JOURNAL OF INTERFERON & CYTOKINE RESEARCH
Volume 29, Number 5, 2009

DOI: 10.1089/jir.2008.0075

Localization of Type I Interferon Receptor Limits Interferon-Induced TLR3 in Epithelial Cells

Jonathan M. Ciencewicki,[1] Luisa E. Brighton,[2] and Ilona Jaspers[2,3]

Previous studies have shown that influenza infections increase Toll-like receptor 3 (TLR3) expression and that type I interferons (IFNs) may play a role in this response. This study aimed to expand on the role of type I IFNs in the influenza-induced upregulation of TLR3 and determine whether and how the localization of the IFN-α/β receptor (IFNAR) in respiratory epithelial cells could modify IFN-induced responses. Using differentiated primary human airway epithelial cells this study demonstrates that soluble mediators secreted in response to influenza infection upregulate TLR3 expression in naive cells. This response was associated with an upregulation of type I IFNs and stimulation with type I, but not type II, IFNs enhanced TLR3 expression. Interestingly, although influenza infection results in IFN-β release both toward the apical and basolateral sides of the epithelium, TLR3 expression is only enhanced in cells stimulated with IFN-β from the basolateral side. Immunohistochemical analysis demonstrates that IFNAR expression is limited to the basolateral side of differentiated human airway epithelial cells. However, non- or poorly differentiated epithelial cells express IFNAR more toward the apical side. These data demonstrate that restricted expression of the IFNAR in the differentiated airway epithelium presents a potential mechanism of regulating type I IFN-induced TLR3 expression.

Introduction

An integral role in the innate immune response to invading pathogens is played by Toll-like receptors (TLRs). These receptors are members of the superfamily of interleukin-1 receptors (IL-1R) and share homology in the cytoplasmic region referred to as the Toll/IL-1R (TIR) domain (Sen and Sarkar 2005). Toll-like receptors recognize conserved pathogen-associated molecular patterns and binding of their respective ligands leads to the production of innate immune defense mediators as well as activation of the adaptive immune response (Aderem and Ulevitch 2000; Akira and others 2001; Janeway and Medzhitov 2002). Toll-like receptor 3 (TLR3) recognizes double-stranded RNA (dsRNA), a molecular pattern commonly associated with viral infection. Double-stranded RNA stimulates TLR3 signaling, which culminates in the activation of numerous downstream signaling proteins and transcription factors and ultimately results in the production of proinflammatory cytokines and type I interferons (IFNs) (Alexopoulou and others 2001; Matsumoto and others 2002; Guillot and others 2005).

The respiratory epithelium is the target of a number of invading pathogens, including influenza. Once infected, these cells secrete various chemokines and cytokines, which elicit an innate antiviral response including the recruitment and activation of inflammatory cells as well the production of a number of antiviral mediators to help limit the spread of infection to neighboring cells. Of these, type I IFNs play an important role in inducing an antiviral State in cells through their induction of numerous genes involved in viral defense, which helps to limit the infection until other responses are mobilized. The family of type I IFNs in humans includes IFN-α, β, ε, κ, and ω, of which IFN-α and β have been the most extensively studied and are known for their potent antiviral effects (Samuel 2001; Platanias 2005). The type I IFNs, including IFN-ω, all bind to a common receptor, the IFN-α/β receptor (IFNAR) (Pestka 1997; Pestka and others 2004), which elicits a signaling cascade upon activation resulting in the transcription of IFN-stimulated genes (ISGs). The IFNAR is composed of 2 subunits (IFNAR1 and IFNAR2), which dimerize upon ligand binding. These subunits are

[1]Laboratory of Respiratory Biology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina.
[2]Center for Environmental Medicine, Asthma, and Lung Biology and [3]Department of Pediatrics, University of North Carolina, Chapel Hill, North Carolina.

each associated with a member of the Janus-activated kinase (Jak) family (Ihle 1996; Darnell 1998), which are responsible for activating the downstream signaling proteins signal transducers and activators of transcription (Stats) (Darnell 1998; Stark and others 1998). Once activated the Stat proteins form homodimers or heterodimers and act as transcription factors regulating the expression of a variety of ISGs (Samuel 2001; Platanias 2005). While the importance of IFNAR in the expression of antiviral defense mediators goes without question, its expression and localization in the respiratory epithelium is not well known.

Both influenza infections and type I IFNs have been shown to upregulate TLR3 expression (Heinz and others 2003; Guillot and others 2005; Tissari and others 2005). Virus-induced IFN-β is known to play a role in a positive-feedback loop whereby viral infection causes increased expression of a number of other ISGs (Takaoka and Yanai 2006), as well as TLR3 (Tanabe and others 2003). However, the localization of the receptor for IFN-β (IFNAR) in the human airway epithelium is not yet known. Previous studies have demonstrated that the expression and activation of the type II IFN receptor (IFNGR1) in the differentiated airway epithelium is limited to the basolateral (Humlicek and others 2007). The results shown here demonstrate that, as expected, influenza-induced type I IFNs are linked with the upregulation of TLR3 expression in influenza infected respiratory epithelial cells and that although influenza-infected airway epithelial cells release IFN-β to both the apical and basolateral side, only stimulation with IFN-β from the basolateral side increases the expression of TLR3. This was likely caused by the fact that because of its restricted localization, access to IFNAR is limited to stimulation from the basolateral side of differentiated human airway epithelial cells.

Materials and Methods

Cell culture

A549 cells as well as differentiated human nasal and bronchial epithelial cells were obtained and cultured as described by us before (Jaspers and others 1999; Jaspers and others 2005; Ciencewicki and others 2006). Briefly, A549 cells, a human pulmonary type II epithelial-like cell line were cultured in F12K medium plus 10% fetal bovine serum and 1% penicillin and streptomycin (all from Invitrogen, Carlsbad, CA). For treatment with IFNs or infection with influenza, A549 cells were grown in 6- or 24-well plates. When the cells reached ~80% confluency and ~18–24 h before exposure to IFNs or infection with influenza, the cell culture media was exchanged for serum-free F12K plus 1.5 μg/mL bovine serum albumin (BSA) plus antibiotics.

