RESEARCH ARTICLE

pISSN: 0126-074X | eISSN: 2338-6223
https://doi.org/10.15395/mkb.v55n2.3143
Majalah Kedokteran Bandung. 2023;55(2):85–91

Majalah Kedokteran Bandung (MKB)

Received: December 27, 2022
Accepted: February 3, 2023
Available online: June 30, 2023

Dose and Time-Dependent Lipopolysaccharide Exposure on A549 Cell Model Influences Pro-Inflammatory Cytokine Interleukin 8

Sarah Sumayyah,[1] Prayoga Suryadarma,[1,2] Rachmawati Noverina,[3] Wireni Ayuningtyas,[3,4] Firman F. Wirakusumah,[5] Ahmad Faried[6]

[1]Biotechnology Study Program, Postgraduate School, Bogor Agricultural Institute, Bogor, Indonesia
[2]Department of Agricultural Industrial Technology, Faculty of Agricultural Technology, Bogor Agricultural Institute, Bogor, Indonesia
[3]Stem Cell Research and Development, Bio Farma, Bandung, Indonesia
[4]Doctoral Program, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia
[5]Department of Obstetrics and Gynecology, Faculty of Medicine Universitas Padjadjaran/Dr. Hasan Sadikin General Hospital Bandung, Indonesia
[6]Department of Neurosurgery, Faculty of Medicine Universitas Padjadjaran/Dr. Hasan Sadikin General Hospital Bandung, Indonesia

Abstract

Hyperinflammation in COVID-19 patients is one of the causes of the high mortality rate of COVID-19. An in vitro model mimicking the inflammatory responses in COVID-19 patients is important in the efforts of finding new drug candidates for this disease. Lipopolysaccharide (LPS) can increase the proinflammatory cytokine interleukin 8 in response to the presence of foreign substances. This preliminary study sought to explore the use of the A549 cells as an in vitro inflammatory model. This study was conducted from August to November 2022 at the stem cell research and development laboratory of Bio Farma Indonesia. The exposure of 100, 500, and 1000 g/mL doses of LPS administered for 24, 72, and 120 hours on the A549 cells was analyzed for cell viability, population doubling time (PDT), and the presence of proinflammatory cytokine IL-8. The group differences were examined using one- and two-way analysis of variance in IBM SPSS Statistics Version 29, with a p-value of 0.05 considered significant. Cells exposed to a dose of 1,000 g/mL LPS had a lower viability and a higher proliferation rate ($p<0.05$) based on the viability and PDT. Viability, PDT, and pro-inflammatory cytokines showed concentration- and time-dependent responses. Therefore, increased levels of the proinflammatory cytokine IL-8 in cells exposed to LPS at a dose of 1000 g/mL for 24 hours can be used as a mimic to study hyperinflammation in COVID-19 patients.

Keywords: A549 cell, inflammation, interleukin 8, lipopolysaccharide exposure

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) refers to the respiratory disease triggered by the coronavirus related to SARS, which is of particular concern after the World Health Organization (WHO) declared it as a pandemic. Although the physiopathology of human coronaviruses (H-CoVs) such as SARS-CoV, MERS, and SARS-CoV-2 is not fully understood, their strong association occurs due to a disproportionately disturbed response of the immune system, especially to cytokine production.[1] Several studies have reported that very high levels of proinflammatory cytokines consumed during crosstalk between epithelial cells and immune cells in COVID-19 are associated with cytokine storms with severe complications.[2]

The involvement of LPS (also known as endotoxin) in ARDS pathology has been reported previously.[3] Toll-like receptor-4 (TLR4) activation with LPS during ARDS induces recruitment of leukocytes to the lung, activation of pro-inflammatory cytokine release, and consequent induction of lung injury, similar to SARS. LPS precisely activates TLRs, leading to activation of the nuclear factor-kappa B (NF-κB) signaling pathway and secretion of pro-inflammatory

