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Cigarette Smoke Inhibits Airway Epithelial Cell Innate Immune Responses to Bacteria▽

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The human upper respiratory tract, including the nasopharynx, is colonized by a diverse array of microorganisms. While the host generally exists in harmony with the commensal microflora, under certain conditions, these organisms may cause local or systemic disease. Respiratory epithelial cells act as local sentinels of the innate immune system, responding to conserved microbial patterns through activation of signal transduction pathways and cytokine production. In addition to colonizing microbes, these cells may also be influenced by environmental agents, including cigarette smoke (CS). Because of the strong relationship among secondhand smoke exposure, bacterial infection, and sinusitis, we hypothesized that components in CS might alter epithelial cell innate immune responses to pathogenic bacteria. We examined the effect of CS condensate (CSC) or extract (CSE) on signal transduction and cytokine production in primary and immortalized epithelial cells of human or murine origin in response to nontypeable *Haemophilus influenzae* and *Staphylococcus aureus*. We observed that epithelial production of interleukin-8 (IL-8) and IL-6 in response to bacterial stimulation was significantly inhibited in the presence of CS ($P < 0.001$ for inhibition by either CSC or CSE). In contrast, epithelial production of beta interferon (IFN-β) was not inhibited. CSC decreased NF-κB activation ($P < 0.05$) and altered the kinetics of mitogen-activated protein kinase phosphorylation in cells exposed to bacteria. Treatment of CSC with antioxidants abrogated CSC-mediated reduction of epithelial IL-8 responses to bacteria ($P > 0.05$ compared to cells without CSC treatment). These results identify a novel oxidant-mediated immunosuppressive role for CS in epithelial cells.

Cigarette smoking and exposure to secondhand smoke are important risk factors for a variety of systemic and respiratory tract diseases that include six of the eight leading causes of death in the world (33). Today, tobacco kills approximately 5 million people every year worldwide, and this number is estimated to rise to 8.3 million by 2030 (22). Smokers are at particular risk of upper and lower respiratory tract infections. Despite decades of research, the molecular mechanisms of cigarette smoke (CS)-induced diseases, including predisposition to infection, remain unclear in many cases. This is in part because CS contains many bioactive compounds, which may act simultaneously and in complex ways on host cells. The human nasopharyngeal epithelium, a sentinel site of respiratory tract immunity, is constantly exposed to microbial patterns and other environmental stimuli. CS brings about structural changes in the respiratory tract as well as alterations in immune responses that may alter these responses, leading to predisposition to infection or exacerbation of existing pathologies. Nontypeable (NT) *Haemophilus influenzae* and *Staphylococcus aureus* are of particular importance in CS-associated respiratory diseases, including chronic obstructive pulmonary disease and chronic rhinosinusitis.

In 2004, Laan et al. reported a CS-mediated downregulation of inflammatory cytokines (granulocyte-macrophage colony-stimulating factor [GM-CSF] and interleukin-8 [IL-8]) in BEAS-2B airway epithelial cells exposed to endotoxin (17). CS has also been reported to inhibit tumor necrosis factor alpha (TNF-α)-induced NF-κB transactivation and IL-8 release in epithelial cells (25). Here, we examined the effect of CS on epithelial cell responses to common respiratory pathogens, including NT *H. influenzae* and *S. aureus*. We noted a dose-dependent, oxidant-mediated immunosuppressive effect of CS on epithelial detection of microbial products conserved across multiple cell lines and active in primary cells. We suggest that this mechanism may play a role in the pathogenesis of the CS-induced predisposition to respiratory infection.

MATERIALS AND METHODS

Epithelial cells. Immortalized human upper airway epithelial cell lines A549 (ATCC CCL-185) and 1HAEo− (6) as well as the human cervical adenocarcinoma cell line HeLa (ATCC CCL-2) were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate (Cellgro), and 10 μg/ml ciprofloxacin (Fisher). Cells were removed from antibiotics and serum (weaned) for 12 to 14 h prior to use in experiments. Differentiated primary murine nasopharyngeal epithelial cells from C57BL/6J mice (Jackson Laboratories) were isolated and grown on Transwell filters as described previously (3) and were used for experiments without weaning.

Bacterial strains. NT *H. influenzae* strain H233, an NT clinical isolate (10, 28), was grown on chocolate agar plates or in brain heart infusion supplemented with 2% Fildes extract (Remel) and 2 μg/ml NAD (Sigma). *S. aureus* RN6390 was grown in tryptic soy (TS) agar or TS broth. Bacteria were washed in sterile phosphate-buffered saline (PBS), and serial dilutions were plated onto the appropriate media for colony counts prior to use. Where indicated, organisms were heat killed for 15 min at 70°C and checked for the absence of live organisms by plating an aliquot onto the appropriate medium.

