Epithelial Cells from Smokers Modify Dendritic Cell Responses in the Context of Influenza Infection

Katherine M. Horvath[1], Luisa E. Brighton[2], Wenli Zhang[2], Johnny L. Carson[2,3], and Ilona Jaspers[1–3]

[1]Curriculum in Toxicology, [2]Center for Environmental Medicine, Asthma, and Lung Biology, and [3]Department of Pediatrics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

Epidemiologic evidence suggests that cigarette smoking is a risk factor for infection with influenza, but the mechanisms underlying this susceptibility remain unknown. To ascertain if airway epithelial cells from smokers demonstrate a decreased ability to orchestrate an influenza-induced immune response, we established a model using differentiated nasal epithelial cells (NECs) from nonsmokers and smokers, co-cultured with peripheral blood monocyte–derived dendritic cells (mono-DCs) from nonsmokers. NEC/mono-DC co-cultures were infected with influenza A virus and analyzed for influenza-induced immune responses 24 hours after infection. We observed that NECs from smokers, as well as mono-DCs co-cultured with NECs from smokers, exhibited suppressed influenza-induced, interferon-related proteins interferon regulatory factor–7, Toll-like receptor–3, and retinoic acid inducible gene–1, likely because of the suppressed production of IFNα from the NECs of smokers. Furthermore, NEC/mono-DC co-cultures using NECs from smokers exhibited suppressed concentrations of T-cell/natural killer cell chemokine interferon gamma–induced protein 10 (IP-10) after infection with influenza, indicating that NECs from smokers may skew early influenza-induced Th1 responses. In contrast, NEC/mono-DC co-cultures using NEC from smokers contained increased influenza-induced concentrations of the Th2 chemokine thymic stromal lymphopoeitin (TSLP). In addition, NECs from smokers cultured alone had increased influenza-induced concentrations of the Th2 chemokine thymus and activation-regulated chemokine (TARC). Using this model, we demonstrated that in the context of infection with influenza, NECs obtained from smokers create an overall cytokine microenvironment that suppresses the interferon-mediated Th1 response and enhances the TSLP–TARC–mediated Th2 response, with the potential to modify the responses of DCs. Smoking-induced alterations in the Th1/Th2 balance may play a role in developing underlying susceptibilities to respiratory viral infections, and may also promote the likelihood of acquiring Th2 proallergic diseases.

Keywords: influenza; cigarette smoke; nasal epithelial cell; dendritic cell; co-culture

CLINICAL RELEVANCE

This research helps elucidate how exposure to cigarette smoke affects antiviral and immune defenses in epithelial cells and dendritic cells of the respiratory epithelium.

Cigarette smoking is a risk factor for infection with respiratory viruses such as influenza A. After the influenza A epidemic in Hong Kong of 1968, Finklea and colleagues reported that smokers who smoked more than 21 cigarettes (~1 pack) per day were subject to a 21% increase in clinical influenza incidence and an increase in illness severity compared with nonsmokers (1). Subsequent epidemiologic studies demonstrated that cigarette smoking leads to an increased incidence and severity of influenza infection in multiple populations, including female military recruits (2) from the United States, male Israeli soldiers (3), and senior citizens in assisted-living communities (4). A meta-analysis in 2004 confirmed that along with influenza infections, smokers are increased risk for invasive pneumococcal disease and tuberculosis (5), and associations were drawn between smoking and the common cold (6). Combined with evidence that smokers have lower influenza vaccination rates than nonsmokers, the increased risk for infection becomes an even more significant public health issue (7, 8). However, the mechanisms mediating the enhanced susceptibility to viral infections in smokers are not known. In both human (9–11) and animal (12) models of influenza, adaptive humoral immunity, as measured by influenza-specific antibody production, was unaffected by exposure to cigarette smoke. Therefore, cigarette smoke more likely affects innate immune mechanisms during an influenza infection.

Exposure to cigarette smoke was shown to suppress the innate immune responses of the respiratory epithelium (13). The influenza virus infects epithelial cells by binding via hemagglutinin and entering cells via residues of sialic acid, using endocytosis. Soon after infection, influenza activates the innate immune system of the respiratory epithelium and stimulates the generation of cytokines and chemokines (14). Pathogen recognition receptors (PRRs), such as Toll-like receptor–3 (TLR-3), and retinoic acid inducible gene–1 protein (RIG-I) recognize viral RNA and activate nuclear transcription factors such as NF-κB, interferon regulatory factor–3 (IRF-3), and IFN regulatory factor–7 (IRF-7) in epithelial cells. The synthesis and binding of IRFs to interferon response element promoter regions induce the production of antiviral Type I IFNs (e.g., IFNα/β) that help prevent further viral infection. We previously showed that the expression of IRF-7 is decreased in nasal epithelial cells (NECs) from smokers after infection with influenza, yielding a suppressed production of Type I IFN in these cells (13). Similarly, media conditioned with cigarette smoke were shown to inhibit the responses of IRF-3, IRF-7, and NF-κB to polyinosinic:polycytidylic acid (poly I:C) in lung epithelial cells and fibroblasts (15).

The interplay between the airway epithelium and resident immune cells such as dendritic cells (DCs) is crucial in mobilizing respiratory immune responses. Activated DCs fulfill the pivotal task of mobilizing both innate and adaptive immune

(*Received in original form May 10, 2010 and in final form September 3, 2010*)

This work was supported in part by grant HL095163 from the National Heart, Lung, and Blood Institute of the National Institutes of Health, by a grant from the Flight Attendant Medical Research Institute, and by grant CR829522 from the Environmental Protection Agency (all to I.J.). This research was also supported by grant 56005708 from the Howard Hughes Medical Institute and by Training Grant 5 T32 ES007126 from the National Institute of Environmental Health Sciences (K.M.H.).

Correspondence and requests for reprints should be addressed to Ilona Jaspers, Ph.D., Department of Pediatrics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7310. E-mail: Ilona_jaspers@med.unc.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 45. pp 237–245, 2011
Originally Published in Press as DOI: 10.1165/rcmb.2010-0190OC on October 8, 2010
Internet address: www.atsjournals.org

cells by secreting chemokines to attract neutrophils, cytotoxic and memory T cells, and natural killer (NK) cells (16). Specifically, DCs produce chemokines such as regulated upon activation, normal T-cell expressed, and secreted (RANTES) chemokine (C-X-C motif) ligand 9 (CXCL9), interferon gamma–induced protein 10 (IP-10), and CXCL11, and drive a Th1 $CD4^+$ T-cell response to expand activated, cytotoxic pools of T cells (17). The direct infection of DCs with influenza virus was shown to activate DC antiviral defenses, including RIG-I, TLR-3, and IRF-7 (18), but whether and how signals derived from the microenvironment, such as surrounding epithelial cells, modify these effects remains unknown.

Respiratory DCs are capable of responding not only to Th1 cytokines produced during a viral infection, but also to Th2 chemokines produced by a dysregulated respiratory epithelium. Thymic stromal lymphopoeitin (TSLP) is an epithelial cell–derived Th2 chemokine that communicates with and induces Th2 responses in DCs. TSLP acts directly on DCs to increase the production of thymus and activation-regulated chemokine (TARC or CCL17) (19), which in turn coaxes naive T cells to secrete proallergic cytokines such as IL-5, IL-4, IL-13, and TNFα, which have the potential to foster the development of an allergic Th2 CD4 T-cell phenotype (20). Although normal human bronchial epithelial cells were demonstrated to up-regulate TSLP in response to stimulation with the viral double stranded RNA (dsRNA) dsRNA mimetic poly-I:C (21), how the virus-induced expression of TSLP or TARC may be altered during an influenza infection in smokers is unknown.

