

# Tobacco Smoke Promotes Lung Tumorigenesis by Triggering IKK $\beta$ - and JNK1-Dependent Inflammation

Hiroyuki Takahashi,<sup>1,3</sup> Hisanobu Ogata,<sup>1</sup> Reiko Nishigaki,<sup>1</sup> David H. Broide,<sup>2</sup> and Michael Karin<sup>1,\*</sup>

<sup>1</sup>Laboratory of Gene Regulation and Signal Transduction, Department of Pharmacology

<sup>2</sup>Department of Medicine

University of California, San Diego, School of Medicine, La Jolla, CA, 92093-0723, USA

<sup>3</sup>Present address: Department of Respiratory Medicine, The University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan

\*Correspondence: [karinoffice@ucsd.edu](mailto:karinoffice@ucsd.edu)

DOI 10.1016/j.ccr.2009.12.008

## SUMMARY

Chronic exposure to tobacco smoke, which contains over 60 tumor-initiating carcinogens, is the major risk factor for development of lung cancer, accounting for a large portion of cancer-related deaths worldwide. It is well established that tobacco smoke is a tumor initiator, but we asked whether it also acts as a tumor promoter once malignant initiation, such as caused by *K-ras* activation, has taken place. Here we demonstrate that repetitive exposure to tobacco smoke promotes tumor development both in carcinogen-treated mice and in transgenic mice undergoing sporadic *K-ras* activation in lung epithelial cells. Tumor promotion is due to induction of inflammation that results in enhanced pneumocyte proliferation and is abrogated by IKK $\beta$  ablation in myeloid cells or inactivation of JNK1.

## INTRODUCTION

Currently, lung cancer is the leading cause of cancer-related mortalities in men and women, and despite extensive anti-smoking campaigns it still accounts for 15% of all new cancers and 29% of all cancer deaths in the U.S. (Jemal et al., 2008). Among lung cancers, pulmonary adenocarcinoma is the predominant histological type (Jemal et al., 2008; Toh, 2009). Tobacco smoking is the major risk factor, estimated to cause 87% of lung cancer cases in the U.S. (Hecht, 2002). Tobacco smoke (TS) contains ~4000 chemical agents, including over 60 carcinogens: polycyclic aromatic hydrocarbons and N-nitrosamines, such as NNK [4-(methylnitrosamino)-1-(3-pyridyl)-butanone] and aromatic amines (Hecht, 2002). Conversion of these compounds to reactive forms (metabolic activation) results in formation of DNA adducts that cause many of the genetic changes underlying lung cancer. Among these changes, *K-ras*-activating mutations are early events in the pathway leading to

lung adenocarcinoma (Herbst et al., 2008). *K-ras* mutations occur in 30%–40% of lung adenocarcinomas, but are infrequent in other lung tumor types or in lung tumors from non-smokers (Berns, 2001). TS also induces pulmonary inflammation (Vlahos et al., 2006), which is believed to play a role in progressive lung destruction in chronic obstructive pulmonary disease (COPD) (Barnes, 2008). Although chronic inflammation was suggested to contribute to tumor initiation through the production of reactive oxygen and nitrogen species that contribute to DNA damage and induction of oncogenic mutations (Hussain et al., 2003), the major effect of inflammation on tumor induction in experimental animals is exerted at the level of tumor promotion (Karin and Greten, 2005). This tumor-promoting effect of inflammation, however, was so far mainly demonstrated in cancers that arise in the context of underlying infections or chronic idiopathic inflammation (Coussens and Werb, 2002; Karin et al., 2006) and its role in tumorigenesis induced by TS or other environmental irritants has not been critically evaluated. Although COPD is known to

## SIGNIFICANCE

Tobacco smoking accounts for the majority of lung cancer-related deaths. Despite identification of numerous tumor-initiating carcinogens, it was not established whether tobacco smoke is also a tumor promoter for initiated lung epithelial cells. We now describe two murine models in which tobacco smoke exposure has a well-defined tumor-promoting effect on both chemically and genetically initiated lung cancers. Induction of low-grade inflammation is likely to be an important contributor to the tumor-promoting activity of tobacco smoke, as it depends on IKK $\beta$  activity in myeloid cells. These results provide new models and mechanistic insights for understanding tumor induction by a major human carcinogen and demonstrate that the tumorigenic activity of an environmental irritant depends on inflammation.

increase lung cancer risk (Tockman et al., 1987), *K-ras* activation in bronchial epithelial cells can cause an inflammatory response (Ji et al., 2006), and autocrine production of the chemokine CXCL-8 (IL-8) stimulates the growth of *K-ras*-transformed lung adenocarcinoma cells (Karin, 2005; Sparmann and Bar-Sagi, 2004), the role of inflammation in smoking-induced lung tumorigenesis remains to be investigated using appropriate in vivo models. However, in none of the mouse models where TS or compounds derived from it can induce lung cancer was TS shown to act on a different step in the tumorigenic process other than initiation (i.e., induction of oncogenic mutations).

Indeed, previous attempts to ascribe tumor-promoting activity to TS in mice have failed (Witschi, 2005). In fact, mice that were first treated with a carcinogen and subsequently exposed to TS for 5 months showed a significant inhibition of tumor development rather than enhancement (Witschi et al., 1997). After a 4 month recovery phase, TS-exposed mice exhibited the same tumor multiplicity as mice treated with carcinogen alone (Witschi et al., 1997). In NNK-treated mice, TS exposure also failed to increase lung tumor multiplicity (Finch et al., 1996). It has been recognized that in these murine models of TS exposure lung tumorigenesis is influenced by smoke-induced toxicity, manifested by weight loss, thereby necessitating the inclusion of a recovery period (Witschi, 2005). It was also noted that long-term exposure desensitizes mice to TS, as maximal induction of pulmonary inflammation and cell proliferation were observed 1 to 3 weeks after initiation of TS exposure (Witschi et al., 1997). Furthermore, TS exposure can inhibit the metabolic activation of NNK and reduces the formation of NNK-induced O<sup>6</sup>-methylguanine DNA adducts (Brown et al., 1999), which strongly correlate with lung tumor yield in A/J mice (Peterson and Hecht, 1991). Taking all of these limitations into consideration, we have devised two murine models in which intermittent, yet prolonged, exposure to mainstream TS (MTS) can successfully promote lung tumor development. We have used these models to determine how the tumor-promoting effect of MTS exposure is accomplished.

## RESULTS

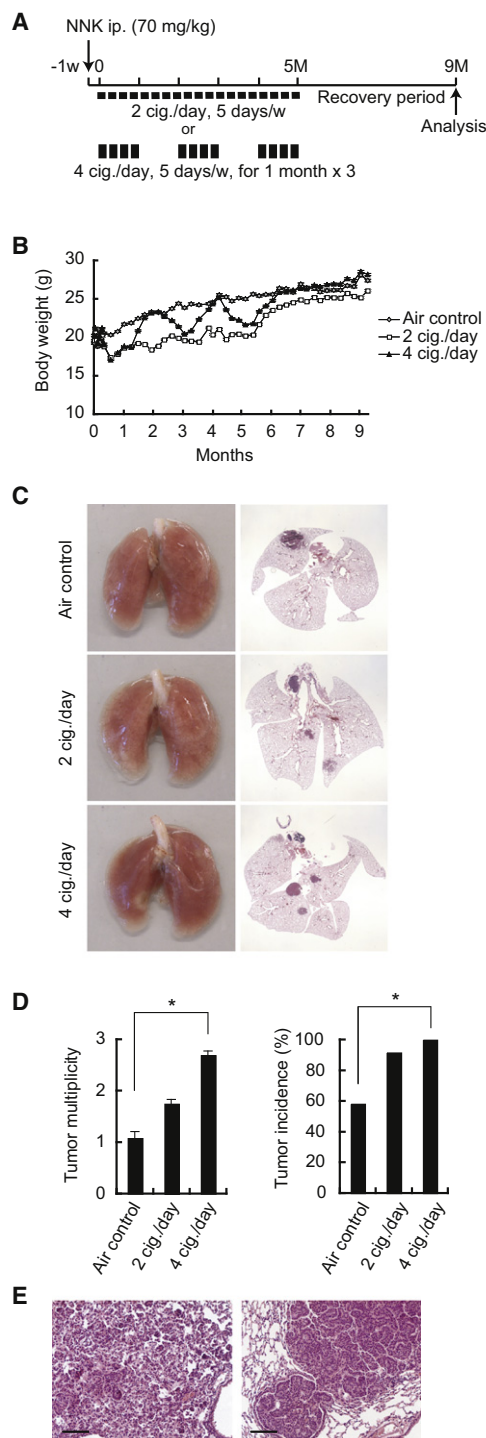
To better understand how exposure to TS, which contains many tumor initiators as well as irritants, promotes development of lung cancer, we sought to develop mouse models in which MTS exposure acts as a tumor promoter in addition to its established tumor initiator activity. In the first model, we used NNK as a tumor initiator and MTS as a tumor promoter. First, we optimized the initiating dose of NNK in A/J mice and found it to be 50–70 mg/kg (see Figure S1A, available online). Next, 1 week after intraperitoneal (i.p.) NNK injection into 7-week-old A/J mice, we exposed one group of mice to MTS generated by burning of 4 cigarettes/day, 5 days per week for 1 month, followed by a 1 month rest interval, and repeated this regimen three times. A second group was exposed to 2 cigarettes/day, 5 days per week for 5 months, whereas the third group received filtered air for the same duration, as a control (Figure 1A). All mice were given a final recovery period of 4 months. Whereas MTS-exposed mice failed to gain weight during the exposure period, mice exposed to 4 cigarettes/day for 1 month rapidly resumed weight gain and caught up with air-exposed mice during the