Reagents. CS condensate (CSC), which is total particulate matter generated by burning University of Kentucky's 1R3E standard cigarettes and extracting into

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dimethyl sulfoxide (DMSO) to prepare a 40 mg/ml stock solution, was purchased from Murty Pharmaceuticals and diluted in tissue culture medium prior to use. CS extract (CSE) was freshly generated by bubbling smoke from one cigarette (Marlboro filter cigarette; Philip Morris) through 25 ml sterile MEM supplemented with 1 mM sodium pyruvate, following which the pH was adjusted to 7.0, and the medium was filter sterilized. CSC was used in all experiments with the exception of those presented in Fig. 1E, which used CSE.

Reagents. Antibodies for total as well as phosphorylated forms of p38, Jun N-terminal protein kinase (JNK), and extracellular signal-regulated mitogen-activated protein kinases (ERK MAPK) were purchased from Cell Signaling Technology. *N-p*-tosyl L-phenylalanyl chloromethyl ketone (TPCK) and *N*-acetyl L-cysteine (NAC) were purchased from Sigma. TNF-α was from PeproTech Inc., and DMSO was from Research Organics.

Treatment of cell lines with CSC. A549 and 1HAEo− human airway and HeLa human cervical epithelial cell lines were grown to confluence in 12- or 96-well plates and were weaned prior to treatment with 50, 100, or 200 μg/ml CSC or an equal volume of DMSO in the presence or absence of bacteria or TNF-α for the times indicated below. Supernatants were harvested for assay of lactate dehydrogenase (LDH) and IL-8. Whole-cell lysates were used for RNA extraction or for separating proteins by SDS-PAGE. Differentiated primary murine airway epithelial cells were similarly treated with 200 μg/ml CSC or an equal volume of DMSO in the presence or absence of bacteria for 2 h and whole-cell lysates used for RNA extraction. Because DMSO is used as a solvent for CSC, control wells were treated with equal volumes of DMSO (vehicle control) in all experiments presented. The amount of LDH released into the cell supernatant was assayed using Cytotoxicity Detection Kit Plus (Roche) per the manufacturer's instructions. Percent cytotoxicity was determined by the following formula: $[(\text{Test LDH} - \text{bc})/(\text{hc} - \text{bc})] \times 100$, where bc (background control) is an estimate of LDH activity in the assay medium, and hc (high control) corresponds to the maximum releasable LDH activity in all cells.

Treatment of cells with TPCK. Weaned A549 monolayers were pretreated with 50 μM TPCK or an equivalent amount of DMSO (vehicle control) for 30 min. The cells were washed with fresh medium and treated with 10^8 heat-killed (HK) NT *H. influenzae*. At the end of 18 h of incubation, IL-8 and LDH concentrations in the supernatants were determined.

Determination of IL-8 by ELISA. Confluent monolayers of epithelial cell lines in 96-well plates were treated overnight with the amounts of HK NT *H. influenzae* indicated below in the presence or absence of CSC, CSE, or vehicle control for 18 h. IL-8 concentrations in supernatants were determined by enzyme-linked immunosorbent assay (ELISA; BD-OptEIA) according to the manufacturer's instructions.

Western blot analysis. Confluent monolayers of A549 cell lines were pretreated with 200 μg/ml CSC or vehicle control for 30 min and stimulated for an additional 30 or 60 min with 10^8 CFU/ml of HK NT *H. influenzae*, keeping the CSC or DMSO concentration constant. Supernatants were then harvested for the LDH assay. Total protein amounts in the cell lysates were estimated using the Bio-Rad protein assay. A total of 20 μg protein per well was separated using SDS-PAGE and transferred onto Immobilon-P membranes (Millipore). Membranes were probed for phosphorylated forms of p38, JNK, or ERK MAP kinases per the manufacturer's instructions. As a loading control, the same membranes were stripped with 0.4 N NaOH for 1 h and reprobed for total p38, JNK, or ERK proteins.

Quantitative real-time PCR. RNA was isolated from cells using an RNeasy kit (Qiagen) or RNAqueous-4PCR (Ambion) according to the manufacturer's instructions. A total of 1 μg RNA was reverse transcribed to cDNA by using the high-capacity cDNA reverse transcription kit (Applied Biosystems). The following primer pairs were used for real-time PCR amplification: human IL-8, 5′-TTG GCA GCC TTC CTG ATT TC-3′ and 5′-TAT GCA CTG ACA TCT AAG TTC TTT AG-3′; human IL-6, 5′-AAG AGT AAC ATG TGT GAA AGC-3′ and 5′-CTA CTC TCA AAT CTG TTC TGG-3′; human GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 5′-GGGCGC CTG GTC ACC AGG GCT G-3′ and 5′-GGG GCC ATC CAC AGT CTT CTG-3′; murine IL-6, 5′-TGA TGC ACT TGC AGA AAA CAA-3′ and 5′-GGT CTT GGT CCT TAG CCA CTC-3′ (21); murine beta interferon (IFN-β), 5′-AAC TCC ACC AGC AGA CAG TG-3′ and 5′-GTG GAG AGC AGT TGA GGA CA-3; murine actin, 5′-CCT TTG AAA AGA AAT TTG TCC-3′ and 5′-AGA AAC CAG AAC TGA AAC TGG-3′; and murine GAPDH, 5′-ACC ACA GTC CAT GCC ATC AC-3′ and 5′-TCC ACC ACC CTG TTG CTG TA-3′. The quantitative real-time PCR was carried out using power SYBR green master mix in a StepOne Plus thermal cycler (Applied Biosystems). The relative quantification (RQ) values were calculated using a comparative threshold cycle ($\Delta\Delta C_T$) program on StepOne software version 2.0.

