

E-cigarette-Induced Pulmonary Inflammation and Dysregulated Repair are Mediated by nAChR $\alpha 7$ Receptor

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Abstract

Electronic cigarette (e-cig) vaping is increasing rapidly in the United States, as e-cigs are considered less harmful than combustible cigarettes. However, limited research has been conducted to understand the mechanism of toxicological and pulmonary effects of e-cigs. We hypothesized that sub-chronic exposure of e-cigs induced inflammatory response and dysregulated repair/extracellular matrix (ECM) remodeling, which occur through the $\alpha 7$ nicotinic acetylcholine receptor (nAChR $\alpha 7$). Adult wild-type (WT), nAChR $\alpha 7$ knockout (KO), and lung epithelial-cell-specific KO (nAChR $\alpha 7$ CreCC10) mice were exposed to e-cig aerosol containing propylene glycol (PG) with or without nicotine. Bronchoalveolar lavage fluids (BALF) and lungs were collected for determination of inflammatory responses and ECM remodeling, respectively. Sub-chronic e-cig exposure with nicotine increased the lung influx of macrophages and T-lymphocytes, and the levels of pro-inflammatory cytokines, while nAChR $\alpha 7$ knockdown blocked the inflammatory responses. Interestingly, matrix metalloproteinases (MMPs), such as MMP2, MMP8, and MMP9, in both sex mice were altered at both protein and gene levels when WT mice were exposed to PG alone in a sex-dependent manner. Moreover, MMP12 increased significantly in male mice exposed to PG with or without nicotine in a nAChR $\alpha 7$ dependent manner. Additionally, the abundance of ECM proteins, such as collagen and fibronectin, was significantly altered after sub-chronic e-cig exposure with or without nicotine in a sex-dependent manner, but nAChR $\alpha 7$ independent manner. Overall, sub-chronic e-cig exposure with or without nicotine affected lung inflammation and repair responses/ECM remodeling, which were mediated by nAChR $\alpha 7$ in a sex-dependent manner.

Introduction

E-cigarettes (e-cigs) are often considered a safer alternative to combustible cigarettes, as well as a method for quitting traditional cigarette smoking [1, 2]. The e-cig delivery system is based on tanks or cartridges that are loaded with e-cig liquid (e-liquid), which is then aerosolized, with inhalation delivering the aerosol to the lungs. Usually, e-liquids are composed of propylene glycol (PG) and/or vegetable glycerin (VG), with varying concentrations of nicotine (up to 100 mg/mL). They may also contain flavors and flavoring chemicals to attract teenagers and young users [3]. While PG and VG are considered safe in food-grade products, adverse health effects have been reported from these substances when they serve as e-cig vehicles [4]. Previous studies have shown that the amount of nicotine delivered by e-cigs is much higher than the amount delivered via cigarette smoke [5, 6]. Our recent research has shown that inflammation and extracellular matrix (ECM) remodeling/dysregulated repair are altered by acute exposure to e-cigs, with or without nicotine [7]. Considering that e-cig vaping is often a long-term habit, research on the effects of chronic exposure to e-cig aerosol is necessary to understand the mechanism of resulting inflammatory responses and ECM remodeling, which are the fundamental basis for most chronic lung diseases, such as idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD).

In our previous studies, acute exposure to e-cig aerosol containing flavoring chemicals was shown to cause lung inflammation, oxidative stress, and dysregulated repair [7, 8]. Further, more severe

viral/bacterial lung inflammation was observed when nicotine was involved, which may have been due to the immune system being suppressed by the nicotine [9, 10]. Nicotinic Acetylcholine Receptors (nAChRs) are largely responsible for activation of acetylcholine neurotransmitter signaling pathways in the central nervous system (CNS) [11, 12]. The nAChRs are widely distributed in the CNS, so they can easily be activated by nicotine, initiating and reinforcing a rewarding feedback loop which might induce nicotine addiction [13, 14]. Interestingly, lung nAChRs activation could help inhibit the inflammation caused by lipopolysaccharides (LPSs) and the receptor knockouts (KOs) that promote inflammatory processes [15, 16]. However, as one of the nAChR-agonists, nicotine could initiate receptor-related pathways, and nicotine aerosol inhalation also induce inflammation [8]. Other chemicals may induce activation of nAChRs, and they may also trigger feedback-loops, similar to flavoring chemicals [17-19]. Although the biological structure and function of nAChRs has been well studied, limited information exists on how nAChRs are affected during e-cig induced lung dysregulation processes.

A common vehicle used for nicotine delivery in e-cigs is PG and/or VG. PG (C₃H₈O₂) is a colorless and odorless organic solvent that is an FDA-approved, food-grade chemical. This is one of the reasons why people originally thought to use PG as a nicotine carrier in e-liquids. Previous studies have shown that aerosolization of PG produces different byproducts, some of which might lead to cancer [20]. While research is ongoing understand the e-cig aerosol causing lung inflammation and dysregulated repair, however it is still not clear the harmful effects of e-cig use, especially in regards to the vehicle/humectant (PG) alone.

In this study, we hypothesized that e-cig aerosol induce lung inflammation and dysregulated repair/ECM remodeling in a nAChR $\alpha 7$ dependent manner. Our results suggest that lung inflammatory responses and dysregulated repair/ECM remodeling induced by e-cig aerosol containing nicotine could potentially be related to the nAChR $\alpha 7$ pathway. On the other hand, PG-induced lung dysregulated repair and inflammatory response occurs in a nAChR $\alpha 7$ -independent manner.

Methods

Animals

Adult C57BL/6J (WT) mice and $\alpha 7$ nicotinic acetylcholine receptor knockout (nAChR $\alpha 7$ KO) mice were both purchased from Jackson Laboratory, weighing 25-35 grams and aged 3-4 months old. The nAChR $\alpha 7$ CreCC10 mice (clara/club-cell-specific nAChR $\alpha 7$ deletion) were generated by crossing nAChR $\alpha 7$ floxed mice (nAChR $\alpha 7$ floxed mutant) from Jackson Laboratory (donated by Dr. Jerry Yakel, NIEHS/NIH) with mice have the Cre recombinase transgene controlled by the CC10 promoter (C57BL/6J; from TJ Mariani, University of Rochester, Rochester, NY). Prior to e-cig exposure, mice were housed in the inhalation core facility at the University of Rochester for 1 week. All experiments performed in this study were in compliance with the standards set by the United States Animal Welfare Act. The Animal Research Committee (UCAR) approved the animal protocol at the University of Rochester Medical Center, Rochester, NY.

Blood gas and exercise ability measurement

Blood gas, including pH, pressure of CO₂ and O₂, concentration of HCO₃, TCO₂, glucose (Glu) and hemoglobin (Hb), percentage of O₂ and hematocrit (Hct) were analyzed by i-STAT system (i-STAT CG8+ cartridge, Cat# 03P88-25; Vet scan i-STAT 1 analyzer; Abaxis Global Diagnostics), as described previously [21]. Exercise ability measurement was done using a motorized animal treadmill (Columbus Instruments) as described in our previous research [22]. Running distance (meters) and running time (minutes) were recorded to present exercise tolerance. Mice stayed on the treadmill for 5 min before start of measurement. The treadmill began at 8.5 m/min with 0° incline for 9 min. Then, the speed was increased to 10 m/min with 5° incline for 3 min. After that, the speed was increased 2.5 m/min every 3 min, and incline was increased 5° every 9 min until mouse exhaustion, judged by observation of failure of running and continuous contact with the electric grid. Following this exercise, blood was collected by submandibular venipuncture for blood gas and cotinine measurement.

E-cig device and e-liquid

The e-cig device and e-liquid used in this study have been described before. The e-liquids used here, PG and PG with nicotine (25mg/mL), were purchased from xtremevaping.com. The e-cig device used is the Joytech eVIC VTCmini with a 0.15Ω atomizer/coil (Kanger Tech). All other components of the e-cig exposure chamber were from SCIREQ. The atomizer/Coil was replaced on a weekly basis to avoid overheating and generation of carbon monoxide.

