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RESEARCH ARTICLE

LJ-529, a partial peroxisome proliferator-activated receptor gamma (PPARγ) agonist and adenosine A_3 receptor agonist, ameliorates elastase-induced pulmonary emphysema in mice

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Abstract Chronic obstructive pulmonary disease (COPD) is the leading cause of human death worldwide. Currently available therapies for COPD mainly relieve symptoms and preserve lung function, suggesting the need to develop novel therapeutic or preventive regimens. Because chronic inflammation is a mechanism of emphysematous lesion formation and because adenosine A_3 receptor signaling and peroxisome proliferator-activated receptor gamma (PPARγ) regulate inflammation, we investigated the effect of LJ-529, a selective adenosine A_3 receptor agonist and partial PPARγ agonist, on inflammation in vitro and elastase-induced pulmonary emphysema in vivo. LJ-529 markedly ameliorated elastase-induced emphysematous lesion formation in the lungs in vivo, as indicated by the restoration of pulmonary function, suppression of airspace enlargement, and downregulation of elastase-induced matrix metalloproteinase activity and apoptotic cell death in the lungs. LJ-529 induced the expression of PPARγ target genes, the activity of PPARγ and several cytokines involved in inhibiting inflammation and inducing anti-inflammatory M2-like phenotypes. Moreover, LJ-529 did not exhibit significant cytotoxicity in normal cell lines derived from various organs in vitro and induced minimal changes in body weight in vivo, suggesting no overt toxicity of LJ-529 in vitro or in vivo. These results indicate the potential of LJ-529 as a novel therapeutic/preventive agent for emphysema with limited toxicity.

Keywords LJ-529 · Emphysema · Elastase · Peroxisome proliferator-activated receptor gamma

Introduction

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of human death worldwide, and the socioeconomic burden of COPD is expected to gradually increase (Lozano et al. 2012; Guarascio et al. 2013; Barnes et al. 2015; Tachkov et al. 2017). COPD is characterized by chronic destructive changes in the lungs that cause gas exchange disturbance, and chronic bronchitis and emphysema are the main symptoms of COPD (Barnes et al. 2015; Van Tho et al. 2015). In patients with emphysema, the alveolar walls are irreversibly destroyed, and the airspace is abnormally enlarged (Horio et al. 2017). Several factors, such as α1-antitrypsin deficiency, oxidative stress, aging, and chronic inflammation, are involved in the pathogenesis of emphysema (Gooptu et al. 2009; Barnes et al. 2015). Several exogenous insults, including smoking and air pollutants, activate macrophages and neutrophils and produce reactive oxygen species and various proteases, leading to tissue damage (Sharafkhaneh et al. 2008; Barnes et al. 2015). Elastin fragments produced by matrix degradation cause chemoattraction and autoimmunity, leading to the further enhancement of pulmonary inflammation (Lee et al. 2007; Sharafkhaneh et al. 2008). Inhaled bronchodilators, anticholinergics, β2-adrenergic agonists, corticosteroids, antibiotics and antioxidants have been used for to relieve symptoms, including respiratory difficulty, and preserve lung function for the treatment of emphysema/COPD (Celli 2018). Although these regimens can alleviate disease progression and cause clinically significant benefits for patients with COPD (Celli 2018), it is difficult to completely cure

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COPD/emphysema using these agents in most cases. Therefore, novel therapeutics for the treatment or prevention of emphysema are urgently needed.

Previous reports have suggested the association of adenosine signaling with the regulation of inflammation and pulmonary homeostasis and its implication in chronic pulmonary disorders such as asthma and COPD (Zhou et al. 2009; Haskó and Cronstein 2013). Adenosine binds to 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 associated with the regulation of ischemia, inflammation, and tumor growth and is considered a target for the development of therapeutic agents (Borea et al. 2015). We have developed potent and selective agonists and antagonists of A_3AR (Jeong et al. 2003, 2006, 2008).

Previous studies have demonstrated the role of peroxisome proliferator-activated receptor (PPAR) signaling in the regulation of inflammation (Daynes and Jones 2002). Among the isoforms of PPAR (PPARα, PPARβ/δ, and PPARγ), PPARγ plays an important role in the regulation of gene expression linked to several pathologic conditions (Tontonoz and Spiegelman 2008). PPARγ agonists exert anti-inflammatory actions by suppressing the production of pro-inflammatory cytokines in macrophages (Tontonoz and Spiegelman 2008) and inducing the polarization of monocytes to the M2 phenotype (Bouhlel et al. 2007) and differentiation into regulatory T cells (Housley et al. 2009). The modulation of the transcriptional activity of nuclear factor-kappa B (NF-κB) is regarded as the mechanism underlying the anti-inflammatory role of PPARγ (Kapadia et al. 2008; Remels et al. 2009). Notably, agonistic PPARγ activation ameliorates pulmonary emphysema in experimental models by suppressing inflammation, inhibiting pathogenic lung dendritic cells and chemokine expression, inducing the polarization of macrophages to the M2 phenotype, and clearing apoptotic neutrophils (Lea et al. 2014; Shan et al. 2014; Solleti et al. 2015). Therefore, the activation of PPARγ may be an effective strategy for controlling COPD/emphysema. To discover novel potential compounds that suppress the development of emphysema, we employed a large chemical library consisting of compounds from various classes. According to our previous report demonstrating the identification of LJ-529 [2-chloro-N^6-(3-iodobenzyl)-5′-*N*-methylcarbomoyl-4′-thioadenosine] as a partial PPARγ agonist (Yu et al. 2017), here we investigated the inhibitory effect of LJ-529 on elastase-induced pulmonary emphysema. Our findings showed that LJ-529 significantly suppressed the elastase-induced deregulation of pulmonary function and lung destruction, matrix metalloproteinase activity, and apoptosis in the lungs. LJ-529 induced the expression of several PPARγ target genes and several cytokines involved in inhibiting inflammation and inducing anti-inflammatory M2-like phenotypes in macrophages in the murine lungs. Moreover, LJ-529 exhibited minimal cytotoxicity in several normal cell lines derived from various organs in vitro and induced no obvious toxic effects in vivo. These findings highlight the potential of LJ-529 as a novel agent for the treatment of patients with emphysema.

