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Research article

Tangeretin inhibits airway inflammatory responses by reducing early growth response 1 (EGR1) expression in mice exposed to cigarette smoke and lipopolysaccharide

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ABSTRACT

Background: Tangeretin, a natural polymethoxyflavone compound, possesses potent anti-inflammatory activity that improves respiratory inflammation in chronic obstructive pulmonary disease (COPD). However, the molecular mechanisms underlying the anti-COPD effects of tangeretin remain unclear. In this study, we aimed to investigate the key molecular mechanisms by which tangeretin suppresses COPD-related inflammatory responses.

Methods: We conducted the investigation in phorbol-12-myristate-13-acetate (PMA)-stimulated human airway epithelial cells (*in vitro*) and cigarette smoke (CS)/lipopolysaccharide (LPS)-exposed mice (*in vivo*).

Results: Tangeretin decreased the release of inflammatory mediators, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and mucin 5AC (MUC5AC), by suppressing early growth response 1 (EGR1) expression *in vitro*. Tangeretin and EGR1 small interfering ribonucleic acid (siRNA) combination showed a synergistic reduction in MUC5AC and TNF-α secretion. Tangeretin administration significantly inhibited the levels of reactive oxygen species (ROS) production, elastase activity, TNF-α, IL-6, and monocyte chemoattractant protein-1 (MCP-1) secretion, and macrophage and neutrophil numbers in the bronchoalveolar lavage fluid of CS/LPS-exposed mice. Tangeretin also prevented CS/LPS-induced abnormal pathological changes and excessive MUC5AC and EGR1 expression in lung tissue.

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Conclusion: Comprehensively, tangeretin inhibits the lung inflammatory response associated with COPD by reducing EGR1 expression in PMA-induced human epithelial cells and in a CS/LPS-stimulated mouse model. This study shows that tangeretin has anti-COPD properties and can be a promising alternative (or complementary) treatment for inflammatory lung disease.

List of abbreviations

BALF	bronchoalveolar lavage fluid
COPD	chronic obstructive pulmonary disease
CS	cigarette smoke
DCF-DA	2′, 7′-dichlorofluorescin diacetate
EGR1	early growth response 1
ICS	inhaled corticosteroids
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein-1
MUC5AC	mucin 5AC
PBS	phosphate-buffered saline
PMA	phorbol 12-myristate 13-acetate
ROF	roflumilast
ROS	reactive oxygen species
siRNA	small interfering ribonucleic acid
TNF-α	tumor necrosis factor-α

1. Introduction

Pulmonary inflammation is characterized by the overproduction of inflammatory cytokines and mucus proteins [1]. The levels of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 are increased in the sputum of patients with asthma and chronic obstructive pulmonary disease (COPD) [2]. The expression of mucin 5AC (MUC5AC), a major mucin protein secreted from the airway surface epithelium, is markedly upregulated in the lung tissue of ovalbumin (OVA) induced asthmatic mice [3] and in the bronchiolar epithelium of patients with COPD [4]. Inhaled corticosteroids (ICS) are currently recommended for managing common respiratory conditions, such as asthma and COPD [5]. ICS are the most effective controllers of potent anti-inflammatory medicines. However, long-term and high-dose ICS treatments exhibit major side effects and have poor activity in disorders such as severe asthma or COPD [6]. Thus, alternative medicinal treatments using medicinal plants that ensure safety and exhibit anti-inflammatory activity are attracting attention [7]. Notably, some herbal remedies have proven to be effective and safe alternatives (or complements) to standard therapies for inflammatory lung diseases [8]. Therefore, it is important to find more effective and safe herbal medicine materials and analyze their potential mechanisms.

Many plant-derived compounds of different classes, such as alkaloids, flavonoids, glycosides, lignans, polyphenols, and saponins, have been studied for their activities against respiratory diseases and inflammation [9]. Specifically, flavonoids (or bioflavonoids), a class of secondary polyphenol metabolites, have demonstrated biological activities, such as anti-inflammatory, anti-allergic, and antioxidant properties, which are important for improving the respiratory system [10]. Tangeretin (4′, 5, 6, 7, 8-pentamethoxyflavone) is a polymethoxylated flavone found in citrus fruit peels and has neuroprotective [11] and anti-inflammatory properties [12]. However, the therapeutic mechanism underlying its anti-COPD effects is yet to be elucidated.

Cigarette smoke (CS) is a prominent risk factor for COPD; it results in airway inflammation and MUC5AC secretion, which contributes to the pathogenesis of COPD [13]. The bronchoalveolar lavage fluid (BALF) from smokers shows increased levels of inflammatory mediators and immune cells [14]. Moreover, CS induces the spillover of inflammatory mediators, such as cytokines (IL-6 and TNF-α) and reactive oxygen species (ROS) in the lungs [15]. In mice, exposure to CS and lipopolysaccharide (LPS) promotes an inflammatory response and induces lung damage similar to that in patients with COPD [16]. This investigation has led to the establishment of a phenotype in an *in vivo* COPD-like mouse model. In addition, phorbol 12-myristate 13-acetate (PMA) induces MUC5AC secretion in NCI-H292 cells, a human pulmonary mucoepidermoid cell line frequently used to study airway mucin production and gene expression *in vitro* [17]. Therefore, COPD mouse models exposed to CS/LPS and lung epithelial cells stimulated with PMA can be used in therapeutic strategies to investigate effective ways to suppress the inflammatory response of patients with COPD.

Early growth response 1 (EGR1) is an important zinc finger transcription factor closely associated with COPD [18]. CS-induced ROS induces EGR1 expression [19], thereby increasing the expression of the target gene *MUC5AC* and cytokines, such as IL-6 and TNF-α [20], resulting in COPD progression and development [18]. Moreover, the down-regulation of EGR1 expression by EGR1-specific small interfering ribonucleic acid (EGR1 siRNA) resulted in a significant reduction in gene expression, such as cytokine, chemokine, and geranylgeranyl diphosphate synthase, which might affect COPD-related lung pathological processes [21,22]. Indeed, the lungs of patients with COPD show increased EGR1 expression [23], whereas EGR1-knockout mice display significantly reduced airway inflammation and mucus hyperproduction [24]. Therefore, understanding the mechanism of suppressing the expression of EGR1 and inflammatory target genes may be an important strategy for ameliorating lung inflammatory disorders caused by exposure to CS.

