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Systematic Examination of Antigen-Specific Recall T Cell Responses to SARS-CoV-2 versus Influenza Virus Reveals a Distinct Inflammatory Profile

Jaclyn C. Law,* Wan Hon Koh,*,† Patrick Budylowski,†,‡ Jonah Lin,* FengYun Yue,† Kento T. Abe,§,¶ Bhavisha Rathod,§ Melanie Girard,* Zhijie Li,¶ James M. Rini,¶,‖ Samira Mubareka,#, Allison McGeer,§,** Adrienne K. Chan,#,†† Anne-Claude Gingras,§,¶ Tania H. Watts,*,1 and Mario A. Ostrowski*,†,‡‡,1**

There is a pressing need for an in-depth understanding of immunity to SARS-CoV-2. In this study, we investigated human T cell recall responses to fully glycosylated spike trimer, recombinant N protein, as well as to S, N, M, and E peptide pools in the early convalescent phase and compared them with influenza-specific memory responses from the same donors. All subjects showed SARS-CoV-2–specific T cell responses to at least one Ag. Both SARS-CoV-2–specific and influenza-specific $CD4^+$ T cell responses were predominantly of the central memory phenotype; however SARS-CoV-2–specific $CD4^+$ T cells exhibited a lower IFN-γ to TNF ratio compared with influenza-specific memory responses from the same donors, independent of disease severity. SARS-CoV-2–specific T cells were less multifunctional than influenza-specific T cells, particularly in severe cases, potentially suggesting exhaustion. Most SARS-CoV-2–convalescent subjects also produced IFN-γ in response to seasonal OC43 S protein. We observed granzyme B^+/IFN-γ^+, $CD4^+$, and $CD8^+$ proliferative responses to peptide pools in most individuals, with $CD4^+$ T cell responses predominating over $CD8^+$ T cell responses. Peripheral T follicular helper (pTfh) responses to S or N strongly correlated with serum neutralization assays as well as receptor binding domain–specific IgA; however, the frequency of pTfh responses to SARS-CoV-2 was lower than the frequency of pTfh responses to influenza virus. Overall, T cell responses to SARS-CoV-2 are robust; however, $CD4^+$ Th1 responses predominate over $CD8^+$ T cell responses, have a more inflammatory profile, and have a weaker pTfh response than the response to influenza virus within the same donors, potentially contributing to COVID-19 disease. ***The Journal of Immunology*****, 2021, 206: 000–000.**

The disease COVID-19, caused by the novel coronavirus (CoV), SARS-CoV-2, emerged in China in late 2019 and is currently causing a devastating pandemic (1–3). Despite the severity of the disease in some individuals, the vast majority of infected people recover, indicating that they have made an effective immune response that clears the virus. Moreover, studies in rhesus macaques demonstrate that SARS-CoV-2 induces protective immunity against rechallenge at least out to 35 d (Ref. 4 and L. Bao, W. Deng, H. Gao, C. Xiao, J. Liu, J. Xue, Q. Lv, J. Liu, P. Yu, Y. Xu et al., manuscript posted on bioRxiv, DOI: 10.1101/2020.03.13.990226). Adaptive immunity, mounted by T and B lymphocytes, is critical for clearance of viral infections and for protection against reinfection. Most studies to date show that people infected with SARS-CoV-2 produce spike (S) and receptor

*Department of Immunology, University of Toronto, Toronto, Ontario M5S 1A8, Canada; †Department of Medicine, University of Toronto, Toronto, Ontario M5S 3H2, Canada; ‡Institute of Medical Science, University of Toronto, Toronto, Ontario M5S 1A8, Canada; §Lunenfeld-Tanenbaum Research Institute at Mt. Sinai Hospital, Sinai Health System, Toronto, Ontario M5G 1X5, Canada; ¶Department of Molecular Genetics, University of Toronto, Toronto, Ontario M5S 1A8, Canada; ‖Department of Biochemistry, University of Toronto, Toronto, Ontario M5S 1A8, Canada; #Sunnybrook Research Institute, Toronto, Ontario M4N 3M5, Canada; **Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario M5S 1A8, Canada; ††Division of Infectious Diseases, Department of Medicine, University of Toronto, Toronto, Ontario M5S 3H2, Canada; and ‡‡Keenan Research Centre for Biomedical Science of St. Michael's Hospital, Unity Health Toronto, Toronto, Ontario M5B 1W8, Canada

[1]T.H.W. and M.A.O. are cosenior authors and contributed equally to the study.

ORCIDs: 0000-0002-0024-9442 (W.H.K.); 0000-0002-2863-6188 (J.L.); 0000-0002-8357-4717 (F.Y.); 0000-0001-9283-6072 (Z.L.); 0000-0002-0952-2409 (J.M.R.); 0000-0002-6090-4437 (A.-C.G.); 0000-0001-7897-4890 (T.H.W.); 0000-0002-7369-1936 (M.A.O.).

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Address correspondence and reprint requests to Dr. Tania H. Watts, Department of Immunology, University of Toronto, Medical Sciences Building, Room 7221, 1 King's College Circle, Toronto, ON M5S 1A8, Canada. E-mail address: tania.watts@utoronto.ca

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Abbreviations used in this article: AUC, area under the curve; CoV, coronavirus; E, envelope; ICC, intracellular cytokine staining; ICU, intensive care unit; M, membrane; N, nucleocapsid; OC43, seasonal human CoV-OC43; PR8, influenza virus strain A/Puerto Rico/8/1934; pTfh, peripheral T follicular helper; RBD, receptor binding domain; REB, research ethics board; S, spike; Tfh, T follicular helper; TIV, trivalent inactivated seasonal influenza vaccine.

binding domain (RBD)–specific IgG and neutralizing Abs within 2–4 wk of infection (Refs. 5–11 and A. Wajnberg, F. Amanat, A. Firpo, D. Altman, M. Bailey, M. Mansour, M. McMahon, P. Meade, D. R. Mendu, K. Muellers et al., manuscript posted on medRxiv, DOI: 10.1101/2020.07.14.20151126). Although some studies have suggested that Ab responses of people with mild or no symptoms can fall off rapidly (7, 12, 13) other studies suggest IgG responses are relatively stable over the first 3–4 mo, with peak responses followed by a gradual decline, as observed in a normal IgG response (Ref. 14, A. Wajnberg, et al., manuscript posted on medRxiv, DOI: 10.1101/2020.07.14.20151126, and L. B. Rodda, J. Netland, L. Shehata, K. B. Pruner, P. M. Morawski, C. Thouvenel, K. K. Takehara, J. Eggenberger, E. A. Hemann, H. R. Waterman et al., manuscript posted on medRxiv, DOI: 10.1101/2020.08.11.20171843). In contrast, IgA responses to SARS-CoV-2 start early and decay rapidly (14). In the absence of complete virus neutralization, T cells are critical for eliminating virus-infected cells. Moreover, $CD4^+$ T cell responses and, in particular, T follicular helper (Tfh) responses are critical for generation of high-affinity long-lived Ab responses (15). Follow-up studies of the SARS-CoV-1 outbreak in 2003 showed that Ab responses fell off substantially between 3 and 5 y in most individuals (16), whereas T cell responses could be detected for more than 11 y (17). Moreover, nucleocapsid (N)-reactive T cells in SARS-CoV-1–recovered patients at 17 y post-infection showed substantial cross-reactivity to SARS-CoV-2 N peptides (18). Thus, T cells likely represent an important part of protective immunity to SARS-CoV-2.

Several studies have examined T cell responses to SARS-CoV-2, with most studies using restimulation with overlapping peptide pools from several SARS-CoV-2 open reading frames (Refs. 18–24 and J. Neidleman, X. Luo, J. Frouard, G. Xie, G. Gill, E. S. Stein, M. McGregor, T. Ma, A. George, A. Kosters et al., manuscript posted on bioRxiv, DOI: 10.1101/2020.06.08.138826). Responses to restimulation with intact N, S-RBD domain and protease proteins have also been reported (10). The studies to date have used a variety of readouts to determine T cell specificity, including activation markers, intracellular cytokine production, IFN-γ ELISpot or measurements of cytokines in the supernatants by multiplex assays. In general, the majority of confirmed SARS-CoV-2 cases have shown $CD4^+$ and $CD8^+$ T cell responses to SARS-CoV-2 Ags in the acute and early convalescent phase, dominated by a Th1 response, with some studies also reporting Th2 or Th17 responses (reviewed in Ref. 25). $CD8^+$ T cell responses have also been detected in the majority of but not all donors. There is also evidence of cross-reactive T cells in 20–50% of donors who donated blood prepandemic. These cross-reactive responses are dominated by $CD4^+$ T cell responses to peptides conserved between seasonal CoVs and SARS-CoV-2 (18, 19, 24, 26, 27).

Given the consistent findings of Th1 and $CD8^+$ T cell responses in the acute and early convalescent stage of SARS-CoV2, often with the strongest responses detected in the more severe cases, it is not yet clear why the immune system fails to rapidly control the virus in some patients. In this study, we undertook a systematic functional examination of T cell responses to SARS-CoV-2 in a cohort of 13 SARS-CoV-2–recovered individuals with a range of disease severity who provided leukapheresis samples in the early convalescent phase (4–12 wk). Specifically, we examined T cell phenotype, cytokine production, and proliferation to SARS-CoV-2 proteins and peptides and compared them with seasonal influenza responses. We also identified peripheral T follicular (pTfh) IL-2–producing $CCR7^+CXCR5^+$ cells in response to SARS-CoV-2 Ags in some donors and found that the frequency of these cells strongly correlated with serum neutralization assays and RBD-specific IgA but were less frequent than those observed in response to influenza. Our study reveals new insights into the recall response of SARS-CoV-2 in the early convalescent phase, highlighting that SARS-CoV-2–specific $CD4^+$ T cell recall responses are more inflammatory and show a weaker pTfh response than influenza A–specific $CD4^+$ memory responses within the same donors.

Materials and Methods

Human subjects and study approval

Written informed consent was obtained from COVID-19–convalescent and healthy blood donors before leukapheresis or peripheral blood samples were obtained. Individuals with recovered COVID-19 infection that was confirmed by positive nasopharyngeal COVID-19 PCR upon presentation were leukapheresed after resolution of symptoms through a research ethics board (REB) approved protocol (St. Michael's Hospital REB20-044c to M.A.O.). Additional healthy donors were recruited at the University of Toronto (REB number 00027673 to T.H.W.). All human subject research was done in compliance with the Declaration of Helsinki.

