



Lunasin influences macrophage–pulmonary epithelial crosstalk in cellular model of hypersensitivity pneumonitis

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Abstract

Introduction and Objective. Hypersensitivity pneumonitis (HP) is a chronic immune-mediated interstitial lung disease driven by persistent antigen exposure, macrophage activation, and progressive lung remodelling. Macrophage-derived transforming growth factor beta (TGF- β) plays a key role in epithelial–mesenchymal transition (EMT), contributing to fibrosis development. Lunasin, a soybean-derived bioactive peptide, exhibits anti-inflammatory and immunomodulatory properties, but its influence on macrophage–epithelial interactions in HP remains unclear. The aim of the study is to evaluate the effects of lunasin on antigen-induced macrophage activation, TGF- β secretion, and macrophage-driven EMT in a long-term *in vitro* model of HP.

Materials and Method. SC macrophages were chronically stimulated with *Pantoea agglomerans* antigen in the presence or absence of lunasin (50 μ M). Viability (MTT), macrophage polarization (flow cytometry), and TGF- β secretion (CBA assay) were assessed. NL20 bronchial epithelial cells were exposed to macrophage-conditioned media, and morphological changes and EMT marker expression were analyzed.

Results. Lunasin attenuated antigen-induced M1 polarization at later time points but did not significantly alter TGF- β levels in PA-stimulated macrophages. Conditioned media from antigen-activated macrophages induced EMT-like changes in epithelial cells, including altered morphology and increased mesenchymal marker expression. Media derived from lunasin-treated macrophages partially preserved epithelial morphology and produced limited, marker-specific changes in EMT-associated proteins.

Conclusions. Lunasin modulates antigen-induced macrophage activation and influences macrophage–epithelial crosstalk *in vitro*, attenuating selected profibrotic and EMT-associated responses relevant to HP-related lung remodelling.

Key words

TGF- β , lunasin, macrophage polarization, hypersensitivity pneumonitis, epithelial–mesenchymal transition (EMT)

INTRODUCTION

Hypersensitivity pneumonitis (HP) is an immune-mediated interstitial lung disease that develops as a consequence of chronic exposure to a wide spectrum of inhaled organic and inorganic antigens, including bacteria, fungi, mycobacteria, plant- and animal-derived proteins, as well as selected chemicals and metals [1–3]. Persistent antigenic stimulation results in prolonged activation of the immune response, leading to sustained inflammation of the respiratory tract. While early stages of HP may remain reversible following antigen avoidance, continued exposure frequently promotes disease progression toward irreversible structural remodelling of the lung parenchyma. In advanced disease, these changes are manifested by alveolar wall thickening, excessive extracellular matrix (ECM) deposition, and a gradual decline in pulmonary function, ultimately resulting in pulmonary fibrosis [4, 5].

Pulmonary fibrosis arising in the course of HP reflects the interplay of multiple pathological processes, including persistent epithelial injury, aberrant activation of fibroblasts, and dysregulation of tissue repair mechanisms [6–8].

Accumulating evidence suggests that impaired regeneration of the respiratory epithelium plays a crucial role in the progression of fibrosis. In this context, increasing attention has been directed toward epithelial-to-mesenchymal transition (EMT) as a biological process linking chronic inflammation with pathological tissue remodelling. EMT represents a phenotypic shift in which epithelial cells lose polarity and intercellular junctions and acquire mesenchymal characteristics. These changes are accompanied by extensive molecular reprogramming, including decreased expression of epithelial markers, such as E-cadherin, and increased expression of mesenchymal markers, notably vimentin, fibronectin, and N-cadherin [9, 10].

Under physiological conditions, EMT occurs as a tightly regulated and transient process that supports wound healing and tissue regeneration. Resolution of inflammation accompanying epithelial injury leads to termination of EMT and restoration of epithelial integrity. In contrast, chronic inflammatory states, such as those observed in HP, sustain EMT signalling, thereby promoting pathological epithelial remodelling and excessive accumulation of ECM components [11]. Prolonged EMT not only compromises epithelial barrier function but also facilitates fibroblast recruitment and activation, further amplifying fibrotic remodelling of lung tissue.

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Macrophages constitute a central cellular link between chronic inflammation and fibrogenesis in HP. They represent one of the key effector cell populations of the pulmonary immune system, and together with epithelial cells, form the first line of defence against inhaled pathogens and allergens. Depending on their activation state, pulmonary macrophages are broadly categorised into two main phenotypes: classically activated M1 and alternatively activated M2. M1 macrophages primarily contribute to inflammatory responses and epithelial injury through the release of pro-inflammatory mediators. Conversely, M2 macrophages are associated with tissue remodelling and fibrosis, largely due to the production of profibrotic factors, among which transforming growth factor beta (TGF- β) plays a dominant role. TGF- β is considered a key regulator of fibrogenesis, as it stimulates ECM synthesis, suppresses epithelial cell proliferation and repair, and acts as a potent inducer of EMT. Of the three TGF- β isoforms, TGF- β 1 appears to be most strongly linked to EMT in both physiological contexts, such as wound healing, and pathological conditions, including pulmonary fibrosis [12, 13].

Given that pulmonary fibrosis in HP develops as a consequence of chronic inflammation-driven impairment of epithelial repair processes, with EMT representing a central pathogenic mechanism, it has been suggested that effective therapeutic approaches should combine modulation of immune responses with inhibition of EMT-associated epithelial remodelling. In this regard, bioactive dietary compounds exhibiting both immunomodulatory and anti-fibrotic properties have attracted increasing interest. Lunasin, a naturally occurring soybean-derived peptide, has been recognized as a particularly promising candidate. A broad range of biological activities has been attributed to lunasin, including antioxidant, anti-inflammatory, immunomodulatory, anticancer, and cardioprotective effects [14–16]. From the perspective of pulmonary fibrosis pathogenesis, its anti-inflammatory activity appears to be of particular relevance. Studies have demonstrated that lunasin attenuates lipopolysaccharide (LPS)-induced production and release of pro-inflammatory mediators by macrophages, including interleukin-6 (IL-6), tumour necrosis factor alpha (TNF- α), inducible cyclooxygenase (COX-2), prostaglandins (PGE2), and nitric oxide [17, 18]. However, despite growing evidence supporting the anti-inflammatory and immunomodulatory properties of lunasin, its influence on the TGF- β , a key driver of EMT [10], has not yet been sufficiently characterized.

