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Roflumilast reduces the number of lung adenocarcinomas, inflammation, and emphysema in a smoking-induced mouse model

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Abstract

Background The prognosis of lung cancer complicated by chronic obstructive pulmonary disease is poor, and effective prophylactic agents have not been established. Given that inflammation is a shared pathogenic mechanism of both diseases, we aimed to evaluate the efficacy of roflumilast, a novel anti-inflammatory drug, in preventing emphysema and lung cancer using a smoking-induced lung cancer mouse model.

Methods Male A/J mice were exposed to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a potent carcinogen, and intermittent mainstream cigarette smoke for 20 weeks. Roflumilast or vehicle was administered via intragastric gavage once daily. Lung tissues were assessed for tumor nodules and emphysema, and bronchoalveolar lavage fluid was collected for cell counting. Emphysema severity and concentrations of inflammatory cytokines (IL-6, IL-1β, and TNF-α) were assessed. RAW 264.7 macrophage cells were used to assess cellular responses to cigarette smoke extract.

Results Roflumilast attenuated the increase in total cells and macrophages in bronchoalveolar lavage fluid induced by intermittent smoking exposure and significantly suppressed smoking-induced expressions of IL-6, IL-1β, and TNF-α. Roflumilast also reduced emphysematous changes and the number of lung tumors. In vitro, roflumilast attenuated cigarette smoke extract-induced expression of IL-6, IL-1β, and TNF-α in RAW 264.7 cells.

Conclusions This study highlights the potential use of roflumilast as a chemopreventive agent for patients with chronic obstructive pulmonary disease who are at risk of lung cancer and underscores its relevance for future clinical application and research on phosphodiesterase-4 inhibitors.

Keywords Chronic obstructive pulmonary disease, Lung neoplasm, PDE4 inhibitor, Smoking, Chemoprevention

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Background

Chronic obstructive pulmonary disease (COPD) is primarily caused by smoking and characterized by emphysema, a condition that causes irreversible structural changes that complicate curative treatment [1]. COPD has various comorbidities, and lung cancer remains a leading cause of death among patients with this condition. Patients with lung cancer who have COPD have poorer prognoses than those who do not [2, 3]. Therefore, chemoprevention for lung cancer in patients with COPD is highly desirable. However, no effective strategies have been established [4].

COPD is characterized by chronic inflammation predominantly driven by neutrophils and macrophages [1]. The addition of inhaled corticosteroid to standard bronchodilator treatment reduces exacerbations, but it fails to completely suppress pulmonary inflammation or prevent lung function decline [5–8]. Attention has recently been shifted to roflumilast, a phosphodiesterase-4 (PDE4) inhibitor with a unique mechanism of action relative to traditional therapies [9]. Roflumilast suppresses smoking-induced emphysema in mouse models [10] and shows superior efficacy to inhaled corticosteroid in reducing pulmonary inflammation, exacerbations, and lung function decline in patients with COPD [11, 12]. Given that smoking-induced pulmonary inflammation plays a central role in both COPD and lung cancer pathogenesis [13], we hypothesized that roflumilast may inhibit not only pulmonary inflammation and emphysema but also the development of smoking-induced lung cancer. Specifically, cytokines such as IL-6, IL-1β, and TNF-α have been reported to play pivotal roles in the pathogenesis of emphysema and lung cancer [14, 15].

This study aimed to evaluate the inhibitory effects of roflumilast on pulmonary inflammation, emphysema, and lung cancer in a smoking-induced lung cancer mouse model. It also aimed to identify the cells targeted by roflumilast through in vitro experiments using cigarette smoke extract.

Methods

Mice

Male A/J mice (7–10 weeks old) were purchased from Sankyo Labo Service (Tokyo, Japan). The mice were housed in plastic cages under a 12:12-h light-dark cycle. All experimental procedures adhered to the National Institutes of Health guidelines and were approved by the Laboratory Animal Center of Keio University School of Medicine.

