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Therapeutic potential of 18-β-glycyrrhetinic acid-loaded poly (lactic-co-glycolic acid) nanoparticles on cigarette smoke-induced *in-vitro* model of COPD

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is strongly linked to cigarette smoke, which contains toxins that induce oxidative stress and airway inflammation, ultimately leading to premature airway epithelial cell senescence and exacerbating COPD progression. Current treatments for COPD are symptomatic and hampered by limited efficacy and severe side effects. This highlights the need to search for an optimal therapeutic candidate to address the root causes of these conditions. This study investigates the possible potential of poly (lactic-co-glycolic acid) (PLGA)-based nanoparticles encapsulating the plant-based bioactive compound 18-β-glycyrrhetinic acid (18βGA) as a strategy to intervene in cigarette smoke extract (CSE)-induced oxidative stress, inflammation, and senescence, *in vitro*. We prepared 18βGA-PLGA nanoparticles, and assessed their effects on cell viability, reactive oxygen species (ROS) production, anti-senescence properties (expression of senescence-associated β galactosidase and p21 mRNA), and expression of pro-inflammatory genes (CXCL-1, IL-6, TNF-α) and inflammation-related proteins (IL-8, IL-15, RANTES, MIF). The highest non-toxic concentration of 18βGA-PLGA nanoparticles to healthy human broncho epithelial cell line BCiNS1.1 was identified as 5 μM. These nanoparticles effectively mitigated cigarette smoke-induced inflammation, reduced ROS production, protected against cellular

Abbreviations: IL-6, Interleukin-6; TNF-α, Tumour necrosis factor-alpha; IL-8, Interleukin-8; IL-15, Interluekin-15; CXCL1, Chemokine C-X-C motif ligand 1; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; MIF, Macrophage inhibitory factor; RANTES, Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted; CDKN1A p21, Cyclin-dependent kinase inhibitor 1; SA-β-Gal, Senescence-Associated β-galactosidase; 18βGA, 18-β-glycyrrhetinic acid; 18βGA-PLGA, 18-β-glycyrrhetinic acid, Poly Lactic-co-Glycolic Acid; NF- κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; PLGA, poly lactic-co-glycolic acid; COPD, Chronic Obstructive Respiratory Diseases; CSE, Cigarette Smoke Extract; ROS, Reactive Oxygen Species; LAMA, Long-acting muscarinic antagonists; LABA, Long-acting beta2 agonists; ICS, Inhaled corticosteroids; Nrf2/HO-1, Nuclear Factor Erythroid 2-Related Factor 2 / Heme Oxygenase-1; PEG, Polyethylene Glycol; PEI, Polyethyleneimine; PEO, Polyethylene Oxide; PVA, Polyvinyl Alcohol; BCi-NS1.1, Human airway-derived epithelial cells; DMSO, Dimethyl sulphoxide; DCF-DA, Dichlorodihydrofluorescein diacetate; X-gal, Beta galactosidase staining; MTT, 3–4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide.

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aging, and counteracted the effects of CSE on the expression of the inflammation-related genes and proteins. This study underscores the potential of 18βGA encapsulated in PLGA nanoparticles as a promising therapeutic approach to alleviate cigarette smoke-induced oxidative stress, inflammation, and senescence. Further research is needed to explore the translational potential of these findings in clinical and *in vivo* settings.

1. Introduction

COPD is a progressive respiratory ailment characterised by chronic bronchitis and emphysema, often leading to persistent airflow limitation and a diminished quality of life [1]. The significance of COPD cannot be overstated, as it ranks among the leading causes of morbidity and mortality worldwide, placing a substantial economic and healthcare burden on society [2]. COPD stands as the third leading cause of global mortality, claiming around 3 million lives annually. It is estimated that the annual deaths of COPD will increase up to 5.6 million by the year 2060 [3]. The Australian Institute of Health and Welfare (AIHW) reported that 638,000 of Australia's population were diagnosed with COPD in 2022, causing around 7691 deaths [4]. In Australia, individuals aged 65 years and over exhibit the highest COPD prevalence, with one in fourteen (7.0 %) affected, surpassing all other age cohorts [5]. According to AIHW, 87 % of those diagnosed with COPD anticipate developing one or more other chronic diseases such as behavioural difficulties (49 %), arthritis (45 %), and asthma (42 %). Additionally, COPD imposes a substantial economic burden with $836 million spent on the management and treatment of COPD in 2021–2022 alone [4]. Cigarette smoke is the primary player in the pathogenesis of COPD, serving as a potent trigger for inflammation and oxidative stress in the lungs [6,7]. Its role in exacerbating COPD is well-documented, and understanding the mechanisms underlying this relationship is crucial for devising effective treatments and preventive measures. Adults aged 18 years and over who currently smoke daily demonstrate a higher likelihood of COPD occurrence compared to ex-smokers (8.1 % versus 4.4 %) or never-smokers (8.1 % versus 1.6 %) [3].

Cigarette smoke contains numerous toxicants that could activate inflammatory, oxidative, and carcinogenic pathways in the lung tissue [6,8,9]. Cigarette smoke-induced COPD progression involves damage or activation of various cells such as airway epithelial cells, neutrophils, and macrophages, that release massive amounts of biological mediators responsible for oxidative stress, inflammation, and senescence [10]. This activation leads to the release of pro-inflammatory cytokines such as interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), interleukin-8 (IL-8), and chemokine (C-X-C motif) ligand 1 (CXCL1) among others [11,12]. These cytokines further amplify the inflammatory response, causing tissue damage, airway remodelling, and the characteristic symptoms of COPD, including shortness of breath, wheezing, and chest tightness [13]. The presence of a sustained and prolonged inflammatory status, which is commonly observed in the airways of cigarette smokers, is one of the main triggers of cell senescence, a status of irreversible growth arrest that severely impairs cell function, leading to disease progression [14]. Cell senescence is manifested by the expression of several molecular markers, including senescence-associated β-galactosidse (SA-β-gal) and p21 [8]. Besides stimulating the production of pro-inflammatory markers, exposure to cigarette smoke has been shown to impair and downregulate the secretion of several cytokines that play a pivotal role in defending the airways from pathogens such as viruses and external insults, further increasing the extent of tissue damage [15]. These include IL-15, a cytokine that stimulates the generation of lung-resident CD4 memory T cells [16], RANTES, a chemoattractant cytokine involved in eosinophile recruitment and the protection of lung epithelia from viral infection [17], and macrophage migration inhibitory factor (MIF), a pleiotropic cytokine whose downregulation has been shown to increase susceptibility to COPD [18].

