Patterns of Inflammatory Cell Infiltration and Expression of STAT6 in the Lungs of Patients With COVID-19: An Autopsy Study

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Background: Severe acute respiratory syndrome coronavirus 2 causes diffuse alveolar damage (DAD), lymphocyte infiltration in the lungs and a cytokine storm. In this study we examined inflammatory cell infiltrates and the expression of signal transducer and activator of transcription (STAT) 6 in the lungs of patients with coronavirus disease 2019 (COVID-19).

Methods: Eighteen COVID-19 autopsy cases, 9 non-COVID cases with DAD, and 11 controls without lung diseases were included. Immunostainings for STAT6, CD3, CD4, CD8, CD68, and broad-spectrum keratins were performed.

Results: The average age of COVID-19 patients was 64.4 ± 2.1 years. The disease duration was 7 to 53 days. The number of pneumocytes, macrophages or CD3+ T cells was significantly increased in the lungs of patients with COVID-19. Patients’ age above 67 years, blood troponin levels > 0.2 ng/mL, platelet count > 100×10^9/L, lung macrophages > 130/high-power field (HPF), CD3+ T cells > 145/HPF, CD8+ T cells <30/HPF, and CD8/CD4 ratio <1 were associated with shorter survival duration after onset of symptoms. In addition, STAT6 staining was much stronger in pneumocytes and lymphocytes in the lungs of patients with COVID-19 than non-COVID DAD patients or controls.

Conclusions: STAT6 expression was increased in pneumocytes and lymphocytes in the lungs of patients with COVID-19, implying a role of STAT6 in cytokine storms.

Key Words: STAT6, CD3, CD4, CD8, COVID-19, diffuse alveolar damage, autopsy

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Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and was first identified in Wuhan, China, in late 2019.1 SARS-CoV-2 belongs to the family Coronaviridae, genus Betacoronavirus, and infects cells through a fusion mechanism driven by the interaction of the Receptor Binding Domain (RBD) of its spike protein with the ACE2 receptor of the host cell.2 It is a highly contagious virus, transmitted primarily through inhalation of respiratory droplets, and has caused millions of deaths worldwide.3 Some variants are even more contagious. For example, the virus variant known as alpha (20I/501Y.V1, VOC 202012/01, or B.1.1.7) was first detected in the United Kingdom, causing one-quarter of the country’s total COVID-19 cases and two-thirds of cases in the UK in December 2020.4,5 This variant was also detected in the United States at the end of December 2020.5 COVID-19 alpha is characterized by an N501Y mutation in the RBD of the spike protein, where asparagine (N) is replaced with glutamic acid (E).6

TABLE 1. Clinical and Pathologic Parameters

| Case number | COVID-19 | Non-COVID | Control |
|-------------|----------|-----------|---------|
| Age         | 64.4 ± 2.1 | 64.2 ± 4.5 | 64.4 ± 4.3 |
| Sex (M/F)   | 9/9      | 4/5        | 5/6      |
| Left lung weight (g) | 936.8 ± 71.4* | 710.8 ± 48 | 584.2 ± 48 |
| Right lung weight (g) | 1065.7 ± 71.9* | 891.5 ± 76.3 | 678.5 ± 54.4 |
| Pneumocytes (N/HPF) | 184.1 ± 17.5* | 87.2 ± 11.1 | 80.5 ± 9.6 |
| Macrophages (N/HPF)  | 137.0 ± 9.9*  | 73.2 ± 13 | 47.3 ± 7.6 |
| CD6 (N/HPF)  | 29.7 ± 5.7  | 15.2 ± 2.6 | 22.5 ± 5.2 |
| CD4 (N/HPF)  | 32.3 ± 4.3  | 24.8 ± 5.8 | 21.5 ± 5 |
| CD3 (N/HPF)  | 113.4 ± 8.5* | 71.9 ± 8.1 | 56.6 ± 9.1 |

