SHORT COMMUNICATION

Nontypeable *Haemophilus influenzae*—Promoted Proliferation of Kras-Induced Early Adenomatous Lesions Is Completely Dependent on Toll-Like Receptor Signaling

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Chronic obstructive pulmonary disease (COPD) is a risk factor for lung cancer. COPD is characterized by chronic airway inflammation and lung infections. The airways of patients with COPD are frequently colonized with bacteria [eg, nontypeable *Haemophilus influenzae* (NTHi)] that cause pulmonary inflammation and exacerbations. Pulmonary adenocarcinomas are frequently associated with an activating mutation in the *KRAS* gene. We determined the function of Toll-like receptor (TLR) signaling on the progression of Kras-induced early adenomatous lesions in the lung. Wild-type (WT) mice and mice doubly deficient in Tlr-2 and -4 (*Tlr2/4*^{-/-}), both with an oncogenic *Kras* allele in lung epithelium, were exposed to NTHi for 4 weeks. Exposure to NTHi resulted in increased tumor proliferation and growth in WT mice, but not in *Tlr2/4*^{-/-} mice. Alveolar adenomatous hyperplasia and adenocarcinoma were significantly increased in WT mice compared with *Tlr2/4*^{-/-} mice. The average size of tumors was significantly larger in WT mice, whereas there was no difference in the number of alveolar lesions between WT and *Tlr2/4*^{-/-} mice. NTHi-induced pulmonary neutrophilic inflammation and tumor-associated neutrophils were reduced in *Tlr2/4*^{-/-} mice. Thus, subsequent to a driver mutation, NTHi-induced inflammation promotes proliferation of early adenomatous lesions in a TLR-dependent manner. (*Am J Pathol* 2017; 187: 973–979; <http://dx.doi.org/10.1016/j.ajpath.2017.01.003>)

Lung cancer is one of the deadliest human cancers and accounts for >1 million deaths per year worldwide.¹ Cigarette smoke (CS) exposure is the most important risk factor for lung cancer and chronic obstructive pulmonary disease (COPD).^{2–4} Forty percent to 70% of lung cancer patients have a coexisting COPD.^{2,5,6} The airways of COPD patients are frequently colonized with bacteria [eg, nontypeable *Haemophilus influenzae* (NTHi)] that cause pulmonary inflammation and exacerbations associated with increased neutrophilic inflammation.^{3,7,8}

Approximately 25% of lung adenocarcinomas are associated with an activating mutation in the *KRAS* gene.^{9–11} Mostly affected by mutations in *KRAS* are smokers. However, *KRAS* mutations are also present in lung

adenocarcinomas in patients who have never smoked.⁹ To study Kras-driven tumorigenesis, Johnson et al¹² generated mouse strains that develop lung cancer due to spontaneous activation of oncogenic *Kras* alleles in the whole animal. In these mice, lung tumors progress through different morphologic stages, such as mild hyperplasia and alveolar adenomas.¹² Using these mice, Takahashi et al¹³ demonstrated that long-term exposure to CS promotes lung cancer development. Studies in a murine lung cancer model with an activated oncogenic *Kras* allele in Clara cell secretory protein-expressing cells have shown that aerosolized NTHi

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increases lung tumor burden and that neutrophils promote NTHi-induced tumor growth.^{14–16}

Here, we demonstrate that NTHi-promoted progression of adenomatous lesions depends entirely on Toll-like receptor (TLR) signaling.