E-cig exposure

The e-cig exposure performed here has been described in our previous studies[23]. Briefly, the *in vivo* e-cig exposure was set up inside a fume hood and based around the SCIREQ InExpose e-cig extension smoking system. The e-cig exposure puffing profile used was based on realistic topographical data from e-cig users, with 3.3 sec/puff and a 70mL puff volume. The aerosolization of e-liquid was performed by a 3rd generation e-cig device (Joytech eVIC VTCmini), which was controlled by the SCIREQ flexware software (V8.0). The whole-body exposure was done for a total of 2 hrs/day, 5 days/week, for 30 days. During exposure, temperature, humidity, oxygen and carbon dioxide percentages were monitored, and carbon monoxide was recorded daily. The e-cig aerosol generated was passed through the condensing chamber and pumped into the mixing chamber with a flowrate of 1.0 L/min. The vapor was diluted with air in the mixing chamber and then delivered into the whole-body exposure chamber where mice stayed, separated by dividers. Simultaneously, the e-cig aerosol in the exposure chamber was exhausted by another pump with 2.0 L/min flowrate. Both pumps were calibrated and adjusted each time before exposure. Pumps were cleaned at the end of each exposure to minimize the effects of nicotine residues. Mice were divided into air (control), PG, and PG with nicotine groups, each with an equal number of males and females, for both the WT and nAChR α7 KO conditions. Air group mice stayed in the inhalation facility in a similar environment during the 30 day exposure. Serum cotinine levels, measured by ELISA (Calbiotech), were ~500 ng/mL for the PG with nicotine group, and ~20 ng/mL for the PG only group, due to nicotine

residue in the pumps and exposure chambers which was difficult to fully clean (**Additional File1: Figure. S1**). As expected, the air group showed no cotinine.

Bronchoalveolar lavage fluid (BALF)

Mice were euthanized with Ketamine/Xylazine 24 hrs after the final exposure. Their tracheas were cannulated and their lungs were lavaged three times with 0.6 mL saline with 1% FBS (1.8 mL total). The recovered fluids were collected and spun down at 1000 x g for 10 min at 4°C for harvesting of BALF cells. The supernatant was stored at -80°C for future analysis. The BALF cells were re-suspended in 1.0mL saline with 1% FBS and stained with acridine orange propidium iodide (AO/PI). Total cell counts per mL were measured from AO/PI stained cells via cellometer.

Inflammatory cell count

The resuspended BALF cells were used for immune-inflammatory cell counts with cell-type-specific monoclonal antibody labeling. Total 1.0×10^5 BALF cells were used for antibody labeling. Before antibody staining, all cells were blocked with purified anti-mouse CD16/32 (Cat# 50-163-432, Fisher Scientific) to prevent non-specific binding, and washed with PBS once. The cells were stained with F4/80 PE-conjugated antibody for macrophages (Cat# 123109, BioLegend), LY6B.2 Alexa fluor488-conjugated antibody for neutrophils (Cat# NBP213077AF488, Novus Biologicals), PE-Cyanine7 antibody for CD4a⁺ T-lymphocytes (Cat# 25-0041-82, Fisher Scientific), and APC conjugated Monoclonal Antibody for CD8a⁺ T-Lymphocytes (Cat# 17-0081-82, Fisher Scientific). The absolute cell numbers of macrophages, neutrophils, and CD4a⁺/CD8a⁺ T-lymphocytes were determined by multiplying the percentage of cells by the total cell counts. Flow cytometry was performed using the Guava® easyCyte™ flow cytometer (Millipore Sigma) and analyzed using Guava® InCyte™ software.

Measurement of pro-inflammatory cytokines by Luminex in BALF

To measure the pro-inflammatory cytokines present in BALF, a Bio-Plex Pro mouse cytokine 23-plex immunoassay kit (Cat#: M60009RDPD, BioRad) was used, according to the manufacturer's instructions. Briefly, the diluted magnetic beads were placed into the assay plate and rinsed with wash buffer. The BALF samples and standards were then added into the wells, and shaken at 850 rpm at room temperature for 30 min. After sample incubation, the plates were washed with wash buffer 3 times, then the detection antibody was added and the plates incubated for 30 min at room temperature, shaking at 850 rpm. Next, plates were washed with wash buffer 3 times, and SA-PE was added for 10 mins at room temperature, shaking at 850 rpm. After this step, the plates were washed 3 times with wash buffer, and the beads were resuspended in assay buffer for reading. Results are determined via the Luminex flexmap 3d (Luminex Corp.)

Protein isolation

Total lung tissues were isolated during sacrifice and stored at -80°C for future analysis. Frozen lungs were cut into roughly 30 mg sections and homogenized mechanically in RIPA buffer with protease inhibitor (Cat#: 78440, ThermoFisher Scientific) for 20 sec, 2 times. After homogenization, lysates were kept on ice for at least 45 min, followed by centrifugation at 15,000 x g for 30 min at 4°C. The supernatant was collected in a new tube. The protein concentration was quantified using the Pierce BCA Protein Assay Kit (Cat#: 23227, ThermoFisher Scientific), based on the manufacturer's protocol. The protein samples were then used for western blot analysis at certain concentrations.

Western blot

The protein samples from lung homogenates were loaded into SDS-polyacrylamide electrophoresis gels (SDS-PAGE) with 20 µg of protein each lane. Proteins were stacked in 4% SDS-PAGE and separated in 10% SDS-PAGE. After electrophoresis, the whole gel was transferred onto a nitrocellulose membrane (Cat# 1620112, BioRad). After protein transfer, the membrane was washed with tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 15 min, then blocked with 5% non-fat dry milk for 1 hr at room temperature. Then the membranes were probed with primary antibodies anti-: MMP2 (1:1000, ab92536, Abcam); MMP9 (1:1000, ab38898, Abcam); MMP8 (1:1000, ab81286, Abcam); MMP12 (1:1000, NBP2-67344, Novus Biologicals); Collagen Ia1 (1:1000, NBP1-30054, Novus Biologicals); Collagen Ia2 (1:500, NBP1-57987, Novus Biologicals); fibronectin (1:2000, ab45688, Abcam); and PAI-1 (1:1000, ab182973, Abcam). The primary antibodies were incubated overnight at 4°C, and then washed 4 times on rotator in TBS-T at room temperature, 15 min per wash. The blots were then probed with a secondary antibody (Goat-Anti-Rabbit, 1:10000, Cat# 1706515, BioRad) for 1 hr at room temperature. Following 4 washes in TBS-T at room temperature, luminescence signals were developed using chemiluminescence substrate (Perkin Elmer, Waltham, MA). The membrane exposure and band intensities were detected via the Bio-Rad ChemiDoc MP imaging system (Bio-Rad Laboratories, Hercules, CA, USA). Protein quantification was done by densitometry analysis, and fold changes were used in this study after normalization to β-actin (1:2500, ab20272, Abcam). All conditioned groups were normalized to the air control group.

RNA isolation and Nanostring quantification

During sacrifice, lung tissues were isolated and snap-frozen for future analysis. Total 100 mg of lung tissues were analyzed for each condition and mechanically homogenized in Trizol for 20s. The RNA was isolated using the Direct-zol™ RNA Miniprep Plus assay kit (Cat# R2073, Zymo Research). Isolated RNA was quantified and checked for quality by spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA). RNA samples were aliquoted and sent out for nanostring myeloid panel screening (NanoString Technologies, Inc.).

Statistical analysis

The statistical analysis was done by either one-way ANOVA or student's t-test, followed by Tukey's multiple comparisons in GraphPad Prism software (Version 8.0, La Jolla, CA). Results were presented as the mean ± SEM. A significant difference was considered significant when $P < 0.05$.

Results

Sub-chronic e-cig exposure altered exercise capacity and blood-gas saturation

After sub-chronic 30 days of e-cig exposure, WT (floxed) mice showed no difference in exercise capacity among the groups (**Additional File 1: Figure S2**). However, mice lacking nAChR $\alpha 7$ showed less capacity to run after being exposed to PG with nicotine compared to the other two exposure conditions. Interestingly, mice with a nAChR $\alpha 7$ lung epithelial cell-specific conditional deletion showed the same results, which indicates that nicotine inhalation with a nAChR $\alpha 7$ KO lowered exercise capacity, and that nAChR $\alpha 7$ in the epithelium played an essential role in this change. Blood pH, O_2 pressure, concentrations of HCO_3 and CO_2 were shown to be dysregulated when WT mice were exposed to PG with nicotine compared to PG alone, while no difference was observed in nAChR $\alpha 7$ deficient mice exposed to PG with or without nicotine (**Additional File 1: Table S1**).

