Early Manifestations of NNK-Induced Lung Cancer

Role of Lung Immunity in Tumor Susceptibility

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A strong correlation exists between smoking and lung cancer; however, susceptibility to lung cancer among smokers is not uniform. Similarly, mice show differential susceptibility to the tobacco carcinogen nitrosamine 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which produces lung tumors in A/J but not in C3H mice. Host immunity may play a role in the susceptibility to cancer, and cigarette smoke/nicotine suppresses the immune system through activation of nicotinic acetylcholine receptors (nAChRs). Mammalian lungs express $\alpha 7$ -nAChRs, and NNK is a high-affinity agonist for $\alpha 7$ -nAChRs. To examine whether NNK differentially modulates lung immunity in susceptible and resistant mouse strains, A/J and C3H mice were treated with NNK and/or immunized with sheep red blood cells. Lung tissues and RNA of treated and untreated animals were analyzed by immunohistochemistry and RT-PCR for $\alpha 7$ -nAChR and COX-2 expression. Spleen- and the lung-associated lymph node cells from control and immunized animals were assessed for immunologic responses, including anti-sheep red blood cell antibody plaque-forming cells, concanavalin A-induced T-cell proliferation, and the anti-CD3/CD28 antibody-induced rise in intracellular calcium. NNK strongly suppressed these responses in A/J but not in C3H mice. Similar NNK-induced immunologic changes were seen in another pair of carcinogen-sensitive (NGP) and relatively carcinogen-resistant (B10.A) mouse strains. Moreover, NNK stimulates a significantly higher expression of COX-2 and $\alpha 7$ -nAChRs in A/J than in C3H lungs. These results suggest that the susceptibility to chemical carcinogenesis among various mouse strains might be influenced by their immunologic response to the carcinogen.

Keywords: COX-2; lung immunity; nAChRs; NNK; tumor susceptibility

Cancer is a multifactorial disease, and genetic and epigenetic factors play a role in the susceptibility and resistance to cancer (1). Increasing evidence suggests that the host immunity, including tumor-specific T-cell responses, plays an important role in tumor growth and metastasis (2). Lung cancer is the most common fatal cancer worldwide, showing a postdiagnosis 5-yr mortality rate of $> 90\%$ (3). Lung cancer is strongly linked to tobacco smoking, and smokers have a 20- to 30-fold higher risk of developing lung cancer than never-smokers (4). However, the susceptibility to lung cancer is not uniform. The lifetime risk of a smoker of developing lung cancer is $< 15\%$ (5), and familial risk for lung cancer has been documented (6, 7). Similarly, different inbred strains of mice exhibit variable susceptibility to spontaneous and carcinogen-induced lung cancer (8, 9). The mouse strains that spontaneously develop lung cancer are also more susceptible

to chemically induced lung carcinogenesis. For example, although there is no absolute resistance to carcinogens, A/J and National Institutes of Health general purpose (NGP) mice are highly susceptible to lung carcinogens (9–13), and C3H and C57BL/6 mice are relatively resistant (8, 9, 13). The tobacco carcinogen 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induces lung tumors in A/J mice and in C3H mice but to a much lower extent in C3H mice (8, 14, 15). Besides being a potent carcinogen, NNK is a high-affinity ligand for $\alpha 7$ -nicotinic acetylcholine receptors (nAChRs) (16), which are expressed in neurons and in some non-neuronal cells, including lung cells, macrophages, and lymphocytes (16, 17; Razani-Boroujerdi and coworkers, unpublished observation). In the lung, $\alpha 7$ -nAChRs are present in airway epithelial cells, alveolar type II cells, pulmonary neuroendocrine cells, and bronchial endothelial cells (17–19). Activation of nAChRs by chronic exposure to cigarette smoke or nicotine suppresses the immune system and inhibits apoptosis (20). Pretreatment with nAChR antagonists reverses several indices of immunosuppression (21). Many tumors, including those induced by NNK, express COX-2, which has been implicated in modulating the immune response, stimulating angiogenesis, inhibiting apoptosis, and promoting tumor invasion (22).

The mechanism(s) of differential susceptibility to NNK-induced lung cancers among mouse strains is not known and may be multifactorial. In this article, we present evidence that NNK suppresses immune responses in the lung of carcinogen-sensitive mouse strains A/J and NGP but not in the relatively carcinogen-resistant mouse strains C3H and B10.A. NNK also causes a significant increase in the expression of $\alpha 7$ -nAChRs and COX-2 in the A/J lung, which might contribute to its higher susceptibility to NNK-induced lung tumorigenesis.

