T cell immunity to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in unexposed donors, possibly due to cross-recognition by T-cells specific for common cold coronaviruses (CCCs). True T-cell cross-reactivity, defined as the recognition by a single TCR of more than one distinct peptide-MHC ligand, has never been shown in the context of SARS-CoV-2.

Methods. We used the ViraFEST platform to identify T cell responses cross-reactive for the spike (S) glycoproteins of SARS-CoV-2 and CCCs at the T cell receptor (TCR) clonotype level in convalescent COVID-19 patients (CCPs) and SARS-CoV-2-unexposed donors. Confirmation of SARS-CoV-2/CCC cross-reactivity and assessments of functional avidity were performed using a TCR cloning and transfection system.

Results. Memory CD4+ T-cell clonotypes that cross-recognized the S proteins of SARS-CoV-2 and at least one other CCC were detected in 65% of CCPs and unexposed donors. Several of these TCRs were shared among multiple donors. Cross-reactive T-cells demonstrated significantly impaired SARS-CoV-2-specific proliferation in vitro relative to mono-specific CD4+ T-cells, which was consistent with lower functional avidity of their TCRs for SARS CoV-2 relative to CCC.

Conclusions. For the first time, our data confirm the existence of unique memory CD4+ T cell clonotypes cross-recognizing SARS-CoV-2 and CCCs. The lower avidity of cross-reactive TCRs for SARS-CoV-2 may be the result of antigenic imprinting, such that pre-existing CCC-specific memory T cells have reduced expansive capacity upon SARS-CoV-2 infection. Further studies are needed to determine how these cross-reactive T-cell responses impact clinical outcomes in COVID-19 patients.

INTRODUCTION

The 2019 SARS-CoV-2 outbreak and ensuing global pandemic has resulted in significant global morbidity and mortality. Coronavirus disease 19 (COVID-19) symptom severity ranges from mild, or even asymptomatic, to the development of acute respiratory distress syndrome (ARDS), hospitalization, and death (1, 2). It is not unusual for viral illnesses to induce a range of symptom severity, and pre-existing immunity to similar but not identical pathogens is generally thought to mitigate disease severity upon re-infection (3–5) . Indeed, Sagar et al., suggested that patients with recent infection by one of the four known endemic human common cold coronaviruses (CCCs; HCoV-NL63, HCoV-OC43, HCoV-229E, and HCoV-HKU1 (6)) experienced less severe COVID-19 illness (7). However, given that 7.5% of people are documented to be infected with a CCC during a given respiratory virus season and that repeated infection with CCCs is common (8–10), it is unlikely that prior CCC exposure alone results in effective viral clearance with minimal disease severity.

One contributing factor to the spectrum of disease severity between patients could be a difference in the development and/or function of SARS-CoV-2-specific T cell-mediated immunity. Ultimately, because B cell responses to antigen (class switching, affinity maturation and memory) are CD4+ T helper dependent, the quality and durability of humoral responses to SARS CoV-2 are intimately linked to the CD4+ T cell response. $CD4^+$ T cell responses are readily detected in the majority of recovered COVID-19 patients (11–13) and SARS-CoV-2-specific T cell responses have been detected in SARS-CoV-2-unexposed donors (14–20). Thus, pre-existing T cell immunity to CCC could impact SARS-CoV-2 immunity and clinical outcomes in COVID-19 patients. True T cell cross-reactivity is defined as the reaction of T cells to more than one distinct peptide-MHC ligand and is mediated by a single T cell clonotype expressing a unique T cell receptor (TCR) heterodimer. A cross-reactive T cell response can be due to a number of things: 1) plasticity of CDR3 loop regions, 2) promiscuous TCR:MHC binding, and 3) flexibility of the peptide:MHC interaction (21). However, a likely cause of a cross-reactive T cells response is significant peptide homology in certain residues that are required for MHC and TCR binding and recognition. In the case of the coronavirus family, there are a multitude of shared epitopes between SARS-COV-2 and other coronaviruses, including those that are endemic to the human population (22). Recognition of one of these shared epitopes may allow for a single T cell to cross-recognize both SARS-CoV-2 and CCCs. The

precise nature of the SARS-CoV-2/CCC cross-reactive response, including whether it is mediated by one or several T cell clonotypes recognizing antigens from different viruses, has not been determined.

In the present study we leverage the ViraFEST assay (23–26) , which identifies TCRs corresponding to memory antigen-specific T cells, to evaluate memory $CD4^+$ T cell CCC/SARS-CoV-2 cross-reactivity at the clonal level in cells obtained before (n=7) or during (n=12) the COVID-19 pandemic. We identified and characterized the TCR clonality and functional avidity of memory T cells cross-reactive for the spike glycoprotein (S) of both SARS-CoV-2 and one or more CCC. We found cross-reactive clones that were shared among several of our CCP and PC donors and were of lower functional avidity than non-cross-reactive clones, together supporting the phenomenon of antigenic imprinting by previous exposure to CCC. The findings presented here underscore the importance of antigen-specificity studies of SARS-CoV-2 to facilitate our understanding of the mechanisms underpinning effective immunity to this virus.

RESULTS

$CD4^+$ T cells from COVID-19 convalescent patients are cross-reactive for SARS-CoV-2 and CCC spike proteins

Our central hypothesis is that unique, individual $CD4^+$ T cell clonotypes recognize epitopes from both CCC and SARS-CoV-2 S proteins. This is supported by prior studies demonstrating that some donors have T cell reactivity to the SARS-CoV-2 S antigen without ever being exposed to the virus (12–14, 16, 17). Bona fide cross-reactivity mediated by a single T cell clonotype expressing a unique T cell receptor (TCR) heterodimer has not yet been shown for T cells that target SARS-CoV-2. To address the hypothesis that the same CD4+ T cell clonotypes recognize both SARS-CoV-2 and CCC S peptides, we used the viral functional expansion of specific T cells (ViraFEST) assay to detect S protein-specific T cell clones in the peripheral blood of COVID convalescent patients (CCPs) and unexposed donors (pre-COVID, PC; **Figure 1, Table S1)**. The ViraFEST assay identifies canonical antigen-specific memory T cell responses and the cognate TCR(s) contributing to this response via a 10-day T cell culture with relevant antigen followed by TCR Vβ complementarity determining region 3 (CDR3) sequencing (23–25, 27, 28). This assay has been successfully used to identify TCRs specific for tumor and viral antigens (25, 27) , and more importantly, TCRs cross-reactive for related viral epitope variants (26). Using this

assay, we tested peripheral blood CD4+ T cells from each donor (**Figure1, Table S1**) for reactivity to pooled peptides comprising the entirety of the S protein from SARS-CoV-2 and four known CCC, as cell number allowed: HCoV-NL63, HCoV-OC43, HCoV-229E, and HCoV-HKU1. Functional CD4+ T cell clonal expansion was measured from each peptide condition in biological replicate wells using quantitative TCR CDR3 Vβ sequencing. Our stringent data-driven our statistical algorithm (23) enabled us to identify significant and specific CD4+ TCR clonotype expansions in response to one or more S proteins. True cross-reactivity was defined by the same CD4+ TCR clonotype seen functionally expanding in response to SARS-CoV-2 S and at least one other CCC S protein.

Consistent with other studies, we detected SARS-CoV-2-specific memory $CD4^+$ T cell responses in 100% of the CCPs tested (n=12; **Figure S1A-L**). Strikingly, eight of these patients (67%) also had TCRs cross-reactive for the S protein from SARS-CoV-2 and at least one other CCC, as evidenced by clonal expansion of the same TCR Vβ CDR3 clonotype in response to multiple S protein peptide pools (**Figure 2A-H**). While responses were also detected against the SARS-CoV-2 nucleocapsid (N) protein and the CMV, EBV, and flu (CEF) control, as expected, none of these TCRs were cross-reactive with any of the S proteins indicating that cross-reactivity relies on protein homology. Cross-reactivity to the S protein of two or more CCCs occurred in all CCP donors in which we tested multiple CCC S peptide pools (**Figure S3A-J**). Surprisingly, SARS-CoV-2/HCoV-NL63 cross-reactive memory responses by three highly homologous TCRβ CDR3 clonotypes (CAWSVQQNYGYTF, CAWSVGGNYGYTF, and CAWSVQGNYGYTF) were independently detected in more than one CCP (red clones, **Figure 2B-D**). TCR sequencing of baseline and CEF-stimulated naïve and memory $CD4^+$ T cells confirmed that the ViraFEST assay is detecting memory T cell responses rather than primary responses induced during the 10-day culture (**Figure S4**), as even in the few cases where antigen-specific clones are detected in the naïve population, these expansions did not replicate and therefore would not have passed our stringent criteria for identifying antigen-specific responses.

To determine if CD4+ cross-reactive clonotypic expansions were also associated with a cross-reactive cytokine profile, CD4+ T cells from CCP6 and CCP12 were cultured separately with SARS-CoV-2 S, HCoV-NL63 S, or without peptide and were each re-challenged with SARS-CoV-2 S, HCoV-NL63 S, or without peptide, following by intracellular cytokine staining. Consistent with our ViraFEST results, CCP6 demonstrated cross-

reactive production of IL-2 and IFN-γ, while CCP12 only had mono-reactive responses **(Figure S5).** Together, these data show that SARS-CoV-2/CCC cross-reactive memory $CD4^+$ T cells are readily detected in the peripheral blood of CCPs, can functionally expand upon antigenic stimulation, and that a subset of TCR Vβ CDR3 clonotypes are shared among patients.

