

Anti-oxidant and anti-inflammatory Effects of Aerosolised microalgal-derived extracellular vesicles in Bronchial Epithelial–Macrophage Co-cultures at the Air-Liquid Interface

Wesam Darwish^{1,2}, Giorgia Adamo³, Mohammad Almasaleekh^{1,2}, Sabrina Picciotto³, Paola Gargano³, Daniele Paolo Romancino³, Samuele Raccosta⁴, Ralf Zimmermann^{1,2}, Mauro Manno⁴, Antonella Bongiovanni^{3,*}, Sebastiano Di Bucchianico^{1,2,*}

¹Department of Life, Light & Matter, University of Rostock, Germany

²Chair of Analytical Chemistry, University of Rostock, Germany

³Cell-Tech HUB and Institute for Research and Biomedical Innovation, National Research Council of Italy (CNR), Italy

⁴Cell-Tech HUB and Institute of Biophysics (IBF) - National Research Council of Italy (CNR), Italy

*corresponding authors: Antonella Bongiovanni, antonella.bongiovanni@irib.cnr.it; Sebastiano Di Bucchianico, s.dibucchianico@uni-rostock.de;

Key words: Nanoalgosomes, oxidative stress, inflammation, air-liquid interface, aerosolized therapy, Microalgal EVs

Abstract

Inflammation and oxidative stress are key drivers in the pathogenesis of chronic lung diseases, including asthma, pulmonary fibrosis, and chronic obstructive pulmonary disease. Extracellular vesicles derived from the marine microalga *Tetraselmis chuii*, referred to as nanoalgosomes, have recently gained attention as natural nanocarriers that possess inherent antioxidant and anti-inflammatory properties. In this study, we investigated the biocompatibility and protective effects of aerosolized nanoalgosomes in a bronchial epithelial–macrophage co-culture model at the air-liquid interface. Co-cultures of CALU-3 epithelial cells and differentiated THP-1 macrophages were primed with aerosolised nanoalgosomes and subsequently exposed to either oxidative stress (tert-butyl hydroperoxide) or an inflammatory stimulus (lipopolysaccharide; LPS). Epithelial barrier integrity and cytotoxicity were evaluated using transepithelial electrical resistance and lactate dehydrogenase release assays, respectively, while intracellular reactive oxygen species levels and cytokine secretion were measured to assess antioxidant and immunomodulatory responses. Nanoalgosomes were non-cytotoxic, preserved epithelial barrier integrity, and significantly reduced oxidative stress. In addition, nanoalgosomes priming attenuated LPS-induced secretion of pro-inflammatory cytokines (IL-1 β , IL-6, IL-8, IL-18, TNF- α) as well as the anti-inflammatory cytokine IL-10, suggesting a balanced immunomodulatory response. Overall, aerosolized nanoalgosomes maintained epithelial homeostasis and mitigated both oxidative and inflammatory stress, underscoring their potential as a safe, sustainable, and effective therapeutic strategy for chronic inflammatory lung diseases. Given their natural origin, excellent biocompatibility, and suitability for aerosol delivery, nanoalgosomes represent a promising class of inhalable biotherapeutics.

Introduction

Chronic lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), and pulmonary fibrosis represent a major global health burden due to their prevalence, morbidity, and mortality. Central to the pathogenesis and progression of these diseases are persistent inflammation and oxidative stress [1]. These processes promote airway remodeling, leading to structural and functional alterations of the epithelial barrier and a reduction in pulmonary macrophage populations [2,3]. The complex interplay among cytokines, signaling pathways, and reactive oxygen species (ROS) contributes significantly to the physiological damage, ultimately exacerbating disease progression [4]. Despite their widespread clinical use, current therapeutics for respiratory diseases largely target symptom relief, providing short-term improvements in airflow or inflammation but failing to address the underlying cellular and molecular drivers of disease [4]. Consequently, there is an urgent need for strategies that simultaneously mitigate oxidative stress and inflammation to preserve epithelial barrier function and restore homeostasis in the lung microenvironment.

Tetraselmis chuii (*T. chuii*) is a non-toxic marine microalga, approved for human consumption as a novel food, and increasingly recognized for its rich content of bioactive compounds, including vitamins, carotenoids, polyphenols, phytosterols, superoxide dismutase (SOD), and polyunsaturated fatty acids (PUFAs) [5,6]. These compounds collectively contribute to the antioxidant and anti-inflammatory properties of *T. chuii*. Preclinical studies suggest that supplementation with *T. chuii* extracts can protect cells against oxidative damage induced by H₂O₂, increase enzymatic antioxidant expression, and reduce oxidative stress markers such as malondialdehyde in vivo [7–11]. Additionally, this alga modulates immune signaling by suppressing pro-inflammatory cytokines, including IFN- γ , TNF- α , and IL-1 β , while regulating anti-inflammatory cytokines like IL-10 [7–9,12]. These multifaceted properties highlight the potential of *T. chuii* as a natural source of therapeutic biomolecules capable of restoring redox and inflammatory balance.

Extracellular vesicles (EVs) are lipid bilayer nanoparticles secreted by diverse cell types across species, expanding their relevance and potential for biomedical applications. They play a crucial role in intercellular communication by mediating the transport of biomolecules, including proteins, lipids, and nucleic acids. Their inherent biocompatibility and capacity for targeted delivery make them attractive candidates for novel biotherapeutics in chronic respiratory diseases [13–15]. EVs have been shown to alleviate inflammatory responses in the lung, reduce oxidative stress, and promote anti-inflammatory macrophage polarization, demonstrating their potential as both therapeutic agents and delivery systems [16–18].

Recently, naturally derived nanosized EVs extracted from *T. chuii* have been termed nanoalgosomes [19,20]. As *T. chuii* is rich in antioxidant and anti-inflammatory biomolecules, its EVs are likely to retain or convey these bioactivities while preserving the intrinsic structural and functional advantages of EVs, including biocompatibility, efficient cellular uptake, and protection of bioactive cargo from enzymatic degradation. Beyond their intrinsic bioactive properties, *T. chuii* offers a cost-effective, scalable, and sustainable source for EV production [21,22]. In vitro studies have shown that nanoalgosomes are non-toxic, decrease intracellular ROS levels, restore cytokine balance, and modulate antioxidant enzyme expression [19,20,22,23]. Using the in vivo model *Caenorhabditis elegans*, nanoalgosome supplementation was shown to significantly attenuate oxidative stress-induced locomotor decline [22]. The preservation of mobility provides compelling evidence for the systemic stability and protective effects of nanoalgosomes in a complex multicellular organism.

Despite these promising findings, the effects of nanoalgosomes in physiologically relevant airway models remain largely unexplored. To date, most studies have focused on simple

monocultures, with only one study investigating pulmonary cells under submerged conditions [24]. This limits our understanding of how nanoalgosomes influence lung-specific responses in models that better recapitulate airway physiology [25]. Because oxidative stress and inflammation are interdependent drivers of lung pathophysiology, it is essential to determine whether nanoalgosomes can modulate intracellular ROS production and cytokine secretion within a more complex airway environment, including bronchial epithelial–macrophage interactions. Addressing this gap will provide critical insight into their potential to prevent or mitigate oxidative injury, immune dysregulation, and barrier dysfunction in chronic lung diseases such as asthma, COPD, and pulmonary fibrosis.

In this study, we evaluate the antioxidant and immunomodulatory properties of *T. chuii*-derived nanoalgosomes using a bronchial epithelial–macrophage co-culture model at the air-liquid interface (ALI). This physiologically relevant system enables assessment of airway barrier integrity, intracellular ROS modulation, and cytokine secretion in response to oxidative and inflammatory stimuli. By investigating the safety, biocompatibility, and functional effects of nanoalgosomes priming, we aim to determine their capacity to restore redox balance and regulate immune responses in the lung. Demonstrating that nanoalgosomes can reduce intracellular ROS, dampen pro-inflammatory cytokine production, and preserve epithelial barrier function would support their development as a biologically active, non-toxic, and scalable therapeutics for chronic respiratory diseases. Moreover, this work lays the groundwork for future translational efforts to leverage nanoalgosomes as inhalable delivery platforms, bridging natural bioactive compounds with next-generation medicine for pulmonary applications.

Materials and methods

Microalgae culture

T. chuii microalgae culture was performed following the established protocol described in Picciotto et al., 2025. Briefly, axenic *T. chuii* cultures were grown under sterile conditions in modified f/2 medium under controlled light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$), temperature ($22 \text{ }^{\circ}\text{C} \pm 2 \text{ }^{\circ}\text{C}$), and a 14:10 h light/dark cycle. Cell growth and viability were monitored weekly. At each time point, 1 mL of culture was sampled and optical density at 600 nm (OD_{600}) was measured spectrophotometrically. Cell concentration was calculated using a linear regression model ($R^2 = 0.998$) derived from a calibration curve correlating OD_{600} values with cell counts obtained using a Thoma chamber from serially diluted cultures. 12 liters of culture were initiated at $\text{OD}_{600} = 0.300$ and grown for 14 days to $\text{OD}_{600} = 0.600$. Half of the culture volume (6 liters) was processed by tangential flow filtration (TFF) for nanoalgosome isolation, while the remaining half was replenished 1:1 with fresh f/2 medium and cultured for an additional two weeks before reprocessing.

Tangential flow filtration

Upon reaching an optical density (OD_{600}) of approximately 0.600 after a two-week growth period, the cultures were processed. Nanoalgosome isolation was performed using TFF, a standard technique for purifying EVs [21]. All procedures were conducted under sterile conditions. The TFF system utilized a sequential three-stage filtration protocol with hollow fiber cartridges (Cytiva), progressing from a 650 nm pore size, to 200 nm, and finally to a 500 kDa molecular weight cut-off (MWCO). The system was equipped with in-line transducers and a peristaltic pump for continuous monitoring and control of permeate flow and transmembrane pressure, which were kept constant to ensure reproducible isolation conditions. During this process, the initial 650 nm microfiltration step removed intact cells, generating a permeate enriched with EVs. The subsequent 200 nm filtration retained larger vesicles while allowing the smaller nanoalgosomes to pass into the permeate. In the final stage, the 500 kDa UF membrane removed soluble proteins, small aggregates, and other non-vesicular contaminants, concentrating the nanoalgosomes in the retentate to a volume of $\sim 150 \text{ mL}$. A final concentration and diafiltration step was performed to exchange the native f/2 medium with calcium- and magnesium-free PBS, thereby further purifying and conditioning the nanoalgosome preparation. The final product was concentrated to an approximate volume of 7 mL.

