CORONAVIRUS

Acetylated K676 TGFBIp as a severity diagnostic blood biomarker for SARS-CoV-2 pneumonia

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The outbreak of the highly contagious and deadly severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), also known as coronavirus disease 2019 (COVID-19), has posed a serious threat to public health across the globe, calling for the development of effective diagnostic markers and therapeutics. Here, we report a highly reliable severity diagnostic biomarker, acetylated 676th lysine transforming growth factor–beta–induced protein (TGFBIp K676Ac). TGFBIp K676Ac was consistently elevated in the blood of patients with SARS-CoV-2 pneumonia ($n = 113$), especially in patients in the intensive care unit (ICU) compared to non-ICU patients. Patients' blood samples showed increased cytokines and lymphopenia, which are exemplary indicators of SARS-CoV-2 pneumonia. Treatment with TGFBIp neutralizing antibodies suppressed the cytokine storm. The increased level of TGFBIp K676Ac in ICU patients suggests the promise of this protein as a reliable severity diagnostic biomarker for severe SARS-CoV-2 disease.

INTRODUCTION

In January 2020, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was identified as the cause of the coronavirus disease 2019 (COVID-19) outbreak and spread around the globe and was recently declared a pandemic by the World Health Organization (1). This novel coronavirus is highly contagious and pathogenic, causing severe pneumonia symptoms in infected patients. Data show that the virus is a serious threat to people, especially for elderly and/or those with underlying diseases, such as cardiovascular disease (2), diabetes (3), chronic respiratory disease (4), and cancer (5). The SARS-CoV-2, although not as lethal as the other previously identified CoVs, has proved much more pervasive with a long incubation period of 14 days (6–8). SARS-CoV-2 is known to induce the life-threatening condition sepsis (9), which accompanies degradation of the blood vessels (10). Because leaky blood vessels cause subsequent damage of other organs, the diagnosis of SARS-CoV-2 infection before the onset of severe organ damage is crucial.

SARS-CoV-2 has five major protein regions for virus structure assembly and viral replications (11), including replicase complex (ORF1ab), spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins (12, 13). However, current diagnostic accuracy remains a problem (14), both because of occasional false-negative outcomes of current tests and because of a lack of indicators of disease severity in patients.

In the current study, we sought to determine a biomarker that can accurately discern the severity of the disease and be a potential therapeutic target for severely symptomatic patients. We analyzed transforming growth factor–beta (TGF-β)–induced protein (TGFBIp) and acetylated 676th lysine TGFBIp (TGFBIp K676Ac) levels in the blood of patients with SARS-CoV-2 to diagnose the severity of SARS-CoV-2 infection. We showed that TGFBIp K676Ac was consistently elevated in the blood of patients with SARS-CoV-2 pneumonia ($n = 113$), and that the levels of this protein were higher in patients in the intensive care unit (ICU) than that in non-ICU cases. Currently, there is an urgent need to find and use a reliable biomarker that can accurately discern the severity of the disease, especially for high-risk patients requiring urgent diagnostic care. As a fresh approach to diagnosis and treatment of patients with SARS-CoV-2 infection, monitoring of TGFBIp K676Ac may ultimately contribute to the development of therapeutic agents.

RESULTS

TGFBIp and TGFBIp K676Ac levels are associated with SARS-CoV-2 infection and severity

