



Silica dioxide nanoparticles aggravate airway inflammation in an asthmatic mouse model via NLRP3 inflammasome activation

Je-Won Ko^{a,b}, Na-Rae Shin^a, Lim Je-Oh^a, Tae-Yang Jung^a, Changjong Moon^a, Tae-Won Kim^b, Jungil Choi^c, In-Sik Shin^a, Jeong-Doo Heo^{c,*}, Jong-Choon Kim^{a,**}

^a College of Veterinary Medicine (BK21 Plus Project Team), Chonnam National University, Gwangju, 61186, Republic of Korea

^b College of Veterinary Medicine, Chungnam National University, Daejeon, 34134, Republic of Korea

^c Gyeongnam Department of Environment and Toxicology, Korea Institute of Toxicology, Gyeongnam, 52834, Republic of Korea

ARTICLE INFO

Keywords:

Silica nanoparticle
Asthma
Inflammation
TXNIP
NLRP3 inflammasome
IL-1 β

ABSTRACT

Silica dioxide nanoparticles (SiONPs) are mainly used in the rubber industry; however, they are a major air pollutant in Asia. Thus, extensive research on this issue is required. In this study, we investigated the effects of SiONPs on asthma aggravation and elucidated the underlying mechanism using ovalbumin (OVA)-induced asthmatic mice model and in NCI-H292 cells. Mice exposed to SiONPs showed markedly increased Penh values, inflammatory cell counts, and inflammatory cytokine levels compared to OVA-induced asthmatic mice. Exposure to SiONPs also induced additional airway inflammation and mucus secretion with increases in protein expression levels of thioredoxin-interacting protein (TXNIP), NOD-like receptor pyrin domain-containing 3 (NLRP3) inflammasome, and interleukin (IL)-1 β compared to those in OVA-induced asthmatic mice. Treatment of SiONPs in NCI-H292 cells also significantly increased mRNA expression levels of inflammatory cytokines accompanied with elevation in the levels of TXNIP, NLRP3 inflammasome, and IL-1 β proteins in a concentration-dependent manner. Taken together, exposure to SiONPs aggravated asthma development, which is closely related to inflammasome activation. Our results provide useful information about the toxicological effects of SiONPs on asthma exacerbation and suggest the need to avoid SiONP exposure especially in individuals with respiratory diseases.

1. Introduction

Nanomaterials are being increasingly applied in various industries due to their specific physicochemical properties (Navya and Daima, 2016). Of these, silica dioxide nanoparticles (SiONPs) are used in a variety of applications in the rubber, food, biomedical tools, and car industries (Mebert et al., 2017). According to Keller and Lazarevar (2014), SiONPs are the most produced nanoparticles in various fields, which reach 185–1400 kilotons of production in 2014. With increasing use of these nanoparticles, concerns regarding their adverse biological impact on human health and the environment have increased inevitably, and various toxicity studies have been reported (Giovannini et al., 2018; Guo et al., 2017; Kim et al., 2017; Lee et al., 2016). Previous studies have shown that SiONPs can cause toxic effects such as lipid peroxidation, oxidative stress, DNA damage, cell membrane destruction, cell death, and inflammation resulting in a variety of diseases including respiratory diseases (Ahmad et al., 2012; Duan et al., 2013).

As the air pollution in Asia increases, the risk of exposure to SiONPs in Asians is increasing gradually. Asian dust originating from China and Mongolia in the spring, contains many particles that can cause respiratory diseases including asthma (Naota et al., 2013). Asian dust contains particulate matter with a diameter of less than 10 μ m, which can reach small airway bronchi. Especially, it is reported that the proportion of silica in this air pollutant is 28%, which is the highest concentration in the pollutant. (Ichinose et al., 2008; Shimada et al., 2015). Importantly, these particles can damage the respiratory system in healthy humans and also exacerbate the clinical symptoms of patients with respiratory disease (Kashima et al., 2014). Considering the amount of use in the industrial fields and the high concentration in polluted air described above, it is difficult to avoid SiONPs exposure in daily life and the risk of subsequent pulmonary disease. Therefore, it is essential to study the mechanisms of disease exacerbation due to SiONPs exposure in patients with respiratory diseases such as asthma.

Inflammasomes play an important role in inflammatory responses

* Corresponding author.

** Corresponding author. College of Veterinary Medicine (BK 21 Plus Project Team), Chonnam National University, Gwangju, 61186, Republic of Korea.

E-mail addresses: jdher@kitox.re.kr (J.-D. Heo), toxkim@jnu.ac.kr (J.-C. Kim).

<https://doi.org/10.1016/j.yrtph.2020.104618>

Received 23 August 2019; Received in revised form 22 January 2020; Accepted 18 February 2020

Available online 19 February 2020

0273-2300/© 2020 Elsevier Inc. All rights reserved.

(Lamkanfi and Dixit, 2014; Skeldon et al., 2014). Among the various inflammasomes, NOD-like receptor pyrin domain-containing 3 (NLRP3) inflammasome, composed of NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC), and caspase-1, has been the most studied in inflammatory responses (Liang et al., 2018). NLRP3 inflammasome activation leads to maturation of pro-interleukin (IL)-1 β cytokines to their active and secreted forms via caspase-1, resulting in an inflammatory response (Wang et al., 2018). In particular, it is reported to play crucial roles in the development of inflammatory respiratory diseases induced by various stimuli (Im and Ammit, 2014; Peng et al., 2018; Shen et al., 2018). The NLRP3 inflammasome can be triggered by numerous environmental fibers and nanoparticles (Sun et al., 2013; Yang et al., 2012). SiONPs are one of the first environmental particles identified to activate the NLRP3 inflammasome (Zhang et al., 2018). Reactive oxygen species (ROS) generation caused by SiONPs is regarded to occur upstream of the NLRP3 activation mechanism (Morishige et al., 2010; Rabolli et al., 2016). However, it is unclear whether NLRP3 inflammasome activation participates in SiONPs-induced asthma aggravation. Furthermore, the mechanisms of SiONPs-mediated activation of the NLRP3 inflammasome remain elusive.

In the present study, we explored the effects of SiONPs on asthma aggravation using an ovalbumin (OVA)-induced asthmatic mouse model. We also investigated the underlying mechanism using SiONPs-treated NCI-H292 cells, focusing on the modulation of NLRP3 inflammasome activation.

2. Materials and methods

2.1. Nanoparticles

SiONPs were purchased from Sigma-Aldrich (particle size < 5–15 nm, 637246, St. Louis, MO, USA). We quantified the morphology and size of SiONPs using transmission electron microscopy (JEM-1210, JEOL, Tokyo, Japan) at an accelerating voltage of 200 kV and scanning electron microscopy (Zeiss EVO-MA10; Carl Zeiss Meditec AG, Jena, Germany) at an accelerating voltage of 15 kV. The samples were deposited on carbon-coated nickel grids, which were air dried overnight. The hydrodynamic size and zeta potential of SiONPs were determined by ELS-8000 (Otsuka Electronic, Tokyo, Japan). The specific surface area of SiONPs was measured by nitrogen absorption methods based on the multipoint Brunauer–Emmett–Teller (BET) method (ASAP2020; Micromeritics, Norcross, GA, USA). The endotoxin levels in the SiONPs suspension were determined using a Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, Waltham, MA, USA). After the experiments, lung tissues were weighed and digested overnight with concentrated nitric acid, and all samples were analyzed for elemental SiONPs using inductively coupled plasma mass spectrometry (ICP-MS; Perkin Elmer, Waltham, MA, USA).

2.2. Experimental procedure for animal experiments

Specific pathogen-free female BALB/c mice (6 weeks old) were purchased from the Koatech Co. (Pyeongtaek, Republic of Korea) and used after 1 week of quarantine and acclimatization. The Institutional Animal Care and Use Committee of Chonnam National University approved the protocols for the animal study. As shown in Fig. 1, mice were divided into five groups: vehicle control group, PBS intranasal instillation; OVA group, OVA challenge + PBS intranasal instillation; SiONPs 5, SiONPs 10, and SiONPs 20 groups, OVA challenge + 5, 10 and 20 mg/kg of SiONPs intranasal instillation, respectively. To induce asthma, the mice were sensitized on days 1 and 15 with an intraperitoneal injection of 20 μ g of OVA (Sigma-Aldrich) emulsified with 2 mg of aluminum hydroxide (Thermo Scientific) in 200 μ L of phosphate-buffered saline (PBS; pH 7.4). On days 21, 23, and 25, the mice received a 1 h airway challenge with a 1% (w/v) OVA solution

aerosolized using an ultrasonic nebulizer (NE-U12; Omron Corp., Tokyo, Japan). On days 20, 22, and 24, the SiONPs treatment groups (5, 10, and 20 mg/kg doses in 50 μ L of PBS, respectively) received the SiONPs via intranasal instillation under slight anesthesia using Zoletil 50 $^{\circ}$ (Virbac Laboratories, Carros, France). The vehicle control and OVA groups received 50 μ L of PBS via intranasal instillation. SiONPs were prepared in PBS and sonicated in an ultrasonicator (VCX-130, Sonics and Materials, Newtown, CT) for 3 min (130 W, 20 kHz, pulse 59/1) before intranasal instillation. Penh values were indirectly assessed at 24 h after the final intranasal instillation via single-chamber, whole body plethysmography (Allmedicus, Seoul, Republic of Korea). The mice were sacrificed at 48 h after the final instillation via an intraperitoneal injection of Zoletil 50 $^{\circ}$, and a tracheostomy was performed. To obtain the bronchoalveolar lavage fluid (BALF), ice-cold PBS (0.7 mL) was infused into the lungs twice and was withdrawn each time using a tracheal cannula (a total volume of 1.4 mL). BALF was centrifuged and its supernatant was collected for the biochemical analysis. Collected cells were resuspended by ice-cold PBS (0.5 mL), 200 μ L of resuspended solution was centrifuged (200 g, 4 $^{\circ}$ C, 10 min) onto slides using a Cytospin (Hanil Science Industrial Co., Ltd., Seoul, Republic of Korea). The slides were dried, and the cells were fixed and stained. Differential cell counts in the BALF were performed using the Diff-Quik $^{\circ}$ staining reagent (Sysmex Corporation, Kobe, Japan) according to the manufacturer's instructions.

