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The Quality of SARS-CoV-2-Specific T Cell Functions Differs in Patients with Mild/Moderate versus Severe Disease, and T Cells Expressing Coinhibitory Receptors Are Highly Activated

Shima Shahbaz,* Lai Xu,* Wendy Sligl†‡§, Mohammed Osman,† Najmeh Bozorgmehr,* Siavash Mashhour,* Desiree Redmond,† Eliana Perez Rosero,* John Walker,‡ and Shokrollah Elahi*†‡§,‖‌#

Understanding the function of SARS-CoV-2 Ag-specific T cells is crucial for the monitoring of antiviral immunity and vaccine design. Currently, both impaired and robust T cell immunity is described in COVID-19 patients. In this study, we explored and compared the effector functions of SARS-CoV-2-reactive T cells expressing coinhibitory receptors and examine the immunogenicity of SARS-CoV-2 S, M, and N peptide pools in regard to specific effector T cell responses, Th1/Th2/Th17, in COVID-19 patients. Analyzing a cohort of 108 COVID-19 patients with mild, moderate, and severe disease, we observed that coinhibitory receptors (e.g., PD-1, CTLA-4, TIM-3, VISTA, Gal-9, and NKG2A) were upregulated on both CD4+ and CD8+ T cells. Importantly, the expression of coinhibitory receptors on T cells recognizing SARS-CoV-2 peptide pools (M/N/S) was associated with increased frequencies of cytokine-producing T cells. Thus, our data refute the concept of pathological T cell exhaustion in COVID-19 patients. Despite interindividual variations in the T cell response to viral peptide pools, a Th2 phenotype was associated with asymptomatic and milder disease, whereas a robust Th17 was associated with severe disease, which may potentiate the hyperinflammatory response in patients admitted to the Intensive Care Unit. Our data demonstrate that T cells may either play a protective or detrimental role in COVID-19 patients. This finding could have important implications for immune correlates of protection, diagnostic, and prophylaxis with respect to COVID-19 management. The Journal of Immunology, 2021, 207: 1–13.

The outbreak of the coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has resulted in a global crisis. Most infected individuals are asymptomatic or present mild to moderate symptoms, but a subgroup will become severely ill (develop acute respiratory distress syndrome) (1), a clinical phenomenon characterized by the development of bilateral infiltrates and hypoxemia (2), septic shock, and organ failure (3, 4). Comorbid conditions, older age, lymphopenia, greater body mass index, and high ferritin levels are associated with higher severity of disease and Intensive Care Unit (ICU) admission (1, 3, 5).

SARS-CoV-2 activates both innate and adaptive immune responses, resulting in unleashed inflammatory responses and, in turn, collateral damage at the site of viral replication such as lung epithelial cells (6). Elevated levels of a variety of inflammatory cytokines were detected in the plasma of COVID-19 patients, in particular, in hospitalized/ICU patients (1). These data suggest that a dysregulated innate immune response and subsequent cytokine storm contribute to the clinical presentation of severe disease. Clearly, viral persistence is linked to disease severity of COVID-19 infection (7), and viral clearance depends on an effective T cell response directed against viral Ags (8). The essential role of T cell–mediated immunity against viral infections is well documented, and Ag-specific CD8+ T cells play a crucial role in eliminating virus-infected cells (9–11). Similarly, CD4+ T cells not only enhance the magnitude and quality of CD8+ T cell responses but also improve their clonal expansion and differentiation into effector and memory CTLs (11–13). Activated T cells are recruited to the infection site, where they eliminate the virally infected cells and may also promote T cell–dependent cytokine production and cytotoxicity, further augmenting the immune pathogenesis (14). Recent evidence suggests that the latter could be the major reason for severe disease manifestations (15). Therefore, transient upregulation of coinhibitory receptors as immune checkpoints may be crucial to...
minimizing immunopathology (16). Given the elevated frequency of NKG2A-expressing CD8+ T cells and upregulation of coinhibitory receptors (e.g., TIM-3 and PD-1), the phenomenon of T cell exhaustion was hypothesized in COVID-19 patients (6, 17). However, overexpression of these coinhibitory receptors could also reflect T cell activation. This has been illustrated by the abundance of CD38+, HLA-DR+, and Ki67+ CD8+ T cells in peripheral blood of COVID-19 patients (18, 19). SARS-CoV-2–specific memory T cells in asymptomatic or mild disease display a robust immune phenotype (20). Recent studies reported that SARS-CoV-2–specific T cells recognize different viral peptides in COVID-19 patient cohorts (21–23). Although the spike (S) protein has received the most attention, there are other viral components with immunological properties such as the membrane (M), the nucleocapsid (N) proteins, and other immunogenic viral components, which may be potential targets for diagnostic or therapeutic interventions (15, 24). We therefore decided to assess T cell responses to the S, M, and N peptide pools and investigate the functionality of Ag-experiencing T cells in relation to the expression of different coinhibitory receptors.

We analyzed blood samples from 108 hospitalized patients including moderate (n = 63), severe/critical (n = 30), and mild non-hospitalized (n = 15) COVID-19 patients. In this study, we show that T cells are decreased in COVID-19 patients, but they are highly activated. In these patients, upregulation of coinhibitory receptors was accompanied by an increased cytokine production capability. In particular, our data show a Th2 immune phenotype in mild illness versus a Th1-skewed immune response after ex vivo stimulation by viral peptide pools in severely ill patients.

