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Decreased neutrophil-mediated bacterial killing in COVID-19 patients

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Abstract

The coronavirus disease COVID-19 was first described in December 2019. The peripheral blood of COVID-19 patients have increased numbers of neutrophils which are important in controlling the bacterial infections observed in COVID-19. We sought to evaluate the cytotoxic capacity of neutrophils in COVID-19 patients. 34 confirmed COVID-19 patients (29 severe, five mild disease), and nine healthy controls were recruited from the Masih Daneshvari Hospital (Tehran, Iran) from March to May 2020. Polymorphonuclear (PMN) cells were isolated from whole blood and incubated with green fluorescent protein (GFP)-labelled methicillin-resistant *Staphylococcus aureus (SA)* and *Pseudomonas aeruginosa (PA)*. Bacterial growth was determined by measuring the florescence of co-cultures of bacteria and neutrophils and reported as the lag time before exponential growth. The number of viable bacteria was determined after 70 hours as colony-forming units (CFU). The immunophenotype of tested cells was evaluated by flow cytometry. Isolated neutrophils have higher surface expression of CD16 and CD62L with negative markers for PMN-MDSC. Bacterial growth in the presence of SA (22 ± 0.9 versus 9.2 ± 0.5 h, $P < .01$) and PA (12.4 ± 0.6 versus 4.5 ± 0.22, $P < .01$) was significantly reduced in COVID-19 patients. After 70 h incubation of PMN with bacteria (SA and PA), CFUs were significant increased in COVID-19 patients SA ($2.6 \pm 0.09 \times 10^8$ CFU/mL-severe patients and $1.4 \pm 0.06 \times 10^8$ CFU/mL-mild patients, $P < .001$) and PA ($2.2 \pm 0.09 \times 10^9$ CFU/mL-severe patients and $1.6 \pm 0.03 \times 10^9$ CFU/mL-mild patients, $P < .001$). Gentamycin proliferation assays confirmed the presence of intracellular bacteria. Reduced bacterial killing by neutrophils from COVID-19 patients may be responsible for the high bacterial yield seen in these patients.

1 | INTRODUCTION

A new coronavirus disease was initially reported in December 2019 (COVID-19), which resulted in a severe acute respiratory disease.[1] COVID-19 caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which is the seventh member of the coronavirus family to infect humans and has a 76.5% amino acid homology with SARS-CoV with more than 85% of its genome sequence being similar to several bat coronaviruses.[1,2] Coronaviruses are RNA viruses with an extensive range of natural hosts.[3] Based on sequence analysis, SARS-CoV-2 may have originated from bats or living animals from the Wuhan seafood market or its surrounding areas.[4] The development of human-to-human transmission was the final

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step in the virus cycle that induced the epidemic and finally the pandemic form of disease.[5] COVID-19 disease spreads through the respiratory tract with lymphopenia and cytokine storms. It mainly happened in severe disease and showed the existence of immunological dysregulation in severe disease.[6]

Activation or suppression of the host immune response is often predictive of disease severity.[7] Neutrophils have significant role in the host defence against micro-organisms and are the important effector cells to combat and killing of pathogens by oxidative burst and phagocytosis.[8,9] Neutrophil granules contain many mediators including antimicrobial peptides, proteolytic enzymes and of reactive oxygen species (ROS) generated via the action of NADPH oxidase.[10] There is a significant increase in circulating neutrophils in patients with severe COVID-19 disease.[11]

Myeloid-derived suppressor cells (MDSC) are a various population of immature myeloid cells with the strong immune-suppressive tasks. MDSC are myeloid cells that are developmentally immature and in different stages of myelopoiesis.[12] MDSCs consist of a mixture of monocytic and granulocytic cells, and based on their morphology, density and cell surface markers are classified into two important groups: polymorphonuclear MDSC (PMN-MDSC) or granulocytic MDSC (G-MDSC) and monocytic MDSC (M-MDSC). G-MDSC has a morphology similar to granulocytes, and M-MDSC has a morphology similar to monocytes.[13-15] Several studies in mice and humans have shown most PMN-MDSCs have a morphology similar to immature granulocytes.[16] An additional group, which lacks macrophage and granulocyte markers, is called early-stage MDSC (e-MDSC) that gather in a few disease settings.[17] A study on COVID-19 patients has illustrated increased production of MDSCs in the PBMC of COVID-19 patients.[18] The development of MDSCs along with a high level of inflammatory mediators can reduce the cytotoxic potential of the immune system.[19]

Since bacterial co-infection is observed in COVID-19 patients,[1,19] we hypothesized that neutrophils from these patients may have functional defects with respect to bacterial killing. Thus, we aimed to compare the ability of peripheral blood PMN obtained from healthy control subjects and COVID-19 patients to evoke bacterial killing ex vivo over time.