SARS-CoV-2-specific TCR avidity and epitope identification

A recent study demonstrated reduced avidity of the SARS-CoV-2/CCC cross-reactive T cell response (29), however this has not been evaluated at the individual clonotype level. To determine the avidity of individual SARS-CoV-2 reactive TCRs, we identified the cognate alpha chain for eight mono-reactive (recognizing only SARS-CoV-2 S) and five cross-reactive TCRs detected in CCP4 using the ViraFEST assay (**Figures 2B and S1D**), including the 3 TCRs that were detected in multiple patients. We then cloned the entire TCR gene block into a CD4-overexpressing Jurkat NFAT-luciferase reporter system, which specifically reads out the quantitative strength of TCR engagement via NFAT activation, commonly referred to as "functional avidity". Mono-reactivity for SARS-CoV-2 S was validated for all 8 TCRs tested (**Figure 3A-H**). To map the precise SARS-CoV-2 and HCoV-NL63 S protein region(s) eliciting these responses, we tested three mono-reactive and all five cross-reactive TCRs for reactivity against mini pools of 10 peptides each to span the entirety of the S protein. TCR-transfected Jurkat reporter cells were stimulated with each pool (**Figure S6A, S6B**) and subsequently with individual 15-mer peptides from the positive pools (SARS-CoV-2 pool 12 and HCoV-NL63 pool 15; **Table S3**). Of the three mono-reactive TCRs (**Figure 4A**), two recognized SARS-CoV-2 – GINITRFQTLLALHRSY (232-248) and one TCR recognized SARS-CoV-2 - QFCNDPFLGVYHKNNK (134-150) (**Figures 4B, S6D,E)**, which are both present in the S1 N-terminal domain (30) (**Figure S7**). The EC50 for these TCRs ranged from 0.37ug/ml to 0.77ug/ml with an average 0.6ug/ml. (**Figure 4B**).

Cross-reactivity was also confirmed for all 5 TCRs tested (**Figure 4C**), with lower NFAT activity induced by SARS-CoV-2 relative to HCoV-NL63 S protein. All cross-reactive TCRs recognized SARS-CoV-2 – SKRSFIEDLLFNKVTLA (amino acids 813-829), which is in the S2 linker region (30) (**Figure S7**) and cross-recognized the IAGRSALEDLLFSKVVT (867-883) region from HCoV-NL63 S (**Figures 4D, S6C, and Table S3**).

Both of these regions have recently been shown to be cross-recognized by antibodies of CCPs (31). The SARS-CoV-2 sequence, EDLLFNKV, within this 17-mer, could represent the core 8-mer responsible for TCR contact and/or MHC class II binding regions and differs from the cognate HCoV-NL63 sequence by only one amino acid substitution, an asparagine to serine at position 6 (EDLLFSKV), which likely does not alter the polarity of the peptide. Peptide titration experiments defined a maximum RLU range of 4,360 to 27,900 for HCoV-NL63 – IAGRSALEDLLFSKVVT (**Figure 4D**). The maximum RLU for SARS-CoV-2- SKRSFIEDLLFNKVTLA was not reached at 20ug/ml but ranged from 2,890 to 18,300. The cross-reactive TCR functional avidity (EC50; peptide concentration required to reach ½ maximum RLU) for HCoV-NL63 S-IAGRSALEDLLFSKVVT ranged from 0.82ug/ml to 1.93ug/ml with mean 1.25ug/ml. The SARS-CoV-2 S- SKRSFIEDLLFNKVTLA functional avidity ranged from 3.80ug/ml to 8.33ug/ml with mean 6.54ug/ml, roughly 5-fold lower than the functional avidity for HCoV-NL63 S peptide (**Figure 4D)**. These data demonstrate significantly reduced functional avidity of the cross-reactive SARS-CoV-2 T cell response relative to the CCC response (p=0.0011; **Figure 4D**), compatible with an antigenic imprinting mechanism. Using genetically diverse patient-derived lymphoblastoid cell lines (LCL), the restricting allele for these cross-reactive epitopes was mapped to HLA-DPA1*01:03/HLA-DPB*04 (**Figure S8A-C, Table S2).** Consistent with this restriction, both HLA-DPA1*01:03/HLA-DPB*04:01 and HLA-DPA1*01:03/HLA-DPB*04:02 are predicted to bind the SARS-CoV-2 and HCoV-NL63 peptides at IC50 <500nM (http://tools.iedb.org/mhcii/).

To further assess the quality of the functional T cell response, we compared the magnitude of SARS-CoV-2/CCC cross-reactive T cell expansion with mono-reactive T cell expansion in the ViraFEST assay (**Figure 5A**). We ordered the TCRs by descending expansion frequency in response to SARS-COV-2 S to visualize the expansive capacity of *in vitro* clonotypic expansion of mono-reactive clones, as well as the clonotypic expansion of cross-reactive TCRs in response to SARS-CoV-2 S or CCC. Cross-reactive clones (n=51; red) had in general less expansion to SARS-CoV-2 S than mono-reactive clones (n=40; blue) as noted by their clustering on the bottom of the chart (**Figure 5A**). To quantify the difference in relative fold change in response to SARS-Cov-2 S and CCC, we compared clones cross-reactive for SARS-CoV-2 S and HCoV-NL63 S (n=46), since HCoV-NL63 S was the only CCC tested in every ViraFEST assay. The CCP cross-reactive clones had a significantly lower level of antigen-specific expansion in response to the SARS-CoV-2 S protein relative to NL63 S protein (p=3.89E-

12; **Figure 5B**). This trend was also observed when comparing the highest CCC-specific expansion (**Figure S9B**). Interestingly, SARS-CoV-2 cross-reactive clonotypic expansions were significantly lower than SARS-CoV-2 mono-reactive expansions in CCPs (p=1.34E-6; **Figure 5C**). Together, these data demonstrate reduced expansion of CCP clones with cross-reactive TCRs for SARS-CoV-2 and support our hypothesis that a subset of observed T cell responses to SARS-CoV-2 likely resulted from pre-existing cross-reactive clones rather than through *de novo* priming by SARS-CoV-2 infection.

Detection of recall SARS-CoV-2/CCC cross-reactive CD4$^+$ T cells in SARS-CoV-2-unexposed donors

The cross-reactivity detected in CCPs could have been generated by recent infection with SARS-CoV-2 or primed by past infection with a CCC. To explore the possibility that memory CD4+ T cells against SARS-CoV-2 resulted from prior CCC exposure, we used the ViraFEST assay to test CD4$^+$ T cells obtained from seven healthy donors between 2017-May 2019, before the COVID-19 pandemic (PC1-PC7; **Table S1, S2 & Figure S2A-G)**. SARS-CoV-2/CCC cross-reactivity was detected in PC3, PC4, and PC5 (**Figure 6**). Notably, the CAWSVGGNYGTYF clone, identified as being SARS-CoV-2/CCC cross-reactive in convalescent patients CCP4, CCP5, and CCP6, also functionally expanded in the PC3 ViraFEST assay (red font, **Figure 6A**), confirming the existence of cross-reactive memory CD4+ T cell responses at the clonal level prior to SARS-CoV-2 exposure. Interestingly, SARS-CoV-2 S-specific CD4+ responses without cross-recognition of a tested CCC were detected in some PC donors (**Figure S2A,B, E-G and Supplementary Data)**, a phenomenon that has been described previously, possibly due to reactivity against untested or undiscovered coronaviruses or other pathogens (29), or low level cross-reactivity not picked up in our assay. Accordingly, the number of mono-reactive clonotypes detected in PC donors ranged from 0 to 6, compared with a range of 7-41 in CCPs (**Supplementary Data**). Taken together, these data support the idea that clonal cross-reactive memory CD4+ T cell responses existed prior to the COVID-19 pandemic, that these clonotypes maintain their antigen-specific in vitro expansive ability, and can be identified by re-stimulation of CCC and SARS-CoV-2 S protein antigens.

SARS-CoV-2/CCC cross-reactive TCRs share strong sequence homology within and among patients.

It has been shown that TCRs with shared viral antigen specificity may converge toward biased distribution of variable gene usage or CDR3 sequence identity (32). This is supported by TCR Vβ CDR3 sequence homology studies and may result from immunodominant epitopes (33), HLA super-families (34), and/or repeated stimulation by epitopes to which there is cross-recognition (35). We therefore investigated TCR Vβ CDR3 sequence homology of the SARS-CoV-2 mono- and cross-reactive TCRs identified in our study. We calculated the Levenshtein distance between each cross-reactive TCR. Seven different cross-reactive TCR Vβ CDR3 sequences with high sequence homology (mutual Levenshtein distance <=3) were found in four patients (PC3, CCP4, CCP5, and CCP6; **Figure 7 & Figure S10**). Similarly, homology between SARS-CoV-2 S reactive TCR Vβ CDR3s was observed both within (CCP5) and between multiple patients (CCP1-3, 5, 7, 8,10, PC1-2; **Figure S11**). This phenomenon is not unexpected and is often seen in response to pathogens (36). Indeed, we observe TCR homology in clones reactive to the CMV, EBV, Flu (CEF) pool we use as a positive control (**Figure S12**). However, when combined with our data demonstrating multiple cross-reactive TCRs converging to recognize a single 17-mer SARS-CoV-2 epitope, the data suggest significant TCR convergent evolution towards recognition of immunodominant epitopes in a TCR Vβ CDR3 sequence-dependent manner.