Primary human bronchial epithelial cells were obtained from healthy nonsmoking adult volunteers by cytologic brushing at bronchoscopy. Primary human nasal epithelial cells were obtained from healthy nonsmoking adult volunteers by gently stroking the inferior surface of the turbinate several times with a Rhino-Probe curette (Arlington Scientific, Arlington, TX), which was inserted through an otoscope with a large aperture. The protocols for the acquisition of both primary human bronchial and nasal epithelial cells were reviewed by the University of North Carolina Institutional Review Board. Both primary human bronchial and nasal epithelial cells were expanded to passage 2 in bronchial epithelial growth medium (Cambrex Bioscience Walkersville, Inc., Walkersville, MD) and then plated on collagen-coated filter supports with a 0.4 μM pore size (Trans-CLR; Costar, Cambridge, MA) and cultured in a 1:1 mixture of bronchial epithelial cell basic medium and Dulbecco's modified Eagles's medium-H with SingleQuot supplements (Cambrex), bovine pituitary extracts (13 mg/mL), BSA (1.5 μg/mL), and nyStatin (20 units). Upon confluency, all-trans retinoic acid was added to the medium and air liquid interface (ALI) culture conditions (removal of the apical medium) were created to promote differentiation. Mucociliary differentiation was achieved after 21 to 30 days after ALI.

For some experiments, cells were treated with 1 μM of the tyrosine kinase inhibitor Jak inhibitor I (Calbiochem, La Jolla, CA).

Infection with influenza or treatment with IFNs

Throughout this study we used influenza A/Bangkok/1/79 (H3N2 serotype) which was propagated in 10-day-old embryonated hen's eggs. The virus was collected in the allantoic fluid and titered by 50% tissue culture infectious dose in Madin–Darby canine kidney cells and hemagglutination as described before (Beck and others 2001). Stock virus was aliquoted and stored at −80°C until use. Unless otherwise indicated, for infection ~5×10^5cells were infected with ~128 (differentiated nasal epithelial cells and A549 cells) or 40 (differentiated bronchial epithelial cells) hemagglutination units of influenza A Bangkok 1/79, which resulted in ~10% of the cells being infected with influenza 24 h after infection. In some experiments, cells were treated with 1 ng/mL of IFN-α, -β, -ω, or -γ (PBL Biomedical Laboratories, Piscataway, NJ), which was added directly to the cell culture medium.

In some experiments, mediators released toward the apical or basolateral side by influenza-infected differentiated epithelial cells were examined. Media contained below the tissue culture inserts were used (see schematic in Fig. 1A) for basolaterally released mediators. Washing of the apical surface with 0.2 mL tissue culture media was used to collect apically released mediators.

RT-PCR

Total RNA was extracted using TRizol (Invitrogen) as per the supplier's instruction. First-strand cDNA synthesis and real-time reverse transcriptase polymerase chain reaction was performed as described previously (Jaspers and others 1999; Jaspers and others 2001). The mRNA analyses were performed using commercially available primer and probe sets (inventoried Taqman Gene Expression Assays) purchased from Applied Biosystems (Foster City, CA).

Western blotting

Whole cell lysates were prepared by lysing the cells in RIPA buffer containing 1% Nonidet P (NP)-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and protease inhibitors (Cocktail Set III; Calbiochem, San Diego, CA) followed by brief sonication. Fifty micrograms of whole cell lysate was separated by SDS–polyacrylamide gel electrophoresis, followed by immunoblotting using specific antibodies to TLR3 (1:500, Imgenex, San Diego, CA), or α-tubulin (1:1000,

Santa Cruz Biotechnology, Santa Cruz, CA). Antigen–antibody complexes were stained with anti-rabbit or anti-mouse, horseradish peroxidase (HRP)-conjugated antibody (1:4000, Santa Cruz Biotechnology) and SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). The chemiluminescent signals were acquired using a 16-bit CCD camera (GeneGnome system; Syngene, Frederick, MD) and visualized using the GeneSnap software (Syngene). Densitometric analysis of the optical densities was performed using GeneTools Software (Syngene).

Immunohistochemistry

Human tracheal tissue was obtained through a tissue procurement agency (Anatomic Gift Foundation, Hanover, MD) and embedded in paraffin. Primary human bronchial epithelial cells on days 1, 6, and 29 after ALI were fixed with 4% paraformaldehyde and embedded in paraffin, as described before (Ross and others 2007). Five micron sections were placed on Superfrost/plus slides (Fisher Scientific) and stained for IFNAR using anti-IFNAR2 antibodies (1:10; Santa Cruz Biotechbnology, Santa Cruz, CA) or cilia using anti-acetylated α-tubulin antibodies (1:800; Zymed). Following incubation with HRP-conjugated secondary antibodies, samples were washed with Tris-buffered saline and evaluated under light microscopy.

ELISA

Basolateral media and apical washes were collected 24 h after infection and analyzed for IFN-β using commercially available enzyme-linked immunosorbent assay (ELISA) kits as per the supplier's instructions (Fujirebio Inc., Tokyo, Japan)

Statistical analysis

Data are expressed as means ± SEM. For all of the studies using differentiated human nasal or bronchial epithelial cells, a minimum of $n = 5$ different subjects were used. Raw data were analyzed using one-way analysis of variance followed by the Newman-Keuls *post hoc* test, or using *t*-tests to compare control and infected/treated groups. Data expressed as fold induction were analyzed using the Wilcoxon Signed Rank Test, assuming a theoretical mean of 1.00 for the control group. A value of $P < 0.05$ was considered to be significant.

