Title

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Journal

Clinical Infectious Diseases, 74(3)

ISSN

1058-4838

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Publication Date

2022-02-11

DOI

10.1093/cid/ciab437

Peer reviewed

Increased peripheral blood neutrophil activation phenotypes and NETosis in critically ill COVID-19 patients: a case series and review of the literature

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Brief Summary: Neutrophils in the circulation of critically ill COVID -19 patients with acute respiratory distress syndrome have functional changes over the course of their disease. These neutrophils have increased antimicrobial and pro-inflammatory functionality, including neutrophil extracellular trap formation (NETosis), phagocytosis and oxidative burst.

Abstract

Background Increased inflammation has been well defined in COVID-19, while definitive pathways driving severe forms of this disease remain uncertain. Neutrophils are known to contribute to immunopathology in infections, inflammatory diseases and acute respiratory distress syndrome (ARDS), a primary cause of morbidity and mortality in COVID-19. Changes in neutrophil function in COVID-19 may give insight into disease pathogenesis and identify therapeutic targets.

Methods Blood was obtained serially from critically ill COVID-19 patients for eleven days. Neutrophil extracellular trap formation (NETosis), oxidative burst, phagocytosis and cytokine levels were assessed. Lung tissue was obtained immediately post-mortem for immunostaining. Pubmed searches for neutrophils, lung and COVID-19 yielded ten peer-reviewed research articles in English.

Results Elevations in neutrophil-associated cytokines IL-8 and IL-6, and general inflammatory cytokines IP-10, GM-CSF, IL-1b, IL-10 and TNF, were identified both at first measurement and across hospitalization ($p<0.0001$). COVID neutrophils had exaggerated oxidative burst ($p<0.0001$), NETosis ($p<0.0001$) and phagocytosis ($p<0.0001$) relative to controls. Increased NETosis correlated with leukocytosis and neutrophilia, and neutrophils and NETs were identified within airways and alveoli in lung parenchyma of 40% of SARS-CoV-2 infected lungs available for examination (2 out of 5). While elevations in IL-8 and ANC correlated with disease severity, plasma IL-8 levels alone correlated with death.

Conclusions Literature to date demonstrates compelling evidence of increased neutrophils in the circulation and lungs of COVID-19 patients. importantly, neutrophil quantity and activation correlates with severity of disease. Similarly, our data shows that circulating neutrophils in COVID-19 exhibit an activated phenotype with enhanced NETosis and oxidative burst.

Keywords: Neutrophil, NETs, NETosis, COVID-19

Background

Since the appearance of SARS-CoV-2 in Wuhan, China in December 2019, to the moment that the World Health Organization (WHO) declared COVID-19 a pandemic on March 11th, 2020 and beyond, there has been a global imperative to better define disease pathogenesis in order to lay the groundwork for rapid development of effective treatments and vaccines. Acute respiratory distress syndrome (ARDS) and systemic inflammation were quickly identified as characteristics of severe forms of COVID-19. Correlations were also found with SARS, as pulmonary failure in SARS-CoV-2 infection has also been found to be due to both viral and host factors leading to cell damage and inflammation [1, 2].

Detrimental host immune responses to infections (immunopathology) are widely recognized and studied in respiratory infections and inflammatory diseases [3-5]. Immunopathology in the context of respiratory viral infections involves increased activity of innate immune cells, including natural killer (NK), NK T-cells (NKT), innate lymphoid cells (ILC), inflammatory monocytes, macrophages and neutrophils [4]. Among these cells, neutrophils are potent early mediators of innate immune responses during infection. Neutrophils rapidly and effectively clear pathogens but sometimes at a cost of collateral inflammatory tissue damage [4, 6]. For example, neutrophil recruitment to and activation in the lungs leads to cytotoxicity, inflammation and overall lung damage [6-8]. Recently, neutrophils have been proposed to play a role in acute lung injury (ALI) and ARDS related to COVID-19 [9-11], based on this concept of collateral lung damage leading to hypoxemic respiratory failure [12]. While several groups have highlighted this potential detrimental effect of neutrophil activation in COVID-19 pathology [9-11, 13], broad, multi-functional assessments of neutrophils isolated from COVID-19 patients are still lacking.

Neutrophils target invading pathogens through various intracellular and extracellular mechanisms, with phagocytosis, production of reactive oxygen species (ROS) and release of neutrophil extracellular traps (NETosis) representing three mechanisms in their arsenal [14]. Neutrophils release cytokines and chemokines that contribute to inflammation during active infections, and ROS and NETosis have been implicated in mediating tissue damage [6, 15, 16]. In COVID-19, two recent studies associated severe disease with circulating neutrophilia [17, 18], and COVID-19 patient neutrophils showed higher activation marker expression than those of healthy controls [18]. Elevated levels of NET components including myeloperoxidase (MPO), citrullinated histone H3, and DNA are seen in COVID-19 patient sera [18, 19]. While treatment of naïve neutrophils from healthy donors with serum from COVID-19 patients stimulated NETosis [19], and NETs are seen in lung tissue of COVID-19 patients [20], assays have not yet been done on neutrophils isolated from COVID-19 patients to define functional capacity and association with disease severity.

Here we sought to review all literature to date related to neutrophil and NET contributions to pulmonary inflammation in COVID-19 as well as to broadly define the functional state of neutrophils from critically ill COVID-19 patients, and put these findings in context of systemic inflammation and disease severity. By obtaining neutrophils and cytokine profiles from multiple days over the course of illness, a longitudinal cohort design, we increased the power of our study to detect corresponding changes in neutrophil function and systemic inflammation, as well as correlations between circulating leukocytes, absolute neutrophil counts, plasma IL-8 levels, neutrophil functions and disease severity. By putting the data from this case series in context with published studies in the literature, the critical role neutrophils are playing in immunopathology in COVID-19 becomes ever clearer.

Methods

Study design and oversight

Critically ill COVID-19 patients hospitalized at the University of California San Diego (UCSD) and Rady Children's Hospital underwent informed consent and blood was drawn on days 1, 3, 5, 7, 9, 11, most commonly between 8-10 am, and immediately prior to discharge. On days where 11 ml of blood or greater was obtained, neutrophils were isolated for functional studies. Healthy controls were recruited from San Diego county and from UCSD and Veterans Affairs San Diego Healthcare System (VASDHS) staff members (for details see: Supplementary Materials).

Subjects

Neutrophil data from 16 hospitalized COVID-19 patients and 15 healthy controls obtained on multiple days were included. Data from an additional 3 hospitalized COVID-19 patients were included for cytokine studies (these patients did not have neutrophil studies done due to <11 cc blood draws). APACHE (acute physiology and chronic health evaluation) II scores were calculated using clinical data within 24 h of blood draws for neutrophil functional assays derived by chart review. COVID-19 patients had APACHE II scores of 7 to 27 on ICU admission. White blood cell count (WBC), absolute neutrophil count (ANC) and neutrophil:lymphocyte ratios were captured from the clinical data of SARs-CoV-2 positive patients throughout their hospital and were closely matched to the timing of blood draws for neutrophil assays. Lung tissue was obtained within 2 h postmortem for NET staining (for details see: Supplementary Materials).

