Cigarette smoke-induced disruption of pulmonary barrier and bacterial translocation drive tumor-associated inflammation and growth


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Jungnickel C, Wonnenberg B, Karabiber O, Wolf A, Voss M, Wolf L, Honecker A, Kamyschnikow A, Herr C, Bals R, Beisswenger C. Cigarette smoke-induced disruption of pulmonary barrier and bacterial translocation drive tumor-associated inflammation and growth. Am J Physiol Lung Cell Mol Physiol 309: L605–L613, 2015.—Microorganisms have an important role in tumorgenesis by the induction of inflammation and by a direct impact on tumor cells. Chronic obstructive pulmonary disease (COPD) is associated with an increased risk for lung cancer and microbial colonization. We asked whether bacterial pathogens act as tumor promoters during CS-induced pulmonary inflammation. In a metastatic lung cancer (LC) model, Lewis lung carcinoma (LLC) cells were injected in mice to initiate the growth of tumors in the lung. Exposure to the combination of cigarette smoke (CS) and nontypeable Haemophilus influenzae (NTHi) synergistically increased metastatic growth. Lung levels of albumin and LDH, translocation of bacterial factors into tumor tissue, tumor inflammation, and tumor proliferation were significantly increased in mice exposed to CS in combination with NTHi. Bacterial pathogens increased the proliferation of cultured LLC cells and human cancer cell lines. Metastatic growth induced by the exposure to CS in combination with NTHi was reduced in mice deficient for IL-17. Our data provide evidence that CS-induced loss of pulmonary barrier integrity allows bacterial factors to translocate into tumor tissue and to regulate tumor-associated inflammation and tumor proliferation. Translocation of bacterial factors in tumor tissue links CS-induced inflammation with tumor proliferation.

lung cancer; COPD; bacterial pathogens; lung metastasis; tumor growth; IL-17; cigarette smoke; bacterial translocation

LUNG CANCER (LC) ACCOUNTS for more than 1,000,000 deaths per year worldwide and is one of the deadliest cancer entities (5). Cigarette smoke (CS) is the most important risk factor for LC and chronic obstructive pulmonary disease (COPD). Moreover, ~40–70% of lung cancer cases have coexisting COPD. Its presence is an important risk factor for LC in smokers (1, 33, 34). COPD is characterized by chronic pulmonary inflammation. Bacterial pathogens, which frequently colonize stable COPD patients and cause exacerbations, contribute to the development of COPD by amplifying pulmonary inflammation (28, 29).

Preclinical and clinical studies suggest that there is a causal relation between inflammation and LC development (12, 14, 15, 22, 31, 33). Studies showed that CS-induced and tumor-associated inflammation in the tumor microenvironment promote LC growth via the release of inflammatory mediators by inflammatory cells (12, 14, 15, 31). We and the Karin group showed that the nuclear factor-κB (NF-κB) pathway in myeloid cells is a key regulator of CS-promoted LC development in metastatic LC and K-ras-induced mouse models. In tumor tissue, NF-κB regulates the expression of inflammatory mediators, such as TNF-α, which promote tumor proliferation (14, 15, 31). As COPD is characterized by chronic inflammation of the lung, it is suggested that COPD-associated airway inflammation also promotes LC (4, 17, 18). Indeed, in K-ras-induced mouse models, COPD-like airway inflammation induced by aerosolized nontypeable Haemophilus influenzae (NTHi) resulted in a Th17 cell- and IL-17-dependent increase in lung tumor burden (4, 17).

Exposure to CS disrupts epithelial barrier function and cell-cell contact in the lung (8, 11, 26). Damage to the airway epithelium leads to an increased alveolar permeability to tracer molecules in smokers and in animal exposed to CS (3, 11, 26). In vitro studies showed that exposure of airway epithelial cells to CS or CS extracts result in a loss of barrier integrity, which is associated with a disassembly of tight junctions and aberrant expression of proteins of tight and adherent junctions (16, 21, 26, 30).