Assay for NF-κB activation. A549 cells were cotransfected with $pNF\kappa B_{luc}$ (Stratagene) and the coreporter pRL-TK (Promega) at a ratio of 10:1, using a Nucleofector II (Amaxa) and appropriate cell line-specific protocols. Confluent monolayers of cotransfected cells in 96-well plates were weaned from FBS overnight and treated with CSC, DMSO, or medium alone with or without HK NT *H. influenzae* for 6 h.

Activation of NF-κB was assessed with the dual-luciferase reporter (DLR) assay system (Promega) by the use of a luminometer (Infinite M200; Tecan). Relative luciferase units (RLU) were calculated by dividing firefly luciferase luminescence by *Renilla* luciferase luminescence for each sample.

Antioxidant treatment of CSC. A total of 2 mg/ml CSC or an equivalent volume of DMSO was treated with 25 mM NAC for 30 min and then diluted 10-fold in fresh medium. A549 cells were exposed to CSC or DMSO (both conditions with or without antioxidant pretreatment) in the presence or absence of NT *H. influenzae*. Supernatants were harvested for IL-8 ELISA as described above.

Statistical analysis. All results are expressed as an average of values from triplicate readings ± standard deviation (SD) from one representative experiment. Each experiment was repeated at least two times. Data were compared by one-way analysis of variance (ANOVA) followed by Tukey's post test by using Prism 4 software (GraphPad). A P value of <0.05 was considered significant.

RESULTS

CSC inhibits epithelial expression of proinflammatory cytokines. To determine the effects of CS on innate immune defenses, upper airway epithelial cells were concurrently exposed to CSC, HK NT *H. influenzae*, or both. Treatment with CSC brought about a dose-dependent, statistically significant reduction in the levels of HK *H. influenzae*-induced IL-8 production in A549 respiratory epithelial cells (Fig. 1A). At the lower dose of HK NT *H. influenzae*, a significant ($P < 0.05$) reduction in IL-8 was seen only at the highest dose of CSC (200 μg/ml), but at the higher NT *H. influenzae* dose, significant inhibition was noted at all CSC doses tested (Fig. 1A). A similar dose-dependent reduction in IL-8 production was observed when A549 (Fig. 1B) and 1HAEo− (data not shown) cells were treated with the Gram-positive upper airway pathogen *S. aureus* in the presence of CSC. CSC inhibited the epithelial IL-8 response to live *S. aureus* (data not shown), live NT *H. influenzae* (Fig. 1C), and purified TNF-α (Fig. 1D). Similar findings were observed when CSE, rather than CSC, was used (Fig. 1E). We also noted that CSC significantly reduced HK NT *H. influenzae*-induced IL-8 production in 1HAEo− cells of airway and in HeLa cells of nonairway (cervical) origin (data not shown), suggesting that CSC-mediated IL-8 downregulation may be conserved among various anatomic sites.

In all experiments, cytotoxicity associated with different treatments was determined by assaying LDH released in cellular supernatants at the end of the incubation periods. No treatment induced cytotoxicity significantly different from that of the corresponding DMSO-alone controls. Percent cytotoxicity (±SD) was as follows, after 4 h treatment of A549 cells with the indicated treatments: DMSO, 6.9 ± 1.6; CSC, 7.5 ± 1.6; DMSO plus 10^8 HK NT *H. influenzae*, 9 ± 4.5; CSC plus 10^8 HK NT *H. influenzae*, 7 ± 1.7 (P values were not significant).