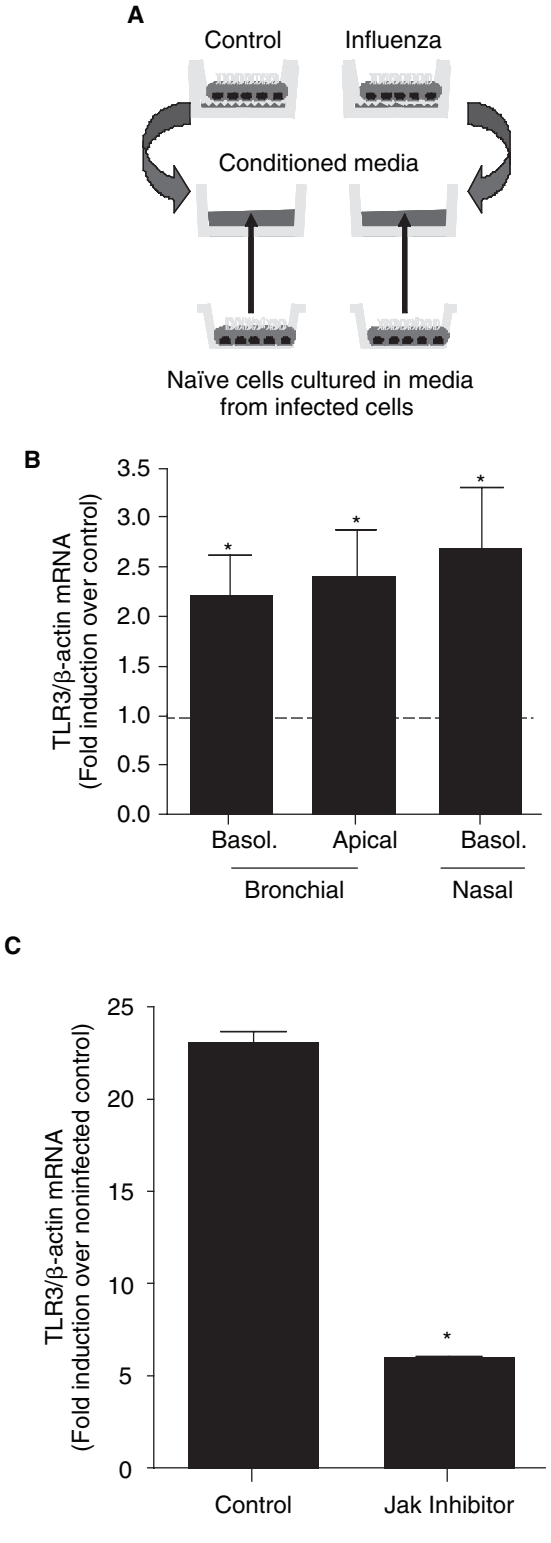

FIG. 1. Effect of soluble mediators on Toll-like receptor 3 (TLR3) expression. (**A**) Schematic of experimental design. Differentiated nasal or bronchial epithelial cells were infected with influenza. Basolateral (Basol.) media and apical washes containing mediators released by epithelial cells were collected 24 h after infection. The conditioned medium was used to stimulate naïve epithelial cells from the basolateral side for 8 h on which TLR3 mRNA expression was quantified using real-time reverse transcriptase polymerase chain reaction (RT-PCR). (**B**) Toll-like receptor 3 (TLR3) mRNA expression in differentiated bronchial and nasal epithelial cells cultured in conditioned media from influenza-infected epithelial cells and expressed as fold induction over TLR3 mRNA levels in epithelial cells cultured in conditioned media from noninfected (control) epithelial cells. (**C**) Differentiated bronchial epithelial cells were treated with 1 μM of a Jak inhibitor or vehicle control before infection with influenza. Toll-like receptor 3 mRNA levels were then quantified 24 h after infection using real-time RT-PCR. *Significantly different from control, $P < 0.05$.

Results

Effect of soluble mediators released by influenza-infected cells on TLR3 expression

Previous studies conducted *in vitro* using human pulmonary epithelial cells and *in vivo* in mice have shown that infection with influenza causes increased expression of TLR3 (Guillot and others 2005; Le Goffic and others 2006). However, to our knowledge it has not been shown if TLR3 expression is only upregulated in influenza-infected cells or if noninfected neighboring cells are also upregulating TLR3 in response to mediators released by infected cells. To determine whether mediators released by infected cells enhance TLR3 expression in a paracrine fashion, TLR3 mRNA was quantified in cells, which were treated with conditioned media from influenza-infected cells (see schematic in Fig. 1A). In this experiment, differentiated primary nasal, and bronchial (Fig. 1B) epithelial cells were first infected with influenza. Basolateral media only (nasal) or basolateral media and apical washes (bronchial) containing mediators released by influenza-infected epithelial cells were collected 24 h after infection. The infection-conditioned medium was used to stimulate naive epithelial cells for 8 h upon which TLR3 mRNA expression was quantified using real-time RT-PCR. To assure that no viral particles contaminated the conditioned medium and enhanced TLR3 expression in naive epithelial cells, viral infection was assessed, by determining the levels of influenza A hemagluttinin (HA) RNA using RT-PCR, as a marker of influenza virus level. Figure 1B shows that differentiated nasal epithelial cells stimulated with virus-conditioned media had significantly higher levels of TLR3 mRNA than cells stimulated with conditioned media from noninfected control cells. Similarly, bronchial epithelial cells stimulated with conditioned media from the basolateral side or media used to wash the apical surface of previously infected bronchial cells significantly enhanced levels of TLR3 mRNA (Fig. 1B). No residual influenza virus was left in either of the conditioned media and had infected these cells, thereby causing the upregulation of TLR3, which was assessed by analyzing the level of HA RNA in these cells using real-time RT-PCR (data not shown).

The next objective was to assess potential involvement of the type I IFN–signaling pathway in the influenza-induced upregulation of TLR3. Binding of type I IFNs to the IFNAR elicits a signaling cascade involving Janus protein tyrosine kinases (Jaks). These kinases are responsible for phosphorylating and activating Stats, which ultimately leads to transcription of ISGs. To determine the role of Jak in influenza-induced TLR3 expression, differentiated bronchial epithelial cells were treated with vehicle control or 1 μM of a Jak inhibitor and TLR3 mRNA levels were quantified 24 h after infection using real-time RT-PCR (Fig. 1C). Cells treated with the vehicle control showed a large increase in TLR3 mRNA levels in response to infection, which was significantly decreased in cells treated with the Jak inhibitor.

As Stated above, type I IFNs have been shown to upregulate TLR3 expression (Miettinen and others 2001; Siren and others 2005). To determine how different IFNs affect TLR3 expression in respiratory epithelial cells, we used a respiratory epithelial cell line to screen the effects of different type I and type II IFNs. First, A549 cells were treated with 0, 0.1, or 1.0 ng/mL of IFN-β, which translates into 0, 40, and 400 U/mL, and examined for changes in TLR3 mRNA levels 6 h afterwards. Figure 2A shows that IFN-β dose-dependently upregulates TLR3 mRNA levels. Time-course experiments showed that the IFN-β-induced increase in TLR3 mRNA levels was maximal between 4 and 6 h (Fig. 2B). Lastly, A549 cells were treated with 1 ng/mL of type I and II IFNs (IFN-α, β, ω, and γ) for 6 h upon which TLR3 mRNA expression was quantified using real-time RT-PCR. All of the type I IFNs tested significantly upregulated TLR3 mRNA, while IFN-γ caused a nonsignificant increase in the levels of TLR3 mRNA (Fig. 2C). Based on these observations, all subsequent studies were conducted using 1 ng/mL IFNs and cells were examined 6 h afterwards for expression of TLR3.