*P < 0.01, ANOVA, compared with control.  
†P < 0.01, ANOVA, compared with non-COVID group.  
ANOVA indicates analysis of variance; COVID-19, coronavirus disease 2019; DAD, diffuse alveolar damage; F, female; HPF, high-power field; M, male.
tyrosine (Y) at the position 501, as well as a 69/70 deletion and P681H mutation. In South Africa, another variant of SARS-CoV-2, known as beta (20H/501Y.V2 or B.1.351), occurred independently of alpha, but shared the same N501Y spike protein mutation, as well as mutations K417N and E484K. Unlike the alpha variant, the beta variant does not have the deletion at 69/70. This beta variant was found in the United States at the end of January 2021. The third COVID-19 variant delta, also called B.1.617.2, was first identified in India and has increased transmissibility.

SARS-CoV-2 causes diffuse alveolar damage (DAD) in the lungs and diffuse infiltration of CD4+ and CD8+ T lymphocytes in the alveolar walls. In the peripheral blood, CD3+, CD4+, and CD8+ T-lymphocyte absolute counts were reduced in severe COVID-19 patients or nonsurvivors and associated with disease severity. CD8+/CD4+ T-cell ratio in the peripheral blood was increased in severe disease of pediatric patients. However, the ratio of CD8+/CD4+ T cells in the lung tissues of COVID-19 patients and its clinical significance is not fully understood.

SARS-CoV-2 may induce a cytokine storm in moderate and severe cases of COVID-19 and cause an increase of inflammatory cytokines such as tumor necrosis factor-α, interleukin (IL)-1, IL-2, interferon-γ, and an imbalance in T helper cells 1 (Th1) and Th2 responses. Naive T cells differentiate into Th2 cells through both TCR-mediated and IL-4-mediated signaling pathways. IL-4-activated signal transducer and activator of transcription (STAT)6 is deemed as a critical step in driving Th2 differentiation. The expression of STAT6 in the lungs of patients with COVID-19 is not known.

In this study, we examined the numbers of CD4+, CD8+, CD3+ T cells, pneumocytes and macrophages, and the expression of STAT6 in the lungs of COVID-19 autopsy cases.

**METHODS**

**Study Population**

The study was approved by the Institutional Review Board (IRB) at Rhode Island Hospital and performed according to our institution IRB guidelines. We searched our pathology database for autopsy cases of COVID-19 from 2020 to 2021. Slides were reviewed to stage the DAD in the lungs and sections were used for immunohistochemical (IHC) staining. Patients with DAD unrelated to COVID-19 were also studied, namely non-COVID group thereafter. Patients without lung disease from the same period were used as controls. Clinicopathologic parameters of COVID-19 patients including age, sex, survival duration from onset of symptoms,


### TABLE 2.

| Group       | Subgroup | Weight (g) | Platelets (×10^9/L) | Lymphocyte (×10^9/L) | CD3 (/HPF) | CD4 (/HPF) | CD8 (/HPF) | Troponin (ng/mL) |
|-------------|----------|------------|---------------------|----------------------|------------|------------|------------|------------------|
|             |          | Left Lung  |                      |                      |            |            |            |                  |
|             |          | Right Lung |                      |                      |            |            |            |                  |
| Age>67     | N=9      | 93.4±10.1  | 1.5±0.4             | 1.6±1.1              | 1.6±1.1    | 1.6±1.1    | 1.6±1.1    | 0.1±0.2          |
| Age<67     | N=9      | 93.6±9.7   | 1.4±0.4             | 1.5±0.4              | 1.5±0.4    | 1.5±0.4    | 1.5±0.4    | 0.1±0.2          |
| P-value     |          | NS         | NS                  | NS                   | NS         | NS         | NS         | NS               |

The numbers of pneumocytes (keratin positive), macrophages (CD68 positive), CD3+, CD4+ or CD8+ lymphocytes were counted in 3 random areas [expressed as the number per high-power field (HPF)] and the average was calculated and used for statistical analysis. The extent of STAT6 expression was scored as negative (0), 1+, 2+, and 3+. The percentage of area was not calculated because STAT6 showed diffuse staining.