Sub-chronic exposure of e-cig aerosols induces upregulation of inflammatory cells

in mouse lung

The total cell counts in WT mice exposed to PG with or without nicotine all increased relative to their respective air group, and total cell counts for mice exposed to PG with nicotine increased significantly (**Figure 1A**). Interestingly, nAChR $\alpha 7$ KO mice showed no change in total cell counts when exposed to PG with nicotine. However, exposure to PG alone insignificantly increased the total cell counts (**Figure 1A**). Macrophage counts followed a similar trend to total cell counts (**Figure 1B**). Neutrophil counts showed a significant increase in PG-exposed nAChR $\alpha 7$ KO mice compared to WT mice (**Figure 1C**). WT and nAChR $\alpha 7$ KO mice exposed to PG with nicotine showed no change in neutrophil counts in BALF (**Figure 1C**). Additionally, $CD4a^+$ and $CD8a^+$ T-lymphocyte counts were significantly increased when WT mice were exposed to PG with nicotine, and lacking nAChR $\alpha 7$ helped to prevent dysregulation of T-lymphocytes (**Figure 1D-E**). Interestingly, PG-exposed nAChR $\alpha 7$ KO mice showed higher levels of neutrophils and $CD4a^+/CD8a^+$ T-lymphocytes than PG-exposed WT mice.

Sub-chronic e-cig exposure augments influx of pro-inflammatory cytokines in BALF

We next determined the level of pro-inflammatory cytokines in BALF. The levels of IL-1 α , MCP-1, TNF α , GM-CSF, MIP-1 β , IL-2, IL-5, IL-9, IFN γ , RANTES, IL-6, IL-12p70, IL-13, KC, IL-1 β , and Eotaxin were significantly increased when exposed to PG with nicotine compared to PG alone and the air control group in WT mice (**Figure 2 and Additional File 1: Table S2**). Interestingly, IL-1 α , GM-CSF, IL-2, IL-9, IFN γ , RANTES, MCP-1, and Eotaxin were decreased significantly with nAChR $\alpha 7$ KO compared to WT mice exposed to PG with nicotine (**Figure 2 and Additional File 1: Table S2**), which is nAChR $\alpha 7$ dependent. Besides, IL-1 β and IL-5 both increased in either nAChR $\alpha 7$ KO or WT mice when exposed to PG with nicotine, which might indicate that these two cytokines are nAChR $\alpha 7$ independent. Additionally, IL-3, IL-4, IL-10, IL-12p40, IL-17A, G-CSF, and MIP-1 α all showed no significant difference among the different conditions (**Additional File 1: Table S2**).

Sub-chronic e-cig exposure alters mRNA expression of inflammation and dysregulated repair response genes in WT and nAChR $\alpha 7$ KO mouse lung

To further understand the inflammatory responses and dysregulated repair/ECM remodeling occurring in this study, a inflammatory-myeloid panel of 734 genes for gene expression analysis was used (Nanostring Inc). The gene datasets from nCounter Mouse Myeloid Innate Immunity Panel were analyzed via Nanostring nSolver and R programming language (**Additional File 2: Table S3**). The boxplots show the distribution of the normalized gene-level among the different experimental conditions (**Additional File 1: Figure S3A**). From the R analysis, 110 and 109 dysregulated genes were found when mice were exposed to PG alone in WT and nAChR $\alpha 7$ KO mice, respectively, and 21 genes were found in common between WT and nAChR $\alpha 7$ KO mice when exposed to PG alone, compared to air controls (**Additional File 1: Figure S3B**). Similarly, a total of 190 and 228 genes were found to be altered in WT and nAChR $\alpha 7$ KO mice, respectively, when exposed to PG with nicotine. Additionally, 89 genes were found in common when mice of different genotypes were exposed to PG with nicotine (**Additional File 1: Figure S3B**). In a comparison between PG alone and PG with nicotine, 146 genes were dysregulated in WT mice, 177 genes were changed nAChR $\alpha 7$ KO mice, and 57 genes were found to be common between WT and nAChR $\alpha 7$ KO mice when exposed to PG alone, compared to PG with nicotine (**Additional File 1: Figure S3B**).

In the WT mice, significantly higher RNA counts were found for multiple targets in both the PG with or without nicotine exposed groups, compared to the air control (**Additional File 1: Figure S4**). When WT mice were exposed to PG alone, compared to air control mice, and mice exposed to PG with nicotine, some inflammatory markers and ECM remodeling markers, including MMP9, MMP8, S100A8, and some collagens (COL17A1 and COL14A1), were dysregulated (**Additional File 1: Figure S4A**). Also in the WT mice, comparisons between the air control and PG with nicotine exposure groups showed differences in more inflammatory focused markers, such as ARG1 and LPL (**Additional File 1: Figure S4A**). As expected, nAChR $\alpha 7$ KO mice exposed to PG or air showed no difference in gene levels compared to WT mice. However, PG with nicotine exposure dysregulated multiple targets with significant differences between WT and nAChR $\alpha 7$ KO mice in levels of gene expression, including both inflammation and dysregulated repaired/ECM remodeling markers, such as SMAD7, KLF4, CDH1, COL4A2, ICAM1, LDLR, IL1B, TLR5, NFKBIA, and CXCL2 (**Additional File 1: Figure S4B**). We then selected specific gene targets and focused on inflammatory responses and dysregulated repair/ECM remodeling for further analysis (**Figures 3**).

Based on the Nanostring nCounter analysis, gene targets with significant differences between WT and nAChR $\alpha 7$ KO mice, when exposed to PG with nicotine, were selected and plotted (**Figure 3A**). Notably, SKIL (Ski-like protein) and LDLR (Low-density lipoprotein receptor) were significantly decreased in WT mice exposed to PG with nicotine, while nAChR $\alpha 7$ KO helped to blocked it. Interestingly, CCL9 (Chemokine ligand 9), KLF4 (Kruppel-like factor 4), DUSP1 (Dual Specificity Phosphatase 1), BTLA (B and T Lymphocyte Associated), and SMAD7 (SMAD Family Member 7) only showed dysregulated gene-level in nAChR $\alpha 7$ KO mice exposed to PG with nicotine compared to WT mice (**Figure 3A**). As for NECTIN1, both WT and nAChR $\alpha 7$ KO mice showed decreased trend, but only WT mice showed a significant difference between the air control and PG with nicotine exposure groups (**Figure 3A**).

Sub-chronic e-cig exposure with nicotine in nAChR α 7 deficient mice prevents dysregulation of p50/p105 in a sex-dependent manner

In order to understand the inflammation responses in protein level, we measured the protein abundance of p50/p105, one of the subunits of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) via Western blot (**Figure 4**). We have observed that the protein levels of both p50 and p105 upregulated in female WT mice exposed to PG with nicotine, while attenuated by lacking nAChR α 7 (**Figure 4A**). However, there was no significant difference in male mice exposed to PG with or without nicotine compared to air controls in either WT or nAChR α 7 KO mice. Notably, in males only, decreased p50/p105 protein abundances were found in nAChR α 7 KO mice compared to WT mice (**Figure 4B**). Female mice exposed to PG alone, whether WT or nAChR α 7 KO, showed similar upregulation of p105, indicating that PG alone could induce pro-inflammatory responses in a nAChR α 7 independent manner (**Figure 4A**).

Sub-chronic e-cig exposure with or without nicotine induced dysregulated repair/ECM remodeling in a sex-dependent but nAChR α 7 independent manner

In recent studies, we have identified that acute e-cig exposure has induced dysregulated repair/ECM remodeling in mouse lungs [7]. We are therefore interested in how ECM remodeling could be induced via sub-chronic exposure. We have selected MMPs and ECM markers to measure in both protein abundance (**Figure 5-6**) and gene expression level (**Figures 3B**). Mice exposed to PG alone showed prominent dysregulation in both protein and gene expression levels, and protein level was in a sex-dependent manner (**Figures 5-6**).

