INFLAMMATORY RESPONSES INDUCED BY CIGARETTE SMOKE IN HEALTHY MICE
AND IN MICE WITH CHRONIC BRONCHITIS

Justine N. Monk

A thesis submitted to the faculty at the University of North Carolina at Chapel Hill in partial
fulfillment of the requirements for the degree of Master of Science in the Department of
Pathology and Laboratory Medicine in the School of Medicine.

Chapel Hill
2015

Approved By:

Claire M. Doerschuk

Stephen L. Tilley

Mehmet Kesimer

ABSTRACT

Justine N. Monk: Acute inflammatory responses induced by cigarette smoke in healthy mice and
in mice with chronic bronchitis
(Under the direction of Claire M. Doerschuk)

Mice overexpressing the β-epithelial sodium channel (βENaC) in pulmonary epithelial

cells have hyperconcentrated airway mucus and develop chronic bronchitis. These mice may

serve as a model for tobacco-related COPD pathogenesis when exposed to cigarette smoke. We

postulated that exposure of βENaC mice to 1 day and 5 days of cigarette smoke activates an

inflammatory response in the lungs. Following smoke exposure, bronchoalveolar lavage fluid

and lung tissue were examined for evidence of inflammation. βENaC mice demonstrated higher

numbers of airspace leukocytes than wild type mice, and smoke exposure resulted in additional

significant alterations. Real-time PCR revealed altered gene expression in βENaC mice with and

without smoke exposure. For several chemokines, cytokines, and matrix metalloproteinases,

female βENaC mice displayed a more pronounced response to smoke exposure compared to

males. Host defense was altered in βENaC females following smoke exposure as demonstrated

by altered immune cell recruitment in response to *H. influenzae*.

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CHAPTER 1: INTRODUCTION TO INFLAMMATION AND CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Introduction and Rationale for Study

Long-term exposure to tobacco smoke from cigarettes contributes to a variety of human diseases, including chronic bronchitis and emphysema, collectively referred to as chronic obstructive pulmonary disease (COPD). In 2010, COPD accounted for over 10 million physician's office visits and more than 1,400,000 emergency department visits in the United States.[1] In the same year, the Global Burden of Disease study concluded that COPD was the 3rd leading cause of death worldwide[2]. In 2011, an estimated 5.7% of Americans were clinically diagnosed with COPD, with females being preferentially affected at a higher rate over males (7.0% versus 4.3%, respectively)[1]. Tobacco smoke is the leading cause of COPD in the United States. Characterizing the inflammatory response associated with cigarette use is essential to unveiling the pathogenesis of tobacco-associated COPD, and will offer a standard to which the effects of new and emerging tobacco products, such as little cigars and e-cigarettes, can be measured and compared.

Inflammation-Associated Gene Expression in COPD and Smoking

Patients with COPD experience chronic airway inflammation that is measurable by increased serum and sputum concentrations of certain inflammatory cytokines. Inflammatory gene profiles with and without COPD are also affected by exposure to cigarette smoke. The serum concentrations of many markers of inflammation, including interleukin (IL)-1β, were found to be decreased in smokers compared to non-smokers,[3] however, in the bronchoalveolar lavage (BAL) fluid of smokers without disease, IL-1β was shown to be increased[4, 5]. Induced overexpression of IL-1β in a murine model resulted in increased BAL cellularity, attributable to

increased numbers of neutrophils and macrophages, and other COPD-like effects, such as disrupted elastin and increased alveolar collagen deposition[6].

IL-6, another inflammatory cytokine, has been shown to be systemically increased in the serum of COPD patients compared to healthy controls, particularly in connection with acute exacerbations of the disease[7, 8]. Wild type mice treated with anti-IL-6 antibodies experienced a diminished inflammatory response to cigarette smoke and poly(I:C) (a synthetic, virus-like stimulus) exposure compared to untreated wild type mice, indicated by reduced BAL cellularity and decreased BAL levels of the neutrophil chemoattractant CXCL1/KC[9]. The role of IL-10, a suppressor of inflammatory mechanisms, in COPD is not currently well understood. In one study, sputum IL-10 was found to be markedly decreased in COPD patients compared to healthy non-smokers[10]. In contrast, another study found IL-10 to be elevated in active- and former-smoking patients with COPD compared to active smokers without a COPD diagnosis[11].

TNFα, a promoter of inflammation produced by macrophages and other immune cells, is increased in the serum of COPD patients compared to healthy controls[7]. Interestingly, active smoking has been shown to increase TNFα in the serum of humans with and without COPD compared to former-smokers with the disease who had not smoked for at least 1 year[11]. Alternatively, increased TNFα in sputum has been demonstrated in smoking and former-smoking COPD patients compared to smokers without a COPD diagnosis[11]. In mice overexpressing pulmonary TNFα, increased mean linear intercepts and decreased alveolar wall elastin were observed in comparison to wild type mice, indicating induction of emphysema[12]. Exposure to cigarette smoke was found to diminish the activity of interferon gamma (IFN- γ), a pro-inflammatory cytokine[13]. T-cells from the lungs of mice exposed to cigarette smoke for 4 weeks produced lower levels of IFN-γ in response to infection with nontypeable *Haemophilus influenzae* (NTHI) compared to the pulmonary T-cells of sham-exposed mice[14].

Secretion of chemokines CXCL1/KC and CXCL2/MIP-2, the presence of which are characteristic of an acute inflammatory process, is increased in the BAL of wild type mice

following short-term exposure to mainstream cigarette smoke, compared to mice exposed to filtered air only[15]. These two chemokines are also increased in a mouse model of pulmonary inflammation induced by IL-1β overexpression[6]. Additionally, an upregulation of CXCL5/LIX has been observed in the bronchial tissues of humans experiencing exacerbations of COPD disease compared to healthy individuals and patients with stable COPD symptoms[16]. Pulmonary matrix metalloproteinases (MMPs) are also variably affected by smoke exposure and COPD disease status. The expression of MMP12, but not MMP9, is increased in response to cigarette smoke exposure in wild type mice[12]. However, mice overexpressing pulmonary TNFα displayed increased expression of MMP9, and not MMP12.

The studies described in this paper examine the gene expression and secretion of the mediators described above (IL-1β, IL-6, IL-10, TNFα, IFNγ, KC, MIP-2, LIX, MMP9, and MMP12) in wild type and βENaC mice (described below) following 1 and 5 days of cigarette smoke exposure. We hypothesized that cigarette smoke induces changes in gene and protein expression consistent with inflammation, including increases in the pro-inflammatory cytokines and matrix metalloproteinases.

Pulmonary Immune Cells in COPD and Smoking

Patients with COPD exhibit increased numbers of macrophages in their pulmonary tissues. The immunologic functions of macrophages are complex and multifaceted. In various tissues, macrophages have a key role in the regulation of the inflammatory response that includes a number of events from pathogen recognition through clearance of microbes and debris. In the lungs, alveolar macrophages serve as a first line of defense against environmental toxins and pathogens, contributing significantly to the pathogenesis of COPD. They secrete proteinases, including MMP9 and MMP12, which dismantle alveolar walls[17]. Examination of BAL and bronchial lavage (BL) cellularity of human smokers indicated an increase in total cell concentration and percentage of alveolar macrophages in the BAL, as well as an increased percentage of neutrophils in the BL, compared to non-smokers[4]. Macrophages stimulated by

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cigarette smoke release inflammatory cytokines, namely IL-1β and TNFα, which in turn promote

the secretion of pro-inflammatory chemokines[18]. These chemokines are responsible for the

recruitment of other immune responders, including neutrophils and circulating monocytes, thus

perpetuating the chronic inflammation that is characteristic of COPD airways.

Wild type mice exposed to short-term cigarette smoke experienced an increase in total

BAL cell counts attributable to increased numbers of macrophages and neutrophils, compared

to mice exposed to filtered air[15]. In fact, increased airway neutrophilia correlates with

exacerbations of COPD[16]. The neutrophilic infiltrate in the lungs of COPD patients has been

found to express anti-apoptotic markers, a characteristic that contributes to their persistent

presence in COPD airspaces[19]. Activated neutrophils in COPD produce proteases, including

MMP9[20]. The role of MMP9 in COPD pathophysiology is not fully understood, but it has been

suggested as a player in airway remodeling.

Host Defense in COPD and Smoking

Though immune cell numbers, particularly alveolar macrophages, are greatly increased

in the lungs of COPD patients, individuals with a COPD diagnosis experience increased

susceptibility to bacterial respiratory infections that contribute to acute exacerbations and

general progression of the disease[21]. This suggests that alveolar macrophage function is altered

in COPD. Nontypeable *Haemophilus influenzae* (NTHI) is the most common pathogen of the

lower airways of COPD patients[22]. The phagocytic capacity of alveolar macrophages, but not

blood-derived macrophages, against NTHI is compromised in former smokers with COPD,

compared to healthy ex-smokers[23]. Interestingly, phagocytosis of inert latex microspheres is

unaffected by COPD or smoking status, suggesting that the impairment of COPD alveolar

macrophages lies in pathogen recognition[24].

Mice exposed to short-term cigarette smoke have been shown to develop marked airway

neutrophilia when sacrificed 16 hours following the final exposure, however, when mice were

given an additional 2 hours of cigarette smoke exposure and harvested 2 hours thereafter, a

significant decrease in airspace neutrophil cell numbers were observed compared to non-smoke exposed mice[25]. Additionally, sputum collected from humans 3 hours after smoking 2 cigarettes showed a similar decrease in neutrophil number, compared to nonsmoking controls[25]. Neutrophils have been shown to undergo necrotic cell death and release damage-associated molecular patterns (DAMPs) upon exposure to cigarette smoke, and these DAMPSs are capable of triggering an innate immune response[25]. These observations imply that the debris released by cigarette smoke-induced necrotic neutrophils have a pro-inflammatory effect in the lungs of smokers.

Gender Variation in COPD

COPD-associated morbidity and mortality are increased among American women compared to men[1, 26]. Women and men with the same COPD burden respond differently, with women experiencing more pronounced symptoms and reporting poorer quality of life than their male counterparts[27]. It has also been reported that women are more likely to develop severe, early onset COPD[28]. A recent survey-based study has implicated the increased susceptibility of women to emotionally driven coping strategies, such as anxiety and depression, as a source of these discrepancies[27]. The rise in the number of female smokers has also been proposed as a large contributor to the surge in female COPD prevalence. However, the difference in lung development and thoracic volume between the sexes may have a role[28], and it has been shown that the airway response to smoke is different between males and females[29]. It is likely that many factors contribute to gender-related differences in COPD in humans, and it is important to consider the influence of gender when investigating the pathogenesis of COPD and other smoking-related diseases, especially in this era of new and emerging tobacco products.

The βENaC Transgenic Mouse Model

The βENaC transgenic mouse, whose overexpression of the β subunit of the epithelial sodium channel in airway epithelium results in hyperconcentration of airway mucus, has been shown to develop a COPD-like phenotype[30]. Though the lung tissue of βENaC mice is

histologically normal at birth[31], bronchioles of βENaC mice quickly become inflamed due to dehydrated airway surface liquid and exhibit significant airway neutrophilia and eosinophilia compared to wild type mice[30]. In addition to this chronic bronchitis, βENaC mice develop an emphysematous phenotype soon after birth: their distal airspaces become enlarged secondary to obstruction from the pathologically thickened mucus[31]. This development of emphysema in βENaC mice is due to a significant upregulation of MMP12 in their pulmonary tissues[32]. As described previously, MMP12 is a matrix metalloproteinase with the capacity to degrade alveolar walls.

Examination of gene expression data, bronchoalveolar lavage fluid, and lung morphology following 6 months of cigarette smoke exposure demonstrated that βENaC mice express immune regulatory genes and response genes to toxins and oxidative stress along with pulmonary inflammation. The lung disease found in these mice thus closely mimics human COPD (Duncan EA, Doerschuk CM, unpublished manuscript). The persistent bronchitis, gradual emphysema, and altered gene expression experienced by the βENaC mice make this genotype a good model for studies of COPD pathogenesis.

The Question and Hypothesis Addressed in this Thesis Work

The disease processes of COPD are highly complex and multifaceted. The mechanisms that take smokers from acute lung injury to chronic pulmonary disease are not yet fully understood. The experiments described in this thesis were performed to elucidate the role that acute pulmonary inflammation plays in the pathologic changes that occur in healthy and chronically inflamed pulmonary tissues upon 1 day and 5 days of tobacco smoke exposure. Wild type and βENaC mice were used in these studies to demonstrate the effect of cigarette smoke exposure on healthy and chronically inflamed tissue, respectively. To my knowledge, the effect of 1 day and 5 days of cigarette smoke exposure on βENaC mice has not yet been investigated.

We hypothesize that βENaC mice exposed to 1 day or 5 days of cigarette smoke will experience an inflammatory response, demonstrated by an influx of innate cellular immune

responders, namely neutrophils, to the lung tissues and airspaces.   We predict that this inflammatory response will be markedly exacerbated in βENaC mice compared to wild type mice. We also expect to see a high amount of variation in the responses of βENaC pulmonary tissues to smoke, but overall we expect a net increase in total BAL cellularity, neutrophil number, and cytokine and MMP expression compared to wild type. This variation may even serve to better exemplify the human experience of COPD in a mouse model, as no two COPD patients present exactly alike, and the very nature of the disease is diverse and multifactorial, with symptoms ranging from mild to very severe.