2 | MATERIALS AND METHODS

2.1 | Patients

34 COVID-19 patients with respiratory system involvement as detected by CT and chest X-ray and 9 healthy control subjects with no indication of lung involvement and a negative polymerase chain reaction (PCR) test were enrolled between March and May 2020 at the Masih Daneshvari Hospital Tehran, Iran. All COVID-19-infected patients were diagnosed based on the World Health Organization (WHO) interim guidance. Patients were confirmed positive for SARS-CoV-2 nucleic acid in the respiratory samples using real-time RT-PCR or serum specific antibodies and chest imaging. Demographic data are presented in Table 1

2.2 | Polymorphonuclear (PMN) cell isolation from COVID-19 patients and healthy subjects

PMN cells were isolated from whole blood using heparin as described earlier.[20] Briefly, phosphate-buffered saline (PBS) was used to dilute blood and the diluted blood put over Ficol-Paque (Sigma-Aldrich- Merck Company) in sterile conical tube and centrifuged at $760 \times g$, for 20 minutes in room temperature. The supernatant was removed, and the pellet re-suspended in lysis buffer (150 mM NH_4CL, 10 mM KHCO3, 0.1 mM EDTA, pH 7.4), re-pelleted and washed with PBS before centrifugation at $350 \times g$, for 5 minutes, at 4℃. Cell pellets were finally re-suspended in HEPESIII buffer (20 mM HEPES) supplemented with 0.5% w/v BSA, 1 mM $CaCl_2$ and 5 mM glucose and counted.

2.3 | Preparation of bacterial strains

The *Methicillin Resistance Staphylococcus Aureus* (SA) strain MW2 and *Pseudomonas Aeruginosa* (PA) (gifted by Prof. L. Koenderman, Utrecht University) were used. These bacteria were previously transformed to express green fluorescent protein (GFP).[21] *SA* was grown in Luria-Bertani (LB) (Sigma-Aldrich) broth with chloramphenicol (10 μg/mL) (Sigma-Aldrich) overnight at 37℃ and $60 \times g$, until the OD600 nm reached 0.5. The suspension was centrifuged and the pellet re-suspended in HEPES buffer to give an $OD_{600nm} = 0.5$. The bacterial suspension was aliquoted and frozen at −80℃. PA was cultured on LB agar with kanamycin (15 μg/mL) and ampicillin (10 μg/mL) overnight at 37℃. The next day, one bacterial colony was cultured in LB broth and grown to an $OD_{600nm} = 0.5$. Bacterial Pellets were washed and re-suspended in HEPES III and diluted again to $OD_{600nm} = 0.5$. GFP-labelled bacteria were detected using flow cytometry (BD FACSCalibur) and florescence evaluated in the FL1 channel (530 nm).

2.4 | Immunophenotyping by flow cytometry

Isolated PMN were stained with FITC-conjugated CD62L (Clone DREG-56) and PE-conjugated CD16 (Clone CB16) for 30 minutes. For characterization of PMN-MDSC cells,

TABLE 1 Demographic information of all COVID-19 participants (PCR +ve and −ve)

	Gender	AGE	PCR	IL-6	CRP	ESR	LDH	Fer	Lymph	IgG	IgM
Patient 1	F	21	+	19.9	-	21	943	118	4	-	-
Patient 2	F	61	+	15.5	29	78	1034	1327	8.7	13.5	10.2
Patient 3	F	58	+	8	19	69	981	288	25	14.3	1.74
Patient 4	F	69	+	14	25	25	977	-	6.6	-	-
Patient 5	F	43	+	8.1	65	95	531	320	-	-	-
Patient 6	M	64	+	12	14	36	384	-	-	-	-
Patient 7	M	71	+	7.9	18	61	245	25	4.3	-	-
Patient 8	M	81	+	28.8	39	104	450	>2000	7	-	-
Patient 9	F	60	+	2.5	37	36	521	285	18	14.83	7.96
Patient 10	F	56	+	9.4	107	35	578		24	14.1	10.1
Patient 11	F	44	+	2.8	62	50	832	466	8	-	-
Patient 12	M	61	+	9.4	-	46	-	347	-	-	-
Patient 13	M	33	+	6.2	107	25	831	1463	18	-	-
Patient 14	F	59	+	13.1	76	45	580	1463	8	-	-
Patient 15	F	28	+	12	47	78	1034	234	12	-	-
Patient 16	M	69	+	13.9	14	36	974	-	58	0.31	1.39
Patient 17	M	71	+	7.5	53	28	521	548	4	13.53	3.38
Patient 18	F	23	+	7.3	-	65	439	132	15	-	-
Patient 19	M	44	+	2.8	23	-	793	1572	-	-	-
Patient 20	M	46	+	13.6	61	36	599	1613	23	-	-
Patient 21	M	63	+	11.7	-	56	569	1888	5.1	-	-
Patient 22	M	66	+	-	56	15	1119	>2000	20	-	-
Patient 23	M	63	+	11.2	38	-	438	827	15	16.22	1.87
Patient 24	M	72	+	-	45	49	713	820	-	-	-
Patient 25	M	39	+	-	41	41	327	437	21	-	-
Patient 26	M	78	+	-	45	-	489	-	-	10.1	0.5
Patient 27	F	39	-	-	-	-	-	-	-	581	95
Patient 28	M	83	-	-	28	31	175	-	11.1	0.47	0.25
Patient 29	M	-	-	-	24	30	1835	-	14	0.27	0.63
Patient 30	M	75	-	-	-	32	-	-	12	-	-
Patient 31	M	81	-	7.8	34	31	429	-	19.7	0.86	0.19
Patient 32	M	81	-	19.5	-	32	245	276	15.6	0.49	0.6
Patient 33	M	59	-	8.1	11	30	363	131	29.9	-	-
Patient 34	M	37	-	-	30	-	623	-	-	8.10	17.8
Control 1	F	34	-	3	12	6	321	26	34	-	-
Control 2	M	35	-	4	9	2	222	44	32	-	-
Control 3	M	37	-	5	8.9	7	311	23	27	-	-
Control 4	F	37	-	2	6.8	1	289	56	21	-	-
Control 5	F	38	-	3	13	11	267	76	38	-	-
Control 6	M	36	-	4.2	7.3	4	378	55	25	-	-
Control 7	M	40	-	3.1	8	7	376	66	26	-	-
Control 8	F	38	-	1.1	11	8	289	47	31	-	-
Control 9	M	29	-	1	19	4	311	58	36	-	-

