There are several limitations in the data from this case series. First, as is common with case series, selection bias is possible. Second, there was no control intervention, and the study sample was small. Third, it is uncertain whether these patients would have improved without prone positioning, although the rapid change, within 1 hour, after proning is suggestive of a favorable impact. Fourth, measures of patient dyspnea or comfort after prone positioning were not collected. Fifth, to minimize the documentation burden on nursing-staff workflow, data on patient adherence to the prone-positioning recommendation beyond the first episode of proning were not collected.

Given the potential of prone positioning as a low-cost, easily implemented, and scalable intervention, particularly in low- and middle-income countries, expeditious yet thorough testing of prone positioning in patients at risk for intubation is warranted (e.g., W. Al-Hazzani and colleagues, unpublished results [clinicaltrials.gov identifier NCT 04350723], among others).

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References

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COVID-19 Severity Correlates with Weaker T-Cell Immunity, Hypercytokinemia, and Lung Epithelium Injury

To the Editor:

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a global pandemic that continues to wreak havoc on people’s lives and livelihoods. As of June 16, 2020, the number of coronavirus disease (COVID-19) cases surpassed 8 million, and the death toll stood at more than 400,000 (1). Although the majority of the patients developed mild symptoms and eventually recovered from this disease, a significant proportion suffered from serious pneumonia and developed acute respiratory distress syndrome, septic shock, and/or multiorgan failure (2, 3). The degree of the disease severity should result from direct viral damages on epithelial surface layer and the host immune response. SARS-CoV-2 infection may trigger a dysfunctional response leading to an overproduction of cytokines (cytokine storm) and the recruitment of more immune cells into the lungs, resulting in greater damages (4). However, the immune effectors that determine or influence the severity of the disease and the reason why immune response mediates recovery in some individuals (5), but not in others, are far from clear. In this study, we addressed these issues by analyzing the blood samples of patients with COVID-19 with varying degrees of disease severity

Figure 2. Respiratory rate before prone positioning and 1 hour after prone positioning of individual patients. Solid symbols represent patients who required intubation. The P value was determined by using the Wilcoxon matched-pairs signed rank test.
<table>
<thead>
<tr>
<th>Group</th>
<th>Patient No.</th>
<th>Age (yr)</th>
<th>Underlying Medical Disorders</th>
<th>SOFA at Last Detected Time Point</th>
<th>Injury in Other Organ(s)</th>
<th>Imaging Score of Radiological Findings</th>
<th>Ventilation Days</th>
<th>Disease Outcome</th>
<th>Oxygenation Index at Last Detected Time Point (mm Hg)</th>
<th>Immune Effectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>R1</td>
<td></td>
<td>Diabetes II, CHD</td>
<td>2</td>
<td>N</td>
<td>None</td>
<td>5</td>
<td>34</td>
<td>D</td>
<td>395</td>
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<tr>
<td>R2</td>
<td>R2</td>
<td></td>
<td>HBV</td>
<td>5</td>
<td>Y</td>
<td>Myocardial</td>
<td>6</td>
<td>30</td>
<td>D</td>
<td>284</td>
</tr>
<tr>
<td>R3</td>
<td>R3</td>
<td></td>
<td>Diabetes, COPD</td>
<td>2</td>
<td>Y</td>
<td>Myocardial</td>
<td>6.5</td>
<td>19</td>
<td>D</td>
<td>238</td>
</tr>
<tr>
<td>R4</td>
<td>R4</td>
<td></td>
<td>Hypertension, diabetes, COPD</td>
<td>4</td>
<td>Y</td>
<td>Kidney</td>
<td>6</td>
<td>38</td>
<td>D</td>
<td>101</td>
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<tr>
<td>R5</td>
<td>R5</td>
<td></td>
<td>Pneumatocele, hepatic cyst, renal cyst</td>
<td>4</td>
<td>N</td>
<td>Kidney</td>
<td>6</td>
<td>34</td>
<td>D</td>
<td>255</td>
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<tr>
<td>R6</td>
<td>R6</td>
<td></td>
<td>Diabetes II, coronary atherosclerotic heart disease, COPD</td>
<td>9</td>
<td>Y</td>
<td>Kidney, myocardial</td>
<td>4</td>
<td>37</td>
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<tr>
<td>Average</td>
<td></td>
<td>59.0</td>
<td></td>
<td>4.3</td>
<td></td>
<td></td>
<td>5.6</td>
<td>2.9</td>
<td>32.0</td>
<td>248.3</td>
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<tr>
<td>S1</td>
<td>S1</td>
<td></td>
<td>None</td>
<td>10</td>
<td>N</td>
<td>None</td>
<td>6.5</td>
<td>71</td>
<td>C</td>
<td>107</td>
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<tr>
<td>S2</td>
<td>S2</td>
<td></td>
<td>None</td>
<td>7</td>
<td>Y</td>
<td>None</td>
<td>7</td>
<td>22</td>
<td>D</td>
<td>108</td>
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<tr>
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<td></td>
<td>Renal cyst</td>
<td>11</td>
<td>N</td>
<td>None</td>
<td>8</td>
<td>83</td>
<td>C</td>
<td>196</td>
</tr>
<tr>
<td>S4</td>
<td>S4</td>
<td></td>
<td>Postoperation of intracranial tumor</td>
<td>10</td>
<td>Y</td>
<td>Myocardial</td>
<td>7</td>
<td>87</td>
<td>C</td>
<td>ECMO 2,731</td>
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<tr>
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<td></td>
<td>HBV, sleep apnea syndrome</td>
<td>14</td>
<td>Y</td>
<td>Myocardial</td>
<td>8</td>
<td>95</td>
<td>C</td>
<td>ECMO 2,678</td>
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<tr>
<td>S6</td>
<td>S6</td>
<td></td>
<td>Hypertension II, diabetes II, hyperuricemia</td>
<td>7</td>
<td>Y</td>
<td>Myocardial</td>
<td>7</td>
<td>92</td>
<td>C</td>
<td>183</td>
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<tr>
<td>Average</td>
<td></td>
<td>62.3</td>
<td></td>
<td>9.8</td>
<td></td>
<td></td>
<td>7.3</td>
<td>6.9</td>
<td>75.0</td>
<td>148.5</td>
</tr>
</tbody>
</table>

