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Acrolein induced both pulmonary inflammation and the death of lung epithelial cells

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HIGHLIGHTS

- Acrolein, a compound found in cigarette smoke, is a major risk factor for respiratory diseases.
- Acrolein increased $CD11c^{+}F4/80^{high}$ macrophages in the lungs.
- Acrolein increased ROS formation *via* induction of NF-κB signaling.
- Acrolein treatment of macrophages induced apoptosis of lung epithelial cells.

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ABSTRACT

Acrolein, a compound found in cigarette smoke, is a major risk factor for respiratory diseases. Previous research determined that both acrolein and cigarette smoke produced reactive oxygen species (ROS). As many types of pulmonary injuries are associated with inflammation, this study sought to ascertain the extent to which exposure to acrolein advanced inflammatory state in the lungs. Our results showed that intranasal exposure of mice to acrolein increased $CD11c^{+}F4/80^{high}$ macrophages in the lungs and increased ROS formation *via* induction of NF-κB signaling. Treatment with acrolein activated macrophages and led to their increased production of ROS and expression of several key pro-inflammatory cytokines. In *in vitro* studies, acrolein treatment of bone marrow-derived GM-CSF-dependent immature macrophages (GM-IMs), activated the cells and led to their increased production of ROS and expression of several key pro-inflammatory cytokines. Acrolein treatment of macrophages induced apoptosis of lung epithelial cells. Inclusion of an inhibitor of ROS formation markedly decreased acrolein-mediated macrophage activation and reduced the extent of epithelial cell death. These results indicate that acrolein can cause lung damage, in great part by mediating the increased release of pro-inflammatory cytokines/factors by macrophages.

1. Introduction

Chronic obstructive pulmonary disease (COPD) remains a major public health problem and is projected to be the fifth leading cause of death worldwide by 2020 (Rabe et al., 2007). Smoking tobacoo is an important cause of COPD and cancers in many countries (Rennard et al., 2006; Bruce et al., 2000; Rivera et al., 2008). Among COPD patients, there is a general increase in the numbers of neutrophils and macrophages in their lung parenchyma (Barnes, 2004; MacNee, 2005). Other pathologic hallmarks in these patients include recurrent infiltration of inflammatory cells to the small airways that, in turn, cause destruction of the alveoli and enlargement of air spaces, leading to wall thickening (Cosio et al., 2009).

Cigarette smoke (CS) is a complex mixture that contains high concentrations of free radicals and other oxidants that are thought to contribute to oxidative damage to the lungs (Rahman and MacNee, 1996; MacNee, 2005; Perrone et al., 2012). Among the >4000 compounds in CS, several can damage DNA and affect apoptosis (Green and Rodgman, 1996; Hoffmann et al., 2001; Rennard et al., 2006). Among these, the important mutagen acrolein can interact with DNA and proteins in lung cells

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Table 1
PCR primers.

Genes		Sequences (5′-3′)
Irf5	Forward	GCTGGCTACAGGGTTCTGAG
	Reverse	CTGCTGGCTTCATTTCTTCC
TNFα	Forward	GCCCATATACCTGGGAGGAG
	Reverse	CACCCATTCCCTTCACAGAG
IL12b	Forward	AGGTCACACTGGACCAAAGG
	Reverse	TGGTTTGATGATGTCCCTGA
IL6	Forward	CCGGAGAGGAGACTTCACAG
	Reverse	TCCACGATTTCCCAGAGAAC
CCR2	Forward	AGAGAGCTGCAGCAAAAAGG
	Reverse	GGAAAGAGGCAGTTGCAAAG
Arg1	Forward	GTGAAGAACCCACGGTCTGT
	Reverse	CTGGTTGTCAGGGGAGTGTT
Retnla	Forward	TGCTGGGATGACTGCTACTG
	Reverse	CTGGGTTCTCCACCTCTTCA
Mrc1	Forward	CAAGGAAGGTTGGCATTTGT
	Reverse	CCTTTCAGTCCTTTGCAAGC
Chi3l3	Forward	GAAGGAGCCACTGAGGTCTG
	Reverse	CACGGCACCTCCTAAATTGT
Gapdh	Forward	AACTTTGGCATTGTGGAAGG
	Reverse	ACACATTGGGGGTAGGAACA

(Esterbauer et al., 1991). The amount of acrolein in a single cigarette can range from 10–500 μg (Fujioka and Shibamoto, 2006). Acrolein is a respiratory irritant and plays a causal role in the pulmonary inflammation and lung injury due to smoking. (Bein and Leikauf, 2011). Acrolein can induce macrophage activation, and it might be a key factor in the induction of pulmonary inflammation associated with cigarette smoke (Facchinetti et al., 2007). Previous studies have noted that cigarette smoke extract (CSE) and acrolein activate the same intracellular pathways that lead to the release of pro-inflammatory cytokines and reactive oxygen species (ROS) proposed to be responsible for the inflammatory processes associated with COPD. Despite intense investigation, a precise mechanistic understanding of acrolein-induced lung injury and inflammation has remained elusive. Recently we established a murine macrophage cell line (GM-IMs) that has the capacity to differentiate to M1 and M2 macrophages (Ito et al., 2013). This cell line enables long-term studies of macrophages treated with crolein. In the present study, we present a relatively simple method for administering acrolein to the lungs and long-term *in vitro* acrolein stimulation of macrophages using our newly established macrophage cell line.

2. Materials and methods

2.1. Mice and acrolein administration

C57BL/6 mice (female, 8 weeks old) were obtained from SLC Japan (Sizuoka, Japan). All mice were maintained in pathogen-free facilities in the Animal Research Center at the Nagoya University Graduate School of Medicine. They were maintained at 25 °C with a 55% relative humidity and a 12-h light: dark cycle. All mice had *ad libitum* access to standard rodent chow and filtered water throughout the study. Nagoya University Animal Experiment Committee approved all protocols used in these studies.

