Healthy donor T cell responses to common cold coronaviruses and SARS-CoV-2

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BACKGROUND. T cell responses to the common cold coronaviruses have not been well characterized. Preexisting T cell immunity to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been reported, and a recent study suggested that this immunity was due to cross-recognition of the novel coronavirus by T cells specific for the common cold coronaviruses.

METHODS. We used the enzyme-linked immunospot (ELISPOT) assay to characterize the T cell responses against peptide pools derived from the spike protein of 3 common cold coronaviruses (HCoV-229E, HCoV-NL63, and HCoV-OC43) and SARS-CoV-2 in 21 healthy donors (HDs) who were seronegative for SARS-CoV-2 and had no known exposure to the virus. An in vitro expansion culture assay was also used to analyze memory T cell responses.

RESULTS. We found responses to the spike protein of the 3 common cold coronaviruses in many of the donors. We then focused on HCoV-NL63 and detected broad T cell responses to the spike protein and identified 22 targeted peptides. Interestingly, only 1 study participant had a significant response to SARS-CoV-2 spike or nucleocapsid protein in the ELISPOT assay. In vitro expansion studies suggested that T cells specific for the HCoV-NL63 spike protein in this individual could also recognize SARS-CoV-2 spike protein peptide pools.

CONCLUSION. HDs have circulating T cells specific for the spike proteins of HCoV-NL63, HCoV-229E, and HCoV-OC43. T cell responses to SARS-CoV-2 spike and nucleocapsid proteins were present in only 1 participant and were potentially the result of cross-recognition by T cells specific for the common cold coronaviruses. Further studies are needed to determine whether this cross-recognition influences coronavirus disease 2019 (COVID-19) outcomes.

Introduction

There are 4 known human common cold coronaviruses (HCoV) that cause mild respiratory disease: HCoV-NL63, HCoV-229E, HCoV-OC43, and HCoV-HKU1 (1). Seroprevalence studies show that a large percentage of adults have been exposed to these viruses (2). Interestingly, surveillance studies have shown that reinfection with these viruses can occur (3, 4), suggesting that immunity is only partially protective. This theory is supported by a challenge study showing that study participants with lower titers of antibodies against HCoV-229E were infected and developed symptoms following experimental inoculation with the virus (5). Some of the same individuals could be reinfected by the same virus 1 year later, but they experienced minimal symptoms and had reduced periods of viral shedding (5). Despite these data, the T cell responses to these viruses in healthy donors (HDs) have not been characterized in an unbiased manner, and it is not known whether T cells contribute to the partial immunity described above.

Severe acute respiratory syndrome coronavirus 2–specific (SARS-CoV-2–specific) T cell responses have been detected in patients with coronavirus disease 2019 (COVID-19) (6–20), and while T cell responses against SARS-CoV have been shown to be long lasting (8), it is not yet known whether SARS-CoV-2–specific T cells will confer protection against reinfection. Recent studies have suggested that preexisting T cell immunity to SARS-CoV-2 is present in some unexposed, HDs (6–11). However, other studies have found no evidence of SARS-CoV-2–specific T cells in unexposed individuals (13, 21). In this study, we sought to characterize the T cell responses to human cold coronaviruses and to determine whether preexisting immunity to SARS-CoV-2 was due to cross-recognition by T cells specific for endemic coronaviruses. To do this, we examined T cell responses to the spike (S) protein of 3 of the 4 common cold coronaviruses (HCoV-NL63, HCoV-229E, and HCoV-OC43) and to SARS-CoV-2 in HDs with no known exposure to SARS-CoV-2. We then focused on HCoV-NL63 and identified what we believe to be 19 novel targeted peptides. We also examined the responses to the SARS-CoV-2 nucleocapsid (N) and membrane (M) proteins and performed experiments to determine whether T cell cross-recognition of HCoV-NL63 and SARS-CoV-2 S peptides was possible. We believe our results further the understanding of the immune response to coronaviruses and may have implications for SARS-CoV-2 vaccine trials.