Once it was confirmed that type I IFNs could increase TLR3 expression in respiratory epithelial cells, the ability to enhance the expression of each of these type I IFNs in response to influenza infection was quantified 24 h after infection. Levels of IFN-α, -β, and -ω mRNA were quantified in influenza-infected differentiated bronchial (Fig. 3A) and nasal (Fig. 3B) epithelial cells using real-time RT-PCR. Levels of IFN-β and IFN-α mRNA were significantly increased above control in both differentiated bronchial and nasal epithelial cells 24 h after infection, while levels of IFN-ω mRNA were increased above control in differentiated bronchial epithelial cells, but not nasal epithelial cells ($P = 0.06$). The next objective was to confirm and expand upon the IFN mRNA results and determine the directionality of IFN-β secretion by differentiated bronchial epithelial cells. Levels of IFN-β protein secreted to the apical and basolateral sides were quantified in differentiated bronchial epithelial cells 24 h after influenza infection. Figure 3C shows that differentiated bronchial epithelial cells secrete IFN-β toward both the apical and basolateral side in response to influenza infection in a dose-dependent manner.

Polarized response of pulmonary epithelial cells to IFN stimulation and localization of IFNAR

It is well known that the airway epithelium is polarized, with the apical and basolateral sides having different morphological as well as biochemical characteristics and functions and that this polarity is maintained in part by tight functional complexes. In addition, recent studies have demonstrated that localization and activation of certain cytokine receptors is limited to the basolateral side of differentiated airway epithelial cells (Humlicek and others 2007). To determine whether there was polarized sensitivity to IFN-β stimulation, TLR3 mRNA levels were quantified in differentiated bronchial epithelial cells treated for 6 h with 1 ng/mL of IFN-β from either the apical or basolateral side or a combination of both. Figure 4A shows that expression of TLR3 was increased only in cells receiving basolateral IFN-β stimulation, either by itself or in combination with apical stimulation. In addition, we also added IFN-γ to both apical and basolateral sides and examined expression of TLR3. Similar to the results observed in Fig. 2C, addition of IFN-γ to the basolateral side of differentiated bronchial epithelial cells did induce a moderate increase in TLR3 mRNA levels, albeit not Statistically significant. Analogous to the changes seen in TLR3 mRNA levels, TLR3 protein levels increased ~2-fold upon stimulation with

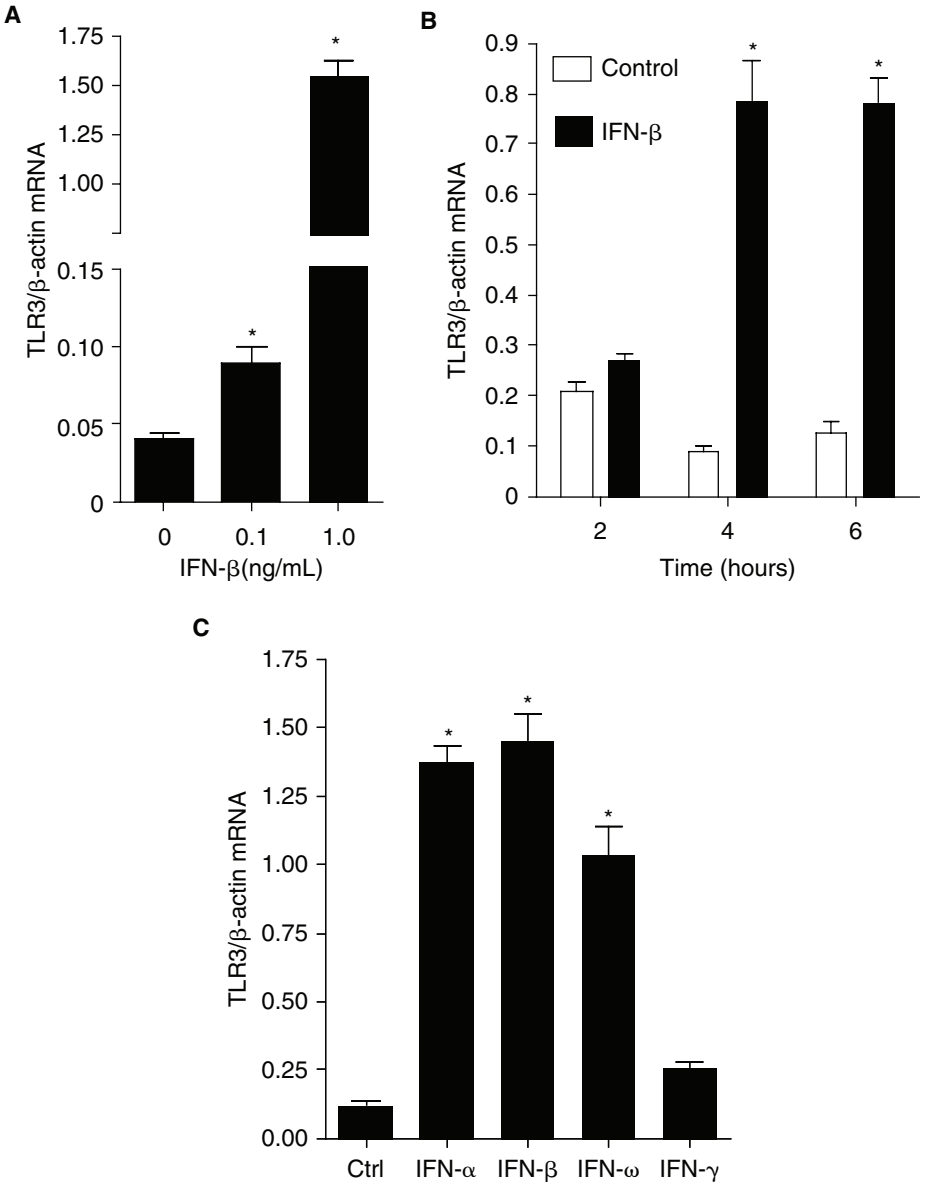

FIG. 2. Effect of interferons (IFNs) on Toll-like receptor 3 (TLR3) expression. A549 cells were (**A**) treated with 0, 0.1, or 1.0 ng/mL IFN-β for 6 h; or (**B**) treated with 1.0 ng/mL IFN-β for different times; or (**C**) treated with 1 ng/mL of IFN-α, β, ω, and γ for 6 h. Following the various treatments TLR3 mRNA expression was quantified using real-time reverse transcriptase polymerase chain reaction (RT-PCR). *Significantly different from control, $P < 0.05$.

