Published in final edited form as:

Int Immunopharmacol. 2010 August ; 10(8): 906–912. doi:10.1016/j.intimp.2010.05.001.

Inactivation of I κ B-kinase- β dependent genes in airway epithelium reduces tobacco smoke induced acute airway inflammation

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Abstract

We have examined the role of NF- κ B regulated genes in airway epithelium in mediating tobacco smoke induced airway inflammation in studies of *CC10-Cre^{tg}/I κ B $\beta^{\Delta/\Delta}$* mice in which NF- κ B signaling through I κ B-kinase- β (IKK- β) is selectively ablated in epithelial cells in the airway. *CC10-Cre^{tg}/I κ B $\beta^{\Delta/\Delta}$* mice exposed to tobacco smoke for seven days had a significant decrease in the number of BAL cells (total cells, neutrophils, macrophages) as well as significantly reduced numbers of peribronchial cells (F4/80+ and myeloperoxidase+) compared to tobacco exposed WT mice. In addition to the reduction in peribronchial cells, *CC10-Cre^{tg}/I κ B $\beta^{\Delta/\Delta}$* mice exposed to tobacco smoke had a significant decrease in the number of macrophages and neutrophils in the alveolar space suggesting that inactivation of NF- κ B in the airway epithelium influenced the number of neutrophils and macrophages recruited to the alveolus. Levels of the NF- κ B regulated chemokines KC and MCP-1 were significantly reduced in lungs of tobacco smoke exposed *CC10-Cre^{tg}/I κ B $\beta^{\Delta/\Delta}$* mice compared to tobacco exposed WT mice. In contrast, there was no significant difference in levels of NF- κ B regulated MIP-1 α between *CC10-Cre^{tg}/I κ B $\beta^{\Delta/\Delta}$* and WT mice. Lung sections of tobacco smoke exposed *CC10-Cre^{tg}/I κ B $\beta^{\Delta/\Delta}$* mice immunostained with KC or MCP-1 antibodies demonstrated reduced expression of these chemokines in the airway epithelium, but not in alveolar epithelium. Overall, these studies demonstrate an important role for NF- κ B regulated genes in airway epithelium in contributing to acute tobacco smoke induced airway inflammation not only in the peribronchial space but also in the alveolar space.

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Keywords

Chemokines; macrophage; neutrophil

1. INTRODUCTION

Inhalation of tobacco smoke induces an inflammatory response in the airway characterized by the recruitment of neutrophils and mononuclear cells [1]. Neutrophils and mononuclear cells are bone marrow derived cells which are recruited to the lung in a multistep process that involves initial leukocyte adhesion to inflamed endothelium, diapedesis of leukocytes between endothelial cells, and subsequent leukocyte chemotaxis into lung tissue [2]. There are likely multiple mechanisms that mediate the recruitment and chemotaxis of these inflammatory cells from the circulation into the airway following inhalation of tobacco smoke including either a direct effect of tobacco smoke on recruitment of inflammatory cells, and/or an indirect effect of tobacco smoke on other cell types (i.e. epithelial or other cell types in the airway) to release inflammatory cell chemoattractants. In this study we have used mice deficient in the ability to activate NF- κ B in airway epithelium [3] to determine the contribution of airway epithelial derived chemoattractants and/or cytokines regulated by NF- κ B to the recruitment of neutrophils and mononuclear cells to the perobronchial and alveolar space following acute exposure to tobacco smoke.