NNK treatment, smoking exposure, and treatment groups

Following 2 weeks of intraperitoneal injection of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (100 mg/kg, Toronto Research Chemicals, Toronto, Ontario, Canada), the mice were exposed to either sham air or mainstream cigarette smoke generated from commercially available filtered cigarettes (Marlboro, 12 mg tar/1.0 mg nicotine, Philip Morris Inc., Richmond, VA, USA) for 1 h a day for 5 days a week for 20 weeks. The mice inhaled cigarette smoke nasally, as previously reported [16]. A cigarette smoke inhalation apparatus (SIS-CS system, SG-300, Shibata Scientific Technology, Tokyo, Japan) was used. Dimethyl sulfoxide (DMSO) (Wako, Osaka, Japan), as a vehicle, or roflumilast (Sigma Aldrich, St. Louis, MO, USA) was administered (5 mg/kg) [17] via intragastric feeding once a day for 5 days a week for 20 weeks. The mice were divided into four groups: DMSO-plus-air, roflumilast-plus-air, DMSO-plus-smoke, and roflumilast-plus-smoke ($n = 20$ per group). At 20 weeks (Fig. 1), all mice were euthanized for analysis by intraperitoneal injection of a combination of medetomidine hydrochloride (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol tartrate (5 mg/kg), administered at 0.1 mL per 10 g body weight [18], followed by exsanguination via the abdominal aorta.

Sampling of mouse lung tissue and bronchoalveolar lavage

The lungs were fixed by an intrabronchial infusion of 4% paraformaldehyde at a pressure of 25 cmH_2O. The lungs were removed, fixed, embedded in paraffin, serially sectioned, and stained with hematoxylin and eosin (H&E) for histopathological assessment of tumor nodules, as previously reported [16]. In a subset of mice, the lungs were lavaged three times with 0.6 mL of phosphate-buffered saline. Total cell counts and differentials in the bronchoalveolar lavage fluid were analyzed as previously described [19].

Pathological assessment of emphysema and lung tumors

The mean linear intercept, a standard parameter of alveolar size, and the destructive index, indicating alveolar destruction, were measured for ten randomly selected fields per mouse [16]. Broncho-alveolar proliferative lesions were pathologically diagnosed as hyperplasia, adenoma, or adenocarcinoma on the H&E-stained sections according to published criteria [20] by a trained pathologist (M.S.). Tumor incidence, multiplicity, and size were calculated as previously described [16]. All sections were evaluated in a blinded manner.

Enzyme-linked immunosorbent assay

The concentrations of IL-6, IL-1β, and TNF-α in lung tissue homogenate were measured using DuoSet® enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems, Minneapolis, MN, USA; DY406, DY401, and DY410).

