Effect of air pollutants on allergic inflammation in structural cells of nasal mucosa

Joo-Hoo Park, PhD[1,2], Jee Won Moon, MD*[3], Hyun-Woo Yang[1,2], Dae Jin Song†[4], and Il-Ho Park, MD, PhD†[1,2,3]*

[1] Upper Airway Chronic Inflammatory Diseases Laboratory, Korea University College of Medicine, Seoul, Republic of Korea

[2] Medical Device Usability Test Center, Korea University Guro Hospital, Seoul, Republic of Korea

[3] Department of Otorhinolaryngology-Head and Neck Surgery, Korea University College of Medicine, Seoul, Republic of Korea

[4] Department of Pediatrics, Korea University College of Medicine, Seoul, Republic of Korea

* These authors equally contributed to this work

† Dae Jin Song and Il-Ho Park are co-corresponding authors

Corresponding author:

Il-Ho Park

Department of Otorhinolaryngology-Head and Neck Surgery, Guro Hospital, Korea University College of Medicine, 148, Gurodong-ro, Guro-gu, Seoul, 08308, South Korea

Telephone: 82-2-2626-1298

Fax: 82-2-868-0475

E-mail: parkil5@korea.ac.kr

Dae Jin Song

Department of Pediatrics, Guro Hospital, Korea University College of Medicine, 148, Gurodong-ro, Guro-gu, Seoul, 08308, South Korea

Telephone: 82-2-2626-3158

Fax: +82-2-2626-1249

E-mail: djsong506@korea.ac.kr

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ORCID

Joo-Hoo Park 0000-0002-3221-640X

Jee Won Moon 0000-0003-2307-1824

Hyun-Woo Yang 0000-0002-8674-3088

Dae Jin Song 0000-0003-0647-3186

Il-Ho Park 0000-0002-7011-6071

Author contributions

Conceptualization: DJS, IHP. Data curation: JHP, HWY. Funding acquisition: IHP. Investigation: JHP, JWM. Methodology: JHP, HWY. Project administration: DJS, IHP. Software: JHP. Supervision: IHP, DJS. Visualization: JHP. Writing - original draft: JHP, JWM. Writing - review & editing: IHP

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HIGHLIGHTS

- PM 2.5 was collected from ambient 24h air of Seoul and component analysis was conducted for metallic constituents.
- We built a 3D-hybrid culture model to test for the effect of aerolized air pollutants on nasal epithelial cells and fibroblasts.
- Ambient air pollutants in the form of aerosols lead to an increased expression of allergic inflammatory cytokines in both nasal epithelial cells and fibroblasts
- Regulations on air pollution will help reduce the burden of allergic diseases worldwide in the future.

Abstract

Objectives

Air pollution is a growing global concern, and its effect on allergic inflammation has attracted the attention of many researchers. Particulate matter (PM) is a major component of ambient air pollution, and heavy metals are the primary toxic constituents of PM. As previous studies on the impact of air pollutants on allergic inflammation lacked physiological resemblance to actual atmospheric exposure, we built an experimental model to investigate the effects of aerosolized air pollutants on nasal epithelial cells and fibroblasts.

Methods

We collected PM 2.5 samples from ambient 24 h air in Seoul from August 2020 to August 2022, and then conducted component analysis for metallic constituents. Primary nasal epithelial cells and nasal fibroblasts, obtained and cultured from the turbinate tissues of human participants, were treated with PM 2.5 and heavy metals were identified from component analysis to observe changes in cytokine expression. 3D-hybrid culture model, a co-culture of an air-liquid interface and nasal fibroblast spheroids, was built to observe the impact of air pollutants in the form of aerosols.

Results

Among the heavy metals, Si was the predominant component of PM 2.5 and Zn showed the highest correlation with the concentration of PM 2.5 in Seoul. PM 2.5, Zn, and Si increased the production of epithelial cell-derived cytokines, with which PM 2.5 and Zn exhibited similar trends with one another. Exposure of 3D-hybrid model to aerosolized PM 2.5 and Zn yielded elevated periostin, α-SMA, and fibronectin expressions from fibroblast spheroids, and those without epithelial barrier exhibited a similar increase in periostin expression.

Conclusions

Ambient air pollutants in the form of aerosols lead to an increased expression of allergic inflammatory cytokines in both nasal epithelial cells and fibroblasts. Regulations on air pollution will help reduce the burden of allergic diseases worldwide in the future.

Keywords: Air pollution, PM2.5, heavy metal, allergic rhinitis, Fine particulates, Indoor pollutant, Allergic inflammation, Epithelial cell, Fibroblasts

Introduction

According to the World Health Organization (WHO), approximately 99% of the global population breathe polluted air that exceeds the WHO guideline limits, and approximately 7 million premature deaths occur annually due to air pollution. Air pollution can be divided into two categories: ambient and outdoor pollution and household and indoor pollution. Although these two types of air pollution have different compositions with spatial and temporal variations, particulate matter (PM) remains as one of the most common components. PM is a heterogeneous mixture of liquid and solid particles, and heavy metals are its main toxic constituents. [1] Despite international efforts to reduce air pollution, PM 2.5 levels have increased 38% globally from 1960 to 2009, and 55% of the global population was exposed to more PM 2.5 in 2016 than that in 2010. [2, 3]

In 2015, the World Health Assembly passed a landmark resolution regarding air quality and health, acknowledging air pollution as a primary factor in noncommunicable diseases (NCDs), which are the leading causes of death worldwide. [4] Air pollution poses a threat to both overall mortality and specific diseases. It can also increase the risk of stroke or ischemic heart disease, aggravate underlying respiratory diseases, and trigger asthma attacks. [5] Even short-term exposure is known to be related to hospitalization, and numerous epidemiologic and experimental studies have demonstrated an association between air pollutants and allergic diseases. [1, 6]

A comparative study conducted in South Korea found that children from industrialized areas had more allergic rhinitis (AR) or asthma-related symptoms than those from non-industrialized areas, and a time-series analysis from Beijing reported a strong association between daily levels of air pollutants and the number of outpatients with AR. [7, 8] A cross-sectional study of two European cohorts involving 1408 adults also concluded that patients with rhinitis residing in

highly polluted areas experienced more severe nasal symptoms. [9] Many laboratory studies have supported these findings. Increased concentrations of air pollutants elevate the deposition of allergens in the airways, and oxidative injury from air pollutants may increase epithelial permeability. [10] PM is thought to serve as an adjuvant and stimulate persistent allergic inflammation in the respiratory tract synergistically with other known pollutants. Heavy metal exposure is known to alter macrophage-related cytokines and surface markers. [11-13]

Although the adverse effects of air pollution on allergic diseases are wellrecognized, the laboratory model to date does not reflect the actual atmospheric environment to which the upper respiratory tract is exposed. Therefore, in this study, we collected and analyzed PM 2.5 Seoul and identified the major heavy metals with the highest correlation with the overall concentration of PM 2.5. We then constructed an experimental model with physiological resemblance and exposed our model to PM 2.5 collected from Seoul, to observe the effects of PM 2.5 and its components on allergic inflammation.