### IHC Staining and Scoring

IHC stainings for STAT6, CD3, CD8, CD4, CD68, and broad-spectrum keratins were performed. Antibodies for CD3, CD4, CD68, and cytokerin cocktail (AE1, AE3, and CAM5.2) were purchased from Dako, Carpenteria, CA. CD8 antibody was from Leica Biosystems (Buffalo Grove, IL) and STAT6 antibody (dilution 1: 200) was from Abcam (Cambridge, MA). IHC was performed on 4 μm thick paraffin sections of the lung tissues on the Ventana Discovery Autostainer (Ventana Medical Systems, Tucson, AZ), Ventana Benchmark Ultra Autostainer (Ventana Medical Systems), and the Dako Autostainer (Dako, Carpenteria, CA). Positive controls included lymph nodes (CD3, CD4, and CD8), lung (CD68) and solitary fibrous tumor (STAT6). The numbers of pneumocytes (keratin positive), macrophages (CD68 positive), CD3+, CD4+ or CD8+ lymphocytes were counted in 3 random areas [expressed as the number per high-power field (HPF)] and the average was calculated and used for statistical analysis. The extent of STAT6 expression was scored as negative (0), 1+, 2+, and 3+. The percentage of area was not calculated because STAT6 showed diffuse staining.

### Statistical Analysis

Data were expressed as mean±SEM. Statistical differences between 2 means were determined by the unpaired Student t test. Analysis of variance analysis was used for multiple mean comparisons. Comparison of clinicopathologic parameters between 2 groups (such as sex differences and STAT6 staining intensity differences) was performed using the χ² test. The Kaplan-Meier method was used for survival analysis. All above statistical analysis was performed by using JMP Statistical Software.

### RESULTS

#### Clinical and Pathologic Parameters

Our study included 18 COVID-19 patients: 9 males and 9 females. The average age was 64.4±2.1 years. All patients died from COVID-19. No lab results were available for analysis in 2 patients; one patient did not have lab work because of sudden death and the other patient lacked clinical information in our system since this patient was from an outside institution. The most common co-morbidities were hypertension (12/17, 70.6%), diabetes mellitus (9/17, 52.9%), and chronic kidney disease (3/17, 17.6%) (Table 1). Some patients had multiple co-morbidities. For example, 8 patients had both diabetes mellitus and hypertension.

The survival duration of 17 patients with available clinical information was 21.2±3.4 days (range: 7 to 53 d) after onset of symptoms. Patients younger than 67 years old (namely young patients thereafter, N=9) survived 26.4±5.9 days after onset of symptoms, which was significantly longer than those above 67 years old (namely older patients thereafter, 15.2±1.4 d, N=8, P<0.05) (Fig. 1A). The younger patients had

lung weights, counts of blood platelets and absolute lymphocytes, D-dimers, and troponins were obtained and analyzed.
significantly lower platelet counts (107.7 ± 33.2×10⁹/L, N = 8) than the older ones (224.6 ± 42.4×10⁹/L, N = 8, P < 0.05, Table 2). Conversely, the younger patients had much higher absolute lymphocyte counts (1.5 ± 0.4×10⁹/L, N = 7) than the older ones (0.7 ± 0.1×10⁹/L, N = 8, P < 0.05, Table 2).

Interestingly, the patients with low platelet counts (<100×10⁹/L) survived longer than those with higher platelet counts (P < 0.05, Fig. 1B). The number of absolute lymphocytes had no correlation with the survival duration.

Troponin levels were measured in 15 patients, among whom 12 patients had elevated troponin. Among these 12 patients, 2 patients had acute myocarditis which was identified during autopsy. Another patient had acute myocardial infarction which was made clinically by troponin level and electrocardiogram, and confirmed by autopsy. Patients with high troponin levels (>0.2 ng/mL) had shorter survival duration after onset of symptoms (16.8 ± 1.9 d) than those with low troponin levels (30.8 ± 8.0 d, P < 0.05, Fig. 1C).

