

Cigarette Smoke Impairs NK Cell-Dependent Tumor Immune Surveillance¹

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In this study, we investigated the impact of cigarette smoke on tumor immune surveillance and its consequences to lung tumor burden in a murine lung metastasis model. Cigarette smoke exposure significantly increased the numbers of lung metastases following B16-MO5 melanoma challenge. This effect was reversible; we observed significantly fewer tumor nodules following smoking cessation. Using RAG2^{-/-} and RAG2^{-/-}γ_c^{-/-} mice, we provide strong evidence that increased tumor incidence was NK cell dependent. Furthermore, we show that cigarette smoke suppressed NK activation and attenuated NK CTL activity, without apparent effect on activating or inhibitory receptor expression. Finally, activation of NK cells through bone marrow-derived dendritic cells conferred protection against lung metastases in smoke-exposed mice; however, protection was not as efficacious as in sham-exposed mice. To our knowledge, this is the first experimental evidence showing that cigarette smoke impairs NK cell-dependent tumor immune surveillance and that altered immunity is associated with increased tumor burden. Our findings suggest that altered innate immunity may contribute to the increased risk of cancer in smokers. *The Journal of Immunology*, 2007, 178: 936–943.

Cigarette smoking is the predominant etiologic factor for lung cancer, accounting for ~90% of all cases (1). Although there is convincing evidence that carcinogens contained within cigarette smoke induce lung tumorigenesis (2–5), additional mechanisms may contribute to the increased cancer risk in smokers.

Host immune defense mechanisms play an important role in eliminating aberrant cells and controlling tumor growth and metastases formation (6–8). For example, specific lymphocyte subsets including CD4, CD8, γδ, and NK T cells as well as NK cells have been shown to recognize and eliminate newly transformed cells (9, 10). Accordingly, immunodeficient animals show increased spontaneous tumor formation with age, and a significant increase in cancer incidence following administration of carcinogens (1, 11). Although mounting evidence suggests that cigarette smoking impairs immune defense mechanisms (12), the consequences of compromised immunity to tumor immune surveillance are poorly understood.

The objective of this study was to investigate the impact of cigarette smoke on tumor immune surveillance in a murine lung metastatic tumor model. We observed an increased tumor burden in smoke-exposed, compared with sham-exposed, mice following

tumor challenge. We provide strong evidence that the increased number of pulmonary metastases associated with cigarette smoke exposure was due to defects within the NK cell compartment. Smoking cessation reversed the detrimental effect of cigarette smoke on tumor immune surveillance. Our study shows for the first time that cigarette smoke impairs NK cell-mediated tumor immune surveillance and that altered immunity is associated with increased lung tumor burden in mice. Our study provides experimental evidence that altered immune status may contribute to the increased cancer risk observed in smokers and is in accord with findings that NK cell activity is decreased in smokers (13, 14).

Materials and Methods

Animals

Female C57BL/6 mice (6–8 wk old) were purchased from Charles River Laboratories. RAG2^{-/-}, RAG2^{-/-}γ_c^{-/-}, and IL-12^{-/-} mice, all on a C57BL/6 background, were bred in-house. Mice were maintained under specific pathogen-free conditions in an access-restricted area, on a 12-h light-dark cycle, with food and water provided ad libitum. The Animal Research Ethics Board of McMaster University approved all experiments described in this study.

Cell culture

C57BL/6 B16 melanoma cells that are stably transfected with OVA (B16-MO5) were cultured in DMEM plus 10% FBS, 2 mM glutamine, 1 mM pyruvate, 50 μM 2-ME, 200 U/ml penicillin, 200 μg/ml streptomycin, and 800 μg/ml G418 (Sigma-Aldrich) at 37°C in 5% CO₂. YAC-1 cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, 200 U/ml penicillin, and 200 μg/ml streptomycin. Dendritic cells were derived from C57BL/6 bone marrow as described previously (15). Briefly, bone marrow cells were cultured in RPMI 1640 in the presence of 40 ng/ml rGM-CSF. Medium was replaced on days 3 and 5. At day 7, nonadherent cells were harvested and washed with PBS three times before injection.

Cigarette smoke exposure protocol

Mice were exposed to mainstream tobacco smoke using a smoke exposure system that was initially developed for guinea pigs (16) and has since been adapted for mice (17). Mice were exposed to two 1R3 reference cigarettes (Tobacco and Health Research Institute, University of Kentucky) daily, 5 days per week following an initial 2-wk lead-up period in which animals