Nanoparticles tracking analysis (NTA)

The size distribution and concentration of three independent batches of nanoalgosomes was evaluated using the NanoSight PRO (Malvern Instruments), equipped with a blue (488 nm) laser and a high-sensitivity camera. Nanoalgosomes samples were diluted in filtered, particle-free water to achieve a particle concentration within the optimal detection range of 20–120 particles per frame, ensuring reliable measurements. Measurements were acquired using the Auto Camera and Auto Focus settings, with a flow rate of $3 \mu\text{L}/\text{min}$. For each sample, five independent video captures were recorded and analyzed. Data were processed using NanoSight software version 2.0.

Dynamic light scattering (DLS)

Nanoalgosomes samples ($n=3$) were diluted 1:5 and centrifuged at $1,000\times g$ for 10 min at $4 \text{ }^{\circ}\text{C}$ to eliminate any dust particles. The supernatant was transferred to a quartz cuvette and incubated at $20 \text{ }^{\circ}\text{C}$ in the thermostatically controlled cell compartment of a BI200-SM goniometer (Brookhaven Instruments) equipped with a He-Ne laser (JDS Uniphase 1136P) tuned at 633 nm and a single-pixel photon counting module (Hamamatsu C11202-050). Scattered light intensity and its autocorrelation function $g_2(t)$ were measured simultaneously at

a scattering angle $\theta = 90^\circ$ using a BI-9000 correlator (Brookhaven Instruments). The electric field autocorrelation function $g_1(t)$ was calculated by using the Siegert relation:

$$g_2(t) = 1 + \beta |g_1(t)|^2 \quad (1)$$

where β is an instrumental parameter and $g_1(t)$ is the field autocorrelation function, associated with the size (σ) of diffusing particles and their size distribution $P_q(\sigma)$ by the relation

$$g_1(t) = \int P_q(\sigma) \exp\{-D(\sigma)q^2t\} d\sigma \quad (2)$$

where $q = 4\pi n \lambda^{-1} \sin(\theta/2)$ is the scattering vector in a medium with n refractive index and $D(\sigma)$ is the diffusion coefficient of a particle of hydrodynamic diameter $D_h = \sigma$, determined by the Stokes–Einstein relation $D(\sigma) = k_B T / [3\pi\eta\sigma]$, with T being the temperature, η the medium viscosity, and k_B the Boltzmann constant. The size distribution $P_q(\sigma)$ was calculated under the assumption that the diffusion coefficient distribution is shaped as a Schultz distribution [21]. Two robust parameters are derived from this analysis: D_z , the z-averaged hydrodynamic diameter, and PDI , the polydispersity index, an estimation of the distribution width. The field autocorrelation function of the samples was fitted with one component or two components if necessary

Zeta-potential

Zeta potential measurements were performed using a Zetasizer Advance (Malvern Panalytical Ltd., UK). EV samples in PBS were suitably diluted in Milli-Q water to achieve comparable concentrations, while maintaining the same final dispersant ($0.1 \times$ PBS). Samples were loaded into folded capillary cells, and measurements were carried out at 25°C . Each measurement consisted of at least 12 automated runs, which were internally averaged by the instrument, was repeated three times (reported values correspond to the mean $\pm$ standard deviation). Zeta potential values were calculated from the measured electrophoretic mobility using the Smoluchowski approximation.

Atomic force microscopy (AFM)

Sample preparation: glass slides were cleaned by boiling in acetone, dried by nitrogen stream and then exposed to UV radiation. The slides were then a) treated with a 0.25 M (3-aminopropyl)-triethoxysilane solution in chloroform for 3 minutes, rinsed with chloroform and dried with nitrogen; b) treated with 0.4 M glutaraldehyde aqueous solution for 3 minutes, rinsed with Milli-Q water and dried with nitrogen. 30 μl of vesicle solution were deposited onto functionalized glass slides at about 5×10^{10} particles/ml and incubated overnight. The samples were then gently rinsed with PBS to remove non-adsorbed vesicles. Vesicle imaging: Quantitative Imaging AFM measurements were performed in PBS using a Nanowizard III scanning probe microscope (JPK Instruments AG, Germany) equipped with a 15- μm z-range scanner, and AC40 (Bruker) silicon cantilevers (spring constant 0.1 N/m, typical tip radius 8 nm). The images (resolution 256x256 pixels) were acquired with a force setpoint of 130 pN and an extension speed of 25 $\mu\text{m/s}$. The cantilever was thermally calibrated by using the tool in JPK software [26].

BCA assay and immunoblotting

The protein concentration of nanoalgosomes was quantified using the micro-bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific). This colorimetric assay determines relative protein concentration by comparison to a bovine serum albumin (BSA) protein standard, from which a calibration curve was generated. The absorbance of the BCA soluble compound was measured at 562 nm using a GloMax Discover Microplate Reader. Protein separation was carried out by sodium dodecyl sulfate–polyacrylamide gel electrophoresis

(SDS–PAGE) using 10% polyacrylamide gels. Equal amounts (~5 µg) of cell lysates (*Tetraselmis chuii* and HEK-293) and nanoalgosome samples were mixed with appropriate volumes of 5× loading buffer (0.25 M Tris-HCl, pH 6.8, 10% SDS, 50% glycerol, 0.25 M dithiothreitol (DTT), and 0.25% bromophenol blue), heated at 100 °C for 5 min, and loaded onto the gels for electrophoretic separation. Cell lysates from *T. chuii* and Human Embryonic Kidney HEK-293 cells were included as internal reference controls to validate antibody specificity and to compare protein expression profiles, thereby supporting the identity of nanoalgosomes [27]. Following electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for 1 h at room temperature in 3% BSA prepared in TBS-T (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). Primary antibodies against H⁺-ATPase (dil. 1:1000 in 5% Milk/TBS-T1X, Agrisera), Alix (clone 3A9, dil. 1:150 in 3% BSA/TBS-T1X, Santa Cruz), were incubated overnight at 4°C and 2 h at room temperature. After washing steps, membranes were incubated for 1 h with species-specific HRP-conjugated secondary antibodies (Cell Signaling). Blots were then washed thoroughly (four washes for 20 min, in TBS-T), and signal detection was carried out using the SuperSignal Pierce ECL (Thermo Fisher Scientific).

DetectEV assay

To assess the membrane integrity and bioactivity of nanoalgosomes, the DetectEV assay [28] was used. This method quantifies the intrinsic esterase-like activity within the lumen of EVs. The assay employs fluorescein diacetate (FDA), a lipophilic, non-fluorescent substrate that passively diffuses across intact lipid bilayers. Within intact vesicles, internal enzymes cleave FDA to produce fluorescein, a green-fluorescent molecule that accumulates in the lumen. For the assay, 2×10^{10} nanoalgosomes were added in triplicate to a 96-well plate and incubated with 18 µM FDA. The final reaction volume was adjusted to 200 µL with 0.2 µm-filtered PBS (without Ca²⁺ and Mg²⁺). Fluorescence (ex/em: 490/514 nm) was monitored continuously for 180 minutes using a GloMax Discover Microplate Reader (Promega). Specific enzymatic activity was quantified by comparison to a standard curve of fluorescein (0–300 nM). Activity is reported in units (U), defined as nM of fluorescein generated per minute (nM/min), calculated from the linear phase of the reaction and normalized to the total assay duration.

Cell culture

A bronchial epithelial–macrophage co-culture model was established using the human bronchial epithelial cell line Calu-3 (ATCC, HTB-55) and differentiated macrophages derived from the human monocytic cell line THP-1 (ATCC, TIB-202) to investigate the anti-inflammatory and antioxidant properties of nanoalgosomes. Calu-3 cells were seeded onto 6-well Transwell inserts (Corning, CLS3450) at a density of 5×10^5 cells per insert and maintained under submerged conditions in Minimum Essential Medium (MEM) GlutaMAX™ (Gibco, 41090093) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich, F7524), 1% (v/v) Penicillin–Streptomycin (Gibco, 15140122), and 1% (v/v) Non-Essential Amino Acids (Gibco, 11140050). Cultures were incubated at 37 °C in a humidified incubator containing 5% CO₂. The medium was replaced every 2–3 days, and the inserts were gently washed with pre-warmed Hank's Balanced Salt Solution (HBSS; Gibco, 14025050) prior to medium change. After seven days of submerged culture, the apical medium was removed to establish ALI conditions. The apical surface was gently rinsed with pre-warmed HBSS the following day and every 2–3 days thereafter until exposure.

THP-1 cells were cultivated in DMEM/F-12 GlutaMAX™ medium (Gibco, 10565018) supplemented with 20% (v/v) FBS and 1% (v/v) Penicillin–Streptomycin under standard incubator conditions (37 °C, 5% CO₂). Differentiation into macrophages was induced by treating cells with 500 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, P1585) for

24 hours, followed by a 24-hour recovery period in fresh medium without PMA. One day prior to exposure, differentiated macrophages were detached using Accutase solution (Sigma-Aldrich, A6964) and 2.2×10^5 cells were carefully seeded onto the apical surface of the CALU-3 epithelial layer. After 2-hour attachment period, the suspension medium was removed, and the co-cultures were maintained overnight under ALI conditions before subsequent treatments.

ALI Exposure and Treatment Conditions

ALI exposures were performed on day 16 post-CALU-3 seeding using the VITROCELL® Cloud Alpha 6 system, which allows uniform and physiologically relevant aerosol deposition onto cultured cells. For nanoalgosome priming, 200 μ L of a freshly prepared nanoalgosome suspension (ALG; 1.2×10^{13} EVs/mL) in calcium- and magnesium-free DPBS (Gibco, 14190144), spiked with 1% HBSS, was nebulized into the exposure chamber. The aerosol cloud was allowed to settle for approximately 5 minutes to ensure even deposition onto the apical surface of the epithelial-macrophage co-cultures. Following exposure, the inserts were incubated with fresh MEM medium without phenol red (Gibco, 51200038) in the basolateral compartment under standard culture conditions for 4 hours to permit cellular uptake and response to nanoalgosomes. To evaluate antioxidant activity, the apical compartment was treated with 1 mL of 1 mM tert-butyl hydroperoxide (TBHP; Thermo Fisher Scientific, A13926) in MEM medium without phenol red for 4 or 24 hours to induce oxidative stress. For assessment of anti-inflammatory effects, 200 μ L of lipopolysaccharide solution (LPS; Santa Cruz Biotechnology, sc-221855) in ultrapure water at 125 or 250 μ g/mL was nebulized onto the apical surface of the co-culture, followed by a 24-hour incubation at 37 °C. Solvent controls (SCs) were prepared in parallel with all treatment conditions. First, all SC inserts were nebulized with 200 μ L of calcium- and magnesium-free DPBS supplemented with 1% HBSS and subsequently incubated for 4 hours. For oxidative-stress experiments, 1 mL of MEM medium without phenol red was then added to the apical compartment and inserts were incubated for 4 or 24 hours. For inflammation studies, SC inserts were instead nebulized with 200 μ L of sterile ultrapure water containing 1% HBSS and incubated for 24 hours at 37 °C. An incubator control (IC) was included by maintaining co-cultures at the ALI under standard culture conditions with fresh basolateral medium only.