MATERIALS AND METHODS

Animals

Pathogen-free A/J, C3H/B6aCr, NGP, and B10 mice were purchased from NCI (Frederick, MD). Animals were housed in filter-top plastic cages and maintained in a 12/12 h light/dark cycle. Food and water were provided *ad libitum*. Lovelace Respiratory Research Institute is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (accreditation number 000,200).

Reagents

NNK was purchased from ChemSyn Labs (San Diego, CA). The $\alpha 7$ -nAChR-specific polyclonal antibody and isotype control immunoglobulins were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents, unless otherwise noted, were obtained from Sigma (St. Louis, MO).

NNK Treatment

Mice were injected intraperitoneally with three doses of NNK (100 mg/kg/d in 0.1 ml PBS) on three alternate days. Control animals received an equivalent volume of PBS. The NNK treatments produced multiple lung tumors in all NNK-treated A/J mice, but under these conditions none of the C3H mice developed visible lung tumors within 7 mo after the NNK treatment. The tumors were often visible to the naked eye as discrete round nodules; microscopically, the tumors exhibited a uniform adenomatous pattern consisting of closely packed columns of cells.

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Immunizations

To determine antibody response, mice were immunized with sheep red blood cells (SRBC) (Colorado Serum Co., Denver, CO). Animals were injected intratracheally with 1×10^8 SRBC in 100 μ l saline 6 d before they were killed and intraperitoneally with 2×10^8 SRBC in 200 μ l saline 4 d before being killed (23).

Preparation of Lung and Lymphoid Tissues

Animals were killed by isoflurane inhalation. The thoracic cavity was opened, and the trachea was exposed. The lung-associated lymph nodes (LALN) become enlarged and clearly visible along the trachea in intratracheally immunized animals (23). Spleens and LALN were quickly collected, and the right branch of the trachea was tied with a thread before the right lung lobes were removed. The lung tissue was collected on liquid nitrogen and stored at -80°C for RNA extraction. The left lung was inflated, fixed with 10% neutral buffered formalin at a constant hydrostatic pressure, sectioned, and used for immunohistochemistry and evaluation of tumor characteristics. Spleen and LALN cells were prepared as described (23). Briefly, spleens and LALN were pressed through stainless steel mesh, and red blood cells in spleen cell preparations were lysed by NH_4Cl treatment. Cells were washed three times with PBS and resuspended in complete tissue culture medium (RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 1% pen/strep).

Immunohistochemistry of $\alpha 7$ -nAChRs in Lung Sections

Sections (10 μm) of formalin-fixed and paraffin-embedded lung tissues were deparaffinized and hydrated. Antigens were retrieved by citrate treatment. Endogenous peroxides and nonspecific immune reactivities were blocked by hydrogen peroxide and normal serum, respectively. Tissue sections were incubated with anti- $\alpha 7$ -nAChR monoclonal antibody or isotype control immunoglobulin. Slides were washed, treated with biotinylated secondary antibody followed by Vectastain Elite ABC Reagent (Vector Laboratories, Burlingame, CA), and developed with the peroxide substrate (3,3'-diaminobenzidine) (Vector). After counterstaining, slides were photographed with a Nikon D X M1200F digital camera mounted on a Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan) and analyzed by MetaMorph software (Universal Imaging Corp., Downingtown, PA).

RNA Extraction and Semiquantitative RT-PCR

Lung tissues were homogenized in the presence of TRI reagent (MRC Molecular Research Center, Cincinnati, OH). Total RNA was isolated using the 1-bromo-3-chloropropane (BCP) phase separation reagent (MRC Molecular Research Center). RNA was precipitated by 2-propanol and washed with 75% ethanol. The RNA pellet was dried for a short time, resuspended in RNase-free water, and quantitated spectrophotometrically. The gene-specific primer sets for $\alpha 7$ -nAChRs and COX-2 were purchased from Sigma Genosys (<https://www.sigma-genosys.com>). The primer set for mouse $\alpha 7$ -nAChRs was as follows: sense primer (5'-GGCCAACGACTCGCAGCCGCTC-3') and antisense primer (5'-GCAGGTCCAAGGACCACCCTC-3'). The primer set for COX-2 was as follows: upstream primer (5'-CATTC TTTGCCAGCACTTCAC-3') and downstream primer (5'-GACC AGGCACCAGACCAAGAC-3'). The primer set for GAPDH was as follows: sense primer (5'-CGTATTGGGCGCCTGGTCACCAG-3') and antisense primer (5'-GTCCTTGCCACAGCCTTGGCAGC-3'). RT-PCR was performed with a Qiagen RT-PCR kit according to the manufacturer's directions. Briefly, cDNA synthesis and predenaturation were performed by one cycle of 50°C for 30 min and 94°C for 1 min. The cDNA was amplified by 35 PCR cycles (denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min), and a final extension of one cycle of 72°C for 10 min in a Thermal Cycler 9600 (Perkin Elmer). The PCR products were electrophoresed on 3% agarose gel with ethidium bromide staining for visualization. The gel was photographed, and the bands were quantified with a Bio-Rad GS-800 scanner and Quantity One software and standardized to the housekeeping gene GAPDH to present the level of gene expression.