From the absolute RNA count, MMP8 and MMP9 were found to be lower in the PG groups than in air control or PG with nicotine exposed WT or nAChR α 7 KO mice (**Figure 3B**). A slightly increased MMP8 level was observed in PG with nicotine-exposed mice compared to air controls, but this difference was not significant (**Figure 3B**). As an inhibitor of MMPs, TIMP3 was shown to be upregulated statistically in both WT and nAChR α 7 KO mice when exposed to PG with nicotine (**Figure 3B**). For the ECM proteins, no change in gene expression was detected in fibronectin (FN1) or Plasminogen activator inhibitor-1 (PAI-1, gene code: SERPINE1), while significant decreases were detected in collagens (COL1A2 and COL4A1), when exposed to PG with or without nicotine in both WT and nAChR α 7 KO mice.

Since we observed dysregulated gene levels, protein abundances were also measured. The protein levels of MMP2, MMP8, MMP9, and MMP12 were selected, and PAI-1, COL1A1, COL1A2, and fibronectin were selected as ECM markers. Both female and male mice were used here to clarify any sex-dependent differences. We noticed similar trends of MMP9 and MMP8 protein levels compared to RNA transcript levels in both female and male mice (**Figure 5**). Decreased MMP8 and MMP9 were observed in PG-exposed WT and nAChR α 7 KO mice, while PG with nicotine-exposed mice showed a lesser decrease when compared to air controls in both females and males (**Figure 5**). Notably, in both females and males, upregulated MMP2 protein abundance was seen in both male and female WT mice when exposed to PG alone, compared to air controls and PG with nicotine-exposed mice. Moreover, there was an increased

MMP2 baseline when female mice lack the nAChR $\alpha 7$ while no such change is seen in male mice (**Figure 5**). Interestingly, MMP9, MMP2, and MMP12 were found to be upregulated in male WT mice exposed to PG with nicotine, while the nAChR $\alpha 7$ deletion helped prevent the dysregulation of these MMPs (**Figure 5B**). For MMP8, female and male mice showed similar protein levels, and nAChR $\alpha 7$ deficiency induced downregulation of protein abundance (**Figure 5**).

ECM proteins/regulator, collagens, fibronectin, and PAI-1, were measured as well (**Figure 6**). The protein abundance of PAI-1 showed no difference among all conditions in females (**Figure 6A**), while it was increased in male WT mice exposed to PG with nicotine, and slightly decreased in nAChR $\alpha 7$ KO males (**Figure 6B**). Further, COL1A1 and COL1A2, two different subunits of type 1 collagens, showed different expression levels among the experimental groups. Interestingly, both female and male WT mice showed upregulated COL1A2 when exposed to PG alone in both WT and nAChR $\alpha 7$ KO mice (**Figure 6**). While, COL1A1 exhibited no changes in either WT or nAChR $\alpha 7$ KO female mice, decreased protein abundances were seen when male WT mice were exposed to PG with or without nicotine, and a lower baseline of COL1A1 was seen in mice lacking nAChR $\alpha 7$. Similarly, we found a lower protein expression level of fibronectin in female WT and nAChR $\alpha 7$ KO mice when exposed to PG with or without nicotine compared to air group (**Figure 6A**). However, decreased fibronectin was seen in the PG-exposed male WT mice, and there was no alteration in male nAChR $\alpha 7$ KO mice among the different exposure conditions (**Figure 6B**).

Sub-chronic e-cig exposure in lung epithelial cell-specific nAChR $\alpha 7$ deletion protects against inflammation

Considering the inflammatory responses seen in WT and nAChR $\alpha 7$ KO mice, we next determined the role of nAChR $\alpha 7$ in the lung epithelial cells when exposed to e-cig with or without nicotine. Surprisingly, we did not see any significant changes in cytokines in PG with nicotine-exposed in nAChR $\alpha 7$ epithelial cell-specific KO mice. However, we did find IL-5, MCP-1, KC, Eotaxin, GM-CSF, and G-CSF to be significantly up-regulated in the PG alone group when compared to air control mice, and inhibited in nAChR $\alpha 7$ epithelial cell-specific KO mice (**Additional File 1: Figure S5A**). The rest of the cytokines, IL-1 α , TNF α , MIP-1 β , IL-2, IL-9, IFN γ , RANTES, IL-6, IL-12p70, IL-13, IL-1 β , Eotaxin, IL-3, IL-4, IL-10, IL-12p40, IL-17A, and MIP-1 α , showed no changes among the different groups (**Additional File 1: Figure S5B-C**).

Discussion

The popularity of e-cig vaping has been rising recently in the United States, and hence the health concerns about vaping have recently attracted public attention [24]. Our previous studies have shown that acute exposure to e-cigarettes could cause inflammatory responses, oxidative stress, and ECM remodeling [7, 8, 25]. Although in past studies, we have shown the adverse health effects caused by unflavored and flavored e-cig exposure, we have only conducted short-term acute exposures. Studies on e-cig/nicotine effects of sub-chronic or chronic, are needed to understand in detail the harmful effects of e-cig aerosol exposure. Based on our knowledge, no study has yet shown that long-term exposure of e-cig aerosol can cause ECM remodeling and inflammatory responses possibly via nAChR $\alpha 7$ receptor.

Similar to our acute exposure results, e-cig aerosol with nicotine induced inflammatory cell counts in BALF compared to air controls, especially increase in macrophages and CD4a⁺/CD8a⁺ T-lymphocytes. Previous report has demonstrated that two weeks of e-cig (with nicotine) exposure increased the number of macrophages but not neutrophils, which supports our findings [26]. Another study reported that acute exposure of e-cig aerosol increased total cell and macrophage counts; even the vehicle exposure alone showed a trend toward upregulation, which also matches our results [27]. More excitingly, based on our results, nAChR $\alpha 7$ deficiency was capable of reducing the upregulation of macrophages and CD4a⁺/CD8a⁺ T-lymphocytes. However, nAChR $\alpha 7$ ablation was shown to promote inflammatory responses by increasing the number of neutrophils and CD4a⁺/CD8a⁺ T-lymphocytes when exposed to PG alone, compared to WT mice. Recently, Uchiyama et al. found aerosol-derived chemicals, such as formaldehyde, acetaldehyde, and methylglyoxal, in nicotine-free aerosol [28], which might be responsible for PG induced inflammation.

Supporting our findings, previous literature has shown that nAChR activation can inhibit inflammation via the NF- κ B pathway [29], which implies that knockdown of nAChR $\alpha 7$ may promote inflammatory responses which are nicotine-independent. Moreover, Zhang et al. [30] and Mucchietto et al. [31] have reported that blockage of nAChR $\alpha 7$ helps to reduce tumor growth and cellular proliferation through the MEK-ERK pathway. Additionally, inhalation of e-cig aerosol with nicotine is well-known for inducing inflammatory responses [7, 8, 27]. As the agonist of nAChR $\alpha 7$, nicotine might be both pro-inflammatory and anti-inflammatory, and detailed research is needed to study the relationship between nicotine and nicotinic receptors.

Apart from the inflammatory cells, some of the pro-inflammatory mediators in BALF were significantly upregulated following exposure to e-cig aerosol containing nicotine. It must be noted that macrophage-driven cytokines, IL-1 α , MCP-1, and GM-CSF, increased in WT mice exposed to PG with nicotine, and attenuated in nAChR $\alpha 7$ KO mice compared to air control. While some other macrophage-driven cytokines, IL-1 β , TNF- α , and MIP-1 β , were increased in both WT mice and nAChR $\alpha 7$ -deficient mice when exposed to PG with nicotine. Moreover, Scott et al. demonstrated that e-cig aerosol contained nicotine induced cytotoxicity in alveolar macrophage and significant release of inflammatory mediators (TNF- α , CXCL-8, MCP-1, and IL-6), while nicotine-free aerosol caused fewer effects [32]. Excluding macrophage-driven pro-inflammatory mediators, T-lymphocytes related to cytokines/chemokines (IL-2, IL-5, IL-9, IFN- γ , and RANTES) were also measured. Recently, studies have shown that IFN- γ is related to cytotoxicity and infiltration of CD8a⁺ T-cells [33, 34]. Similarly, Lim et al. discovered that asthmatic airway inflammation induced by e-cig aerosol with or without nicotine is accompanied by the production of Th2 cytokines, such as IL-5 [10]. Commonly, IL-2, IL-9, and RANTES are all associated with T-cells, and all exhibited a higher concentration in BALF of WT mice exposed to PG with nicotine [35-37]. Interestingly, we observed increased levels of IL-2, IL-9, IFN- γ , and RANTES in mice exposed to PG with nicotine, and we found that this effect was blocked by the nAChR $\alpha 7$ KO. Changes in cytokine expression are partially corroborates with our inflammatory cell counts for mice exposed to PG with nicotine. However, PG-induced upregulation of CD4a⁺/CD8a⁺ T-lymphocytes does not corroborate with the cytokine levels we showed

here. Further studies are required to determine the toxicological effects of nicotine-free e-liquid over a long-term exposure period.