the following conjugated Abs were used: CD11b-APC (Clone ICRF44), HLA-DR-PE (clone LN3), CD14-PerCP-Cy^{TM} (Clone MφP-9) and CD15-FITC (Clone MMA) all from BD Biosciences (San jose, CA95131, USA). PMN-MDSCs were HLA-DR$^-$, CD11b$^+$, CD14$^-$ and CD15$^+$ whilst M-MDSCs were HLA-DR$^-$, CD11b$^+$, CD14$^+$ and CD15$^-$. For all staining, cells (5×10^6 cell/mL) were incubated for 30 minutes at 4℃ with Abs before being washed with FACS buffer and 10 000 events analysed by flow cytometry (BD FACSCalibur). Flow cytometry results were analysed using FlowJo™ software (7.6 version) and presented as MFI.

2.5 | Neutrophil lag time and counting of colony-forming units (CFU)

Measurements of GFP fluorescence intensity of bacteria and cells were determined using a FLUOstar Optima (BMG Labtech) as described previously.[22] Isolated PMN were co-cultured with PA and SA in 96-well imaging plates (black, clear bottom; Corning Life Sciences, Tewskbury). Briefly, 5×10^6 cell/mL in human pooled serum (Sigma-Aldrich) with HEPES III buffer was incubated with 2×10^6CFU/mL of bacteria at a final level of 40% serum (vol/vol). The plate was placed in the FLUOstar at 37℃ with constant shaking (150 rpm) for 72 hours as previously described,[23] and GFP fluorescence was measured every 20 minutes (at excitation 485 nm/emission 520 nm). After 72 hours, wells were processed for CFU analysis. Cells were removed from plates and 20 μl of supernatant diluted in serum and cultured on UTI-Agar plates (HiCrome™-HIMEDIA) overnight at 37℃.

Gentamicin (Caspian Tamin Pharmaceutical Co. Iran, 1, 3, 5, 10 and 20 mg/L) was incubated with PA or SA in the presence or absence of cells to inhibit bacterial growth for up to 48 hours.[24] Bacterial growth as assessed by GFP-RFU was calculated using Graph Pad Prism 8 software (Graph Pad Software Inc, San Diego).

2.6 | Cell viability analysis

To determine PMN viability, PMN (5×10^5 cell/well) were cultured with bacteria in a FLUOstar Optima for 24 and 60 hours and then stained with Annexin V-FITC and PI (Invitrogen™ 88-8005-72) (UK) based on the manufacturer's instructions (eBioscience™ Annexin V Apoptosis Detection Kit FITC). 10,000 events were analysed by flow cytometry (BD FACSCalibur), and the result data were calculated based on FL1 and FL2 (FlowJo™ software). In addition, we performed an analysis of PMN cell counts 24 h after seeding in RPMI by counting under light microscopy of live cells (5×10^6) from both control and COVID-19 patients.

2.7 | Statistical analysis

All analyses were performed in triplicate, and all experiments were repeated up to five times. Results are presented as mean ± SEM. Statistical tests (Kruskal-Wallis) were analysed using GraphPad Prism Software (Version 8). Results were considered statistically significant when *P < .05, **P < .01 and ***P < .001.

3 | RESULTS

3.1 | Determination of Immunophenotyping of isolated cells

Neutrophils isolated from peripheral blood of both healthy and COVID-19 subjects were positively stained for CD62L and CD16 (Figure 1A). Subsequently, G-MDSCs were gated according to their staining for HLA-DR, CD11b, CD14 and CD15 (Figure 1B) as previously described.[25] There were more G-MDSCs present in COVID-19 patients than healthy control isolated cells. (P < .05) (Figure 1C).