DISCUSSION

Most individuals infected with SARS-CoV-2 recover quickly and without long-term complications. Unfortunately, COVID-19 leads to ARDS, chronic health complications, and death in other patients. The immunologic correlates of distinct clinical outcomes remain incompletely defined. CD4+ T cells are requisite effectors of antiviral immunity and critical to formation of high affinity neutralizing antibodies, shown to prevent SARS-CoV-2 infection and limit viral spread (37). Not surprisingly, SARS-CoV-2-specific CD4+ T cell responses are detected in most COVID-19 patients and studies have estimated that a significant proportion of SARS-CoV-2-unexposed individuals have memory T cell responses against SARS-CoV-2. This reactivity has been shown to be the result of cross-reactivity for one or more CCC which are responsible for mild and sometimes asymptomatic upper respiratory illnesses (16–18). However, whether this is true cross-reactivity mediated by one or several T cell clonotypes had not been explored. In addition, whether specific cross-reactive clones expand less efficiently in response to SARS-CoV is unknown. We studied the clonal nature of cross-reactive

CD4+ T cell responses to SARS-CoV-2 using the ViraFEST assay. This assay uniquely identifies antigen-specific TCR Vβ clonotypes and enables concurrent evaluation for T cell cross-reactivity at the clonal level. Additionally, this is the first report using the ViraFEST assay to query $CD4^+$ T cell responses. Our data show that $CD4^+$ T cells that cross-recognize CCC and SARS-CoV-2 S peptides existed as memory T cell clones in prior to the COVID-19 pandemic.

Bacher, *et al* recently studied the functional avidity of the SARS-CoV-2/CCC cross-reactive T cell response in unexposed individuals and COVID-19 patients (29). Our findings presented here align with the primary conclusion of this study that cross-reactive T cells exhibit lower avidity relative to mono-reactive T cells. However, in contrast to this study, we were able to detect cross-reactive responses in COVID-19 patients, likely the result of using the T cell proliferation readout of the ViraFEST assay compared with activation marker upregulation. Nonetheless, we present here the first report of the identity and avidity of cross-reactive T cell receptor clonotypes and their prevalence. These findings suggest some of the SARS-CoV-2-specific $CD4^+$ T-cell responses detected in COVID 19 patients may be the result of pre-existing cross-reactivity rather than de novo priming by SARS-CoV-2 infection. Although we are limited by the number of patients and number of TCRs tested, the patterns of functional avidity and support by other recent literature provide strength to our claim (13, 16, 17, 29, 31). It is worth noting that of only 12 COVID-19 convalescent patients in whom ViraFEST assays were performed, 8 patients possessed cross-reactive T cell responses and 3 patients had shared TCRs, thus making it likely that SARS-CoV-2/CCC cross-reactive responses are common and that these are mediated by public TCR clonotypes. It is notable that in four unrelated donors (3 CCPs and one unexposed donor), identical TCRs contributed to their endogenous SARS-CoV-2/CCC cross-reactive memory CD4+ T cell response. Similarly, TCR cloning was based on single cell TCR sequencing of a single subject's T cells, however the cloned TCRs corresponded to memory CD4+ T cells detected in four different donors (CCP4, CCP5, CCP6, and PC3), reflecting a shared SARS-CoV-2-specific memory T cell repertoire. While these patients all share a common restricting allele, specifically HLA DPA1*01:03 and DPB1*04 was shared between CCP4, CCP5, CCP6, and PC3 (**Table S2**), it could also be the result of HLA/epitope degeneracy such that one or several immunodominant epitopes bind to multiple related MHC class II alleles. The epitope recognized by these TCRs is located in a conserved motif that is present in CCCs and in avian coronaviruses (22), and is targeted by cross-reactive

antibodies (31). CCP4 has several different CD4+ T cell clonotypes recognizing this same conserved immunodominant epitope. Thus, this epitope may represent a target for a universal coronavirus vaccine. It is intriguing that the cross-reactive TCRs in our study targeted this region, whereas the mapped mono-reactive responses targeted epitopes in the less conserved N terminal domain. This may represent true biology, i.e. more conserved regions are more likely to induce cross-reactive responses, or could be an observation limited to the TCRs that were mapped for epitope specificity in our study.

It is possible that SARS-CoV-2 infection in a subset of patients leads to preferential expansion of pre-existing cross-reactive memory T cells that were generated in response to prior CCC infections. Our data suggest these cross-reactive T cells have lower functional avidity TCRs for SARS-CoV-2 and do not proliferate to the same degree as mono-reactive SARS-CoV-2-specific CD4+ T cells. These findings strongly suggest a mechanism of antigenic imprinting (sometimes termed “original antigenic sin”), in which naïve high affinity T cell clones are outcompeted by lower affinity cross-reactive memory T cell clones in individuals previously infected with a viral strain expressing the cross-reactive epitope. This has been demonstrated for other viral infections (38, 39), but not for SARS-CoV-2. Accordingly, SARS-CoV-2 “mono-reactive” responses were detected in cells obtained prior to the COVD-19 pandemic, possibly due to missed cross-reactive responses to unknown CCCs or other pathogens (29). A limitation of the present study is the lack of matched pre-COVID and COVID biospecimens from the same donor, which may have enabled us to further understand the source of pre-COVID SARS-CoV-2-specific responses. In contrast to the antigenic imprinting hypothesis, the cross-reactive responses could alternatively lead to an earlier adaptive immune response to SARS-CoV-2 resulting in earlier control of viral replication, as has been described with some cross-reactive vaccine-induced influenza-specific T cell responses (39, 40).

A limitation of our approach is that we focused on the S protein, whereas other studies have characterized cross-reactive T cells that recognize other viral proteins (17, 18). However, to our knowledge, this is the first time cross-reactive TCRs that recognize SARS-CoV-2 and CCC antigens have been identified and characterized at the clonal level. While cross-reactive T cells have been shown to influence disease outcomes (41), more systematic analyses should be performed to determine how best to modulate this response to improve clinical

outcome or treatment/vaccine efficacy. Taken together, our data have major implications for coronavirus vaccine design and could potentially partially explain heterogeneous clinical outcomes in COVID-19 patients.

METHODS

Study participants, biospecimens, and HLA haplotyping

This study was conducted according to Declaration of Helsinki principles. COVID-19 convalescent patients (CCPs) refer to patients who tested positive for SARS-CoV-2 by nasal swab PCR test, were symptomatic but not hospitalized, and have since recovered from COVID-19. Pre-COVID healthy donors (PCs) refer to peripheral blood mononuclear cell (PBMC) donors whose blood was drawn and processed prior to the SARS-CoV-2 epidemic (between 2017 and May 2019) (42). The median age of the 12 CCPs was 38.5 (range 21 to 72). There were 8 males and 4 females. 2 of the subjects were Hispanic. There were 8 Caucasians, 2 African Americans, 1 Asian, and 1 multiracial individual. All the subjects except CCP2 had mild disease and were not hospitalized. CCP2 has well controlled HIV on antiretroviral therapy and developed severe disease. Leukapheresis product was commercially purchased for all unexposed donors between 2011-2018. CCPs were enrolled to protocols approved by the Johns Hopkins University IRB and provided written informed consent. Peripheral Blood Mononuclear Cells (PBMCs) from each study participant and unexposed donor were isolated from leukapheresis product or whole blood via Ficoll-Paque Plus gradient centrifugation and were viably cryopreserved at -140C or were used immediately in FEST assay. CCP4 lymphoblastoid cell lines (LCL) were conducted via EBV transformation of peripheral blood B cells was at the Genetic Resources Core Facility, Johns Hopkins Institute of Genetic Medicine, Baltimore, MD. Low resolution MHC class I and II haplotyping was performed on DNA from each subject at the Johns Hopkins Hospital Immunogenetics Laboratory. High resolution was done for CCP4, CCP5, CCP6, PC3, PC5, PC6, PC7, and all additional LCL at the Johns Hopkins Hospital Immunogenetics Laboratory.

Identification of human coronavirus-specific T cells

Overlapping peptide pools spanning the S protein of four common human coronaviruses (HCoV-NL63, HCoV-OC43, and HCoV-229E; BEI and jpt and HCoV-HKU1; jpt), as well as overlapping peptide pools spanning

the S and N proteins of SARS-CoV-2 (BEI and jpt) were used to stimulate CD4+ T cells in the ViraFEST assay as described previously (23), with minor modifications. Briefly, 2 x 10^6 PBMC were plated in culture medium (IMDM, 5% human AB serum, 10 IU/ml IL-2, 50 ug/mL gentamicin) with 10ug/ml of individual HCoV and CoV-2 peptide pools, a positive control CEFX Ultra SuperStim consisting of pooled CMV, EBV, and Flu MHC-II restricted epitopes (jpt, PM-CEFX-3), a negative control HIV-1 Nef peptide pool (NIH AIDS Reagents), or without peptide. Each assay condition was performed in triplicate unless otherwise noted. On day 3, half the media was replaced with fresh culture media containing IL-2 (final concentration of 10 IU/mL Il-2). On day 7, half the media was replaced with fresh culture media containing IL-2 (final concentration of 10 IU/ml IL-2). On day 10, cells were harvested and CD4+ T cells were isolated using the EasySep CD4+ T cell isolation kit (STEMCELL, 17952). DNA was extracted from cultured CD4+ T cells using the Qiamp micro-DNA kit according to the manufacturer's instructions. TCRseq of DNA extracted from cultured $CD4^+$ T cells was performed by the Johns Hopkins FEST and TCR Immunogenomics Core Facility (FTIC) using the Oncomine TCR Beta short-read assay (Illumina, Inc). Samples were pooled and sequenced on an Illumina iSeq 100 using unique dual indexes.