CHAPTER 2: METHODS AND MATERIALS FOR THE EVALUATION OF THE IMMUNOLOGIC RESPONSE TO SMOKE IN WILD TYPE AND βENaC MICE

βENaC Transgenic Mice

The mouse model utilized in these experiments is the *Scnn1b* transgenic model (*Scnn1b-tg)* backcrossed to a C57BL/6N background. The colony is maintained by breeding Scnn1b transgenic mice to wild type mice.   *Scnn1b* is the gene that codes for the βENaC epithelial sodium channel subunit. In this paper, the *Scnn1b* transgenic mice will be referred to as βENaC mice. Generation of this βENaC mouse model has been previously described[33]. All protocols involving the use of animals were reviewed and approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill.

Cigarette Smoke Exposure

Wild type and βENaC animals were divided into groups to be given either cigarette smoke or sham treatment (Table 1). Two separate time points were used for analysis of response to smoke: 1-day and 5-days. Each day of smoke consisted of exposure to 6 University of Kentucky research cigarettes. Smoke was delivered every 60 seconds in puffs of 2-second duration. Each cigarette was "puffed" 10 times for a total exposure time of 60 minutes. WT and βENaC control groups were not exposed to smoke or to the smoky chamber, but they were equally handled and subjected to the noise of the smoking pumps while remaining in their filtered-air cages to keep stress-related variability at a minimum. The smoking and control groups were harvested 16 hours following the end of the 1-day exposure, and 24 hours following the 5th day of exposure in the 5-day exposure groups.

For the experiments assessing the clearance of *H. influenzae*, βENaC and wild type mice were subjected to daily smoke or sham treatments for 2 weeks (10 total days of exposure)

following the same daily exposure protocol described above. Clearance experiments commenced 1 hour following the final exposure.

Bronchoalveolar Lavage Collection and Analysis

After tying off and removing the left lung for other analyses (see Chapter 2), cold 1% Dulbecco's Phosphate Buffered Saline (D-PBS) solution at a volume adjusted for each mouse's body weight (microliters D-PBS equal to 0.17% of body weight, in grams) was instilled into the right lung and removed. This was repeated three times and pooled. The pooled BAL fluid from each mouse was then spun down, the supernatant was removed, and the cells were resuspended in 100uL D-PBS. BAL cells were then counted manually with a hemacytometer, and 100,000 cells were transferred onto a cytospin prep and stained (Protocol Hema 3 Stain Set, Fisher Diagnostics) for manual differential counting via light microscopy.

Digestion of Lung Tissue

Following BAL collection, the right lung of each mouse was instilled with a 2x Dispase solution, submerged in D-PBS, and incubated for 30 minutes at 37C. Following initial incubation, the lungs were transferred to a solution of collagenase/dispase and DNase II in D-PBS, minced with scissors, and incubated for an additional 10 minutes at 37C. Each tissue solution was then gently forced through an 18-gauge needle to achieve confluent solution, treated for lysis of red blood cells, washed and filtered several times before analysis.

Cell Staining and Analysis by Flow Cytometry

Digested lung tissue was washed and stained with Ly6G-FITC for neutrophil identification and CD11b-PE Cy5 as a marker of leukocyte activation. Stained cells were then run through a Beckman Coulter CyAn ADP flow cytometry instrument, and analyzed using Summit 4.3 software.

Gene Expression Analysis by qPCR

The left lung of each mouse was removed and flash-frozen for RNA isolation. Whole lung tissue was homogenized for isolation of total RNA using a QIAGEN miRNeasy kit. Total

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RNA was then used to make cDNA by reverse transcription polymerase chain reaction. Gene expression data was then obtained by qPCR.

Clearance of *Haemophilus influenzae*

Nontypeable *Haemophilus* influenzae (NTHI) was grown up on Chocolate II Agar (CHOC II) plates and re-plated after 16-18 hours. A suspension of 1.0 optical density (OD) at 600nm was made from the re-plated isolate. The NTHI suspension was plated on CHOC II to determine the number of colony forming units (CFUs) per milliliter of suspension. One hour following the 10th day of smoke or sham exposure, βENaC and wild type female mice were instilled with the NTHI suspension at a volume proportional to their individual body weight (microliters of suspension equal to 0.227% of body weight, in grams). A unilateral instillation was performed, with the suspension being directed into the left lung only. Twenty hours after the instillation, the mice were harvested. The left lung was lavaged 6 times, following the previously described protocol, before being homogenized. The BALF and homogenate were then plated on CHOC II at varying dilutions for clearance. After 24 hours, the CFUs on each plate were counted and compared to the CFU/mL concentration of the NTHI instillate to determine bacterial clearance.

CHAPTER 3: EXPERIMENTAL RESULTS

Effect of Smoke Exposure on Airway Inflammation

The BAL fluid collected from βENaC mice yielded variable numbers of total leukocytes by manual hemacytometer counting. Though the numbers did not achieve significance, there was a slight trend towards increased total BAL leukocytes in βENaC mice after 1 day of exposure, particularly in the males, regardless of smoke or sham treatment (Figures 1A, E). In the groups exposed to smoke and sham for 5 days, total leukocytes were significantly elevated in βENaC mice exposed to smoke compared to both smoke- and sham-exposed wild type mice (Figures 2A, E). Sham-exposed βENaC mice had higher numbers of total leukocytes when compared to wild type groups, indicating that the βENaC genotype was solely responsible for the increase in leukocyte number, but this trend only reached significance in the male groups. There was no significant change in the number of total BAL leukocytes singly attributable to smoke exposure in either the wild type or βENaC mice for either duration of smoke exposure.

In addition to total leukocytes, the numbers of leukocyte subtypes, namely macrophages, neutrophils and lymphocytes, were determined by examination of cytospin preparations. BAL macrophages in mice exposed to smoke for 1 day did not significantly change with genotype or smoke exposure (Figures 1B, F). Male βENaC mice exposed to cigarette smoke for 5 days experienced a significant increase in BAL macrophages, compared to sham-exposed wild type males (Figure 2F). Female mice exposed to 5 days of smoke, demonstrated a similar trend, but not to a significant degree (Figure 2B).

Both treatment durations revealed a significant increase in the percentage of BAL neutrophils in sham-exposed βENaC mice (Figures 1C, G, 2C, G). This observed increase in

neutrophils is consistent with what has been previously demonstrated by the βENaC genotype[30]. Interestingly, following 5 days of cigarette smoke exposure, male and female βENaC mice exhibited significantly diminished numbers of BAL neutrophils when compared to sham-exposed βENaC mice (Figures 2C, G), though the presence of neutrophils in these smoke-exposed βENaC groups was still greater than that of both the sham- and smoke-exposed wild type mice. This trend was demonstrated by both genders, but was significant only in the female groups. The smoke-induced decrease in neutrophils was not observed to a significant degree in the βENaC mice exposed to a single day of smoke (Figures 1C, G).   In general, neutrophils were observed to be elevated in βENaC mice regardless of gender, treatment, or treatment duration compared to their wild type counterparts.

Total BAL lymphocytes were significantly elevated in βENaC males and females exposed to smoke for 5 days (Figures 2D, H). No significant trends were observed in the total lymphocytes recovered in the BAL fluid of 1-day exposed mice (Figures 1D, H).

Gender Differences in Smoke-Induced Airway Inflammation

When directly compared, male and female mice receiving the same smoke or sham treatment demonstrated some significantly different responses in cellular airway inflammation. Female βENaC mice exposed to smoke for 1 day had fewer total BAL leukocytes than βENaC males given the same smoke treatment (Figure 3A). In addition, 1-day sham-treated βENaC females had fewer BAL neutrophils than their male counterparts. This was also observed in the 5-day sham-treated βENaC females, but not to a significant degree. Five days of smoke exposure induced a greater number of BAL lymphocytes in βENaC females compared to βENaC males given the same treatment (Figure 3B).

Effect of Smoking Duration on Airway Inflammation

The duration of smoke or sham treatment also had an effect on some parameters of the BAL cell counts. Female βENaC mice exposed to smoke for 5 days demonstrated higher numbers of total leukocytes, macrophages, and lymphocytes compared to βENaC females

exposed to only a single day of smoke (Figure 4A). In addition, βENaC females receiving 5 days of sham treatment had increased numbers of total leukocytes and neutrophils compared to βENaC females given only 1 day of sham treatment. It is unclear to what the differences in airspace leukocyte numbers between 1- and 5-days of sham treatment can be attributed. βENaC males did not demonstrate any significant changes in airspace leukocyte numbers between 1 and 5 days of smoke exposure (Figure 4B). In the male groups, the trend in total neutrophil number between 1 and 5 days resembled that observed in the female groups, but did not achieve significance.

Smoke Exposure Effects on Inflammatory Cells Within Lung Tissues

Neutrophils residing in the pulmonary tissue were quantified by Ly6G expression as a percentage of the total number of cells in the lung digest using flow cytometry. There was no significant trend in tissue neutrophil percentage resulting from genotype or smoking treatment in either the 1-day (Figures 5A, D) or the 5-day (Figures 6A, D) groups. Similar to what was observed of airspace neutrophils in BAL fluid, tissue neutrophil presence was highly variable in βENaC mice compared to wild type.

Cells staining negatively for Ly6G but expressing measurable CD11b (CD11b(+), Ly6G(-)) were categorized as non-neutrophils. Though there were no differences in the percentage of these non-neutrophils in response to genotype or smoking treatment (Figures 5B, E, 6B, E), it was noted that CD11b(+), Ly6G(-) cells consistently presented in two distinct subpopulations of high and low expression (Figures 5C, F, 6C, F). Significant trends were observed in these proportions when data from males and females were compared to each other. Following 5 days of sham or smoke exposures, wild type and βENaC females had significantly fewer low-expressing Ly6G(-)CD11b(+) cells than their male counterparts (Figure 7C). This trend was not observed to a significant degree in the groups exposed to cigarette smoke for 1-day (Figure 7A). Neither gender nor smoke exposure had a significant effect on high CD11b-expressing cells (Figures 7B, D).

Smoke Exposure Effects on Pulmonary Cytokine Expression

The expression of select cytokines and matrix metalloproteinases was examined after exposure to 1 and 5 days of smoke or sham air and measured by ΔΔCt normalized to sham-exposed, wild type 18S expression (Tables 2 and 3). Among the cytokines, three chemokines of interest, KC, MIP-2, and LIX, demonstrated significant changes in the 1 day exposure group: both female and male βENaC mice exposed to sham air showed increased expression of KC, MIP-2, and LIX compared to wild type sham- and smoke-exposed mice (Figure 8A), indicating that the increased chemokines were a result of the βENaC genotype. Interestingly, KC and MIP-2 expression decreased in female βENaC mice upon a single day of smoke exposure compared to sham-exposed βENaC females. KC expression increased in wild type females exposed to 1 day of smoke compared to wild type sham-exposed mice, and in fact this was one of very few instances in which smoke had a measureable effect on a wild type group. MIP-2 and LIX expression were increased in smoke-exposed βENaC males compared to wild type smoke- and sham-exposed males, but were not significantly altered in comparison to sham-exposed βENaC males.

Following 5 days of smoke or sham exposure, no significant trends were observed in the expression of KC and LIX in the female groups (Figure 8B). A significant increase in MIP-2 expression was observed in female βENaC smoke-exposed mice compared to wild type sham-exposed females. Male βENaC mice exposed to 5 days of smoke experienced a significant increase in KC expression compared to wild type sham. MIP-2 expression was significantly increased in sham-exposed βENaC males compared to wild type sham, and in smoke-exposed male βENaC mice compared to both sham- and smoke-exposed wild type mice. Smoke-exposed βENaC male mice demonstrated significantly increased expression of LIX compared to wild type males exposed to sham and smoke air.

The relative expression of 5 other cytokines was also measured: TNFα, IFNγ, IL-1β, IL-6, and IL-10. Following 1 day of exposure, βENaC sham-exposed females had increased expression of TNFα, IL-1 β, and IL-6 compared to wild type sham females (Figure 9A). IL-6 expression was

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significantly decreased in βENaC females exposed to smoke compared to βENaC females exposed to sham air. There were no significant trends in the expression of IFNγ and IL-10 in the 1-day female groups. Examination of these cytokines in male wild type and βENaC mice exposed to 1 day of smoke or sham air did not reveal any significant trends in expression.

Female βENaC mice exposed to sham air for 5 days had increased expression of TNFα compared to their wild type sham-exposed counterparts (Figure 9B). βENaC males demonstrated elevated TNFα compared to wild type sham-exposed males in response to 5 days of smoke exposure. No significant trends were observed in the expression of IFNγ, IL-1β, IL-6, and IL-10 in the 5-day exposure group for either gender.

Smoke Exposure Effects on Pulmonary MMP Expression

The measurement of MMP expression following a single day of smoke exposure revealed interesting changes. Expression of MMP9 was increased in smoke-exposed wild type females compared to wild type females exposed to sham air (Figure 10A). MMP12 expression in wild type females exposed to 1 day of smoke was increased compared to wild type sham-exposed females. βENaC sham-exposed females expressed higher levels of MMP12 compared to wild type females exposed to sham or smoke. Interestingly, MMP12 expression was decreased in βENaC females exposed to smoke compared to βENaC females given sham air treatment. Male mice exposed to 1-day of sham or smoke revealed no trends in MMP9 expression, but showed increased MMP12 expression in βENaC sham-exposed mice compared to both sham- and smoke-exposed wild type mice. The measurement of expression data for MMP9 and MMP12 following 5 days of smoke or sham exposure did not reveal any significant trends in either gender (Figure 10B).

Gender Differences in Cytokine and MMP Expression Following Smoke Exposure

Male and female βENaC mice presented with distinct expression patterns of select inflammation-associated genes. In the 1-day exposed group, female βENaC mice exposed to sham air expressed higher levels of cytokines KC, MIP-2, and IL-6 compared to male βENaC

mice exposed to sham air (Figure 11A). In addition, sham-exposed female βENaC mice demonstrated higher expression of MMP12 compared to sham-exposed βENaC males. βENaC females exposed to 5 days of cigarette smoke had higher KC and MIP-2 expression than βENaC males with the same smoke exposure (Figure 11B).