NF- κ B, a transcription factor expressed in most cell types including airway epithelium, regulates gene transcription by binding to specific κ B elements in the promoter regions of target genes [4]. NF- κ B regulates the epithelial expression of multiple genes that may be important to airway inflammation, including genes encoding cytokines, chemokines, and adhesion molecules [4]. In unstimulated epithelial cells, NF- κ B is not active as the vast majority of NF- κ B dimers are retained in the cytoplasm through association with specific inhibitors termed I κ Bs [4]. Exposure of airway epithelial cells to tobacco smoke is one of many stimuli that are known to activate NF- κ B [5]. Upon epithelial cell exposure to NF- κ B activating stimuli such as tobacco smoke, the I κ Bs in the cytoplasm are phosphorylated and degraded and liberated NF- κ B dimers enter the nucleus to upregulate genes containing κ B elements in their regulatory regions. The degradation of I κ Bs activates NF- κ B and is mediated by the multi-subunit I κ B kinase (IKK) complex comprised of the catalytic subunits IKK α and IKK β , and the regulatory subunit IKK γ (also known as NEMO)[4]. One of the major functions of the IKK complex is thus to phosphorylate I κ B molecules, thereby triggering their degradation, and activation of NF- κ B. While both IKK α and IKK β can phosphorylate all three I κ B proteins *in vitro*, studies in mice deficient in IKK subunits demonstrate that in most cells IKK β plays the dominant role in signal-induced I κ B phosphorylation and degradation [4]. We have recently used the Cre/*loxP* approach [6] to generate *CC10-Cre^{tg}/Ikk β ^{Δ/Δ}* mice (deficient in I κ B-kinase β (IKK β) in airway epithelium and thus deficient in the ability to activate NF- κ B in these cells) to demonstrate that NF- κ B regulated genes expressed by airway epithelium contribute to eosinophilic airway inflammation in a model of lung disease induced by inhaled allergen [3]. *CC10-Cre^{tg}/Ikk β ^{Δ/Δ}* mice are deficient in the ability to activate NF- κ B in airway epithelium, but are able to activate NF- κ B in alveolar epithelium [3]. In this study we have utilized the *CC10-Cre^{tg}/Ikk β ^{Δ/Δ}* mice to determine the importance of airway epithelial expressed genes regulated by NF- κ B to the recruitment of neutrophils and mononuclear cells to either the airway epithelium (deficient in the ability to activate NF- κ B) or to the alveolar epithelium (able to activate NF- κ B) following acute tobacco smoke exposure. These studies shed light on the contribution of NF- κ B regulated genes in the airway epithelium (compared to other cell types) to recruitment of neutrophils and mononuclear cells to the airway or alveolus in response to acute exposure to tobacco smoke.

2. MATERIALS AND METHODS

2.1 CC10-Cre^{tg}/Ikk $\beta^{\Delta/\Delta}$ Mice

We used *CC10-Cre^{tg}/Ikk $\beta^{\Delta/\Delta}$* in which NF- κ B signaling through IKK- β is selectively ablated in the airway epithelium by conditional Cre-recombinase expression from the Clara cell (CC10) promoter [3,7,8,9]. The transgenic mice and the constructs that were used to generate the *CC10-Cre^{tg}/Ikk $\beta^{\Delta/\Delta}$* mice in this laboratory have been described previously in detail [3,7,8,9]. The CC10 promoter is expressed selectively in Clara cells which represent approximately 70% of the adult mouse airway epithelium [10]. In situ hybridization studies in mouse lung have demonstrated that CC10 is expressed primarily in the airway (bronchi and bronchioles) and not in the alveolar epithelium [11]. Confirmation of IKK- β deletion in airway epithelium in *CC10-Cre^{tg}/Ikk $\beta^{\Delta/\Delta}$* was demonstrated using TaqMan PCR of laser capture dissected airway epithelium [3], as well as in immunofluorescence microscopy studies in which impaired translocation of the NF- κ B component RelA, from the cytoplasm to the nucleus in airway epithelial cells but not non-airway epithelial cells was demonstrated in *CC10-Cre^{tg}/Ikk $\beta^{\Delta/\Delta}$* mice [3].