Quantitative Real-Time PCR

Lung tissue RNA (50 ng) from C3H and A/J mice treated with NNK or vehicle for 72 h as described previously was subjected to qRT-PCR

on the ABI PRISM 7900HT Real-Time PCR System using the one-step RT-PCR master mix (Applied Biosystems, Foster City, CA). The following primers and probe sets were used for mouse and rat $\alpha 7$ -nAChRs, respectively: forward (TGC TGC TTG TGG CTG AGA TC), reverse (CTG GCG AAG TAC TGT GCT ATC AA), and probe (FAM TGC CAG CAA CAT CTG ATT CCG TGCTAMRA); and rat/mouse β -actin: forward (TTCAACACCCAGCCATGT), reverse (GTGGTACGACCAGAGGCATACA), and probe (VIC-CGTAGC CATCCAGGCTGTGTTGTCC-TAMRA). All results were derived from the linear amplification curve and normalized to β -actin. The $\Delta\Delta\text{CT}$ method was used to calculate the fold change in $\alpha 7$ -nAChR expression.

Immunoprecipitation and Western Blot Analysis of $\alpha 7$ -nAChR

Tissues were homogenized in lysis buffer containing 50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a cocktail of protease inhibitors. Supernatants were immunoprecipitated with $\alpha 7$ -nAChR-specific polyclonal antibody or the isotype control immunoglobulin. The immunoprecipitates were captured by Protein A/G agarose beads. After elution by boiling, samples were run on a 10% Tris-HCl gel along with Kaleidoscope Prestained Standards (Bio-Rad, Hercules, CA) and electrophoresed on a Midi Format Criterion Cell system (Bio-Rad) at 200 V for 45 min. The gel was blotted onto a nitrocellulose paper, and the blot was stained with Ponceu S to confirm the transfer of proteins from the gel to the paper and the equal loading of proteins in each well. The blots were probed with anti- $\alpha 7$ -nAChR monoclonal antibody and developed by alkaline phosphate-labeled second antibody. Blots were photographed and quantitated by a Fluor-S MultiImager system (Bio-Rad).

Antibody Forming Cells Assay

The anti-SRBC antibody forming cells (AFC) assay was performed as described (17). Briefly, LALN and spleen cells (2×10^5 in 100 μ l complete medium) were mixed with 20 μ l of SRBC solution (25×10^6 SRBC in PBS) and 20 μ l of guinea pig complement (Cederlane, Hornby, ON, Canada) preabsorbed on SRBC. Aliquots were distributed in duplicate on Cunningham slides and incubated for 45 min at 37°C . The AFC plaques were counted and normalized to 1×10^6 cells.

T-Cell Proliferation Assay

LALN or spleen cells (2×10^5 cells) were cultured in 0.2 ml of complete medium in the presence of various concentrations of Con A or anti-CD3 in microtiter wells. The cultures were incubated at 37°C in the presence of 5% CO_2 , and cells were harvested after 3 d by a Skatron cell harvester (Skatron Inc., Sterling, VA). Cell proliferation was assayed by pulsing the culture wells with 0.5 μCi of [^3H]-thymidine (ICN, Irvine, CA) at 18 h before harvest.

Assay for Intracellular Ionized Calcium

The intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) of LALN and splenocytes in response to stimulation with anti-CD3+anti-CD28 were determined as described (18). Briefly, cells (5×10^6) were loaded with indo-1 (Molecular Probes, Eugene, OR) and suspended in PBS containing 2% FCS, 2 mM Ca^{2+} , and 1 mM Mg^{2+} ; $[\text{Ca}^{2+}]_i$ was determined by spectrofluorometry (Deltascan Model 4000; Photon Technology International, South Brunswick, NJ). The baseline $[\text{Ca}^{2+}]_i$ of cells was recorded before the addition of antibodies, and changes in $[\text{Ca}^{2+}]_i$ were calculated as described (24).