For the treatment regimens, mice were randomly allocated into 3 groups ($N = 8$/group). In these studies, mice were instilled intranasally with acrolein (Sigma) at doses of 1 or 5 μmol/kg body weight. Control mice received PBS alone. Instillation volumes never exceeded 100 μl/mouse. In the study, the mice were treated daily for 5 d/week for up to 4 weeks. To permit analysis of the endpoints outlined below, and to ascertain any time dependent effects, subsets of mice ($N = 4$/group) were sacrificed at the end of 1 and 4 weeks. At necropsy, a lung was removed from each mouse and one-half was used for histological analysis and the other half was used for flow cytometry.

2.2. Cell culture and treatment

Murine culture macrophages (GM-IMs), were established previously by Ito et al. (2013). The cells were cultured in RPM1640 (Sigma) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) and 10% GM-CSF which was produced by murine GMCSF-producing Chinese hamster ovary (CHO) cells (GM-CSFCM). Cells (5×10^5/well) were seeded in 6-well plates and treated with 1, 5 and 10 μM acrolein (Sigma) for 1 week for *in vitro* study.

Lewis lung carcinoma (LLC) cells were obtained from Riken (Bertram and Janik, 1980). Cells were cultured in HEPES-buffered Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (Hyclone). LLC cells (1×10^5) were co-cultured overnight with 2×10^5 GM-IMs that had been treated with acrolein for 1 week.

2.3. Histology and immunohistochemistry

Mouse lungs were isolated and embedded in Tissue-Tek OCT compound (Sakura, Japan). The 5 μm frozen lung sections were stained with hematoxylin and eosin (HE) (Zhang et al., 2011). Alternatively, cryosections were postfixed in cold acetone. After blockade with 2% BSA-PBS, sections were incubated with rabbit anti- ProSpC (epithelial Type II cells marker) overnight. After 3 washes in PBS, the sections were stained with Alexa Flour 448 goat anti-rabbit IgG for 1 h. All sections were counterstained with DAPI.

2.4. Flow cytometry

The whole lung was minced, and RBCs were lysed with tris-NH_4Cl red blood cell lysing buffer (150 mM sodium chloride, 1% Triton, 0.5% sodium deoxycholate 0.1% SDS 50 mM Tris). Cells (1×10^5) were blocked in 50 μl PBS with 0.2% BSA, stained with PE-conjugated anti-CD11b, anti-CD11c and APC-conjugated anti-F4/80 (BD Biosciences) for 30 min at 4 °C, and analyzed by flow cytometry using a FACS Canto flow cytometer (BD Biosciences).

2.5. ROS measurements and inhibition of ROS production

Lung tissue was isolated from mice and fully minced, after which RBCs were lysed. Cells (1×10^5) were incubated with PE-conjugated CD11c and APC-conjugated F4/80 for 30 min at 4 °C, after which the cells were seeded in 12-well plates, stained with 2 μM carboxy-2′,7′-dihydrofluorescein diacetate (H2DCFDA) (Invitrogen) for 30 min at 37 °C. The cells were collected and ROS generation was determined by flow cytometry as descrived previously (Kimura et al., 2008).

Cells (GM-IMs) were plated in 12-well dishes and treated with 1 or 5 μM acrolein for 1 week as previously described. Culture medium was removed and cells were washed with PBS. Next, cells were incubated with 2 μM H2DCFDA for 30 min at 37 °C. Collected cells and analyzed for ROS generation by flow cytometry. ROS inhibitor *N*-acetyl-L-cysteine (NAC) (Sigma–Aldrich) was dissolved at 1 mol/L in deionized water and neutralized by titration with NaOH until pH 7.

2.6. Western blotting

Proteins were extracted from the lung tissue and GM-IMs with RIPA lysis buffer (0.1 M PBS pH 7.4 containing 1% deoxycholic acid

