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Original Article

Response biomarkers of inhalation exposure to cigarette smoke in the mouse lung

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Abstract: Cigarette smoking is known to increase the risk of cancer and chronic obstructive pulmonary disease (COPD). In this study, we evaluated the effects of short-term nose-only inhalation exposure to cigarette smoke in mice. Male 10-week-old C57BL mice were exposed to clean air (control) or mainstream cigarette smoke for 1 h/day, 5 days/week, for 2 or 4 weeks. Exposure to cigarette smoke increased the number of inflammatory cells, especially neutrophils, in the bronchoalveolar lavage fluid, increased inflammatory cell infiltration foci, and caused an increase in the thickness of the peripheral bronchial epithelium. Microarray gene expression analysis indicated that smoke exposure induced inflammatory responses, including leukocyte migration and activation of phagocytes and myeloid cells, as early as two weeks after the initiation of exposure. Importantly, chemokine (C-C motif) ligand 17, resistin-like alpha, and lipocalin 2 were upregulated and may serve as useful markers of the toxic effects of exposure to cigarette smoke before pulmonary histological changes become evident. (DOI: 10.1293/tox.2021-0077; J Toxicol Pathol 2022; 35: 247–254)

Key words: response marker, cigarette smoke, lung, mice

Introduction

Epidemiological studies have demonstrated that cigarette smoking increases the risk of developing several cancers such as lung cancer[1, 2] and chronic obstructive pulmonary disease (COPD) that includes emphysema and chronic bronchitis[3]. COPD is a serious threat to public health because of its prevalence, economic cost, and impact on death and disability. The pathobiology of COPD encompasses multiple injurious processes, including inflammation, cellular apoptosis, impaired alveolar maintenance, abnormal cell repair, extracellular matrix destruction, and oxidative stress[4]. Several animal models of cigarette smoking-induced pulmonary emphysema have been established[5–8]. Understanding the mechanisms by which cigarette smoke induces pulmonary emphysema will facilitate the development of strategies for the treatment and prevention of smoking-related diseases. Neutrophilic inflammation is characteristic of pulmonary emphysema, and airway neutrophilia is associated with a decline in lung function which leads to pulmonary emphysema[9]. In our previous 12-week inhalation studies, we demonstrated that cigarette smoke induces emphysema in mice via the induction of inflammation and oxidative stress[10, 11].

In the present study, we evaluated the effects of short-term inhalation exposure to cigarette smoke on mouse lungs and identified response genes to cigarette smoke in a mouse pulmonary emphysema model[10, 11].

Materials and Methods

Animal experiments

All animal studies were approved (Permit Number: 17023) by the Institutional Animal Care and Use Committee of Osaka Metropolitan University Graduate School of Medicine and were conducted in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006). The Laboratory Animal Center of the Osaka Metropolitan University Graduate School of Medicine is accredited by the Center for Accreditation

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of Laboratory Animal Care and Use, Japan Health Sciences Foundation.

Nine-week-old C57BL/6J male mice were purchased from Charles River Laboratories, Japan (Atsugi, Japan). They were housed 5 per cage on wood-chip bedding in an air-conditioned animal room at 22 ± 2°C and 55 ± 10% humidity. All mice were allowed free access to a pelleted diet (CE2; Clea Japan Inc., Tokyo, Japan) and tap water, except for exposure to cigarette smoke or clean air. All mice were acclimatized for 1 week prior to the beginning of the study.

Twenty mice were divided into two groups (control and cigarette smoke-exposed groups). The animals were exposed to clean air (control group) or mainstream cigarette smoke (cigarette smoke-exposed group) for 1 h/day (10–11 am), 5 days/week, for 2 or 4 weeks. During exposure, each mouse in the exposure group was placed in an acrylic holder attached to an inhalation exposure chamber (SIS; Sibata Scientific Technology Ltd., Saitama, Japan) and nose-only exposed to mainstream cigarette smoke. Similarly, each mouse in the control group was placed in an acrylic holder and exposed to clear air. Cigarette smoke was generated with a cigarette smoke generator (SG-300, Sibata Scientific Technology Ltd.) using commercially marketed Peace non-filter cigarettes (2.3 mg nicotine and 28 mg tar/cigarette; Japan Tobacco, Tokyo, Japan). Cigarette smoke was diluted with an airflow of 39.0 L/h. Wet total particulate matter (WTPM) was measured using a Glass Fiber Filter (Advantec, Tokyo, Japan) and minipump (MP-Σ100HN, Sibata Scientific Technology, Ltd.) twice per week. The average WTPM in the cigarette smoke-exposed group during the experiment was 1375 ± 98 ($\mu g/m^3$). This method of exposure to tobacco smoke has been reported to induce emphysema in mouse lungs after 12 weeks of exposure[10, 11].