1 month rest interval (Figure 1B). Yet, mice that were continuously exposed to 2 cigarettes/day showed a sustained reduction in body weight.

We analyzed lung tumor multiplicity and incidence by microscopic examination after serial sectioning of lungs at 350  $\mu$ m intervals as described previously (Curtin et al., 2004). Mice administered 70 mg/kg NNK and exposed to 4 cigarettes/day exhibited significantly increased NNK-initiated lung tumor multiplicity, as well as increased tumor incidence (Figures 1C and 1D). Exposure to 2 cigarettes/day MTS also increased tumor multiplicity and the effect was most pronounced when combined with the highest NNK dose that was tested—70 mg/kg (Figure S1A). Alveolar adenoma was the most common histological type in both air- and MTS-exposed mice, seen in about 70% of each cohort. About 30% of the tumors in both air- and MTS-exposed mice showed features of well-differentiated human papillary adenocarcinoma, including nuclear enlargement, prominent nucleoli, increased mitotic rate, and bronchial invasion (Figure 1E and Figure S1B).

Because the most common mutation found in murine lung tumors induced by NNK is a GGT  $\rightarrow$  GAT transition in codon 12 of the *K-ras* gene (Hecht, 1998), we developed a second model in which we investigated whether intermittent MTS exposure could promote *K-ras*-driven lung tumorigenesis using *K-ras*<sup>LA2/+</sup> (*K-ras*<sup>LA2</sup>) mice, which develop lung cancer in response to sporadic activation of an oncogenic *K-ras*<sup>asp12</sup> allele through a spontaneous homologous recombination event (Johnson et al., 2001). Six- to eight-week-old, sex-matched *K-ras*<sup>LA2</sup> mice were exposed to MTS at 4 cigarettes/day for 2–3 weeks followed by 2 week rest intervals on air alone, three times, and lung tumors were analyzed at 5 months of age (Figure 2A). In contrast to A/J mice, weight reduction in *K-ras*<sup>LA2</sup> mice, which are in the C57BL6 background, was more modest and occurred only during the initial two cycles of MTS exposure (Figure 2B). As found in NNK-initiated mice, MTS exposure increased tumor multiplicity and maximal tumor sizes (Figures 2C and 2D). No tumors were found in MTS-exposed wild-type (WT) mice (Figure 2C), indicating that increased tumor number in MTS-exposed *K-ras*<sup>LA2</sup> mice is not due to MTS-induced tumor initiation. Similar effects were observed in male and female mice (Figures S2A and S2B). *K-ras*<sup>LA2</sup> mice also develop thymic lymphoma due to *K-ras*<sup>asp12</sup> activation in thymocytes (Johnson et al., 2001). No statistically significant differences in the incidence of thymic lymphoma were detected between air- and MTS-exposed *K-ras*<sup>LA2</sup> mice (Figure S2C), suggesting that the tumor-promoting effect of MTS is limited to the primary site of exposure—the lung. Most pulmonary tumors in air- or MTS-exposed *K-ras*<sup>LA2</sup> mice were alveolar adenomas (Figure 2E and Figure S2D), corresponding to a relatively early stage of lung cancer development, as reported previously (Johnson et al., 2001). Interestingly, tumors in MTS-exposed mice were more vascularized than those in air-exposed controls (Figures S2E and S2F). Thus, MTS exposure promotes development of both chemically and genetically induced lung cancer and this constitutes strong evidence that TS acts as a tumor promoter and not only as an initiator.

We investigated whether the tumor-promoting effect of MTS exposure is due to induction of inflammation. We examined the subacute inflammatory pulmonary response of C57BL6 male



**Figure 1. Tobacco Smoke Exposure Promotes Development of Chemically Induced Lung Cancer**

(A) Experimental protocols for promotion of NNK-induced lung cancer. Seven-week-old male A/J mice were MTS exposed using two different regimens starting 1 week after NNK injection. Tumor development was analyzed 9 months later.

(B) Effect of MTS exposure on weight gain in A/J mice. Results are means  $\pm$  SEM (air control:  $n = 12$ ; 2 cigarettes/day:  $n = 12$ ; 4 cigarettes/day:  $n = 13$ ).

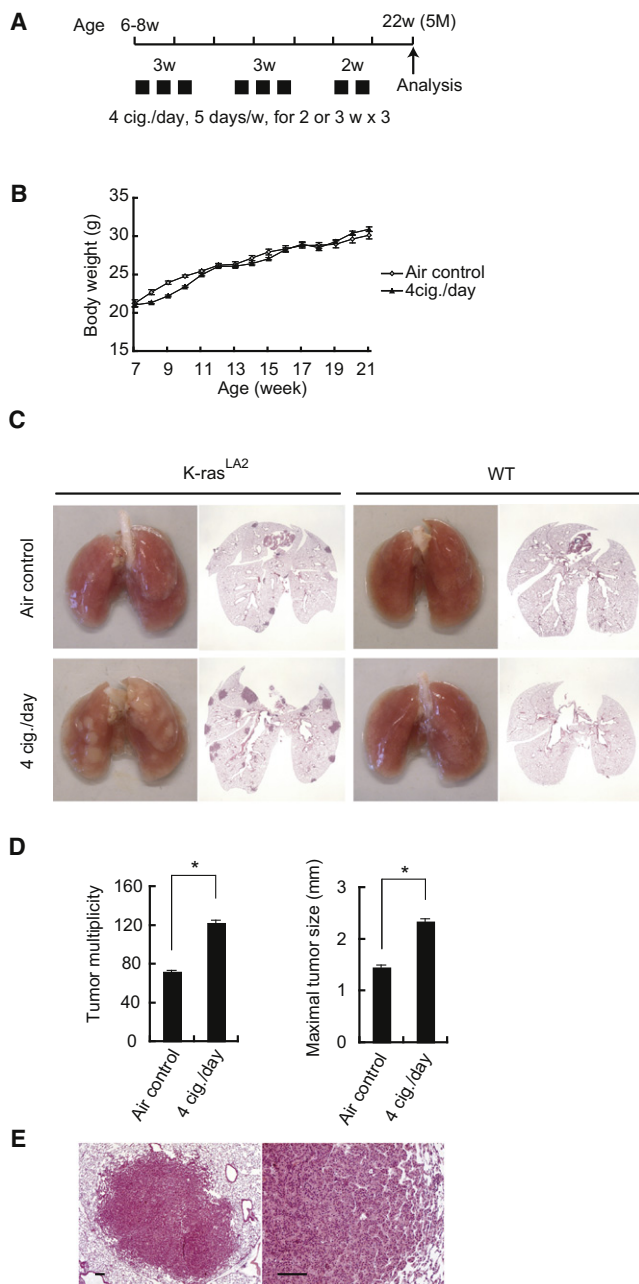
(C) Lung appearance (left) and histology (H&E stain; right) in A/J mice 9 months after initiation of the NNK + MTS protocol.