Materials and methods

Reagents

LJ-529 was synthesized as described previously (Jeong et al. 2003). 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.

Cell culture

Human retinal pigment epithelial (RPE) cells were kindly provided by Dr. Jeong Hun Kim (Seoul National University, Seoul, Republic of Korea). The murine hippocampal neuronal cell line HT-22 was kindly provided by Dr. Dong Gyu Jo (Sungkyunkwan University, Suwon, Republic of Korea). Human bronchial epithelial (HBE) cells were kindly provided by Dr. John Minna (University of Texas Southwestern Medical Center, Dallas, TX, USA). The murine macrophage cell line RAW 264.7 was kindly provided by Dr. Sang Kook Lee (Seoul National University, Seoul, Republic of Korea). RPE, HT-22, and RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS and antibiotics (all from Welgene, Inc., Gyeongssan-si, Republic of Korea). HBE cells were cultured in keratinocyte-SFM (Invitrogen, Grand Island, NY, USA) supplemented with 5 ng/ml recombinant epidermal growth factor (EGF), 50 µg/ml bovine pituitary extract, and antibiotics. The 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 LJ-529 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 percentage of the control group.

Real-time PCR

Total RNA was isolated from frozen lung tissues using a phenol–chloroform extraction method, reverse-transcribed using a first-strand cDNA synthesis kit (TransGen Biotech, Beijing, China), and analyzed by real-time PCR using a

SYBR Green-based qPCR master mix solution (Enzynomics, Daejeon, Republic of Korea) and gene-specific primers. The primer sequences used for the PCR analyses are listed in Table 1. 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 20 s; and melting curve analysis to determine reaction specificity. The quantification or 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 provided standard mouse chow and water ad libitum and housed in temperature- and humidity-controlled facilities under 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-529 (50 μg/kg) by oral gavage 6 times per week for 6 weeks. One week after drug treatment, 0.25 units of PPE was intratracheally instilled into the lungs of the mice. Body weight changes were monitored during the treatment. Changes in pulmonary function in vehicle- and LJ-529-treated mice were analyzed using the FlexiVent system (Scireq, EMKA Technologies, Montreal, Canada) (Vanoirbeek et al. 2010) according to the protocol recommended by the manufacturer. The mice were euthanized by inhalation of an overdose of isoflurane, and the lungs were excised after perfusion with ice-cold PBS and then embedded in OCT compound (Sakura Finetek, USA, Inc., Torrance, CA, USA). Frozen blocks were used for further analyses. H&E-stained tissues were used to determine structural changes in the lungs and histological changes in the kidneys, liver, and brain. The quantification of structural changes was determined 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 (Thermo Fisher Scientific, Carlsbad, CA, USA) diluted in low-gelling temperature agarose for 3 h at room temperature. Fluorescence was observed under a fluorescence microscope, and the sections were photographed.

TUNEL staining

TUNEL staining was performed using the TMR Red In Situ Cell Death Detection Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's provided protocol.

Immunofluorescence staining

Cryosections (8 μm) were prepared for immunofluorescent 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. The slides were incubated with anti-CD206 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) or anti-Arginase 1 antibody (Cell Signaling Technology, Danvers, MA, USA) at 4 °C overnight. After washing, sections were incubated with alexa flour-conjugated secondary antibody for 1 h at room temperature. The nuclei were stained with DAPI and analyzed by confocal microscope (LSM 700; Carl Zeiss Microscopy, Jena, Germany).

Luciferase reporter gene assays

Raw264.7 cells were seeded in a 24-well plate and transiently transfected with PPARγ, PPRE-luciferase and the

Table 1 Primer sequences used in this study

Gene	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
Il4	CCT CAC AGC AAC GAA GAA CA	ATC GAA AAG CCC GAA AGA GT
Il10	TAA GGC TGG CCA CAC TTG AG	GTT TTC AGG GAT GAA GCG GC
Tgfb1	TGA CGT CAC TGG AGT TGT ACG G	GGT TCA TGT CAT GGA TGG TGC
Mrc1	TGA TTA CGA GCA GTG GAA GC	GTT CAC CGT AAG CCC AAT TT
Arg1	GAA CAC GGC AGT GGC TTT AAC	TGC TTA GCT CTG TCT GCT TTG C
Cd36	TGG AGG CAT TCT CAT GCC AG	CTG TAC ACA GTG GTG CCT GT
Adrp	CAG CCA ACG TCC GAG ATT G	CAC ATC CTT CGC CCC AGT T
Abcg1	TTC ATC GTC CTG GGC ATC TT	CGG ATT TTG TAT CTG AGG ACG AA
Lpl	CCA ATG GAG GCA CTT TCC AG	CCA CGT CTC CGA GTC CTC TC
Rn18s	GTA ACC CGT TGA ACC CCA TT	CCA TCC AAT CGG TAG TAG CG
Actb	TGT CCA CCT TCC AGC AGA TGT	AGC TCA GTA ACA GTC CGC CTA G

CH110-Lac-Z plasmid vector using jetPRIME transfection reagent (Polyplus Transfection, Illkirch, France). Luciferase reporter gene assays were performed using the p.j.k Luciferase Assay Kit (PJK GmbH, Kleinbittersdorf, Germany). The PPARγ and PPRE-luciferase reporter plasmid vector were kindly provided by Professor Keon Wook Kang of Seoul National University (Seoul, Korea).

Migration assay

Migration assays were performed in a Boyden chamber (Corning, Inc., Corning, NY, USA) with 8-μm inserts coated with gelatin. MLE12 cells were seeded into the lower wells. After 24 h, Raw 264.7 cells were loaded into the upper wells. Cells were incubated for 20 h. After the incubation, the cells were fixed with 100% cold methanol and stained with hematoxylin solution. The number of stained cells was counted using a microscope.

Statistical analysis

The data are presented as the mean ± SD. Statistical significance of difference was determined using a two-tailed Student's *t*-test or one-way analysis of variance with Dunnett's multiple comparisons test using GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA). A *P* value less than 0.05 was considered statistically significant.