3.2 | Effect of neutrophils on bacterial growth lag time

Isolated cells were incubated with GFP-SA and GFP-PA and the lag time before the bacterial population starts exponential growth in a new environment were calculated as described previously.[26] PMN from COVID-19 patients were significantly less efficient reducing the lag time of GFP-SA (22 ± 0.9 versus 9.2 ± 0.5 hours, P < .01) (Figure 2A,B) and GFP-PA (12.4 ± 0.6 versus 4.5 ± 0.22 hours, P < .01) (Figure 2C,D) than cells from healthy control subjects. The lag time as a deviation from the growth curve observed in the no-PMN samples. (Figure 2).

3.3 | Determination of CFU in treated cells by bacteria

Neutrophils from COVID-19 patients and healthy control subjects were cultured for 72 h with GFP-SA and GFP-PA and CFU assessed as described in the methods. There was an increase in GFP-SA growth ($2.6 \pm 0.09 \times 10^8$ CFU/mL-severe patients) and ($1.4 \pm 0.06 \times 10^8$ CFU/mL-moderate patients) compared with neutrophils from healthy control subjects ($0.8 \pm 0.04 \times 10^8$ CFU/mL) (Figure 3A). This indicated that a greater number of GFP-SA were alive in the cultures with cells from severe COVID-19 patients. In the absence of any neutrophils, GFP-SA numbers reached $2.8 \pm 0.03 \times 10^8$ CFU/mL (Figure 3A).