Methods

Sampling collection and analysis

A high-capacity air sampler was employed to capture particulate matter with a diameter of 2.5 μm or less in Seoul. The collected PM2.5 with phosphate-buffered saline (PBS) was filtered through a 2.5 μm pore filter, effectively sieving out particles with a diameter of 2.5 μm or less. The particles smaller than 2.5μm were separated through centrifugation. Subsequently, the collected particulate matter was transferred to a tube, subjected to a 15 minute heating at 121°C, and then stored at -20°C. Quantification of 19 trace elements (PM2.5, Al, Ti, V, Mn, Fe, Ni, Co, Cu, Zn, As, Sr, Mo, Cd, Ba, Pb, P, Cr, Si) was performed using Atomic Absorption Spectroscopy (AAS, GBC Avanta PM, Australia). The entire process was designed to ensure the accurate measurement of trace elements associated with PM2.5 in the air quality assessment in Seoul.

Patients and specimens

The inferior turbinate tissues were harvested from individuals who exhibited no indications of inflammation, allergic reactions, asthma, or sensitivity to aspirin. during rhinoplasty. Patients were recruited from the Department of Otorhinolaryngology at Korea University Medical Center, following the principles outlined in the Declaration of Helsinki, with prior informed consent obtained. This study received approval from the Institutional Review Board for Bioethics at Korea University Medical Center. (approval number: 2023GR0179). Ethical handling of patients and their tissue specimens followed established protocols and guidelines.

Aerosol exposure

ALI culture in 12 well transwell plates were placed on VitroCell Cloud12 (VitroCell Systems, Waldkirch, Germany) and treated with the exposure system as an aerosol that exposes cells to 200 µL of PM2.5 and $ZnCl_2$ (Sigma-Aldrich, St. Louis, MO, USA) (Fig. 1A). The exposure to aerosol was conducted single time, and real-time measurements of the deposited particles were performed using a quartz microbalance (QCM).

Primary nasal epithelial cell cultures

Primary nasal epithelial cells were collected using a brush and incubated with neumaCult-Ex Plus medium (Stemcell Technologies, Vancouver, Canada) on plates coated with collagen type 1 (Corning Incorporated, Corning, NY, USA). After cells were adherent, red blood cells (RBCs) were removed by washing with PBS. When cells reached > 90% confluence, cells were isolated using an animal-free cell isolation kit (Stemcell Technologies) and cultured in 75T flasks.

Air-Liquid Interface (ALI) culture of primary nasal epithelial cells

Human nasal epithelial cells (HNECs) were expanded, washed with Dulbecco's Phosphate-Buffered Saline (DPBS), and dissociated using the Animal Component-Free Cell Dissociation Kit. For ALI culture, 0.5 mL of PneumaCult™-Ex medium (Stemcell) containing HNECs is seeded into the upper chamber of a 0.4 µm Transwell insert (Corning MediTech) and only medium is added to the lower chamber. The media in both the upper and lower chambers were refreshed every 2 to 4 days. Upon reaching 100% cellular confluence, the medium in both the upper and lower chambers was aspirated. Subsequently, 1 mL of PneumaCult™-ALI Maintenance Medium was added only to the lower chamber, with subsequent medium changes every 2 days.

Nasal fibroblasts spheroid formation

The inferior turbinate, obtained from the patient, are finely chopped and placed in DMEM media containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). The chopped tissue is then evenly distributed onto a plate to isolate. The cells around the tissue are dissociated using 0.05% trypsin and cultured to isolate fibroblasts. When the fibroblasts reach 100% confluence, they are detached from the plate using trypsin and cultured at a density of 5x10^4 cells per well in a 96-well round-bottom ultra-low attachment plate (Corning). Each well formed a single sphere and was cultured for 7 days, with the medium changed every 3 days. For co-culture with ALI and nasal fibroblast spheroids, spheroids were transferred to Transwell bottom plates, inserted into the upper chamber containing ALI culture, and incubated at 37°C with 5% CO_2 (Fig. 1B). RPMI 1640 medium (Hyclone, Logan, UT, USA) was added only to the bottom plate.

Cell cytotoxicity assay

Cellular cytotoxicity induced by PM2.5, $ZnCl_2$, and SiO_2 (Sigma-Aldrich) was evaluated using a WST-1 assay. Nasal epithelial cells or nasal fibroblasts were plated in 96-well plates and exposed to various concentrations of PM2.5, ZnCl2, or SiO2 for 72 hours. WST-1 solution is then added to the medium. Incubation at 37°C facilitated the enzymatic conversion of WST-1 to formazan by metabolically active cells. The absorbance at 450 nm (with reference to 650 nm) was measured using a microplate reader (Bio-Rad).

Real-time PCR

PM2.5, $ZnCl_2$, and SiO_2 were treated with epithelial cells and fibroblasts for 24 hours. RNA was isolated from the samples using the TRIzol reagent (Invitrogen) according to

the manufacturer's instructions. Concentration and purity of the extracted RNA were determined using a NanoDrop spectrophotometer. For cDNA synthesis, 2 μg of total RNA was reverse transcribed into cDNA using a mixture of M-MLV reverse transcriptase (Invitrogen), oligo dT primers, and a ribonuclease inhibitor. The synthesized cDNA was used for gene expression analysis by real-time PCR. Power SYBR Green PCR Master Mix (Applied Biosystems) was mixed with the target-specific primers and a cDNA template. The target-specific primers are described in Table 1. Real-time PCR was performed using Quantstudio3 to measure PCR amplification. Gene expression levels were quantified using the ΔΔCt method. The Ct values obtained from target gene amplification were normalized to the expression of the reference gene, GAPDH. The relative fold change in gene expression was calculated by comparing the normalized target gene Ct values between experimental and control samples.

Enzyme-linked immunosorbent assays (ELISA)

PM2.5, $ZnCl_2$, and SiO_2 were treated with epithelial cells and fibroblasts for 72 hours. The concentrations of IL-6, IL-25, IL-33, TSLP, and Periostin in the culture media were determined using ELISA (R&D Systems, Minneapolis, MN, USA). Standards and samples were added to the wells and incubated at room temperature for 2 hours. After washing the wells three times, the appropriate conjugate (IL-6, IL-25, IL-33, TSLP, or Periostin) was added and incubated for 2 hours at room temperature. The reaction was then stopped with a stop solution, and the optical densities of the standards and samples were measured at 450 nm using a microplate reader (Bio-Rad).