This study aimed to explore the inhibitory effect of tangeretin on COPD pathogenesis and its molecular mechanisms in human lung epithelial cells stimulated with PMA and in animals exposed to CS/LPS.

2. Materials and methods

2.1. Chemicals and reagents

We purchased a unique collection library of 131 natural products (#L1400) for MUC5AC enzyme linked immunosorbent assay (ELISA) screening and tangeretin (#S2363) from Selleckchem (Houston, TX, USA). Tangeretin was dissolved using 2 % dimethyl sulfoxide (DMSO) and 2 % Tween 20 in phosphate-buffered saline (PBS), the solvent composition of which was determined based on previous reports [25–28]. The 3R4F cigarettes were purchased from the University of Kentucky (Lexington, KY, USA). PMA (#P1585) and LPS (from *E. coli* serotype 0111:B4) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-EGR1 antibody (#4154) was acquired from Cell Signaling Technology (Danvers, MA, USA) using Western blot. Anti-EGR1 (#sc-101033) for immunohistochemistry and anti-β-Actin (#sc-47778) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-MUC5AC antibody (#ab3649) was purchased from Abcam (Cambridge, UK). The siRNA targeting EGR1 (#sc-29303) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA), and a non-targeting siRNA (#SN-1012) as a negative control was obtained from Bioneer (Daejeon, South Korea).

2.2. Cell lines and culture conditions

Human airway epithelial cells (NCI-H292, #CRL-1848) and human embryonic kidney cells (HEK293T, #CRL-3216) were obtained from the American Type Culture Collection. NCI-H292 and HEK293T cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 (#LM011-01) and Dulbecco's Modified Eagle Medium (DMEM, #LM001-05, WELGENE, Daegu, South Korea), respectively, and added to 10 % fetal bovine serum (#16000044) and 100 μg/ml streptomycin plus 100 units/ml penicillin (#15140122) (Thermo Fisher Scientific, Waltham, MA, USA). Human bronchial epithelial (NHBE) cells (#CC-2540) were purchased from LONZA (Basel, Switzerland). NHBE cells were maintained in bronchial epithelial cell growth basal medium (#CC-3171) supplemented with SingleQuots™ bronchial epithelial cell growth medium supplements and growth factors (#CC-4175). All cells were cultured at 37°C and in a humidified 5 % CO_2 atmosphere.

2.3. Cytotoxicity analysis

NCI-H292 and NHBE cells (1×10^4 cells/well) were grown in 96-well culture plates for 24 h. For cell starvation, the medium was changed to a low serum medium for 16 h. The cells were incubated with applicable concentrations of tangeretin for 2 h and treated with PMA. After 24 h, cell counting kit-8 (CCK-8) solution (#CK04, Dojindo Laboratories, Kumamoto, Japan) was added to the culture medium, and cell viability was measured at 450 nm using an Epoch microplate reader (Biotek Instruments, Winooski, Vermont, USA).

2.4. Enzyme-linked immunosorbent assays

The cells were seeded in 96-well microplates for 24 h. The complete growth medium was replaced with reduced serum, and the cells were incubated for 16 h. The cells were treated with tangeretin for 2 h, PMA was added to the medium for 6 h. We measured TNF-α (#555212) and IL-6 (#555220) protein levels in the supernatants according to the manufacturer's instructions (BD PharMingen, San Diego, CA, USA). We measured MUC5AC protein levels in cell culture supernatants and lung tissue lysates, as described elsewhere [29]. The absorbance was measured at 450 nm using an Epoch microplate reader. The measured absorbance was converted to a percentage (%) of the control value.

2.5. Construction of luciferase reporter gene for MUC5AC gene promoter

The construction of a reporter clone for the promoter region (−1384 to +31) of *MUC5AC* (*MUC5AC* promoter-Luc) has been described previously [29]. EGR1 binding sites within the *MUC5AC* promoter (−1384 to +31) were predicted to be R1 (−1090 to −1079) and R2 (−796 to −785) using TFBIND (http://tfbind.hgc.jp/) software. Polymerase chain reaction-amplified from genomic DNA isolated from NCI-H292 cells was used as a primer to generate a reporter clone for the promoter region (−974 to +31) of *MUC5AC* promoter-Luc. Human *MUC5AC*-specific products were amplified using the following parameters: (forward) 5′-AGATCTGGACCTTTCTGTGCTGGG-3′ and (reverse) 5′-AAGCTTGAGGGACCCAAGGTGGCA-3'. The amplified *MUC5AC* promoter region (−974 to +31) was cloned between the pGL4.14 vector (#E6691; Promega, Madison, WI, USA) with *Bgl*II (#R0144; NEB, Ipswich, MA, USA) and *Hin*dIII (#R3104; NEB, Ipswich, MA, USA) restriction sites for luciferase expression. HEK293T cells were seeded in 96-well plates at a density of 2×10^4 cells/well and cultured for 24 h to measure the luciferase activity. The cells were transiently transfected with a reporter plasmid using Lipofectamine 3000 (#L3000015; Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Briefly, 200 ng firefly luciferase reporter plasmid and 4 ng Renilla reporter plasmid were mixed with Lipofectamine 3000 and added to each well. After transfection for 16 h, the cells were pretreated with different concentrations of tangeretin for 2 h. PMA (50 nM) was then administered for 6 h, and luciferase reporter activity was evaluated using a dual-luciferase assay system (#E2940, Promega, Madison, WI) and a plate reader (SpectraMax M4; Molecular Devices, Sunnyvale, CA, USA). The luciferase reporter activity (the ratio of firefly luciferase activity normalized to Renilla luciferase activity) was calculated as a percentage (%) of the control value (the group treated with PMA).

2.6. Western blot analysis

The cells (NCI-H292) were washed twice with PBS and lysed in CETi lysis buffer (#TLP-121; TransLab, Daejeon, South Korea). After centrifugation, equal amounts of protein lysates were subjected to electrophoresis and electrotransferred onto polyvinylidene fluoride membranes. The membrane was reacted with primary antibodies (anti-EGR1 or anti-β-Actin) and incubated with secondary antibodies. We analyzed data using an Amersham Imager 600 (Amersham, Little Chalfont, UK), and measured quantifiable data using Multi Gauge software (version 3.0; Fujifilm, Tokyo, Japan). Supplementary Figs. S1–S2 show the original images for all relevant western blots.