In vitro studies that treat DCs directly with cigarette smoke may not provide the most realistic model system, because signals derived from the respiratory epithelium are important in generating the proper microenvironment for the maturation of DCs *in vivo*. To address the limitations of human *in vitro* airway epithelia models, co-culture systems of airway epithelial cells and DCs were developed to study the effects of particles on the airway epithelium (22–24). These models have the potential to explore the mechanisms of airway epithelial and immune cell communication during immunologic responses, including respiratory viral infections. We expanded upon these models to develop a co-culture system of human NECs obtained from nonsmokers and smokers and monocyte-derived DCs (mono-DCs) obtained from healthy nonsmokers, to determine how smoking-induced changes at the epithelial level affect communication with resident immune cells.

MATERIALS AND METHODS

Culture of NECs

NECs were obtained from smoking and nonsmoking healthy human volunteers and differentiated *in vitro* on 0.4-μM pore size membrane support, as described previously (13). The criteria for recruiting subjects were similar to those described previously (13, 25). Smoking status was assessed via questionnaire and confirmed through measurements of urine cotinine (25). All smokers recruited for the study were current smokers.

Culture of Mono-DCs, NEC–Mono-DC Co-culture System, and Infection with Influenza

Peripheral blood monocytes were isolated from healthy nonsmoking volunteers. Mono-DCs were generated by culturing peripheral blood monocytes with 30 ng/ml granulocyte/macrophage colony–stimulating factor (GM-CSF) (Peprotech, Rocky Hill, NJ) and 30 ng/ml IL-4 (Peprotech) for 5–7 days. The differentiation of mono-DCs was confirmed using flow cytometry to identify the expression of DC markers CD86, CD40, CD209, human leukocyte antigen DR (HLA-DR), and CD11c, as described in the online supplement (BD Biosciences, San Jose, CA).

Our NEC–mono-DC co-culture system was based on a three-dimensional cell culture model described previously (22). We applied 1.5×10^5 mono-DCs to the basolateral side of inverted, differentiated NECs grown on a membrane support of approximately 1.13 cm^2 (see Figure E1 in the online supplement). Mono-DCs were adhered for 2 hours, and then NECs were infected with influenza A/Bangkok/1/79 (H3N2 serotype), as described previously (26). All samples were collected 24 hours after infection, unless otherwise indicated.

Supernatant Concentrations of Cytokine

The apical surfaces of co-cultured NECs were washed with Hanks' balanced salt solution. Apical washes and basolateral supernatants were collected and analyzed for cytokine secretions of IP-10 (BD Biosciences), RANTES, TARC, and TSLP (all from R&D Systems, Minneapolis, MN), using commercially available ELISA kits.

Analysis of mRNA from NECs and Mono-DCs

NECs and mono-DCs were removed from the membrane and added to Trizol (Invitrogen, Carlsbad, CA) for isolation of total RNA. Real-time, quantitative RT-PCR was performed as described previously (26), using commercially available primers and probes for TLR-3, RIG-I, IRF-7, IP-10, and RANTES (Applied Biosystems, Foster City, CA).

Western Blotting

Whole-cell lysates were prepared and analyzed as described elsewhere (13), using specific antibodies to IRF-7 (Santa Cruz Biotechnology, Santa Cruz, CA) or β-actin (1:2,000, US Biological, Swampscott, MA).

Visualization of Co-culture System

Co-cultures were fixed with ice-cold methanol for 20 minutes. Antibodies to CD11c (eBioscience, San Diego, CA) and acetylated α-tubulin (Abcam, Cambridge, MA) were used to identify mono-DCs and the cilia of NECs, respectively, followed by incubation with Alexa-488–conjugated and Alexa-596–conjugated secondary antibodies (Invitrogen). Samples were visualized using a Nikon C1Si laser scanning confocal microscope, and images were processed using EZ-C1 FreeViewer software (Nikon Instruments, Melville, NY).

Statistical Analysis

Data regarding mRNA and mono-DC maturation from co-culture experiments are expressed as fold induction over noninfected control samples to determine influenza-induced responses, and were analyzed using the Wilcoxon signed-rank test. Differences in influenza-induced and baseline responses between nonsmoker and smoker NECs were analyzed using a nonparametric Mann-Whitney U test. Protein supernatant data were analyzed using two-way ANOVA, followed by Bonferroni *post hoc* analysis. All data are expressed as mean ± SEM, with $P < 0.05$ considered significant.

RESULTS

Development and Visualization of Co-culture Model

To develop mono-DCs, peripheral blood monocytes were incubated with IL-4 and GM-CSF and analyzed for the positive expression of characteristic DC surface markers. Before co-culture with NECs, mono-DCs were analyzed by flow cytometry and shown to demonstrate positive expression of the DC maturation markers CD11b, CD11c, CD86, CD209, and HLA-DR (data not shown).

To determine whether mono-DCs form networks on the basolateral side of NECs in our co-culture model, we visualized the different cell types, using confocal microscopy. Figure 1 (*top*) provides an *en face* visualization of the apical border of the epithelium (*left*) and the mono-DCs on the basolateral side. To stain the cilia of NECs, we used mouse anti-acetylated α tubulin, followed by an Alexa-596–conjugated secondary antibody (*red*), as described previously (26, 27). DCs were identified using murine anti-CD11c, followed by an Alexa-488–conjugated secondary antibody (Figure 1, *green*). Figure 1 (*bottom*) depicts

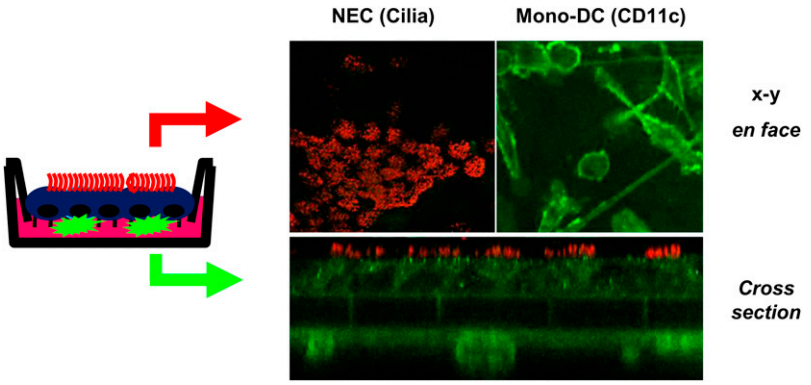

Figure 1. Visualization of the nasal epithelial cell (NEC)/mono-dendritic cell (DC) co-culture model. Co-culture membranes were fixed in ice-cold methanol for 20 minutes and stored in 70% ethanol until analysis. Antibodies to CD11c and acetylated α-tubulin were used to identify mono-DCs and cilia of NECs, respectively. After incubation with secondary Alexa-596 (NECs, *red*) and Alexa-488 (mono-DCs, *green*), conjugated antibody samples were visualized using confocal microscopy. Z-stack analyses were used to visualize cross sections of the co-culture system.

an *x–y–z* optical cross section of the co-cultureco-culture model, with the mono-DCs lying basolaterally to the nasal NECs, a polarization that resembles their *in vivo* orientation.