Materials and Methods

Study subjects and ethics

Blood samples were collected from 108 hospitalized COVID-19 patients in Edmonton, Alberta, Canada (Table I). Thirty were critically ill patients admitted to the ICU, whom we defined as having severe disease. The remaining 63 patients were hospitalized on a hospital ward with moderate disease. ICU patients were older and 60% male (18 out of 30), whereas non-ICU patients were 54% male (34 out of 63). The mean age average for men and women in the ICU were 71.25 ± 11.41 and 66.13 ± 18.86, respectively, and the mean age average for men and women on a hospital ward were 66.88 ± 15.31 and 62.69 ± 21.04, respectively. Patient age ranged from17–95 y. We also recruited 15 patients with mild illness who were not hospitalized (age 57 ± 7.2). Healthy controls (HCs) (n = 20) were HIV, hepatitis C virus, and hepatitis B virus seronegative without any history of respiratory infections in recent months (age 58 ± 10).

All COVID-19 patients were SARS-CoV-2 positive by quantitative RT-PCR assay specific for viral RNA-dependent RNA polymerase and envelope transcripts, detected using a nasopharyngeal swab. The Human Research Ethics Board (HREB) at the University of Alberta approved this study (Pro00099502). Waiver of consent was obtained by the HREB for those patients admitted to the ICU. Verbal consent was required from all other patients. Wet consent was not required because of logistics and the possible risk of viral transmission. However, written consent was obtained from patients with mild disease (HREB no. Pro00100207).

Sample collection and processing

Fresh blood samples were processed and subjected to Ficoll–Hypaque gradients for the isolation of PBMCs. Mainly freshly isolated PBMCs but occasionally following overnight incubation at 37°C were used for our studies. However, we did not observe any impairment or reduced frequency of Ag-specific T cells in overnight-stored PBMCs.

Immune phenotyping and T cell stimulation

Flow cytometry was performed on freshly isolated PBMCs for T and B cell frequency/phenotype, T cell activation markers, and coinhibitory receptors. For in vitro intracellular cytokine staining (ICS), according to our previous protocols (25, 26), PBMCs were cultured and stimulated with anti-CD3, CD28, and/or SARS-CoV-2 peptides in RPMI-1640 media supplemented with 10% FBS for 6 h in the presence of brefeldin A (10 µg/ml). Peptides consisted of 15-mer sequences with 11 aa overlapping peptide pools covering the complete sequence of the M glycoprotein, the immunodominant sequence domains of the surface glycoprotein S, and the N phosphoprotein of SARS-CoV-2 (Milltenyi Biotec). SARS-CoV-2 peptide pools of S, N, and M were used at 2 µg/ml.

Abs and flow cytometry

Fluorophore or biotin-conjugated Abs with specificity to human cell surface Ags and cytokines were purchased mainly from BD Biosciences or Thermo Fisher Scientific and, in some occasions, from other suppliers as indicated below. Specifically, the following Abs were used: anti-CD3 (HTI3a), anti-CD4 (RPA-T4), anti-CD8 (RPA-TK), anti-CD45 (H-130 or 2D1), anti-VISTA (B7H5DS8), anti-TIM-3 (TD3), anti-PD-1 (MIH4), anti-CD160 (BY55), anti-CD244 (DM244), anti-galactin-9 (Gal-9) (9M1-3), anti-TIGIT (MB-SA43), anti-CD29 (TU66), anti-CD73 (AD2), anti-CD26 (M-A261), anti-CTLA-4 (BNI3), anti-TOX-1 (TXRX10), CD62L (DREG-56), anti-CR7 (2-L1-A), anti-CD45RA (H1000), anti-CD45RO (UCHL1), anti-perforin (6G9), anti-granzyme B (GB11), anti-ICOS (C938-A4), anti-NKG2A (131411), anti-CD19 (HIB19), anti-CD27 (O323), anti-CD38 (HIT2), anti-CD138 (M15), anti-IGG (G18-145), anti-IgM (RMM-1), anti-CD107a (H4A3), anti–HLA-DR (LN3), anti-CD15 (H9F8), anti-CD14 (M5E2), anti-CD11b (M1/70), anti–IL-2 (Miq1-1H12), anti–TNF-α (MAB11), and anti–IFN-γ (4S.B5). LIVE/DEAD Fixable Dead Cell Stains (Thermo Fisher Scientific) were used to exclude dead cells in flow cytometry. Paraformaldehyde fixed cells were acquired by flow cytometry using a LSR Fortessa flow cytometer (Becton Dickinson) and analyzed with FlowJo software (version 10).

ImageStream cytometry. PBMCs obtained from patients were stained with the indicated Abs and fixed with paraformaldehyde for ImageStream analysis. We collected a minimum of 3000 images for each condition using Annis ImageStream Mark II (EMD Millipore). Analysis was performed by choosing a high aspect ratio, choosing only in-focus images, and calculating maximum pixel intensity of the fluorochrome dye according to our previous reports (27, 28).

ELISA and ELISPOT assay. Frozen plasma from HCs and patients were subjected to ELISA using the V-PLEX Plus Kit from Meso Scale Discovery. For ELISPOT, we cultured ≥ 106 cells per well and stimulated with 2 µg/ml of peptides pools. Positive responses were designated when the number of spot-forming cells was twice background and at least 50 spot-forming cells/106 PBMC, as we have described elsewhere (9, 29).

Statistical analysis

For statistical analysis, we first determined the distribution of data using the Wilks–Shapiro test and then, based on the distribution of data, the appropriate test was used. When data were not normally distributed, the nonparametric tests such as the Mann–Whitney U test or Kruskal–Wallis one-way ANOVA was used. Results are expressed as mean ± SEM. A p value < 0.05 was considered statistically significant.