Corresponding Author:
Rachmawati Noverina,
Stem Cell Research and Development, Bio Farma, Jl. Pasteur 28 Bandung, Indonesia,
Email: rnoverina@gmail.com

cytokines and chemokines, such as interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor α (TNF-α) and type 1 interferon.[4] Endotoxins have been shown to reduce alveolar epithelial cell viability in an animal model of LPS-induced acute lung injury (ALI)[5] and in vitro.[6]

Primary alveolar epithelial cell culture is currently considered the most representative in vitro model for alveolar studies. Still, the problem is tissue availability which requires ethical approval and patient consent to diagnose lung carcinoma.[7] In addition, using these cells has a short period because they will differentiate spontaneously within 1–2 weeks.[8] Therefore, cell lines such as A549 cells with lasting properties (provided they are correctly maintained) have their advantages. These continuous cells have the main advantages of ease of cultivation, reproducibility, and relatively unlimited supply.[9]

The critical parameter for successfully modeling hyperinflammatory reactions in vitro is the increased concentration of cytokines and chemokines in the conditioned culture medium. Intraperitoneal or intravenous injection of high doses of LPS into animals induces systemic inflammatory cytokine production, ultimately leading to tissue damage, body temperature dysregulation, and death.[10] Because of this, high-dose LPS injection has been used as an experimental model of septic shock. However, low-dose (sublethal) injection of LPS into animals can induce a state of "LPS tolerance" that alters the subsequent response to induction with LPS or other inflammatory stimuli. Long-term exposure to endotoxin at low doses has little or no effect on immune function, indicating that endotoxin can be rapidly eliminated by the immune system.[11]

The lack of effective target-specific therapies has been increasingly highlighted during the COVID-19 pandemic, resulting in severe acute respiratory failure and ARDS. Mortality and morbidity from this clinical condition remain high, so there is an urgent need to find new effective therapies to reduce mortality.[12] A549 cells, as an in vitro model exposed to LPS, will be the basis for making an inflammatory model so that later it will become the basis of targeted supporting therapy for COVID-19 patients.

Methods

This study was conducted from August to November 2022 at the stem cell research and development laboratory, Bio Farma. This research did not contain any studies involving animal or human participants because it uses the commercial cell line A549, nor did it take place on any private or protected areas. No specific permissions were required for corresponding locations.

The A549 human lung adenocarcinoma cell line (CLL-185™) were purchased from American Type Culture Collection (ATCC). A549 cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (Sigma Aldrich, Dorset, United Kingdom) supplemented with 10% (v/v) Fetal Bovine Serum (Gibco, Invitrogen, USA) and 1% (v/v) pen-strep (Gibco, Invitrogen, USA) seeded at an optimal cell density of 2×10^3 cells/cm^2 in a humidified atmosphere with 5% CO_2 at 37°C. The culture medium was renewed every third day. After the cells have 85% confluence, change the medium with the treatment medium.

Based on the analysis, LPS was dissolved in a culture medium and used for the cell treatment in a concentration and time-dependent manner. LPS-induced cultures with doses of 100, 500, and 1,000 µg/mL for 24 hours, and in the follow-up study, one dose was taken, which experienced a significant difference based on viability, PDT, and IL-8 using time intervals of 24, 72, and 120 hours.

To measure the viability and population doubling time of A549 cells, the A549 cells Passage 54 were plated at a density of $5x10^3$ cells/cm^2 and were seeded into four-well plates (Nunc, Thermo Scientific, Massachusetts, USA) supplemented with 2 mL of growth medium, grown to confluence, and incubated around 4 days in the growth medium at 37°, 5% CO_2. On the fourth day, media were replaced by culture media with LPS in varied concentrations in the presence of 10% FBS, and cells were incubated for 24, 72, and 120 h. A549 cells were harvested according to the time interval and dissociated with trypsin porcine for 3 minutes and centrifuged at 300 g for 4 minutes. The pellet was resuspended with a culture medium, and cells were counted with a hemocytometer. The viability cells were determined as a ratio of viable cells to the number of death cells and expressed as a percentage. The population doubling (PD) was calculated as described previously[13] using the equation of PD $=\log_2 / \log (C_H/C_S)$, where CH is the number of viable cells at harvest and CS is the number of cells seeded. The population doubling time (PDT) was calculated using the interval between cell seeding and harvest divided by the number of PDs. All experiments

