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LJ-2698, an Adenosine A_3 Receptor Antagonist, Alleviates Elastase-Induced Pulmonary Emphysema in Mice

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Abstract

Emphysema, a major component of chronic obstructive pulmonary disease (COPD), is a leading cause of human death worldwide. The progressive deterioration of lung function that occurs in the disease is caused by chronic inflammation of the airway and destruction of the lung parenchyma. Despite the main impact of inflammation on the pathogenesis of emphysema, current therapeutic regimens mainly offer symptomatic relief and preservation of lung function with little therapeutic impact. In the present study, we aimed to discover novel therapeutics that suppress the pathogenesis of emphysema. Here, we show that LJ-2698, a novel and highly selective antagonist of the adenosine A_3 receptor, a G protein-coupled receptor involved in various inflammatory diseases, significantly reversed the elastase-induced destructive changes in murine lungs. We found that LJ-2698 significantly prevented elastase-induced airspace enlargement, resulting in restoration of pulmonary function without causing any obvious changes in body weight in mice. LJ-2698 was found to inhibit matrix metalloproteinase activity and pulmonary cell apoptosis in the murine lung. LJ-2698 treatment induced increases in anti-inflammatory cytokines in macrophages at doses that displayed no significant cytotoxicity in normal cell lines derived from various organs. Treatment with LJ-2698 significantly increased the number of anti-inflammatory M2 macrophages in the lungs. These results implicate the adenosine A_3 receptor in the pathogenesis of emphysema. Our findings also demonstrate the potential of LJ-2698 as a novel therapeutic/preventive agent in suppressing disease development with limited toxicity.

Key Words: Adenosine A_3 receptor, LJ-2698, Emphysema

INTRODUCTION

Air pollution is a global environmental problem, and the incidence, mortality, and socioeconomic burden of pulmonary diseases are expected to gradually increase. Among the various pulmonary disorders, chronic obstructive pulmonary disease (COPD) is the third leading cause of human death worldwide (Lozano *et al*., 2012; Guarascio *et al*., 2013; Barnes *et al*., 2015; Tachkov *et al*., 2017) and is characterized by chronic destructive changes in the airways and lung parenchyma that result in gas exchange disturbances (Barnes *et al*., 2015; Van Tho *et al*., 2015). Emphysema is a symptom of COPD and is defined as abnormal enlargement of the airspace caused by irreversible destruction of the alveolar walls (Horio *et al*., 2017). Several factors, including α1-antitrypsin deficiency, oxidative stress, aging, and chronic inflammation, are involved in the pathogenesis of emphysema (Gooptu *et al*., 2009; Barnes *et al*., 2015). As the pathologic mechanism, several causative factors such as cigarette smoking and air pollutants induce the activation of macrophages and neutrophils, leading to tissue destruction by producing reactive oxygen species and various proteases, such as neutrophil elastase and matrix metalloproteases (Sharafkhaneh *et al*., 2008; Barnes *et al*., 2015). Matrix degradation generates elastin fragments and further enhances inflammatory processes in the lungs through elastin fragment-mediated chemoattraction and autoimmunity (Lee *et al*., 2007; Sharafkhaneh *et al*., 2008). Several inhaled bronchodilators and anti-inflammatory agents, including anticholinergics, β2-adrenergic agonists, corticosteroids, antibiotics and antioxidants, have been used to treat emphysema/COPD (Celli, 2018). These medications can alleviate disease progression and show marginal but clinically significant benefits

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for patients with COPD (Celli, 2018). However, in most cases, these regimens offer only symptomatic relief and preservation of lung function. To date, there are no therapeutic options to completely reverse disease progression and cure emphysema. Therefore, it is necessary to develop novel therapeutics for the treatment or prevention of emphysema.

Previous reports have suggested the role of adenosine-mediated signaling in the maintenance of homeostasis in the lungs by regulating defense against injury and regeneration, indicating the association of adenosine-mediated signaling with chronic pulmonary disorders such as asthma and COPD (Zhou *et al*., 2009). Adenosine transduces signals through one of four G protein-coupled adenosine receptors (A_1AR, A_{2A}AR, A_{2B}AR, and A_3AR). Of these, the adenosine A_3 receptor (A_3AR) is the only adenosine receptor subtype to be overexpressed in inflammatory and cancer cells and is considered a target for the development of therapeutic agents for inflammation and cancer (Borea *et al*., 2015). Previous reports have demonstrated the involvement of A_3AR in several pathophysiological conditions, and depending on the experimental system, A_3AR has displayed conflicting roles in the regulation of ischemic conditions, inflammation, and tumor growth (Borea *et al*., 2015). The affinity and expression density of A_3AR were significantly altered in lung tissues from patients with COPD, which were correlated with pulmonary function in matched patients (Varani *et al*., 2006). In addition, experimental models have demonstrated that A_3AR signaling upregulates inflammation, eosinophil trafficking, degranulation of mast cells, and mucus secretion, suggesting that blockade of A_3AR might be beneficial for relieving airway inflammation (Polosa and Blackburn, 2009). Considering the association of chronic inflammation with the pathogenesis of COPD, it is possible that blockade of A_3AR can alleviate emphysema/COPD; however the precise roles of A_3AR signaling in emphysema remain to be elucidated, and the potential of adenosine A_3 receptor agonists as therapeutic or preventive agents in emphysema/COPD has not been investigated thoroughly.

Based on these findings, we investigated the effect of the highly selective A_3AR antagonist LJ-2698 (Jeong *et al*., 2007) in an *in vivo* experimental model of emphysema. LJ-2698 significantly suppressed elastase-mediated pulmonary dysfunction and lung degeneration in mice, as indicated by restoration of lung function, reduction in emphysematous lesions, and decreases in matrix metalloproteinase activity and apoptosis in the lungs. LJ-2698 significantly upregulated several cytokines associated with the repair of injured tissues. Moreover, LJ-2698 exhibited minimal toxicity *in vitro* and *in vivo*. These findings highlight the potential of LJ-2698 as a novel agent for the treatment or prevention of emphysema.

MATERIALS AND METHODS

Reagents

Fluorescein-conjugated DQ-gelatin was purchased from Thermo Fisher Scientific (Carlsbad, CA, USA). Porcine pancreatic elastase (PPE), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. LJ-2698 was synthesized as described previously (Jeong *et al*., 2007).

Cell culture

Human retinal pigment epithelial (RPE) cells were kindly provided by Dr. Jeong Hun Kim (College of Medicine, Seoul National University, Seoul, Korea). The murine hippocampal neuronal cell line HT-22 was kindly provided by Dr. Dong Gyu Jo (College of Pharmacy, Sungkyunkwan University, Suwon, Korea). The murine macrophage cell line RAW 264.7 was kindly provided by Dr. Sang Kook Lee (Seoul National University). Murine alveolar epithelial MLE-12 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). RPE, HT-22, and RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS and antibiotics (all from Welgene, Inc., Gyeongssan, Korea). MLE12 cells were cultured in HITES medium [DMEM-F12 media (Welgene) containing 1x insulin-transferrin-selenium solution (Thermo Fisher Scientific), 10 nM hydrocortisone, 10 nM β-estradiol, 10 mM HEPES, and 2 mM L-glutamine (Welgene)] supplemented with 2% FBS and antibiotics. Cells were incubated at 37°C with 5% CO_2 in a humidified atmosphere.