Results

Patient demographics and baseline characteristics

Patients demographics, laboratory findings, and comorbidities of patients are described in Table I. Mortality was significantly higher in men versus women, and similar to previous reports, we observed comorbidities such as asthma, type 2 diabetes, cardiovascular diseases, obesity, and chronic obstructive pulmonary disease (COPD) in our patients. Overall, severe patients (ICU) had a lower lymphocyte count (p = 0.003), increased C-reactive protein (p = 0.01), ferritin (p = 0.03), D-dimer (p = 0.01), and Troponin I (p = 0.003) compared with moderate patients (non-ICU) (Table I). HCs were age matched with a ratio of 60% males to mimic COVID-19 patients. All patients were diagnosed SARS-CoV-2 positive by quantitative RT-PCR assay specific for viral RNA-dependent RNA polymerase and envelope transcripts using a nasopharyngeal swab. Because the kinetics, phenotype, and T cell function change dramatically over time, we performed our studies on specimens collected from COVID-19 patients 2 wk after onset of symptoms and/or SARS-CoV-2 diagnosis.

Altered frequency and phenotype of T cells in COVID-19 patients

Although the mechanism of lymphopenia in COVID-19 patients remains to be explored, the decline in the number of T and B cells in the periphery is an established feature of COVID-19 infection...
(18). Consistent with this, we observed a significantly reduced percentage of CD3+ CD4+, and CD8+ T and B cells in the peripheral blood of COVID-19 patients compared with HCs (Fig. 1A, 1B). However, the ratio of CD4+ and CD8+ T cells in COVID-19 patients compared with HCs remained unchanged (Fig. 1C). Of note, the absolute blood lymphocyte count was significantly declined in severe (ICU) patients (Fig. 1D) as was the percentages of T cells (Fig. 1E) and CD4+ and CD8+ T cells compared with those patients with moderate disease (Supplemental Fig. 1A). We noted that lymphopenia was more CD8+ T cell biased, especially in those in the ICU (Fig. 1A, Supplemental Fig. 1A). We observed that deceased patients had significantly lower lymphocyte count compared with those who survived (Supplemental Fig. 1B). Because IL-7 is crucial for T cell development and maintenance (30), we analyzed the concentration of IL-7 in the plasma of COVID-19 patients (the transferrin receptor resembles Ki67 as a marker of T cell activation and/or proliferation) (32) were also observed in most but not all of COVID-19 patients (Fig. 1H–I). These data support the presence of lymphopenia, lower plasma IL-7 concentrations, and highly activated and skewed T cell phenotypes in COVID-19 patients.

Cytokine response of CD4+ and CD8+ T cells after stimulation with SARS-CoV-2 peptides

Because viral infection can impact the cytokine production of T cells, we measured Ag-specific production of IFN-γ, TNF-α, and IL-2 in T cells after stimulation with different peptide pools using ICS (33). Fresh PBMCs were stimulated with SARS-CoV-2 peptide pools consisting of 15-mer sequences with 11 aa overlap, covering the entire sequence of the N phosphoprotein, SARS-CoV-2 M glycoprotein, or S protein. Stimulation of PBMCs from COVID-19 patients with different peptide pools led to dominant TNF-α production by both CD4+ and CD8+ T cells compared with those patients with moderate disease (Supplemental Fig. 1A). The same trend was observed for IFN-γ and TNF-α/IFN-γ.

Table I. Demographics and baseline characteristics of COVID-19 patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>ICU Patients (#)</th>
<th>ICU Patients (%)</th>
<th>Non-ICU Patients (#)</th>
<th>Non-ICU Patients (%)</th>
<th>p Value</th>
</tr>
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<tbody>
<tr>
<td>Males</td>
<td>18/30</td>
<td>60</td>
<td>34/63</td>
<td>54</td>
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<tr>
<td>Total deceased</td>
<td>3/30</td>
<td>10</td>
<td>10/63</td>
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<tr>
<td>Male deceased</td>
<td>3/0</td>
<td>100</td>
<td>6/10</td>
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<tr>
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<td>0</td>
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<tr>
<td>Type 2 diabetes</td>
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<td>25</td>
<td>18/63</td>
<td>28.5</td>
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<tr>
<td>Dyslipidemia</td>
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<td>25</td>
<td>15/63</td>
<td>23.8</td>
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<td>3/20</td>
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<td>7/63</td>
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<tr>
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<td>0</td>
<td>10/63</td>
<td>15.8</td>
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<tr>
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<td>15</td>
<td>9/63</td>
<td>14.3</td>
<td></td>
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<tr>
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<td>5</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cancer</td>
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<td>5</td>
<td>6/63</td>
<td>9.5</td>
<td></td>
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<tr>
<td>EtOH use disorders</td>
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<td>5</td>
<td>15/63</td>
<td>23.8</td>
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<tr>
<td>Obesity</td>
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<td>20</td>
<td>6/63</td>
<td>9.5</td>
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<tr>
<td>Pneumonia</td>
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<td>15</td>
<td>3/63</td>
<td>4.76</td>
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<td>5</td>
<td>7/63</td>
<td>11.11</td>
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</tr>
<tr>
<td>Anemia</td>
<td>1/20</td>
<td>5</td>
<td>8/63</td>
<td>12.7</td>
<td></td>
</tr>
<tr>
<td>Respiratory support</td>
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<td>100</td>
<td>32/63</td>
<td>50.8</td>
<td></td>
</tr>
<tr>
<td>Chronic liver disease</td>
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<td>5</td>
<td>5/63</td>
<td>7.9</td>
<td></td>
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<tr>
<td>Ventilation</td>
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<td>50</td>
<td>5/63</td>
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<td></td>
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<tr>
<td>Creatinine</td>
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<td>90.714</td>
<td>7.56</td>
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<tr>
<td>WBC (10^9/L)</td>
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<td>5.65</td>
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<td>0.9567</td>
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</tr>
<tr>
<td>Neutrophils (10^9/L)</td>
<td>6.472</td>
<td>5.657</td>
<td>1.2</td>
<td>0.003</td>
<td></td>
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<tr>
<td>Lymphocytes (10^9/L)</td>
<td>0.68</td>
<td>1.2</td>
<td>73.5</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>165</td>
<td>73.5</td>
<td>211.86</td>
<td>0.7361</td>
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</tr>
<tr>
<td>CPK (U/L)</td>
<td>431.125</td>
<td>286.26</td>
<td>389.68</td>
<td>0.1563</td>
<td></td>
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<tr>
<td>LDH (U/L)</td>
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<td>1177</td>
<td>1.44</td>
<td>0.01</td>
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<tr>
<td>Ferritin (μg/L)</td>
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<td>0.05</td>
<td>132</td>
<td>0.4</td>
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</tr>
<tr>
<td>D-dimer (mg/L)</td>
<td>0.57</td>
<td>130</td>
<td>132</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>8/20</td>
<td>40</td>
<td>6/63</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td>Troponin I (μg/L)</td>
<td>10/20</td>
<td>50</td>
<td>16/63</td>
<td>25.4</td>
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<tr>
<td>Antiretroviral therapies</td>
<td>17/20</td>
<td>85</td>
<td>26/63</td>
<td>41.2</td>
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</table>