The Effect of Smoke Exposure Duration on Inflammatory Gene Expression

Examination of gene expression changes between the 1- and 5-day duration of smoke or sham exposure revealed interesting changes: among female βENaC mice, MIP-2 expression significantly increased from 1 to 5 days of smoke exposure (Figure 12A). A similar trend in MIP-2 expression was discovered in wild type female mice, but the observed increase did not reach significance. Interestingly, βENaC sham-exposed females demonstrated a decrease in IFNγ expression between 1 and 5 days of exposure. MMP9 expression decreased from 1 to 5 days of smoke exposure in wild type females. A similar trend was observed in βENaC females, though it did not reach statistical significance. Also between 1 and 5 days of smoke exposure, MMP12 expression in βENaC females appeared to increase with a high degree of variability, though the trend did not achieve significance. MMP12 expression between 1 and 5 days of smoke exposure in βENaC males revealed the same interesting, though insignificant trend as that observed in the βENaC females: increased expression with high variability at 5 days of smoke, compared to 1 day. Curiously, in the male groups, KC and MIP-2 expression decreased between 1 and 5 days of sham exposure in βENaC mice (Figure 12B).

Effect of Smoke Exposure on Host Defense Against *Haemophilus influenzae*

After 2 weeks, 10 individual days, of smoke or sham exposure, female βENaC and wild type mice received an intrapulmonary instillation of suspension of NTHI. The dose of NTHI delivered was insufficient to determine bacterial capacity 20 hours after the instillation; all genotypes and treatment groups demonstrated over 99% clearance capacity at the 20-hour time point. However, the cell counts performed on BAL fluid collected 20 hours after instillation with NTHI provided significant findings. Interestingly, smoke-exposed mice of either genotype

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had more macrophages than their sham-exposed counterparts (Figure 13B). βENaC females exposed to sham air prior to instillation with bacteria had fewer total leukocytes in their BAL fluid when compared to wild type females that received smoke treatment before instillation (Figure 13A). This change was due to less macrophages (Figure 13B) and neutrophils (Figure 13C). There were no significant trends in total number of lymphocytes in BAL fluid following bacterial instillation in either smoke or sham treatment groups or genotype (Figure 13D).

CHAPTER 4: Discussion

The addition of long-term smoke exposure to the βENaC transgenic mouse model results in a pulmonary disease that mimics human tobacco-related COPD. This study sought to characterize the cellular immune response following 1 day and 5 days of smoke exposure in an effort to better understand how smoke exposure specifically contributes to the development of disease in these mice. The failure of wild type mice to recruit increased numbers of airspace leukocytes indicates that short durations of exposure to cigarette smoke are insufficient to initiate this recruitment in healthy wild type mice. This explanation is supported by previous work that demonstrated an increase in BAL cellularity of wild type mice following 6 months of smoke exposure (Duncan EA, Doerschuk CM, unpublished manuscript). While βENaC mice exposed to 1 and 5 days of smoke did have more airspace leukocytes compared to their wild type counterparts, the sham-exposed βENaC mice showed similarly larger numbers of the same leukocyte subtypes. Thus, the βENaC genotype is responsible for the greater number of airspace immune cells.

The results of this study also suggest that cigarette smoke exposure suppresses neutrophil presence in the airspaces of βENaC lungs. This observation is supported by BALF cell counts and by gene expression data for chemokines KC and MIP-2, particularly in the female groups, which shows a decrease in the expression of these neutrophil chemotactic cytokines after a single smoke exposure. It is interesting to note that while a single day of exposure did not induce a significant phenotype for airspace neutrophils, as evident by BALF cell counts, qPCR data indicated alterations at the level of gene expression. The phenotype of diminished neutrophil numbers in the smoke-exposed βENaC mice reached significance later, after 5 days of smoke exposure.

The reduced expression of MMP12, the main culprit in the destruction of alveolar septae and development of emphysema, following 1 day of smoke in the female group is an interesting observation. Five days of smoke exposure resulted in a 10-fold increase in MMP12 mRNA expression. This increase is unlikely to be explained by increasing neutrophil numbers, because neutrophil numbers were suppressed by smoke. Macrophages are also a source of this MMP.

The mechanism through which cigarette smoke is acting in βENaC mice, therefore, may be to impair the function of neutrophils first, as demonstrated by decreased expression of KC, MIP-2 and MMP12 after 1 day, which in turn impairs the recruitment of additional neutrophils, resulting in a significant decrease in neutrophil number after 5 days of smoke exposure. Alternatively, neutrophil survival may be compromised (shortened) by prolonged smoke exposure, however this would be in contrast with the results of the long-term exposure study, which did not observe a significant decrease in neutrophils after 6 months of smoke exposure (Duncan EA, Doerschuk CM, unpublished manuscript).

In contrast with the airspace neutrophils, the number of lung tissue neutrophils was not changed by smoke exposure for either duration of smoke. Interestingly, the βENaC genotype did not induce increased tissue neutrophils as it did with airspace neutrophils. Two distinct non-neutrophil CD11b-positive populations were found, expressing low or high levels of CD11b. These Ly6G negative-CD11b-positive cells are mostly like to be bone marrow-derived macrophages and a population of dendritic cells. A greater number of CD11b high cells were recovered from the smoked βENaC mice after 5 days. Furthermore, the females had fewer CD11b low cells after 5 days of smoke or sham compared to males in both genotypes. These results were unexpected and may have significant implications for further studies, especially considering the clear differences in the high and low CD11b populations between males and females. Further investigations into the identity and function of the low-expressing CD11b-positive cells may yield answers as to why these cells are consistently lower in female wild type and βENaC mice compared to their male counterparts.

The clearance of *H. influenzae* was measured in female βENaC and wild type mice to determine the effect of smoke on leukocyte recruitment. Interestingly, macrophages were increased in response to infection in smoke-treated wild type and βENaC mice compared to their sham-exposed counterparts. This observation may have significant implications, in that 2 weeks of smoke may be sufficient to prime the tissue for an amplified response to stimuli. However, studies to further investigate the effect of 2 weeks of smoke on wild type and βENaC lungs without bacterial infection are needed to draw meaningful conclusions from these data. In addition, the decreased number of macrophages in the airspaces of female βENaC mice in response to infection with NTHI following 10 days of sham treatment, compared to wild type females who received smoke treatment, is interesting to note, considering the observed tendency of βENaC mice to have slightly increased, or at least comparable numbers of BAL macrophages regardless of smoke or sham exposure in both the 1 and 5 day studies.

As previously described, COPD patients experience high rates of respiratory infection despite having increased numbers of alveolar macrophages in their pulmonary tissue, indicating impaired function of the macrophage population of COPD lungs. The decrease in IL-6 in βENaC female mice coupled with the lack of significant changes in BAL macrophage number seen after a single day of smoke exposure may implicate cigarette smoke as an early suppressor of macrophage function in chronically inflamed lungs, as macrophages are a source of this cytokine. This finding, coupled with the increased macrophage number in βENaC smoke-exposed females after infection with NTHI, may prove to be very interesting as studies continue. A possible mechanism may be that although macrophage number is increased in response to infection in smoke-treated βENaC females, the function of these macrophages is compromised, measurable by diminished clearance capacity against NTHI. In addition to determining the effect of 2 weeks of smoke exposure on wild type and βENaC females without bacterial infection, it will be essential to optimize the bacterial dose so that the clearance capacity of smoked and sham-treated βENaC females can be tested.

The distinct responses of males and females to similar smoking conditions and the exacerbated effects of the βENaC transgene in females compared to males, as demonstrated by higher expression of KC, MIP-2, IL-6, and MMP12 by sham-exposed βENaC females over their male counterparts, suggests that the increased susceptibility to COPD development and the severity of symptoms experienced by human females with COPD may be reproducible in a mouse model. This would be helpful as future strategies for disease management and treatment options are developed. As personalized medicine becomes the new standard of care, the understanding of the influence of gender on an individual's unique experience of an established disease like COPD becomes increasingly important.

More work is needed to characterize the mechanisms of pathologic change that take place between 1 and 5 days of smoke exposure, but this study suggests that within a short window of smoke exposure, 1 to 5 days, significant inflammatory responses occur in the pulmonary tissues, and that these effects depend heavily on the pre-existing health status of the tissue as well as the subject's gender. The results described in these studies demonstrate the complexity of COPD pathogenesis by describing the significant alterations in numerous parameters, from the gene expression to the cellular level, that occur after just 1 or 5 days of exposure to cigarette smoke, and highlight the value of the βENaC mouse model for use in future investigations of the disease.

Table 1: Study Animal Information

MALES		n	Age (weeks) ± SEM	Weight (g) ± SEM
1-Day Exposure	**Wild Type Sham**	5	7.1 ± 0.2	22.4 ± 0.5
	Wild Type Smoke	5	7.2 ± 0.2	22.7 ± 1.7
	ßENaC Sham	6	7.0 ± 0.1	22.0 ± 0.8
	ßENaC Smoke	6	7.1 ± 0.2	23.1 ± 0.5
1-Day Exposure*	**Wild Type Sham**	5	6.9 ± 0.4	23.0 ± 0.8
	Wild Type Smoke	4	7.3 ± 0.5	22.3 ± 1.5
	ßENaC Sham	5	6.9 ± 0.4	20.6 ± 0.5
	ßENaC Smoke	4	7.3 ± 0.5	21.0 ± 1.1
5-Day Exposure	**Wild Type Sham**	7	6.2 ± 0.2	22.0 ± 0.7
	Wild Type Smoke	7	6.4 ± 0.1	19.5 ± 0.8
	ßENaC Sham	7	6.2 ± 0.2	20.6 ± 0.5
	ßENaC Smoke	7	6.4 ± 0.1	19.6 ± 0.9

FEMALES		n	Age (weeks) ± SEM	Weight (g) ± SEM
1-Day Exposure	**Wild Type Sham**	5	7.0 ± 0.1	17.7 ± 0.9
	Wild Type Smoke	5	7.0 ± 0.2	16.4 ± 0.6
	ßENaC Sham	5	7.0 ± 0.1	18.0 ± 0.5
	ßENaC Smoke	5	7.0 ± 0.2	17.2 ± 0.7
1-Day Exposure*	**Wild Type Sham**	4	8.3 ± 0.6	18.5 ± 1.0
	Wild Type Smoke	5	7.6 ± 0.4	17.8 ± 0.6
	ßENaC Sham	4	7.9 ± 0.8	16.8 ± 1.4
	ßENaC Smoke	5	7.3 ± 0.5	18.0 ± 1.0
5-Day Exposure	**Wild Type Sham**	5	6.7 ± 0.4	18.2 ± 0.5
	Wild Type Smoke	5	6.4 ± 0.3	17.5 ± 0.3
	ßENaC Sham	5	6.7 ± 0.4	16.8 ± 0.5
	ßENaC Smoke	5	6.0 ± 0.4	15.8 ± 0.8

*These 1-Day exposed mice were used exclusively for gene expression analysis by qPCR

Table 2: ΔΔCT Values by qPCR following 1-Day Cigarette Smoke Exposure

	WT SHAM		WT SMOKE		BENaC SHAM		BENaC SMOKE	
	Male	Female	Male	Female	Male	Female	Male	Female
KC	1.24 ± 0.46	1.13 ± 0.28	1.11 ± 0.37	$2.71 \pm 0.54^{\S}$	$7.25 \pm 1.31^{\dagger\dagger,**}$	$14.50 \pm 3.63^{\dagger\dagger,**}$	4.61 ± 1.09	$5.19 \pm 1.81^{\ddagger}$
MIP-2	1.51 ± 0.48	1.08 ± 0.18	1.98 ± 0.45	1.42 ± 0.13	$7.97 \pm 0.75^{\dagger\dagger\dagger,***}$	$15.09 \pm 4.12^{\dagger\dagger,**}$	$6.91 \pm 0.75^{\dagger\dagger,***}$	$4.47 \pm 1.44^{\ddagger}$
LIX	1.03 ± 0.14	1.20 ± 0.32	0.92 ± 0.32	1.54 ± 0.53	$9.80 \pm 0.61^{\P\P,\ \S\S\S}$	$9.39 \pm 2.06^{\dagger,**}$	$11.09 \pm 3.83^{\dagger,*}$	4.95 ± 1.80
IFNγ	1.76 ± 0.89	1.21 ± 0.35	4.22 ± 1.17	2.96 ± 1.11	1.88 ± 0.30	4.87 ± 1.63	1.44 ± 0.21	1.90 ± 1.32
TNFα	1.66 ± 0.67	1.02 ± 0.10	1.11 ± 0.33	3.06 ± 0.48	3.10 ± 0.59	$4.06 \pm 1.07^{*}$	1.73 ± 0.33	2.57 ± 0.54
IL-1β	1.41 ± 0.48	1.25 ± 0.44	2.91 ± 2.06	8.43 ± 3.70	1.14 ± 0.18	$3.17 \pm 0.48^{\S}$	0.91 ± 0.26	6.41 ± 4.22
IL-6	1.15 ± 0.33	1.31 ± 0.45	0.96 ± 0.34	6.77 ± 2.88	1.93 ± 0.40	$8.68 \pm 2.45^{\S}$	5.26 ± 1.83	$2.70 \pm 0.74^{\|}$
IL-10	1.51 ± 0.79	1.12 ± 0.26	1.19 ± 0.30	1.03 ± 0.22	0.88 ± 0.15	0.49 ± 0.12	0.88 ± 0.22	0.76 ± 0.16
MMP9	1.51 ± 0.52	1.15 ± 0.35	2.51 ± 1.44	$5.81 \pm 1.51^{\S}$	0.96 ± 0.23	$1.39 \pm 0.38^{\P}$	0.74 ± 0.34	3.94 ± 2.13
MMP12	1.24 ± 0.37	1.24 ± 0.40	0.93 ± 0.24	$4.90 \pm 0.98^{\S}$	$8.79 \pm 1.08^{\P\P,\S\S}$	$27.95 \pm 4.20^{\dagger\dagger\dagger,***}$	9.25 ± 3.67	$6.73 \pm 1.90^{\ddagger\ddagger\ddagger,\S}$

Table 2: ΔΔCT values normalized to sham-exposed, wild type 18S expression following 1 day of sham or smoke exposure. Analysis by 1- or 2-way ANOVA with Bonferroni's post-test: significance compared to (*)sham-exposed wild type, (†) smoke-exposed wild type, (‡) sham-exposed βENaC. Analysis by unpaired t-test: significance compared to (§) sham-exposed wild type, (¶) smoke-exposed wild type, (||) sham-exposed βENaC. Single symbols indicate p values <0.05, double symbols indicate p values <0.01, and triple symbols indicate p values <0.001.