Ex vivo assays

For details on plasma cytokine quantification, neutrophil isolation from blood, neutrophil functional assays [21], literature review methods, and statistical analyses, please see Supplementary Materials.

Results

Characteristics of the COVID-19 patients and healthy controls

Mean age was 58 years for COVID-19 patients (n=19) and 42 years for controls (n=15; p=0.01; Table 1). Control and COVID-19 cohorts included similar numbers of females and males (Table 1). All COVID-19 patients had pre-existing conditions, with hypertension being the most prevalent (50%), followed by diabetes mellitus (25%), COPD (25%), congestive heart failure (12.5%), ischemic stroke (6%) and asthma (6%).

Eighteen out of 19 COVID-19 patients were admitted to the ICU (95%) with average APACHE II score of 14 (range of 7-27). Patients averaged 7 days from first symptoms of COVID-19 to ICU admission and remained in the ICU for an average of 13 days (range 4-32). The mortality rate for this cohort was 16% (n=3). Fifteen patients required vasopressors (79%) and 15 required invasive mechanical ventilation. Ventilated patients had an average Murray score of 2.6 and an average P/F ratio of 120 prior to intubation and 229 at extubation. The average PEEP requirement at intubation was 10 and peak PEEP was 14. Only three patients received steroids. A wide variety of antimicrobials and antivirals were given (Supplementary Table 1).

Increased neutrophil and general inflammatory cytokines in COVID-19

Plasma cytokine profiles and complete blood counts of our COVID-19 patient cohort demonstrated similarities to published cohorts [22-25], with elevations in IL-8, IL-6, IP-10, and of the neutrophil:lymphocyte ratio (mean 9.3). Profiling of specific cytokines relevant to neutrophil activity showed broad elevations across IP-10, IL-6, IL-8, GM-CSF, IL-1β, IL-10 and TNFα in the circulation of critically ill COVID-19 patients both early in their hospitalization (Fig. 1A) and were persistently elevated across their hospitalization, assessed at multiple time points (Fig. 1B). Cytokine levels of individual subjects 9 and 20 were assessed relative to APACHE score over time (Supplementary Figure 2). SOFA score was also assessed relative to cytokine levels (Supplementary Figure 3). Five COVID-19 subjects received Tocilizumab, and were found to have increased levels of plasma IL-6 (Supplementary Figure 6).

Circulating neutrophils from COVID-19 patients have higher rates of NETosis than healthy individuals

Circulating neutrophils receive inflammatory signals prior to entering tissues that impact their potential detrimental activity once in the tissue. There is mounting evidence of the role of NETs in systemic and pulmonary inflammation in COVID-19 [20, 26]. We investigated NET production by circulating neutrophils *ex vivo* by relative fluorescence of released dsDNA that may parallel exacerbated inflammation in the lungs. Neutrophils isolated from COVID-19 patients produced more NETs in steady state (without stimulation) than neutrophils from healthy individuals, and such differences were consistent even upon stimulation with different concentrations of PMA (Fig. 2A). We observed the same trend when using fluorescence microscopy staining for myeloperoxidase, a major protein component of NETs (Fig. 2B). These data show that circulating neutrophils from

COVID-19 patients are more prone to produce NETs than neutrophils from healthy individuals, and thus may be primed to cause significant NET-mediated tissue injury once they enter into lung tissue.

Neutrophils and NETs present in terminal bronchioles and alveoli of COVID-19 lung tissue.

Beside their antimicrobial function, release of NETs has been associated with collateral host tissue damage, including the lungs [10, 15]. Deterioration of lung function due to cellular damage and inflammation with subsequent hypoxemia is a hallmark feature of severe COVID-19, although the underlying mechanisms remain unknown [27]. To investigate the presence of neutrophils and NETs in COVID-19 lung tissue, we performed rapid autopsies on five deceased patients, which demonstrated a multitude of neutrophils across airways and alveoli by H&E staining of lung tissue in two of the five patients (Fig. 3A). Specifically, alveolar lung parenchyma showed extensive organizing pneumonia with areas of interstitial fibrosis, consistent with prior diffuse alveolar damage, with the majority of neutrophils within the terminal/respiratory bronchioles with focal areas with neutrophils within the alveolar lung parenchyma (Fig. 3A). Bronchiolar metaplasia was also seen. Positive staining for citrullinated Histone H3, CD66b, and DAPI confirmed the presence of neutrophils and NETs within bronchioles, alveoli and lung parenchyma (Fig. 3B); NETs were furthermore positive for MPO (Supplemental Figure 1). In the two SARS-CoV-2 infected lungs found to have NETs, the majority of NETs were found within terminal respiratory bronchioles, with focal areas within the alveolar lung parenchyma.

Circulating neutrophils from COVID-19 patients have increased production of reactive oxygen species and increased phagocytosis

ROS production is an antimicrobial mechanism mechanistically linked to NETosis and known to cause tissue damage [28]. Mirroring the NETosis phenotype, ROS production was increased in COVID-19 patients as compared to healthy controls at steady state over time (no stimulation; Fig. 4A), and

functional capacity increased upon stimulation with PMA at 2.5 nM (Fig. 4B), 25 nM (Fig. 4C) and 250 nM (Fig. 4D). Circulating neutrophils from COVID-19 patients also demonstrated increased phagocytosis of *S. aureus* bioparticles (Fig. 4E), suggesting that multiple key innate antimicrobial and inflammatory functions of circulating neutrophils patients are significantly increased in COVID-19 patients. No significant difference in neutrophil phagocytosis was noted in subjects that developed superimposed bacterial infections (Supplementary Figure 5).

Increased NETosis is associated with leukocytosis and neutrophilia, and leukocytosis, neutrophilia and plasma IL-8 correlate with COVID-19 disease severity

To assess absolute neutrophil count (ANC) and leukocytosis as potential biomarkers for disease severity in COVID-19, we assessed the relationship between WBC and ANC with our *ex vivo* data for NETosis at steady state (without stimulation) while controlling for neutrophil numbers in our experiments. We found a positive correlation of WBC to NETosis (Fig. 5A). Patients with leukocytosis (WBC >11x10^3 cells/mm^3) produced more NETs per neutrophil than patients without leukocytosis (r^2=0.48, p<0.0001; Fig. 5B). Similarly, we found a positive correlation of ANC to NETosis (r^2=0.53, p <0.0001; Fig. 5C) and patients with neutrophilia (ANC >8x10^3 cells/mm^3) produced more NETs per neutrophil than patients without neutrophilia (Fig. 5D). Elevated NETosis also correlated with circulating immature neutrophil numbers (Supplemental Figure 4). Higher levels of neutrophils correlated with disease severity, as measured by APACHE II (r^2=0.24, p=0.005; Fig. 5E), while lymphocyte counts fell as disease severity increased (r^2=0.19, p<0.001; Fig 5F). These data demonstrate that COVID-19 patients with leukocytosis, neutrophilia and lymphopenia are highly likely to have increased inflammatory and antimicrobial functions, including NETosis.