Results

Inhibitory effect of LJ-529 on elastase-induced pulmonary emphysema

Based on the inhibitory effect of PPARγ agonists on pulmonary emphysema (Lea et al. 2014; Shan et al. 2014), we examined the effect of LJ-529 on elastase-induced emphysema in mice (Mahadeva and Shapiro 2002). As shown in Fig. 1a, 1 week after LJ-529 treatment, porcine pancreatic elastase (PPE) was instilled into the murine lungs, and the mice were treated with LJ-529 for an additional 4 weeks. Because pulmonary dysfunction is a main characteristic of emphysema (Barnes et al. 2015), we examined whether treatment with LJ-529 prevents the PPE-mediated deregulation of lung function by determining the compliance and tissue elastance of the lungs (Papandrinopoulou et al. 2012). In PPE-instilled mice, a reduction in lung tissue elastance caused elevated lung compliance, and treatment with LJ-529 significantly restored the PPE-induced deregulation of lung function in mice (Fig. 1b). Consistently, microscopic analysis of the mean linear intercept (MLI) of H&E-stained lung tissues also showed that LJ-529 treatment significantly suppressed PPE-induced airspace enlargement (Fig. 1c). These results suggest that LJ-529 has the capacity to suppress elastase-induced pulmonary emphysema.

Inhibitory effect of LJ-529 on elastase-induced apoptotic death of alveolar epithelial cells

Based on the importance of the protease-antiprotease imbalance in the development of emphysema (Sharafkhaneh et al. 2008) and the activation of matrix metalloproteinases by elastase (Ferry et al. 1997), we next investigated the effects of LJ-529 on elastase-induced gelatinase activity. We observed a marked increase in gelatinase activity in the lungs of PPE-instilled mice, and treatment with LJ-529 significantly suppressed PPE-induced gelatinase activity (Fig. 2a). In addition, the PPE-induced increase in the number of TUNEL-positive cells in the lungs, an indicator of PPE-mediated lung damage, was also significantly attenuated by treatment with LJ-529 (Fig. 2b). These results indicate the suppressive effect of LJ-529 on emphysema formation.

Induction of anti-inflammatory cytokines by treatment with LJ-529

Macrophages play an important role in the development of emphysema upon exposure to causative factors (Yamasaki and Eeden 2018). M1 macrophages are classical pro-inflammatory macrophages that produce high levels of pro-inflammatory cytokines and reactive oxygen and nitrogen species (Yamasaki and Eeden 2018). Macrophages polarized to the M2 type are known to mediate damage repair by secreting anti-inflammatory cytokines, such as IL4 and IL10 (Krzyszczyk et al. 2018). Therefore, we examined whether LJ-529 modulates the expression of several factors associated with the polarization of macrophages to the M2 type and the inhibition of inflammation under pro-inflammatory environments in vitro. To mimic in vivo experimental conditions, murine macrophage cells were stimulated with lipopolysaccharide (LPS), a potent activator of innate immunity (Raetz and Whitfield 2002) which is also known to induce elastase production in macrophages (Duc Dodon and Vogel 1985), in the presence or absence of LJ-529 (1 μM). In LPS-stimulated RAW 264.7 cells, the expression of anti-inflammatory cytokines such as *Il4* and *Il10* was significantly decreased, which was substantially restored by treatment with LJ-529 (Fig. 3a). The *Tgfb1* mRNA expression was moderately increased upon stimulation with LPS, which was presumably due to context-dependent roles of TGF-β1 in innate immunity (Wahl 2007). Consistent with the effect on the expression of *Il4* and *Il10*, treatment with LJ-529 significantly enhanced *Tgfb1* expression in LPS-stimulated macrophage cells (Fig. 3a). Moreover, LJ-529 significantly ameliorated LPS-mediated downregulation