Table 3: ΔΔCT Values by qPCR following 5-Day Cigarette Smoke Exposure

	WT SHAM		WT SMOKE		BENaC SHAM		BENaC SMOKE	
	Male	Female	Male	Female	Male	Female	Male	Female
KC	1.12 ± 0.18	1.10 ± 0.21	1.44 ± 0.20	2.36 ± 0.74	2.36 ± 0.92	4.72 ± 2.25	3.09 ± 0.86§	15.29 ± 7.86
MIP-2	1.07 ± 0.14	1.08 ± 0.21	1.50 ± 0.30	3.17 ± 0.91	2.87 ± 0.64§	5.75 ± 3.10	4.62 ± 1.22†,*	17.87 ± 6.90*
LIX	1.47 ± 0.60	1.08 ±0.22	1.80 ± 0.49	1.53 ± 1.00	9.56 ± 4.87	9.43 ± 3.38	7.36 ± 1.86	8.57 ± 3.49¶,§
IFNγ	1.02 ± 0.10	1.26 ± 0.45	1.60 ± 0.34	1.96 ± 0.78	1.59 ± 0.53	1.55 ± 0.24	3.00 ± 1.22	1.89 ± 0.67
TNFα	1.01 ± 0.07	1.04 ± 0.15	1.50 ± 0.25	1.32 ± 0.28	2.10 ± 0.74	2.69 ± 0.62§	2.59 ± 0.48§§	2.54 ± 0.95
IL-1β	1.30 ± 0.36	1.39 ± 0.53	2.85 ± 1.18	2.77 ± 1.02	1.63 ± 0.51	2.18 ± 0.50	5.22 ± 3.69	4.69 ± 2.9
IL-6	1.26 ±0.32	1.17 ± 0.39	2.56 ± 0.71	2.16 ± 0.96	2.04 ± 1.07	3.72 ± 1.42	3.55 ± 1.72	4.63 ± 3.19
IL-10	1.30 ± 0.42	1.23 ± 0.41	1.03 ± 0.33	1.02 ± 0.23	0.86 ± 0.17	1.33 ± 0.20	1.28 ± 0.40	1.43 ± 0.32
MMP9	1.82 ± 0.74	1.06 ± 0.17	1.49 ± 0.55	1.11 ± 0.21	0.87 ± 0.39	1.02 ± 0.20	1.91 ± 1.43	0.59 ± 0.21
MMP12	1.68 ± 0.66	1.26 ± 0.40	2.31 ± 1.11	2.77 ± 0.80	12.20 ± 5.70	37.95 ± 29.04	28.26 ± 13.14	92.68 ± 69.69

Table 3: ΔΔCT values normalized to sham-exposed, wild type 18S expression following 5 days of sham or smoke exposure. Analysis by 1- or 2-way ANOVA with Bonferroni's post-test: significance compared to (*)sham-exposed wild type, (†) smoke-exposed wild type, (‡) sham-exposed βENaC. Analysis by unpaired t-test: significance compared to (§) sham-exposed wild type, (¶) smoke-exposed wild type, (||) sham-exposed βENaC. Single symbols indicate p values <0.05, double symbols indicate p values <0.01, and triple symbols indicate p values <0.001.

Figure 1. 1-Day BAL Data

Females, 1-Day Exposure
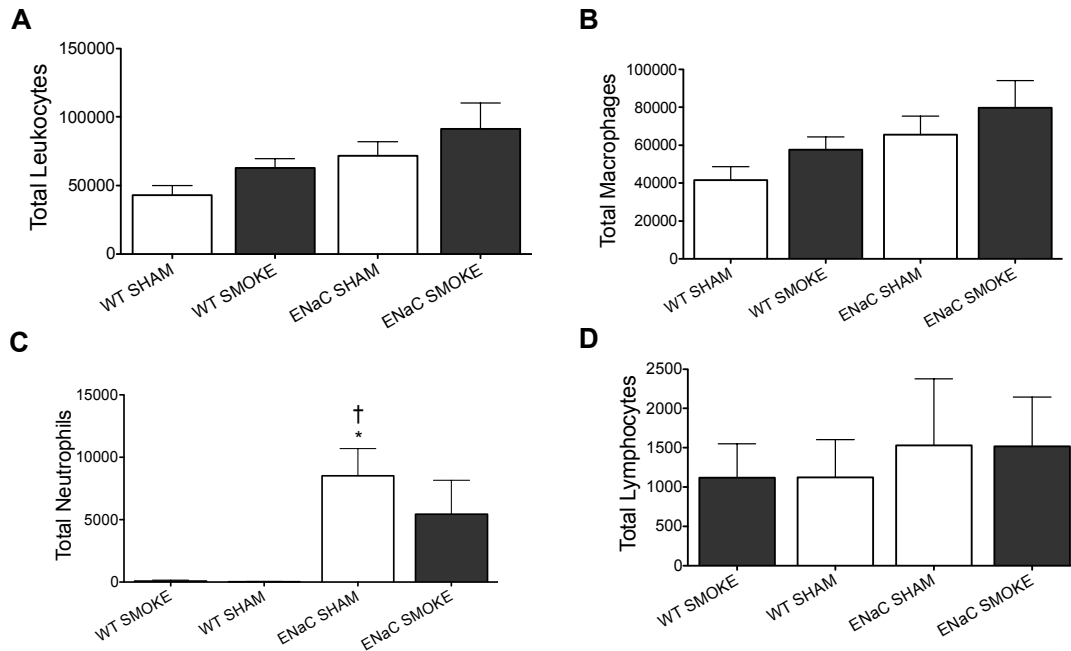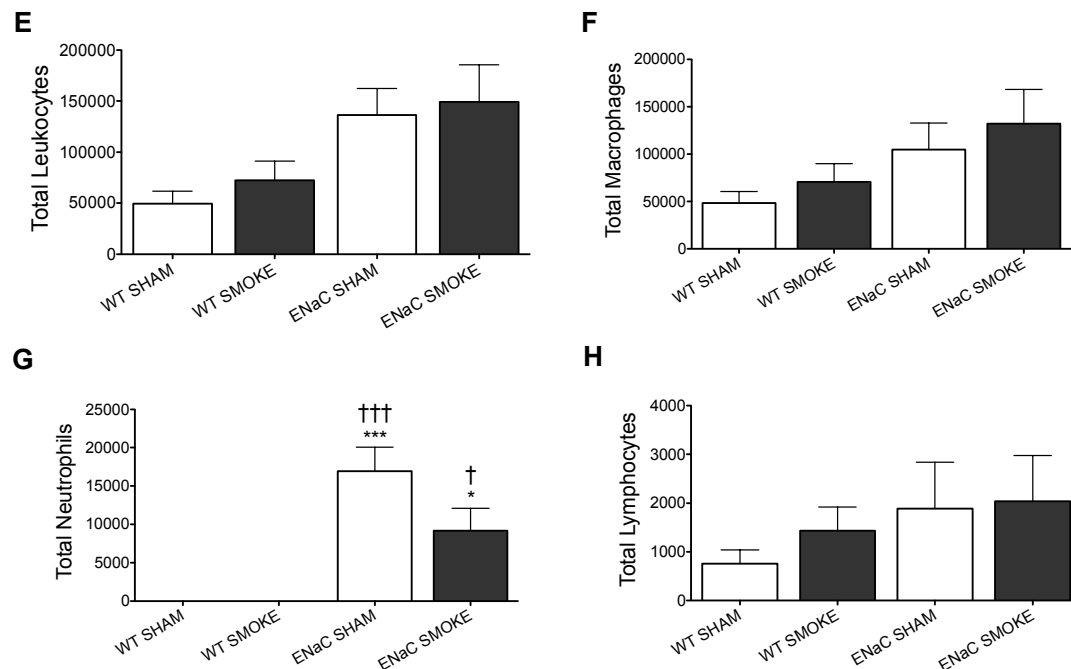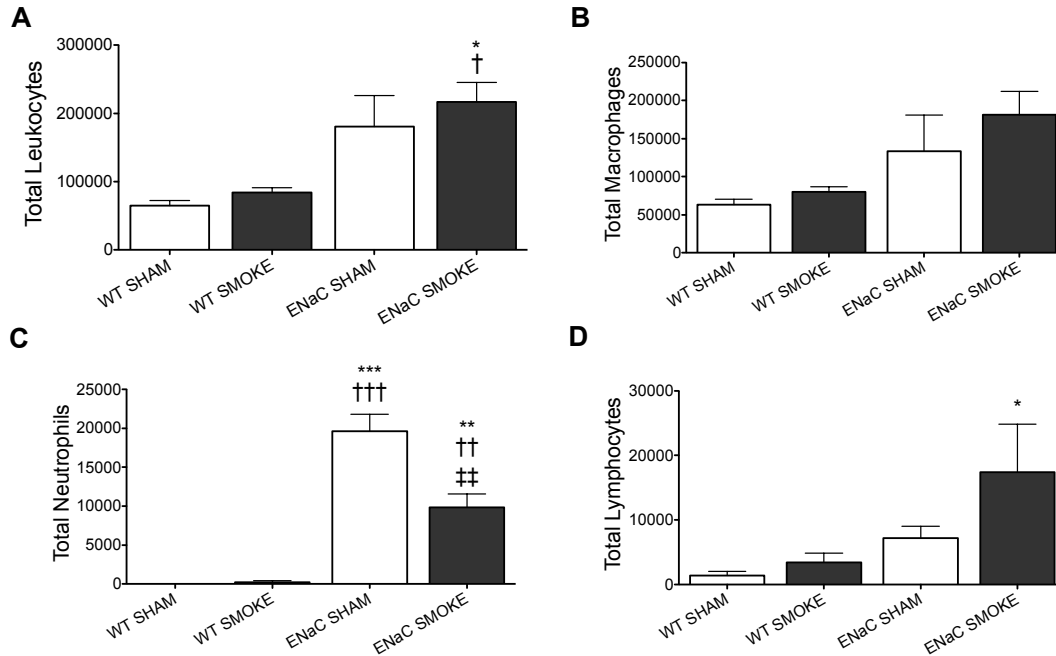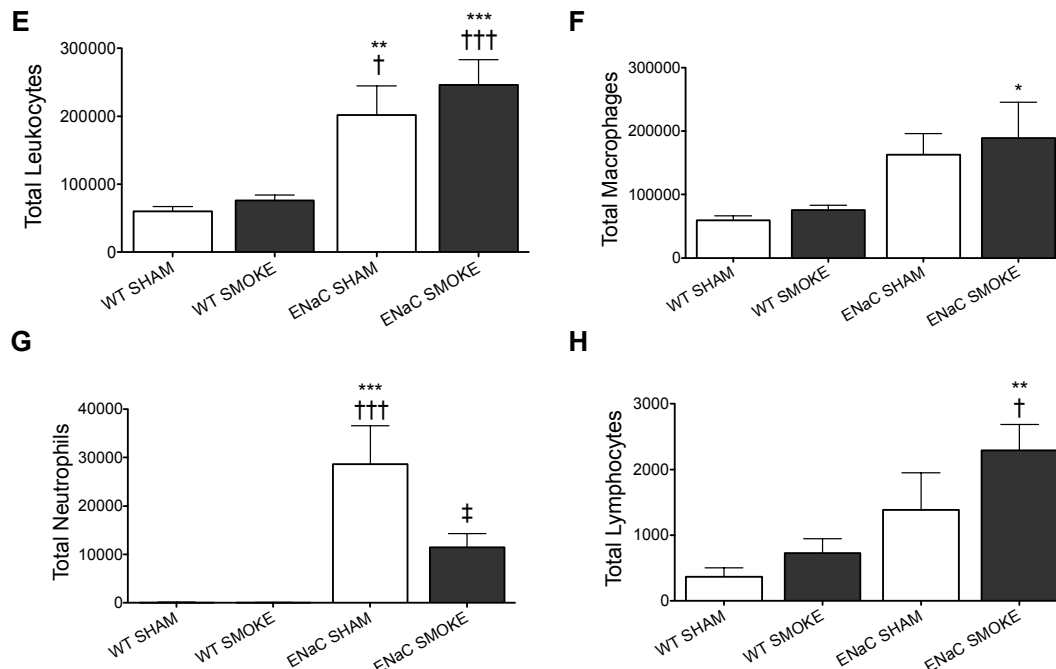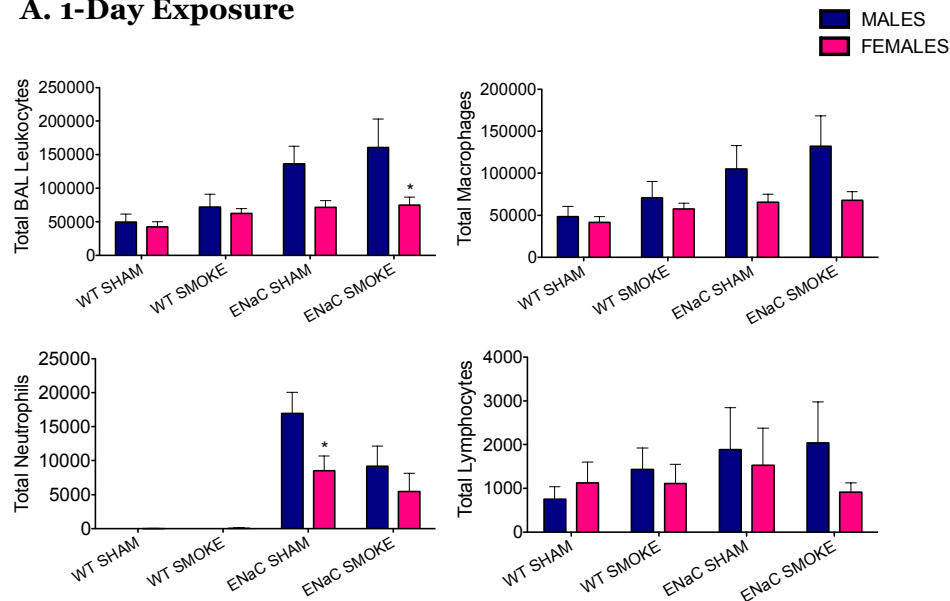