mice exposed to MTS or air. Total cell number and numbers of various leukocyte types in bronchoalveolar lavage fluid (BALF) were significantly increased 1 and 2 weeks after MTS exposure (Figure 3A). MTS exposure induced the expression of various inflammatory cytokine and chemokine mRNAs, such as *Il1 $\beta$* , *Il6*, *Tnf $\alpha$* , *Ccl3* (*Mip1 $\alpha$* ), and *Ccl2* (*Mcp1*) in the lung (Figure 3B). Induction of most of these transcripts was maximal within 2 weeks of exposure initiation and declined at later time points (Figure S3A). MTS exposure also induced secretion of TNF- $\alpha$  and IL-6 by lung cells (Figure 3C) and stimulated mitogen-activated protein kinases, including extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) (Figure 3D). Similar induction of inflammatory response genes was seen after MTS exposure of *K-ras*<sup>LA2</sup> tumor-bearing mice (Figure S3B). MTS exposure modestly increased NF- $\kappa$ B DNA binding activity (Figure 3D) and increased cell proliferation in the lung (Figure 3E), without affecting basal rate of apoptosis (Figure S3C). Proliferating cells in the alveolar zone of MTS-exposed lungs were mainly alveolar epithelial cells and immune cells (Figure S3D). As shown below, MTS exposure also increased cell proliferation within lung adenomas. Both IL-6- and TNF- $\alpha$ -deficient mice showed decreased MTS-enhanced lung cell proliferation (Figure S3E), indicating the importance of these inflammatory cytokines in MTS-induced cell proliferation. Lung tumors in MTS-exposed *K-ras*<sup>LA2</sup> mice exhibited enhanced infiltration with IL-6-producing macrophages (Figure 3F and Figure S3F).

I $\kappa$ B kinase  $\beta$  (IKK $\beta$ ), required for NF- $\kappa$ B activation, links chronic inflammation with carcinogenesis in a mouse model of colitis-associated cancer (Greten et al., 2004) and is a critical regulator of inflammatory cytokine production (Karin, 2006). To examine the contribution of IKK $\beta$  to MTS-induced subacute pulmonary inflammation, we used *Ikk $\beta$*  <sup>$\Delta$ mye</sup> (*LysM-Cre*; *Ikk $\beta$* <sup>F/F</sup>) mice that specifically lack IKK $\beta$  in the myeloid lineage (Greten et al., 2004) because myeloid cells, including alveolar macrophages, are a major source of inflammatory cytokines needed for initiation and propagation of inflammatory responses (Barnes, 2008). The efficiency of IKK $\beta$  deletion in alveolar macrophages was  $\sim$ 80%–90% (Figure 4A) and previous experiments have shown that IKK $\beta$  is not deleted in any cell type other than mature macrophages and neutrophils (Greten et al., 2004). *Ikk $\beta$*  <sup>$\Delta$ mye</sup> mice showed impaired MTS-induced cell recruitment into BALF, which mostly affected neutrophils (Figure 4B), decreased induction of *Il6*, *Tnf $\alpha$* , *Ccl3*, and *Ccl2* mRNAs, as well as decreased secretion of IL-6 and TNF- $\alpha$  by MTS-exposed lungs, relative to MTS-exposed *Ikk $\beta$* <sup>F/F</sup> mice (Figures 4C and 4D). *Ikk $\beta$*  <sup>$\Delta$ mye</sup> mice also exhibited decreased MTS-induced cell proliferation (Figure 4E). We also deleted IKK $\beta$  in Clara cells that account for 70% of the airway epithelium using *CC10-Cre*; *Ikk $\beta$* <sup>F/F</sup> mice (Broide et al., 2005). Clara cell-specific deletion of IKK $\beta$  had only a modest and statistically insignificant effect on MTS-induced cytokine

(D) Lung tumor multiplicity and incidence were determined by serial sectioning at 350  $\mu$ m intervals. Incidence equals the number of tumor-bearing mice divided by the number of mice in each group. Results are means  $\pm$  SEM ( $n$  is as described in B). Significant difference, \* $p < 0.03$ .

(E) Histological appearance of adenoma (left) and adenocarcinoma with bronchial invasion (right) in A/J mice given NNK and 4 cigarettes/day MTS. The scale bar represents 100  $\mu$ m. See also Figure S1.



**Figure 2. Tobacco Smoke Exposure Promotes Development of Genetically Induced Lung Cancer**

(A) Outline of the experimental protocol in which *K-ras*<sup>LA2</sup> mice were exposed to MTS and analyzed at 5 months of age.

(B) Effect of MTS exposure on weight gain in *K-ras*<sup>LA2</sup> mice. Results are means  $\pm$  SEM (air control: *n* = 4; 4 cigarettes/day: *n* = 8).

(C) Lung appearance (left side) and histology (H&E stain; right side) in 5-month-old *K-ras*<sup>LA2</sup> or WT mice with or without MTS exposure.

(D) Lung tumor multiplicity and maximal tumor sizes were determined as above. Results are means  $\pm$  SEM (air control: *n* = 16; 4 cigarettes/day: *n* = 18). Significant difference, \**p* < 0.002.

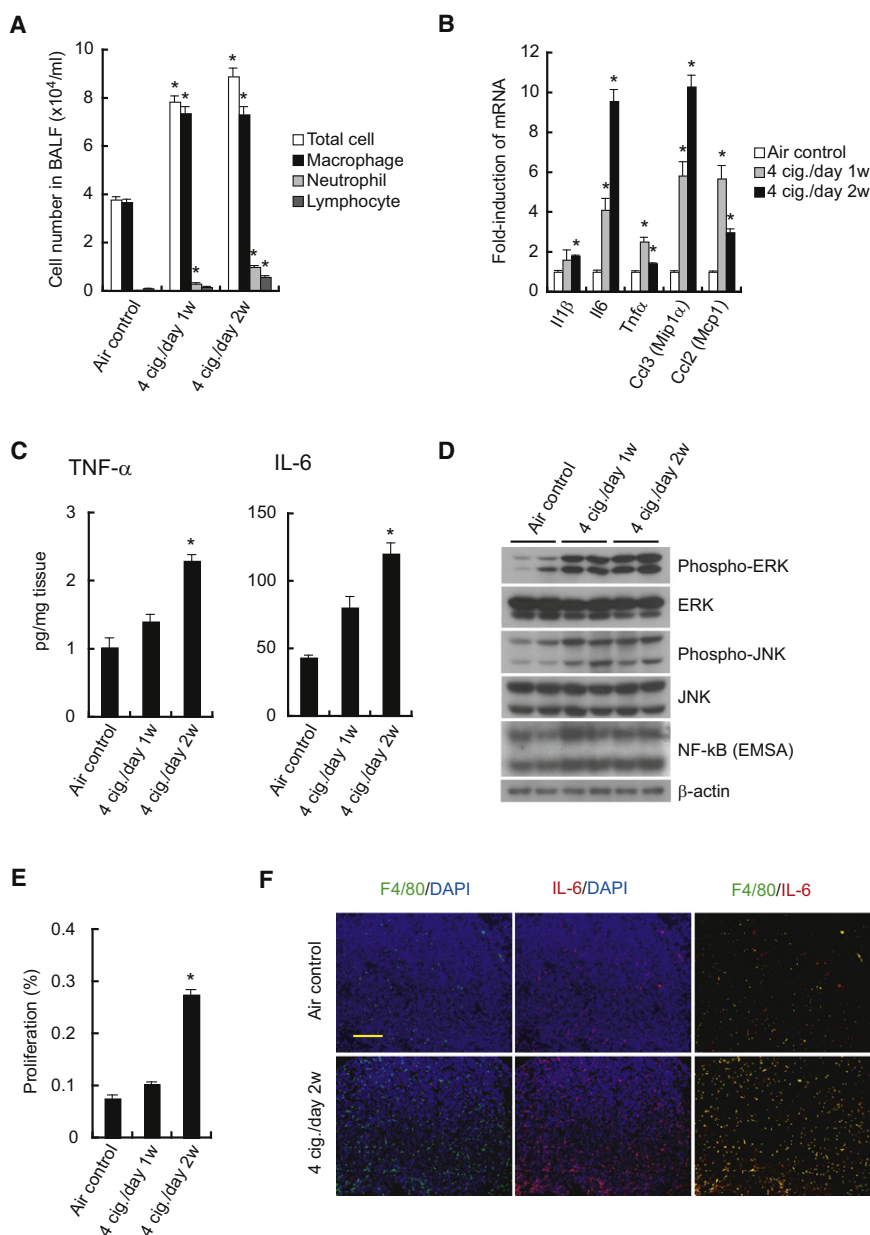
(E) Histological appearance of adenoma in MTS-exposed *K-ras*<sup>LA2</sup> mice. The scale bar represents 100 μm. See also Figure S2.

secretion and cell proliferation (Figures S4A and S4B). Its effect on MTS-induced tumor promotion was therefore not explored.