Current treatment options for COPD primarily revolve around symptom management and include bronchodilators, corticosteroids, and lifestyle modifications including but not limited to smoking cessation, and avoiding air pollutants such as pollen, dust, and environmental chemicals [19]. For mild cases, short-acting bronchodilators like albuterol or ipratropium are typically used as needed to relieve symptoms. As the disease progresses from mild to moderate stage, long-acting bronchodilators such as tiotropium or salmeterol are often prescribed for regular use. In severe cases, inhaled corticosteroids (ICS) are added to reduce inflammation, often in combination with long-acting beta-agonists (LABAs) or muscarinic antagonists (LAMAs) [20]. For extremely severe COPD cases, patients may require triple therapy (ICS, LABA, LAMA) and even long-term oxygen therapy [21]. Additionally, roflumilast, a phosphodiesterase-4 inhibitor, may be prescribed to reduce exacerbations in patients with chronic bronchitis. The choice of treatment is guided by symptom severity, exacerbation history, and lung function decline [22].

These therapies offer valuable relief by alleviating bronchoconstriction and reducing airway inflammation. However, their limitations become apparent in their inability to halt the disease's progression or address its fundamental causes and pathophysiological features [23]. Furthermore, patients with COPD experience side effects from these treatments, like a higher risk of pneumonia from inhaled corticosteroids or from other medications. These side effects can make overall management more difficult and have an influence on adherence [24]. On top of that, the present therapeutic treatments tend to lack personalisation, failing to account for individual differences in disease phenotype and concomitant diseases, which can result in inadequate care for specific patients [25].

Over the past few decades, there has been a growing interest in the use of herbal-based medicine and isolated phytochemicals for lung disease treatment, especially asthma, COPD, and lung cancer [13,26–33]. One promising candidate that has garnered much attention is 18βGA, a compound found in liquorice root and various other medicinal herbs [26,34]. The studies reported highlight that 18βGA exerts anti-inflammatory effects by inhibiting NF-κB nuclear translocation, reducing cytokine expression, and impacting various signalling pathways [35]. One study demonstrated its potential in reducing lung inflammation and fibrosis induced by carbon nanotubes, offering hope for pulmonary fibrosis treatment [36]. In addition, Liu et al. showed 18βGA's ability to prevent NF-κB phosphorylation and activate the Nrf2/HO-1 pathway, suggesting its potential for treating allergic airway inflammation [37]. These findings underscore 18βGA's potential as a therapeutic agent in controlling pro-inflammatory cytokines and immune-inflammatory conditions. The effect of 18βGA in COPD is also notable as it increases the expression of antioxidant enzymes such as superoxide dismutase resulting in reduced oxidative stress [38]. By reducing airway inflammation and oxidative stress, 18βGA shows potential in alleviating COPD symptoms and slowing down the disease progression.

Despite extensive research into the therapeutic potential of 18βGA, it has not yet been integrated into clinical settings due to challenges related to its formulation and drug delivery, stemming from its low water solubility which significantly limits its bioavailability [38]. Polymeric nanoparticles, however, present a promising avenue to overcome these hurdles, as exemplified by the work of Vij et al., who employed a novel polymeric vesicle composed of a PLGA and PEG mixture to encapsulate prednisolone and theophylline, a corticosteroid and anti-inflammatory bronchodilator respectively [39]. This dual therapeutic strategy has been shown to enhance the therapeutic outcomes in COPD, demonstrating the potential of polymeric nanoparticles

in delivering therapeutics effectively to the target site [40]. The use of biocompatible and biodegradable polymers such as Polyethylene Glycol (PEG), Polyethyleneimine (PEI), Polyethylene Oxide (PEO), and Polyvinyl Alcohol (PVA) for coating nanoparticles further enhances their drug delivery capabilities, mitigating cytotoxic and immunogenic effects while facilitating evasion from the reticuloendothelial system [41]. Additionally, the encapsulation of steroid drugs in polymeric micelles has significantly reduced inflammatory cell counts in the bronchoalveolar region in a COPD rat model [42]. These advancements highlight the potential of polymeric nanoparticles as an innovative and efficient delivery system for therapeutics in COPD, paving the way for compounds incorporation like 18βGA into clinical practice.

The present study aims to assess the therapeutic potential of 18βGA loaded in PLGA nanoparticles for alleviating COPD features induced by cigarette smoke extract (CSE) in the BCi-NS1.1 minimally immortalized human bronchial epithelial cell line. Our objectives include the *in vitro* evaluation of these nanoparticles to determine their impact on reducing inflammation, senescence, and oxidative stress in CSE-exposed cells. Our hypothesis posits that delivering 18βGA *via* PLGA nanoparticles will enhance its therapeutic effects, providing a potential innovative strategy for COPD treatment by significantly alleviating the associated adverse effects.

2. Materials & methods

2.1. Formulation and physicochemical characterisation of 18βGA-PLGA nanoparticles

Nanoparticles were produced using an adapted emulsion-evaporation method. Initially, a PLGA solution was prepared by dissolving 50 mg of PLGA in a mixture of 5 mL dichloromethane and acetone. Then, 10 mg of 18βGA was added, and the mixture was sonicated to form a primary emulsion. This emulsion was combined with the Poloxamer solution and sonicated again to create the final oil/water emulsion. After adding deionized water, the nanoparticles were separated through centrifugation, washed multiple times, freeze-dried, and stored. The nanoparticles were characterized for physicochemical properties such as particle size, morphology, encapsulation efficiency, and *in vitro* drug release profile as reported in a previous work [43].

2.2. Cell culture, reagents, and treatment

Human airway-derived epithelial cells (BCi-NS1.1) from Weill Cornell were cultured in bronchial epithelial basal medium (BEBM, Lonza, USA) supplemented with specific growth factors [44]. MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), dimethyl sulphoxide (DMSO), dichlorodihydrofluorescein diacetate (DCF-DA), were obtained from Sigma-Aldrich, St. Louis, MO. Anti. Beta galactosidase staining (X-gal) kit (ab102534) was purchased from Abcam, Victoria, Australia. All pre-design primers (KiCqStart® SYBR® Green Primers, Table 1) were purchased from Sigma Aldrich. Cells were maintained at 37°C in a humidified 5 % CO_2 atmosphere. For experimental purposes, the cells were seeded onto either a 96-well plate (Corning, USA) or a 6-well plate (Corning, USA). The cells underwent a one-hour pre-treatment with 18βGA-PLGAs at the specified concentrations, followed by exposure to either 5 % cigarette smoke extract (CSE) or control treatment (complete cell culture media) for a duration of 24 hours.