From 113 blood samples of patients with SARS-CoV-2 (85 of non-ICU and 10 of ICU cases; patient information is shown in table S1), the levels of TGFBIp were measured using an enzyme-linked immunosorbent assay (ELISA). The median serum TGFBIp value was 122.72 (77.2 to 195.65 ng/ml) in 20 healthy volunteers. The level of TGFBIp was increased to 428.65 (109.64 to 726.14 ng/ml) in non-ICU cases and further increased to 757.69 (622.56 to 977.1 ng/ml) in ICU cases (Fig. 1A). The recovered cases (designated as discharged) showed a reduced level of TGFBIp of 239.48 (128.34 to 393.09 ng/ml). When we analyzed TGFBIp K676Ac, the difference was much clearer, 11.81 (5.22 to 23.02 ng/ml) for normal, 212.1 (22.06 to 459.89 ng/ml) for non-ICU, 724.63 (605.47 to 952.14 ng/ml) for ICU, and 66.62 (49.99 to 107.92 ng/ml) for discharged patients (Fig. 1B). The acetylation ratio, defined as the ratio of TGFBIp K676Ac and total TGFBIp, was significantly higher in SARS-CoV-2 ICU cases than in other cases and even twofold higher than that of non-ICU cases (fig. S1, A and B). Using the molecular weight of TGFBIp, the concentration measurements
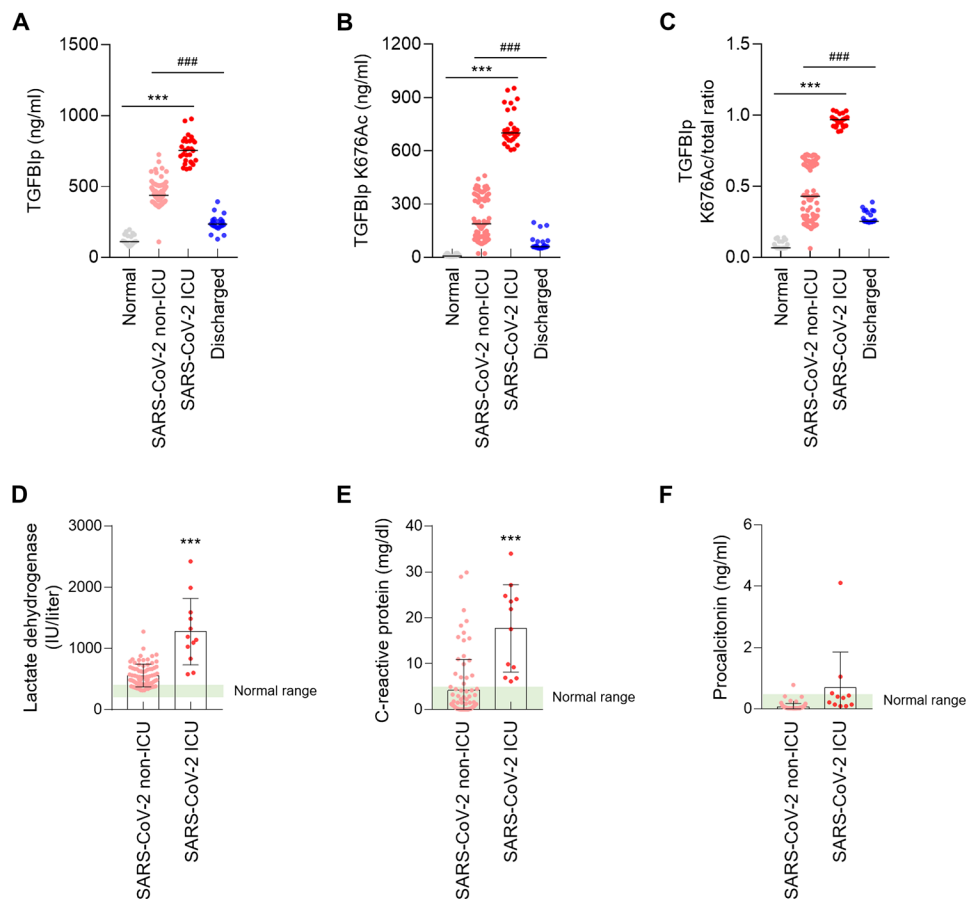
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Fig. 1. Circulating TGFBIp and TGFBIp K676Ac levels are associated with SARS-CoV-2 infection and severity. (**A** and **B**) Plasma was obtained from 20 healthy individuals, 113 patients with SARS-CoV-2, and 25 discharged patients. TGFBIp and TGFBIp K676Ac levels were measured by ELISA. (**C**) In patients with SARS-CoV-2, the ratio of TGFBIp K676Ac/TGFBIp may be a diagnostic and prognostic factor. LDH (**D**) and CRP (**E**) in SARS-CoV-2 ICU patients were particularly elevated, and PCT (**F**) was not altered. ***$P < 0.001$ versus normal; ###$P < 0.001$ versus patients with SARS-CoV-2.

of TGFBIp and TGFBIp K676Ac in patient blood samples (fig. S1A) support those shown in Fig. 1 (A and B), and comparison with the control group from the immunoprecipitation (IP) and immuno-blotting experiments (fig. S1B) is accurate. Note that the highest TGFBIp K676 level was observed in 10 deceased patients (fig. S2). The TGFBIp K676Ac clearly discriminated at cutoff values that separate the degree of severity and recovery of symptoms, ~532.68 ng/ml for the determination of severity between ICU and non-ICU cases and ~212.10 ng/ml for the determination of recovery. These results indicated that the TGFBIp Ac not only can serve as a biomarker for identifying the SARS-CoV-2–induced disease but also can diagnose the severity of diseases. For the diagnosis of pneumonia, lactate dehydrogenase (LDH), complement-reactive protein (CRP), and procalcitonin (PCT) have been verified (15). LDH and CRP showed a sharp increase in the ICU cases of the blood samples of patients with SARS-CoV-2 pneumonia, with no significant difference in PCT (Fig. 1, D to F). Although LDH and CRP showed statistically different values between non-ICU and ICU cases, the ranges of distribution in each case were overlapped, and thus, determination of cutoff value for diagnosis was unavailable. The results indicate that the conventional biomarkers of pneumonia cannot be used for the determination of severity of SARS-Cov-2 pneumonia.

We analyzed the expression of pneumonia markers including TGFBIp K676Ac, TGFBIp, and LDH in plasma of nine SARS-CoV-2 ICU patients using Western blot analysis. As shown in fig. S1A, the pneumonia markers were detected in all patient plasmas. We also confirmed the detection of the pneumonia markers with IP technique (fig. S1B). TGFBIp K676Ac and TGFBIp were up-regulated in SARS-CoV-2 cases, and such high level was rescued in the discharged cases. Considering the experimental results of ELISA, Western blot, and IP, the acetylated form of TGFBIp K676Ac was consistently expressed in patients with SARS-CoV-2 and thus proven to be used as a severity diagnostic biomarker for SARS-CoV-2 pneumonia.