FIGURE 1 (A) Representative flow cytometry plots of CD16(bright) and CD62L(dim) expression on isolated neutrophils (PMNs) from a healthy subject and a COVID-19 patient. (B) Representative flow cytometry plots of HLA-DR⁻, CD11b⁺, and the CD14⁻ and CD15⁺ PMN-myeloid-derived suppressor cells (PMN-MDSCs) and HLA-DR⁻, CD11b⁺, and CD14⁺ and CD15⁻ monocytic MDSCs (M-MDSCs) in a healthy subject and a COVID-19 patient. (C) Graphical representation of the percentage of PMN, G-MDSCs and M-MDSCs in the peripheral blood of healthy subjects (n = 6) and COVID-19 patients (n = 9). Data are presented as a dot plot per patient together with a median and 95% confidence intervals. *P < .05
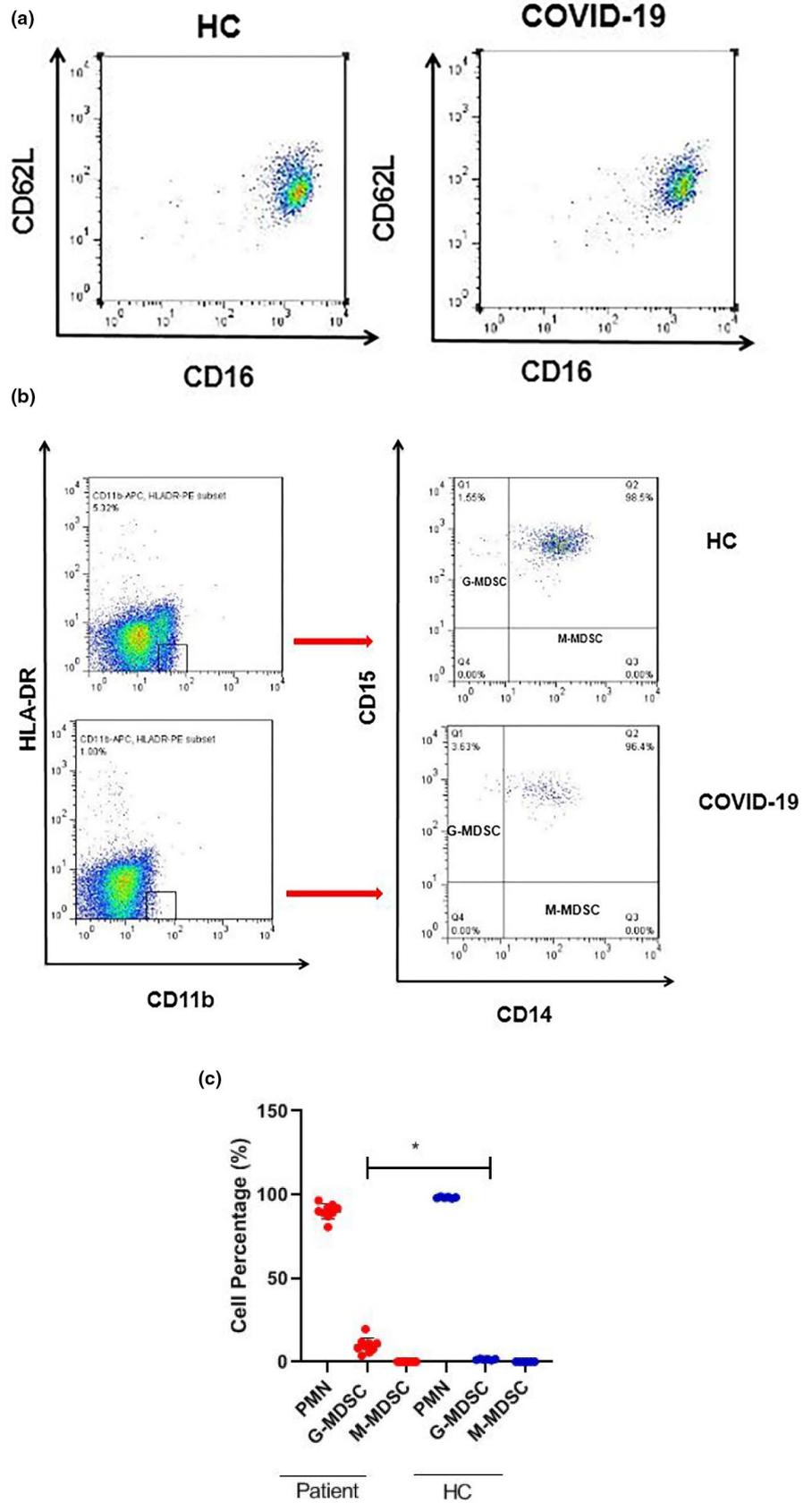
Similarly, GFP-PA growth with neutrophils from healthy subjects (1.0 ± 0.06 × 10⁹ CFU/mL) was reduced compared to growth with cells from severe COVID-19 patients (2.2 ± 0.09 × 10⁹ CFU/mL) and cells from mild COVID-19 patients (1.6 ± 0.03 × 10⁹ CFU/mL) (Figure 3B). The growth of PA seen with severe COVID-19 patient cells was
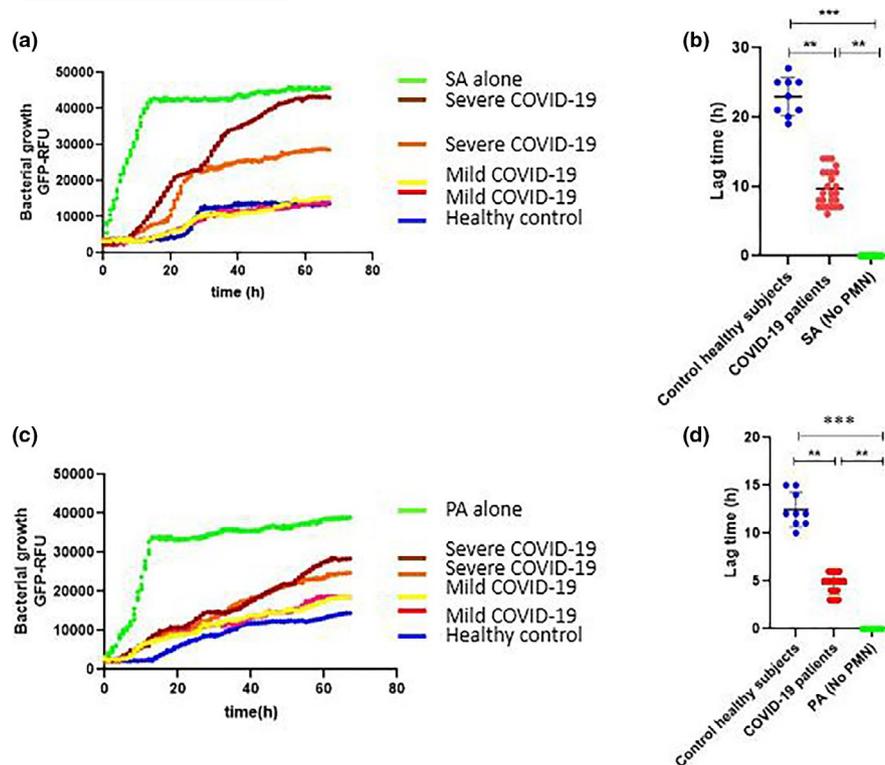
FIGURE 2 (A) Representative growth curves of green fluorescent protein (GFP)-labelled methicillin-resistant Staphylococcus aureus (SA) alone and in culture with polymorphonuclear (PMN) cells from 2 mild, 2 severe COVID-19 patients and from 1 healthy control subject. The bacterial GFP-signal was recorded every 20 minutes by FLOUstar Optima. (B) Graphical analysis of lag time for SA growth in the presence of PMN from COVID-19 patients and healthy subjects. Results are presented as individual patient results with bars as mean ±SEM. (C) Representative growth curves of GFP-labelled Pseudomonas aeruginosa (PA) alone and in culture with PMN from mild and severe COVID-19 patients and a healthy control subject (control). (D) Graphical analysis of lag time for PA growth in the presence of PMN from COVID-19 patients and healthy subjects. Results are presented as individual patient results with bars as mean ± SEM. **$P < .01$, ***$P < .001$

similar to that seen in the absence of any cells ($2.4 \pm 0.09 \times 10^9$ CFU/mL) (Figure 3B). Dead bacteria did not emit detectable light by the FLUOstar.

We evaluated whether this was due, at least in part, to bacterial uptake by neutrophils or by incubation with gentamicin. Gentamicin induced a concentration-dependent suppression of SA and PA bacterial growth in the absence of cells with PA being more sensitive than SA (Figure 4).