2.2 Acute tobacco smoke exposure

CC10-Cre^{tg}/Ikk $\beta^{\Delta/\Delta}$ and littermate IKK- β -expressing WT control mice (n= 10 mice/group) were used at 8–10 wk of age. Mice were exposed twice a day for 7 days, to either room air or tobacco smoke derived from five non-filtered standard research cigarettes (2R4F, Tobacco Research Institute, University of Kentucky, Lexington, KY) using a tobacco smoking machine (McChesney-Jaeger CSM-SSM Single Cigarette Machine, CH Technologies USA Inc, Westwood, NJ) regulated by programmable controls provided with JASPER Windows 9x/2000 software over RS-232 communication ports (CH Technologies USA Inc, Westwood, NJ) as previously described in this laboratory [8]. Each smoldering cigarette is puffed for approximately 2s, once every 25 seconds, for a total of twelve puffs/cigarette, at a flow rate of 5 l/min. The mice were exposed to the main stream tobacco smoke (100%) in a 12 port nose-only directed flow inhalation exposure system (Jaeger-NYU 12 port). We have previously reported that the concentration of total suspended particulates (TSP) in the nose port assessed using a gravimetric method is $173 \pm 5.3 \text{ mg/m}^3$ which is similar to the range of 100–250 mg TSP/m³ which other investigators report in mouse models of main stream tobacco smoke exposure [8]. Mice were killed 24 hours after the last tobacco smoke exposure, and BAL fluid and lungs were analyzed. All animal experimental protocols were approved by the University of California, San Diego, Animal Subjects Committee.

2.3 Quantitation of BAL cells (total cells, neutrophils and macrophages)

Bronchoalveolar lavage (BAL) fluid was collected by lavaging the lung with 1 mL PBS via a tracheal catheter [8]. The total number of BAL cells, as well as the total number of BAL neutrophils and macrophages, were counted in Wright-Giemsa stained cytopspins as described previously in this laboratory [8].

2.4 Quantitation of lung neutrophils and macrophages in different lung compartments (peribronchial and alveolar)

Lungs from the different experimental groups were processed as a batch for histologic immunostaining under identical conditions. Immunostained slides were all quantified under identical light microscope conditions, including magnification (20 $\times$), gain, camera position, and background illumination. Lung sections were immunostained with primary Abs directed against either F4/80 (rat anti-mouse F4/80 antigen; Accurate Chemical & Scientific Corporation, Westbury, NY), or myeloperoxidase (rabbit anti-mouse MPO Ab-1; Thermo

Fisher Scientific, Fremont, CA) using the immunoperoxidase method as previously described in this laboratory [8]. The number of F4/80+ macrophages or MPO+ neutrophils was counted in both the peribronchial space (in bronchioles of 150–200 μm of internal diameter) as well as in the alveolar space. The peribronchial space, or the alveolar area, were outlined and the number of F4/80+ and MPO+ cells in each of these spaces quantitated using image analysis (Image-Pro Plus; Media Cybernetics, Silver Springs, Md). Cell counts were quantitated in at least ten bronchioles (for the peribronchial space), and ten random fields (for the alveolar space) in each slide. The total number of F4/80+ or MPO+ cells within and surrounding alveolar structures in this 20x field (area $\sim 0.18 \text{ mm}^2$) were counted. Results are expressed as the number of F4/80+ or MPO+ cells/ mm^2 of the peribronchial or alveolar space.

2.5 ELISA for NF- κB regulated chemokines expressed by airway epithelium (KC, MCP-1, MIP-1 α)

Levels of NF- κB regulated chemokines (i.e. KC, MCP-1, MIP-1 α) known to be expressed by airway epithelium [12] and to mediate neutrophil or mononuclear cell recruitment [2] were measured in lung tissue supernatants by ELISA according to the manufacturer's instructions (R&D System Inc, Minneapolis, Minn). To obtain lung tissue supernatants, lung tissue was homogenized in lysis buffer and lung supernatants (obtained by centrifugation $10,000 \times g$ for 20 min) were passed through a 0.8 μm -pore size filter, and frozen at -80°C in polypropylene tubes until used in assays as previously described in this laboratory [13]. The KC assay has a sensitivity of 15.6 pg/ml, the MCP-1 assay has a sensitivity of 3.9 pg/ml, and the MIP-1 α assay has a sensitivity of 7.5 pg/ml. Lung protein levels were quantitated using a BCA protein assay (Pierce, Rockford, IL)[13]. Results are expressed as either pg KC, pg MCP-1, or pg MIP-1 α / μg lung protein.