Cell viability assay

Cells (2×10^3 cells/well in 96-well plates) were treated with various concentrations of LJ-2698 for three days. The cells were incubated with an MTT solution for 4 h at 37°C. The formazan products were dissolved in DMSO, and the absorbance was measured at 570 nm. The data are presented as a percentage of the control group.

Real-time polymerase chain reaction (PCR)

Total RNA was isolated from cultured cells or frozen lung tissues using a phenol-chloroform extraction method, reverse-transcribed using a first-strand cDNA synthesis kit (TransGen Biotech, Beijing, China), and analyzed with real-time PCR on the Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific) using a SYBR Green-based qPCR master mix solution (Enzynomics, Daejeon, Korea) and gene-specific primers. The primer sequences used for the real-time PCR are as follows: Mouse *Il4* forward, CCT CAC AGC AAC GAA GAA CA; mouse *Il4* reverse, ATC GAA AAG CCC GAA AGA GT; mouse *Il10* forward, TAA GGC TGG CCA CAC TTG AG; mouse *Il10* reverse, GTT TTC AGG GAT GAA GCG GC; mouse *Arg1* forward, GAA CAC GGC AGT GGC TTT AAC; mouse *Arg1* reverse, TGC TTA GCT CTG TCT GCT TTG C; mouse *Mrc1* forward, TGA TTA CGA GCA GTG GAA GC; mouse *Mrc1* reverse, GTT CAC CGT AAG CCC AAT TT; mouse *Actb* forward, TGT CCA CCT TCC AGC AGA TGT; mouse *Actb* reverse, AGC TCA GTA ACA GTC CGC CTA G; and mouse *Rn18s* forward, GTA ACC CGT TGA ACC CCA TT; mouse *Rn18s* reverse, CCA TCC AAT CGG TAG TAG CG. The thermocycler conditions were as follows: preincubation at 95°C for 15 min; 50 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 30 s; and melting curve analysis to determine reaction specificity. The quantification of relative mRNA expression was performed using the comparative cycle threshold (CT) method as described previously (Livak and Schmittgen, 2001).

Animal experiment

The animal experiment was performed according to a protocol approved by the Seoul National University Institutional Animal Care and Use Committee. Mice were given standard mouse chow and water *ad libitum* and housed in a tempera-

ture- and humidity-controlled facility with a 12-h light/12-h dark cycle. Eight-week-old FVB mice were administered vehicle [20% DMSO dissolved in sterile distilled water containing 20% polyethylene glycol (PEG)] or LJ-2698 (50 μg/kg) by oral gavage 6 times per week for 5 weeks. One week after drug treatment, 0.25 units of PPE was intratracheally instilled into the lungs of mice. Body weight changes were monitored during the treatment. Changes in pulmonary function in vehicle- or LJ-2698-treated mice were analyzed using the FlexiVent (Scireq, EMKA Technologies, Montreal, Canada) (Vanoirbeek *et al*., 2010) according to the protocol recommended by the manufacturer. Mice were euthanized by inhalation of an overdose of isoflurane, and lungs were excised after perfusion with ice-cold PBS and then embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc., Torrance, CA, USA). Frozen tissue blocks were used for further analyses. H&E-stained tissues were used to determine structural changes in the lungs. Structural changes were quantified by calculating the mean linear intercept (MLI) as previously described (Dunnill, 1962; Chen *et al*., 2010).

In situ zymography

Dried cryosections of the lungs were incubated with fluorescein-conjugated DQ-gelatin diluted in low gelling temperature agarose for 3 h at room temperature. Fluorescein isothiocyanate (FITC) fluorescence was detected at an excitation wavelength of 460-500 nm and an emission wavelength of 512-542 nm under a fluorescence microscope and photographed.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

Analysis of apoptotic DNA fragmentation was performed by using a TUNEL assay kit (Millipore, MA, USA) according to the manufacturer's instructions. Briefly, cryosections (8 μm) were fixed in 1% paraformaldehyde for 10 min at room temperature. After washing, sections were postfixed in precooled ethanol:acetic acid (2:1) for 5 min at –20°C. Then, the sections were incubated with working strength TdT enzyme for 1 h at 37°C. The sections were incubated with working strength stop/wash buffer for 10 min at room temperature. Anti-digoxigenin conjugate was then applied to the sections for 30 min at room temperature. The slides were counterstained with DAPI.

Immunofluorescence staining

Cryosections (8 μm) were prepared for immunofluorescence analysis. The sections were fixed in 4% paraformaldehyde (PFA) for 30 min followed by permeabilization in 0.2% Triton X-100 for 15 min at room temperature. Next, the slides were incubated with anti-CD206 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) at 4°C overnight. After washing, sections were incubated with Alexa Fluor-conjugated secondary antibody for 1 h at room temperature. The nuclei were stained with DAPI and analyzed by confocal microscopy (LSM 700; Carl Zeiss Microscopy, Jena, Germany).

Statistical analysis

The data are presented as the mean ± SD. The statistical significance was determined with a two-tailed Student's *t*-test