Mono-DCs from Nonsmoker and Smoker Co-cultures Have Similar Levels of DC Maturation after Infection with Influenza

To determine whether surface markers indicating the maturation of DCs are changed in mono-DCs cultured with NECs from smokers and nonsmokers, we analyzed the surface marker expression of mono-DCs by flow cytometry after infection with influenza. Cells were gated (P1) for the positive expression of leukocyte marker CD45 (Figure 2A). Mono-DCs co-cultureco-cultured with NECs exhibited a positive baseline expression of CD11b, CD40, CD209, CD11c, and CD86 (representative histograms in Figures 2B–2F). Figures 2G–2I show that infection with influenza did not change the expression of CD11b, CD40, and CD209 in mono-DCs cultured with NECs from either smokers or nonsmokers. In contrast, the expression of CD11c was only up-regulated after infection with influenza in mono-DCs derived from co-cultureco-cultures using NECs from non-

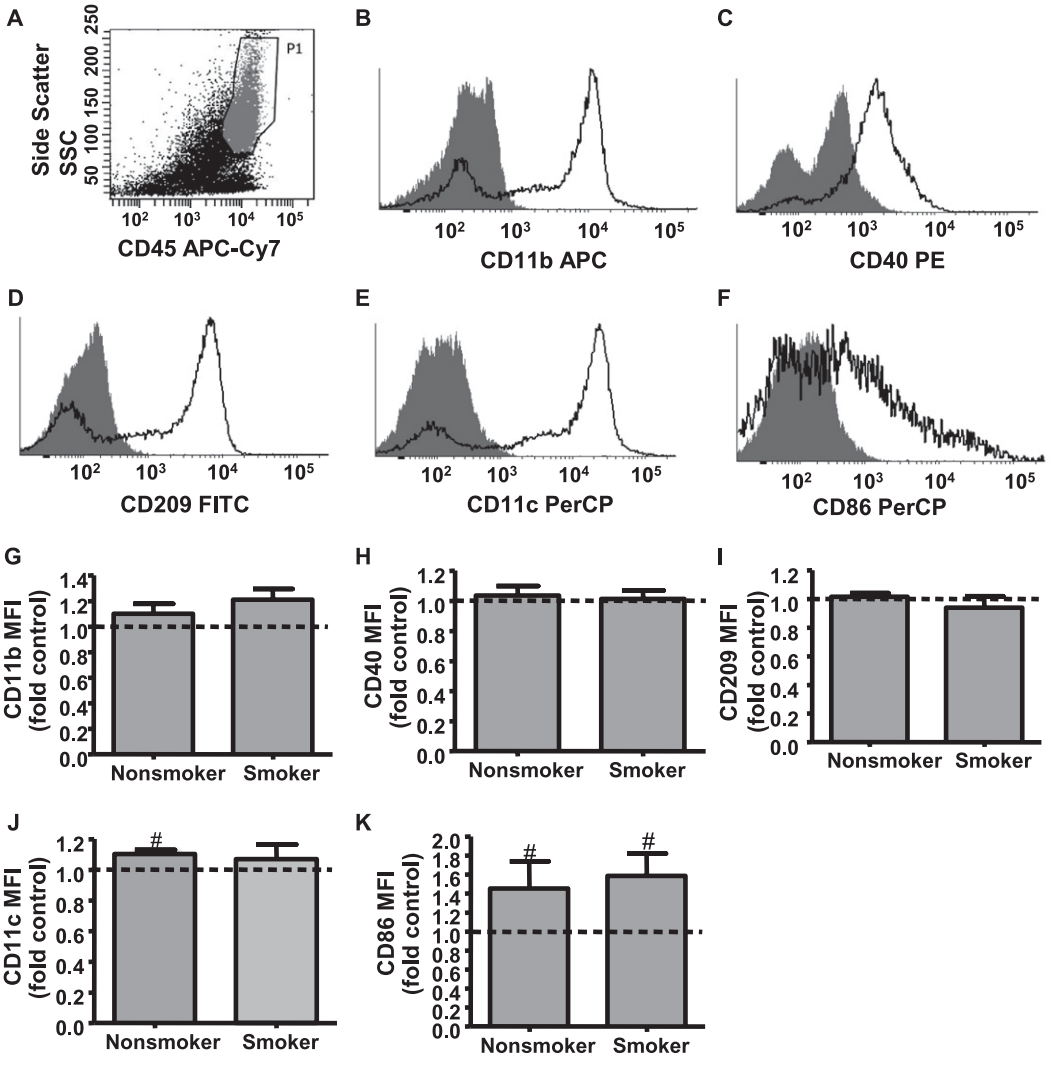

Figure 2. Mono-DCs co-cultured with either nonsmoker or smoker NECs have similar influenza-induced changes in DC maturation markers. Twenty-four hours after infection with influenza, mono-DCs were harvested from co-cultures and stained for DC markers, using flow cytometry. (*A*) $CD45^+$ leukocytes are identified in the P1 gate. Representative histogram plots show the expression of DC markers (*B*) CD11b, (*C*) CD40, (*D*) CD209, (*E*) CD11c, and (*F*) CD86, and the response to influenza (fold induction) of DC markers (*G*) CD11b, (*H*) CD40, (*I*) CD209, (*J*) CD11c, and (*K*) CD86. CD11b, CD40, CD209, and CD11c: nonsmokers, $n = 6$; smokers, $n = 5$; CD86: nonsmokers, $n = 7$; smokers, $n = 6$. Data are expressed as fold induction over noninfected control samples, and as mean ± SEM. $^{\#}P < 0.05$ versus noninfected control samples. MFI, mean fluorescence intensity; APC-Cy-7, allophycocyanin cyanine 7; APC, allophycocyanin; PE, phycoerythrin; FITC, fluorescein isothiocyanate; PerCP, peridinin chlorophyll protein.

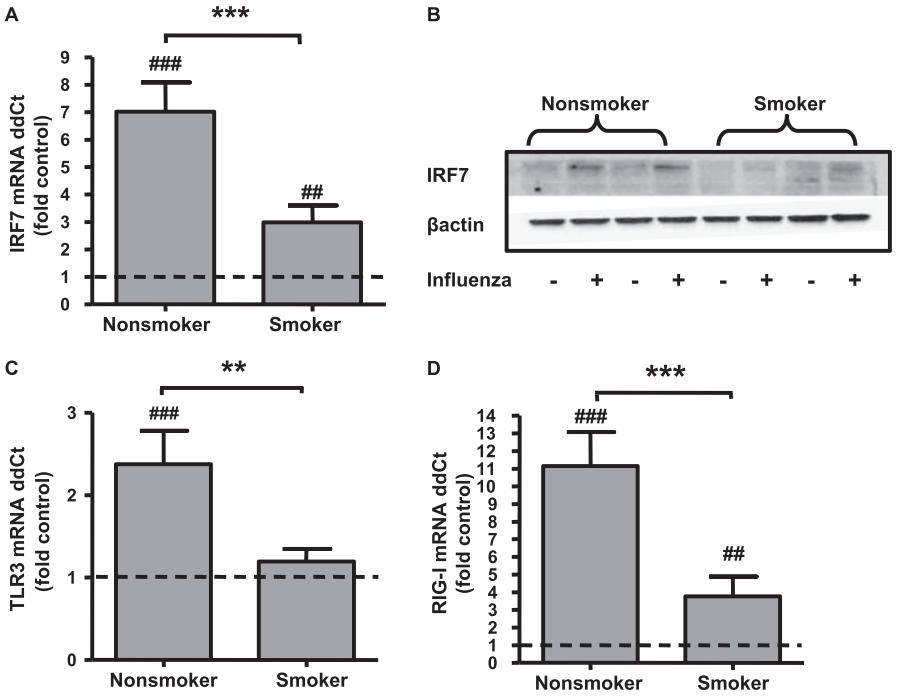

Figure 3. NECs from smokers have suppressed influenza-induced antiviral responses. NEC total RNA and whole-cell lysates from nonsmoker and smoker NEC/mono-DC co-cultures were collected 24 hours after infection with influenza. (*A*) Real-time quantitative RT-PCR was performed for interferon regulatory factor–7 (IRF-7). (*B*) NEC whole-cell lysates were analyzed by Western blotting for IRF-7 and then stripped and probed for β-actin. Real-time quantitative RT-PCR was performed for (*C*) Toll-like receptor–3 (TLR-3) and (*D*) retinoic acid inducible gene–1 protein (RIG-I). The mRNA expression of targets was normalized to β-actin, quantified using the delta delta Ct (ΔΔ Ct) method, and expressed as fold induction over noninfected control samples. mRNA data are expressed as mean ± SEM: nonsmokers, $n = 6$; smokers, $n = 6$. For Western blots, representative immunoblots are shown: nonsmokers, $n = 2$; smokers, $n = 2$. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus noninfected control samples. $^{**}P < 0.01$, $^{***}P < 0.001$, nonsmokers versus smokers.

smokers (Figure 2J). CD86 was enhanced after infection with influenza in mono-DCs derived from both nonsmoker and smoker co-cultureco-cultures (Figure 2K). However, changes in CD11c and CD86 did not appear to be robust.