To investigate the role of myeloid cell IKK $\beta$  in MTS-induced lung tumor promotion, we crossed *K-ras*<sup>LA2</sup> mice with *Ikk $\beta$*  <sup>$\Delta$ mye</sup> or *Ikk $\beta$* <sup>F/F</sup> mice and exposed the hybrid strains to MTS as described above. Deletion of IKK $\beta$  in myeloid cells significantly reduced the effect of MTS exposure on lung tumor multiplicity and maximal tumor sizes (Figures 5A and 5B). IKK $\beta$  deletion in myeloid cells also reduced tumor angiogenesis, especially in MTS-exposed mice (Figures S2E and S2F). In agreement with the reduction in IL-6 production, we observed decreased MTS-induced STAT3 activation in *K-ras*<sup>LA2</sup>; *Ikk $\beta$*  <sup>$\Delta$ mye</sup> mice (Figures S5A and S5B). As indicated above, IL-6 and TNF- $\alpha$ , whose induction in MTS-exposed lungs requires IKK $\beta$  in myeloid cells in both tumor-free (Figure 4C) and in *K-ras*<sup>LA2</sup> tumor-bearing mice (Figure S3B), are essential for MTS-induced epithelial cell proliferation (Figure S3E). Consistent with these findings, IKK $\beta$  ablation in myeloid cells reduced MTS-induced proliferation of lung adenoma cells (Figure 5C). Myeloid cell IKK $\beta$  ablation, however, did not have a significant effect on apoptosis in lung adenomas, which was slightly elevated upon MTS exposure (Figure 5D). We also examined the effect of IKK $\beta$  deletion in myeloid cells on MTS-induced recruitment of various immune cell types into lungs of *K-ras*<sup>LA2</sup> tumor-bearing mice. As seen in naive mice, IKK $\beta$  ablation reduced the number of recruited neutrophils (Gr1<sup>+</sup> cells) but did not significantly impact the MTS-induced recruitment of macrophages (F4/80<sup>+</sup> cells) and CD4<sup>+</sup> T cells (Figures S5C and S5D). The reduction in neutrophil count was supported by a marked reduction in expression of *myeloperoxidase* mRNA that encodes a neutrophil-specific oxidative enzyme. MTS exposure and IKK $\beta$  deletion had no significant effect on the abundance of lung CD8<sup>+</sup> T cells. Thus, IKK $\beta$  in myeloid cells plays a critical role in the promotion of *K-ras*<sup>asp12</sup>-induced lung cancer development by MTS, most likely through the induction of inflammation, angiogenesis, and tumor-promoting inflammatory cytokines that stimulate the proliferation of both premalignant and malignant cells. Our results rule out reduced apoptosis as a component of the tumor-promoting effect of MTS exposure.

It has been suggested that activation of mitogen-activated protein kinases, including JNK, contributes to COPD-associated mucus overproduction and inflammation (Mercer and D'Armiento, 2006). JNK activation is also required for optimal induction of inflammatory cytokines (Karin and Gallagher, 2005). To investigate the role of JNK in MTS-induced inflammation, we analyzed the subacute response to MTS exposure in *Jnk1*<sup>-/-</sup> mice. *Jnk1*<sup>-/-</sup> mice showed reduced induction of *Il6*, *Tnf $\alpha$* , *Ccl3*, and *Ccl2* mRNAs, as well as decreased secretion of IL-6 and TNF- $\alpha$  by lung tissue upon MTS exposure, compared to WT mice (Figures 6A and 6B). *Jnk1*<sup>-/-</sup> mice also exhibited decreased induction of pneumocyte proliferation upon MTS exposure (Figure 6C).

To examine the role of JNK1 in MTS-induced lung tumor promotion, we crossed *K-ras*<sup>LA2</sup> mice with *Jnk1*<sup>-/-</sup> mice and generated *K-ras*<sup>LA2</sup>; *Jnk1*<sup>-/-</sup> hybrids that were exposed to MTS. Ablation of JNK1 reduced the enhancing effect of MTS on lung tumor multiplicity and maximal tumor sizes (Figures 6D and 6E). We also found decreased MTS-induced STAT3



**Figure 3. Tobacco Smoke Induces Pulmonary Inflammation and Cell Proliferation**

(A) Total cell number and leukocyte populations in BALF collected from C57BL6 males 24 hr after last MTS or air exposure. Cellular composition was analyzed using cytopsin preparations. Results are means  $\pm$  SEM (n = 8 for each group). Significant difference, \*p < 0.02 versus air control.

(B) Induction of inflammatory cytokine and chemokine mRNAs in lungs of MTS-exposed C57BL6 males. Lung RNA was isolated 24 hr after last MTS exposure and analyzed by real-time PCR. Results are means  $\pm$  SEM (n = 5 for each group). Significant difference, \*p < 0.03 versus air control. (C) Elevated cytokine secretion by lungs of MTS-exposed male mice. Fresh lungs were cut into small pieces and incubated in medium at 37°C for 48 hr. Cytokines in culture supernatants were measured by ELISA. Results are means  $\pm$  SEM (air control: n = 7; 4 cigarettes/day 1w: n = 9; 4 cigarettes/day 2w: n = 9). Significant difference, \*p < 0.02 versus air control.

(D) ERK and JNK activation and NF-κB DNA binding activity in lungs of MTS-exposed mice. Lung lysates and nuclear extracts prepared 4 hr after last MTS exposure were analyzed by immunoblotting and EMSA. Nuclear protein content was determined by immunoblotting with β-actin antibody. Shown are results from two representative mice per group.

(E) Cell proliferation in lungs of air- or MTS-exposed mice was determined by BrdU labeling. Results are means  $\pm$  SEM (air control: n = 7; 4 cigarettes/day 1w: n = 5; 4 cigarettes/day 2w: n = 8). Significant difference, \*p < 4.0  $\times$  10<sup>-5</sup> versus air control.

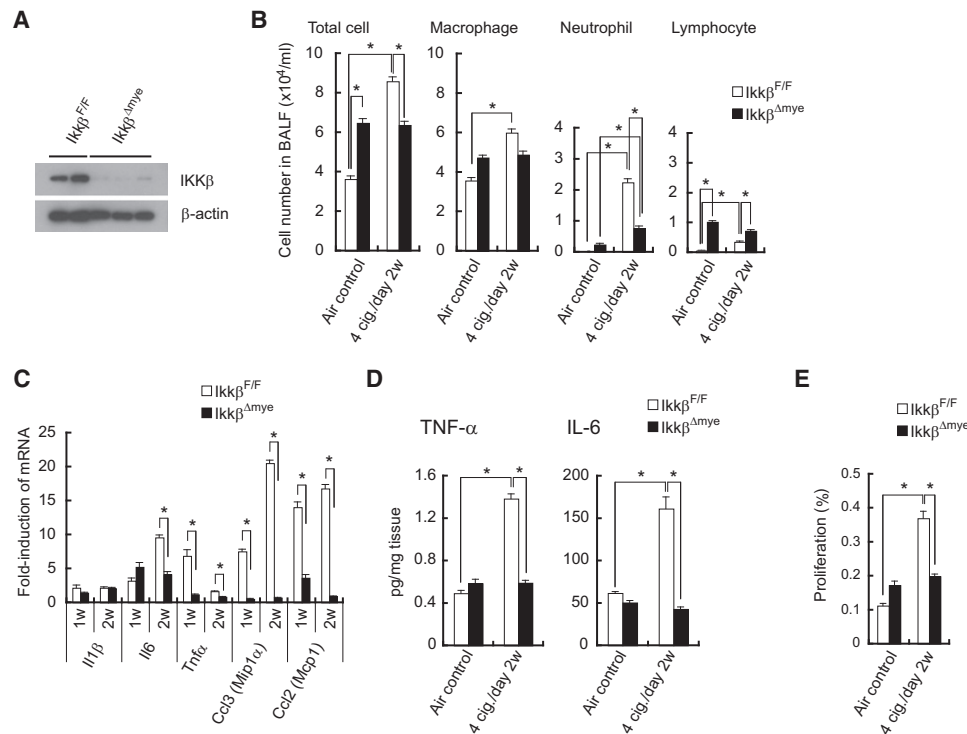
(F) Infiltration of IL-6-positive macrophages into *K-ras*<sup>LA2</sup> lung tumors 2 weeks after initiation of MTS exposure. Lung sections prepared 24 hr after last MTS exposure were analyzed by immunostaining for F4/80 (green) and IL-6 (red). Nuclei were counterstained by DAPI (blue). Scale bar = 100 μm. Results shown are for one representative mouse. For quantitation of the entire experiment see Figure S3F. See also Figure S3.

activation in *K-ras*<sup>LA2</sup>; *Jnk1*<sup>-/-</sup> mice (Figures S6A and S6B). Therefore, JNK1 also plays a significant role in the promotion of lung inflammation and tumorigenesis subsequent to MTS exposure.