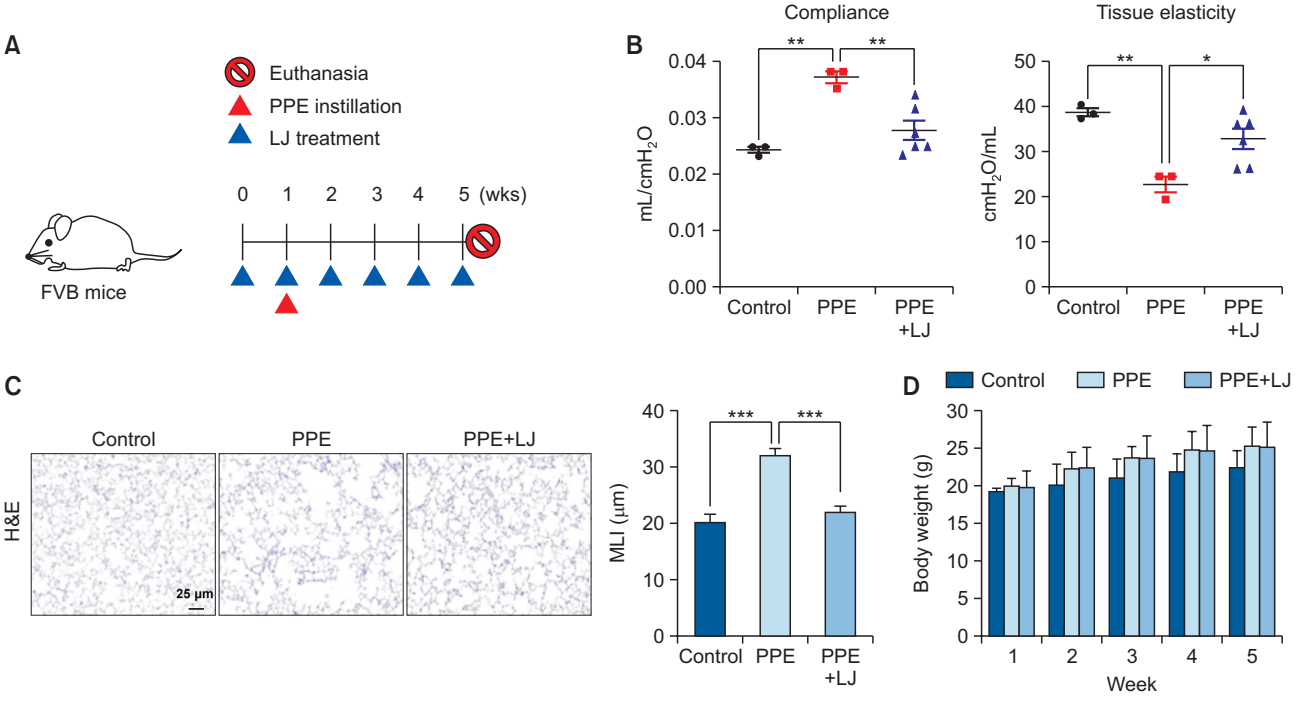

Fig. 1. Suppression of elastase-induced emphysema by oral administration of LJ-2698 with minimal toxicity. (A) Schematic diagram of the experimental schedule. (B) Inhibition of elastase-induced pulmonary dysfunction by treatment with LJ-2698 (50 μg/kg). Changes in lung function were monitored using FlexiVent. (C) Alleviation of elastase-mediated pulmonary destruction in lungs from LJ-2698-treated mice. *Right*. Quantification of structural changes in the airspace in each treatment group. (D) Body weight changes in each treatment group during the animal experiment. The bars represent the mean ± SD; **p*<0.05, ***p*<0.01, and ****p*<0.001 compared with the PPE-treated group. PPE, porcine pancreatic elastase; LJ, LJ-2698; MLI, mean linear intercept.

using Microsoft Excel software (Microsoft Corp., Redmond, MA, USA) or GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). A *p*-value less than 0.05 was considered statistically significant.

RESULTS

Inhibitory effect of LJ-2698 on elastase-induced pulmonary emphysema

Based on the possible association of A_3AR signaling with the development of emphysema/COPD and to discover a novel active compound to suppress emphysema, we examined the inhibitory effect of LJ-2698 on elastase-induced emphysema in mice, a widely used animal model for pulmonary emphysema (Mahadeva and Shapiro, 2002). LJ-2698 was orally administered to mice for 5 weeks. One week after LJ-2698 treatment, PPE was instilled into the murine lungs (Fig. 1A). Because deregulation in lung function is a main characteristic of emphysema (Barnes *et al*., 2015), we examined whether treatment with LJ-2698 attenuates PPE-mediated pulmonary dysfunction. Murine lung function was determined as compliance, with the static value indicating the change in lung volume per unit change in the transpulmonary pressure (Papandrinopoulou *et al*., 2012), and tissue elasticity. As shown in Fig. 1B, increased lung compliance as a result of reduced lung tissue elasticity, a typical pulmonary dysfunction found in patients with emphysema, was observed in PPE-instilled mice compared with the control group, and treatment with LJ-2698 significantly alleviated PPE-induced pulmonary dysfunction in mice. Microscopic observation of H&E-stained tissues clearly demonstrated that LJ-2698 markedly suppressed PPE-induced airspace enlargement (Fig. 1C). These results suggest that LJ-2698 significantly attenuates elastase-induced pulmonary emphysema. Importantly, during the animal experiment, the body weight of mice in the LJ-2698-treated group was not significantly different compared with that of the vehicle- or the PPE-treated groups (Fig. 1D). Therefore, although additional *in vivo* investigation is needed for a precise determination of the toxicity of LJ-2698, these findings indicate the minimal toxicity of LJ-2698.

The protease-antiprotease imbalance plays an important role in the development of emphysema (Sharafkhaneh *et al*., 2008). Because elastase further activates matrix metalloproteinases (Ferry *et al*., 1997), we examined the suppressive effect of LJ-2698 on elastase-induced gelatinase activity by *in situ* zymography using fluorochrome-conjugated DQ-gelatin. As expected, upregulation of matrix metalloproteinase activity was observed in the lungs of PPE-instilled mice, and the PPE-mediated elevation in matrix metalloproteinase activity in the lungs was markedly suppressed by treatment with LJ-2698 (Fig. 2A, 2B). In addition, the increase in the number of TUNEL-positive cells in the lungs as a consequence of PPE-mediated lung damage was also significantly downregulated in the lungs of LJ-2698-treated mice (Fig. 2C, 2D). These results confirmed the suppressive effect of LJ-2698 on the formation of pulmonary emphysema.

Anti-inflammatory action of LJ-2698

Macrophages play important roles in inflammation and tissue repair. Upon stimulation by various pathogenic foreign elements, macrophages produce a number of cytokines, chemokines, and growth factors, resulting in the induction of inflammatory responses (Abdulkhaleq *et al*., 2018). In addition