2.3. Measurements of proinflammatory cytokines in BALF and immunoglobulin E in serum

The levels of IL-1 β (BD Biosciences, San Jose, CA, USA), IL-5 (R&D System, Minneapolis, MN, USA), IL-6 (BD Biosciences), IL-13 (R&D System), and tumor necrosis factor- α (TNF- α) (BD Biosciences) were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocols. The level of OVA-specific immunoglobulin E (IgE) in the serum was determined using an ELISA kit (BioLegend, San Diego, CA, USA). Absorbance was measured at 450 nm using an ELISA reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.4. Histopathology and immunohistochemistry (IHC)

After collecting the BALF samples were collected, the lung tissue was fixed by inflated with 4% (v/v) paraformaldehyde for 48 h. The tissues were paraffin-embedded, sectioned at a thickness of 4 μ m and stained using a hematoxylin and eosin solution (Sigma-Aldrich) or the periodic acid-Schiff solution (IMEB Inc., San Marcos, CA, USA) to estimate the amount of inflammation or mucus production, respectively. Each slide was examined manually in a totally blinded manner using a light microscope (Leica, Wetzlar, Germany) with 10 and 20x objective lenses and a 100x oil immersion lens. Ten randomly selected non-overlapping areas per slide were captured with digital camera (IMTcamCCD5; IMT Inc., Daejeon, Republic of Korea), and quantitative analyses of airway inflammation and mucus production were performed using an image analyzer (IMT i-Solution software, Vancouver, BC, Canada). For IHC, the paraffin-embedded sections were deparaffinized, dehydrated, washed using PBS with 0.3% Tween-20 (PBST), and incubated for 10 min at room temperature with 10% goat serum to block non-specific staining. Subsequently, slides were incubated overnight at 4 $^{\circ}$ C with a primary antibodies against thioredoxin-interacting protein (TXNIP; NBP1-54578; 1:200 dilution; Novus Biologicals, Littleton, CO, USA) and IL-1 β (ab9722; 1:200 dilution; Abcam, Cambridge, MA, USA). After removing the primary antibodies, the sections were washed and incubated with a biotinylated secondary antibody at 37 $^{\circ}$ C for 1 h, followed by incubation with the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. The excess complex was removed and sections were washed with PBST prior to incubation in 0.05% diaminobenzidine

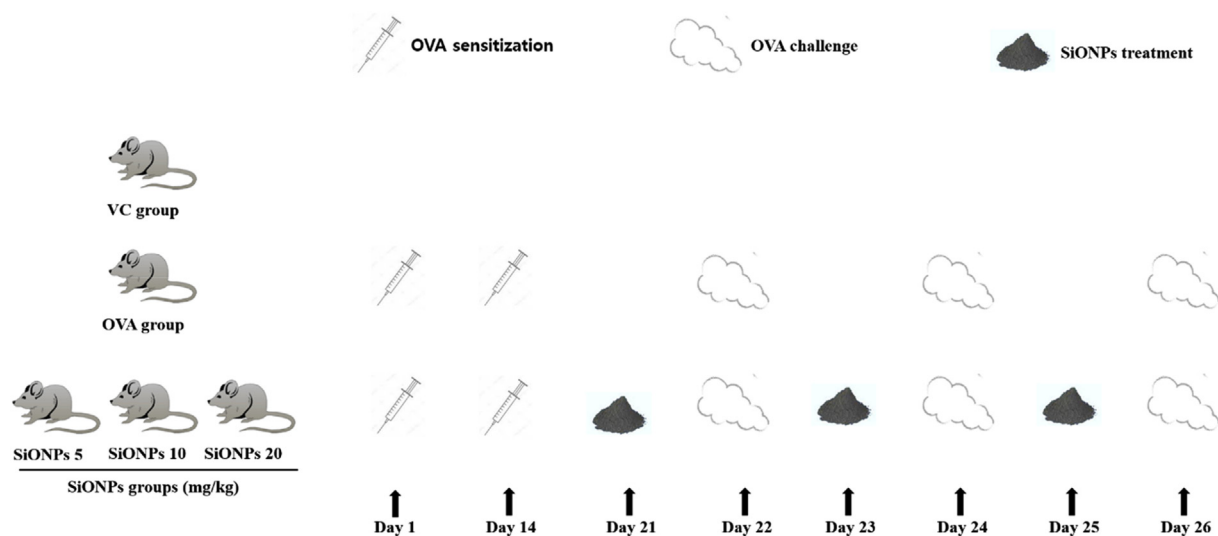


Fig. 1. Schematic diagram of the experimental procedure.

(1:200; Millipore Co., Bedford, MA, USA) for additional 10 min. The sections were counterstained, rinsed with PBST to terminate the reaction, and protected with coverslips prior to microscopic examination.

2.5. Immunoblotting for measurements of inflammatory protein in lung tissue

To quantify protein expression, we performed immunoblotting as described previously (Ko et al., 2017). The following primary antibodies were used: TXNIP (NBP1-54578; Novus Biologicals), NLRP3 (ab214185; Abcam), ASC (AG-25B-0006; AdipoGen Co., San Diego, CA, USA), caspase-1 (ab207802; Abcam), IL-1 β (ab9722; Abcam), and β -actin (#4967; Cell signaling, Danvers, MA, USA). Densitometric analysis of each protein was performed using Chemi-Doc (Bio-Rad Laboratories).

2.6. Cell culture

The human airway epithelial cell line NCI-H292, was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum in the presence of streptomycin (100 μ g/mL), penicillin (100 U/mL), and HEPES (25 mM), at 37 °C with 5% CO₂.

2.7. Cytotoxicity assay

Cell viability in response to SiONPs was measured using an EZ-Cytox cell viability assay kit (DAELIL lab, Seoul, Republic of Korea). The NCI-H292 cells were cultured in 96-well plates at a density of 5×10^4 cells/well for 24 h. The cells were subsequently treated with SiONPs at concentrations of 0, 1.5625, 3.125, 6.25, and 12.5 μ g/mL. After culturing for 24 h, 10 μ L of the kit solution was added to each well and incubated for 4 h at 37 °C and 5% CO₂. Cell viability relative to that of the untreated control cells was determined by measuring absorbance at 450 nm using an ELISA reader (Bio-Rad Laboratories).

2.8. Measurement of proinflammatory cytokines mRNA expression in NCI-H292 cells

Cells were seeded into 6-well plates at a density of 3×10^5 cells/well, treated with a nontoxic concentration of SiONPs (1.5625, 3.125, 6.25, and 12.5 μ g/mL), and incubated for 12 h. Reverse-transcription polymerase chain reaction (RT-PCR) and real-time PCR were used to measure the mRNA expression of proinflammatory mediators. Total

RNA was isolated using Trizol™ reagent (Invitrogen, Carlsbad, CA, USA) as instructed by the manufacturer, and was reverse transcribed using a cDNA kit (Qiagen, Hilden, Germany). RT-PCR experiments were performed using specific forward and reverse primers (IL-8, forward, 5'-AGA TAT TGC ACG GGA GAA-3', reverse, 5'-GAA ATA AAG GAG AAA CCA-3'; TNF- α , forward, 5'-CAA AGT AGA CCT GCC CAG AC-3', reverse, 5'-GAC CTC TCT CTA ATC AGC CC-3'; IL-6, forward, 5'-GAC AGC CAC TCA CCT CTT CA-5', reverse, 5'-AGT GCC TCT TTG CTG CTT TC-3'; IL-1 β , forward, 5'-AAA AGC TTG GTG ATG TCT GG-3', reverse, 5'-TTT CAA CAC GCA GGA CAG G-3'; GAPDH, forward, 5'-CAA AAG GGT CAT CTC TG-3', reverse, 5'-CCT GCT TCA CCA CCT TCT TG-3') and a premixed solution according to the manufacturer's instructions (Bioneer, Daejeon, Republic of Korea). Real-time PCR experiments were performed using specific forward and reverse primers (IL-8, forward, 5'-ATG ACT TCC AAG CTG GCC GTG GCT-3', reverse, 5'-TTA TGA ATT CTC AGC CCT CTT CAA AAA-3'; TNF- α , forward, 5'-CAA AGT AGA CCT GCC CAG AC-3', reverse, 5'-GAC CTC TCT CTA ATC AGC CC-3'; IL-6, forward, 5'-ATG CAA TAA CCA CCC CTG AC-5', reverse, 5'-ATC TGA GGT GCC CAT GCT AC-3'; IL-1 β , forward, 5'-AGC CAG GAC AGT CAG CTC TC-3', reverse, 5'-ACT TCT TGC CCC CTT TGA AT-3'; GAPDH, forward, 5'-CAA AAG GGT CAT CTC TG-3', reverse, 5'-CCT GCT TCA CCA CCT TCT TG-3').