The p value is comparing patients admitted to the ICU versus non-ICU admitted patients. CPK, creatine phosphokinase; CRP, C-reactive protein; LDH, lactate dehydrogenase.
FIGURE 1. Percentages of T cells, their phenotype, and activation status in COVID-19 patients. (A) Percentages of CD3, CD4, and CD8 in PBMCs of HCs versus COVID-19 patients. (B) Percentages of B cells (CD19 cells) in PBMCs of HCs versus COVID-19 patients. (C) Cumulative data of CD4 and CD8 T cell ratios in COVID-19 and HCs. (D) Absolute blood lymphocyte count in moderate versus severe patients. (E) Percentages of CD3 T cells in patients with moderate and severe COVID-19 infection. (F) Plasma IL-7 levels in HCs versus COVID-19 patients, and (G) moderate versus severe patients measured by ELISA. (H) Flow cytometry analysis of central memory, naive, effector memory, and TEMRA T cells from a representative COVID-19 patient and a HC, and (I) their cumulative data for CD4 T cells. (J) Flow cytometry analysis of central memory, naive, effector memory, and TEMRA T cells from a representative COVID-19 patient and a HC, and (K) their cumulative data for CD8 T cells. (L) Flow cytometry analysis of CD cell activation markers (CD38 and HLA-DR) from a representative COVID-19 patient and a HC. (M) Cumulative data of CD38- and HLA-DR-expressing CD4 T cells and (N) CD8 T cells in COVID-19 patients versus HCs. Each point represents data from cells of a patient. Bar, mean ± one SE. Red and black symbols represent COVID-19 patients and HCs, respectively.
FIGURE 2. Cytokine response and expression of coinhibitory receptors on T cells of COVID-19 patients. (A) Flow cytometry analysis of IFN-γ and TNF-α production as measured by ICS in CD4^+ and CD8^+ T cells after 24-h polyclonal stimulation with anti-CD3/CD28 Abs or SARS-CoV-2 peptide pools (M, N, or S peptide pools or their combination) from a representative patient. (B and C) Cumulative data of the percentage TNF-α, IFN-γ, or TNF-α/IFN-γ production by CD4^+ T cells and CD8^+ T cells following stimulation with anti-CD3/CD28 Abs or different peptide pools as shown. (D and E) Percentages of CD4^+ and CD8^+ T cells expressing TIM-3, VISTA, 2B4, CD160, Gal-9, PD-1, CD39, CD73, TIGIT, and CD26 in HCs versus COVID-19 patients. (F) Flow cytometry plots of Gal-9 expression on CD4^+ and CD8^+ T cells of a representative COVID-19 patient and a HC. (G) ImageStream plots of CD44 and Gal-9 expression on CD3^+ T cells. Each point represents data from cells of a patient. Bar, mean ± one SE. *p < 0.05, **p < 0.001, ***p < 0.0001.
producing CD4+ T cells in response to the S and M peptide pools (Fig. 2B, 2C). Interestingly, detectable but very low T cell response to the viral peptides was observed in HCs (Supplemental Fig. 1D, 1E), possibly because of the cross-reactivity with previous exposure to other coronavirus Ags (22). Moreover, we evaluated IL-2 production in response to stimulation with SARS-CoV-2 peptide pools. Overall, the M and S peptide pools induced similar levels of IL-2 in CD4+ but significantly higher than the N peptide pool (Supplemental Fig. 1F). However, we observed significantly higher IL-2 production only by the S peptide pool in CD8+ T cells (Supplemental Fig. 1G). To allow comparison between COVID-19 patients in the ICU and non-ICU, we quantified TNF-α as the dominant cytokine in response to the SARS-CoV-2 S peptide pool. Both CD4+ and CD8+ T cells from COVID-19 patients in the ICU (severe disease) exhibited impaired TNF-α production capacity compared with those with a moderate disease (non-ICU hospitalized patients) (Supplemental Fig. 1H–J), which is in agreement with our previous report (36), in which we demonstrated the decreased cytokine production ability and proliferative capacity of T cells in severe cases of SARS-CoV-2 infection were related to the abundance of immunosuppressive erythroid precursors/progenitors (CD71+ erythroid cells [CECs]) rather than intrinsic defect (36). To control for the confounding effect of CECs and to be able to have a robust and consistent approach to evaluate the T cell response to viral peptide pools, CECs were simply lysed by using an RBC lysis buffer for subsequent studies. This approach has extensively been used without any adverse effects on T cell phenotype/functions (29, 37–39). Taken together, our observations indicate that responses to the S peptide pool were higher with regards to inducing TNF-α expression in T cells, and the magnitude of response was significantly greater upon in vitro stimulation of PBMCs of COVID-19 patients to the S versus other peptide pools.