3.4 | Cell viability of PMN over time

Annexin-V and PI staining was used to assess cell death in neutrophils incubated over time (Figure 5). Cell death increased over time in both healthy subjects and COVID-19 patients (Figure 5). This was significantly greater in cells from COVID-19 patients compared with healthy controls after 60hrs. Cell counting 24 hours after seeding at a density of 5×10^6 cells demonstrated that 2×10^6 cells from COVID-19 subjects remained whilst only 6×10^5 cells from healthy control subjects remained.

4 | DISCUSSION

This pilot study demonstrated a decreased bacterial killing capacity of neutrophils isolated from the systemic circulation of COVID-19 patients in comparison with control healthy subjects against both Gram-positive and Gram-negative bacteria. Importantly, both SA and PA infection are associated with patients whose immune systems are suppressed.[27,28] Neutrophil-mediated killing is important in reducing bacterial growth,[21] and this is supported by our limited data examining SA and PA CFUs. A decreased lag time until bacterial outgrowth occurs also reflects a reduced antibacterial capacity of the neutrophils.[22] Overall, the reduced neutrophil killing of PA and SA in COVID-19 patients compared to healthy subjects enables greater bacterial colonization. Although there was a small significant difference in PMN survival between cells from healthy subjects and COVID-19 patients, it is unlikely that this accounts for the differences in bacterial growth reported here.

Neutrophil phagocytosis is most efficient in the presence of opsonins such as specific immunoglobulin (Ig) G and

FIGURE 3 Growth of green fluorescent protein (GFP)-labelled methicillin-resistant Staphylococcus aureus (SA)(A) and Pseudomonas aeruginosa (PA)(B) in the presence or absence of polymorphonuclear cells (PMN) isolated from the peripheral blood of COVID-19 patients (6 severe and 3 moderate out of 34 COVID-19 patients) and four healthy subjects. Results are presented as colony-forming units (CFUs/mL) for each individual patient with bars as mean ± SEM. **$P < .01$, ***$P < .001$, ns—not significant
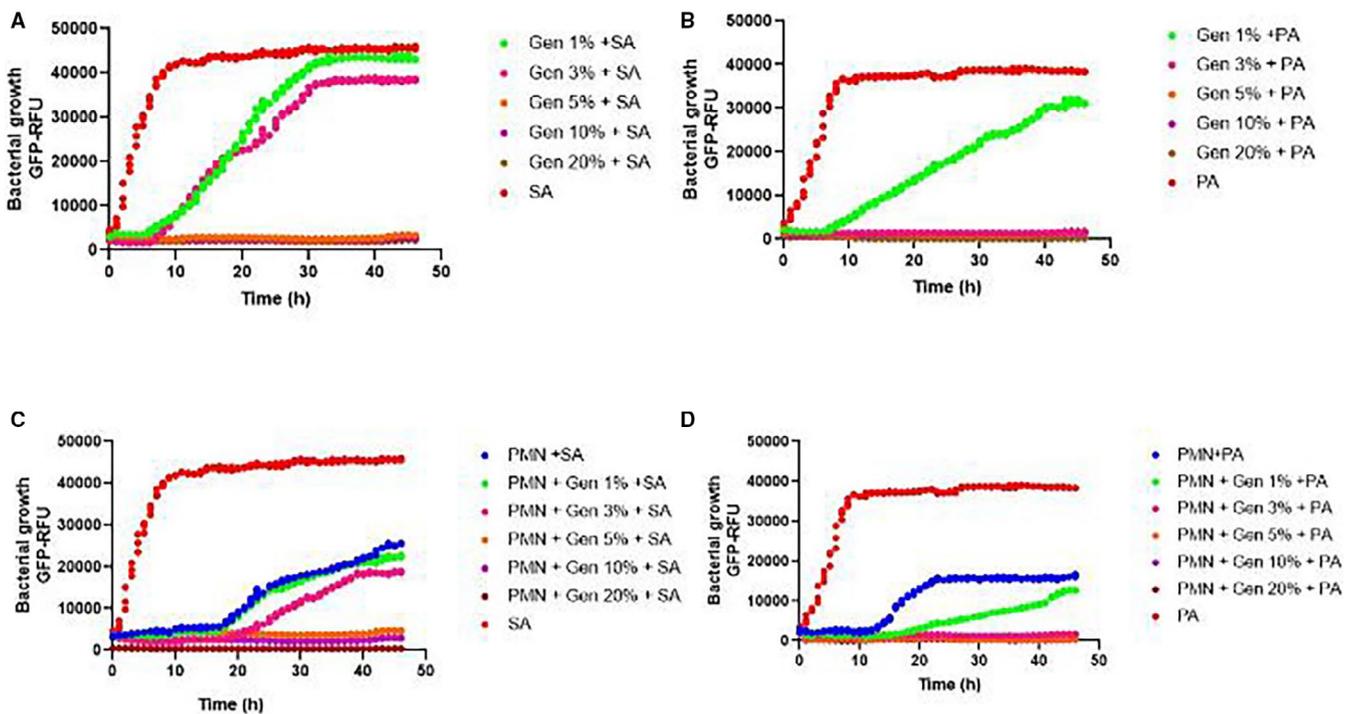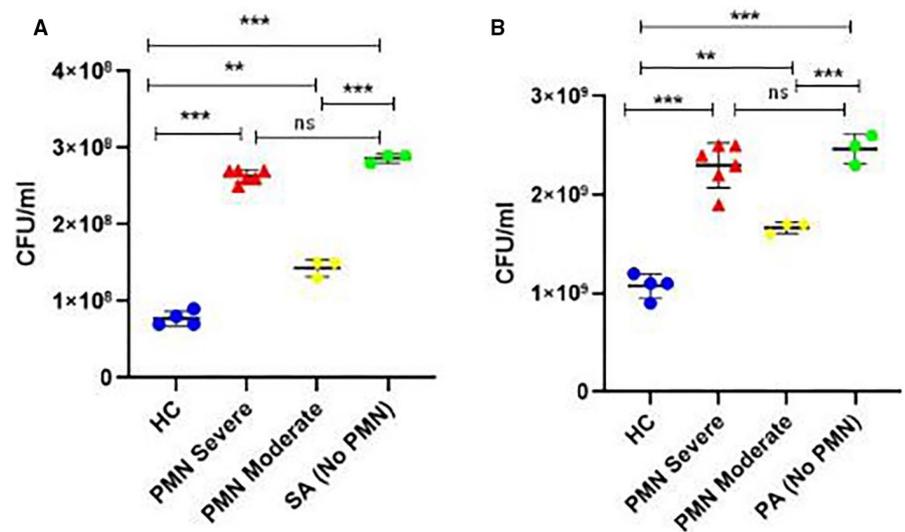