**Definition of abbreviations:** CHD = coronary heart disease; COPD = chronic obstructive pulmonary disease; COVID-19 = coronavirus disease; ECMO = extracorporeal membrane oxygenation; HBV = hepatitis B virus; nAbs = neutralizing antibodies; R = recovering group; S = severe persistence group; SOFA = Sequential Organ Failure Assessment.

\*R group: six males; S group: two females and four males.

\†Y: sepsis; N: no sepsis.

\‡Imaging scores observed within the 6 weeks after disease onset; regression of scores from “maximum to latest” was used as an indicator of grouping.

\§Days of ventilation from the initiation to May 8, including invasive ventilation and noninvasive ventilation less than 12 h/d.

\D: discharged from hospital; C: continued hospitalization as of May 8.

\¶Integral average of immune effectors within 5 weeks after disease onset. Activated CD8\(^+\) is defined by CD38\(^+\)-HLA-DR\(^+\)-CD8\(^+\) T cells (n/ml blood); activated CD4\(^+\) is defined by CD38\(^+\)-HLA-DR\(^+\)-CD4\(^+\) T cells (n/ml blood); and nAbs is defined by units of neutralizing antibodies in 1 ml blood.
and by collecting their clinical data over a period of more than 3 months. Our findings highlight the importance of T-cell immunity in COVID-19 recovery.

Methods

Longitudinal peripheral blood mononuclear cells from 12 patients with severe COVID-19 hospitalized at the First Affiliated Hospital, Guangzhou Medical University, (Guangzhou, China), 6 with regressing imaging scores (recovering group [R]: R1, R2, R3, R4, R5, and R6) and 6 with no improvements in imaging scores within 6 weeks after disease onset (severe persistence group [S]: S1, S2, S3, S4, S5, and S6), were analyzed (Ethics No. 202051).