Twenty-four hours after the last exposure to cigarette smoke, 5 mice per group were euthanized via transection of the abdominal aorta while under deep anesthesia induced by inhalation of an overdose of isoflurane (Abbott Japan Co., Ltd., Tokyo, Japan) using a small animal anesthetizer (MK-A110D, Muromachi Kikai Co., Ltd., Tokyo, Japan) coupled with an anesthetic gas scavenging system (MK-T 100E, Muromachi Kikai Co., Ltd.). The mice were tracheotomized and cannulated, and the lungs were lavaged thrice with 0.5 mL phosphate-buffered saline (PBS) for collection of bronchoalveolar lavage fluid (BALF) as described previously[10, 11]. After bronchoalveolar lavage, the left lung was excised and submerged in RNA*later*® Stabilization Solution (Thermo Fisher Scientific, Baltics UAB, Vilnius, Luthuania), kept at 4°C for one day, and then stored at −80°C until RNA isolation. The right lungs were excised and inflated with phosphate-buffered formalin at a water pressure of 25 cm, fixed by immersion in phosphate-buffered formalin for 48 h, embedded in paraffin, and processed for hematoxylin/eosin staining and immunohistochemical analysis. To determine the thickness of the peripheral bronchial epithelium, the length from the base to the cilia of the bronchoalveolar epithelium on the peripheral side without tracheal cartilage was measured. Three photos with three measurements for each were taken per mouse, and the average of the nine measurements was taken as the thickness of the bronchial epithelium of the mouse.

Bronchoalveolar lavage fluid analysis

The collected BALF was centrifuged at 1,200 × g at 4°C for 10 min, and the cell pellet resuspended in 1 mL of PBS. Samples (500 μL) from the 2 week treatment group and 200 μL samples from the 4 week treatment group were subjected to a cytospin procedure using a Shandon Cytospin 3 centrifuge (Shandon Scientific Co., London, England). The slides were stained with Diff-Quick (Sysmex Corp., Kobe, Japan), and enumeration of cells and differential cell counts (neutrophils, lymphocytes, and macrophages) performed according to the standard hematological criteria.

Immunohistochemical staining

Mouse lung specimens were examined for expression of Ki67 and nuclear factor-κB (NF-κB) by immunohistochemical staining using the avidin–biotin–peroxidase complex (ABC) method. Briefly, the antigen was retrieved by microwaving at 98°C for 20 min in 0.01 M citrate buffer (pH 6.0). Next, the endogenous peroxidase activity was blocked with 0.3% H_2O_2 in distilled water for 5 min, followed by blocking of non-specific binding with 10% goat serum at room temperature for 20 min. Sections were then incubated with rabbit monoclonal anti-Ki67 (SP6, dilution 1:500; Abcam plc, Cambridge, UK) or anti-NF-κB p65 (D14E12, dilution 1:800; Cell Signaling Technology, Inc., Danvers, MA, USA) antibodies overnight at 4°C. Reactivity with the primary antibody was detected by incubating the sections with biotin-labeled goat anti-rabbit IgG, followed by treatment with the Vectastain ABC kit (Vector, Burlingame, CA, USA) and diaminobenzidine tetrahydrochloride. Tissue sections were counterstained with hematoxylin. To determine Ki67 labeling indices, a minimum of 500 epithelial cells in each section were counted.

Microarray analysis in lungs

Total RNA was isolated from the RNAlater-preserved lung tissue of five mice from each group using an RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Microarray analysis was performed using a Clariom D mouse array (Affymetrix Inc. Santa Clara, CA, USA) obtained from Cell Innovator Inc. (Fukuoka, Japan). Raw expression data were deposited with accession number GSE178480. After global median normalization, data cleansing was performed to remove values for which the fluorescence intensity was <100. We established the criteria for regulated genes as a Z score≥2.0 and a ratio≥1.5 fold for upregulated genes, and a Z score≤−2.0 with a ratio≤0.67 for downregulated genes. To investigate the functional significance of the up and downregulated genes in the cigarette smoke-exposed group, differentially expressed genes were analyzed using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Inc., Mountain View, CA, USA).