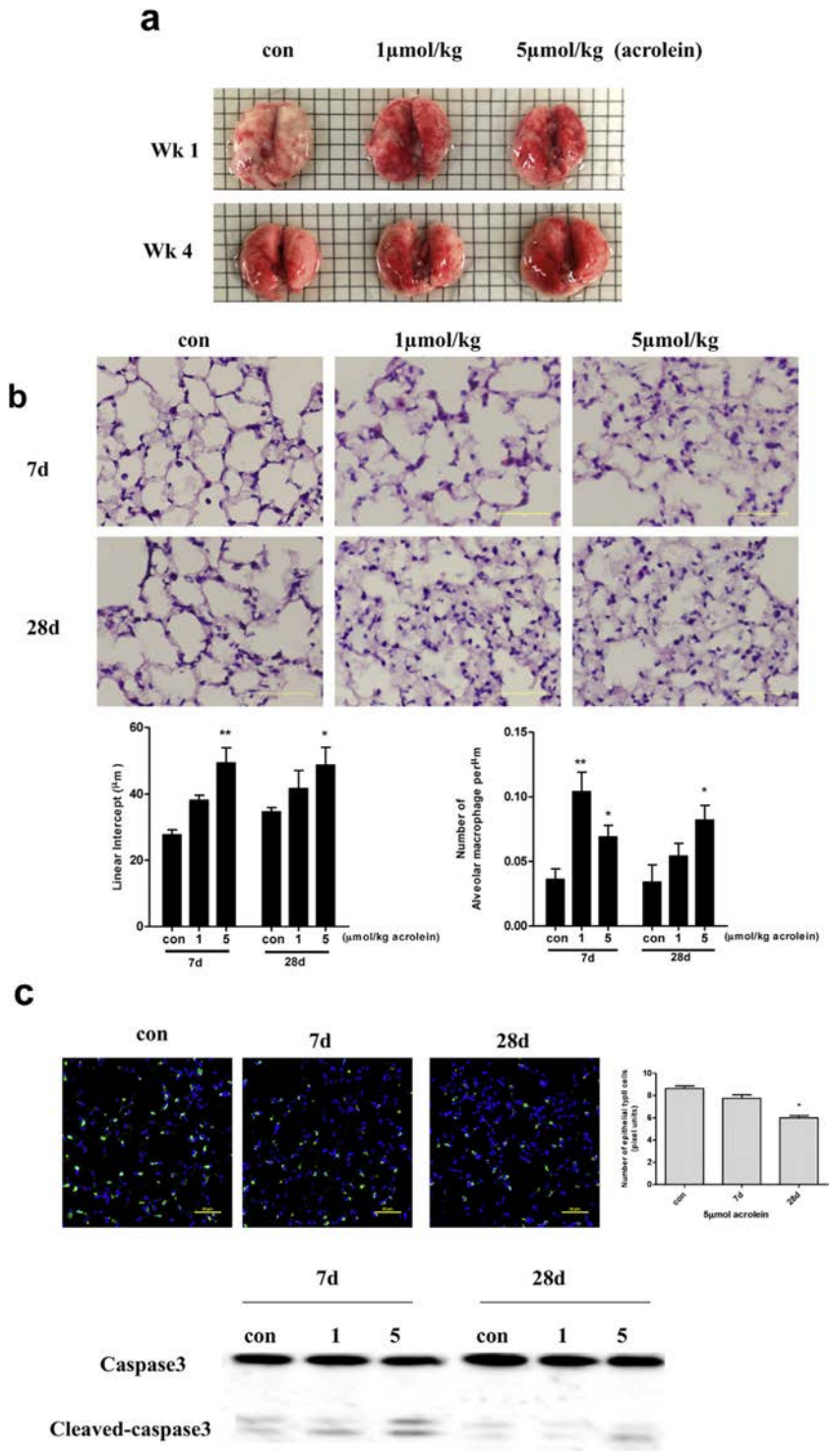

Fig. 1. Acrolein induced structural damage in the lung. Mice were instilled with 1 or 5 μmol/kg acrolein daily for 5 d/week for 1 or 4 week. After 1 or 4 week, subsets of mice were euthanized and lung tissues collected. (a) Representative lung showing hemorrhage at the end of the first week. (b) Representative H&E staining of lung tissue shows airspace enlargement at the end of 1 and 4 weeks of exposures. Scale bar = 50 μm. Assessed linear intercepts and number of alveolar macrophages ($n = 4$ mice/group). *, $p < 0.05$;**, $p < 0.01$ *vs.* control mouse. (c) Left: immunofluorescence staining of epithelial type II cells (ProSpC green). Right: the number of epithelial type II+ cells (10 fields, $n = 4$ mice/group). *, $p < 0.05$ *vs.* control mouse. Scale bar = 50 μm. (d) Expression of cleaved caspase3 in whole lung after acrolein treatment. Data show means ± SE. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sodium, 0.2% SDS, and protease inhibitors). After measurement of protein concentration, the samples were loaded and separated by SDS-PAGE and then transferred to Immobilon tansfer membranes. The membranes were incubated with primary anti-phospho-NF-κB P65 (Ser536) antibody and anti-caspase3 (Cell signaling) overnight at 4 °C. Then the membranes were incubated with the secondary anti-rabbit IgG for 1 h. Blots were developed with Western Blot Detection Reagent (GE Healthcare).