NECs and Mono-DCs from Smoker Co-cultures Both Have Suppressed Antiviral Responses to Infection with Influenza

To determine how antiviral defense responses are modified, NEC/mono-DC cocultures using NECs from nonsmokers and smokers were infected from the epithelial side with one multiplicity of infection of influenza A virus. Total RNA was collected separately from both cell types 24 hours after infection and analyzed for antiviral responses. To determine whether our previous observations of suppressed influenza-induced IRF-7 expression in NECs from smokers (13) were also evident in this coculture model, we analyzed the expression of IRF-7 mRNA (Figure 3A) and protein (Figure 3B) in NECs from cocultures. Similar to our previous study (13), the influenza-induced expression of IRF-7 was suppressed in NECs from smokers. In addition to IRF-7, the influenza-induced expression of PRRs is important in antiviral defense responses. Figures 3C and 3D show that infection with influenza did not induce the expression of TLR-3 mRNA in NECs from smokers (Figure 3C), and that the influenza-induced expression of RIG-I mRNA was suppressed in NECs from smokers (Figure 3D). The baseline expression of RIG-I, TLR-3, and IRF-7 was similar in nonsmoker and smoker NECs, as shown in Table E1.

To determine whether changes in antiviral defense responses in NECs from smokers also led to suppressed responses in mono-DCs, we analyzed the same antiviral defense markers in the mono-DCs of the co-cultureco-cultures. Infection with influenza increased the concentrations of IRF-7 (Figure 4A) and RIG-I (Figure 4B) mRNA in mono-DCs co-cultureco-cultured with NECs from either nonsmokers or smokers, but this induction was significantly greater in mono-DCs cultured with NECs from nonsmokers. Infection with influenza did cause an increase in the concentrations of TLR-3 mRNA in mono-DCs co-cultured with NECs from nonsmokers, although this increase did not reach statistical significance ($P = 0.06$) (Figure 4C).

NECs and Mono-DCs from Smoker Co-cultures Exhibit Suppressed Up-regulation of Th1 Chemokines in Response to Infection with Influenza

The activation of TLR-3 and RIG-I prompts the production of inflammatory and immune mediators, including chemokines such as IP-10 and RANTES (28, 29), which are important for the migration of immune cells. Therefore, we examined the expression of these two chemokines in our co-cultures. The baseline expression of RANTES and IP-10 mRNA is similar in nonsmoker and smoker NECs from co-cultures (Table E1). The expression of influenza-induced IP-10 mRNA is suppressed in both NECs (Figure 5A) and mono-DCs (Figure 5B) from co-cultures using NECs from smokers. The use of transwell inserts allowed for separate analyses of protein secretion from the apical (upper) and basolateral (lower) compartments of the mono-DC/NEC co-culture system (Figure 1), as reported previously (27, 30). In both apical washes (Figure 6A) and basolateral supernatants (Figure 6B), the secretion of IP-10 in influenza-infected co-cultures using smoker NECs was suppressed compared with nonsmoker control samples. Infection with influenza induces the expression of RANTES in NECs from both nonsmokers and smokers (Figure 5C), but not in mono-DCs derived from co-cultures (Figure 5D). Interestingly, the secretion of RANTES protein is increased in both apical washes and basolateral supernatants from co-cultures using influenza-infected NECs from nonsmokers but not from smokers. Overall, these data indicate that influenza-induced IP-10 and potentially RANTES are suppressed in both NEC and mono-DCs co-cultures using NECs from smokers.

Infection with Influenza Increases Th2 Chemokines in NECs from Smokers

Exposure to cigarette smoke can lead to a Th2 phenotypic immune response (31). Therefore, after demonstrating that co-cultures using NECs from smokers show a suppressed expression of chemokines associated with Th1 responses (e.g., IP-10), we determined whether chemokines associated with Th2 phenotypes were altered. We first analyzed the expression of TSLP, a Th2 chemokine that is secreted by NECs and is up-regulated

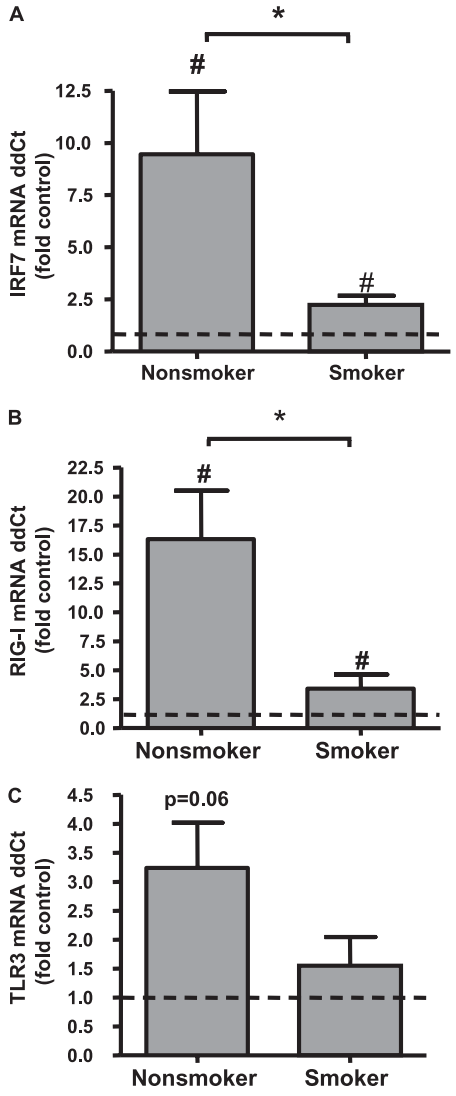

Figure 4. Mono-DCs co-cultured with smoker NECs have suppressed influenza-induced antiviral responses. RNA isolated from co-cultured mono-DCs was collected 24 hours after infection with influenza. Real time quantitative RT-PCR was performed for (*A*) IRF-7, (*B*) RIG-I, and (*C*) TLR-3. The mRNA expression of targets was normalized to β-actin, quantified using the ΔΔCt method, and expressed as fold induction over noninfected control samples. Data are expressed as mean ± SEM. Nonsmokers, $n = 5$; smokers, $n = 5$. $^{\#}P < 0.05$ versus noninfected control samples. $^{*}P < 0.05$, nonsmokers versus smokers.

in murine lungs after exposure to cigarette smoke extract (32). Infection with influenza increased the secretion of TSLP into the basolateral supernatant in co-cultures using NECs from smokers compared with nonsmokers (Figure 7A). TSLP was not detectable in the apical compartment (data not shown). TSLP acts upon DCs to stimulate the production of TARC (19). Therefore, we determined whether the changes in expression of TSLP were reflected in an increased expression of TARC in basolateral supernatants from cocultures using NECs from smokers. Overall, infection with influenza did not increase concentrations of TARC in the basolateral supernatants from NEC/mono-DCs co-cultures using NECs from nonsmokers or smokers (Figure 7B), and concentrations of TARC were below detection in the apical compartment (data not shown). Considering that mono-DCs from healthy individuals contain high constitutive levels of TARC expression (33), mono-DCs are likely to contribute heavily to the overall high concentrations of TARC in the basolateral supernatants. Previous studies showed that in addition to DCs, TARC can be expressed in other respiratory cell types, including epithelial cells (34). To determine further if the influenza-induced expression of TARC is altered in NECs from smokers alone, we designed experiments in which NECs in the absence of mono-DCs were analyzed. Figure 7 shows that infection with influenza significantly increased the production of TARC in NECs from smokers, but not in NECs from nonsmokers, indicating that NECs may be significant producers of TARC in smokers but not nonsmokers.

DISCUSSION

Communication between DCs and other cell types during an immune response is crucial for the activation of DCs, and single-cell culture models using DCs cannot be used to study this interaction. Our two-cell co-culture model of NECs and DCs allowed us to determine the interplay between the virally infected epithelium and DCs, and also to determine how smoking may interrupt this communication. Using this model, we demonstrated that in the context of a viral infection, NECs obtained from smokers demonstrate altered communication with underlying mono-DCs, creating an overall cytokine microenvironment that suppresses the interferon-mediated Th1 response and enhances the TSLP–TARC–mediated Th2 response.