IFN-γ and over 4-fold in differentiated bronchial epithelial cells stimulated with IFN-β from the basolateral side (Fig. 4B). Thus, although stimulation with IFN-γ does increase TLR3 expression in bronchial epithelial cells, stimulation with IFN-β results in much greater augmentation of TLR3 expression.

Given the polarized response of airway epithelial cells to IFN-β stimulation despite the fact that IFN-β is released toward both the apical and the basolateral side, it seemed likely that the observed responses were due to restricted expression of the INFAR in these cells. To test this hypothesis IFNAR expression was immunohistochemically examined in airway epithelial cells *in vitro* and human tracheal tissue *in vivo*. Figure 5A demonstrates that in contrast to cilia, which line the apical side of airway epithelial cells, expression of IFNAR is predominantly located on the basolateral side of human tracheal epithelial cells. Similarly, fully differentiated bronchial epithelial cells *in vitro* express IFNAR predominantly toward the basolateral side (Fig. 5B, bottom panel). Interestingly, nondifferentiated bronchial epithelial cells (days 1 and 6 after ALI) express IFNAR more apically (Fig. 5B; top 2 panels). Morphological changes induced upon differentiation, including presence of cilia and columnar morphology of the cells is shown in Fig. 5C, which has also been described in more detail by us before (Ross and others 2007).

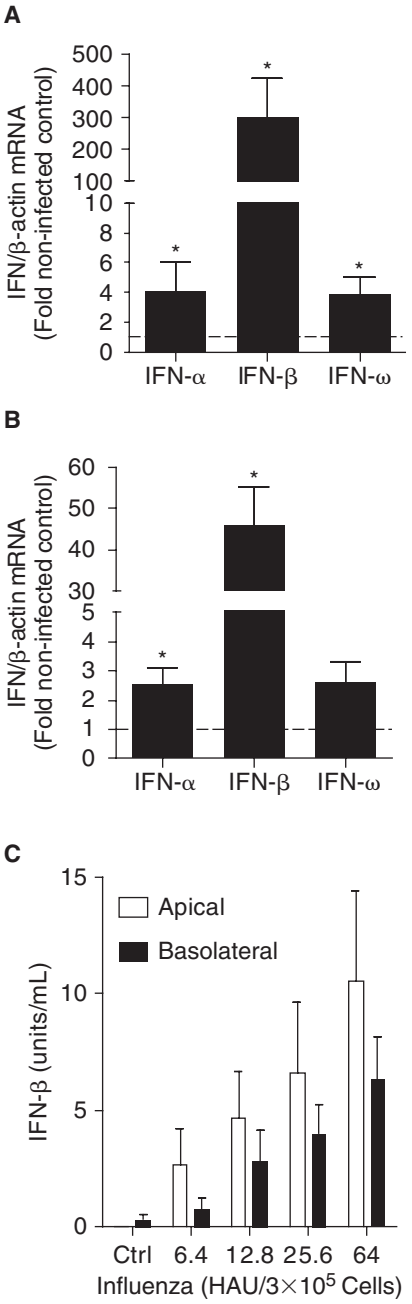

FIG. 3. Influenza-induced interferon (IFN) expression and release. Differentiated nasal and bronchial epithelial were infected with influenza and examined for IFN expression 24 h after infection. (**A**) IFN-α, β, and ω mRNA levels in differentiated bronchial epithelial cells and (**B**) IFN-α, β, and ω mRNA levels in differentiated nasal epithelial cells were quantified using real-time reverse transcriptase polymerase chain reaction (RT-PCR). *Significantly different from control, $P < 0.05$. (**C**) Levels of IFN-β protein secreted to the apical and basolateral sides were quantified by enzyme-linked immunosorbent assay in bronchial epithelial cells 24 h after infection with various doses of influenza.

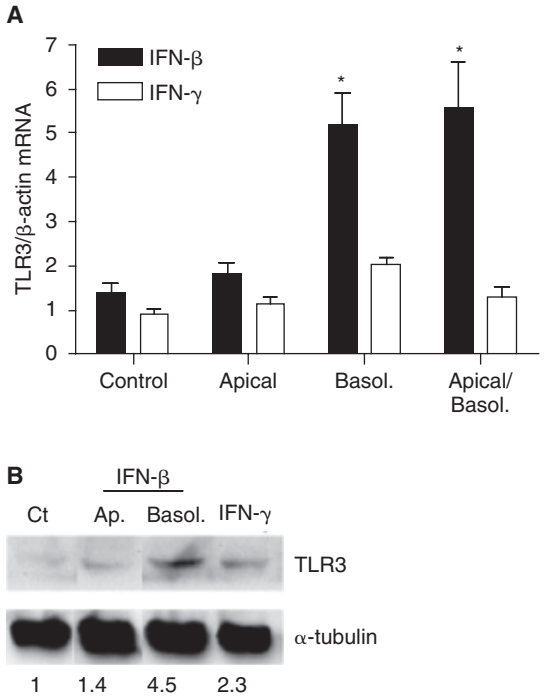

FIG. 4. Polarized response of pulmonary epithelial cells to interferon (IFN) stimulation. (**A**) Toll-like receptor 3 (TLR3) mRNA levels were quantified using real-time reverse transcriptase polymerase chain reaction in differentiated bronchial epithelial cells treated for 6 h with 1 ng/mL of either IFN-β or IFN-γ from either the apical or basolateral side or a combination of both. *Significantly different from control, $P < 0.05$. (**B**) Whole cell lysates of differentiated bronchial epithelial cells treated with 1 ng/mL of IFN-β from apical or basolateral side or 1 ng/mL of IFN-γ from the basolateral side for 24 h were analyzed for TLR3 (*top*) and α-tubulin (*bottom*) protein levels by Western blotting. Densitometric analysis indicating fold change over control is shown below.