We also explored a potential direct relationship between NETosis and disease severity, using the APACHE II score as an indicator for illness severity, but found no such correlation (r^2=0.10, p=0.07; Fig. 5G). To be thorough, we also assessed the relationship of NETosis with SOFA and COVID-GRAM and found no associations. However, plasma levels of IL-8 significantly correlated with higher APACHE II scores, both across hospitalization (Fig. 5H) and on the first check (Fig. 5I). All other elevated cytokines were also assessed for a disease severity correlation, but none was found. The neutrophil-associated cytokine was highest in the plasma of the most critically ill patients as compared to healthy controls and those less severely ill (Fig. 5J), and IL-8 was significantly higher in those who succumbed to the disease (Fig. 5K). Plasma IL-8 also correlated with SOFA score and N:L ratio, which are additional indexes of clinical severity (Supplementary Figure 7).

Conclusions

There has been much interest and focus on the role of neutrophils in COVID-19 immunopathology. In aggregate, our study has placed functional assessments of circulating neutrophils isolated from critically ill COVID-19 patients in context of plasma cytokine levels, disease severity, and neutrophil counts. Cytokine storm has been characteristic of severe forms of COVID-19 [29] and we found that all cytokines tested were elevated in COVID-19 subjects across their hospitalizations, longitudinally. Each cytokine was also significantly elevated at the first check. Related to this, plasma IL-6, TNF-α and IL-8 levels at the time of hospitalization have shown to be strong and independent predictors of patient survival [30]. In particular, IL-8 seems to be significantly enhanced during the course of COVID-19 [30, 31], which was true in our cohort as well and fits with the pattern of elevated neutrophil counts also seen [18, 20, 26].

We found that IL-8 positively correlated with increased severity of disease, assessed by APACHE II scores. Other scores were also considered, including APACHE III and IV which are more complex scores with additional variables, but yielded no additional sensitivity to detecting changes in disease severity in this patient population. APACHE II is known to have consistent calibration compared to III and IV, and more research studies globally use the APACHE II. Published studies to date have associated the presence and activity of neutrophils with the severity of COVID-19 using different parameters such as comparing patients on room air versus patients requiring oxygen and mechanical ventilation [19], hospitalized non-ICU versus ICU patients [18], intubated versus non-intubated patients [31] and a classification of mild, moderate and severe based on different conditions (epidemiological history, fever, respiratory symptoms, frequency of CT image abnormalities of viral pneumonia, and positive RT-PCR for SARS-CoV-2 RNA)[17]. However, in terms of actual scores for mortality prediction, while a few studies have used SOFA scores [30, 31], which range from 0-4, our study, to the best of our knowledge, is the first to use the APACHE II score to estimate severity of COVID-19 in context of a variety of biomarkers, including neutrophils and IL-8. Unlike SOFA scores, APACHE II scores have a broad range from 0-71, which allows delineation of finer differences in severity of disease.

IL-8 is a powerful neutrophil chemoattractant and activating chemokine. Elevations in systemic IL-8 during COVID-19 [30, 31], in context of an association with IL-8 and COVID-19 severity and mortality found in our study, supports the hypothesis that neutrophils are a driver of disease pathogenesis in this disease. We found that COVID-19 patients have leukocytosis and neutrophilia, and both are associated with the severity of disease, which again supports the detrimental role of neutrophils in COVID-19. Across studies published to date, neutrophils have been found to be elevated in the circulation [18, 20, 26] and in the lungs [17, 20, 26, 32] of critically ill COVID-19 patients. Additionally,

the data presented here demonstrates that absolute neutrophils counts are associated with severity of disease.

By studying multiple cellular functions in parallel, we were able to confirm that the function of circulating neutrophils in patients suffering from COVID-19 are broadly affected, with increased production of NETs, increased reactive oxygen species production (oxidative burst), and enhanced phagocytosis. While the combination of these three assays, and the phagocytosis in particular, is new information, many studies have defined the changes in NETosis and oxidative burst in this disease state to date. Notably, Veras *et al* found an increase in NET production from neutrophils obtained from COVID-19 subjects as compared to neutrophils from healthy subjects [26] and Zuo *et al* showed that serum from COVID-19 can trigger control neutrophils to release NETs [19]. In alignment with those findings, several studies have found an increase in NET components in serum and plasma of COVID-19 patients as compared to healthy subjects, including cell-free DNA [18, 19], myeloperoxidase-DNA complexes (MPO-DNA) [18, 19, 26, 31], citrullinated histone H3 (citH3) [18, 19] and neutrophil elastase-DNA complexes [18]. However, when comparing severity of disease with any of these NET components, only two found significant associations. Leppkes *et al* showed that critically ill COVID-19 patients had significantly higher cell-free DNA versus non-ICU patients [18]; Middleton *et al* found that MPO-DNA was increased in severe COVID-19 and was associated with increased mortality [31]. Interestingly, our study found a lack of NETs in the lungs of some COVID-19 patients. Although surviving and non-surviving COVID-19 patients had elevated NETosis by circulating neutrophils, the major factor that correlated with mortality was serum IL-8. This suggests that NETosis within the lungs may not be driving pathogenesis.

Besides the presence of enhanced neutrophil and NET components in circulation, studies have found increased neutrophils and NETs in airways and lung tissue [18, 20, 26, 31, 32]. Veras *et al* found increased MPO-DNA in tracheal aspirates of COVID-19 as compared to healthy subjects [26] and Middleton *et al* found higher NET levels in tracheal aspirates relative to plasma in COVID-19 patients [31], which indicates relevance to airway inflammation in this disease. In lung parenchyma, concurring with our findings, significantly higher numbers of neutrophils have been found in the lungs of COVID-19 patients by conventional H&E staining [18, 20, 32]. Using immunohistochemistry (CD66b staining of neutrophils, and MPO, citH3 and neutrophil elastase staining of NETs), ours and other studies found increased NETs within lung tissue [18, 20, 26, 31, 32]. Presence of multiple, widely distributed NET-infiltrating areas have been identified in lungs of COVID-19 patients [20]. Our study shows neutrophils and NETs in bronchioles and alveoli, although the absence of NETs within lung parenchyma in three deceased COVID-19 patients suggests heterogeneity in the patterns of immunopathology in this disease. This matches the current concept of different subtypes of ARDS [33], and confirms that not all COVID-19 ARDS is the same [34]. Moreover, others have found that neutrophils and NETs associated with intermediate-sized pulmonary vessels were frequently clogged pulmonary blood vessels [18]. Furthermore, transcriptomic analyses have shown significant enrichment for annotated genes involved in neutrophil activation pathways, including NET formation [17, 32]. Interestingly, it has been also shown that SARS-CoV-2 itself can induce the release of NETs in healthy neutrophils [26].

neutrophils from COVID-19 patients also had increased oxidative burst and phagocytic activity. It has been proposed that tissue damage in COVID-19 may be mediated by neutrophil-induced oxidative stress [28], although no formal studies have addressed ROS production in lung tissue (Table 2)[28]. It was reassuring that phagocytosis was retained and even enhanced by these cells of host defense, as critically ill patients frequently develop secondary bacterial infections and the bactericidal

mechanism of phagocytosis clears bacterial pathogens without causing increased inflammation and tissue damage. Nevertheless, no correlation of our neutrophil functional assays (NETosis, oxidative burst and phagocytosis) with severity of disease was found. It is important to mention that this lack of correlation does not mean that these neutrophil-mediated activities are not playing a key detrimental role within the lung tissue, where different inflammatory signals are received.