Sixteen patients had hemoglobin measurement and were all anemic. Their hemoglobin ranged from 4.3 to 11.9 g/dL (average 8.6 ± 0.6 g/dL). Fourteen patients had d-dimer measurements, ranging from 303 to 19,461 ng/mL (normal value 0 to 300 ng/mL), and averaging 3408.2 ± 1429.6 ng/mL.

**FIGURE 2.** Effect of macrophage and CD3+ or CD8+ T cells in the lungs on survival. A, Patients with macrophages >130/high-power field (HPF) survived shorter than those with macrophages <130/HPF (P < 0.01). B, Patients with CD3+ T cells >145/HPF survived shorter than those with CD3+ T cells <145/HPF (P < 0.05). C, Patients with CD8+ T cells <30/HPF survived shorter than those with CD8+ T cells >30/HPF (P < 0.05). D, Patients with CD8+/CD4+ ratio <1 survived shorter time than those with CD4+/CD8+ ratio >1 (P < 0.03).

**FIGURE 3.** Representative image of STAT6 staining showing nuclear and cytoplasmic staining in pneumocytes (×600).
mL. The hemoglobin and d-dimer levels were not correlated with the survival duration after onset of symptoms.

### Inflammatory Cell Infiltrate and Pathologic Findings in Patients With COVID-19

Microscopic examination showed DAD in bilateral lungs. DAD can be divided into 3 phases: acute (exudate), proliferative, and repair (scarring) phase.15 Of 18 patients, 14 reached the repair (scarring) phase and 4 were at proliferative phase. Different phases may coexist in the same patient. As we expected, lungs were significantly heavier in patients with COVID-19 than in patients without lung diseases (control) but had no significant difference from non-COVID patients with DAD (non-COVID group) associated with acute bacterial pneumonia (8/9) or acute renal failure (1/9) (Table 1).

We also counted pneumocytes, macrophages, CD3+, CD4+, and CD8+ T cells by IHC staining. We found that the number of pneumocytes, macrophages, and CD3+ T cells were significantly increased when compared with control or non-COVID group (Table 1). CD4+ and CD8+ T cells were also increased, but the difference did not have statistical significance.

In addition, we examined the survival duration in COVID-19 patients with different numbers of macrophages, CD3+ and CD8+ T cells, or different ratios of CD8+/CD4+. Patients with macrophages > 130/HPF, CD3+ T cells > 145/HPF, and CD8+ T cells < 30/HPF had a shorter survival time compared with those with macrophages < 130/HPF, CD3+ T cells < 145/HPF, and CD8+ T cells > 30/HPF, respectively (Figs. 2A–C). We also calculated the ratio of CD8+ over CD4+ T cells and separated the patients into 2 groups: CD8+/CD4+ ratio < 1 and > 1. We found that patients with CD8+/CD4+ ratio < 1 survived shorter than those with CD8+/CD4+ ratio > 1 (P < 0.05, Fig. 2D).

Furthermore, we found that CD4+ T cells were significantly increased in COVID-19 patients with CD3+ T cells > 145/HPF, when compared with those with CD3+ T cells < 145/HPF. The number of macrophages or CD3+ T cells in the lungs was significantly higher in patients with troponin > 0.2 ng/mL than in those with troponin < 0.2 ng/mL (Table 2).

### Expression of STAT6

STAT6 immunostaining showed cytoplasmic and nuclear staining in pneumocytes (Fig. 3). 94.4% (17/18) cases demonstrated moderate to strong staining in pneumocytes, and 55.6% cases showed moderate to strong staining in lymphocytes, both of which were significantly higher than controls or non-COVID group (Table 3, Fig. 4). The staining intensity in macrophages was 1+ to 3+ in COVID-19 and not significantly different from controls. 100% patients (9/9) in non-COVID group had moderate to strong STAT6 staining in macrophages, which was significantly higher than in COVID-19 group or control (Table 3). The staining intensity in endothelial cells was 0 to 1+, which was similar to controls.