Males, 1-Day Exposure

Figure 1. BAL total cell counts following 1 day of cigarette smoke exposure, separated by gender. (A, E) Total leukocytes (B, F) Total macrophages (C, G) Total neutrophils (D, H) Total lymphocytes. Analysis by 1- or 2-way ANOVA with Bonferroni's post-test: significance compared to (*)sham-exposed wild type, (†) smoke-exposed wild type, (‡) sham-exposed βENaC. Analysis by unpaired t-test: significance compared to (§) sham-exposed wild type, (¶) smoke-exposed wild type, (||) sham-exposed βENaC. Single symbols indicate p values <0.05, double symbols indicate p values <0.01, and triple symbols indicate p values <0.001. Bars represent SEM.

Figure 2. 5-Day BAL Data

Females, 5-Day Exposure

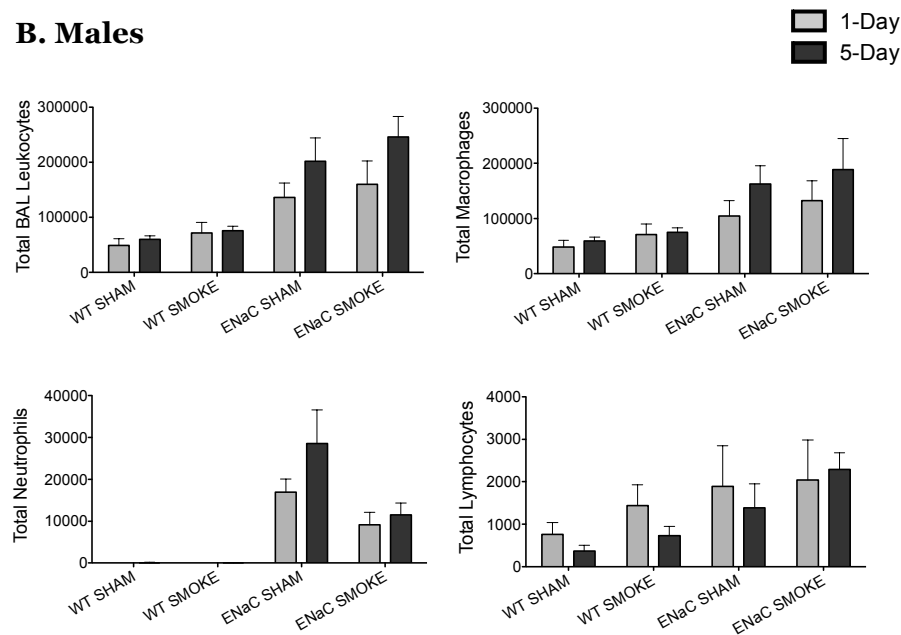**Males, 5-Day Exposure**

Figure 2. BAL total cell counts following 5 days of cigarette smoke exposure, separated by gender. (A, E) Total leukocytes (B, F) Total macrophages (C, G) Total neutrophils (D, H) Total lymphocytes. Analysis by 1- or 2-way ANOVA with Bonferroni's post-test: significance compared to (*)sham-exposed wild type, (†) smoke-exposed wild type, (‡) sham-exposed βENaC. Analysis by unpaired t-test: significance compared to (§) sham-exposed wild type, (¶) smoke-exposed wild type, (||) sham-exposed βENaC. Single symbols indicate p values <0.05, double symbols indicate p values <0.01, and triple symbols indicate p values <0.001. Bars represent SEM.

Figure 3. Males vs. Females BAL Data

A. 1-Day Exposure

B. 5-Day Exposure
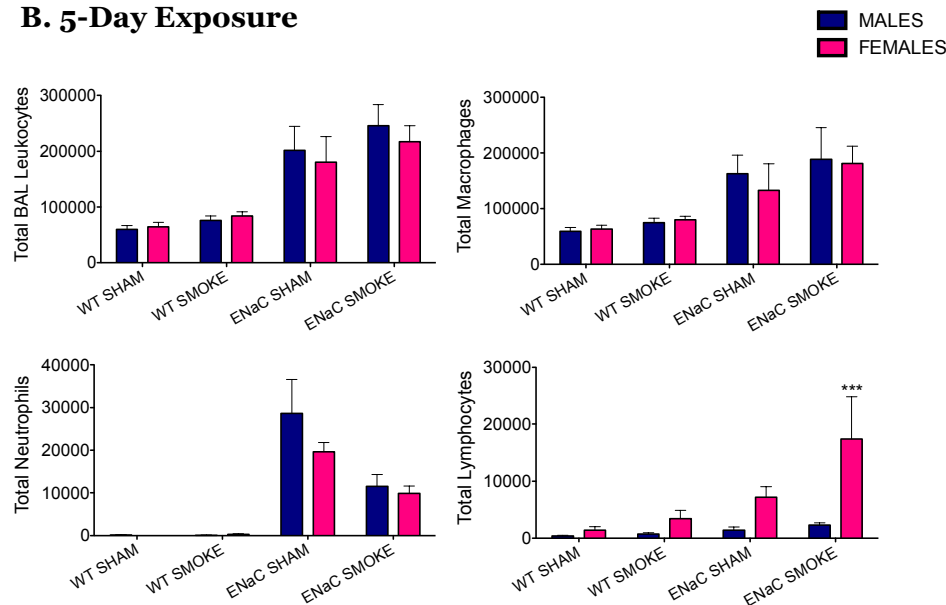
Figure 3. BAL total cell counts in males (blue) and females (pink) following 1- (A) and 5- (B) days of cigarette smoke exposure. Total leukocytes, macrophages, neutrophils, and lymphocytes are shown. Analysis by 1- or 2-way ANOVA with Bonferroni's post-test: significance compared to (*)sham-exposed wild type, (†) smoke-exposed wild type, (‡) sham-exposed βENaC. Analysis by unpaired t-test: significance compared to (§) sham-exposed wild type, (¶) smoke-exposed wild type, (||) sham-exposed βENaC. Single symbols indicate p values <0.05, double symbols indicate p values <0.01, and triple symbols indicate p values <0.001. Bars represent SEM.

Figure 4. 1-Day vs. 5-Day BAL Data

A. Females
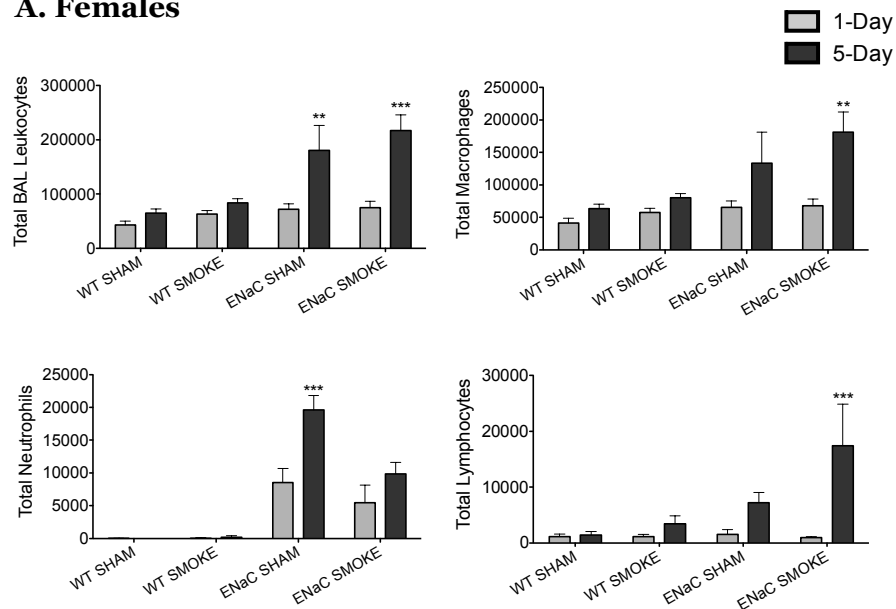
B. Males

Figure 4. BAL total cell counts for both durations, separated by gender. Total leukocytes, macrophages, neutrophils, and lymphocytes in female (A) and male (B) mice following 1- (light gray) vs. 5-day (dark gray) exposures. Analysis by 1- or 2-way ANOVA with Bonferroni's post-test: significance compared to (*)sham-exposed wild type, (†) smoke-exposed wild type, (‡) sham-exposed βENaC. Analysis by unpaired t-test: significance compared to (§) sham-exposed wild type, (¶) smoke-exposed wild type, (||) sham-exposed βENaC. Single symbols indicate p values <0.05, double symbols indicate p values <0.01, and triple symbols indicate p values <0.001. Bars represent SEM.