The method used for scoring computed tomographic and X-ray images was similar to the previous report (6), in which one point...
was assigned to the presence of a single lesion observed in the lung. A score was marked up or down by 0.5 points when consolidation was increased or resolved, respectively. Flow cytometric analysis for T-cell immune effectors was done using a FACSaria III instrument (BD Bioscience) and analyzed with FlowJo software (Treestar). Cytokines were measured by using Cytometric Bead Array kits (BD Bioscience). Focus reduction neutralization test was performed to evaluate the levels of neutralizing antibodies (nAbs) using Vero E6 cells infected with SARS-CoV-2 and rabbit anti-SARS-CoV-2 nucleocapsid protein polyclonal antibody (Sino Biological). The foci were visualized by TrueBlue reagent and counted with an ELISPOT reader (CTL S6 Ultra).

Results
The clinical data and immune status of patients examined are shown in Table 1. The comparison of oxygenation indexes (PAO2/FAO2) shows that the R group was better than the S group, which includes two extracorporeal membrane oxygenation users (P = 0.03). Furthermore, the S group had significantly higher Sequential Organ Failure Assessment scores than the R group (P = 0.002). At Days 95–110 after disease onset, five patients from the S group remained hospitalized in the ICU, whereas all six patients from the R group had long been discharged.

Longitudinal changes in plasma levels of sTM (soluble thrombomodulin), syndecan-1, MMP2, and MMP9 were analyzed to evaluate the damages to the epithelial surface layer in SARS-CoV-2 infection. Meanwhile, cytokines IL-6, IL-8, IP-10, MCP-1, and MIG were measured as inflammatory injury markers (7). Our data showed that the levels of syndecan-1 and IL-6 were significantly higher in the S than the R group (Figure 1A), suggesting that these effectors could be used as potential severity markers.

To dissect immune recovery mechanisms in severe COVID-19 cases, the frequency of activated CD8+y and CD4+y T cells was analyzed based on the expression of CD38 and HLA-DR. nAbs were also measured at corresponding time points. The data in Table 1 showed that S6, who had the highest level of CD8+y activation among all the samples (22,112 CD38+y HLA-DR+y CD8+y cells/ml) and a very strong CD4+y activation (33,879 CD38+y HLA-DR+y CD4+y cells/ml), developed more severe disease. However, this patient also exhibited an extreme low level of nAbs (74.8 U, compared with 3240–786.0 U in the rest of S group) (Table 1). Obviously, S6 whose immune response is distinctive from that of the others in the S group forms a separate category in terms of the T-cell and B-cell immunity and demands an independent assessment. As such, the data from S6 were not included in the subsequent analysis.

Marked differences between the R and S groups were seen for the number of CD38+y HLA-DR+y CD8+y (P = 0.0072) and CD38+y HLA-DR+y CD4+y (P = 0.0055), whereas no significant differences were observed for nAbs (Figure 1B, left and middle panels). Regression analyses show that activation of CD8+y (R² = 0.328, P = 0.002) and CD4+y (R² = 0.430, P = 0.0002) T cells are strongly and inversely correlated to the severity of COVID-19 in patients (Figure 1B, right panel).

Discussion
The key findings of this study are 1) the lung injury and inflammation effectors (syndecan-1 and IL-6) are associated with disease severity, and 2) CD8+y and CD4+y T cells play a major role in the recovery of patients with critical COVID-19 under the caveat that adequate amounts of nAbs must also be present. These are consistent with the observations made in the studies of other severe infections with emerging viruses such as Ebola and influenza A virus H7N9 (8, 9). The T-cell immunity and lung injury markers were analyzed at a relatively early stage of COVID-19 (within Day 33 after disease onset). The updated fact that 6/6 of the R group had long been discharged while 5/6 of S group still suffered acute respiratory distress syndrome and had a prolonged use of ventilators in ICU (Table 1) strongly suggests that T-cell immunity can be used as a prognostic marker for COVID-19. Nevertheless, because of the small sample size, our findings warrant further verifications with larger cohorts.

Importantly, our study emphasizes that a balance between T-cell immunity and neutralizing antibodies is required for the COVID-19 recovery. The variability of T-cell immunity in individuals suggests that patients with a different balance of immune activation may require tailored treatments. For example, convalescent serum antibody therapy may benefit those patients who have strong T-cell immunity but low levels of nAbs (as in the case of S6), whereas other patients with insufficient T-cell activation may need a T-cell immunity boost strategy and should be cautiously treated with corticosteroids to suppress the cytokine storm.