**Upregulation of coinhibitory receptors on CD4+ and CD8+ T cells in COVID-19 patients**

To determine the expression level of different coinhibitory receptors, PBMCs from COVID-19 patients and HCs were subjected to flow cytometry analysis. We observed significant upregulation of TIM-3, VISTA, 2B4 (CD244), CD160, PD-1, CD39, and TIGIT on CD4+ T cells of COVID-19 patients versus controls (Fig. 2D, Supplemental Fig. 2A). A similar pattern was observed for the expression of these coinhibitory receptors on CD8+ T cells except CD160, which did not appear to be significantly different from controls (Fig. 2E). The expression level of coinhibitory receptors was not significantly different on total T cells between patients with moderate/mild versus severe disease. We and others have reported recruitment of Gal-9, a TIM-3 ligand, into immune synapsis upon T cell activation in chronic and acute conditions (26, 40). In agreement, we observed the upregulation of Gal-9 on T cells of COVID-19 patients compared with HCs (Fig. 2D–F). Using ImageStream analysis, we found that Gal-9 was dispersed from the cytoplasm to the cell membrane (Fig. 2G). This may suggest the recruitment of exogenous Gal-9 to immune synapses through T cell activation as reported elsewhere (40). The increased soluble Gal-9 in the plasma of COVID-19 patients may contribute to T cell activation (41). These results indicate that COVID-19 infection results in the upregulation of a wide range of coinhibitory receptors on both CD4+ and CD8+ T cells. Conversely, CD73+ T cells expressing CD4+ or CD8+ were significantly lower in COVID-19 patients compared with controls (Fig. 2D, 2E, Supplemental Fig. 2B), but the expression of CD26 remained unchanged (Fig. 2D, 2E). More importantly, we observed an inverse correlation between CD73 expression on CD8+ T cells and activation markers CD38 and HLA-DR (Supplemental Fig. 2C, 2D). Taken together, our data indicate the upregulation of a wide range of coinhibitory receptors on total CD4+ and CD8+ T cells in COVID-19 patients.

The enhanced functionality of SARS-CoV-2–specific CD4+ and CD8+ T cells is concomitant with the upregulation of coinhibitory receptors

After evaluating the expression of coinhibitory receptors on total CD4+ and CD8+ T cells, we aimed to determine if the upregulation of these coinhibitory receptors was associated with impaired effector functions as reported in chronic conditions such as cancer and HIV (16, 42). In particular, we focused on protective cytokines (TNF-α and IFN-γ) using ICS. To ensure a robust and meaningful analysis of T cell functionality and to mimic viral stimulation in vivo, we stimulated PBMCs of patients with overlapping SARS-CoV-2 peptide pools (M, N, or S) followed by measuring Ag-specific cells using activation-induced markers (AIM) (43). By using SARS-CoV-2 peptide pools, we demonstrated that Ag-specific T cells can be identified by AIM (e.g., CD137 and CD154 markers) for CD8+ and CD4+ T cells, respectively, as reported elsewhere (9, 44) (Fig. 3A, 3B). Also, CD69 can be used for the identification of Ag-specific T cells (45), and occasionally, this marker was used. Of note, we observed a significant reduction in the frequency of Ag-specific T cells in previously frozen PBMCs compared with their fresh counterparts (Fig. 3C, 3D). Therefore, all Ag-specific experiments were performed on freshly isolated PBMCs. Next, we measured the expression of coinhibitory receptors on Ag-specific T cells using AIM as illustrated in Fig. 3E. For instance, we found substantial expression of PD-1 on Ag-specific CD4+ and CD8+ T cells following stimulation with different peptide pools (Fig. 3F). It was noted that a significantly increased frequency of CD4+ T cells recognized the S versus the M and N peptide pools (Fig. 3F); therefore, we decided to quantify the expression of coinhibitory receptors on Ag-specific T cells following the stimulation of PBMCs with the S peptide pool.

We found a substantial level of coinhibitory molecules expression on CD154+CD4+ and CD137+CD8+ T cells following stimulation of PBMCs for 6 h with the S peptide pool (Fig. 3G, 3H). As shown in Fig. 2D and 2E, T cells from COVID-19 patients already have an elevated expression level of different coinhibitory molecules, and thus, 6-h stimulation did not substantially change their expression. Interestingly, we observed a significantly higher expression level of PD-1, TIM-3, CD39, VISTA, and Gal-9 in CD4+ but PD-1, TIM-3, CD39, VISTA, Gal-9, NKG2A, and CTLA-4 in CD8+ T cells of patients with severe disease versus those with a mild/moderate disease (Fig. 3G, 3H). However, the expression of LAG-3 was very low in Ag-specific T cells in our cohort. Then, we decided to correlate the magnitude of cytokine production (TNF-α and IFN-γ) by Ag-specific T cells in regard to the expression of different coinhibitory receptors using ICS. Freshly isolated PBMCs were stimulated with the S peptide pool for 6 h in the presence of the Golgi block. Because all peptides were water soluble, we used the same volume of water (2 μl/well) for background reactivity, which was always very low (Supplemental Fig. 3C). We consistently observed that the expression of different coinhibitory receptors such as PD-1, TIM-3, VISTA, CD160, NKG2A, and Gal-9 on CD154+CD4+ T cells was associated with significantly higher TNF-α and IFN-γ production compared with their negative counterparts (Fig. 4A, 4B). The same pattern was observed for CD137+CD8+ T cells expressing the same coinhibitory receptors (Fig. 4C, 4D, Supplemental Fig. 4A, 4B). Although the expression of a coinhibitory receptor does not necessarily define Ag specificity, overexpression of an coinhibitory receptor on Ag-specific T cells reflected an activated rather than exhausted T cell phenotype in COVID-19 patients in response to the S peptide pool.