Data pre-processing was performed to eliminate non-productive TCR sequences and to align and trim the nucleotide sequences to obtain only the CDR3 region. Sequences not beginning with "C" or ending with "F" or "W" and having less than 7 amino acids in the CDR3 were eliminated. Resultant processed data files were uploaded to our publicly available MANAFEST analysis web app (www.stat-apps.onc.jhmi.edu) to bioinformatically identify antigen-specific T cell clonotypes. Clones were considered positive based on the following criteria: 1) significantly expanded in the culture of interest (in two of three replicate wells) compared to the reference culture (PBMC cultured with 10 IU/ml IL-2 and HIV-1 Nef pool or media without peptide for HIV+ donor CCP2) at an FDR less than the specified threshold (<0.05; default value), 2) significantly expanded in the culture wells of interest compared to every other culture well performed in tandem (FDR<0.05; default value), and 3) have an odds ratio >5 (default value). To identify cross-reactive responses, we used statistical criteria established previously (29)(26).

Detection of antigen-specific T cell clonotypes in naïve and memory CD4+ T cells

Naïve and memory CD4+ T cells were isolated from cryopreserved PBMC by first isolating CD4+ T cells from bulk PBMC using the Easy Sep™ Human CD4+ T cell Enrichment kit (STEMCELL, 19052) followed by using the Easy Sep™ Human Naïve CD4+ T cell Enrichment kit (STEMCELL, 19555). DNA was extracted from CD4+ T cells using the Qiamp micro-DNA kit according to the manufacturer's instructions. Deep TCR sequencing of DNA extracted from $CD4^+$ T cells was performed by the Johns Hopkins FEST and TCR Immunogenomics Core Facility (FTIC) using the Adaptive Biotechnologies immunoSEQ human TCRβ kit.

Separately, 10^5 naïve or memory CD4s isolated using the methods above were plated separately at a 1:1 ratio with T-cell depleted PBMC and cultured with a CEF peptide pool (5μg/ml). The ViraFEST culture was performed as described above. After 10 days of culture, all cells were harvested, and DNA was extracted from cultured cells using the Qiamp micro-DNA kit according to the manufacturer's instructions. Survey TCRseq of DNA extracted from cultured CD4+ T cells was performed by the Johns Hopkins FEST and TCR Immunogenomics Core Facility (FTIC) using the Adaptive Biotechnologies immunoSEQ human TCRβ kit. Data pre-processing was performed, and all data were uploaded to our publicly available MANAFEST analysis web app (www.stat-apps.onc.jhmi.edu) to bioinformatically identify antigen-specific T cell clonotypes. The same statistical criteria mentioned above was used to determine CEF-specific T cell clonotypes. All SARS-CoV-2 S and CEF reactive TCR Vβ CDR3 sequences identified in previous ViraFEST assays were searched for in the TCR-seq TSV data files from cultured and uncultured naïve and memory $CD4^+$ T cells. The frequency of clones present in at least one naïve or memory population were plotted using GraphPad Prism (version 9.0.2).

Intracellular staining of T cell activation cytokines

10^7 PBMCs from CCP6 and CCP12 were cultured in R10 media with 10 IU/ml IL-2 and 5ug/ml SARS-CoV-2 S, HCoV-NL63 S, or without peptide for 10-12 days. At the end of culture period, the cells were washed and plated in fresh R10 media with 10 IU/ml IL-2 and rested overnight prior to re-stimulation with 5ug/ml SARS-CoV-2 S, HCoV-NL63 S, and without peptide. Protein transport inhibitors (GolgiPlug, 1ug/ml; GolgiStrop, 0.7 ug/ml) as well as antibodies against CD28 and CD49d (BD Biosciences). Following a 12-hour stimulation, the cells were washed and stained with annexin V (BV-421, BD Biosciences, 563973), CD3 (APC-Cy7, BioLegend, 300426), and CD4 (PerCP-Cy-5.5, BioLegend, 300530). We then fixed, permeabilized, and stained the cells

intracellularly for cytokinesIFN-γ (APC, BD Biosciences, 506510), and IL-2 (PE, BioLegend, 500307). Flow cytometry was done on a BD FACS LSR Fortessa flow cytometer, and data was analyzed with FlowJo, version 10. A minimum of 100,000 lymphocytes were collected and analyzed.

Identification of the cognate TCRα for SARS-CoV-2 and HCoV-CCC specific Vβ CDR3s

PBMC from CCP4 were cultured for 10 days with SARS-CoV-2 S, SARS-CoV-2 N, and HCoV-NL63 S peptide pools as described above. On day 10, live CD4+ T cells were FACS-sorted and subjected to single cell 5' VDJ sequencing to identify phased TCRα and TCRβ chain sequences at single cell resolution using the 10X Genomics Chromium Single Cell 5' VDJ sequencing platform on a Chromium Controller (10X Genomics) to achieve a target cell capture rate of 10,000 individual cells per sample. All samples were processed simultaneously, and the resulting libraries were prepared in a single batch following the manufacturer's instructions for VDJ library preparation. The resulting 5' VDJ libraries were subjected to next generation sequencing at the Sidney Kimmel Comprehensive Cancer Center Experimental and Computational Genomics Core. Resulting data were pre-processed and analyzed using cellranger VDJ software (10X Genomics) and visualized using Loupe V(D)J browser (10X Genomics) to identify the paired TCR Vα chain for the cognate CDR3 Vβ chains identified by ViraFEST. IMGT Repertoire was used to identify the full amino acid sequence for each V and J gene for both the TCRα and TCRβ chains.

Generation of a Jurkat reporter cell line

A gBlock was created with the full TCRα and TCRβ chains separately and human constant regions and was synthesized (Integrated DNA Technologies, IDT). To generate a Jurkat reporter cell in which we could transfer our TCRs of interest, the endogenous T cell receptor (TCR) α and β chains were knocked out of a specific Jurkat line that contains a luciferase reporter driven by an NFAT-response element (Promega) using the Alt-R CRISPR system (Integrated DNA Technologies, IDT). Two sequential rounds of CRISPR knockout were performed using crDNA targeting the TCRα constant region (AGAGTCTCTCAGCTGGTACA) and the TCRβ constant region (AGAAGGTGGCCGAGACCCTC). crDNA and tracrRNA (IDT) were resuspended at 100uM with Nuclear-Free Duplex Buffer. They were duplexed at a 1:1 molar ratio according to the manufacturer's

instructions. The duplexed RNA was cooled to room temperature before mixing with Cas9 Nuclease at a 1.2:1 molar ratio for 15 minutes. 40pmols of Cas9 RNP complexed with gRNA were mixed with 500,000 cells in 20ul of OptiMEM, loaded into a 0.1cm cuvette (Bio-Rad) and electroporated at 90V and 15ms using an ECM 2001 (BTX, Holliston, MA). Cells were transferred to complete growth medium and expanded for 7 days. Limiting dilution was used to acquire single cell clones and gDNA was harvested using the Quick-DNA™96 Kit (Zymo Research, Irvine, CA). The regions flanking the CRISPIR cut sites were PCR amplified (TCRα forward primer: GCCTAAGTTGGGGAGACCAC, reverse primer: GAAGCAAGGAAACAGCCTGC; TCRβ forward primer: TCGCTGTGTTTGAGCCATCAGA, reverse primer: ATGAACCACAGGTGCCCAATTC) and Sanger Sequenced. Only TCRα$^-$/β$^-$ clones were selected. Complete knockout was confirmed by failure to restore CD3 expression on electroporation with only a TCRα or TCRβ chain, and successful CD3 expression on electroporation with both TCR chains.

CD8 was transduced into the TCRα$^-$/β$^-$ Jurkat reporter cells using the MSCV retroviral expression system (Clontech). gBlocks (IDT) encoding CD8α and CD8β chains separated by a T2A self-cleaving peptide was cloned into the pMSCVpuro retroviral vector by HiFi DNA assembly (New England Biolabs). The plasmid was then co-transfected with a pVSV-G envelope vector into the GP2-293 packaging cell line per the manufacturer's instructions. Viral supernatant was harvested 48 hours after transfection and concentrated 20-fold using Retro-X Concentrator (Clontech). For transduction, non-tissue cultured treated 48-well plates were coated with 150 µL retronectin (Clontech) in PBS at 10 ug/mL overnight at 4°C. Plates were then blocked with 10% FBS for 1hr at RT followed by washing once with PBS. After removing PBS, viral particles and 2x10^5 of TCRα$^-$/β$^-$ Jurkat reporter cells were added to each well in a total volume of 500 µL cell culture media. Plates were spun at 2000 g for 1hr at 20°C then incubated at 37°C. Selection with 1 µg/mL puromycin (Thermo Fisher Scientific) began three days later. Single cell clones were established by limiting dilution and clones were subsequently screened for CD8 expression by flow cytometry. To generate a Jurkat reporter line that expresses both CD4 and CD8, CD4 viral particles were produced and transduced into the CD8-expressing Jurkat reporter cells using similar procedures.