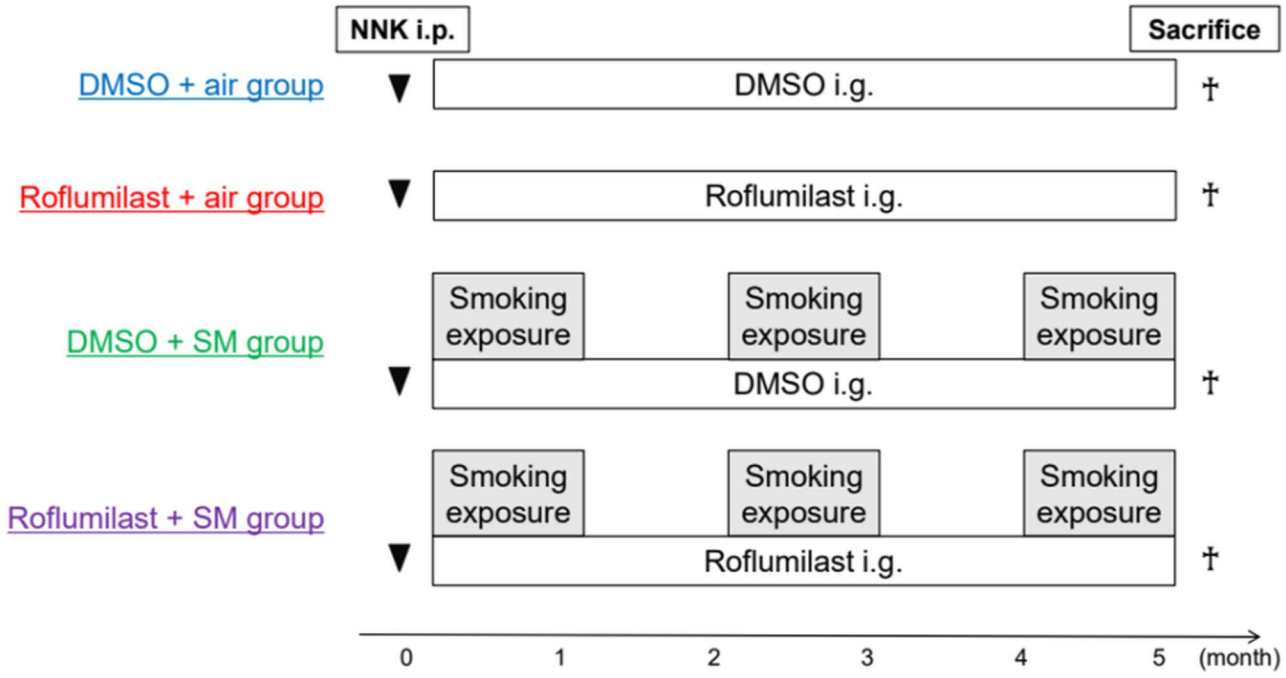

Fig. 1 Study design and experimental protocols. Male A/J mice (7–10 weeks old) are exposed to either sham air or cigarette smoke, beginning 2 weeks after injection with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Vehicle (dimethyl sulfoxide [DMSO]) or roflumilast was administered via intragastric feeding (i.g.). Tumor development and emphysema are assessed at 20 weeks. SM, smoke

Cell culture

The murine macrophage cell line RAW 264.7 (ATCC, Manassas, VA, USA; TIB-71) was used. RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA, USA) and supplemented with 10% fetal bovine serum (Sigma Aldrich) and 1% penicillin-streptomycin in a humidified 5% CO_2 incubator at 37 °C, according to the supplier's protocol.

Preparation of cigarette smoke extract

Cigarette smoke extract (CSE) was prepared fresh for each experiment by drawing the smoke from one whole cigarette (Marlboro, 12 mg tar/1.0 mg nicotine, Philip Morris Inc.) into the medium [21].

Treatment protocols

RAW 264.7 cells were seeded into 24-well plates at a density of 1×10^5 cells/well. Cells were treated with CSE (5%) and roflumilast (10 nM, 100 nM) [22, 23] for 24 h. Control cells received an equivalent concentration of the DMSO. Experiments were performed in triplicate. Total RNA was isolated after incubation.

Quantitative real-time polymerase chain reaction analysis

Total RNA was isolated from whole lungs or cells using an RNeasy Mini Kit (Qiagen, Hilden, NRW, Germany). RNA was reverse-transcribed using the High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific), following the manufacturer's protocol. Real-time quantitative polymerase chain reaction (PCR) analysis was performed with SYBR Green assays on the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific). GAPDH was used as an endogenous control for normalization. The primer sequences were as follows: GAPDH (forward: 5'-AATGGATTTGGACGCATTGGT-3', reverse: 5'-TTTG CACTGGTACGTGTTGAT-3'), IL-6 (forward: 5'- GAG GATACCACTCCCAACAGACC-3', reverse: 5'- AAGTG CATCATCGTTGTTCATACA-3'), IL-1β (forward: 5'-C AACCAACAAGTGATATTCTCCATG-3', reverse: 5'- G ATCCACACTCTCCAGCTGCA-3'), and TNF-α (forward: 5'-GACCAGGCTGTCGCTACATCA-3', reverse: 5'-CGTAGGCGATTACAGTCACGG-3'). The relative levels of expression were calculated using the delta-delta Ct method. Experiments were performed at least three times, with representative data shown.

Statistical analysis

Data are expressed as mean ± SE or median (interquartile range). Statistical analyses were conducted using Student's t-test and one-way analysis of variance (ANOVA) followed by Tukey-Kramer and Dunnet's post hoc test or the Kruskal–Wallis test followed by the Steel–Dwass test. Categorical data were analyzed using the χ^2 test. All analyses were performed using JMP Pro version 17 (SAS Institute, Cary, NC, USA). All p-values were two-sided, and statistical significance was set at $p < 0.05$.