Human PBMC isolation

PBMCs were isolated from whole blood of healthy human donors by density centrifugation using Ficoll-Paque PLUS (GE Healthcare Mississauga, ON, Canada). PBMCs were cryopreserved in 10% DMSO in AIM-V Medium (Life Technologies, Thermo Fisher Scientific, Mississauga, ON, Canada) before use.

Virus and viral Ags

The human codon-optimized cDNA encoding the seasonal human CoV-OC43 (OC43) S protein (AAT84354.1) was synthesized by GeneArt (Thermo Fisher Scientific). The soluble OC43 S construct includes residues 15–1295, followed by a T4 fibritin trimerization motif, a TEV cleavage site, and a 6xHis-tag. The 20 aa human cystatin secretion signal was added N-terminal to the S sequence. To stabilize the prefusion state of the OC43 S trimer, residues 1070–1071 (AL) were mutated to two proline residues (PP) as described for other S proteins (28). The human codon-optimized cDNA encoding the SARS-CoV-2 S protein (YP_009724390) was synthesized by GenScript (Piscataway, NJ). The soluble S trimer construct includes residues 1–1211, followed by a T4 fibritin trimerization motif, a 6xHis-tag and an AviTag biotinylation motif (29). Residues 682–685 (RRAR) were mutated to SSAS to remove the furin cleavage site on the SARS-CoV-2 S protein. Residues 986–987 (KV) were mutated to two proline residues (PP) to stabilize the prefusion form.

The S proteins were cloned into a piggyBac-based inducible expression vector PB-T-PAF. Inducible stable cell lines were generated in FreeStyle 293-F cells (Thermo Fisher Scientific) as previously described (30, 31). For the OC43 S protein, the stable cells were grown as an adherent culture in DMEM/F12 medium supplemented with 3% (v/v) FBS. For the SARS-CoV-2 S protein, the stable cells were grown in suspension culture in FreeStyle 293 Expression Medium (Thermo Fisher Scientific). Protein expression was induced by the addition of 1 μg/ml doxycycline. The secreted proteins were purified from the tissue culture medium using Ni-NTA Resin. The proteins were further purified by size-exclusion chromatography using a Superose 6 Increase column (GE Healthcare). The quality of the purified S protein trimers was assessed using negative stain electron microscopy.

Nucleocapsid$^{1\text{-}419}$ (N) expressed as a N-terminally tagged HIS-GST-TEV fusion was purified from bacteria and kindly provided by Frank Sicheri, Mt. Sinai Hospital, Toronto, ON, Candada, as described in Ref. 14.

Endotoxin levels were measured in S and N proteins using the ToxinSensor Chromogenic LAL Endotoxin Assay Kit, from GenScript/VWR (catalog no. L00350C). Final concentrations of LPS were <1 endotoxin unit per well (0.18 for S, 0.43 for N). Influenza virus strain A/Puerto Rico/8/1934 (PR8) was grown in embryonated chicken eggs, and tissue culture infectious dose was determined by infection of MDCK cells (32).

15-mer peptides overlapped by 11 aa spanning most of the full protein sequence of N, membrane (M), envelope (E), and RBD/transmembrane/cytoplasmic domains of S protein of SARS-CoV-2 were synthesized (GenScript). To stimulate PBMC, an N master peptide pool with102 peptides, an E master peptide pool with 12 peptides, an M master peptide pool with 49 peptides, and an S master peptide pool with 49 peptides were used in the study.

T cell stimulation assay

For all stimulation assays, cryopreserved PBMCs were thawed at 37°C, washed twice with PBS, and cultured in complete media (RPMI 1640

supplemented with 10% FBS, 2-ME, sodium pyruvate, penicillin, streptomycin, and nonessential amino acids; Life Technologies/Thermo Fisher Scientific) at 37˚C with 5% CO_2. A total of 2×10^6 PBMCs were plated per well in 96-well round-bottom plates for 18 h with 1 μg/ml S, 1 μg/ml N, 3 μg/ml OC43 S, or 100 HAU/ml live PR8. PBMCs were cultured with 1 μg/ml BSA (Sigma-Aldrich, Oakville, ON, Canada) as a negative control. GolgiStop (BD Biosciences, San Jose, CA) containing monensin and GolgiPlug (BD Biosciences) containing brefeldin A were added in the last 6 h of the culture. As a positive control, 50 ng/ml PMA (Sigma-Aldrich), 1 μg/ml ionomycin (Sigma-Aldrich), GolgiStop, and GolgiPlug were added to PBMCs cultured with complete media in the last 6 h of culture.

To assess T cell recall responses to live PR8 compared with trivalent inactivated seasonal influenza vaccine (TIV; Fluzone High-Dose), cells were either cultured with complete media, 100 HAU/ml PR8, or 1 μg/ml TIV for 18 h. To determine whether the addition of agonistic costimulatory Abs increased the sensitivity of detection of intracellular cytokine staining (ICC) by flow cytometry, PBMCs were stimulated with 1 μg/ml S or 1 μg/ml BSA, either with or without 2 μg/ml anti-CD28 and 2 μg/ml anti-CD49d (BD Biosciences) for 18 h. GolgiStop and GolgiPlug were added in the last 6 h of these cultures.

Intracellular cytokine staining

After culture, PBMCs were washed with PBS containing 2% FBS (FACS buffer). Cells were first stained with anti-human CCR7 (clone G043H7; BioLegend, San Diego, CA) at 37˚C for 10 min, followed by staining with Fixable Viability Dye eFluor 506 (eBiosciences, Thermo Fisher Scientific) to discern viable cells, anti-human CD3 (clone UCHT1; BioLegend), CXCR5 (clone J252D4; BioLegend), 4-1BB (clone 4B4-1; BioLegend), HLA-DR (clone L243; BioLegend), CD4 (clone SK3; BD Biosciences), CD27 (clone L128; BD Biosciences), and CD45RA (clone HI100; BD Biosciences) for 20 min at 4˚C. Cells were washed twice with FACS buffer, then fixed with BD Cytofix/Cytoperm buffer (BD Biosciences) for 20 min. Following fixation and permeabilization, cells were washed twice with 1× BD Perm/Wash buffer (BD Biosciences) and stained with anti-human IFN-γ (clone 4S.B3; BioLegend), TNF (clone Mab11; BioLegend), IL-2 (clone MQ1-17H12; eBioscience), and IL-17A (clone eBio64DEC17; eBioscience] for 15 min at 4˚C. Samples were washed twice, then resuspended in FACS buffer and acquired on the BD LSRFortessa X-20 flow cytometer using FACSDiva software.

Multiplex cytokine bead assay

A total of 2×10^6 PBMCs were seeded per well in 96-well round-bottom plates with 1 μg/ml S, 1 μg/ml N, 3 μg/ml OC43 S, 1 μg/ml BSA, or 100 HAU/ml PR8. Cell culture supernatants were collected after 48 h of incubation. Cytokines in the supernatants were measured using the LEGENDplex Human Th Cytokine Panel (12-plex) Kit (BioLegend) with capture reagents specific for IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-22, IFN-γ, and TNF. The assay was performed per the manufacturer's instructions using a V-bottom plate. Samples were acquired on the BD LSRFortessa X-20 flow cytometer.

CFSE T cell proliferation assay

PBMC (2×10^6 cells/ml) were prelabeled with 5 μM CFSE (Thermo Fisher Scientific) in PBS with 2.5% FBS for 8 min in a 37˚C water bath. Excessive CFSE dye was removed by using 100% FBS and further rinsed with R-10 (RPMI 1640, FBS, penicillin/streptomycin; [Thermo Fisher Scientific], Glutamax [Thermo Fisher Scientific], and sodium pyruvate [Thermo Fisher Scientific]). Cells were then resuspended in R-10 (supplemented with 1 U IL-2 [BioLegend] and 2-ME [Thermo Fisher Scientific]) and plated at 0.4×10^6 cells per well in a 96-well round-bottom polystyrene plate at a final volume of 200 μl. These cells were prestimulated with 0.1 μg of S, E, N, and M master peptide pools or DMSO (negative control) or staphylococcal enterotoxin B (positive control) for 5 d. At day 6, cells were restimulated with 1 μg/ml of master peptide pools, and the exocytosis was blocked by the addition of BD GolgiStop and BD GolgiPlug for another 24 h. At day 7, cells were prepared for flow cytometry staining. LIVE/DEAD Fixable Blue Dead Cell Stain (Thermo Fisher Scientific) was used to determine the viability of cells and then preblocked with Fc receptor blocking solution (Human TruStain FcX; BioLegend) prior to extracellular staining with anti-human CD3 (clone SK7; BD Biosciences), anti-human CD4 (clone SK3; BD Biosciences) and anti-human CD8 (clone HIT8a; BD Biosciences). Cells were then fixed with BD Cytofix and permeabilized with BD Perm/Wash per the manufacturer's protocol and stained with anti-human IFN-γ (clone 4S.B3; BD Biosciences) and anti-human granzyme B (clone GB11; BD Biosciences). Samples were acquired on the BD LSRFortessa X-20 flow cytometer. Net peptide pool-induced $CFSE^{low}$ responses were calculated as the percentage of $CFSE^{low}$ cells after stimulation with master pool peptides minus the percentage of $CFSE^{low}$ cells after stimulation with DMSO.

Wild-type SARS-CoV-2 neutralization assay

One hundred microliters of Vero E6 cells were seeded into a 96-well plate at 0.3×10^6 cells/ml and were incubated overnight for attachment. On the following day, patient serum was heat inactivated at 56˚C for 30 min, then serially diluted eight times, 2-fold downward, starting at 1:10. Equal volumes of SARS-CoV-2 were added to all wells, with a final concentration of 100 tissue culture infective dose per well. The plate was incubated for 1 h, shaking every 15 min. After incubation, all the media from the Vero E6 cells was removed, and 50 μl of the SARS-CoV-2/serum coculture was used to inoculate the Vero E6 cells. The infection was done for 1 h, shaking every 15 min. Postinfection, the inoculum was removed, and growth media was added. Cytopathic effect was tracked over the course of 5 d. Samples were run in quadruplicates.