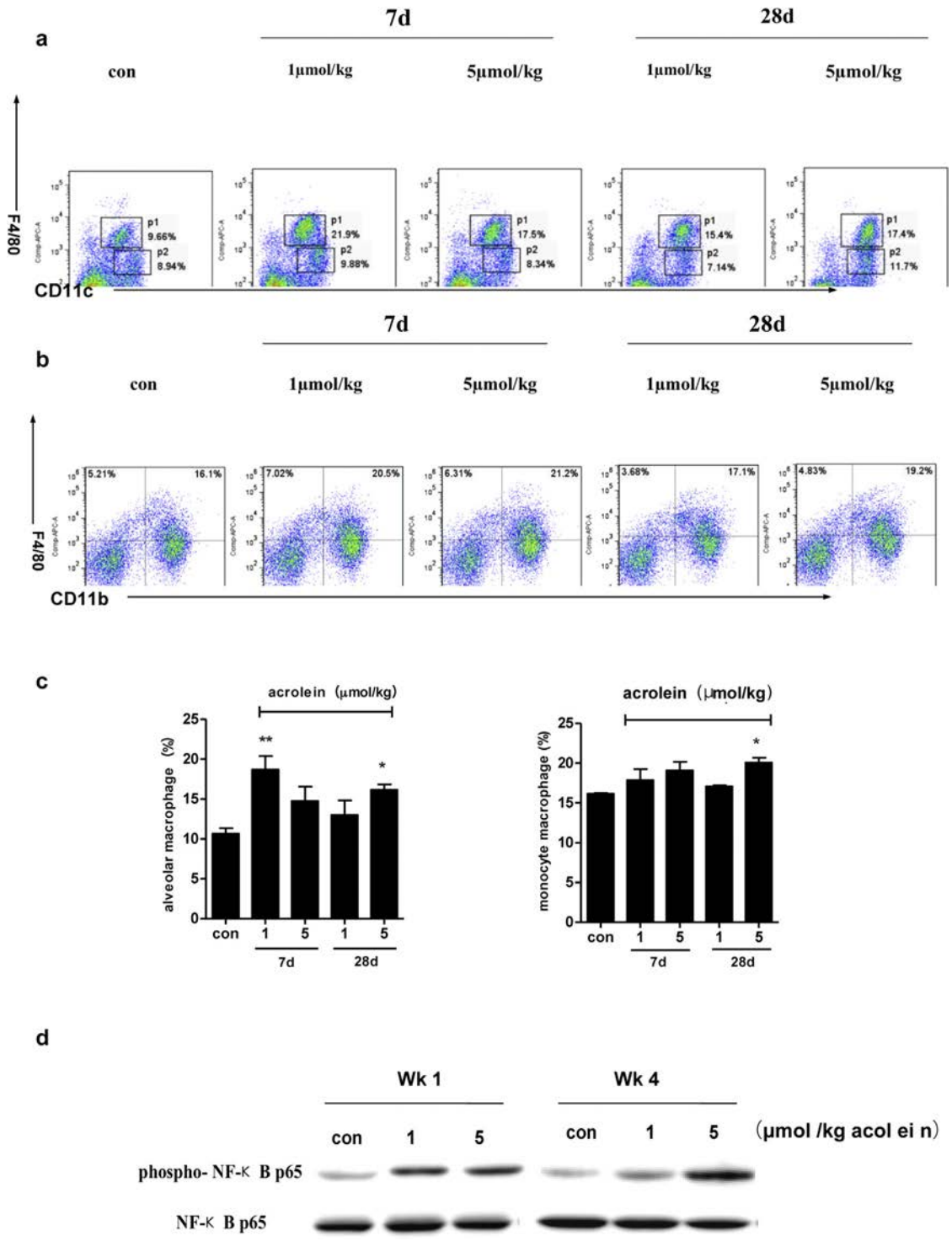

Fig. 2. Analysis of infiltrating macrophages after acrolein challenge in mice. (a) Lung tissues and (b) bone marrow were collected 1 and 4 weeks after installation 1 or 5 μmol/kg acrolein. The recruitment of $CD11c^{+}F4/80^{high}$ alveolar macrophages to lungs and $CD11b^{+}$ $F4/80^{+}$ macrophages to bone marrow were confirmed by flow cytometry. (c) The number of $CD11c^{+}$ $F4/80^{high}$ alveolar macrophages and monocyte- macrophages. (d) The expression of phospho-NF-κB p65 was determined by Western Blot. Data show means ± SEM ($n = 4$). *, $p < 0.05$; **, $p < 0.01$.

2.7. Real-time PCR

Total RNA was extracted with TRIzol Reagent and reverse-transcribed using Hiah Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative Real-time PCR was performed using the MX3000P QPCR System (Agilent) according to the manufacturer's protocol. The sequences of the RT-PCR primers for each pair are listed in Table 1.

2.8. Apoptosis

GM-IMs were treated by 10 μM acrolein for 7 days then they were washed with PBS twice and they were co-cultured with LLC cells overnight. Both cells were collected and incubated with APC-conjugated F4/80 for 30 min at 4 °C and stained with FITC-labeled Annexin V and 7-AAD (BD Bioscience) for 15 min. Cells were analyzed by flow cytometry on

a FACS Calibur. Data were analyzed by FlowJo software (TreeStar).

2.9. Statistics

Data are expressed as means ± standard error of the mean (SEM). Statistical comparisons were performed by ANOVA followed by Fisher's post hoc test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Acrolein-induced pulmonary damage

Two different doses of acrolein were administered intranasally 5 times/week for 4 weeks. This regimen led to extensive hemorrhaging in the lung tissue of the treated mice at 1 week after instilled and it continued to week 4 (Fig. 1a). The histological analysis of lung tissue showed that acrolein caused enlargement of alveoli and immune cell infiltration. This was reflected in a significant increase in linear intercept and the number of macrophages. We also found that alveolar fusion and alveolar walls thickened after 4 weeks (Fig. 1b). In addition, when the lung tissues were stained with FITC-labeled-anti-pro-surfactant protein-C (SPC; green) specific for type II epithelial, they constitute major alveolar epithelial cells and 10–15% of all lung cells (Castranova et al., 1988). It was observed that a significantly reduced type II epithelial cells as compared to in the lungs of control mice on 5 μmol/kg acrolein (Fig. 1c). We also found greater expression of cleaved caspase 3 in the lungs of acrolein-treated mice than in the control mice (Fig. 1d).

3.2. Acrolein caused pulmonary inflammation and activated NF-κB p65 signaling in situ

To examine the association between lung damage and immune responses, the inflammatory cells that migrated into the acrolein-damaged lung were examined by staining for immune cell markers and analyszed by flow cytometry. Specifically, cells prepared from the lungs and bone marrows of treated mice were stained with anti-CD11c, -CD11b, or -F4/80 to identify the presence of macrophages. The results indicated there was significant increases in the levels of $CD11c^{+}F4/80^{high}$ alveolar macrophages in the lungs 1 week after the beginning of acrolein administration. Increased levels of alveolar macrophages continued until 4 weeks at 5 μmol/kg (Fig. 2a and c). The data also showed that there was an increase in $CD11b^{+}F4/80^{+}$ macrophages in the bone marrow of the acrolein-treated mice (Fig. 2b and c). As the induction of macrophage infiltration into the lungs might in turn induce NF-κB signaling, the latter was also examined. The results showed that acrolein activated *p*-NF-κB p65 in the lungs after 1 and 4 weeks of acrolein administration (Fig. 2d).