Despite these promising attributes, the effects of lunasin on key fibrotic mechanisms in HP – particularly macrophage polarization and EMT – remain largely unexplored. Therefore, the present study was designed to investigate the potential role of lunasin in modulating mechanisms involved in lung fibrosis. A unique *in vitro* experimental model was employed, in which EMT was induced by chronic exposure of respiratory epithelial cells to factors secreted by macrophages activated with *Pantoea agglomerans*, a well-documented etiological agent of HP [19]. Within this model, lunasin cytotoxicity in macrophages was evaluated, and its effects on macrophage polarization and TGF- β secretion were analyzed. Furthermore, the influence of lunasin on EMT induction in human bronchial epithelial NL20 cells was assessed by examining changes in cell morphology as well as the expression of mesenchymal markers (vimentin,

fibronectin, and N-cadherin) and the epithelial marker E-cadherin.

MATERIALS AND METHOD

Reagents. Unless otherwise specified, all reagents used in the experiments were sourced from Sigma-Aldrich Co. LLC.

Lunasin. Lunasin (amino acid sequence: SKWQHQQDS-CRKQLQGVNLTTPCEKHIMEKIQGRGDDDDDDDDDD) was obtained from Novazym Polska. The peptide purity was 95.53%. Lunasin was dissolved in sterile distilled water supplemented with 1% bovine serum albumin (BSA) to obtain a stock solution with a final concentration of 1 μ g/mL. Aliquots (10 μ L) were prepared and stored at -80°C . Working solutions were freshly prepared in phosphate-buffered saline (PBS) immediately before use.

***Pantoea agglomerans* antigen.** The *P. agglomerans* antigen was prepared by mechanical disintegration of bacterial cells from the M-10–3 strain, following the protocol described by Lemieszek et al. (2020) [20]. For all experiments, the antigen was used at a final concentration of 250 μ g/mL.

Cell lines and culture conditions. Human respiratory epithelial cells (NL20) and macrophages (SC) were obtained from the American Type Culture Collection (ATCC). SC macrophages were cultured in Iscove's Modified Dulbecco Medium (IMDM, ATCC), supplemented with 0.1 mM hypoxanthine, 0.016 mM thymidine, and 10% foetal bovine serum (FBS). NL20 cells were maintained in F12-Knight's Modified Medium (Thermo Scientific), supplemented with 2.6 g/L glucose, 0.1 mM nonessential amino acids, 5 μ g/mL insulin, 10 ng/mL epidermal growth factor (EGF), 1 μ g/mL transferrin, 500 ng/mL hydrocortisone, and 4% FBS. All media were supplemented with 100 U/mL penicillin and 100 μ g/mL streptomycin. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 .

Cell viability evaluation – MTT assay. SC cells were seeded in 96-well plates at a density of 1×10^5 cells/mL and exposed to lunasin (10, 25, or 50 μ M), *P. agglomerans* antigen (250 μ g/mL), or a combination of both for 1, 7, 14, or 21 days. For extended treatments, the culture medium was completely replaced every 2–3 days with fresh medium containing newly prepared test substances at the designated concentrations to ensure consistent experimental conditions and uniform exposure throughout the experiment. At each specified time point, 15 μ L per well of MTT solution (5 mg/mL) was added, and cells were incubated for 6 hours at 37°C . Subsequently, 100 μ L per well of SDS-HCl buffer (pH 7.4) was added to dissolve the formazan crystals. After an additional 24-hour incubation at 37°C , absorbance was measured at 570 nm using a BioTek ELx800 microplate reader (BioTek, Winooski, VT, USA).

Examination of SC cells differentiation-flow cytometry. SC cells were seeded in 24-well culture plates at a density of 1×10^5 cells/mL and cultured under standard conditions with lunasin (50 μ M), *P. agglomerans* antigen (250 μ g/mL), or a combination of both for 7, 14, or 21 days. At the specified time points, cells were harvested by centrifugation (500 $\times$ g, 10

minutes, room temperature), washed twice with phosphate-buffered saline (PBS), and subjected to immunophenotypic analysis. Cell surface staining was performed, according to manufacturer's protocols, using fluorochrome-conjugated monoclonal antibodies specific for macrophage polarization markers, including CD80 (PerCP-Cy5.5) and CD86 (APC) as markers of the M1 phenotype, and CD163 (PE) and CD206 (FITC) as markers of the M2 phenotype (BD Biosciences). Antibody incubation was carried out for 20 minutes at room temperature in the dark. After staining, cells were washed to remove unbound antibodies and resuspended in PBS. Samples were analysed using a BD Accuri C6 Plus flow cytometer (BD Biosciences). Data acquisition and analysis were performed using BD Accuri C6 Plus software. For each sample, a minimum of 30,000 events were collected. The acquired data were analysed to determine the percentage of double-positive cells.

Quantification of TGF- β – cytometric bead assay. To quantify TGF- β secretion, SC cells were seeded in 24-well culture plates at a density of 1×10^5 cells/mL and cultured with lunasin (50 μ M), *P. agglomerans* antigen (250 μ g/mL), or their combination for 7, 14, or 21 days under standard conditions. At the indicated time points, culture plates were centrifuged (500 $\times$ g, 10 minutes, room temperature), and cell-free supernatants were collected and stored at -80°C until analysis. Quantification of TGF- β levels was performed using a cytometric bead assay according to the manufacturer's protocol. Briefly, aliquots of supernatants (50 μ L) were incubated with 50 μ L of TGF- β -specific capture beads for 1 hour at room temperature in the dark. Subsequently, 50 μ L of PE-conjugated detection reagent was added, and samples were incubated for an additional 2 hours under the same conditions. Following incubation, unbound reagents were removed by washing with assay buffer and centrifugation (500 $\times$ g, 10 minutes, room temperature). The bead pellets were resuspended in 300 μ L sample buffer and were analyzed using an Accuri C6 Plus flow cytometer. Flow cytometric acquisition was performed until a minimum of 300 singlet bead events per analyte was collected, in accordance with the manufacturer's recommendations. Data acquisition and analysis were performed using BD Accuri C6 Plus Software. TGF- β concentrations were determined based on standard curves generated using recombinant TGF- β standards.