Protein-based surrogate neutralization ELISA

A protein-based surrogate neutralization ELISA was performed as described in Ref. 30. Essentially, 100 ng of purified RBD expressed in FreeStyle 293-F cells was immobilized overnight onto 96-well Immulon HBX plates (2 μg/ml), blocked, and incubated with four, 2-fold dilutions of patient samples, starting at 4 μl. Biotinylated ACE2 purified from FreeStyle 293-F cells was added (50 ng/well, incubated for 1 h), followed by streptavidin poly-HRP (catalog no. S2438, 22 ng; Sigma-Aldrich). 1-Step Ultra TMB-ELISA Substrate Solution (catalog no. 34029; Thermo Fisher Scientific) was added for 7.5 min at room temperature, the reaction was quenched with 50 μl of stop solution containing 0.16 N sulfuric acid (catalog no. N600; Thermo Fisher Scientific), and the OD at 450 nm was read. The area under the curve (AUC) of each dilution series for each patient plasma sample was calculated in R (version 4.0.1).

Data and statistical analysis

Flow cytometry data were analyzed using FlowJo v10 (BD Biosciences). Multiplex cytokine bead data were analyzed using the LEGENDplex data analysis software (v8). All statistical and graphical analyses were performed using GraphPad Prism v6. Illustrations were created with BioRender.com. When data are shown in the absence of the control group, the values are calculated by subtracting background signal, as indicated by "Δ" in panel labels. Background signal is defined by the frequency of cells expressing a particular cytokine or concentration of an analyte in wells cultured with BSA. The response was considered positive if the response to SARS-CoV-2 Ag was 10% higher than the response to BSA. For multiplex cytokine data, the limits of detection are indicated with dashed lines. Pairwise comparisons were made by a two-tailed Wilcoxon test, one-way ANOVA with Holm–Sidak multiple comparisons test, or a nonparametric Dunn multiple comparisons test, as indicated in figure legends. Correlation analyses were performed by computing the Pearson or Spearman correlation coefficient. Statistical outliers were excluded from analyses by a Grubb test, but all data points are displayed in figure panels.

Results

Patient characteristics

Thirteen COVID-19–convalescent donors, who had recently tested positive for SARS-CoV-2 by PCR, and a single SARS-CoV-1 patient from 2003 consented for leukapheresis to obtain plasma and PBMC (Table I). Samples for SARS-CoV-2–convalescent individuals were collected from 27 d to 90 d after onset of symptoms. Disease severity ranged from asymptomatic to mild (nonhospitalized) to moderate (hospitalized, not intensive care unit [ICU]) to severe (ICU). The average age was 53 (range 31–72), and eight out of 13 were male (Table I).

Ex vivo intracellular cytokine responses to S and N proteins in convalescent COVID-19 patients

To date, most studies have used overlapping peptide pools to assess Ag-specific T cell responses to SARS-CoV-2. In this study, we used intact glycosylated S trimer from SARS-CoV-2 and OC43, as well as recombinant *Escherichia coli*–expressed SARS-CoV-2 N to determine how T cells respond functionally to SARS-CoV-2 under conditions in which Ag processing is required. ICC of ex vivo PBMC was conducted to determine the frequency of IFN-γ–, TNF-, and

Table I. Clinical characteristics of participants

Participant Identification	Days from Onset of Symptoms	Clinical Features[a]	Age	Sex
OM8072	50	COVID-19 recovered; moderate	56	Male
OM8073	50	COVID-19 recovered; asymptomatic	56	Female
OM8074	25	COVID-19 recovered; mild	27	Male
OM8076	41	COVID-19 recovered; mild	61	Male
OM8077	37	COVID-19 recovered; mild	31	Male
OM8078	36	COVID-19 recovered; mild	64	Female
OM8081	31	COVID-19 recovered; mild	60	Male
OM8082	35	COVID-19 recovered; mild	42	Female
OM8083	29	COVID-19 recovered; severe	50	Male
OM8086	27	COVID-19 recovered; severe	54	Male
OM8087	65	COVID-19 recovered; moderate	62	Male
OM8094	~90	COVID-19 recovered; moderate	72	Female
OM8099	86	COVID-19 recovered; severe	43	Male
OM8085	17 y	SARS-1, recovered, mild	67	Female

[a]Mild illness, not admitted to hospital; moderate illness, required hospital admission; severe illness, ICU admission.

IL-2–producing cells with the gating strategy shown in Fig. 1A. Following 18 h of stimulation, with GolgiStop and GolgiPlug added for the last 6 h, SARS-CoV-2 S-specific $CD4^+$ T cells were detected in 54% of donors based on IFN-γ production, 75% of donors based on TNF production, and 85% of donors based on IL-2 production. N-specific $CD4^+$ T cells were detected in 38% of donors based on production of IFN-γ, 58% based on TNF, and 54% of donors based on IL-2 (Fig. 1B–D, Supplemental Table I). Overall, 92% of COVID-19–convalescent donors showed a specific $CD4^+$ T cell response to at least one SARS-CoV-2 protein based on production of at least one cytokine, in which a positive response was defined as a 10% increase over control-stimulated samples. No responses were detected to OC43 S protein by ICC in the same donors, whereas 100% of donors produced cytokines in response to PMA/ionomycin (data not shown). In addition, no responses to SARS-CoV-2 S, N, or OC43 were detected in five healthy donor samples collected in early March 2020, demonstrating the specificity of these S- and N-specific cytokine responses (Supplemental Fig. 1). We did not observe cytokine production in $CD8^+$ T cells from any of the donors, albeit $CD8^+$ T cells from all donors responded to influenza A virus as well as to PMA/ionomycin (data not shown). This is likely because $CD8^+$ T cells respond poorly to whole protein Ags. Taken together, our data show that the vast majority of SARS-CoV-2–convalescent individuals have recall $CD4^+$ responses to SARS-CoV-2 S or N proteins at 4–12 wk after initial symptoms, with IL-2– and TNF-producing T cells predominating over IFN-γ–producing T cells.

Comparison of the $CD4^+$ T cell response to SARS-CoV-2 versus influenza A virus by multiparameter flow cytometry

As most adults are expected to have memory T cells specific for influenza virus, we compared recall responses to the PR8 strain of influenza A virus for all donors within the same experiments. Although the influenza-specific responses have the caveat that they represent a lifetime of exposure to influenza virus or vaccine (whereas the SARS-CoV-2 response is based on recent exposure), we reasoned that the influenza-specific responses provided a good internal control for the quality of the samples and to demonstrate the ability of these particular subjects to mount a normal recall response. Ninety-two percent of SARS-CoV-2–convalescent patient samples showed strong $CD4^+$ recall responses to PR8 stimulation based on IFN-γ–producing T cells, and the frequency of these responding cells was substantially higher than responses to S and N proteins (Fig. 1B, Supplemental Table I). This was not due to insufficient S protein, as increasing the dose from 1 to 5 μg/ml did not increase the frequency of responses (Supplemental Fig. 2A). We also obtained similar responses using TIV, which contains only influenza proteins (Supplemental Fig. 2B). Thus, the weaker response to SARS-CoV-2 S protein compared with influenza proteins is unlikely because of the use of live influenza virus versus recombinant SARS-CoV-2 proteins, albeit it could be impacted by an incomplete set of SARS-CoV-2 epitopes covered by including only two of the SARS-CoV-2 proteins. Some human T cell studies use costimulation with anti-CD49d and anti-CD28 to increase the sensitivity of detection with ICC (33); however, we found no difference in the frequency of response to S with or without additional costimulation (Supplemental Fig. 2C). We also repeated the assays three times for two of the donors and obtained a similar frequency of responding T cells each time (Supplemental Fig. 2D).

Analysis of T cell production of multiple cytokines showed that 80% of S-specific $CD4^+$ T cells produced only one cytokine. Influenza-specific $CD4^+$ T cell responses were more multifunctional, with 8.7% of PR8-specific $CD4^+$ T cells as compared with 3.4% of S-specific T cells producing all three cytokines (Fig. 2A). We also noted that the ratio of IFN-γ/TNF-producing cells was significantly higher among the PR8-specific $CD4^+$ T cells than the S-specific $CD4^+$ T cells (Fig. 2B) and this was independent of disease severity.

Analysis of CD27 and CD45RA expression on the S-specific and PR8-specific TNF-producing $CD4^+$ T cells indicated that the responding T cells were predominantly central memory T cells (Fig. 2C). The activation markers HLA-DR and 4-1BB are frequently used to determine specific recall responses. Based on these markers, 100% of donors responded to influenza PR8, whereas 69% responded to S and 85% to N (Supplemental Table I). Examination of HLA- DR/4-1BB double-positive cells for cytokine production showed some discordance between activation markers and cytokine-producing cells (Fig. 2D, Supplemental Table I), with neither approach identifying 100% of the responding $CD4^+$ T cells.

Although there appeared to be a trend toward higher responses in donors with severe illness in the first 4 wk, using either activation markers and/or production of cytokines as a measure of response, differences in ICC response based on disease severity were NS (Fig. 2E). Taken together, our data show that both SARS-CoV-2 and influenza-specific recall T cell responses come from T cells that are predominantly of the central memory phenotype; however, SARS-CoV-2–specific T cells from patients in the early convalescent phase respond to S and N proteins with a higher ratio of TNF/IFN-γ–producing cells compared with the memory response to influenza virus, which shows a more typical antiviral IFN-γ dominant response.