Discussion

Previous studies have shown that infection with influenza results in enhanced TLR3 expression (Guillot and others 2005; Le Goffic and others 2006), but the mechanism and potential limiting factors whereby this upregulation occurs had not been completely elucidated. The results of this study show that as expected influenza-induced upregulation of TLR3 in respiratory epithelial cells is associated with release of type I IFNs, which can act in a paracrine manner to induce TLR3 expression in neighboring cells. However, more importantly our data demonstrate that while IFN-β is secreted to both apical and basolateral sides of the epithelium, localization of the type I IFN receptor (IFNAR) limits access to the basolateral side, which is similar to previous studies identifying the limited localization and activation of the type II IFN receptor (IFNGR1) (Humlicek and others 2007). Together these data provide a potential mechanism whereby the structural integrity of the host epithelium could regulate IFN-induced TLR3 expression in responses to viral infections.

The involvement of type I IFNs in the influenza-induced upregulation of TLR3 is not surprising considering that the role of these cytokines is to induce the expression of genes involved in antiviral defenses. In order for type I IFNs to exert their effects they must bind

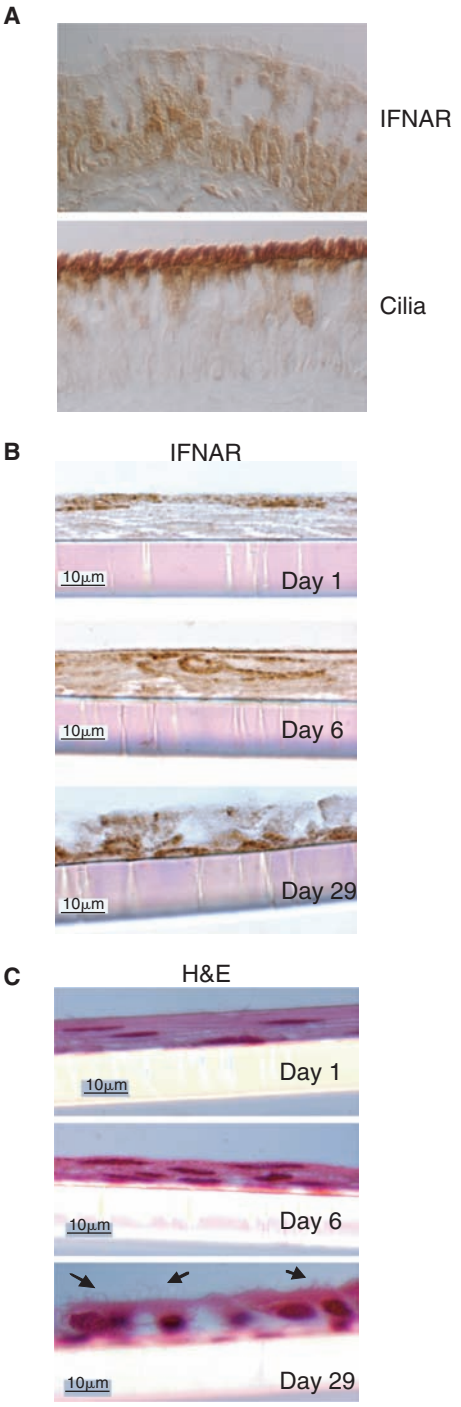

FIG. 5. Localization of the interferon-α/β receptor (IFNAR) in epithelial cells. (**A**) Human tracheal tissue was stained with antibodies against IFNAR (*top panel*) and acetylated α-tubulin (*bottom panel*). Bronchial epithelial cells on Days 1, 6, and 29 after air liquid interface were stained with (**B**) rabbit anti-IFNAR antibodies or (**C**) hematoxylin and eosin (H&E) with arrows pointing at cilia covering the apical side. Sections were examined by light microscopy under 100× magnification.

to the IFNAR, which then activates a signaling cascade that ultimately results in the induction of various ISGs. An increase in TLR3 expression in differentiated human airway epithelial cells was only observed in cells stimulated with IFN-β from the basolateral side suggesting a polarized localization of IFNAR in airway epithelial cells. Immunohistochemical staining confirmed that the apical surface was devoid of any IFNAR in fully differentiated bronchial epithelial cells *in vitro* and tracheal epithelium *in vivo* and was predominantly localized on the basolateral side of the epithelium. This specialized localization of IFNAR in fully differentiated human airway epithelial cells is very similar to that of other receptors, including the receptors for type II IFNs, IL-4, and IL-9 (Humlicek and others 2007) as well as coxsackievirus and adenovirus receptor (CAR) (Cohen and others 2001). This restricted localization is part of the reason why adenovirus-mediated gene transfer can only occur when the viral vector is added to the basolateral side or after disruption of the tight junctions (Pickles and others 1996; Pickles and others 1998) and why *in vivo* epithelial cells respond poorly to luminal stimulation with IFN-γ unless epithelial permeability is increased (Humlicek and others 2007). Similar to CAR and IFNGR1, the localization of IFNAR would clearly prevent access from the apical side and limit stimulation of IFNAR by type I IFNs from the basolateral side. Consequently, activation of IFNAR in an intact and healthy respiratory epithelium would be limited to type I IFNs secreted by epithelial cells to the basolateral side. However, dendritic cells, which produce a great deal of type I IFNs in response to viral infections, usually reside near the basolateral side of the epithelium (Stumbles and others 2003). Therefore, the basolateral access to IFNAR could play an important role during epithelial cell–dendritic cell communications following respiratory virus infections.

In addition, it is conceivable that under certain circumstances respiratory epithelial cells increase apical expression of the IFNAR or allow access of apically released IFNs to the basolateral side of the cell through changes in epithelial cell permeability. For example, chemically induced disruption of tight junctions significantly enhances activation of CAR and IFNGR1 from either apical or basolateral side (Cohen and others 2001; Humlicek and others 2007). A similar regulatory mechanism is employed endogenously by airway epithelial cells to control binding of the growth factor heregulin to its receptors (Vermeer and others 2003). Under normal conditions, heregulin, which is located on the apical surface, cannot bind to its receptors, which are located on the basolateral membrane. However, when the integrity of the epithelium is compromised, such as during injury, heregulin can bind its receptors on the basolateral portion of the cell and promote repair of the damaged epithelium. In the case of INFAR and type I IFNs, epithelial cells may prevent excess stimulation under normal conditions through sequestration of receptor and ligand, but when virus-induced injury compromises epithelial integrity there may be a need for enhanced antiviral responses to prevent viruses from permeating the epithelial barrier.