Further, lung epithelial cell-specific nAChR $\alpha 7$ KO mice were used to investigate the role of the nicotinic receptor in the lung epithelium, which is directly in contact with e-cig aerosol in the lungs. For the first time, we have observed increased cytokines (IL-5, MCP-1, KC, Eotaxin, GM-CSF, and G-CSF) in the PG only exposure group, but no change in the PG with nicotine exposure group in lung epithelial nAChR $\alpha 7$ KO. Surprisingly, we noticed that PG-induced inflammation is ameliorated by the lung epithelial nAChR $\alpha 7$ KO, indicating that nAChRs $\alpha 7$ in the lung epithelial cells play a significant role in inhaled nicotine-induced inflammation. However, we observed varied results when compared to WT mice in these mice by PG with nicotine exposures. Further studies are required to fully understand the variability of e-cig exposure in animal models in a chronic studies, in particularly using different flavors.

We measured the absolute RNA count (mRNA expression) using the mouse myeloid innate immunity panel from Nanostring technology to screen the potential critical targets, such as SKIL and LDLR. Our results showed dysregulation of these targets in PG with nicotine-exposed WT mice, but no dysregulation was observed in nAChR $\alpha 7$ KO mice compared to PG or air control groups. Notably, SKIL inhibits the TGF β /SMAD signaling pathway by binding to SMAD molecules [38, 39]. Interestingly, Yang et al. found that SKI significantly prevents TGF β -induced epithelial–mesenchymal transition (EMT) through observation of more severe dysregulation of E-cadherin, N-cadherin, SMAD3, and PAI-1 when SKI is silenced in lung epithelial cells [39]. From our study, we found PAI-1 increased in WT mice exposed to PG with nicotine, but this increase was significantly reduced in KO mice, indicating that the SKIL-TGF β -PAI-1 connection may be regulated by nAChR $\alpha 7$ when challenged with e-cig aerosol containing nicotine. In relation to the other significantly altered target, LDLR, previous publications have proved that it is related to inflammatory processes [40, 41]. Ricci et al. identified that LDLR is associated with macrophage differentiation [40]; and that LDLR deficiency is capable of reducing inflammatory responses in THP-1 cells [40], as well as inhibiting the pro-inflammatory cytokines in the central nervous system [41]. In our study, downregulation of LDLR was seen in PG with nicotine exposed WT mice while this effect was significantly reduced in mice with the nAChR $\alpha 7$ deletion. These findings suggest that nAChR $\alpha 7$ may be an important gateway to the disruption of specific genes and further inflammatory responses/ECM remodeling in the case of long-term exposure to e-cig aerosol with nicotine.

To further study the inflammatory responses, we measured the protein abundance of NF- κ B (p50/p105) in both female and male mice. The activation of NF- κ B is well documented in inflammatory responses, especially in cigarette smoke-induced inflammation, however research on e-cig aerosol-related NF- κ B dysregulation is relatively scarce [42, 43]. Interestingly, while the anti-inflammatory effects (i.e. inhibition of NF- κ B signaling) via activation of nAChRs is well-known and has been studied in-depth, nicotine also induces inflammation through activation of the same NF- κ B signaling pathway [29, 44]. Our findings suggest that the increase in p50 and p105 protein abundance seen in female mice exposed to e-cig aerosol containing nicotine was nAChR $\alpha 7$ dependent. We believe that the nicotine-induced inflammation occurring via activated nAChR $\alpha 7$ was stronger than the anti-inflammatory effects of the activated

nAChR-related signaling pathway, so lack of nAChR $\alpha 7$ inhibited the inflammatory response caused by nicotine inhalation. However, nicotine-free e-cig exposure induced upregulation of one NF- κ B subunit (p105), which suggest this subunit may act through a different pathway that is nAChR $\alpha 7$ independent. We have for the first time shown that the inflammation caused by e-cig aerosol containing nicotine is nAChR $\alpha 7$ -dependent in a sex-dependent manner. However, more detailed studies are needed to illustrate how sex-differences in inflammatory responses caused by e-cig aerosol are nicotinic receptor-related.

As we recently reported, acute exposure to an e-cig aerosol with or without nicotine resulted in dysregulated repair and ECM remodeling [7]. We have noticed similar effects in our sub-chronic exposure. E-cig aerosol with or without nicotine disrupted MMPs and ECM proteins in a sex-dependent manner, most of them appearing to be nAChR $\alpha 7$ receptor independent. It is known that nicotine exposure causes harmful respiratory effects, such as airway remodeling in airway smooth muscle cells [45], which is a key feature of emphysema [46, 47]. However, dysregulation of MMPs serves not only as a factor for ECM remodeling, but also as a marker of inflammation. Interestingly, we found that nicotine-free e-cig aerosol induced augmented dysregulated MMPs than e-cig aerosol with nicotine. Increased MMP2/MMP12, along with decreased MMP9/MMP8, might suggest a compensatory feedback loop [48].

One of the major substrate groups of MMPs is ECM related proteins. From our previous studies of mice exposed to e-cig aerosols for 3 days, we noticed that ECM remodeling occurred via altered fibronectin and E-cadherin levels in a sex-dependent manner [7]. The current study shows that sub-chronic exposure of e-cig aerosol with or without nicotine dysregulated ECM remodeling to a certain extent dependent on nAChR $\alpha 7$, which also occurred in a sex-dependent manner. Increased PAI-1 was found only in male WT mice exposed to PG with nicotine, with this effect appearing to be blocked in nAChR $\alpha 7$ KO mice. PAI-1 is a primary ECM regulator and pro-fibrotic marker [49]. Therefore, increased PAI-1 following inhalation of e-cig aerosol with nicotine indicates that e-cig vaping, especially with nicotine, could increase risk of chronic fibrotic diseases, with nAChR $\alpha 7$ playing an important role. Some other ECM proteins, such as type 1 collagen and fibronectin, also showed dysregulated levels after exposure of e-cig aerosol with or without nicotine. Altered ECM protein synthesis has been associated with dysregulated wound healing and fibrotic disease [50]. The observed Increase in COL1A2 following PG exposure in both male and female mice demonstrates that PG alone poses a significant health risk, in addition to the risks associated with nicotine. In mice lacking nAChR $\alpha 7$, this COL1A2 dysregulation was partially blocked, but still showed some up-regulation. As mentioned above, nicotine is not the only stimulus for nAChR $\alpha 7$, and PG alone may alter nicotinic receptors, is in agreement with our previous study [7, 17]. Recently, Kicic et al. reported that asthmatic epithelial cells were unable to synthesize fibronectin and thus had less ability in wound healing [51]. It's well known that collagen, fibronectin, and smooth muscle actin are increased in interstitial lung diseases, such as interstitial pneumonia and pulmonary fibrosis. We have noticed dysregulation of collagen and fibronectin following e-cig exposure, but further studies are required to understand the mechanisms underlying how e-cigarette usage may induce lung diseases.

Conclusion

In conclusion, nAChR $\alpha 7$ ablation attenuates the inflammation induced by sub-chronic e-cig exposure, but partially involved in e-cig induced ECM remodeling/dysregulated repair. In a sub-chronic e-cig exposure, nAChR $\alpha 7$ was found to play a novel role in an anti-inflammatory response induced by nicotine. We show a significant difference in terms of altered genes (including genes related to inflammation and cytokine-chemokine signaling) in WT mice exposed to PG with nicotine compared to nAChR $\alpha 7$ KO mice exposed to PG with nicotine. The differential inflammatory cell counts and cytokine expression in BALF presented similar results, corroborating our findings. Hence, nAChR $\alpha 7$ is vital to inflammatory responses induced by nicotine-containing e-cig aerosols. However, the ECM remodeling/dysregulated repair caused by e-cig vaping is mostly nAChR $\alpha 7$ independent, and occurs in a sex-dependent manner. Only PAI-1, MMP2, and MMP12 in male mice were found to be nAChR $\alpha 7$ dependent; the rest of the targets were found to be independent of nAChR $\alpha 7$. Overall, the inflammation caused by e-cig aerosol with nicotine is mediated by nAChR $\alpha 7$, and the deletion of this receptor helped attenuate the inflammatory responses. Hence, nAChR $\alpha 7$ is considered a valid target for inflammation induced by e-cig aerosol with nicotine in the lung.