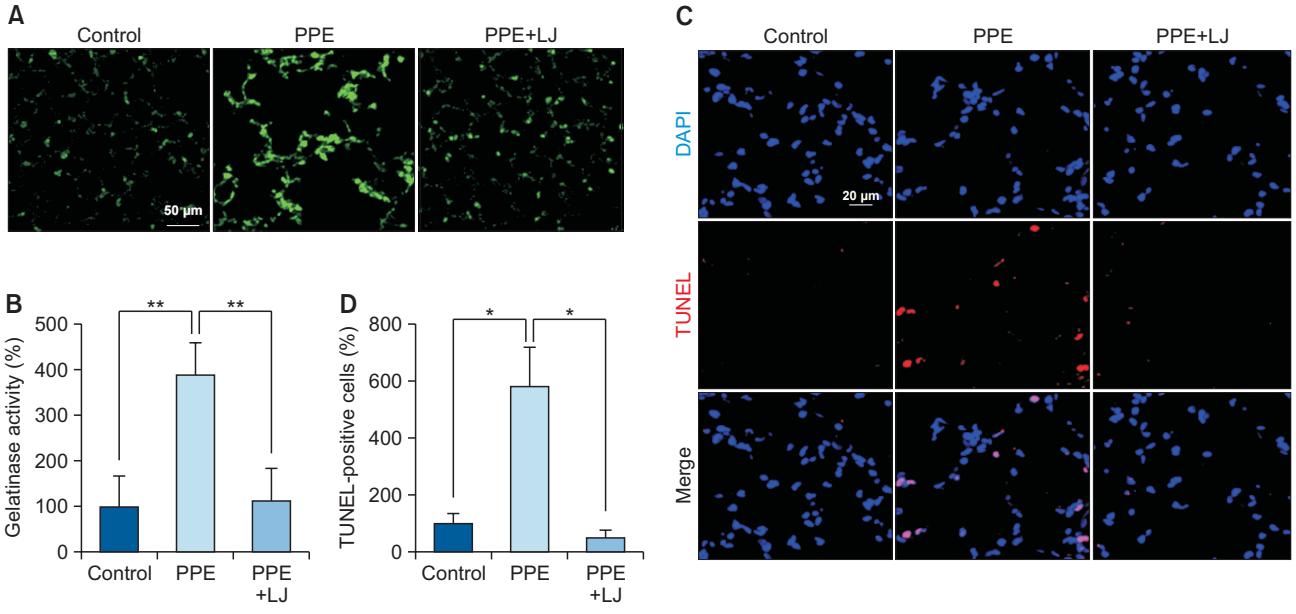

Fig. 2. Inhibitory effects of LJ-2698 on elastase-induced increases in matrix metalloproteinase activity and apoptosis in the lungs. (A) Attenuation of elastase-mediated matrix metalloproteinase (MMP) activity by treatment with LJ-2698. MMP activity was determined by using fluorescein-conjugated DQ-gelatin. (B) Quantification of gelatinase activity in each treatment group versus vehicle-treated controls. (C) Inhibition of elastase-mediated apoptosis in the lungs by treatment with LJ-2698 as determined by TUNEL staining of lung tissue cryosections. (D) Quantification of TUNEL-positive cells in each treatment group versus vehicle-treated controls. The bars represent the mean ± SD; **p*<0.05 and ***p*<0.01 versus the PPE-treated group. PPE, porcine pancreatic elastase; LJ, LJ-2698.

to inducing inflammation and acute lung injury in response to causative factors, macrophages also mediate tissue damage repair following polarization into M2 macrophages and the secretion of several anti-inflammatory cytokines such as IL4 and IL10 (Krzyszczyk *et al*., 2018). Because LJ-2698 markedly suppressed elastase-induced formation of emphysematous lesions in the lungs, we next investigated whether LJ-2698 can modulate the expression of several cytokines associated with the inhibition of inflammation and the resolution of lung injury by M2 macrophages. We first examined the effect of LJ-2698 *in vitro* using RAW 264.7 murine macrophage cells. Previous reports demonstrated secretion of elastase by activated macrophages (Werb and Gordon, 1975), activation of macrophages by stimulation with lipopolysaccharide (LPS) (Meng and Lowell, 1997), and induction of elastase production in macrophages by LPS stimulation (Duc Dodon and Vogel, 1985). Therefore, for consistency with the *in vivo* experimental conditions, we examined the effect of LJ-2698 on the regulation of the expression of anti-inflammatory cytokines in LPS-stimulated macrophages. Stimulation of macrophages with LPS (100 ng/mL) for 24 h reduced the expression of anti-inflammatory cytokines such as *Il4* and *Il10* in mouse macrophages, whereas treatment with LJ-2698 (0.1 μM) significantly ameliorated LPS-mediated downregulation of these anti-inflammatory cytokines (Fig. 3A). The mRNA expression of M2 macrophage-associated markers, including *Arg1* [encoding arginase 1, an M2 macrophage-specific marker (Yang and Ming, 2014)] and *Mrc1* [encoding mannose receptor C-type 1 (also known as CD206), a receptor highly expressed in M2 macrophages (Zhang *et al*., 2017)], was also significantly downregulated in LPS-stimulated macrophages, which was significantly restored by treatment with LJ-2698 (Fig. 3B). Moreover, consistent with the *in vivo* results, LJ-2698 had minimal effects on the viability of three normal cell lines derived from alveolar epithelium (MLE-12), RPE, and the hippocampus (HT-22); the viability was over 50% even at the highest concentration of LJ-2698 (1 μM), a 10-fold higher concentration than that regulating the expression of several anti-inflammatory cytokines in mouse macrophages (Fig. 3C), indicating minimal toxic effects of LJ-2698 *in vitro*.

We also determined the effect of LJ-2698 on the expression of these anti-inflammatory factors in murine lungs *in vivo*. Consistent with the *in vitro* findings, downregulation of the transcription of *Il4*, *Il10*, *Arg1*, and *Mrc1* by elastase treatment was significantly restored in the lungs in the LJ-2698-treated mice (Fig. 4A). Immunofluorescence (IF) analysis confirmed that the population of M2 macrophages in the lungs was also significantly elevated by the administration of LJ-2698 (Fig. 4B, 4C). Upregulation of the recruitment of CD206-positive M2 macrophages in the lungs from elastase-instilled mice appeared to be an autonomous resolution mechanism in the host in response to elastase-induced extensive lung injuries. In addition, the enhanced recruitment of M2 macrophages by administration of LJ-2698 compared with elastase treatment may contribute to the inhibitory effect of LJ-2698 on elastase-induced pulmonary emphysema. Collectively, these results indicate that LJ-2698 exerts anti-inflammatory action by regulating cytokine expression and inducing the recruitment of M2 macrophages; these anti-inflammatory effects of LJ-2698 may be associated with the inhibitory effect of LJ-2698 on elastase-induced pulmonary emphysema. In addition, the minimal effects of LJ-2698 on the viability of various normal cells and changes in body weight of mice also indicate no overt toxicity of LJ-2698 *in vitro* or *in vivo*.

DISCUSSION

Although the socioeconomic burden caused by COPD/emphysema has gradually increased, the pathophysiologic