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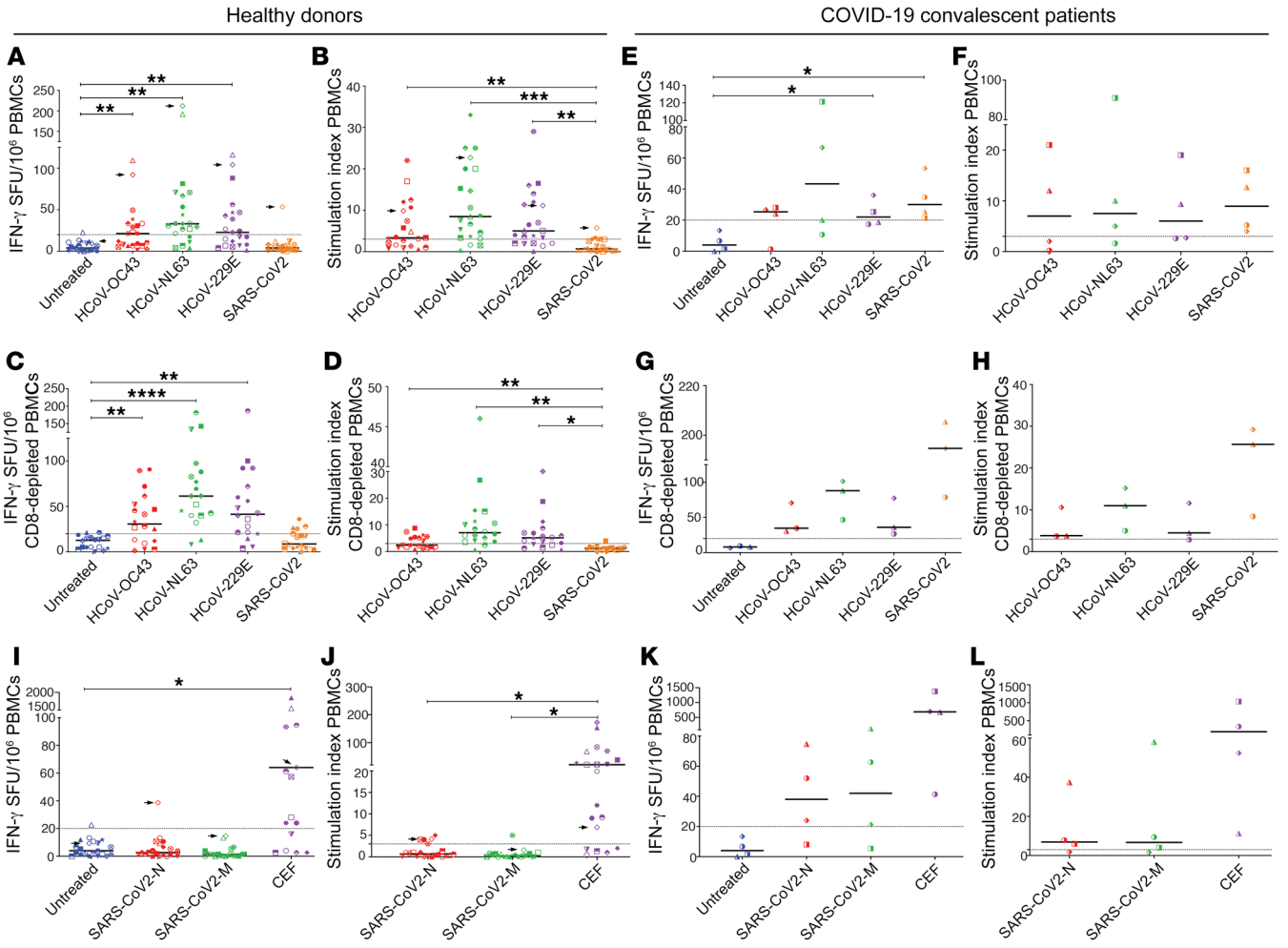

Figure 1. IFN-γ responses to viral peptide pools from HDs and CCPs. The number of SFU from unfractioned PBMCs (**A** and **E**) and CD8⁺ T cell–depleted PBMCs (**C** and **G**) and the corresponding stimulation indices (**B**, **D**, **F**, and **H**) in response to S protein peptide pools from different viruses are shown. The number of SFU (**I** and **K**) and the stimulation indices (**J** and **L**) from unfractioned PBMCs in response to CEF and SARS-CoV-2 M and N peptide pools are also shown. Arrows indicate HD9. Each data point represents the mean of 3 replicate values. Horizontal bars represent the median. Statistical comparisons were performed using 1-way ANOVA with Geisser-Greenhouse correction and Dunnett's multiple-comparison test (n = 19–21 for samples from HDs; n = 3–4 for samples from patients with COVID-19). *P = 0.0332, **P = 0.0021, ***P = 0.0002, and ****P < 0.0001.

Results

HDs have circulating CD4⁺ T cell responses to 3 common cold coronaviruses but not to SARS-CoV-2. For the purposes of this publication, the term "HDs" refers to individuals not previously exposed to SARS-Cov-2. To quantify responses in these individuals, we performed IFN-γ ELISPOT assays to measure the frequency of T cells that secreted IFN-γ in response to peptides from the S protein from the common cold coronaviruses and SARS-CoV-2. A stimulation index (SI) was calculated by dividing the spot-forming units (SFU) per million PBMCs elicited by a peptide pool by the SFU present in wells treated with media alone. A positive response was defined as a SI of greater than 3 and an absolute value of greater than 20 SFU per million PBMCs. The median frequency of T cells reactive to HCoV-NL63, HCoV-229E, and HCoV-OC43 S proteins was 33, 23, and 21 cells per million PBMCs, respectively. In contrast, the median response to SARS-CoV-2 was just 3 T cells per million PBMCs, which was not statistically different from the response to media alone (Figure 1A). Of the 21 HDs tested, 15, 10, and 10 individuals met both criteria for positive responses to HCoV-NL63, HCoV-229E, and HCoV-OC43 S peptides, respectively, whereas only 1 HD (HD9, indicated by the arrowheads in Figure 1) met both criteria for a positive response to the SARS-CoV-2 S peptide pool (Figure 1B).

In order to determine whether CD4⁺ or CD8⁺ T cells were responding to the peptides, we depleted CD8⁺ T cells from PBMCs and used the residual cells in an ELISPOT assay. In virtually all study participants, CD8⁺ T cell depletion increased the number of SFU in all conditions. The median responses elicited by HCoV-NL63, HCoV-229E, and HCoV-OC43 S peptide pools were 61, 41, and 31 SFU per million cells, respectively (Figure 1C), and while the median responses to the SARS-CoV-2 S peptide pools were also higher, none of the participants met both the absolute count and the SI criteria for a positive response (Figure 1, C and D). In contrast, T cells from COVID-19 convalescent patients (CCPs) recognized peptide pools from the SARS-CoV-2 S protein (Figure 1, E–H). The increase in responses to the common cold coronavi-