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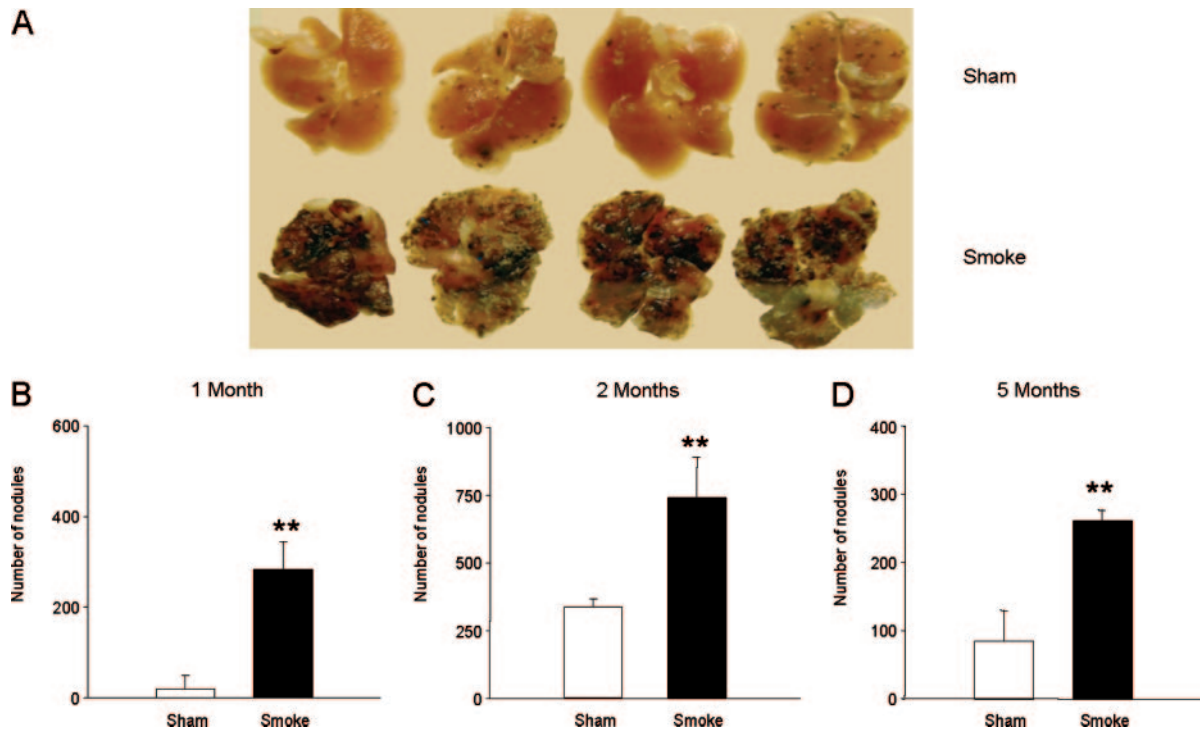


FIGURE 1. Increased numbers of lung metastasis in cigarette smoke-exposed mice. C57BL/6 mice were exposed to mainstream tobacco smoke for 1 (A and B), 2 (C), and 5 (D) mo. To control for handling, groups of mice were placed into restrainers only (sham exposure). Animals were challenged with 10^6 B16-MO5 melanoma cells via the tail vein. Seventeen to 21 days following tumor infusion, animals were killed and numbers of tumor foci were counted using a dissection microscope. A, Tumor metastasis formation in mice that were exposed to cigarette smoke for 1 mo before tumor challenge. B–D, Quantification of metastatic tumor nodules in mice exposed to cigarette smoke for 1, 2, and 5 mo. Data represent mean \pm SD, n denotes individual animals (1 mo: $n = 4$, 2 mo: $n = 5$, 5 mo: $n = 4$); **, $p < 0.01$, one representative experiment of three shown for 1 and 2 mo.

were accustomed to one cigarette in the first and to two cigarettes in the second week. The number of months for which animals were exposed to cigarette smoke is specified in *Results*. To control for handling, additional groups of mice were placed into restrainers only (sham exposure).

Serum carboxyhemoglobin (COHb)⁴ and cotinine levels

COHb levels were measured at the Hamilton Regional Laboratory Medicine Program (McMaster University Medical Centre, Hamilton, Ontario, Canada) using standard spectrophotometry in venous blood samples obtained by retro-orbital bleeding into heparinized clintubes (Radiometer). Samples were obtained immediately following exposure to cigarette smoke of mice exposed to cigarette smoke for 2 mo and 24 h after the last smoke exposure. Cotinine levels were measured by ELISA (Bio-Quant) in serum obtained by incubating whole blood for 30 min at 37°C, followed by centrifugation.

Tumor challenge

C57BL/6, RAG2^{-/-}, RAG2^{-/-}γ_c^{-/-}, and IL-12^{-/-} mice were challenged i.v. with B16-MO5 cells in 0.2 ml of PBS via the tail vein. Cell numbers used and times of sacrifice are specified in *Results*. Animals were anesthetized and euthanized via exsanguinations through abdominal vein puncture. Lungs and livers were removed and placed in PBS. Tumor nodules were counted using a dissecting microscope by a researcher blinded to the treatment groups. In some cases, lungs were immersed in a 67% ethanol, 9% formaldehyde, and 4% glacial acetic acid solution and the number of tumor foci was enumerated 24–48 h later.

Isolation of lung mononuclear cells

Lung mononuclear cells were isolated as described previously (18). Briefly, lungs were perfused with prewarmed HBSS from the right ventricle. Cell suspensions from excised organs were generated by collagenase digestion and followed by mechanical mincing. Cell debris were removed by passing through nylon mesh. Cells were washed and resuspended in

HBSS. Nonparenchymal cells were isolated by density-gradient centrifugation with Lympholyte-M (Cedarlane Laboratories).

Flow cytometric analysis

All Abs for flow cytometric analysis except FITC-conjugated anti-Ly49I (YL1-90) and allophycocyanin-conjugated anti-NKG2D (CX5) (both from eBioscience) were purchased from BD Pharmingen, including FITC-conjugated anti-LY49A (A1), FITC-conjugated anti-CD11c (HL3), PE-conjugated anti-NK1.1 (PK136), PerCP-conjugated anti-CD3 (145-2C11), allophycocyanin-conjugated anti-B220 (RA3-6B2), PE-Cy7-conjugated anti-CD69 (H1.2F3), allophycocyanin-Cy7-conjugated anti-CD25 (PC61), and anti-FcγRII/III (2.4G2). For flow cytometric analysis, cells were incubated with anti-FcγRII/III Abs on ice for 15 min to block nonspecific binding. Subsequently, cells were stained with combinations of indicated fluorochrome-conjugated mAbs on ice for 30 min and washed. Data were collected using a FACSCanto or LSRII (BD Biosciences) and analyzed using FlowJo 6.2 (Tree Star).