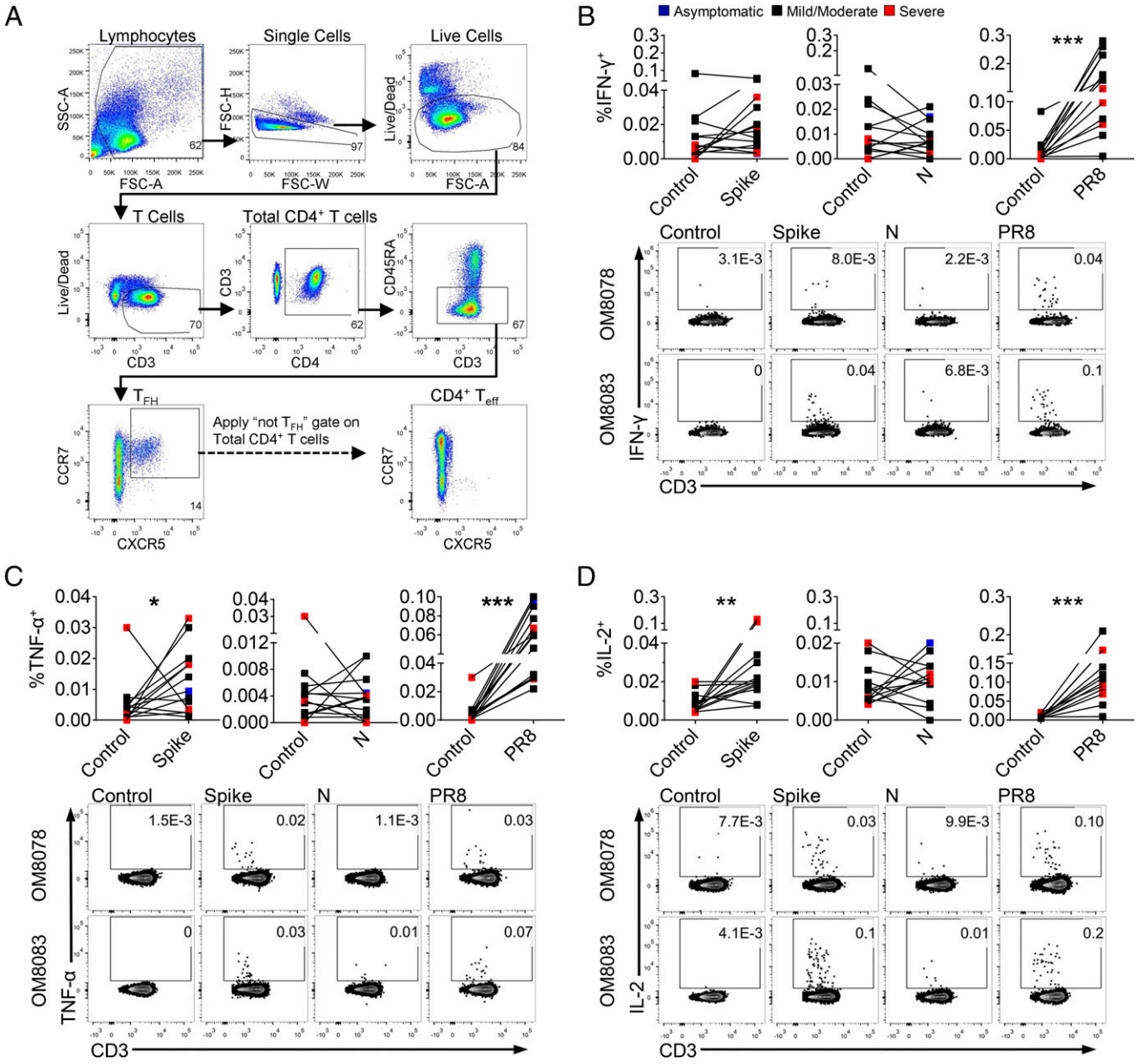

FIGURE 1. Intracellular cytokine responses to S and N proteins in convalescent COVID-19 subjects by flow cytometry. Cytokine production by SARS-CoV-2–specific $CD4^+$ T cells after 18 h of incubation with S, N, or PR8. (**A**) Representative gating strategy for pTfh and non-pTfh $CD4^+$ T cells. Graphs and representative flow cytometry plots show the frequency of $CD4^+$ T cells expressing (**B**) IFN-γ ($n = 13$), (**C**) TNF ($n = 12$), and (**D**) IL-2 ($n = 13$). One donor exhibited high background TNF^+ $CD4^+$ T cells and was determined to be an outlier by the Grubb test. Although this data point is shown in all panels, it was excluded from statistical analysis of TNF^+ $CD4^+$ T cells. Pairwise comparisons were made in (B)–(D) by two-tailed Wilcoxon test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Recall responses of SARS-CoV-2–convalescent PBMC based on cytokine secretion

To further analyze cytokine production during recall responses to SARS-CoV-2, we collected supernatants from ex vivo PBMC 48 h poststimulation with S, N, or influenza PR8 by multiplex bead array analysis of 13 cytokines (Fig. 3, Supplemental Fig. 3, Supplemental Table I). Ninety-two percent of SARS-CoV-2–convalescent donors showed IFN-γ production in response to SARS-CoV-2 S, whereas 100% showed IFN-γ responses to SARS-CoV-2 N and PR8, albeit the median level of IFN-γ in response PR8 was higher than that observed in response to SARS-CoV-2 N ($p = 0.033$) or S ($p = 0.0003$) (Fig. 3A). One hundred percent of patient PBMC produced specific TNF responses in response to N, whereas 50% produced TNF in response to S and 92% in response to PR8. N-specific responses showed substantially higher TNF responses than S- ($p < 0.0001$) or PR8-specific responses ($p = 0.043$) (Fig. 3B). IL-2 was produced in response to S or PR8, but not in response to N stimulation, whereas IL-10 was produced in all cases, albeit the highest amount of IL-10 was observed in the N-stimulated cultures (Fig. 3C, 3D). IL-13 was produced in response to S and PR8, but not N, whereas IL-6 was only observed with S and N restimulation and not with PR8 (Fig. 3E, 3F). Similar to our findings with ICC, the ratio of IFN-γ/TNF or IL-10 was highest in cultures stimulated with influenza A virus (Fig. 3G), and these responses did not appear to correlate with disease severity (Fig. 3H). We did not detect IL-4, -5, or -17F in any of the cultures (data not shown). IL-9 and -17A were detected in some cultures but did not show consistent

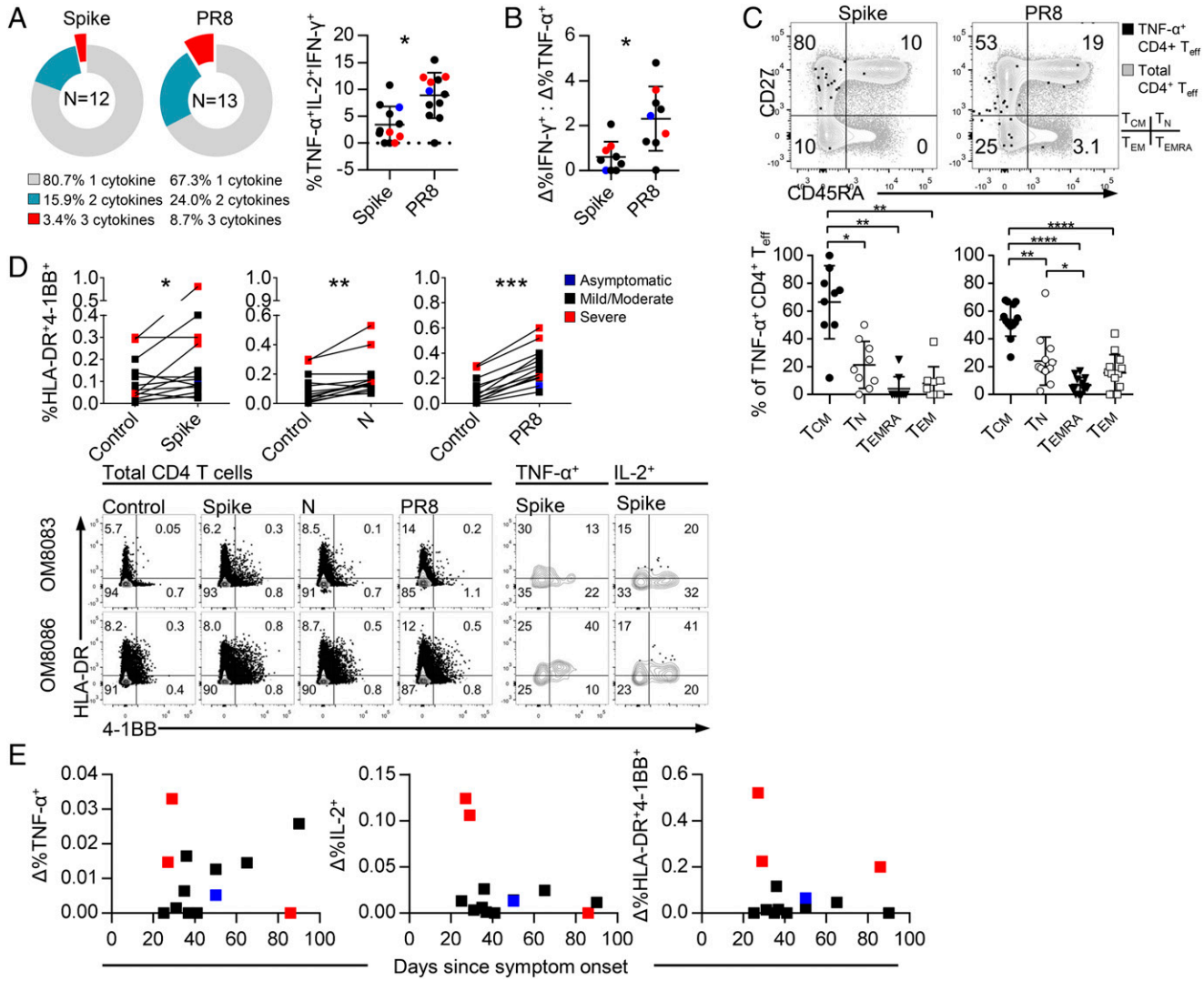

FIGURE 2. Comparison of CD4$^+$ T cell responses to SARS-CoV-2 or influenza A virus. (**A**) Frequency of cells expressing IFN-γ, TNF, and/or IL-2 as a proportion of total cytokine-producing cells. (**B**) Ratio of the %IFN-γ$^+$/TNF$^+$ CD4$^+$ T cells in donors producing both cytokines (n = 9). (**C**) Representative flow cytometry plot of CD27 and CD45RA expression by total CD4$^+$ T cells and TNF$^+$ CD4$^+$ T cells (n = 9). The distribution of memory subsets of TNF$^+$ CD4$^+$ T cells is shown for the donors with a TNF response. Graphs show mean ± SD. (**D**) Graphs show the percentage of CD4$^+$ T cells coexpressing HLA-DR and 4-1BB. Representative flow cytometry plots show the expression of HLA-DR and 4-1BB by total CD4$^+$ T cells and TNF$^+$ and IL-2$^+$ CD4$^+$ T cells after stimulation with S. (**E**) The frequency of TNF$^+$, IFN-γ$^+$, and HLA-DR$^+$4-1BB$^+$ CD4$^+$ T cells versus days since symptom onset. Pairwise comparisons were made in (A), (B), and (D) by two-tailed Wilcoxon test and in (C) by one-way ANOVA with Holm–Sidak multiple comparisons test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

increases with S or N stimulation, whereas IL-22 was produced in response to S stimulation for some donors (Supplemental Fig. 3).