3.3. Expression of inflammatory cytokines and ROS generation due to acrolein

We used RT-PCR to examine the effects of acrolein instillation on the levels of inflammatory cytokine gene expression in the lungs. Gene expression levels for inflammatory cytokines associated with M1 macrophages, including TNFα, IL-6, Irf5, and IL-12, were upregulated by the end of first week of acrolein exposure (Fig. 3). However, levels of TNFα and Irf5 decreased (from week 1 values) after 4 weeks. In contrast, IL6 and IL12 levels remained high

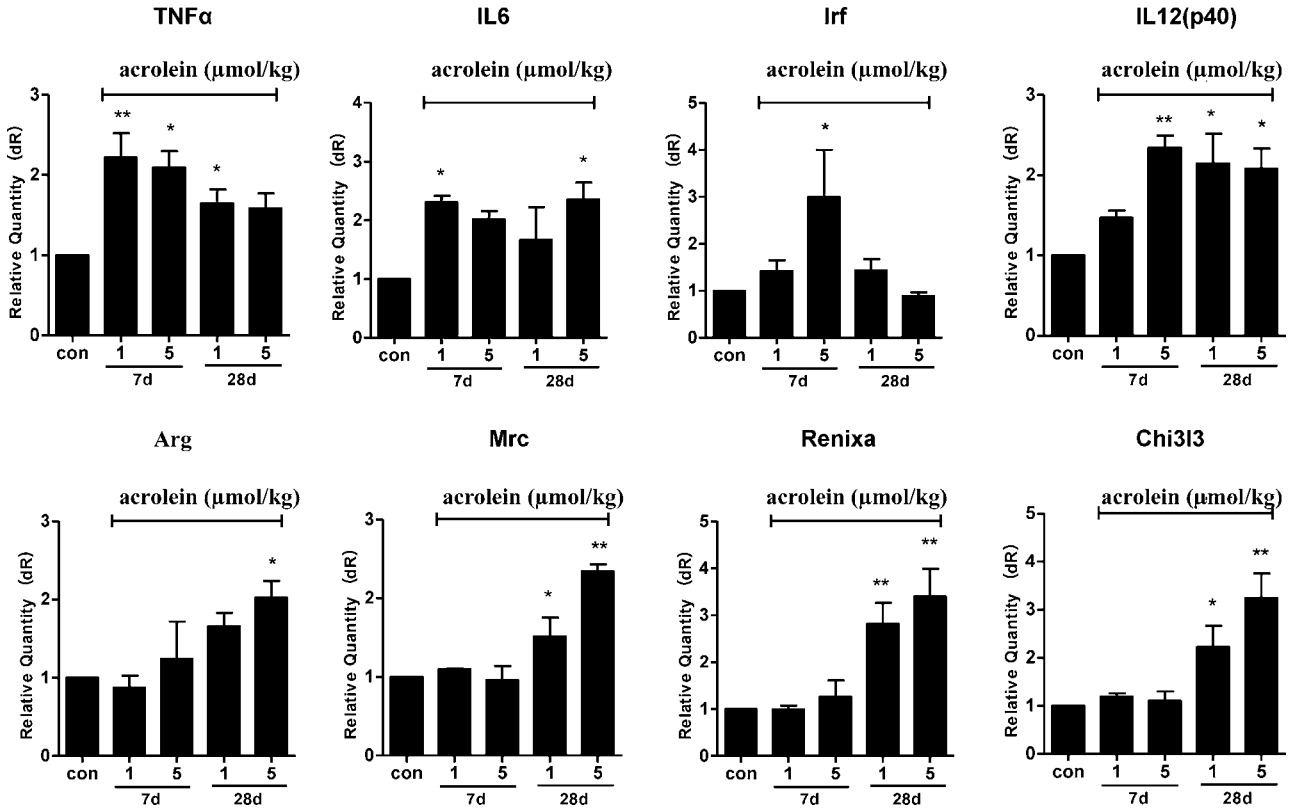

Fig. 3. Acrolein stimulated the expression of inflammatory cytokines in the lung. Lungs were treated with acrolein for 1 or 4 weeks. Expression of (a, b) macrophage typeI markers, TNFα, IL6, Irf5 and IL12 and macrophage typeII markers, Arg-1, Mrc-2, Retnla and Chi3l3 was determined by quantitative Real-time PCR. Each histogram is the mean ± SEM of $n = 3$ or 4 independent experiments. *, $p < 0.05$; **, $p < 0.01$.