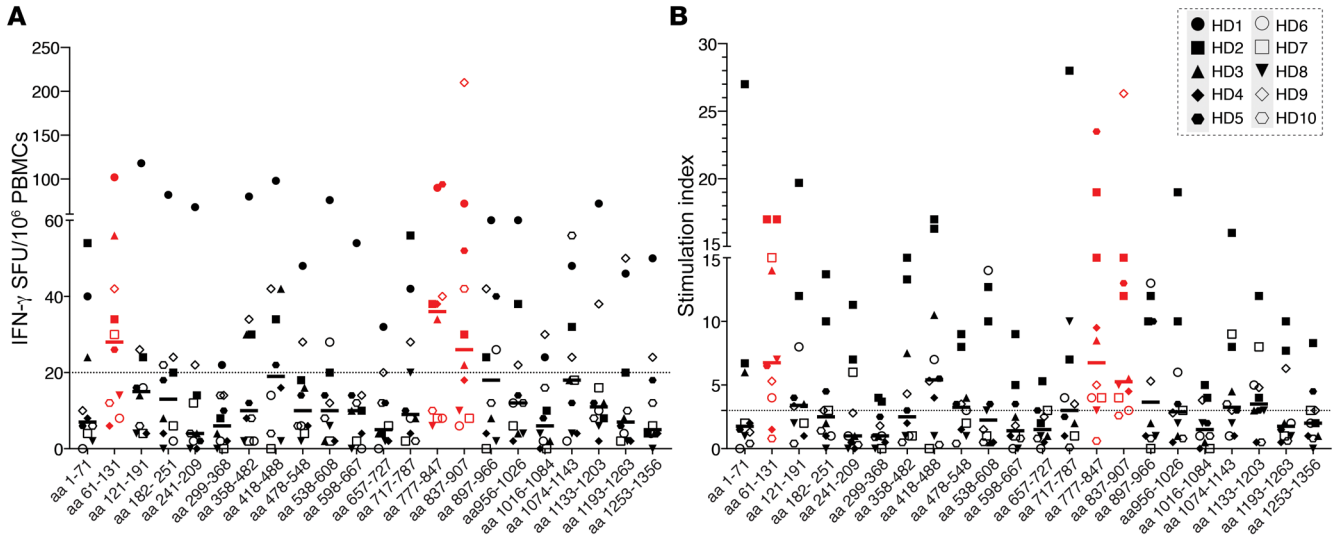

Figure 2. Breadth of T cell responses to HCoV-NL63 S protein. The numbers of SFU per million PBMCs (**A**) and stimulation indices (**B**) generated for pools of 10 peptides are shown for 10 HDs. Horizontal bars indicate the median. Pools that elicited the most potent responses are highlighted in red.

rus S peptide pools following CD8⁺ T cell depletion suggests that CD4⁺ T cells were the major effector cells in our assay, especially since depletion of CD8⁺ T cells abrogated the responses to MHC class I–restricted peptide pools from CMV, EBV, and influenza (CEF) (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI143120DS1). However, it is likely that CD4⁺ T cells were more apt to be induced by the relatively long peptides used in our assay.

We then asked whether HD T cells were better able to recognize other SARS-CoV-2 peptides, including those from the N and M proteins. As shown in Figure 1, I and J, although the majority of HDs responded to CEF peptides, only HD9 had a robust response to peptides from the N protein, and no individual responded to peptides from the M protein. Although some of the other HDs had T cell responses that met the criteria for a positive response according to the SI, the absolute number of responding cells was less than the 20 SFU cutoff. In contrast, T cells from 2 of the 4 CCPs recognized peptide pools from the SARS-CoV-2 M and N proteins (Figure 1, K and L).

T cells target various regions of the HCoV-NL63 S protein. The most robust T cell responses were directed against the S protein of HCoV-NL63, so we focused on this virus for epitope-mapping studies. In order to determine which regions of the S protein were targeted by HD T cells, we performed ELISPOT assays with sequential peptide pools consisting of 10 overlapping peptides. As shown in Figure 2, we observed broad responses to the S peptide pools, and every pool was targeted by T cells from at least 1 individual. However, the most potent responses were elicited by pools 14 (amino acids 777–847), 2 (amino acids 61–131), and 15 (amino acids 837–907), with a median of 36, 28, and 26 T cells producing IFN-γ, respectively (marked in red on Figure 2). In order to define the targeted peptides, we repeated the ELISPOT assay with individual peptides from the pools that were targeted by the 6 HDs for whom we had sufficient numbers of PBMCs. Table 1 contains the list of the 22 peptides we were able to identify and the potential optimal epitopes and restricting HLA alleles. Interestingly, peptides 16 (amino acids 91–107), 132 (amino acids 783–799), and 141 (amino acids 837–853) were each targeted in 2 individuals.

Expansion of memory T cells and cross-recognition of HCoV-NL63 and SARS-CoV-2 S protein peptide pools. We cultured PBMCs with peptide pools from different viral proteins to determine whether we could detect memory CD4⁺ T cell responses that were not seen when PBMCs were assayed directly after isolation. As shown in Figure 3, preculturing of PBMCs with the HCoV-NL63 S peptide pool caused an increase in the percentage of HD and CCP CD4⁺ T cells that coexpressed either IFN-γ and IL-2 (Figure 3, A and B) or IFN-γ and TNF-α (Figure 3, C and D) when the cells were restimulated with the same peptide pool. Interestingly, a modest but significant increase was also seen when cells from HDs were precultured and stimulated with SARS-CoV-2 S peptide pools, suggesting that memory responses to these peptides could be amplified in some HDs. HD9, the only individual who had a positive ELISPOT response to the SARS-CoV-2 peptide pool, also had the most robust memory T cell responses to both HCoV-NL63 and SARS-CoV-2 S peptide pools. We performed the preculture expansion assay to determine whether cross-recognition could potentially explain this observation. As shown in Figure 4, PBMCs cultured in the absence of antigen for 10 days did not produce responses to HCoV-NL63 or SARS-CoV-2 S proteins or to the SARS-CoV-2 N protein that were above background levels (Figure 4A, plots 1–4) following a 12-hour restimulation with each peptide pool. In contrast, following 10 days of culturing with HCoV-NL63 S protein peptides, a 12-hour restimulation with the same peptides induced coexpression of IFN-γ and IL-2 from 1.25% of CD4⁺ T cells, a 9.6-fold increase over the response obtained when the cells were precultured without peptide (Figure 4A, plot 6 vs. plot 2). Interestingly, when cells that were cultured with the HCoV-NL63 S peptide pool for 10 days were restimulated with SARS-CoV-2 S peptides, we detected coexpression of IFN-γ and IL-2 in 0.41% of CD4⁺ T cells (Figure 4A, plot 8). This represents a 2.6-fold increase over cells that were precultured for 10 days in the absence of peptide and then stimulated with SARS-CoV-2 S peptides (Figure 4A, plot