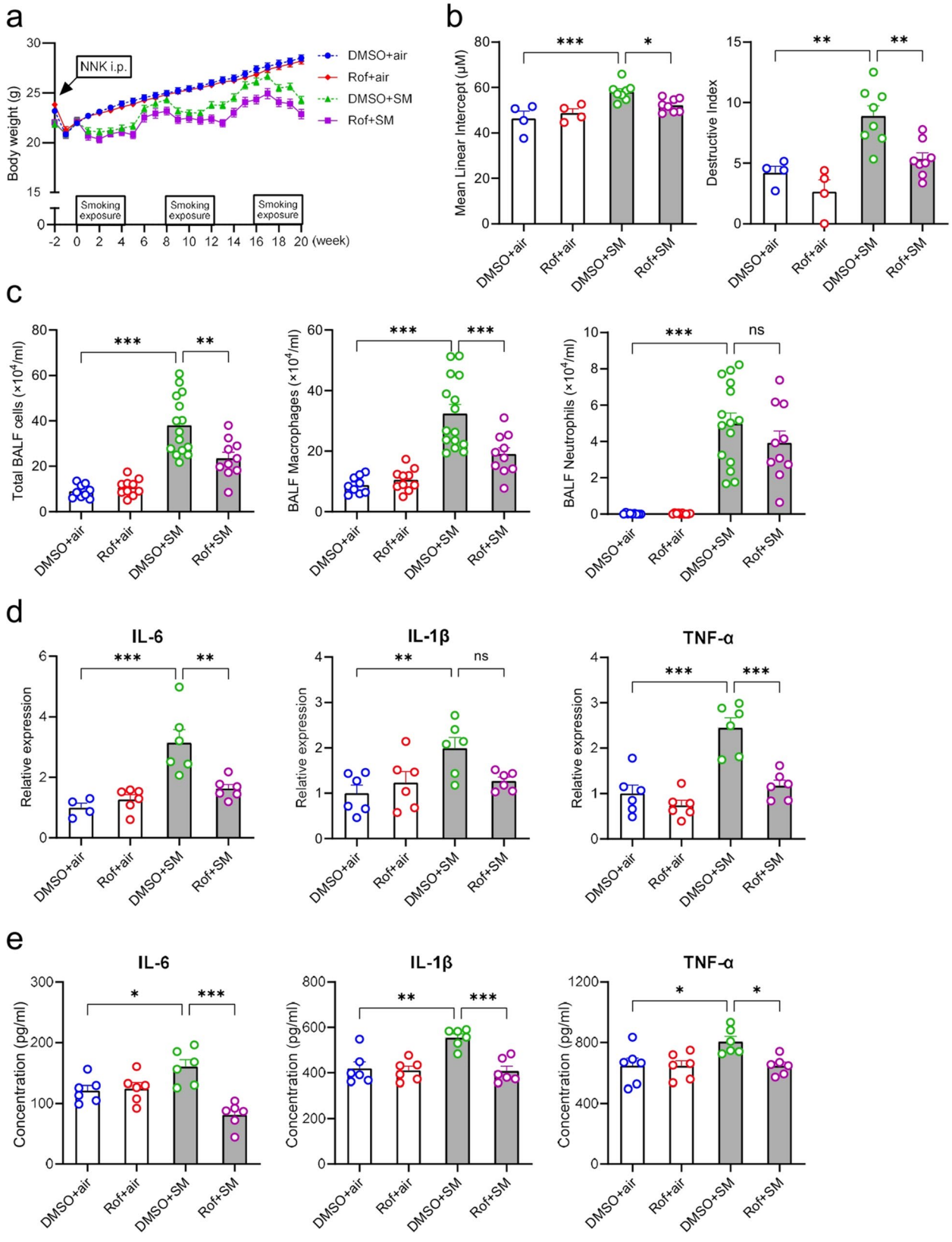

Fig. 2 (See legend on next page.)

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Fig. 2 Effects of smoking exposure and roflumilast administration. (**a**) Changes in body weight over 5 months. (**b**) Comparison of mean linear intercept and destructive index. (**c**) Total cell numbers and cell fractionation in bronchoalveolar lavage fluid (BALF). (**d**, **e**) mRNA and protein concentrations of IL-6, IL-1β, and TNF-α, measured using RT-qPCR (normalized to GAPDH levels) and enzyme-linked immunosorbent assay. Data are shown as mean ± SE. Sample sizes: (**b**) $n = 4–8$; (**c**) $n = 10–15$; (**d**, **e**) $n = 4–6$. Statistical analysis is performed using ANOVA, followed by the Tukey–Kramer post hoc test. ns: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p \leq 0.001$. Dimethyl sulfoxide (DMSO)-plus-air group (blue); Roflumilast-plus-air group (red); DMSO-plus-smoke group (green); Roflumilast-plus-smoke group (purple). Rof, roflumilast; SM, smoke

Results

Roflumilast suppresses inflammation and lung emphysematous changes caused by intermittent smoking exposure

The body weight of the smoking group consistently decreased throughout the exposure periods. However, roflumilast had no effect on body weight in either the air or intermittent smoking groups (Fig. 2a). The mean linear intercept and destructive index were significantly higher for the DMSO-plus-smoke group than for the DMSO-plus-air group. These indices were significantly lower for the roflumilast-plus-smoke group than for the DMSO-plus-smoke group (Fig. 2b, Supplementary Fig. 1).