In vitro cytotoxicity assay

Cytotoxic activity of lung mononuclear cells was assessed using an Apoptosis Detection kit from BD Bioscience with minor modifications. Briefly, YAC-1 cells, an NK cell-specific target, were labeled with the tracking dye PKH-26 (Sigma-Aldrich). Labeled target cells were cocultured with lung mononuclear cells (effector cells) at different E:T ratios for 6 h. Subsequently, cells were stained with annexin V and 7-aminoactinomycin D (7-AAD) according to the protocol provided by BD Biosciences and data were acquired on a FACSCanto within an hour. Annexin V and 7-AAD expression was assessed on gated PKH-26-stained YAC-1 cells. Apoptotic cells were defined as annexin V single positive, while annexin V and 7-AAD double positive were defined as dead cells.

Transfer studies

Lung mononuclear cells were isolated from cigarette smoke- and sham-exposed RAG2^{-/-} mice. The proportion of NK1.1-positive cells contained within the lung mononuclear preparations was assessed by flow cytometry. An equivalent of 8×10^5 NK1.1 cells were mixed with 1×10^6 B16-MO5

⁴ Abbreviations used in this paper: COHb, carboxyhemoglobin; 7-AAD, 7-aminoactinomycin D.

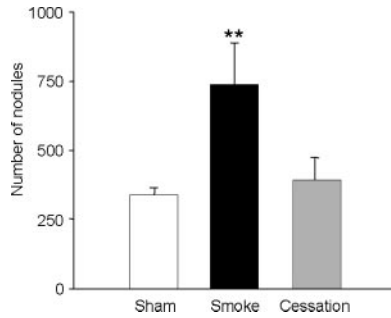


FIGURE 2. Smoking cessation and lung metastatic tumor burden. C57BL/6 mice were cigarette smoke- or sham-exposed for 2 mo and then challenged with 1×10^6 B16-MO5 cells. Following tumor infusion, smoke-exposed mice were divided into two groups. The first group was no longer cigarette smoke exposed and placed in restrainers only (cessation), while the other group continued their smoke exposure regimen until the day of sacrifice. Seventeen days following tumor challenge, animals were sacrificed and numbers of tumor foci were counted using a dissecting microscope. Data represent mean \pm SD, $n = 5$ (n denotes individual animals, one representative experiment of two shown); **, $p < 0.001$.

in 200 μ l of PBS and injected i.v. into RAG2^{-/-} γ_c ^{-/-} mice. Twenty-one days later, tumor burden in recipient mice was assessed.

Data analysis

Data are expressed as means \pm SD. Statistical analysis was performed using SigmaStat statistical software, version 2.0 (SPSS). t tests were used for two-group comparisons, and one-way ANOVA for three-group comparisons. Statistical interpretation of results is indicated in figure legends. Differences were considered statistically significant when $p < 0.05$.

Results

Cigarette smoke and lung metastasis

To investigate the impact of cigarette smoke on the lung metastatic tumor burden, C57BL/6 mice were cigarette smoke-exposed for 1 mo and then challenged i.v. with 1×10^6 B16-MO5 melanoma cells. Animals were continuously exposed to cigarette smoke until the day of sacrifice (21 days posttumor challenge). Cigarette smoke exposure results in elevated COHb saturation levels immediately following smoke exposure of 13.6% as compared with 3.8% for sham-exposed animals. COHb saturation fell to sham-exposed levels within 24 h of smoke exposure. Serum cotinine

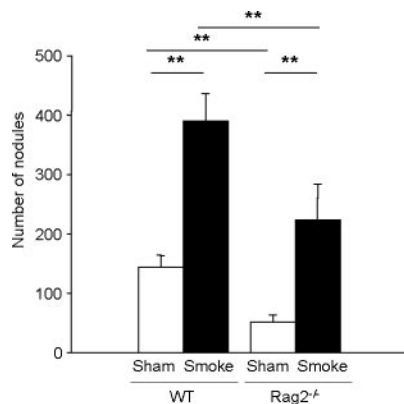


FIGURE 3. Cigarette smoke and lung metastasis formation in RAG2^{-/-} mice. Wild-type and RAG2^{-/-} mice were cigarette smoke or sham exposed for 1 mo and then infused i.v. with 1×10^6 B16-MO5 cells. Twenty-one days following tumor challenge, mice were sacrificed and tumor nodules were counted using a dissecting microscope. Data represent mean \pm SD, $n = 5$, (n denotes individual animals); **, $p < 0.001$.

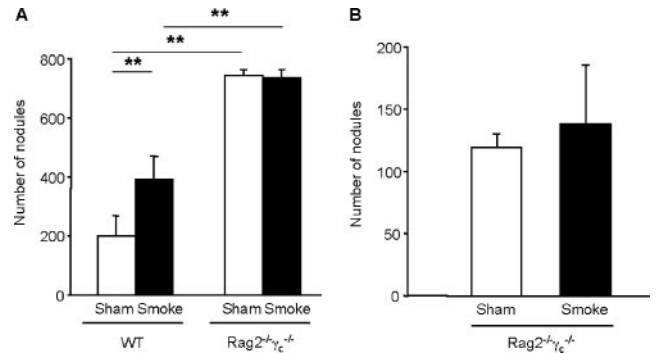


FIGURE 4. Cigarette smoke and lung metastasis formation in RAG2^{-/-} γ_c ^{-/-} mice. **A**, Wild-type and RAG2^{-/-} γ_c ^{-/-} mice were smoke- or sham-exposed for 1 mo and infused i.v. with 8×10^5 B16-MO5 cells. Mice were sacrificed 21 days following tumor challenge. Data displayed represent number of lung tumor nodules per lung (mean \pm SD, $n = 5$ (n denotes individual animals); **, $p < 0.001$). No significant difference was observed between smoke- and sham-exposed RAG2^{-/-} γ_c ^{-/-} mice ($p = 0.63$). **B**, RAG2^{-/-} γ_c ^{-/-} mice were cigarette smoke or sham exposed for 1 mo and then infused i.v. with 2×10^5 B16-MO5 cells. Twenty-one days later, mice were sacrificed and lung metastasis nodules were counted. Data represent mean \pm SD, $n = 4$ (n denotes individual animals), $p = 0.118$.