2.6 Immunohistochemistry to detect KC and MCP-1 expression in airway epithelium

Lungs in the different groups of mice were equivalently inflated with an intratracheal injection of a similar volume of 4% paraformaldehyde solution (Sigma Chemicals, St. Louis, MO) to preserve the pulmonary architecture. Lung sections were processed for KC or MCP-1 immunohistochemistry, using either an anti-mouse KC Ab (R & D Systems, Minneapolis, MN), or an anti-MCP-1 Ab (Abcam, Cambridge, MA). The area of airway epithelial staining for KC or MCP-1 was outlined and quantified under a light microscope (Leica DMLS, Leica Microsystems) attached to an image analysis system (Image-Pro plus, Media Cybernetics) as previously described [8]. Results are expressed as the area of KC or MCP-1 immunostaining per μm length of basement membrane of bronchioles 150–200 μm of internal diameter.

2.7 Statistical Analysis

Results in the different groups of mice were compared by ANOVA using the non-parametric Kruskal-Wallis test followed by post-testing using Dunn's multiple comparison of means. All results are presented as mean $\pm$ SEM. A statistical software package (Graph Pad Prism, San Diego, CA) was used for the analysis. P values of < 0.05 were considered statistically significant.

3. RESULTS

3.1 Tobacco exposed CC10-Cre^{tg}/Ikk $\beta^{\Delta\Delta}$ mice have reduced levels of BAL cells (total, neutrophils, and macrophages) compared to WT mice

Exposure of WT mice to tobacco smoke induced a significant increase in the total number of BAL cells ($p=0.0001$)(Fig 1A), BAL neutrophils ($p=0.001$)(Fig 1B), and BAL macrophages

($p=0.01$)(WT tobacco smoke vs WT no tobacco smoke)(Fig 1C). In comparison to tobacco exposed WT mice, *CC10-Cre^{tg}/Ikk β ^{Δ/Δ}* mice exposed to tobacco smoke had significantly reduced levels of total BAL cells (3.0 ± 0.4 vs 10.4 ± 1.4 total BAL cells $\times 10^4$) ($p=0.0001$) (Fig 1A), BAL neutrophils (1.2 ± 0.2 vs 7.2 ± 1.1 total BAL neutrophils $\times 10^4$) ($p=0.0001$) (Fig 1B), and BAL macrophages (1.4 ± 0.2 vs 2.8 ± 0.5 total BAL macrophages $\times 10^4$) ($p=0.01$)(Fig 1C).

3.2 Tobacco exposed *CC10-Cre^{tg}/Ikk β ^{Δ/Δ}* mice have reduced levels of neutrophils in separate lung compartments (peribronchial and alveolar) compared to WT mice

Exposure of WT mice to tobacco smoke induced a significant increase in the total number of cells immunostaining positive for MPO in the peribronchial region (9.3 ± 3.3 vs 270.8 ± 37.5 total peribronchial neutrophils/mm²)(WT mice no tobacco smoke vs WT mice tobacco smoke)($p=0.0001$)(Fig 2A), as well as a significant increase in the total number of cells immunostaining positive for MPO in the alveolar region (4.5 ± 1.5 vs 82.5 ± 14.3 total alveolar neutrophils/mm²)($p=0.0001$)(Fig 2B). In comparison to tobacco exposed WT mice, *CC10-Cre^{tg}/Ikk β ^{Δ/Δ}* mice exposed to tobacco smoke had significantly reduced levels of neutrophils in the peribronchial space (36.6 ± 8.6 vs 270.8 ± 37.5 peribronchial neutrophils/mm²)($p=0.0001$)(Fig 2A), as well as a significant reduction in neutrophils in the alveolar space (26.9 ± 6.3 vs 82.6 ± 14.3 total neutrophils/mm² alveolar space)($p=0.01$)(Fig 2B).