Cell morphology examination – MGG staining. To examine morphological changes associated with EMT, NL20 cells were seeded at a density of 5×10^4 cells/mL on Lab-Tek Chambers Slide (Nunc). Cells were cultured with macrophage-conditioned media 7, 14 and 21 days. After treatment, cells were stained using the May–Grünwald–Giemsa (MGG) method. Following medium removal and PBS washing, cells were stained with May–Grünwald solution for 3 minutes, diluted 1:1 with distilled water, and incubated for an additional 3 minutes. This was followed by staining with Giemsa solution (1:20 dilution) for 30 minutes. Excess stain was removed, and preparations were air-dried. Morphological assessment was carried out using an MW 50 light microscope (OPTA-TECH, Warsaw, Poland), and images captured using Capture v2.0 software.

Expression of EMT-related surface proteins – flow cytometry. NL20 human bronchial epithelial cells at a

density of 1×10^5 cells/mL cells were cultured in 24-well culture plates under standard conditions with macrophage-conditioned media containing tested substances for 7, 14 and 21 days. After treatment, cells were detached using 5 mM EDTA in phosphate-buffered saline (PBS) at 37°C for 10 minutes, collected by centrifugation (500 $\times$ g, 10 minutes), and washed with PBS. Cells were then stained with fluorochrome-conjugated monoclonal antibodies against EMT-related markers, including E-cadherin (CD324, PerCP-Cy5.5), fibronectin (Alexa Fluor 488), N-cadherin (Alexa Fluor 647), and vimentin (PE). Antibody staining was performed for 20 minutes at room temperature in the dark. Following removal of unbound antibodies by washing with PBS, cells were resuspended in 100 μ L PBS. Flow cytometric analysis was performed using an Accuri C6 Plus flow cytometer, and data acquisition and analysis were carried out using BD Accuri C6 Plus Software. For each sample, a minimum of 30,000 events were collected. The acquired data were analysed to determine the percentage of cells positive for each investigated surface marker.

Statistical analysis. Data were analyzed using GraphPad Prism 5.0 and BD Accuri C6 Plus Software. Results are expressed as mean $\pm$ standard error of the mean (SEM). Statistical significance was determined using one-way ANOVA followed by Tukey's *post hoc* test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Lunasin effect on viability of macrophages. The impact of lunasin on SC macrophage viability was evaluated using the MTT assay following 1, 7, 14, and 21 days of exposure to lunasin at concentrations of 10, 25, and 50 μ M, administered either alone or with *P. agglomerans* (PA) antigen (Fig. 1). In the absence of antigen stimulation, lunasin induced a transient, concentration-dependent reduction in macrophage viability after 1 day of treatment (Fig. 1A). Compared to the untreated control, normalized to 100%, cell viability significantly decreased to 94.75%, 89.80%, and 82.25% after exposure to 10, 25, and 50 μ M lunasin, respectively. This reduction was not sustained during prolonged culture. After 7 days, viability values slightly exceeded the control, reaching 103.08%, 103.18%, and 104.30% at 10, 25, and 50 μ M lunasin, respectively, with no significant differences from untreated cells. A similar trend was observed after 14 days, with viability at 101.63%, 102.65%, and 102.72%, respectively. After 21 days, viability remained close to control levels, with values of 101.30%, 102.35%, and 103.27% for 10, 25, and 50 μ M lunasin, respectively. Therefore, while lunasin transiently reduced macrophage viability during the first 24 hours of exposure, no sustained reduction was observed during extended incubation.

Macrophages showed a distinct response when exposed to *P. agglomerans* antigen (Fig. 1B). After 1 day, antigen stimulation increased cell viability from 100% in the non-stimulated control to 130.80%. In the presence of lunasin, viability remained elevated but decreased progressively with increasing lunasin concentration, reaching 129.98%, 126.05%, and 115.20% at 10, 25, and 50 μ M, respectively. The reduction relative to the antigen-only group was significant at 25 μ M and more pronounced at 50 μ M. After 7 days,

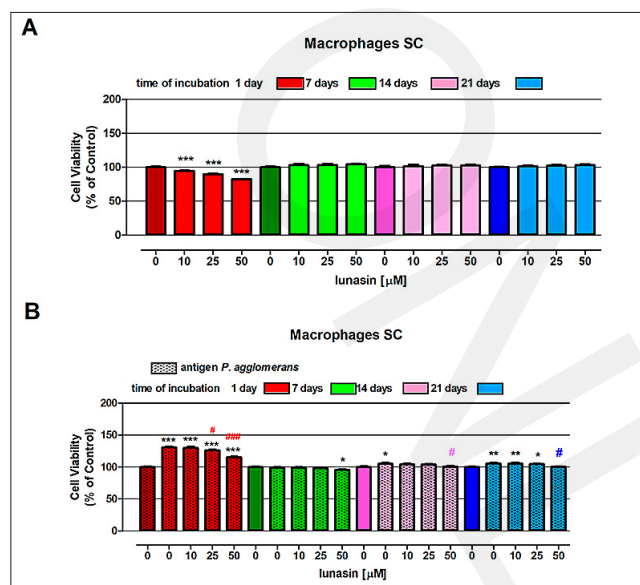


Figure 1. Effect of long-term exposure to lunasin (A) and *P. agglomerans* antigen (B) on macrophage viability. The MTT assay assessed macrophage viability after 1, 7, 14, and 21 days of incubation with lunasin at a concentration of 10 μM, 25 μM and 50 μM, administered alone (A) or in combination with *P. agglomerans* antigen at a concentration of 250 μg/mL (B). Results are expressed as mean ± SEM. Statistically significant differences compared to the control group (SC cells cultured in basic medium for the respective incubation period) are indicated as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Statistically significant differences in macrophage viability induced by lunasin compared to macrophages cultured in medium containing only antigen for the corresponding incubation period are indicated as # $p < 0.05$, and ### $p < 0.001$. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test

antigen-treated macrophages had 98.75% viability, while 10, 25, and 50 μM lunasin groups had 98.75%, 97.97%, and 95.43% viability, respectively. At this time point, only the group treated with PA and 50 μM lunasin showed a significant decrease relative to the corresponding non-

stimulated control, while no significant difference was detected between the PA and PA plus lunasin groups. After 14 days, antigen exposure increased viability modestly but significantly to 105.00% compared with 100.02% in the non-stimulated control. Addition of 10 and 25 μM lunasin resulted in values of 104.15% and 104.00%, respectively, whereas 50 μM lunasin reduced viability to 100.53%, significantly lower than that observed in the antigen-only group. A similar pattern was evident after 21 days. Viability reached 105.40% in macrophages exposed to PA alone and remained elevated at 105.33% and 104.45% in the presence of 10 and 25 μM lunasin, respectively. In contrast, treatment with 50 μM lunasin reduced viability to 100.38%, a value close to that of the non-stimulated control and significantly lower than that observed following antigen treatment alone.