2.3. Preparation of cigarette-smoke extract

We created 100 % CSE by burning a 3R4F reference cigarette and bubbling the smoke in 10 mL cell culture media. This was diluted to 5 % by further addition of cell culture media. Absorbance measurements ensured uniformity, with 5 % CSE chosen based on our previous work to induce cell inflammation, oxidative stress, and senescence [8,44–46].

2.4. Cell viability assay

To determine the highest concentration of 18βGA-PLGA nanoparticles to use without significantly impacting BCi-NS1.1 cell viability, the cell viability of BCiNS1.1 cells was determined by employing 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), following a previously established procedure [47]. The cells were exposed to various concentrations of 18βGA-PLGAs (ranging from 2.5 to 10 μM) for a duration of 24 hours in a 96-well plate. Subsequently, MTT solution was introduced into each well and allowed to incubate for 4 hours. Following this incubation, the liquid medium was aspirated, and the resulting formazan crystals, created as part of the reaction, were dissolved with 100 μL of dimethyl sulfoxide. The optical absorbance at a wavelength of 540 nm was measured using a POLARstar Omega microplate reader.

2.5. Total cellular reactive oxygen species assay

2.5.1. Fluorescence intensity quantification – DCF-DA assay

BCiNS1.1 cells were seeded in a 96-well plate and treated with various concentrations of 18βGA-PLGAs for 1 hour, followed by a 5 % CSE exposure for 24 hours. After adding 10 μM of DCF-DA and incubating for 30 minutes in the dark, fluorescence was measured with a FLUOstar Omega reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm [48,49].

2.5.2. Fluorescence imaging – DCF-DA

BCiNS1.1 cells were cultivated on a cover slip within a 6-well plate. Following overnight attachment, these cells were pre-treated with various concentrations of 18βGA-PLGAs for 1 hour, and then exposed to 5 % CSE for 24 hours. After a double wash with PBS, the cells were incubated with 10 μM of DCF-DA for 30 minutes. Post incubation, the cells underwent two additional PBS washes, and microscopic images were captured immediately. A fluorescence microscope was employed for this purpose, with images taken at 20× magnification and an excitation wavelength of 485 nm and an emission wavelength of 530 nm [48].

2.6. Senescence assay (X-Gal staining)

BCiNS1.1 cells were grown in 6-well plates. Cells were pre-treated with 18βGA-PLGAs at 5 μM concentration for 1 h followed by 5 % CSE treatment for another 24 h. Cells were then washed with phosphate buffered saline (PBS) and fixed with fixative solution for 10 min. After washing with PBS, the cells were stained with the X-gal staining mixture overnight at 37 °C inside the incubator. Cover slips were transferred from 6-well plates to glass slides and images of cells were captured with Zeiss Axio [50].

2.7. Real time qPCR (Inflammation and senescence-related genes)

BCiNS1.1 cells were cultured in six-well plates (100,000 cells/well) and were subjected to a 1-hour pre-treatment with varying concentrations of 18βGA-PLGAs, followed by exposure to 5 % CSE for an additional 24 hours. Total RNA was then extracted using the Trizol method, and subsequent cDNA synthesis from 200 ng of RNA was carried out through reverse transcription. Real-time quantitative PCR analysis was subsequently performed to measure gene expression. Gene expressions were quantified using the $2^{-[\Delta\Delta]Ct}$ method, relative to appropriate reference genes (GADPH). The primers used for the qPCR are listed in Table 1. The results were presented as the relative abundance compared to untreated control cells [51].

2.8. Human cytokine protein array

BCiNS1.1 cells were seeded in a 6-well plate at a density of 100,000

Table 1
List of primers used.

Gene Name	FW Sequence	RV Sequence
IL−6	GCAGAAAAAGGCAAAGAATC	CTACATTTGCCGAAGAGC
TNF-α	AGGCAGTCAGATCATCTTC	TTATCTCTCAGCTCCACG
CXCL1	ATGCTGAACAGTGACAAATC	TCTTCTGTTCCTATAAGGGC
GAPDH	TCGGAGTCAACGGATTTG	CAACAATATCCACTTTACCAGAG
CDKN1A (p21)	CAGCATGACAGATTTCTACC	CAGGGTATGTACATGAGGAG

cells/well and subjected to a 1-hour pre-treatment with varying concentrations of 18βGA-PLGAs, followed by exposure to 5 % CSE for an additional 24 hours. The cells were then lysed with 400 µL of RIPA buffer (ThermoFisher Scientific, Australia) supplemented with protease inhibitor tablets (Roche Diagnostics, Germany). The lysate was incubated for 15 minutes on ice, followed by centrifugation at 15,000 RPM for 5 minutes at 4°C to remove cell debris. The protein content of the clarified protein extract obtained was then quantified using the BCA Assay kit (ThermoFisher Scientific, Australia) following the manufacturer's instructions. For the Human Cytokine Protein Array experiment, the Proteome Profiler Human XL Cytokine Array Kit (R%D Systems, Australia) was used, loading 300 µg of proteins from each group on the respective membranes. The subsequent incubation stages with antibodies and chemiluminescent reagents were carried out according to the manufacturer's instructions. The arrays were imaged with a ChemiDoc MP (Bio-Rad, Hercules, CA, USA), and the pixel density of each spot was calculated using ImageJ (version 1.53c, Bethesda, MD, USA) [52].

2.9. Statistical analysis

The data was expressed as mean ± SEM. Statistical analysis was conducted using one-way ANOVA, followed by either Dunnett's or Tukey's multiple comparison test, utilising Graph Pad Prism software (version 9.3). Statistical significance was considered at $p < 0.05$.

3. Results

18βGA-PLGA nanoparticles were formulated using the adapted emulsion-evaporation method and characterised as described in our previously published report [43].