FIGURE 4 Representative growth curves of Staphylococcus aureus (SA) (A, C) and Pseudomonas aeruginosa (PA) (B, D) at different concentrations of gentamycin (1, 3, 5, 10, and 20 mg/L) alone and in the presence of polymorphonuclear cells (PMN) isolated from peripheral blood (C, D)

IgM. IgG or IgM bound to the microbial surface is recognized by C1q which activates the classical complement pathway. PMNs express receptors for IgG (FcγRI, FcγRII and FcγRIII) and opsonic complement molecules C3b and iC3b (CR1, CR3 and CR4).[29] The improvement seen in COVID-19 patients with intravenous immunoglobulins[30] or by reducing complement activation[31] suggests that the reduced neutrophil-mediated bacterial killing seen in COVID-19 patients may result from lower levels of immunoglobulins in these subjects. The innate immune response is associated with COVID-19

immunopathogenesis, and neutrophils are thought to be effective cells against COVID-19.[32] Lung autopsies of patients who died from COVID-19 have shown PMN penetration in pulmonary capillaries with extravasation to alveolar space and neutrophilic mucositis.[33]

Immunophenotyping of CD16 and CD62L positive cells showed that these were associated with segmented neutrophils. Mature neutrophils are recognized based on CD16 (FcγRIII receptor) and CD62L (L-selectin) expression.[34] Immature neutrophils (CD16lowCD62Lhigh) probably occur
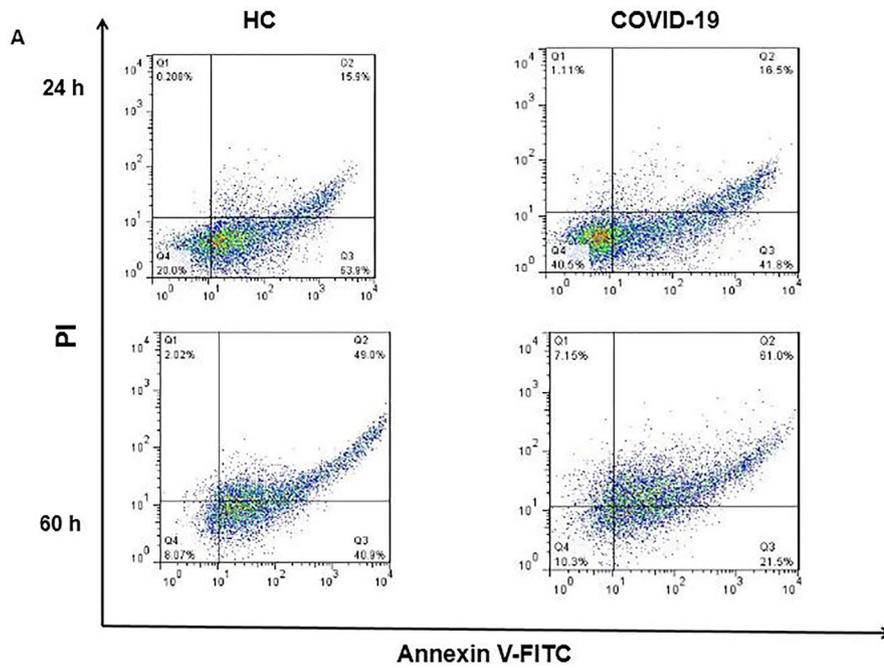
FIGURE 5 Representative cell viability assay of neutrophils using FITC-labelled anti-Annexin-V antibody and propidium iodide (PI) staining at 24 and 60 hours (A)