Statistical Analysis

Data were analyzed for statistical significance by Prism Software 3.0 (Graphpad Inc., San Diego, CA) using the Student's *t* test or by two-way ANOVA. Values were considered significant at $P \leq 0.05$.

RESULTS

NNK Treatment Induces High Levels of $\alpha 7$ -nAChRs in the A/J Lung

Lung tissues from C3H and A/J mice were visualized for $\alpha 7$ -nAChR expression by immunohistochemical staining, RT-PCR,

and Western blot analysis. Figure 1A shows the immunohistochemical staining for the expression of $\alpha 7$ -nAChRs. The basal density of $\alpha 7$ -nAChRs in the lungs of age-matched A/J mice is higher than in C3H mice (Figure 1A, *left panel*). The density of $\alpha 7$ -nAChRs is dramatically upregulated in the A/J lung and lung tumors but not in C3H lung tissue at 6 mo after NNK treatment (Figure 1A, *middle panel*). The increased expression of $\alpha 7$ -nAChRs is seen in the lung Type-II and epithelial cells (Figure 1A, *right panel*). RT-PCR analysis confirmed the increased RNA expression of $\alpha 7$ -nAChRs in NNK-induced lung tissue/tumors in A/J mice. Figure 1B (*right panel*) shows data from a representative RT-PCR analysis using a control (CON) and an NNK-treated (NNK) A/J mouse. Densitometric quantitation of the RT-PCR data from four control and four NNK-treated A/J mice is shown in Figure 1B (*right panel*). To ascertain whether the increased expression of $\alpha 7$ -nAChR-specific RNA expression in NNK-treated A/J mice was accompanied with increased protein expression, lung lysates from three control and three NNK-treated A/J mice were analyzed by Western blot analysis. Figure 1C shows that $\alpha 7$ -nAChR protein was increased by NNK treat-

ment. These results suggest that NNK increases transcription and translation of the $\alpha 7$ -nAChR gene in A/J lung tissues and lung tumors.

$\alpha 7$ -nAChR Expression Is Upregulated in A/J Lungs Shortly after NNK Treatment

To determine whether the NNK-induced increased expression of $\alpha 7$ -nAChRs in the A/J lung is an early event after NNK treatment or associated with lung tumor formation, A/J and C3H mice were treated with PBS (CON) or NNK and killed 72 h and 3 wk after the treatment. Lung tissues were examined for $\alpha 7$ -nAChR expression by semiquantitative RT-PCR analysis. An aliquot of 2 μ g of lung RNA/reaction, found to be within the linear range for the RT-PCR analysis of nAChRs (Figure 2A), was used subsequently for the various samples for the RT-PCR analysis. Figure 2B shows that NNK increases the mRNA expression of $\alpha 7$ -nAChRs in both mouse strains after 72 h. However, the upregulation of $\alpha 7$ -nAChR expression is much stronger in A/J than in C3H lungs. These results were confirmed using a real-time RT-PCR analysis of the RNA lung samples (Figure 2C). Moreover, although the moderate increase in $\alpha 7$ -nAChR expression in the C3H lung waned by 3 wk after NNK treatment, the increased expression of $\alpha 7$ -nAChRs in the A/J lung remained high at 3 wk (Figure 2B) and 6 mo (Figure 1B) after the treatment. These results suggest that in the A/J lung, the receptors were sharply and permanently upregulated shortly (within 3 d) after NNK treatment, but the increased expression of the receptors in the C3H mouse was smaller and transitory.

NNK Stimulates the Expression of COX-2 in the A/J Lung

In most cancers, increased expression of COX-2 correlates with carcinogenesis and metastasis, and COX-2 blockers reduce the risk for development of many types of cancers, including lung cancer (25, 26). COX-2 inhibitors also moderate the growth of NNK-induced tumors in mice (25). To determine whether NNK induces COX-2 differentially in NNK-sensitive and NNK-resistant mouse strains, lung COX-2 expression in A/J and C3H mice was determined by RT-PCR analysis at 72 h and 3 wk after NNK treatment. Although the base (untreated) level of COX-2 was low in C3H and A/J lungs, as with $\alpha 7$ -nAChRs, NNK induced a much higher expression of COX-2 in A/J than in C3H lungs (Figure 3). Moreover, unlike A/J lungs, COX-2 expression in the C3H lungs was lost within 3 wk after NNK treatment. These results indicate that a strong and sustained COX-2 expression might be an early manifestation of NNK-induced lung carcinogenesis.