levels averaged 150 ng/ml. Both COHb and cotinine levels are consistent with the levels observed in human smokers. Fig. 1, **A** and **B**, show a significantly increased lung tumor burden in cigarette smoke- compared with sham-exposed mice. Similarly, we observed an increased tumor burden when C57BL/6 mice were challenged with 1×10^6 B16-MO5 melanoma cells following 2 and 5 mo of cigarette smoke exposure (Fig. 1, **C** and **D**). In comparison, when mice were challenged with B16-MO5 cells at the

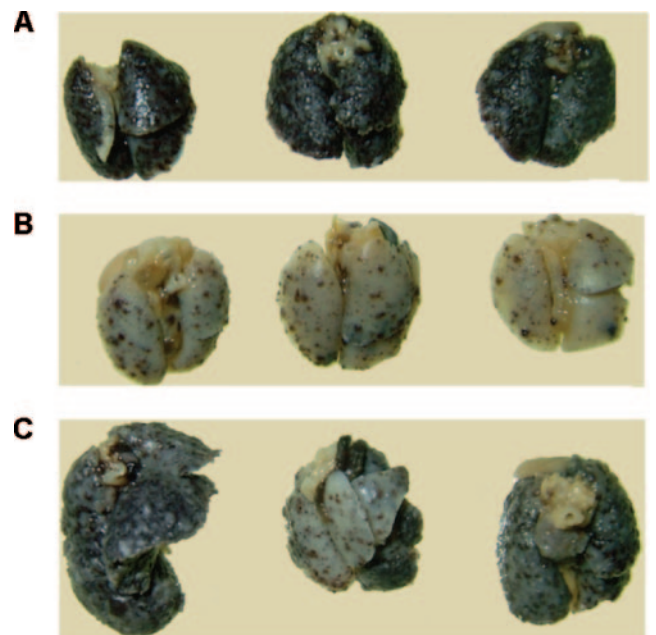
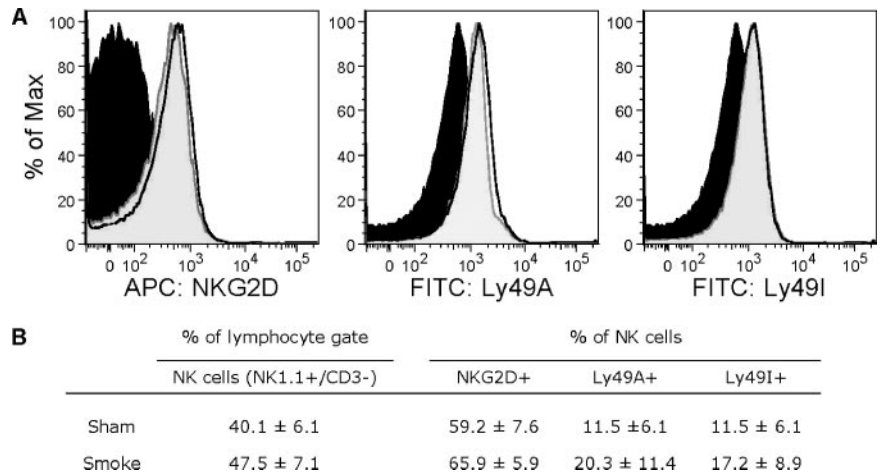


FIGURE 5. Lung mononuclear cell transfer. RAG2^{-/-} mice were sham- or cigarette smoke-exposed for 2 mo and lung mononuclear cells were isolated. A total of 8×10^5 NK1.1⁺ cells (contained within a lung mononuclear cell suspension) were mixed with 1×10^6 B16-MO5 cells, and injected i.v. into recipient RAG2^{-/-} γ_c ^{-/-} mice. Twenty-one days later, animals were sacrificed and lungs excised. Photographs depict lungs from tumor-challenged RAG2^{-/-} γ_c ^{-/-} mice, receiving no cells (**A**) or lung mononuclear cells from sham- (**B**) or smoke-exposed animals (**C**). Data displayed represent one of two experiments.

FIGURE 6. Stimulatory and inhibitory receptor expression by lung NK cells. Rag2^{-/-} mice were sham- or cigarette smoke-exposed smoke for 4 mo. Animals were killed and lung mononuclear cells were isolated. NKG2D, Ly49A, and Ly49I-positive cells were quantified within the CD3⁻/NK1.1⁺ gate by flow cytometry. Isotype controls (black) and stained cells from smoke- (gray) and sham-exposed (open) lungs are shown in representative histograms (A) and summarized (B). Data represent mean ± SD, n = 4–6/group (n denotes individual animals, one representative experiment of two shown). APC, Allophycocyanin.



onset of cigarette smoke exposure, there was no difference in lung metastatic tumor burden between cigarette smoke- and sham-exposed animals (data not shown). Finally, we observed an increased metastatic tumor burden in smoke- compared with sham-exposed animals following challenge with 2.5 × 10⁵ and 5 × 10⁵ B16-MO5 cells (data not shown).

Although smoke-exposed mice consistently showed an increased tumor burden compared with sham-exposed animals, we observed experiment-to-experiment variability in the number of lung tumor nodules. This variability in tumor burden in between experiments likely reflects differences in the biological behavior of tumor cells of different passages and exact cell numbers injected. To account for this intraexperimental variability, we only compared tumor burden between mice that were injected with the same preparation of B16-MO5 cells throughout the manuscript. Of note, data presented for 1, 2, and 5 mo of smoke exposure were generated in independent experiments, precluding a direct comparison between Fig. 1, B–D.