3.3 Tobacco exposed *CC10-Cre^{tg}/Ikk β ^{Δ/Δ}* mice have reduced levels of macrophages in separate lung compartments (peribronchial and alveolar) compared to WT mice

Exposure of WT mice to tobacco smoke induced a significant increase in the total number of peribronchial cells immunostaining positive for F4/80 in the peribronchial region (34.5 ± 8.9 vs 160.6 ± 24.7 total peribronchial macrophages/mm²)(WT mice no tobacco smoke vs WT mice tobacco smoke)($p=0.0005$)(Fig 2C), as well as a significant increase in the total number of cells immunostaining positive for F4/80 in the alveolar region (24.3 ± 2.2 vs 69.3 ± 6.9 total alveolar macrophages/mm²)($p=0.0001$)(Fig 2D). In comparison to tobacco exposed WT mice, *CC10-Cre^{tg}/Ikk β ^{Δ/Δ}* mice exposed to tobacco smoke had significantly reduced levels of total peribronchial macrophages (53.8 ± 6.3 vs 160.6 ± 24.7 total peribronchial macrophages/mm²)($p=0.002$)(Fig 2C), and alveolar macrophages (25.3 ± 2.3 vs 69.4 ± 6.9 total alveolar macrophages/mm²)($p=0.001$)(Fig 2D).

3.4 Tobacco exposed *CC10-Cre^{tg}/Ikk β ^{Δ/Δ}* mice have reduced lung levels of NF-kB regulated chemokines (KC) that attract neutrophils

The level of KC expression in the lungs of WT mice exposed to tobacco smoke was significantly increased compared with non-tobacco exposed WT mice (0.49 ± 0.05 vs. 8.57 ± 1.55 pg KC/ μ g lung protein; $p=0.0001$; Fig 3A). In comparison to tobacco exposed WT mice, *CC10-Cre^{tg}/Ikk β ^{Δ/Δ}* mice exposed to tobacco smoke had significantly reduced levels of KC (3.67 ± 0.63 vs 8.57 ± 1.55 pg KC/ μ g lung protein)($p=0.01$)(Fig 3A).

3.5 Tobacco exposed *CC10-Cre^{tg}/Ikk β ^{Δ/Δ}* mice have reduced lung levels of NF-kB regulated chemokines (MCP-1) that attract mononuclear cells

The level of MCP-1 expression in the lungs of WT mice exposed to tobacco smoke was significantly increased compared with non-tobacco exposed WT mice (0.50 ± 0.06 vs. 2.54 ± 0.34 pg MCP-1/ μ g lung protein; $p=0.0001$; Fig 3B). In comparison to tobacco exposed WT mice, *CC10-Cre^{tg}/Ikk β ^{Δ/Δ}* mice exposed to tobacco smoke had significantly reduced levels of MCP-1 (1.75 ± 0.19 vs 2.54 ± 0.34 pg MCP-1/ μ g lung protein)($p=0.04$)(Fig 3B).

Levels of MIP-1 α were also significantly increased in WT mice exposed to tobacco smoke ($p=0.0001$; vs non-tobacco smoke exposed WT mice) (Fig 3C). Although there was a trend

for *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice exposed to tobacco smoke to have lower levels of MIP-1α, this was not statistically significant ($p = \text{NS}$; vs tobacco smoke exposed WT mice) (Fig 3C).

3.6 Levels of KC and MCP-1 immunostaining in airway epithelium and alveolar epithelium in tobacco exposed *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice and WT mice

Immunohistochemistry studies demonstrated that tobacco smoke exposure induced significantly increased expression of both KC (Fig 4) and MCP-1 (Fig 5) in WT mice in both airway and alveolar epithelium. In contrast, *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice exposed to tobacco smoke had reduced levels of KC (Fig 4) and MCP-1 (Fig 5) immunostaining of airway epithelium, but significant expression of KC (Fig 4) and MCP-1 (Fig 5) in alveolar epithelium.