2.9. Measurement of inflammatory proteins expression in NCI-H292 cells

The cells were treated with various concentrations of SiONPs (0, 0.78125, 1.5625, 3.125, 6.25, and 12.5 μ g/mL), and then incubated for the indicated times. The cells were then collected by centrifugation, washed twice with PBS, and resuspended in a tissue lysis/extraction reagent (Sigma Aldrich) with a protease inhibitor (Roche, Basel, Switzerland). Immunoblot was performed according to a previous report (Shin et al., 2018). The following primary antibodies were used: TXNIP (Novus Biologicals), NLRP3 (Abcam), ASC (AdipoGen company), caspase-1 (Abcam), IL-1 β (Abcam), and β -actin (Cell signaling). Relative protein expression was determined using ChemiDoc (Bio-Rad Laboratories).

2.10. Statistical analysis

Data are expressed as means $\pm$ standard deviation (SD). Statistical significance was determined using analysis of variance followed by Dunnett's test for multiple comparisons. *P* values less than 0.05 were considered statistically significant.

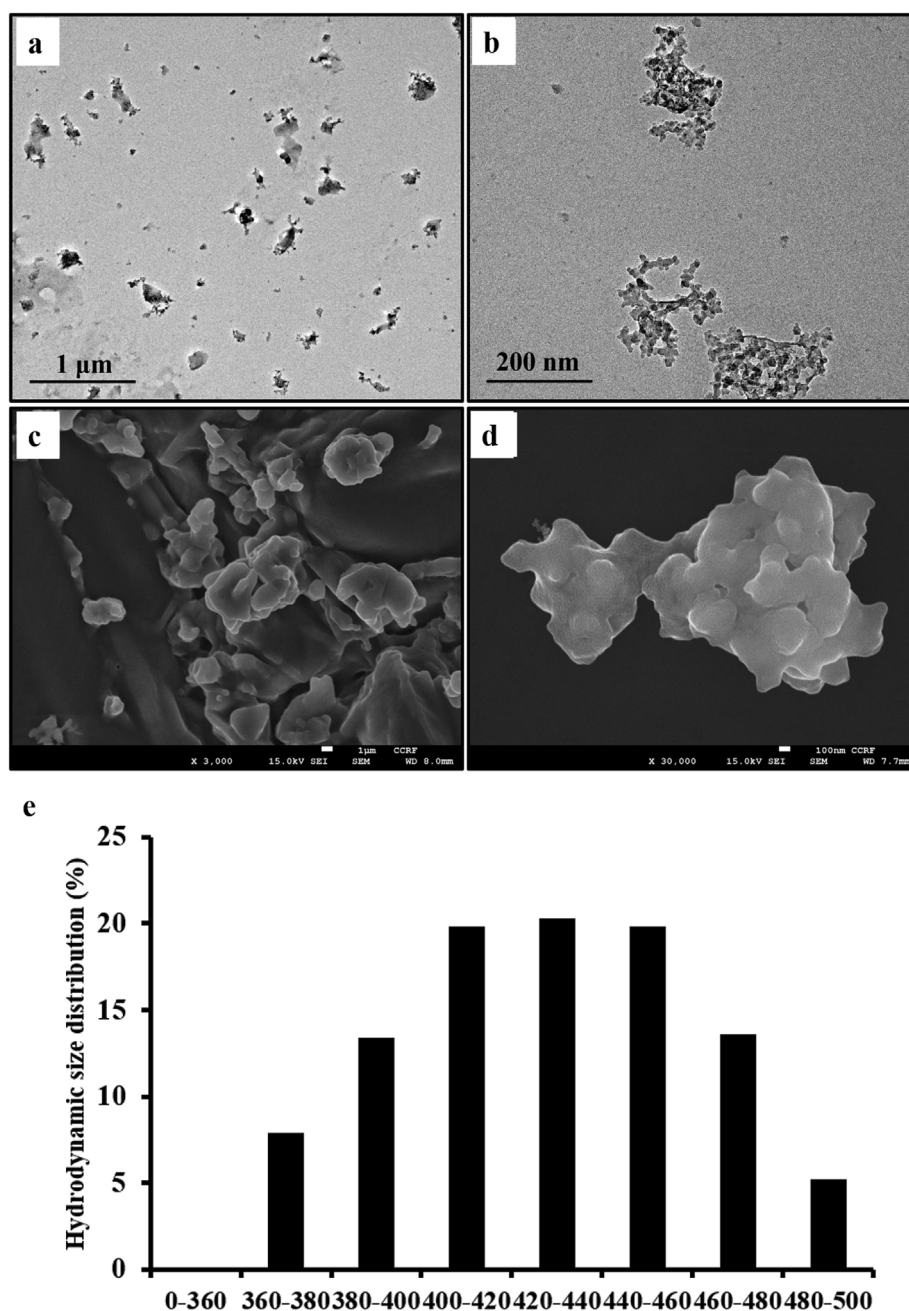


Fig. 2. Morphology and hydrodynamic size distribution of SiONPs. (a, b) Morphology of SiONPs measured by transmission electron microscopy. (c, d) Morphology of SiONPs measured by scanning electron microscopy. (e) Hydrodynamic size distribution of SiONPs, 440.0 ± 38.8 nm.

3. Results

3.1. Physicochemical characterization of SiONPs

The morphology, primary size, and hydrodynamic size of SiONPs are shown in Fig. 2. The primary size and hydrodynamic size in PBS were 48.89 ± 15.49 nm and 440.0 ± 38.8 nm, respectively. The specific surface area of SiONPs was $365 \text{ m}^2/\text{g}$ as determined using BET methods and the zeta potential of the SiONPs was -19.69 mV (Table 1). The SiONPs suspensions did not show detectable endotoxin levels (data not shown). After experiments, the assessed SiONPs concentrations in lung tissues were determined using ICP-MS (Table 1).

3.2. Effects of SiONPs on Penh values and inflammatory cell counts

Penh values were increased in the OVA group compared to that in the vehicle control group. Exposure to SiONPs markedly elevated the Penh values compared to that in the OVA group in a dose-dependent manner (Fig. 3a). The OVA group also showed increased inflammatory cell counts in the BALF, particularly that of eosinophils, compared to the vehicle control group. Similar to the results of AHR, exposure to SiONPs markedly increased the inflammatory cell counts, including eosinophils, neutrophils, and macrophages in a dose-dependent manner compared to those in the OVA group (Fig. 3b).

3.3. Effects of SiONPs on the pathophysiological factors in BALF and serum

The production of IL-1 β , IL-6, and TNF- α was increased in OVA

Table 1
Physicochemical characterization of SiONPs.

BET Surface area (m ² /g)	Zeta potential (mV)	ICP-MS (mg/g)				
365	-19.69	VC	OVA	SiONPs 5	SiONPs 10	SiONPs 20
		0.37 ± 0.053	0.39 ± 0.047	0.53 ± 0.097	0.69 ± 0.107	1.31 ± 0.113

Abbreviations: BET, Brunauer–Emmett–Teller; ICP-MS, Inductively coupled plasma mass spectrometry; VC, vehicle control group; OVA, ovalbumin challenge + phosphate-buffered saline intranasal instillation group; SiONPs 5, 10, and 20, ovalbumin challenge + 5, 10, and 20 mg/kg of SiONPs intranasal instillation groups, respectively.

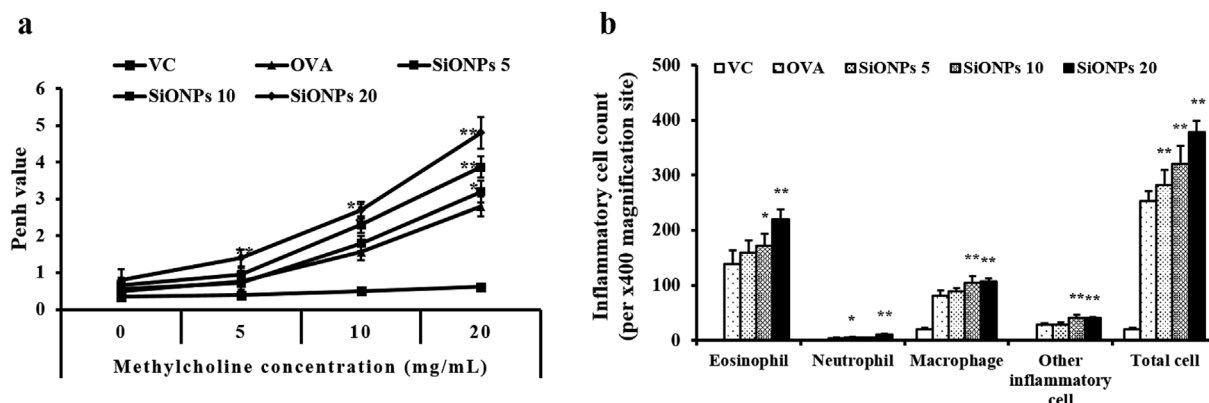


Fig. 3. Effects of SiONPs exposure on Penh values and inflammatory cell counts in BALF. (a) Penh values. (b) Inflammatory cell counts. Data are represented as means ± SD, n = 6. *, ***P* < 0.05 and *p* < 0.01 vs. OVA group. VC, PBS intranasal instillation; OVA, OVA challenge + PBS intranasal instillation; SiONPs 5, 10, and 20, OVA challenge + 5, 10, and 20 mg/kg of SiONPs intranasal instillation, respectively.