mRNA expression analysis in the lungs

cDNA synthesis was performed with 1 μg of RNA using an Advantage RT-for-PCR kit (Takara Bio Inc., Otsu, Japan). The mRNA expression levels of chemokine (C-C motif) ligand 17 (CCL17; Mm01244826_g1), resistin-like alpha (Retnla; Mm00445109_m1), and lipocalin-2 (LCN2; Mm01324470_m1) were determined using TaqMan real-time quantitative PCR. PCR reagents, sequence-specific primers, and probes for each gene (TaqMan Gene Expression Assay) were purchased from Thermo Fisher Scientific. The mRNA expression assays were performed using a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). 18S ribosomal RNA was used as an internal control. Values for the target genes were normalized to 18S rRNA.

Statistics

All mean values are reported as the mean ± standard deviation (SD). Statistical analyses were performed using Prism 9 (GraphPad Software, Inc., San Diego, CA, USA). Differences in the mean values between the control and cigarette smoke-exposed groups were evaluated a 2-tailed Student's t-test. Differences in the multiplicities of histopathological lesions between the control and treatment groups were evaluated by the 2-tailed Mann–Whitney test. $P<0.05$ was considered significant.

Results

Body and organ weights and food and water consumption

Fifteen days after the start of the experiment, one mouse in the cigarette exposure group died during exposure. As the death was not due to exposure to cigarette smoke, the mouse was excluded from the study. There were no significant differences in the final body weights of the groups exposed to cigarette smoke for 2 and 4 weeks compared with the controls (Table 1). Water consumption in control mice tended to be higher than that in the cigarette smoke-exposed group at weeks 2 and 4 (Table 1).

Histological and proliferation analyses of the lung

Representative microscopic images of the lung tissues are presented in Fig. 1A. No emphysematous changes in the alveoli were observed in any animal. Mixed inflammatory cell and perivascular lymphocytic infiltrates were observed in the lungs of mice in the 2 and 4 week smoke-exposed groups (Fig. 1B). The number of inflammatory cell-infiltrated foci tended to increase in the 2 week smoke-exposed group and significantly increased in the 4 week smoke-exposed group (Fig. 1C). The thickness of the peripheral bronchial epithelium was significantly higher in both the 2 and 4 week smoke-exposed groups (Fig. 1D and 1E).

Staining for NF-κB p65 was detected in the bronchial epithelium but not in the alveolar epithelium in the lungs of animals in both the groups (Fig. 1F). Staining of NF-κB p65 in infiltrating inflammatory cells, such as lymphocytes and neutrophils, in the lungs was also observed.

Indices of Ki67 in both bronchial and alveolar epithelia of the lungs tended to be higher in both the 2 and 4 week smoke-exposed groups compared with their respective controls, albeit without statistical significance (Fig. 2).

Inflammatory cells in BALF

Representative microscopic images of BALF are depicted in Fig. 1G. Some dust cells were present in the cigarette smoke-exposed group but not in the control group. The number of macrophages, neutrophils, and total inflammatory cells in the BALF was significantly higher in the groups exposed to cigarette smoke for 2 and 4 weeks than in their respective control groups (Table 2). The number of lymphocytes in BALF in the group exposed to cigarette smoke for 4 weeks, but not at 2 weeks, was also significantly higher than that in the control group (Table 2).

Table 1. Body Weight, Food and Water Consumption

Treatment	No. of total mice	Final body weight (g)		Average food consumption (g/BW/day)		Average water consumption (g/BW/day)	
		2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
Control	10	24.5 ± 1.1	26.0 ± 1.4	0.15	0.15	0.26	0.27
Cigarette smoke	9	24.4 ± 1.6	25.2 ± 1.0	0.13	0.14	0.20	0.23

Table 2. Inflammatory Cells in the BALF

Treatment	No of mice	Macrophages ($\times10^4$/mouse)	Neutrophils ($\times10^3$/mouse)	Lymphocytes ($\times10^3$/mouse)	Total cells ($\times10^4$/mouse)
2 weeks					
Control	5	1.7 ± 0.5	0.2 ± 0.4	0.7 ± 0.1	1.8 ± 0.5
Cigarette smoke	5	2.9 ± 1.0*	5.7 ± 2.9**	0.4 ± 0.3	3.5 ± 1.2*
4 weeks					
Control	5	1.6 ± 0.4	0.6 ± 0.2	0.2 ± 0.3	1.6 ± 0.4
Cigarette smoke	4	2.3 ± 0.4*	21.9 ± 6.4***	1.7 ± 1.4*	4.7 ± 1.1***

*, **, ***: Significantly different from respective control group, at $p<0.05$, 0.01, 0.001.