Materials and Methods

Kras-Induced Lung Cancer Model

The protocols of all of these animal experiments were approved by the Landesamt für Soziales, Gesundheit, und Verbraucherschutz (Hamburg, Germany; reference number 42/2013) following the national guidelines for animal treatment. Eight- to 10-week-old female wild-type (WT) mice with an oncogenic *Kras* allele (*Kras*^{LA1})¹² and *Kras*^{LA1} mice doubly deficient in Tlr-2 and -4 (*Tlr2/4*^{−/−}) were exposed to a clinical isolate of heat-inactivated NTHi 3 days/week. C57BL/6 *Tlr2/4*^{−/−} mice^{17–19} were a gift from Prof. Dr. Markus Schnare (Institute for Immunology, Philipps-University Marburg, Marburg, Germany). C57BL/6 KRas mice from our own breeding were crossed with C57BL/6 *Tlr2/4*^{−/−} mice from our own breeding, resulting in KRas mice heterozygous for *Tlr2* and -4. These mice were crossed to obtain KRas *Tlr2/4*^{−/−} mice. Breeding pairs of KRas *Tlr2/4*^{−/−} mice were set to obtain sufficient mice for the experiments. KRas *Tlr2/4*^{−/−} mice were used in second to fourth generations. NTHi were grown on selective chocolate agar with IsoVitalX (Becton, Dickinson and Company, Heidelberg, Germany) at 300 μL per 10-cm plate for 24 hours at 37°C in 5% CO₂, harvested and incubated for 24 hours in 500 mL of brain-heart infusion broth (Becton, Dickinson and Company) containing 3.5 μg/mL NAD (Sigma-Aldrich, Hamburg, Germany) and 2% Difco Supplement B (Becton, Dickinson and Company). The culture was centrifuged at 2500 × *g* for 15 minutes at 4°C, washed and resuspended in 20 mL of phosphate-buffered saline, heat-inactivated at 70°C on a mechanical shaker for 45 minutes, and sonicated three times for 30 seconds. The protein concentration was adjusted to 2.5 mg/mL in phosphate-buffered saline using the Pierce BCA protein assay (Thermo Fisher Scientific Inc., Rockford, IL). Mice were placed in a plexiglass box connected to a Pari Master nebulizer (Pari GmbH, Starnberg, Germany) and exposed to bacterial lysate for 40 minutes, three times a week.

Histopathology

All histologic analyses were performed on formalin-fixed and paraffin-embedded sections. Briefly, lungs were fixed by instillation of phosphate-buffered saline—buffered 4% formalin under a constant hydrostatic pressure of 30 cm for 15 minutes and placed in phosphate-buffered saline—buffered 4% formalin. The fixed lungs were embedded in 1% agarose and cut into regular slices of identical thickness and embedded in paraffin. Paraffin sections were stained with hematoxylin

and eosin. The sizes of the individual lesions and tumor areas from total lung area on hematoxylin and eosin—stained slides were calculated using cellSens Dimension software version 1.5 (Olympus Corp., Shinjuku, Japan) and Visiopharm Integrator System software version 4.2.7.0 (Visiopharm, Hørsholm, Denmark). The lesions were classified as alveolar hyperplasia, alveolar adenomatous hyperplasia, or adenocarcinoma. *Alveolar hyperplasia* was defined as an increase in cells along the interalveolar septa in the alveolar space. Alveolar hyperplasia developed from single-layered lesions into *alveolar adenomatous hyperplasia*, characterized by a further increase in cellularity, with the structure of the alveoli still recognizable. *Adenocarcinoma* was defined as a solid, acinar, or papillary growth pattern. Cells within adenocarcinomas were characterized by a round shape and an appearance not fully differentiated.²⁰ Primary antibodies for Ki-67 (catalog number ab15580; Abcam plc, Cambridge, UK) and Ly6B (AbD Serotec, Kidlington, UK) were used for immunohistochemistry analysis, as described previously.²¹ Imaging was performed using cellSens Dimension software version 1.5 (Olympus Corp.).

Determination of Inflammatory Cells

Bronchoalveolar lavage (BAL) fluids were obtained from mice as described previously.²² Percentages of leukocyte subpopulations were determined by counting 100 leukocytes in a randomly selected portion of the cytopsin slide. Numbers of inflammatory cells in the BAL fluid were determined by using a hemocytometer (Innovatis AG, Reutlingen, Germany). The concentration of lactate dehydrogenase in BAL fluid was determined using a lactate dehydrogenase—Cytotoxicity Assay Kit according to the manufacturer's instructions (catalog number ab65393; Abcam). Mouse albumin concentration in BAL fluid was determined using a Mouse Albumin Enzyme-Linked Immunosorbent Assay Quantitation Set (Bethyl Laboratories, Montgomery, TX).