Microorganisms and bacterial products have an important role in tumor progression in diverse tumor entities by the induction of inflammation and by a direct impact on tumor cells (27). Helicobacter pylori, for instance, is associated with gastric inflammation and a strong risk factor for gastric adenocarcinoma (25). It also has been suggested that bacterial products entering tumor tissue promote colorectal cancer (6). The purpose of this study was to investigate whether bacterial lung pathogens act as tumor promoters during CS-induced pulmonary inflammation. Using a murine metastatic model, we provide evidence that translocation of bacterial factors in tumor tissue links CS-induced inflammation with tumor proliferation.

METHODS

CS and microbial exposure. All animal experiments were approved by the Landesamt für Soziales, Gesundheit und Verbraucherschutz of the State of Saarland following the national guidelines for animal treatment. Mice were maintained under a pathogen-free condition. A lung cancer metastasis model in mouse was generated by intravenous injection of Lewis lung carcinoma (LLC) cells in 7- to 9-wk-old female wild-type (WT) C57BL/6 and IL-17A-deficient C57BL/L6 mice as described before (14, 15). IL-17A-deficient mice were a gift from Dr. Yoichiro Iwakura (Institute of Medical Science, University of Tokyo) (19). Subconfluent LLC cells were harvested and passed through a 40-μm cell strainer (BD Biosciences, Bedford, MA), washed three times in PBS, resuspended in serum free DMEM, and inoculated at 2 × 10⁵ or 5 × 10⁵ cells per animal by the tail vein.

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The CS and NTHi exposure protocol is graphically displayed in Fig. 1A. Mice were exposed to CS (3R4F; College of Agriculture, Reference Cigarette Program, University of Kentucky, Lexington, KY) in a TE-10 smoking machine (Teague Enterprises, Woodland, CA) for a total of 100 min/day, 7 days/wk for 7 days after the injection of LLC cells. The CS concentration was 120 mg/m³ total suspended particles. CS- or air-exposed mice were exposed to a clinical isolate of NTHi 7, 10, and 13 days after injection of LLC cells. NTHi were grown on selective chocolate agar with IsoVitaleX at 300 μg/ml NAD (Sigma-Aldrich) and 2% Difco Supplement B (BD Biosciences). The culture was centrifuged at 2,500 g for 15 min at 4°C, washed and resuspended in 20 ml PBS, and heat inactivated at 70°C on a mechanical shaker for 45 min and sonicated three times for 30 s. The protein concentration was adjusted to 2.5 mg/ml in PBS using the Pierce BCA-protein assay (Thermo Fisher Scientific, Rockford, IL). CS- or air-exposed mice were exposed to a clinical isolate of NTHi for 40 min.

Cytokine, albumin, and lactate dehydrogenase measurements. Bronchoalveolar lavage fluids (BALFs) were obtained as described previously (10). Percentages of leukocyte subpopulations were determined by counting 100 leukocytes in a randomly selected portion of the cytospin slide. The total number of leukocytes in the BALFs was determined by using a hemocytometer (Innovatis, Reutlingen, Germany). Cytokines in BALFs were measured using cytometric beads for regulated on activation normal T-expressed and presumably secreted (RANTES), macrophage inflammatory protein-1 (MIP-1), and IL-6 (BD Biosciences) on a BD FACS Canto II system. Keratinocyte-derived chemokine (KC) and IL-17A were measured with an ELISA Development Kit by R&D Systems (R&D Systems). Lactate dehydrogenase (LDH) in BALFs and serum was determined using a LDH-Cytotoxicity Assay Kit according to the manufacturer’s manual (ab65393; Abcam). Mouse albumin in BAL fluids was determined by ELISA Mouse Albumin ELISA Quantitation Set (Bethyl Laboratories).

Real time RT-PCR. RNA was isolated from blood-free lungs using a Trizol Reagent (Life Technologies) and reversely transcribed using a cDNA Synthesis Kit (ThermoScientific). Quantitative RT-PCRs were performed with a SYBR Kit (Bioline). Primers were as follows: mβ-actin: 5′-AGC CAT GTA CGT AGC CAT CC-3′ and 5′-CTC TCA GCT GTG GTG AA-3′; mOccludin: 5′-CCC TGA AAT ACA AAG GCA 3′ and 5′-GAG TTA ACG TCA AGC GTG ACC 3′; and mZo-1: 5′-GCC ATT CCT GCT GGT TAC-3′ and 5′-AGG ACA CCA AAG CAT GTG-3′. Specificity of amplification was controlled by melt curve analysis and gel electrophoresis. RT-PCR results were analyzed with the ΔΔCT method (23).