Figure 5. 1-Day Tissue Neutrophils by Flow Cytometry

Females, 1-Day Exposure

Males, 1-Day Exposure
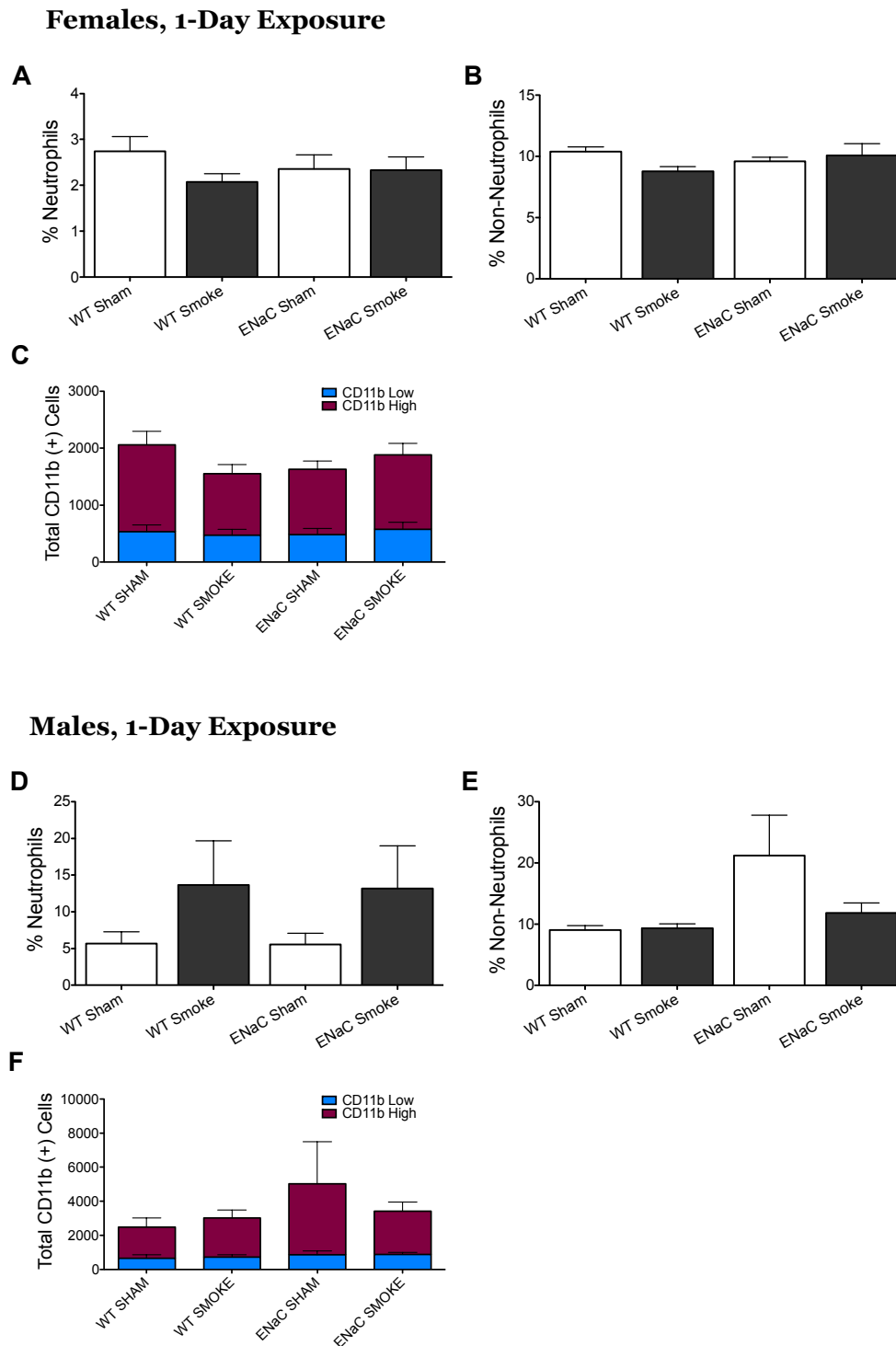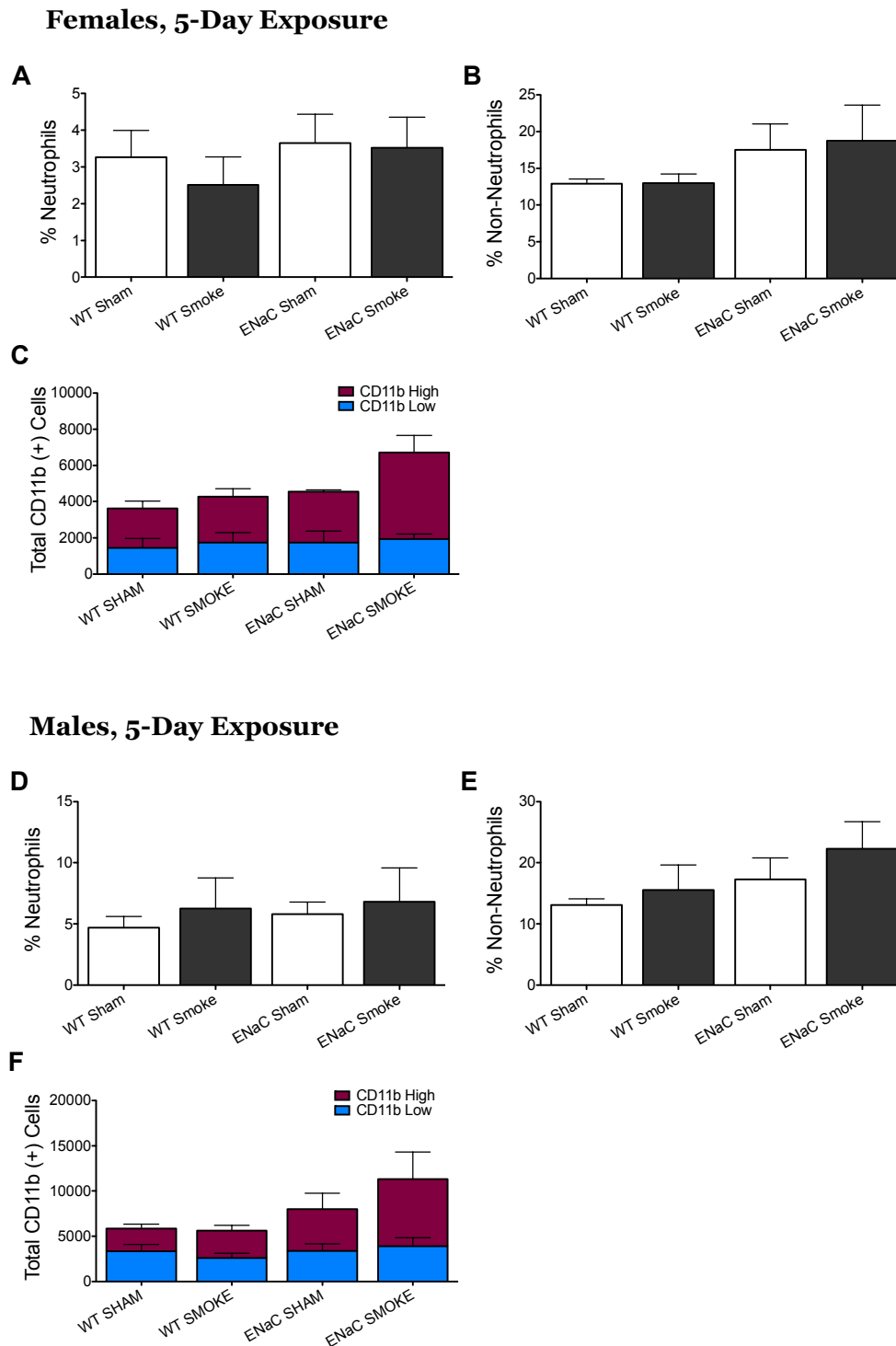

Figure 5. Lung tissue leukocytes recovered after 1 day of cigarette smoke exposure, measured by flow cytometry, sorted by gender. A, D) % Neutrophils by Ly6G expression, B, E) % Non-neutrophils by Ly6G(-), CD11b (+) expression, C, F) Total Ly6G(-), CD11b(+) cells classified as high (red) or low (blue) expression of CD11b. Analysis by 1- or 2-way ANOVA with Bonferroni's post-test: significance compared to (*)sham-exposed wild type, (†) smoke-exposed wild type, (‡) sham-exposed βENaC. Analysis by unpaired t-test: significance compared to (§) sham-exposed wild type, (¶) smoke-exposed wild type, (||) sham-exposed βENaC. Single symbols indicate p values <0.05, double symbols indicate p values <0.01, and triple symbols indicate p values <0.001. Bars represent SEM.

Figure 6. 5-Day Tissue Neutrophils by Flow Cytometry

Females, 5-Day Exposure

Males, 5-Day Exposure

Figure 6. Lung tissue leukocytes recovered after 5 days of cigarette smoke exposure, measured by flow cytometry, sorted by gender. A, D) % Neutrophils by Ly6G expression, B, E) % Non-neutrophils by Ly6G(-), CD11b (+) expression, C, F) Total Ly6G(-), CD11b(+) cells classified as high (red) or low (blue) expression of CD11b. Analysis by 1- or 2-way ANOVA with Bonferroni's post-test: significance compared to (*)sham-exposed wild type, (†) smoke-exposed wild type, (‡) sham-exposed βENaC. Analysis by unpaired t-test: significance compared to (§) sham-exposed wild type, (¶) smoke-exposed wild type, (||) sham-exposed βENaC. Single symbols indicate p values <0.05, double symbols indicate p values <0.01, and triple symbols indicate p values <0.001. Bars represent SEM.

Figure 7. Males vs. Females CD11b Expression

Males vs. Females, 1-Day Exposure
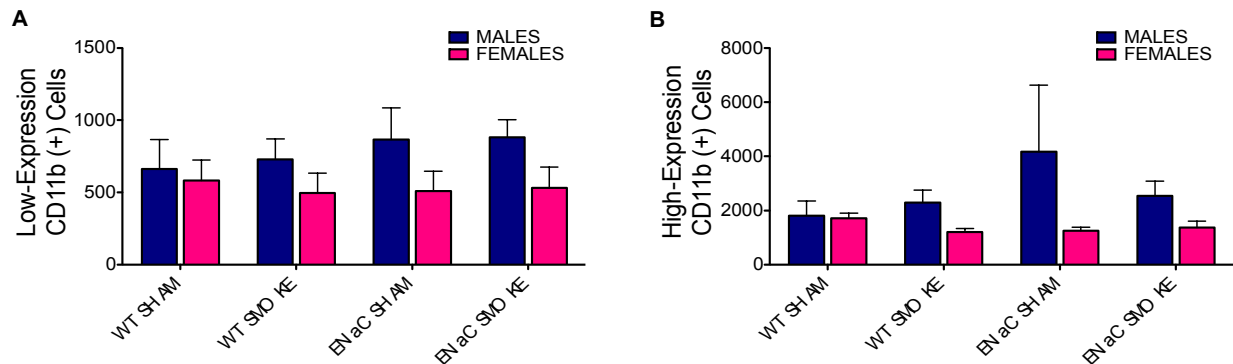
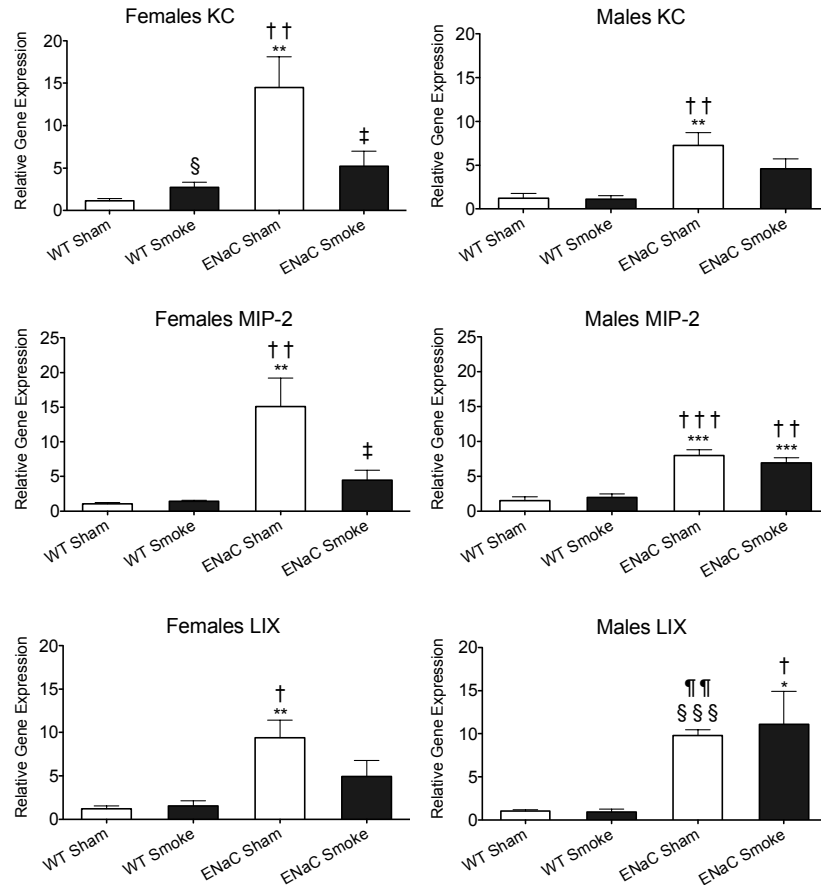**Males vs. Females, 5-Day Exposure**
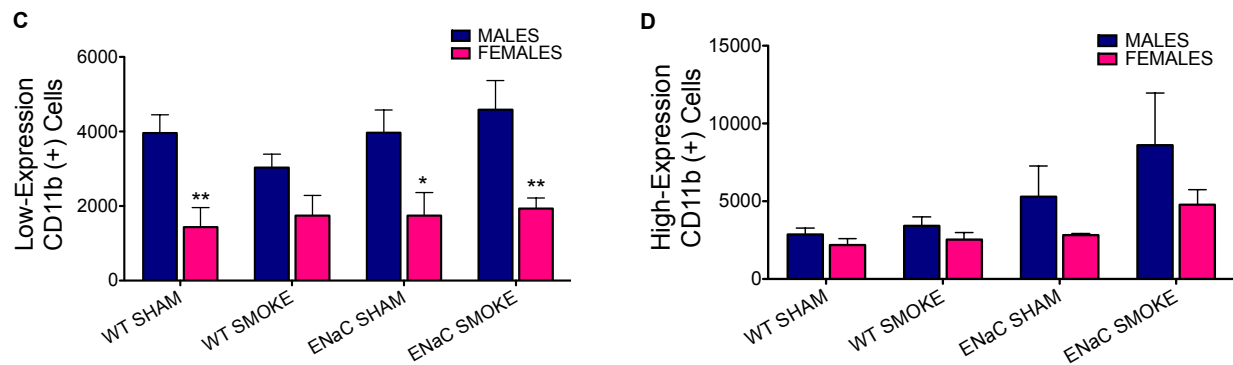
Figure 7. Lung tissue CD11b expression in males (blue) and females (pink) following 1- (A, B) and 5- (C, D) day cigarette smoke exposures, measured by flow cytometry. A, C) Total number of cells with low expression of CD11b. B, D) Total number of cells with high expression of CD11b. Analysis by 1- or 2-way ANOVA with Bonferroni's post-test: significance compared to (*)sham-exposed wild type, (†) smoke-exposed wild type, (‡) sham-exposed βENaC. Analysis by unpaired t-test: significance compared to (§) sham-exposed wild type, (¶) smoke-exposed wild type, (||) sham-exposed βENaC. Single symbols indicate p values <0.05, double symbols indicate p values <0.01, and triple symbols indicate p values <0.001. Bars represent SEM.