Up-regulation of plasma inflammatory cytokine levels in patients with SARS-CoV-2

Acute respiratory distress syndrome (ARDS), sepsis, and septic shock are the main causes of death of patients with SARS-CoV-2 (16, 17). Among them, ARDS and sepsis are the common immuno-pathological phenomena for SARS-CoV-2 infection. One of the main mechanisms for ARDS and sepsis in SARS-CoV-2 infection is the cytokine storm, a deadly uncontrollable systemic inflammatory response resulting from the release of large amounts of proinflammatory cytokines and chemokines by immune effector cells (16–18).
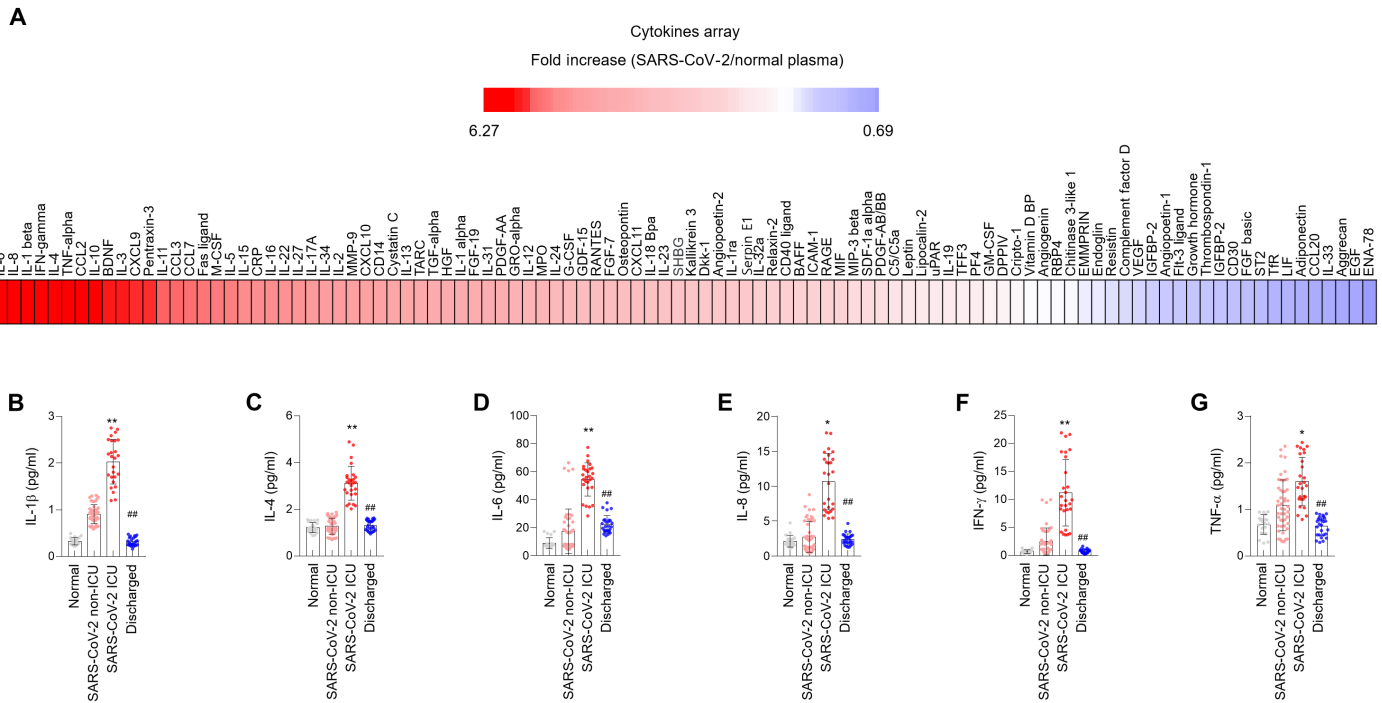
Fig. 2. Plasma cytokine levels in patients with SARS-CoV-2. (**A**) Proteome analysis of inflammatory factors in plasma from healthy volunteers ($n = 2$) and patients with SARS-CoV-2 ($n = 2$). (**B** to **G**) Series of comparisons of plasma cytokine levels in SARS-CoV-2 non-ICU, SARS-CoV-2 ICU, and discharged patients. *$P < 0.05$ versus normal; **$P < 0.01$ versus normal; ##$P < 0.01$ versus patients with SARS-CoV-2.

According to the cytokine array analysis, patients with SARS-CoV-2 showed changes in the level of cytokines (Fig. 2A). The up-regulated cytokines include interleukin-6 (IL-6), IL-8, IL-1β, interferon-γ (IFN-γ), IL-4, tumor necrosis factor–α (TNF-α), chemokine C-C motif ligand 2 (CCL2), IL-10, etc. The down-regulated cytokines include epidermal-derived neutrophil-activating peptide-78 (ENA-78), epidermal growth factor, aggrecan, IL-33, CCL-20, etc. In particular, the levels of IL-1β, IL-4, IL-6, IL-8, and IFN-γ were significantly increased in 10 plasma samples obtained from SARS-CoV-2 ICU patients (Fig. 2, B to G). These increased cytokine levels were consistent in all patient samples. Furthermore, the high levels of cytokines observed in patient blood support the sudden rise in the systemic inflammatory response "cytokine storm" after the SARS-CoV-2 infection.

Lymphopenia symptoms in patients with SARS-CoV-2
It has been reported that the patients with SARS-CoV-2 pneumonia lose their immune activity and thus aggravate their symptoms as a result of negative feedback. The number of each immune cell was counted to explain the loss of immune activity in ICU patients. In the ICU case of patients with SARS-CoV-2, the number of lymphocytes was decreased, indicating the lymphopenia symptom (Fig. 3D). However, the number of white blood cells, neutrophils, monocytes, and platelets did not show a significant difference between non-ICU and ICU cases.

TGFBIp neutralizing antibody suppresses SARS-CoV-2–mediated cytokine storm
Peripheral blood mononuclear cells (PBMCs) are known to actively regulate the immune response upon invasion of pathogens, such as viruses, by secreting various anti-inflammatory cytokines. Consid-

ering that cytokine storm is also observed in patients with SARS-CoV-2 without underlying diseases, it is hypothesized that PBMCs are affected by the SARS-CoV-2 virus. In this regard, the expression of TGFBIp K676Ac as well as TGFBIp from the PBMCs of SARS-CoV-2 ICU patients was determined using immunofluorescence (Fig. 4A). The fluorescence for TGFBIp K676Ac was rescued in the PBMCs of the discharged patients. Furthermore, the acetylation of TGFBIp-related mRNAs was quantified (fig. S3). Although the mRNA level of CBP/p300 that acetylates the TGFBIp does not show a significant difference, the mRNA levels of TGFBIp and IL-6 were significantly up-regulated in the SARS-CoV-2 ICU patients.