We showed here that coculturing mono-DCs with NECs from smokers alters the mono-DC response to infection with influenza. The nature of this communication is likely through "wireless" cytokine secretion, although other possibilities exist. Activated DCs were shown to release exovesicles, small membrane-bound vesicles that can contain cytokine and immune receptors (35). These exovesicles are capable of activating TNF-α pathways in airway epithelial cells in a co-culture model (36). Thus, in our model, NEC and mono-DCs may have communicated through exovesicle-mediated pathways. Moreover, a co-culture model of lung epithelial A549 cells and mono-DCs suggested that DCs are capable of forming intraepithelial cell projections through the pores of the cell culture membrane, to engage in direct epithelial cell–DC contact (24). Although we cannot rule out such interactions because of the smaller pore size we must use for efficient cultures of differentiated NECs (0.4-μM versus the 3.0-μM size used by Blank and colleagues [24]), we doubt that mono-DC projections play a large role in their activation. Moreover, the mono-DCs used in our studies did not exhibit any markers of direct infection with influenza (i.e., no detectable influenza hemagglutinin RNA; data not shown), suggesting that they may not have access to the apical surface where infection is occurring. Therefore, in our model, antiviral mediator and cytokine expression may have been initiated by epithelial-derived cytokines and chemokines acting on mono-DCs. For example, NEC-derived IFNs are secreted by virally infected epithelial cells (13) and can activate nearby NECs in an autocrine fashion, to induce the expression of interferon-related gene products, including TLR-3 (27). Similarly, influenza-infected NECs could also activate nearby DCs in a paracrine fashion via IFNα secretion to act upon mono-DC IFNα receptors, culminating in the transcription of IRF-7, TLR-3, RIG-I, and IP-10. Thus, the reduced expression of IFNα seen in NECs from smokers, as shown in our previous study (13), may lead to suppressed IFN-induced antiviral defense responses in underlying immune cells, such as DCs. Overall, these data demonstrate that in addition to the release of Type I IFN and IRF-7 expression (13), the expression of PRRs such as RIG-I and TLR-3 are suppressed in smoker NECs, potentially resulting in the suppressed activation of resident immune cells that

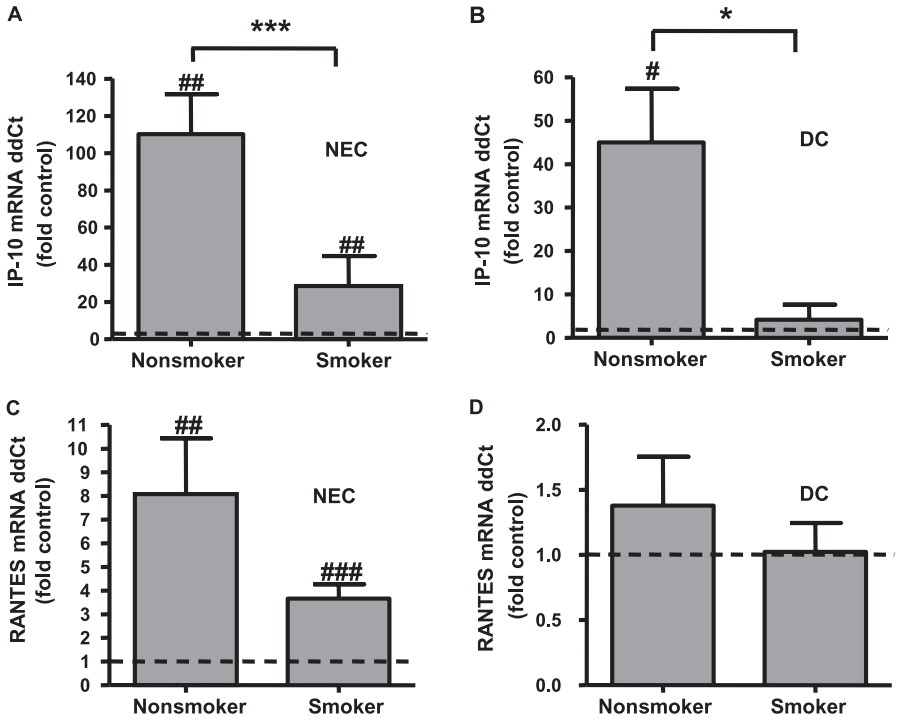

Figure 5. NECs from smokers as well as mono-DCs derived from co-cultures using smoker NECs have suppressed influenza-induced Th1 responses. NEC and mono-DC total RNA from nonsmoker and smoker NEC/mono-DC cocultures was collected 24 hours after infection with influenza. Real-time quantitative RT-PCR was performed for interferon gamma–induced protein 10 (IP-10) in (*A*) NECs and (*B*) mono-DCs. Real-time quantitative RT-PCR was also performed for regulated upon activation, normal T-cell expressed, and secreted (RANTES) in (*C*) NECs and (*D*) mono-DCs. The mRNA expression of targets was normalized to β-actin, quantified using the ΔΔCt method, and expressed as fold induction over noninfected control samples. Data are expressed as mean ± SEM. Nonsmokers, $n = 5$; smokers, $n = 5$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus noninfected control samples. $^{*}P < 0.05$, $^{***}P < 0.001$, nonsmokers versus smokers.

communicate with the respiratory epithelium during an influenza infection.

Our data demonstrate that the influenza-induced expression of IP-10 is reduced in both NECs from smokers and in mono-DCs co-cultured with NECs from smokers, thereby leading to overall reductions in the secretion of IP-10 protein in both the apical and basolateral compartments of co-culture systems. During an influenza infection, IP-10 is released from NECs to attract lymphocytes and T cells to the site of infection (37). Smoke-exposed mice infected with influenza have suppressed levels of whole-lung IP-10 mRNA, with modified profiles of CD4/CD8 T cells in their bronchoalveolar lavage fluid and draining lymph nodes (38). Data from our own laboratory showed that smokers inoculated with live, attenuated influenza virus (LAIV) have reduced concentrations of IP-10 in nasal lavage fluid compared with nonsmoker control samples (T.L. Noah, personal communication). In addition to T cells, the suppressed production of IP-10 by the respiratory epithelium could lead to a suppressed recruitment of other immune cells such as NK cells. NK cells express the IP-10 receptor CXC