Statistical Analysis

Statistical significance in experiments with more than two subgroups was calculated by analysis of variance (Tukey test). Results were considered statistically significant if *P* values were <0.05. All statistical tests were performed using Prism software version 5 (GraphPad, San Diego, CA).

Results

TLR Signaling Mediates NTHi-Induced Tumor Growth and Proliferation

COPD is associated with aberrant microbiota of the lung. NTHi is often found in the lungs of stable COPD patients and is the major pathogen triggering exacerbations associated with neutrophilic inflammation.³ As lung adenocarcinomas are frequently associated with an activating mutation in *KRAS*

in humans,^{9–11} we studied the effect of COPD-like inflammation on the progression of *Kras*-induced early adenomatous lesions. Therefore, WT and *Tlr2/4*^{-/-} mice, both with somatic *Kras* activation due to a spontaneous recombination event on *Kras*^{LA1},¹² were exposed to air or inactivated NTHi three times per week for 4 weeks. WT mice exposed to NTHi showed a significantly larger lung area covered by alveolar lesions compared with that in air-exposed control mice (Figure 1A). The NTHi-induced tumor growth was completely absent in the *Tlr2/4*^{-/-} mice. The deficiency in Tlr-2/4 did not affect tumor growth in air-exposed mice. Lung

tumors carrying an oncogenic *Kras* allele progress through different morphologic stages, such as mild hyperplasia and alveolar adenomas in mice.^{12,20} We analyzed the outcome of NTHi-induced inflammation on the progression of adenomatous lesions by differentiating alveolar hyperplasia, alveolar adenomatous hyperplasia, and adenocarcinoma (Figure 1B). Exposure to NTHi resulted in significantly increased growth of alveolar adenomatous hyperplasias and adenocarcinomas in WT mice compared with air-exposed WT mice and NTHi-exposed *Tlr2/4*^{-/-} mice (Figure 1B). We further examined proliferation of tumor cells by

Figure 1 Nontypeable *Haemophilus influenzae* (NTHi) promotes tumor growth in a Toll-like receptor (Tlr)-dependent manner in mice. **A:** Microscopic pathology (hematoxylin and eosin) and percentage of lung area covered by alveolar lesions. **B:** Percentages of lung area covered by alveolar hyperplasia, alveolar adenomatous hyperplasia (AAH), and adenocarcinoma. **C:** Tumors were examined by immunostaining with anti-Ki-67 antibodies to detect proliferating cells. Data are expressed as means $\pm$ SEM. $n \geq 6$ per group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars: 550 μ m (A); 100 μ m (B and C). WT, wild type.

immunostaining for Ki-67 (Figure 1C). The numbers of Ki-67–positive cells were significantly increased in tumors in WT mice exposed to NTHi. Exposure to NTHi did not increase tumor proliferation in *Tlr2/4^{-/-}* mice. There were no significant differences in the numbers of Ki-67–positive epithelial cells in the parenchyma (data not shown). No tumors could be detected in other organs, such as the liver and colon, in 12- to 14-week-old mice.

TLR Deficiency Affects Tumor Size

We further analyzed the sizes of individual alveolar lesions. Exposure to NTHi resulted in a significantly increased average size of alveolar lesions in WT mice, whereas NTHi exposure did not affect the mean size of alveolar lesions in *Tlr2/4^{-/-}* mice (Figure 2A). We also analyzed the sizes of cancerous lesions by evaluating the fraction of small lesions (<0.25 mm²), medium lesions (0.25 to 0.75 mm²), and large lesions (>0.75 mm²) (Figure 2, B–D). The fraction of small lesions was significantly increased in NTHi-exposed *Tlr2/4^{-/-}* mice compared with NTHi-exposed WT mice (Figure 1B), and the fraction of medium lesions was significantly increased in the lungs of WT mice exposed to NTHi compared with *Tlr2/4^{-/-}* mice (Figure 2C). There were no significant differences in the fractions of large lesions between the different groups (Figure 2D). Exposure to NTHi resulted in increased numbers of alveolar lesions. However, there were no significant differences in the numbers of alveolar lesions between NTHi-exposed WT and *Tlr2/4^{-/-}* mice (Figure 2E). These data further indicate that, in our disease model, deficiency in Tlr-2/4 results in reduced tumor proliferation and growth and not in decreased tumor initiation.