Table 1. HCoV-NL63 T cell–targeted peptides detected by ELISPOT assay and HLA-binding predictions

HD ID (HLA alleles)	Peptide number	Amino acid number	Targeted peptide (predicted optimal epitope underlined)	Predicted HLA-restricting alleles	SFU/10^6 PBMCs
HD2 DRB1*01:01, 07:01, DRB4* 01:01, DQA1*02:01/DQB1*02:02 DQA1*01:01/DQB1*05:01 DPA1*01:03/DPB1*04:01	16[A]	91–107	VTNEIGLNASVTLKICK	DRB1*07:01, DRB1*01:01	26
	75	442–458	FEKLQCEHLQFGLQDGF	DRB4*01:01, DPA1*01:03/DPB1*04:01	48
	133	789–805	CATYVCNGNPRCKNLLK	DRB1*01:01, DRB1*07:01	30
	161	956–972	ARLNYVALQTDVLQENQ	DQA1*02:01/DQB1*02:02, DRB1*01:01	30
	166	986–1002	IVASFSSVNDAITQTAE	DQA1*02:01/DQB1*02:02, DRB1*07:01	26
HD4 DRB1*01:03, 13:05, DRB3* 02:02, DQA1*01:01/DQB1*05:01, DQA1*05:05/DQB1*03:01, DPA1*01:03/DPB1*02:01, DPA1*01:03/DPB1*04:01	134[B]	795–811	NGNPRCKNLLKQYTSAC	DRB3*02:02	28
	142	843–859	ANVTSFGDYNLSSVLPQ	DQA1*01:01/DQB1*05:01	36
	147	873–889	LEDLLFSKVVTSGLGTV	DRB1*01:03, DRB1*13:05	30
HD5 DRB1*07:01,11:01, DRB3*0202, DQA1*02:01/DQB1*03:03, DQA1*05:05/DQB1*03:01, DPA1*01:03/DPB1*04:01, DPA1*02:01/DPB1*13:01	16[A]	91–107	VTNEIGLNASVTLKICK	DRB3*02:02, DRB1*07:01	44
	132	783–799	TPIVVDCATYVCNGNPR	DRB3*02:02, DQA1*02:01/DQB1*03:03	30
HD7 DRB1*04:04,11:01, DRB3*0202, DRB4*0103, DQA1*03:01/DQB1*03:02, DQA1*05:05/DQB1*03:01, DPA1*01:03/DPB1*06:01, DPA1*02:01/DPB1*14:01	17	97–113	LNASVTLKICKFSRNTT		64
	20	115–131	DFLSNASSSFDCIVNLL	DRB3*02:02, DQA1*05:05/DQB1*03:01	38
	150	891–907	VDYKSCTKGLSIADLAC	DRB1*11:01 DQA1*05:05/DQB1*03:01	68
HD9 DRB1*11:01,14:02, DRB3*0101, 0202, DQA1*05:03/DQB1*03:01, DQA1*05:05/DQB1*03:01, DPA1*01:03/DPB1*04:01, DPA1*01:03/DPB1*04:02	18	103–119	LKICKFSRNTTFDFLSN	DRB3*02:02	80
	62	364–380	TFVGILPPTVREIVVAR		35
	71	418–434	ATFVDVLVNVSATNIQN		40
	132	783–799	TPIVVDCATYVCNGNPR	DRB3*01:01	65
	141	837–853	SNAFSLANVTSFGDYNL	DRB3*02:02, DRB1*14:02	180
	146[C]	867–883	IAGRSALEDLLFSKVVT		108
	158	938–954	VLGGLTSAAAIPFSLAL	DQA1*05:03/DQB1*03:01	48
	160	950–966	FSLALQARLNYVALQTD	DRB1*14:02	45
	192	1139–1155	KNVKAWSGICVDGIYGY	DQA1*05:05/DQB1*03:01	70
	196	1163–1179	VLYSDNGVFRVTSRVMF		55
	205	1217–1233	VNKTLQEFAQNLPKYVK	DRB3*02:02, DRB1*14:02	50
HD10 DRB1*04:05,04:07, DRB4*0103, DQA1*03:03/DQB1*03:01, DQA1*03:03/DQB*03:02, DPA1*01:03/DPB1*03:01, DPA1*01:03/DPB1*04:01	141	837–853	SNAFSLANVTSFGDYNL	DRB1*04:07, DQA1*03:03/DQB1*03:01	38

[A]Peptide 16 partially overlaps with an HCoV-NL63 peptide that was found to be homologous to a SARS-CoV-2 peptide targeted by unexposed HDs (S 96-110) (16). [B]Peptide 134 partially overlaps with an HCoV-NL63 peptide that was found to be homologous to a SARS-CoV-2 peptide targeted by unexposed HDs (S 802-816) (16). [C]Peptide 146 partially overlaps with an HCoV-NL63 peptide that was found to be homologous to a SARS-CoV-2 peptide targeted by unexposed HDs (S 861-880) (16).