Image analysis quantitation of the area of KC immunoreactivity in airway epithelial cells demonstrated that WT mice exposed to tobacco smoke had a significant increase in the area of KC immunostaining compared to WT mice not exposed to tobacco smoke (1.16 ± 0.11 vs $0.25 \pm 0.05 \mu\text{m}^2/\mu\text{m}$ area of KC immunoreactivity in airway epithelium) ($p < 0.0001$) (Fig 6A). In contrast, *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice exposed to tobacco smoke had significantly reduced levels of airway epithelial KC immunoreactivity compared to WT mice exposed to tobacco smoke (0.52 ± 0.06 vs $1.16 \pm 0.11 \mu\text{m}^2/\mu\text{m}$ area of KC immunoreactivity in airway epithelium) ($p < 0.0001$) (Fig 6A).

Image analysis quantitation of the area of MCP-1 immunoreactivity in airway epithelial cells (Fig 6B) demonstrated similar results to that observed with studies of KC. The area of MCP-1 immunoreactivity in airway epithelial cells in WT mice exposed to tobacco smoke was significantly higher compared to WT mice not exposed to tobacco smoke ($p < 0.0001$) (Fig 6B). In contrast, *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice exposed to tobacco smoke had significantly reduced levels of airway epithelial MCP-1 immunoreactivity compared to WT mice exposed to tobacco smoke ($p < 0.0001$) (Fig 6B).

4. DISCUSSION

In this study we have demonstrated that NF-κB dependent genes expressed in airway epithelium are important to the recruitment of both neutrophils and mononuclear cells into the lung in mice exposed to tobacco smoke. Interestingly, inactivation of NF-κB in the airway epithelium not only significantly reduced levels of neutrophils and mononuclear cells in the peribronchial space, but also significantly reduced levels of neutrophils and mononuclear cells in the alveolar space. As *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice have NF-κB inactivated only in airway epithelium, but not in alveolar epithelium, these results suggest that NF-κB dependent genes in the airway epithelium also play an important role in modulating levels of neutrophils and mononuclear cells in the alveolar space and not only the peribronchial space following exposure of mice to acute tobacco smoke. As baseline levels of total cells, neutrophils and mononuclear cells were similar in WT and *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice in both BAL, as well as the peribronchial and alveolar space, these studies suggest that inactivation of NF-κB in airway epithelium does not influence baseline trafficking of these cell types into the airway or alveolus.

The mechanism by which inactivation of NF-κB in airway epithelium reduces neutrophil and mononuclear cell levels following exposure to tobacco smoke may involve one or more NF-κB regulated genes expressed in airway epithelium which include chemokines, cytokines, adhesion molecules, and/or other NF-κB regulated genes [4]. Our study is unable to definitively address which NF-κB regulated gene(s) in airway epithelium are responsible for the reduced number of lung neutrophils and macrophages in *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice exposed to tobacco smoke, but provides evidence for a significant association between

reduced levels of lung KC and reduced levels of lung neutrophils [14]. KC (also known as keratinocyte chemoattractant or CXCL1) is a potent neutrophil chemottractant binding preferentially to CXCR2, rather than CXCR1, expressed by neutrophils. KC in the mouse, and IL-8 in humans, are both potent CXC chemokines, although they are not structural homologues as mice do not have a structural homologue for human IL-8 [15]. In addition to the strong correlation between reduced levels of KC and reduced levels of neutrophils, we also noted a statistically significant reduction in levels of MCP-1, a mononuclear cell chemoattractant, in *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice exposed to tobacco smoke. MCP-1 is a CC chemokine that attracts monocytes in vitro and in vivo [16]. Although studies in MCP-1 deficient mice demonstrate that MCP-1 is solely responsible for mononuclear cell recruitment in several inflammatory cell settings [17,18], the reduced mononuclear cell recruitment to the lung in *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice exposed to tobacco smoke is likely to be partially, but not completely, mediated by the reduced levels of MCP-1 noted in *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice. In contrast, to the significant reductions in levels of two NF-κB regulated genes expressed in airway epithelium (i.e. KC and MCP-1) noted in *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice exposed to tobacco smoke, we did not note a significant reduction in levels of another NF-κB regulated genes MIP-1α. MIP-1α was induced at similar levels in WT mice and *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice exposed to tobacco smoke. This suggests that cell types other than airway epithelium continued to express significant levels of MIP-1α when NF-κB was inactivated in airway epithelium. MIP-1α (also known as CCL3) is a CC chemokine expressed by multiple cell types in addition to airway epithelium including monocytes, macrophages, mast cells, fibroblasts, eosinophils, and neutrophils [19]. For NF-κB regulated genes which are not reduced in *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice (e.g. MIP-1α), the activity of other transcription factors might prevail over that of NF-κB in regulating their expression.