Time-dependent reduction in cell viability of PBMCs isolated from SARS-CoV-2 ICU patients was observed, unlike the healthy group. However, the cell viability was significantly increased when PBMCs isolated from SARS-CoV-2 ICU patients were cocultured with the TGFBIp neutralizing antibody (Fig. 4B).

To demonstrate the effects of neutralizing antibody on nuclear factor κB (NF-κB) activation and cytokine secretion, we treated the PBMCs of SARS-CoV-2 patients with TGFBIp monoclonal antibody (50 ug/ml; 6 hours). Data showed that the neutralizing TGFBIp antibody significantly reduced activation of NF-κB (Fig. 4C) and secretion of cytokines (IL-1β, IL-4, IL-6, IL-8, IFN-γ, and TNF-α) (Fig. 4, D to H).

DISCUSSION
TGFBIp is a 68-kDa-sized protein in the extracellular matrix, usually found near the blood vessel (*19*). It contains an RGD domain after Lys[676], a binding motif that modulates cell adhesion and serves as a ligand recognition site for several integrins (*20*). In our recent
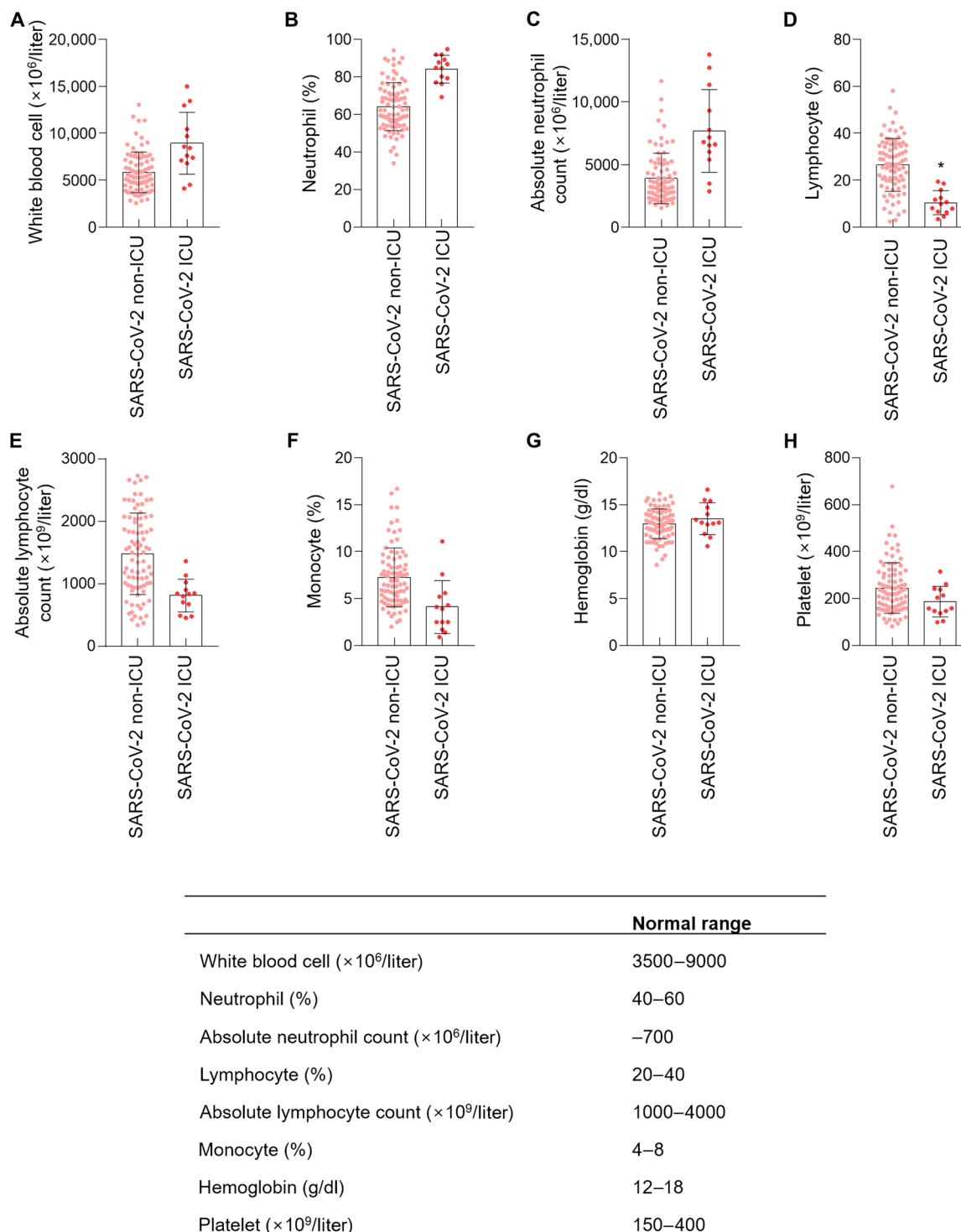
Fig. 3. Number of blood cell subsets in patients with SARS-CoV-2. A series of comparisons of white blood cells (**A**), neutrophils (**B**), absolute neutrophil count (**C**), lymphocytes (**D**), absolute lymphocyte count (**E**), monocytes (**F**), hemoglobin (**G**), and platelets (**H**) between SARS-CoV-2 non-ICU and SARS-CoV-2 ICU patients. *$P < 0.05$ versus SARS-CoV-2 non-ICU.