Based on published data, the VITROCELL® Cloud Alpha 6 system provides a consistent aerosol deposition efficiency of approximately $15.6\% \pm 0.6\%$, ensuring uniform and reproducible delivery of nebulized materials across different experimental conditions [29]. Accordingly, the estimated deposition for nanoalgosome priming is 1.34×10^{10} EVs/cm², while for LPS concentrations of 125 and 250 μ g/mL, the corresponding deposited doses are approximately 140 and 280 ng/cm², respectively.

Cytotoxicity

At each experimental endpoint, transwell inserts were gently washed with 1 mL of pre-warmed HBSS to collect residual lactate dehydrogenase (LDH). Both the apical wash and basolateral medium were then collected and centrifuged at $250 \times g$ for 5 minutes. Cytotoxicity under all experimental conditions was subsequently evaluated using the Cytotoxicity Detection Kit^{PLUS} (Roche, 4744926001) according to the manufacturer's instructions. LDH release, which serves as a reliable indicator of membrane damage and overall cytotoxicity, was quantified by transferring 50 μ L of each sample in duplicate into a 96-well plate, followed by the addition of 50 μ L of the LDH reaction mixture. The plates were incubated at room temperature for 20 minutes to allow for color development, after which the optical density was measured at 492 nm and 620 nm using a Varioskan LUX Multimode Microplate Reader. As a positive control (PC), one insert was treated with 2% (v/v) Triton X-100 (Sigma-Aldrich, T8787) for 20 minutes prior to the end of the exposure. The combined optical density of the apical and basolateral

fractions was then used to calculate cytotoxicity as a percentage relative to the PC. All measurements were performed in three independent experiments, and each condition was analyzed in duplicate to ensure reproducibility and statistical reliability.

Transepithelial Electrical Resistance

At each experimental time point, the integrity of the CALU-3/dTHP-1 layer was assessed using an EVOM™ Epithelial Volt/Ohm Meter 3 (World Precision Instruments) equipped with 4 mm wide chopstick electrodes. This method was employed to monitor the tight junction formation and barrier function of the bronchial epithelial monolayer under different treatment conditions. Prior to the measurements, 1 mL of pre-warmed HBSS was added to both the apical and basolateral compartments of each Transwell insert to maintain electrical continuity across the membrane. The electrodes were carefully inserted, and stabilized transepithelial electrical resistance (TEER) values were recorded in triplicate from three separate points on each insert to obtain representative measurements from different regions of the insert. To normalize the TEER measurements, the resistance of a cell-free insert was subtracted from each recorded value. The corrected resistance was then multiplied by the surface area of the insert to obtain TEER values expressed in $\Omega \cdot \text{cm}^2$. The mean of the resulting data was calculated from four independent experiments and used as an indicator of epithelial barrier integrity and viability following exposure to oxidative or inflammatory stimuli.

Intracellular reactive oxygen species

The 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA; Sigma-Aldrich, 287810) assay was used to evaluate the anti-oxidative effects of nanoalgosomes in CALU-3/THP-1 co-cultures. Prior to nanoalgosome priming, transwell inserts were incubated with 50 μM H₂DCFDA in MEM without phenol red (Gibco, 51200038) for 1 hour at 37 °C to allow intracellular uptake. Following this preloading step, the inserts were exposed to nanoalgosomes and treated with TBHP as previously described to induce oxidative stress. After the treatment period, the inserts were washed, and the cells were detached using 0.25% Trypsin-EDTA (Gibco, 15400054) for 3 minutes. The cell suspensions were collected and centrifuged at $200 \times g$ for 7 minutes to pellet the cells. The resulting pellets were resuspended in DPBS and transferred to a black 96-well plate for fluorescence measurement at 488 nm excitation and 530 nm emission every 5 minutes. ROS levels were calculated as relative fluorescence units and expressed relative to control samples. Measurements were taken within the linear range of the signal (0–20 minutes), with data acquired from three technical replicates per condition across three independent experiments.

IL-8 and IL-18 quantification

The levels of the pro-inflammatory cytokine IL-8 and IL-18 were measured using the Human IL-8/CXCL8 DuoSet ELISA Kit (R&D Systems, DY208) and Human Total IL-18 DuoSet ELISA (R&D Systems, DY318) following the manufacturer's recommendations. Both kits follow identical experimental procedures. Briefly, a 96-well plate was coated with 100 μL of capture antibody and incubated overnight at room temperature. Plates were then washed three times with wash buffer consisting of 0.05% Tween-20 (Sigma-Aldrich, P9416) in DPBS (Gibco, 14190144). Wells were blocked for 1 hour with 300 μL of blocking buffer containing 10 mg/mL BSA (Sigma-Aldrich, A2153) in DPBS. Following an additional wash step, 100 μL of apical washes or standards were added to the wells and incubated for 2 hours. Standards were prepared as serial two-fold dilutions, ranging from 31.2 to 2000 pg/mL for IL-8 and from 11.7 to 750 pg/mL for IL-18. Plates were washed three times before adding 100 μL of detection antibody and incubating for an additional 2 hours. Following a fourth wash, 100 μL of

streptavidin–horseradish peroxidase was added and incubated for 20 minutes. After a final wash, 100 μ L of 3,3',5,5'-tetramethylbenzidine (TMB; Sigma-Aldrich, T0440) substrate was added and incubated for 20 minutes. The enzymatic reaction was stopped by adding 50 μ L of 2 N H₂SO₄ (Fisher Chemical, S/9240/PB15), and absorbance was measured at 450 nm with a reference wavelength of 570 nm.

Luminescent ELISA

Luminescence-based ELISA was used to quantify the levels of IL-1 β , IL-6, IL-10, and TNF- α (Promega; W6010, W6030, W6070, W6050) according to the manufacturer's guidelines. In a white 96-well plate, 50 μ L of apical washes were added in duplicate alongside seven standards, with standard curves ranging from 18.2 pg/mL to 25,000 pg/mL prepared using 3.33-fold serial dilutions. Subsequently, 50 μ L of freshly prepared antibody mixture was added to each well, and the plate contents were mixed at 300 rpm for 10 seconds. Plates were then incubated at 37 °C in a CO₂ incubator for 60 minutes. Following incubation, the plate was equilibrated to room temperature, and 25 μ L of luminescence detection reagent was added. The plate was briefly shaken at 400 rpm for 10 seconds and incubated for 4 minutes before measuring luminescence using a Varioskan LUX Multimode microplate reader (Thermo Scientific). For IL-1 β specifically, 50 μ L of each sample was assayed with seven standards ranging from 21.7 pg/mL to 40,000 pg/mL, prepared by 3.5-fold serial dilutions. The plate was also incubated for 75 minutes instead of 60 minutes, following the manufacturer's recommendations.

Statistical analysis

All assays were conducted in at least three independent experiments. Data are presented as the mean $\pm$ standard error of the mean (SEM). Statistical significance was determined using one-way analysis of variance (ANOVA) with Bonferroni multiple comparison test. Graphs and data analyses were generated using GraphPad Prism for Windows (version 10.2.3; GraphPad Software).

Results

Production and quality assessment of EV derived from the microalga *Tetraselmis chuii*

To generate a reproducible source of nanoalgosomes, an axenic culture of *T. chuii* was successfully cultivated under strictly controlled sterile conditions. At harvest, *T. chuii* culture were processed for nanoalgosome isolation using TFF. This optimized preparative process achieved over a 1000-fold enrichment of the small extracellular vesicle fraction, yielding 5 mL of purified small EVs in PBS from the initial 6 L culture volume. After TFF-based isolation, nanoalgosomes were systematically characterized using standardized quality assessment assays, as recommended by the MISEV2023 guidelines and reported in the literature [22,28,30,31] (Figure 1).

Analysis of nanoalgosomes preparations ($n = 3$) by BCA revealed a protein content of 148 ± 9 $\mu\text{g/mL}$. NTA in scattering mode showed a particle concentration of $5.6 \times 10^{12} \pm 8.7 \times 10^{11}$ particles/mL, as well as a size distribution characterized by a modal size of 123 ± 3 nm (Figure 1A). The distribution percentiles were $D_{10} = 80 \pm 4$ nm, $D_{50} = 137 \pm 3$ nm, and $D_{90} = 253 \pm 5$ nm, indicating that 10%, 50%, and 90% of the particles were below these diameters, respectively. The NTA profile also suggested a narrow size distribution, consistent with a population of small EVs. This was confirmed by DLS measurements, which showed hydrodynamic diameters (D_z) ranging from 105 ± 5 nm to 123 ± 5 nm with polydispersity indices (PDI) between 0.47 ± 0.01 and 0.49 ± 0.01 (Figure 1B).

AFM further supported these results, confirming the characteristic small EV-like morphology of nanoalgosomes (Figure 1C). In addition, protein analysis by SDS-PAGE and immunoblotting detected canonical nanoalgosome biomarkers, including H^+ -ATPase and Alix proteins (Figure 1D) [19]. Furthermore, nanoalgosomes dispersed in PBS exhibited a zeta potential value of -30 ± 4 mV, confirming the expectation of a negative surface charge, as for EVs from other cell sources (Figure 1E). Overall, these findings demonstrated that nanoalgosomes derived from *T. chuii* exhibit the characteristic size, morphology, surface charge, and molecular markers of small EVs, with consistently low batch-to-batch variability.

Moreover, to verify the functionality of the nanoalgosomes batches used in this study, the DetectEV assay was performed. The DetectEV assay quantify the enzymatic activity of intraluminal esterase-like enzymes, that is contextually a measure of vesicle membrane integrity. The results showed a bioactivity equal to 1 ± 0.1 nM/min in nanoalgosomes samples used in this study ($n = 3$). This value is fully consistent with the bioactivity range reported for high-quality nanoalgosomes preparations [28], confirming the reproducibility of the production process and the suitability of the batches used for subsequent cell experiments.