**Differential cytokine response to SARS-CoV-2 peptide pools in COVID-19 patients**

Polyfunctional T cells that express more cytokines and effector functions have been associated with protective immunity against...
FIGURE 3. The expression of coinhibitory receptors on Ag-specific CD4⁺ and CD8⁺ T cells in COVID-19 patients. (A) Representative flow cytometry analysis of CD4⁺ T cells expressing CD154 and (B) CD8⁺ T cells expressing CD137 in response to the S, M, and N peptide pools in HC versus COVID-19 patients with mild/moderate or severe disease. (C) Representative flow cytometry plots and (D) cumulative data showing the frequency of Ag-specific T cells in fresh versus previously frozen PBMCs of COVID-19 patients following stimulation with the S peptide pool for 6 h. (E) Representative flow cytometry analysis and (F) cumulative data of PD-1 expression on Ag-specific CD4⁺ T cells (CD154) following stimulation with the M, N, and S peptide pools for 6 h versus unstimulated (Unstim) wells. (G) Cumulative data showing the percentage of Ag-specific CD4⁺ T cells and (H) CD8⁺ T cells expressing different coinhibitory receptors in patients with mild/moderate (blue) and severe (red) disease. Each point represents data from cells of a patient. Bar, mean ± one SE. *p < 0.05, **p < 0.001, ***p < 0.0001.
Addressing this point, we analyzed the TNF-α, IFN-γ, IL-10, IL-13, and IL-17 responses to SARS-CoV-2 peptide pools. Fresh PBMCs (CECs depleted) from healthy individuals, patients with mild disease (not admitted to the hospital), and those with moderate disease (admitted to hospital wards) or severe disease (admitted to ICU) were stimulated with anti-CD3/CD28 Abs (polyclonal) and SARS-CoV-2 peptide pools (M, N, and S). We observed that whereas the M, S, and the M\textsubscript{1}N\textsubscript{1}S peptide pools induced a greater TNF-α expression in CD4\textsuperscript{+} from patients with severe disease compared with those with mild/moderate disease (Fig. 5A), only the S and the M+N+S peptide pools induced such effect in CD8\textsuperscript{+} T cells of severe patients (Fig. 5B). In contrast, CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells from patients with severe disease exhibited a lower IFN-γ expression in response to the M+N+S peptide pools compared with those with a mild or moderate disease (Fig. 5C, 5D). In addition, the expression of IFN-γ was significantly lower in CD8\textsuperscript{+} T cells of severe patients compared with other groups when stimulated with the S or M+N+S peptide pools (Fig. 5D). However, the polyclonal response to anti-CD3/CD28 Abs did not show any difference between the groups in terms of TNF-α and IFN-γ expression (Fig. 5A, 5B). This indicates that T cells from COVID-19 patients with severe disease exhibit a higher TNF-α production.

FIGURE 4. Cytokine response in CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells expressing coinhibitory receptors compared with their negative counterparts in COVID-19 patients. (A) Representative flow cytometry analysis of TNF-α expression in CD4\textsuperscript{+}CD154\textsuperscript{+}PD-1\textsuperscript{-} versus CD4\textsuperscript{+}CD154\textsuperscript{-}PD-1\textsuperscript{+} T cells. (B) Cumulative data of TNF-α expression in CD4\textsuperscript{+}CD154\textsuperscript{+} T cells expressing PD-1, TIM-3, VISTA, CD160, NKG2A, and Gal-9 versus their negative counterparts in COVID-19 patients following stimulation of PBMCs with the S peptide pool for 24 h. (C) Representative flow cytometry analysis of TNF-α expression in CD8\textsuperscript{+}CD137\textsuperscript{+}TIM-3\textsuperscript{-} versus CD8\textsuperscript{+}CD137\textsuperscript{-}TIM-3\textsuperscript{+} T cells. (D) Cumulative data of TNF-α expression in CD8\textsuperscript{+}CD137\textsuperscript{+} T cells expressing PD-1, TIM-3, VISTA, CD160, NKG2A, and Gal-9 versus their negative counterparts in COVID-19 patients. Each point represents data from cells of a patient. Bar, mean ± one SE. *p < 0.05, **p < 0.001, ***p < 0.0001.
capability versus others in response to in vitro stimulation, which is consistent with other reports (46). To better characterize IFN-γ secretion by Ag-specific T cells, we decided to use ELISPOT assay, which is a more sensitive method as we have reported elsewhere (9, 33). Interestingly, we found a significant deficiency in IFN-γ secretion by Ag-specific T cells in patients with severe versus those with a mild/moderate disease when stimulated with different peptide pools (Supplemental Fig. 4C). Moreover, CD4+ T cells from patients with mild and moderate disease produced significantly higher levels of IL-10 compared with those with severe disease once their PBMCs were stimulated with the S and the M+N+S peptide pools (Fig. 5E). However, no significant difference was observed for IL-10 production by CD8+ T cells stimulated with different peptide pools (Fig. 5F). Substantial IL-13 levels were detected...
by both CD4\(^+\) and CD8\(^+\) T cells when PBMCs of patients with milder or moderate disease were activated with the S peptide pool compared with those with severe disease (Fig. 5G–I, Supplemental Fig. 4D). However, IL-13 expression in both CD4\(^+\) and CD8\(^+\) T cells remained significantly elevated only in milder patients when the S+M+N peptide pool was used (Fig. 5H, 5I). Of note, the N peptide pool induced a significantly higher IL-13 response in CD4\(^+\) T cells of patients with milder disease compared with those with severe disease (Fig. 5I). These observations were confirmed as we detected significantly higher plasma IL-13 in mild patients compared with moderate and severe patients (Supplemental Fig. 4E, 4F).