	Normal range
White blood cell (×10⁶/liter)	3500–9000
Neutrophil (%)	40–60
Absolute neutrophil count (×10⁶/liter)	−700
Lymphocyte (%)	20–40
Absolute lymphocyte count (×10⁹/liter)	1000–4000
Monocyte (%)	4–8
Hemoglobin (g/dl)	12–18
Platelet (×10⁹/liter)	150–400

study, we found that TGFBIp is acetylated after infection and undergoes secretion into the circulation system (*21*). Previous reports have demonstrated that TGFBIp can induce signaling activation and up-regulation of NF-κB (*21, 22*). NF-κB is a family of inducible transcrip-

tion factors that plays a vital role in different aspects of immune responses, and it is normally sequestered in the cytoplasm as inactive complexes via physical association with inhibitory proteins (*23*). In response to stress and/or immune stimuli, NF-κB gets activated
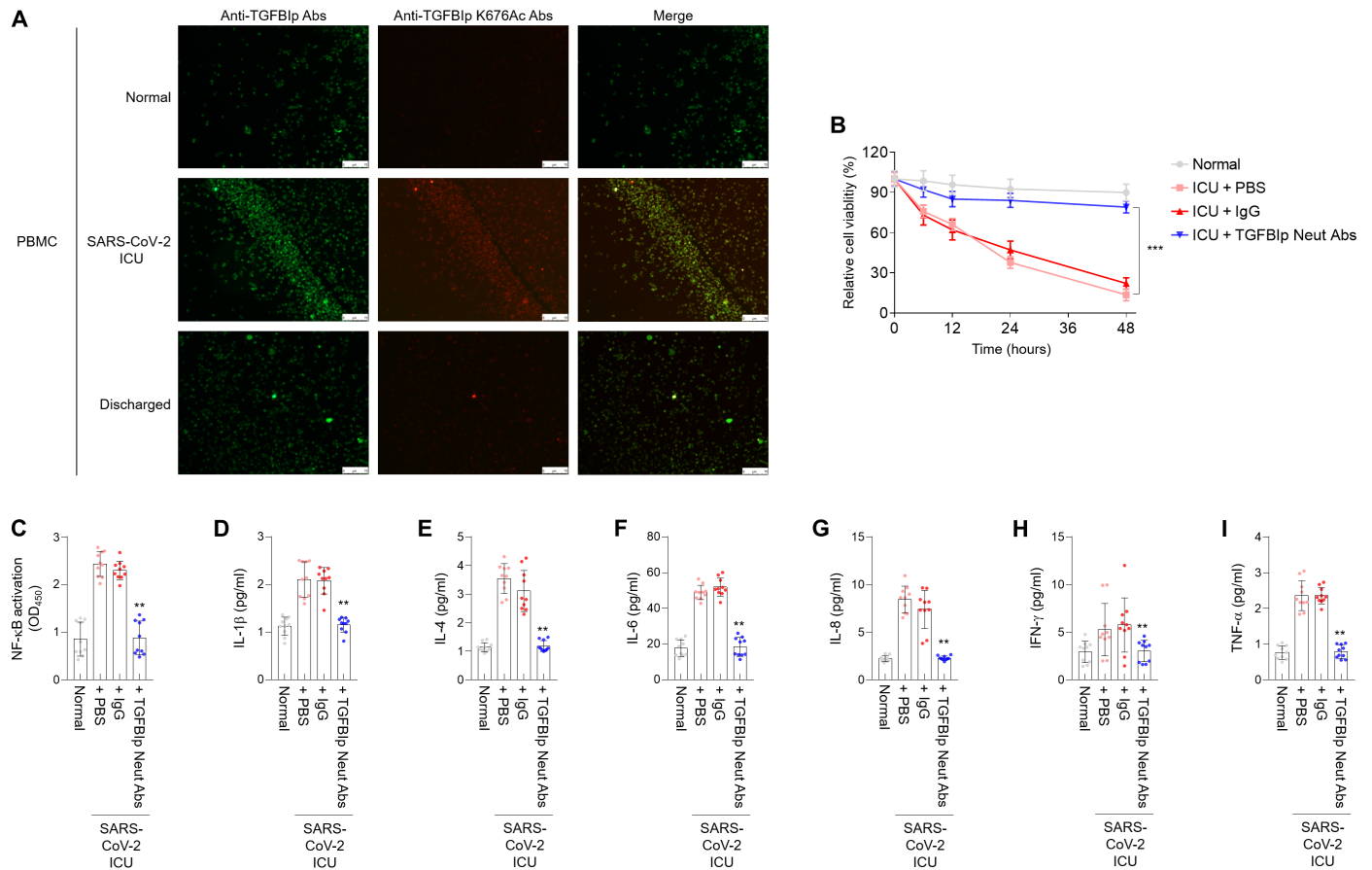
Fig. 4. Imaging of TGFBIp K676Ac and anti-inflammatory effect of TGFBIp neutralizing antibody in PBMCs of patients with SARS-CoV-2. (**A**) Expression and acetylation of TGFBIp was shown in isolated PBMCs from patients with SARS-CoV-2 by immunofluorescence staining (×200) (green: TGFBIp; red: TGFBIp K676Ac). (**B**) Relative cell viability of PBMCs after the treatment of TGFBIp neutralizing antibody (Ab). (**C**) Binding activity of NF-κB p65 in PBMCs cultured with PBS, mouse immunoglobulin G (IgG) antibodies (50 μg/ml; 6 hours), or TGFBIp neutralizing antibodies (50 μg/ml; 6 hours) was measured using the TransAM NF-κB p65 assay kit. (**D** to **I**) Series of comparisons of plasma cytokine levels in PBS-, mouse IgG–, or TGFBIp neutralizing antibody–treated PBMCs of patients with SARS-CoV-2. **$P < 0.01$ versus IgG-treated group; ***$P < 0.001$ versus ICU patients PBMC + IgG–treated group.

and moves to the nucleus to exert transcriptional functions (*24*). Although NF-κB is vital for normal immune responses against infections, nuclear accumulation of activated NF-κB can lead to an increase in endothelial cell apoptosis, vascular permeability, and secretion of endotoxins (*25*).