after depletion of mature neutrophils from the bone marrow and are inappropriate in antimicrobial immune function. Suppressive neutrophils (CD16highCD62Llow) illustrate a hypersegmented nucleus indicative of enhanced maturation compared to mature neutrophils. The suppressive neutrophils have a significant function in modulating severe inflammatory responses and suppressing immune defence against pathogens.[35,36] Pillay et al have shown the presence of these neutrophil subsets based on CD16 and CD62L expression in studies with healthy adults challenged with systemic LPS and in patients with severe inflammation originating from bacterial sepsis and trauma.[34]

In cancer, G-MDSCs are functionally distinct from neutrophils even though they possess similar morphological and phenotypic features and are pathologically activated precursors of neutrophils. PMN-MDSCs and neutrophils share a similar phenotype: $CD11b^+CD14^-CD15^+$ (or $CD66b^+CD33^+$). In healthy individuals, PMN-MDSCs are undetectable. Lectin-type oxidized LDL receptor 1 (LOX-1) allows for better distinction between human neutrophils and PMN-MDSCs.[37,38] Neutrophils of tumour-free mice have higher phagocytic activity, expression of lysosomal proteins and TNF-α than G-MDSCs of tumour-bearing mice.[16] We show in this study, an increase in the percentage of peripheral blood PMN-MDSCs in severe COVID-19 patients confirming a previous report.[39] In this previous study, as severe COVID-19 patients recovered, the percentage of MDSCs decreased and this was associated with enhanced levels of plasma cytokines illustrating a relationship between MDSC development and a decrease in inflammation.[39]

Neutrophils compose 60% of the leukocyte population present in blood and are the most important phagocytic cells. These cells can defend the host against bacterial infection. Neutrophils kill Gram-positive bacteria such as SA using a combination of bombardment with reactive oxygen species (an event called oxidative burst) and the induction of antimicrobial peptides (AMPs) and several enzymes.[40] PA is a Gram-negative opportunistic pathogen capable of infecting humans with compromised natural defences and causing severe pulmonary disease. PA can facilitate adhesion, modulate or disrupt host cell pathways, and target the extracellular matrix. This bacterium be able to form biofilms that protect bacteria from antibiotics and the host immune system.[41] Inflammation as a result of PA infection is mediated by neutrophils either directly by the release of chemoattractant factors or indirectly through stimulation of other cell types such as epithelial cells. The presence of neutrophils in PA infections helps mediate bacterial killing through phagocytosis, NET formation as well as the release of neutrophil microvesicles.[42] However, co-infection can harm the host immune system by the development of anti-bacterial intolerance.[43]

Treatment of SA and PA with gentamicin showed both a time- and dose-dependent inhibition of bacterial growth. Aminoglycosides, such as gentamicin, are considered as concentration-dependent killers of Gram-negative rather than Gram-positive organisms,[44-47] and they are often prescribed with other antimicrobial agents such as beta-lactams or glycopeptides for the treatment of serious infections with Gram-negative and Gram-positive organisms. However, our results showing a differential time- and dose-dependent killing of

both SA and PA are in agreement with previous data.[45] Our data showing that the presence of PMNs affected the ability of gentamicin to kill PA and SA suggested bacterial uptake by PMNs. However, further experiments are required to show whether this was due to phagocytosis and that PMNs from COVID-19 had defective phagocytosis. In addition, although our data suggest that PMN from COVID-19 patients underwent reduced apoptosis compared to PMN from uninfected subjects we cannot rule out that cells from COVID-19 patients at baseline are dying quicker and cannot handle the bacterial infection. The numbers of live cells present 24hrs after culture suggest that this is unlikely and that PMNs from COVID-19 patients have reduced apoptosis. Future studies will need to be performed to address the precise mechanism mediating this effect on apoptosis.

In viral pneumonia, bacterial co-infection is a key factor driving mortality.[48] Furthermore, 94.2% of COVID-19 patients are co-infected with further pathogens including 9 viruses, 11 bacteria and 4 fungi with bacterial co-infection being the predominant pathogens in all COVID-19 patients.[1] In this study, we show a reduced capacity of neutrophils COVID-19 patients to kill two important pathogenic bacteria. A limitation of this study is the lack of analysis of neutrophils from patients with different COVID-19 severity and the restriction to SA and PA. However, SA and PA are key pathogenic organisms in lung disease and may be important in the pathophysiology of severe COVID-19.

CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS
M.N and EM performed experiments and analysed results and wrote the paper. MV, PT, HJ, MM and SMRH provided the patients samples. MG designed bacterial analysis. IMA edited and finalized the paper.

DATA AVAILABILITY STATEMENT
Research data are not shared.

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