Jurkat TCR transfer

TCRs of interest were introduced into the CD4/CD8 TCRα^{-}/β^{-} Jurkat reporter line by cloning the TCRα and TCRβ chains separately into the pCI vector (Promega) by HiFi DNA assembly (New England Biolabs). The two plasmids were co-electroporated into the TCRα^{-}/β^{-} Jurkat reporter line using 4mm cuvettes (Bio-Rad) and 275V for 10ms for 3 pulses at 0.1 interval between pulses. Cells were rested in RPMI 10% FBS at 37 ° for 24 hours. TCR expression efficiency was assessed by CD3 expression using flow cytometry. After rest live Jurkat cells were counted and plated at 1:1 ratio with a patient matched lymphoblastoid cell line (LCL) and peptide pools. Peptide titrations were carried out from 50ug/ml to 1.25ug/ml to assess TCR reactivity to peptide pools. Cells and peptide were co-cultured for 24 hours. TCR activity was assessed by NFAT-luciferase reporter readout using Bio-Glo™ Luciferase Assay System (Promega).

Epitope identification and avidity analysis

Cross-reactive and mono-reactive TCRs were cloned into the Jurkat reporter cell line and plated at a 1:1 ratio with patient-derived lymphoblastic cell lines (LCL) and first with mini pools consisting of 10 peptides all together comprising the entirety of the SARS-CoV-2 or HCoV-NL63 S protein. Once the stimulating mini pool was identified, the same TCRs were again transfected into Jurkats and plated with LCL and the individual SARS-CoV-2 and HCoV-NL63 peptides representing the stimulating mini pool. Once the specific peptide was identified we did peptide titrations from 20ug/ml to 0.15ug/ml to assess TCR avidity for each stimulating peptide. TCR activity was again assessed by NFAT-luciferase reporter readout using Bio-Glo™ Luciferase Assay System (Promega). TCR EC50 was calculated by identifying the peptide concentration (ug/ml) required to reach ½ plateaued RLU. If 20ug/ml of peptide was insufficient to maximize Jurkat-TCR activation, then we estimated EC50 by calculating the peptide concentration (ug/ml) required to read ½ maximum RLU reached in our assay. TCR EC50 was then used as a metric to estimate TCR relative avidity for individual 17-mer peptides. A two-tailed student's t test was performed using the mean of the EC50 of cross-reactive TCRs for SARS-CoV-2 and the mean of the EC50 of mono-reactive TCRs for SARS-CoV-2.

Heatmaps and unrooted phylogenetic trees

The non-redundant TCR sequences were defined by excluding the first 3 and last 3 amino acids of the TCR Vβ CDR3 region due to significant sequence overlap at the beginning and end of the CDR3 sequence (36, 43). The levenshtein distance between each pair of TCR sequences was calculated based on non-redundant TCRs, using the 'stringdist' R package (44). The TCR sequence homology pattern was visualized in a heatmap and an unrooted phylogenetic tree, where each row of the heatmap and each leaf of the unrooted phylogenetic tree represented a TCR Vβ CDR3 sequence from a sample. The heatmap and unrooted phylogenetic tree were generated using the 'pheatmap' and 'ape' R packages respectively. All analyses were performed using R software, version 3.6.1.

Statistics

Wilcoxon signed-rank test was performed to compare the fold change of cross-reactive clones in response to NL63 S vs. SARS-CoV-2 S and CCC S vs. SARS-CoV-2 S. Mann-Whitney U test was used to compare the fold change of SARS-CoV-2 S mono-reactive vs. cross-reactive clones. $P<0.05$ was considered statistically significant.

Study approval

This study was approved by the IRB of Johns Hopkins University. All study participants gave written informed consent before their inclusion in this study.

Data availability

All raw TCR sequencing data has been made publicly available. Illumina data has been uploaded to SRA under the BioProject accession: PRJNA705196 CD4+ T Cell Receptor Sequencing of COVID-19 Convalescent, Vaccinated, or Pre-COVID Healthy Donors. PC5-7 and additional naïve vs. memory experiments were sequenced using the Adaptive Biotechnologies TCR-sequencing kit and those data can be accessed here: 10.21417/AGDBZ2021JCI.

AUTHOR CONTRIBUTIONS

AGD, BAW, CCG, LSC, DC, SB, RR, JXC, EH-CH, KC, EAT, AKK, DS, and ST performed the experiments and acquired the data. BZ, LA, and HJ led the bioinformatic and computational analyses. KNS and JNB led study design and data interpretation. All authors contributed to experimental planning, data analysis and interpretation, and manuscript preparation.

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FIGURES AND LEGENDS

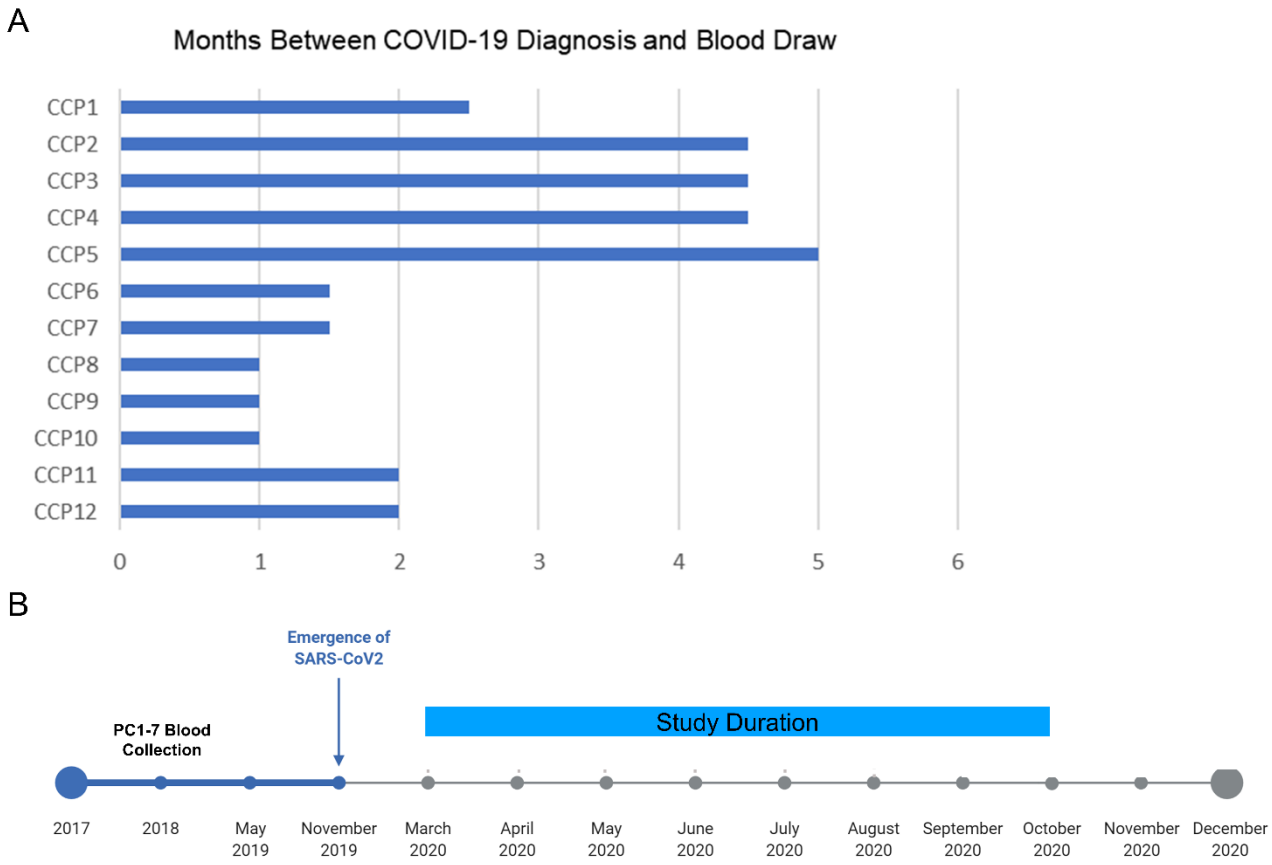

Figure 1. COVID-19 diagnosis to blood draw timeline. The number of months between COVID-19 diagnosis to blood sample (x-axis) matched to COVID-19 convalescent patient on y-axis (A). The study timeline indicates the timeframe of pre-COVID (PC) blood collection prior to SARS-CoV-2 emergence and the duration of our study timeline from the first COVID-19 diagnosis to the last blood collection date (B).