Interestingly, our analysis demonstrated that in non- or poorly differentiated bronchial epithelial cells *in vitro* IFNAR is predominantly localized toward the apical side. Thus, it is possible that differential expression of IFNAR over the course of epithelial cell differentiation confers different susceptibility to influenza-induced expression of TLR3 or other functional responses. This notion is supported by previous studies which have demonstrated that well-differentiated

airway epithelial cells are much more resistant to rhinovirus infections and virus-induced inflammatory responses than poorly or nondifferentiated epithelial cells (Lopez-Souza and others 2004). These observations also highlight the notion that cell culture techniques need to be considered when interpreting and designing studies aimed to understand responses of the airway epithelium in the context of viral infections.

Despite existing reports demonstrating that influenza infection upregulates TLR3 expression (Miettinen and others 2001; Guillot and others 2005), it was unknown whether this effect occurred only in infected cells, and was therefore dependent on virus replication, or whether infected cells could signal uninfected neighboring cells to upregulate TLR3. Upregulation of TLR3 not only in infected cells, but also in neighboring uninfected cells would provide a more robust antiviral defense response and contribute to keeping the infection under control. Our results demonstrate that differentiated human bronchial and nasal epithelial cells, which were infected with influenza, did in fact secrete mediators that caused an upregulation of TLR3 in naive uninfected cells. This response was not due to residual virus left in the cellular media from infected cells, which was confirmed by the absence of HA RNA in the uninfected cells. The fact that mediators released by epithelial cells in response to influenza infection can act in a paracrine manner to enhance the expression of TLR3 in neighboring cells is important for amplifying the inflammatory and innate immune response against viral infection.

Previous studies have also demonstrated that both IFN-α and IFN-β have the ability to stimulate TLR3 expression (Miettinen and others 2001; Tissari and others 2005). To confirm and expand upon these findings the ability of the type I IFNs α, β, and ω as well as the type II IFN γ, to stimulate TLR3 expression in respiratory epithelial cells were examined. Similar to previous results treatment with IFN-α and -β resulted in a significant increase in TLR3 mRNA expression. Interestingly, IFN-ω was also able to significantly induce TLR3 mRNA expression indicating that this effect is not specific to just IFN-α and -β, but that other type I IFNs exert a similar effect. Interestingly, stimulation with IFN-γ did not result in significant upregulation of TLR3 in respiratory epithelial cells. This is in contrast to previous studies that have demonstrated that stimulation of macrophages with IFN-γ enhanced the expression of TLR3 (Flandin and others 2006). Hence the ability of IFN-γ to stimulate expression of TLR3 is likely cell type dependent.

Taken together, the data presented here demonstrate localization of IFNAR to the basolateral domain of cell membranes limits IFN-dependent activation of TLR3 expression to stimulation from the basolateral side. Recent evidence indicates that in influenza-infected epithelial cells, TLR3 primarily regulates proinflammatory responses, while RIG-I mediates both type I IFN and proinflammatory responses (Le Goffic and others 2007). Indeed, TLR3-deficient mice have a survival advantage after influenza infection, which was associated with decreased pulmonary inflammation in these mice (Le Goffic and others 2006). Thus, while TLR3 is necessary for efficient production of inflammatory mediators, such as MCP-1, that are important for an efficient antiviral defense response (Ritter and others 2005; Dessing and others 2007), excessive expression and activation of TLR3 could enhance virus-induced inflammation and injury. Air pollutants, such as cigarette smoke and diesel exhaust, enhance the severity of influenza infections and can also increase epithelial permeability (Olivera and others 2007). It remains to be examined whether chemical or pollutant-induced disruption of epithelial integrity facilitates access to IFNAR and possibly other cytokine receptors whose localization is limited to the basolateral side (Humlicek and others 2007) and consequently enhances influenza-induced expression of TLR3 and TLR3-dependent inflammation in the context of a viral infection.

Acknowledgments

We thank Wenli Zhang for her technical assistance, and Robert Silbajoris for his help with the real-time RT-PCR. We also thank Drs Devlin and Ross and Ms Dailey for help with the epithelial differentiation time course. The project described was supported by grant number ES013611 from the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH), as well as a grant from the Environmental Protection Agency (EPA) (CR829522) (all I.J.) and, finally, the EPA-UNC Curriculum in Toxicology Training agreement (No. T829472). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS, NIH. Although the research described in this article has been 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. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

References

Aderem A, Ulevitch RJ. 2000. Toll-like receptors in the induction of the innate immune response. Nature 406:782–787.

Akira S, Takeda K, Kaisho T. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2:675–680.

Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. 2001. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. Nature 413:732–738.

Beck MA, Nelson HK, Shi Q, Van Dael P, Schiffrin EJ, Blum S, Barclay D, Levander OA. 2001. Selenium deficiency increases the pathology of an influenza virus infection. FASEB J 15(8):1481–1483.

Ciencewicki J, Brighton L, Wu W-D, Madden M, Jaspers I. 2006. Diesel exhaust enhances virus- and poly(I:C)-induced Toll-like receptor 3 expression and signaling in respiratory epithelial cells. Am J Physiol Lung Cell Mol Physiol 290:L1154–L1163.

Cohen CJ, Shieh JT, Pickles RJ, Okegawa T, Hsieh JT, Bergelson JM. 2001. The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. Proc Natl Acad Sci USA 98(26):15191–15196.

Darnell JE Jr. 1998. Studies of IFN-induced transcriptional activation uncover the Jak-Stat pathway. J Interferon Cytokine Res 18(8):549–554.

Dessing MC, van der Sluijs KF, Florquin S, van der Poll T. 2007. Monocyte chemoattractant protein 1 contributes to an adequate immune response in influenza pneumonia. Clin Immunol 125(3):328–336.