NNK Inhibits the AFC Response in A/J but Not in C3H Mice

Activation of nAChRs causes immunosuppression (20), and NNK is a potent agonist of $\alpha 7$ -nAChRs (16). To ascertain whether the increased NNK-induced expression of $\alpha 7$ -nAChRs in the A/J lung is associated with increased immunosuppression, control and NNK-treated A/J and C3H mice were immunized with SRBC, a T-cell-dependent antigen, intratracheally and intraperitoneally to facilitate antibody response in the LALN and spleen, respectively. NNK treatment strongly suppressed the anti-SRBC AFC response in the spleen and LALN cells of A/J mice (Figures 4A and 4B). On the other hand, NNK treatment did not significantly change the AFC response of C3H mice in the spleen (Figure 4C) or in the LALN cells (Figure 4D). These results suggest that NNK suppressed the humoral immunity in highly susceptible A/J but not in the resistant C3H strain.

NNK Suppresses T-Cell Proliferative Responses to Mitogens and Antigens

T cells play an important role in the resistance to tumorigenesis (2). To determine whether NNK selectively or preferentially

Figure 1. NNK treatment induces high levels of $\alpha 7$ -nAChRs in the A/J lung. (A) Lungs from three animals per group were stained immunohistochemically for $\alpha 7$ -nAChR expression. A representative set of lung sections from A/J (*top*) and C3H (*bottom*) mice, before (*left panel*) and after 27 wk of NNK treatment (*middle panel* [original magnification: $\times 10$]; *right panel* [original magnification: $\times 40$]) is shown. Notice the high level of staining in the epithelial cells and tumor cells of NNK-treated A/J lung compared with NNK-treated C3H lungs. (B) A representative RT-PCR analysis for $\alpha 7$ -nAChRs in control and NNK-treated A/J lung (*left panel*). Differences in the RT-PCR expression were quantitated by densitometry of the gels (*right panel*; $n = 4$). (C) Western blot analysis of lung lysates from control (CON) and NNK-treated A/J mice killed 27 wk after the carcinogen treatment. $\alpha 7$ -nAChR was immunoprecipitated and run on a Tris-HCl gel. Proteins were transferred on a nitrocellulose paper, and the blot was stained with Ponceau S to confirm equal loading of the receptor in each well. The blot was developed with monoclonal antibody specific for anti- $\alpha 7$ -nAChR as described in MATERIALS AND METHODS.

Figure 2. Short-term NNK exposure markedly increases $\alpha 7$ -nAChR expression in the A/J lung. (A) To establish an RNA concentration that produced a linear response in the RT-PCR for $\alpha 7$ -nAChR, different quantities of RNA (1, 2, and 4 μ g/reaction) from NNK-treated A/J mice were analyzed by RT-PCR for $\alpha 7$ -nAChR expression. An RNA concentration of 2 μ g/reaction is within the linear range, and this concentration was subsequently used for comparing the expression of $\alpha 7$ -nAChR expression in various RNA samples. (B) C3H and A/J mice (six mice per group) were treated with NNK or vehicle as described in MATERIALS AND METHODS and killed at 72 h and 3 wk after NNK treatment. RNA was isolated from various lungs and analyzed by RT-PCR for $\alpha 7$ -nAChR expression. The RT-PCR analysis is a representative set of control (CON) and NNK-treated samples from A/J and C3H mice. (C) RNA (50 ng) from C3H and A/J mice (six mice per group) treated with NNK or vehicle for 72 h was subjected to qRT-PCR as described in MATERIALS AND METHODS. Results for each sample were derived from the linear amplification curve and were normalized to β -actin. The bar graph presents the mean $\pm$ SEM of fold increase in $\alpha 7$ -nAChR expression after NNK treatment in A/J or C3H mice.

affected T-cell responses in the NNK-susceptible mice, LALN cells from NNK-treated A/J and C3H mice were cultured with the T-cell mitogen Con A. The proliferative response to Con A, an index of cell-mediated immunity, was significantly decreased in A/J but not in C3H LALN cells (Figures 5A and 5B). Similarly, T-cell proliferation by ligation of the T-cell receptor (TCR) with anti-CD3 (a model for antigen-mediated T-cell responses) was significantly decreased in NNK-treated A/J (Figure 5A) but not in C3H mice (Figure 5B). These results suggest that NNK downregulated T-cell mitogenesis and the antigen-specific T-cell responses in the sensitive strain.