One limitation of our longitudinal cohort study is that our control subjects were younger than our COVID-19 patients (42 vs 58, respectively; p=0.01; Table 1). This was primarily due to limitations in conducting human subjects research during the COVID-19 pandemic, such that we were limited to recruiting from the pool of employees allowed into the hospital. It is as yet unknown whether hypertension, cardiac disease, and ancestry impact neutrophil function. Diabetes and increasing age are associated with diminished neutrophil function [35-37]; thus, we do not believe that the increased neutrophil function in the COVID cohort is due to higher levels of these comorbidities. A second limitation is the size of our cohort. By utilizing a longitudinal design with neutrophils, and plasma and clinical data obtained at multiple points over time, we were able to increase the power of our study. A third limitation is the lack of African American/Black subjects, preventing us from defining neutrophil functional changes in this population. The fourth limitation was that we could not analyze samples from non-COVID critically ill ARDS controls due to the pandemic.

In aggregate, work to date provides strong and compelling evidence of the contribution of neutrophils in immunopathology in COVID-19 (summarized in Table 2). Published studies and the data presented here highlight the hyperactivated phenotype of neutrophils during COVID-19 and demonstrate that increased neutrophils and IL-8 in the circulation function as predictors for severe COVID-19. These data support the ongoing efforts to therapeutically target neutrophil-related mechanisms and propose neutrophil-centric biomarkers to utilize in the continuing battle against this devastating disease.

Notes

Author Contributions

Study design by LCA, AM, JAMS, AM, NC, ML and JD. Experiments performed by JAMS, AM, JO, JD, LCA, VP and LX. Data analysis by JMS, AM, LCA, SP, LM, JP, AP, JAMS, ALF, JO, TA, XS, ALF and GL. The manuscript was written by LCA, JAMS, SC, TA, AM and AP.

Acknowledgements

This work would not have been possible without numerous staff members at the University of California San Diego, Rady Childrens Hospital (RCH), the VA San Diego Healthcare System, and the Salk Institute. A special thank you to the IRB members at all institutions, to Victor Nizet, MD, for his valuable insight, and to Jennifer Foley, RN, the RCH PICU Research nurse who helped with consents and documentations.

Funding

This work was supported by the Department of Veterans Affairs VA Merit Award [1I01BX004767 to L.C.A.] and the National Institutes of Health National Heart, Lung, and Blood Institute [R01HL147326 to LCA].

Conflicts of Interests

The authors do not have an association that might pose a conflict of interest.

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Tables

Table 1. Demographics by Group Category

	Control n=15(%)	COVID-19 Patients n=19(%)	Overall n=34(%)	p value
Gender				
Female	5	7	12	
Male	10	12	22	0.8317
Total	15	19	34	
Race				
African American/Black	0	1	1	
Caucasian	11	17	28	0.1599
Asian	4	1	5	
Total	15	19	34	
Ethnicity				
Hispanic	4	12	16	
Non-Hispanic	7	5	12	0.0739
Total of Caucasian	11	17	28	
Age				
Mean (range)	41.93 (25-70)	57.68 (17-88)	50.74 (17-88)	0.0097

Demographic data was taken from healthy controls as well as SARS-CoV-2 positive patients (COVID-19). Chi-squared test was used to analyze the variety of subset groups present in Gender, Race and Ethnicity. There was no significant difference between our control group and our COVID-19 patients' group. An unpaired T-Test was used to compare Controls with COVID-19 patients regarding age, with a statistically significant difference existing between the two groups with a p-value of 0.0097.

Table 2. Summary of published data of circulating and lung neutrophils in COVID-19

Reference	Increased Neutrophil Number		Increased IL-8		Increased Neutrophil Activation							Association with COVID-19 severity
					NETosis		Oxidative burst		Phagocytosis	Activation markers		
	Blood	Lung	Blood	Lung	Blood	Lung	Blood	Lung		Blood	Lung	
Zuo *et al* Jun, 2020 [19]					X							X
Wang *et al* Aug, 2020 [17]		X									X	
Leppkes *et al* Aug, 2020 [18]	X				X	X	X			X		
Del Valle *et al* Oct, 2020 [30]			X									
Radermecker *et al* Sep, 2020 [20]	X	X				X						
Middleton *et al* Sep, 2020 [31]	X		X		X	X						X
Busch *et al* Nov, 2020 [35]					X							X
Wu *et al* Nov, 2020 [32]		X				X					X	X
Veras *et al* Dec, 2020 [26]	X	X			X	X						
Zuo *et al* Feb, 2021 [36]					X							
This Work	X	X	X		X	X	X		X			X

Figure Legends

Figure 1. **Pro-inflammatory cytokines are increased in the plasma of critically ill COVID-19 patients.** Consistent with prior reports, cytokines in the circulation of COVID-19 patients were elevated at the first timepoint assessed (**A**) and remained elevated persistently across their hospital courses (**B**). At the time of enrollment in the study, most typically at the time of ICU admission, all cytokines were significantly elevated relative to controls at this first timepoint (**A**). IP-10, IL-6, IL-8, GM-CSF, IL-1β, IL-10 and TNFα were elevated in the plasma of COVID-19 patients (n = 18) when averaged across all timepoints tested, relative to healthy controls (n = 12; **B**). Error bars represent standard error of the mean. **p < 0.01, ***p < 0.001, ****p < 0.0001.

Figure 2. **Increased NETosis by circulating neutrophils from COVID-19 patients.** Functional NETosis assays on neutrophils isolated from the blood of critically ill COVID-19 patients on multiple days across their hospitalization (n = 11 subjects; total timepoints represented = 33) and healthy controls over time (n = 14; total timepoints represented = 42) demonstrate that neutrophils from COVID-19 patients have higher NETosis at steady state and also upon stimulation with PMA (both 25 nM and 250 nM), assessed by A) quantification of dsDNA by fluorescence, and B) fluorescence microscopy of neutrophils stained for Myeloperoxidase (green) and DNA (blue). Error bars represent standard error of the mean. **p < 0.01, ***p < 0.001, ****p < 0.0001.