## DISCUSSION

TS exposure accounts for the majority of lung cancer-related deaths in men and women (Hecht, 1999). Despite the identification of numerous tumor-initiating carcinogens, such as NNK (Hecht, 1999), the mechanism by which prolonged TS exposure, either active or passive, enhances lung tumorigenesis is not well understood due to the paucity of proper experimental models that faithfully replicate different features of TS-induced lung tumorigenesis in a small animal species amenable to genetic

analysis, i.e., the laboratory mouse. We now describe two mouse models in which TS exposure has a well-defined tumor-promoting effect on both chemically (NNK) and genetically (*K-ras*<sup>asp12</sup>) induced lung cancer. We show that in both NNK-initiated A/J mice and C57BL6 mice in which lung cancer has been initiated through expression of an oncogenic *K-ras*<sup>asp12</sup> allele, MTS exposure increases tumor multiplicity, incidence, and size. Consistent with being a tumor promoter under our experimental conditions, MTS exposure had no discernable effect on tumor histology, although tumor size was clearly increased along with an enhanced tumor angiogenesis. Although tumor promotion may be attributed to several factors, induction of inflammation, which elevates the production of inflammatory cytokines that enhance the proliferation and survival of initiated epithelial cells, is likely to be a major tumor-promoting mechanism (Karin,



**Figure 4. Myeloid Cell IKK $\beta$  Deletion Decreases MTS-Induced Inflammation and Cell Proliferation**

(A) Expression of IKK $\beta$  in alveolar macrophages of 7-week-old *Ikk $\beta$ <sup>F/F</sup>* and *Ikk $\beta$  <sup>$\Delta$ mye</sup>* mice.

(B) BALF cellular composition in air- or MTS-exposed *Ikk $\beta$ <sup>F/F</sup>* and *Ikk $\beta$  <sup>$\Delta$ mye</sup>* mice 24 hr after last MTS exposure. Results are means  $\pm$  SEM (*Ikk $\beta$ <sup>F/F</sup>* air control: n = 9; *Ikk $\beta$ <sup>F/F</sup>* 4 cigarettes/day 2w: n = 9; *Ikk $\beta$  <sup>$\Delta$ mye</sup>* air control: n = 9; *Ikk $\beta$  <sup>$\Delta$ mye</sup>* 4 cigarettes/day 2w: n = 13). Significant difference, \*p < 0.03.

(C) Induction of inflammatory cytokine and chemokine mRNAs in lungs of MTS-exposed *Ikk $\beta$ <sup>F/F</sup>* and *Ikk $\beta$  <sup>$\Delta$ mye</sup>* mice 24 hr after last 1 or 2 weeks of MTS exposure. Results are means  $\pm$  SEM (n = 5 for each group). Significant difference, \*p < 0.05.

(D) Secretion of cytokines by lungs of MTS-exposed mice was analyzed as in Figure 3C. Results are means  $\pm$  SEM (*Ikk $\beta$ <sup>F/F</sup>* air control: n = 14; *Ikk $\beta$ <sup>F/F</sup>* 4 cigarettes/day 2w: n = 8; *Ikk $\beta$  <sup>$\Delta$ mye</sup>* air control: n = 12; *Ikk $\beta$  <sup>$\Delta$ mye</sup>* 4 cigarettes/day 2w: n = 13). Significant difference, \*p < 0.04.

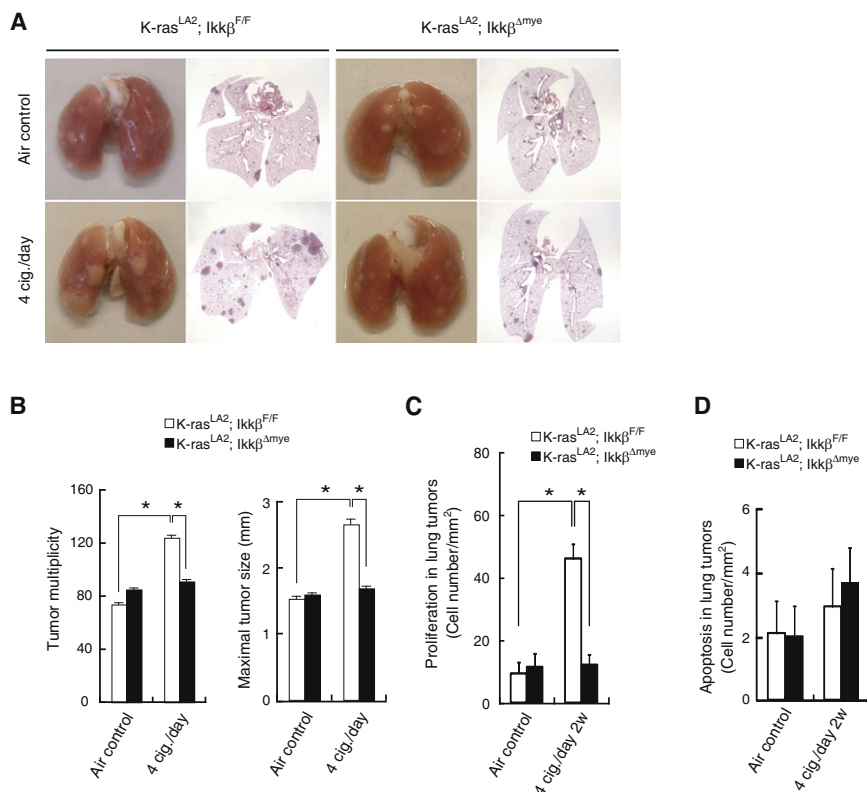
(E) Cell proliferation in lungs of air- or MTS-exposed mice was analyzed as in Figure 3E. Results are means  $\pm$  SEM (n = 7 for each group). Significant difference, \*p < 0.03. See also Figure S4.

2006). Here we demonstrate that induction of a low grade (subacute) inflammatory response is an important contributor to the tumor-promoting activity of TS, leading to the enhanced proliferation of both premalignant and malignant pulmonary epithelial cells.

Interference with this inflammatory response through myeloid cell-specific IKK $\beta$  ablation resulted in nearly complete abrogation of MTS-induced tumor promotion, but had little effect, if any, on *K-ras*<sup>asp12</sup>-induced tumor initiation. These findings should be contrasted with the importance of autocrine chemokine production for the growth and progression of already established and transplantable lung adenocarcinomas (Karin, 2005; Sparmann and Bar-Sagi, 2004). Although Ras-induced CXCL-8 (IL-8) produced by lung adenocarcinoma cells is a chemoattractant for myeloid cells (macrophages and neutrophils) and it and other *K-ras*-induced chemokines can cause severe pulmonary inflammation in mice (Ji et al., 2006), the mechanism through which such chemokines exert their tumor-promoting effect was not defined (Karin, 2005). Our results suggest that one possible mechanism by which myeloid cells promote tumor growth is through the IKK $\beta$ /NF- $\kappa$ B-dependent production of cytokines, such as IL-6 and TNF- $\alpha$ , both of which stimulate the

proliferation of pulmonary epithelial cells. These or other cytokines and chemokines may also enhance tumor angiogenesis.