NNK Suppresses the Rise in $[Ca^{2+}]_i$ in Response to TCR Ligation

Increased $[Ca^{2+}]_i$ is an early critical event in the activation of T cells through the TCR and is associated with the activation of Src-like tyrosine kinases in the TCR signaling cascade (27). To ascertain whether NNK affected T-cell function by impairing

antigen-mediated T-cell signaling pathway(s), indo-1-labeled LALN and spleen cells from control and NNK-treated A/J mice were treated with anti-CD3 + anti-CD28; the increase in $[Ca^{2+}]_i$ was quantitated by spectrofluorometry. Figure 6 shows that the increased $[Ca^{2+}]_i$ response after anti-CD3/CD28 treatment was significantly lower in the LALN (Figure 6A) and spleen cells (Figure 6B) from NNK-treated A/J mice. The NNK treatment did not significantly affect the $[Ca^{2+}]_i$ response of C3H LALN or spleen cells (not shown). Thus, NNK might affect the T-cell function in susceptible mice by impairing the component(s) of the antigen-mediated signaling pathway upstream of the antigen-induced Ca^{2+} response.

NNK Is Immunosuppressive in NGP, Another Carcinogen-Susceptible Mouse Strain

To determine whether the immunosuppressive effects of NNK would be seen in other mouse strains that are sensitive to

Figure 3. Short-term NNK exposure upregulates COX-2 expression in the A/J lung. C3H and A/J mice (six mice per group) were treated with NNK and killed at 72 h and 3 wk after NNK treatment as described in Figure 2. RNA was isolated from lung tissues and analyzed by RT-PCR for COX-2 and GAPDH expression. RT-PCR analysis of a representative set of control (CON) and NNK-treated samples from A/J and C3H mice is presented.

Figure 4. NNK treatment inhibits the anti-SRBC AFC response in the LALNs and spleens from A/J mice. A/J and C3H mice (five animals per group) were exposed to NNK and immunized with SRBC intratracheally and intraperitoneally as described in MATERIALS AND METHODS. The AFC response of the LALN and spleen cells was determined by the Cunningham-Szenberg technique and expressed as AFC/10⁶ LALN or spleen cells.

Figure 5. NNK inhibits Con A- and anti-CD3-induced T-cell proliferative responses in A/J mice. A/J and C3H mice were treated with NNK. At 72 h after the treatment, LALN cells were cultured with Con A or rat anti-mouse anti-CD3 antibody for 3 d. [³H]-thymidine was added to culture wells 18 h before cell harvesting. Results represent mean $\pm$ SEM ($n = 6$). Values were significantly different ($P < 0.05$) between control and NNK-treated A/J cells (A) but not between control and NNK-treated C3H cells (B).

carcinogen-induced lung tumorigenesis, we compared the immunologic effects of NNK in NGP (high lung tumor susceptibility) with B10.A (less sensitive to carcinogen-induced lung tumorigenesis) mice. Animals were treated with saline or NNK (see Figure 4). At 3 d after NNK treatment, animals were immunized with SRBC intratracheally, followed by intraperitoneal injection of SRBC 2 d later. Animals were killed 4 d after the second SRBC injection and analyzed for the anti-SRBC AFC and Con A- and anti-CD3-induced proliferative responses. NNK treatment significantly impaired the antibody and T-cell proliferative responses in NPG but not in B10.A mice (Figure 7). Thus, the two mouse strains (A/J and NPG) susceptible to carcinogen-induced lung cancers exhibit immunosuppression in response to NNK, but mouse strains (C3H and B10.A) that are relatively less sensitive to lung carcinogenesis do not show significant immunosuppression in response to NNK.

DISCUSSION

The susceptibility to cancer development is a multifactorial process (1); however, increasing evidence indicates that the immune system plays an important role in the control of malignancy. The immune surveillance theory suggested that tumors arise spontaneously in normal individuals but are eliminated by immune mechanisms, and "immunological failure" may promote tumor development. Patients with breast and lung cancer have

Figure 6. NNK inhibits the CD3/CD28-induced rise in [Ca^{2+}]_i in A/J mice. Indo-1-labeled LALN (A) and spleen (B) cells from control (CON) and NNK-treated A/J mice were treated with rat anti-mouse CD3 monoclonal antibody followed by hamster anti-mouse CD28 antibody. Changes in the [Ca^{2+}]_i were analyzed by fluorometry. The graph is representative of the [Ca^{2+}]_i response in cells from six different control and NNK-treated mice.