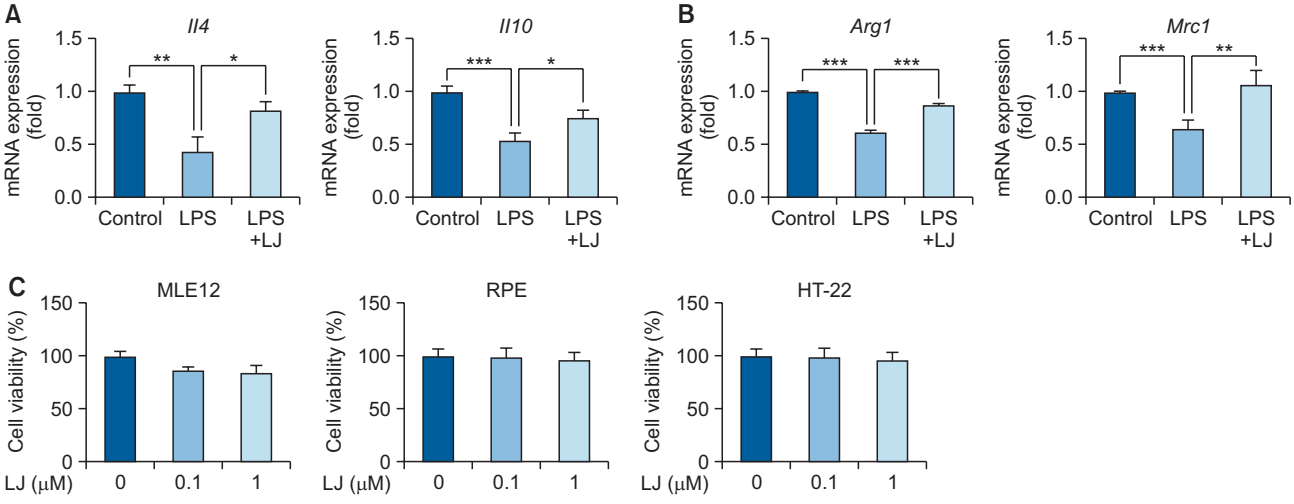

Fig. 3. Amelioration of lipopolysaccharide-mediated decreases in the expression of anti-inflammatory cytokines and M2 macrophage-related markers in murine macrophages by treatment with LJ-2698. (A, B) RAW 264.7 cells were pretreated with vehicle (DMSO) or LJ-2698 (0.1 μM) for 48 h; cells were then stimulated with LPS (100 ng/mL) in the presence of LJ-2698 for 24 h. The effects of LJ-2698 on the mRNA expression of anti-inflammatory cytokines and M2 macrophage-associated markers were determined by real-time PCR, as described in the Materials and methods. (C) No significant cytotoxic effects of LJ-2698 in normal cell lines from various organs. MLE-12, RPE, and HT-22 cells were treated with vehicle (DMSO) or LJ-2698 (0.1 and 1 μM) for 3 days. Cell viability was determined by the MTT assay. The bars represent the mean ± SD; *p<0.05, **p<0.01, and ***p<0.001 compared with the LPS-treated group. LPS, lipopolysaccharide; LJ, LJ-2698.

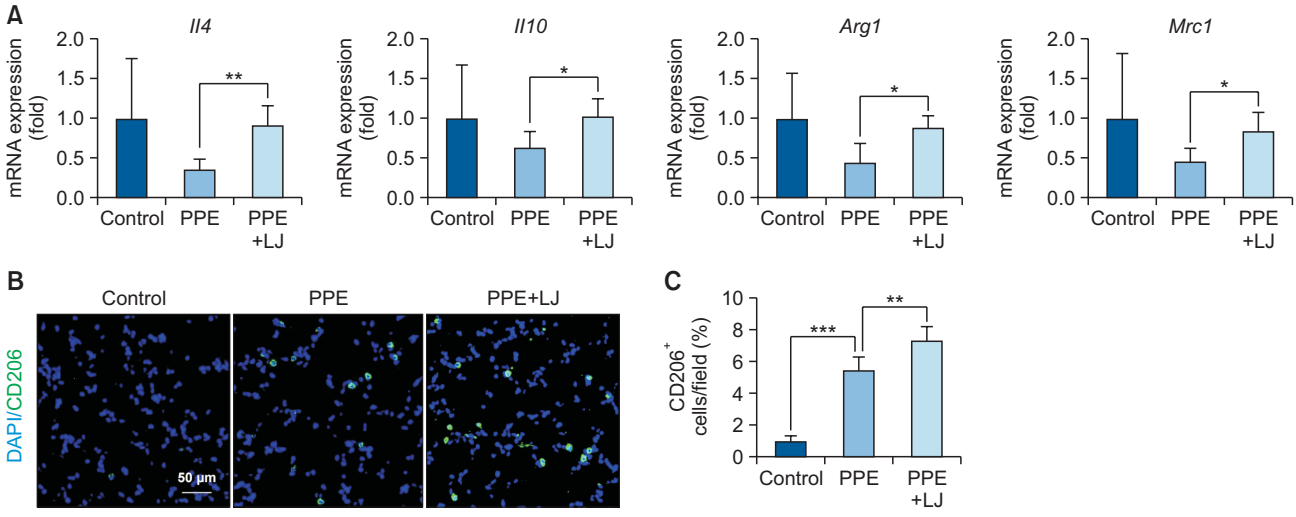

Fig. 4. Alleviation of elastase-induced decreases in the expression of anti-inflammatory cytokines and M2 macrophage-related markers in lungs from LJ-2698-treated mice. (A) The transcription of several anti-inflammatory cytokines and M2 macrophage-related markers in the lungs from each treatment group was determined by real-time PCR. (B) The recruitment of M2 macrophages (CD206-positive cells) was determined by immunofluorescence analysis. (C) Quantification of the recruitment of CD206-positive cells in each treatment group versus vehicle-treated controls. The bars represent the mean ± SD; **p*<0.05, ***p*<0.01, and ****p*<0.001 versus the PPE-treated group. PPE, porcine pancreatic elastase; LJ, LJ-2698.

mechanism of emphysema development is largely unknown, and only symptomatic regimens have been used for the treatment of emphysema. Therefore, the need for the development of efficacious therapeutic or preventive agents is unmet in the management of emphysema. In the present study, we investigated the inhibitory effect of the A_3AR antagonist LJ-2698 on elastase-induced pulmonary emphysema. LJ-2698 significantly suppressed elastase-induced structural and functional deregulation in the lungs by blocking elastase-induced matrix metalloproteinase activation and apoptosis in the lungs and inducing M2 macrophage-mediated anti-inflammatory action. Moreover, LJ-2698 displayed minimal toxic effects *in vitro* and *in vivo*. These results suggest the potential of LJ-2698 as a novel therapeutic/preventive agent for pulmonary emphysema.

By utilizing the elastase-induced murine emphysema model, we demonstrated the inhibitory effect of the A_3AR antagonist LJ-2698 on the formation of emphysematous lesions in the lungs *in vivo* and identified LJ-2698 as a potential antiemphysema agent. Consistent with previous studies suggesting elastase-induced lung tissue damage caused by degradation of the extracellular matrix, mucins, surfactant proteins, and host defensive cytokines and the generation of bradykinin (von Bredow *et al*., 2003; Henke *et al*., 2011; Sahoo *et al*., 2014), instillation of elastase markedly degraded the airspace and induced apoptosis in the lungs. Our results showed that LJ-2698 significantly suppressed elastase-induced apoptosis of pulmonary epithelial cells and lung tissue damage. Previous studies have demonstrated that the elastase-induced apoptosis of pulmonary epithelial cells was mediated by activation of protease-activated receptor-1 (PAR-1) (Suzuki *et al*., 2005), induction of alterations in mitochondrial membrane permeability (Ginzberg *et al*., 2004), and placenta growth factor (PlGF)-mediated activation of c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38 MAPK), and protein kinase C delta (PKCδ) signaling pathways (Hou *et al*., 2013, 2014). Modulation of these signaling pathways might be associated with the mechanism underlying the LJ-2698-mediated inhibitory effect on elastase-induced apoptosis in the lungs.