CSC downregulates the levels of IL-8 mRNA transcript in epithelial cells. NT *H. influenzae*-associated molecular patterns interact with various host pattern recognition receptors, such as Toll-like receptors, leading to the induction of proinflammatory cytokines and type I IFNs (18). IL-8 protein production may be regulated both at the level of transcription and post-translationally (13). Because CSC dampened the production of

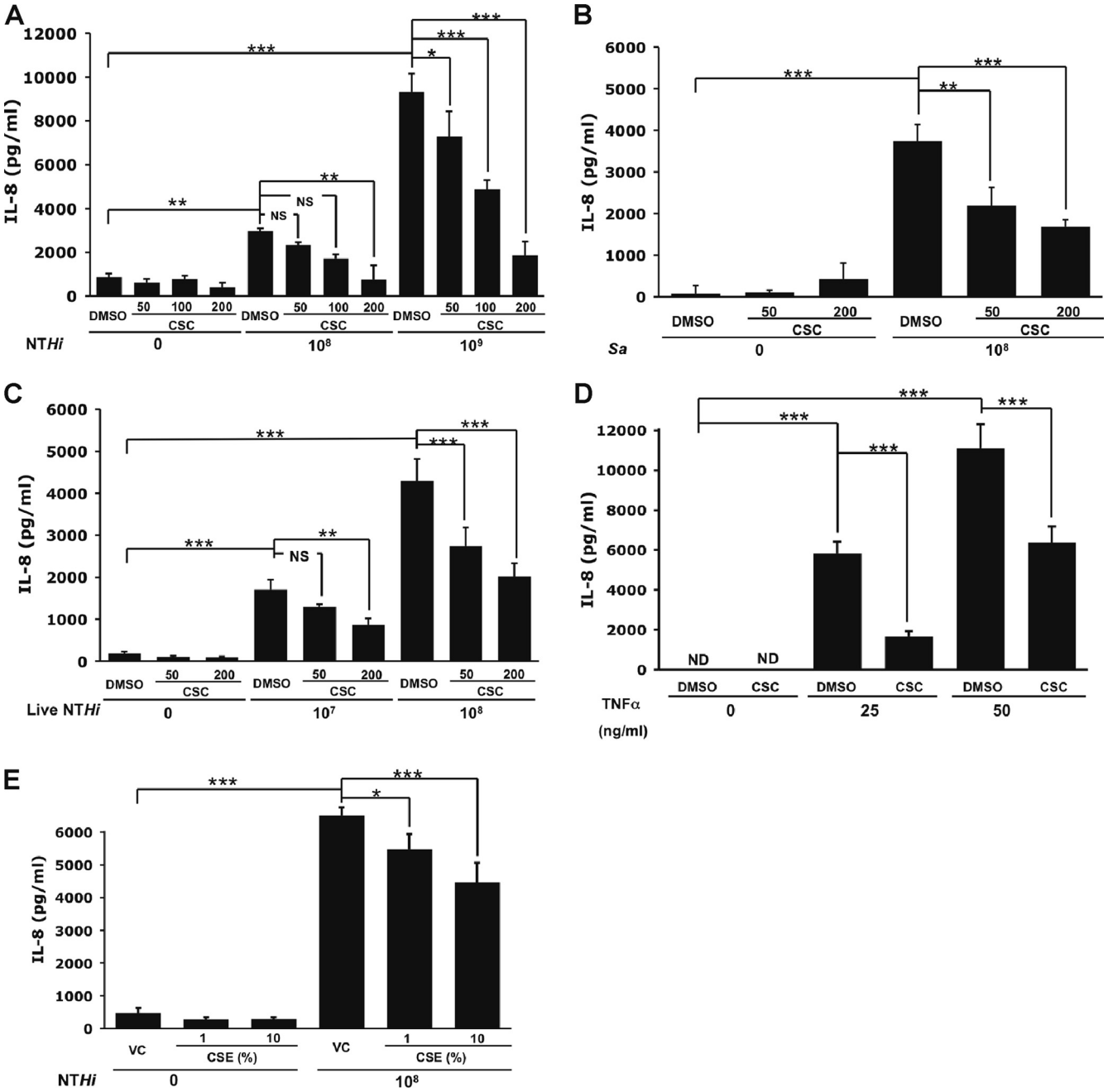

FIG. 1. CSC inhibits pathogen-induced epithelial cell innate immune responses. A549 human airway epithelial cells were exposed to *H. influenzae* (*Hi*) strain H233 or *S. aureus* (*Sa*) strain RN6390 in the presence of CSC, CSE, or vehicle control (DMSO for CSC; medium [VC] for CSE) for 18 h, and levels of IL-8 in the supernatants were determined by ELISA. CSC induced dose-dependent inhibition of IL-8 responses to HK NT *H. influenzae* bacteria (A), HK *S. aureus* (B), or live NT *H. influenzae* (C). Similar effects were observed when cells were treated with CSC and proinflammatory cytokine TNF-α (D) or with CSE and HK NT *H. influenzae* (E). *P* values for this and subsequent figures are as follows: *, <0.05, **, <0.01, and ***, <0.001, while NS (not significant) refers to a *P* value of >0.05. ND, not detected.