Histologic and immunohistochemical analysis. All histologic and immunohistochemical analysis were performed on formalin-fixed and paraffin-embedded sections. Briefly, lungs were fixed by instillation of PBS-buffer 4% formalin under a constant hydrostatic pressure of 30 cm for 15 min and placed in PBS-buffered 4% formalin. The fixed lungs were embedded in 1% agarose and cut into regular slices of exactly the same thickness and embedded in paraffin. The primary antibodies used for immunohistochemistry were TNF-α (ab6671; Abcam plc), Ki67 (ab15580), and NTHi (ab65270). Imaging was performed using the software Cell Sense Dimension (Olympus, Germany). TNF-α and NTHi stainings were quantified (%) area using ImageJ software.

Fig. 1. Concomitant exposure to cigarette smoke (CS) and nontypeable Haemophilus influenzae (NTHi) promotes metastatic growth in the lung. Mice were injected with 5 × 10⁵ Lewis lung carcinoma (LLC) cells and were exposed to air, CS, NTHi, or the combination of CS and NTHi. A: schema of the experimental protocol to study CS- and NTHi induced metastatic growth. B: survival curves of mice. Statistical differences compared with the air-exposed mice were determined by the log-rank (Mantel-Cox) test. *P < 0.05 at day 19 and **P < 0.05 at day 217 days after the injection of LLC cells; n = 5 per group. Numbers of tumor nodules detectable on the lung surface (C), tumor area from total lung area (D), and macroscopic and microscopic pathology (hematoxylin and eosin stain) (E) were determined in the remaining mice 21 days after the injection of LLC cells. Scale bar = 250 μm. *P < 0.05, by Mann-Whitney test; n ≥ 3 per group.
Cell culture. The human lung adenocarcinoma cell line A549 and mouse LLC cells were cultured in DMEM (Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Invitrogen), 100 U/ml penicillin, and 100 U/ml streptomycin (PAA Laboratories, Pasching, Austria). U1810 cells were cultured in RPMI (Invitrogen) supplemented with 10% FCS, 100 U/ml penicillin, and 100 U/ml streptomycin. Primary human bronchial epithelial cells (HBECs) were cultured and polarized as air-liquid interface cultures as described previously (2, 24). The protocol was approved by the Institutional Review Board (Ethics Committee) of the Landesärztekammer des Saarlandes and informed consent was obtained from the patients. Polarized HBECs grown on Transwell inserts were exposed to volatile smoke for 10 min (2 cigarettes) as described before (2). The transepithelial resistance was >450 Ωcm² before exposure to CS. Directly after exposure to air or CS, media were replaced at the basolateral compartment (1 ml/well) and 200 μl PBS containing 10 mg/ml FITC-conjugated dextran (70 kDa) with and without heat-inactivated NTHi [10^7 colony-forming units (CFU)/ml] were applied to the apical side of the Transwells. Fluorescence intensity was measured in 100-μl media samples collected after 2 h. A standard curve was generated and concentrations of FITC-dextran were calculated. Cytotoxicity was determined using a LDH-Cytotoxicity Assay Kit (ab65393; Abcam). Proliferation of the cell lines was determined using a Quick Cell Proliferation Assay Kit (ab65473; Abcam) according to the manual. Cells were stimulated with heat-inactivated bacteria and IL-17A (R&D Systems) as indicated in the figure legends for 24 h before assay evaluation. OD_{440} was measured 2 h after addition of WST-1. Proliferation of the cell lines was determined using a LDH-Cytotoxicity Assay Kit (ab65393; Abcam). Cytotoxicity was determined in mice 14 days after the injection of LLC cells. *P < 0.05 and ***P < 0.001, by one-way ANOVA test; n = 5 per group. C: macroscopic and microscopic pathology (hematoxylin and eosin stain) of lungs 14 days after the injection of LLC cells. Scale bar = 250 μm.