3.1. Finding the optimal concentration of 18βGA-PLGA nanoparticles for treatment in CSE-induced BCiNS1.1 cells

The toxicity studies, as depicted in Fig. 1, examined various concentrations of 18βGA-PLGAs on BCiNS1.1 cells and their respective impact on cell viability. It was determined that 18βGA-PLGAs at concentrations of 2.5–5 µM exhibited no adverse effects on BCiNS1.1 cells (Fig. 1A). Conversely, the concentration of 10 µM exhibited toxicity, leading to a significant reduction in cell viability (approximately 17 %, Fig. 1A). Consequently, all *in vitro* assays were conducted using 18βGA-PLGAs at concentrations not exceeding 5 µM. The empty-PLGA nanoparticles showed no significant toxic effects at concentrations up to 50 µg/mL (Fig. 1B).

3.2. Inhibition of CSE-induced ROS formation in BCiNS1.1 cells by 18βGA-PLGA nanoparticles

The assessment of total ROS formation induced by 5 % CSE and the reduction of ROS by 18βGA-PLGA in BCiNS1.1 cells was performed by measuring DCF-DA fluorescence intensity and by fluorescence microscopy. The results are shown in Fig. 2. Our observations revealed that 5 % CSE significantly elevated ROS production in BCiNS1.1 cells by 2.65-fold when compared to the untreated control group (Fig. 2A). In contrast, treatment with 18βGA-PLGA nanoparticles resulted in a significant reduction (39.5 %) in ROS generation compared to the CSE-treated group (Fig. 2A). Treatment with concentration-matched empty PLGA nanoparticles also resulted in a slighter, but statistically significant, reduction of CSE-induced ROS production (7.8 %, Fig. 2A). In line with these fluorescence intensity findings, we also noted a similar pattern in fluorescence imaging, with 18βGA-PLGA strongly diminishing the level of ROS intensity, evident through the decrease in green fluorescence compared to cells exposed to 5 % CSE alone (Fig. 2B).

3.3. Inhibition of CSE-induced senescence of BCiNS1.1 cells by 18βGA-PLGA

We utilised beta galactosidase staining to assess the occurrence of cellular senescence triggered by a 24-hour exposure to 5 % CSE in BCiNS1.1 cells and to evaluate the protective effect exerted by 18βGA-PLGA nanoparticles pre-treatment. As illustrated in Fig. 3A, the microscopic imaging demonstrated that, 24-hour exposure to 5 % CSE, BCiNS1.1 cells showed marked senescence, evident in the form of blue-stained, senescence-positive cells expressing SA-β-Gal. Conversely, when treated with 18βGA-PLGAs for 24 hours, there was a noticeable decrease in the number of senescence-positive cells (Fig. 3A). Treatment with empty PLGA nanoparticles did not induce a noticeable decrease of cell senescence (Fig. 3A). From a mechanistic point of view, we performed the RT-qPCR to quantify the mRNA expression of the gene p21 (senescence marker). The gene expression of p21 was significantly

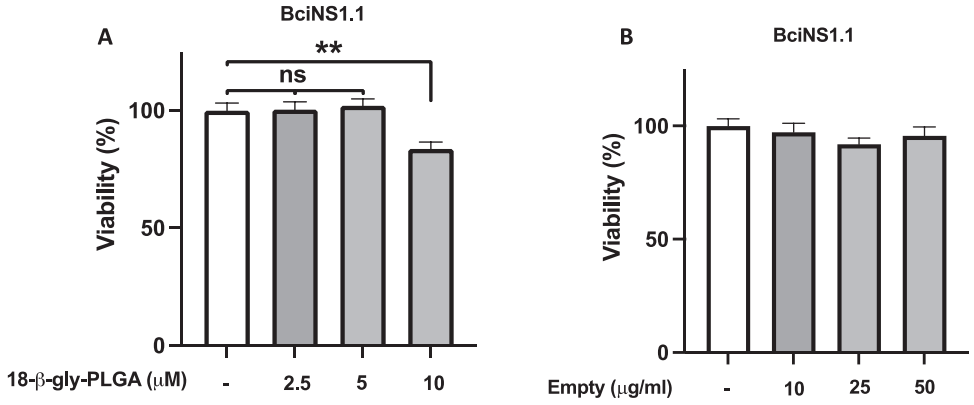

Fig. 1. Effect of 18βGA-PLGA nanoparticles and empty-PLGA nanoparticles in BCiNS1.1 cell viability. This was quantified by MTT colorimetric assay through measuring the absorbance of purple formazan at 540 nm upon exposure to increasing concentrations of 18βGA-PLGA nanoparticles **(A)** or empty PLGA nanoparticles **(B)**. ** $p < 0.01$ vs control (without 18βGA-PLGA nanoparticles), ns = not significant. Values are shown as mean ± SEM, n = 3 independent experiments. Analysis was performed with one-way ANOVA followed by Dunnett's multiple comparison test. CSE: Cigarette smoke extract; 18βGA-PLGA: 18-β-Glycyrrhetinic Acid-Poly (lactic-co-glycolic acid) nanoparticles.