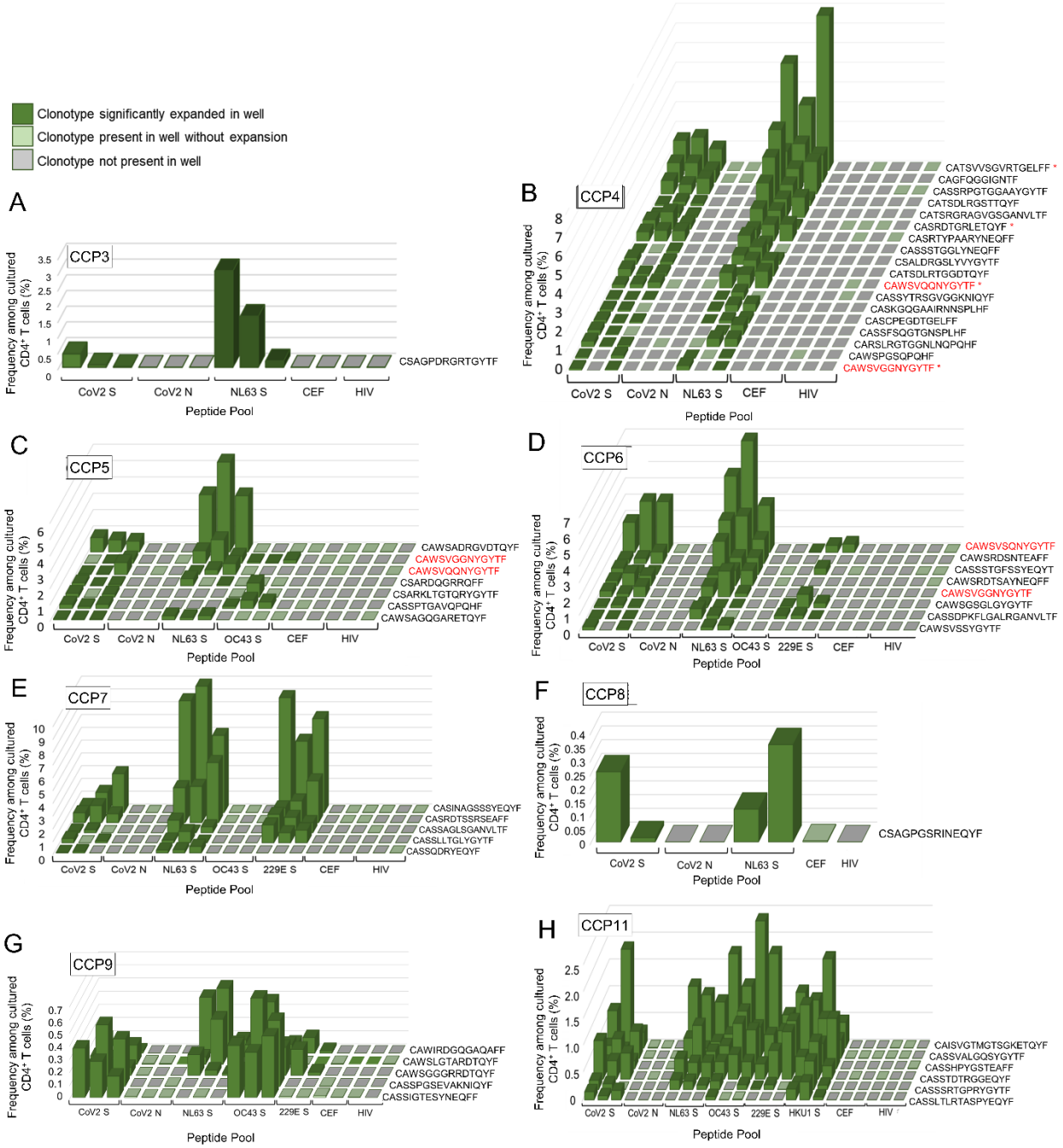

Figure 2. Detection of memory CD4⁺ T cell clonotypes that cross-recognize SARS-CoV-2 and CCC spike proteins. The ViraFEST assay detected cross-reactivity to the S protein from SARS-CoV-2 and at least one other CCC (HCoV-NL63, HCoV-OC43, HCoV-229E, and HCoV-HKU1) in 8 out of 12 CCPs tested (A-H). Peptide co-culture was done in triplicate unless otherwise noted. Data are shown as the frequency (%) after culture (y-axis) of antigen-specific CD4+ T cell clonotypes (z-axis) for all peptide pools tested (x-axis). The top five mono-reactive TCR Vβ CDR3 amino acid clonotypes for each CCP are shown. Solid green bars represent significant (FDR<0.05) clonotypic expansion in response to the indicated antigenic peptide pool(s), whereas translucent green color indicates the clonotype was present at low frequency in the well, but did not significantly expand. Grey indicates the relevant TCR clonotype was not detected in that well. An identical, shared TCR Vβ CDR3 amino acid clonotype corresponding to cross-reactive memory CD4+ T cells in CCP4, CCP5, and CCP6 is shown in red (B, C, D). Single cell TCR sequencing was performed on stimulated CD4+ T cells from CCP4 to identify the cognate TCR α chain for TCR β chains of interest (*).

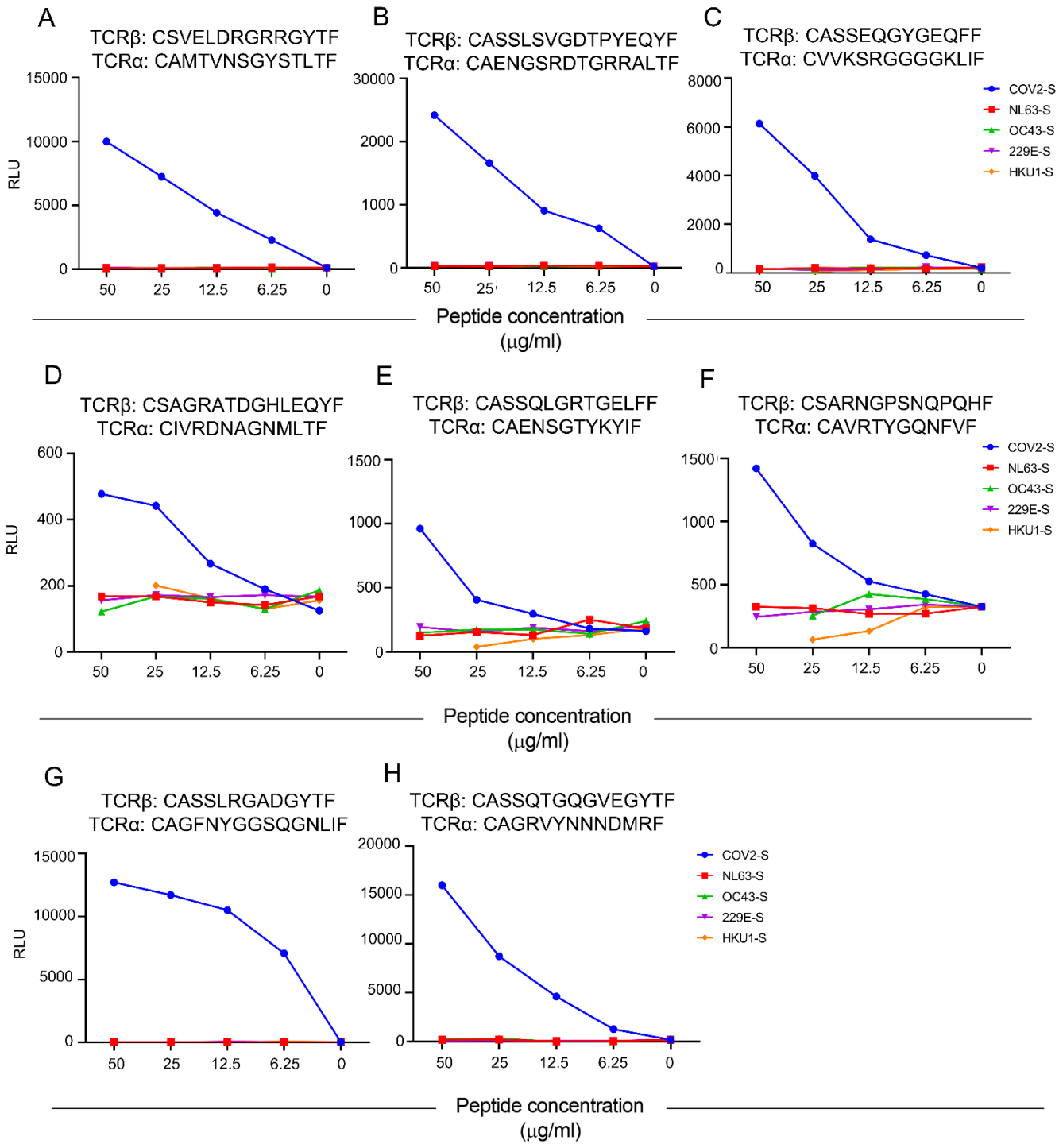

Figure 3: Confirmation of SARS-CoV-2 mono-reactivity. SARS-CoV-2 mono-reactive TCRs as determined by ViraFEST were cloned and transfected into Jurkat reporter cell line. Jurkats expressing the TCRs of interest were co-cultured at 1:1 ration with CCP4 LCL and SARS-CoV-2 or all CCC S protein peptide pools in titrating concentration or without peptide. Data are shown as relative luminescence units (RLU) at each pool concentration. These data are representative of all experiments repeated twice.