Based on these results, 50 μM lunasin was selected for further experiments, as this concentration produced the most pronounced effect in antigen-stimulated macrophages without causing a sustained reduction in viability when administered alone.

Modulatory effects of lunasin on macrophage polarization into M1 and M2 subpopulations. The effects of lunasin on macrophage polarization were assessed by quantifying the proportions of CD80⁺CD86⁺ M1 and CD163⁺CD206⁺ M2 macrophages after 7, 14, and 21 days of culture (Fig. 2). In non-stimulated macrophage cultures, the impact of lunasin on the M1 population varied substantially depending on the duration of exposure. After 7 days, the percentage of M1 macrophages was 4.38% in the control and 5.31% following treatment with 50 μM lunasin, with no significant difference between groups. At day 14, lunasin significantly reduced the proportion of M1 macrophages from 8.26% in the control to 5.24%. By contrast, after 21 days of exposure, lunasin markedly increased the M1 population from 4.02% in the

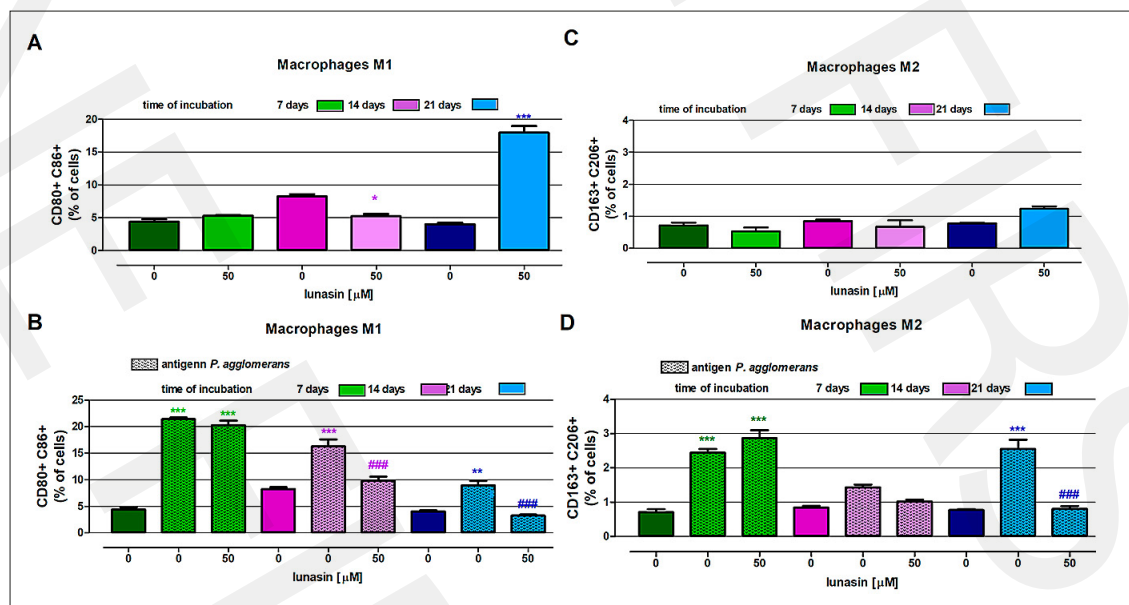


Figure 2. Effect of long-term exposure to lunasin on macrophage differentiation. Macrophage phenotype was determined by flow cytometry using antibodies specific for surface antigens characteristic of M1-type cells (CD80+ CD86+) or M2-type cells (CD163+ CD206+). Analysis was conducted after 7, 14, and 21 days of incubation of SC cells with lunasin at a concentration of 50 μM, administered either alone or in combination with *P. agglomerans* antigen at a concentration of 250 μg/mL. Results are expressed as mean ± SEM. Statistically significant differences compared to the control group (SC cells cultured in basic medium for the corresponding incubation period) are indicated as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Statistically significant differences in macrophage differentiation induced by lunasin compared to macrophages cultured in medium containing only antigen for the corresponding incubation period are indicated as ### $p < 0.001$. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test

control to 17.97%. These findings indicate that the effect of lunasin on M1 polarization was time-dependent, with opposing responses observed at 14 and 21 days. Lunasin did not significantly affect M2 polarization in non-stimulated macrophages. The proportion of M2 cells was 0.71% in the control and 0.52% in the lunasin-treated group after 7 days, 0.85% and 0.67% after 14 days, and 0.78% and 1.23% after 21 days, respectively.

Stimulation with *P. agglomerans* antigen strongly promoted M1 polarization, particularly at earlier time points (Fig. 2B). After 7 days, the proportion of M1 macrophages increased from 4.38% in the non-stimulated control to 21.40% following PA exposure. A similarly high proportion of M1 cells, 20.27%, was observed in the PA plus lunasin group, with no significant difference between PA alone and PA combined with lunasin. At day 14, PA increased the percentage of M1 macrophages from 8.26% in the control to 16.25%, whereas the addition of 50 μ M lunasin significantly reduced this proportion to 9.75%. A similar effect was observed after 21 days, when M1 macrophages accounted for 4.02% of the non-stimulated control population and 8.92% of cells exposed to PA, while the addition of lunasin significantly reduced the M1 population to 3.24%. These results indicate that lunasin did not substantially affect the early PA-induced M1 response at day 7 but significantly attenuated M1 polarization during prolonged antigen exposure at days 14 and 21.