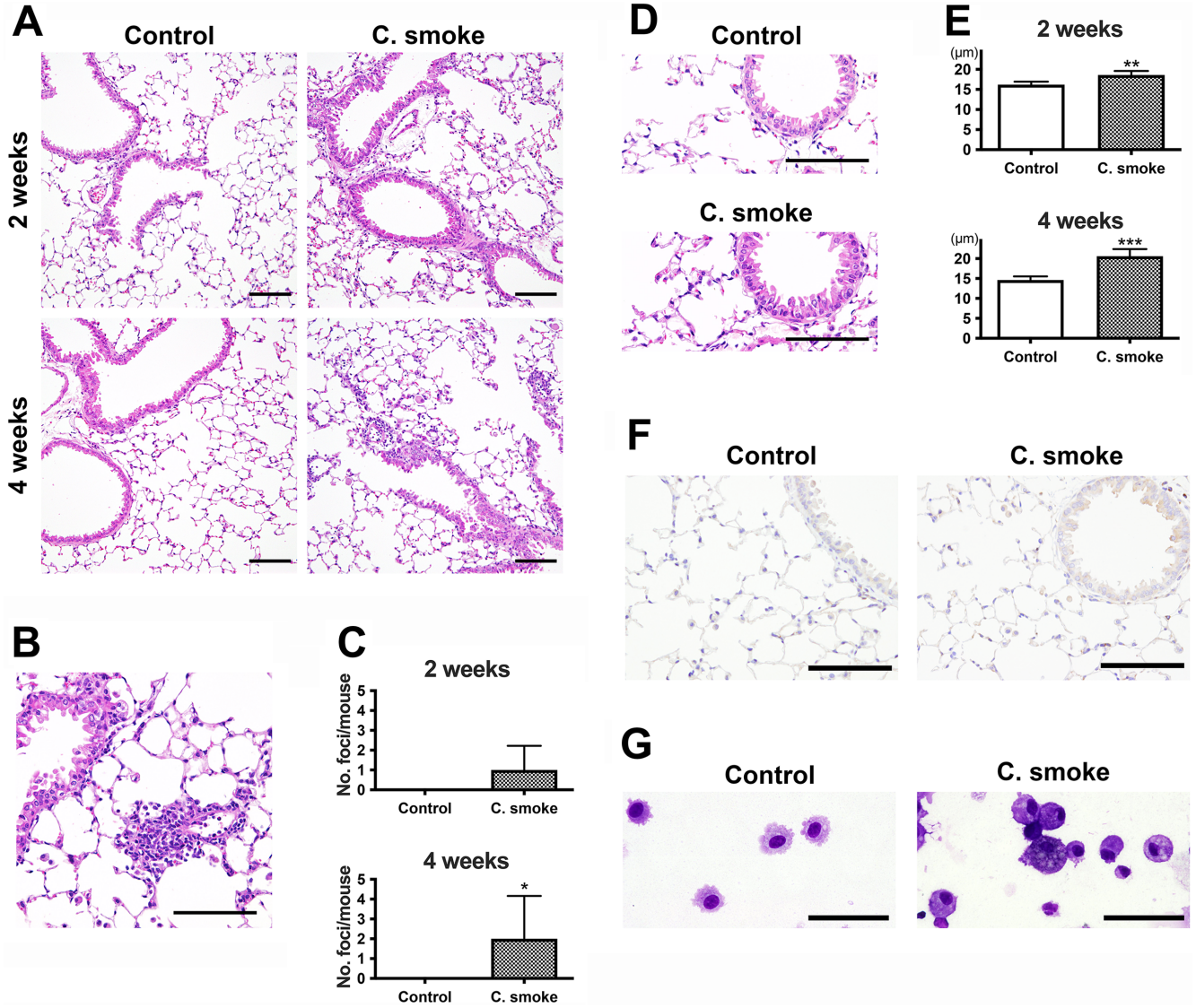

Fig. 1. Histology and immunostaining of mouse lung and BALF samples A: Lungs of control and smoke-exposed mice at 2 and 4 weeks. B: Perivascular mixed inflammatory cell infiltration was detected in the lungs of mice exposed to cigarette smoke for 4 weeks. C: The number of infiltrating inflammatory cell foci per mouse at 2 and 4 weeks. D: Photographs of the peripheral bronchial epithelium in the lungs of control and smoke-exposed mice at 4 weeks. E: Thickness of the bronchial epithelium in the lungs of control and smoke-exposed mice at 2 and 4 weeks. F: Immunostaining of NF-κB p65 in control and smoke-exposed mice at four weeks. G: BALF of control and smoke-exposed mice at 4 weeks. Significantly different from the control group, **p<0.01, ***p<0.001. Bar=100 μm for A, B, D, F, and 50 μm for G. C. smoke, cigarette smoke; BALF, bronchoalveolar lavage fluid; NF-κB, nuclear factor-κB.

Microarray data analysis of the lung

A total of 591 genes (393 upregulated and 198 downregulated) and 802 genes (515 upregulated and 287 downregulated) were differentially expressed in the lungs of mice exposed to cigarette smoke