2.7. Transfection of siRNA

NCI-H292 cells were transfected with siRNA targeting EGR1 or non-targeting siRNA using Lipofectamine RNAiMAX (#13778150; Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Briefly, NCI-H292 cells were seeded into 24 well plates. The cells were transfected with 25 nM siRNA in opti-MEM (#31985070, Thermo Fisher Scientific, Waltham, MA, USA) and grown in a 37°C incubator for 48 h. The cells were treated with tangeretin for 2 h and then stimulated with PMA.

2.8. Lung inflammation mice model induced by CS exposure and LPS treatment

We placed the six-week-old male mice (Koatech Co., Pyeongtaek, Republic of Korea) in a smoking chamber (SciTech Korea, Inc., Republic of Korea) for 7 d [whole-body cigarette smoke (CS) exposure: 1 h daily]. On day 6, the mice were intranasally injected with 5 μg LPS dissolved in 30 μl distilled water. On days 1–7, tangeretin and roflumilast (ROF), dissolved in PBS with 2 % DMSO and 2 % Tween 20, were administered orally (p.o.) to the mice [27]. The experimental groups were as follows: normal control (NC), COPD [CS-exposed and LPS administered mice], ROF (COPD + 5 mg/kg roflumilast, p.o.), T 7.5 (COPD + 7.5 mg/kg tangeretin, p.o), and T 15 (COPD + 15 mg/kg tangeretin, p.o).

We empirically determine the drug dosage for *in vivo* tests, which is about 1000 times higher than for *in vitro* tests. For example, to get a 7.5 mg/kg dosage, we first identified the *in vitro* effective dose of tangeretin, which ranged from 5 to 20 μM (Fig. 1). These values

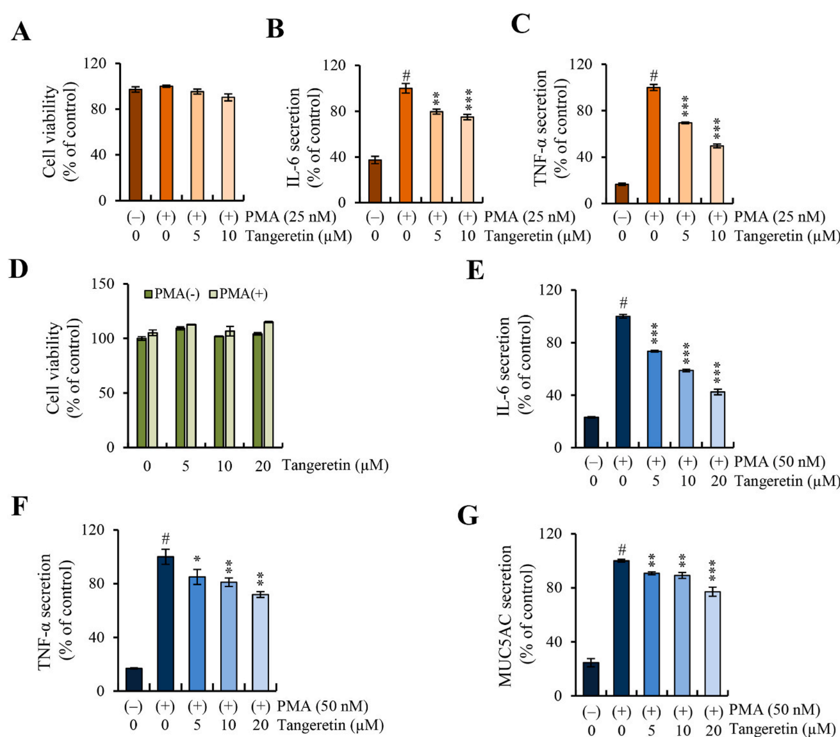

Fig. 1. Tangeretin inhibited inflammatory responses in PMA-stimulated normal human bronchial epithelial cells (NHBE) and human airway epithelial cells (NCI-H292). (A) The effect of tangeretin on the viability of PMA-treated NHBE cells was investigated using a cell counting kit 8 (CCK-8). (B–C) The levels of inflammatory cytokines (IL-6 and TNF-α) in the supernatants of PMA-treated NHBE cells were measured using enzyme linked immunosorbent assay (ELISA). (D) The viability of NCI-H292 cells was assessed following treatment with tangeretin in the absence or presence of PMA using the CCK-8 assay. (E–G) The effect of tangeretin on the secretion of IL-6, TNF-α, and MUC5AC in PMA-stimulated NCI-H292 cells was assayed by ELISA. Error bars indicate the mean ± standard deviation of values from three independent experiments. #$p < 0.01$ (compared to the group without PMA treatment); *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (compared to the PMA-only treated group).

were then converted to the volume-based values of 1.87–7.5 μg/ml. Next, the volume-based values are roughly converted to the weight-based values of 1.87–7.5 μg/g, assuming 1 ml is equivalent to 1 g. Finally, 1.87–7.5 mg/kg was obtained by multiplying 1000.

2.9. Analysis of bronchoalveolar lavage fluid in a mouse model

On day 8, the zoletil/xylazine mixture was injected intraperitoneally (i.p.) into the mice for anesthesia, and cannula tracheal insertion/BALF collection was performed based on a previous protocol [30]. ROS production and elastase activity in BALF cells were detected using 2′,7′-dichlorofluorescin diacetate (DCF-DA) and N-succinyl-(Ala)3-p-nitroanilide (#D6883, Sigma-Aldrich, CA, USA), respectively, based on previous protocol [31]. The BALF cells were were transferred onto glass using a cytospin to count the immune cells. The cells were placed in Diff-Quik® solution (#38721, SYSMEX, Kobe, Japan), and the stained cells were observed under a light microscope (×400 magnification) [32]. Cytokine and chemokine expression in BALF supernatant was detected using ELISA kits (TNF-α/IL-6/MCP-1, all: BD Biosciences, CA, USA).

2.10. Hematoxylin and eosin (H&E) analysis

Each lung was fixed in formalin, embedded in paraffin, and cut using a microtome (each section: 4 μm) to detect histological changes. Mouse lung paraffin sections were placed in a hematoxylin and eosin solution (Sigma-Aldrich, CA, USA). The lung tissue samples were visualized using a light microscope (H&E staining: magnification ×200; scale bar 50 μm and magnification ×400; scale bar 25 μm).