4). Of note, we observed no increase in the percentage of cells that recognized SARS-CoV-2 N peptides following preculturing with the HCoV-NL63 S peptide pool (Figure 4A, plot 7 vs. plot 3), suggesting that the increase in SARS-CoV-2 S peptide–reactive cells was not due to nonspecific stimulation. Thus, it is likely that there was $CD4^+$ T cell cross-recognition of S peptides from the 2 viruses. We observed similar 2.6- and 3-fold increases in antigen-responsive $CD4^+$ T cells when PBMCs precultured with SARS-CoV-2 S peptides for 10 days were restimulated with SARS-CoV-2 (Figure 4A, plot 16 vs. plot 4) and HCoV-NL63 (Figure 4A, plot 14 vs. plot 2) S peptide pools, respectively, which is further evidence of T cell cross-recognition in HD9.

We performed the same experiment with PBMCs from a CCP (CCP2). Preculturing of PBMCs with HCoV-NL63 S pep-

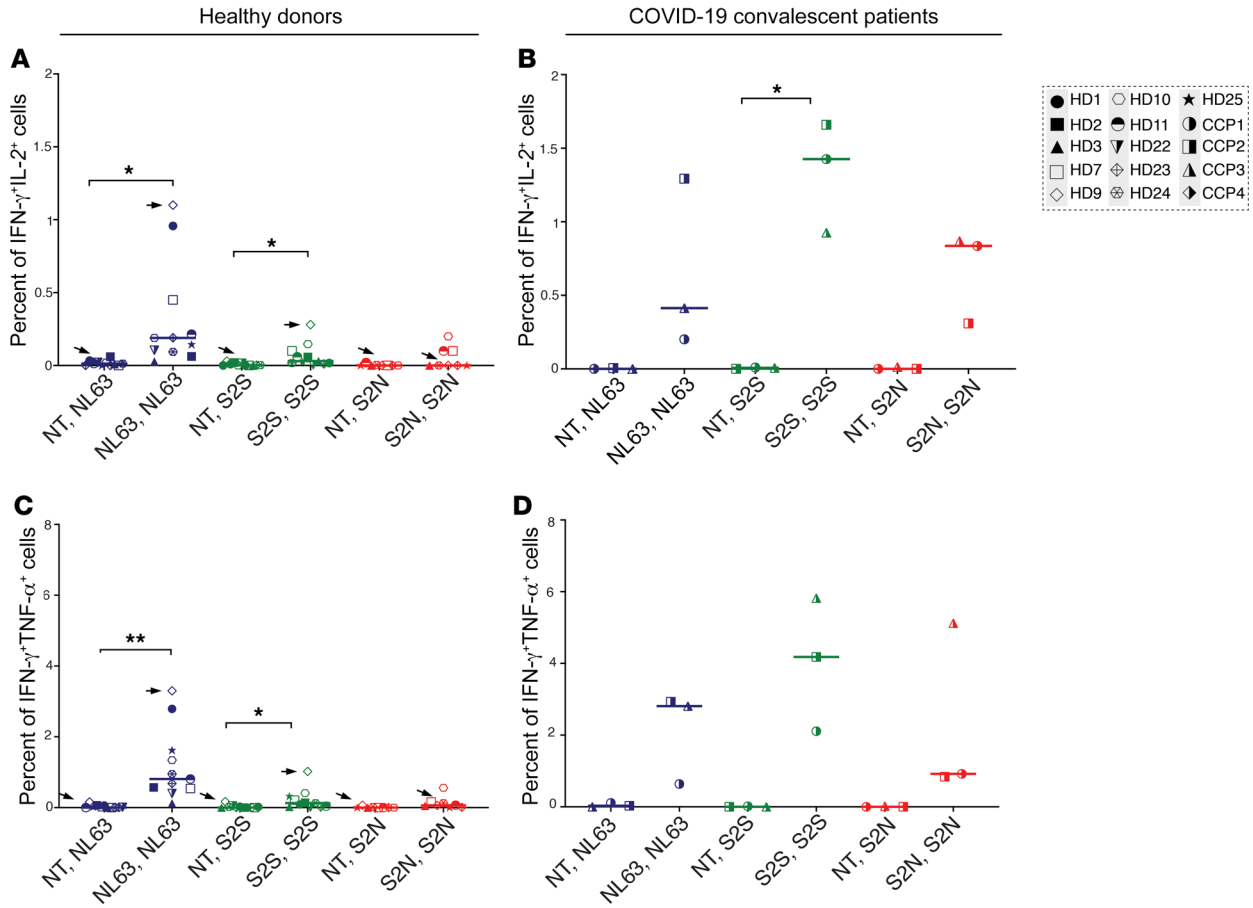

Figure 3. Expansion of antigen-specific CD4⁺ T cell responses. The percentages of cells that coexpressed either IL-2 and IFN-γ (**A** and **B**) or TNF-α and IFN-γ (**C** and **D**) are shown for cells from HDs (**A** and **C**) and CCPs (**B** and **D**) following preculturing for 10–12 days and stimulation for 12 hours with varied peptide pools (n = 11 HDs; n = 3 CCPs). In each panel, the peptide pool used for preculturing is shown first, followed by the peptide pool used in the 12-hour stimulation. *P = 0.0332 and **P = 0.0021, by 2-tailed, paired Student's t test. Horizontal bars represent the median. NT, untreated; NL63, HCoV-NL63; S2N, SARS-CoV-2-N; S2S, SARS-CoV-2-S.

tides resulted in 42.6- and 10.0-fold increases in the percentage of cells that responded to restimulation with HCoV-NL63 (Figure 4B, plot 22 vs. plot 18) and SARS-CoV-2 S peptides (Figure 4B, plot 24 vs. plot 20), respectively. Interestingly, while preculturing of the PBMCs with the SARS-CoV-2 S peptide resulted in a 76.8-fold increase in the percentage of cells that responded to restimulation with the SARS-CoV-2 S peptide pool (Figure 4B, plot 32 vs. plot 20), no such increase was seen in the percentage of CD4⁺ T cells that responded to restimulation with HCoV-NL63 S peptides (Figure 4B, plot 30 vs. plot 18). Thus, the memory CD4⁺ T cells that were amplified by the S peptides from the 2 viruses most likely had different T cell receptor repertoires with different cross-recognition capacities. We obtained similar cross-recognition results with PBMCs from another CCP, CCP3 (Supplemental Figure 2), and in this case, preculturing of PBMCs with SARS-CoV-2 S peptides also resulted in cross-recognition of the NL63 S peptide pool.