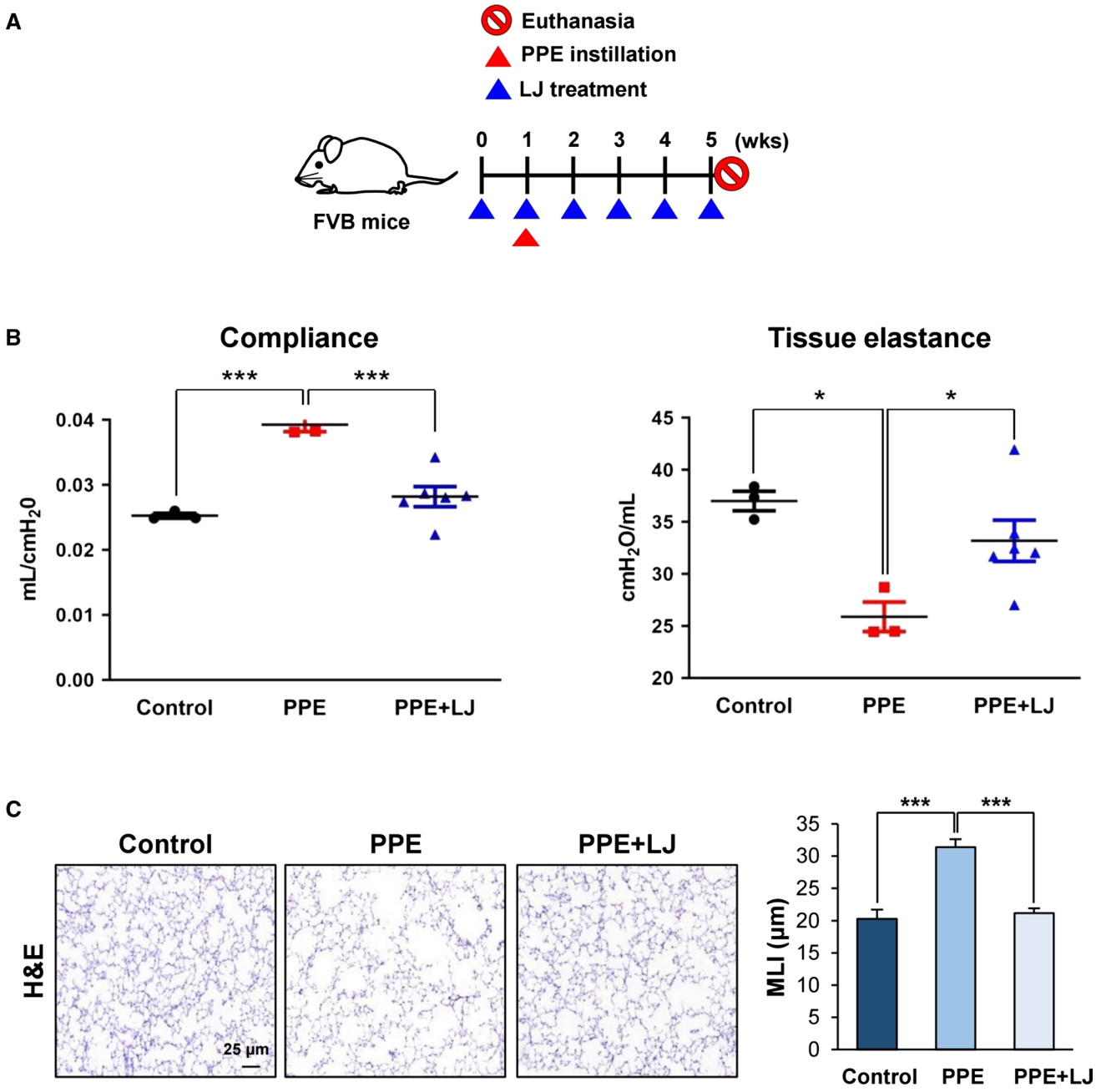


Fig. 1 Suppression of elastase-induced emphysema by the oral administration of LJ-529. **a** Schematic diagram of the experimental schedule. **b** Inhibition of elastase-induced pulmonary dysfunction by treatment with LJ-529 (50 μg/kg). Changes in lung function were monitored using the FlexiVent system. **c** Alleviation of elastase-mediated pulmonary destruction in the lungs of LJ-529-treated mice. *Right*. Quantification of structural changes in the airspace in each treatment group. The bars represent the mean ± SD; $*P < 0.05$ and $***P < 0.001$ compared with the PPE-treated group. *PPE* porcine pancreatic elastase, *LJ* LJ-529, *MLI* mean linear intercept

of markers of M2 macrophages, including *Arg1* (encodes arginase 1, an enzyme involved in nitric oxide metabolism (Yang and Ming 2014) and *Mrc1* (encodes CD206 the mannose receptor C-type 1 present on the surface of M2 macrophages (Trombetta et al. 2018) (Fig. 3b). Consistent with in vitro results, we observed the transcription of *Il4, Il10,* and *Tgfb1* was restored or enhanced in the lungs of LJ-529-treated mice compared with those of PPE-instilled mice (Fig. 3c). Moreover, LJ-529 treatment significantly alleviated elastase-induced downregulation of the mRNA levels of *Arg1* and *Mrc1* in the lungs (Fig. 3d). We also asked whether LJ-529 treatment affects M1 macrophage parameters including TNF-α, IL-1β, and iNOS. Unlike M2 macrophage parameters, M1 macrophage parameters did not