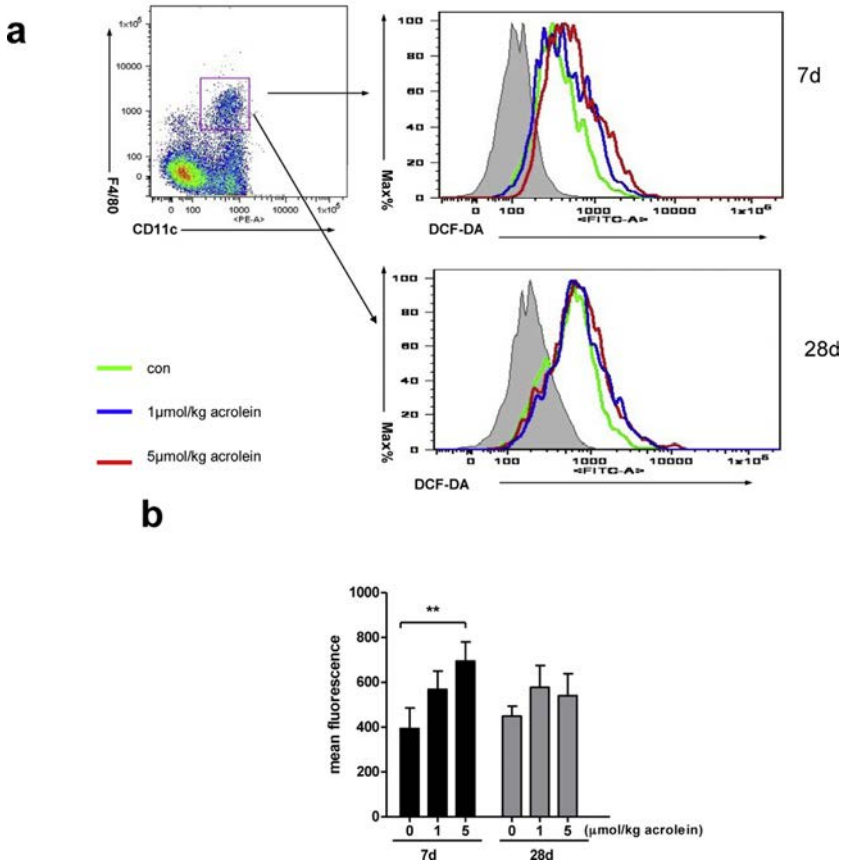

Fig. 4. ROS was generated after acrolein challeng in the lung. (a) Lung homogenates were collected at 1 and 4 weeks after instilled of 1 or 5 μmol/kg acrolein. ROS levels in $CD11c^{+}F4/80^{high}$ alveolar macrophages were measured with DCFH-DA fluorescence by flow cytometry. (b) The graph represents the percentage of mean fluorescence after subtracting autofluorescence ($n = 3$). **, $p < 0.01$.

at 4 weeks. Expression of M2 macrophage markers, like Arg1 and Mrc-2, were upregulated by the end of week 1 and continued to remain elevated after 4 weeks of treatment. In contrast, expression of M2 macrophage marker Retnla and Chi3L3 were increased after 4 weeks of exposure.

Acrolein has been shown to cause alveolar cell apoptosis, in part, by inducing the formation of ROS, Thus, we assessed ROS generation in the lungs after acrolein administration. We observed that increased mean fluorescence of DCFH-DA from alveolar macrophages increased after 5 μmol/kg acrolein was instilled. Thus, a single week of exposure to acrolein induced ROS from macrophages in the lung (Fig. 4a and b). The results indicate that ROS generation was increased by the end of the first week of treatment and decreased gradually thereafter.

3.4. Effects of acrolein on macrophage activation and role of the NF-κB pathway

In vivo administration of acrolein showed that macrophages were activated by acrolein and produced pro-inflammatory cytokines. Although bone marrow-derived macrophages taken from mice can be used for short-term stimulation, such macrophages are not suitable for the present research because of their short lifespan. Recently, we established a normal macrophage line (GM-IMs) that is capable of long-term cultivation. Thus, we used them to analyze prolonged exposure to acrolein. GM-IMs have both M1 and M2 characteristics depending on the level of stress in the microenvironment. We found that acrolein induced ROS production by GM-IMs and that the presence of the anti-oxidant/inhibitor *N*-acetyl-L-cysteine (NAC) blocked ROS formation (Fig. 5a). Acrolein also induced expression of genes for pro-inflammatory cytokines TNFα and IL-6; the increase of IL-1β was not significant. Acrolein also caused an increase in expression of the macrophage surface makers CD86 and MHC-II. As with the ROS, these increases were abrogated by NAC (Fig. 5b and c).

Acrolein-associated oxidative stress might promote inflammation through NF-κB signaling. After treatment with acrolein, the GM-IM displayed a sizable NF-κB response, with inceases in the levels of phosphorylation of p65 on Ser536; this response was abolished by NAC (Fig. 5d).

3.5. Acrolein- activated macrophages induce apoptosis of lung epithelial cells

In order to know whether GM-IMs, which were treated with acrolein, induce apoptosis of LLC cells, we first treated GM-IMs for 7 days with 10 μM of acrolein and then co-cultured with LLC cells. When LLC cells (a lung epithelial carcinoma cell line) were co-cultured with acrolein-treated GM-IM for 24 h, the number of Annexin V^{+} LLC was increased. By the treatment of NAC to GM-IMs apoptosis of LLC cells was reduced. (Fig. 6). These results show that acrolein-activated macrophages induced apoptosis of LLC cells in a manner that seems dependent on ROS generation.

4. Discussion

Chronic inhalation of cigarette alters immunological functions (Holt, 1987; Sopori and Kozak, 1998). Cigarette smoke contains carcinogens such as methylcholanthrene, benzo-α-pyrenes and acrolein (Stämpfli and Anderson, 2009), which might also induce