for 2 and 4 weeks, respectively, compared with the controls. The numbers of commonly up and downregulated genes after 2 and 4 weeks of exposure were 226 and 50, respectively (Supplementary Table 1). IPA analysis indicated that these genes were associated with the activation of leukocyte migration, cell movement of leukocytes, and the inflammatory response (Supplementary Table 2), and the top regulator effects network analysis predicted that the inflammatory response genes NF-κB, tumor necrosis factor, and interleukin 1 alpha are primary upstream regulators (Supplementary Table 3). These findings were consistent with the histopathological findings of the lungs and showed that the major gene expression alterations were associated with inflammatory responses.

Identification of the early response biomarker genes for cigarette smoke exposure

To identify the response biomarker genes of cigarette smoke exposure in the lungs, we analyzed genes that were expressed at levels at least 2 times higher in cigarette smoke-

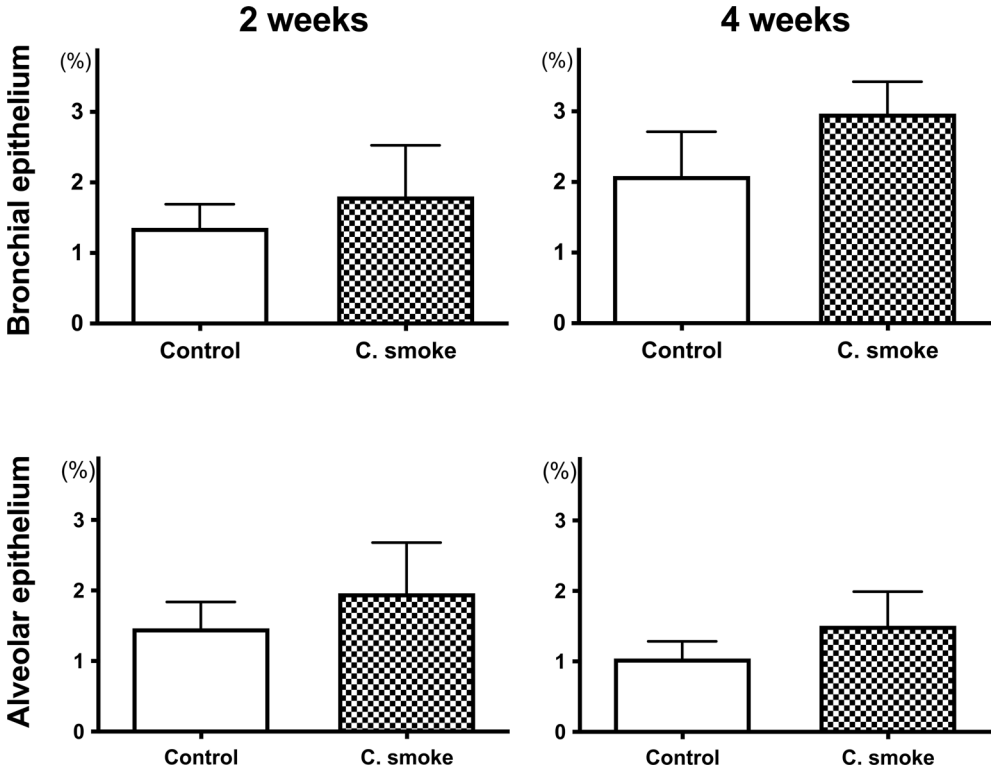

Fig. 2. Ki67 labeling index in the bronchial and alveolar epithelium. C. smoke, cigarette smoke.