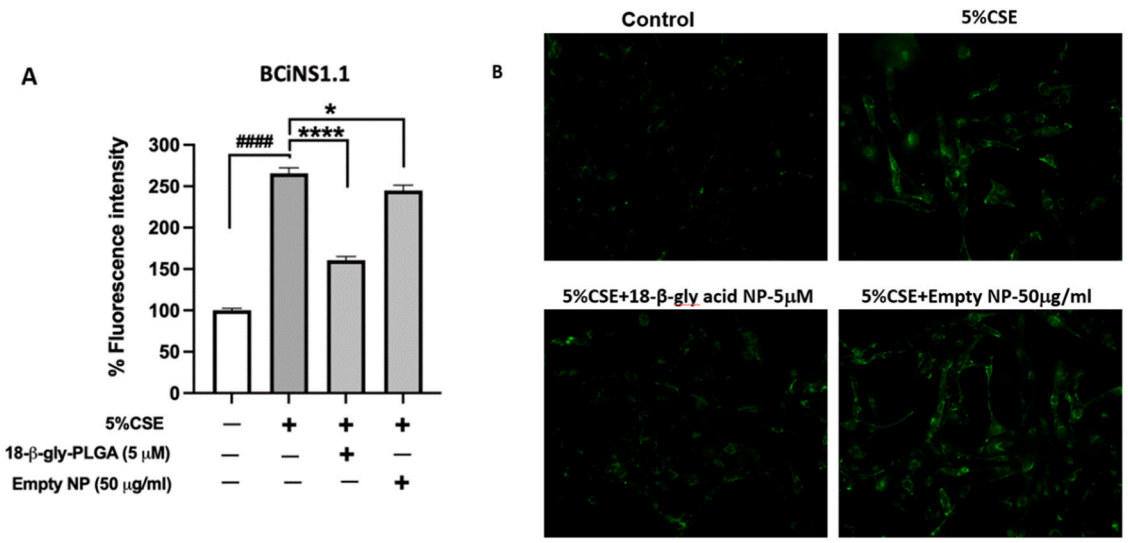

Fig. 2. Impact of 18βGA-PLGA nanoparticles on CSE-induced ROS generation in BCiNS1.1 cells. **(A)** Fluorescence intensity, captured at specific wavelengths, showed significant differences: #### $p < 0.0001$ vs. control and * $p < 0.05$, **** $p < 0.0001$ vs. 5 % CSE across three independent experiments with four replicates each. **(B)** DCF-DA fluorescence images of cells with varied treatments were taken at 20X magnification using a Zeiss Axio Imager Z2. CSE denotes Cigarette smoke extract; 18βGA-PLGA signifies 18-β-Glycyrrhetinic Acid-Poly nanoparticles.

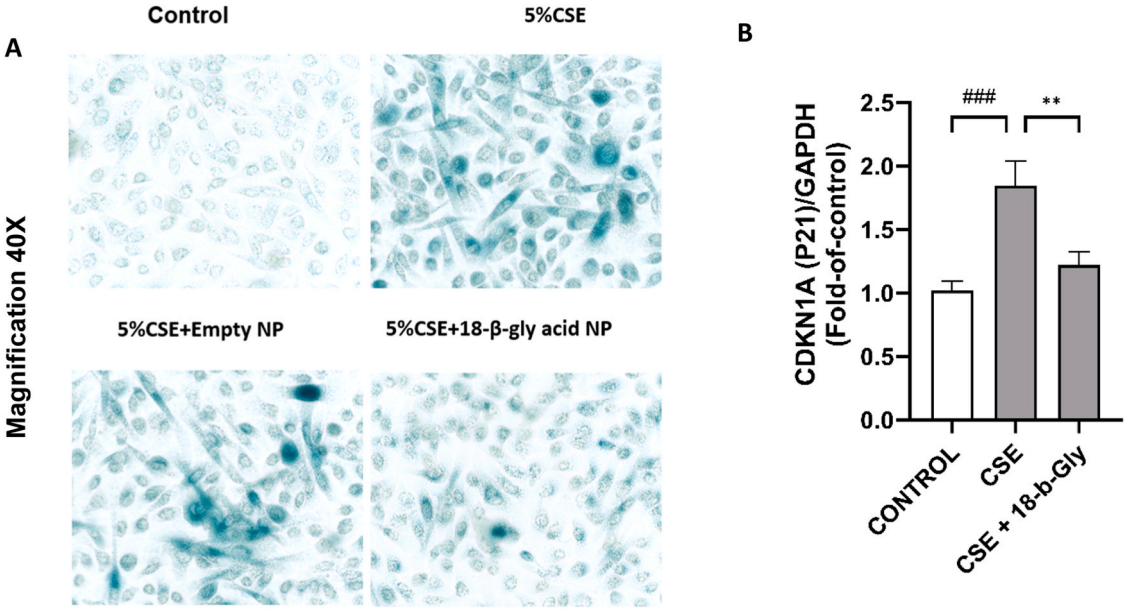

Fig. 3. Effect of 18βGA-PLGA on 5 % CSE-induced senescence of BCiNS1.1 cells. **A)** BciNS1.1 cells treated with 18βGA-PLGA and 5 % CSE for 24 h. Cells were stained with X-Gal. Senescence positive cells are represented with blue-colour positive staining of x-gal as seen more on 5 %CSE group and 5 %CSE+empty nanoparticles group. The microscopic images were captured under a 40 X magnification. **B)** Gene expression of CDKN1A (p21), ### $p < 0.001$ vs. control (without 18βGA-PLGAs and 5 % CSE treatment) and ** $p < 0.01$ vs. 5 % CSE.

upregulated by 5 % CSE (1.8-fold) compared to untreated control (Fig. 3B). In contrast, 18βGA-PLGAs significantly decreased the 5 % CSE-induced expression of p21 by 33.7 % (Fig. 3B).

3.4. Inhibition of inflammation-related gene expression in CSE-stimulated BCiNS1.1 cells by 18βGA-PLGA

CXCL1, IL-6, and TNF-α are some of the primary inflammatory cytokines whose expressions are triggered in the airways by cigarette smoking. In our results, BCiNS1.1 cells exposed to 5 % CSE showed an increase in *IL-6* (2.1-fold — Fig. 4A), *CXCL1* (1.5-fold — Fig. 4B), and *TNF-α* (1.3-fold — Fig. 4C) gene expressions compared to the untreated control, while 18βGA-PLGAs treatment at 5 μM decreased the mRNA expressions of these genes compared to 5 % CSE. In particular, 18βGA-PLGAs treatment resulted in a significant reduction of the expression of *CXCL1* (11.2 %, Fig. 4B), and in a non-statistically significant drop of *IL-6* and *TNF-α* expression (10.1 % - Fig. 4A and 11.5 % - Fig. 4C, respectively). These results demonstrated that 18βGA-PLGA nanoparticles significantly downregulate the expression of the CSE-induced pro-inflammatory gene *CXCL1* (Fig. 4B). Furthermore, 18βGA-PLGA nanoparticles downregulated the CSE-induced expression of *IL-6* and *TNF-α* to a lesser extent, but not statistically significant (Fig. 4A and 4C, respectively).