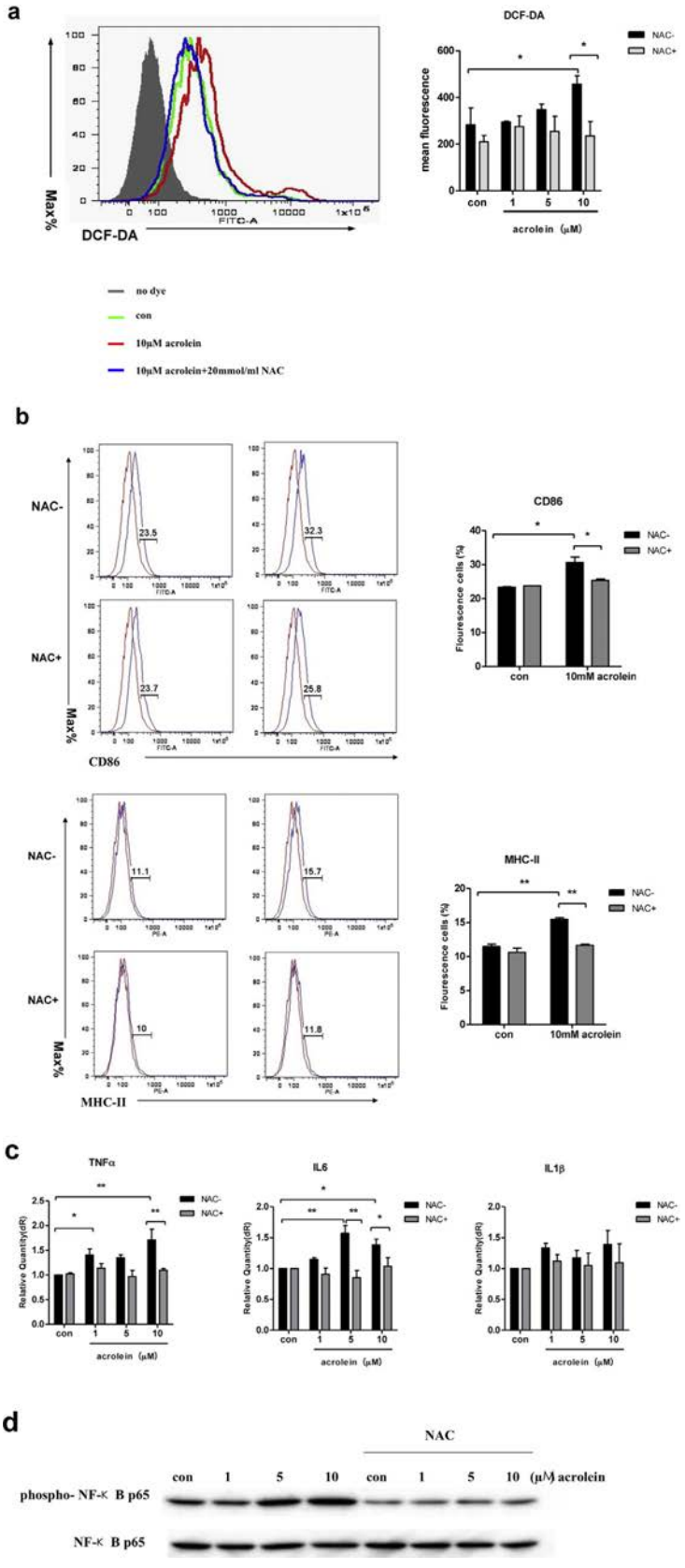

Fig. 5. ROS affect the activation of GM-IMs though NF-κB pathway. (a) GM-IMs were cultured by 10 μM acrolien for 1 week, after which ROS levels were measured by DCFH-DA fluorescence by flow cytometry. Right, mean fluorescence after subtracting autofluorescence. (b, c) After stimulation by 10 μM acrolein and treatmend with 20 mM NAC for 1 week, GM-IMs were collected and stained with CD86 and MHCII antibodies and analyzed by flow cytometry. The expression of genes for TNFα, IL6 and IL-1ß was analyzed by Real-time quantitative PCR. (d) The NF-κB p65 pathway was determined by western blot in GM-IMs after stimulated with 10 μM acrolein and 20 mM NAC. Data show means ± SEM ($n = 3$ or 4). *, $p < 0.05$; **, $p < 0.01$.