Abbreviations

E-cig: Electronic cigarette

ECM: extracellular matrix

nAChRs: Nicotinic Acetylcholine Receptors

WT: wild-type

nAChR $\alpha 7$ KO: nAChR $\alpha 7$ knockout

nAChR $\alpha 7$ CreCC10: lung epithelial-cell-specific nAChR $\alpha 7$ KO

PG: propylene glycol

VG: vegetable glycerin

BALF: Bronchoalveolar lavage fluids

MMPs: matrix metalloproteinases

IPF: idiopathic pulmonary fibrosis

COPD: chronic obstructive pulmonary disease

CNS: central nervous system

LPSs: lipopolysaccharides

Glu: glucose

Hb: hemoglobin

Hct: hematocrit

AO/PI: acridine orange propidium iodide

CCL9: Chemokine ligand 9

KLF4: Kruppel-like factor 4

DUSP1: Dual Specificity Phosphatase 1

BTLA: B and T Lymphocyte Associated

SMAD7: SMAD Family Member 7

NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells

PAI-1: Plasminogen activator inhibitor-1

COL1A1: type 1 collagens chain 1

COL1A2: type 1 collagens chain 2

Declarations

Ethical approval

This study was performed according to the standards from the United States Animal Welfare Act, National Institutes of Health (NIH). All the animal experiments were followed the protocol approved by The Animal Research Committee of the University of Rochester (UCAR).

Human participants, human data and human tissue: Not applicable

Human ethical approval: Not applicable

Consent for publication: Not applicable.

Competing interests

The authors have declared that no competing interest.

Availability of data and material: All data and materials are described in the manuscript.

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Competing interests: None

Author Contributions

QW, IKS, IR conceived and designed the experiments. QW, DL, JHL, TM, SRM conducted the experiments. QW, IKS, DL, TM, SRM analyzed the data. QW, IKS, SRM, IR wrote and revised the manuscript.

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Figures

Figure.1

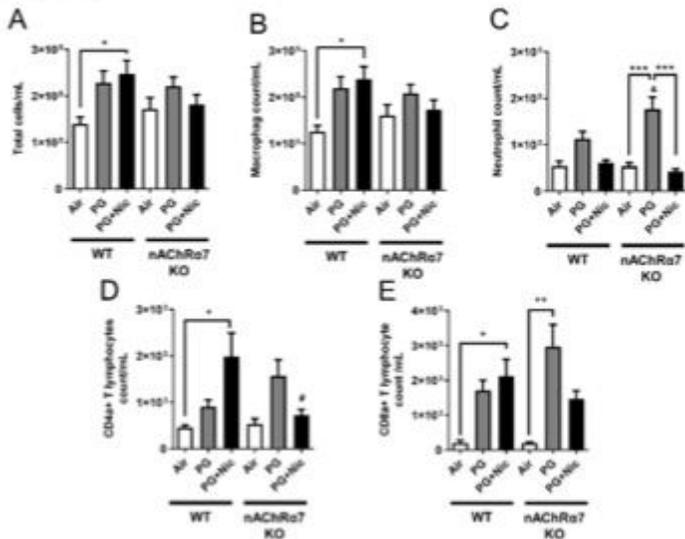


Figure 1

Sub-chronic e-cig exposure augments inflammatory cell influx in BALF. Differential inflammatory cell counts were measured in BALF from mice exposed to air, PG or PG with nicotine (PG+Nic) for 30 days (2 hrs/day). (A) Total inflammatory cell count was found via cellometer using AO/PI staining. Differential inflammatory cell counts were determined as percentages by flow cytometry. Absolute cell counts for (B) F4/80+ macrophages, (C) Ly6B.2+ neutrophils, (D) CD4a + T-lymphocytes, and (E) CD8a + T-lymphocytes were normalized to the total cell count. Data are shown as mean \pm SEM (n=4-6/group; equal number of male and female mice). * P < 0.05, ** P < 0.01 between groups; & P < 0.05, compared to PG exposed WT group; # P < 0.05, compared to PG+Nic exposed WT group.

Figure.2

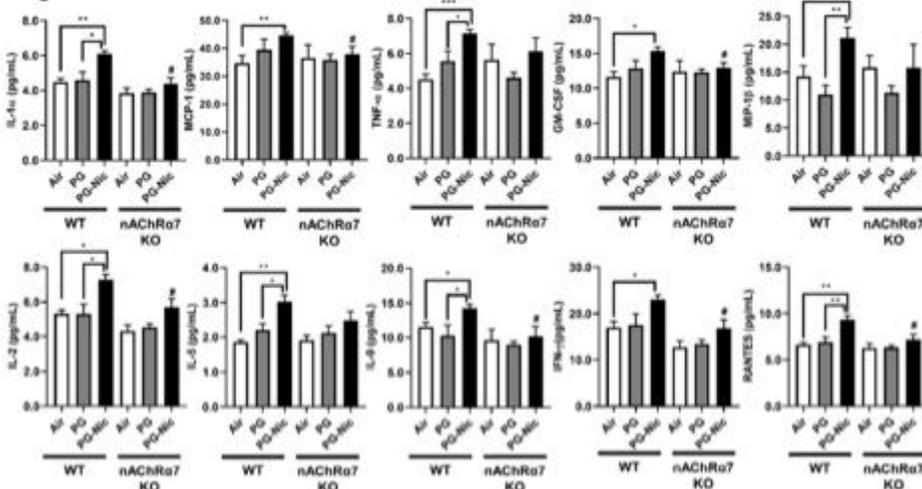


Figure 2

Sub-chronic e-cig exposure-induced pro-inflammatory mediators in BALF. Bio-Plex Pro mouse cytokine 23-plex assay kit (Bio-Rad) was used to determine levels of pro-inflammatory cytokines/chemokines in BALF from mice exposed to e-cig with or without nicotine for 30 days (2 hrs/day). Significant changes were found in cytokines related to macrophages (IL-1 α , MCP-1, TNF- α , GM-CSF, and MIP-1 β) and T-lymphocytes (IL-2, IL-5, IL-9, RANTES, and IFN- γ). Data are shown as mean \pm SEM (n=6-10/group; equal number of male and female mice). * P < 0.05, ** P < 0.01, *** P < 0.001, compared to air control; # P < 0.05, compared to PG+Nic exposed WT mice.