The effects of PA and lunasin on the M2 population were also dependent on the duration of exposure (Fig. 2D). After 7 days, PA significantly increased the proportion of M2 macrophages from 0.71% in the non-stimulated control to 2.44%. The PA plus lunasin group showed a similar increase to 2.87%, with no significant difference between the two 2 antigen-stimulated groups. At day 14, M2 macrophages represented 0.85% of the control population, 1.44% of the PA-treated population, and 1.02% of the PA plus lunasin group, with no significant differences observed. A distinct response was observed after 21 days, when PA significantly increased the M2 population from 0.78% in the non-stimulated control to 2.55%. The addition of lunasin reduced the proportion of M2 macrophages to 0.81%, which was significantly lower than that observed following PA treatment alone, and was comparable to the non-stimulated control. Collectively, these results demonstrate that the influence of lunasin on macrophage polarization was both time- and activation-state-dependent. In non-stimulated macrophages, prolonged exposure to lunasin markedly increased M1 polarization at day 21. In contrast, in PA-stimulated macrophages, lunasin reduced M1 polarization at days 14 and 21, and attenuated the PA-associated increase in M2 macrophages at day 21.

Effect of lunasin on TGF- β release by SC macrophages.

TGF- β concentrations in SC macrophage culture supernatants were measured after 7, 14, and 21 days of incubation (Fig. 3). In non-stimulated cultures, lunasin influenced TGF- β release in a time-dependent manner (Fig. 3A). After 7 days, treatment with 50 μ M lunasin significantly reduced TGF- β concentration from 22.33 pg/mL in the control group to 12.63 pg/mL. At day 14, TGF- β concentration increased significantly from 23.97 pg/mL in control cultures to 32.93 pg/mL following lunasin treatment. After 21 days, TGF- β levels were 44.20 pg/mL in the control group and 33.80 pg/mL in the lunasin-treated group; this difference was not statistically significant. These findings indicate that

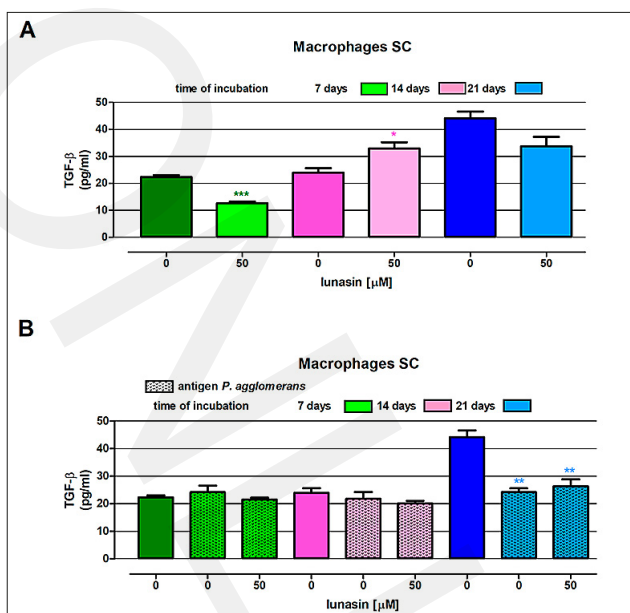


Figure 3. Effect of exposure of macrophages to lunasin on the production and release of TGF- β . The concentration of TGF- β was assessed by flow cytometry using CBA detection beads. Analysis was performed after 7, 14, and 21 days of macrophage incubation with lunasin at a concentration of 50 μ M, administered either alone or in combination with *P. agglomerans* antigen at a concentration of 250 μ g/mL. Results are expressed as mean $\pm$ SEM. Statistically significant differences compared to the control group (SC cells cultured in basic medium for the corresponding incubation period) are indicated as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Statistical analysis was conducted using one-way ANOVA followed by Tukey's *post-hoc* test

lunasin's effect on TGF- β release was time-dependent and not consistently suppressive.

In macrophages stimulated with *P. agglomerans* antigen, TGF- β concentrations were 24.20, 21.83, and 24.20 pg/mL after 7, 14, and 21 days, respectively (Fig. 3B). The corresponding values in cultures treated with PA and 50 μ M lunasin were 21.50, 20.10, and 26.37 pg/mL. No significant differences were detected between the PA and PA plus lunasin groups at any time point. After 21 days, TGF- β concentrations in both the PA and PA plus lunasin groups were significantly lower than in the non-stimulated control, which reached 44.20 pg/mL.

Impact of lunasin on the transformation of bronchial epithelial cells into mesenchymal cells in response to chronic exposure to factors released by macrophages activated with *P. agglomerans* antigen. Chronic exposure of NL20 bronchial epithelial cells to macrophage-conditioned media resulted in marked morphological alterations consistent with EMT (Fig. 4). Observed changes included reduced cell recovery after detachment, progressive loss of intercellular junctions, and acquisition of an elongated, spindle-like cell morphology. Similar morphological alterations were observed in cultures exposed to conditioned media derived from macrophages stimulated with *P. agglomerans* antigen or TGF- β 1.

The addition of lunasin at a concentration of 50 μ M to macrophage cultures attenuated these morphological changes. NL20 cells exposed to conditioned media derived from lunasin-treated macrophages demonstrated improved preservation of epithelial morphology and higher cell recovery compared to cells exposed to conditioned media from antigen-activated macrophages without lunasin. The protective effect was most pronounced after 21 days of incubation. Although EMT-associated changes were not