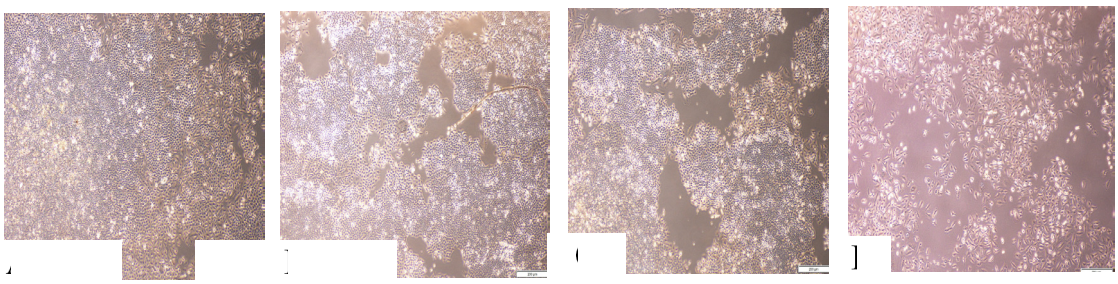

Figure 1 Morphology of A549 Cells After Exposure to LPS at Various Concentrations for 24 Hours. (A) 0 µg/mL LPS, (B) 100 µg/mL LPS, (C) 500 µg/mL LPS, (D) 1,000 µg/mL LPS

were performed in triplicate.

The IL-8 proinflammatory cytokine of A549 cells were evaluated by flow cytometry (BD FACSLyric, Becton, USA). A549 cells were seeded in four-well plates (2,000 cells/cm^2) and cultured in media with 10% FBS. On the fourth day, cells were treated with LPS of varied concentration of LPS in the presence of 10% FBS for 24, 72 and 120 h. The analysis phase was carried out by following the procedure of the FACS commercial kit. The secretome was transferred to a 1.5 mL tube, then 1µL/sample was given a cytokine bead capture. PE antibody was prepared with the same calculation as the capture cytokine bead and then stored in the dark. A 50 µL secretome sample was put into a 1.5 mL tube, then 50 µL of cytokine bead capture mix was added. Vortex briefly to make sure everything is homogeneous. Incubate in the dark, room temperature for 1 hour. The 50µL prepared PE antibody was added into the mixture of cytokine capture bead and secretome samples. Vortex at medium speed, then incubate in the dark, room temperature for 2 hours. 1 mL of wash buffer was added, then centrifuged at 1,600 rpm for 5 minutes. The supernatant was carefully removed. After that, it was vortexed, and 300 µL wash buffer was added and ended with another vortex.

Data of viability and PDT were presented as mean±standard deviation (SD) and analyzed using analysis of variance (ANOVA). If there were differences between the treatments, the Tukey Post Hoc Test was continued ($p<0.05$)data analysis using IBM SPSS statistics.

Results

The morphological observations showed a decrease in cell density with increasing LPS doses without changing the morphology of A549 cells (Figure 1). The phenomenon aligns with decreased cell viability (Figure 2A) and prolonged PDT (Figure 2B). Cell viability decreased to 96% after exposure with 100 µg/mL LPS, to 92% after exposure with 500 µg/mL LPS and to 84% after exposure with 1,000 µg/mL LPS for 24 hours. Proliferation in A549