As inflammatory cytokines cannot be used as an infection biomarker because of very short half-life and are also increased in other diseases (*26*, *27*), finding a good biomarker that can predict the symptoms from infectious disease is essential. We have observed that TGFBIp K676Ac is continuously secreted by SARS-CoV-2 and thus can be a suitable biomarker for diagnosis of the severity of infection. From the analysis of blood samples derived from patients with SARS-CoV-2, the level of TGFBIp K676Ac was significantly higher, indicating the possibility of increase in NF-κB activation. We found that an increase in the cytokine level was consistent in all patient samples, supporting the rise in the systemic inflammatory response as a result of activation of the NF-κB and secretion of endotoxins. In the most severe cases, subsequent up-regulation of the cytokines may elicit cytokine storm, leading to sepsis-induced organ failures and death. Because NF-κB occupies a central role in signaling pathways important in sepsis, modulation of NF-κB activity via use of antibodies such as acetylation shadow inhibitors may be an appropriate therapeutic target in patients with SARS-CoV-2.

In most patients with COVID-19, the primary cause of death is lung failure due to severe ARDS (*16*, *17*). The syndrome is largely attributed to uncontrolled inflammatory responses characterized by cytokine storm and edema and fibrosis in the lungs at the end stages. The fibrosis in the lung may be caused mainly by TGF-β activation (*28*). In addition, TGF-β is involved in the fluid homeostasis in the lung as well. This leads to the functional failure of the lungs and death of the patients. Thus, massive increase in active TGF-β in the lungs may be observed (*29*).

In our previous study, it was observed that under inflammatory conditions, the secreted TGFBIp becomes acetylated by CBP/p300, and then the RGD domain of the secreted TGFBIp K676Ac binds with integrin αvβ5 and activates NF-κB to induce inflammation (*21*, *30*). When the secretion of TGFBIp is elevated by SARS-CoV-2, it was noticed that the p300 mRNA levels are increased by more than 1.5-fold, and we confirmed that TGFBIp K676Ac gets secreted (*31*, *32*). Moreover, CBP/p300 gets activated and up-regulated upon inflammatory

responses (*33*). In addition, with the expression of TGFBIp, IL-6 is regulated by NF-κB activation by infection (*21*). Here, we found that CBP/p300 acetylates K676 of TGFBIp and gets secreted by SARS-CoV-2 infection, suggesting that it may be a diagnostic and therapeutic target for SARS-CoV-2. The data show that the CBP/p300 level in SARS-CoV-2 ICU was increased to a statistically significant level ($P < 0.05$) in response to the SARS-CoV-2 infection (fig. S3).

Previously, it was observed that the expression and extracellular secretion of TGFBIp is controlled by TGF-β and histone methylation (*34*). TGF-β increases recruitment of the H3K4 methyltransferase mixed-lineage leukemia 1 (MLL1) and of SET domain-containing protein 7 (SET7/9) and the binding of Smad3 to the promoters (*34*). SARS-CoV-2 virus infection and consequent strong immune and inflammatory responses induce massive activation of the TGF-β in the lungs (*29*). TGF-β can up-regulate TGFBIp expression through Smad transcription factors and E-box–dependent mechanisms (*35*, *36*). Therefore, the possible mechanism in patients with SARS-CoV-2 may be that, after the level of TGFBIp is increased by severe infectious responses, the TGFBIp becomes acetylated and accumulates in the granules, and then the acetylated TGFBIp is secreted out of the cells (*21*, *30*).

There lies a possibility that TGFBIp K676Ac may be a blood biomarker in common pneumonia and other diseases. Previously, we found that TGFBIp and TGFBIp K676Ac are associated with sepsis (*21*, *30*). In this study, we verified that TGFBIp K676Ac could be used as a biomarker for severe disease caused by SARS-CoV-2 pneumonia. Although it may seem that TGFBIp K676Ac is SARS-CoV-2 specific, we should not rule out the possibility of TGFBIp K676Ac in association with other pathogens. There are reports of SARS-CoV-2 mutants being found, and also, because of inaccurate diagnostics, there are reports of recurrence of the infection as a result of poor antibody formation in the patient blood. Therefore, finding a good biomarker that can predict the severity of the SARS-CoV-2–mediated symptom is essential. Here, we suggest the TGFBIp K676Ac protein in blood as an additional factor that can be used to diagnose the severity of SARS-CoV-2 infection. We believe that TGFBIp K676Ac could be used as a suitable biomarker as the level is increased only in patients with SARS-CoV-2, and the level remains low in normal and discharged patients. In our cohort study, mild patients include the SARS-CoV-2–positive but not nonsymptomatic patients (SARS-CoV-2 non-ICU patients). In the case of young patients, they showed no symptoms other than mild fever. As shown in Fig. 1 (D and E), some SARS-CoV-2 non-ICU patients displayed normal ranges of LDH and CRP, which are the markers for COVID-19 diagnosis (dots in green part, normal range). An association between confounding factors such as age, sex, gender, and comorbidities in patients with SARS-CoV-2 and the statistically significant increase of TGFBIp K676 secretion was confirmed in elderly patients and those with diabetes (table S1). Thus, the TGFBIp may not be an early diagnostic marker, but it can be a reliable severity diagnostic marker for SARS-CoV-2.