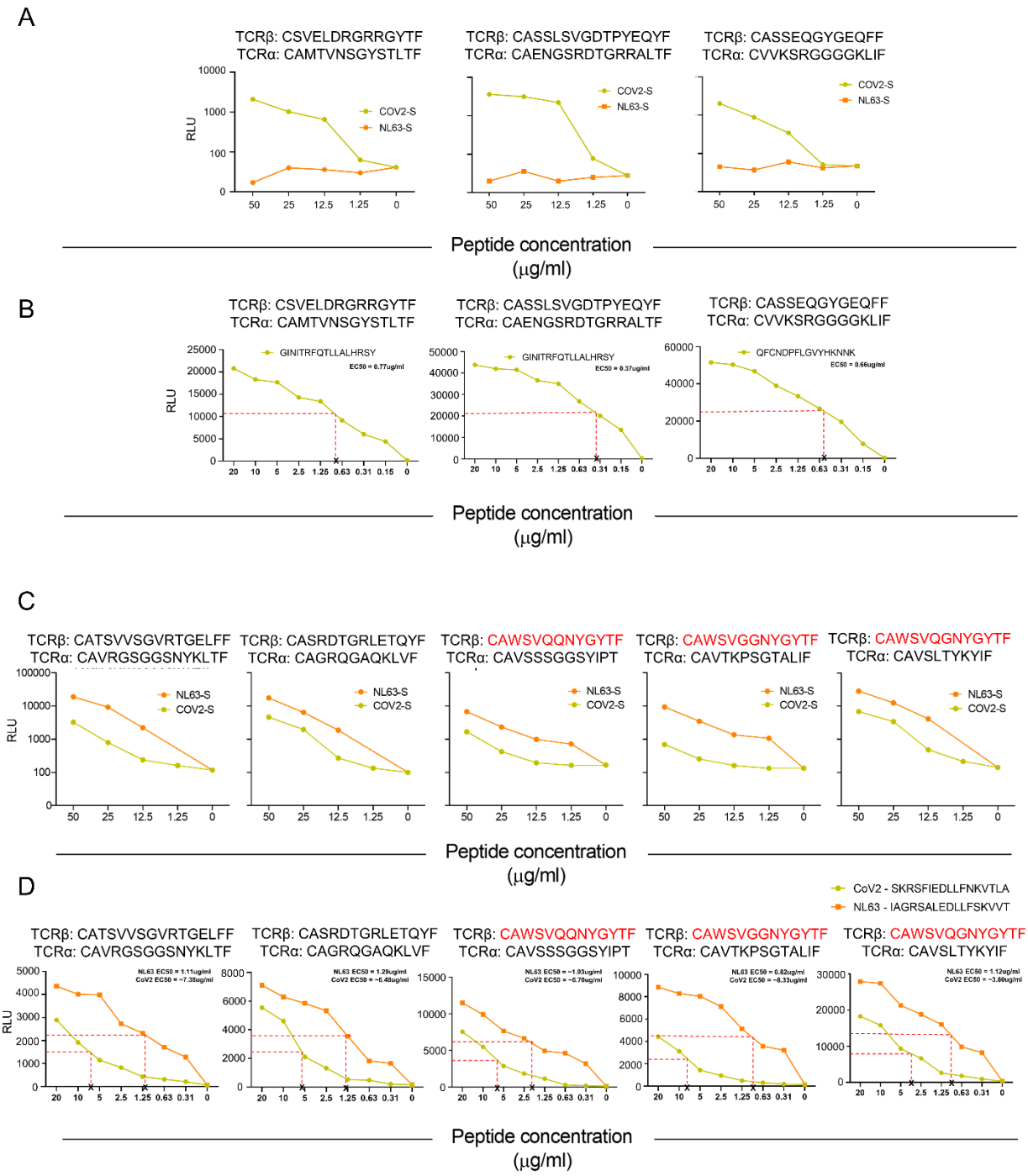

Figure 4. Functional validation and avidity of SARS-CoV-2 and HCoV-NL63 S-reactive TCRs. Single cell TCR sequencing was performed to identify the cognate TCRα of SARS-CoV-2 mono-reactive (A,B) and SARS-CoV-2/HCoV-NL63 cross-reactive (C&D) and TCRβ clonotypes. TCRs were cloned and transfected into a Jurkat NFAT-luciferase reporter cell line. Jurkat cells expressing TCRs of interest were co-cultured at 1:1 ratio with patient lymphoblastic cell lines (LCL) and SARS-CoV-2 S or HCoV-NL63 S peptide pools in titrating concentrations of S protein peptide pools (A and C) or the mapped 17-mer (B and D). Data are shown as relative luminescence units (RLU) at each pool or peptide concentration. Functional avidity for the specific 17-mer epitope was determined by calculating an EC50 (concentration at which response was ½ of maximum RLU, black x). These data are representative of all experiments repeated twice.

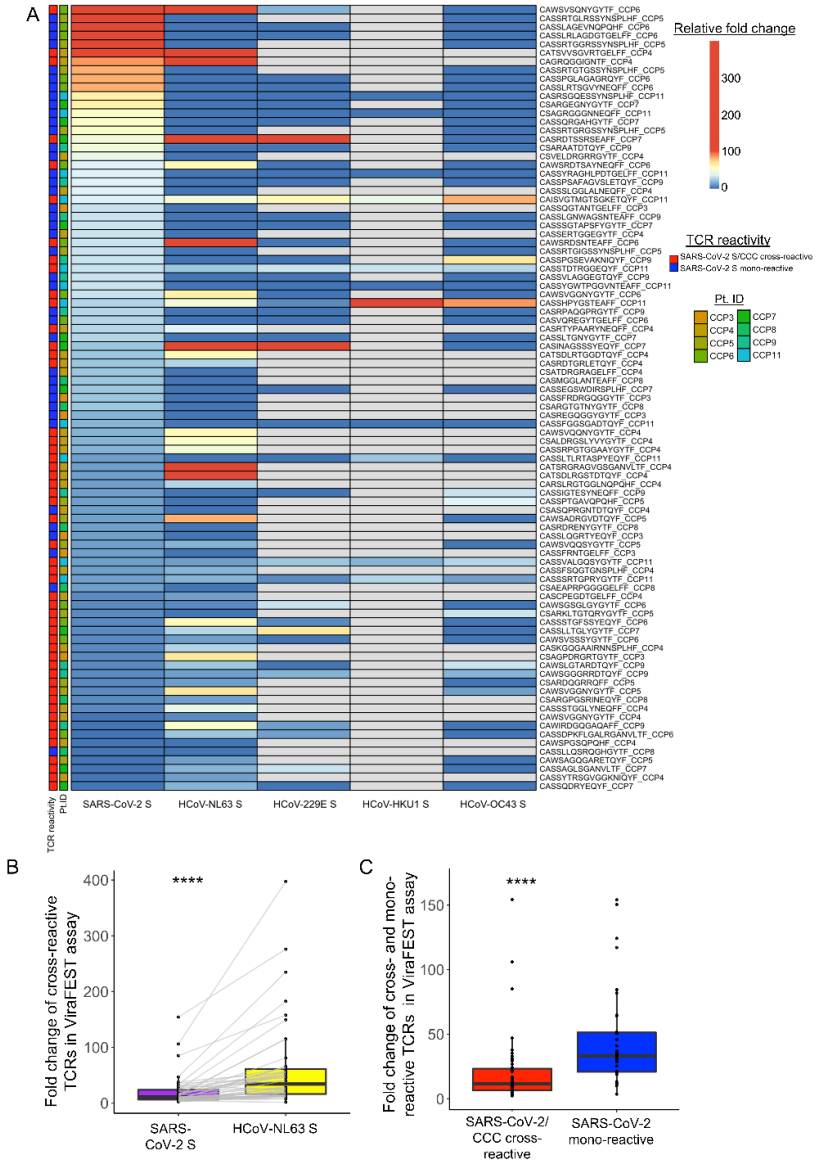

Figure 5. Functional activity of SARS-CoV-2 S cross-reactive and mono-reactive CD4+ T cell responses in CCP donors. A heatmap was generated to visualize the fold change of SARS-CoV-2/CCC cross-reactive (red) and SARS-CoV-2 mono-reactive (blue) T cell clonotypes relative to the negative control (A) as detected in the VIraFEST assays of CCP donors. Each row represents a single TCR Vβ CDR3 clonotype and each column represents a peptide pool. To account for clones that were only present in one condition, a pseudocount of 1 was added to all clonotype counts and frequency was recalculated. Fold change was then computed by dividing frequency in response to a peptide of interest (i.e., SARS-CoV-2 S or CCC S) over the negative control (HIV). Rows were ordered by fold change of SARS-CoV-2 S in a descending order. Red represents high relative fold change and blue represents low relative fold change. The average fold change was calculated to compare overall functional avidity of cross-reactive clones for SARS-CoV-2 S relative to HCoV-NL63 S for each clonotype (n=46; B), and to compare relative functional activity for the SARS-CoV-2 pool between cross-reactive (n=51) and mono-reactive clones (n=40; C). Each dot represents a SARS-CoV-2 S-reactive clonotype from a sample with grey line indicating a pair. Wilcoxon signed-rank test was performed to compare the fold change of cross-reactive clones in response to HCoV-NL63 S vs. SARS-CoV-2 S. Mann-Whitney U test was used to compare the fold change of SARS-CoV-2 S mono-reactive vs. cross-reactive clones. ****: P≤0.0001