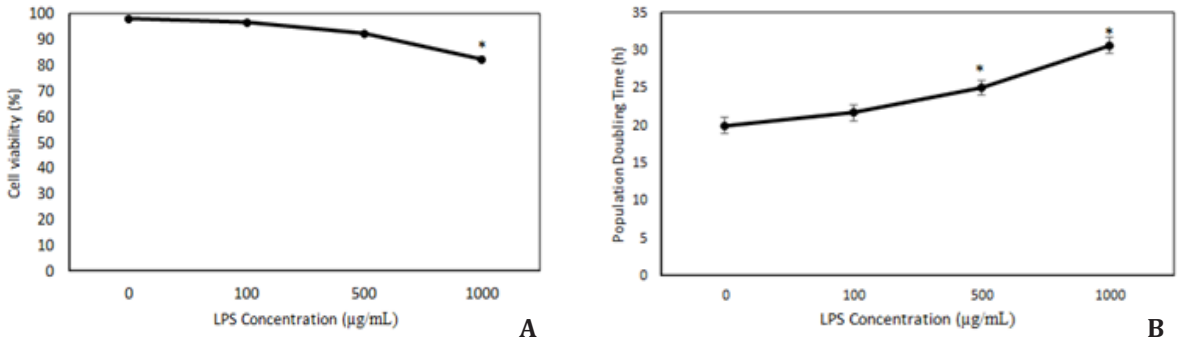

Figure 2 Viability and PDT After Exposure to LPS at Various Concentrations for 24 Hours. (A) Viability cell, (B) Population Doubling Time. Bars Represent the Mean±SD. Significant Differences were Considered when *p<0.05

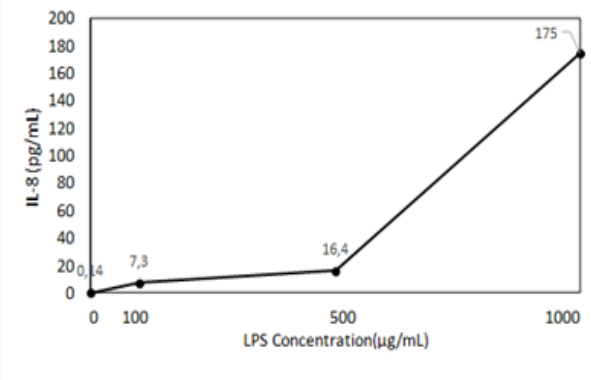

Figure 3 The Concentration of the Proinflammatory Cytokine IL-8 at LPS Concentrations Varied 24 Hours

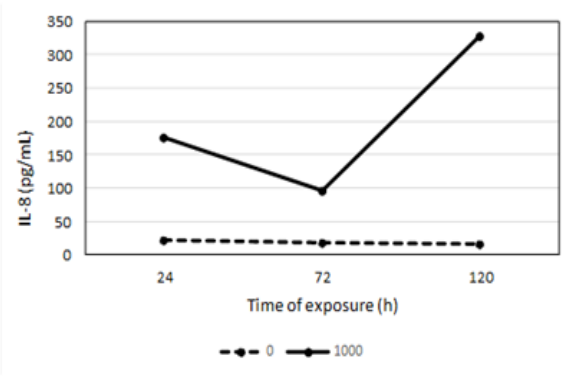

Figure 5 Concentration of Proinflammatory Cytokine IL-8 at LPS Concentrations of 1,000 µg/mL with Different Length of Exposure

cells exposed to LPS was significantly increased compared to cells not exposed to LPS.

A549 cells were analyzed for their secretome to determine the concentration of the pro-inflammatory cytokine IL-8. The increase in the pro-inflammatory cytokine IL-8 was marked when compared with cells that were not exposed (Figure 3). A sharp increase occurred with exposure to LPS with a 1,000 µg/mL concentration. So that in future studies, a dose of 1,000 µg/mL was used to see the effect of LPS exposure time.

Based on the morphology, viability, and PDT on the effect of LPS exposure time, there was a significant difference between cells exposed to LPS and those not exposed to LPS (Figure 4). The concentration of the pro-inflammatory cytokine IL-8 produced by cells exposed to LPS increased compared to those not exposed (Figure 5). Although the LPS exposure time of 72 hours decreased, the concentration of IL-8 produced was still much higher compared to cells that were not exposed to LPS.