Table 3. Candidate Key Genes in Response to Cigarette Smoke Exposure

Gene Symbol	Gene Description	Ratio (vs. Contol)	
		2 weeks	4 weeks
CCL17	chemokine (C-C motif) ligand 17	6.9	9.2
Retnla	resistin like alpha	5.5	5.0
LCN2	lipocalin 2	6.0	4.4
CCL22	chemokine (C-C motif) ligand 22	3.0	3.3
CSF2	colony stimulating factor 2 (granulocyte-macrophage)	3.0	3.1
Cxcl9	chemokine (C-X-C motif) ligand 9	2.3	2.6
IL1RN	interleukin 1 receptor antagonist	2.5	2.5
CD14	CD14 antigen	2.1	2.1

exposed mice than in the controls in both the 2 and 4 week smoke-exposed groups. Among these genes, eight (Table 3) were predicted as key genes associated with the IPA Diseases and Disorders categories (Supplementary Table 2) and top regulator effects network analysis (Supplementary Table 3).

Notably, three of these genes, CCL17, Retnla, and LCN2, were associated with the NF-κB pathway (Fig. 3). NF-κB is a key regulator of genes associated with the inflammatory response. The expression levels of these genes were confirmed using quantitative RT-PCR. Similar to the microarray analysis, the expression of these genes in the lungs of cigarette smoke-exposed mice was markedly higher than that in control mice in both the 2 and 4 week smoke-exposed groups (Fig. 4).

Discussion

Neutrophilic inflammation is a characteristic of COPD, and airway neutrophilia is associated with a decline in lung function, leading to pulmonary emphysema[9]. In a previous study, using the same cigarette smoke exposure method as in the present study, we reported that isoflavone aglycones attenuated cigarette smoke-induced emphysema via the suppression of neutrophilic inflammation[10]. In the present study, the number of neutrophils in the BALF was significantly increased in mice after 2 weeks of exposure to cigarette smoke, indicating that neutrophilic inflammation occurs relatively early after the initiation of exposure to cigarette smoke and is an important event in the development of COPD. There was a marginal effect of cigarette smoke