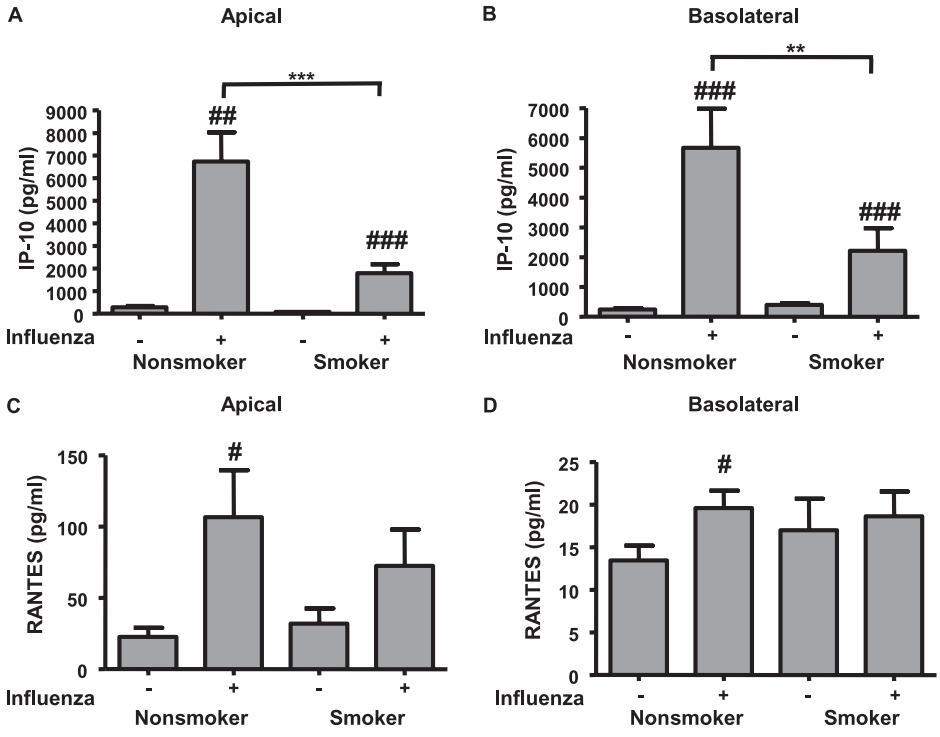

Figure 6. NEC/mono-DC co-culture apical washes and basolateral supernatants using NECs from smokers have suppressed influenza-induced Th1 chemokines. NEC/mono-DC co-cultures using NECs from nonsmokers and smokers were harvested 24 hours after infection with influenza. IP-10 protein concentrations in (*A*) apical washes (nonsmokers, $n = 8$; smokers, $n = 7$) and (*B*) basolateral supernatants (nonsmokers, $n = 5$; smokers, $= 7$) and RANTES protein concentrations in (*C*) apical washes (nonsmokers, $n = 8$; smokers, $n = 7$) and (*D*) basolateral supernatants (nonsmokers, $n = 9$; smokers, $n = 7$) were measured by ELISA. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus noninfected control samples. $^{*}P < 0.05$, $^{***}P < 0.001$, nonsmokers versus smokers.

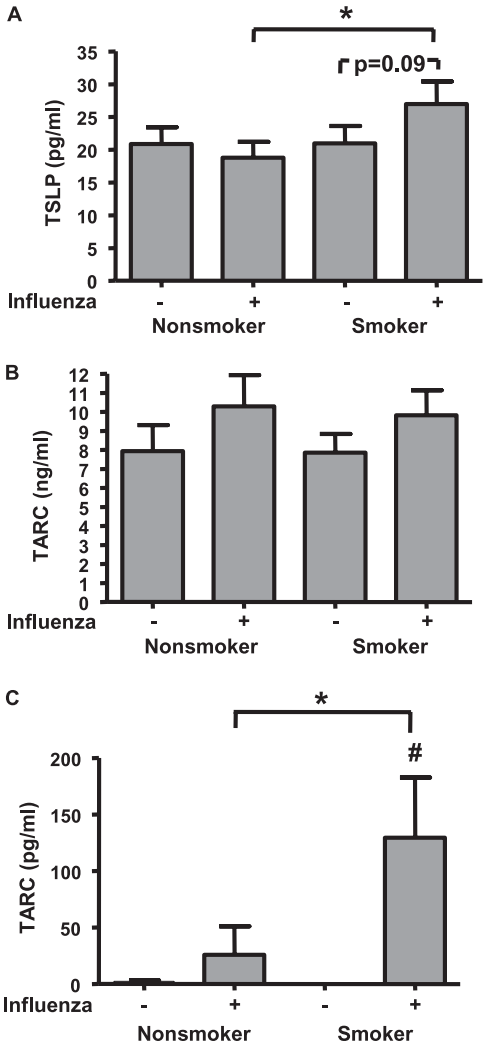

Figure 7. NEC/mono-DC co-culture supernatants using NECs from smokers have increased influenza-induced concentrations of the Th2 chemokine thymic stromal lymphopoeitin (TSLP). Basolateral supernatants from NEC/mono-DC co-cultures were collected 24 hours after infection with influenza, and were analyzed via ELISA for (*A*) TSLP (nonsmokers, $n = 9$; smokers, $n = 8$) and (*B*) TARC (nonsmokers, $n = 10$; smokers, $n = 8$). Data are expressed as fold induction over noninfected control samples, and as mean ± SEM. $^{\#}P < 0.05$ versus noninfected control samples. $^{*}P < 0.01$, nonsmokers versus smokers. (*C*) Basolateral supernatants from NECs cultured alone were collected 24 hours after infection with influenza, and were analyzed for TARC concentrations via ELISA. Data are expressed as mean ± SEM. Nonsmokers, $n = 7$; smokers, $n = 5$. $^{\#}P < 0.05$ versus noninfected control samples.

chemokine receptor 3 and migrate to the respiratory epithelium during an influenza infection (39). Decreased IP-10 responses could be responsible for decreased cytotoxic NK cell numbers, and could explain why levels of granzymes B and K, important mediators of cytotoxic NK cells, are reduced after influenza infections of mice exposed to cigarette smoke (38). Thus, the reduced production of IP-10 by either NECs or DCs after viral infections could exert a significant impact on the ability to fight and clear infections. Infection with influenza increases the secretion of RANTES into the apical and basolateral compartments in nonsmokers but not in smokers, although no difference was evident between groups. Like IP-10, RANTES is an important chemokine that is released during infection with influenza (37) A lack of RANTES response in smokers may indicate that the NECs of smokers have decreased communication with immune cells, highlighting the importance of non-immune cells in orchestrating both innate and adaptive immune responses to viral infections.

Exposure to cigarette smoke was shown to shift immune responses from a Th1 to a Th2 phenotype (31). TSLP, a Th2 chemokine, is secreted by epithelial cells and triggers the expression of TARC in nearby DCs (19). TSLP in epithelial cells, as well as TARC in DCs, is up-regulated with exposure to airborne pollutants, including diesel exhaust (40) and cigarette smoke extract (32). Exposure to cigarette smoke was also shown to up-regulate the expression of TARC mRNA in whole murine lungs (41). We showed here that infection with influenza up-regulates the secretion of TSLP in co-cultures using NECs from smokers, but not nonsmokers, suggesting that in smokers, the activation of the TSLP pathway may occur in concert with the suppression of Th1 chemokines such as IP-10. Smokers' NECs also have elevated concentrations of TARC, as shown in the broncheoalveolar lavage of current and ex-smokers (34). Both TSLP and TARC were undetectable in apical washes (data not shown), which also suggests that differentiated NECs may manifest polarized protein secretion patterns. Our present data may offer the first example of TSLP and TARC up-regulation during infection with influenza in the context of smoking, and suggest that in smokers, Th2 chemokines that alter the viral immune response from a predominant Th1 phenotype to a Th2 phenotype may originate from both airway epithelial cells and myeloid cells (e.g., DCs).

In our model, virally infected NECs can communicate with underlying, immature mono-DCs through cytokine secretion. Immature DCs develop from monocyte precursors after treatment with GM-CSF and IL-4, and only progress to maturation through two steps: (*1*) exposure to a combination of inflammatory cytokines from the respiratory epithelium, and (*2*) the acquisition of antigen, with associated increases in costimulatory molecules that engage naive T cells (42). Our model of virally infected NECs did produce inflammatory and immune-activating cytokines such as Type I IFNs and IL-6 (13), but may not provide other signals such as TNF-α that are necessary to induce the production of IL-12 from mature DCs (43). In the case of an influenza infection, DCs are thought to process antigen through the phagocytosis of apoptotic cells (42). In our model, the cell culture membrane that separated NECs from mono-DCs likely impaired antigen capture by DCs. This may explain the limited influenza-induced changes in maturation markers CD11c and CD86 and the lack of influenza-induced changes in CD40, CD209, and CD11b that we observed during infection with influenza in both nonsmoker and smoker co-cultures, despite drastic differences in antiviral responses. In addition, our model examined the effects of smoking on the ability of NECs to communicate with mono-DCs from healthy nonsmoking donors. Peripheral blood mononuclear cells from smokers demonstrated an overall suppressed Type I IFN response to poly-I:C, a mimetic of double-stranded viral RNA (44). Thus, generating mono-DCs by using monocytes from smokers in this co-culture model could provide information on how signals emanating from influenza-infected NECs result in the different activations and maturation of mono-DCs obtained from smokers.