Western blot analysis

PM2.5, $ZnCl_2$, and SiO_2 were treated with epithelial cells and fibroblasts for 72

hours. Proteins extracted using RIPA buffer were measured for concentration using the Bradford assay. Samples were prepared by mixing equal amounts of proteins with 5X SDS-PAGE and resolved by electrophoresis on 10% SDS-PAGE gels. The proteins were transferred to polyvinyl difluoride membranes and then blocked in 3% skim milk). The membranes were probed with anti-α-SMA (Abcam, Cambridge, MA, USA), anti-E-cadherin, anti-fibronectin, anti-GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and anti-vimentin (Cell Signaling Technology, Danvers, MA, USA) primary antibodies overnight at 4°C. The horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Vector Laboratories, Burlingame, CA, USA) were diluted in 3% skim milk for 1 hour before measurement using an ECL system (Pierce, Rockford, IL, USA). Subsequently, acquired images were analyzed with ImageJ software (NIH, Rockville, MD, USA) to quantify the observed signals.

Immunofluorescence staining

Cellular fixation was achieved by treating cells with 4% paraformaldehyde for 30 minutes, followed by permeabilization using 0.1% Triton X-100 for 10 minutes. Subsequently, a blocking step with 5% BSA for 1 hour was implemented before incubating the cells with primary antibodies against E-cadherin, α-SMA, or periostin (Santa Cruz Biotechnology, Inc., 1:100 dilution in blocking buffer) at 4°C overnight. Goat anti-mouse IgG (H+L) Alexa 488 or goat anti-rabbit IgG (H+L) Alexa 555 (Invitrogen) was diluted 1:200 in blocking solution and cells were incubated in this mixture for 1 hour at room temperature. Nuclei were counterstained with DAPI (Invitrogen) for 10 min. After washing with PBS, the coverslips were mounted on glass slides using a mounting medium. A confocal laser scanning microscope LSM700 (Zeiss, Oberkochen, Germany) was used to capture images of the stained cells.

Measurement of transepithelial electrical resistance

Epithelial electrical resistance (TEER) measurements were performed by inserting sterile electrodes of an EVOM2 epithelial volt-ohmmeter (World Precision Instruments) into both the upper and lower chambers of the transwell system. The background resistance of the cell-free insert membrane devoid of cells was subtracted from each measurement. TEER values were recorded in ohms (Ω), to provide insight into the barrier integrity of the epithelial cell layer.

Statistical analysis

The data are presented as the mean ± standard deviation of at least three experiments performed in duplicate. Statistical analysis was performed by unpaired t-test or one-way analysis of variance (ANOVA) followed by Tukey's test using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Statistical significance was set at $P < 0.05$.

Results

Correlation between PM2.5 and Zn and Si

Correlations were observed between concentrations of PM2.5, and concentrations of heavy metals. Among the elements comprising PM2.5, the order of abundance was Si, and aluminum (Al) (Fig. 1C). The correlation coefficient between PM2.5, and heavy metal concentrations was the highest for Zn, followed by iron (Fe) and nickel (Ni). The correlation coefficients between Zn concentration and PM2.5 and Si concentration and PM2.5, were 0.8 and 0.54, respectively, which were statistically significant (Fig. 1D and 1E). Considering these results, Zn and Si were selected as target heavy metals for further research.

PM2.5 stimulates the production of inflammatory cytokines in nasal epithelial cells

Primary nasal epithelial cells treated with different concentrations of PM2.5 showed no cytotoxicity until 1000 μg/mL (Fig. 2A). We evaluated the mRNA and protein expression of markers associated with allergic inflammation, including IL-6, IL-25, IL-33, TSLP, and periostin. The results showed that IL-6, IL-25, IL-33, and TSLP levels significantly increased in a concentration-dependent manner in both mRNA (Fig. 2B-2E) and protein expression (Fig. 2G–2J) in response to PM2.5 The expression of periostin mRNA and protein slightly increased at a concentration of 100 μg/mL of PM2.5. (Fig 2F and 2K), respectively. Under ALI culture conditions, the expression levels of IL-6, IL-25, IL-33, and TSLP increased in response to PM2.5 exposure, whereas periostin expression did not change (Fig. 2M-2V).

Effect of Zn on allergic inflammation in nasal epithelial cells

Primary nasal epithelial cells treated with various concentrations of $ZnCl_2$ showed no cytotoxicity at concentrations <100 uM (Fig. 3A). We evaluated the mRNA and protein expression of markers associated with allergic inflammation, including IL-6, IL-25, IL-33,

TSLP, and periostin. The results showed that IL-6, IL-25, IL-33, and TSLP significantly increased in both mRNA (Fig. 3B-3E) and protein expression (Fig. 3G-3J) in a concentration-dependent manner in response to $ZnCl_2$ exposure. Periostin expression weakly increased at a concentration of 50 μg/mL of $ZnCl_2$, but significantly increased at a concentration of 100 μg/mL of $ZnCl_2$. (Fig 3F and 3K). In ALI culture conditions, $ZnCl_2$ did not lead to cytotoxicity at concentrations less than 100 uM (Fig. 3L), and expression of IL-6, IL-25, IL-33, and TSLP increased in response to $ZnCl_2$ exposure, while periostin expression remained unchanged (Fig. 3M-3V).

Effect of Si on allergic inflammation in nasal epithelial cells

Primary nasal epithelial cells treated with various concentrations of SiO_2 showed no cytotoxicity until a concentration of 100 μM (Fig. 4A). We evaluated the mRNA and protein expression of markers associated with allergic inflammation, including IL-6, IL-25, IL-33, TSLP, and periostin. The results showed that IL-6, IL-25, IL-33, and TSLP significantly increased both mRNA (Fig. 4B–4E) and protein expression (Fig. 4G–4J) in a concentration-dependent manner in response to SiO_2 exposure. However, the periostin mRNA and protein expression levels did not change (Fig. 4F and 4K). Consistently, in ALI culture conditions, SiO_2 did not lead to cytotoxicity at concentrations <40 μM (Fig. 4L), and the expressions of IL-6, IL-25, IL-33, and TSLP increased in response to SiO_2 exposure, whereas periostin expression remained unchanged (Fig. 4M-4V).