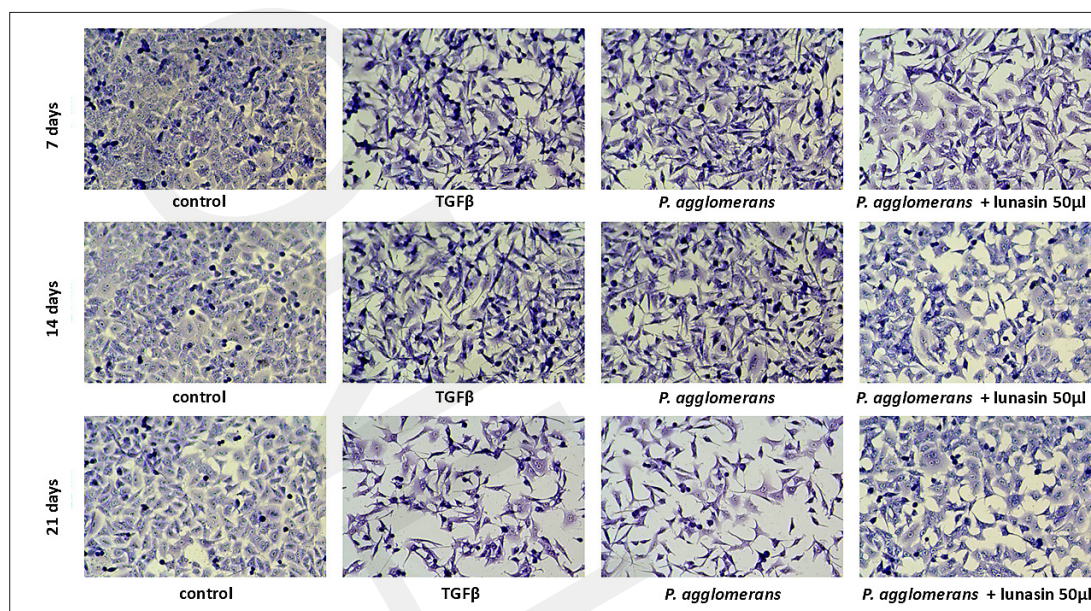


Figure 4. Changes in the morphology of NL20 bronchial epithelial cells after incubation with substances released by macrophages activated by *P. agglomerans* antigen in the presence or absence of lunasin. Morphological analysis was conducted after 7, 14, and 21 days of incubation of NL20 cells with conditioned media collected from SC cells stimulated with 10 ng/mL TGF- β 1, *P. agglomerans* antigen at a concentration of 250 μ g/mL, and *P. agglomerans* antigen (250 μ g/mL) with lunasin at a concentration of 50 μ M. The control group consisted of NL20 cells incubated with media derived from non-activated SC macrophages. Cell morphology was visualised using light microscopy following May-Grünwald-Giemsa staining at 200 $\times$ magnification

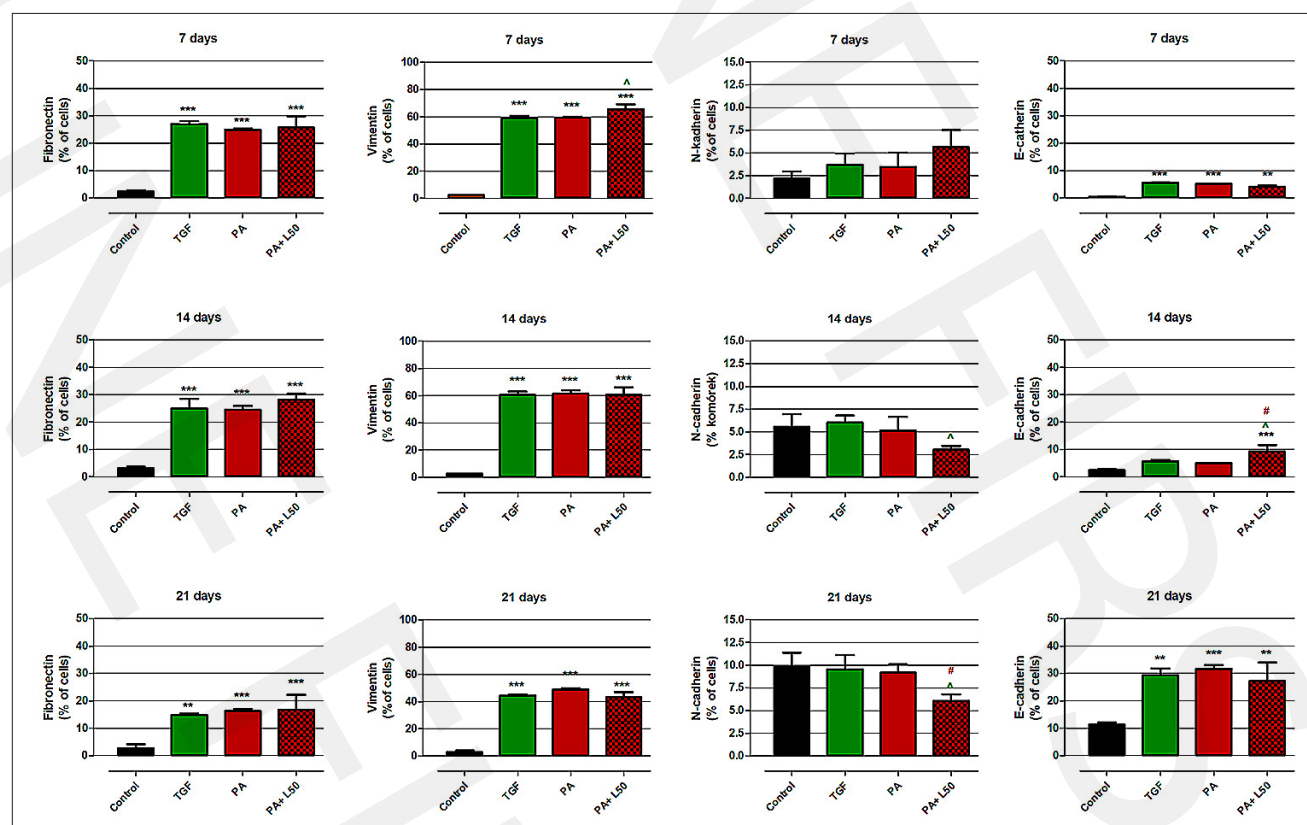


Figure 5. Changes in EMT marker expression levels in NL20 airway epithelial cells after incubation with substances released by macrophages activated by *P. agglomerans* antigen in the presence or absence of lunasin. Expression levels of epithelial-to-mesenchymal transition (EMT) surface markers were assessed by flow cytometry using specific antibodies. The analysis was conducted after 7, 14, and 21 days of incubation of NL20 cells with conditioned media from SC macrophages stimulated with 10 ng/mL TGF- β 1 (TGF), *P. agglomerans* antigen (PA) at a concentration of 250 μ g/mL, and lunasin at a concentration of 50 μ M (L50). Results are presented as mean $\pm$ SEM from three independent measurements. Statistically significant differences compared to the control group (NL20 cells incubated with media from non-activated macrophages) are indicated as ** $p < 0.01$, and *** $p < 0.001$. Differences compared to the positive control group (TGF: NL20 cells exposed to media from TGF- β 1-activated macrophages) are marked as # $p < 0.05$. Differences between NL20 cells treated with media from antigen-activated macrophages with or without lunasin are indicated as # $p < 0.05$. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post-hoc* test

completely abolished, their extent was visibly reduced in the presence of lunasin.