2.11. (IHC) analysis

The lung tissues were fixed, paraffin-embedded, and cut to 6–7 μm thickness, and the sections were deparaffinized. Antigen retrieval was performed using Tris-ethylene diamine tetraacetic acid buffer (pH 9.0) for 15 min at 120°C in a pressure cooker. After washing with water, the endogenous peroxidase activity was blocked by treating the tissue with 3 % H_2O_2 for 15 min. After blocking for 15 min, the tissues were incubated overnight at 4°C with anti-EGR1 and Vectastain ABC reagent according to the Vectastain ABC kit instruction (#PK-4000; Vector Laboratories, Newark, CA, USA). Targeted staining for anti-EGR1 was visualized with 3,3′-Diaminobenzidine. Next, counterstaining was performed using Mayer's hematoxylin solution for 3 min. The sections were dehydrated, cleared, and mounted on coverslips. The lung tissue samples were visualized using a light microscope with magnifications of approximately ×200 and ×400.

2.12. Statistical analysis

Data are presented as mean ± standard deviation. Statistical significance was analyzed using a two-tailed Student's *t*-test for in *vitro* experiments. A one-way analysis of variance, followed by Dunnett's multiple comparison test, was used to analyze the differences between multiple groups for in *vivo* experiments using the statistical package for social sciences software (version 20.0; IBM Corp., NY, USA). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Tangeretin suppressed inflammatory responses in PMA-induced human lung epithelial cells

MUC5AC is a pathological marker in lung inflammatory diseases such as COPD [4]. Hence, we performed MUC5AC ELISA in PMA-stimulated airway epithelial cells (NCI-H292) using the 131 natural products library. Tangeretin significantly inhibited MUC5AC secretion and was selected for subsequent experiments (data not shown). We evaluated the anti-inflammatory effects of tangeretin using two types of human epithelial cells: primary NHBE and NCI-H292 cells. First, we determined whether tangeretin was cytotoxic to NHBE and NCI-H292 cells. Tangeretin treated at < 10 μM in NHBE cells (Fig. 1A) and 20 μM in NCI-H292 cells (Fig. 1D) did not affect on cell growth, regardless of the presence of PMA. The secretion levels of inflammatory cytokines, such as IL-6 and TNF-α in PMA-stimulated NHBE and NCI-H292 cells were measured using ELISA within the range of no tangeretin cytotoxicity. PMA stimulation increased IL-6 and TNF-α secretion levels, whereas tangeretin significantly inhibited their secretion in NHBE (Fig. 1B and C) and NCI-H292 cells (Fig. 1E and F). MUC5AC was detected in NCI-H292 cells but not in NHBE cells and was detected only in NCI-H292 cells stimulated with PMA. Increasing tangeretin concentrations significantly suppressed the secretion level of MUC5AC (Fig. 1G). These results show that tangeretin reduced the secretion of inflammatory mediators from both types of human lung epithelial cells, suggesting that tangeretin exerts anti-inflammatory effects on human lung epithelial cells in response to external stimuli.

3.2. Tangeretin decreased EGR1 and MUC5AC expression in PMA-stimulated NCI-H292 cells

We performed ELISA to measure the suppressive effect of tangeretin on MUC5AC expression depending on PMA stimulation time. MUC5AC secretion level significantly increased starting 8 h after PMA stimulation, whereas tangeretin decreased this secretion starting 12 h after adding PMA (Fig. 2A).

Since EGR1 induces MUC5AC expression, exacerbating COPD pathogenesis [18], we assessed whether tangeretin affects EGR1

expression using western blotting. PMA stimulation increased EGR1 expression level (Fig. 2B, lane 2), whereas tangeretin significantly inhibited this increase (Fig. 2B, lanes 3–5), as shown by the relative quantitative changes (Fig. 2C).

We evaluated the activation of the *MUC5AC* promoter by tangeretin to determine whether tangeretin affects the transcriptional level of MUC5AC. HEK293T cells were transiently transfected with plasmid DNA containing the *MUC5AC* promoter (HEK293T/*MUC5AC* promoter-Luc, −1384 to +31), and the luciferase reporter activation was measured at each concentration in the presence of PMA. PMA stimulation increased the activation of the *MUC5AC* promoter-Luc reporter gene, whereas this activity was significantly decreased by tangeretin pretreatment in a dose-dependent manner (Fig. 2D).

We analyzed the activity of luciferase reporter based on the two predicted binding sites of EGR1 (R1 and R2) within the *MUC5AC* promoter to investigate further whether the activity of EGR1 affects the transcriptional level of *MUC5AC*. Similar to the result indicated in Fig. 2D, PMA increased luciferase activity of *MUC5AC* promoter (HEK293T/*MUC5AC* promoter-Luc, −1384 to +31) containing R1 (−1090 to −1079) and R2 (−796 to −785), whereas tangeretin significantly decreased these activities. However, luciferase activity was slightly increased in the truncated short plasmid DNA containing only R2 (HEK293T/*MUC5AC* promoter-Luc, −974 to +31). It showed little change after treatment with tangeretin (Fig. 2E). These results show that tangeretin inhibited the transcriptional level of MUC5AC by reducing EGR1 expression binding activity of R1, but not R2, within the *MUC5AC* promoter (HEK293T/*MUC5AC* promoter-Luc).

3.3. Tangeretin suppresses EGR1-mediated MUC5AC and TNF-α expression in PMA-stimulated NCI-H292 cells

We analyzed the inflammatory response due to EGR1 downregulation using EGR1 siRNA and analyzed changes in combination with tangeretin to measure the effect of tangeretin on EGR1 expression and the inflammatory response more accurately. Before