IL-8 by epithelial cell lines in response to bacterial pathogens, we assessed whether this effect was due to the reduction in the level of IL-8 transcription. Levels of IL-8 mRNA transcripts from A549 cells exposed to CSC or DMSO in the presence or absence of HK NT *H. influenzae* were determined using quantitative real-time PCR. At 2 and 4 h postexposure, *H. influenzae* stimulation led to a 15- to 20-fold increase in IL-8 mRNA levels ($P < 0.001$) (Fig. 2A). When CSC was present along with *H. influenzae*, there was a statistically significant (*P* value of <0.05 at 2 h and <0.001 at 4 h) decrease in IL-8 mRNA transcript levels compared to those from cells treated with *H. influenzae* and DMSO. IL-6 is another important neutrophil chemokine secreted by the cells of airway epithelium in response to NT *H. influenzae* (16). We used differentiated pri-

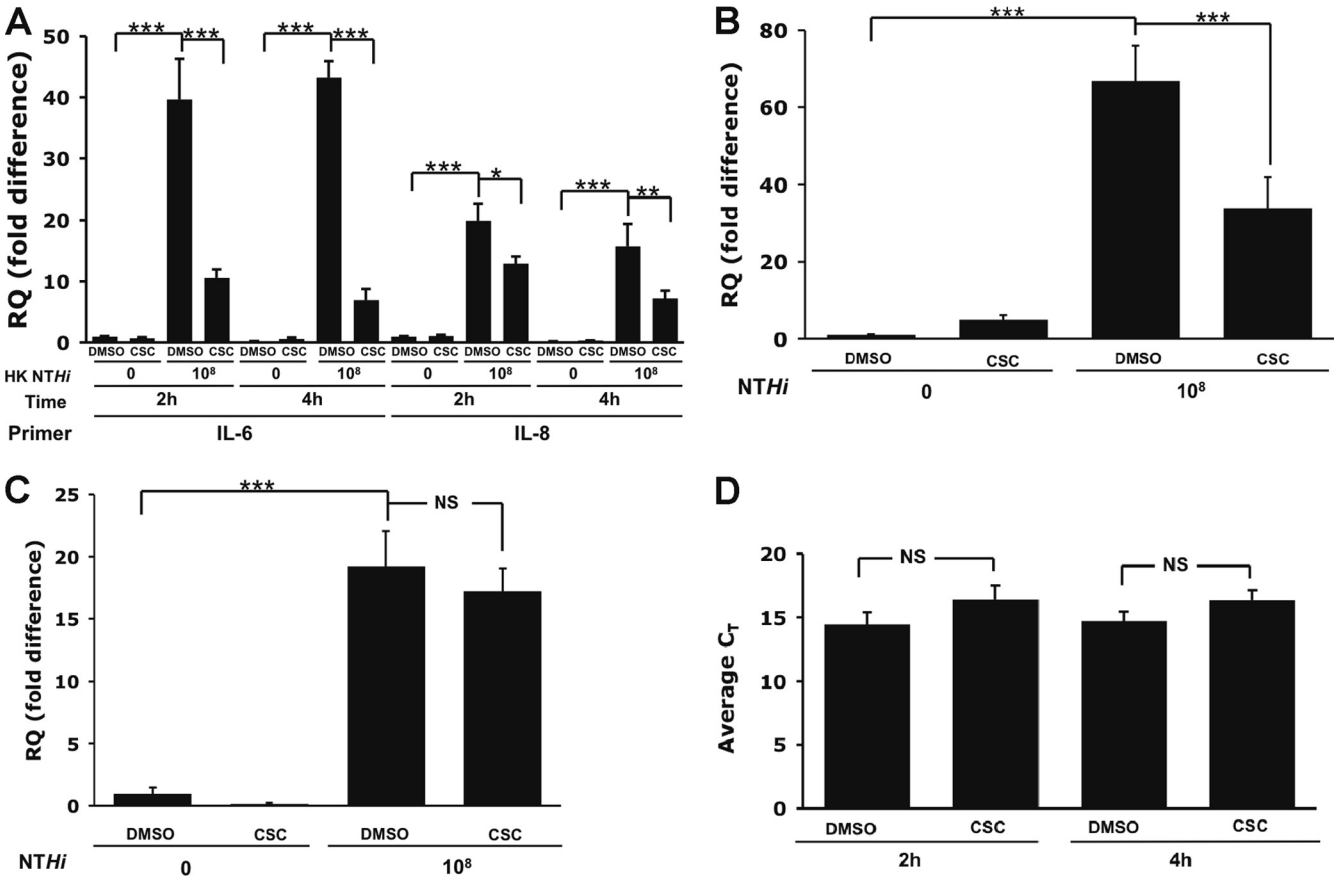

FIG. 2. CSC decreases IL-6 and IL-8 mRNA transcription in response to bacteria. Immortalized A549 (A) or differentiated primary murine respiratory epithelial (B and C) cells were concurrently treated with CSC (200 μg/ml) or DMSO and 10^8 CFU/ml of HK NT *H. influenzae*. The amount of IL-6 (A and B), IL-8 (A), or IFN-β (C) mRNA was determined using real-time PCR with normalization to GAPDH (A and B) or actin (C) transcript levels. RQ values obtained from comparative C_T analysis ($\Delta\Delta C_T$) are shown. The RQ value for DMSO alone was standardized to 1. Comparison of C_T values representing GAPDH transcript levels in A549 cells treated with DMSO or CSC (200 μg/ml) for 2 or 4 h (D) demonstrates that CSC does not affect transcription globally.