### RESULTS

**CS and NTHi exposure synergistically promote growth of lung metastasis.** Smokers and COPD patients have an increased risk for lung cancer and microbial infections (12, 14, 15, 22, 31, 33). COPD patients are frequently colonized with bacterial pathogens, such as NTHi, which perpetuate ongoing inflammation of the lung (18, 28). To examine the effect of bacterial lung pathogens on the growth of lung tumors during tobacco smoke-induced inflammation in a metastatic lung cancer model, LLC cells were injected in WT C57BL/6 mice. Mice were exposed to air or CS in combination with and without exposure to inactivated NTHi 7 days after the LLC injection for additional 7 days. Mice were analyzed 14 and 21 days after the LLC injection (Fig. 1A). Mice exposed to NTHi or the combination of CS and NTHi showed reduced survival compared with air-exposed mice. The survival of mice exposed to the combination of CS and NTHi was significantly reduced 19 and 21 days after LLC injection and the survival of mice exposed only to NTHi was significantly reduced 21 days after LLC injection compared with the survival of air-exposed control mice (Fig. 1B). The number of macroscopic lung nodules detectable on the lung surface (A) and tumor area from total lung area (B) were determined in mice 14 days after the injection of LLC cells. *P < 0.05 and ***P < 0.001, by one-way ANOVA test; n = 5 per group. C: macroscopic and microscopic pathology (hematoxylin and eosin stain) of lungs 14 days after the injection of LLC cells. Scale bar = 250 μm.
nODULES (Fig. 1B) AND THE PERCENTAGE OF TUMOR AREA FROM TOTAL LUNG AREA (Fig. 1C) WERE INCREASED IN THE REMAINING MICE EXPOSED TO CS OR NTHI COMPARED WITH AIR-EXPOSED MICE. MACROSCOPIC AND MICROSCOPIC ANALYSIS SHOWED THAT THE TUMOR LOAD WAS INCREASED IN THE REMAINING MICE EXPOSED TO CS, NTHI, OR THE COMBINATION OF CS AND NTHI (Fig. 1D).

AS TUMOR GROWTH WAS STRONGLY INCREASED AND THE Survival WAS DECREASED IN MICE EXPOSED TO THE COMBINATION OF CS AND NTHI 21 DAYS AFTER LLC INJECTION, WE DECIDED TO ANALYZE TUMOR GROWTH 14 DAYS AFTER LLC INJECTION. NO MOUSE DIED WITHIN THE FIRST 14 DAYS AFTER LLC INJECTION. THE NUMBER OF MACROSCOPIC LUNG NODULES WAS SIGNIFICANTLY INCREASED IN MICE EXPOSED TO THE COMBINATION OF CS AND NTHI COMPARED WITH AIR-, CS-, AND NTHI-EXPOSED MICE, WHEREAS EXPOSURE TO CS OR NTHI ALONE DID NOT RESULT IN SIGNIFICANTLY INCREASED NUMBERS OF LUNG NODULES (Fig. 2A). THE PERCENTAGE OF TUMOR AREA FROM THE TOTAL LUNG AREA WAS SIGNIFICANTLY INCREASED IN MICE EXPOSED TO THE COMBINATION OF CS AND NTHI COMPARED WITH AIR- AND CS-EXPOSED MICE (Fig. 2B). MACROSCOPIC AND MICROSCOPIC ANALYSIS SHOWED THAT THE TUMOR LOAD WAS INCREASED IN MICE EXPOSED TO THE COMBINATION OF CS AND NTHI 14 DAYS AFTER LLC INJECTION (Fig. 2C). THESE RESULTS SHOW THAT CS AND NTHI SYNERGISTICALLY PROMOTE GROWTH OF LUNG METASTASIS.