Here, we report that the acetylated TGFBIp can serve as a more reliable severity diagnostic biomarker for SARS-CoV-2 pneumonia. This is the first evidence that shows the acetylation site K676 of TGFBIp as a novel biomarker for SARS-CoV-2. We have demonstrated that TGFBIp K676Ac is the key player in the up-regulation of cytokines via the NF-κB pathway. Recent reports have shown frequent cases of false-negative diagnoses associated with the SARS-CoV-2, raising concerns over the reliability of commercially available test kits (*37*). There have been reports that SARS-like coronaviruses have a high rate of mutation (*38*). For patients requiring urgent diagnostic care, it is essential to use a reliable biomarker that can accurately discriminate the severity of the disease. Our experimental results from the patient blood sample support the idea that TGFBIp K676Ac is a more suitable diagnostic target for SARS-CoV-2 disease and that TGFBIp K676Ac neutralizing antibody is a potential therapeutic drug for severely symptomatic patients.

MATERIALS AND METHODS
Experimental design
The purpose of this study was to identify and evaluate the reliability of TGFBIp K676Ac as a severity diagnostic biomarker for the SARS-CoV-2 pneumonia. A cohort study was designed for patients admitted to the Yeungnam University Medical Center. First, the SARS-CoV-2–infected patients were classified according to their symptoms (mild disease and sepsis). Blood was collected and analyzed from patients that were admitted to the hospital with mild disease and from those in the ICU because of septic symptoms. The progression of sepsis was assessed by monitoring increases in the absolute number of neutrophils, lymphopenia, and cytokine levels. In addition, TGFBIp and TGFBIp K676Ac were analyzed from patient blood samples as indicators of severity of the disease caused by the viral infection. We treated the patients' PBMCs with TGFBIp neutralizing antibodies to analyze suppression of SARS-CoV-2–mediated cytokine storm. Blood and immunohistochemical analyses were performed in a blinded manner. The study protocol (YUH 2020-03-057) was approved by the Institutional Review Board of Yeungnam University Hospital at Daegu in Korea.

Plasma sample
The patients were admitted to the Department of Internal Medicine, Yeungnam University Medical Center, after SARS-CoV-2 infection was confirmed at a public health center in Daegu. SARS-CoV-2 ICU is a group that has progressed to ARDS or sepsis and receives respiratory intensive care in ICU. Healthy volunteers were used as controls, and clinical data of all patients were collected. Plasma samples were generated by centrifugation at 2000*g* for 5 min within 48 hours after whole blood collection. The study protocol (YUH 2020-03-057) was approved by the Institutional Review Board of Yeungnam University Hospital at Daegu in Korea.

ELISA for TGFBIp/TGFBIp K676Ac
Rabbit anti-human TGFBIp K676 acetylation or nonacetylation antibody was produced by Abclon (Seoul, Korea). For immunoprecipitation (IP), anti-TGFBIp antibody (10188-1-AP) was purchased from Proteintech (Rosemont, IL), and for immunoblot (IB), anti-TGFBIp antibody (ab89062) was purchased from Abcam (MA, USA). We performed competitive ELISA using antibodies that recognize the acetylated peptide RLAPVYQ(K-Ac)LLERMK or nonacetylated RLAPVYQKLLERMK (amino acid sequence 669 to 682 of hTGFBIp). TGFBIp peptide (RLAPVYQKLLERMK, 669 to 682 amino acids) or TGFBIp K676 acetylation peptide [RLAPVYQ(K-Ac)LLERMK] was 2 μg/100 μl diluted and coated onto Nunc-Immuno MicroWell 96-well plates and incubated overnight at 4°C. Before use, the plates were washed three times with phosphate-buffered saline with Tween 20 (PBST) and blocked with 3% bovine serum albumin (BSA) in PBS for 30 min 37°C. Primary antibody (1:1000 dilution) and plasma sample (20 μg) were preincubated for 1 hour at 37°C, and then the preincubated sample

was transferred to a peptide-coated plate and incubated for 1 hour at 37°C. The plate was washed five times with PBST. Five secondary antibodies (1:3000 dilution) were incubated for 30 min at 37°C, and then the plate was washed five times with PBST. The washed plate was treated with trimethylboron ELISA substrate 100 μl per well for 10 min at 37°C, and then stop solution 100 μl per well was added. The detection was performed at 450 nm using a microplate reader (TECAN).

Laboratory tests

PCT was determined with an enzyme-linked fluorescent immunoassay (B.R.A.H.M.S. Diagnostica, Berlin, Germany). The test needs 20 min and 200 μl of serum. CRP and LDH were determined using a Roche/Hitachi Modular DP Chemistry Analyzer. Blood cell count was performed using the Sysmex XE-2100 Automated Hematology System.

MPO ELISA

To quantify the release of granule matrix proteins upon degranulation in PBMCs of SARS-CoV-2–infected patients and mice, plasma was analyzed using a human myeloperoxidase (MPO) ELISA kit (BMS2038INST, Invitrogen) and a mouse MPO ELISA kit (MBS700747, MyBioSource), respectively.