Effect of PM2.5 or $ZnCl_2$ on fibroblast spheroids in allergic inflammatory response

The effects of PM2.5 and Zn on nasal fibroblast spheroids from the perspective of allergic inflammation were examined. Fibroblast spheroids were exposed to PM 2.5 and $ZnCl_2$ by aerosolization under two conditions: with and without ALI on top, to examine the

expression of alpha-smooth muscle actin (α-SMA), a marker associated with myofibroblast differentiation, and fibronectin, an extracellular matrix component. In the presence of ALI, ALI-cultured cells were exposed to aerosolized PM2.5 (24.2 μg/cm^2) and $ZnCl_2$ (46.3 ng/cm^2) to investigate their effects on nasal fibroblast spheroids and allergic inflammatory responses. Both mRNA and protein expression of α-SMA and fibronectin were significantly increased following exposure to PM2.5 or $ZnCl_2$ aerosols (Fig. 5A-5C). Additionally, both mRNA and protein levels of periostin, an important factor in allergic inflammatory responses, were dramatically increased after exposure to PM2.5 or $ZnCl_2$ aerosols (Fig 5D and 5E). Immunofluorescence staining confirmed the increased expression of α-SMA and periostin, with increased periostin expression in α-SMA -positive cells (Fig 5F). Furthermore, direct aerosol exposure to PM2.5 or $ZnCl_2$ significantly elevated periostin mRNA and protein expression in nasal fibroblast spheroids without ALI (Fig 5G and 5H). These results suggest that exposure to PM2.5 and $ZnCl_2$, whether directly or indirectly, increases the activation of nasal fibroblast spheroids and consequently upregulates periostin expression.

Effect of PM2.5 and $ZnCl_2$ on the functional impairment of epithelial cells in ALI culture with nasal fibroblasts spheroids

In experiments conducted in ALI culture and nasal fibroblast spheroids, exposure to aerosolized PM2.5 and $ZnCl_2$ was examined for its effect on the expression of epithelial cell-derived cytokines and epithelial-mesenchymal transition (EMT). The measurement of TEER, used to quantify the barrier integrity of the cells, was found to decrease upon exposure to PM2.5 and $ZnCl_2$ (Fig 6A). Both mRNA and protein expression levels of IL-6, IL-25, IL-33, and TSLP were significantly increased in response to PM2.5 and $ZnCl_2$ exposure (Fig 6B - 6E and Fig 6G - 6J). Furthermore, periostin mRNA expression was upregulated by exposure to PM2.5 and $ZnCl_2$ (Fig 6F). Notably, exposure to PM2.5 and $ZnCl_2$ resulted in a reduction

in the expression of E-cadherin, a marker of epithelial cells, along with an increase in the expression of mesenchymal cell markers, including vimentin and fibronectin (Fig 6K–6N). Immunofluorescence staining supported these findings and revealed decreased E-cadherin and increased periostin expression (Fig 6O). Aerosol treatment with PM2.5 or $ZnCl_2$ showed similar trends in their effect on ALI in the presence of nasal fibroblast spheroids, but with increased reactivity.

Discussion

Over the past few decades, the global prevalence of allergic diseases has significantly increased. Because it is a relatively short time for the natural properties of the immune system to change, external causes for such phenomena have been investigated, and air pollution has attracted the attention of many researchers. With rapid urbanization and industrialization, an increase in allergic diseases was first observed in Europe and North America. This phenomenon is now being observed in rapidly growing Southeast Asian countries, and a meta-analysis conducted in 2021 confirmed that exposure to air pollutants may increase the likelihood of IgE-mediated allergic diseases. [6]

PM is a pollutant with compelling evidence for its impact on public health. [4] PM is classified based on the diameter of the particles, and its toxicity depends on its size and chemical composition. Because smaller particles tend to remain longer in the atmosphere, PM 2.5 has a greater impact on respiratory diseases than PM 10, and heavy metals are considered as the major chemical constituents. [1, 14] In our concentration analysis and correlation study, Si was the most abundant heavy metal in PM 2.5, and the concentration of Zn showed the highest correlation with the level of PM 2.5 among others. Therefore, we commenced further experiments using PM 2.5, Zn, and Si.

Allergic inflammation of the upper airway is caused by IgE-mediated reactions to inhaled allergens. In addition to type 2 inflammatory cells, nasal epithelial cells and fibroblasts play important roles in this process. The nasal epithelium is the first line of defense against inhaled allergens, and it releases inflammatory cytokines such as IL-6, IL-25, IL-33, and TSLP, which triggers Th2-mediated inflammation. [15] These cytokines subsequently stimulate nasal fibroblasts, leading to the production of inflammatory mediators and extracellular matrix (ECM) proteins with eosinophil infiltration. [16] In our experiment on PNEC and ALI cultures,

treatment with PM 2.5, Zn, and Si solutions led to an increased expression of epithelial cell-derived cytokines. Overall, Zn exhibited more similar trends in the increase of cytokine expression with PM 2.5 compared with Si.

Periostin, a matricellular and an extracellular matrix protein involved in the tissue remodeling, plays an important role in inflammation and allergy, and it is known to be induced by various stimuli such as TGF- β, angiotensin II, or other cancer-derived factors. [17] In allergy, it is mainly produced by fibroblasts stimulated by IL-4 or IL-13 and is also expressed by some endothelial or epithelial cells. [18, 19] In 2011, it was first reported that serum periostin level can be utilized as a predictor for efficacy of a monoclonal antibody for severe asthma, and its role as a potential biomarker for type 2 inflammation in allergic diseases has been investigated ever since. [20] Hoshino *et al* reported its utility as a marker of response to sublingual immunotherapy, and Krasilnikova *et al* found that exacerbation of AR was associated with increased periostin in nasal secretion. [21, 22]

In our experiment with PNEC and ALI cultures, treatment with PM 2.5, Zn, and Si solutions at high concentrations led to elevated expression of periostin. In an experiment using our 3D-hybrid model, PM 2.5 and Zn aerosol treatment also led to a dramatic increase in periostin expression in fibroblast spheroids compared to the control. Notably, fibroblast spheroids directly exposed to PM 2.5 and Zn aerosols without the upper epithelial cell barrier also exhibited similar results. The pollutant itself may easily penetrate the epithelial barrier and reach the submucosa, or it may impair the epithelial barrier and ease its way to the submucosal layer. In further experiment the 3D-hybrid culture model, the upper ALI culture showed reduced TEER and E-cadherin expression after exposure to PM 2.5 and Zn aerosol, whereas vimentin, a marker of epithelial-to-mesenchymal transition, increased after exposure. Altogether, it can be assumed that the upper ALI culture was damaged and lost its function as

an epithelial barrier, with the reduction and collapse of tight junctions after atmospheric exposure to air pollutants.