Moreover, the N, S, and M+N+S peptide pools induced a significantly stronger IL-4 response by CD4\(^+\) T cells of mild patients compared with those with moderate or severe disease (Fig. 5J, Supplemental Fig. 4G). Although the magnitude of IL-4 expression in CD8\(^+\) T cells appeared to be lower than CD4\(^+\) T cells, we observed significantly higher levels of IL-4 expression in CD8\(^+\) T cells of mild patients compared with severe patients once activated with the S and M+N+S peptide pools (Fig. 5K).

Interestingly, we noted that the N, S, and N+M+S peptide pools induced a significant IL-17 response in the severe patients compared with those with a mild disease (Fig. 5L). Of note, IL-17 expression also was significantly higher in CD4\(^+\) T cells of severe patients compared with those with a moderate disease when stimulated with the N peptide pool (Fig. 5L). Moreover, we observed the elevation of IL-17 expression in CD8\(^+\) T cells of severe patients once stimulated with the N peptide pool compared with mild patients and the S and M+N+S peptide pools versus those with mild or moderate disease (Fig. 5M).

Overall, these data indicate that the nature of cytokine response varies with COVID-19 illness severity, and what is more, the type of T cell reactivity to viral Ags may predict/impact the disease outcome.

**Discussion**

CTLs play an important role in viral infection control (9, 33, 47). Similarly, CD4\(^+\) T cells enhance the magnitude and quality of CD8\(^+\) T cell responses and boost their clonal expansion and differentiation (12). Although functional impairment of T cells is associated with disease progression (9, 16, 48, 49), exaggerated T cell response can result in collateral damage (50). We noted that SARS-CoV-2 infection causes a significant reduction in the percentages of circulating T cells and B cells compared with uninfected controls as reported elsewhere (31, 51). However, the proportion of CD4\(^+\) and CD8\(^+\) T cell subsets in the peripheral blood did not differ from healthy individuals as was demonstrated in a recent study (34).

Although the relative frequency of T cells was significantly reduced in both moderate and severe cases, this observation was more prominent in severe patients and deceased patients. At present, the mechanisms underlying the lymphopenia in SARS-CoV-2 infection is not fully understood. Several potential mechanisms could be involved, including direct or indirect effects of viral infection on promoting apoptosis of hematopoietic stem and progenitor cells.

The use of different medications such as glucocorticoids might also be a contributing element, as reported in SARS patients (52). In this study, we could not exclude the potential effects of medications during hospitalization. We observed a significant decline in the plasma IL-7 concentration in COVID-19 patients. It has been reported that IL-7 is required for T cell development and for the maintenance of mature T cells, and it also contributes to B cell development (30). Stromal cells as the major source of IL-7 (53) could be damaged as a result of SARS-CoV-2–induced effects. Moreover, IL-7 has been reported in mediating protective roles in the setting of septic shock (30), which frequently has been observed in severe cases of SARS-CoV-2 infection (3). Thus, profoundly lymphopenic COVID-19 patients may potentially benefit from IL-7 therapy. Further research is needed to determine the mechanism(s) underlying the lymphopenia and therapeutic effects of IL-7 therapy.

In agreement with other reports (31, 43, J. Neidleman, X. Luo, J. Fouard, G. Xie, G. Gill, E. S. Stein, M. McGregor, T. Ma, A. George, A. Kosters, et al, manuscript posted on bioRxiv), we found a marked increase in central memory CD4\(^+\) T cells but a significant reduction in naive and central memory CD8\(^+\) T cells and a parallel increase in the abundance of TEMRA effector CD8 T cells in the setting of COVID-19 infection. Further analysis enabled us to detect a marked increase in the frequency of activated T cells exhibiting CD71, CD38, and HLA-DR in COVID-19 patients. We found significant upregulation of TIM-3, VISTA, 2B4, CD160, PD-1, CD39, TIGIT, and Gal-9 on both T cell subsets in COVID-19 patients. It has previously been reported that the functional exhaustion of T cells is associated with the upregulation of coinhibitory receptors and viral disease progression (16, 54, 55); however, we found that the expression of these coinhibitory receptors was not associated with an impaired T cell phenotype but, rather, highly activated T cells capable of super response after reactivation with SARS-CoV-2 peptide pools in vitro. Our data are in stark contrast with previous studies performed in COVID-19 patients that reported NKG2A, TIGIT, PD-1, and CTLA-4 expression as signs of functional T cell exhaustion (17, 56). T cell exhaustion is characterized by functional impairment of T cells (16); therefore, overexpression of coinhibitory receptors does not necessarily indicate an exhausted T cell phenotype (57). More accurately, transient expression of coinhibitory receptors on activated T cells is meant to prevent a massive and deleterious hyperimmune response. For example, the PD-1/PDL-1 axis can mediate potent inhibitory signals to reduce T effector cell function as a protective mechanism of preventing collateral damage, without compromising antiviral immunity in an acute setting (58).

The massive production and release of inflammatory cytokines in T cells expressing coinhibitory receptors compared with their counterparts lacking these receptors are very similar to what occurs during T cell activation (59).

More importantly, we investigated the functional phenotype of Ag-specific T cells in relation to the expression of coinhibitory receptors. It was striking that both Ag-specific CD4\(^+\) (CD154\(^+\)) and CD8\(^+\) (CD137\(^+\)) T cells expressing PD-1, VISTA, TIM-3, CD160, Gal-9, NKG2A, and CTLA-4 exhibited significantly higher levels of cytokines (e.g., TNF-α and IFN-γ) compared with their negative counterparts. Therefore, the method of T cell stimulation, i.e., anti-CD4/CD28 Abs or PMA versus peptide Ags derived from SARS-CoV-2, can result in different outcomes and should be taken into consideration when T cell effector functions are analyzed.