mary murine respiratory epithelial cells grown at an air-liquid interface as a model that mimics many characteristics of murine airway epithelium *in vivo*. In this system, we observed that HK NT *H. influenzae* induces a robust upregulation of IL-6 (50- to 100-fold) as well as IFN-β (20-fold) transcription ($P < 0.001$). When CSC was present along with HK NT *H. influenzae*, there was a 3-fold reduction in the level of IL-6 mRNA transcript (Fig. 2B). A similar effect was observed in A549 cells (Fig. 2A). It is important to note that treatment with CSC does not nonspecifically affect all transcription, as CSC did not reduce NT *H. influenzae*-induced IFN-β transcription in primary murine respiratory epithelial cells (Fig. 2C) or the level of GAPDH housekeeping gene transcript in A549 cells (Fig. 2D). Taken together, these results suggested the existence of a selective immunosuppressive effect of CS on epithelial innate immune responses to bacteria.

CSC has a minor effect on the kinetics of MAPK activation in response to bacteria. NT *H. influenzae* induces the activation of p38 and JNK MAPK leading to the upregulation of a variety of key inflammatory mediators, including IL-8 (7, 8, 29). To assess the effects of CSC on NT *H. influenzae*-induced MAPK activation, A549 airway epithelial cells were treated with HK *H. influenzae* in either the presence or absence of 200 μg/ml CSC for 30 or 60 min. Western blot analysis of the lysates revealed that a concurrent addition of CSC did not affect the extent of p38 or JNK phosphorylation in response to NT *H. influenzae* at 30 min. There was a minor but reproducible decrease in the intensity of p38 and JNK phosphorylation at 60 min compared to those of the matched controls without CSC exposure. ERK phosphorylation was not affected by CSC exposure. (Fig. 3).

CSC inhibits epithelial NF-κB activation. NF-κB binding sites in promoter regions are essential for transcriptional activation of IL-6 and IL-8 genes (13, 23). NT *H. influenzae* is a known inducer of NF-κB in epithelial cells (29). To confirm the role of NF-κB in NT *H. influenzae*-mediated IL-8 production, we used TPCK, a chemical inhibitor that prevents IκB degradation and nuclear localization of NF-κB. Pretreatment of A549 cells with 50 μM TPCK inhibited IL-8 production 4-fold (Fig. 4A). After establishing the importance of NF-κB in the NT *H. influenzae*-mediated IL-8 response, we analyzed whether CSC had an inhibitory effect on *H. influenzae*-induced NF-κB activation. A549 cells transfected with a plasmid carrying a firefly luciferase reporter gene downstream of NF-κB

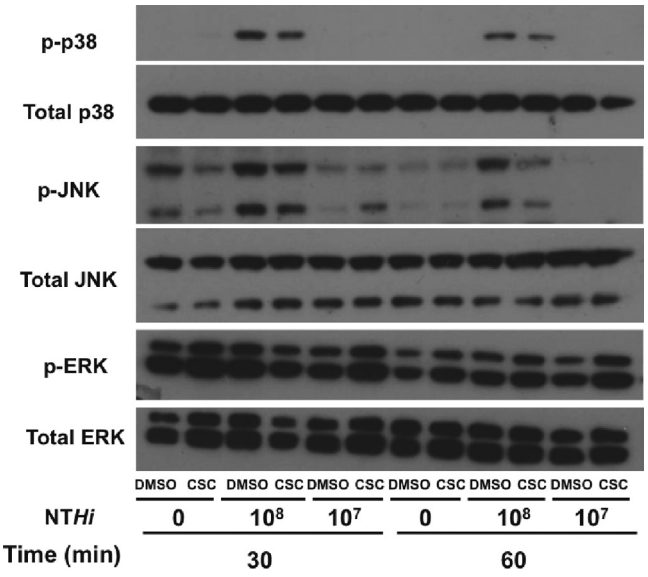

FIG. 3. MAPK activation in response to bacteria with or without CSC exposure. A549 cells were treated with 200 μg/ml CSC or DMSO with or without HK NT *H. influenzae* for 30 or 60 min, and cell lysates were separated on SDS-PAGE and probed for phosphorylated and total forms of p38, JNK, and ERK MAP kinases. Results for total MAPK are shown as loading controls.