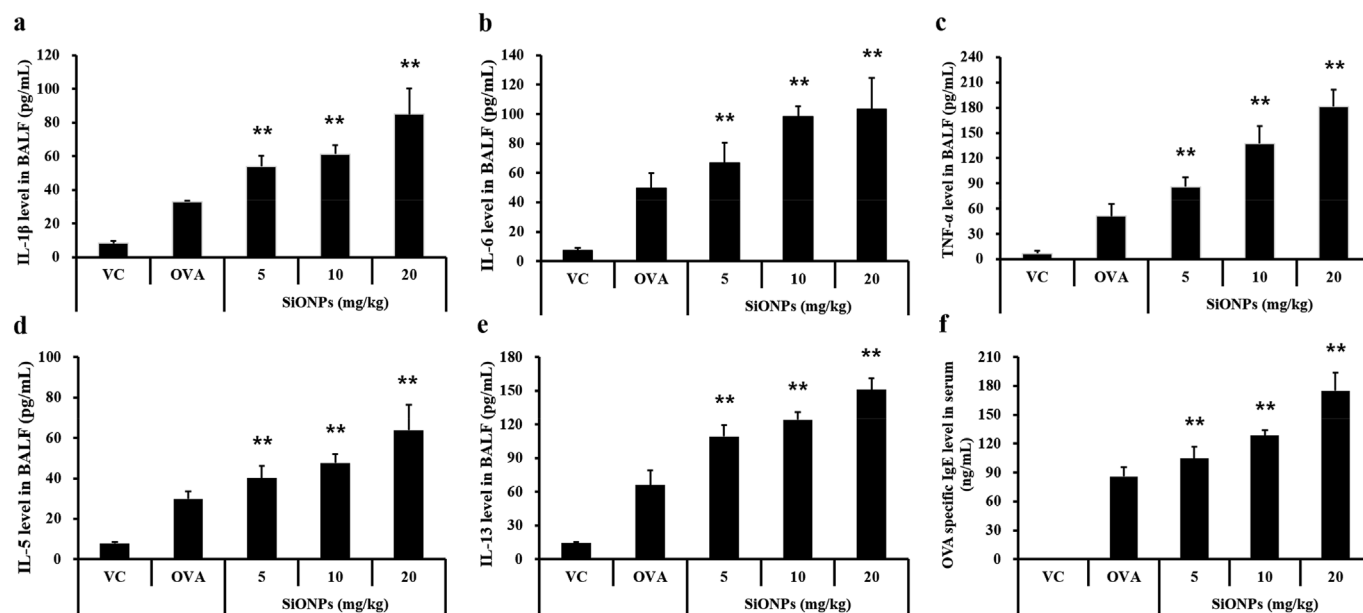


Fig. 4. Effects of SiONPs exposure on cytokines levels in BALF and OVA-specific IgE levels in serum. (a) IL-1β level in BALF. (b) IL-6 level in BALF. (c) TNF-α level in BALF. (d) IL-5 level in BALF. (e) IL-13 level in BALF. (f) OVA-specific IgE level in serum. Data are represented as means ± SD, n = 6. ***P* < 0.01 vs. OVA group. VC, PBS intranasal instillation; OVA, OVA challenge + PBS intranasal instillation; SiONPs 5, 10, and 20, OVA challenge + 5, 10 and 20 mg/kg of SiONPs intranasal instillation, respectively.

group compared to that in the vehicle control group. Exposure to SiONPs increased the IL-1β, IL-6, and TNF-α production in a dose-dependent manner, and these increases were higher than those observed in the OVA group (Fig. 4a–c). Production of the Th2 cytokines, IL-5 and IL-13, was consistent with the results of IL-1β, IL-6, and TNF-α production. The OVA group showed increases in IL-5 and IL-13 production compared to the vehicle control group, which was observed to a greater extent in the SiONPs groups than in the OVA group (Fig. 4d and e). A

greater increase in OVA-specific IgE level was also observed in the SiONPs groups compared to that in the OVA group (Fig. 4f).

3.4. Effects of SiONPs on airway inflammation and mucus secretion

The OVA group showed increased airway inflammation compared to the vehicle control group. Exposure to SiONPs showed a marked increase in airway inflammation in a dose-dependent manner, and this

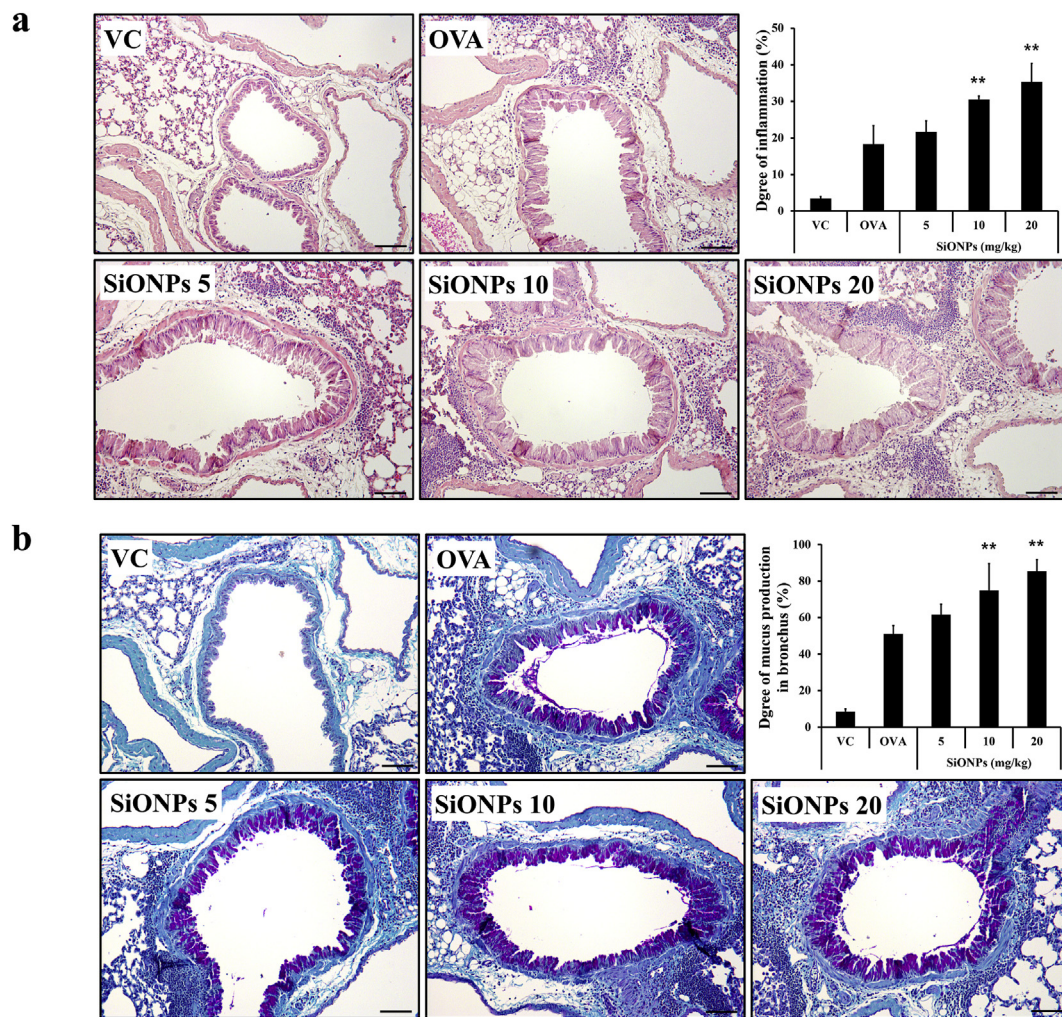


Fig. 5. Effects of SiONPs exposure on inflammatory responses and mucus production in lung tissue. (a) Lung tissue stained with hematoxylin and eosin ($\times 200$). (b) Lung tissue stained with periodic acid-Schiff stain ($\times 200$). Data are represented as means $\pm$ SD, $n = 6$. ** $P < 0.01$ vs. OVA group. VC, PBS intranasal instillation; OVA, OVA challenge + PBS intranasal instillation; SiONPs 5, 10, and 20, OVA challenge + 5, 10 and 20 mg/kg of SiONPs intranasal instillation, respectively. Bar = 50 μ m.

increase was higher than that observed in the OVA group (Fig. 5a). Similarly, mucus secretion was increased in the OVA group compared to that in the vehicle control group. Exposure to SiONPs further increased the mucus secretion in a dose-dependent manner compared to that in the OVA group (Fig. 5b).

3.5. Effects of SiONPs on TXNIP and IL-1 β expression in lung tissue

To evaluate the TXNIP and IL-1 β expression in lung tissues, we performed IHC in lung tissues from mice. The OVA group showed elevated TXNIP expression compared to the vehicle control group, this expression increased to a greater extent in the SiONPs groups compared to that in the OVA group (Fig. 6a). Similarly, IL-1 β expression was increased in the OVA group compared to the vehicle control group, and this increase was greater in the SiONPs groups (Fig. 6b).

3.6. Effects of SiONPs TXNIP, IL-1 β , and inflammasome proteins expression

Next, we measured the levels of TXNIP, IL-1 β , and inflammasome proteins by immunoblotting. As shown in Fig. 7, the OVA group showed a marked increase in the TXNIP levels in the lung tissue compared to that in the vehicle control group. Exposure to SiONPs also induced a marked increase in TXNIP levels in a dose-dependent manner, and this

increase was higher than that observed in the OVA group. Similarly, the levels of inflammasome proteins including NLRP3, ASC, and cleaved caspase-1 were elevated in the OVA group compared to those in the vehicle control group, and this increase was greater in the SiONPs groups compared to that observed in the OVA group. Levels of cleaved IL-1 β also showed a tendency similar that of other protein levels. Increased levels of cleaved IL-1 β observed in the OVA group showed even higher levels in a dose-dependent manner in the SiONPs groups.