More importantly, using cryopreserved PBMCs can significantly reduce the magnitude of Ag-specific T cell responses, possibly because of an altered functionality of APCs (60), and should be considered. Although not all coinhibitory-expressing T cells were SARS-CoV-2 Ag specific, we observed that Ag-specific T cells, defined by CD154 and CD137 expression, were highly enriched with coinhibitory receptors.

The purinergic system fine-tunes immune cell functions via CD39 and CD73 ecto-enzymes. This system shifts an ATP-driven proinflammatory to an anti-inflammatory milieu induced by adenosine (61). Patients with COVID-19 infection had significantly higher CD39- and lower CD73-expressing T cells. The dysregulated expression level of these ecto-enzymes may exacerbate an inflammatory response and impact lymphocyte homing to draining lymph nodes (62). In COVID-19 patients with severe disease, we observed an increased capability of CD4\(^+\) and CD8\(^+\) T cells to produce IL-17 in response to SARS-CoV-2 peptide pools; this would presumably
heighten inflammation and the subsequent activation of neutrophils. Previous studies have shown similar results, in particular, cells present in the lung exhibited more IL-17 production capacity than those in the peripheral blood (21, 63). These data further support a potentially crucial role for IL-17 in COVID-19 pathogenesis and may lead to novel therapeutic approaches based on IL-17 blockade. However, further investigations are required to determine whether IL-17 is truly increasing tissue pathology in COVID-19 patients.

Although the S glycoprotein has been reported as an efficient Ag, CD4+ and CD8+ T cells reactive to N and M peptide pools were also observed in our cohort. As expected, the majority of T cells in response to viral peptides produced TNF-α but very low levels of IFN-γ as reported elsewhere (34, 64). With respect to the relatively low sample numbers and multiple correction testing, the lack of statistically significant differences is not surprising. More importantly, SARS-CoV-2–reactive T cells in our moderate and severe patients were predominantly of the Th1 phenotype. Although the observed Th1-mediated response is regarded as protective immunity (9, 65), it also can contribute to immunopathogenesis (66). In this context, our finding of a dominant Th2 response of SARS-CoV-2–reactive T cells in mild disease patients raises the question about the beneficial effect of Th1 immunity. One could speculate that even though the Th1 response is generally protective against viral infections, a more balanced combination of Th1/Th2 responses could lead to a milder form of illness among COVID-19 patients. This was illustrated in SARS-CoV-2–responding T cells in patients with mild disease exhibiting a dominant Th2 phenotype (e.g., IL-10, IL-4, and IL-13). It has been shown that COVID-19 infection is more severe in elderly patients with different comorbidities that are generally associated with an inflammatory state and Th1 response (67), whereas children have a tendency to develop an anti-inflammatory Th2 predominant response, which may in part explain a milder course of illness seen after SARS-CoV-2 infection (68, 69). The abundance of IL-4–secreting CD8+ T cells in a group of older adults may not only counterbalance the overproduction of Th1 cytokines but has also been associated with an intact humoral immunity in old age (70). In our study, there was no indication of Th2 response in severe patients but instead a Th17 phenotype was observed. The presence of IL-17+CD8+ T cells in COVID-19 patients with severe disease suggests that these cells may be present in inflamed tissues as reported in the lungs of COPD patients (71).

We are aware of the limitations of our study. The relatively low number of mild disease patients did not allow us to have sufficient statistical power for further desired analyses. Analyzing the coexpression of coinhibitory receptors on Ag-specific T cells is very valuable, but we were unable to run all the coinhibitory receptors on a single panel. Also, we were unable to conduct longitudinal studies to compare T cells response over time; such studies are highly valuable for assessing the fate and functionality of memory T cells. Furthermore, because of the limitations of blood volume collection among COVID-19 patients, we were unable to conduct all of the presented studies on all of the patients. To abrogate this possible confounding factor, patient samples were used randomly for each experiment to prevent any bias in data collection. Having a low number of deceased patients in our cohort did not allow us to provide a meaningful correlation between the disease outcome and immunological responses.

Taken together, the reactivity of T cells expressing different coinhibitory receptors to viral peptides and their polyfunctionality demonstrate the existence of T cell activation but not exhaustion in COVID-19 patients. It is worth noting that T cells are not created equally, and there is a diversity in the nature of T cells reactive to SARS-CoV-2 peptides. Although the upregulation of coinhibitory molecules on Ag-reactive T cells suggest an exhaustion phenotype, SARS-CoV-2–responding T cells from patients with severe disease displayed features that support the generation of robust T cell memory response. Whether these memory cells provide a long-term benefit in severe versus moderate/mild patients is yet to be determined.

Novel SARS-CoV-2 vaccines are developed or in development, and studies are underway to investigate the immunogenicity of vaccine components. The S glycoprotein as an efficient Ag has been the main focus; however, our study shows that T cells in different patient groups also recognize peptides belonging to M and N peptide pools. A unique immune response in mild disease patients, a Th2-biased anti-inflammatory response, may limit excessive inflammation and play a protective and regulatory role in homeostasis in the lungs. A balanced Th1/Th2 response of Ag-specific T cells could be essential in host defense against SARS-CoV-2 infection. In contrast, T cell activation and a skewed immune response toward the Th1/Th17 phenotype could exacerbate illness. Therefore, a robust Ag-specific T cell response can be protective, and uncoordinated or skewed responses fail to control virus with a connection between pathology and impaired adaptive immune responses to SARS-CoV-2.

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Disclosures

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