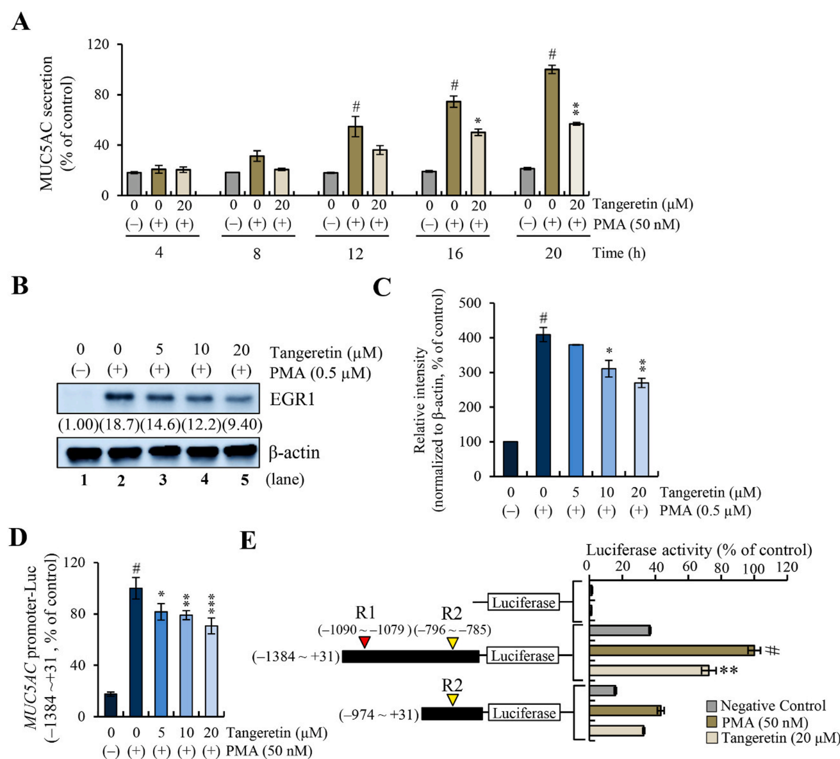

Fig. 2. Tangeretin suppressed EGR1 and MUC5AC expression in PMA-stimulated NCI-H292 cells. (A) NCI-H292 cells were pretreated with the tangeretin (20 μM) for 2 h and then treated with PMA (50 nM). MUC5AC secretion levels were assessed at various time points using ELISA. (B–C) The NCI-H292 cells were treated with each concentration of tangeretin for 2 h and then with PMA (0.5 μM) for 30 min. Total cell lysates were analyzed through western blotting using EGR1 antibody (B), and the bar graph shows relative quantification changes in the EGR1 protein (C). Representative blots of three independently performed experiments are shown. Original images for Western blot are provided as supplementary file. Anti-β-Actin was chosen as an internal loading control. The numbers below the bands indicate relative band intensity (calculated fold change compared to control without added PMA). (D) Effect of tangeretin on the luciferase activity of *MUC5AC* promoter in HEK293T cells. The cells were transiently transfected with the *MUC5AC* promoter (HEK293T/*MUC5AC* promoter-Luc), a luciferase reporter gene fused to the *MUC5AC* promoter region (−1384 to +31), for 16 h. The cells were then pretreated with tangeretin for 2 h, followed by incubation with PMA (50 nM) for 6 h. (E) The effect of tangeretin on two *MUC5AC* promoter sequences (−1384 to +31 and −974 to +31) constructed based on two regions (R1 and R2) predicted to bind to EGR1 was measured using a luciferase activity assay. Error bars indicate the mean ± standard deviation of values from three independent experiments. #, $p < 0.01$ (compared with the group without PMA treatment); *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ (compared with the group with PMA only).

analysis, a CCK-8 assay was performed to determine whether EGR1 siRNA and tangeretin combination affected cytotoxicity. Treatment with EGR1 siRNA alone or in combination with tangeretin did not affect NCI-H292 cells (Fig. 3A). We measured MUC5AC and TNF-α secretion levels using ELISA to determine whether EGR1 downregulation and tangeretin combination affect inflammatory mediators. The downregulation of EGR1 by EGR1 siRNA suppressed the PMA-induced levels of MUC5AC and TNF-α. Moreover, EGR1 siRNA and tangeretin combination potently reduced the PMA-induced secretion of MUC5AC and TNF-α compared with EGR1 siRNA or tangeretin alone (Fig. 3B). At this time, we performed western blotting to confirm EGR1 expression in NCI-H292 cells. This result showed that EGR1 expression was downregulated by EGR1 siRNA and more significantly reduced by EGR1 siRNA and tangeretin combination compared to non-targeting RNA under PMA alone or in combination with PMA and tangeretin (Fig. 3C and D). These results show that tangeretin inhibits the expression of inflammatory mediators, including MUC5AC and TNF-α, by decreasing the EGR1 expression.

3.4. Tangeretin reduces the secretion of inflammatory mediators in CS/LPS-induced COPD mouse model

CS is the most prominent risk factor for COPD due to increased inflammatory mediator production and mucin secretion in the sputum of patients with COPD [13]. Moreover, LPS promotes lung inflammation *in vivo*, similar to chronic lung inflammatory diseases in humans [16]. Hence, we examined whether tangeretin administration could reduce the inflammatory responses in the lungs of mice stimulated with a mixture of CS and LPS (CS/LPS), according to the experimental design shown in Fig. 4A.

BALF acquired from the lungs of mice with COPD caused by CS/LPS significantly increased inflammatory mediators, including ROS production, elastase activation, and pro-inflammatory cytokine/chemokine levels (such as TNF-α, IL-6, and MCP-1), compared to NC mice (Fig. 4, # in panels B–F). However, the administration of tangeretin and ROF significantly suppressed this inflammatory response. ROF, a drug approved by the United States Food and Drug Administration for chronic airway inflammatory disorders, was used in this experiment, and it served as a positive control [33]. These results support the potential anti-inflammatory activity of tangeretin in the CS/LPS mouse model.

3.5. Tangeretin reduces the recruitment of inflammatory cells and the expression of EGR1 and MUC5AC in the COPD mice model

Since CS induces the increased recruitment of immune cells and abnormal histopathology in the lungs *in vivo* [34], we tested the effect of tangeretin administration on immune cell recruitment in the BALF of CS/LPS-exposed mice. The results showed an increased accumulation of neutrophils (Fig. 5A, #) and macrophages (Fig. 5B, #) in the BALF of CS/LPS-exposed mice. However, ROF and