Roflumilast attenuated the increase in total cells and macrophages in bronchoalveolar lavage fluid induced by intermittent smoking exposure (Fig. 2c). Inflammatory cytokines were evaluated by real-time PCR analysis and ELISA of whole-lung mRNA and homogenate. Roflumilast significantly suppressed smoking-induced expressions of IL-6, IL-1β, and TNF-α (Fig. 2d, e).

Roflumilast reduces the number of lung adenocarcinomas induced by intermittent smoking exposure

Intermittent smoking exposure induced the formation of lung adenomas (Fig. 3a) and adenocarcinomas (Fig. 3b), which was consistent with a previous report [16]. We compared the incidence, multiplicity, and sizes of the lung tumors among the four groups (Fig. 3c). The DMSO-plus-smoke group had higher lung tumor multiplicity than the vehicle-plus-air group. The roflumilast-plus-smoke group tended to have a lower tumor incidence than the DMSO-plus-smoke group and fewer tumors. However, there was no significant difference in lung tumor size between the two groups. These findings suggest that roflumilast may impact the initiation phase of smoking-induced lung tumor development, rather than on the growth of existing tumors.

Effect of roflumilast on inflammatory mediators in CSE-stimulated cells

Further investigation of the target cells revealed an increase in macrophages following intermittent smoking exposure and subsequent suppression after roflumilast administration. In vitro experiments using RAW 264.7 cells revealed significant increase in the expressions of the inflammatory cytokines IL-6, IL-1β, and TNF-α after CSE exposure. Roflumilast treatment at concentrations of 10 nM and 100 nM effectively reduced the expression levels of these cytokines in the control group without CSE exposure (Fig. 4). For IL-1β, an increase in expression relative to that in the CSE-free control group was observed after the administration of a higher concentration (100 nM) of roflumilast.

Discussion

To the best of our knowledge, this is the first study to demonstrate the suppressive effect of a PDE4 inhibitor on lung cancer development using a smoking-induced lung cancer mouse model. This study has two significant clinical implications. First, lung cancer complicated by COPD accounts for a substantial number of deaths and has a poorer prognosis than lung cancer without COPD [24]. The potential of roflumilast to prevent lung cancer onset could be clinically valuable in improving the survival outcomes of patients with COPD. Second, roflumilast has already demonstrated benefits in COPD management and is routinely used in clinical practice [25]. The findings of this study support the rationale for future clinical trials in humans, highlighting its potential applicability to clinical settings.

The phosphodiesterase superfamily comprises 11 enzyme families, with PDE4 being of particular interest. PDE4 is strongly expressed in airway epithelial cells and several inflammatory cell types implicated in COPD [26, 27]. Furthermore, smoking exposure increases the expression of PDE4 in the lung tissue [28]. Roflumilast, a selective PDE4 inhibitor, inhibits PDE4 activity, leading to elevated intracellular cAMP concentrations and subsequent downregulation of inflammatory signal pathways [28]. In the present study, long-term administration of roflumilast suppressed macrophage infiltration into the alveoli and prevented intermittent smoking-induced emphysema. This finding is consistent with that of a previous report indicating that roflumilast suppresses acute neutrophil infiltration, chronic macrophage infiltration, and emphysema with continuous smoking exposure [10]. The observed reduction in lung inflammation and emphysema under different smoking patterns further supports the anti-inflammatory and emphysema-preventive effects of roflumilast.

Smoking exposure induces a temporal production of various cytokines and chemokines within the lung. The smoking-exposed mouse model is well-suited for evaluating the dynamic changes in inflammatory cytokines