Figure 8. Chemokine Expression
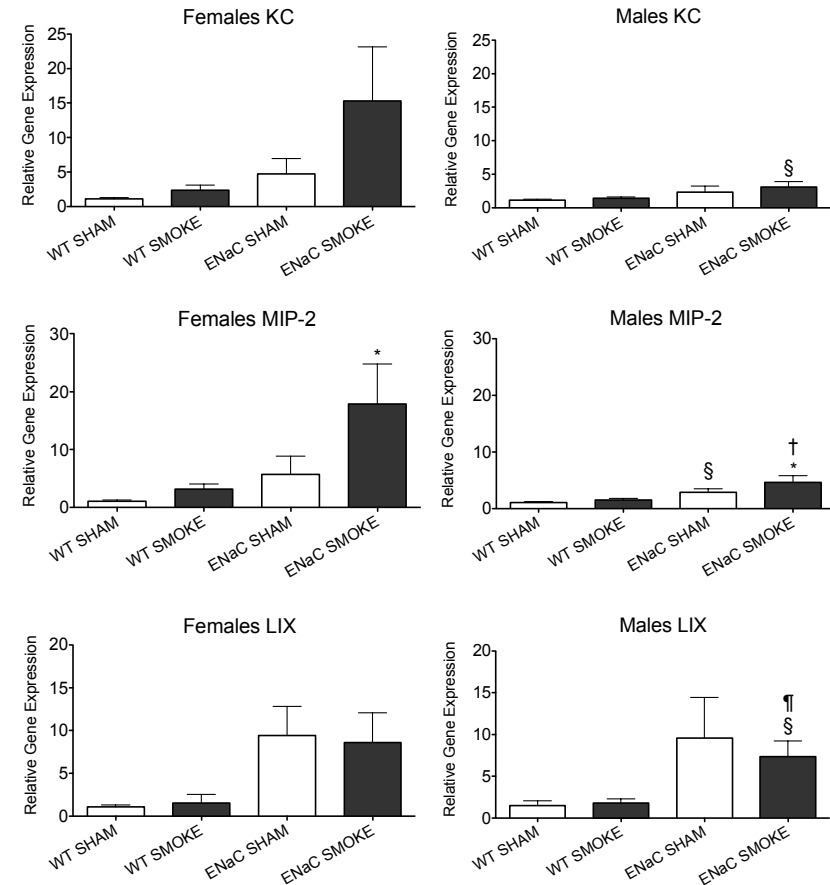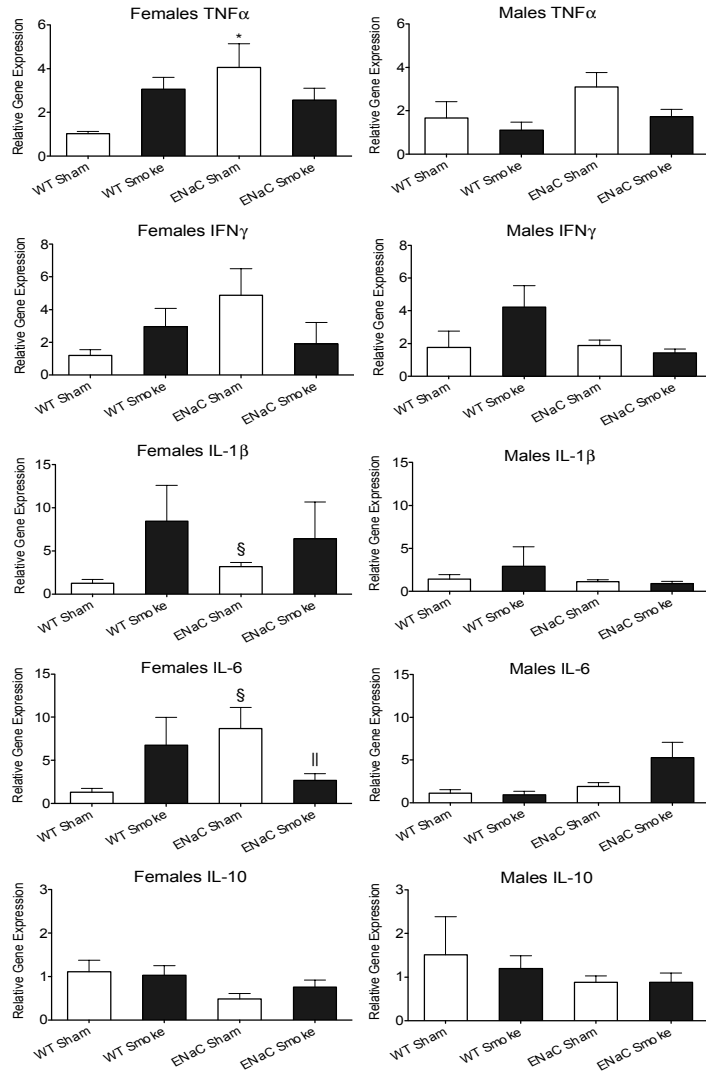
A. 1-Day Exposure

B. 5-Day Exposure

Figure 8. Relative expression of chemokines measured by qPCR in males and females following 1- (A) and 5- (B) day exposures to cigarette smoke. Relative gene expression is in the form of ΔΔCt normalized to wild type, sham-exposed, 18S expression. Analysis by 1- or 2-way ANOVA with Bonferroni's post-test: significance compared to (*)sham-exposed wild type, (†) smoke-exposed wild type, (‡) sham-exposed βENaC. Analysis by unpaired t-test: significance compared to (§) sham-exposed wild type, (¶) smoke-exposed wild type, (||) sham-exposed βENaC. Single symbols indicate p values <0.05, double symbols indicate p values <0.01, and triple symbols indicate p values <0.001. Bars represent SEM.

Figure 9. Cytokine Expression

A. 1-Day Exposure
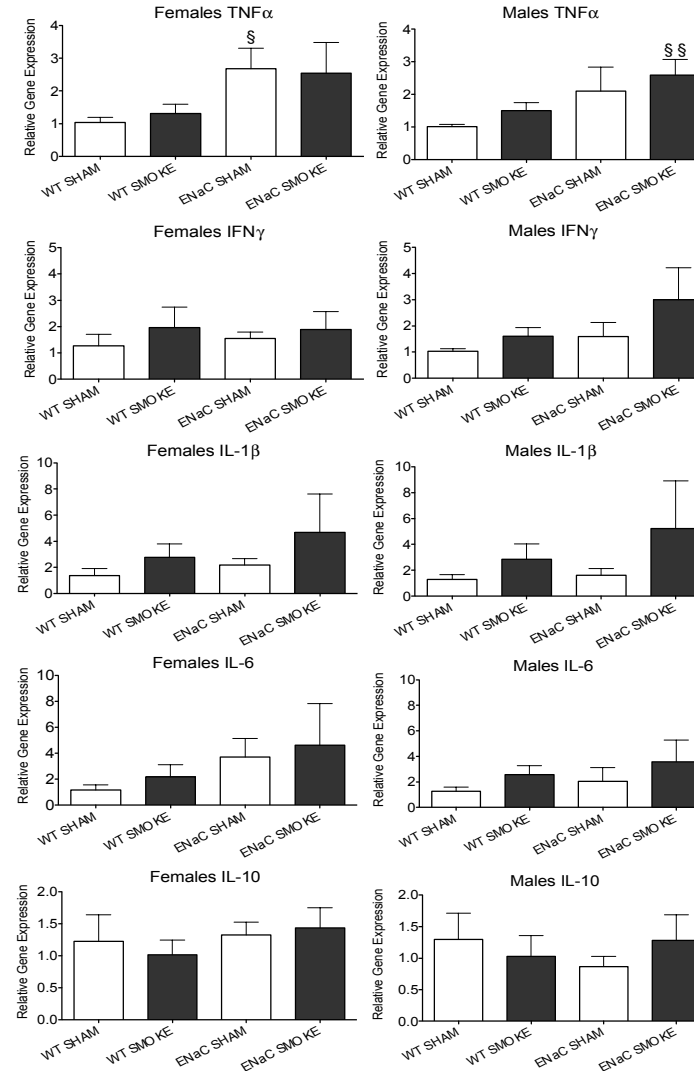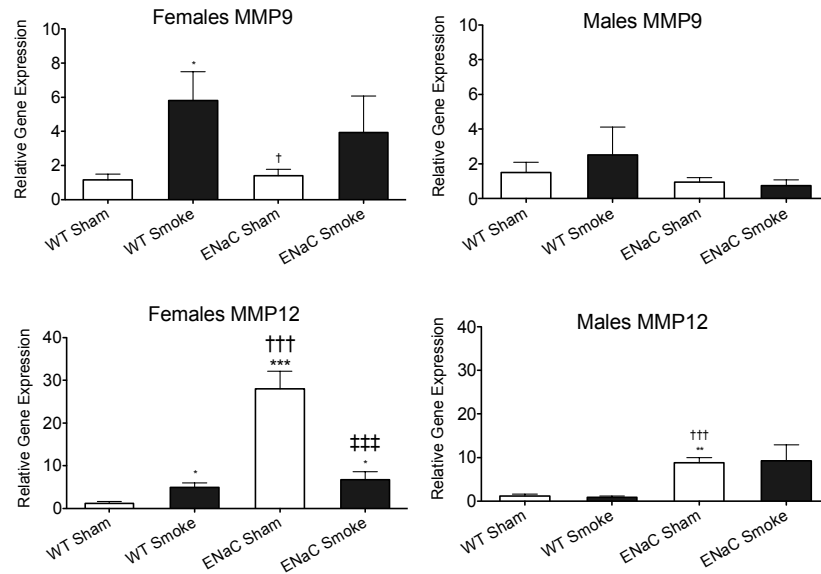

B. 5-Day Exposure

Figure 9. Relative expression of selected cytokines measured by qPCR in males and females following 1- (A) and 5- (B) day exposures to cigarette smoke. Relative gene expression is in the form of ΔΔCt normalized to wild type, sham-exposed, 18S expression. Analysis by 1- or 2-way ANOVA with Bonferroni's post-test: significance compared to (*)sham-exposed wild type, (†) smoke-exposed wild type, (‡) sham-exposed βENaC. Analysis by unpaired t-test: significance compared to (§) sham-exposed wild type, (¶) smoke-exposed wild type, (||) sham-exposed βENaC. Single symbols indicate p values <0.05, double symbols indicate p values <0.01, and triple symbols indicate p values <0.001. Bars represent SEM.

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Figure 10. MMP Expression

A. 1-Day Exposure
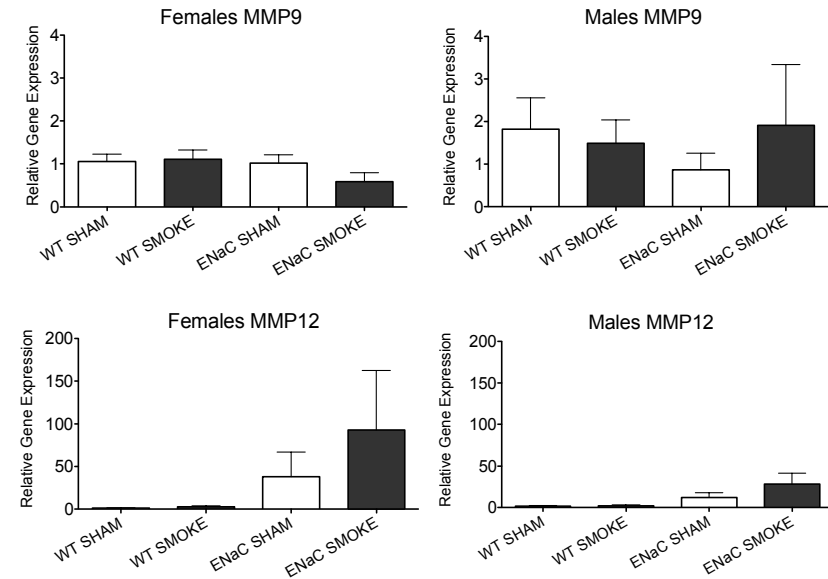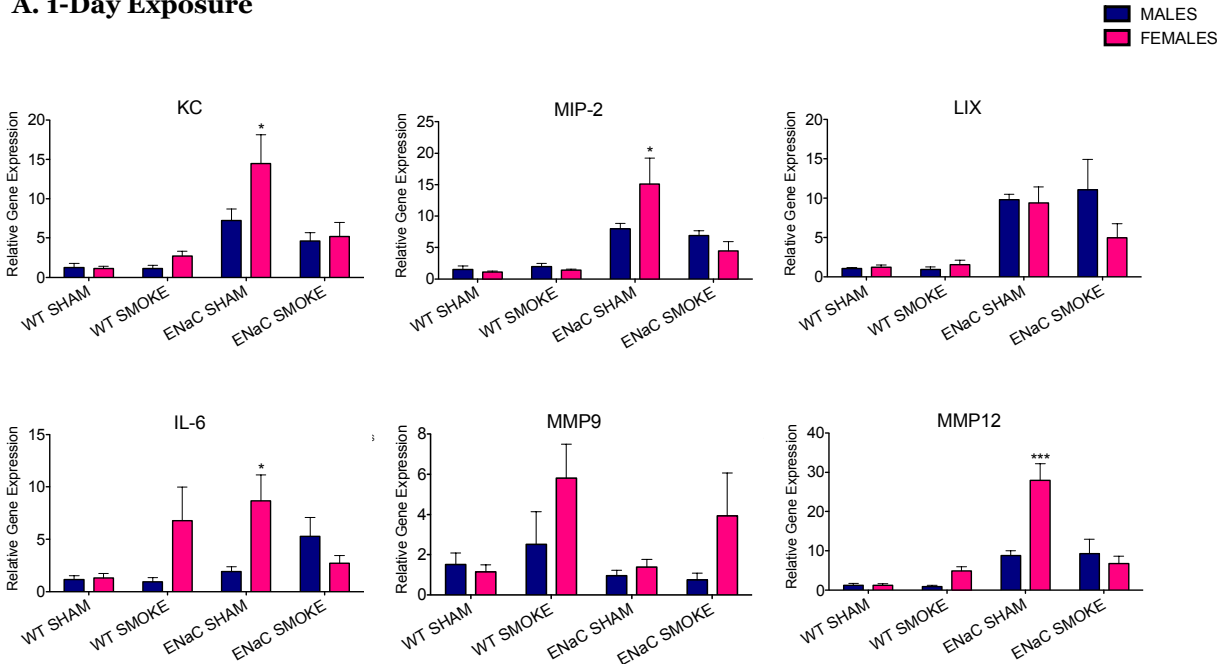

B. 5-Day Exposure

Figure 10. Relative expression of matrix metalloproteinases measured by qPCR in males and females following 1- (A) and 5- (B) day exposures to cigarette smoke. Relative gene expression is in the form of ΔΔCt normalized to wild type, sham-exposed, 18S expression. Analysis by 1- or 2-way ANOVA with Bonferroni's post-test: significance compared to (*)sham-exposed wild type, (†) smoke-exposed wild type, (‡) sham-exposed βENaC. Analysis by unpaired t-test: significance compared to (§) sham-exposed wild type, (¶) smoke-exposed wild type, (||) sham-exposed βENaC. Single symbols indicate p values <0.05, double symbols indicate p values <0.01, and triple symbols indicate p values <0.001. Bars represent SEM.