NTHi-Induced Neutrophilic Inflammation Depends on TLR-2/4 Signaling

Inflammation is suggested to promote the growth of lung cancers.^{13,21,23,24} Exposure of WT mice to NTHi resulted in increased numbers of inflammatory cells, mainly neutrophils, in BAL fluids (Figure 3, A–C). There were no significant increases in the numbers of inflammatory cells in BAL fluids in *Tlr2/4^{-/-}* mice after exposure to NTHi (Figure 3A). The NTHi-induced influx of neutrophils into the lung was strongly reduced in *Tlr2/4^{-/-}* mice compared with WT mice (Figure 3B). Exposure of WT mice to NTHi resulted in increased numbers of macrophages in BAL fluids (Figure 3C). We also detected tumor-associated neutrophils by immunostaining. Figure 3D shows that numbers of tumor-associated neutrophils were significantly reduced in NTHi-exposed *Tlr2/4^{-/-}* mice compared with NTHi-exposed WT mice. To determine loss of pulmonary barrier integrity and lung damage, we measured the albumin and lactate dehydrogenase content in BAL fluids. Exposure to NTHi resulted in significantly increased concentrations of albumin (Figure 3E) and lactate dehydrogenase (Figure 3F) in BAL fluids in WT mice compared with *Tlr2/4^{-/-}* mice.

Figure 2 Deficiency in Toll-like receptor (TLR)-2/4 results in smaller tumors in mice. **A:** Average sizes of individual tumor lesions. **B–D:** Adenomatous lesions were analyzed by evaluation of small lesions (<0.25 mm²; **B**), medium lesions (0.25 to 0.75 mm²; **C**), and large lesions (>0.75 mm²; **D**). **E:** Numbers of lesions per square millimeter of lung. Data are expressed as means $\pm$ SEM. $n \geq 34$ tumors per group. ** $P < 0.01$, *** $P < 0.001$. NTHi, nontypeable *Haemophilus influenzae*; WT, wild type.

Discussion

COPD patients have an increased risk for lung cancer and are frequently colonized with bacterial pathogens that perpetuate ongoing inflammation of the lung.^{3,6,25} In

Figure 3 Nontypeable *Haemophilus influenzae* (NTHi)-induced neutrophilic inflammation depends on Toll-like receptor (Tlr)-2/4 in mice. **A–C:** Numbers of total inflammatory cells (**A**), neutrophils (**B**), and macrophages (**C**) in bronchoalveolar lavage fluid (BALF). **D:** Immunohistochemistry analysis and quantification of Ly6B expression in lung tumors. **E and F:** Concentrations of serum albumin (**E**) and lactate dehydrogenase (LDH) (**F**) in BALF. Data are expressed as means $\pm$ SEM. $n \geq 6$ per group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars = 100 μ m. WT, wild type.

particular, NTHi colonizes in stable COPD patients and causes exacerbations associated with neutrophilic inflammation.³ Therefore, it is important to understand whether and how bacteria contribute to the development and progression of lung cancer.

As adenocarcinomas are frequently associated with an activating mutation in *KRAS* in humans,^{9–11} we decided to study the effect of NTHi on the tumor proliferation and growth in a *KRAS*-dependent lung cancer model. We found that mice exposed to NTHi showed significantly larger lung areas covered by alveolar lesions compared with those in air-exposed control mice. Remarkably, the tumor-promoting effect of NTHi was completely dependent on Tlr signaling, as NTHi did not increase tumor proliferation and growth in *Tlr2/4*^{-/-} mice, and deficiency in *Tlr-2/4* did not affect tumor growth in air-exposed mice. Moreover, the average tumor size was significantly larger in a Tlr signaling-dependent manner after exposure to NTHi. Thus, our data indicate that NTHi promotes the proliferation and growth of *Kras*-induced early adenomatous lesions in a TLR-dependent manner once a driver-mutation occurs. However, our data do not prove that the effect of TLR-2/4 deficiency on NTHi-induced tumor proliferation and growth is necessarily linked to an activating mutation in *KRAS*. The tumor-promoting function of TLR signaling also might be relevant in *KRAS* WT tumors.