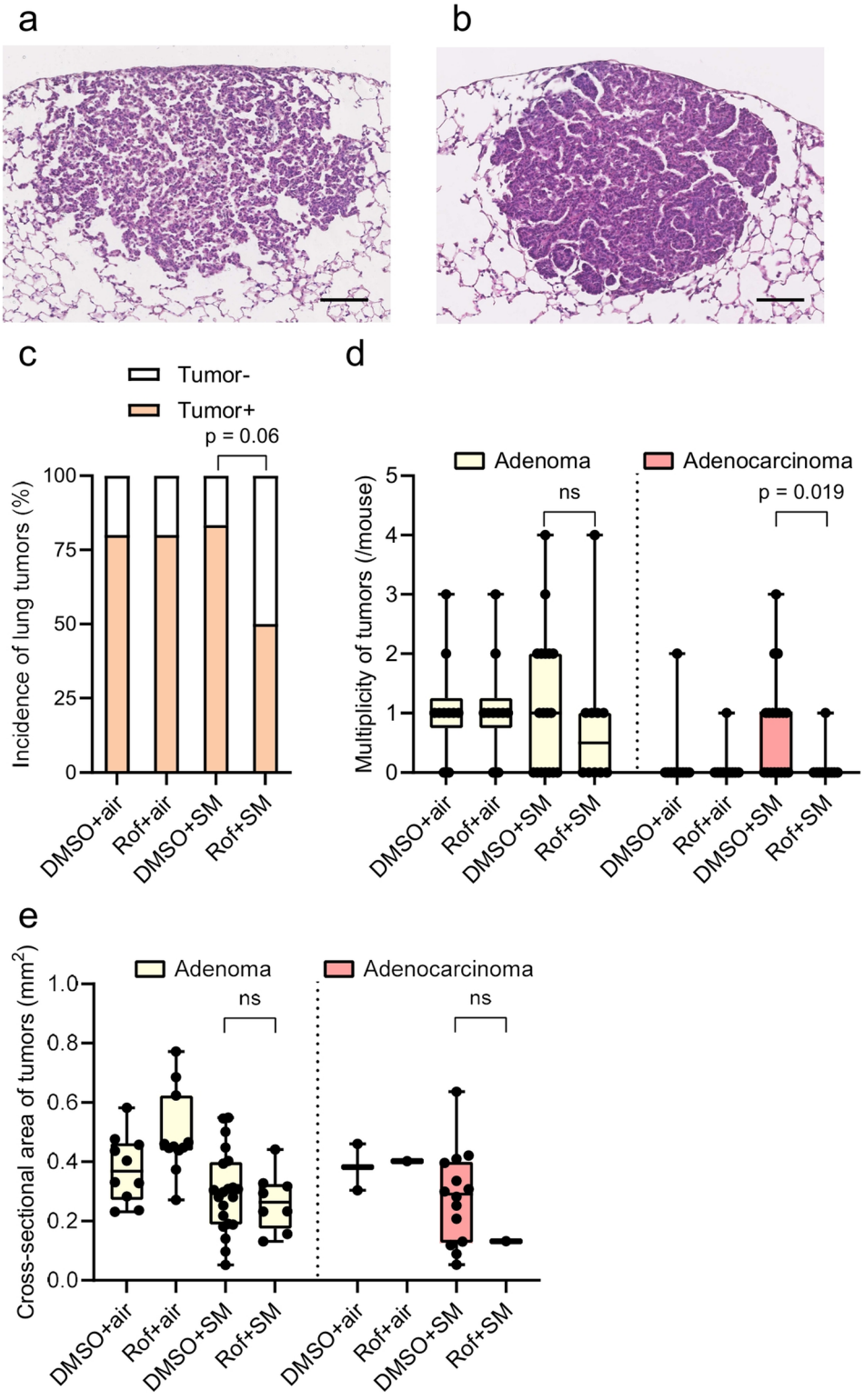

Fig. 3 Histological and morphological findings of lung tumors. Representative (**a**) lung adenoma and (**b**) lung adenocarcinoma stained with hematoxylin and eosin. Scale bars: 100 μm. (**c**–**e**) Incidence, multiplicity, and size of lung tumors. (**c**) Statistical analysis of tumor incidence is performed using the χ^2 test. (**d**, **e**) Data are presented as median and interquartile range ($n = 10$–18 per group). Statistical analysis of multiplicity and size of tumors are performed using the Kruskal–Wallis test, followed by Steel–Dwass test. ns: not significant. DMSO, dimethyl sulfoxide; Rof, roflumilast; SM, smoke