Protein profiling

Plasma pools of normal patients or patients with SARS-CoV-2 were processed as indicated in the Human XL Cytokine Array Kit (R&D Systems). Developed films were scanned, and the obtained images were analyzed using ImageJ version 1.43.

ELISA for IL-1β, IL-4, IL-6, IL-10, IFN-γ, and TNF-α

The concentrations of cytokines in the plasma of patients with SARS-CoV-2 were quantified according to the manufacturer's instructions using a commercially available ELISA kit. Values were measured using an ELISA plate reader (Tecan, Austria GmbH, Austria). Human IL-1β Quantikine ELISA Kit (DLB50, R&D Systems, Minneapolis, MN, USA), Human IL-4 Quantikine ELISA Kit (D4050, R&D Systems, Minneapolis, MN, USA), Human IL-6 Quantikine ELISA Kit (D6050, R&D Systems, Minneapolis, MN, USA), Human IL-10 Quantikine ELISA Kit (D1000B, R&D Systems, Minneapolis, MN, USA), Human IFN-γ Quantikine ELISA Kit (DIF50, R&D Systems, Minneapolis, MN, USA), and Human TNF-α Quantikine ELISA Kit (DTA00D, R&D Systems, Minneapolis, MN, USA) were used.

PBMC isolation and culture

Samples from healthy volunteers, patients with SARS-CoV-2 pneumonia, and discharged patients were obtained from the Yeungnam University Medical Center. The relevant local Institutional Review Boards and Ethics Committees approved the study. Heparinized blood samples were used fresh within 4 hours, and PBMCs were separated from blood using Ficoll-Hypaque or NycoPrep according to the manufacturer's recommendations. Following this, more refined PBMCs were obtained via the MACSprep PBMC Isolation Kit and cultured in RPMI 1640 with 1 mM sodium pyruvate, 2 mM L-glutamine, glucose (4.5 mg/liter), 10 mM Hepes, and sodium bicarbonate (2 mg/liter).

Polyclonal rabbit anti-TGFBIp neutralizing antibody is obtained and purified as described previously (39). To verify the effect of TGFBIp neutralizing antibodies on suppressing cytokine secretion, PBMCs isolated from patients with SARS-CoV-2 and the TGFBIp neutralizing antibodies (50 μg/ml) were cultured for 6 hours. The supernatant was used for analysis of cytokine ELISA, and lysate was used for NF-κB activity analysis.

Immunofluorescence staining

For TGFBIp/TGFBIp K676Ac staining, PBMCs were fixed in 4% formaldehyde in PBS (v/v) for 15 min at room temperature. After fixation, cells were blocked in blocking buffer (5% BSA in PBS) overnight at 4°C. Then, cells were then incubated with primary mouse monoclonal TGFBIp antibody, anti-mouse Alexa 488 (green), primary rabbit polyclonal TGFBIp K676Ac antibody, and anti-mouse Alexa 594 (red). In addition, PBMCs were visualized by fluorescence microscopy at a ×200 magnification (Leica Microsystems, Germany).

WST-1 cell proliferation assay

Following exposure of PBMCs to TGFBIp neutralizing antibody, 10 μl of WST-1 reagent was added per well and incubated at 37°C with 5% CO_2. At indicated time points, measurements of absorbance were taken at 480 and 600 nm (background) on a Tecan Spark microplate reader.

NF-κB activity kit

Nuclear extract preparation and TransAM assay were performed as previously described (33). The activity of individual NF-κB subunits was detected by an ELISA-based NF-κB family transcription factor assay kit (43296, Active Motif, Carlsbad, CA, USA). Briefly, nuclear extracts (2 μg) were incubated in a 96-well plate, which were immobilized by NF-κB consensus oligonucleotides. The captured complexes were incubated with specific NF-κB primary antibodies and subsequently detected with horseradish peroxidase–conjugated secondary antibodies (included with the kit). Last, the optical density (OD) value at 450 nm was measured by the Tecan Spark microplate reader.

Western blotting and IP

TGFBIp, acetylated lysine (K), and acetylated K676 TGFBIp in plasma of healthy volunteers, patients with SARS-CoV-2, and discharged patients were detected by immunoblotting. After SDS–polyacrylamide gel electrophoresis (PAGE), we performed an immunoblotting assay with each antibody. Plasmas were incubated with the anti-TGFBIp antibody at 4°C overnight. Immunoprecipitates were recovered using protein A/G-S magnetic beads, washed four times with IP buffer, and resuspended in the sample buffer of SDS-PAGE, followed by boiling for 10 min. Bound proteins were then analyzed by immunoblotting using anti-TGFBIp or anti-acetylated K676 TGFBIp antibodies.

Real-time PCR of TGFBIp, CBP/p300, and IL-6

To generate complementary DNA from PBMC incubation with or without TGFBIp neutralizing antibody samples, 1 μg of total RNA was reverse-transcribed with random hexamers using expand reverse transcription polymerase (Roche). Real-time polymerase chain reaction (PCR) was performed using the LightCycler FastStart DNA Master SYBR Green I from Roche Diagnostics GmbH according to the manufacturer's protocol. The following LightCycler conditions were used: initial denaturation at 95°C for 10 min, followed by 45 cycles with denaturation at 95°C for 10 min, annealing at 60°C for 5 min, and elongation at 72°C for 15 min. Quantities of specific mRNA in the sample were measured according to the corresponding gene-specific standard curves.

Statistical analysis

All experiments were performed independently at least three times. Statistically significant differences were determined using unpaired *t* test. Prism software was used for statistical analyses. Data are reported as means ± SEM with significance set at $P < 0.05$. P values for each experiment are provided in the figure legends.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/31/eabc1564/DC1

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