3.7. Effects of SiONPs on the production of proinflammatory cytokines in NCI-H292 cells

In these experiments, we used a nontoxic concentration of SiONPs based on the results of cytotoxicity analysis (Fig. 8a). SiONPs treatment markedly increased the levels of IL-1 β in NCI-H292 cells in a concentration-dependent manner (Fig. 8b). The levels of IL-6 and TNF- α were consistent with those of IL-1 β (Fig. 8c and d).

3.8. Effects of SiONPs on proinflammatory cytokines mRNA expression in NCI-H292 cells

As shown in Fig. 9 and Fig. 10, SiONPs treatment significantly increased the mRNA expression levels of IL-8 and IL-6 in NCI-H292 cells in a concentration-dependent manner. The mRNA expression levels of

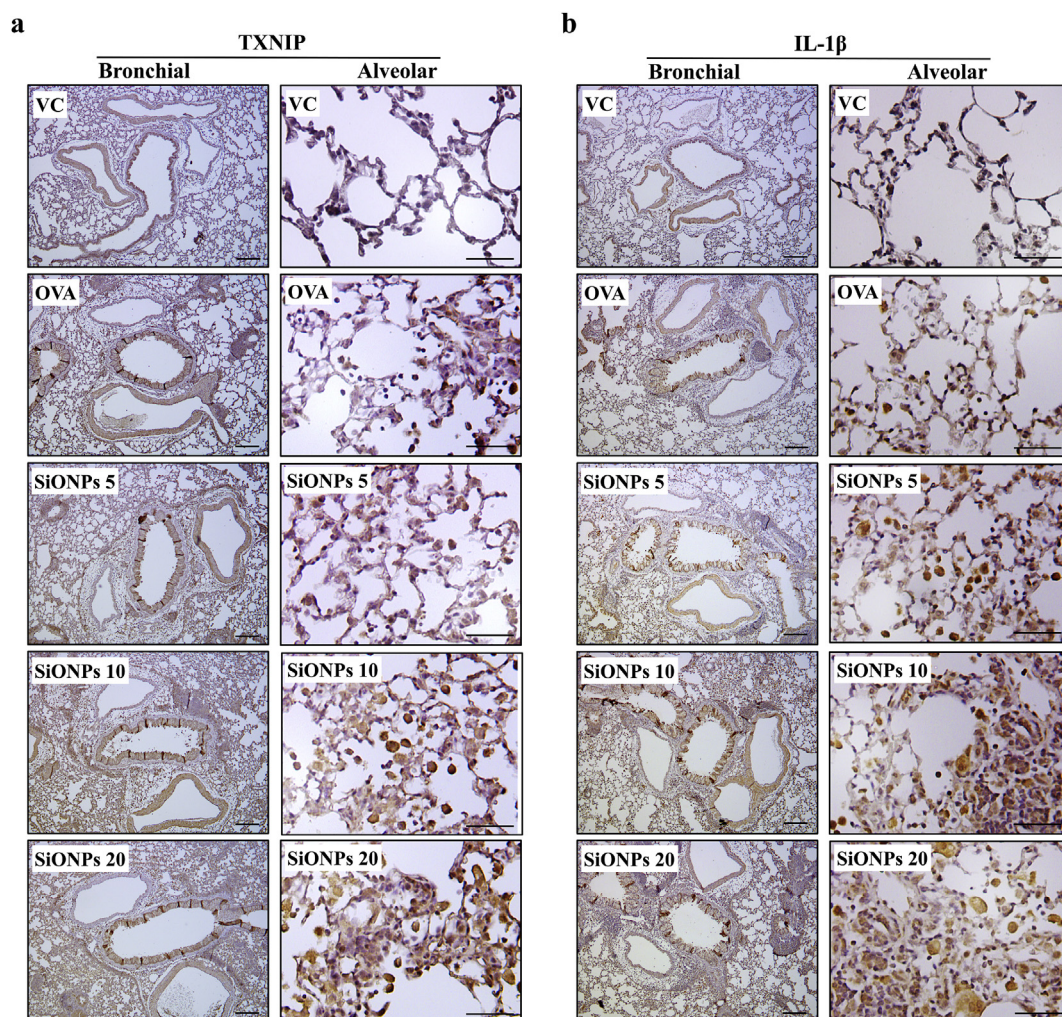


Fig. 6. Effects of SiONPs exposure on positivity of TXNIP and IL-1β in lung tissue. (a) Positivity of TXNIP ($\times 100$, bronchial; and $\times 400$, alveolar, respectively). (b) Positivity of IL-1β ($\times 100$, bronchial; and $\times 400$, alveolar, respectively). VC, PBS intranasal instillation; OVA, OVA challenge + PBS intranasal instillation; SiONPs 5, 10, and 20, OVA challenge + 5, 10 and 20 mg/kg of SiONPs intranasal instillation, respectively. Bar = 50 μ m.

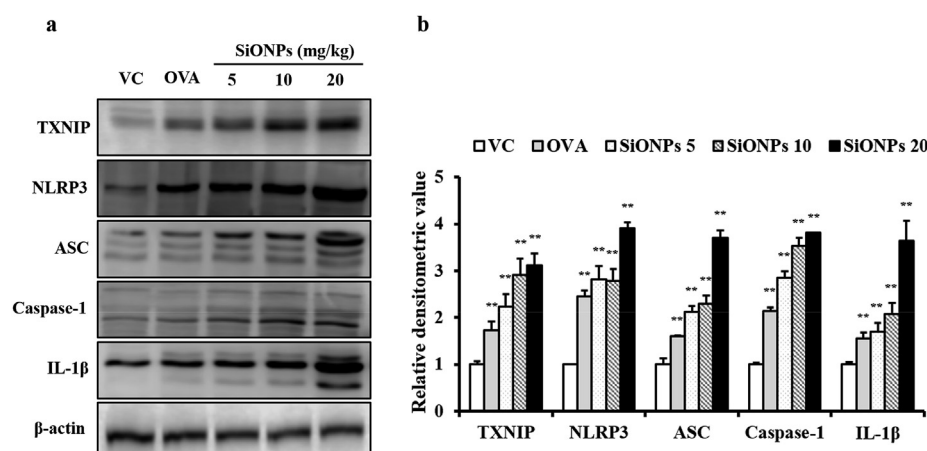


Fig. 7. Effects of SiONPs exposure on TXNIP, inflammasome protein, and IL-1β expression in lung tissue. (a) Proteins expression. (b) Relative densitometric values of proteins expression. Data are represented as means $\pm$ SD, $n = 6$. ** $P < 0.01$ vs. OVA group. VC, PBS intranasal instillation; OVA, OVA challenge + PBS intranasal instillation; SiONPs 5, 10, and 20, OVA challenge + 5, 10 and 20 mg/kg of SiONPs intranasal instillation, respectively.

TNF- α and IL-1β were consistent with those of IL-8 and IL-6.

3.9. Effects of SiONPs on TXNIP, IL-1β, and inflammasome proteins expression

SiONPs treatment increased the TXNIP expression in NCI-H292 cells in a concentration-dependent manner (Fig. 11).

Additionally, the levels of inflammasome proteins, NLRP3, ASC, and cleaved caspase-1, were markedly increased by SiONPs in a concentration-dependent manner. IL-1β expression was also markedly increased in a concentration-dependent manner.

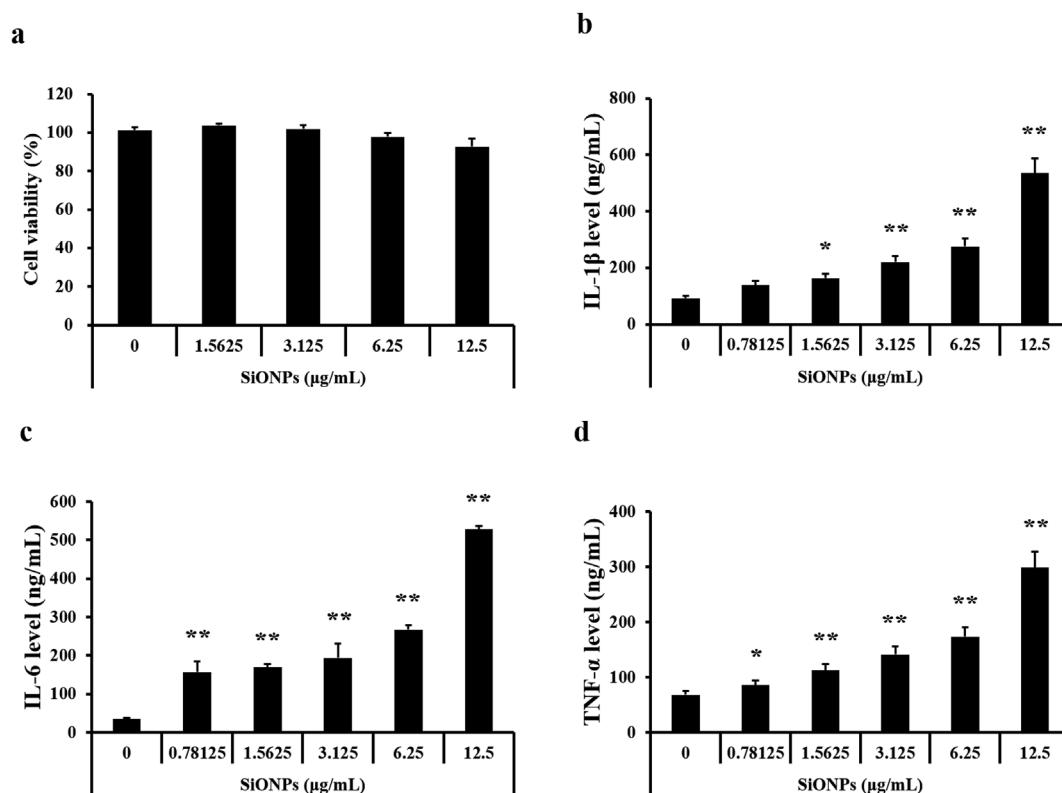


Fig. 8. Effects of SiONPs treatment on cell viability and inflammatory cytokines in NCI-H292 cells. (a) Cell viability. (b) IL-1 β level. (c) IL-6 level. (d) TNF- α level. Data are represented as means $\pm$ SD, $n = 3$. *, ** $P < 0.05$ and $p < 0.01$ vs. control, respectively. Control, PBS treatment; 0.78125, 1.5625, 3.125, 6.25, and 12.5, 0.78125, 1.5625, 3.125, 6.25, and 12.5 $\mu\text{g/mL}$ of SiONPs treatment; respectively.