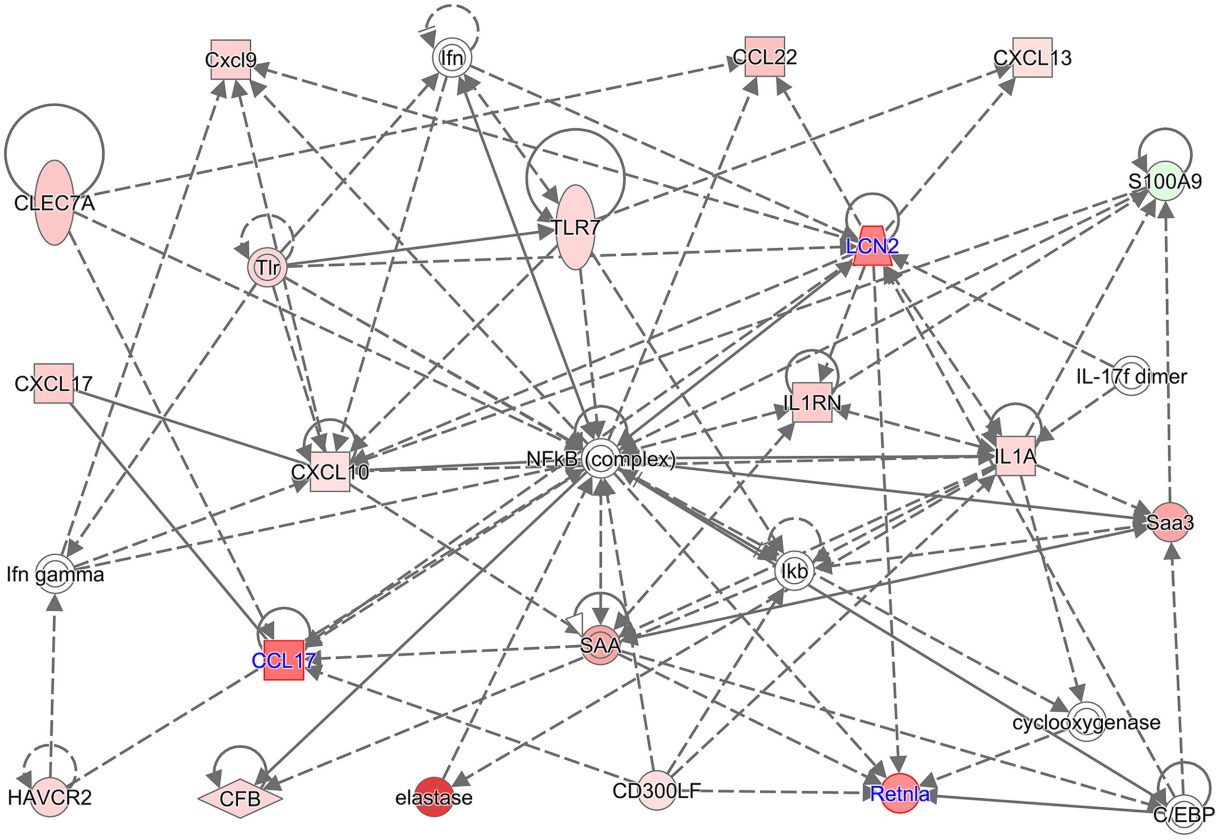

Fig. 3. Pathway analysis with RNA expression changes in the lungs of smoke-exposed mice. Software-assisted identification of putative biological networks affected by smoke exposure, as revealed by IPA. Differentially-expressed RNA genes showing a +/− 1.5-fold change or greater were selected and entered into IPA software to illustrate potential interactions. Solid arrows represent known physical interactions, dotted arrows represent indirect interactions. Red- or green-colored shapes indicate up- or down-regulated genes in the lungs of smoke-exposed mice. IPA, Ingenuity Pathway Analysis.

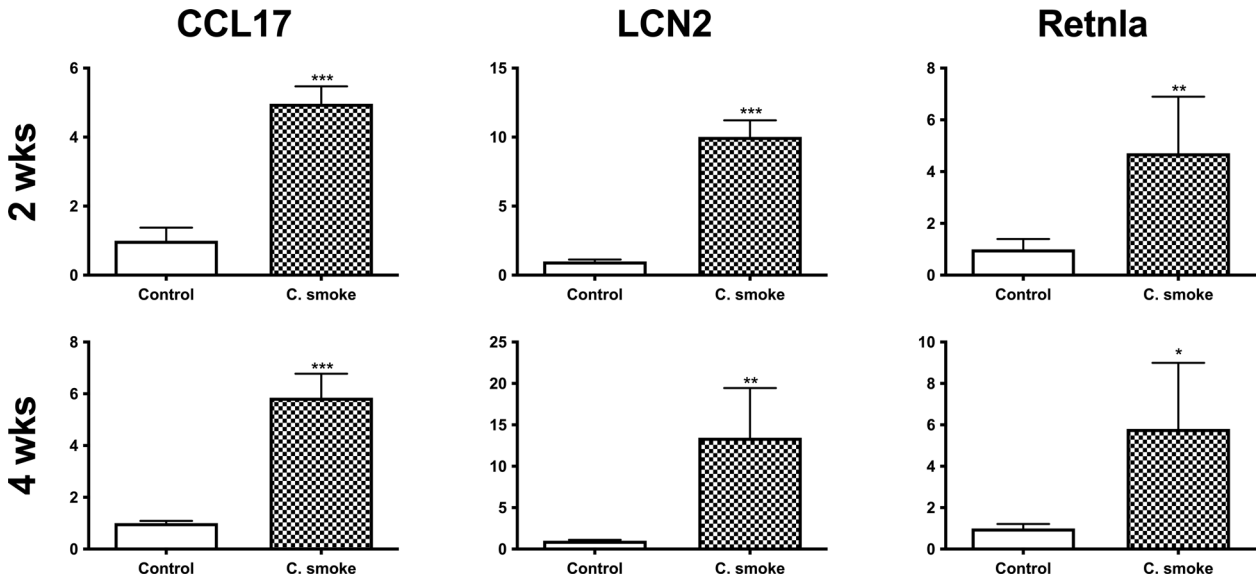

Fig. 4. RNA expression of Ccl17, Retnla, and Lcn2 in the lungs of control and smoke-exposed mice at 2 and 4 weeks. Significantly different from the control group, *p<0.05; **p<0.01; ***p<0.001. C. smoke, cigarette smoke; CCL17, chemokine (C-C motif) ligand 17; Retnla, resistin-like alpha; LCN2, lipocalin-2.

exposure on the alveolar epithelium; cigarette smoke exposure tended to induce cell proliferation in both bronchial and alveolar epithelia. However, this was not significant. These results suggest that while inflammatory responses were detected after 2 and 4 weeks of exposure to cigarette smoke, more than 4 weeks of exposure may be necessary for the detection of effects on the alveolar epithelium.