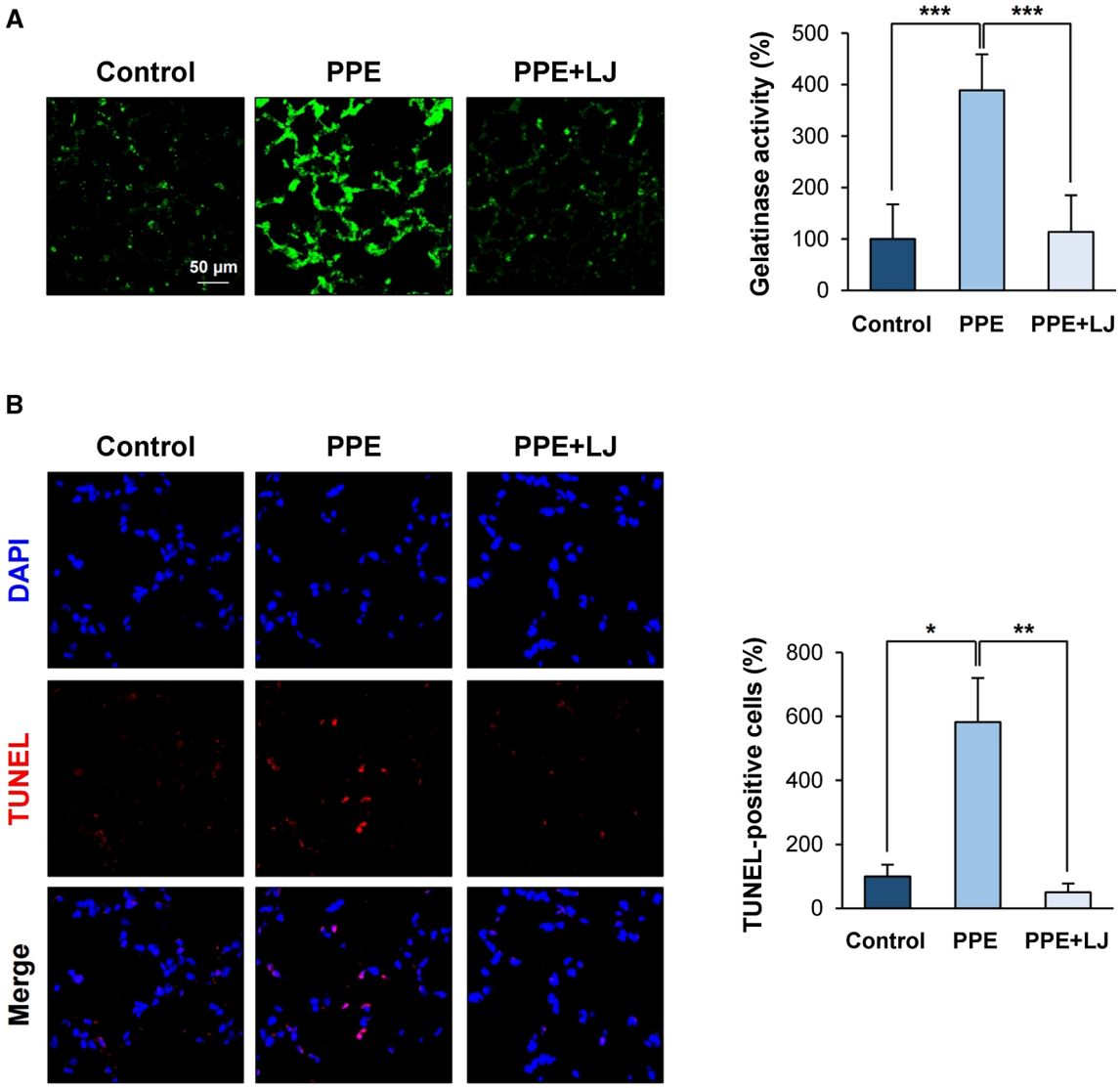


Fig. 2 Inhibitory effects of LJ-529 on elastase-induced increases in matrix metalloproteinase activity and apoptotic cell death in the lungs. **a** Attenuation of elastase-mediated matrix metalloproteinase (MMP) activity by treatment with LJ-529. MMP activity was determined by using fluorescein-conjugated DQ-gelatin. *Right*. Quantification of gelatinase activity versus that in vehicle-treated controls. **b** Inhibition of elastase-mediated apoptotic cell death in the lungs by treatment with LJ-529, as determined by TUNEL staining of lung tissue cryosections. *Right*. Quantification of the number of TUNEL-positive cells versus that in 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-529

show significant differences by LJ-529 in the lungs (Fig. 3e). These results suggest that the inhibitory effects of LJ-529 on PPE-induced emphysema development are mediated by its anti-inflammatory actions in M2 macrophages rather than regulating pro-inflammatory actions of M1 macrophages.

Next, we examined whether M2 macrophages are recruited by LJ-529 treatment in the lungs. Treatment of LJ-529 resulted in the enhanced recruitment of M2 macrophages, as determined by the increase of the $CD206^{+}$ and $Arg1^{+}$ cells in the lungs (Fig. 3f). Unexpectedly, we found that the recruitment of CD206-positive M2 macrophages is upregulated by elastase instillation in the lungs. According to previous studies, CD206 is generally expressed by dendritic cells, and lymphatic or endothelial cells as well as M2 macrophages (Haque et al. 2019). And also, CD206 positive cells can be enhanced in the elastase-induced extensive injuries as a result of autonomous resolution mechanism (Shibata et al. 2018; Yan et al. 2019). Both differentiation and recruitment of macrophages are likely responsible for PPE-induced pathologies and LJ-529-induced suppression against the pathologies. We next evaluated the role of LJ-529 in macrophage migration by using Boyden chambers. LPS-stimulated alveolar epithelial cells promoted macrophage migration. Moreover,

LJ-529 treatment more significantly enhanced macrophage migration than LPS-stimulated epithelial cells (Fig. 3g). Collectively, these findings suggest that LJ-529 exhibit anti-inflammatory actions by inducing recruitment of macrophages and their differentiation into M2 in an inflammatory alveolar space.

Induction of PPARγ target genes by treatment with LJ-529

LJ-529 has been shown to activate the PPARγ signaling pathway (Yu et al. 2017). Based on the role of PPARγ signaling in the regulation of inflammation (Kapadia et al. 2008; Martin 2010), we assessed whether LJ-529 increases the expression of target genes of the PPARγ signaling pathway. We found that LJ-529 upregulated the expression of PPARγ target genes, including *Adrp* (Fig. 4a), *Cd36* (Fig. 4b), *Abcg1* (Fig. 4c), and *Lpl* (Fig. 4d), in the lungs of mice. We next asked whether LJ-529 treatment enhances PPARγ activity. To assess PPARγ activity following LJ-529 treatment, we performed simultaneous co-transfection of PPARγ and PPRE-Luc and then conducted PPRE luciferase reporter assay. LJ-529 significantly increased PPARγ activity inhibited by LPS stimulation (Fig. 4e). These results suggest that the anti-inflammatory effect of LJ-529 (the inhibitory effect of LJ-529 on elastase-induced pulmonary emphysema) is mediated through the induction of PPARγ activity.