Healthy donor PBMC produced IL-6, -10, IFN-γ, and TNF in response to N but not S proteins (Fig. 3I). We also assessed stimulation of SARS-CoV-2–convalescent PBMC as well as healthy donor PBMC with OC43 S protein from seasonal CoV and detected Ag-specific induction of IFN-γ in 85% of convalescent donors, TNF in 100% of convalescent donors, IL-10 in 100% of convalescent donors, and IL-6 in 85% of convalescent donors, but no IL-2. Healthy donors did not show significant increases in any of the cytokines, albeit the lack of significance may be due to small sample size (Fig. 4A, 4B, Supplemental Table I).

Overall, the multiplex cytokine assays show a predominant Th1 profile based on restimulation with SARS-CoV-2 or seasonal human CoV-OC43 S protein as well as reactivity of healthy donor PBMC to SARS-CoV-2 N and OC43 S protein. SARS-CoV-2–convalescent patients' PBMC showed a lower IFN-γ/TNF or IFN-γ/IL-10 ratio in response to SARS-CoV-2 proteins compared with influenza virus restimulation.

pTfh as well as T effector responses to SARS-CoV-2 Ags correlate with serum Abs and neutralization titers

Tfh responses are important for the generation of long-lived Ab responses (34). Although fully differentiated Tfh are normally found in the lymphoid organs, their peripheral blood precursors, pTfh, can be detected in the blood based on expression of CCR7 and CXCR5 (35, 36). In this study, we used expression of IL-2 by ICC, combined with CCR7 and CXCR5 expression, to detect Ag-specific pTfh cells in SARS-CoV-2–convalescent patient PBMC following restimulation with S, N, or PR8 (Fig. 5A). Forty-six percent of samples showed S-specific IL-2–producing pTfh, 54% of N-specific and 100% of PBMC samples showed PR8-specific pTfh (Fig. 5A, Supplemental Table I). For samples collected during the first 4 wk postsymptoms, pTfh responses to S

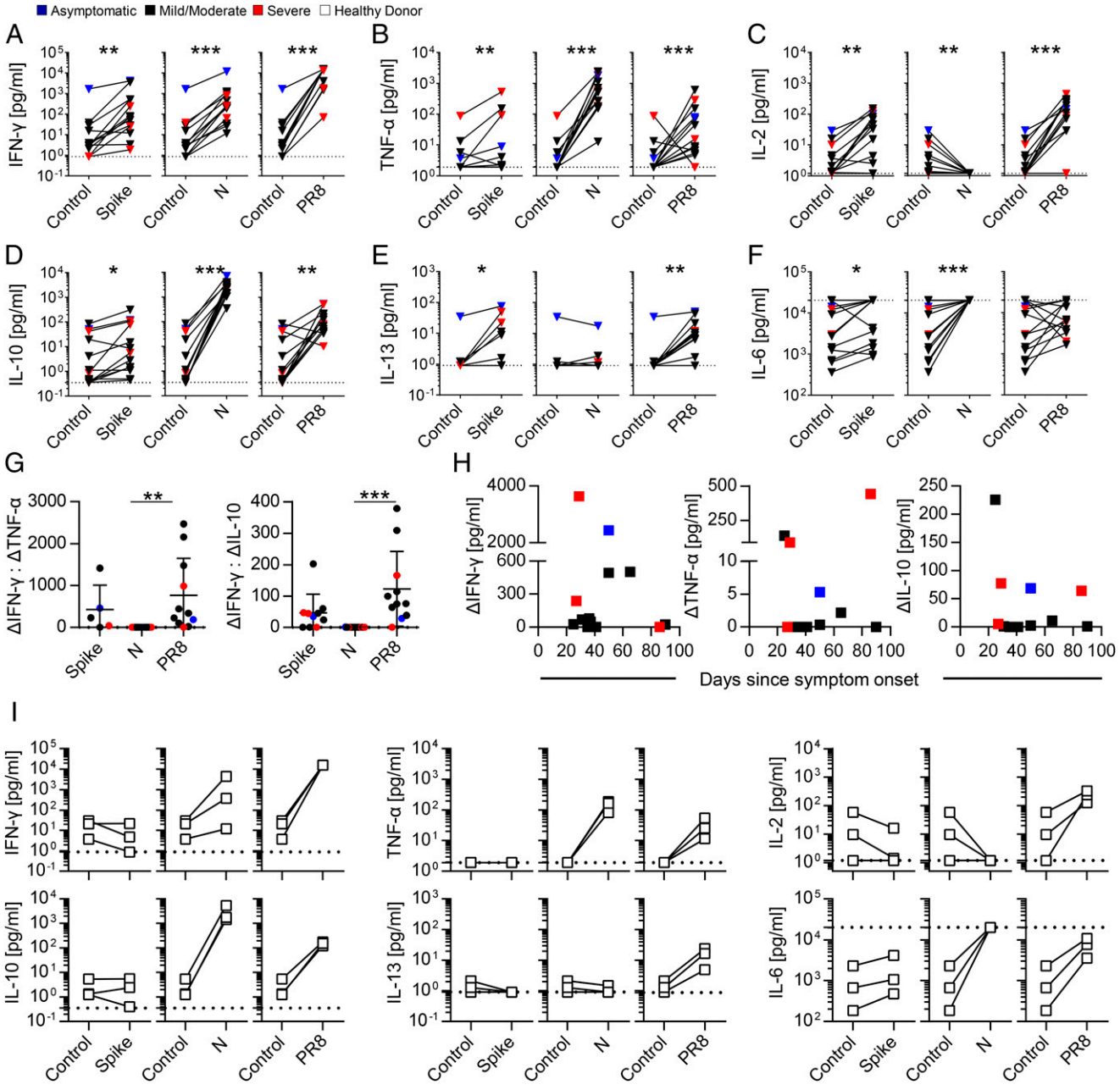

FIGURE 3. Recall responses of SARS-CoV-2–convalescent and healthy donor PBMC based on cytokine secretion. Cytokines in SARS-CoV-2–convalescent PBMC culture (n = 13) supernatants after 48-h stimulation with S, N, or PR8 as quantified by the multiplex cytokine bead assay (n = 13). Graphs show (**A**) IFN-γ, (**B**) TNF, (**C**) IL-2, (**D**) IL-10, (**E**) IL-13, and (**F**) IL-6. (**G**) Ratio of IFN-γ/TNF and IFN-γ/IL-10 in SARS-CoV-2–convalescent PBMC culture supernatants (S, n = 5; N, n = 11; PR8, n = 11). Graphs show mean ± SD. (**H**) The levels of IFN-γ, TNF, and IL-10 versus days since symptom onset. (**I**) The levels of IFN-γ, TNF, IL-2, IL-10, IL-13, and IL-6 in healthy donor PBMC cultures (n = 3). OM8099 exhibited high background TNF and was determined to be an outlier by the Grubb test. Although this data point is shown in (B), it was excluded from statistical analysis of TNF responses. Pairwise comparisons were made by two-tailed Wilcoxon test for (A)–(F) and (I). Nonparametric Dunn multiple comparisons test was performed for (G). Graphs show mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

were higher in severe compared with mild cases (Fig. 5A, 5B). We also compared pTfh responses of PBMC from the SARS-CoV-2–convalescent patients with IgG and IgA responses to N and RBD, based on serum ELISA (30) and neutralization data (Fig. 5C, 5D). There was a positive correlation between the frequency of IL-2$^+$pTfh and N-specific IgG (R = 0.57, $p < 0.05$). The correlation between IL-2$^+$pTfh and RBD-specific IgG showed a similar positive trend (R = 0.29) but did not reach statistical significance. Similarly, no significant correlation with S-specific IgG was observed (data not shown). There was a significant positive correlation between the pTfh response and RBD-specific IgA (R = 0.66, $p < 0.05$) (Fig. 5D). There was also a strong correlation between wild-type SARS-CoV-2 IC_{50} neutralization (modified plaque reduction neutralization assay) by patient sera and the pTfh response (R = 0.84, $p < 0.001$) (Fig. 5E). Similar correlations were obtained by calculating the AUC in a surrogate neutralization ELISA with patient sera, human ACE2, and immobilized S-RBD ($R = -0.75$, $p < 0.01$) (Fig. 5E).

Similar to the analysis of the pTfh results, there was a positive correlation between the frequency of IL-2$^+$ CD4$^+$ effector T cells and N-specific IgG (R = 0.61, $p < 0.05$) and a positive trend between IL-2$^+$ CD4$^+$ T cells and RBD-specific IgG (R = 0.36),

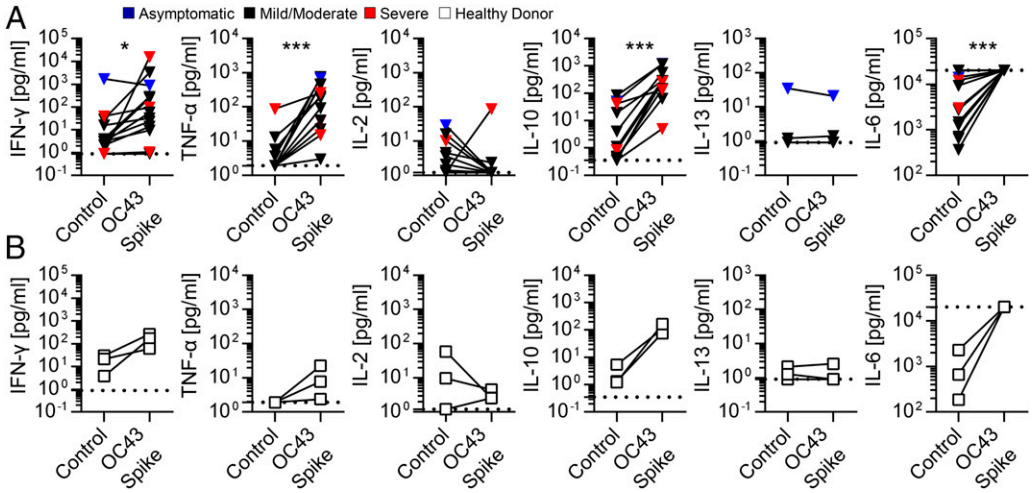

FIGURE 4. Cytokine secretion in response to OC43 S by all donors. The levels of IFN-γ, TNF, IL-2, IL-10, IL-13, and IL-6 were measured in the supernatants of (**A**) SARS-CoV-2–convalescent PBMC cultures ($n = 13$) and (**B**) healthy donor PBMC cultures ($n = 3$) after 48 h of incubation with OC43 S. Pairwise comparisons were made by two-tailed Wilcoxon test. Graphs show mean ± SD. $*p < 0.05$, $***p < 0.001$.

albeit NS (Fig. 6A). There was also a positive correlation between IL-2$^+$ CD4$^+$ and RBD-specific IgA ($R = 0.60$, $p < 0.05$) (Fig. 6B). A strong positive correlation was also found between virus neutralization and IL-2$^+$ CD4$^+$ T cells ($R = 0.82$, $p < 0.001$), and between S-RBD IgG AUC and IL-2$^+$ CD4$^+$ T cells (Fig. 6C). There was also a positive correlation between virus neutralization and disease severity (Fig. 6D).