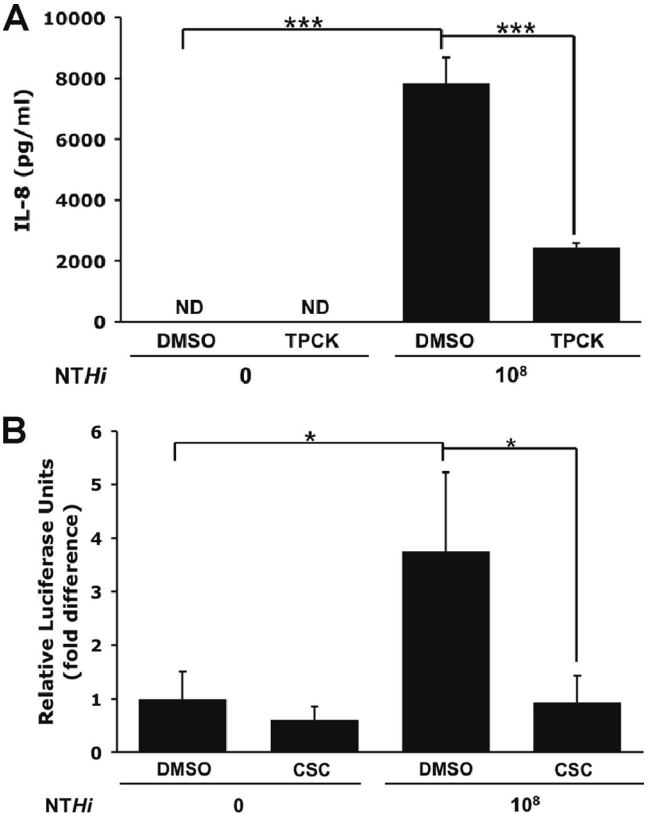

FIG. 4. CSC decreases NT *H. influenzae*-mediated NF-κB activation. (A) A549 monolayers were treated with 50 μM TPCK or DMSO for 30 min, washed with fresh medium, and treated with HK NT *H. influenzae* for 18 h prior to determination of IL-8 concentrations in cell-free supernatants. (B) Luciferase-based assay of NF-κB activity in A549 cells treated with 200 μg/ml CSC with or without HK NT *H. influenzae* for 6 h prior to cell lysis.

binding site repeats ($pNF\kappa B_{luc}$) were treated with HK *H. influenzae* with or without CSC, and luminescence was measured. Treatment with CSC reduced the *H. influenzae*-induced epithelial NF-κB activation to levels indistinguishable from those in untreated cells (Fig. 4B).

Antioxidants prevent CSC-mediated IL-8 downregulation. Free radicals and reactive oxygen species (such as O_2^-, NO, OH^-, and H_2O_2) contained in CS increase oxidative stress in the respiratory tract, leading to pathology (14). To analyze whether the CSC-mediated IL-8 downregulation in human airway epithelial cell lines is a function of free radicals, we treated CSC with the antioxidant NAC. Following treatment with 25 mM NAC (Fig. 5), CSC had no suppressive effect on *H. influenzae*-mediated upregulation of epithelial IL-8, indicating a potential role for oxidative stress in the immunosuppressive nature of CS.

DISCUSSION

The epithelial lining of the human upper respiratory tract is normally inhabited by a wide variety of organisms, many of which are potential pathogens. Epithelial cells contribute to the defense of the upper respiratory tract by at least three mechanisms: (i) tight junctions between epithelial cells form a barrier to pathogen entry; (ii) a mucociliary escalator clears foreign substances from the airway; and (iii) epithelial innate immune signaling leads to local secretion of proinflammatory cytokines and recruitment of professional immune cells. Bacterial products and environmental insults, such as inhalation of CS, may affect the upper respiratory tract by damaging the physical structure of the epithelial lining or by altering innate immune responses of epithelial cells. We examined the effects of concurrent exposure to CS and bacterial products on airway epithelial innate immune signaling and found that CS suppressed innate immune responses to common respiratory pathogens, such as NT *H. influenzae* and *S. aureus*.

Diverse microbial pathogens stimulate airway epithelial

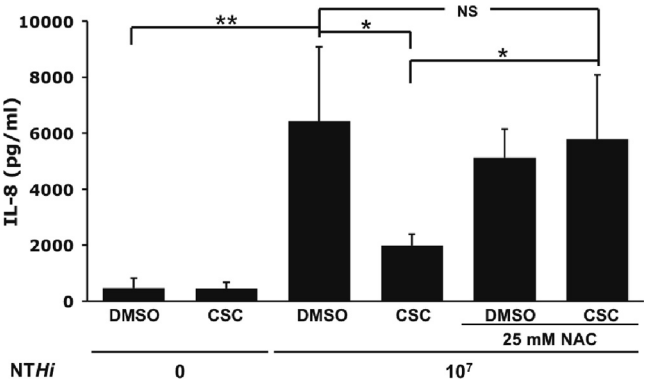

FIG. 5. Antioxidant treatment abrogates CSC-mediated IL-8 suppression. A549 monolayers were treated with CSC and/or HK NT *H. influenzae* for 18 h. CSC or DMSO was pretreated with 25 mM NAC for 30 min. IL-8 concentrations in supernatants were assayed by ELISA. Similar results were obtained with *H. influenzae* at a concentration of 10^8 CFU/ml (not shown).