Figure 3. **Neutrophils and NETs throughout airways and parenchyma of COVID-19 lung tissue. A**) H&E staining demonstrates diffuse inflammation and fibrosis with the presence of neutrophils throughout alveoli and bronchioles. The majority of neutrophils were found within terminal bronchioles, with focal areas of neutrophils within the alveolar lung parenchyma. 1. Terminal bronchiole and alveoli with neutrophils throughout. 2. Neutrophils within fibrotic lung parenchyma with bronchiolar metaplasia. 3. Neutrophils present throughout areas of organizing pneumonia. **B**) Immunofluorescence staining of nuclei with DAPI (blue), CD66b (magenta) and H3-cit (green) demonstrate the presence of neutrophils and NETs within the lung parenchyma. Bronchioles, alveoli and pulmonary arteries are indicated with colored arrows and squares. Three areas of NETs are highlighted and shown at 20x magnification, with H&E images for reference.

Figure 4. **Circulating neutrophils from COVID-19 patients produce more reactive oxygen species and have increased phagocytosis than those from healthy subjects.** Reactive oxygen species (ROS) production and phagocytosis by neutrophils isolated from the blood of COVID-19 patients and healthy controls were assessed by relative fluorescence. ROS production was significantly higher at A) steady state, B) 2.5 nM PMA, C) 25 nM PMA and D) 250 nM PMA. E) Phagocytosis was significantly higher in COVID-19 neutrophils relative to neutrophils from healthy controls. Data was analyzed with mixed-effects models with Geisser-Greenhouse correction, giving $p < 0.0001$ for all analyses. Error bars represent 95% CI. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Figure 5. **Increased NETosis is positively correlated with leukocytosis and neutrophilia, and neutrophilia and IL-8 are positively correlated with COVID-19 severity.** Levels of NETosis were correlated to white blood cell counts (WBC) and absolute neutrophil counts (ANC) from COVID-19 patients. A) Scatter plot demonstrated positive correlation of WBC and NETosis ($r^2 = 0.48$, p

<0.0001). B) Patients with leukocytosis produced significantly more NETs than patients with WBC levels under $11x10^3/mm^3$. C) Scatter plot showed positive correlation of ANC and NETosis ($r^2 = 0.53$, p <0.0001). D) Patients with neutrophilia produced significantly more NETs than patients with ANC levels under $8x10^3/mm^3$. E) Scatter plot showing that severity of COVID-19 positively correlated with neutrophilia ($r^2 = 0.24$, p = 0.005). F) Decreasing lymphocyte counts were found as COVID-19 severity rose. G) NETosis was not found to correlate with severity of disease ($r^2 = 0.11$, p = 0.069). NETosis was also not significantly different in COVID-19 subjects that were diagnosed with a DVT (deep vein thrombosis) or PE (pulmonary embolism) during their admission for treatment for COVID-19. (Supplementary Figure 8). H) Severity of COVID-19 correlated with IL-8 levels quantified on multiple days across hospitalization across patients, from timepoint 0 to Day 11 ($r^2 = 0.403$, p = 0.006). I) IL-8 levels at the first time point, on entry to the study, also correlated with disease severity ($r^2 = 0.449$, p = 0.034). J) Plasma IL-8 levels were highest in COVID-19 patients with the greatest disease severity (APACHE II >15). K) Patients who succumbed to their disease had greater IL-8 plasma levels than those who survived. Error bars represent 95% CI. ****p < 0.0001.

Figures

Figure 1.

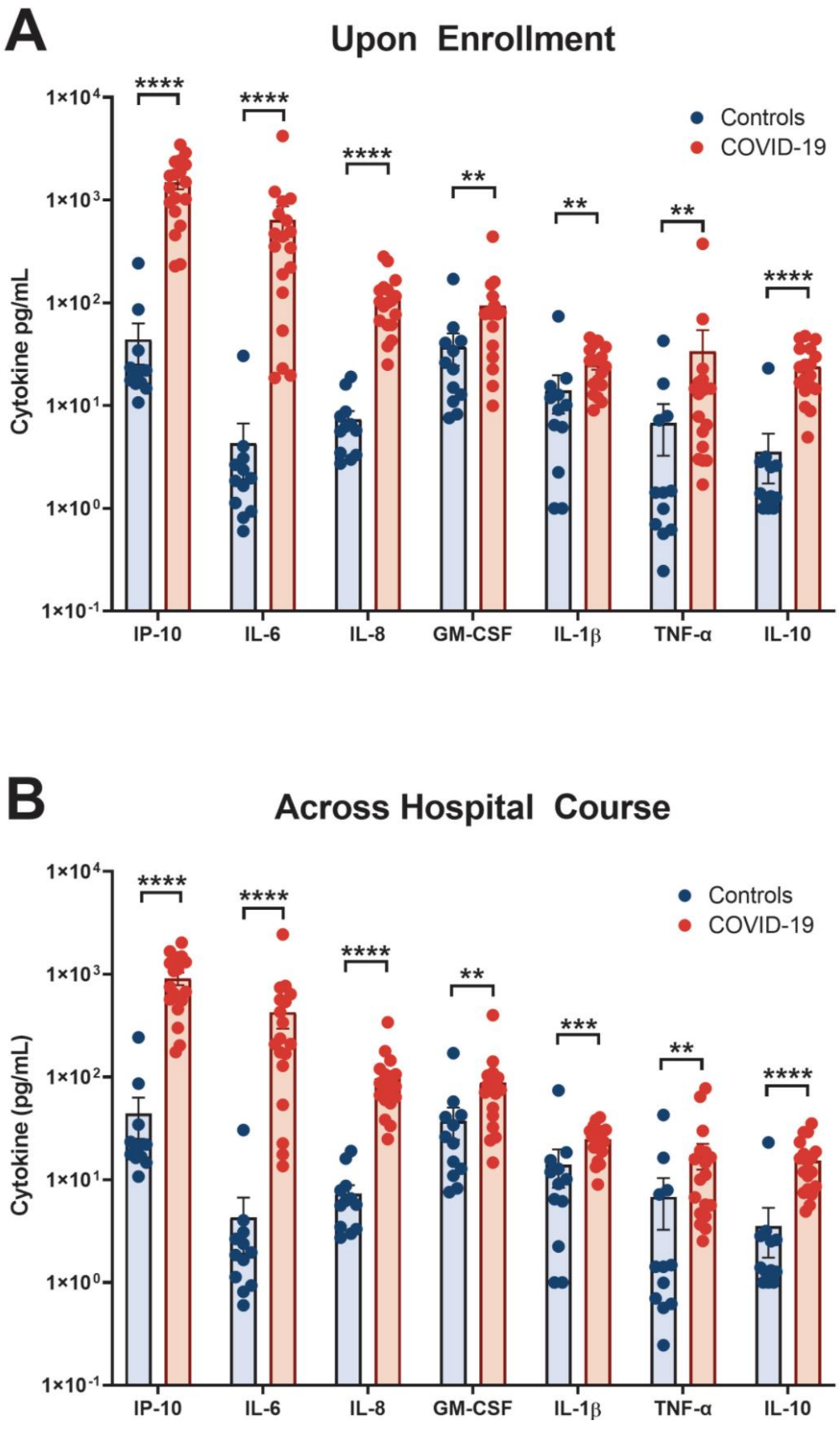


Figure 2.