We also show that LJ-2698 exerts an anti-inflammatory effect by inducing the expression of anti-inflammatory cytokines and the recruitment of M2 macrophages in the lungs. The anti-inflammatory effect of LJ-2698 was also revealed in murine macrophages. Since chronic inflammation is a major pathogenic cause of the development of pulmonary emphysema, targeting lung inflammation is considered a therapeutic approach for emphysema (Yao *et al*., 2008; Cazzola *et al*., 2012). In support of this notion, the orally available anti-inflammatory phosphodiesterase 4 (PDE4) inhibitor roflumilast has been utilized for the treatment of COPD in the clinic (Wedzicha *et al*., 2016). Therefore, the anti-inflammatory action of LJ-2698 appears to be closely associated with its inhibitory effect on pulmonary emphysema. Moreover, the anti-inflammatory effect of LJ-2698 is consistent with a recent finding of an anti-inflammatory effect following treatment with an A_3AR antagonist (Min *et al*., 2016). Because adenosine signaling via A_3AR plays a role in airway inflammation (Tilley *et al*., 2003), targeting A_3AR may be a reasonable approach to controlling pulmonary inflammation. However, in recent studies, A_3AR agonists exhibited anti-inflammatory effects in a bleomycin model of pulmonary inflammation and fibrosis (Morschl *et al*., 2008) and attenuated lung ischemia-reperfusion injury (Mulloy *et al*., 2013). Therefore, A_3AR is associated with both pro- and anti-inflammatory actions depending on the model system, and additional mechanisms might also be involved in the anti-inflammatory effect of A_3AR antagonism. One possible mechanism is the involvement of peroxisome proliferator-activated receptor (PPAR). Our recent study suggests that both A_3AR agonists and antagonists act as partial PPARγ agonists and PPARδ antagonists (Yu *et al*., 2017). Considering the involve-

ment of PPARγ in the anti-inflammatory action and differentiation of monocytes into an M2 macrophage phenotype (Bouhlel *et al*., 2007; Kapadia *et al*., 2008), the effect of LJ-2698 on the promotion of anti-inflammatory cytokine production might be through activation of PPARγ in addition to A_3AR blockade. In addition, elastase and other proteases activate protease-activated receptor-mediated signal transduction. Protease-activated receptors (PARs) are G protein-coupled receptors activated by proteolytical cleavage of the amino-terminus, acting as sensors for extracellular proteases (Rothmeier and Ruf, 2012). Among several PARs, PAR-2 is activated by elastase and associated with inflammation (Rothmeier and Ruf, 2012; Muley *et al*., 2016). Therefore, additional studies are necessary to investigate the involvement of the regulation of PPARγ and/or PAR-2 in the inhibitory effect of LJ-2698 on elastase-induced emphysema in the lungs.

Finally, consistent with a previous finding (Dorotea *et al*., 2018), we observed minimal toxicity of LJ-2698 in several normal cell lines derived from different organs and in mice. These results suggest the potential of LJ-2698 to be efficacious in attenuating pulmonary emphysema with limited toxicity. Importantly, these effects were achieved by oral administration of a very low dose (50 μg/kg) of LJ-2698, suggesting high potency of LJ-2698 in the suppression of emphysema. Emphysema is a chronic disease, and thus long-term therapy is required. Indeed, most agents that were effective in preclinical models of COPD/emphysema, such as anti-inflammatory agents, have not been approved for the treatment of emphysema/COPD in the clinic due to a lack of effectiveness, low potency, side effects, and/or toxicity (Wedzicha *et al*., 2016). Therefore, high potency, oral bioavailability and limited toxicity by LJ-2698 would strengthen its utility for the treatment or prevention of emphysema. Further investigation is required to elucidate the mechanism of action of LJ-2698 in the blockade of emphysema.

In summary, in the present study, we found that LJ-2698 suppressed elastase-induced pulmonary emphysema by inducing the production of anti-inflammatory cytokines and the recruitment of M2 macrophages. In addition, LJ-2698 displayed no overt toxicity in several normal cell lines and in mice. These results collectively suggest the potential of LJ-2698 as an efficacious therapeutic and/or preventive agent for pulmonary emphysema with limited toxicity. Further studies are warranted to investigate the detailed mechanism of action of LJ-2698 and its potential additional biological activities in advanced preclinical and clinical settings.

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REFERENCES

Abdulkhaleq, L. A., Assi, M. A., Abdullah, R., Zamri-Saad, M., Taufiq-Yap, Y. H. and Hezmee, M. N. M. (2018) The crucial roles of inflammatory mediators in inflammation: a review. *Vet. World* **11**, 627-635.

Barnes, P. J., Burney, P. G., Silverman, E. K., Celli, B. R., Vestbo, J., Wedzicha, J. A. and Wouters, E. F. (2015) Chronic obstructive pulmonary disease. *Nat. Rev. Dis. Primers* **1**, 15076.

Borea, P. A., Varani, K., Vincenzi, F., Baraldi, P. G., Tabrizi, M. A., Merighi, S. and Gessi, S. (2015) The A3 adenosine receptor: history and perspectives. *Pharmacol. Rev.* **67**, 74-102.

Bouhlel, M. A., Derudas, B., Rigamonti, E., Dievart, R., Brozek, J., Haulon, S., Zawadzki, C., Jude, B., Torpier, G., Marx, N., Staels, B. and Chinetti-Gbaguidi, G. (2007) PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell Metab.* **6**, 137-143.

Cazzola, M., Page, C. P., Calzetta, L. and Matera, M. G. (2012) Emerging anti-inflammatory strategies for COPD. *Eur. Respir. J.* **40**, 724-741.

Celli, B. R. (2018) Pharmacological therapy of COPD: reasons for optimism. *Chest* **154**, 1404-1415.

Chen, Z. H., Lam, H. C., Jin, Y., Kim, H. P., Cao, J., Lee, S. J., Ifedigbo, E., Parameswaran, H., Ryter, S. W. and Choi, A. M. K. (2010) Autophagy protein microtubule-associated protein 1 light chain-3B (LC3B) activates extrinsic apoptosis during cigarette smoke-induced emphysema. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 18880-18885.

Dorotea, D., Cho, A., Lee, G., Kwon, G., Lee, J., Sahu, P. K., Jeong, L. S., Cha, D. R. and Ha, H. (2018) Orally active, species-independent novel A3 adenosine receptor antagonist protects against kidney injury in db/db mice. *Exp. Mol. Med.* **50**, 38.

Duc Dodon, M. and Vogel, S. N. (1985) Analysis of effects of lipopolysaccharide and interferon on murine macrophages: modulation of elastase secretion *in vitro*. *Infect. Immun.* **49**, 709-714.

Dunnill, M. S. (1962) Quantitative methods in the study of pulmonary pathology. *Thorax* **17**, 320-328.

Ferry, G., Lonchampt, M., Pennel, L., de Nanteuil, G., Canet, E. and Tucker, G. C. (1997) Activation of MMP-9 by neutrophil elastase in an *in vivo* model of acute lung injury. *FEBS Lett.* **402**, 111-115.

Ginzberg, H. H., Shannon, P. T., Suzuki, T., Hong, O., Vachon, E., Moraes, T., Abreu, M. T., Cherepanov, V., Wang, X., Chow, C. W. and Downey, G. P. (2004) Leukocyte elastase induces epithelial apoptosis: role of mitochondial permeability changes and Akt. *Am. J. Physiol. Gastrointest. Liver Physiol.* **287**, G286-G298.

Gooptu, B., Ekeowa, U. I. and Lomas, D. A. (2009) Mechanisms of emphysema in alpha1-antitrypsin deficiency: molecular and cellular insights. *Eur. Respir. J.* **34**, 475-488.