Minimal toxic effects of LJ-529 in vitro and in vivo

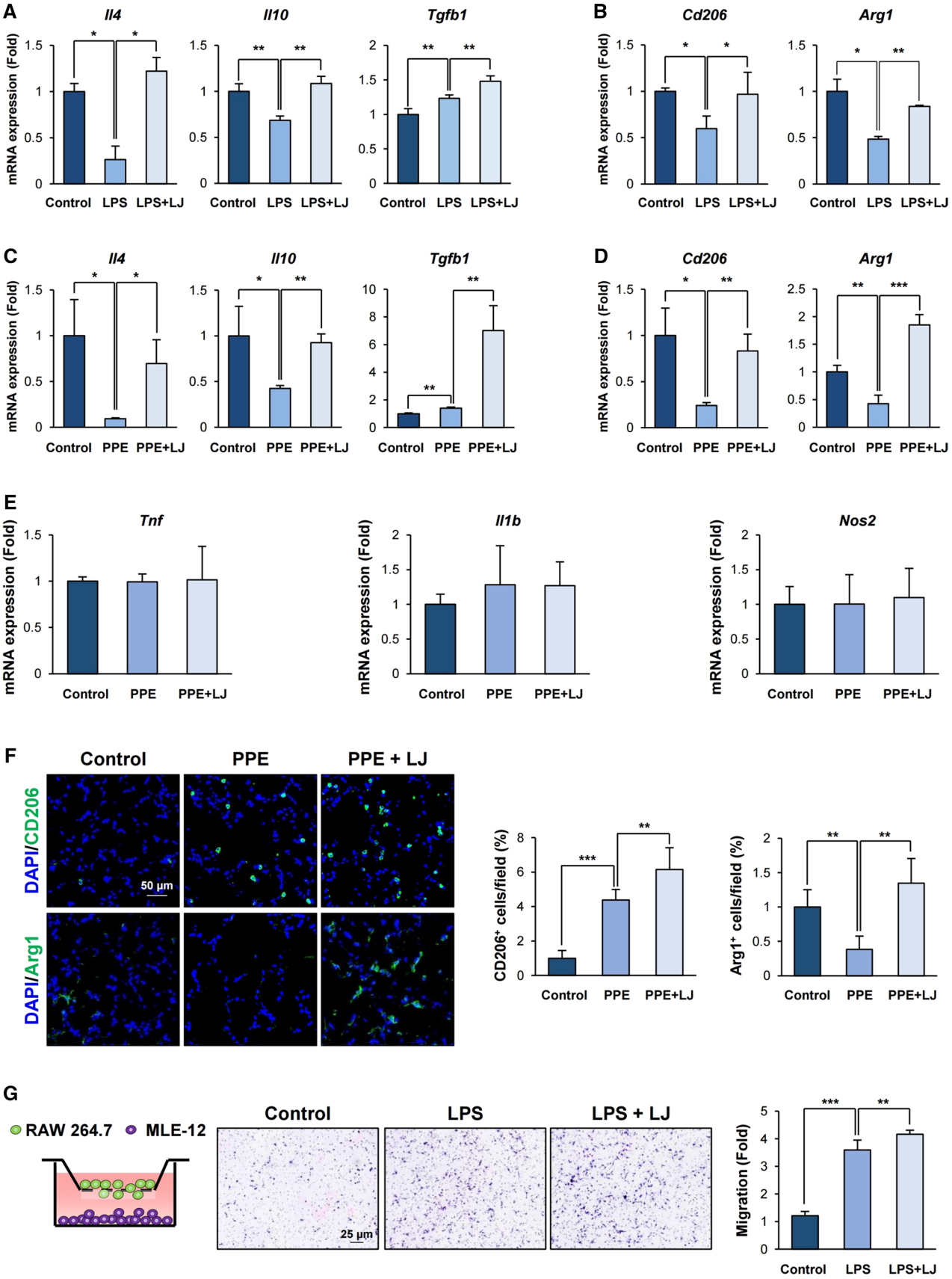
We determined the toxicity of LJ-529 in vitro and in vivo. LJ-529 had minimal effects on the viability of three normal epithelial cell lines derived from the lung bronchus (HBE), retinal pigment epithelium (RPE), and hippocampus (HT-22). The viability of these cells was minimally affected by the drug even at a concentration of 1 μM, which induced a transcriptional increase in anti-inflammatory and M2 macrophage-associated markers in macrophage cells (Fig. 5a). Consistent with previous findings indicating no side effects of LJ-529 in in vivo experiment (Chung et al. 2006), the administration of LJ-529 caused no significant body weight changes in the mice (Fig. 5b). Moreover, histological examination also revealed that the administration of LJ-529 did not cause any changes in major organs, such as the brain, kidneys, and liver (data not shown). Therefore, although additional in vitro and in vivo investigations are needed for a precise determination of the toxicity of LJ-529, these findings indicate that LJ-529 has minimal toxicity and suggest the potential for LJ-529 as an efficacious agent for attenuating pulmonary emphysema with limited toxicity.

Discussion

The socioeconomic burden of COPD has increased globally. However, therapeutic options for COPD are still limited, and symptomatic alleviation using inhaled bronchodilators or corticosteroids is the main therapeutic approach for COPD/emphysema in the clinic. Therefore, novel therapeutic or preventive agents for COPD/emphysema are urgently needed. In the present study, we aimed to discover new drugs with a safe toxicity profile that effectively suppress the development of emphysema. We demonstrated herein that LJ-529 is a drug that inhibits elastase-induced pulmonary emphysema in mice without overt toxicity. We further demonstrated that LJ-529 significantly suppressed the expression of anti-inflammatory cytokines through the activation of the PPARγ signaling pathway. These results suggest the potential of LJ-529 as an efficacious agent for the treatment of emphysema with minimal toxicity.

As chronic inflammation is one of the crucial pathogenic causes of the development of pulmonary emphysema, the inhibition of the inflammatory response is considered a logical approach for the treatment of COPD (Cazzola et al. 2012). Indeed, the oral phosphodiesterase 4 (PDE4) inhibitor roflumilast displays anti-inflammatory effects and has been approved for the treatment of COPD in the clinic (Wedzicha et al. 2016). However, most anti-inflammatory agents, which have various modes of action, have not reached clinical application due to a lack of effectiveness, low potency, side effects, and/or toxicity (Wedzicha et al. 2016). Therefore, it is necessary to develop potent drugs with safe toxicity profiles for the treatment of COPD/emphysema. PPARγ is considered a cellular target for the development of anti-COPD/emphysema agents due to its role in the negative regulation of inflammation. PPAR agonists exhibit excellent inhibitory effects on inflammation and COPD; however, several drawbacks, including the need for a high dose and increased cardiovascular risk in patients with diabetes (Cazzola et al. 2012), limit their use in the clinic. In this regard, our study may be of importance for the development of drugs for treating patients with emphysema.