We generally did not see amplification of HD CD8⁺ T cell responses after preculturing with HCoV-NL63 S or SARS-CoV-2 S or N peptide pools (Supplemental Figure 3). However, CD8⁺ T cells coexpressing TNF-α and IFN-γ in response to SARS-CoV S and N peptide pools were amplified in CCP3 in the expansion assay, and there was again evidence of cross-recognition of HCoV-NL63 and SARS-CoV-2 S peptides, suggesting that this phenomenon was not limited to CD4⁺ T cells (Supplemental Figure 2).

Discussion

In this study, we characterized the frequency of circulating common cold coronavirus–specific CD4⁺ T cells in COVID-19–negative individuals. We show that many HDs who had not had upper respiratory syndromes in the past few months had a significant percentage of T cells that targeted the S protein of 3 common cold coronaviruses. The response to the HCoV-NL63 S protein appeared to be broad, and we identified 22 targeted peptides in this protein.

Several studies have looked for the presence of SARS-CoV-2–specific T cells in HDs. Peng et al. found no responses to various peptide pools by ELISPOT assays in 15 HDs from the United Kingdom (13), and Zhu et al. did not detect any baseline ELISPOT responses to S protein peptides in 108 vaccine recipients in China (21). In contrast, using an ELISPOT assay, Sekine et al. found T cells specific for the S and M, but not N, proteins in HDs in Sweden who donated blood prior to the pandemic (7). Le Bert et al. detected responses to the N and nonstructural proteins in at least 30% of HDs in Singapore, also with the ELISPOT assay (8). Using upregulation of Ox40 and CD137 to detect T cell responses in PBMCs col-

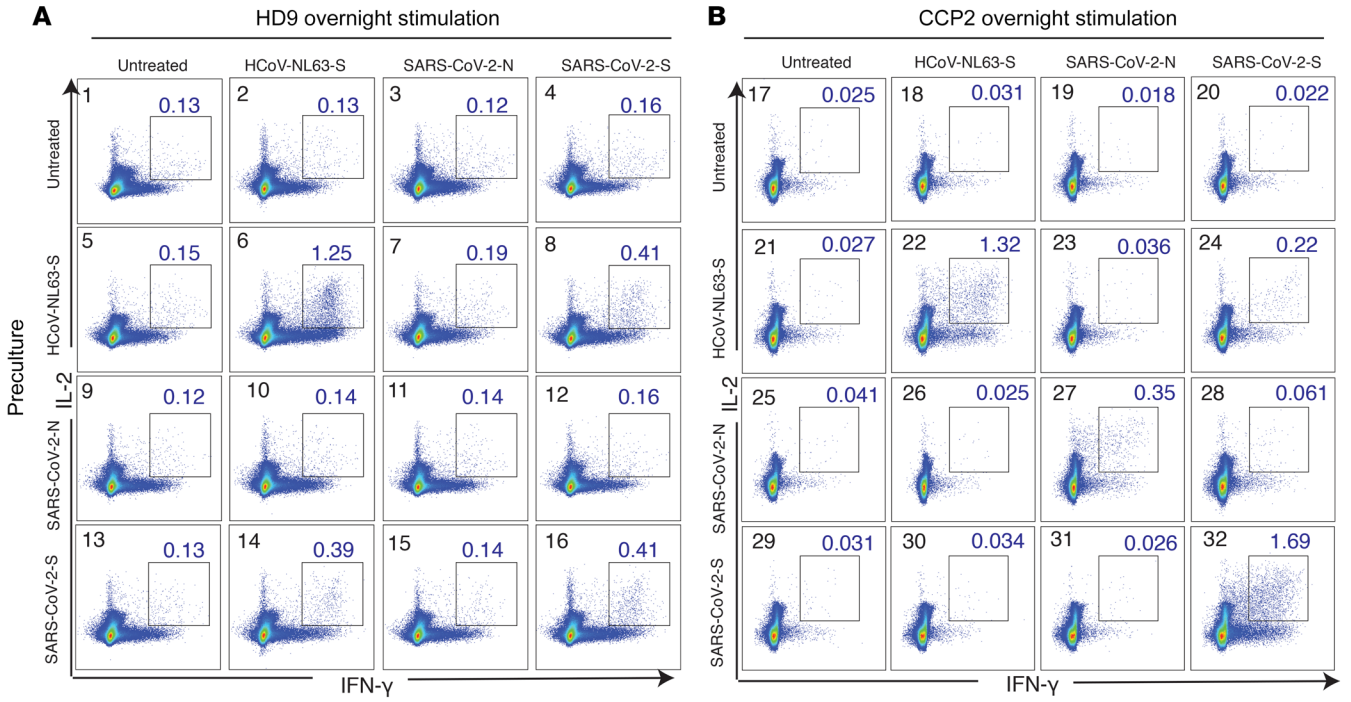

Figure 4. Cross-recognition of HCoV-NL63 and SARS-CoV-2 S protein peptide pools in HD9 and CCP2. PBMCs from HD9 (**A**) and CCP2 (**B**) were precultured with peptide pools (shown in rows) for 10–12 days and then stimulated for 12 hours with peptide pools (shown in columns). The percentage of cells that coexpressed IL-2 (*y* axis) and IFN-γ (*x* axis) is shown above the gated box in the upper right corner of each plot.