Thus, pTfh responses, although low in frequency, can be detected in six out of 13 SARS-CoV-2–PBMC responding to S protein and seven out of 13 in response to N, whereas 13 out of 13 showed a Tfh response to influenza A virus. Both pTfh and T effector responses correlated strongly with the neutralization titers observed in the same donors, with the highest neutralization activity correlating with disease severity.

CD4$^+$ and CD8$^+$ T cell proliferative responses to peptide pools

Much of the published work on SARS-CoV-2–specific T cells has focused on peptide pools, and these are more effective in inducing CD8$^+$ recall responses than intact proteins. Therefore, we used peptide pools encompassing the RBD, transmembrane, and cytoplasmic

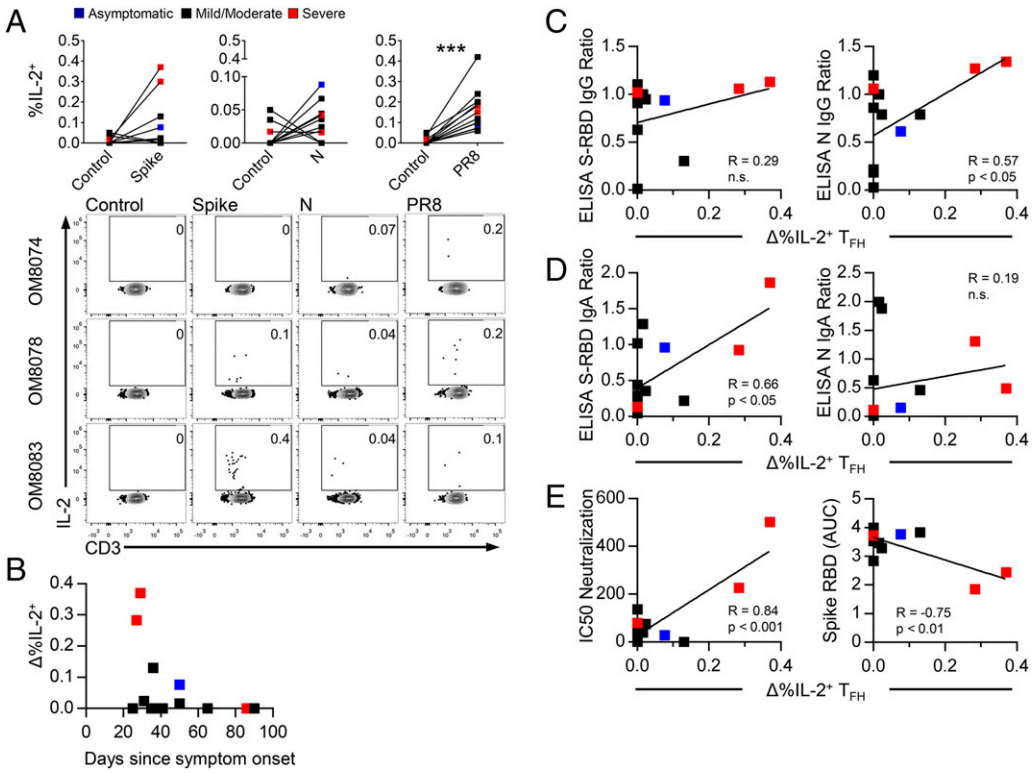

FIGURE 5. pTfh responses to SARS-CoV-2 Ags. (**A**) Graphs and representative flow cytometry plots show percentage IL-2$^+$ pTfh cells in response to S, N, and PR8 after 18 h of stimulation ($n = 13$). (**B**) Percentage IL-2$^+$ pTfh versus days since symptom onset. (**C**) Correlation between S-RBD or N serum IgG and percentage of IL-2$^+$ pTfh. (**D**) Correlation between S-RBD or N serum IgA and %IL-2$^+$ pTfh. (**E**) Correlation between viral neutralization titers and percentage IL-2$^+$ pTfh or between S-RBD IgG AUC in a surrogate neutralization ELISA with human ACE2 and percentage IL-2$^+$ pTfh. Serum Ab titers were normalized to a positive control well. Pairwise comparisons were made by two-tailed Wilcoxon test for (A). Correlation analysis for (C)–(E) was performed by Pearson correlation. $***p < 0.001$.

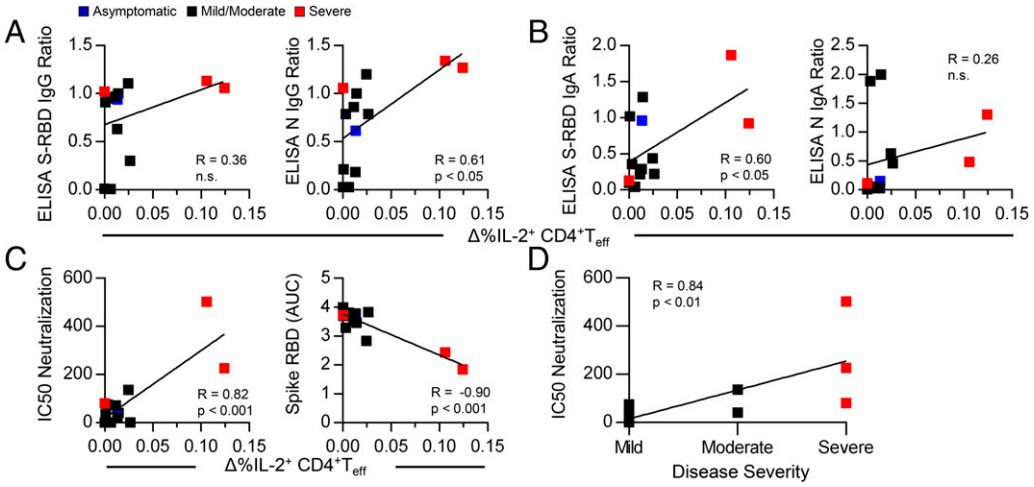

FIGURE 6. Correlation analysis between effector $CD4^+$ T cell responses, serum Abs, and virus neutralization. Correlation analysis was performed between (**A**) S-RBD or N serum IgG and percentage IL-2^+ $CD4^+$ T cells, (**B**) between S-RBD or N serum IgA and percentage IL-2^+ $CD4^+$ T cells, (**C**) between virus neutralization titers or S-RBD IgG AUC and percentage IL-2^+ $CD4^+$ T cells, and (**D**) between virus neutralization titers and disease severity. Serum Ab titers were normalized to a positive control well. Correlation analysis was performed by Pearson correlation in (A)–(C), and by Spearman correlation in (D).

regions of S as well as N, E, and M to stimulate PBMC from the same patient samples used for ICC. As T cell proliferation to virus Ags has previously been associated with the ability to control the virus (37, 38), we labeled PBMC with CFSE and assessed the proliferation of the T cells in response to each peptide pool after 7 d by flow cytometry (gating strategy shown in Fig. 7A). Proliferation responses of total T cells to at least one Ag was observed in 12 out of 13 donors (Fig. 7B, 7C, Supplemental Fig. 2, Supplemental Tables I and II), albeit there was considerable variability between donors. Generally, donors who made strong proliferative responses had strong responses to all Ags tested. However, Ag-specific proliferation did not correlate significantly with disease severity (Fig. 7B–D). We also examined PBMC from a SARS-CoV-1 patient taken 17 y after illness and observed modest reactivity to the N peptide pool (Fig. 7B, 7C). We next broke down responses into $CD4^+$ and $CD8^+$ T cell responses for each peptide pool. For most subjects and Ags, $CD4^+$ T cell proliferative responses were substantially higher than $CD8^+$ T cell responses, independent of disease severity (Fig. 7E).

Although $CD8^+$ cytotoxic T cells are classically associated with virus-infected cell killing, $CD4^+$ granzyme$^+$ cytotoxic T cells can be a significant part of the human antiviral T cell responses (37, 39–41). Therefore, we also assessed IFN-γ and granzyme B levels by flow cytometry in the $CFSE^{low}$ responding $CD4^+$ and $CD8^+$ T cells (Fig. 8A, 8B). Of note, the samples from subjects with severe and moderate disease tended to have a higher proportion of $CD4^+$ IFN-γ/granzyme B–coproducing T cells than subjects with mild disease; however, this was not universally the case, as we also saw a high proportion of IFN-γ/granzyme B–expressing T cells in the asymptomatic donor.

The frequency of proliferating IFN-γ/granzyme B–coproducing T cells in response to S peptide pools correlated with the frequency of IL-2–producing pTfh in response to intact S (Fig. 8C) as well as with virus neutralization titers (Fig. 8D). In addition, proliferating IFN-γ/granzyme–producing cells in response to E or M peptide pools correlated with serum neutralization titers (Fig. 8E, 8F). Thus, a strong $CD4^+$ response overall, whether based on analysis of whole protein or peptide stimulation, correlates with strong neutralization responses.