First, our study identified a potential drug that suppresses elastase-induced pulmonary emphysema, a chronic disease that requires long-term treatment. This effect was achieved by the administration of a very low dose (50 μg/kg) of LJ-529 by oral gavage, suggesting the high potency of oral LJ-529 for suppressing emphysema. In a previous study on the antitumor effect of LJ-529 in breast cancer, the intraperitoneal administration of LJ-529 for up to 1 month did not cause toxicity in mice (Chung et al. 2006). Consistently, LJ-529 did not cause significant cytotoxicity in a panel of normal cell lines derived from various organs. More importantly, mice administered LJ-529 did not exhibit detectable changes in body weight or side


A
Il4
Il10
Tgfb1
B
Cd206
Arg1
mRNA expression (Fold)
Control
LPS
LPS+LJ
C
Il4
Il10
Tgfb1
D
Cd206
Arg1
Control
PPE
PPE+LJ
E
Tnf
Il1b
Nos2
F
Control
PPE
PPE + LJ
DAPI/CD206
DAPI/Arg1
50 μm
CD206⁺ cells/field (%)
Arg1⁺ cells/field (%)
G
RAW 264.7
MLE-12
Control
LPS
LPS + LJ
25 μm
Migration (Fold)

◂**Fig. 3** Upregulation of anti-inflammatory cytokines and a M2 macrophage-related marker in the lungs from LJ-529-treated mice. **a**–**d** Changes in the transcription of cytokines and markers of M2 macrophages in murine macrophages (**a** and **b**) and in the lungs (**c** and **d**) of each treatment group were determined by real-time PCR. **e** Changes in the M1 macrophage parameters in the lungs of each treatment group were determined by real-time PCR. **f** The recruitment of M2 macrophages in the lungs of each treatment group were determined by immunofluorescence analysis. *Right.* Quantification of the recruitment of CD206- or Arginase 1-positive cells in each treatment group versus vehicle-treated controls. **g** Changes of the macrophages migration were determined by migration assay. 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-529

effects or toxicity in various organs. Hence, our data showing the high potency and oral bioavailability of LJ-529 as well as its limited toxicity indicate its use for the treatment of emphysema.

Second, our study proposes LJ-529 as a novel PPARγ agonist with clinical applicability. A_3AR agonists have been shown to suppress bleomycin-induced pulmonary inflammation and fibrosis (Morschl et al. 2008) and lung ischemia–reperfusion injury (Mulloy et al. 2013) through its anti-inflammatory activities. Consistently, the A_3AR agonist IB-MECA also inhibits dextran sulfate sodium (DSS)-induced colitis in mice via modulating the NF-κB signaling pathway (Ren et al. 2015). Interestingly, the A_3AR modulator has been found to act as a partial peroxisome proliferator activated receptor gamma (PPARγ) agonist and PPARδ antagonist (Yu et al. 2017). A recent study reported that an A_3AR agonist, LJ529, has the capacity to act as a partial PPARγ agonist (Jeong et al. 2003; Yu et al. 2017). Previous studies have shown that PPARγ exerts anti-inflammatory effects via inhibiting the expression of inflammatory cytokines (Martin 2010) and modulating the transcriptional activity of NF-κB (Kapadia et al. 2008; Remels et al. 2009). Furthermore, previous studies have suggested that PPARγ exerts anti-inflammatory properties by inducing differentiation into M2 macrophages (Bouhlel et al. 2007) and the stimulation of A3 receptors can support survival of anti-inflammatory monocytes (Haskó and Pacher 2012). Hence, we reasoned that the inhibitory effects of LJ-529 on pulmonary emphysema and the induction of anti-inflammatory gene expression are attributed to the activation of PPARγ signaling. Indeed, in our cell line and animal models, LJ-529 upregulated the expression of several PPARγ target genes and enhanced PPARγ activity, as indicated by the augmentation of PPRE-luciferase reporter gene. These findings suggest that both PPARγ and A_3AR are potential mechanisms by which LJ-529 inhibits the development of pulmonary emphysema by triggering differentiation into M2 macrophages via PPAR-γ activity and supporting survival of anti-inflammatory monocytes through the stimulation of A3 receptors.

In the present study, we used porcine pancreatic elastase (PPE)-induced emphysema mouse model. Unlike cigarette smoke-induced emphysema mouse model, the PPE-induced emphysema mouse model requires less than on month to induce disease development and has been used to screen multiple reagents within 1 month. Despite the advantage, the main weakness of the PPE-induced model is lack of all the featured clinical signs of COPD and emphysema, including changes in body weight (Wright et al. 2008; Antunes and Rocco 2011; Rodrigues et al. 2017). Therefore, further studies are warranted to validate the efficacy of LJ-529 in animal models, wherein the disease development is induced by chronic exposure to cigarette smoking.

In summary, the present study demonstrates the potential of LJ-529 as an inhibitor of elastase-induced emphysema with limited toxicity. Further investigation of the biological activities and toxicity of LJ-529 in various preclinical and clinical settings is warranted. In addition, extensive studies are required to elucidate the detailed mechanisms underlying the LJ-529-mediated blockade of pulmonary emphysema.