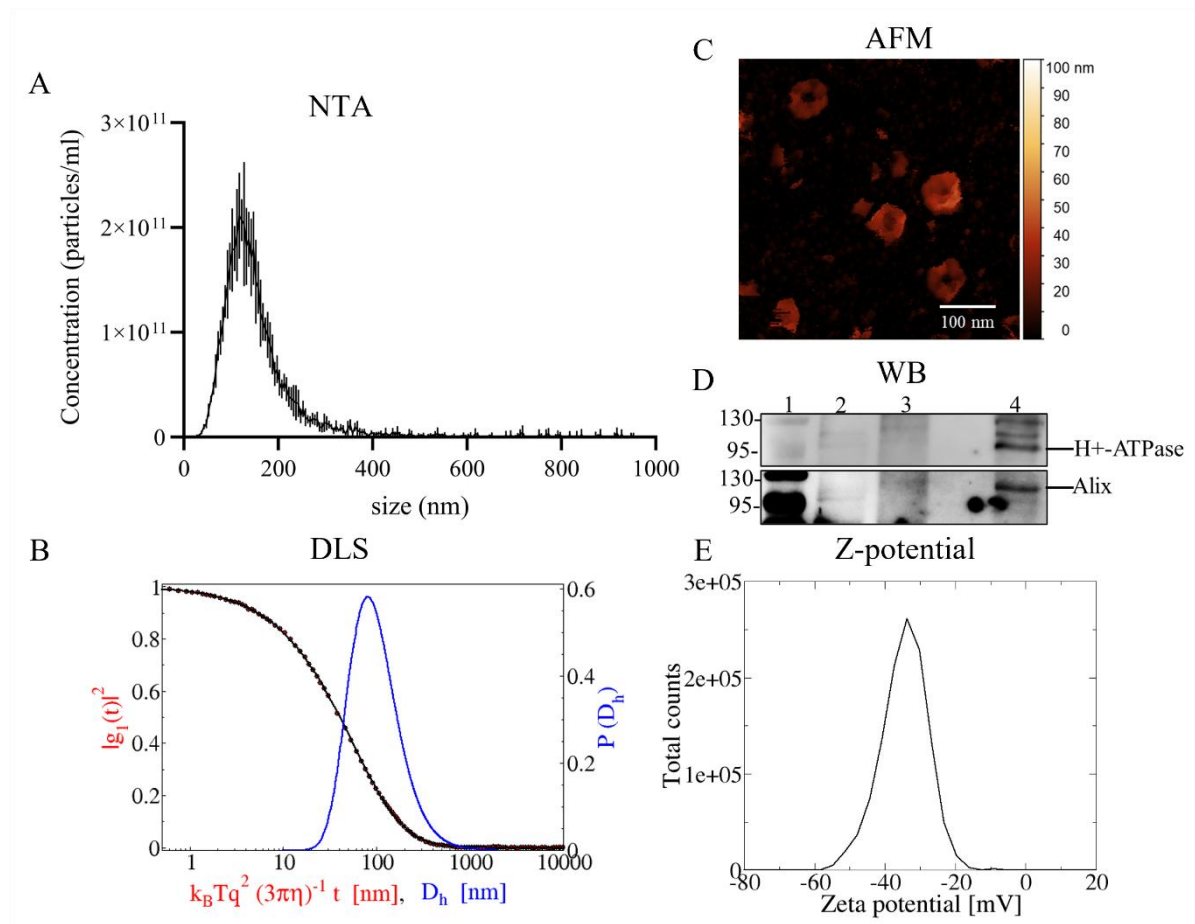


Figure 1. Nanoalgosome features. (A) concentration and size distribution were determined by NTA (error bars represent five measurements per sample) and (B) confirmed by DLS; (C) morphology was assessed by AFM; (D) expression of canonical EV markers (H⁺-ATPase and Alix) was analyzed by immunoblot in the following samples: 1) marker; 2) HEK-293 lysate; 3) *T. chuii* lysate; 4) nanoalgosomes; (E) nanoalgosome surface charge was measured as zeta potential. Representative curves and images for all analyses are shown for three independent samples.

Cytotoxicity assessment of the CALU-3/THP-1 Co-culture

Cytotoxicity was assessed by measuring LDH release as an indicator of plasma membrane damage, which results in the leakage of the cytoplasmic LDH enzyme. Pro-oxidant stimulation with TBHP caused a modest increase in LDH release after 4 hours of exposure, an effect that was attenuated when cells were pre-treated with nanoalgosomes, which on their own did not affect cell viability (Figure 2A). No significant cytotoxicity was detected after 24 hours (Figure 2B). LPS nebulization at 125 and 250 $\mu\text{g/mL}$, corresponding to a deposition of approximately 140 and 280 ng/cm^2 , resulted in an increase in cytotoxicity after 24 hours, with the effect at 250 $\mu\text{g/mL}$ reaching statistical significance compared with SC (Figure 2C). Notably, nanoalgosomes priming did not enhance cytotoxicity and instead restored LPS-induced changes to control levels.

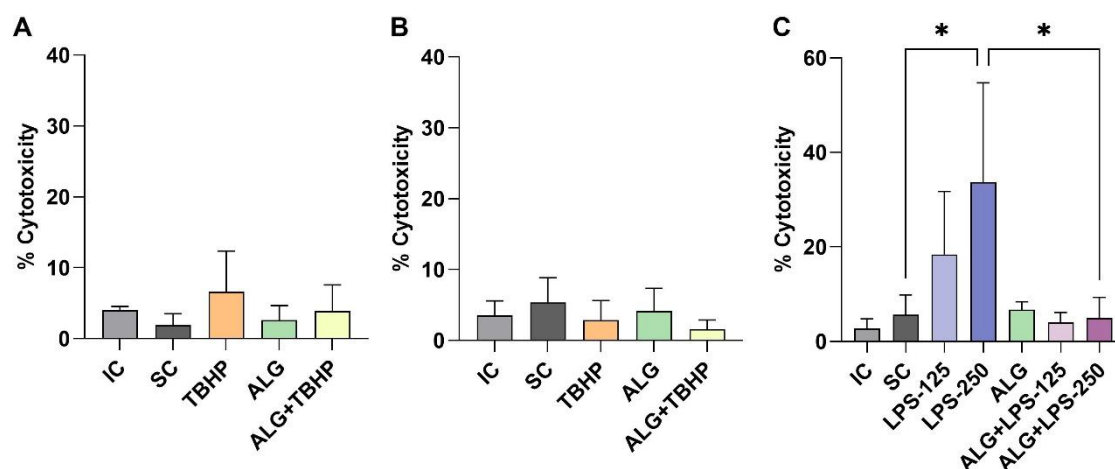


Figure 2. Cytotoxicity of co-cultured CALU-3 and dTHP-1 cells as determined by LDH release following oxidative stimuli (1mM TBHP) for 4 hours (A), 24 hours (B), or following inflammatory stimuli (125 and 250 $\mu\text{g/mL}$ LPS) for 24 hours (C). Data are expressed as mean $\pm$ SEM of at least three independent experiments. One-way ANOVA was used to determine statistically significant differences among the tested groups. (* $p < 0.05$).

Overall, neither nanoalgosome priming nor subsequent oxidative stimuli elicited significant cytotoxic effects, in contrast to the more pronounced cytotoxicity observed under inflammatory challenge. These findings highlight the safety and biocompatibility of nanoalgosomes, and support their potential suitability for airway-targeted therapeutic applications.

Integrity of the Bronchial Epithelial Barrier at the Air-Liquid Interface

Epithelial barrier integrity was evaluated by measuring the TEER in CALU-3/dTHP-1 co-cultures exposed for 4- or 24 hours to SC, TBHP, nanoalgosomes, or TBHP following nanoalgosome priming. TEER values in SC treated co-cultures remained stable at both time points, indicating intact tight junctions and functional epithelial barrier. Priming with aerosolized nanoalgosomes did not significantly alter TEER, confirming their biocompatibility and absence of disruption of airway epithelial integrity (Figure 3A, B). Exposure to 1 mM TBHP for 4- or 24 hours resulted in TEER values similar to SC, with only a modest, non-significant reduction observed in TBHP-treated cultures (SC: $1537 \pm 119.5 \Omega \cdot \text{cm}^2$ vs. TBHP: $1401 \pm 90.5 \Omega \cdot \text{cm}^2$) (Figure 3A). This indicates that oxidative stress at this concentration did not compromise epithelial barrier function, either acutely or after extended exposure. Nebulization of LPS at 125 $\mu\text{g/mL}$ and 250 $\mu\text{g/mL}$ for 24 hours did not significantly affect TEER compared to SC, with only a slight increase observed at the highest concentration (Figure 3C). Notably, this minor effect was absent in co-cultures primed with nanoalgosomes. Collectively, these results demonstrate that nanoalgosomes preserve epithelial barrier integrity under both oxidative or inflammatory challenges, further supporting their biocompatibility.

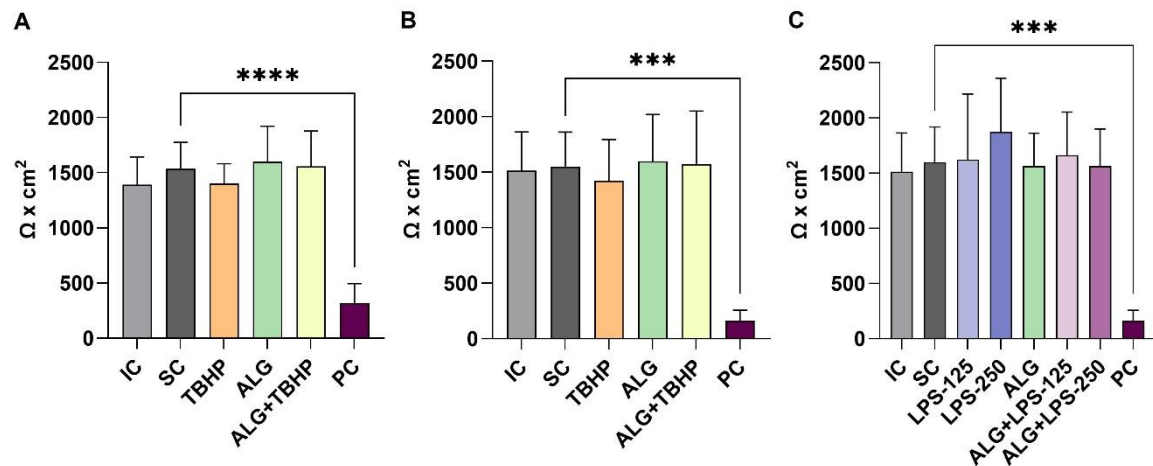


Figure 3. Effect of nanoalgosome priming on epithelial barrier integrity in CALU-3/dTHP-1 coculture. TEER values after 1mM TBHP treatment for 4 hours (A) and 24 hours (B), and in response to nebulized LPS (125 and 250 µg/mL) after 24 hours (C). Data are presented as mean ± SEM of four independent experiments. Statistical significance was determined using one-way ANOVA (**p < 0.01, ****p < 0.0001).