Effect of lunasin on EMT marker expression in NL20 epithelial cells. The expression of fibronectin, vimentin, N-cadherin, and E-cadherin were assessed in NL20 cells cultured with macrophage-conditioned media for 7, 14, and 21 days (Fig. 5). Fibronectin expression was markedly elevated in all experimental groups compared to untreated controls. After 7 days, the proportion of fibronectin-positive cells was 26.94% in the TGF group, 24.87% in the PA group, and 25.75% in the PA + lunasin group, whereas only 2.34% of control cells were positive. This trend persisted at day 14, with values of 24.94%, 24.45%, and 28.23%, respectively, compared to 3.18% in the control group. By day 21, fibronectin expression decreased but remained substantially higher in all treated groups, reaching 14.78% in the TGF group, 16.28% in the PA group, and 16.89% in the PA + lunasin group, compared to 2.75% in untreated cells. No significant differences were observed between the PA and PA + lunasin groups at any time point.

Vimentin expression exhibited a comparable pattern. After 7 days, vimentin-positive cells increased from 2.41% in the control group to 58.98% in the TGF group, 59.43% in the PA group, and 65.62% in the PA + lunasin group. At this time point, the PA + lunasin group demonstrated significantly higher vimentin expression than the TGF group. By day 14, the proportions of vimentin-positive cells were nearly identical among the experimental groups, at 60.48% for TGF, 61.47% for PA, and 60.71% for PA + lunasin, compared with 2.59% in the control group. At day 21, vimentin expression remained substantially elevated, reaching 44.50%, 49.00%, and 43.30% in the TGF, PA, and PA + lunasin groups, respectively, while control cells exhibited 2.70% positivity. Although the PA + lunasin group showed a numerically lower value than the PA group at day 21, this difference was not statistically significant.

N-cadherin expression displayed greater variability across groups. After 7 days, expression ranged from 2.19% in the control group to 3.71% in the TGF group, 3.47% in the PA group, and 5.66% in the PA + lunasin group, with no significant differences observed. At day 14, N-cadherin expression was 5.55% in control cells, 6.02% in the TGF group, and 5.14% in the PA group, while the PA + lunasin group exhibited a lower value of 3.03%. This reduction was significant compared with the TGF group, but not compared with the PA group. By day 21, a more pronounced effect was evident: N-cadherin expression was 9.89% in the control group, 9.55% in the TGF group, and 9.18% in the PA group, whereas the PA + lunasin group showed a decrease to 6.05%. At this time point, N-cadherin expression in the PA + lunasin group was significantly lower than in both the PA and TGF groups.

E-cadherin expression also varied throughout the experiment. After 7 days, the proportion of E-cadherin-positive cells increased from 0.45% in the control group to 5.53% in the TGF group, 5.10% in the PA group, and 4.05% in the PA + lunasin group. No significant difference was observed between the PA and PA + lunasin groups. The most pronounced lunasin-associated effect was observed at day 14, when E-cadherin expression reached 9.32% in the PA + lunasin group, compared to 2.43% in the control, 5.57% in the TGF group, and 4.98% in the PA group; this

value was significantly higher than in each of the other groups. At day 21, E-cadherin expression remained elevated in all experimental groups, reaching 29.38% in the TGF group, 31.68% in the PA group, and 27.25% in the PA + lunasin group, compared to 11.21% in the control group. No significant difference was detected between the PA and PA + lunasin groups at this time point.

DISCUSSION

Persistent antigen exposure is a defining feature of hypersensitivity pneumonitis (HP) and establishes a chronic inflammatory microenvironment in which innate immune cells, particularly macrophages, play a central regulatory role [5]. Sustained macrophage activation contributes not only to cytokine production but also to the release of growth factors that influence epithelial plasticity and tissue remodeling [21]. Among these mediators, transforming growth factor beta (TGF- β) represents a key link between inflammation and fibrogenesis, acting as a major inducer of epithelial–mesenchymal transition (EMT) [22]. Modulation of the macrophage–epithelial axis may therefore represent an important regulatory point in inflammation-driven structural changes of the lung.

In the present study, the biological activity of lunasin was evaluated in a long-term *in vitro* model integrating chronic stimulation of macrophages with *P. agglomerans* antigen and subsequent analysis of macrophage-driven effects on bronchial epithelial cells. This bacterium is a well-documented component of organic dusts that triggers intense pulmonary inflammatory responses due to its high endotoxin content [19]. Moreover, this approach enabled simultaneous assessment of immune activation, profibrotic mediator release, and epithelial responses within a unified experimental framework relevant to HP-associated remodeling.

At a concentration of 50 μ M, the effect of lunasin on macrophage polarization depended on the duration of exposure and the activation state of the cells. In non-stimulated cultures, lunasin reduced the proportion of CD80⁺CD86⁺ macrophages at day 14, whereas prolonged exposure for 21 days resulted in a marked increase in the M1 population. This time-dependent response indicates that the effect of lunasin on macrophage phenotype is not unidirectional. In non-stimulated conditions, lunasin promoted M1 polarisation, typically associated with Th1-type responses and antimicrobial defence [23]. In macrophages chronically stimulated with *P. agglomerans* antigen, lunasin significantly reduced M1 polarisation at both day 14 and day 21. These contrasting responses indicate that lunasin does not function as a universal suppressor of M1 differentiation, but instead modulates macrophage activation according to the activation state of the cells and duration of exposure. Hsieh et al. reported a similarly complex immunomodulatory profile, describing anti-inflammatory effects of lunasin accompanied by modulation of Th1-associated immune responses in an obesity-related inflammatory model [24]. Although their experimental system differs substantially from the present macrophage model, these observations support the view that lunasin may exert context-dependent immunomodulatory effects rather than acting as a broadly suppressive agent. Another crucial aspect of this study was

evaluating lunasin's effect on the secretion of TGF- β , a master inducer of EMT undergoing pathological tissue regeneration leading to fibrosis [25–26].