In summary, in this study we have demonstrated an important role for airway epithelial genes regulated by NF-κB in contributing to neutrophil and mononuclear cell inflammation not only in the airway but also in the alveolar space of mice exposed to tobacco smoke. In addition, we have demonstrated that chemokines regulated by NF-κB important to neutrophil recruitment (i.e. KC), and to mononuclear cell recruitment (i.e. MCP-1) are reduced in *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice exposed to tobacco smoke. Airway epithelial NF-κB regulated genes are thus important to airway inflammation induced by tobacco smoke as well as to inflammation induced by alternate stimuli including allergen induced eosinophil inflammation and remodeling [3,20,21], LPS induced lung inflammation [12,22], and nitrogen dioxide induced lung injury [23]. Overall, these studies suggest that activation of NF-κB regulated genes in airway epithelial cells plays an important role in mediating airway inflammation from a diverse array of epithelial stimuli. As NF-κB is activated in human subjects with tobacco smoke induced COPD [24] who have increased levels of lung neutrophils, mononuclear cells and chemokines such as IL-8 and MCP-1 [24], developing an inhaled small molecule inhibitor of NF-κB [25] to target airway epithelium may allow for reduced levels of airway inflammation with potential for reduced side effects associated with systemic therapy targeting NF-κB.

Acknowledgments

This study was supported by NIH grants AI 38425, AI 70535, and AI 72115 (DHB)

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Figure 1. Levels of BAL total cells, neutrophils, and alveolar macrophages in *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice and WT mice exposed to tobacco smoke

Different groups of *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice or WT mice were exposed to room air or tobacco smoke for 7 days (n=10 mice/group). The number of total BAL cells (Fig 1A), total BAL neutrophils (Fig 1B), and total BAL macrophages (Fig 1C) were quantitated in Wright-Giemsa stained BAL cytopins. In WT mice tobacco smoke exposure increased levels of total BAL cells (*p=0.0001)(Fig 1A), total BAL neutrophils (*p=0.0001)(Fig 1B), and total BAL alveolar macrophages (*p=0.002)(Fig 1C) compared to non-tobacco exposed WT mice. *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice exposed to tobacco smoke had significantly reduced levels of total BAL cells (# p=0.0001)(Fig 1A), total BAL neutrophils (# p=0.0001)(Fig 1B), and total BAL alveolar macrophages (# p=0.01)(Fig 1C) compared to tobacco exposed WT mice.

Figure 2. Levels of MPO+ neutrophils and F4/80+ macrophages in peribronchial and alveolar spaces in *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice and WT mice exposed to tobacco smoke

Different groups of *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice or WT mice were exposed to room air or tobacco smoke for 7 days (n=10 mice/group). Lung sections were processed for immunohistology using either an anti-MPO or F4/80 Ab and the number of MPO+ or F4/80 positive cells in the peribronchial region, or the alveolar space, quantitated using image analysis. Results are expressed as the number of MPO+ neutrophils or F4/80+ macrophages per mm². In WT mice, tobacco smoke exposure increased levels of MPO+ cells in the peribronchial region (*p=0.0001)(Fig 2A), as well as the number of MPO+ cells in the alveolar space (*p=0.0001)(Fig 2B) compared to non-tobacco exposed WT mice. Similarly, in WT mice, tobacco smoke exposure increased levels of F4/80+ cells in the peribronchial region (*p=0.0005)(Fig 2C), as well as the number of F4/80+ cells in the alveolar space (*p=0.0001)(Fig 2D) compared to non-tobacco exposed WT mice. *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice exposed to tobacco smoke had significantly reduced levels of MPO+ cells (# p=0.0001)(Fig 2A) and F4/80+ cells (# p=0.002)(Fig 2C) in the peribronchial region, as well as significantly reduced numbers of MPO+ cells (# p=0.0005)(Fig 2B) and F4/80+ cells (# p=0.0001)(Fig 2D) in the alveolar space compared to tobacco exposed WT mice.