Figure 7. NNK treatment inhibits the anti-SRBC AFC and Con A-induced proliferative response in LALN and spleen cells from NGP mice. NGP and B10.A mice (six animals per group) were exposed to NNK for 72 h and immunized with SRBC intratracheally and intraperitoneally as described in MATERIALS AND METHODS. The anti-SRBC AFC response is expressed as AFC/10⁶ LALN or spleen cells and the proliferative response mean cpm $\pm$ SEM. The AFC responses of LALN and spleens from NGP and B10.A are shown in A and B, respectively. The mitogenic responses to Con A (1 $\mu\text{g}/\text{ml}$) and anti-CD3 (1 $\mu\text{g}/\text{ml}$) of NGP and B10.A spleen cells are shown in C and D, respectively.

shown defective immune responses (28). It has been demonstrated in animal models that chemically induced tumors are strongly rejected in syngeneic hosts by preimmunization with tumor antigens (29, 30). Similarly, there is convincing evidence for tumor immunogenicity and immune surveillance in virally induced neoplasms (31). The importance of the immune system in cancer therapy has been recently underscored by the use of dendritic cells (DCs) in cancer vaccines (32), and successful immunization with tumor antigen-presenting DCs or genetically modified DCs has been reported in animal models and clinical trials (33, 34).

In a variety of human cancers, the presence of tumor-infiltrating T lymphocytes is associated with tumor regression and favorable prognosis (35). On the other hand, immunosuppression accelerates tumor growth; for example, the incidence of lung cancers has increased dramatically among relatively young HIV-1-positive smokers (36). Moreover, T-cell cytokines such as IL-12 along with overexpression of the costimulatory molecule B7 have boosted cytotoxic T cells against preexisting tumors (37). Thus, although other factors, including carcinogen metabolism, DNA hypermethylation, oncogene expression, and cell-cycle defects, play an important role in tumorigenesis (38, 39), effects of carcinogens on T-cell-mediated immunity may contribute to overall tumor growth and metastases (2, 40). Immunotherapy directed to improve T-cell responses is a promising approach to manage malignancies (41, 42).

NNK is a potent tobacco-specific carcinogen that requires activation by the cytochrome P450 enzyme system for its tumorigenic activity, whereas inhibitors of the enzyme system reduce its lung tumorigenesis (43). However, NNK is also a strong agonist of $\alpha 7$ -nAChRs (16), and these receptors are present on neuronal and non-neuronal cells, including leukocytes (17; Razani-Boroujerdi and coworkers, unpublished) and the lung (17–19). The activation of nAChRs modulates cell proliferation, differentiation, and survival (44, 45) and leads to immunosuppression (20). Activation of nAChRs also promotes angiogenesis, and pharmacologic or genetic disruption of $\alpha 7$ -nAChRs significantly reduces angiogenesis and tumor growth (46).

The susceptibility to carcinogen-induced lung tumor formation is not uniform among various inbred mouse strains (9). For example, chronic NNK exposures (three times per week for 7 wk) induces lung tumors in A/J mice within 8 mo after NNK treatment (14, 15), whereas a similar NNK treatment induces lung tumors in a much smaller percentage of C3H at 17 to 19 mo post NNK exposure (14, 15), indicating that tumor incidence and latency are significantly different in these two mouse strains. In general, inbred strains susceptible to spontaneous development of lung tumors are also sensitive to a variety of chemical carcinogens (47), and A/J and NGP strains are highly sensitive to developing lung tumors in response to multiple chemical carcinogens tested to date (9, 10, 12, 13).

Activation of nAChRs causes immunosuppression (20), and NNK is a strong agonist of these receptors. We speculated that differences in tumorigenesis might in part relate to differences in the immunologic response of the sensitive and resistant mouse strains to NNK. Therefore, we selected mice that are highly sensitive (A/J and NGP) and relatively resistant (C3H and B10.A) to chemical carcinogens (9, 48) and determined their immunologic status after NNK treatment. Because the lung immune responses are initiated in the LALN and do not necessarily overlap with systemic (e.g., spleen) immunity (23), we analyzed the immune responses of LALN and spleen cells. Our results show that NNK treatment decreases T-cell mitogenesis and T-dependent antibody responses in the spleen and LALN of A/J but not C3H mice. Similarly, antigen-mediated T-cell proliferation in response to TCR ligation is inhibited only in A/J but not C3H mice. Moreover, it seems that NNK affects the TCR-linked signaling process that results in a significantly reduced rise in $[Ca^{2+}]_i$ in A/J T cells. These studies indicate that NNK is a potent immunosuppressant and affects T-cell function through the TCR-mediated signaling upstream of the Ca^{2+} response. T-cell immunity is an important component in tumor resistance and regression (41). Thus, by selective suppression of T-cell responses, NNK may promote tumorigenesis in susceptible animals.