INFLAMMATION IN THE ALVEOLAR SPACE IS NOT LINKED WITH TUMOR GROWTH. NEXT, WE INVESTIGATED WHETHER THE INFLAMMATORY RESPONSE IN THE LUNG INDUCED BY CS AND NTHI ASSOCIATES WITH LUNG METASTASIS GROWTH 14 DAYS AFTER LLC INJECTION. EXPOSURE TO NTHI RESULTED IN ENHANCED NUMBERS OF NEUTROPHILS IN BALF (Fig. 3A). THE NUMBERS OF MACROPHAGES IN BALF WERE NOT INCREASED AFTER NTHI EXPOSURE (Fig. 3B). CONCENTRATIONS OF THE INFLAMMATORY MEDIATORS IL-17A, RANTES, MIP-1β, AND KC WERE SIGNIFICANTLY INCREASED IN BALF MICE EXPOSED NTHI (Fig. 3C). EXPOSURE TO CS DID NOT AFFECT THE NTHI-INDUCED INFUX OF NEUTROPHILS AND EXPRESSION OF IL-17A AND RANTES, WHEREAS CONCENTRATIONS OF MIP-1β AND KC WERE SLIGHTLY REDUCED BY CS EXPOSURE. THESE RESULTS INDICATE THAT THE ENHANCED GROWTH OF LUNG METASTASIS IN MICE EXPOSED TO CS IN COMBINATION WITH NTHI IS NOT DUE TO INCREASED INFLAMMATION IN THE ALVEOLAR SPACE.

CS PROMOTES BACTERIAL TRANSLLOCATION IN TUMOR TISSUE. WE FURTHER ANALYZED WHETHER EXPOSURE TO CS AND NTHI RESULTED IN AN INCREASED LUNG DAMAGE AND TRANSLLOCATION FROM BACTERIAL FACTORS INTO TUMOR TISSUE 14 DAYS AFTER LLC INJECTION. TO DETERMINE LOSS OF PULMONARY BARRIER INTEGRITY AND LUNG DAMAGE, WE MEASURED THE ALBUMIN CONTENT IN BALF AND DETERMINED THE RATIO OF LDH IN BALF TO LDH IN SERUM. ALBUMIN CONCENTRATIONS WERE SIGNIFICANTLY INCREASED IN BALF MICE EXPOSED TO CS IN COMBINATION WITH NTHI COMPARED WITH THOSE OF AIR-EXPOSED, CS-EXPOSED, AND NTHI-EXPOSED MICE (Fig. 4A). THE RATIO OF LDH (BALF) TO LDH (SERUM) WAS SIGNIFICANTLY INCREASED IN MICE EXPOSED TO CS IN COMBINATION WITH NTHI BUT NOT IN MICE EXPOSED TO CS OR NTHI ALONE (Fig. 4B).
To test whether exposure to CS impacts the expression of tight junction factors in cancer-free lungs, we analyzed the expression of occludin and Zo-1 in lungs of LLC-free animals exposed to air or CS for 3 days. Exposure to CS resulted in reduced mRNA levels of occludin and Zo-1 in whole lung tissue (Fig. 4C). We further determined whether fragments of NTHi are present in tumor tissue by immunostaining (Fig. 4D). Staining for NTHi in tumor tissue was significantly increased in mice exposed to CS in combination with NTHi compared with NTHi-exposed control mice (Fig. 4E). It has been shown before that CS decreases the barrier function of HBECs measured by transepithelial electrical resistance within 6 h (26). To determined whether CS affects barrier permeability of cultured respiratory epithelial cells we exposed polarized primary HBECs cultured as air-liquid interface cultures to volatile CS in vitro. Exposure of HBECs to volatile CS for 10 min resulted in an increased permeability for FITC-conjugated dextran (70 kDa) determined two h after exposure to CS (Fig. 4F). A LDH release assay showed that the observed effects were not due to cytotoxicity (data not shown). Together, these results indicate that CS-induced loss of pulmonary integrity and lung damage favors translocation of bacterial factors in lung tumor tissue.

Concomitant exposure to CS and NTHi results in increased tumor inflammation and proliferation. Next, we determined TNF-α expression in tumor tissue by immunostaining (Fig. 5A). In tumor tissue of mice exposed to CS in combination with NTHi, expression of TNF-α was significantly increased compared with those of control mice and mice treated with either CS or NTHi (Fig. 5B). We further examined proliferation of tumor cells by immunostaining for Ki-67 (Fig. 5C). The numbers of Ki-67-positive cells were significantly increased in tumors of mice exposed to CS in combination with NTHi, even though exposure to CS or NTHi alone also resulted in significantly increased numbers of Ki-67-positive cells compared with air-exposed control mice (Fig. 5D). These results show that the exposure to a combination of CS and NTHi leads to enhanced tumor inflammation and proliferation.