Smoking cessation and tumor burden

We next assessed the impact of smoking cessation on lung metastatic tumor burden. Mice were exposed to cigarette smoke for 2 mo and then challenged with 1 × 10⁶ B16-MO5 cells. Following challenge, mice were divided into two groups. The first group (cessation) was no longer exposed to cigarette smoke and was placed in restrainers only to control for handling. The other group was continuously exposed to cigarette smoke until the day of sacrifice. We also delivered B16-MO5 cells to control animals that were sham-exposed through the entire protocol. We observed significantly greater metastatic tumor burden in smoke-exposed mice compared with both the cessation group and sham-exposed mice (Fig. 2). A similar number of tumor nodules were observed between the cessation group and sham-exposed mice. Similarly, we observed significantly less tumor burden, if animals were challenged following 1 mo of smoking cessation (data not shown).

Table I. Numbers of liver metastasis in Rag2^{-/-}γc^{-/-} mice^a

Dose of MO5 Cells	Sham	Smoke	p Value
2 × 10 ⁵	38 ± 4	33 ± 8	0.59
8 × 10 ⁵	100 ± 9	113 ± 5	0.25

^a Rag2^{-/-}γc^{-/-} mice were cigarette smoke- or sham-exposed for 1 mo and infused i.v. with either 2 × 10⁵ or 8 × 10⁵ B16-MO5 cells. After 21 days, mice were sacrificed and liver tumor nodules were counted. Data represent mean ± SD (n = 4–5). No significant differences were observed following low- or high-dose B16-MO5 challenge.

Cigarette smoke and tumor immune surveillance

To investigate whether the increased tumor susceptibility in smoke-exposed animals was T and/or B cell dependent, we exposed T and B cell-deficient Rag2^{-/-} and wild type mice to cigarette smoke for 1 mo. Knockout and wild-type mice were then challenged i.v. with 1 × 10⁶ B16-MO5 cells. Following tumor challenge, animals were continuously exposed to cigarette smoke and sacrificed 21 days later. Fig. 3 shows that cigarette smoke exposed Rag2^{-/-} mice had a significantly greater lung metastatic tumor burden compared with sham-exposed Rag2^{-/-} mice, a pattern similar to that seen in wild-type mice. It is noteworthy that the number of tumor nodules was significantly lower in Rag2^{-/-} mice compared with their wild-type controls regardless of whether animals were sham or smoke exposed.

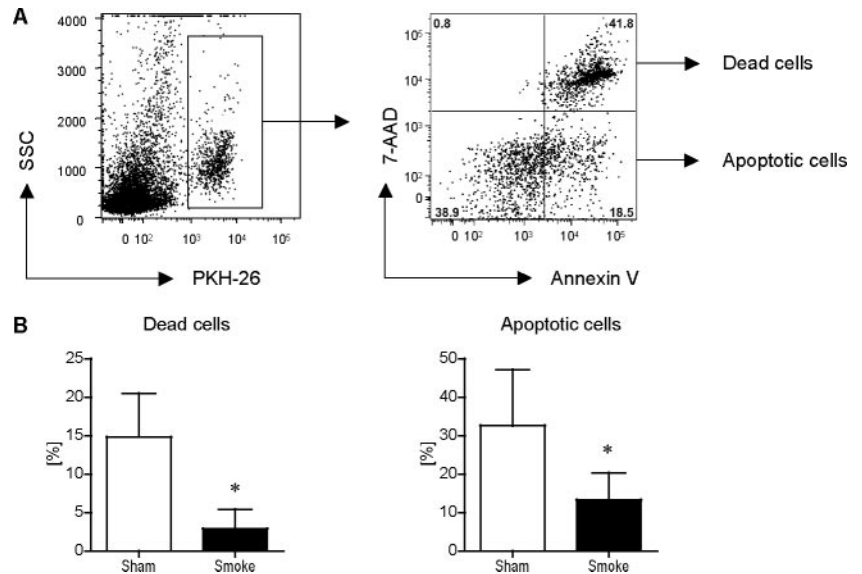
Cigarette smoke and NK-mediated tumor immune surveillance

That NK cells recognize tumor cells and are thought to aid in the elimination of B16 melanoma cells (19–21) led us to investigate whether increased tumor burden was due to deficiencies within the NK cell population. To this end, we exposed Rag2^{-/-}γc^{-/-} mice (which lack NK cells in addition to T and B cells (22)) to cigarette smoke for 1 mo. Mice were then injected with 8 × 10⁵ B16-MO5 cells and continuously exposed to cigarette smoke until the day of sacrifice (21 days posttumor injection). Fig. 4A shows similar numbers of tumor nodules in sham- and smoke-exposed Rag2^{-/-}γc^{-/-} mice, contrasting the pattern observed in wild-type or Rag2^{-/-} mice. Furthermore, Rag2^{-/-}γc^{-/-} mice had significantly greater numbers of tumor nodules compared with



FIGURE 7. CD69 expression by lung NK cells. C57BL/6 mice were sham- or cigarette smoke-exposed smoke for 2 mo. Animals were challenged with 10⁶ B16-MO5 melanoma cells via the tail vein. Twenty-four hours following tumor infusion, animals were killed and lung mononuclear cells were isolated. CD69 expression on NK1.1-positive cells was assessed by flow cytometry. Data represent mean ± SD, n = 5 (n denotes individual animals, one representative experiment of two shown), p = 0.036.