To the best of our knowledge, most previous *in vitro* studies demonstrating the association between PM and allergic inflammation have used nasal epithelial cells and fibroblasts submerged in PM solutions. [23, 24] Additionally, the effect of heavy metals on allergies is mostly investigated by measuring the concentration of heavy metals in serum or urine. [25] However, in the human body, the nasal epithelium is directly exposed to pollutants through inhaled air, whereas fibroblasts in the submucosal layer are indirectly affected by various cytokines from the epithelium. In addition, whether atmospheric exposure to heavy metals directly affects their concentrations in body fluids remains unclear. Therefore, we built a model containing ALI cells co-cultured with fibroblast spheroids underneath, namely 3D-hybrid culture model, which is more anatomically and physiologically relevant than any other experimental method to date. Furthermore, air pollutants can be directly sprayed over this model, such that the epithelial cytokines would spread to the fibroblast spheroids in the lower chamber through solution media, which reflects the real-world effect of air pollutants on the upper respiratory tract.

In this study, we confirmed that Si and Zn were the most predominant and highly correlated heavy metal with PM 2.5 in Seoul, and that PM 2.5, Zn, and Si increased the expression of inflammatory cytokines in nasal epithelial cells and fibroblasts. In PNEC and ALI cultures, increased production of epithelial cell-derived and proinflammatory cytokines was observed, and impaired barrier function was confirmed through decreased TEER and elevated EMT markers. In an experiment on fibroblast spheroids, fibroblast activation and increased production of ECM and periostin were observed after exposure to PM 2.5. In conclusion, we demonstrated the effect of ambient air pollutants on allergic inflammation in nasal epithelial

cells and fibroblasts and confirmed these findings through both physiologically and anatomically relevant experimental models. As air pollution worsens worldwide, it is important to restrict exposure to air pollutants to prevent and manage allergic diseases. Further *in vivo* studies are required to investigate the clinical effects of air pollutants on allergic inflammation.

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Table 1. Sequences of PCR primers

Gene Name		Sequences (quantitative RT-PCR)
IL-6	Forward	5′-ACT CAC CTC TTC AGA ACG AAT TG-3′
	Reverse	5′-CCA TCT TTG GAA GGT TCA GGT TG-3′
IL-25	Forward	5′-CAG GTG GTT GCA TTC TTG GC-3′
	Reverse	5′-GAG CCG GTT CAA GTC TCT GT-3′
IL-33	Forward	5′-GTG ACG GTG TTG ATG GTA AGA T-3′
	Reverse	5′-AGC TCC ACA GAG TGT TCC TTG-3′
TSLP	Forward	5′-TAT GAG TGG GAC CAA AAG TAC CG-3′
	Reverse	5′-GGG ATT GAA GGT TAGGCT CTG G-3′
Periostin	Forward	5′-GCT ATT CTG ACG CCT CAA AAC T-3′
	Reverse	5′-AGC CTC ATT ACT CGG TGC AAA-3′
α-SMA	Forward	5′-GGC TCT GGG CTC TGG GCT TCA TC-3′
	Reverse	5′-CTC TTG CTC TGG GCT TCA TC-3′
Fibronectin	Forward	5′-CTT TGG TGC AGC ACA ACT TC-3′
	Reverse	5′-CCT CCT CGA GTC TGA ACC AA-3′
E-cadherin	Forward	5′-TGC TCT TGC TGT TTC TTC GG-3′
	Reverse	5′-TGC CCC ATT CGT TCA AGT AG-3′
Vimentin	Forward	5′-CTC TTG CTC TGG GCT TCA TC-3′
	Reverse	5′-CTC TTG CTC TGG GCT TCA TC-3′
GAPDH	Forward	5′-GTG GAT ATT GTT GCC ATC AAT GAC C-3′
	Reverse	5′-GCC CCA GCC TTC TTC ATG GTG GT-3′

Figure legends

Figure 1. Schematic drawing of aerosolized PM2.5 and $ZnCl_2$ treatment of a co-culture of ALI and fibroblast spheroids. (A) Aerosol exposure of PM2.5, $ZnCl_2$ to epithelial cells, (B) Co-culture of ALI and nasal fibroblasts spheroids. Illustration of the correlation between PM2.5 and the heavy metals Zn and Si. (C) The list of the order of abundance of heavy metals in PM2.5. (D) Heatmap of correlation coefficients between PM2.5 concentrations and heavy metal concentrations. (E) The correlation coefficients between Zn concentrations and PM2.5 concentrations and Si concentrations and PM2.5 concentrations.

Figure 2. PM2.5 affects allergic inflammation in primary nasal epithelial cells. (A) Primary nasal epithelial cells were treated by different concentrations of PM2.5 (0–1000 μg/mL) and cytotoxicity was measured using WST-1. (B-E) The mRNA levels of allergic inflammation markers, including IL-6, IL-25, IL-33, and TSLP, were measured by real-time PCR after treatment of PM2.5. (G-J) Protein expression of these markers was measured by ELISA. (F, K) The expression of periostin mRNA and protein was measured. (L) ALI culture treated with different concentrations of PM2.5 (0-1000 μg/mL) was analyzed for cytotoxicity using WST-1. (M-V) IL-6, IL-25, IL-33, TSLP, and periostin mRNA and protein expression were measured after treatment of PM2.5. *$P < 0.05$ compared to Control. Data are presented as the mean ± SD of three independent experiments.

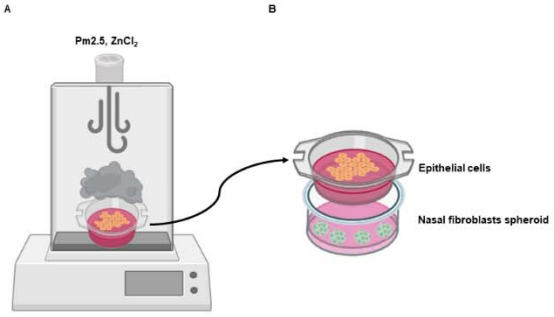
Figure 3. $ZnCl_2$ affects allergic inflammation in primary nasal epithelial cells. (A) Primary nasal epithelial cells treated with different concentrations of $ZnCl_2$ (0–1000 μM) were measured for cytotoxicity using WST-1. (B-E) The mRNA levels of allergic inflammation markers including IL-6, IL-25, IL-33, and TSLP were measured by real-time PCR after treatment of $ZnCl_2$. (G-J) Protein expression of these markers was measured by ELISA. (F, K) The expression levels of periostin mRNA and protein were measured. (L) ALI culture treated with different concentrations of $ZnCl_2$ (0–1000 μM) were measured for cytotoxicity using WST-1. (M-V) IL-6, IL-25, IL-33, TSLP, and periostin mRNA and protein expression were measured after treatment of $ZnCl_2$. *P < 0.05 compared to Control. Data are presented as the mean ± SD of three independent experiments.