In non-stimulated cultures, the effect of lunasin was time-dependent. TGF- β concentration was significantly reduced after 7 days of exposure, whereas a significant increase was observed at day 14. At day 21, TGF- β levels were numerically lower in lunasin-treated cultures than in the corresponding controls, but the difference was not statistically significant. Thus, the present data do not support a sustained suppressive effect of lunasin on TGF- β production. In PA-stimulated macrophages, TGF- β concentrations were similar between the PA and PA + lunasin groups throughout the experiment, with no statistically significant differences between these groups at any time point. After 21 days, TGF- β concentrations were lower in both antigen-stimulated groups than in the corresponding non-stimulated control. Because the PA and PA + lunasin groups did not differ significantly, this reduction cannot be attributed directly to lunasin. As TGF- β is a central mediator of fibrotic remodeling and EMT induction, modulation of its secretion by macrophages may influence downstream epithelial responses [22, 26]. It should be emphasized that the present findings reflect modulation of TGF- β production at the macrophage level and do not demonstrate direct inhibition of TGF- β signalling pathways within epithelial cells. Nevertheless, in HP, chronic antigen exposure sustains macrophage activation and continuous release of profibrotic mediators. Regulation at this upstream level may alter the composition of the local microenvironment and thereby affect epithelial behaviour.

In the context of fibrosis development in the course of HP, the next features of lunasin discovered in the presented study seems to be crucial. The results suggest that lunasin exerted a limited and time-dependent influence on selected EMT-associated features in NL20 cells. Chronic exposure of NL20 cells to macrophage-conditioned media induced morphological changes characteristic of EMT, including progressive loss of intercellular junctions, spindle-like morphology, and reduced cell recovery. Similar alterations were observed in the TGF- β 1 positive-control condition, confirming that the system reproduced key EMT-driving stimuli. Conditioned media derived from lunasin-treated macrophages induced less pronounced morphological disruption, particularly after prolonged incubation. Although EMT-like changes were not completely prevented, epithelial morphology was partially preserved and cell recovery improved [27]. Flow-cytometric analysis of epithelial–mesenchymal transition (EMT)-associated markers demonstrated that lunasin exerted limited and variable effects depending on the specific marker and time point. Conditioned media derived from *P. agglomerans* antigen-stimulated macrophages substantially increased fibronectin and vimentin expression, and lunasin treatment did not significantly attenuate these elevations. More selective effects were observed for cadherin family proteins. E-cadherin expression was significantly higher in the PA plus lunasin group compared to the PA group at day 14, while N-cadherin expression was significantly reduced following lunasin treatment at day 21. These results suggest that lunasin modulates specific aspects of the EMT-associated phenotype; however, the effects were modest, marker-dependent, and influenced by the duration of exposure. The absence of a consistent response across all EMT markers aligns with the complex, multi-step progression of

EMT [28]. During this process, epithelial cells may acquire partial or intermediate hybrid phenotypes rather than undergoing a complete phenotypic transition [28]. Given the heterogeneous composition of macrophage-conditioned media, these findings imply that lunasin may selectively modulate macrophage–epithelial crosstalk rather than broadly reversing EMT-associated alterations.

To the best of the authors' knowledge, lunasin has not been previously investigated in a model integrating chronic stimulation with an HP-related antigen, longitudinal assessment of macrophage polarization, quantification of macrophage-derived TGF- β , and evaluation of macrophage-driven EMT in bronchial epithelial cells. While the anti-inflammatory properties of lunasin have been documented in other cellular contexts [29, 30], its potential influence on the immune–epithelial crosstalk relevant to fibrotic lung remodelling has remained unexplored. The present study therefore expands the current understanding of lunasin's bioactivity into a disease-relevant framework, linking chronic inflammation with epithelial plasticity. These findings underscore the pleiotropic nature of lunasin's activity. By influencing both immune and structural cells within the lung, lunasin appears to target multiple stages in the pathogenesis of pulmonary fibrosis. Significantly, its modulatory effects are not limited to a singular molecular axis but extend across inflammatory cytokine production, growth factor signalling, and epithelial plasticity.

Limitations of the study. The study also has some limitations which should be taken into account. Since all the experiments were carried out *in vitro*, they do not fully reflect the structural and immunological complexity of the lung microenvironment. Furthermore, the effects of lunasin were only partial and depended on the particular endpoint and the length of exposure. It might be useful to carry out comparative studies with well-established antifibrotic agents in order to see whether lunasin has any complementary action in more advanced experimental systems. Also, the intracellular signalling pathways in macrophages and epithelial cells were not directly examined; as a result, the molecular mechanisms responsible for the effects observed still remain unclear and will need further investigation.

This study indicates that lunasin has time- and context-dependent effects on antigen-induced macrophage activation and results in only minor alterations in certain aspects of the epithelial response to media conditioned by macrophages. While lunasin had a time-dependent influence on the release of TGF- β in non-stimulated macrophages, it did not significantly affect TGF- β levels when the macrophages were stimulated with PA. In NL20 cells, the impact on EMT-related markers was small and primarily consisted of an increase in E-cadherin expression by day 14 and a decrease in N-cadherin expression by day 21. Within the constraints of the current *in vitro* model, these results suggest that lunasin can alter some of the components of macrophage–epithelial crosstalk associated with chronic antigen exposure and offer a foundation for further studies.

CONCLUSIONS

The present study demonstrates that lunasin modulates antigen-induced macrophage activation and influences

macrophage–epithelial crosstalk in a long-term *in vitro* model relevant to hypersensitivity pneumonitis (HP). At a concentration of 50 μ M lunasin attenuated antigen-driven M1 polarization at later time points; however, it did not significantly alter macrophage-derived TGF- β levels during prolonged stimulation with *Pantoea agglomerans* antigen. These alterations were accompanied by a partial preservation of epithelial morphology and a moderate normalization of selected EMT markers in NL20 bronchial epithelial cells exposed to macrophage-conditioned media. These findings indicate that lunasin influences selected immune and epithelial responses within a framework of chronic, antigen-driven inflammation. While the observed effects were partial, they suggest that lunasin can modify selected components of the cellular crosstalk linking sustained immune activation to epithelial plasticity. Given the pivotal role of macrophage-derived mediators and EMT in inflammation-associated lung remodelling, further evaluation of lunasin in more sophisticated experimental systems, including *in vivo* models, is warranted.

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