FIGURE 8. Cigarette smoke and NK cell-dependent CTL activity. C57BL/6 mice were sham or smoke exposed for 2 mo. Lung mononuclear cells were isolated and cocultured with PKH26-labeled YAC-1 target cells at 5:1 E:T ratio for 6 h. Subsequently, cells were stained with annexin V and 7-ADD. **A**, Annexin V and 7-ADD expression was assessed on gated PKH-26-stained YAC-1 cells. Apoptotic cells were defined as annexin V single positive, while annexin V and 7-ADD double positive were defined as dead cells. **B**, Data displayed show percentage of dead and apoptotic cells (mean \pm SD, $n = 4$ (n denotes individual animals, one representative experiment of three shown); *, $p < 0.05$).



wild-type mice, confirming previous reports of increased tumor susceptibility of NK-deficient animals (22, 23). To exclude the possibility that the lack of difference in tumor burden was because numbers of tumor nodules had reached a saturation plateau, smoke- and sham-exposed RAG2^{-/-} γ_c ^{-/-} mice were injected with fewer B16-MO5 cells (2×10^5). Similar to the high challenge dose, we observed equal numbers of tumor nodules between sham- and smoke-exposed animals (Fig. 4B), suggesting that increased tumor burden observed in smoke-exposed animals is due to NK cell deficiencies.

We next transferred lung mononuclear cells isolated from smoke- and sham-exposed RAG2^{-/-} donors into naive RAG2^{-/-} γ_c ^{-/-} recipients at the time of B16-MO5 tumor challenge. Recipient RAG2^{-/-} γ_c ^{-/-} animals were not exposed to cigarette smoke. Flow cytometric analysis was performed, and the number of cells delivered was adjusted to provide 8×10^5 NK cells per animal. Fig. 5 shows the increased tumor burden in RAG2^{-/-} γ_c ^{-/-} mice that received lung mononuclear cells from smoke- compared with sham-exposed RAG2^{-/-} mice. Together, the data shown in Figs. 3–5 provide strong evidence that the increased tumor burden observed in cigarette smoke-exposed mice is due to NK cell deficiencies.

To investigate potential mechanisms underlying this phenomenon, mononuclear cells were isolated from the lungs of naive smoke- and sham-exposed RAG2^{-/-} animals, and levels of activating (NKG2D) and inhibitory (Ly49A, Ly49I) receptors were analyzed by flow cytometry. No differences were observed in the frequencies of NK cells expressing either class of receptors between smoke- and sham-exposed mice (Fig. 6). Similar results were found in wild type animals (data not shown). Interestingly, we noted significantly higher frequencies of Ly49I and NKG2D-positive NK cells in the spleens of both smoke- and sham-exposed animals compared with the lungs (data not shown).

Finally, we observed the formation of liver metastases in RAG2^{-/-} γ_c ^{-/-} mice following challenge with 2×10^5 and 8×10^5 B16-MO5 cells (Table I). The number of nodules was similar between sham- and smoke-exposed animals. No liver metastases were observed in sham- or smoke-exposed WT and RAG2^{-/-} mice (data not shown).

NK cell activation and CTL activity

We next investigated whether exposure to cigarette smoke resulted in impaired NK cell activation, and whether this was associated

with compromised CTL activity. C57BL/6 mice were sham or smoke exposed for 2 mo and challenged with 1×10^6 B16-MO5 cells. 24 h following tumor challenge, expression of the early activation marker CD69 was assessed on lung NK cells (NK1.1⁺/CD3⁻). Although we observed similar numbers of lung NK cells in sham- and smoke-exposed animals (2.5 ± 0.3 and 2.4 ± 0.3 , $p = 0.63$), Fig. 7 shows decreased expression of CD69 on NK cells from smoke- compared with sham-exposed animals. Furthermore, cigarette smoke compromised CTL activity by NK cells. Decreased percentages of apoptotic (annexin 5 single positive) and dead (annexin 5 and 7-AAD double positive) YAC-1 target cells were observed following incubation with NK cells from smoke- compared with sham-exposed animals (Fig. 8).

Tumor burden in IL-12^{-/-} mice

To investigate the molecular mechanisms underlying the reduced activation and killing abilities of NK cells from smoke-exposed animals, we exposed IL-12^{-/-} mice to cigarette smoke for 1 mo, then challenged with 1×10^6 B16-MO5 cells. Fig. 9 shows that cigarette smoke-exposed IL-12^{-/-} mice had a significantly greater

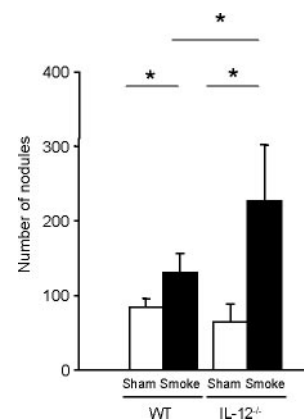
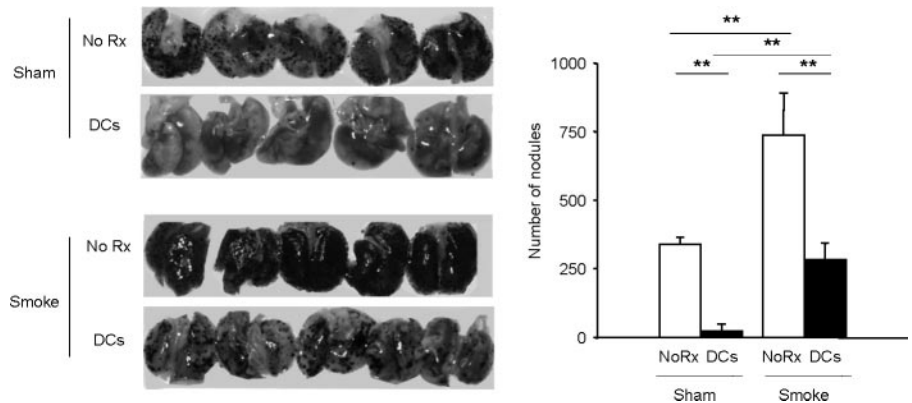


FIGURE 9. Cigarette smoke and lung metastasis formation in IL-12^{-/-} mice. WT and IL-12^{-/-} mice were smoke- or sham-exposed for 1 mo and then challenged with 1×10^6 B16-MO5 melanoma cells. Animals were sacrificed 21 days after challenge. Data show numbers of tumor nodules per lung (mean \pm SD, $n = 4$ WT, $n = 4$ IL-12, n denotes individual animals; *, $p < 0.05$; **, $p < 0.05$).