Bacterial lung pathogens increase proliferation of cultured cancer cell lines. NTHi possibly increase growth of LC by the regulation of the tumor-associated inflammation and by direct induction of tumor proliferation. Thus we examined...
whether bacterial lung pathogens promote proliferation in cultured lung cancer cell lines. We exposed LLC, A549, and U1810 cells to a high and low dose of heat-inactivated NTHi and Pseudomonas aeruginosa. NTHi and P. aeruginosa dose dependently promote proliferation of cultured cancer cell lines as determined by WST-1 proliferation assay (Fig. 6). We further determined cell numbers of LLC and A549 cells incubated with inactivated NTHi for 24 h. The numbers of LLC (14.1%, ± 2.9; P = 0.004) and A549 (4.6%, ± 1.6 SD; P = 0.005) cells were increased when incubated with NTHi compared with control cells incubated with media without NTHi.

Growth of lung metastasis is decreased in IL-17A-deficient mice. Studies showed that tumor proliferation is mediated by tumor-associated inflammation (12, 14, 15). Because in K-ras-induced mouse models COPD-like airway inflammation induced by aerosolized NTHi resulted in an IL-17-dependent increase in lung tumor burden (4, 17), we examined whether IL-17 mediates metastatic growth in our model of CS- and NTHi-induced inflammation by injecting LLC in IL-17-deficient (IL-17A−/−) mice. There was no difference in the tumor growth between CS-exposed WT and IL-17A−/− mice (data not shown). To ensure survival of mice exposed to the combination of CS and NTHi, we injected a low dose of LLC in IL-17A−/− and WT mice and exposed the mice to the combination of CS and NTHi. The numbers of macroscopic lung nodules were slightly, however, not significantly, decreased (Fig. 7A) and the percentage of tumor area from total lung area was significantly decreased (Fig. 7B) in IL-17A−/− mice compared with those of WT mice. The expression of TNF-α (Fig. 7, C and D) and the numbers of Ki-67-positive cells (Fig. 7, E and F) were significantly decreased in tumor tissue of IL-17A−/− mice. It has been shown before that IL-17A has no direct effect on the in vitro growth rate of human cancer cells, including A549 cells (20). We further examined whether IL-17A modulates the direct effect of bacteria on the proliferation of cultured LLC and A549 cells. IL-17A did not modify the proliferation of LLC cells incubated with inactivated NTHi and reduced the proliferation of A549 cells incubated with and without NTHi (Fig. 7G).
Together, these results indicate that bacteria increase tumor inflammation in an IL-17A-dependent manner.

**DISCUSSION**

The main finding of the present study is that NTHi act as tumor promoters during CS-induced pulmonary inflammation. We show that concomitant exposure to NTHi and CS results in the disruption of pulmonary integrity, translocation of bacterial factors in tumor tissue, and enhanced tumor-associated inflammation and tumor proliferation. We demonstrate that bacterial pathogens directly induced tumor proliferation in cultured lung cancer cells. Using IL-17-deficient mice, we further show that tumor proliferation induced by concomitant exposure to NTHi and CS is also mediated by tumor-associated inflammation.

COPD is characterized by chronic pulmonary inflammation, which, in most cases, is provoked by smoking. Preclinical
models indicate a mechanistic relation between pulmonary and tumor-associated inflammation induced by CS and the development of LC. We and other groups showed that myeloid cells regulate tumor-associated inflammation and promote growth of LC in CS-exposed mice (14, 15, 31). Pulmonary inflammation in COPD patients is also associated with bacterial colonization and infection of the lung (28). Stable COPD patients are abundantly colonized with bacterial pathogens (e.g., NTHi, *P. aeruginosa*) and bacterial infections of the lung play an important role in the initiation of acute exacerbations of COPD (18, 28). Bacterial colonization and infection of the lung contribute to the development of COPD by damaging epithelial surfaces and amplifying pulmonary inflammation (18, 29, 32). Moghaddam et al. (17) demonstrated that pulmonary inflammation triggered by NTHi increases the growth of tumors in a K-ras-induced mouse model. In line with the mentioned studies, we found that exposure to both CS and NTHi promotes growth of metastatic LC. However, we also show that bacterial pathogens are strong tumor promoters during CS-induced inflammation. In our metastatic model, concomitant exposure of mice to CS and NTHi synergistically promoted growth of LC, which suggests that bacterial pathogens significantly contribute to the increased risk of LC and tumor growth in smokers and COPD patients.