Effects of Nanoalgosome Priming on Intracellular ROS Production Following Oxidative Stimuli

The antioxidant activity of nanoalgosomes was evaluated by measuring intracellular ROS in CALU-3/dTHP-1 co-cultures using the H₂DCFDA assay. Nanoalgosomes treatment alone did not affect basal ROS levels (Figure 4A, B), indicating that the vesicles are non-oxidant with both macrophages and bronchial epithelial cells [22]. As expected, exposure to 1 mM TBHP for 4 hours induced a robust oxidative response, increasing intracellular ROS by approximately 4.8-fold relative to the SC. Notably, 4 hours of nanoalgosome priming markedly mitigated this response, reducing TBHP-induced ROS accumulation by 46% (Figure 4A). Although ROS levels in the ALG+TBHP group remained above control values, the reduction reflects a substantial protective effect against acute oxidative stress. At 24 hours post-treatment, ROS levels in TBHP-exposed cultures declined toward baseline (Figure 4B), consistent with endogenous antioxidant defense mechanisms over time. Even at this later time point, nanoalgosomes-primed cultures exhibited approximately 55% lower ROS levels than TBHP-treated cells, demonstrating a sustained antioxidant effect. While the magnitude of protection differed between the 4- and 24 hours measurements, the overall trend indicates that nanoalgosomes confer both early and prolonged resistance to oxidative stress.

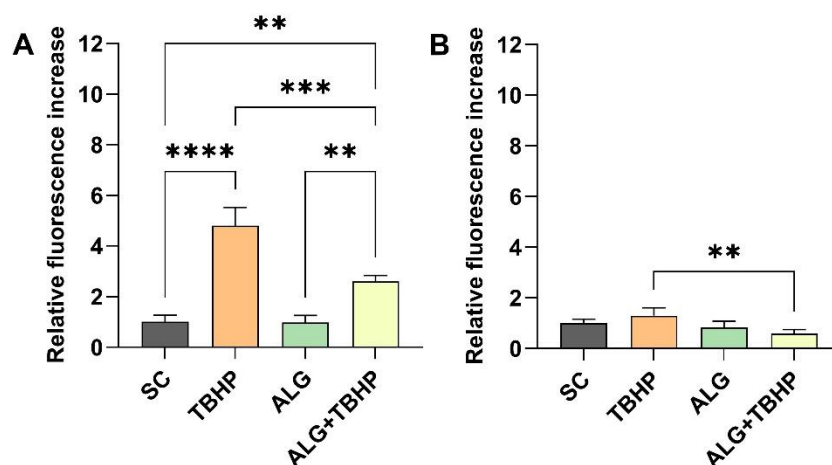


Figure 4. Intracellular ROS levels following TBHP treatment. Intracellular ROS generation was quantified using the H₂DCFDA assay and expressed as relative fluorescence intensity after exposure to 1 mM TBHP for 4 hours (A) and 24 hours (B). Data are presented as mean $\pm$ SEM from four independent experiments. Statistical significance was determined using one-way ANOVA (**p < 0.01, ***p < 0.001, ****p < 0.0001).

Taken together, these findings indicate that nanoalgosome pre-exposure significantly mitigates intracellular ROS generation in response to TBHP-induced oxidative stress. This supports the intrinsic antioxidant capacity of nanoalgosomes and their potential to preserve redox balance in airway epithelial systems.

Effects of Nanoalgosome Priming on Cytokine Secretion Induced by LPS Nebulization

To investigate the immunomodulatory properties of nanoalgosomes, cytokine secretion was quantified 24 hours after exposure to nebulized LPS (125 and 250 μ g/m). Nanoalgosome priming alone did not significantly alter the secretion of any of the six cytokines measured relative to SCs, indicating that nanoalgosomes do not disrupt basal immune signalling (Figure 5).

IL-6 levels remained statistically unchanged across all conditions, showing only a slight upward trend (Figure 5A). A similar pattern was observed for IL-8, where modest LPS-induced increases were attenuated following nanoalgosome pre-treatment (Figure 5B). In contrast, more robust pro-inflammatory responses were detected for IL-1 β , IL-18 and TNF- α : both concentrations of LPS elevated their secretion, confirming activation of the inflammatory pathway in the co-culture (Figures 5C-E). Nanoalgosome priming effectively mitigated these increases. At the higher LPS concentration (250 μ g/mL), IL-1 β , IL-18 and TNF- α levels were reduced by approximately 1.6-fold, 2.2-fold and 3.6-fold, respectively, relative to LPS alone. The anti-inflammatory cytokine IL-10 was also significantly elevated following LPS exposure (Figure 5F). Notably, nanoalgosome priming restored IL-10 secretion toward baseline, reducing LPS-induced elevations by 1.9 and 1.7-fold at 125 μ g/mL and 250 μ g/mL LPS, respectively.

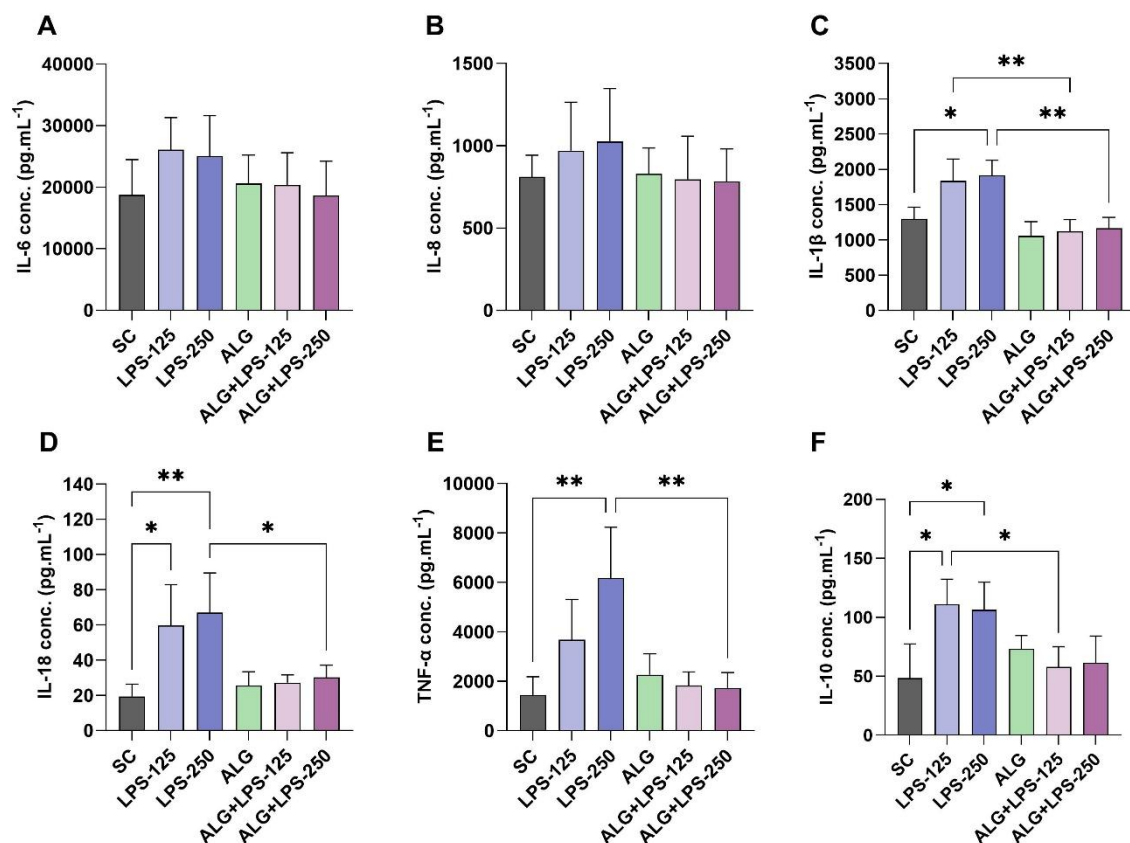


Figure 5. Cytokine secretion profile of CALU-3/dTHP-1 coculture following exposure to nebulized LPS. (A) IL-6, (B) IL-8, (C) IL-1 β , (D) IL-18, (E) TNF- α , and (F) IL-10 were quantified after exposure to 125 and 250 μ g/mL nebulized LPS solution for 24 hours. Data are presented as mean $\pm$ SEM from three independent experiments. Statistical significance was determined using one-way ANOVA (* p < 0.05, ** p < 0.01).

Overall, these findings indicate that nanoalgosomes exert a modulatory effect on both pro- and anti-inflammatory cytokine response. By attenuating both pro- and anti-inflammatory cytokine dysregulation, nanoalgosomes seem to promote a more balanced cytokine milieu, likely through the modulation of macrophage-epithelial interactions and the restoration of airway inflammatory homeostasis.

Discussion

This study demonstrates that EVs derived from *Tetraselmis chuii*, referred to as nanoalgosomes, are biocompatible and exert protective antioxidant and anti-inflammatory effects in a physiologically relevant bronchial epithelial–macrophage co-culture model. Using CALU-3 epithelial cells and PMA-differentiated THP-1 macrophages at the ALI, we showed that nanoalgosome priming did not compromise cell viability or epithelial barrier integrity, which are essential parameters for preventing pathogen invasion and progressive lung damage. These findings confirm the non-cytotoxic nature of nanoalgosomes and their compatibility with airway cellular systems. Similar observations have been observed in vitro in diverse cell lines, as well as in *C. elegans* and mice, where nanoalgosomes did not compromise cell or organism viability [19,32]. Likewise, *T. chuii* extracts have been shown to preserve endothelial barrier function in human brain microvascular endothelial cells, reinforcing the notion that *T. chuii*-derived biomaterials are non-disruptive to epithelial and endothelial structures regardless of their form [11].

The maintenance of epithelial integrity following nanoalgosome exposure may relate to the regulation of tight junction proteins, including E-cadherin, which is essential for epithelial cohesion and proliferation, in addition to the production of pro-inflammatory mediators and growth factors [33]. Recent evidence indicates that nanoalgosomes upregulate E-cadherin mRNA expression, suggesting a potential mechanism by which they may actively reinforce epithelial barrier stability [24]. This feature is particularly relevant for chronic lung diseases such as COPD, asthma, and pulmonary fibrosis, in which epithelial dysfunction and loss of barrier integrity are key pathogenic events.