Previous work on the tumor-promoting role of inflammation has been focused on cancers that are associated with either chronic inflammatory disease, such as colitis associated cancer (Greten et al., 2004; Grivennikov et al., 2009), or persistent viral and bacterial infections, such as hepatocellular carcinoma (Maeda et al., 2005; Pikarsky et al., 2004) and gastric cancer (Tu et al., 2008). The present work has addressed the role of inflammation in the tumorigenic effect of an environmental irritant, TS, with a major public health impact as being responsible for ~25% of all cancer-related deaths in the U.S. Although tobacco smoking increases colonization of the airways with inflammation-causing bacteria (Saetta et al., 1994), it also delivers irritants that are capable of direct induction of inflammation, a property shared with other inhaled microparticles, such as asbestos and silica (Borm and Driscoll, 1996). Whereas chronic inflammation lasting for decades, such as in long term smokers, may lead to induction of oncogenic mutations through the production of reactive oxygen and nitrogen species (Hussain et al., 2003), it also leads to activation of NF- $\kappa$ B, AP-1, and other transcription factors in airway myeloid cells, resulting in elevated



**Figure 5. IKK $\beta$  Deletion in Myeloid Cells Inhibits MTS-Induced Lung Tumor Promotion and Malignant Cell Proliferation in *K-ras*<sup>LA2</sup> Mice**

(A) Lung appearance (left) and histology (H&E stain; right) in 5-month-old *K-ras*<sup>LA2</sup>; *Ikkβ*<sup>F/F</sup> and *K-ras*<sup>LA2</sup>; *Ikkβ*<sup>Δmye</sup> mice with or without MTS exposure.

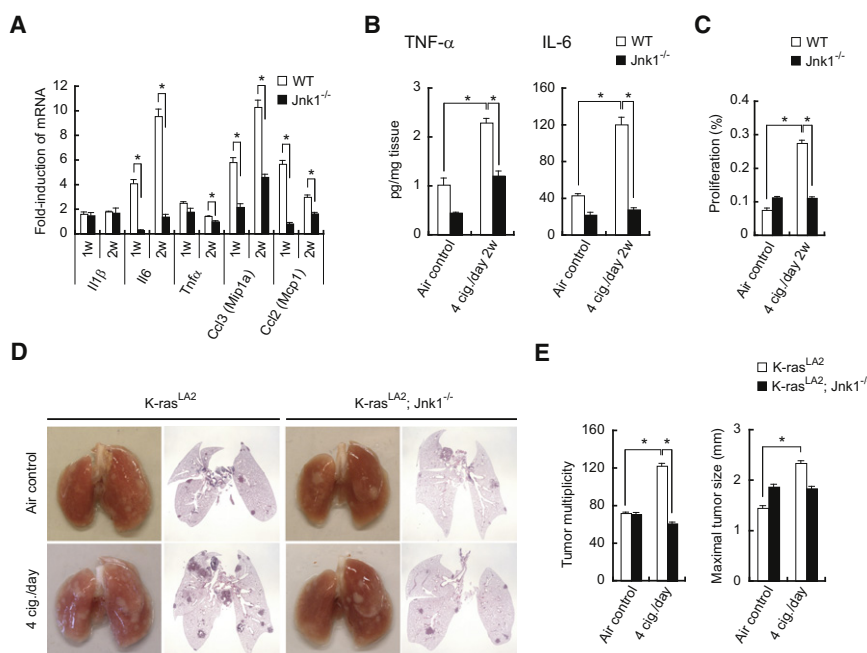
(B) Lung tumor multiplicity and maximal tumor sizes were determined as in Figure 2D in *K-ras*<sup>LA2</sup>; *Ikkβ*<sup>F/F</sup> and *K-ras*<sup>LA2</sup>; *Ikkβ*<sup>Δmye</sup> mice that were either air or MTS exposed. Results are means  $\pm$  SEM (*K-ras*<sup>LA2</sup>; *Ikkβ*<sup>F/F</sup> air control:  $n = 18$ ; *K-ras*<sup>LA2</sup>; *Ikkβ*<sup>F/F</sup> 4 cigarettes/day:  $n = 17$ ; *K-ras*<sup>LA2</sup>; *Ikkβ*<sup>Δmye</sup> air control:  $n = 20$ ; *K-ras*<sup>LA2</sup>; *Ikkβ*<sup>Δmye</sup> 4 cigarettes/day:  $n = 18$ ). Significant difference, \* $p < 0.02$ .

(C) Cell proliferation in lung adenomas from air- or MTS-exposed mice of indicated genotypes was determined by BrdU labeling. Results are means  $\pm$  SEM ( $n = 5$ – $6$  for each group). Significant difference, \* $p < 0.001$ .

(D) Apoptosis in lung adenomas from air- or MTS-exposed mice of indicated genotypes was determined by TUNEL staining. Results are means  $\pm$  SEM ( $n = 5$ – $6$  for each group). See also Figure S5.

cytokine production (Barnes, 2008). Our work based on shorter term TS exposure in mice suggests that activation of IKK $\beta$  and JNK signaling pathways and induction of inflammatory cytokine

production in myeloid cells by TS-born irritants results in a strong tumor-promoting effect that enhances the development of lung cancer, at least in mice. Although so far we have only examined



**Figure 6. Deletion of JNK1 Decreases MTS-Induced Inflammation, Cell Proliferation, and Lung Tumor Promotion**

(A) Induction of inflammatory cytokine and chemokine mRNAs in lungs of MTS-exposed *Jnk1*<sup>-/-</sup> mice was analyzed as in Figure 3B. Results are means  $\pm$  SEM ( $n = 5$  for each group). Significant difference, \* $p < 0.05$ .

(B) Secretion of cytokines in lungs of MTS-exposed *Jnk1*<sup>-/-</sup> mice was analyzed as in Figure 3C. Results are means  $\pm$  SEM (WT air control:  $n = 12$ ; WT 4 cigarettes/day:  $n = 9$ ; *Jnk1*<sup>-/-</sup> air control:  $n = 9$ ; *Jnk1*<sup>-/-</sup> 4 cigarettes/day:  $n = 9$ ). Significant difference, \* $p < 0.05$ .

(C) JNK1 deletion decreases MTS-induced lung cell proliferation. Results are means  $\pm$  SEM (WT air control:  $n = 7$ ; WT 4 cigarettes/day:  $n = 8$ ; *Jnk1*<sup>-/-</sup> air control:  $n = 10$ ; *Jnk1*<sup>-/-</sup> 4 cigarettes/day:  $n = 9$ ). Significant difference, \* $p < 0.0003$ .

(D) Lung appearance (left side) and histology (H&E stain; right side) in 5-month-old *K-ras*<sup>LA2</sup> or *K-ras*<sup>LA2</sup>; *Jnk1*<sup>-/-</sup> mice with or without MTS exposure.

(E) Lung tumor multiplicity and maximal tumor sizes were determined as in Figure 2D. Results are means  $\pm$  SEM (*K-ras*<sup>LA2</sup> air control:  $n = 16$ ; *K-ras*<sup>LA2</sup> 4 cigarettes/day:  $n = 18$ ; *K-ras*<sup>LA2</sup>; *Jnk1*<sup>-/-</sup> air control:  $n = 13$ ; *K-ras*<sup>LA2</sup>; *Jnk1*<sup>-/-</sup> 4 cigarettes/day:  $n = 14$ ). Significant difference, \* $p < 0.002$ . See also Figure S6.

the prophylactic value of anti-inflammatory intervention in TS-enhanced tumorigenesis, it is likely that appropriate anti-inflammatory therapy may slow down the development and progression of lung cancer if administered early enough and may interfere not only with tumor promotion but also with the accumulation of further genetic changes. Our findings suggest that it is also worthwhile to determine whether the great variation in the incidence of lung cancer among smokers is associated with genetic differences in TS-induced inflammation, as many genetic polymorphisms are known to affect the inflammatory response in the airways and other tissues (Cook et al., 2004). Identification of the long suspected, but heretofore unproven, tumor-promoting effect of TS provides a sound scientific rationale to the benefits of anti-inflammatory therapy or intervention as well as for smoking cessation, which has to be practiced as early as possible after detection of the smallest primary lung tumor, if not earlier.