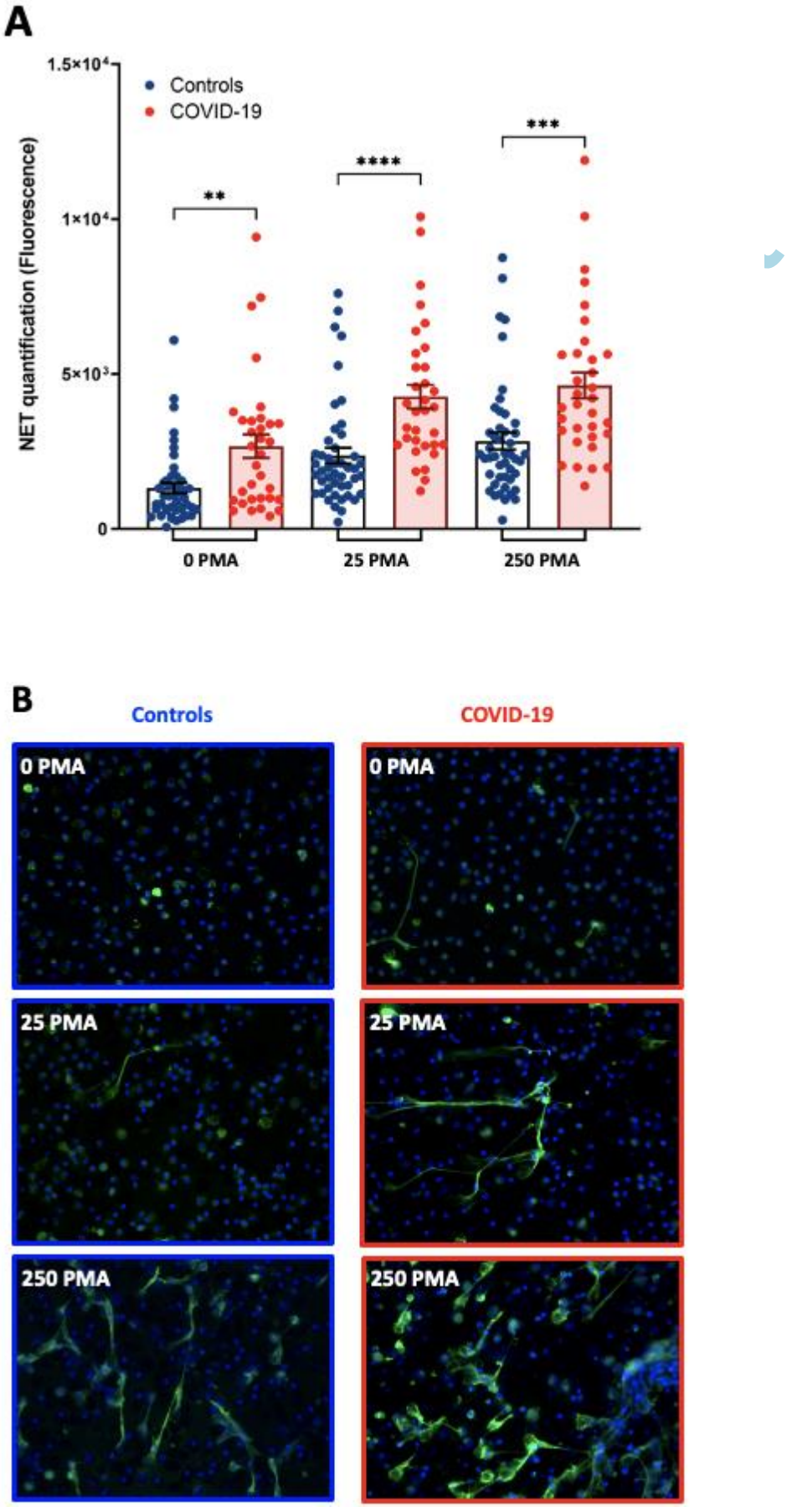
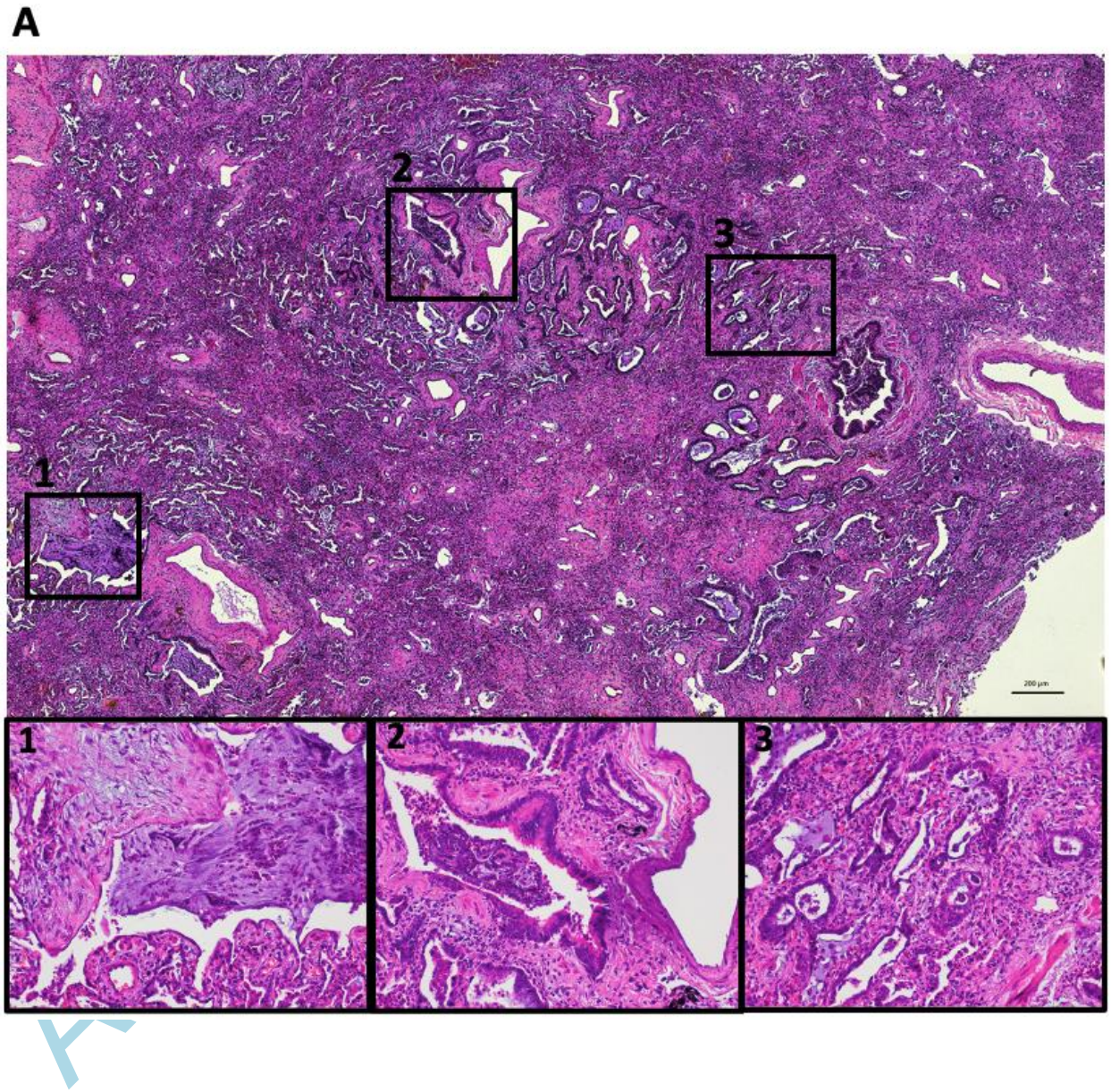

A
1.5×10⁴
1×10⁴
5×10³
0
NET quantification (Fluorescence)
Controls
COVID-19
**
****
***
0 PMA
25 PMA
250 PMA
B
Controls
COVID-19
0 PMA
0 PMA
25 PMA
25 PMA
250 PMA
250 PMA

Figure 3.