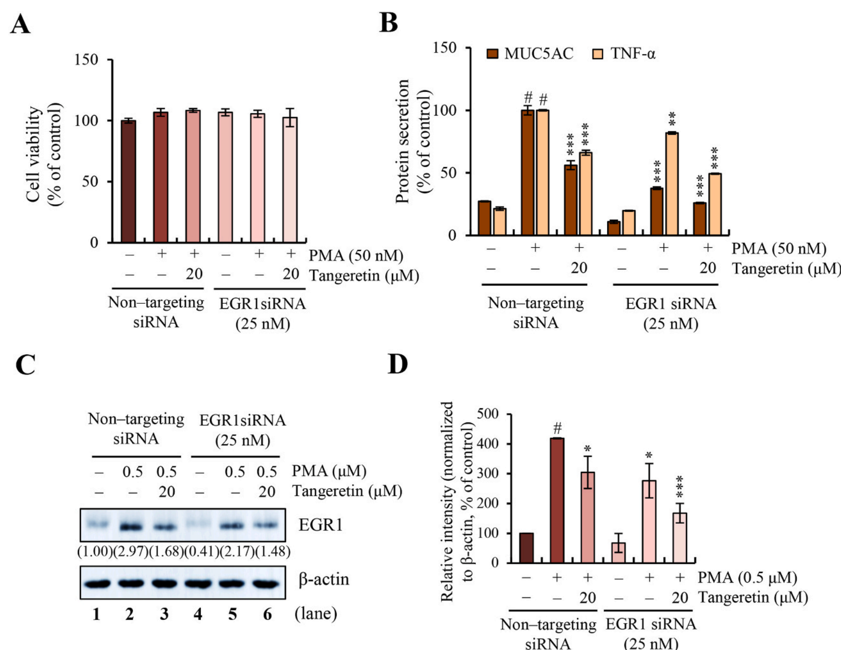

Fig. 3. Tangeretin reduced the secretion levels of MUC5AC and TNF-α by downregulating EGR1 in PMA-stimulated NCI-H292 cells. (A) The cell viability of NCI-H292 cells was measured by CCK-8 after tangeretin treatment following transfection with non-targeting RNA or EGR1 siRNA in the presence or absence of PMA. (B) The effect of EGR1 siRNA and tangeretin combination on the secretion levels of MUC5AC and TNF-α proteins, respectively, was measured using ELISA. (C–D) The effect of EGR1 siRNA and tangeretin combination on EGR1 protein expression using Western blot analysis (C). A bar graph showing relative quantitative changes in EGR1 protein expression in panel C (D). Representative blots of three independently performed experiments are shown. Original images for Western blot are provided as supplementary file. Error bars indicate the mean ± standard deviation of values from three independent experiments. #, $p < 0.01$ (compared to the group without PMA treatment in non-targeting siRNA); *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ (compared to PMA-only treated group in non-targeting RNA).

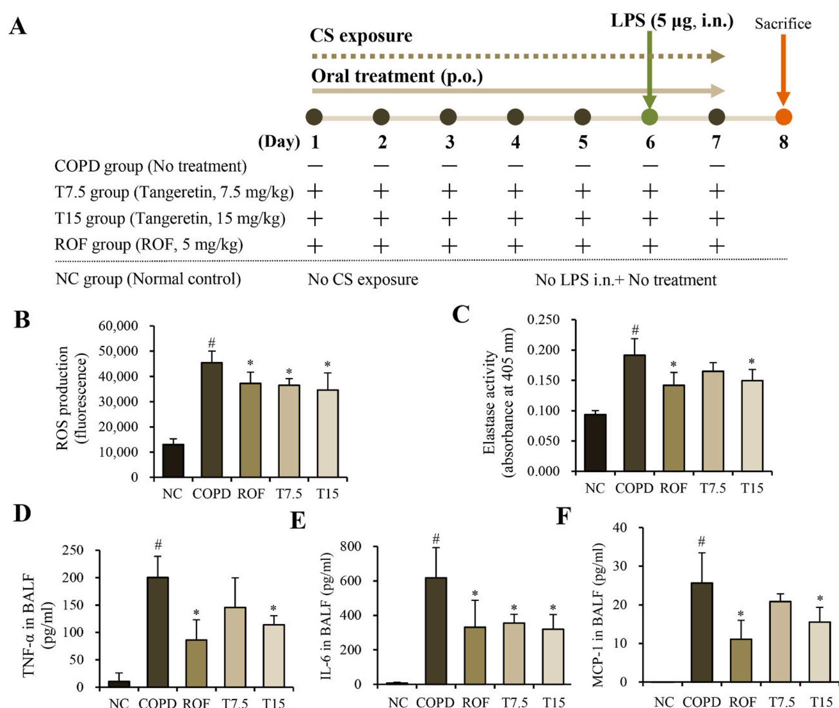

Fig. 4. Tangeretin suppressed the inflammatory response in the airways of a cigarette smoke (CS)/lipopolysaccharide (LPS)-induced COPD mouse model. (A) Experimental schematic representation of tangeretin administration in a CS/ LPS-induced COPD mouse model. (B–F) The levels of inflammatory mediators such as ROS (B), elastase activity (C), TNF-α (D), IL-6 (E), and MCP-1 (F) significantly increased in the bronchoalveolar lavage fluid (BALF) of COPD mice. Tangeretin effectively decreased the levels of these increased inflammatory mediators. The reduction effect of 15 mg/kg tangeretin was similar to that of 5 mg/kg roflumilast (ROF). NC, normal control mice; COPD, CS and LPS-exposed mice; ROF, CS and LPS-exposed mice treated with ROF (5 mg/kg); T 7.5 and 15, CS and LPS-exposed mice treated with tangeretin (7.5 and 15 mg/kg, respectively); #, significantly different from NC, $p < 0.01$; *, significantly different from COPD, $p < 0.05$. i.n., intranasal; p.o., per oral.; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1. Values are the mean ± standard deviation (SD; $n = 6$).

tangeretin significantly inhibited this increase in inflammatory cells (Fig. 5A–B, asterisk). H&E staining showed the recruitment of immune cells with dense peribronchial infiltrates in the lung tissue of mice exposed to CS/LPS (Fig. 5C, white arrows shown in enlarged image of black square). However, these histopathological changes were attenuated by ROF or tangeretin administration.

We monitored EGR1 expression in the lungs of mice by immunostaining because tangeretin exerts anti-inflammatory activity by inhibiting EGR1 and MUC5AC expression *in vitro* (Fig. 6A, black square). Increased EGR1 expression in the lungs of CS/LPS-exposed mice was observed mainly in the airway epithelium (Fig. 6A, black arrows shown in enlarged image of black square). In contrast, ROF or tangeretin suppressed EGR1 expression (Fig. 6A, bottom panels). In addition, lung lysate data targeting MUC5AC using ELISA showed that MUC5AC expression increased in the CS/LPS-induced group (Fig. 6B) but was inhibited by ROF and tangeretin treatment. These findings suggest that tangeretin ameliorates lung inflammatory responses by inhibiting the expression of inflammatory mediators, including MUC5AC, and by reducing EGR1 expression in PMA-stimulated human epithelial cells and CS/LPS-exposed mouse models.