NECs comprise a useful model for studying innate immune responses of the airways. During a respiratory viral infection, the secretion of antiviral and immune-activating mediators from the respiratory epithelium engages accessory cells (e.g., NK cells, DCs, or monocytes) to induce an innate immune response (45). Mucosal epithelial cells in the nasopharynx act as a first line of defense, where they must differentiate between harmless

and disease-causing pathogens such as influenza, therefore setting the stage for a respiratory immune response (45). Compared with bronchial cells of the lower airways, NECs have similar profiles of baseline as well as cytokine-stimulated inflammatory mediators (46). Recent genome-wide expression analyses showed that gene expression patterns of epithelial cells from the nose and bronchial region overlap significantly at baseline, and that similar smoking-induced changes are reflected in both cell types (47). Previously, we showed that nasal and bronchial epithelial cells have comparable influenza-induced IFNα, IFNβ, and IFNΩ expression, albeit with differing magnitudes of response (27), further supporting the notion that the smoking-induced changes we observed in NECs are likely to be present in the lower airways as well.

As discussed previously (13), NECs from smokers and nonsmokers, when differentiated *in vitro*, appear to resemble their *in vivo* counterparts, and this resemblance includes the persistence of high expression of mucin 5B over time (13). As demonstrated in our previous study, smoking-induced changes in phenotypes of NECs were associated with epigenetic changes in these cells. Specifically, we previously showed that suppression of the influenza-induced expression of IRF-7 in NECs from smokers was associated with enhanced DNA methylation of the *IRF-7* gene in these cells (13), which also correlates with our findings here. In addition to *IRF-7*, DNA methylation patterns of other genes are likely altered in NECs from smokers, which may affect influenza-induced defense responses. We are conducting genome-wide analyses of DNA methylation patterns in NECs from smokers and nonsmokers, to identify other genes with potential roles during influenza-induced defense responses whose expression may be modified in NECs from smokers because of changes in DNA methylation.

The implication that cigarette smoke enhances susceptibility to infection with influenza extends beyond active smokers, to affect those nonsmoking individuals exposed to second-hand smoke (SHS), especially children. Exposure to SHS is estimated to cause over $10 billion in increased costs of mortality, morbidity, and medical care annually in the United States (48). Exposure to SHS is a risk factor for respiratory viral infections, including respiratory syncytial virus, upper and lower respiratory tract infections, and the common cold (49). Data from our laboratory showed that individuals exposed to SHS also have suppressed antiviral responses to LAIV *in vivo* (T.L. Noah, personal communication), and to infection with influenza *in vitro* (unpublished data). Our data here and in previous studies (31) suggest that chronic exposure to cigarette smoke may decrease Th1 antiviral responses over time, and increase the likelihood of developing Th2 responses. Indeed, exposure to cigarette smoke early in life is a risk factor for the development of allergic diseases, including atopic asthma (50). Respiratory viral infections, including influenza, were shown to exacerbate existing asthma (51). Therefore, exposure to cigarette smoke can exert a two-pronged deleterious effect on children, first by increasing the risk of developing asthma, and then by increasing the likelihood that a child will suffer virus-related complications of asthma.

Author Disclosure: L.E.B. received a sponsored grant from the National Institutes of Health for $10,001–$50,000. J.L.C. received sponsored grants from the Flight Attendant Medical Research Institute for $50,001–$100,000 and from the National Institutes of Health for more than $100,001. I.J. received a sponsored grant from Entegrion for $10,001–$50,000, from the Flight Attendant Medical Research Institute for $50,001–$100,000, and from the National Institutes of Health for more than $100,001. K.M.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. W.Z. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank Sally Ivins and Margret Herbst for their assistance in developing an institutional review board–approved protocol and for recruiting subjects into the present study. This manuscript's content is solely the responsibility of the authors, and does not necessarily represent the official views of the National Heart, Lung, and Blood Institute, the National Institutes of Health, the National Institute of Environmental Health Sciences, or the Howard Hughes Medical Institute (HHMI). Although the research described in this article was funded wholly or in part by the United States Environmental Protection Agency through cooperative agreement CR829522 with the Center for Environmental Medicine, Asthma, and Lung Biology, it has not been subjected to the agency's required peer and policy review, and therefore does not necessarily reflect the views of the agency, and no official endorsement should be inferred. The mention of trade names or commercial products does not constitute an endorsement or recommendation for use.

References

1. Finklea JF, Sandifer SH, Smith DD. Cigarette smoking and epidemic influenza. *Am J Epidemiol* 1969;90:390–399.
2. Kark JD, Lebiush M. Smoking and epidemic influenza-like illness in female military recruits: a brief survey. *Am J Public Health* 1981;71:530–532.
3. Kark JD, Lebiush M, Rannon L. Cigarette smoking as a risk factor for epidemic A(H1N1) influenza in young men. *N Engl J Med* 1982;307:1042–1046.
4. Nicholson KG, Kent J, Hammersley V. Influenza A among community-dwelling elderly persons in Leicestershire during winter 1993–4: cigarette smoking as a risk factor and the efficacy of influenza vaccination. *Epidemiol Infect* 1999;123:103–108.
5. Arcavi L, Benowitz NL. Cigarette smoking and infection. *Arch Intern Med* 2004;164:2206–2216.
6. Bensenor IM, Cook NR, Lee IM, Chown MJ, Hennekens CH, Buring JE, Manson JE. Active and passive smoking and risk of colds in women. *Ann Epidemiol* 2001;11:225–231.
7. de Andres AL, Garrido PC, Hernandez-Barrera V, Del Pozo SV, de Miguel AG, Jimenez-Garcia R. Influenza vaccination among the elderly Spanish population: trend from 1993 to 2003 and vaccination-related factors. *Eur J Public Health* 2007;17:272–277.
8. Johansen H, Nguyen K, Mao L, Marcoux R, Gao RN, Nair C. Influenza vaccination. *Health Rep* 2004;15:33–43.
9. MacKenzie JS, MacKenzie IH, Holt PG. The effect of cigarette smoking on susceptibility to epidemic influenza and on serological responses to live attenuated and killed subunit influenza vaccines. *J Hyg (Lond)* 1976;77:409–417.
10. Pyhala R, Takala J, Turunen A, Aho K. Smoking and influenza in the elderly: a sero-epidemiological study. *Eur J Respir Dis* 1983;64:212–216.
11. Mancini DA, Mendonca RM, Mendonca RZ, do Prado JA, Andrade Cde M. Immune response to vaccine against influenza in smokers, non-smokers, and in individuals holding respiratory complications. *Boll Chim Farm* 1998;137:21–25.
12. Robbins CS, Bauer CM, Vujicic N, Gaschler GJ, Lichty BD, Brown EG, Stampfli MR. Cigarette smoke impacts immune inflammatory responses to influenza in mice. *Am J Respir Crit Care Med* 2006;174:1342–1351.
13. Jaspers I, Horvath KM, Zhang W, Brighton LE, Carson JL, Noah TL. Reduced expression of IRF7 in nasal epithelial cells from smokers after infection with influenza. *Am J Respir Cell Mol Biol* 2010;43:368–375.
14. Sladkova T, Kostolansky F. The role of cytokines in the immune response to influenza A virus infection. *Acta Virol* 2006;50:151–162.
15. Bauer CM, Dewitte-Orr SJ, Hornby KR, Zavitz CC, Lichty BD, Stampfli MR, Mossman KL. Cigarette smoke suppresses Type I interferon–mediated antiviral immunity in lung fibroblast and epithelial cells. *J Interferon Cytokine Res* 2008;28:167–179.
16. Piqueras B, Connolly J, Freitas H, Palucka AK, Banchereau J. Upon viral exposure, myeloid and plasmacytoid dendritic cells produce 3 waves of distinct chemokines to recruit immune effectors. *Blood* 2006;107:2613–2618.
17. Grayson MH, Holtzman MJ. Emerging role of dendritic cells in respiratory viral infection. *J Mol Med* 2007;85:1057–1068.
18. Osterlund P, Veckman V, Siren J, Klucher KM, Hiscott J, Matikainen S, Julkunen I. Gene expression and antiviral activity of alpha/beta interferons and interleukin-29 in virus-infected human myeloid dendritic cells. *J Virol* 2005;79:9608–9617.
19. Lin Y, Wang W, Jin H, Zhong Y, Di J, Zeng S, Saito S. Comparison of murine thymic stromal lymphopoietin- and polyinosinic polycytidylic acid–mediated placental dendritic cell activation. *J Reprod Immunol* 2009;79:119–128.
20. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, Gilliet M, Ho S, Antonenko S, Lauerma A, *et al.* Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* 2002;3:673–680.