lected prior to the pandemic, Grifoni et al. found that CD4⁺ T cells from 40%–60% of donors in the United States reacted to SARS-CoV-2 peptides (6). Weiskopf et al. found that CD4⁺ T cells from 2 of 10 HDs in the Netherlands upregulated CD69 and CD137 in response to SARS-CoV-2 peptides (11). Similarly, Braun et al. found that 35% of their HDs in Germany had CD4⁺ T cell responses to SARS-CoV-2 spike protein peptide pools as determined by upregulation of 4-1BB and CD40L (9). The reason for this baseline reactivity and the difference in the frequency of HDs with preexisting immunity to SARS-CoV-2 is unclear, but differences in exposure to common cold coronaviruses and potential cross-reactivity between T cells specific for these viruses and SARS-CoV-2 have been postulated as a possible explanation. Although we did not analyze responses to the nonstructural proteins, we show here that most of our HDs did not have detectable responses to SARS-CoV-2 M, N, or S peptide pools by ELISPOT in spite of having detectable responses to 2 or 3 common cold coronaviruses. However, preculturing of cells with S peptide pools resulted in a modest but significant ($P = 0.03$) increase in the frequency of T cells that responded to these peptides, suggesting that memory T cell responses existed in some HDs. Although it is also possible that these were de novo responses, the expansion assay we used did not involve the stimulation of T cells with isolated DCs, and in prior experiments, we were unable to generate de novo responses to peptides (22).

Mateus et al. recently mapped out thirty-one SARS-CoV-2 S protein epitopes that were targeted by T cell lines from unexposed HDs (16). They showed that the homologous peptides in the S protein from the common cold coronaviruses were also recognized. These data suggest that this cross-recognition of viral epitopes by T cells can explain the preexisting immunity seen in some of their study participants. Notably, 28 of 31 of the homologous HCoV-NL63 S protein peptides identified by this approach were not targeted by CD4⁺ T cells from the 6 HDs we tested, and this difference may partially explain the low number of individuals with preexisting SARS-CoV-2 immunity in our cohort. Interestingly, HD9, the only participant in our cohort who responded to SARS-CoV-2 peptide pools, had T cells that made a robust response to an HCoV-NL63 peptide (S 867-883) that overlaps significantly with a homologous HCoV-NL63 peptide (S 861-880) found to be targeted in 2 individuals in the Mateus et al. cohort (16). The S 861-880 peptide was found to have 53% homology to the SARS-CoV-2 S peptide (S 811-825) that elicited T cell responses in unexposed individuals.

A strength of our study is that we used an unbiased approach and examined the responses to overlapping peptides spanning the entire HCoV-NL63 S protein to determine targeted peptides. This approach is distinct from, and complimentary to, the approach used by Mateus et al., in which epitopes in the 4 common cold coronaviruses were detected by analyzing peptides that had homology to 142 SARS-CoV-2 epitopes (16). Our study is limited by the fact that we did not look at responses to HCoV-HKU1 protein and that we analyzed the responses to just the S protein. However, in studies in individuals with SARS (23) and COVID-19 (6, 7, 9–16), the S protein is quite immunodominant, so it is likely that the responses to the S protein peptides of the common cold coronaviruses we observed were representative of the responses to the entire viral proteome. Another limitation is that, although we analyzed HD responses to SARS-CoV-2 S, M, and N peptide pools, we did not test for reactivity to SARS-CoV-2 nonstructur-

al proteins. This is important, because some studies have shown responses to peptides from these antigens in unexposed donors (6, 8, 16). We may also not have detected SARS-CoV-2–specific memory CD4+ T cell responses in more HDs because we used a low concentration of IL-2 in our expansion assay in an effort to minimize nonspecific activation. Finally, we characterized cross-reactive T cell responses in just 1 unexposed HD, because HD9 was the only unexposed HD in our cohort with preexisting immunity to SARS-CoV-2. Although we screened this individual using 2 different SARS-CoV-2 antibody tests with stated sensitivities of 100% among hospitalized patients by 3 weeks after symptom onset (24, 25), negative findings do not definitively prove that this individual did not have asymptomatic infection. This is important, given the studies showing that seronegative exposed individuals can have SARS-specific T cell responses (7, 26), although the cross-reactivity we describe here may also explain those results.

We believe our data are important, because we interpreted the frequency of circulating SARS-CoV-2–specific effector T cells in HDs in the context of the frequency of HCoV-specific effector T cells. Furthermore, we show directly that in HD9, cross-recognition of SARS-CoV-2 peptides by HCoV-NL63–specific CD4+ T cells could occur, and this can potentially explain previously described reports of preexisting immunity to SARS-CoV-2 in unexposed individuals, and is consistent with the results of Mateus et al. (16). Further studies in larger cohorts will be needed to determine how common these cross-reactive responses are. It will also be important to determine whether these responses lead to more rapid control of viral replication, thus conferring protection, or whether they contribute to inflammation or suboptimal priming of SARS-CoV-2–naive T cells and lead to poor outcomes.