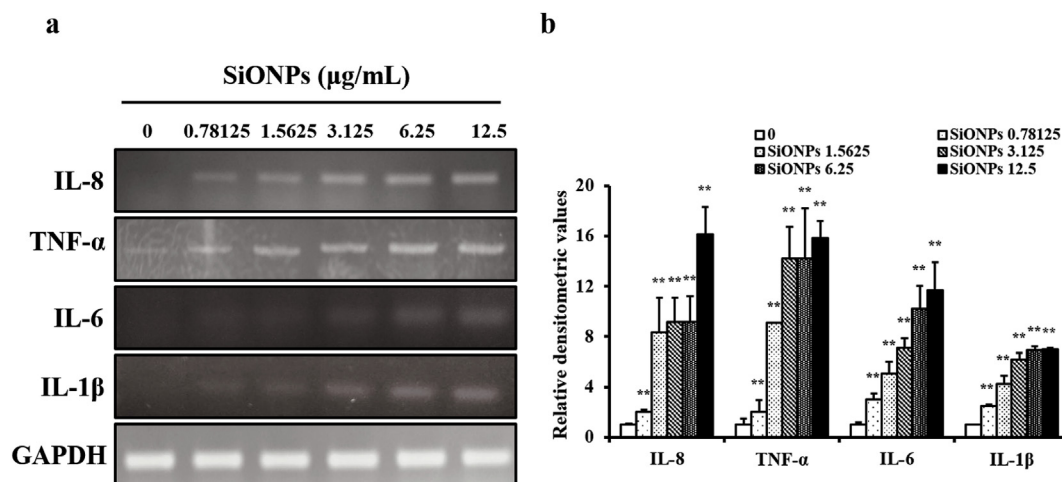


Fig. 9. Effects of SiONPs treatment on mRNA expression of inflammatory cytokines measured by RT-PCR in NCI-H292 cells. (a) Gel image of inflammatory cytokines mRNA expression. (b) Densitometric values of inflammatory cytokines mRNA expression. Data are represented as means $\pm$ SD, $n = 3$. ** $P < 0.01$ vs. control. Control, PBS treatment; 0.78125, 1.5625, 3.125, 6.25, and 12.5, 0.78125, 1.5625, 3.125, 6.25, and 12.5 $\mu\text{g/mL}$ of SiONPs treatment; respectively.

4. Discussion

As the market related for nanomaterials is expanding rapidly, concerns of potential toxicity induced by exposure to nanomaterials are increasing (Jain et al., 2018). In particular, SiONPs constitute a large proportion of nanoparticles in industrial and biomedical applications, and there is a growing concern about the toxicological effects of SiONPs on human health and the environment (Meibert et al., 2017). Although toxic effects of SiONPs have been explored both *in vitro* and *in vivo*, there is still a lack of research on its disease-causing mechanisms, especially in pulmonary disease, which is the main route of exposure to

SiONPs (Giovannini et al., 2018; Guo et al., 2017; Shin et al., 2017). In addition to attempting to limit SiONPs exposure in the industrial environment, the effect of SiONPs on exacerbation of existing respiratory diseases should also be concurrently investigated when considering the inevitable exposure in daily life to pollutants like Asian dust, which contains a large amount of SiONPs (Naota et al., 2013; Shimada et al., 2015). In this study, we evaluated the potential toxicity and the mechanism of SiONPs toxicity *in vitro* and explored the effects of SiONPs on asthma aggravation using OVA-induced asthmatic mice model focusing on inflammasome activation. SiONPs treatment markedly increased the levels of inflammatory mediators and expression of TXNIP

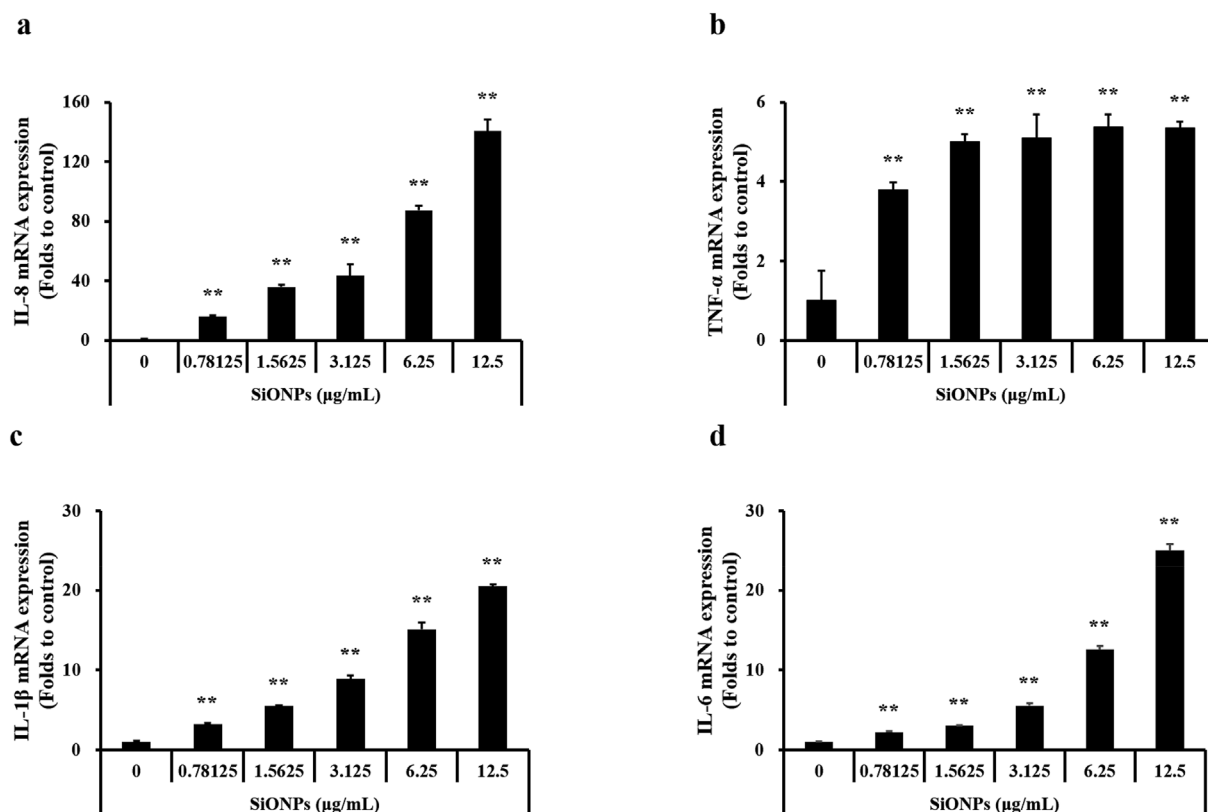


Fig. 10. Effects of SiONPs treatment on mRNA expression of inflammatory cytokines measured by real-time PCR in NCI-H292 cells. (a) mRNA expression level of IL-8. (b) mRNA expression level of TNF-α. (c) mRNA expression level of IL-1β. (d) mRNA expression level of IL-6. Data are represented as means $\pm$ SD, $n = 3$. ** $P < 0.01$ vs. control. Control, PBS treatment; 0.78125, 1.5625, 3.125, 6.25, and 12.5, 0.78125, 1.5625, 3.125, 6.25, and 12.5 μ g/mL of SiONPs treatment; respectively.

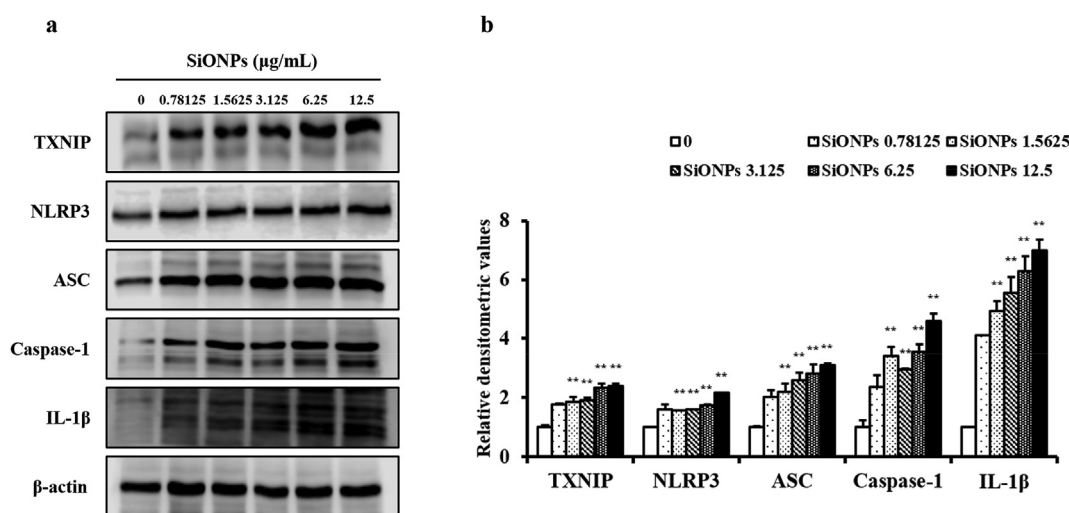


Fig. 11. Effects of SiONPs treatment on TXNIP, inflammasome protein, and IL-1β expression in NCI-H292 cells. (a) Proteins expression. (b) Relative densitometric values of proteins expression. Data are represented as means $\pm$ SD, $n = 6$. ** $P < 0.01$ vs. control. Control, PBS treatment; 0.78125, 1.5625, 3.125, 6.25, and 12.5, 0.78125, 1.5625, 3.125, 6.25, and 12.5 μ g/mL of SiONPs treatment; respectively.

and inflammasome proteins in NCI-H292 cells. Exposure to SiONPs in OVA induced asthmatic mice increased the levels of inflammatory markers compared to those in OVA-induced asthmatic mice, concurrent with the elevation of TXNIP and inflammasome proteins expression in lung tissues.