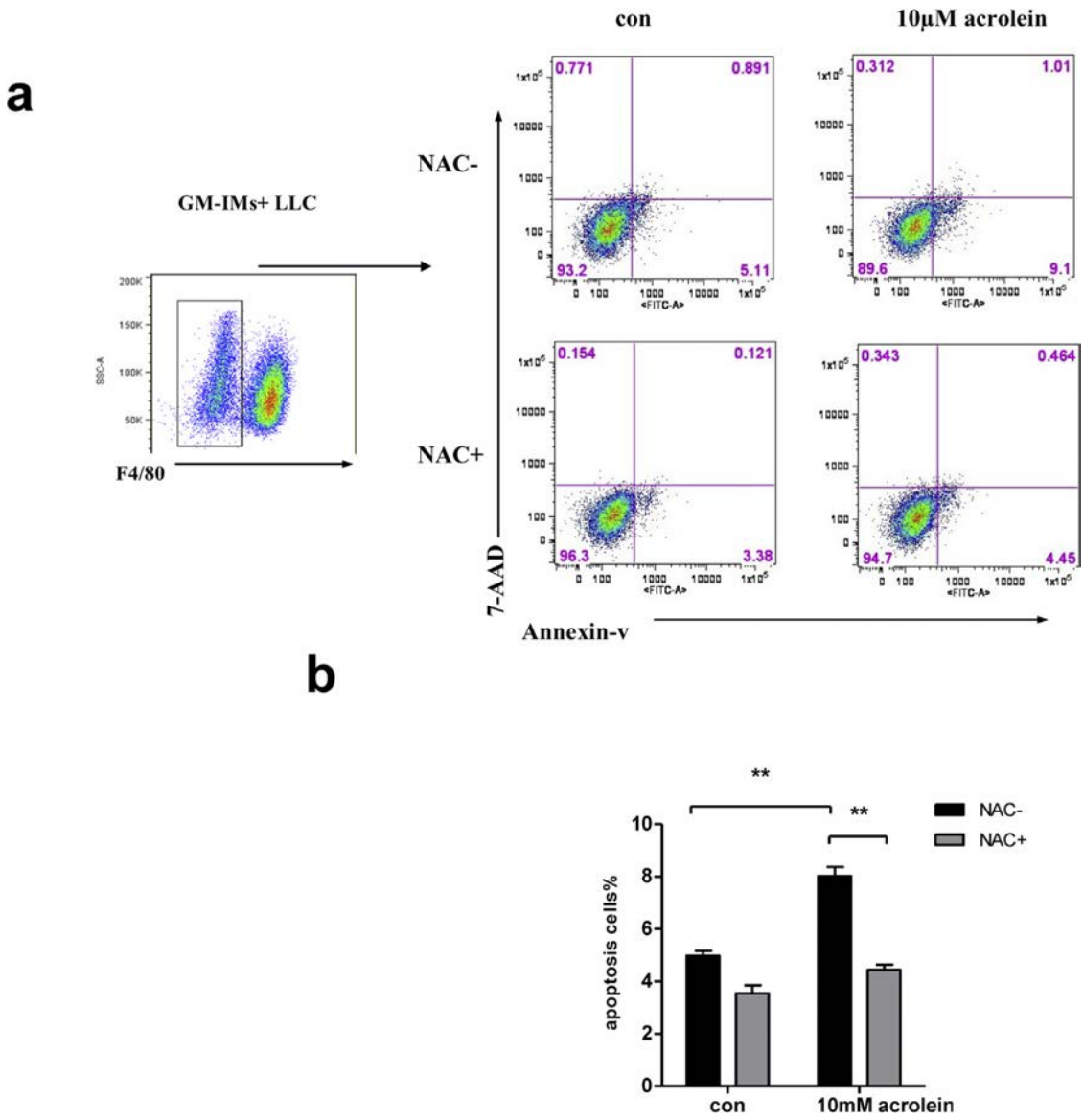

Fig. 6. Acrolein-treated GM-IMs caused apoptosis in Lewis lung carcinoma cells (LLC). (a) GM-IMs were treated with acrolein for 1 week, and were then co-cultured with LLC cells for 24 h. Both cells were collected and incubated with APC-conjugated F4/80 for 30 min at 4 °C and stained with FITC-labeled Annexin V and 7-AAD (BD Bioscience) for 15 min. Then F4/80 negative LLC cells were gated and apoptotic cells were analyzed by flow cytometry. (b) The graph represents the percentage of apoptotic cells. Data include means ± SEM ($n = 4$). **, $p < 0.01$.

inflammation. Here we examined the effects of acrolein to lung epithelial cells and lung innate immunity. Acrolein is an α,β-unsaturated aldehyde that is highly irritating to the respiratory system. Acrolein contains an active carbonyl group and an electrophilic α-carbon and thus is highly reactive with biological macromolecules (Bein and Leikauf, 2011). High concentration of acrolein are cytotoxic to many cell types including hepatocytes, neurocyte and alveolar epithelial cells (Mohammad et al., 2012).

The pathological hallmarks of COPD are destruction of the lung parenchyma, inflammation of the peripheral airways and inflammation of the central airways, which are characteristics of emphysema, bronchiolitis and chronic bronchitis, respectively (Turato et al., 2001). Acrolein also induces pulmonary injury and inflammation, but the underlying molecular mechanism is not completely understood. Alveolar type II epithelial cells (AE2) play various roles in alveolar fluid balance and host defense (Fehrenbach, 2001). Here, the numbers of AE2 cells were decreased by acrolein- induced apoptosis; this outcome may be relevant to overall lung destruction.

Our studies demonstrated that intranasal instillation of acrolein induced lung damage, including hemorrhage and macrophage infiltration. Acrolein-induced lung injury might induce DAMPs (Kono and Rock, 2008), that promote pulmonary inflammation through NF-κB signaling. $CD11c^{+}F4/80^{high}$ alveolar macrophages and $CD11b^{+}F4/80^{+}$ bone marrow-derived monocyte/macrophages were elevated after 1 week of exposure. We found that acrolein induced acute inflammatory responses, including M1 macrophages infiltration. Those cells produced pro-inflammatory cytokines such as TNFα, IL6, Irf5, and IL12. Chronic administration of acrolein causes enlargement of alveolar air space accompanied by a reduction of alveolar inflammatory responses. By the long term expression of acrolein, M1 macrophages returned to baseline and the M2 macrophages population increased. The latter might reflect tissue repair or tumor formation.

COPD is associated with oxidative stress. That stress is directly linked to the increased burden of smoke-derived inhaled oxidants and to the high amount of ROS generated by the increased number of activated alveolar macrophages and neutrophils present in the

alveolar spaces (Tuder et al., 2003; Lee et al., 2007). Cigarette smoke-produced oxidative stress might promote lung inflammation through NF-κB signaling (Marwick et al., 2004; Vlahos et al., 2006). Consistent with cigarette smoke's elevation of ROS, our results also indicate that ROS was significantly generated by acrolein during early inflammatory responses. We examined whether these macrophages could mediate direct tissue injury and lung epithelial cell apoptosis through secretion of pro-inflammatory mediators and generation of ROS. Thus, we conducted *in vitro* experiments by co-cultivation of macrophages and epithelial cells. Our results showed that 7 days of culture, GM-IMs were activated by low concentration of acrolein through the NF-κB signaling pathway. We also found that the ROS inhibitor NAC prevented acrolein-induced stimulatory effects on GM-IMs, presumably due to a blockade of NF-κB activation. In contrast to our results, Li et al. reported that acrolein inhibited NF-κB activation in lung macrophages and epithelial cells (Li et al., 1999). Further work is needed to resolve this discrepancy. Acrolein-activated GM-IMs induced early apoptosis of LLC cells co-cultured with GM-IMs. Inactivation of GM-IMs reduced early apoptosis of LLC cells. On acrolein challenge, lung-infiltrating macrophages have both pathogenic and protective functions during tissue damage, depending on the cells' phenotype (Murray and Wynn, 2011).

In conclusion, this study demonstrated that macrophages play an important roles during acrolein-induced lung injury and inflammation. Acute lung injury led to recruitment of M1 type macrophages to the lungs that, in turn, induced alveolar inflammation secondary to increases in the formation of ROS. As such, it is likely that oxidative stress is one key mechanism underlying acrolein-induced lung inflammation and tissue injury.

Conflict of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

Transparency document

The Transparency document associated with this article can be found in the online version.

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