We asked how NTHi promotes growth of LC during CS-exposed inflammation. One prerequisite of the impacts of lung microorganisms on tumor growth is increased access into the tumor environment. It has been demonstrated that exposure to CS disrupts epithelial barrier function and increases alveolar permeability (3, 8, 11, 26). In line with a study showing that exposure to CS extracts decreases the barrier function of HBECs measured by transepithelial electrical resistance (26), we found that exposure of cultured HBECs to volatile CS increases the permeability for FITC-conjugated dextran (70 kDa). Moreover, our mouse model demonstrated that concomitant exposure to CS and NTHi results in a loss of pulmonary integrity determined by levels of LDH and concentrations of albumin in BALFs. Thus we suggest that disruption of pulmonary integrity allows bacteria to translocate in tumor tissue. Once translocated in the tumor tissue, bacterial factors regulate tumor-associated inflammation, which drives growth of LC. In line with this hypothesis, combined exposure to CS and NTHi resulted in an increased expression of tumor-associated TNF-α, whereas NTHi-triggered inflammation in the alveolar space was not affected by exposure to CS. TNF-α has a central role in the regulation of cancer cell biology and has been shown to mediate LC growth (12, 14, 31). Because concentrations of albumin and levels of LDH in BALFs were associated with the tumor burden at the time point measured, it is conceivable that enhanced tumor growth also contributes to the tissue injury in mice exposed to the combination of CS and NTHi. Thus increased tumor growth in lungs of mice exposed to the combination of CS and NTHi may further promote access of bacterial factors into the tumor microenvironment.

Using mice deficient for IL-17, we further demonstrate that tumor-associated inflammation mediates NTHi-triggered metastatic growth in the lungs exposed to CS. Th17 cells and IL-17 are key mediators of antibacterial host defense and are suggested promoting tumor growth in various tissues (4, 6). However, the role of Th17 cells and IL-17 in the development of LC is discussed controversial. Kryczek et al. (13) showed that the metastatic growth of MC38 cells in mice deficient for IL-17 was enhanced compared with WT mice. However, Kryczek et al. did not expose tumor-bearing mice to inflammatory stimuli, which provoke IL-17-dependent immune mechanisms, such as NTHi. In opposite, Chang et al. (4) using a K-ras-induced mouse model of LC showed that Th17 cells are enriched in tumor tissue and that deficiency for IL-17 reduced tumor growth in both nontreated and NTHi-exposed mice. Moreover, deficiency for IL-17 resulted in reduced tumor proliferation and tumor-associated inflammation (4). In line with Chang et al., our data obtained with IL-17-deficient mice demonstrate that IL-17 deficiency associates with reduced metastatic LC growth after exposure to CS in combination with NTHi. As in the K-ras-induced mouse model, in our metastatic LC model, tumor inflammation and proliferation is at least partially regulated by IL-17. As IL-17 did not modify NTHi-induced growth of LLC cells in vitro we suggest that IL-17 promotes tumor growth by regulating tumor-associated inflammation in our metastatic LC model.

Besides the regulation of tumor-associated inflammation, bacterial products also possibly promote growth of LC by direct induction of tumor proliferation. It has been shown before by MTS assay and cell counting that *Escherichia coli*-derived endotoxin induces proliferation of nonsmall cell lung cancer including A549 cells (7). In line with this study, we showed by WST-1 assay and cell counting that bacterial lung pathogens directly increase the proliferation of LLC and A549 cells. Thus we suggest that direct induction of cancer cell proliferation is another mechanism by which bacterial factors promote growth of LC once they invaded tumor tissue.

In summary, our data indicate that bacterial lung pathogens promote tumor development in a model of CS and NTHi exposure, similar to that observed in smokers with COPD (9). Mechanistically, we suggest that CS-induced loss of pulmonary barrier integrity allows bacterial factors to invade tumor tissue. Bacterial lung pathogens enhance growth of LC by regulating tumor-associated inflammation but also by direct promotion of proliferation of cancer cells.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

BACTERIAL PATHOGENS PROMOTE GROWTH OF LUNG METASTASIS