Discussion

In this study, we have conducted a systematic examination of T cell recall responses of PBMC taken in the early convalescent phase (4–12 wk postsymptoms) of COVID-19 in response to SARS-CoV-2 recombinant proteins as well as to peptide pools. The use of recombinant proteins is relevant because it allows us to assess the response in the context of Ag presentation, and the use of the fully glycosylated S trimer is important in mimicking the form of Ag that is presented by intact virus. A T cell response was detected ex vivo in all SARS-CoV-2–convalescent patients, with 92% responding based on ICC responses to recombinant SARS-CoV-2 proteins and 100% responding based on multiplex cytokine assays. $CD8^+$ T cell responses were not detected in response to whole protein restimulation by ICC but were identified in 12 out of 13 patients based on proliferation in response to restimulation with peptide pools encompassing N, E, M, or S, albeit with various frequencies. Although several other studies have identified Th1 responses in response to SARS-CoV-2 peptide or protein stimulation (Refs. 10, 20, 22, 23, and 25 and J. Neidleman et al., manuscript posted on medRxiv, DOI: 10.1101/2020.06.08.138826), our comparison of memory responses to influenza within the same donors highlights some key differences in SARS-CoV-2 versus influenza-specific T cell responses.

ICC revealed that SARS-CoV-2–specific $CD4^+$ recall responses exhibit a hierarchy of IL-2 > TNF > IFN-γ, whereas influenza A virus-specific T cells show IFN-γ > IL-2 > TNF based on the frequency of cytokine-producing cells. This altered Th1 profile in SARS-CoV-2–specific T cells could contribute to increased inflammation with poorer viral control compared with influenza virus-specific T cells. It was possible that these differences are due to the use of whole influenza A virus compared with recombinant proteins. However, control experiments showed indistinguishable frequencies of responding $CD4^+$ T cells producing IFN-γ, TNF, and IL-2, whether samples were restimulated with live influenza virus or with TIV, the inactivated influenza vaccine that is dominated by the hemagglutinin protein. It is unlikely that the altered cytokine response in response to SARS-CoV-2 Ags is driven by disease severity, as mild and severe patients were distributed throughout the plots showing this altered ratio (Figs. 2B, 3G). In contrast, the lower frequency of multifunctional cells in the SARS-CoV-2–specific as compared with influenza A–specific

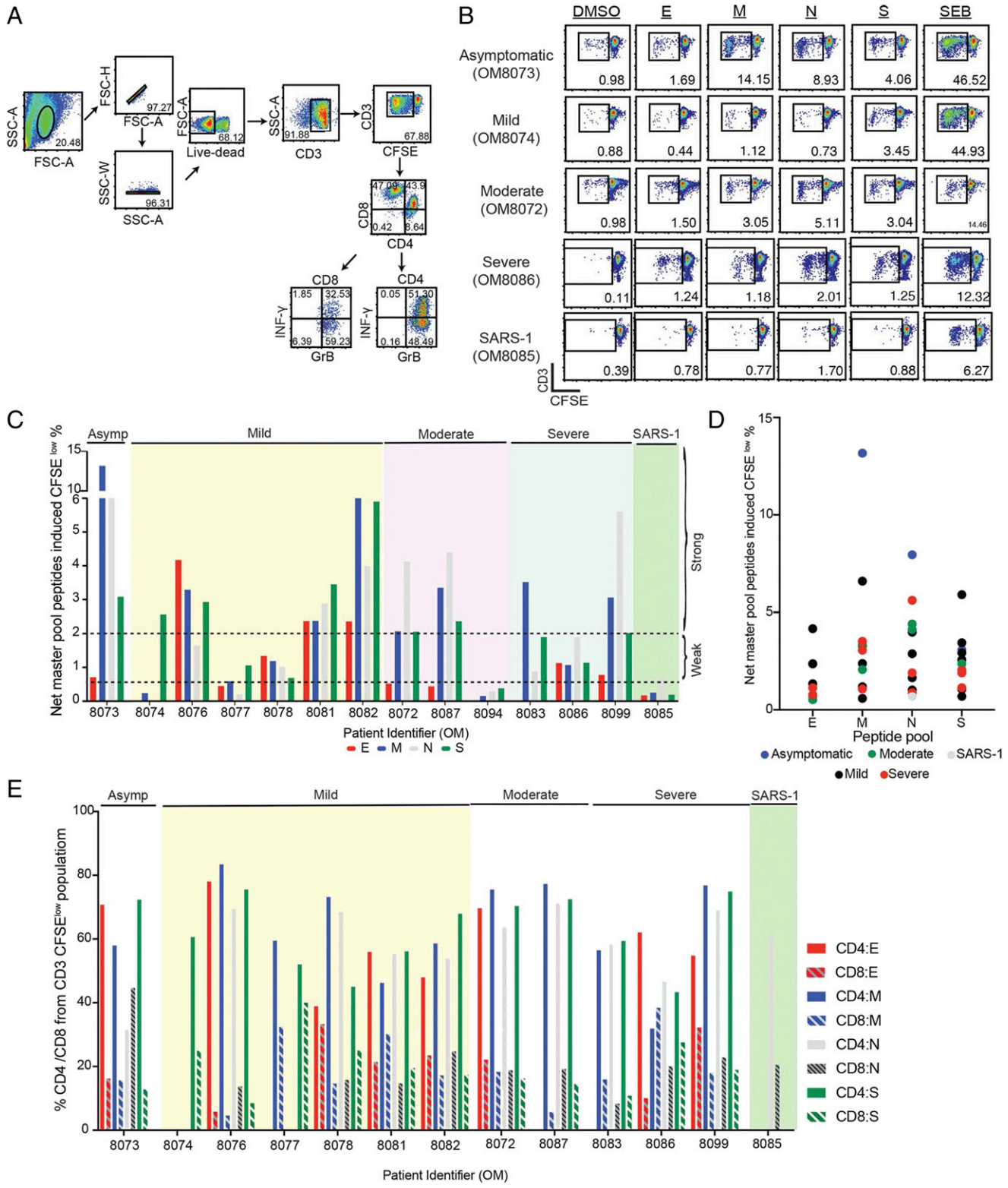

FIGURE 7. T cell proliferation responses induced by master pool peptides (E, M, N, S) in convalescent COVID-19 patients. PBMCs were prelabeled with CFSE, prestimulated with 0.1 μg/ml of master pool peptides for 5 d, then restimulated with 1 μg of master pool peptides on day 6 for 24 h. (**A**) Representative gating strategy for $CFSE^{low}$ $CD3^+$ T cells, $CD4^+/CD8^+$ T cells, and IFN-γ/granzyme B–producing $CD4^+/CD8^+$ T cells. (**B**) Representative flow cytometry plots of CFSE fluorescence by $CD3^+$ T cells. (**C**) Net master pool peptides induced T cell proliferative responses from convalescent asymptomatic (Asymp; $n = 1$), mild ($n = 6$), moderate ($n = 3$), severe ($n = 3$) and SARS-1 ($n = 1$) patients. Net $CFSE^{low}$ percentages were calculated by subtracting the DMSO-stimulated percentages from the master pool peptides. The horizontal dashed line at 0.5 and 2.0% indicates weak and strong positive responses, respectively. (**D**) Comparison of T cell proliferative responses from Asymp ($n = 1$), mild ($n = 6$), moderate ($n = 2$), severe ($n = 3$), and SARS-1–convalescent patients ($n = 1$) against master pool peptides (E, M, N, and S). (**E**) The frequency of $CD4^+$ and $CD8^+$ T cells within $CFSE^{low}$ $CD3^+$ T cells in each patient.