Our findings are consistent with those from studies showing that exposure to NTHi results in increased tumor numbers in lung cancer models in which an oncogenic *KRAS* allele is activated in Clara cell secretory protein-expressing cells.^{14,15} Using a *Kras*-dependent lung cancer model, Chang et al¹⁵ demonstrated that COPD-like inflammation induced by NTHi accelerates tumor growth and that type 17 helper T cells infiltrating into tumor tissue are crucial for lung cancer development. In addition, we have shown that COPD-like inflammation promotes metastatic growth in an IL-17A-dependent manner.²¹ In our model of pulmonary inflammation, exposure to NTHi resulted in neutrophilic lung inflammation that was dependent on Tlr signaling. Recent studies have shown a function of neutrophils in lung cancer development. El Rayes et al²⁶ showed that pulmonary inflammation induced by intranasal instillation of lipopolysaccharides results in enhanced metastatic growth mediated by neutrophil-derived proteases. Using a *Kras*-driven lung cancer model, Koyama et al²⁷ showed that tumor-associated neutrophils can display T cell-suppressive effects. As a dramatically reduced influx of neutrophils into the lungs of NTHi-exposed *Tlr2/4*^{-/-} mice was associated with a complete absence of NTHi-induced tumor proliferation and growth, our data suggest that pulmonary neutrophilic inflammation evoked by bacteria may promote cancer progression in the lung. Our data are consistent with those from a study by Gong et al¹⁶ in a *Kras*-dependent lung cancer model

that showed that the tumor-promoting effects of NTHi are dependent on the presence of neutrophils, the specific neutrophil receptor chemokine (C-X-C motif) receptor 2, and neutrophil elastase. We found reduced numbers of neutrophils in the tumor microenvironment in Tlr-2/4-deficient mice. Therefore, TLR-dependent recruitment of neutrophils into the tumor microenvironment likely promotes tumor proliferation during COPD-like inflammation. However, as TLR signaling acts upstream of a variety of inflammatory mediators and contributes to the activation of many inflammatory cells, it is likely that deficiency in TLR-2/4 also affects additional tumor-promoting mechanisms, such as the development of tumor-associated IL-17-expressing immune cells.

As there is a strong connection between smoking and COPD, it is of interest how smoking relates to NTHi-induced inflammation and TLR activation. A study by Doz et al²⁸ revealed a function for TLR-4 in CS-induced pulmonary inflammation. Pulmonary neutrophilic inflammation was significantly reduced in mice deficient in Tlr-4, myeloid differentiation primary response 88 protein, or Il-1R1 in a model of acute CS exposure.²⁸ In addition, the Tlr-4 inhibitor TAK-242 decreased pulmonary inflammation in a mouse model of acute CS exposure.²⁹ Mouse studies also have shown that NTHi exacerbates CS-induced neutrophilic inflammation.^{8,30} However, it is controversial whether CS increases or dampens TLR signaling in different cell types. It has been shown, for instance, that CS increases the expression of TLR-4 and the release of IL-8 induced by lipopolysaccharide or Gram-negative bacteria in airway epithelial cells *in vitro*.^{31,32} However, other *in vitro* studies have shown that CS extract also inhibits the NTHi-induced epithelial expression of IL-8.^{33,34} Alveolar macrophages obtained from mice exposed to CS for 8 weeks did not show any difference in the expression of Tlr-4 but produced fewer inflammatory cytokines, such as tumor necrosis factor- α and Il-6.³⁵ Additional studies are needed to clarify the function of TLR signaling in tumor growth in the context of CS-induced pulmonary inflammation.

In summary, our data further strengthen the concept that pulmonary inflammation induced by bacteria accounts for the poor outcome of lung cancer in COPD.³⁶ Lung cancer patients may benefit from the elimination of bacterial pathogens from the lung. In addition, reducing pulmonary and tumor-associated inflammation by therapeutic intervention in TLR-signaling cascades with the help of TLR antagonists might improve disease outcomes in lung cancer patients and support cancer therapies, such as immunotherapies with checkpoint inhibitors.

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