Guarascio, A. J., Ray, S. M., Finch, C. K. and Self, T. H. (2013) The clinical and economic burden of chronic obstructive pulmonary disease in the USA. *Clinicoecon. Outcomes Res.* **5**, 235-245.

Henke, M. O., John, G., Rheineck, C., Chillappagari, S., Naehrlich, L. and Rubin, B. K. (2011) Serine proteases degrade airway mucins in cystic fibrosis. *Infect. Immun.* **79**, 3438-3444.

Horio, Y., Ichiyasu, H., Kojima, K., Saita, N., Migiyama, Y., Iriki, T., Fujii, K., Niki, T., Hirashima, M. and Kohrogi, H. (2017) Protective effect of Galectin-9 in murine model of lung emphysema: involvement of neutrophil migration and MMP-9 production. *PLoS ONE* **12**, e0180742.

Hou, H. H., Cheng, S. L., Chung, K. P., Wei, S. C., Tsao, P. N., Lu, H. H., Wang, H. C. and Yu, C. J. (2014) PlGF mediates neutrophil elastase-induced airway epithelial cell apoptosis and emphysema. *Respir. Res.* **15**, 106.

Hou, H. H., Cheng, S. L., Liu, H. T., Yang, F. Z., Wang, H. C. and Yu, C. J. (2013) Elastase induced lung epithelial cell apoptosis and emphysema through placenta growth factor. *Cell Death Dis.* **4**, e793.

Jeong, L. S., Choe, S. A., Gunaga, P., Kim, H. O., Lee, H. W., Lee, S. K., Tosh, D. K., Patel, A., Palaniappan, K. K., Gao, Z. G., Jacobson, K. A. and Moon, H. R. (2007) Discovery of a new nucleoside template for human A3 adenosine receptor ligands: D-4'-thioadenosine derivatives without 4'-hydroxymethyl group as highly potent and selective antagonists. *J. Med. Chem.* **50**, 3159-3162.

Kapadia, R., Yi, J. H. and Vemuganti, R. (2008) Mechanisms of anti-inflammatory and neuroprotective actions of PPAR-gamma agonists. *Front. Biosci.* **13**, 1813-1826.

Krzyszczyk, P., Schloss, R., Palmer, A. and Berthiaume, F. (2018) The Role of macrophages in acute and chronic wound healing and interventions to promote pro-wound healing phenotypes. *Front.*