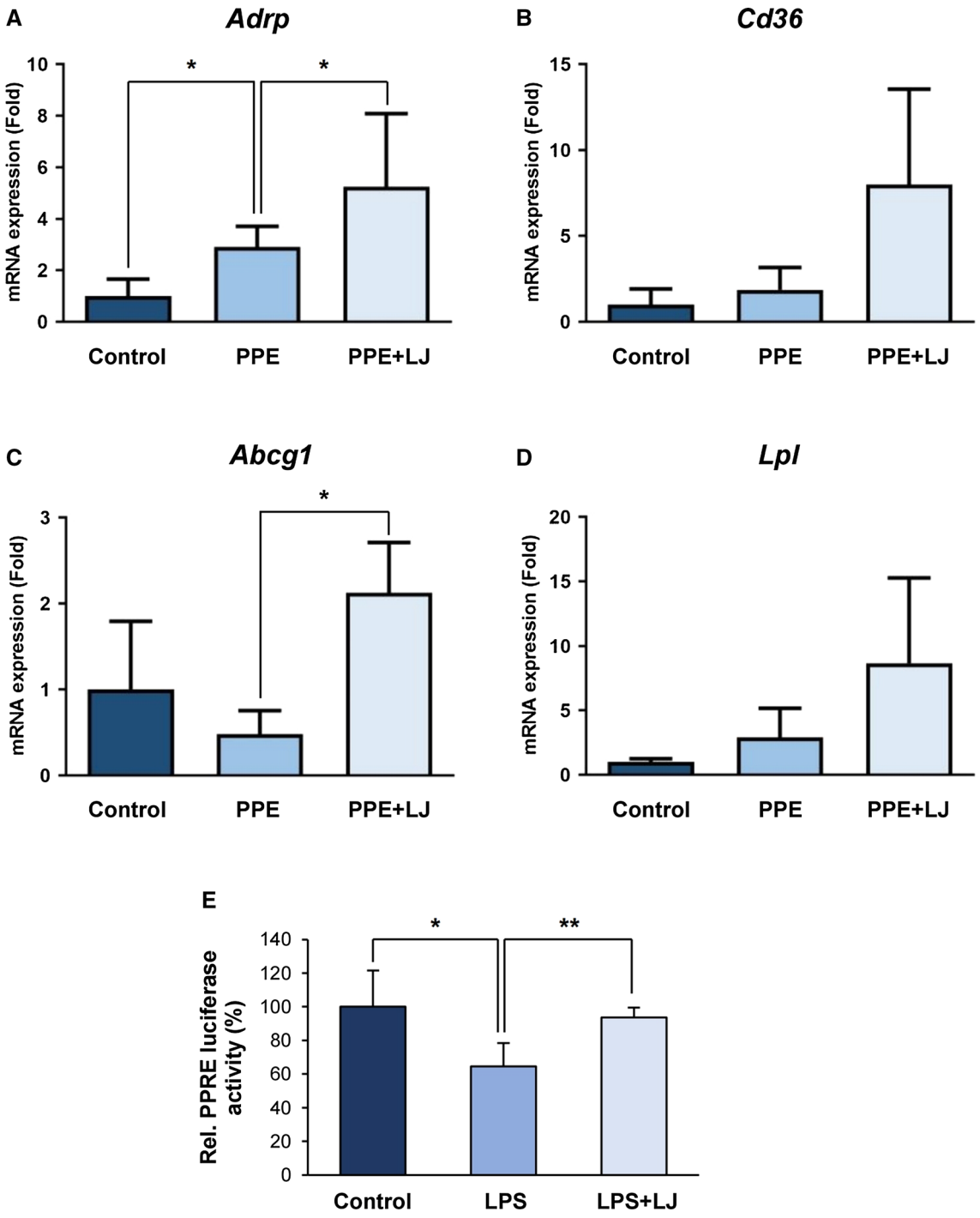


Fig. 4 Activation of PPARγ by treatment with LJ-529. **a**–**d** Expression of PPARγ target genes in the lungs of mice, as determined by real-time PCR. **e** PPARγ activation was assessed by PPRE-Luc reporter assay in murine macrophages. The bars represent the mean ± SD; $*P < 0.05$ and $**P < 0.01$ compared with the indicated control group. *PPE* porcine pancreatic elastase, *LJ* LJ-529

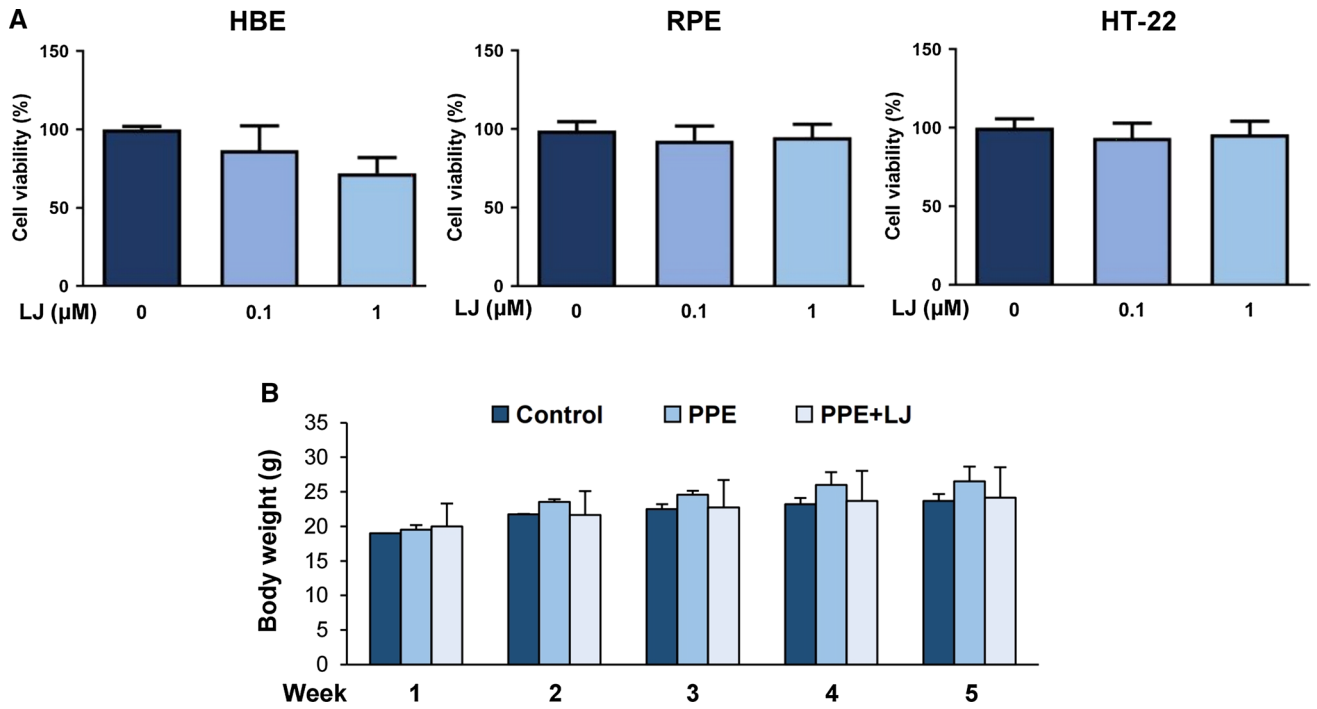


Fig. 5 Minimal toxic effects of LJ-529 in vitro and in vivo. **a** LJ-529 had no significant cytotoxic effects in normal cell lines from various organs. HBE, RPE, and HT-22 cells were treated with vehicle (DMSO) or LJ-529 (0.1 or 1 μM) for 3 days. Cell viability was determined by the MTT assay. **b** Body weight changes in each treatment group during the animal experiment. *PPE* porcine pancreatic elastase, *LJ* LJ-529

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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