In this study, exposure to SiONPs increased the inflammatory cell counts especially that of eosinophils, levels of inflammatory cytokines, OVA-specific IgE levels, and AHR compared to those in the OVA group.

These findings are consistent with the histological analyses results. Exposure to SiONPs in OVA-induced asthmatic mice elevated inflammatory cell infiltration and mucus production in comparison to that in the OVA group. In addition, treatment with SiONPs markedly increased the mRNA expression levels of proinflammatory cytokines in NCI-H292 cells, in a concentration-dependent manner. These results indicated that exposure to SiONPs can induce airway inflammatory responses and accelerate asthma development. In asthma development,

elevation of eosinophil count is an important feature and is closely associated with asthma pathogenesis (Park et al., 2016). The recruitment, maturation, and activation of eosinophils is controlled by various factors, particularly Th2 cytokines and IgE, resulting in elevated AHR, mucus overproduction, and the increased inflammatory response in asthma progression (Foster et al., 2017). Park et al. (2015) reported that acute exposure to silica nanoparticles induced significant airway inflammation and AHR. Exposure to SiONPs during OVA sensitization caused a dose-dependent aggravation in allergic airway disease compared to that by challenge with OVA alone, which was manifested as significantly greater OVA-specific serum IgE level, airway eosinophil infiltration, mucus cell metaplasia, and elevated Th2 and Th17 cytokine gene and protein expression (Brandenberger et al., 2013; Han et al., 2016). Therefore, exposure to SiONPs aggravates asthma pathogenesis represented by eosinophilic inflammation.

It has been reported that innate immune cells produce pro-inflammatory cytokines such as IL-1 β (Kusaka et al., 2014). This process involves a cytosolic multiprotein complex called the inflammasome, which plays a major role in innate immunity (Gómez et al., 2017). Among the various inflammasomes, one of the best characterized is the NLRP3 inflammasome comprising the NLRP3, ASC, and caspase-1 proteins. NLRP3 inflammasome activation leads to the maturation of pro-IL-1 β into its active and secreted form via caspase-1 (Naji et al., 2016). Recently, numerous studies have indicated that NLRP3 inflammasome is associated with various inflammatory diseases, such as acute lung injury, diabetes, and obesity (Cao et al., 2016). In particular, it is reported that NLRP3 inflammasome plays crucial roles in asthma pathogenesis (Im and Ammit, 2014). A large number of stimuli, such as viral and bacterial infection, UV radiation, and particles including many nanomaterials are known to activate the NLRP3 inflammasome (Yao et al., 2016). Of these, silica was one of the first environmental particles identified to activate the NLRP3 inflammasome (Jessop et al., 2017). One of the proposed mechanisms of SiONP-induced NLRP3 activation is ROS production (Cassel et al., 2008). The complex formed by thioredoxin (TRX) and TXNIP has also been proposed to link ROS and NLRP3 activation (Dostert et al., 2008). Under normal conditions, TXNIP is associated with TRX. However, free radicals oxidize TRX, and the dissociated TXNIP then interacts with and activates NLRP3 (Rabolli et al., 2016). TRX overexpression or treatment with recombinant TRX is reported to attenuate caspase-1 enzymatic activity and secretion of IL-1 β in silica-exposed epithelial cell or macrophage cultures (Peeters et al., 2014). In this study, exposure to SiONPs increased expression levels of TXNIP and inflammasome proteins accompanied by elevation of IL-1 β expression in a dose-dependent manner compared to that in the OVA group. In addition, SiONPs treatment also increased these proteins expression levels in NCI-H292 cells in a concentration-dependent manner, which were confirmed in the *in vivo* experiment. Therefore, these results indicated that asthma aggravation by SiONPs may be related to inflammasome activation.

In conclusion, exposure to SiONPs significantly elevated the characteristic markers of asthma including AHR, levels of inflammatory mediators and IgE, inflammatory cell infiltration, and mucus production. Our results indicate that SiONPs exposure has the potential to aggravate asthmatic inflammation via activation of the NLRP3 inflammasome. These effects of SiONPs were also confirmed in SiONPs-stimulated NCI-H292 cells. Our experimental results provide information on the respiratory toxicity and disease exacerbation caused by SiONPs and provide information on the target signal in the therapeutic aspect.

Funding sources

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education [NRF-2016R1D1A2B04936124].

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.

Transparency document

Transparency document related to this article can be found online at <https://doi.org/10.1016/j.yrtph.2020.104618>.

References

- Ahmad, J., Ahamed, M., Akhtar, M.J., Alrokayan, S.A., Siddiqui, M.A., Musarrat, J., Al-Khedhairi, A.A., 2012. Apoptosis induction by silica nanoparticles mediated through reactive oxygen species in human liver cell line HepG2. *Toxicol. Appl. Pharmacol.* 259, 160–168.
- Brandenberger, C., Rowley, N.L., Jackson-Humbles, D.N., Zhang, Q., Bramble, L.A., Lewandowski, R.P., Wagner, J.G., Chen, W., Kaplan, B.L., Kaminski, N.E., Bake, r G.L., Worden, R.M., Harkema, J.R., 2013. Engineered silica nanoparticles act as adjuvants to enhance allergic airway disease in mice. *Part. Fibre Toxicol.* 10, e26. <https://doi.org/10.1186/1743-8977-10-26>.
- Cao, Z., Fang, Y., Lu, Y., Qian, F., Ma, Q., He, M., Pi, H., Yu, Z., Zhou, Z., 2016. Exposure to nickel oxide nanoparticles induces pulmonary inflammation through NLRP3 inflammasome activation in rats. *Int. J. Nanomed.* 11, 3331–3346.
- Cassel, S.L., Eisenbarth, S.C., Iyer, S.S., Sadler, J.J., Colegio, O.R., Tephly, L.A., Carter, A.B., Rothman, P.B., Flavell, R.A., Sutterwala, F.S., 2008. The Nalp3 inflammasome is essential for the development of silicosis. *Proc. Natl. Acad. Sci.* 105, 9035–9040.
- Dostert, C., Pétrilli, V., Van Bruggen, R., Steele, C., Mossman, B.T., Tschopp, J., 2008. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 320, 674–677.
- Duan, J., Yu, Y., Li, Y., Yu, Y., Li, Y., Zhou, X., Huang, P., Sun, Z., 2013. Toxic effect of silica nanoparticles on endothelial cells through DNA damage response via Chk1-dependent G2/M checkpoint. *PloS One* 8, e62087. <https://doi.org/10.1371/journal.pone.0062087>.
- Foster, P.S., Maltby, S., Rosenberg, H.F., Tay, H.L., Hogan, S.P., Collison, A.M., Yang, M., Kaiko, G.E., Hansbro, P.M., Kumar, R.K., Mattes, J., 2017. Modeling TH 2 responses and airway inflammation to understand fundamental mechanisms regulating the pathogenesis of asthma. *Immunol. Rev.* 278, 20–40.
- Giovannini, G., Warncke, P., Fischer, D., Stranik, O., Hall, A.J., Gubala, V., 2018. Improving colloidal stability of silica nanoparticles when stored in responsive gel: application and toxicity study. *Nanotoxicology* 12, 407–422.
- Gómez, D.M., Urcuqui-Inchima, S., Hernandez, J.C., 2017. Silica nanoparticles induce NLRP3 inflammasome activation in human primary immune cells. *Innate Immun.* 23, 697–708.
- Guo, C., Wang, J., Yang, M., Li, Y., Cui, S., Zhou, X., Li, Y., Sun, Z., 2017. Amorphous silica nanoparticles induce malignant transformation and tumorigenesis of human lung epithelial cells via P53 signaling. *Nanotoxicology* 11, 1176–1194.
- Han, H., Park, Y.H., Park, H.J., Lee, K., Um, K., Park, J.W., Lee, J.H., 2016. Toxic and adjuvant effects of silica nanoparticles on ovalbumin-induced allergic airway inflammation in mice. *Respir. Res.* 17, e60. <https://doi.org/10.1186/s12931-016-0376-x>.
- Ichinose, T., Yoshida, S., Sadakane, K., Takano, H., Yanagisawa, R., Inoue, K., Nishikawa, M., Mori, I., Kawazato, H., Yasuda, A., Shibamoto, T., 2008. Effects of Asian sand dust, Arizona sand dust, amorphous silica and aluminum oxide on allergic inflammation in the murine lung. *Inhal. Toxicol.* 20, 685–694.
- Im, H., Ammit, A.J., 2014. The NLRP3 inflammasome: role in airway inflammation. *Clin. Exp. Allergy* 44, 160–172.
- Jain, A., Ranjan, S., Dasgupta, N., Ramalingam, C., 2018. Nanomaterials in food and agriculture: an overview on their safety concerns and regulatory issues. *Crit. Rev. Food Sci. Nutr.* 58, 297–317.
- Jessop, F., Hamilton Jr., R.F., Rhoderick, J.F., Fletcher, P., Holian, A., 2017. Phagolysosome acidification is required for silica and engineered nanoparticle-induced lysosome membrane permeabilization and resultant NLRP3 inflammasome activity. *Toxicol. Appl. Pharmacol.* 318, 58–68.
- Kashima, S., Yorifuji, T., Suzuki, E., 2014. Asian dust and daily emergency ambulance calls among elderly people in Japan: an analysis of its double role as a direct cause and as an effect modifier. *J. Occup. Environ. Med.* 56, 1277–1283.
- Keller, A.A., Lazarevar, A., 2014. A predicted releases of engineered nanomaterials: from global to regional to local. *Environ. Sci. Technol. Lett.* 1, 65–70.
- Kim, Y.S., Lim, C.H., Shin, S.H., Kim, J.C., 2017. Twenty-eight-day repeated inhalation toxicity study of nano-sized neodymium oxide in male Sprague-Dawley rats. *Toxicol. Res.* 33, 239–253.
- Ko, J.W., Shin, N.R., Park, S.H., Kim, J.S., Cho, Y.K., Kim, J.C., Shin, I.S., Shin, D.H., 2017. Pine bark extract (Pycnogenol®) suppresses cigarette smoke-induced fibrotic response via transforming growth factor- β 1/Smad family member 2/3 signaling. *Lab. Anim. Res.* 33, 76–83.
- Kusaka, T., Nakayama, M., Nakamura, K., Ishimiya, M., Furusawa, E., Ogasawara, K., 2014. Effect of silica particle size on macrophage inflammatory responses. *PloS One* 9, e26234. <https://doi.org/10.1371/journal.pone.0092634>.
- Lamkanfi, M., Dixit, V.M., 2014. Mechanisms and functions of inflammasomes. *Cell* 157, 1013–1022.