Physiol. **9**, 419.
Lee, S. H., Goswami, S., Grudo, A., Song, L. Z., Bandi, V., Goodnight-White, S., Green, L., Hacken-Bitar, J., Huh, J., Bakaeen, F., Coxson, H. O., Cogswell, S., Storness-Bliss, C., Corry, D. B. and Kheradmand, F. (2007) Antielastin autoimmunity in tobacco smoking-induced emphysema. *Nat. Med.* **13**, 567-569.
Livak, K. J. and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**, 402-408.
Lozano, R., Naghavi, M., Foreman, K., Lim, S., Shibuya, K., Aboyans, V., Abraham, J., Adair, T., Aggarwal, R., Ahn, S. Y., Alvarado, M., Anderson, H. R., Anderson, L. M., Andrews, K. G., Atkinson, C., Baddour, L. M., Barker-Collo, S., Bartels, D. H., Bell, M. L., Benjamin, E. J., Bennett, D., Bhalla, K., Bikbov, B., Bin Abdulhak, A., Birbeck, G., Blyth, F., Bolliger, I., Boufous, S., Bucello, C., Burch, M., Burney, P., Carapetis, J., Chen, H., Chou, D., Chugh, S. S., Coffeng, L. E., Colan, S. D., Colquhoun, S., Colson, K. E., Condon, J., Connor, M. D., Cooper, L. T., Corriere, M., Cortinovis, M., de Vaccaro, K. C., Couser, W., Cowie, B. C., Criqui, M. H., Cross, M., Dabhadkar, K. C., Dahodwala, N., De Leo, D., Degenhardt, L., Delossantos, A., Denenberg, J., Des Jarlais, D. C., Dharmaratne, S. D., Dorsey, E. R., Driscoll, T., Duber, H., Ebel, B., Erwin, P. J., Espindola, P., Ezzati, M., Feigin, V., Flaxman, A. D., Forouzanfar, M. H., Fowkes, F. G., Franklin, R., Fransen, M., Freeman, M. K., Gabriel, S. E., Gakidou, E., Gaspari, F., Gillum, R. F., Gonzalez-Medina, D., Halasa, Y. A., Haring, D., Harrison, J. E., Havmoeller, R., Hay, R. J., Hoen, B., Hotez, P. J., Hoy, D., Jacobsen, K. H., James, S. L., Jasrasaria, R., Jayaraman, S., Johns, N., Karthikeyan, G., Kassebaum, N., Keren, A., Khoo, J. P., Knowlton, L. M., Kobusingye, O., Koranteng, A., Krishnamurthi, R., Lipnick, M., Lipshultz, S. E., Ohno, S. L., Mabweijano, J., MacIntyre, M. F., Mallinger, L., March, L., Marks, G. B., Marks, R., Matsumori, A., Matzopoulos, R., Mayosi, B. M., McAnulty, J. H., McDermott, M. M., McGrath, J., Mensah, G. A., Merriman, T. R., Michaud, C., Miller, M., Miller, T. R., Mock, C., Mocumbi, A. O., Mokdad, A. A., Moran, A., Mulholland, K., Nair, M. N., Naldi, L., Narayan, K. M., Nasseri, K., Norman, P., O'Donnell, M., Omer, S. B., Ortblad, K., Osborne, R., Ozgediz, D., Pahari, B., Pandian, J. D., Rivero, A. P., Padilla, R. P., Perez-Ruiz, F., Perico, N., Phillips, D., Pierce, K., Pope, C. A., 3rd, Porrini, E., Pourmalek, F., Raju, M., Ranganathan, D., Rehm, J. T., Rein, D. B., Remuzzi, G., Rivara, F. P., Roberts, T., De Leon, F. R., Rosenfeld, L. C., Rushton, L., Sacco, R. L., Salomon, J. A., Sampson, U., Sanman, E., Schwebel, D. C., Segui-Gomez, M., Shepard, D. S., Singh, D., Singleton, J., Sliwa, K., Smith, E., Steer, A., Taylor, J. A., Thomas, B., Tleyjeh, I. M., Towbin, J. A., Truelsen, T., Undurraga, E. A., Venketasubramanian, N., Vijayakumar, L., Vos, T., Wagner, G. R., Wang, M., Wang, W., Watt, K., Weinstock, M. A., Weintraub, R., Wilkinson, J. D., Woolf, A. D., Wulf, S., Yeh, P. H., Yip, P., Zabetian, A., Zheng, Z. J., Lopez, A. D., Murray, C. J., AlMazroa, M. A. and Memish, Z. A. (2012) Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* **380**, 2095-2128.
Mahadeva, R. and Shapiro, S. D. (2002) Chronic obstructive pulmonary disease • 3: experimental animal models of pulmonary emphysema. *Thorax* **57**, 908-914.
Meng, F. and Lowell, C. A. (1997) Lipopolysaccharide (LPS)-induced macrophage activation and signal transduction in the absence of Src-family kinases Hck, Fgr, and Lyn. *J. Exp. Med.* **185**, 1661-1670.
Min, H. S., Cha, J. J., Kim, K., Kim, J. E., Ghee, J. Y., Kim, H., Lee, J. E., Han, J. Y., Jeong, L. S., Cha, D. R. and Kang, Y. S. (2016) Renoprotective effects of a highly selective A3 adenosine receptor antagonist in a mouse model of adriamycin-induced nephropathy. *J. Korean Med. Sci.* **31**, 1403-1412.
Morschl, E., Molina, J. G., Volmer, J. B., Mohsenin, A., Pero, R. S., Hong, J. S., Kheradmand, F., Lee, J. J. and Blackburn, M. R. (2008) A3 adenosine receptor signaling influences pulmonary inflammation and fibrosis. *Am. J. Respir. Cell Mol. Biol.* **39**, 697-705.
Muley, M. M., Reid, A. R., Botz, B., Bolcskei, K., Helyes, Z. and McDougall, J. J. (2016) Neutrophil elastase induces inflammation and pain in mouse knee joints via activation of proteinase-activated receptor-2. *Br. J. Pharmacol.* **173**, 766-777.
Mulloy, D. P., Sharma, A. K., Fernandez, L. G., Zhao, Y., Lau, C. L., Kron, I. L. and Laubach, V. E. (2013) Adenosine A3 receptor activation attenuates lung ischemia-reperfusion injury. *Ann. Thorac. Surg.* **95**, 1762-1767.
Papandrinopoulou, D., Tzouda, V. and Tsoukalas, G. (2012) Lung compliance and chronic obstructive pulmonary disease. *Pulm. Med.* **2012**, 542769.
Polosa, R. and Blackburn, M. R. (2009) Adenosine receptors as targets for therapeutic intervention in asthma and chronic obstructive pulmonary disease. *Trends Pharmacol. Sci.* **30**, 528-535.
Rothmeier, A. S. and Ruf, W. (2012) Protease-activated receptor 2 signaling in inflammation. *Semin. Immunopathol.* **34**, 133-149.
Sahoo, M., Del Barrio, L., Miller, M. A. and Re, F. (2014) Neutrophil elastase causes tissue damage that decreases host tolerance to lung infection with burkholderia species. *PLoS Pathog.* **10**, e1004327.
Sharafkhaneh, A., Hanania, N. A. and Kim, V. (2008) Pathogenesis of emphysema: from the bench to the bedside. *Proc. Am. Thorac. Soc.* **5**, 475-477.
Suzuki, T., Moraes, T. J., Vachon, E., Ginzberg, H. H., Huang, T. T., Matthay, M. A., Hollenberg, M. D., Marshall, J., McCulloch, C. A., Abreu, M. T., Chow, C. W. and Downey, G. P. (2005) Proteinase-activated receptor-1 mediates elastase-induced apoptosis of human lung epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **33**, 231-247.
Tachkov, K., Kamusheva, M., Pencheva, V. and Mitov, K. (2017) Evaluation of the economic and social burden of chronic obstructive pulmonary disease (COPD). *Biotechnol. Biotechnol. Equip.* **31**, 855-861.
Tilley, S. L., Tsai, M., Williams, C. M., Wang, Z.-S., Erikson, C. J., Galli, S. J. and Koller, B. H. (2003) Identification of A3 receptor- and mast cell-dependent and -independent components of adenosine-mediated airway responsiveness in mice. *J. Immunol.* **171**, 331-337.
Van Tho, N., Ogawa, E., Trang le, T. H., Ryujin, Y., Kanda, R., Nakagawa, H., Goto, K., Fukunaga, K., Higami, Y., Seto, R., Wada, H., Yamaguchi, M., Nagao, T., Lan le, T. T. and Nakano, Y. (2015) A mixed phenotype of airway wall thickening and emphysema is associated with dyspnea and hospitalization for chronic obstructive pulmonary disease. *Ann. Am. Thorac. Soc.* **12**, 988-996.
Vanoirbeek, J. A., Rinaldi, M., De Vooght, V., Haenen, S., Bobic, S., Gayan-Ramirez, G., Hoet, P. H., Verbeken, E., Decramer, M., Nemery, B. and Janssens, W. (2010) Noninvasive and invasive pulmonary function in mouse models of obstructive and restrictive respiratory diseases. *Am. J. Respir. Cell Mol. Biol.* **42**, 96-104.
Varani, K., Caramori, G., Vincenzi, F., Adcock, I., Casolari, P., Leung, E., Maclennan, S., Gessi, S., Morello, S., Barnes, P. J., Ito, K., Chung, K. F., Cavallesco, G., Azzena, G., Papi, A. and Borea, P. A. (2006) Alteration of adenosine receptors in patients with chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* **173**, 398-406.
von Bredow, C., Wiesener, A. and Griese, M. (2003) Proteolysis of surfactant protein D by cystic fibrosis relevant proteases. *Lung* **181**, 79-88.
Wedzicha, J. A., Calverley, P. M. and Rabe, K. F. (2016) Roflumilast: a review of its use in the treatment of COPD. *Int. J. Chron. Obstruct. Pulmon. Dis.* **11**, 81-90.
Werb, Z. and Gordon, S. (1975) Elastase secretion by stimulated macrophages. Characterization and regulation. *J. Exp. Med.* **142**, 361-377.
Yang, Z. and Ming, X. F. (2014) Functions of arginase isoforms in macrophage inflammatory responses: impact on cardiovascular diseases and metabolic disorders. *Front. Immunol.* **5**, 533.
Yao, H., de Boer, W. I. and Rahman, I. (2008) Targeting lung inflammation: novel therapies for the treatment of COPD. *Curr. Respir. Med. Rev.* **4**, 57-68.
Yu, J., Ahn, S., Kim, H. J., Lee, M., Ahn, S., Kim, J., Jin, S. H., Lee, E., Kim, G., Cheong, J. H., Jacobson, K. A., Jeong, L. S. and Noh, M. (2017) Polypharmacology of N6-(3-iodobenzyl)adenosine-5′-N-methyluronamide (IB-MECA) and related A3 adenosine receptor ligands: peroxisome proliferator activated receptor (PPAR) γ partial agonist and PPARδ antagonist activity suggests their antidiabetic potential. *J. Med. Chem.* **60**, 7459-7475.
Zhang, C., Yu, X., Gao, L., Zhao, Y., Lai, J., Lu, D., Bao, R., Jia, B., Zhong, L., Wang, F. and Liu, Z. (2017) Noninvasive imaging of

CD206-positive M2 macrophages as an early biomarker for post-chemotherapy tumor relapse and lymph node metastasis. *Theranostics* **7**, 4276-4288.

Zhou, Y., Schneider, D. J. and Blackburn, M. R. (2009) Adenosine signaling and the regulation of chronic lung disease. *Pharmacol. Ther.* **123**, 105-116.