Figure 11. Males vs. Females Gene Expression

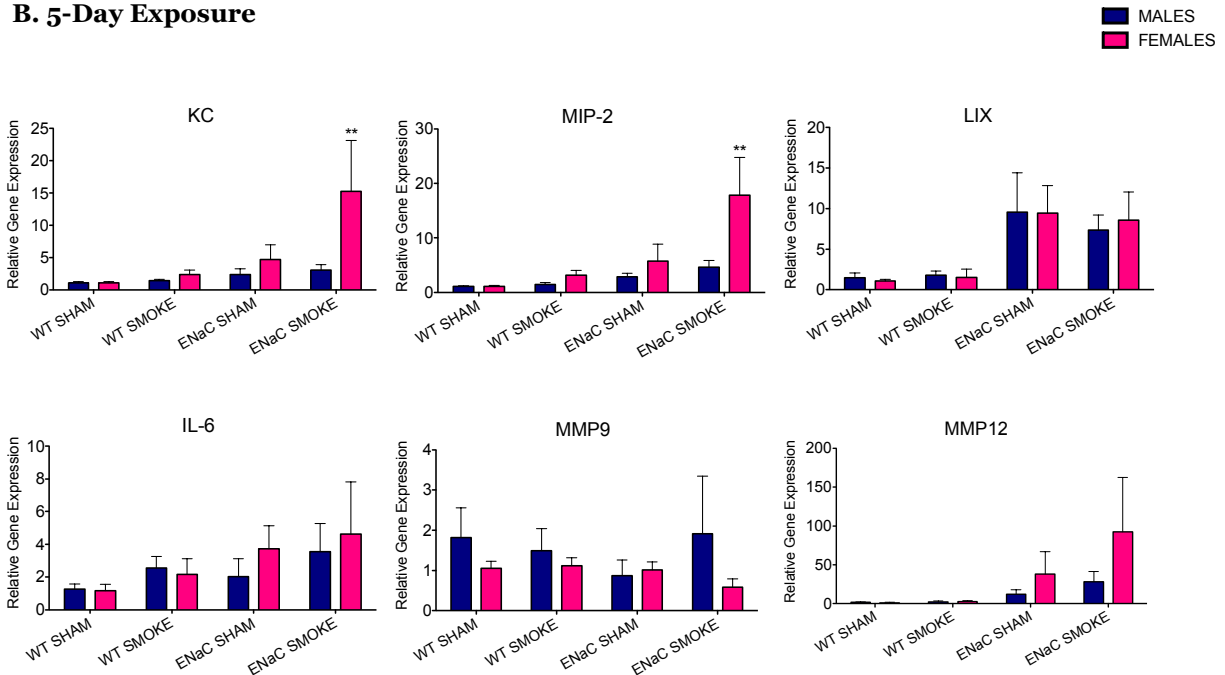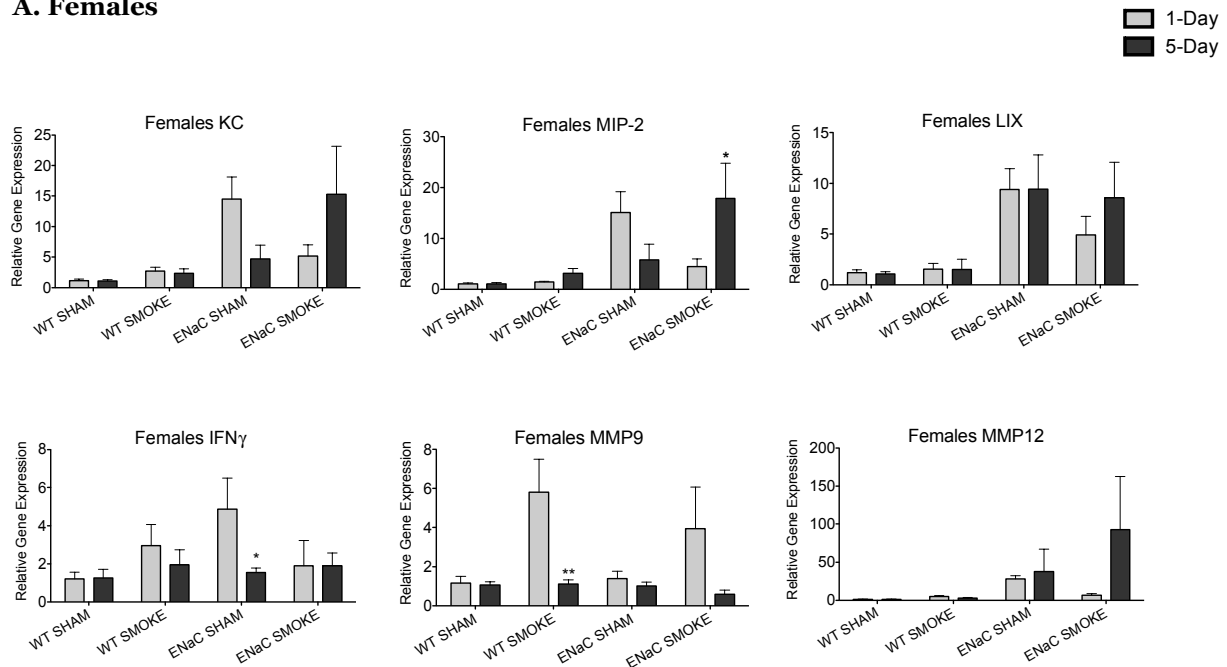## A. 1-Day Exposure

B. 5-Day Exposure

Figure 11. Relative gene expression in males (blue) and females (pink) following 1- (A) and 5- (B) day exposures to cigarette smoke. Relative gene expression is in the form of $\Delta\Delta Ct$ normalized to wild type, sham-exposed, 18S expression. Analysis by 1- or 2-way ANOVA with Bonferroni's post-test: significance compared to (*)sham-exposed wild type, (†) smoke-exposed wild type, (‡) sham-exposed βENaC. Analysis by unpaired t-test: significance compared to (§) sham-exposed wild type, (¶) smoke-exposed wild type, (||) sham-exposed βENaC. Single symbols indicate p values <0.05, double symbols indicate p values <0.01, and triple symbols indicate p values <0.001. Bars represent SEM.

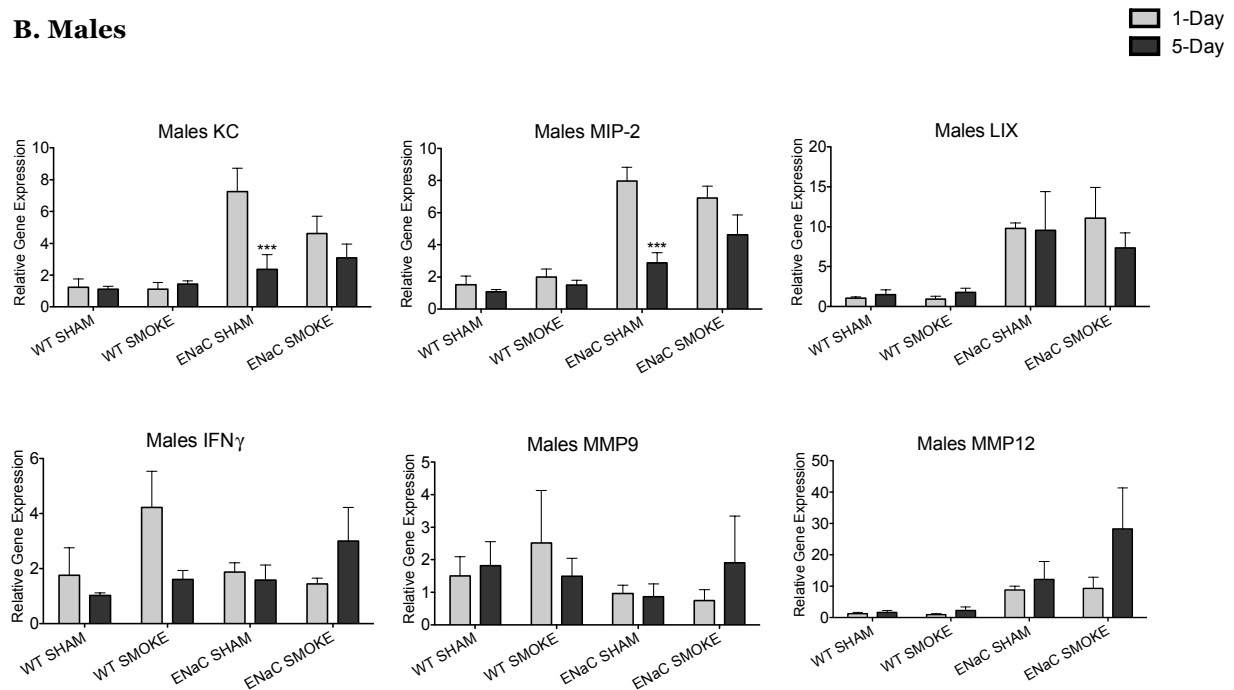# Figure 12. 1-Day vs. 5-Day Gene Expression

A. Females

B. Males

Figure 12. Relative gene expression for 1- (light gray) vs. 5-day (dark gray) exposures in females (A) and males (B). Analysis by 1- or 2-way ANOVA with Bonferroni's post-test: significance compared to (*)sham-exposed wild type, (†) smoke-exposed wild type, (‡) sham-exposed βENaC. Analysis by unpaired t-test: significance compared to (§) sham-exposed wild type, (¶) smoke-exposed wild type, (||) sham-exposed βENaC. Single symbols indicate p values <0.05, double symbols indicate p values <0.01, and triple symbols indicate p values <0.001. Bars represent SEM.
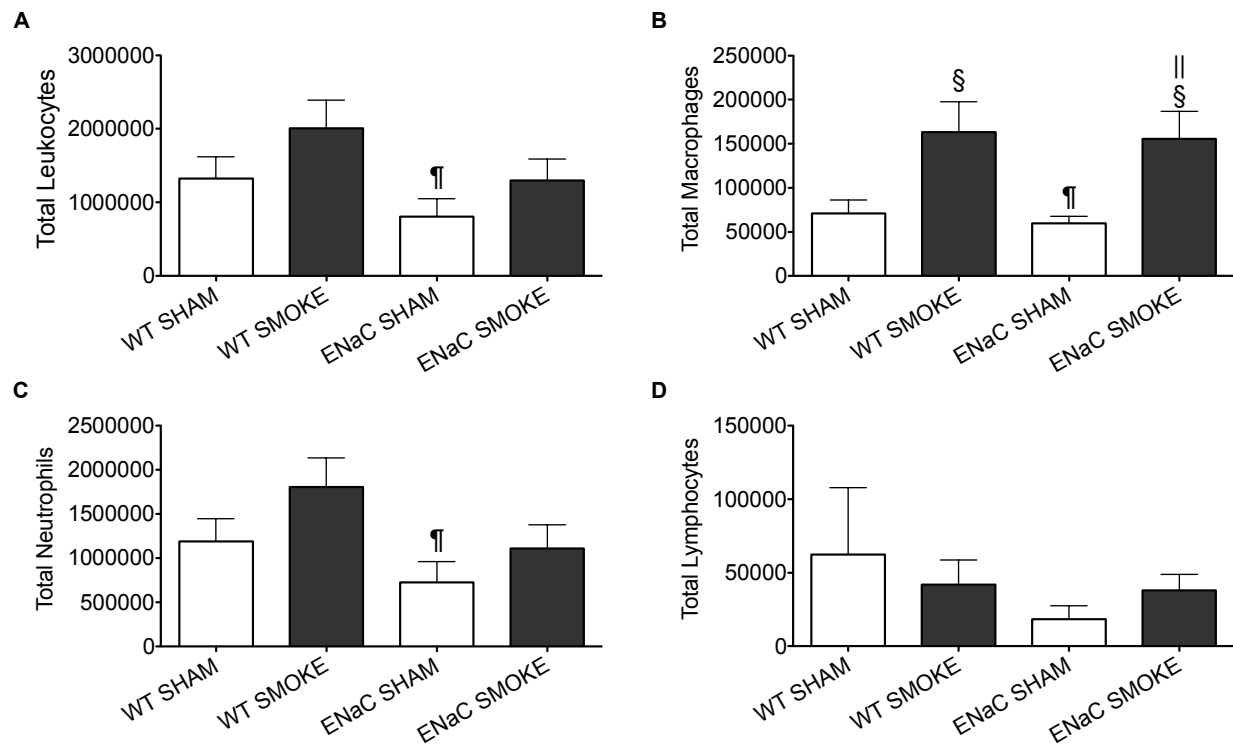
Figure 13. 20-Hour NTHI BAL Data

Figure 13. Recovered BAL leukocytes following 20-hour infection with NTHI. Cells counted include (a) total leukocytes, (B) macrophages, (C) neutrophils, and (D) lymphocytes. Analysis by 1- or 2-way ANOVA with Bonferroni's post-test: significance compared to (*)sham-exposed wild type, (†) smoke-exposed wild type, (‡) sham-exposed βENaC. Analysis by unpaired t-test: significance compared to (§) sham-exposed wild type, (¶) smoke-exposed wild type, (||) sham-exposed βENaC. Single symbols indicate p values <0.05, double symbols indicate p values <0.01, and triple symbols indicate p values <0.001. Bars represent SEM.

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