## EXPERIMENTAL PROCEDURES

### Animals

*Ilk $\beta$ <sup>F/F</sup>*, *LysM-Cre*; *Ilk $\beta$ <sup>F/F</sup>* (referred to as *Ilk $\beta$  <sup>$\Delta$ mye</sup>*), *CC10-Cre*; *Ilk $\beta$ <sup>F/F</sup>*, and *Jnk1<sup>-/-</sup>* mice were described (Broide et al., 2005; Maeda et al., 2005). *K-ras<sup>LA2</sup>* mice were provided by T. Jacks (Massachusetts Institute of Technology) through the National Cancer Institute. *Tnf $\alpha$ <sup>-/-</sup>*, *Il6<sup>-/-</sup>*, and A/J mice were from the Jackson Laboratory. All strains, except A/J mice, used in this study were backcrossed to the C57BL6 background for more than six times. Mice were maintained under specific pathogen-free conditions, and experimental protocols were approved by University of California, San Diego Animal Care Program, following National Institutes of Health guidelines.

### NNK Treatment and Tobacco Smoke Exposure

For examining promotion of chemically-induced lung cancer, 7-week-old male A/J mice were i.p. injected with 70 mg/kg NNK (Toronto Research Chemicals) 1 week before initiation of MTS exposure. Mice were exposed to MTS generated by burning 3R4F reference cigarettes (College of Agriculture, Reference Cigarette Program, University of Kentucky) using a smoking machine (McChesney-Jaeger CSM-SSM Single Cigarette Machine; CH Technologies) regulated by programmable controls provided with JASPER windows 9x/2000 software over RS-232 communication ports (CH Technologies). Each smoldering cigarette was puffed for ~2 s, once every 25 s, for a total of 12 puffs/cigarette, at a flow rate of 5 liters/min. Fresh smoke was diluted with filtered air and delivered to a 12 port nose-only directed flow inhalation exposure system (Jaeger-NYU 12 port). The average total suspended particulate matter monitored at nose ports was  $173 \pm 5.3$  mg/m<sup>3</sup>. In the 4 cigarettes/day exposure group, mice were exposed to MTS from 4 cigarettes/day for ~5 min per cigarette with a 5 min break between cigarettes, 5 days/week for 1 month, followed by 1 month of rest. This cycle was repeated two more times. In the 2 cigarettes/day group, mice were exposed to MTS from 2 cigarettes/day, administered over 5 min per cigarette with 5 min break between cigarettes, 5 days/week for 5 months. Control mice were kept on filtered air. Lung tumors were analyzed 9 months after initiation of NNK plus MTS exposure. For examining promotion of genetically induced lung tumors, 6- to 8-week-old *K-ras<sup>LA2</sup>* mutant mice were exposed to MTS at 4 cigarettes/day, 5 days/week for 3 weeks, followed by a 2 week rest interval on air alone, two times and exposed to MTS at 4 cigarettes/day, 5 days/week for 2 more weeks. Sex-matched control *K-ras<sup>LA2</sup>* mutant mice were kept on filtered air. Lung tumors were analyzed at 5 months of age. For the NNK dose optimization study, 7-week-old male A/J mice were i.p. injected with 30, 50, or 70 mg/kg NNK one week before initiation of MTS exposure. Mice were exposed to 2 cigarettes/day MTS for 2 months and 1 cigarettes/day for 3 months. Control mice were kept on filtered air. Lung tumors were analyzed 9 months after NNK injection. In the subacute inflammation study, 7- to 8-week-old sex-matched mice were exposed to 4 cigarettes/day MTS, 5 days/week for 1 or 2 weeks. Control mice were kept on filtered air.

### BrdU Labeling

For examining pulmonary cell proliferation, randomly chosen mice were i.p. injected with 100 mg/kg BrdU (Sigma-Aldrich) 2 hr prior to sacrifice, and paraffin sections were stained with a BrdU in situ detection kit (BD Biosciences). 3000 to 5000 cells per lung were counted in randomly selected fields. BrdU labeling index was calculated as the percentage of labeled cells per total cells counted.

### BALF Leukocyte Counts and Alveolar Macrophage Isolation

Number of total cells and leukocyte types in BALF were determined as described previously (Ikeda et al., 2003). In brief, after instillation of 800  $\mu$ l sterile PBS through the trachea, BALF was withdrawn, and cytospin preparations of BALF cells were prepared using Shandon Cytospin centrifuge (Shandon Lipshaw). BALF cells were visualized by Wright-Giemsa staining and percentages of leukocyte types were determined by counting 400 leukocytes in a randomly selected portion of the slide under light microscopy. BALF total leukocyte counts were performed using a hemocytometer. Alveolar macrophages were isolated as previously described (Vlahos et al., 2006).

### Evaluation of Lung Tumors

For determining tumor incidence, multiplicity, and maximal sizes, whole lungs were manually inflated with and fixed in 4% paraformaldehyde for at least 24 hr and paraffin embedded. Paraffin-embedded lungs were serially sectioned at 350  $\mu$ m and histologically examined with hematoxylin and eosin (H&E) stained sections as previously described (Curtin et al., 2004).

### Cytokine ELISA

Fresh lung lobes were cut into small pieces and incubated in 1 ml RPMI medium (Invitrogen) at 37°C for 48 hr. IL-1 $\beta$ , IL-6, and TNF- $\alpha$  were measured by enzyme-linked immunosorbent assay (BD Biosciences, eBioscience, and R&D) with culture supernatants.

### Analysis of Gene Expression

Total tissue RNA was prepared with RNeasy plus mini kit (QIAGEN). Quantitative PCR was performed as described (Kim et al., 2009). RNA was reverse-transcribed with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR was performed using SYBR green (Bio-Rad) on a Bio-Rad iQ5 machine. Expression data were normalized to  $\beta$ -actin mRNA amounts. Primer pairs for *Il1 $\beta$* , *Il6*, *Tnf $\alpha$* , *Ccl2*, and *Ccl3* were as follows: 5'-CAACCAAGTGATATTCTCCATG-3'/5'-GATCCACACTCTCCAGCTGCA-3'; 5'-GAGGATACCACTCCCAACAGACC-3'/5'-AAGTGCATCATCGTTGTTCATA-3'; 5'-GACCAAGGCTGTCGCTACATCA-3'/5'-CGTAGGCGATTACAGTCACGG-3'; 5'-GCAGGTCCCTGTCTGCTTC-3'/5'-TCCAGCCTACTCATTGGGATCA-3'; 5'-TGGAGCTGACACCCGAC-3'/5'-ACGATGAATTGGCGTGGAA-3', respectively. Primer pairs for *myeloperoxidase* were 5'-CACCTCTTTGTTCGAGAGC-3'/5'-CAACCAAGGGCAGGTAGT-3'.

### Biochemical and Immunohistochemical Analyses

Nuclear proteins were isolated from lung tissues using Nuclear Extract Kit (Active Motif), and EMSA was performed using Gelshift Kit (Active Motif) and a  $\kappa$ B site consensus oligonucleotide (Promega). Nuclear extracts were analyzed by SDS-PAGE and immunoblotting with antibody to  $\beta$ -actin (Sigma-Aldrich). Total tissue lysates were prepared and analyzed by SDS-PAGE and immunoblotting with antibodies to P-JNK, ERK, P-ERK (all from Cell Signaling), JNK (BD Biosciences), and IKK $\beta$  (Millipore). Apoptosis was determined with ApopTag Red In Situ Apoptosis Detection Kit (Millipore). For immunohistochemistry, paraffin-embedded slides were deparaffinized and antigens were unmasked by incubation at 94°C for 40 min in Target Retrieval Solution S1700 (Dako). Slides were incubated with primary antibodies in 4°C overnight. Signals were detected with fluorescent microscopy or VECTASTIN ABC Elite kit (Vector Laboratories), DAB Substrate Kit (Vector Laboratories), and light microscopy. The following antibodies were used: anti-IL-6-PE, anti-P-STAT3 (both from Cell Signaling), anti-F4/80-Alexa488 (Caltag), anti-CD31 anti-CD4, anti-CD8 (all three from BD Biosciences), anti-F4/80, and anti-Gr-1 (both from eBioscience).

### Statistical Analysis

Results are expressed as means  $\pm$  SEM. Data were analyzed by Student's t test and Fisher's exact test. p values < 0.05 were considered significant.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at doi:10.1016/j.ccr.2009.12.008.