Figure.3

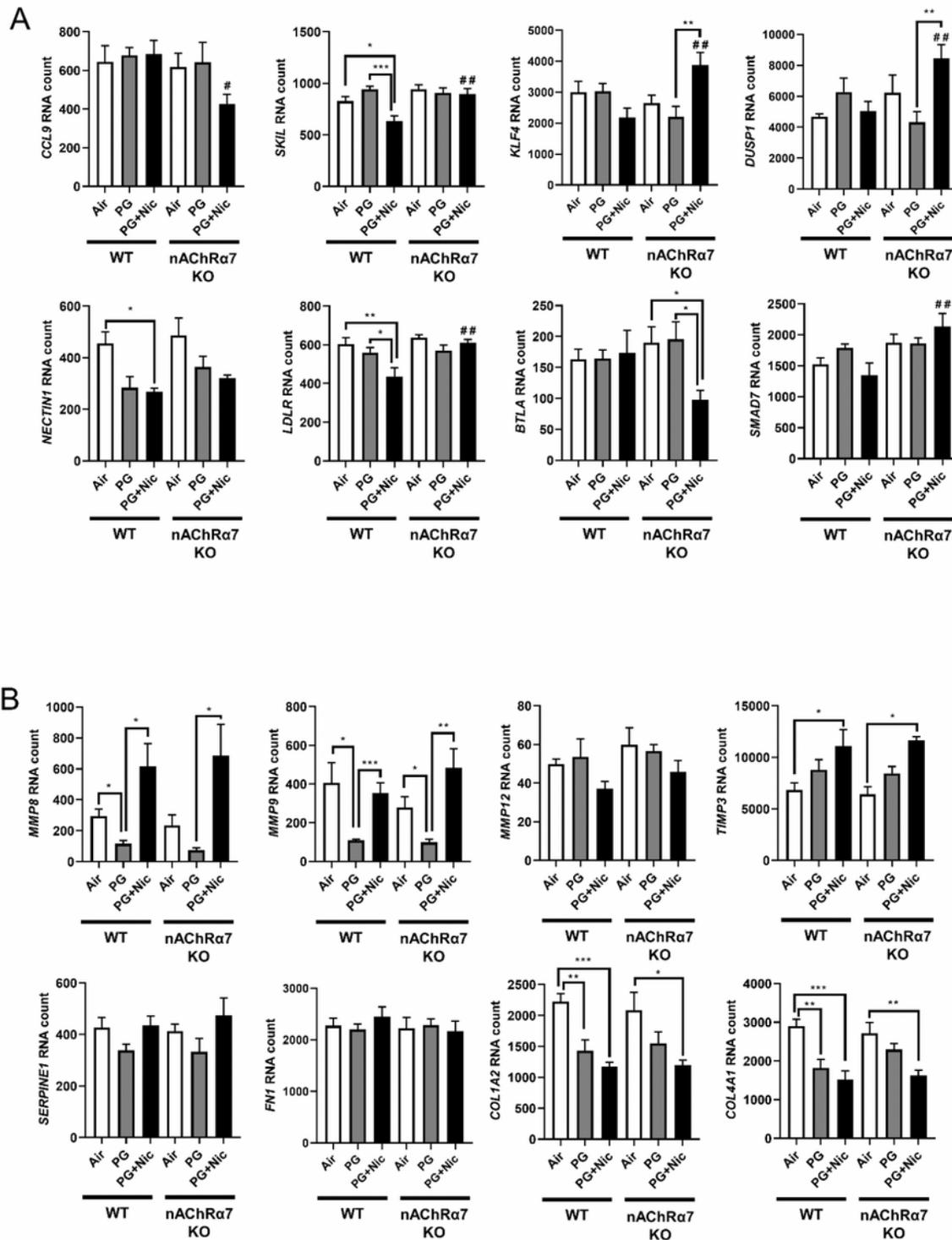


Figure 3

Sub-chronic e-cig exposure affects mRNA expression analyzed by Nanostring analysis. Mice were exposed to e-cig aerosol with or without nicotine for 30 days (2 hrs/day), and sacrificed 24 hrs after final exposure. RNA was isolated from lungs and screened via nCounter Mouse Myeloid Innate Immunity Panel using Nanostring analysis. Targets were selected based on significant differences, especially (A) between PG+Nic exposed WT and nAChRa7 KO mice, or (B) ECM remodeling focused. RNA counts were

normalized to multiple housekeeping genes and quantified using nSolver. Data are shown as mean \pm SEM (n=6/group; equal number of male and female mice). * P < 0.05, ** P < 0.01, *** P < 0.001 between groups; # P < 0.05, # # P < 0.01 compared with PG+Nic exposed WT group.

Figure.4

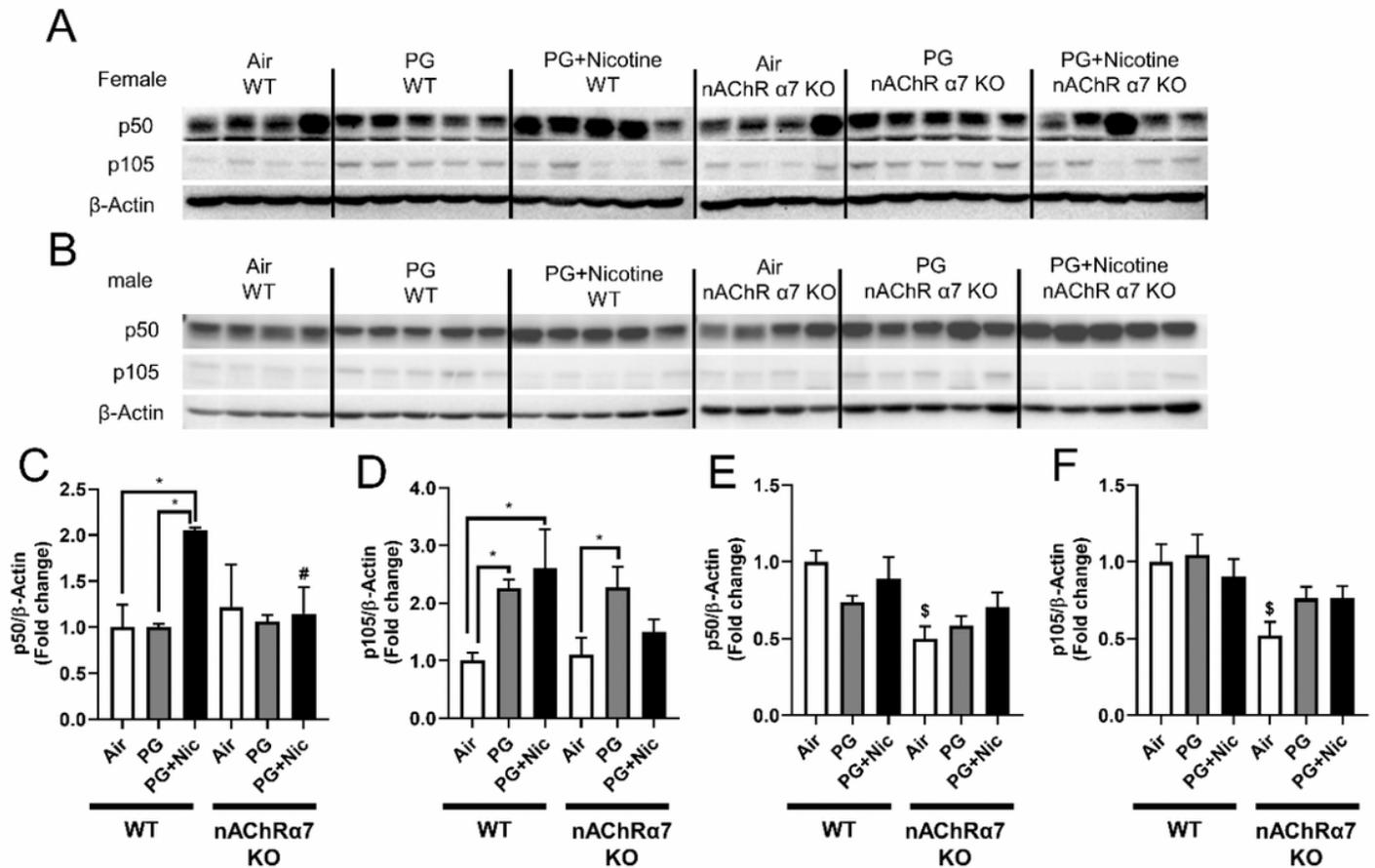


Figure 4

Sub-chronic e-cig exposure lead to dysregulated protein abundance of NF- κ B subunits (p50/p105) in mouse lungs with sex differences. The protein abundance of p50/p105 was measured in whole lung homogenates via Western blot. Representative blot images for female (A) and male (B) mice are shown. Densitometry analysis of individual blots was performed for female p50 (C) and p105 (D), and male p50 (E) and p105 (F), and β -actin was used as an endogenous control. Data are shown as mean \pm SEM. (n=4-5/group). * P < 0.05 significant compared between groups; \$ P < 0.05, compared with air exposed WT group; # P < 0.05, compared with PG+Nic exposed WT group.