Methods

Subjects. Blood samples from healthy laboratory donors and 4 individuals who recovered from COVID-19 were obtained between April and July 2020. All the HDs reported no known exposure to COVID-19 patients and no upper respiratory tract infections over the preceding 3 months. Twelve of the HDs were between the ages of 20 and 29 years, 3 were between the ages of 30 and 39 years, 5 were between the ages of 40 and 49 years, and 1 was between the ages of 50 and 59 years. Thirteen of the HDs were men and 8 were women. We also studied 4 patients who had recovered from COVID-19. Blood was drawn 3 months after the onset of their symptoms. Three were previously healthy and had mild disease courses (CCP1, CCP3, CCP4), and 1 participant with well-controlled HIV-1 infection on antiretroviral therapy had a severe disease course (CCP2). For all experiments, PBMCs were collected from whole blood after Ficoll-Paque PLUS gradient centrifugation (GE Healthcare Life Sciences). For some experiments, CD8+ T cells were depleted using Miltenyi Biotec CD8+ T Cell Positive Selection Kits. High-resolution class II typing was performed on PBMCs from 6 HDs at the Johns Hopkins Hospital Immunogenetics Laboratory. The Immune Epitope Database and Analysis Resource (http://www.iedb.org) was used for optimal epitope and HLA-binding predictions using recommended parameters (27).

Peptides and ELISPOT assays. Peptides for the S protein of HCoV-NL63, HCoV-229E, HCoV-OC43, and SARS-Cov-2, as well as the M and N proteins of SARS-CoV-2 were obtained from BEI Resources and were reconstituted with DMSO at a concentration of 10 mg/mL. The HCoV-229E S protein peptide pool has 195 peptides consisting of 17 mer with 11 amino acid overlaps. The HCoV-NL63 S protein peptide pool has 226 peptides made up of 14–17 mer with 11–13 amino acid overlaps. The HCoV-OC43 S protein peptide pool has 226 peptides made up of 17 or 18 mer with 11 amino acid overlaps. The SARS-CoV-2 peptides are 12 mer, 13 mer, or 17 mer, with 10 amino acid overlaps. The S protein peptide pool was made up of 181 peptides, the N protein peptide pool was made up of 59 peptides, and the M peptide pool was made up of 31 peptides. All the peptides were combined into 1 pool for each viral protein. Pools of 10 peptides were made for the HCoV-NL63 S protein, and 1 pool had 17 peptides. Peptides for CEF were obtained from Anaspec. The pool consisted of thirty-two 8–12 mer peptides. Stimulation with anti-CD3 antibody (Mabtech, 1 μg/mL) was used as a positive control for each study participant.

IFN-γ ELISPOT assays were performed as previously described (28, 29). Briefly ELISPOT Pro and ELISPOT Plus kits with precoated plates were purchased from Mabtech. The wells were plated with unfractionated PBMCs or CD8+ T cell–depleted PBMCs at 250,000 cells/well, and the cells were cultured for 22–24 hours with HCoV peptides at a concentration of 10 μg/mL or with CEF peptides at a concentration of 3 μg/mL. The plates were then processed according to the manufacturer's protocol and read by a blinded independent investigator using an automated reading system. Four replicates per pool were run for the comparison of the different viral proteins. The replicate furthest from the median was not used. If 2 values were equally distant from the median, then the higher value was discarded. Two replicates were run for the HCoV-NL63 S protein pools that examined the breadth of the T cell responses. For epitope mapping, each individual peptide present in a pool was tested in duplicate wells. A peptide was only considered to be positive if both wells had values that were at least twice the average of the untreated wells and the average stimulation index was above 3 and more than 20 SFU/10^6 cells were present.

Expansion culture assay. PBMCs (10^7 cells) were cultured in R10 media with 10 U/mL IL-2 and 5 μg/mL peptides for 10–12 days in a modified version of a previously described assay (22). The media were not changed during this period. The cells were then washed and replated in fresh R10 with 10 U/mL IL-2 and rested 1 day before they were stimulated again with 5 μg/mL peptide with protein transport inhibitors (GolgiPlug, 1 μg/mL; GolgiStop, 0.7 μg/mL) as well as an antibody against CD107a (FITC, clone H4A3) and antibodies against CD28 and CD49d (all from BD Biosciences). After a 12-hour incubation, the cells were washed and stained with annexin V (BV-421, BD Biosciences, 563973) and antibodies against CD3 (APC-Cy-7, BioLegend, 300426), CD4 (PerCP-CY-5.5, BioLegend, 300530), CD8 (BV-605, BioLegend, 301040), and CD107a (FITC, BD Biosciences, 555800). The cells were then fixed, permeabilized, and stained intracellularly for the following cytokines: TNF-α (PE-Cy-7, BD Biosciences, 557647), IFN-γ (APC, BD Biosciences, 506510), and IL-2 (PE, BioLegend, 500307). Flow cytometry was performed on a BD FACS LSR Fortessa flow cytometer, and data were analyzed using FlowJo, version 10. Data on a minimum of 100,000 events in the lymphocyte gate were collected and analyzed.

Serology. Donors were tested for SARS-CoV-2–specific antibodies with a rapid IgG/IgM combined antibody prescreening kit (sensing.self). Plasma from HD9 was also tested for SARS-CoV-2 IgG and IgA antibodies at the Johns Hopkins Hospital clinical laboratory to confirm seronegative status.

Statistics. All statistical analyses were performed using GraphPad Prism 8.4.3 (GraphPad Software). For experiments requiring multiple comparisons, a 1-way ANOVA with Geisser-Greenhouse correction was used. Dunnett's multiple-comparison test was used to determine difference between groups. For experiments requiring comparisons between 2 groups, a 2-tailed, paired Student's *t* test was used to determine significance. A *P* value of less than 0.05 was considered statistically significant.

Study approval. The study was approved by the IRB of Johns Hopkins University. Written informed consent was obtained from all study participants prior to their inclusion in the study.

Author contributions

BAW and AKK performed the experiments and wrote the manuscript. CCG performed experiments. OL provided reagents. SCR analyzed data. JNB supervised experiments and wrote the manuscript.

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