Exposure to TBHP induced a pronounced increase in intracellular ROS after 4 hours, consistent with oxidative stress-mediated injury commonly reported in airway epithelial cells exposed to pollutants or inflammatory mediators [34,35]. The lack of ROS elevation at 24 hours likely reflects the activation of endogenous antioxidant defense systems over time, as ROS generation is typically an early and transient event [36,37]. Remarkably, nanoalgosome priming significantly attenuated this oxidative response, suggesting an intrinsic antioxidant capacity capable of mitigating oxidative insults. This protective effect likely arises from the bioactive cargo naturally present in *T. chuii*, including carotenoids, polyphenols, and PUFAs which are known to enhance cellular antioxidant defenses [5,6].

These antioxidant mechanisms may involve Nrf2 activation, upregulation of enzymatic defenses such as SOD, catalase, and glutathione peroxidase, and mitigation of lipid peroxidation, as reflected by reduction in malondialdehyde levels [7–9,38,10]. Consistent with this, nanoalgosomes have been shown to modulate oxidative stress-related enzymes including AKR1C2 (aldo-keto reductase family 1 member C2), FTH1 (ferritin heavy chain 1), Alox12 (arachidonate 12-Lipoxygenase), CAT (catalase), GPX1 (glutathione peroxidase 1) and GSR (glutathione reductase) in mammary epithelial cells [22]. In alveolar epithelial cells, nanoalgosomes also restored glutathione peroxidase 4 levels, further supporting their role in redox regulation [24]. Notably, the magnitude of intracellular ROS reduction observed here was comparable to that achieved using liposomes encapsulating drugs such as pirfenidone or quercetin—agents known to enhance treatment outcomes in lung fibrosis and non-small cell lung cancer [24,39–41]. Together, these findings suggest that natural nanoalgosomes can function as biologically active nanocarriers with intrinsic antioxidant properties.

The cytokine profiling further highlights the immunomodulatory potential of nanoalgosomes. LPS nebulization induced a typical pro-inflammatory response characterized by elevated IL-

1 β , IL-6, IL-8, IL-18 and TNF- α secretion, reflecting macrophage and epithelial activation [42–49]. The minimal increase in IL-6 and IL-8 may be attributed to apical LPS exposure, since Toll-Like Receptor 4, the primary receptor for LPS in bronchial epithelial cells [50], is typically expressed at low levels and predominantly localized on the basolateral side of the cells [51]. High concentrations of pro-inflammatory cytokines are characteristic of airway inflammatory conditions such as COPD, asthma, and pulmonary fibrosis [52–59]. Nanoalgosome priming markedly reduced the secretion of these cytokines, particularly IL-1 β , IL-18, and TNF- α , indicating a robust anti-inflammatory response. This attenuation is likely mediated by the inhibition of NF- κ B and MAPK signaling cascades, pathways that are known to be modulated by the polyphenolic and fatty acid components of *T. chuii* [6]. In macrophage cultures, nanoalgosomes have been shown to downregulate IL-6 expression, which aligns with our findings in a more complex epithelial–macrophage system [22]. Importantly, by normalizing TNF- α release, nanoalgosomes may help preserve epithelial tight junction integrity, since excessive TNF- α is known to disrupt barrier function and contribute to airway hyperresponsiveness and fibrosis [60].

IL-10 is a key anti-inflammatory cytokine released by both macrophages and bronchial epithelial cells in the lung [61,62]. Interestingly, IL-10 levels were elevated following LPS exposure but normalized upon nanoalgosome priming. This normalization suggests that nanoalgosomes facilitate a return to immune homeostasis by preventing excessive compensatory anti-inflammatory signaling. Given that IL-10 dysregulation is associated with chronic inflammation, COPD, and cancer, the ability of nanoalgosomes to stabilize IL-10 levels highlights their potential in rebalancing immune responses in diseased airways [63–65].

The anti-inflammatory properties of nanoalgosomes likely stem from their distinctive bioactive composition. *T. chuii* is rich in vitamins, carotenoids, polyphenols, phytosterols, and PUFAs, which may act synergistically to modulate inflammatory signaling pathways [66]. These compounds have been shown to suppress the production of key pro-inflammatory cytokines, including IFN- γ , TNF- α , and IL-1 β [7–9,12]. Recent studies further demonstrated that *T. chuii*-derived nanoalgosomes downregulate the expression of transforming growth factor- β (TGF- β) in alveolar epithelial cells, highlighting their potential to mitigate inflammation at the cellular level [24]. Given that cytokine modulation is an emerging therapeutic strategy for managing inflammatory lung diseases, nanoalgosomes may represent a multifaceted alternative to conventional cytokine-blocking therapies, offering a natural means of attenuating excessive cytokine responses [67–69].

From the perspective of therapeutic development, the natural origin, nanoscale size, and aerosolization compatibility of nanoalgosomes render them particularly suited for inhalation delivery. Aerosolized administration allows for targeted deposition in the respiratory tract, maximizing local bioavailability and minimizing systemic side effects—a major advantage over conventional systemic anti-inflammatory or antioxidant drugs. Moreover, their innate antioxidant and anti-inflammatory properties could complement or enhance the therapeutic performance of encapsulated drugs, offering opportunities for combination strategies in targeted pulmonary delivery. Notably, the effects of native nanoalgosomes were comparable to those of liposomes loaded with established drugs such as pirfenidone and quercetin, further highlighting their therapeutic promise even in the absence of additional cargo [24].

Further research is warranted to decode the molecular cargo of nanoalgosomes, including proteins, lipids, and microRNAs, which likely orchestrate their protective actions through modulating cellular signaling pathways related to oxidative stress response, inflammation, and tissue repair. Additional studies employing in vivo animal inhalation models will be essential

to validate the therapeutic efficacy, optimize dosing regimens, and evaluate safety profiles and pharmacokinetics under physiologically relevant conditions.

In summary, *T. chuii*-derived nanoalgosomes represent a new class of naturally sourced EVs capable of modulating oxidative and inflammatory processes in airway systems at the ALI. Their intrinsic bioactivity, combined with safety and scalability, highlights their potential as both therapeutic agents and delivery vehicles in the treatment of chronic lung diseases characterized by oxidative stress and inflammation, including asthma, COPD, and pulmonary fibrosis.

Conclusion

This study demonstrates that *Tetraselmis chuii*-derived nanoalgosomes are biologically active EVs following aerosolization with potent antioxidant and anti-inflammatory properties relevant to chronic lung diseases characterized by epithelial barrier disruption, oxidative stress and immune dysregulation. Nanoalgosome priming effectively protected bronchial epithelial-macrophage co-cultures at the ALI by reducing intracellular ROS and modulating key cytokines, including lowering pro-inflammatory IL-1 β , IL-18 and TNF- α levels while normalizing anti-inflammatory IL-10 under inflammatory challenges. Importantly, nanoalgosomes exhibited excellent biocompatibility and no detectable cytotoxicity, underscoring their suitability for airway-targeted applications.

The antioxidant and immunomodulatory activities are likely attributed to the rich cargo of *T. chuii* bioactive molecules—such as carotenoids, polyphenols, and polyunsaturated fatty acids—combined with the intrinsic protective and delivery properties of EVs. Given their natural origin, scalability, and compatibility with aerosol-based administration, nanoalgosomes represent a promising therapeutic nanoplatform capable of restoring redox balance and immune homeostasis within the lung microenvironment.

Further investigations should delineate the molecular pathways underlying nanoalgosome-mediated bioactivity and confirm their therapeutic efficacy, pharmacokinetics, and safety in vivo using preclinical inhalation models. Collectively, these findings lay the groundwork for developing nanoalgosome-based inhalable therapeutics as a novel, biologically derived strategy for managing chronic inflammatory and oxidative lung diseases such as asthma, COPD, and pulmonary fibrosis.

Acknowledgments

The authors would like to thank Nicolas Touzet for his assistance in establishing the microalgal cultures and the TFF setup, and Paolo Bergese for his insightful discussions on technical and conceptual aspects throughout the H2020 BOW project.

Funding

This work was supported by the BOW - Biogenic Organotropic Wetsuits - project funded by the European Union's Horizon 2020 research and innovation programme, under grant agreements no. 952183.

Author contributions

Wesam Darwish: Methodology, Validation, Formal analysis, Investigation, Writing – Original Draft, Visualization. **Giorgia Adamo:** Methodology, Validation, Investigation, Writing –

Review & Editing. **Mohammad Almasaleekh**: Validation, Investigation, Writing – Review & Editing. **Sabrina Picciotto**: Validation, Investigation, Writing – Review & Editing, Visualization. **Paola Gargano**: Validation, Investigation, Writing – Review & Editing. **Daniele P. Romancino**: Formal analysis, Writing – Review & Editing. **Samuele Raccosta**: Validation, Formal analysis, Investigation, Writing – Review & Editing. **Ralf Zimmermann**: Resources, Writing – Review & Editing, Supervision. **Mauro Manno**: Resources, Writing – Review & Editing, Supervision. **Antonella Bongiovanni**: Conceptualization, Methodology, Validation, Resources, Writing – Review & Editing, Supervision, Project administration, Funding acquisition. **Sebastiano Di Bucchianico**: Conceptualization, Methodology, Validation, Formal analysis, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