Figure.5

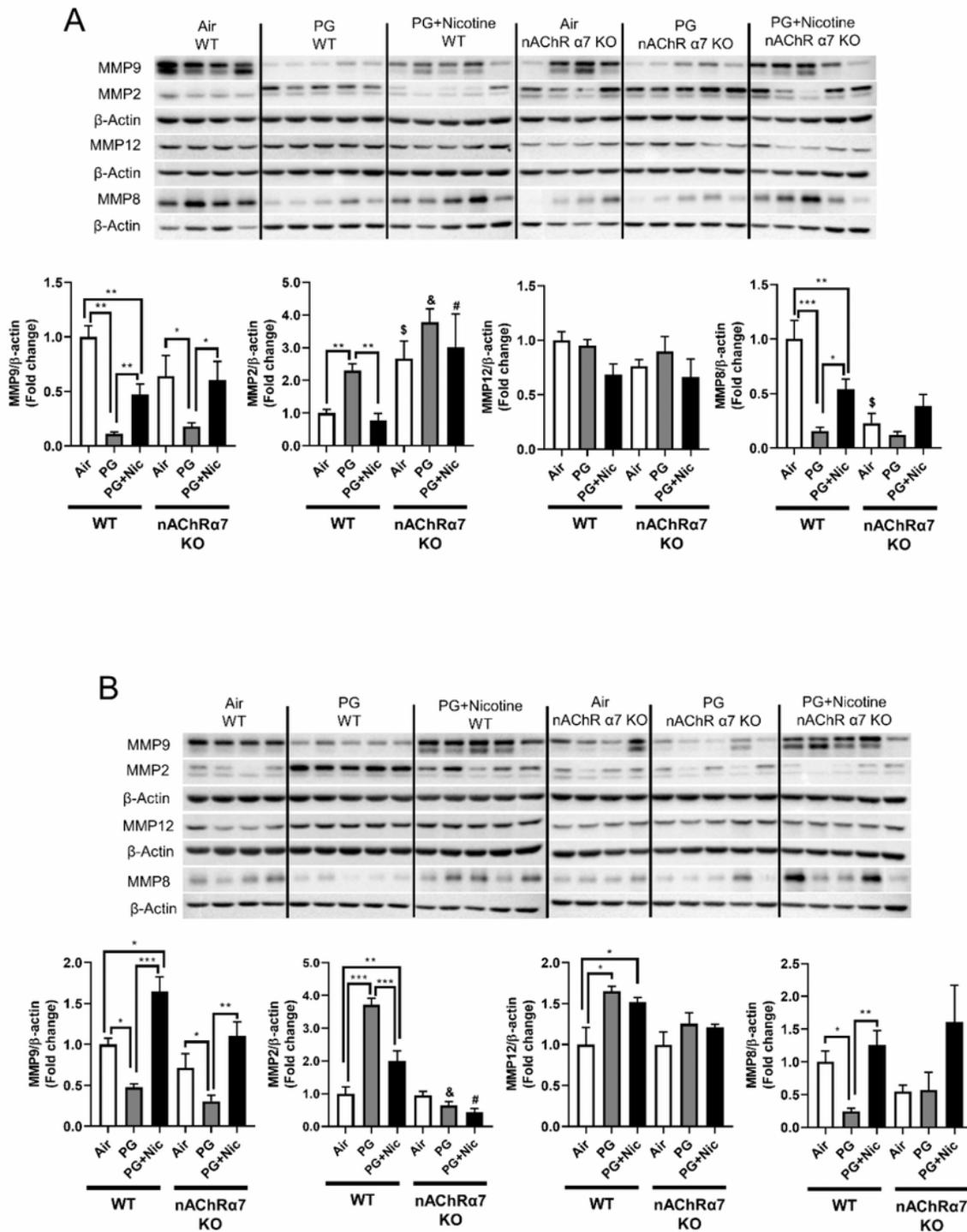


Figure 5

Sub-chronic e-cig exposure affects protein abundance of matrix metalloproteinases (MMPs) in mouse lungs with sex differences. Protein levels of several MMPs (MMP9, MMP2, MMP12, and MMP8) were measured in whole mouse lung homogenates via Western blot. Representative blot images and densitometry analyses for female (A) and male (B) mice are shown. β -actin was used as an endogenous control. Data are shown as mean \pm SEM. (n=4-5/group). * $P < 0.05$ between groups; \$ $P < 0.05$, compared

with air exposed WT group; & P < 0.05, compared with PG exposed WT group. # P < 0.05, compared with PG+Nic exposed WT group.

Figure.6

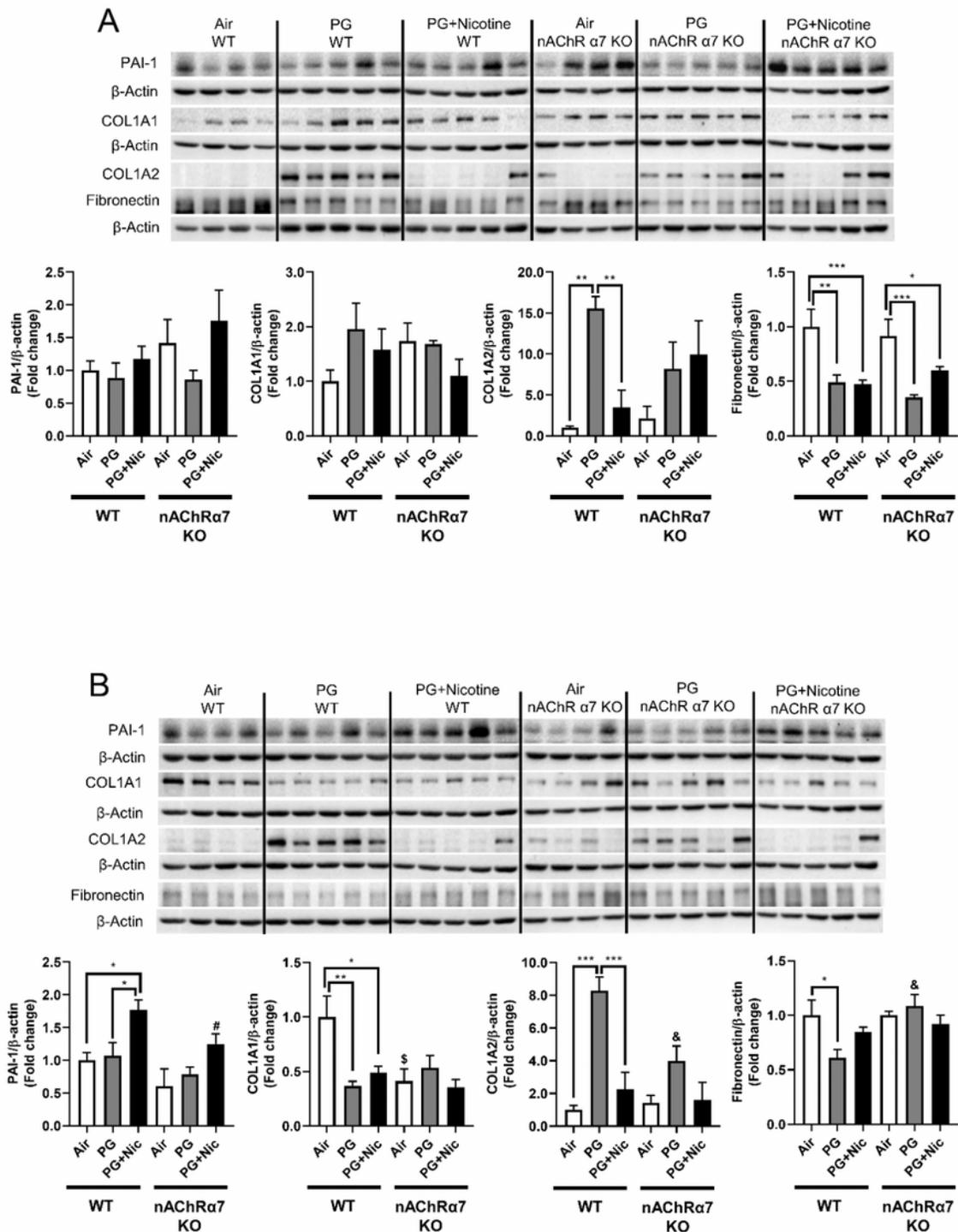


Figure 6

Sub-chronic e-cig exposure affects protein abundance of ECM-related markers in mouse lungs with sex differences. The abundance of ECM proteins (PAI-1, COL1A1, COL1A2, and fibronectin) were measured in whole mouse lung homogenates via Western blot. Representative blot images and densitometry analyses

for female (A) and male (B) mice are shown. β -actin was used as an endogenous control. Data are shown as mean \pm SEM. (n=4-5/group). (* P < 0.05 between groups; \$ P < 0.05, compared with air exposed WT group; & P < 0.05, compared with PG exposed WT group. # P < 0.05, compared with PG+Nic exposed WT group).

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