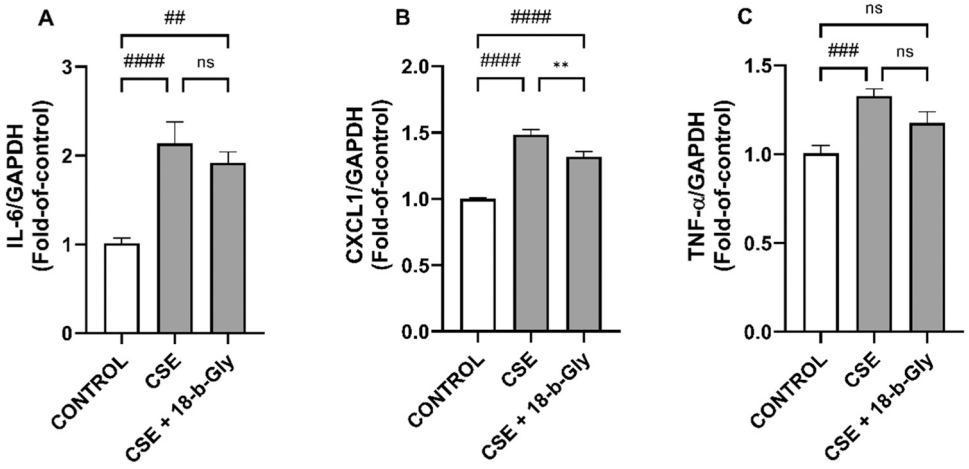

Fig. 4. : Effect of 18βGA-PLGAs on the CSE-induced expression of *IL-6*, *CXCL1,* and *TNF-α* mRNAs. BCi-NS1.1 cells were pre-incubated for 1 h in the presence of at 5 μM 18βGA-PLGAs, followed by exposure to 5 % CSE for 24 h. The mRNA levels of *IL-6*, *CXCL1* and *TNF-α* were determined via RT-qPCR. Values are expressed as mean ± SEM (n = 4, **: $p < 0.01$; ###: $p < 0.001$, ####: $p < 0.0001$), ns = not significant. Analysis was carried out using one-way ANOVA followed by Dunnett's multiple comparison test.

3.5. Impact of 18βGA-PLGA on the protein expression of cytokines and other mediators in CSE-stimulated BCiNS1.1 Cells

The relative protein expressions of IL-8, IL-15, RANTES, and MIF are shown in Fig. 5, as detected using the Human XL Cytokine Protein Array. Treatment of BCiNS1.1 with 5 % CSE significantly increased the relative expression of IL-8 (2.7-fold, Fig. 5A). Conversely, the treatment of BCiNS1.1 with 5 % CSE significantly decreased the relative expressions of IL-15, RANTES, and MIF by 55.5 % (Fig. 5B), 64.6 % (Fig. 5C), and 12.0 % (Fig. 5C), respectively. Pre-treatment with a 5 μM 18βGA-PLGAs decreased the level of expression of IL-8 by 46.9 % compared to the 5 % CSE-treated group ($p = 0.0573$, Fig. 5A). Similarly, the expression levels of IL-15, RANTES, and MIF were significantly increased upon treatment with 5 μM 18βGA-PLGAs, and the expression levels of these proteins were restored to levels comparable to the untreated control group (Fig. 5B, 5C, and 5D, respectively).

4. Discussion

Liquorice, a herb commonly utilised in Eastern medicinal practices and recognised for its low toxicity, is endorsed by the US Food and Drug Administration (FDA) as a food supplement, finding its way into numerous products. As highlighted previously, the principal active compound in liquorice is glycyrrhizic acid, which undergoes a prompt transformation into 18βGA within the human body, manifesting a variety of effects including antioxidant, anti-inflammatory, and anti-senescence, as referenced in numerous studies [34,53–55]. The results depicted in the present work establishes the capability of 18βGA to counteract CSE-induced expression of oxidative stress, inflammatory status, and senescence markers *in vitro* in BCiNS1.1 cells.

As illustrated in Fig. 2, 18βGA-PLGA exhibited a significant reduction in the CSE-induced ROS generation. This is particularly crucial in the context of COPD as when the ROS levels are uncontrolled, they can initiate lipid peroxidation, producing harmful by-products such as malondialdehyde [56,57]. This compound is capable of inactivating numerous cellular proteins through the formation of protein cross-linkages, potentially exacerbating pulmonary inflammation, and furthering the damage to alveolar walls, eventually leading to emphysema [58]. Moreover, another lipid peroxidation product, 4-hydroxy-2, 3-nonenal, has a range of cytotoxic effects, including induction of

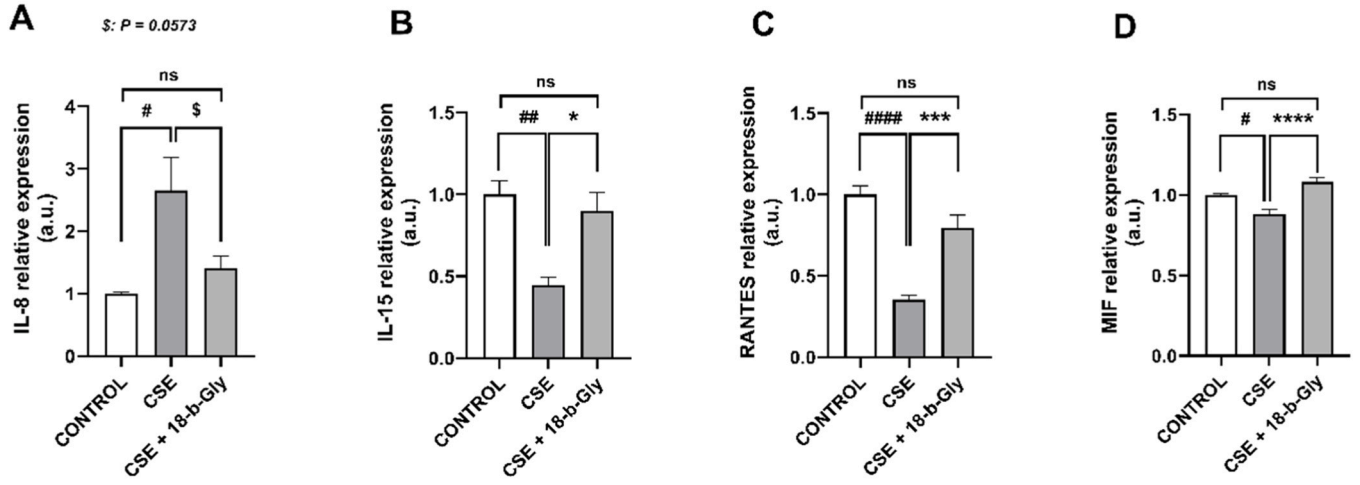

Fig. 5. The impact of 18βGA-PLGA nanoparticles on the expression of inflammatory proteins induced by 5 % CSE. BCi-NS1.1 cells treated with 18βGA-PLGA and 5 % CSE for 24 h and pretreated with or without 5 μM 18βGA-PLGA nanoparticles. The relative protein expression levels of **A)** IL-8, **B)** IL-15, **C)** RANTES, **D)** MIF were determined using Human XL cytokine protein array. Values are expressed as mean ± SEM (n = 4, * or #: $p<0.05$; ##: $p < 0.01$; ***: $p< 0.001$, **** or ####: $p < 0.0001$; $: $p = 0.0573$, ns: not significant). Analysis was carried out using one-way ANOVA followed by Dunnett's multiple comparison test.

pro-inflammatory cytokines, mitochondrial dysfunction, and apoptosis, as well as causing an accumulation of cytoplasmic Ca^{2+} and activation of NF-κB, which in turn reinforces inflammation in a feedback loop [59].