Figure 3. Levels of lung KC, MCP-1, and MIP-1α in *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice and WT mice exposed to tobacco smoke

Levels of KC, MCP-1, and MIP-1α were measured by ELISA in lung tissue derived from *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice and WT mice exposed to room air or tobacco smoke (n=10 mice/group). Results are expressed as pg KC, pg MCP-1 or pg MIP-1α per μg lung protein. WT mice exposed to tobacco smoke had a significant increase in levels of lung KC (*p=0.0001) (Fig 3A), lung MCP-1 (*p=0.0001) (Fig 3B), and MIP-1α (*p=0.0001) (Fig 3C) (WT tobacco smoke vs WT no tobacco smoke). Levels of lung KC (#p=0.01) (Fig 3A) and MCP-1 (#p=0.04) (Fig 3B) were significantly reduced in *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice exposed to tobacco smoke vs WT mice exposed to tobacco smoke. There was a statistically non-significant trend for reduced MIP-1α in *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice exposed to tobacco smoke (p= NS; vs WT tobacco smoke) (Fig 3C).

Figure 4. Immunostaining of lung sections with an anti-KC Ab

Lungs from WT mice (Fig 4A–D) or *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice (Fig 4E–H) were immunostained with an anti-KC Ab using the immunoperoxidase method. Lung sections from non-tobacco exposed WT mice (Fig 4A and 4B) and non-tobacco exposed *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice (Fig 4E and 4F) have minimal KC staining of bronchial epithelium or alveolar epithelium. In contrast, in tobacco exposed WT mice both bronchial epithelium (Fig 4C) and alveolar epithelium (Fig 4D) demonstrate significant KC immunostaining. In *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice, tobacco smoke induced KC expression in alveolar epithelium (Fig 4H), but minimal induction of KC expression in bronchial epithelium (Fig 4G).

Figure 5. Immunostaining of lung sections with an anti-MCP-1 Ab

Lungs from WT mice (Fig 5A–D) or *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice (Fig 5E–H) were immunostained with an anti-MCP-1 Ab using the immunoperoxidase method. Lung sections from non-tobacco exposed WT mice (Fig 5A and 5B) and non-tobacco exposed *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice (Fig 5E and 5F) have minimal MCP-1 staining of bronchial epithelium or alveolar epithelium. In contrast, in tobacco exposed WT mice both bronchial epithelium (Fig 5C) and alveolar epithelium (Fig 5D) demonstrate significant MCP-1 immunostaining. In *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice, tobacco smoke induced MCP-1 expression in alveolar epithelium (Fig 5H), but minimal induction of MCP-1 expression in bronchial epithelium (Fig 5G).

Figure 6. Image analysis quantitation of area of airway epithelial KC or MCP-1 immunostaining
 The area of airway epithelial immunostaining was quantitated by image analysis in lung sections immunostained with either an anti-KC Ab (Fig 6A) or an anti-MCP-1 Ab (Fig 6B) in WT mice and *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* exposed to either no tobacco smoke or tobacco smoke. In WT mice, tobacco smoke exposure significantly increased levels of KC (* $p < 0.0001$ vs no tobacco smoke WT)(Fig 6A) and MCP-1 (* $p < 0.0001$ vs no tobacco smoke WT)(Fig 6B) expression in bronchial epithelium. In contrast tobacco smoke exposure of *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}* mice induced significantly less expression of KC (# $p < 0.0001$ vs tobacco smoke *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}*)(Fig 6A) and MCP-1 (# $p < 0.0001$ vs tobacco smoke *CC10-Cre^{tg}/Ikkβ^{Δ/Δ}*)(Fig 6B) expression in bronchial epithelium.