4. Discussion

ICS is an effective drug for improving chronic lung inflammatory disorders such as COPD and asthma [5]. However, ICS is limited in terms of long-term use and high–doses due to adverse effects, including osteoporosis and pneumonia [35]. Therefore, there is increasing recognition of the significance of medicinal plants and their bioactive compounds with anti-inflammatory effects for the safe and effective treatment of chronic lung inflammation symptoms. Various herbal therapies may be used as an alternative to standard treatments for lung inflammatory diseases to overcome the limitations of ICS [36].

Recent studies have shown that some flavonoids can treat pulmonary inflammatory diseases [37], and this approach is effective in relieving systemic and respiratory inflammation [10,38]. Thus, it is important to identify the classes of flavonoids with anti-inflammatory effects and identify their mechanisms.

Notably, studies using lung epithelial cells stimulated with PMA and a COPD mouse model exposed to CS/LPS were performed to identify the molecular mechanism underlying the anti-inflammatory activity of tangeretin (a flavonoid).

It is well known that CS is a major risk factor for COPD development [13]. Patients with COPD who smoke have worse lung function compared to non-smokers due to impaired airflow limitation, emphysema, chronic cough, and mucus secretion [39,40]. Moreover, the

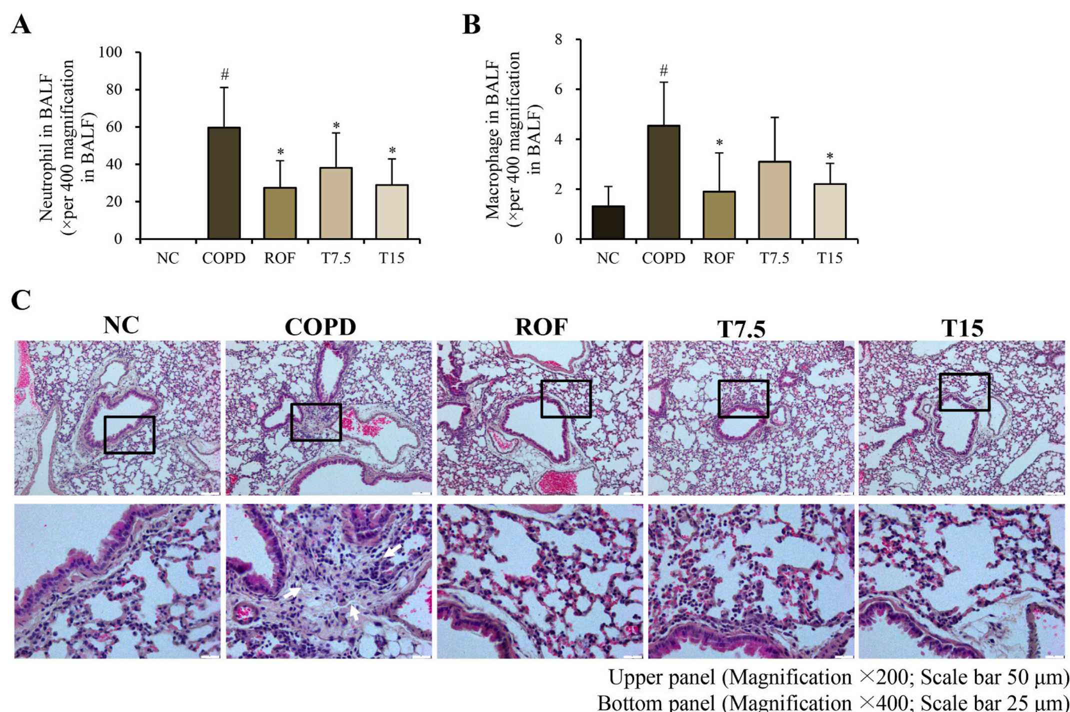

Fig. 5. Tangeretin reduced inflammatory cell recruitment and abnormal histological changes in the lung tissues of the COPD mouse model. (A–B) The number of infiltrated inflammatory cells in the BALF was calculated using the Diff-Quik reagent. Increased numbers of neutrophils (A) and macrophages (B) in the BALF of COPD mice significantly reduced after tangeretin administration. (C) Hematoxylin and eosin (H&E) staining of the peribronchial region of the lungs. The bottom panel is an enlarged version of the black square in the upper panel (upper panel: magnification ×200, scale bar 50 μm; bottom panel: magnification ×400, scale bar 25 μm). A dense accumulation of immune cells was detected around the airway tissues of COPD mice (white arrows). In contrast, there is no significant accumulation of inflammatory cells around the airway tissues in the normal control (NC), 7.5 mg/kg tangeretin (T 7.5), 15 mg/kg tangeretin (T 15), or ROF groups. #, significantly different from NC, $p < 0.01$; *, significantly different from COPD, $p < 0.05$.

lungs of CS-exposed mice had increased levels of inflammatory mediators (TNF-α, IL-6, MCP-1, ROS, and elastase) and an increased number of immune cells (mainly neutrophils and macrophages) [41]. Thus, drugs that inhibit these inflammatory mediators are believed to have potential efficacy in managing COPD.

This study showed that tangeretin inhibited IL-6, TNF-α, and MUC5AC secretion in PMA-induced human airway epithelial cells. In the lungs of mice with CS/LPS-induced COPD, tangeretin administration suppressed the infiltration of immune cells (especially neutrophils and macrophages) and the production of inflammatory mediators, including TNF-α, IL-6, ROS, and elastase activity. These results suggest that tangeretin can alleviate lung inflammatory diseases, such as COPD.

EGR1 is considered a potential target for treating COPD [18,42]. EGR1 regulates the expression of the MUC5AC protein, which is a serious hallmark of the exacerbation or pathogenesis of respiratory lung diseases, such as asthma [43] and COPD [44]. Thus, inhibiting EGR1 and its target genes is an important signaling pathway for managing asthma and COPD [45].