- Lee, I.C., Ko, J.W., Park, S.H., Shin, N.R., Shin, I.S., Moon, C., Kim, J.H., Kim, H.C., Kim, J.C., 2016. Comparative toxicity and biodistribution assessments in rats following subchronic oral exposure to copper nanoparticles and microparticles. *Part. Fibre Toxicol.* 13 (1), 56. <https://doi.org/10.1186/s12989-016-0169-x>.
- Liang, Y., Zhang, Y., Deng, Y., Liang, S., He, Y., Chen, Y., Liu, C., Lin, C., Han, L., Tu, G., Yang, Q., 2018. Chaihu-Shugan-San decoction modulates intestinal microbe dysbiosis and alleviates chronic metabolic inflammation in NAFLD rats via the NLRP3 inflammasome pathway. *Evid. base Compl. Alternative Med.* 2018 <https://doi.org/10.1155/2018/9390786>. e9390786.
- Mebert, A.M., Baglolle, C.J., Desimone, M.F., Maysinger, D., 2017. Nanoengineered silica: properties, applications and toxicity. *Food Chem. Toxicol.* 109, 753–770.
- Morishige, T., Yoshioka, Y., Tanabe, A., Yao, X., Tsunoda, S., Tsutsumi, Y., Mukai, Y., Okada, N., Nakagawa, S., 2010. Titanium dioxide induces different levels of IL-1 β production dependent on its particle characteristics through caspase-1 activation mediated by reactive oxygen species and cathepsin B. *Biochem. Biophys. Res. Commun.* 392, 160–165.
- Naji, A., Muzembo, B.A., Yagyu, K., Baba, N., Deschaseaux, F., Sensebé, L., Suganuma, N., 2016. Endocytosis of indium-tin-oxide nanoparticles by macrophages provokes pyroptosis requiring NLRP3-ASC-Caspase1 axis that can be prevented by mesenchymal stem cells. *Sci. Rep.* 6, e26162. <https://doi.org/10.1038/srep26162>.
- Naota, M., Shiotsu, S., Shimada, A., Kohara, Y., Morita, T., Inoue, K., Takano, H., 2013. Pathological study of chronic pulmonary toxicity induced by intratracheally instilled Asian sand dust (kosa). *Toxicol. Pathol.* 41, 48–62.
- Navya, P.N., Daima, H.K., 2016. Rational engineering of physicochemical properties of nanomaterials for biomedical applications with nanotoxicological perspectives. *Nano Convergence* 3, e1. <https://doi.org/10.1186/s40580-016-0064-z>.
- Park, H.J., Sohn, J.H., Kim, Y.J., Park, Y.H., Han, H., Park, K.H., Lee, K., Choi, H., Um, K., Choi, I.H., Park, J.W., Lee, J.H., 2015. Acute exposure to silica nanoparticles aggravate airway inflammation: different effects according to surface characteristics. *Exp. Mol. Med.* 47, e173. <https://doi.org/10.1038/emmm.2015.50>.
- Park, J.W., Lee, I.C., Shin, N.R., Jeon, C.M., Kwon, O.K., Ko, J.W., Kim, J.C., Oh, S.R., Shin, I.S., Ahn, K.S., 2016. Copper oxide nanoparticles aggravate airway inflammation and mucus production in asthmatic mice via MAPK signaling. *Nanotoxicology* 10, 445–452.
- Peeters, P.M., Eurlings, I.M., Perkins, T.N., Wouters, E.F., Schins, R.P., Borm, P.J., Drommer, W., Reynaert, N.L., Albrecht, C., 2014. Silica-induced NLRP3 inflammasome activation in vitro and in rat lungs. *Part. Fibre Toxicol.* 11, e58. <https://doi.org/10.1186/s12989-014-0058-0>.
- Peng, Z., Zhang, W., Qiao, J., He, B., 2018. Melatonin attenuates airway inflammation via SIRT1 dependent inhibition of NLRP3 inflammasome and IL-1 β in rats with COPD. *Int. Immunopharm.* 62, 23–28.
- Rabolli, V., Lison, D., Huaux, F., 2016. The complex cascade of cellular events governing inflammasome activation and IL-1 β processing in response to inhaled particles. *Part. Fibre Toxicol.* 13, e40. <https://doi.org/10.1186/s12989-016-0150-8>.
- Shen, C., Zhang, Z., Xie, T., Xu, J., Yan, J., Kang, A., Dai, Q., Wang, S., Ji, J., Shan, J., 2018. Jinxin oral liquid inhibits human respiratory syncytial virus-induced excessive inflammation associated with blockade of the NLRP3/ASC/Caspase-1 pathway. *Biomed. Pharmacother.* 103, 1376–1383.
- Shimada, A., Kohara, Y., Naota, M., Kobayashi, Y., Morita, T., Inoue, K., Takano, H., 2015. Pathological study of chronic pulmonary toxicity induced by intratracheally instilled Asian sand dust (Kosa): possible association of fibrosis with the development of granulomatous lesions. *Folia Histochem. Cytobiol.* 53, 294–306.
- Shin, J.H., Jeon, K., Kim, J.K., Kim, Y., Jo, M.S., Lee, J.S., Baek, J.E., Park, H.S., An, H.J., Park, J.D., Ahn, K., Oh, S.M., Yu, I.J., 2017. Subacute inhalation toxicity study of synthetic amorphous silica nanoparticles in Sprague-Dawley rats. *Inhal. Toxicol.* 29, 567–576.
- Shin, N.R., Jung, T.Y., Seo, C.S., Park, S.W., Ko, J.W., Kim, J.C., Shin, I.S., 2018. Protective effect of water extract of guibi-tang against pulmonary inflammation induced by cigarette smoke and lipopolysaccharide. *Lab. Anim. Res.* 34, 92–100.
- Skeldon, A.M., Faraj, M., Saleh, M., 2014. Caspases and inflammasomes in metabolic inflammation. *Immunol. Cell Biol.* 92, 304–313.
- Sun, B., Wang, X., Ji, Z., Li, R., Xia, T., 2013. NLRP3 inflammasome activation induced by engineered nanomaterials. *Small* 9, 1595–1607.
- Wang, X., Gong, P., Zhang, X., Li, S., Lu, X., Zhao, C., Yu, Q., Wei, Z., Yang, Y., Liu, Q., Yang, Z., Li, J., Zhang, X., 2018. NLRP3 inflammasome participates in host response to *Neospora caninum* infection. *Front. Immunol.* 9, e1791. <https://doi.org/10.3389/fimmu.2018.01791>.
- Yang, E.J., Kim, S., Kim, J.S., Choi, I.H., 2012. Inflammasome formation and IL-1 β release by human blood monocytes in response to silver nanoparticles. *Biomaterials* 33, 6858–6867.
- Yao, H., Zhang, Y., Liu, L., Xu, Y., Liu, X., Lin, J., Zhou, W., Wei, P., Jin, P., Wen, L.P., 2016. Inhibition of lanthanide nanocrystal-induced inflammasome activation in macrophages by a surface coating peptide through abrogation of ROS production and TRPM2-mediated Ca²⁺ influx. *Biomaterials* 108, 143–156.
- Zhang, X., Luan, J., Chen, W., Fan, J., Nan, Y., Wang, Y., Liang, Y., Meng, G., Ju, D., 2018. Mesoporous silica nanoparticles induced hepatotoxicity via NLRP3 inflammasome activation and caspase-1-dependent pyroptosis. *Nanoscale* 10, 9141–9152.