Discussion

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infects lung tissue, significantly accumulating immune cells and causing an inflammatory cytokine storm. The excess of inflammatory cytokines then leads to the infiltration of immune cells into the inflamed lung to induce alveolar damage and reduced lung function.[14] There was an increase in the pro-inflammatory cytokine IL-8 compared to cells that were not exposed to LPS. A sharp

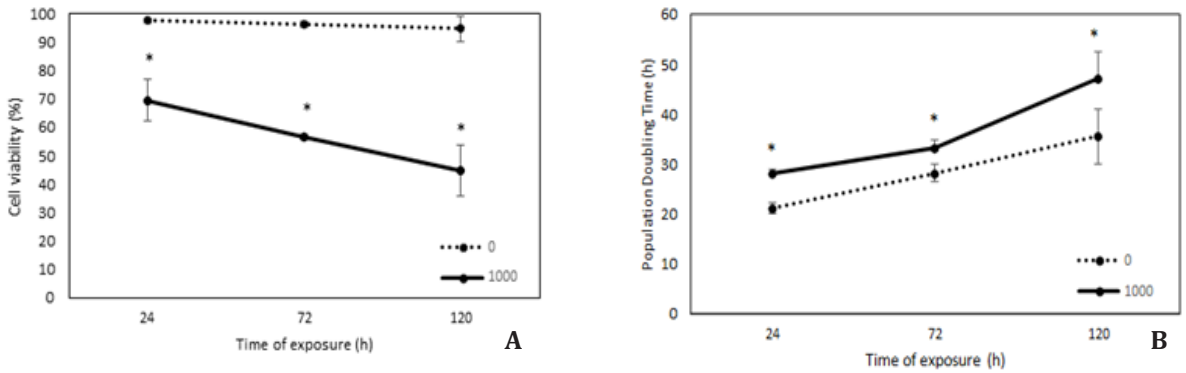

Figure 4 Viability and PDT of A549 Cells After Exposure to LPS at a Dose of 1000 µg/mL with Differences in Exposure Time. After Exposure to LPS at Various Concentrations for 24 Hours. (A) Viability cell, (B) Population Doubling Time. Bars Represent the mean±SD. Significant Differences were considered when *p<0.05

increase occurred with exposure to LPS with a concentration of 1000 µg/mL. IL-8 is one of the main mediators of the inflammatory response.[15] These results are in line with research conducted by Li et al. which showed an increase in IL-8 in COVID-19 patients,[16] which means that acute inflammation had occurred in A549 cells in the presence of LPS exposure. Lipopolysaccharide (LPS), the main component of the cell wall of Gram-negative bacteria, is the central stimulus for releasing inflammatory mediators. This is supported by changes in morphology, viability, and PDT with exposure to LPS at various concentrations.

Based on morphological observations, it was shown that there was a decrease in cell density with increasing exposure to LPS doses without changing the morphology of A549 cells. This is in line with the decrease in cell viability and prolonging PDT. Literature data regarding the responsiveness of A549 cells to LPS is highly controversial. Several studies have reported a significant decrease in A549 cell viability of around 50% at a low LPS concentration of 1 µg/mL after 24 hours.[17] Meanwhile, the minimal cytotoxic dose of endotoxin for A549 cells at a 50 µg/mL concentration.[18] However, cell viability did not fall below 80%. A potential reason for the different responses of A549 cells could be the type of LPS used for the experiment. Previous studies showed that the LPS substructure could modulate its endotoxic properties, possibly through different interactions of the LPS molecule with the TLR4 receptor complex, leading to different activation of subsequent inflammatory pathways.[19] This is supported by studies that found that structural differences in the O-antigen LPS molecule were able to modulate its recognition and phagocytosis by macrophages.[20]

The results of PDT showed that the longer the time needed to double the number of cells as the exposure to LPS dose increased. In cells that were not exposed to LPS, they showed a PDT of 21.9 hours; this is in line with the literature, which states that the PDT of A549 cells is 21.8 hours.[13] Based on analysis with one way ANOVA, it showed significance in cells exposed to LPS at a dose of 1000 µg/mL. This means that cells exposed to LPS experience disturbances in their proliferation process, so the time needed becomes longer. LPS with a concentration of 1000 µg/mL greatly affected the viability, PDT, and the concentration of the pro-inflammatory cytokine IL-8 in A549 cells. Therefore, an LPS concentration of 1000 µg/mL was set as the concentration to induce inflammation in the follow-up experiments.