FIGURE 10. Dendritic cell treatment and metastasis formation. C57BL/6 mice were exposed to cigarette smoke for 2 mo. Smoke- and sham-exposed animals were injected i.v. with dendritic cells 7 days before challenge with 10^6 B16-MO5 via the tail vein. Seventeen days following tumor infusion, animals were killed and numbers of tumor foci were counted using a dissection microscope. Data represent mean \pm SD, $n = 5$ (n denotes individual animals); **, $p < 0.001$.



lung metastatic tumor burden compared with sham-exposed IL-12^{-/-} mice, a pattern similar to that seen in wild-type mice. Interestingly, cigarette smoke-exposed IL-12^{-/-} mice displayed a significantly greater tumor burden than smoke-exposed wild-type mice.

Cigarette smoke and dendritic cell-mediated activation of NK antitumor immunity

It has been shown that administration of syngeneic dendritic cells reduces lung tumor formation through NK-dependent mechanisms (24). Based on our observations that cigarette smoke impairs NK-mediated tumor immune surveillance, we investigated whether dendritic cell-based intervention strategies could protect cigarette smoke-exposed animals against tumor challenge. C57BL/6 mice were sham or smoke exposed for 2 mo, and infused with 1×10^6 bone marrow-derived dendritic cells 1 wk before challenge with 1×10^6 B16-MO5 cells. Although administration of dendritic cells significantly reduced the number of metastatic tumor nodules in both smoke- and sham-exposed mice, the number of tumor nodules even after dendritic cell administration was significantly greater in smoke-compared with sham-exposed animals (Fig. 10). Moreover, NK cells isolated from smoke-exposed animals displayed decreased CTL activity compared with NK cells isolated from sham-exposed animals (Table II). No differences in the number of lung NK cells were observed between sham- and smoke-exposed mice (5.3 ± 1.8 and 4.9 ± 1.2 , $n = 5$, $p = 0.32$).

Discussion

Clinical evidence and strong experimental data derived from murine tumor models holds that the immune system protects against cancers, lending credence to the concept of tumor immune surveillance (25). Although tobacco smoke impacts a wide range of immune functions, encompassing both innate and adaptive immunity (12), the consequences of compromised immune function to tumor immune surveillance are poorly understood. The objective of the present study was to investigate whether cigarette smoke impairs tumor immune surveillance and whether altered immunity

Table II. NK CTL activity^a

	Sham	Smoke	<i>p</i> Value
Dead cells (%)	41.4 \pm 7.8	19.9 \pm 10.4	0.023
Apoptotic cells (%)	36.7 \pm 6.3	23.3 \pm 7.1	0.036

^a C57BL/6 mice were cigarette smoke- or sham-exposed for 2 mo and infused i.v. with bone marrow-derived dendritic cells prior to challenge with B16-MO5 cells. Three days after tumor challenge, lung mononuclear cells were isolated, and CTL activity was assessed using labeled YAC-1 cells. Data represent mean \pm SD, $n = 3$ (one of two representative experiments is shown).

leads to an increased tumor burden in a murine model of metastatic lung cancer.

To address this question, we exposed mice to cigarette smoke using a smoke exposure system that is widely used as murine model of emphysema (17). We have previously reported on changes in the immune status in these animals. Cigarette smoke exposure decreases dendritic cell numbers in the lungs and impairs immune responses against viral and bacterial agents (18, 26). These immunological changes precede the formation of emphysematous lesions that are observed following 4–6 mo of cigarette smoke exposure (27).

To investigate whether these immunological changes leave animals more susceptible to tumors, we used a well-established experimental lung metastasis model (28). Animals were challenged i.v. with B16-MO5 cells, a B16 melanoma clone of C57BL/6 origin that are stably transfected with OVA. We observed a significantly increased metastatic tumor burden in smoke-exposed animals, if challenged following as little as 1 mo of cigarette smoke exposure. An increased tumor burden was also observed when animals were challenged following 2 and 5 mo of smoke exposure, demonstrating that increased tumor susceptibility persisted during chronic exposure. Of note, smoking cessation completely reversed the detrimental effects of cigarette smoke, providing experimental evidence that smoking cessation is beneficial and reduces tumor susceptibility.

To investigate whether smoke exposure impairs T cell-mediated tumor immune surveillance, we exposed RAG2^{-/-} mice to cigarette smoke. RAG2^{-/-} mice lack mature T, NK T, and B cells due to deficiencies in the recombination of the T and B cell receptors. We observed increased numbers of metastatic lung tumor nodules in smoke-exposed RAG2^{-/-} mice compared with sham-exposed animals, suggesting that increased tumor susceptibility is not due to altered B, T, or NK T cell function. Remarkably, RAG2^{-/-} mice displayed fewer nodules (144.8 ± 19.2) compared with wild-type animals (223 ± 11.8) despite deficiencies in T and B lymphocytes. This observation is in agreement with previous reports and may be a consequence of the increased proportion of NK cells in RAG2^{-/-} mouse strain (29). Indeed, numerous studies have demonstrated that NK cells have powerful antitumor activity and that a lack of NK cells is associated with increased cancer susceptibility (8, 19, 30–33). Our data further indicate that NK T cell-mediated tumor protection is redundant under our experimental conditions. Specifically, tumor immune surveillance is intact in RAG2^{-/-} animals, a mouse strain that is deficient in NK T cells, but sufficient in NK cells.