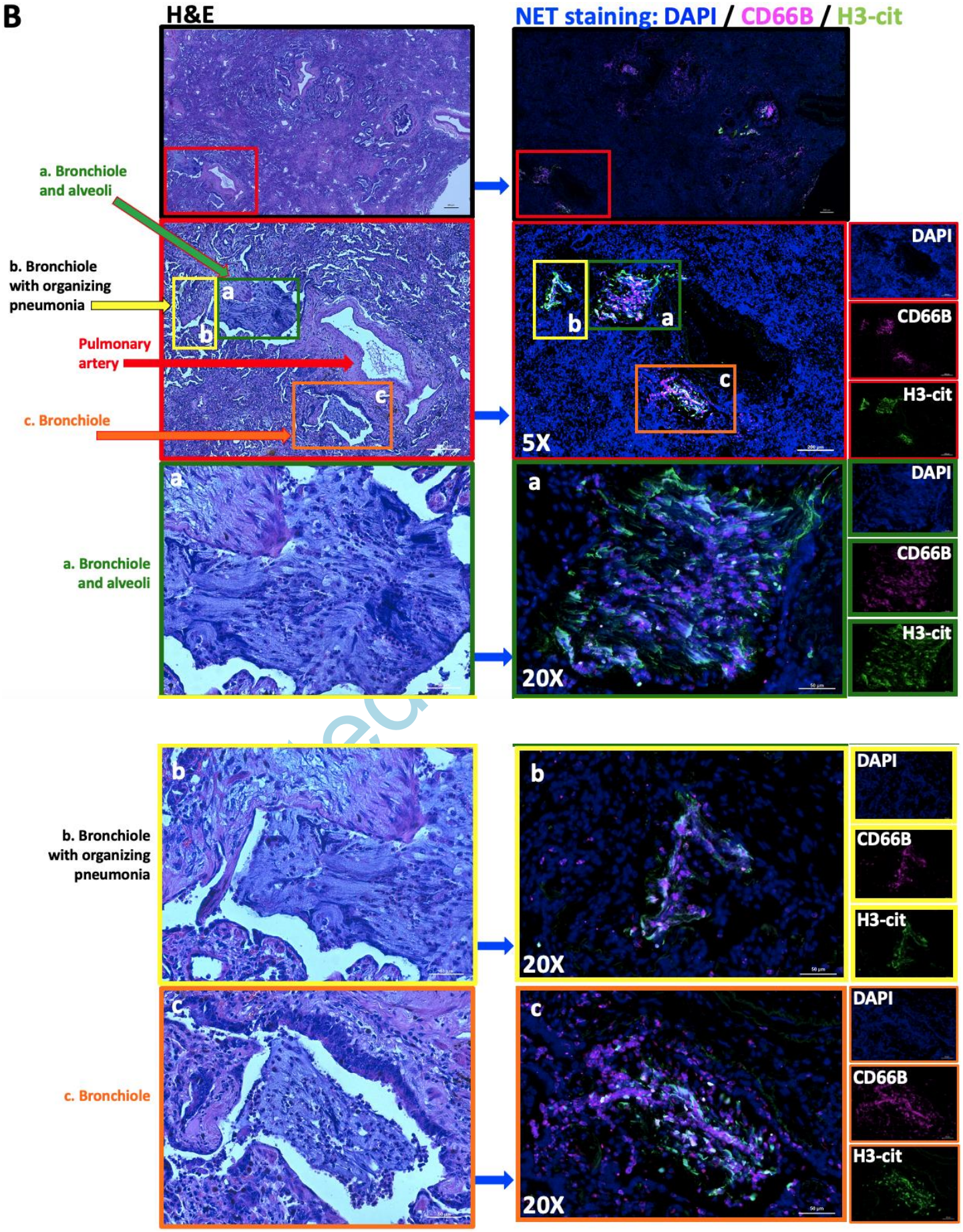

B
H&E
NET staining: DAPI / CD66B / H3-cit
a. Bronchiole and alveoli
b. Bronchiole with organizing pneumonia
Pulmonary artery
c. Bronchiole
a
b
c
e
5X
20X
DAPI
CD66B
H3-cit
a. Bronchiole and alveoli
b. Bronchiole with organizing pneumonia
c. Bronchiole

Figure 4.