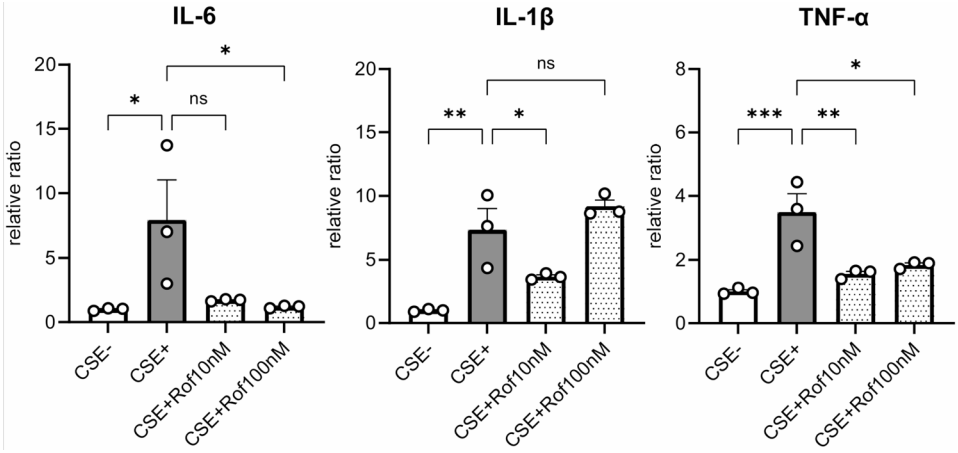

Fig. 4 Effect of roflumilast on inflammatory mediators in cigarette smoke extract (CSE)-stimulated cells. mRNA levels of IL-6, IL-1β, and TNF-α are measured using RT-qPCR and normalized to GAPDH concentrations. Data are presented as mean ± SE ($n = 3$ per group). Statistical analysis is performed using ANOVA, followed by post hoc analysis with the Dunnett's test. ns: not significant, *: $p < 0.05$; **: $p < 0.01$; ***: $p \leq 0.001$. Rof, roflumilast

and chemokines over time, which has been extensively studied [29]. The effects of PDE4 inhibitors on the dynamics of chemokines and cytokines in the lungs during smoking exposure have not been thoroughly studied. In this study, roflumilast administration significantly suppressed the expressions of IL-6, IL-1β, and TNF-α, which were increased by intermittent smoking exposure. IL-6, IL-1β, and TNF-α are key factors associated with smoking-induced emphysema, as demonstrated in knockout mice [30–32], and our findings are consistent with these reports.

Several studies have investigated the relationship between lung cancer and PDE4. PDE4 is overexpressed in lung cancer cells relative to normal lung cells [33], and PDE4 inhibitors have anti-proliferative and apoptosis-inducing effects on lung cancer cells [33, 34]. Additionally, PDE4 inhibitors have been shown to reduce lung tumor burden in a benzo(a)pyrene-induced lung cancer model [17]. In the present study, the administration of roflumilast suppressed the number of lung tumors, particularly adenocarcinomas, associated with intermittent smoking exposure. However, no inhibitory effect was observed on the size of the lung tumors. These findings suggest that roflumilast may influence early events in tumor development, such as initiation, rather than exerting a strong inhibitory effect on the growth of existing tumors in this model.

As mentioned above, our findings suggest that roflumilast exerts anti-inflammatory effects by acting on alveolar macrophages. Lung inflammation is a critical factor in creating a permissive environment for tumor development [35]. Chronic inflammation not only contributes to tumor growth and progression [36], but also increases the risk of carcinogenesis and is associated with tumor initiation [37]. TNF-α and IL-6 have been implicated in the lung tumor initiation in Kras transgenic mice [38]. Furthermore, IL-1β is a key regulator of immune responses and inflammation [39], and there is evidence of its overexpression in various malignancies [40]. Therefore, the suppression of IL-6, IL-1β, and TNF-α by roflumilast may represent one of the mechanisms underlying its anti-tumor effects. Moreover, the anti-inflammatory properties of roflumilast could lead to the suppression of tumor initiation by addressing the inflammatory microenvironment associated with cancer development [41]. Alveolar macrophages are broadly categorized into M1 and M2 phenotypes, with M1 macrophages involved in tumor initiation and M2 macrophages—also known as tumor-associated macrophages—contributing to tumor promotion [42]. While our study demonstrated that roflumilast reduced the number of macrophages in BALF, whether it affects the phenotypic polarization of these macrophages remains unclear and warrants further investigation. In our study, roflumilast administration exhibited a more pronounced tumor-suppressive effect in the smoke-exposed group than in air-exposed group. This finding suggests that roflumilast may be more effective in preventing tumor development in the context of chronic inflammation induced by cigarette smoke, rather than in tumors arising in the absence of pulmonary inflammation caused by chemical induction alone. Given that smoking-induced pulmonary inflammation plays a central role in both COPD and lung cancer pathogenesis [13], these findings highlight the potential importance of the inflammatory microenvironment in modulating the anti-tumor effects of roflumilast.