Figure 4. SiO_2 affects allergic inflammation in primary nasal epithelial cells. (A) Primary nasal epithelial cells treated with different concentrations of SiO_2 (0–100 μM) were measured for cytotoxicity using WST-1. (B-E) The mRNA levels of allergic inflammation markers including IL-6, IL-25, IL-33, and TSLP were measured by real-time PCR after treatment of SiO_2. (G-J) Protein expression of these markers was measured by ELISA. (F, K) The expression of periostin mRNA and protein was measured. (L) ALI culture treated with different concentrations of $ZnCl_2$ (0–100 μM) was measured for cytotoxicity using WST-1. (M-V) IL-6, IL-25, IL-33, TSLP, and periostin mRNA and protein expression levels were measured after SiO_2 treatment. *P < 0.05 compared to Control. Data are presented as the mean ± SD of three independent experiments.

Figure 5. Aerosolized PM2.5 and $ZnCl_2$ induced fibroblast activation and allergic inflammatory response in spheroids of nasal fibroblasts co-cultured with ALI cultures. (A-C) The mRNA and protein levels of alpha-smooth muscle actin (α-SMA) and fibronectin were determined after exposure to PM2.5 or $ZnCl_2$ aerosols. (D, E) The expression of periostin was measured by mRNA and protein expression after exposure to PM2.5 or $ZnCl_2$ aerosols. (F) Immunofluorescence staining data confirmed the expression of α-SMA (green) and periostin (red). The nuclei of the cells were stained with DAPI (blue). (G, H) Periostin mRNA and protein expression was measured in nasal fibroblast spheroids upon direct aerosol exposure to PM2.5 or $ZnCl_2$. *$P < 0.05$ compared to Control. Data are presented as the mean ± SD of three independent experiments. Scale bar = 50 μM.

Figure 6. Aerosolized PM2.5 and $ZnCl_2$ induced an allergic inflammatory response of epithelial cells in co-cultures of ALI and nasal fibroblast spheroids. (A) TEER, which determines cell barrier integrity, was measured. Cell exposure to aerosolized PM2.5 or $ZnCl_2$ was examined for (B-E) mRNA and (G-J) protein expression of IL-6, epithelial cell-derived cytokines IL-25, IL-33, TSLP, (F) mRNA of periostin. (K-N) The mRNA and protein expression of E-cadherin, vimentin, and fibronectin involved in epithelial-mesenchymal transition (EMT) by aerosolized PM2.5 or $ZnCl_2$ were measured. (O) Immunofluorescence staining data confirmed the expression of E-cadherin (green) and periostin (red). The nuclei of the cells were stained with DAPI (blue). *$P < 0.05$ compared to Control. Data are presented as the mean ± SD of three independent experiments. Scale bar = 50 μM.

Figure 1.



C

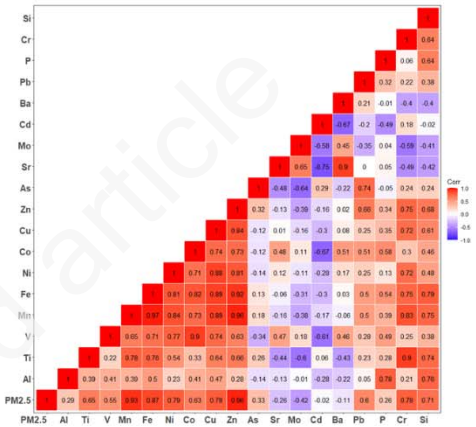
PM2.5 and heavy metal concentrations in Seoul

Heavy metal	Concentration (ng/m³)				Mass fraction(%)*
	Mean	SD	Min.	Max.	
$PM_{2.5}$	22271.87	13.29	3.2	90.89	-
Al	346.90	444.74	5.58	2230.22	0.372
Ti	12.75	11.83	0.05	120.96	0.057
V	1.04	0.75	0.05	5.08	0.004
Mn	12.64	7.67	1.09	56.92	0.057
Fe	184.34	114.10	21.48	885.64	0.828
Ni	1.36	1.91	0.05	47.38	0.006
Co	1.02	0.68	0.05	6.03	0.004
Cu	7.84	5.82	0.6	60.36	0.035
Zn	45.20	29.45	3.71	446.1	0.203
As	4.09	5.03	0.05	60.34	0.018
Sr	1.32	1.01	0.05	5.83	0.004
Mo	1.78	1.81	0.02	16.41	0.003
Cd	3.54	3.80	0.03	19.71	0.010
Ba	13.03	10.18	0.05	88.9	0.042
Pb	15.79	10.40	0.1	55.09	0.069
P	10.10	8.09	0.15	54.28	0.035
Cr	2.85	5.59	0.05	161.65	0.013
Si	396.71	466.48	10.54	4162.72	1.761

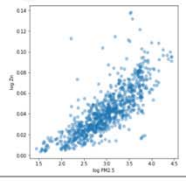
*Mass fraction includes up to N.D. value among heavy metal values

D

Correlation analysis heatmap



E

Substance	Mean	S.D.	Medium	Max
$PM_{2.5}$	22.11	13.08	20.97	83.69
Zn	0.04	0.02	0.04	0.15
Correlation coefficient	0.80			
p-value	p<0.01 (1.63×10⁻⁹⁵)			
scatter plot				

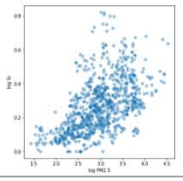
Substance	Mean	S.D.	Medium	Max
$PM_{2.5}$	21.99	13.32	18.68	90.89
Si	0.32	0.22	0.29	1.27
Correlation coefficient	0.54			
p-value	p<0.01 (2.85×10⁻⁶⁵)			
scatter plot				

Figure 2.