Competing interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Antonella Bongiovanni reports a relationship with EVEBiofactory srl that includes: board membership. Mauro Manno reports a relationship with EVEBiofactory srl that includes: board membership. Antonella Bongiovanni has patent #PCT/EP2020/086622 issued to CNR. Mauro Manno has patent #PCT/EP2020/086622 issued to CNR. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Albano GD, Gagliardo RP, Montalbano AM, Profita M. Overview of the Mechanisms of Oxidative Stress: Impact in Inflammation of the Airway Diseases. *Antioxidants*. 2022;11:2237. <https://doi.org/10.3390/antiox11112237>
2. Boulet L-P. Airway remodeling in asthma: update on mechanisms and therapeutic approaches. *Curr Opin Pulm Med*. 2018;24:56–62. <https://doi.org/10.1097/MCP.0000000000000441>
3. Tiotiu A, Zounemat Kermani N, Badi Y, Pavlidis S, Hansbro PM, Guo Y-K, et al. Sputum macrophage diversity and activation in asthma: Role of severity and inflammatory phenotype. *Allergy*. 2021;76:775–88. <https://doi.org/10.1111/all.14535>
4. Bezerra FS, Lanzetti M, Nesi RT, Nagato AC, Silva CP e, Kennedy-Feitosa E, et al. Oxidative Stress and Inflammation in Acute and Chronic Lung Injuries. *Antioxidants*. 2023;12:548. <https://doi.org/10.3390/antiox12030548>
5. Cokdinleyen M, Alvarez-Rivera G, Tejera JLG, Mendiola JA, Valdés A, Kara H, et al. Tetraselmis chuii Edible Microalga as a New Source of Neuroprotective Compounds Obtained Using Fast Biosolvent Extraction. *Int J Mol Sci*. 2024;25:3897. <https://doi.org/10.3390/ijms25073897>
6. Cocksedge SP, Mantecón L, Castaño E, Infante C, Bailey SJ. The Potential of Superoxide Dismutase-Rich Tetraselmis chuii as a Promoter of Cellular Health. *Int J Mol Sci*. 2025;26:1693. <https://doi.org/10.3390/ijms26041693>
7. Sharp M, Sahin K, Stefan M, Orhan C, Gheith R, Reber D, et al. Phytoplankton Supplementation Lowers Muscle Damage and Sustains Performance across Repeated Exercise Bouts in Humans and Improves Antioxidant Capacity in a Mechanistic Animal. *Nutrients*. 2020;12:1990. <https://doi.org/10.3390/nu12071990>
8. Sharp M, Wilson J, Stefan M, Gheith R, Lowery R, Ottinger C, et al. Marine phytoplankton improves recovery and sustains immune function in humans and lowers proinflammatory immunoregulatory cytokines in a rat model. *Phys Act Nutr*. 2021;25:42–55. <https://doi.org/10.20463/pan.2021.0007>
9. Gil-Cardoso K, Del Bas JM, Caimari A, Lama C, Torres S, Mantecón L, et al. TetraSOD®, a Unique Marine Microalgae Ingredient, Promotes an Antioxidant and Anti-Inflammatory Status in a Metabolic Syndrome-Induced Model in Rats. *Nutrients*. 2022;14:4028. <https://doi.org/10.3390/nu14194028>
10. Cocksedge SP, Infante C, Torres S, Lama C, Mantecón L, Manchado M, et al. Tetraselmis chuii Supplementation Increases Skeletal Muscle Nuclear Factor Erythroid 2-Related Factor 2 and Antioxidant Enzyme Gene Expression, and Peak Oxygen Uptake in Healthy Adults: A Randomised Crossover Trial. *Antioxidants*. 2025;14:435. <https://doi.org/10.3390/antiox14040435>
11. Cokdinleyen M, Valdés A, Kara H, Ibáñez E, Cifuentes A. Neuroprotective Potential of Tetraselmis chuii Compounds: Insights into Blood–Brain Barrier Permeability and Intestinal Transport. *Pharmaceuticals*. 2025;18:629. <https://doi.org/10.3390/ph18050629>
12. Hong S-C, Kim J-W, Lee EH, Hong CR, Choi J, Chang P-S, et al. Mechanism of dry eye syndrome improvement by Tetraselmis chuii: Anti-inflammatory effects and ocular mucous membrane restoration. *J Appl Phycol*. 2025;37:3865–3880. <https://doi.org/10.1007/s10811-025-03598-7>
13. Jung JH, Kang SA, Park J-H, Kim S-D, Yu HS, Mun SJ, et al. Immunomodulatory Effect of Adipose Stem Cell-Derived Extra-Cellular Vesicles on Cytokine Expression and Regulatory T Cells in Patients with Asthma. *Int J Mol Sci*. 2024;25:10524. <https://doi.org/10.3390/ijms251910524>

14. Meng Q, Winston T, Ma J, Song Y, Wang C, Yang J, et al. Induced pluripotent stem cell-derived mesenchymal stem cells-derived extracellular vesicles attenuate LPS-induced lung injury and endotoxemia in mice. *Shock* Augusta Ga. 2024;62:294–303.
<https://doi.org/10.1097/SHK.0000000000002381>
15. Gao Y, Liu M-F, Li Y, Liu X, Cao Y-J, Long Q-F, et al. Mesenchymal stem cells-extracellular vesicles alleviate pulmonary fibrosis by regulating immunomodulators. *World J Stem Cells*. 2024;16:670–89.
<https://doi.org/10.4252/wjsc.v16.i6.670>
16. Xu N, Shao Y, Ye K, Qu Y, Memet O, He D, et al. Mesenchymal stem cell-derived exosomes attenuate phosgene-induced acute lung injury in rats. *Inhal Toxicol*. 2019;31:52–60.
<https://doi.org/10.1080/08958378.2019.1597220>
17. Gao Y, Huang X, Lin H, Zhao M, Liu W, Li W, et al. Adipose mesenchymal stem cell-derived antioxidative extracellular vesicles exhibit anti-oxidative stress and immunomodulatory effects under PM2.5 exposure. *Toxicology*. 2021;447:152627. <https://doi.org/10.1016/j.tox.2020.152627>
18. Liu C, Xi L, Liu Y, Mak JCW, Mao S, Wang Z, et al. An Inhalable Hybrid Biomimetic Nanoplatfrom for Sequential Drug Release and Remodeling Lung Immune Homeostasis in Acute Lung Injury Treatment. *ACS Nano*. 2023;17:11626–44. <https://doi.org/10.1021/acsnano.3c02075>
19. Adamo G, Fierli D, Romancino DP, Picciotto S, Barone ME, Aranyos A, et al. Nanoalgosomes: Introducing extracellular vesicles produced by microalgae. *J Extracell Vesicles*. 2021;10:e12081. <https://doi.org/10.1002/jev2.12081>
20. Picciotto S, Barone ME, Fierli D, Aranyos A, Adamo G, Božič D, et al. Isolation of extracellular vesicles from microalgae: towards the production of sustainable and natural nanocarriers of bioactive compounds. *Biomater Sci*. 2021;9:2917–30. <https://doi.org/10.1039/D0BM01696A>
21. Paterna A, Rao E, Adamo G, Raccosta S, Picciotto S, Romancino D, et al. Isolation of Extracellular Vesicles From Microalgae: A Renewable and Scalable Bioprocess. *Front Bioeng Biotechnol*. 2022;10:836747. <https://doi.org/10.3389/fbioe.2022.836747>
22. Adamo G, Santonicola P, Picciotto S, Gargano P, Nicosia A, Longo V, et al. Extracellular vesicles from the microalga *Tetraselmis chuii* are biocompatible and exhibit unique bone tropism along with antioxidant and anti-inflammatory properties. *Commun Biol*. 2024;7:941.
<https://doi.org/10.1038/s42003-024-06612-9>
23. Gargano P, Picciotto S, Paterna A, Raccosta S, Rao E, Romancino DP, et al. Extracellular vesicles from microalgae as natural bioactive agents for UVB protection, anti-aging, and skin depigmentation. *Biomed Pharmacother*. 2026;194:118961. <https://doi.org/10.1016/j.biopha.2025.118961>
24. Conlon T, Schaaf M, Mateos-Maroto A, Picciotto S, Morsbach S, Adamo G, et al. Comparative effects of extracellular vesicles and liposomal nanocarriers on bleomycin-induced stress in A549 human adenocarcinoma cells. *Biomed Pharmacother*. 2025;187:118081.
<https://doi.org/10.1016/j.biopha.2025.118081>
25. Braakhuis HM, Gremmer ER, Bannuscher A, Drasler B, Keshavan S, Rothen-Rutishauser B, et al. Transferability and reproducibility of exposed air-liquid interface co-culture lung models. *NanoImpact*. 2023;31:100466. <https://doi.org/10.1016/j.impact.2023.100466>
26. Hutter JL, Bechhoefer J. Calibration of atomic-force microscope tips. *Rev Sci Instrum*. 1993;64:1868–73. <https://doi.org/10.1063/1.1143970>

27. Picciotto S, Belmonte D, Gargano P, Adamo G, Paterna A, Rao E, et al. Optimizing sustainable production of high-quality microalgae-derived extracellular vesicles through batch-refeed perfusion cultivation. *J Biotechnol.* 2025;406:236–43. <https://doi.org/10.1016/j.jbiotec.2025.07.016>
28. Adamo G, Picciotto S, Gargano P, Paterna A, Raccosta S, Rao E, et al. DetectEV: A functional enzymatic assay to assess integrity and bioactivity of extracellular vesicles. *J Extracell Vesicles.* 2025;14:e70030. <https://doi.org/10.1002/jev2.70030>
29. Zimmermann EJ, Candeias J, Gawlitta N, Bisig C, Binder S, Pantzke J, et al. Biological impact of sequential exposures to allergens and ultrafine particle-rich combustion aerosol on human bronchial epithelial BEAS-2B cells at the air liquid interface. *J Appl Toxicol.* 2023;43:1225–41. <https://doi.org/10.1002/jat.4458>
30. Welsh JA, Goberdhan DCI, O'Driscoll L, Buzas EI, Blenkiron C, Bussolati B, et al. Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. *J Extracell Vesicles.* 2024;13:e12404. <https://doi.org/10.1002/jev2.12404>
31. Loria F, Picciotto S, Adamo G, Zendrini A, Raccosta S, Manno M, et al. A decision-making tool for navigating extracellular vesicle research and product development. *J Extracell Vesicles.* 2024;13:e70021. <https://doi.org/10.1002/jev2.70021>
32. Picciotto S, Santonicola P, Paterna A, Rao E, Raccosta S, Romancino DP, et al. Extracellular Vesicles From Microalgae: Uptake Studies in Human Cells and *Caenorhabditis elegans*. *Front Bioeng Biotechnol.* 2022;10:830189. <https://doi.org/10.3389/fbioe.2022.830189>
33. Nawijn MC, Hackett TL, Postma DS, van Oosterhout AJM, Heijink IH. E-cadherin: gatekeeper of airway mucosa and allergic sensitization. *Trends Immunol.* 2011;32:248–55. <https://doi.org/10.1016/j.it.2011.03.004>
34. van der Vliet A, Janssen-Heininger YMW, Anathy V. Oxidative stress in chronic lung disease: From mitochondrial dysfunction to dysregulated redox signaling. *Mol Aspects Med.* 2018;63:59–69. <https://doi.org/10.1016/j.mam.2018.08.001>
35. Hammond J, Maher BA, Gonet T, Bautista F, Allsop D. Oxidative Stress, Cytotoxic and Inflammatory Effects of Urban Ultrafine Road-Deposited Dust from the UK and Mexico in Human Epithelial Lung (Calu-3) Cells. *Antioxidants.* 2022;11:1814. <https://doi.org/10.3390/antiox11091814>
36. Oh JM, Jung YS, Jeon BS, Yoon BI, Lee KS, Kim BH, et al. Evaluation of hepatotoxicity and oxidative stress in rats treated with *tert*-butyl hydroperoxide. *Food Chem Toxicol.* 2012;50:1215–21. <https://doi.org/10.1016/j.fct.2012.01.031>
37. Váňová N, Múčková L, Kalíšková T, Lochman L, Bzonek P, Švec F. In Vitro Evaluation of Oxidative Stress Induced by Oxime Reactivators of Acetylcholinesterase in HepG2 Cells. *Chem Res Toxicol.* 2023;36:1912–20. <https://doi.org/10.1021/acs.chemrestox.3c00203>
38. Andrés CMC, Pérez de la Lastra JM, Juan CA, Plou FJ, Pérez-Lebeña E. Antioxidant Metabolism Pathways in Vitamins, Polyphenols, and Selenium: Parallels and Divergences. *Int J Mol Sci.* 2024;25:2600. <https://doi.org/10.3390/ijms25052600>
39. Meng H, Xu Y. Pirfenidone-loaded liposomes for lung targeting: preparation and in vitro/in vivo evaluation. *Drug Des Devel Ther.* 2015;9:3369–76. <https://doi.org/10.2147/DDDT.S84046>
40. Riaz MK, Zhang X, Wong KH, Chen H, Liu Q, Chen X, et al. Pulmonary delivery of transferrin receptors targeting peptide surface-functionalized liposomes augments the chemotherapeutic effect

of quercetin in lung cancer therapy. *Int J Nanomedicine*. 2019;14:2879–902.
<https://doi.org/10.2147/IJN.S192219>