Compared with C3H, NNK treatment strongly upregulates the expression of $\alpha 7$ -nAChRs in the A/J lung. Although the increased NNK-induced expression of the receptors in the A/J lung and lung tumors is indefinitely sustained at high levels, the increased expression of the receptors in C3H is temporary and is lost within 3 wk after NNK treatment. Similarly, the expression of COX-2 is significantly elevated within 3 d after NNK treatment, and the expression is much higher in the A/J than in the C3H lung. Also, as with $\alpha 7$ -nAChRs, A/J lungs retain the increased COX-2 expression, but that expression in C3H lungs is lost within 3 wk after NNK treatment. COX-2 is a well established marker for many types of cancer cells, including NNK-induced lung tumors (22, 49), and COX-2 inhibitors have shown promise in suppressing tumorigenesis in humans and animal models (50). Increased COX-2 expression is associated with tumor proliferation, tumor invasion, angiogenesis, and resistance to apoptosis (22, 51). Decreased apoptosis is a hallmark of tumor growth, and COX-2 expression has stabilized survivin, an inhibitor of apoptosis (52). Expression of COX-2 also has significant implications on the immune system. COX-2 is important in the production of prostaglandin (PG) E_2 produced by a wide variety of malignancies, including lung cancers. PGE $_2$ affects DC function, and recent evidence suggests that DCs cultured in the presence of PGE $_2$ -producing lung cancer cells exhibit a dramatic decrease in their migration through the extracellular matrix (53). Decreased migration is likely to impair the ability of DCs to reach tumor cells and present tumor antigens to activate antitumor T-cell immunity (54). Although we have not tested whether COX-2 inhibitors affect NNK-induced changes in $\alpha 7$ -nAChR

expression, we have shown that nicotine stimulates COX-2 and PGE $_2$ in human Jurkat T cells, and the effects are significantly blunted by $\alpha 7$ -nAChR knockdown by siRNA (S. P. Singh and coworkers, unpublished results). We believe a similar relationship might exist between $\alpha 7$ -nAChRs and COX-2 in NNK-treated animals. The increased and sustained expressions of $\alpha 7$ -nAChRs and COX-2 might serve as early biomarkers for lung tumorigenesis; some of the NNK-induced changes in the susceptible lung might start early, stay longer, and promote lung tumorigenesis.

Many non-neuronal cells express nAChRs, and activation of these receptors by nicotinic agonists inhibits cytokine/chemokine production by these cells, which are important in innate immunity (55). The paucity of chemokines/cytokines may affect leukocyte migration, an important factor in tumor surveillance. Thus, the NNK-induced overexpression of $\alpha 7$ -nAChRs might impair the innate and adaptive immunities and may facilitate tumor growth. In addition to NNK, A/J mice are also sensitive to a number of other carcinogens (e.g., urethane). $\alpha 7$ -nAChRs seem to bind compounds that are not structurally typical nAChR agonists, such as the β -amyloid peptide Abeta (56). Our preliminary experiments show that urethane also upregulates $\alpha 7$ -nAChRs in the lung cells (S. Razani-Boroujerdi and colleagues, unpublished results). Although the mechanism by which various lung carcinogens upregulate $\alpha 7$ -nAChRs in the lung is not known, the receptors could be targets for exogenous (e.g., cigarette smoke/nicotine) and endogenous compounds (e.g., acetylcholine). In that context, pulmonary neuroendocrine cells, small-cell lung carcinoma cells, malignant pleural mesothelioma cells, and bronchial epithelial cells express $\alpha 7$ -nAChRs and secrete acetylcholine and serotonin (57, 58), which act as autocrine growth factors for these cells. Moreover, NNK stimulates the release of these growth factors from normal lung and from lung cancer cells (58). Thus, $\alpha 7$ -nAChRs may be an important target for NNK-induced immunosuppression and an important nicotinic receptor subtype for acetylcholine that is the natural *in vivo* ligand for nAChRs and an autocrine growth factor for tumor cells (57, 58).

Conflict of Interest Statement: Neither author has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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