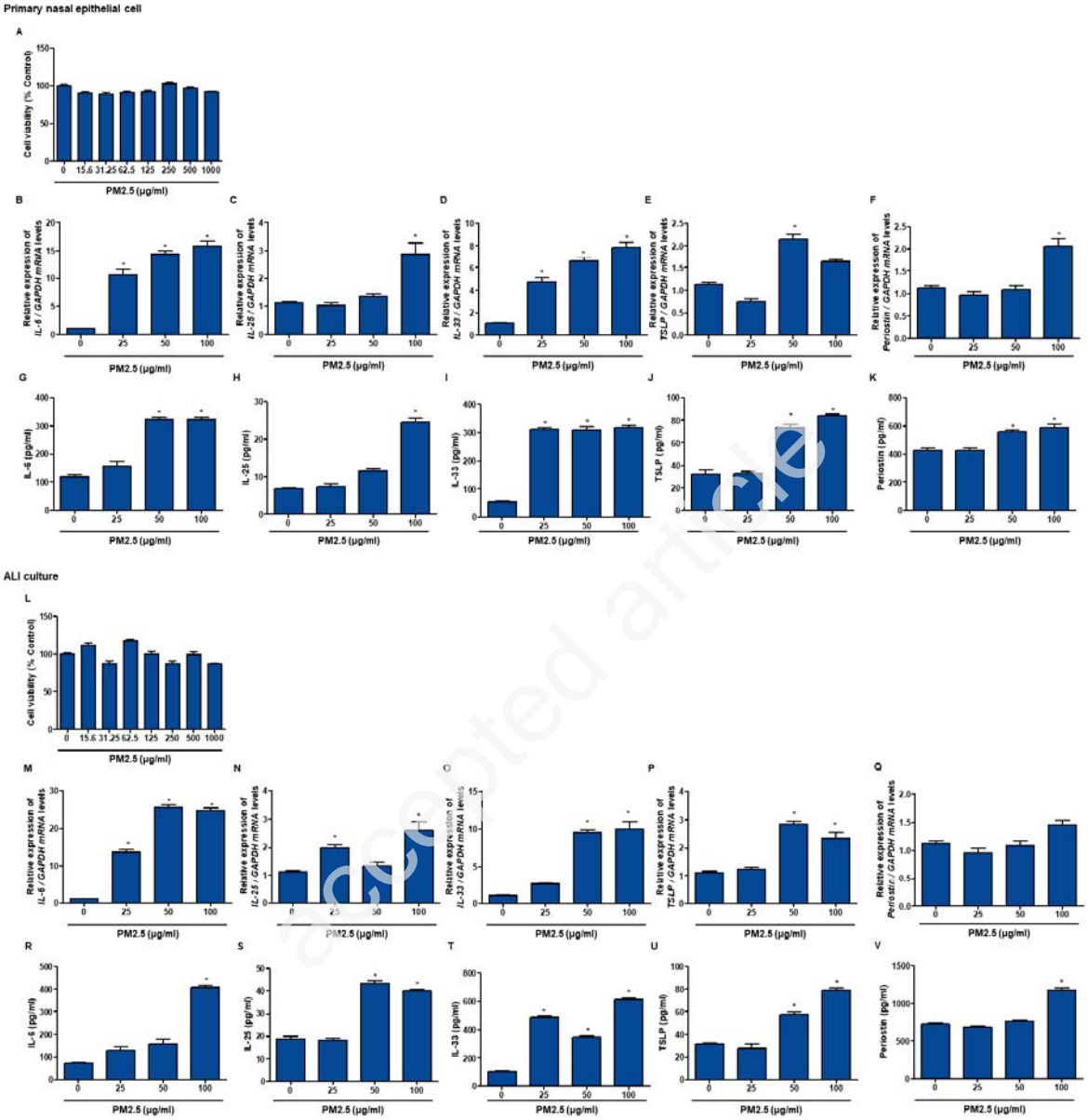
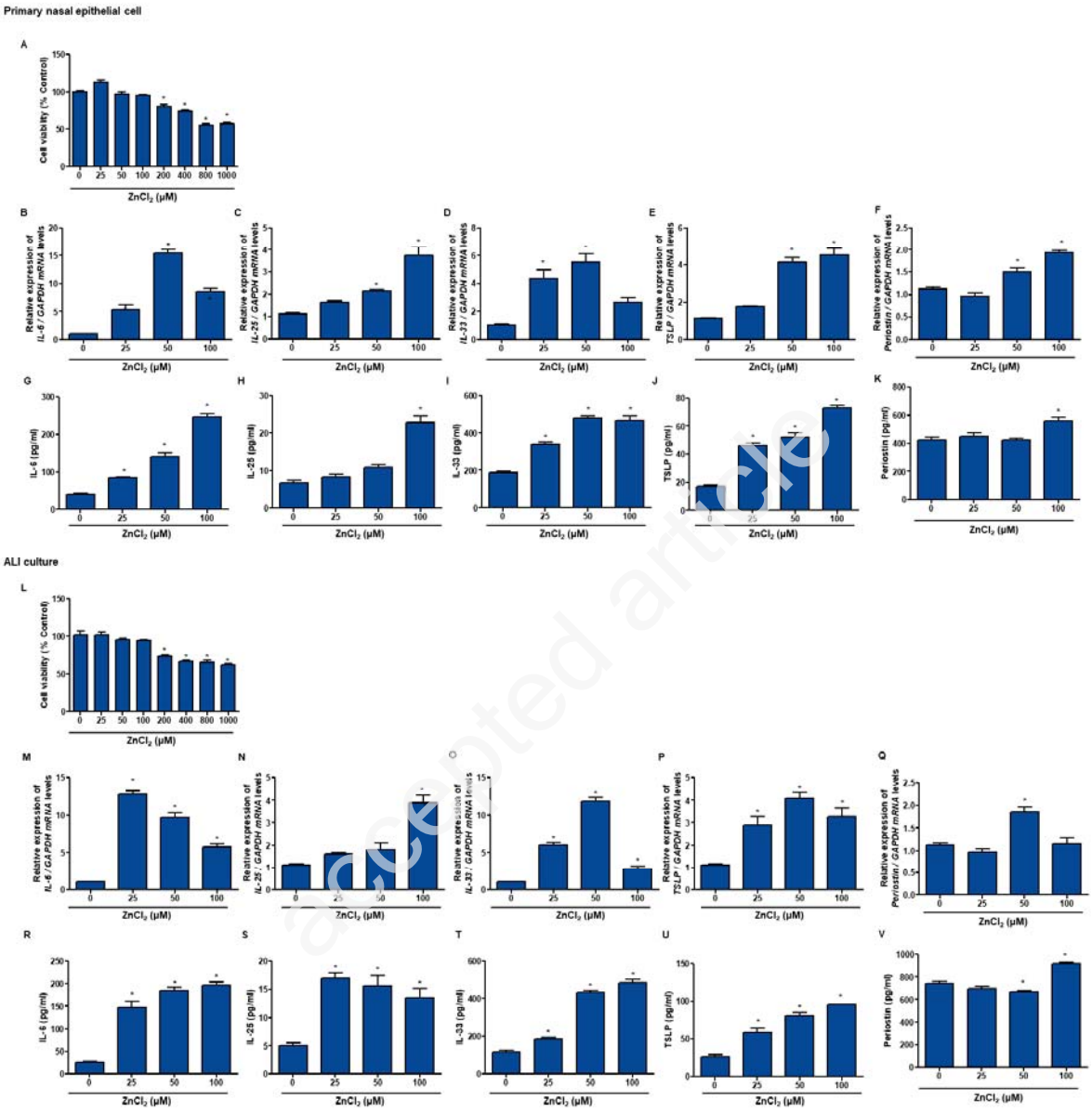


Figure 3.