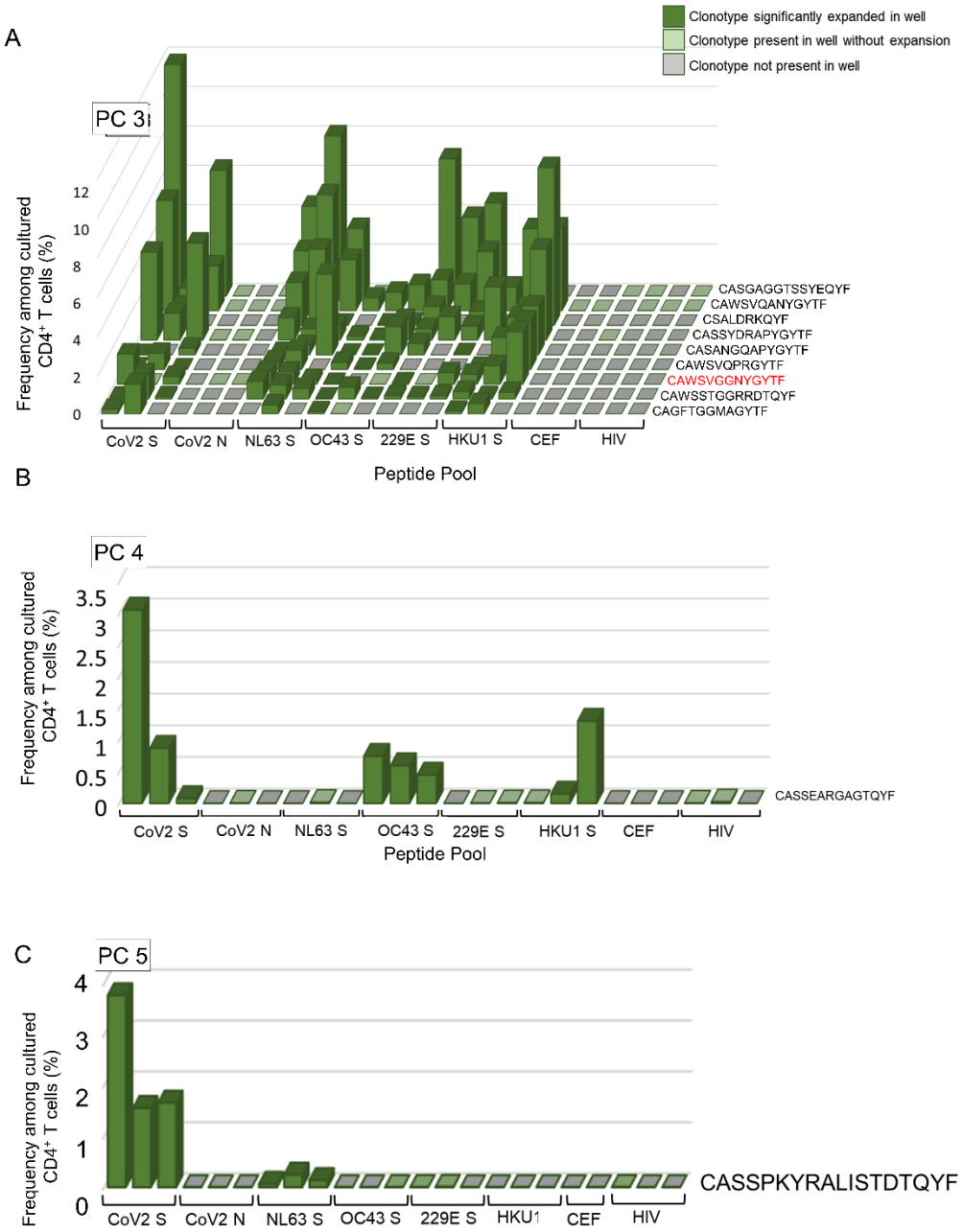

Figure 6. Detection of recall SARS-CoV-2/CCC cross-reactive CD4⁺ T cells in unexposed donors. The ViraFEST assay was used to probe peripheral blood CD4+ T cells obtained prior to the COVID-19 pandemic (2011-2018) for reactivity to SARS-CoV-2 S and N as well as CCCs (HCoV-NL63, HCoV-OC43, HCoV-229E, and HCoV-HKU1) as cell number allowed. The assay was performed in triplicate unless otherwise noted. Responses were detected in PC3 (A), PC4 (B), and PC5 (C).). Data are shown as the frequency (%) after culture (y-axis) of antigen-specific CD4+ T cell clonotypes (z-axis) for all peptide pools tested (x-axis). Solid green bars represent significant (FDR<0.05) clonotypic expansion in response to the indicated antigenic peptide pool(s), whereas translucent green color indicates the clonotype was present at low frequency in the well but did not significantly expand. Grey indicates the relevant TCR clonotype was not detected in that well. An identical, shared TCR Vβ CDR3 amino acid clonotype corresponding to cross-reactive memory CD4+ T cells, previously identified in CCP4, CCP5, and CCP6, is shown in red.

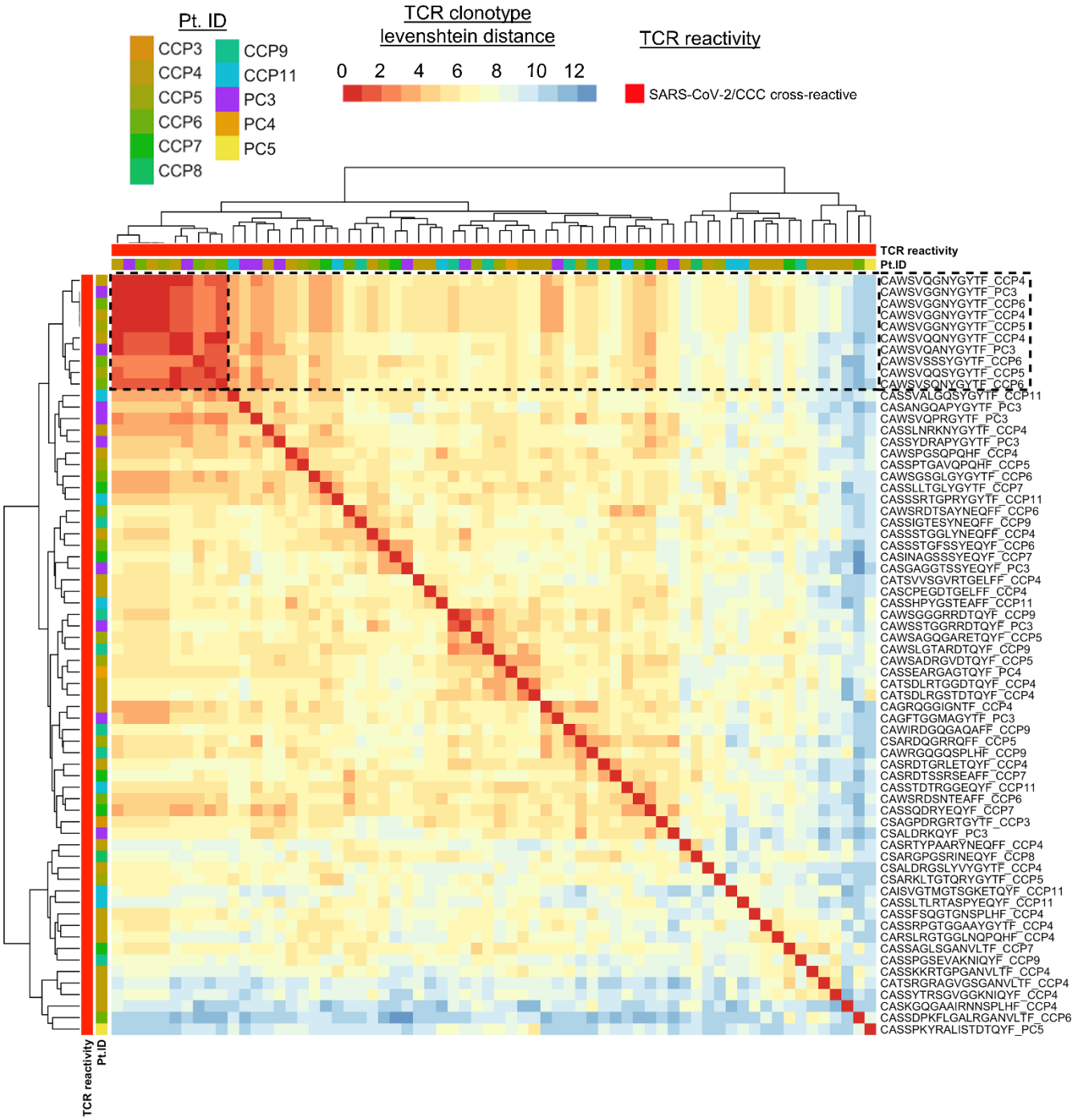

Figure 7: Shared sequence homology of SARS-CoV-2/CCC cross-reactive TCRs within and among patients. A heatmap was generated to visualize sequence homology of cross-reactive clones within and between the patients in our study. Each row/column represents a cross-reactive clonotype in a sample. The color represents the levels of sequence homology using Levenshtein distance, with red indicating high degree of sequence homology and blue for less sequence similarity. Different colors were used to distinguish patient samples in the color sidebar. The black box highlights an area of high sequence homology across multiple patients with Levenshtein distance ≤ 3.