To assess whether increased metastasis formation in smoke exposed animals is due to defects in NK cell tumor immune surveillance, we challenged smoke-exposed RAG2^{-/-} γ_c ^{-/-} mice with

B16-MO5 melanoma cells. In addition to T and B cell deficiencies, this mouse strain also lacks NK cells. We observed similar numbers of lung metastatic nodules in cigarette smoke- and sham-exposed RAG2^{-/-} γ_c ^{-/-} mice. The fact that removal of NK cells from our experimental system obviates the effects of smoke on tumor burden provides evidence that cigarette smoke impairs NK cell-mediated tumor surveillance. This was corroborated in transfer experiments, in which lung mononuclear cells isolated from sham- and smoke-exposed RAG2^{-/-} mice were transferred into RAG2^{-/-} γ_c ^{-/-} recipients. Greater tumor burden was observed in RAG2^{-/-} γ_c ^{-/-} mice receiving lung mononuclear cells isolated from smoke- compared with sham-exposed animals.

NK cells are predominantly compartmentalized in the vascular system. In response to inflammatory stimuli, NK cells are mobilized from the bone marrow, subsequently infiltrating into the hepatic and pulmonary parenchyma (34). Numbers of NK cells were similar between sham- and smoke-exposed animals, suggesting that cigarette smoke impairs neither NK cell mobilization, nor transmigration, nor survival, because interference with any of these events would result in decreased numbers of NK cells in the lungs. Of note, cigarette smoke attenuated NK cell activation and suppressed NK-dependent CTL activity, suggesting that the increased tumor burden in smoke-exposed animals is due to impaired NK cell effector function. That expression of the inhibitory NK cell receptors Ly49A and Ly49I, as well as the activating receptor NKG2D, was unchanged between smoke- and sham-exposed animals may indicate that cellular signaling events downstream of these receptors are affected by cigarette smoke, resulting in reduced antitumor activity. Alternatively, given the large array of activating and inhibitory receptors expressed by NK cells, smoke may have a direct effect on the expression of receptors other than the ones we have examined here.

We observed the formation of liver metastasis in both sham- and smoke-exposed RAG2^{-/-} γ_c ^{-/-} animals. This predisposition to metastatic tumor formation in multiple organs is in agreement with a previous report showing that depletion of NK cells using an anti-NK1.1 Ab was associated with liver metastasis formation (23). The absence of liver metastases in smoke-exposed wild-type and RAG2^{-/-} mice suggests that effect of cigarette smoke on NK cells was lung specific as opposed to the global deficit seen in knockout (Table I) or depletion models (23).

IL-12 is a heterodimeric cytokine that is produced primarily by APCs (35). IL-12 induces proliferation and activation of NK cells. That we observed an increased tumor burden in smoke- compared with sham-exposed IL-12^{-/-} mice suggests that the effect of cigarette smoke is not a result of impaired activation of NK cells through IL-12-dependent mechanisms. In contrary, our data suggest that IL-12 confers protection in smoke-exposed wild-type mice; we observed a greater susceptibility of smoke-exposed IL-12^{-/-} mice compared with smoke-exposed wild-type mice.

It is well-established that dendritic cells activate both cytotoxic and IFN- γ -secreting NK cells. Given the potent antitumor activity of NK cells, dendritic cell-mediated activation of NK cells has been used as an antitumor immunotherapy (36–38). Because our data suggest that cigarette smoke impairs NK cell-mediated tumor immune surveillance, such an intervention strategy may not be feasible in the context of cigarette smoke. To this end, we transfused smoke- and sham-exposed mice with dendritic cells before tumor challenge. Dendritic cells conferred strong protection in sham-exposed animals; mice were almost tumor free. Although dendritic cells significantly curtailed the tumor burden in smoke-exposed animals, these animals had greater tumor burdens than their sham-exposed controls in both the presence and absence of exogenously delivered dendritic cells. This finding is in agreement

with our observation that cigarette smoke impairs NK-mediated antitumor immunity and suggests that preclinical validation of immune-based interventions should be performed in the context of cigarette smoke exposure.

The effect of cigarette smoke on the immune system and its implications for smoking-related diseases has been largely ignored and is an understudied area. Our findings clearly show that altered NK cell activity is associated with increased tumor burden mice. This is of clinical relevance, because it has been shown that NK cell CTL activity is suppressed in smokers (13, 14). Consequently, while it is well-established that carcinogens contained within tobacco smoke lead to cell transformation and cancer formation, we postulate that the effect of cigarette smoke on the immune system further increases the risk for cancer development in smokers.

In summary, we show that cigarette smoke exposure increases tumor burden in a murine model of lung metastasis. We provide strong evidence that the detrimental effects of cigarette smoke on tumor immune surveillance are due to weakened NK cell activity. Perhaps the most striking finding of this study is that smoking cessation completely reversed the detrimental effects of cigarette smoke, suggesting that the impact of cigarette smoke on NK cells is reversible. This finding provides experimental credence to campaigns encouraging smokers to quit. Our data also suggest that immune-based intervention strategies must be accompanied by strong efforts to quit smoking before the onset of therapy. To our knowledge this is the first experimental evidence showing that smoking cessation could significantly restore tumor immune surveillance and should serve as impetus for the continued promotion of smoking cessation to reduce the risk of the development of smoking-related diseases.

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Disclosures

The authors have no financial conflict of interest.

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