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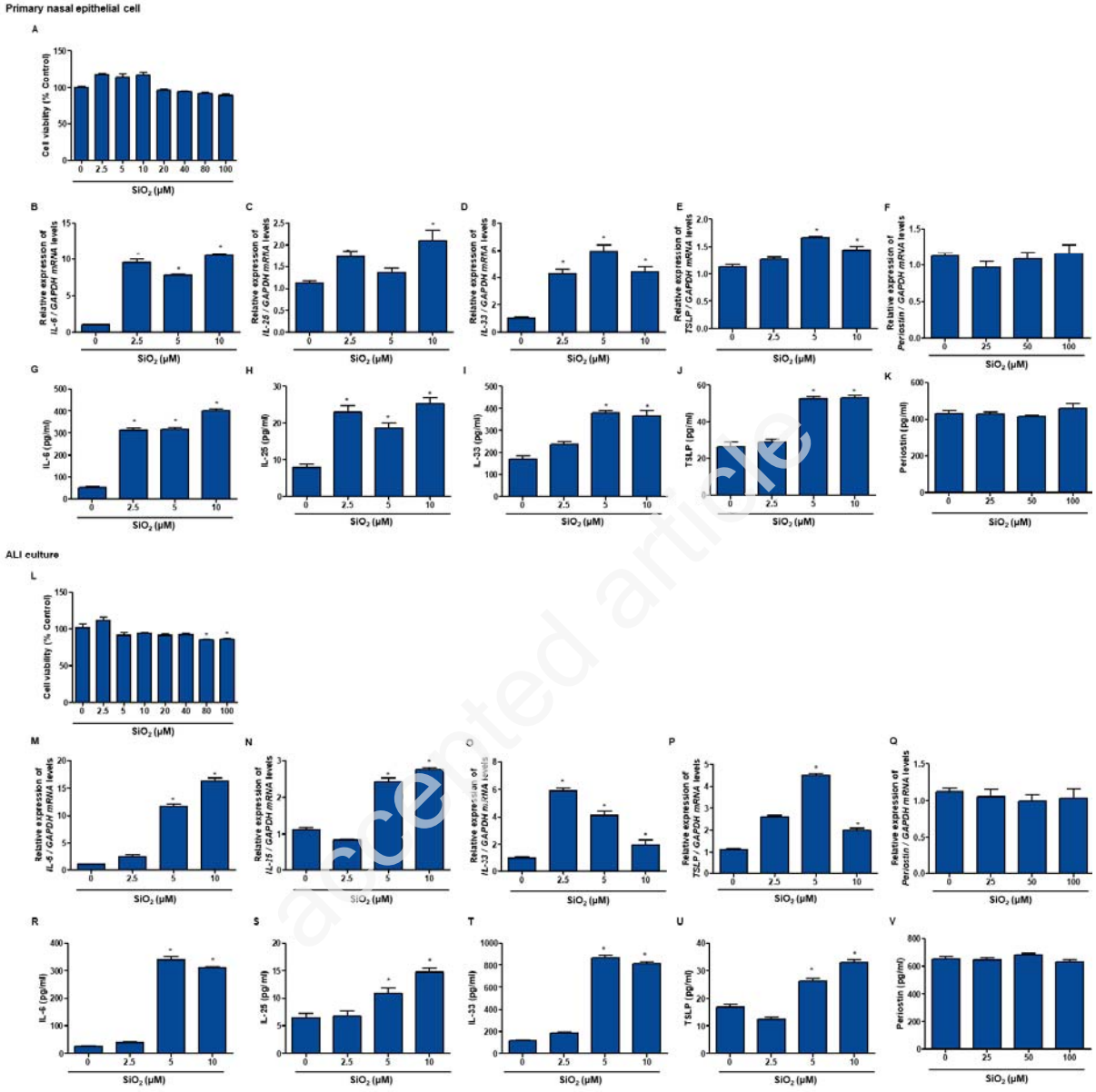
Primary nasal epithelial cell
A
Cell viability (% Control)
SiO2 (μM)
B
Relative expression of IL-6 / GAPDH mRNA levels
C
Relative expression of IL-25 / GAPDH mRNA levels
D
Relative expression of IL-33 / GAPDH mRNA levels
E
Relative expression of TSLP / GAPDH mRNA levels
F
Relative expression of Periostin / GAPDH mRNA levels
G
IL-6 (pg/ml)
H
IL-25 (pg/ml)
I
IL-33 (pg/ml)
J
TSLP (pg/ml)
K
Periostin (pg/ml)
ALI culture
L
M
N
O
P
Q
R
S
T
U
V

Figure 5.

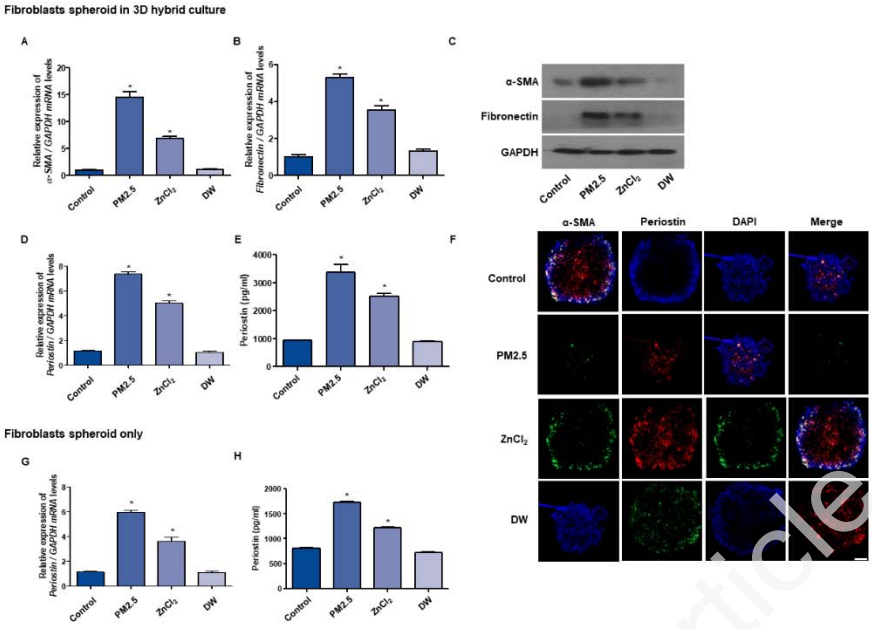

Figure 6.