In this study, we used lung samples subjected to BALF for microarray and mRNA expression analyses. Microarray analysis revealed that most genes showing altered expression after inhalation exposure to cigarette smoke were associated with inflammation, including migration and/or activation of inflammatory cells (Supplementary Tables 2 and 3). NF-κB is a well-known regulator of genes associated with the inflammatory response[12], and three of the genes (CCL17, Retnla, and LCN2) that showed the highest upregulation in response to cigarette smoke exposure were associated with the NF-κB pathway (Fig. 3 and Supplementary Table 3). Interestingly, the expression levels of inhibitory protein κB alpha (IκBα) are significantly reduced in healthy smokers and in current and ex-smoking patients with COPD compared with non-smokers[13]. In addition, it has been reported that NF-κB is activated in human lung epithelial BEAS-2B cells treated with tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)[14]. These data support the premise that exposure to cigarette smoke activates the NF-κB pathway to induce an inflammatory response and that this activation is associated with COPD and development of lung tumors. In addition, NF-κB expression was detected in the bronchial epithelium (Fig. 1F), and thickening of the bronchial epithelium was observed in the smoking-exposed group (Fig. 1D and 1E), suggesting that NF-κB pathway activity may be associated with bronchial epithelial cell hypertrophy. NF-κB expression was also detected in inflammatory cells in the lungs, suggesting that this pathway may also be associated with inflammatory cells in the lungs of mice exposed to cigarette smoke in this study.

Our results suggest that CCL17, Retnla, and LCN2 might be useful response biomarker genes for the toxic effects of exposure to cigarette smoke from as short as 2 or 4 weeks, earlier than the 12 weeks of cigarette smoke-induced emphysema in mice[10]. CCL17, also known as thymus and activation-regulated chemokine, is a CC chemokine that is constitutively expressed in the thymus and inducible in peripheral blood mononuclear cells, macrophages, bronchial epithelial cells, endothelial cells, dendritic cells, and keratinocytes. Expression of CCL17 has been reported to be upregulated in the lungs of rat models of idiopathic pulmonary fibrosis, acute asthma, and cigarette smoke-induced inflammation[15]. The serum level of CCL17 is a predictive biomarker for the rapid decline in forced expiratory volume in one second in COPD patients[16]. Serum levels of CCL17 were also positively associated with lung cancer risk[17]. It was also elevated in former or current smokers compared to never smokers[17]. Retnla, also known as the inflammatory zone, is a cysteine-rich secreted protein belonging to the resistin-like molecule family[18]. Retnla expression is increased in hypertrophic, hyperplastic bronchial epithelium and appears in type II alveolar pneumocytes during allergic pulmonary inflammation[19]. In NNK-induced pulmonary tumorigenesis, Retnla expression was highly upregulated in NNK-susceptible A/J mice, but not in NNK-resistant C3H mice[20]. LCN2 belongs to the lipocalin family of proteins involved in the transport of steroids and lipids into cells. LCN2 expression was continuously upregulated in the lungs of rats and mice exposed to cigarette smoke for 13 and 6 months, respectively[20, 21]. LCN2 has also been reported to be associated with pulmonary diseases and infection[22]. Notably, serum levels of LCN2 in COPD patients was significantly higher than those in healthy controls[23]. Collectively, these data suggest that the aberrant expression of these three genes is associated with the inhalation exposure to cigarette smoke that is detectable even after a relatively short exposure period of 2 weeks in mice. In addition, continuously high expression of these three genes may be associated with lung diseases, such as COPD and cancer.

In conclusion, the results of the present study demonstrated that neutrophilic inflammation is an early event caused by inhalation exposure to cigarette smoke in mice, and most genes showing altered expression were associated with inflammation, including migration and/or activation of inflammatory cells. CCL17, Retnla, and LCN2 may serve as useful markers of the toxic effects of cigarette smoke exposure before pulmonary histological changes become evident.

Disclosure of Potential Conflicts of interest: The authors declare that they have no conflict of interest.

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