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References
Measurement of Short-Chain Fatty Acids in Respiratory Samples: Keep Your Assay above the Water Line

To the Editor:

Short-chain fatty acids (SCFAs) are bacterial products that have important biological functions, including maintenance of immune homeostasis (1). Growing evidence indicates that bacteria residing in the airways of patients with numerous pulmonary diseases as well as in those of healthy individuals (2, 3) are capable of making SCFAs (4). Therefore, there is growing interest in measuring respiratory SCFA concentrations because they could provide insight into biological processes in the lungs.

Sampling the lungs is challenging; the most common biospecimen is BAL fluid, which requires bronchoscopy under sedation. This invasive procedure is labor intensive and costly, and it may not be feasible in unstable critically ill patients. Exhaled breath condensate (EBC) is an easily acquired, abundant biospecimen that could be used as an alternative to BAL. To test the utility of EBC for this purpose, we measured SCFAs and 16S ribosomal (r)RNA in paired BAL and EBC samples acquired from healthy control subjects.

Methods
The study (clinicaltrials.gov NCT03034642) and its consent procedures were performed in accordance with the Declaration of Helsinki at the Veterans Affairs Ann Arbor Healthcare System and the University of Michigan; protocols were reviewed and approved by the respective Institutional Review Boards (Federalwide Assurance [FWA] 000,000,348 [Veterans Affairs] and FWA 000,049,969 [University of Michigan]). All participants gave written consent.

Participants, who could be never-smokers, current smokers, or former smokers, had normal chest radiographs and post-bronchodilator spirometry and were free of respiratory and gastrointestinal disease. We excluded those with unstable cardiovascular disease, significant renal or hepatic dysfunction, mental incompetence, active psychiatric illness, or infection. We also excluded pregnant women and those on immunosuppression or antibiotic therapy.

On the day of study, EBC (RTube breath condensate collection device; Respiratory Research) was acquired in accordance with the manufacturer’s instructions and American Thoracic Society guidelines, and paired BAL was acquired as previously described (5). Total bacterial DNA from each sample was extracted (6), sequenced (7), and quantified for the bacterial 16S rRNA gene (8) as previously described.

Assay of EBC and BAL fluid for SCFAs. Remaining volumes of samples were assayed for SCFAs by gas chromatography (GC)–mass spectroscopy (MS) without derivatization using a previously published protocol (9). A liquid chromatography (LC)–MS method was also used to confirm results (see supplemental material at https://doi.org/10.7302/wk4r-7x52). Methyl tert-butyl ether (MTBE) was used for liquid–liquid extraction of SCFAs from acidified EBC. Negative control samples, including MTBE alone, MTBE used to extract acidified water and internal standards, a water wash of the EBC acquisition equipment, and a representative sample of normal saline acquired from the bronchoscopy suite, were also assayed. A postmortem porcine portal vein plasma sample was assayed as a positive control.

Data analysis. The SCFA concentrations for each subject were summed, and Pearson correlation was used to assess the association between the total SCFA concentration and the microbiome signal within each medium. The summed SCFA concentrations of BAL and EBC were compared with a Mann–Whitney U test.

Results
Twenty subjects were enrolled into the study. Of these, 13 subjects had sufficient volumes of both EBC and BAL available for SCFA and microbiome assays. The median (interquartile range [IQR]) age of our sample was 59 (48–64) years, 59% of subjects were female, and 85% were white. Four subjects were current smokers, two were former smokers, and seven had never smoked; the median (IQR) pack-years of smokers was 20 (11–21). The median FEV1% predicted (IQR) was 91% (83–104%), and the FEV1/FVC ratio (IQR) was 0.87 (0.82–0.95).

SCFAs are in the water. All water samples (negative control samples) had detectable SCFA concentrations as measured by GC-MS (Figure 1); this finding was corroborated by an LC-MS assay (https://doi.org/10.7302/wk4r-7x52). Acetate was profoundly

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