21. Harada M, Hirota T, Jodo AI, Doi S, Kameda M, Fujita K, Miyatake A, Enomoto T, Noguchi E, Yoshihara S, *et al.* Functional analysis of the thymic stromal lymphopoietin variants in human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 2009;40:368–374.
22. Rothen-Rutishauser BM, Kiama SG, Gehr P. A three-dimensional cellular model of the human respiratory tract to study the interaction with particles. *Am J Respir Cell Mol Biol* 2005;32:281–289.
23. Rothen-Rutishauser B, Muhlfeld C, Blank F, Musso C, Gehr P. Translocation of particles and inflammatory responses after exposure to fine particles and nanoparticles in an epithelial airway model. *Part Fibre Toxicol* 2007;4:9.
24. Blank F, Rothen-Rutishauser B, Gehr P. Dendritic cells and macrophages form a transepithelial network against foreign particulate antigens. *Am J Respir Cell Mol Biol* 2007;36:669–677.
25. Zhou H, Wang X, Brighton L, Hazucha M, Jaspers I, Carson JL. Increased nasal epithelial ciliary beat frequency associated with lifestyle tobacco smoke exposure. *Inhal Toxicol* 2009;21:875–881.
26. Jaspers I, Ciencewicki JM, Zhang W, Brighton LE, Carson JL, Beck MA, Madden MC. Diesel exhaust enhances influenza virus infections in respiratory epithelial cells. *Toxicol Sci* 2005;85:990–1002.
27. Ciencewicki JM, Brighton LE, Jaspers I. Localization of Type I interferon receptor limits interferon-induced TLR3 in epithelial cells. *J Interferon Cytokine Res* 2009;29:289–297.
28. Taima K, Imaizumi T, Yamashita K, Ishikawa A, Fujita T, Yoshida H, Takanashi S, Okumura K, Satoh K. Expression of IP-10/CXCL10 is upregulated by double-stranded RNA in BEAS-2B bronchial epithelial cells. *Respiration.* 2006;73:360–364.
29. Guillot L, Le Goffic R, Bloch S, Escriou N, Akira S, Chignard M, Si-Tahar M. Involvement of Toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J Biol Chem* 2005;280:5571–5580.
30. Jaspers I, Flescher E, Chen LC. Respiratory epithelial cells display polarity in their release of the chemokine IL-8 after exposure to ozone. *Inflamm Res* 1997;46:S173–S174.
31. Robays LJ, Maes T, Joos GF, Vermaelen KY. Between a cough and a wheeze: dendritic cells at the nexus of tobacco smoke–induced allergic airway sensitization. *Mucosal Immunol* 2009;2:206–219.
32. Nakamura Y, Miyata M, Ohba T, Ando T, Hatsushika K, Suenaga F, Shimokawa N, Ohnuma Y, Katoh R, Ogawa H, *et al.* Cigarette smoke extract induces thymic stromal lymphopoietin expression, leading to T(H)2-type immune responses and airway inflammation. *J Allergy Clin Immunol* 2008;122:1208–1214.
33. Vissers JL, Hartgers FC, Lindhout E, Teunissen MB, Figdor CG, Adema GJ. Quantitative analysis of chemokine expression by dendritic cell subsets *in vitro* and *in vivo*. *J Leukoc Biol* 2001;69:785–793.
34. Ying S, O'Connor B, Ratoff J, Meng Q, Mallett K, Cousins D, Robinson D, Zhang G, Zhao J, Lee TH, *et al.* Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity. *J Immunol* 2005;174:8183–8190.
35. Obregon C, Rothen-Rutishauser B, Gitahi SK, Gehr P, Nicod LP. Exovesicles from human activated dendritic cells fuse with resting dendritic cells, allowing them to present alloantigens. *Am J Pathol* 2006;169:2127–2136.
36. Obregon C, Rothen-Rutishauser B, Gerber P, Gehr P, Nicod LP. Active uptake of dendritic cell–derived exovesicles by epithelial cells induces the release of inflammatory mediators through a TNF-alpha–mediated pathway. *Am J Pathol* 2009;175:696–705.
37. Julkunen I, Sareneva T, Pirhonen J, Ronni T, Melen K, Matikainen S. Molecular pathogenesis of influenza A virus infection and virus-induced regulation of cytokine gene expression. *Cytokine Growth Factor Rev* 2001;12:171–180.
38. Gualano RC, Hansen MJ, Vlahos R, Jones JE, Park-Jones RA, Deliyannis G, Turner SJ, Duca KA, Anderson GP. Cigarette smoke worsens lung inflammation and impairs resolution of influenza infection in mice. *Respir Res* 2008;9:53.
39. Toapanta FR, Ross TM. Impaired immune responses in the lungs of aged mice following influenza infection. *Respir Res* 2009;10:112.
40. Bleck B, Tse DB, Curotto de Lafaille MA, Zhang F, Reibman J. Diesel exhaust particle–exposed human bronchial epithelial cells induce dendritic cell maturation and polarization via thymic stromal lymphopoietin. *J Clin Immunol* 2008;28:147–156.
41. Ritter M, Goggel R, Chaudhary N, Wiedenmann A, Jung B, Weith A, Seither P. Elevated expression of TARC (CCL17) and MDC (CCL22) in models of cigarette smoke–induced pulmonary inflammation. *Biochem Biophys Res Commun* 2005;334:254–262.
42. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K. Immunobiology of dendritic cells. *Annu Rev Immunol* 2000;18:767–811.
43. Kim HJ, Kim HO, Lee K, Baek EJ, Kim HS. Two-step maturation of immature DCs with proinflammatory cytokine cocktail and poly(I:C) enhances migratory and T cell stimulatory capacity. *Vaccine* 2010;28:2877–2886.
44. Mian MF, Stampfli MR, Mossman KL, Ashkar AA. Cigarette smoke attenuation of poly I:C–induced innate antiviral responses in human PBMC is mainly due to inhibition of IFN-beta production. *Mol Immunol* 2009;46:821–829.
45. Reynolds HY. Modulating airway defenses against microbes. *Curr Opin Pulm Med* 2002;8:154–165.
46. McDougall CM, Blaylock MG, Douglas JG, Brooker RJ, Helms PJ, Walsh GM. Nasal epithelial cells as surrogates for bronchial epithelial cells in airway inflammation studies. *Am J Respir Cell Mol Biol* 2008;39:560–568.
47. Sridhar S, Schembri F, Zeskind J, Shah V, Gustafson AM, Steiling K, Liu G, Dumas YM, Zhang X, Brody JS, *et al.* Smoking-induced gene expression changes in the bronchial airway are reflected in nasal and buccal epithelium. *BMC Genomics* 2008;9:259.
48. Behan DF, Eriksen MP, Lin Y. Economic effects of environmental tobacco smoke. Schaumburg, IL: Society of Actuaries; 2005.
49. Ciencewicki J, Jaspers I. Air pollution and respiratory viral infection. *Inhal Toxicol* 2007;19:1135–1146.
50. Metsios GS, Flouris AD, Koutedakis Y. Passive smoking, asthma and allergy in children. *Inflamm Allergy Drug Targets* 2009;8:348–352.
51. Hansbro NG, Horvat JC, Wark PA, Hansbro PM. Understanding the mechanisms of viral induced asthma: new therapeutic directions. *Pharmacol Ther* 2008;117:313–353.