## ACKNOWLEDGMENTS

The authors thank Cell Signaling Technology for the gifts of antibodies. Research was supported by Grants from the National Institutes of Health, including the Superfund Research Program (P42ES010337 to M.K.) and Tobacco-Related Disease Research Program (15RT-0197 to M.K. and 12RT-0071 to D.H.B). H.T. and H.O. were supported by the Japanese Respiratory Society and Kanzawa Medical Research Foundation, respectively. M.K. is an American Cancer Society research professor.

Received: July 9, 2009

Revised: October 14, 2009

Accepted: December 4, 2009

Published: January 19, 2010

## REFERENCES

- Barnes, P.J. (2008). The cytokine network in asthma and chronic obstructive pulmonary disease. *J. Clin. Invest.* 118, 3546–3556.
- Berns, A. (2001). Cancer. Improved mouse models. *Nature* 410, 1043–1044.
- Borm, P.J., and Driscoll, K. (1996). Particles, inflammation and respiratory tract carcinogenesis. *Toxicol. Lett.* 88, 109–113.
- Broide, D.H., Lawrence, T., Doherty, T., Cho, J.Y., Miller, M., McElwain, K., McElwain, S., and Karin, M. (2005). Allergen-induced peribronchial fibrosis and mucus production mediated by IkappaB kinase beta-dependent genes in airway epithelium. *Proc. Natl. Acad. Sci. USA* 102, 17723–17728.
- Brown, B.G., Chang, C.J., Ayres, P.H., Lee, C.K., and Doolittle, D.J. (1999). The effect of cotinine or cigarette smoke co-administration on the formation of O6-methylguanine adducts in the lung and liver of A/J mice treated with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). *Toxicol. Sci.* 47, 33–39.
- Cook, D.N., Pisetsky, D.S., and Schwartz, D.A. (2004). Toll-like receptors in the pathogenesis of human disease. *Nat. Immunol.* 5, 975–979.
- Coussens, L.M., and Werb, Z. (2002). Inflammation and cancer. *Nature* 420, 860–867.
- Curtin, G.M., Higuchi, M.A., Ayres, P.H., Swauger, J.E., and Mosberg, A.T. (2004). Lung tumorigenicity in A/J and rasH2 transgenic mice following mainstream tobacco smoke inhalation. *Toxicol. Sci.* 81, 26–34.
- Finch, G.L., Nikula, K.J., Belinsky, S.A., Barr, E.B., Stoner, G.D., and Lechner, J.F. (1996). Failure of cigarette smoke to induce or promote lung cancer in the A/J mouse. *Cancer Lett.* 99, 161–167.
- Greten, F.R., Eckmann, L., Greten, T.F., Park, J.M., Li, Z.W., Egan, L.J., Kagnoff, M.F., and Karin, M. (2004). IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell* 118, 285–296.
- Grivennikov, S., Karin, E., Terzic, J., Mucida, D., Yu, G.Y., Vallabhapurapu, S., Scheller, J., Rose-John, S., Cheroutre, H., Eckmann, L., and Karin, M. (2009). IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell* 15, 103–113.
- Hecht, S.S. (1998). Biochemistry, biology, and carcinogenicity of tobacco-specific N-nitrosamines. *Chem. Res. Toxicol.* 11, 559–603.
- Hecht, S.S. (1999). Tobacco smoke carcinogens and lung cancer. *J. Natl. Cancer Inst.* 91, 1194–1210.
- Hecht, S.S. (2002). Cigarette smoking and lung cancer: chemical mechanisms and approaches to prevention. *Lancet Oncol.* 3, 461–469.
- Herbst, R.S., Heymach, J.V., and Lippman, S.M. (2008). Lung cancer. *N. Engl. J. Med.* 359, 1367–1380.
- Hussain, S.P., Hofseth, L.J., and Harris, C.C. (2003). Radical causes of cancer. *Nat. Rev. Cancer* 3, 276–285.
- Ikeda, R.K., Nayar, J., Cho, J.Y., Miller, M., Rodriguez, M., Raz, E., and Broide, D.H. (2003). Resolution of airway inflammation following ovalbumin inhalation: comparison of ISS DNA and corticosteroids. *Am. J. Respir. Cell Mol. Biol.* 28, 655–663.
- Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Murray, T., and Thun, M.J. (2008). Cancer statistics, 2008. *CA Cancer J. Clin.* 58, 71–96.
- Ji, H., Houghton, A.M., Mariani, T.J., Perera, S., Kim, C.B., Padera, R., Tonon, G., McNamara, K., Marconini, L.A., Hezel, A., et al. (2006). K-ras activation generates an inflammatory response in lung tumors. *Oncogene* 25, 2105–2112.
- Johnson, L., Mercer, K., Greenbaum, D., Bronson, R.T., Crowley, D., Tuveson, D.A., and Jacks, T. (2001). Somatic activation of the K-ras oncogene causes early onset lung cancer in mice. *Nature* 410, 1111–1116.
- Karin, M. (2005). Inflammation and cancer: the long reach of Ras. *Nat. Med.* 11, 20–21.
- Karin, M. (2006). Nuclear factor-kappaB in cancer development and progression. *Nature* 441, 431–436.
- Karin, M., and Gallagher, E. (2005). From JNK to pay dirt: jun kinases, their biochemistry, physiology and clinical importance. *IUBMB Life* 57, 283–295.
- Karin, M., and Greten, F.R. (2005). NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat. Rev. Immunol.* 5, 749–759.
- Karin, M., Lawrence, T., and Nizet, V. (2006). Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. *Cell* 124, 823–835.
- Kim, S., Takahashi, H., Lin, W.W., Descargues, P., Grievnikov, S., Kim, Y., Luo, J.L., and Karin, M. (2009). Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. *Nature* 457, 102–106.
- Maeda, S., Kamata, H., Luo, J.L., Leffert, H., and Karin, M. (2005). IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. *Cell* 121, 977–990.
- Mercer, B.A., and D'Armiento, J.M. (2006). Emerging role of MAP kinase pathways as therapeutic targets in COPD. *Int. J. Chron. Obstruct. Pulmon. Dis.* 1, 137–150.
- Peterson, L.A., and Hecht, S.S. (1991). O6-methylguanine is a critical determinant of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone tumorigenesis in A/J mouse lung. *Cancer Res.* 51, 5557–5564.
- Pikarsky, E., Porat, R.M., Stein, I., Abramovitch, R., Amit, S., Kasem, S., Gutkovich-Pyest, E., Urieli-Shoval, S., Galun, E., and Ben-Neriah, Y. (2004). NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature* 431, 461–466.
- Saetta, M., Di Stefano, A., Maestrelli, P., Turato, G., Ruggieri, M.P., Roggeri, A., Calcagni, P., Mapp, C.E., Ciaccia, A., and Fabbri, L.M. (1994). Airway eosinophilia in chronic bronchitis during exacerbations. *Am. J. Respir. Crit. Care Med.* 150, 1646–1652.
- Sparmann, A., and Bar-Sagi, D. (2004). Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. *Cancer Cell* 6, 447–458.
- Tockman, M.S., Anthonisen, N.R., Wright, E.C., and Donithan, M.G. (1987). Airways obstruction and the risk for lung cancer. *Ann. Intern. Med.* 106, 512–518.
- Toh, C.K. (2009). The changing epidemiology of lung cancer. *Methods Mol. Biol.* 472, 397–411.
- Tu, S., Bhagat, G., Cui, G., Takaishi, S., Kurt-Jones, E.A., Rickman, B., Betz, K.S., Penz-Oesterreicher, M., Bjorkdahl, O., Fox, J.G., and Wang, T.C. (2008). Overexpression of interleukin-1beta induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. *Cancer Cell* 14, 408–419.
- Vlahos, R., Bozinovski, S., Jones, J.E., Powell, J., Gras, J., Lilja, A., Hansen, M.J., Gualano, R.C., Irving, L., and Anderson, G.P. (2006). Differential protease, innate immunity, and NF-kappaB induction profiles during lung inflammation induced by subchronic cigarette smoke exposure in mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 290, L931–L945.
- Witschi, H. (2005). The complexities of an apparently simple lung tumor model: The A/J mouse. *Exp. Toxicol. Pathol.* 57 (Suppl 1), 171–181.
- Witschi, H., Espiritu, I., Peake, J.L., Wu, K., Maronpot, R.R., and Pinkerton, K.E. (1997). The carcinogenicity of environmental tobacco smoke. *Carcinogenesis* 18, 575–586.