Given this backdrop, the capacity of 18βGA-PLGA nanoparticles to substantially decrease ROS production acquires a heightened significance. The observed effects are attributed to the enhanced bioavailability and prolonged release of 18βGA from the PLGA nanoparticles, allowing for an efficient intracellular delivery and antioxidant action [60]. By doing so, they not only prevent the initial oxidative damage but also disrupt the cascade of events leading to lipid peroxidation and its subsequent harmful effects. The elevation of lipid peroxidation end products in the breath and serum of COPD patients further underscores the potential clinical relevance of using 18βGA-PLGA nanoparticles as a therapeutic strategy in this context [57].

Upon evaluating the impact of 18βGA-PLGA nanoparticles on cellular senescence in BCiNS1.1 cells exposed to 5 % CSE, our results, as illustrated in Fig. 3A, revealed a notable reduction in senescence-positive cells. This finding is particularly significant in the context of COPD, where an increase in senescent cells has been consistently observed across various studies. Previous research has established a higher prevalence of senescent cells in the lungs and airways of COPD patients compared to age-matched smokers or healthy non-smokers, with increased senescence markers such as p21 found in type II pneumocytes, endothelial cells, and pulmonary artery smooth muscle cells in lung tissue from COPD patients [61]. These findings were corroborated by increased senescence-associated β-gal staining and reduced population doubling levels in endothelial and smooth muscle cells from COPD patients [62], aligning with what was observed in our *in vitro* model experiments in this study. Similar trends were observed in small airway epithelial cells and endothelial progenitor cells from COPD patients, further validating the link between cellular senescence and COPD [61].

With regards to, the pro-inflammatory genes involved in the pathogenesis of COPD, the most significant effect observed when treating the cells with 18βGA-PLGA was on the *CXCL1* gene. The downregulation of CXCL1 gene expression through 18βGA treatment has been previously demonstrated and tested by Jiang et al. in the context of renal fibrosis and chronic kidney disease [63]. The study has also demonstrated that 18βGA can supress immune response, cytokine–cytokine receptor interaction, and chemokine signalling pathway [63]. Our findings additionally reveal that 18βGA encapsulated in PLGA nanoparticles can decrease the expression of TNF-α and IL-6 genes. While the observed reduction in TNF-α and IL-6 expression upon treatment with 18βGA-PLGA did not reach statistical significance in comparison to treatment with 18βGA alone, these findings remain consistent with prior research [38,64]. Cigarette smoking has been established to significantly elevate the levels of inflammatory cytokines such as TNF-α, IL-6, and IL-10, while reducing the levels of IL-2 and IFN-γ, which are crucial for T cell function and anti-tumour immune responses [65]. Although we did not test all these mentioned genes, this pattern was consistent with our findings, showing a significant increase in inflammatory cytokines in response to cigarette smoke exposure in BCiNS1.1 cells, and their subsequent reduction upon treatment with 18βGA-PLGA nanoparticles.

In addition to evaluating inflammatory markers at the gene level, the protein array was also employed to assess the expression of relevant biomarkers at the protein level in this study. The protein array studies provided a mechanistic understanding about how CSE and 18βGA-PLGAs impact various signalling pathways and exert anti-inflammatory effects. CSE is the primary component of ROS that enhances the Ca^{2+} sensor which increases the production of IL-8 in the human alveolar macrophages [66]. According to Mio et al., the release of IL-8 from cultivated human bronchial epithelial cells was increased by CSE which aligns with the results of this study (Fig. 5A) [67]. On the other hand, IL-15 is a multifunctional cytokine produced by both antigen-presenting cells (APCs) and epithelial cells, such as dendritic cells and macrophages [68]. IL-15 accumulates in respiratory virus infections and plays a crucial role in the antiviral immune response. CSE significantly reduces IL-15 mRNA as well as membrane-bound and intracellular IL-15 protein levels. This consequently prevents natural killer NK cell activation characteristics and cytotoxic potential [69]. In a study conducted by Shiels et al., IL-15 was shown to be an essential cytokine that regulated immunological responses, and changes in its levels can have a variety of impacts on the immune system [70]. Smoking can have a wide range of consequences on immunological function, including alterations in the expression level of IL-15. This is consistent with our data, which shows that CSE induced a significant downregulation of the expression of IL-15 (Fig. 5B). Considering the role played by IL-15 in NK cells function and in the protection of the respiratory system from viral infections, the fact that 18βGA-PLGA nanoparticles restore the expression of this cytokine to normal levels, counteracting the effect of CSE, is particularly relevant as it suggests that, besides suppressing inflammation, senescence, and oxidative stress, 18βGA-PLGA nanoparticles may restore the immunological defense mechanisms of the airways, which are significantly impacted by cigarette smoking.

CSE can trigger the production of IL-8 from human airway smooth muscle cells, which is further amplified by overexpressed TNF-α in COPD patients. Cigarette smoke inhibits eotaxin and RANTES, which is consistent with COPD's primary neutrophilic rather than eosinophilic inflammation, being RANTES a pivotal mediator of eosinophile recruitment [71]. In the present study (Fig. 5C) the expression of RANTES was downregulated by CSE exposure and was restored by the treatment with 18βGA-PLGA nanoparticles, suggesting that 18βGA-PLGA nanoparticles may restore a physiological balance in immune cells in the lungs and airways of COPD patients. Furthermore, there is a significant role played by RANTES in promoting the antiviral response in lung epithelia [17]. Macrophage migration inhibitory factor (MIF) is a cytokine with dual functions that inhibits both apoptosis and premature senescence, and it plays a crucial role in the pathophysiology of COPD [18]. Fallica et al. reported that the serum level of MIF was reduced in COPD patients and, in addition, a study has shown that MIF expression was decreased in epithelial tissues when they are exposed to CSE [72]. With regards to the effect of 18βGA in the expression of the aforementioned proteins, one study has shown that treatment of epithelial cells with 18βGA inhibits the expression of pro-inflammatory cytokines such as IL-8. The underlying mechanism involves 18βGA blocking the phosphorylation of MAPKs after extracellular-regulated kinases (IκBα) breakdown and NF-κB activation, which inhibits TNF-α-resulting in inducing IL-8 production in intestinal epithelial cells [73]. To the best of our knowledge, this present study is the first to report that 18βGA impacts the level of expression of IL-15, RANTES, and MIF, restoring their expression and counteracting the inhibitory effect exerted by CSE treatment.