In this study, among the two regions where EGR1 is predicted to bind to the *MUC5AC* promoter (−1384 to +31), tangeretin inhibited EGR1 activity by interfering with EGR1 binding to the R1 (−1090 to−1079) region, but not to the R2 region (−796 to −785). Moreover, the downregulation of EGR1 by siRNA suppressed the expression of inflammatory mediators such as TNF-α and MUC5AC, and the combination with tangeretin further enhanced this effect.We demonstrated for the first time that tangeretin alleviates EGR1 and MUC5AC expression in PMA-induced human airway epithelial cells and lungs in a CS/LPS-exposed COPD mouse model. These results suggest that tangeretin exerts its anti-COPD activity by inhibiting EGR1 expression. Interestingly, as shown in Fig. 3B, a smaller amount of EGR1 siRNA more effectively reduced MUC5AC secretion than a larger amount of tangeretin's effect on MUC5AC secretion. The suppressive effect of EGR1 siRNA was more specific for MUC5AC secretion than for TNF-α secretion, while tangeretin equally suppressed both MUC5AC and TNF-α secretion. These data suggest that the anti-inflammatory effect of EGR1 siRNA is specific to the EGR1-MUC5AC axis, while that of tangeretin has broader targets, including MUC5AC and TNF-α. Since EGR1 siRNA and tangeretin have distinct anti-inflammatory properties, we can surmise that combining treatment with both tangeretin and EGR1 siRNA could be an effective way to manage COPD.

Recently, Li et al. demonstrated that tangeretin reduces the activity of Th17 cells to attenuate lung inflammation [46]. They found that tangeretin accomplishes this by suppressing the activity of Notch signaling in Th17 cells, which is necessary for the transcriptional expression of RORγt and IL-23R genes. Since RORγt and IL-23R are crucial for Th17 cell identity and differentiation, the authors suggest that tangeretin inhibits Th17 differentiation, ultimately reducing airway inflammation.

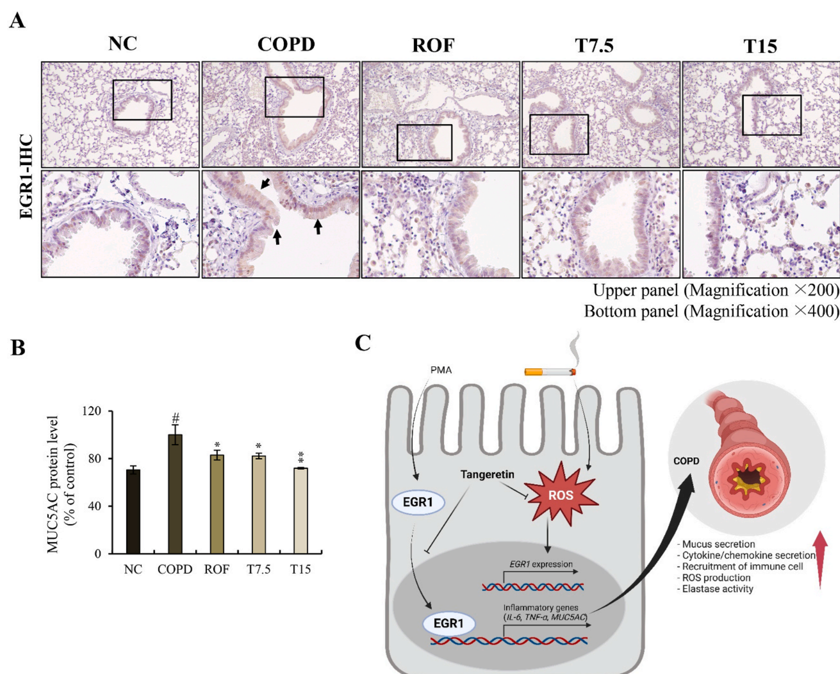

Fig. 6. Tangeretin reduced the MUC5AC and EGR1 expression in the lung tissue of the COPD mouse model and a schematic illustration of the effect of tangeretin on inflammatory response on PMA-induced human epithelial cells and CS/LPS-exposed COPD mice. (A) Immunostaining of EGR1 in lung tissues. The increased expression of EGR1 in the airway epithelium of the COPD group lung tissue (black arrows in the bottom image) was decreased by administering tangeretin or ROF. The bottom panels (magnification ×400) are enlarged from the black squares in the upper panels (magnification ×200). (B) The levels of MUC5AC protein in lung tissues were assayed using ELISA. #, significantly different from NC, $p < 0.01$; *, $p < 0.05$, **, $p < 0.01$, significantly different from COPD. (C) PMA and CS induce EGR1 expression and activation, which is considered a therapeutic target for inflammation-related lung diseases. Tangeretin inhibits EGR1 expression, thereby reducing inflammatory cytokine and MUC5AC secretion in PMA-induced human airway epithelial cells. Furthermore, tangeretin reduces the airway inflammatory response such as mucus secretion, cytokine/chemokine secretion, recruitment of immune cells, ROS production, and elastase activity in CS/LPS-exposed COPD mice, and in this case, the expression of EGR1 and MUC5AC is suppressed in lung tissue. This figure was generated with BioRender (https://biorender.com/).

Our results showed that tangeretin effectively inhibited the inflammatory response induced by CS, suggesting that tangeretin may be utilized as a supplement for smokers and people with respiratory diseases exposed to irritants or air pollution. Therefore, we propose that tangeretin from natural sources is a promising treatment for inflammatory lung diseases, including COPD.

5. Conclusions

Our findings are the first to demonstrate that tangeretin alleviates EGR1 expression and inflammatory response, both *in vitro* and *in vivo*. Tangeretin inhibited the increased expression of EGR1 induced by either PMA or CS, thereby reducing the levels of inflammatory mediators, inflammatory cells, mucus secretion, and abnormal histopathology associated with COPD (Fig. 6C). Therefore, we propose that tangeretin may be a promising anti-COPD drug candidate for the replacement (or complement) of ICS.

CRediT authorship contribution statement

Eun Sol Oh: Writing – original draft, Visualization, Validation, Investigation. **Jae-Won Lee:** Writing – original draft, Visualization, Validation. **Yu Na Song:** Visualization, Validation, Methodology, Investigation. **Mun-Ock Kim:** Visualization, Validation, Supervision. **Ro Woon Lee:** Validation, Methodology, Investigation. **Myung-Ji Kang:** Validation, Methodology, Investigation. **Juhyun Lee:** Validation, Methodology. **Seok Han Yun:** Validation, Methodology. **Sung-Tae Hong:** Writing – review & editing, Resources. **Hyunju Ro:** Writing – review & editing. **Su Ui Lee:** Writing – original draft, Project administration, Funding acquisition, Conceptualization.

Ethics statement

The procedures for the animal experiments was approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology approved the on April 10, 2020 (KRIBB, Chungbuk, Korea; KRIBB-AEC-20216). We performed the procedure in compliance with the National Institute of Health guidelines for the Care and Use of Laboratory Animals and the Korean National Laws for Animal Welfare.

Data availability

Data will be made available on request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e39797.

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