Morphological observations showed a decrease in cell density with increasing exposure to LPS doses without changing the morphology of A549 cells. This is in line with the decrease in cell viability and prolonging PDT. The results of the PDT calculation also show an extension of the time needed for cells to double their cell number. PDT increases with increasing exposure time. Cells not exposed to LPS showed PDT 21 hours, then increased to 28 hours, and at the end, showed 35 hours. In control cells at 24 hours, the cells were still growing well with normal PDT. An increase in PDT in control cells indicates the occurrence of confluence, which causes the cells to experience abnormalities. Cells exposed to LPS showed abnormalities after exposure to LPS for 24 hours and then increased with the length of exposure, the difference in time needed for cells not exposed to LPS compared to cells exposed to LPS for 120 hours reached 11 hours.

Scoring cytokine storm by levels of MCP-3 and IL-8 can accurately stratify COVID-19 patients for high mortality risk.[21] The pro-inflammatory cytokine IL-8 showed a sharp increase during LPS exposure for 120 hours. Hyperinflammation of the lungs of severe COVID-19 patients is fueled by excessive production of chemokines Chemokines like CXCL1 (GROα) and IL-8 were found to be 30 times more abundant in BALF than in plasma and 200 times more abundant than IL-6 and TNF-α; consistent with the levels of these chemotactic molecules, BALF was rich in neutrophils, lymphocytes, and eosinophils.[22]

Neutrophils are essential effector cells in the innate immune defense against human infection. IL-8, secreted by macrophages and lung epithelial cells, is a neutrophil chemoattractant. IL-8 contributes to neutrophil activation and NET formation after binding to CXCR2 on neutrophils, causing hyperinflammation.[23] The results showed that the concentration of the pro-inflammatory cytokine IL-8 increased significantly after 1,000 µg/mL LPS exposure, indicating that the LPS-induced A549 cell inflammation model was successful.

References

1. Darif D, Hammi I, Kihel A, el Idrissi Saik I, Guessous F, Akarid K. The pro-inflammatory cytokines in COVID-19 pathogenesis: What goes wrong?. Microb Pathog. 2021;153:104799.