### Multivariate Survival Analysis in Patients With COVID-19

Multivariate survival analysis performed by using the Cox proportional hazards model showed that age (above 67 y), macrophages (> 130/HPF), CD8 (< 30/HPF), CD8/CD4 ratio (< 1), CD3 (> 145/HPF), platelets (> 100×109/L), and troponin (> 0.2 ng/mL) were not independent factors affecting survival duration (Table 4).

### DISCUSSION

We found that the number of pneumocytes, macrophages and CD3+ T cells were significantly increased in patients with COVID-19. COVID-19 patients with macrophages > 130/HPF, CD3+ T cells > 145/HPF, and CD8+ T cells < 30/HPF had a shorter survival time than those with macrophages < 130/HPF, CD3+ T cells < 145/HPF, and CD8+ T cells > 30/HPF, respectively. In addition, the expression of STAT6 was increased in pneumocytes and lymphocytes.

The most common co-morbidities in our cohort were hypertension (70.6%) and diabetes mellitus (52.9%). Diabetes is a known risk factor for death in COVID-196 and the crude mortality rate for hospitalized patients with diabetes was 25.2%.16 One study showed that 49% ICU COVID-19 patients had hypertension.17 As we expected, young patients (below 67 y old) lived longer after onset of symptoms than older patients, which is consistent with the literature showing that old patients had higher mortality.
Interestingly, patients with low platelet counts (<100×10^9/L) survived longer than those with high platelet counts, which might be related to a relatively low coagulation state in these patients with thrombocytopenia.

In addition, higher troponin levels were associated with shorter survival duration after onset of symptoms. Higher blood troponin levels indicate more severe cardiac injury, thus causing a poorer outcome. In fact, of 12 troponin-elevated

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**FIGURE 4.** Representative images of STAT6 staining. A, Strong STAT6 staining was observed in pneumocytes of a patient with coronavirus disease 2019 (COVID-19). Moderate staining was seen in macrophages located in the middle of alveoli. B, Moderate to strong STAT6 staining was observed in lymphocytes of a patient with COVID-19. C, Weak STAT6 staining was observed in endothelial cells of a patient with COVID-19. D, Weak STAT6 staining was observed in pneumocytes of a patient with diffuse alveolar damage and without COVID-19. Strong staining was seen in macrophages located in the middle of alveoli. E, Weak STAT6 staining was observed in lymphocytes of a patient with diffuse alveolar damage and without COVID-19. Please note the cells with strongest staining were macrophages. F, Weak STAT6 staining was observed in endothelial cells of a patient with diffuse alveolar damage and without COVID-19. G, Weak STAT6 staining was observed in pneumocytes of a control patient. Moderate staining was seen in macrophages located in the middle of an alveolus. H, Weak STAT6 staining was observed in lymphocytes of a control patient. I, Weak STAT6 staining was observed in endothelial cells of a control patient.
patients, 2 patients had acute myocarditis and 1 patient had acute myocardial infarction. The result is consistent with the literature showing that only 13% SARS-COV-2-positive hearts showed myocarditis.18

Sars-CoV-2 caused DAD in bilateral lungs of all our patients. Most of these patients (77.8%) were in a repair (scarring) phase. Different phases of DAD may coexist in the same patient. Type II pneumocyte proliferation is a known phenomenon in DAD, whereas type I pneumocytes do not have the ability to proliferate.15 Therefore, the increased pneumocytes in the lungs of COVID-19 were likely type II pneumocytes.