41. Parvathaneni V, Kulkarni NS, Shukla SK, Farrales PT, Kunda NK, Muth A, et al. Systematic Development and Optimization of Inhalable Pirfenidone Liposomes for Non-Small Cell Lung Cancer Treatment. *Pharmaceutics*. 2020;12:206. <https://doi.org/10.3390/pharmaceutics12030206>
42. Mattoli S, Colotta F, Fincato G, Mezzetti M, Mantovani A, Patalano F, et al. Time course of IL1 and IL6 synthesis and release in human bronchial epithelial cell cultures exposed to toluene diisocyanate. *J Cell Physiol*. 1991;149:260–8. <https://doi.org/10.1002/jcp.1041490212>
43. Pirhonen J, Sareneva T, Julkunen I, Matikainen S. Virus infection induces proteolytic processing of IL-18 in human macrophages via caspase-1 and caspase-3 activation. *Eur J Immunol*. 2001;31:726–33. [https://doi.org/10.1002/1521-4141\(200103\)31:3%253C726::AID-IMMU726%253E3.0.CO;2-5](https://doi.org/10.1002/1521-4141(200103)31:3%253C726::AID-IMMU726%253E3.0.CO;2-5)
44. Pease JE, Sabroe I. The Role of Interleukin-8 and its Receptors in Inflammatory Lung Disease. *Am J Respir Med*. 2002;1:19–25. <https://doi.org/10.1007/BF03257159>
45. Fehrenbach H, Zissel G, Goldmann T, Tschernig T, Vollmer E, Pabst R, et al. Alveolar macrophages are the main source for tumour necrosis factor- α in patients with sarcoidosis. *Eur Respir J*. 2003;21:421–8. <https://doi.org/10.1183/09031936.03.00083002>
46. Gubernatorova EO, Gorshkova EA, Namakanova OA, Zvartsev RV, Hidalgo J, Drutskaya MS, et al. Non-redundant Functions of IL-6 Produced by Macrophages and Dendritic Cells in Allergic Airway Inflammation. *Front Immunol*. 2018;9:2718. <https://doi.org/10.3389/fimmu.2018.02718>
47. Peiró T, Patel DF, Akthar S, Gregory LG, Pyle CJ, Harker JA, et al. Neutrophils drive alveolar macrophage IL-1 β release during respiratory viral infection. *Thorax*. 2018;73:546–56. <https://doi.org/10.1136/thoraxjnl-2017-210010>
48. Li M, Li A, Huang H, Munson J, Obadan A, Fuller DH, et al. Impact of progesterone on innate immunity and cell death after influenza A virus H1N1 2009 infection of lung and placental cells in vitro. *Front Virol*. 2022;2:953208. <https://doi.org/10.3389/fviro.2022.953208>
49. Okuma N, Ito M, Shimizu T, Hasegawa A, Ohmori S, Yoshida K, et al. Amplification of poly(I:C)-induced interleukin-6 production in human bronchial epithelial cells by priming with interferon- γ . *Sci Rep*. 2023;13:21067. <https://doi.org/10.1038/s41598-023-48422-9>
50. Guillot L, Medjane S, Le-Barillec K, Balloy V, Danel C, Chignard M, et al. Response of Human Pulmonary Epithelial Cells to Lipopolysaccharide Involves Toll-like Receptor 4 (TLR4)-dependent Signaling Pathways: Evidence for an intracellular compartmentalization of TLR4*. *J Biol Chem*. 2004;279:2712–8. <https://doi.org/10.1074/jbc.M305790200>
51. McClure R, Massari P. TLR-Dependent Human Mucosal Epithelial Cell Responses to Microbial Pathogens. *Front Immunol*. 2014;5:386. <https://doi.org/10.3389/fimmu.2014.00386>
52. Yamamoto C, Yoneda T, Yoshikawa M, Fu A, Tokayama T, Tsukaguchi K, et al. Airway Inflammation in COPD Assessed by Sputum Levels of Interleukin-8*. *Chest*. 1997;112:505–10. <https://doi.org/10.1378/chest.112.2.505>
53. Gibson PG, Simpson JL, Saltos N. Heterogeneity of Airway Inflammation in Persistent Asthma: Evidence of Neutrophilic Inflammation and Increased Sputum Interleukin-8. *Chest*. 2001;119:1329–36. <https://doi.org/10.1378/chest.119.5.1329>

54. Mukhopadhyay S, Hoidal JR, Mukherjee TK. Role of TNF α in pulmonary pathophysiology. *Respir Res.* 2006;7:125. <https://doi.org/10.1186/1465-9921-7-125>
55. Rincon M, Irvin CG. Role of IL-6 in Asthma and Other Inflammatory Pulmonary Diseases. *Int J Biol Sci.* 2012;8:1281–90. <https://doi.org/10.7150/ijbs.4874>
56. Dima E, Koltsida O, Katsaounou P, Vakali S, Koutsoukou A, Koulouris NG, et al. Implication of Interleukin (IL)-18 in the pathogenesis of chronic obstructive pulmonary disease (COPD). *Cytokine.* 2015;74:313–7. <https://doi.org/10.1016/j.cyto.2015.04.008>
57. Yang L, Herrera J, Gilbertsen A, Xia H, Smith K, Benyumov A, et al. IL-8 mediates idiopathic pulmonary fibrosis mesenchymal progenitor cell fibrogenicity. *Am J Physiol - Lung Cell Mol Physiol.* 2018;314:L127–36. <https://doi.org/10.1152/ajplung.00200.2017>
58. Osei ET, Brandsma C-A, Timens W, Heijink IH, Hackett T-L. Current perspectives on the role of interleukin-1 signalling in the pathogenesis of asthma and COPD. *Eur Respir J.* 2020;55:1900563. <https://doi.org/10.1183/13993003.00563-2019>
59. Stancil IT, Michalski JE, Hennessy CE, Hatakka KL, Yang IV, Kurche JS, et al. Interleukin-6–dependent epithelial fluidization initiates fibrotic lung remodeling. *Sci Transl Med.* 2022;14:eabo5254. <https://doi.org/10.1126/scitranslmed.abo5254>
60. Hardyman MA, Wilkinson E, Martin E, Jayasekera NP, Blume C, Swindle EJ, et al. TNF- α –mediated bronchial barrier disruption and regulation by src-family kinase activation. *J Allergy Clin Immunol.* 2013;132:665–675.e8. <https://doi.org/10.1016/j.jaci.2013.03.005>
61. Bonfield TL, Konstan MW, Burfeind P, Panuska JR, Hilliard JB, Berger M. Normal bronchial epithelial cells constitutively produce the anti-inflammatory cytokine interleukin-10, which is downregulated in cystic fibrosis. *Am J Respir Cell Mol Biol.* 1995;13:257–61. <https://doi.org/10.1165/ajrcmb.13.3.7544594>
62. Kawano H, Kayama H, Nakama T, Hashimoto T, Umemoto E, Takeda K. IL-10-producing lung interstitial macrophages prevent neutrophilic asthma. *Int Immunol.* 2016;28:489–501. <https://doi.org/10.1093/intimm/dxw012>
63. Haase MG, Klawitter A, Geyer P, Baretton GB. Expression of the Immunomodulator IL-10 in Type I Pneumocytes of the Rat: Alterations of IL-10 Expression in Radiation-induced Lung Damage. *J Histochem Cytochem.* 2007;55:1167–72. <https://doi.org/10.1369/jhc.7A7173.2007>
64. Sun L, Louie MC, Vannella KM, Wilke CA, LeVine AM, Moore BB, et al. New concepts of IL-10-induced lung fibrosis: fibrocyte recruitment and M2 activation in a CCL2/CCR2 axis. *Am J Physiol - Lung Cell Mol Physiol.* 2011;300:L341–53. <https://doi.org/10.1152/ajplung.00122.2010>
65. Jiang S, Shan F, Zhang Y, Jiang L, Cheng Z. Increased serum IL-17 and decreased serum IL-10 and IL-35 levels correlate with the progression of COPD. *Int J Chron Obstruct Pulmon Dis.* 2018;13:2483–94. <https://doi.org/10.2147/COPD.S167192>
66. Conlon T, Herbert H, Campion E, Touzet N. Bioactivity of autotrophic and mixotrophic *Tetraselmis chuii* (CCAP 66/21B) extracts against H₂O₂-mediated stress in A549 cells. *Process Biochem.* 2025;155:117–27. <https://doi.org/10.1016/j.procbio.2025.05.007>
67. Malaviya R, Laskin JD, Laskin DL. Anti-TNF α Therapy in Inflammatory Lung Diseases. *Pharmacol Ther.* 2017;180:90–8. <https://doi.org/10.1016/j.pharmthera.2017.06.008>

68. Yuan B, Clowers MJ, Velasco WV, Peng S, Peng Q, Shi Y, et al. Targeting IL-1 β as an immunopreventive and therapeutic modality for *K-ras*–mutant lung cancer. JCI Insight. 2022;7:e157788. <https://doi.org/10.1172/jci.insight.157788>

69. Zhang J, Veeramachaneni N. Targeting interleukin-1 β and inflammation in lung cancer. Biomark Res. 2022;10. <https://doi.org/10.1186/s40364-021-00341-5>