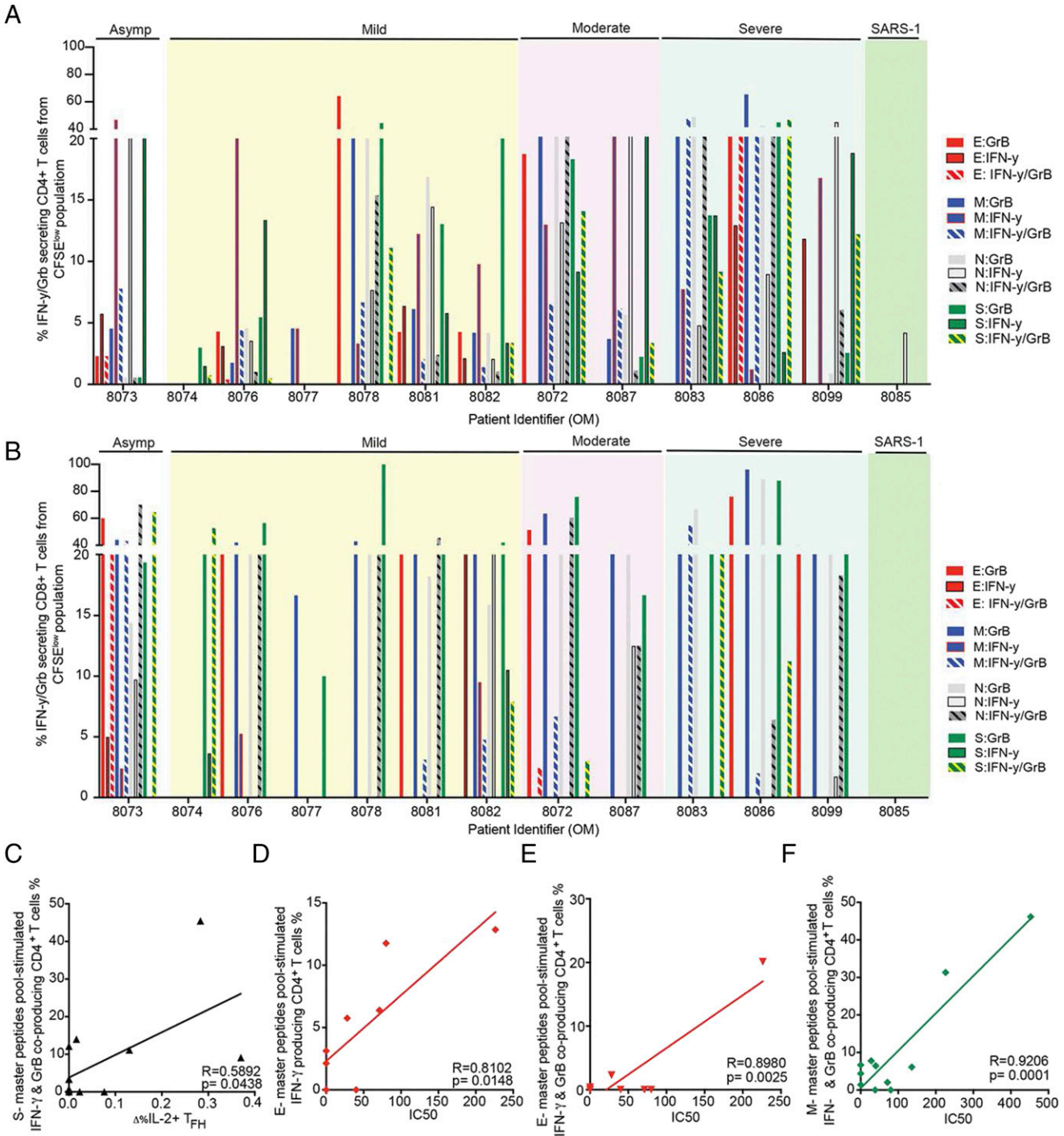

FIGURE 8. IFN-γ/granzyme B–producing $CD4^+$ T cells. The percentage of IFN-γ/granzyme B–coproducing (**A**) $CD4^+$ or (**B**) $CD8^+$ from $CD3^+$ $CFSE^{low}$ T cells from convalescent asymptomatic (n = 1), mild (n = 6), moderate (n = 2), severe (n = 3), and SARS-1 (n = 1) patients. (**C**) Correlation analysis between S master peptide pool-stimulated IFN-γ and granzyme B–coproducing $CD4^+$ T cells and percentage IL-2^+ pTfh cells in response to S. Correlation analysis between virus neutralization titers (IC_{50}) and (**D**) E master peptides pool-stimulated IFN-γ–producing $CD4^+$ T cells or (**E**) E master peptides pool-stimulated IFN-γ and granzyme B–coproducing $CD4^+$ T cells or (**F**) M master peptides pool-stimulated IFN-γ and granzyme B–coproducing $CD4^+$ T cells. Pearson correlation test (n = 12, SARS-1 patient excluded). Asymp: asymptomatic.

$CD4^+$ recall responses seems to be heavily weighted by the severe cases (Fig. 2B) and could reflect COVID-19–specific exhaustion as has been suggested by other studies based on activation/exhaustion markers (42, 43). A limitation of our study is that the influenza-specific recall responses we observed are likely due to a lifetime of seasonal exposure and/or vaccination, whereas the SARS-CoV-2–specific responses are more recent and represent the early recall response at 4–12 wk postexposure. However, both the influenza-specific and SARS-CoV-2–specific CD4 T cell responses were of the central memory phenotype, arguing that they had fully acquired the features of memory T cells. Moreover, it is unlikely that the time since exposure is driving the altered cytokine profile we observe, as the cytokine profile observed in recall responses generally reflects the epigenetic profile imprinted during priming (44). Our previous studies of subjects recently recovered from H1N1 influenza 2009 found that the response to

influenza virus showed a predominant CD8 response over the CD4 response. The majority of those CD8 T cells produced only IFN-γ or IFN-γ and granzyme B with minimal TNF production (45). Thus, the altered CD4/CD8 ratio observed in SARS-CoV-2–specific responses is unlikely due to time since exposure, but rather reflects an altered response compared with the influenza response.

Multiplex analysis of cytokines in supernatants of PBMC following SARS-CoV-2 Ag stimulation also revealed lower IFN-γ/TNF ratios of SARS-CoV-2 compared with influenza-specific responses as well as higher levels of IL-10 and IL-6. The ICC flow cytometry assay allows one to clearly identify the source of the cytokines as $CD4^+$ T cells, whereas the multiplex cytokine assay reflects the total amount of secreted cytokine and can also reflect cytokines secreted from other cells, such as monocytes or NK cells, in response to the activated T cells. IL-10 can be produced by both T cells and Ag-presenting cells, whereas IL-6 is likely coming from monocytes. Particularly striking in our study was the high level of IL-10 detected in the supernatants of N-stimulated cultures, which could contribute to impaired Ag presentation and immunosuppression (46). Further investigation is required to determine whether N-specific responses are immunosuppressive, which would have significant implications for vaccine design.

A potential caveat to our findings is that we included only two of the SARS-CoV-2 proteins in our cytokine analysis and not the full spectrum of SARS-CoV-2 Ags. However, the cytokine profile we observed in the supernatants of S- and N-stimulated PBMC is quite similar to that reported by Weiskopf et al. (20) for SARS-CoV-2 acute respiratory distress syndrome patient PBMC collected 3 wk after ICU admission and stimulated with peptide megapeptide pools covering most of the SARS-CoV-2 proteins. There were, however, some differences noted, such as their detection of IL-17A, which we did not detect in response to SARS-CoV-2 proteins in our assays.

The cytokine profile we detect in the supernatants of SARS-CoV-2–convalescent PBMC after Ag stimulation is similar to the overall cytokine profile reported at the acute phase of infection, including high levels of IL-6, IL-10, and TNF (47, 48). This is consistent with the evidence that memory T cells are imprinted by the acute inflammatory milieu (44). Schultheiss et al. (49) recently analyzed total PBMC from SARS-CoV-2–active and early convalescent patients and also noted that total $CD4^+$ T cells showed an altered nonclassical Th1 profile, similar to what we observe in this study with Ag-specific T cell responses. They also noted Th17 responses, which were not consistently observed in the Ag-specific T cells in our cohort.

Of note, we observed a disconnect between ICC responses and analysis of T cell responses to S and N based on activation markers. This was not unique to SARS-CoV-2, however, as we observed a similar disconnect with influenza A–specific T cell cytokine response and activation markers (data not shown). It is possible that some Ag-specific T cells are not making cytokines in the time frame analyzed or that some of these activation markers are induced on memory T cells by bystander effects (cytokines). We suggest that functional readouts based on cytokines may be more relevant to understanding protective immunity to SARS-CoV-2 than use of activation markers.

Although low in frequency, pTfh responses ($IL\text{-}2^+CCR7^+CXCR5^+$) were detected in 62% of PBMC after S or N stimulation and strongly correlated with virus neutralization activity of sera based on neutralization of SARS-CoV-2 as well as a surrogate neutralization ELISA for binding to RBD. The strong correlation with IgA might reflect the recently reported role for IgA in SARS-CoV-2 neutralization (D. Sterlin, A. Mathian, M. Miyara, A. Mohr, F. Anna, L. Claer, P. Quentric, J. Fadlallah, P. Ghillani, C. Gunn, et al., manuscript posted on medRxiv, DOI: 10.1101/2020.06.10.20126532). Our findings are similar to those of Ni et al. (10), who showed a correlation between total N-specific T cells measured by ELISpot and neutralizing Ab titers. Of note, total T effector responses to N and S as well as IFN-γ or IFN-γ and granzyme B responses to E and M peptide pools also correlated with virus neutralization, suggesting that a strong $CD4^+$ T cell response, in general, correlated with effective virus neutralization whether we used peptide pools or intact Ags for these recall assays. Of note, 100% of donors showed pTfh responses to influenza virus and the response was generally of higher frequency than the pTfh response to SARS-CoV-2. Thus, the Tfh response to SARS-CoV-2 in convalescent subjects is weaker than that observed in response to influenza virus restimulation.

Several recent studies have revealed responses of healthy donors to SARS-CoV-2 peptides (18–20, 24, 27). It has also been suggested that prior exposure to seasonal CoVs might allow some cross-protective immunity to SARS-CoV-2 (26). Our ICC assays did not reveal responses of SARS-CoV-2–convalescent patients or healthy donors to seasonal OC43 S protein. However, such responses were detected in supernatants based on the cytokines IFN-γ, TNF, IL-6, and IL-10 but not IL-2. This may reflect the lower sensitivity of the overnight ICC assay compared with assessment of cytokines in the supernatant at 48 hrs. Healthy donors similarly responded to OC43 S based on multiplex cytokine assays but with ∼10-fold weaker responses than SARS-CoV-2–convalescent patients, suggesting that recent boosting with SARS-CoV-2 might enhance such responses. Healthy donors also responded to SARS-CoV-2 N but not S based on release of IFN-γ, TNF, and IL-6 in the supernatant. We also detected proliferative responses to the N peptide pool of a SARS-CoV-1 patient, 17 y after illness, similar to results recently reported (18).

Proliferative responses to SARS-CoV-2 peptide pools showed that $CD4^+$ T cell responses predominated over $CD8^+$ T cell responses, which might contribute to the pathophysiology of COVID-19. In contrast, many of these $CD4^+$ T cells coproduced IFN-γ and granzyme B, suggesting cytotoxic potential. As airway epithelial cells, the target of SARS-CoV-2 infection, can express MHC class II (50–52), these granzyme B^+ cells may be relevant to viral control.

A recent study of early T cell responses to SARS-CoV-2 showed delayed T cell responses compared with Ab responses in the first 2 wk after symptom onset but with T cell responses increasing at >3 wk (53). Although we did not do a kinetic analysis, the data on convalescent samples collected at 4–12 wk postsymptoms are consistent with a peak response around 4 wk and falling off thereafter. These kinetics are similar to what was observed in the recall response to the 2009 influenza virus pandemic, in which peripheral blood $CD4^+$ and $CD8^+$ T cell responses to whole H1N1 restimulation peaked at ∼3–4 wk postsymptoms and then fell off gradually (45). We did not see a consistent difference between severe and mild cases in terms of magnitude of the T cell response, albeit this may be limited by sample size.

In sum, our study shows robust T cell recall responses in SARS-CoV-2–convalescent subjects at 4–12 wk postsymptoms. Based on proliferation, ICC, or multiplex ELISA, all donors showed SARS-CoV-2–specific T cell responses. By 4 wk post–SARS-CoV-2 infection, most subjects exhibit a strong $CD4^+$ Th1 recall response, predominantly of the central memory phenotype, with a less predominant $CD8^+$ T cell response and an altered cytokine profile with more TNF and less IFN-γ compared with responses to influenza virus of the same donors. In addition, pTfh responses to SARS-CoV-2 were weaker than that to influenza

A virus. Taken together, these results suggest that $CD4^+$ T cell responses are more inflammatory than influenza-specific recall responses and show a weaker Tfh response, potentially contributing to disease. The strong correlation between the N- or S-specific pTfh response or the IFN-γ/granzyme B^+ proliferative response and neutralization capacity suggests that these responses should be incorporated into vaccine design and testing.

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