cells, leading to activation of the transcriptional activator NF-κB and production of proinflammatory cytokines, such as IL-6 and IL-8 (5, 15, 20, 28, 30). In this study, we observed that CS inhibited IL-8 production brought about by NT *H. influenzae* and *S. aureus* in a dose-dependent manner. This effect was conserved in physiologically relevant human upper airway cell lines, such as A549 and 1HAEo−, as well as in the nonairway HeLa cell line. We saw similar effects when we treated A549 cells with freshly prepared CSE and NT *H. influenzae*, which confirmed that epithelial immunosuppression mediated by CSC was not due to a component introduced during the manufacturing of CSC. We also noted that CSC decreased the epithelial cytokine response to TNF-α but did not affect the overall transcription of IFN-β or the GAPDH housekeeping gene, suggesting that a global transcriptional block was not the mechanism involved. These findings are consistent with prior studies demonstrating an inhibitory effect of CSC on lipopolysaccharide (LPS)-induced IL-8 production in lung epithelial cells (17, 26) and with a *Caenorhabditis elegans* model of CS inhibition of innate immunity (11).

Production of IL-8 may be controlled either by regulating its transcription or by altering the rate of degradation of unstable IL-8 mRNA (13). Real-time PCR analysis of A549 lung epithelial cells revealed a significant reduction in the levels of IL-8 mRNA transcripts in cells treated with CSC and HK NT *H. influenzae* compared to those treated with HK NT *H. influenzae* alone.

The IL-6 and IL-8 promoter elements contain a binding site for the transcription factor NF-κB (12, 23). NT *H. influenzae* activates NF-κB in different human epithelial cell lines, including A549 and HeLa, leading to the upregulation of IL-8 in a Toll-like receptor 2-dependent manner (29). CS by itself has been reported to bring about modest (2) to no (25, 26) activation of NF-κB in airway epithelial cells, though this effect may be cell type dependent, as myeloid cells appear to have more significant proinflammatory responses to CSC (2). We noted an increase in activity of NF-κB-dependent transcription when cells were treated with HK NT *H. influenzae*. This was reduced to levels indistinguishable from background in the presence of CSC. This demonstrates potent NF-κB inhibition by CSC and suggests that this mechanism may be partially responsible for the transcriptional repression of IL-6 and IL-8 genes in epithelial cells concurrently exposed to CSC and bacteria.

MAPK signaling can be induced in response to bacterial products and may regulate epithelial cytokine production (1, 9, 27). We noted a minor but reproducible inhibitory effect of CS on the kinetics of NT *H. influenzae*-induced MAPK phosphorylation. This suggests that repression of proinflammatory signaling leading to IL-6 and IL-8 production in CS-treated cells may occur both through modulation of MAPK signaling and through effects on gene transcription, though it appears likely that transcriptional effects dominate. In addition, the striking difference between the effect of CSC on NT *H. influenzae*-induced NF-κB activation (Fig. 4B) and its effect on either IFN-β induction (Fig. 2C) or MAPK phosphorylation (Fig. 3) indicates that there is some specificity to the signaling pathways affected. We do not argue that the NFκB pathway is the only affected pathway in CSC-exposed epithelial cells, but it appears to be more sensitive than are some others, with consequences for bacterium-induced cytokine production.

CS contains a high concentration of free radicals and other oxidants, such as nitric oxide (NO), superoxide anions, hydroxyl radical, and hydrogen peroxide, which cause direct and indirect damage to a variety of cell types (4, 14, 19, 24, 31, 32). Treatment of CSC with 25 mM antioxidant NAC abrogated the inhibitory effect of CSC on NT *H. influenzae*-induced IL-8 production. This finding implicates reactive oxygen species in the immunosuppressive effects of CS on airway epithelial cells.

Exposure to CS leads to a predisposition to a variety of adverse health conditions, including respiratory tract infections. On the basis of the results described in this report, we conclude that CS may have local immunosuppressive properties, affecting the innate immune ability of airway epithelial cells to detect potential pathogens and produce proinflammatory cytokines, such as IL-6 and IL-8, to attract neutrophils, and to successfully ward off respiratory pathogens, such as NT *H. influenzae* and *S. aureus*. This may aid colonization of the respiratory mucosa and subsequent infection of both the lower airways and distant sites.

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