In COVID-19, lungs were infiltrated by inflammatory cells, including macrophages and lymphocytes. The increased macrophages may be good or bad for the host. On one hand, they play a critical role in the body’s defense against viral infections by producing inflammatory mediators, which remove pathogens and repair tissue injury. In contrast, they may cause a cytokine storm, which is harmful to the host.19 In fact, patients with macrophages > 130/HPF lived shorter after onset of symptoms than those with macrophages <130/HPF. In addition, CD8+ T cells were significantly increased in the lungs of COVID-19, an increase which correlated with shorter survival time. Interestingly, patients with fewer CD8+ T cells in the lungs showed poorer outcome since CD8+ T cells <30/HPF had a shorter survival time than those with CD8+ T cells >30/HPF. CD8+ T cells recognizes infected cells and causes apoptosis, thus preventing the virus from spreading.20 Therefore, more CD8+ T cells in the lungs are likely beneficial to the host. Similarly, patients with CD8+/CD4− ratio <1 had a shorter survival time than those with CD8+/CD4+ ratio >1.

SARS-CoV-2 may induce an imbalance in Th1 and Th2 responses11,12 and IL-4-induced activation of STAT6 drives Th2 polarization. STAT6 plays an important role in adaptive immunity in response to virus infection.21 We found that STAT6 was significantly increased in the cytosol and nuclei of pneumocytes, implying a role of STAT6 in cytokine storms. Cytosolic location of STAT6 after viral infection has been reported in the literature.21 Although the staining intensity was not increased in the macrophages, the number of macrophages in the lungs nearly doubled (compared with non-COVID group) or tripled (when compared with control). Dr Chen et al found that STING (also named MITA/ERIS) triggered by viruses recruits STAT6 to the endoplasmic reticulum and activates it. STAT6 was also found to be associated with mitochondria.22 In non-COVID DAD group, STAT6 staining was stronger in macrophages, but not in pneumocytes and lymphocytes, than in COVID-19 group or control, indicating a different pattern in non-COVID DAD group, when compared with COVID-19.

We also tried to detect SARS-COV-2 virus in the lungs by IHC using 5 SARS-COV-2 antibodies from 2 companies (Genetex and Nova Biologocals) at different dilutions (up to 1:100), but failed. One reason was that the level of the virus was too low to be detected by IHC. In fact, one of our cases was sent to The Centers for Disease Control and Prevention (CDC). Polymerase chain reaction test performed at CDC was positive in the lungs and bronchi, but IHC staining and in situ hybridization performed at CDC were negative in both bronchi and lungs. The other reason was that majority of our cases were at repair (late) phase of DAD. According to the literature, SARS-COV-2 virus is detectable in the early phase of DAD by in situ hybridization, but not at the organizing (late) phase.23 In addition, levels of SARS-COV-2 virus decrease over the time since nasopharyngeal swab SARS-COV-2 polymerase chain reaction positivity decreases over the time (down to 50% in cases over 11 d after diagnosis).24

In conclusion, the number of pneumocytes, macrophages or CD3+ T cells was significantly increased in lungs of patients with COVID-19. Older age, high blood troponin level, high number of macrophages, and low number of CD8+ T cells in the lungs of patients with COVID-19 were associated with poorer outcome. STAT6 expression was increased in pneumocytes and lymphocytes in the lungs of patients with COVID-19, implying a role of STAT6 in cytokine storms.

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### TABLE 4. Multivariate Survival Analysis in Patients With COVID-19 Performed by Using the Cox Proportional Hazards Model

| RR   | Confidence Interval | P    |
|------|---------------------|------|
| Age > 67 | 4.28               | 0.2-206.9 | > 0.05 |
| Macrophage > 130 | 1.85           | 0.1-56.2     | > 0.05 |
| CD8 < 30 | 1.29             | 0.1-15.6     | > 0.05 |
| CD8/CD4 < 1 | 4.33           | 0.3-16.1     | > 0.05 |
| CD3 > 145 | 1.8              | 0.3-11.1     | > 0.05 |
| Platelet > 100 | 0.72         | 0.1-4.7      | > 0.05 |
| Troponin > 0.2 | 4.21         | 0.5-61.1     | > 0.05 |

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