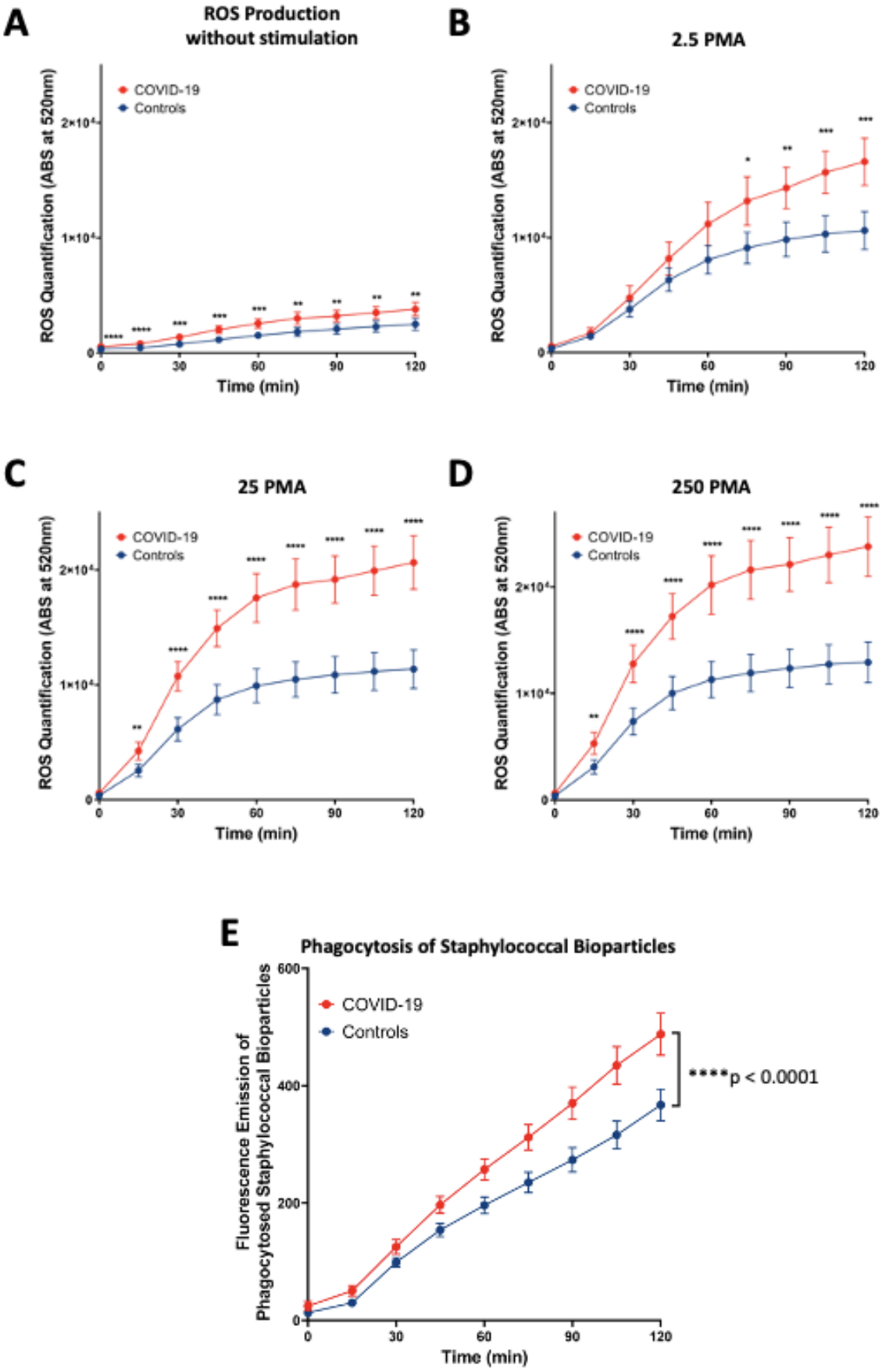
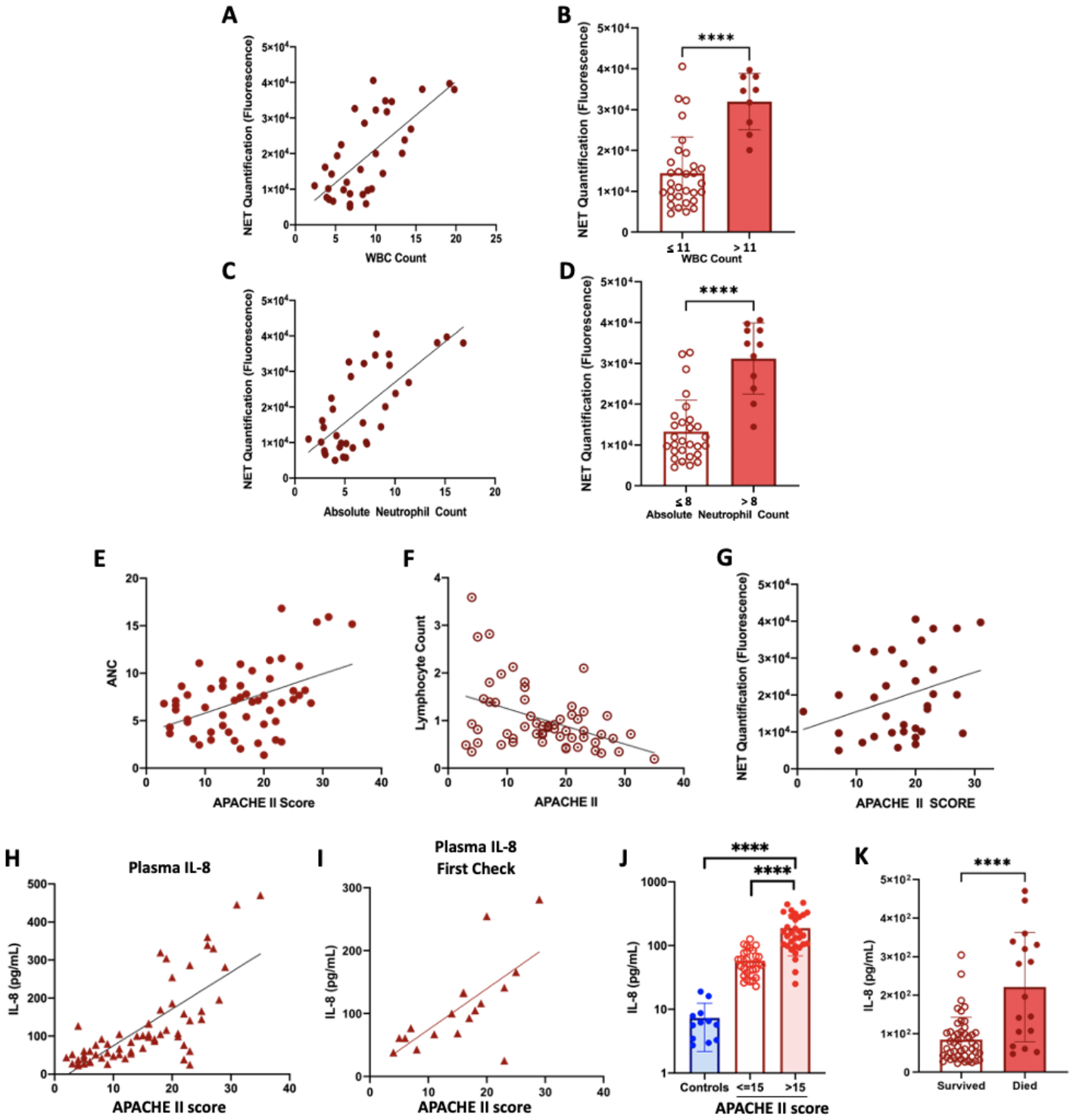

A
ROS Production without stimulation
COVID-19
Controls
ROS Quantification (ABS at 520nm)
Time (min)
B
2.5 PMA
C
25 PMA
D
250 PMA
E
Phagocytosis of Staphylococcal Bioparticles
Fluorescence Emission of Phagocytosed Staphylococcal Bioparticles
****p < 0.0001

Figure 5.


A
NET Quantification (Fluorescence)
WBC Count
B
NET Quantification (Fluorescence)
****
≤ 11
> 11
WBC Count
C
NET Quantification (Fluorescence)
Absolute Neutrophil Count
D
NET Quantification (Fluorescence)
****
≤ 8
> 8
Absolute Neutrophil Count
E
ANC
APACHE II Score
F
Lymphocyte Count
APACHE II
G
NET Quantification (Fluorescence)
APACHE II SCORE
H
Plasma IL-8
IL-8 (pg/mL)
APACHE II score
I
Plasma IL-8
First Check
IL-8 (pg/mL)
APACHE II score
J
****
****
IL-8 (pg/mL)
Controls
<=15
>15
APACHE II score
K
****
IL-8 (pg/mL)
Survived
Died