It is notable that the therapeutic potential of 18βGA-PLGAs is not only limited to anti-inflammatory, antioxidant, and antisenescence effects. In a study conducted by Darvishi et al., the comparative antimicrobial assessment revealed a superior efficacy of the 18βGA-PLGAs over pure 18βGA when tested against *S. aureus*, *S. epidermidis*, and *P. aeruginosa,* the most common bacterial strains causing respiratory-related infections, which often represent complications of COPD [74]. These findings suggest the potential of amplifying 18βGA's clinical effectiveness through its nanoparticle formulation, offering a more potent therapeutic alternative than the traditional antibiotics that are currently available [74].

Pertaining to the potentially toxic effects linked with polymeric nanoparticles, Voigt et al. highlighted that the biocompatibility of these particles is dependent on a variety of elements, including the type of polymer used, the size of the particles, their chemical makeup, surface charge, rate of degradation, and the kinetics of drug release [75]. These aspects are crucial as they could influence immune system reactions, interactions at the cellular level, toxicity specific to certain organs, and the cumulative impact of the nanoparticles [75]. This underscores the critical need for meticulous attention in the design and implementation

of nanoparticle-based therapeutic strategies in our future studies. Furthermore, although 18βGA and related compounds exhibit numerous therapeutic benefits, they have rarely progressed to clinical trials, as highlighted by Shinu et al. [38]. While 18βGA has undergone testing for conditions like immune thrombocytopenia, end-stage renal disease, and excess apparent mineralocorticoids, there is a significant gap in clinical and *in-vivo* research regarding its effects on respiratory health [38]. Therefore, nanoencapsulation of 18βGA can be considered a promising strategy for accelerating the transition of 18βGA into *in-vivo* studies and clinical settings.

Our study, while providing crucial insights, has limitations due to the absence of specific tests that could have enhanced the comprehensiveness of our findings. One of the significant omissions is the lack of p16 and p21 immunofluorescence staining in BCiNS1.1 cells treated with 5 % CSE and 18βGA nanoparticles. These proteins are well-known markers for cellular senescence and aging, and their visual representation through immunofluorescent staining, together with the study of their expression at the transcript/protein level, could have provided a direct and more complete assessment of the treatment's impact on cellular aging processes, which is particularly relevant in the context of COPD [76]. Furthermore, our study lacks qPCR data on oxidative stress genes and apoptosis-related genes in the treated cells. To broaden the impact and understanding of the current findings, it is crucial to conduct the abovementioned tests in future studies and assess the effects of 18βGA-PLGA on various cell types, including macrophages and other lung-resident cells. This would offer a comprehensive and accurate portrayal of 18βGA-PLGA's versatile actions in counteracting inflammation and oxidative stress. The development of inhalation formulations of 18βGA to combat lung diseases would be another future perspective of this study [77]. Given the array of pathways influenced by 18βGA-PLGAs treatment, exploring their efficacy against other inflammatory diseases such as asthma presents an intriguing avenue for future research. To pave the way for clinical applications, further validation through appropriate *in-vivo* animal models of COPD is imperative.

5. Conclusion

In summary, our study underscores the promising *in vitro* therapeutic potential of 18βGA, a major active component of liquorice, encapsulated in PLGA nanoparticles (18βGA-PLGA), against COPD-related cellular damage. Liquorice has been recognised for its low toxicity and is widely accepted as a food supplement, supported by the FDA. Our findings have demonstrated the efficacy of 18βGA-PLGA in reducing ROS, cellular senescence, pro-inflammatory gene expression, and in counteracting the effect of CSE on the expression of pro-inflammatory cytokines and other immune modulators in BCiNS1.1 cells. This is particularly relevant in COPD, where oxidative stress, chronic inflammation, and cellular senescence play a crucial role. The ability of 18βGA-PLGA nanoparticles to mitigate these processes highlights their antioxidant and anti-inflammatory properties and their potential to address the senescence-associated aspects of COPD. While our findings are promising, our study acknowledges certain limitations due to the absence of specific tests such as nitric oxide assay, p16 and p21 staining and qPCR analysis of oxidative stress and apoptosis-related genes, which are areas that should be addressed in future studies. Additionally, expanding the scope of research to include various lung-resident cell types and *in vivo* animal models of COPD is imperative for a comprehensive evaluation and to facilitate the transition of 18βGA-PLGA from laboratory research to clinical settings.

Ethical approval

All studies were conducted *in vitro* using commercially available human cell line. No ethical approval required.

Author statement

The authors of the Research manuscript titled "Therapeutic Potential of 18-β-Glycyrrhetinic Acid-loaded Poly (lactic-co-glycolic acid) Nanoparticles on Cigarette Smoke-Induced in-vitro model of COPD", resubmitted after revisions to the journal "Pathology – Research and Practice", declare that they all have read and agreed with the finalized submitted version of the manuscript. The following table indicates the contribution of each author to the study.

CRediT authorship contribution statement

Brian Oliver: Writing – review & editing, Visualization, Supervision, Methodology, Conceptualization. **Tammam El-Sherkawi:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Philip Michael Hansbro:** Writing – review & editing, Visualization, Supervision, Methodology, Conceptualization. **Stewart Yeung:** Writing – review & editing, Visualization, Supervision, Methodology, Conceptualization. **Gabriele De Rubis:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Ayeh Bani Saeid:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sachin Kumar Singh:** Writing – review & editing, Visualization, Methodology, Conceptualization. **Swathi Sudhakar:** Writing – review & editing, Validation, Methodology, Funding acquisition, Data curation, Conceptualization. **Keshav Raj Paudel:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Gaurav Gupta:** Writing – review & editing, Visualization, Methodology, Conceptualization. **Dinesh Kumar Chellappan:** Writing – review & editing, Validation, Resources, Methodology, Investigation. **Kamal Dua:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Sofia Kokkinis:** Writing – review & editing, Visualization, Methodology, Conceptualization. **Siddiq Mohamad:** Writing – review & editing, Validation, Resources, Methodology, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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