In this study, we investigated the effects of roflumilast on macrophages using in vitro experiments. The impact of roflumilast on inflammatory cytokine dynamics in macrophages treated with CSE has not been previously studied. However, PDE4 inhibitors suppress IL-6 and TNF-α production in alveolar and airway epithelial cells

stimulated with lipopolysaccharide [43], as well as TNF-α and IL-1β production in RAW264.7 cells [44]. Roflumilast also inhibits the CSE-induced increase in IL-6 and TNF-α expressions in lung cancer cell lines [30, 45] and reduces MMP-12 production in RAW264.7 cells [46]. Our findings are consistent with these reports. In this study, roflumilast suppressed the CSE-induced increase in macrophages associated with intermittent smoking exposure and directly influenced macrophage function, thereby reducing inflammation following CSE exposure. Chronic inflammation driven by macrophages plays a central role in the pathogenesis of smoking-induced emphysema [1], supporting the view that macrophages are primary target cells for the anti-inflammatory effects of roflumilast.

In this study, the anti-inflammatory effects of roflumilast did not exhibit clear dose-dependency. Similar nonlinear or plateaued responses have been observed with other pharmacologic agents, often attributed to off-target effects at higher concentrations [47–49]. Although such effects have not been specifically reported for roflumilast, it is possible that high concentrations may engage unintended molecular targets, thereby diminishing the net anti-inflammatory response. Alternatively, the lack of dose-dependency may be due to saturation of the cAMP signaling pathway [23]. For example, excessive accumulation of the intracellular cAMP might lead to a saturation or downregulation of cAMP-responsive receptors or downstream signaling elements, limiting further anti-inflammatory effects. These possibilities highlight the importance of further research to determine the optimal dosing range of roflumilast for therapeutic applications, ensuring maximal efficacy while minimizing potential off-target effects.

Roflumilast reduces exacerbations, improves quality of life, and enhances lung function in patients with COPD [11, 12, 25], and it has received an FDA approval. The GOLD statement recommends its use in a limited patient population, specifically in those with an FEV1 < 50% and features of chronic bronchitis [50]. However, further validation in humans may expand the eligible patient population for this drug based on our results. Oral PDE4 inhibitors are associated with side effects such as nausea, diarrhea, and weight loss, which have limited its clinical use [9]. Inhaled forms of these drugs have been developed to reduce such side effects. This has improved tolerability and holds promise for new therapeutic agents [9, 51, 52]. Future studies are needed to investigate the preventive effects of roflumilast and inhaled PDE4 inhibitors on lung cancer development in human patients with COPD.

Conclusions

In an intermittent smoking-induced lung cancer mouse model, roflumilast suppressed pulmonary inflammation and emphysema and prevented lung cancer development. In vitro analyses suggest that alveolar macrophages may be crucial targets of the anti-inflammatory effects of roflumilast. Further investigations in human patients with COPD are warranted.

Abbreviations

ANOVA	Aanalysis of variance
COPD	Chronic obstructive pulmonary disease
CSE	Cigarette smoke extract
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
H&E	Hematoxylin and eosin
PCR	Polymerase chain reaction
PDE4	Phosphodiesterase-4

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12890-025-03730-w.

Supplementary Material 1: Figure 1: Histological assessment of emphysema in the lungs: Hematoxylin and eosin-stained lung sections illustrating representative morphological changes due to emphysema and the effects of roflumilast treatment. Scale bars: 100 μm. DMSO, dimethyl sulfoxide; Rof, roflumilast; SM, smoke

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Author contributions

K.S. conducted methodology, validation, formal analysis, investigation, original draft writing, and visualization. S.N. contributed to investigation, review & editing, and funding acquisition. S.C. was responsible for conceptualization, original draft writing, supervision, project administration, and funding acquisition. S.O., H.I., and A.T. participated in investigation and review & editing. T.S. and A.E.H. contributed to review & editing and visualization. N.K. and M.S. were involved in methodology, investigation, and review & editing. J.H. contributed to validation, investigation, and review & editing. H.T. and H.Y. participated in methodology and review & editing. Y.K. and K.F. provided resources and contributed to review & editing, with K.F. also supervising the study. All the authors have read and approved the final version of the manuscript.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

All experimental procedures involving animals were approved by the Laboratory Animal Center of Keio University School of Medicine, which serves as the institutional ethics committee for animal research (Approval Number:

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Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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