2. Henderson LA, Canna SW, Schulert GS, Volpi S, Lee PY, Kernan KF, et al. On the alert for cytokine storm: immunopathology in COVID-19. Arthritis and Rheumatology. 2020;72(7):1059–63.
3. Al-Ani B, ShamsEldeen AM, Kamar SS, Haidara MA, Al-Hashem F, Alshahrani MY. Lipopolysaccharide induces acute lung injury and alveolar haemorrhage in association with the cytokine storm, coagulopathy and AT1R/JAK/STAT augmentation in a rat model that mimics moderate and severe Covid-19 pathology. Clin Exp Pharmacol Physiol. 2022;49(4):483–91.
4. von Schéele I, Larsson K, Palmberg L. Interactions between alveolar epithelial cells and neutrophils under pro-inflammatory conditions. Eur Clin Respir J. 2014;1(1):24545.
5. Ogata-Suetsugu S, Yanagihara T, Hamada N, Ikeda-Harada C, Yokoyama T, Suzuki K, et al. Amphiregulin suppresses epithelial cell apoptosis in lipopolysaccharide-induced lung injury in mice. Biochem Biophys Res Commun. 2017;484(2):422–8.
6. Nova Z, Skovierova H, Strnadel J, Halasova E, Calkovska A. Short-term versus long-term culture of A549 cells for evaluating the effects of lipopolysaccharide on oxidative stress, surfactant proteins and cathelicidin LL-37. Int J Mol Sci. 2020;21(3):1148.
7. Thorley AJ, Ford PA, Giembycz MA, Goldstraw P, Young A, Tetley TD. Differential regulation of cytokine release and leukocyte migration by lipopolysaccharide-stimulated primary human lung alveolar type II epithelial cells and macrophages. J Immunol. 2007;178(1):463–73.
8. Fuchs S, Hollins AJ, Laue M, Schaefer UF, Roemer K, Gumbleton M, et al. Differentiation of human alveolar epithelial cells in primary culture: Morphological characterization and synthesis of caveolin-1 and surfactant protein-C. Cell Tissue Res. 2003;311(1):31–45.
9. Cooper JR, Abdullatif MB, Burnett EC, Kempsell KE, Conforti F, Tolley H, et al. Long term culture of the a549 cancer cell line promotes multilamellar body formation and differentiation towards an alveolar type II Pneumocyte phenotype. PLoS One. 2016;11(10):e0164438.
10. Nova Z, Skovierova H, Calkovska A. Alveolar-capillary membrane-related pulmonary cells as a target in endotoxin-induced acute lung injury. Int J Mol Sci. 2019;20(4):831.
11. Li C, Ma D, Zhang M, An L, Wu C, Zhou H. Effects of long-time exposure to lipopolysaccharide on intestinal lymph node immune cells and antibodies level in mice. Iranian J Immunol. 2020;17(3):175–84.
12. Cesta MC, Zippoli M, Marsiglia C, et al. The role of interleukin-8 in lung inflammation and injury: implications for the management of COVID-19 and hyperinflammatory acute respiratory distress syndrome. Front Pharmacol. 2022;12:808797.
13. Wiesmann N, Gieringer R, Viel M, Eckrich J, Tremel W, Brieger J. Zinc oxide nanoparticles can intervene in radiation-induced senescence and eradicate residual tumor cells. Cancers (Basel). 2021;13(12):2989.
14. McElvaney OJ, McEvoy NL, McElvaney OF, Carroll TP, Murphy MP, Dunlea DM, et al. Characterization of the inflammatory response to severe COVID-19 Illness. Am J Respir Crit Care Med. 2020;202(6):812–21.
15. Long X, Ye Y, Zhang L, Liu P, Yu W, Wei F, et al. IL-8, a novel messenger to cross-link inflammation and tumor EMT via autocrine and paracrine pathways (Review). Int J Oncol. 2016;48(1):5–12.
16. Li L, Li J, Gao M, Fan H, Wang Y, Xu X, et al. Interleukin-8 as a biomarker for disease prognosis of coronavirus disease-2019 Patients. Front Immunol. 2021;11:602395.
17. Zhao J, Li X, Zou M, He J, Han Y, Wu D, et al. MiR-135a inhibition protects A549 cells from LPS-induced apoptosis by targeting Bcl-2. Biochem Biophys Res Commun. 2014;452(4):951–7.
18. Li S, Guo L, Qian P, Zhao Y, Liu A, Ji F, Chen L, et al. Lipopolysaccharide induces autophagic cell death through the PERK-dependent branch of the unfolded protein response in human alveolar epithelial A549 cells. Cell Physiol Biochem. 2015;36(6):2403–17.
19. Migale R, Herbert BR, Lee YS, Sykes L, Waddington SN, Peebles D, et al. Specific lipopolysaccharide serotypes induce differential maternal and neonatal inflammatory responses in a murine model of preterm labor. Am J Pathol. 2015;185(9):2390–401.
20. Eder K, Vizler C, Kusz E, Karcagi I, Glavinas H, Balogh GE, et al. The role of lipopolysaccharide moieties in macrophage response to Escherichia coli. Biochem Biophys Res Commun. 2009;389(1):46–51.
21. Chen L, Wang G, Tan J, Cao Y, Long X, Luo H, et al. Scoring cytokine storm by the levels of MCP-3 and IL-8 accurately distinguished

COVID-19 patients with high mortality. Signal Transduct Target Ther. 2020;5(1):292.
22. Bendib I, Beldi-Ferchiou A, Schlemmer F, Surenaud M, Maitre B, Plonquet A, et al. Alveolar compartmentalization of inflammatory and immune cell biomarkers in pneumonia-related ARDS. Crit Care. 2021;25(1):23.
23. Chiang CC, Korinek M, Cheng WJ, Hwang TL. Targeting neutrophils to treat acute respiratory distress syndrome in coronavirus disease. Front Pharmacol. 2020;11:572009.