Flandin JF, Chano F, Descoteaux A. 2006. RNA interference reveals a role for TLR2 and TLR3 in the recognition of Leishmania

donovani promastigotes by interferon-gamma-primed macrophages. Eur J Immunol 36(2):411–420.
Guillot L, Le Goffic R, Bloch S, Escriou N, Akira S, Chinard M, Si-Tahar M. 2005. 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 280(7):5571–5580.
Heinz S, Haehnel V, Karaghiosoff M, Schwarzfischer L, Muller M, Krause SW, Rehli M. 2003. Species-specific regulation of Toll-like receptor 3 genes in men and mice. J Biol Chem 278(24):21502–21509.
Humlicek AL, Manzel LJ, Chin CL, Shi L, Excoffon KJDA, Winter MC, Shasby DM, Look DC. 2007. Paracellular permeability restricts airway epithelial responses to selectively allow activation by mediators at the basolateral surface. J Immunol 178:6395–6403.
Ihle JN. 1996. Janus kinases in cytokine signalling. Philos Trans R Soc Lond B Biol Sci 351:159–166.
Janeway CA Jr, Medzhitov R. 2002. Innate immune recognition. Annu Rev Immunol 20:197–216.
Jaspers I, Ciencewicki JM, Zhang W, Brighton LE, Carson JL, Beck MA, Madden MC. 2005. Diesel exhaust enhance Influenza virus infections in respiratory epithelial cells. Toxicol Sci 85:990–1002.
Jaspers I, Samet JM, Reed W. 1999. Arsenite exposure of cultured airway epithelial cells activates kappaB-dependent interleukin-8 gene expression in the absence of nuclear factor-kappaB nuclear translocation. J Biol Chem 274(43):31025–31033.
Jaspers I, Zhang W, Fraser A, Samet JM, Reed W. 2001. Hydrogen peroxide has opposing effects on IKK activity and IkappaBalpha breakdown in airway epithelial cells. Am J Respir Cell Mol Biol 24(6):769–777.
Le Goffic R, Balloy V, Lagranderie M, Alexopoulou L, Escriou N, Flavell R, Chignard M, Si-Tahar M. 2006. Detrimental contribution of the Toll-like receptor (TLR)3 to influenza A virus-induced acute pneumonia. PLoS Pathog 2(6):e53.
Le Goffic R, Pothlichet J, Vitour D, Fujita T, Meurs E, Chignard M, Si-Tahar M. 2007. Cutting edge: influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells. J Immunol 178(6):3368–3372.
Lopez-Souza N, Dolganov G, Dubin R, Sachs LA, Sassina L, Sporer H, Yagi S, Schnurr D, Boushey HA, Widdicombe JH. 2004. Resistance of differentiated human airway epithelium to infection by rhinovirus. Am J Physiol Lung Cell Mol Physiol 286(2):L373–L381.
Matsumoto M, Kikkawa S, Kohase M, Miyake K, Seya T. 2002. Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. Biochem Biophys Res Comm 293:1364–1369.
Miettinen M, Sareneva T, Julkunen I, Matikainen S. 2001. IFNs activate toll-like receptor gene expression in viral infections. Genes Immun 2(6):349–355.
Olivera DS, Boggs SE, Beenhouwer C, Aden J, Knall C. 2007. Cellular mechanisms of mainstream cigarette smoke-induced lung epithelial tight junction permeability changes in vitro. Inhal Toxicol 19(1):13–22.
Pestka S. 1997. The interferon receptors. Semin Oncol 24(3 Suppl 9):S9–18–S9–40.
Pestka S, Krause CD, Walter MR. 2004. Interferons, interferon-like cytokines, and their receptors. Immunol Rev 202:8–32.
Pickles RJ, Barker PM, Ye H, Boucher RC. 1996. Efficient adenovirus-mediated gene transfer to basal but not columnar cells of cartilaginous airway epithelia. Hum Gene Ther 7(8):921–931.
Pickles RJ, McCarty D, Matsui H, Hart PJ, Randell SH, Boucher RC. 1998. Limited entry of adenovirus vectors into well-differentiated airway epithelium is responsible for inefficient gene transfer. J Virol 72(7):6014–6023.
Platanias LC. 2005. Mechanisms of type I-and type II-interferon-mediated signaling. Nat Rev Immunol 5(5):375–386.
Ritter M, Mennerich D, Weith A, Seither P. 2005. Characterization of Toll-like receptors in primary lung epithelial cells: strong impact of the TLR3 ligand poly(I:C) on the regulation of Toll-like receptors, adaptor proteins and inflammatory response. J Inflamm (Lond) 2(1):16.
Ross AJ, Dailey LA, Brighton LE, Devlin RB. 2007. Transcriptional profiling of mucociliary differentiation in human airway epithelial cells. Am J Respir Cell Mol Biol 37(2):169–185.
Samuel CE. 2001. Antiviral actions of Interferons. Clin Microbiol Rev 14:778–809.
Sen GC, Sarkar SN. 2005. Transcriptional signaling by double-stranded RNA: role of TLR3. Cytokine Growth Factor Rev 16: 1–14.
Siren J, Pirhonen J, Julkunen I, Matikainen S. 2005. IFN-alpha regulates TLR-dependent gene expression of IFN-alpha, IFN-beta, IL-28, and IL-29. J Immunol 174(4):1932–1937.
Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. 1998. How cells respond to interferons. Annu Rev Biochem 67:227–264.
Stumbles PA, Upham JW, Holt PG. 2003. Airway dendritic cells: coordinators of immunological homeostasis and immunity in the respiratory tract. APMIS 111(7–8):741–755.
Takaoka A, Yanai H. 2006. Interferon signalling network in innate defence. Cell Microbiol 8(6):907–922.
Tanabe M, Kurita-Taniguchi M, Takeuchi K, Takeda M, Ayata M, Ogura H, Matsumoto M, Seya T. 2003. Mechanism of up-regulation of human Toll-like receptor 3 secondary to infection of measles virus-attenuated strains. Biochem Biophys Res Comm 311:39–48.
Tissari J, Siren J, Meri S, Julkunen I, Matikainen S. 2005. IFN-α enhances TLR3-mediated antiviral cytokine expression in human endothelial and epithelial cells by up-regulating TLR3 expression. J Immunol 174:4289–4294.
Vermeer PD, Einwalter LA, Moninger TO, Rokhlina T, Kern JA, Zabner J, Welsh MJ. 2003. Segregation of receptor and ligand regulates activation of epithelial growth factor receptor. Nature 422(6929):322–326.

Address reprint requests or correspondence to:
Ilona Jaspers, Ph.D.
Center for Environmental Medicine, Asthma, and Lung Biology
University of North Carolina at Chapel Hill
104 Mason Farm Rd; CB 7310
Chapel Hill, NC 27599-7310

Tel: (919) 966-8657
Fax: (919) 966-9863
E-mail: ilona_jaspers@med.unc.edu

Received 25 September 2008/Accepted 26 November 2008