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Involvement of Toll-like Receptor 3 in the Immune Response of Lung Epithelial Cells to Double-stranded RNA and Influenza A Virus*

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Influenza A is a highly contagious single-stranded RNA virus that infects both the upper and lower respiratory tracts of humans. The host innate immune Toll-like receptor (TLR) 3 was shown previously in cells of myeloid origin to recognize the viral replicative, intermediate double-stranded RNA (dsRNA). Thus, dsRNA may be critical for the outcome of the infection. Here we first compared the activation triggered by either influenza A virus or dsRNA in pulmonary epithelial cells. We established that TLR3 is constitutively expressed in human alveolar and bronchial epithelial cells, and we describe its intracellular localization. Expression of TLR3 was positively regulated by the influenza A virus and by dsRNA but not by other inflammatory mediators, including bacterial lipopolysaccharide, the cytokines tumor necrosis factor- α and interleukin (IL)-1 β , and the protein kinase C activator phorbol 12-myristate 13-acetate. We also demonstrated that TLR3 contributes directly to the immune response of respiratory epithelial cells to influenza A virus and dsRNA, and we propose a molecular mechanism by which these stimuli induce epithelial cell activation. This model involves mitogen-activated protein kinases, phosphatidylinositol 3-kinase/Akt signaling, and the TLR3-associated adaptor molecule TRIF but not MyD88-dependent activation of the transcription factors NF- κ B or interferon regulatory factor/interferon-sensitive response-element pathways. Ultimately, this signal transduction elicits an epithelial response that includes the secretion of the cytokines IL-8, IL-6, RANTES (regulated on activation normal T cell expressed and secreted), and interferon- β and the up-regulation of the major adhesion molecule ICAM-1.

Influenza is a highly contagious, acute respiratory disease that affects all age groups and that can promote exacerbations of obstructive airways disorders, including asthma and cystic fibrosis. The etiological agent of the disease, the single-

stranded RNA influenza viruses, are responsible for an average of 114,000 hospitalizations and 20,000 deaths each year, in the United States alone (1). Influenza viruses are classified into three types (A, B, and C) of which influenza A is the most important clinically (2). The major problem in fighting influenza is the high genetic variability of the virus, resulting in the rapid emergence of variants that escape the acquired immunity induced by the available vaccines or the resistance of the pathogen to antiviral agents (1, 3). In that context, it would be valuable to unravel the mechanisms of virus-host cell interactions that are responsible for the “flu” syndrome. Indeed, several studies suggest that the inflammatory response to respiratory viral infections contributes to the pathogenesis of the airway symptoms. In that regard, it is of note that the viral replicative intermediate double-stranded RNA (dsRNA)¹ is critical for the outcome of the infection (reviewed in Refs. 4 and 5). For instance, synthetic dsRNA and dsRNA isolated from influenza virus-infected lungs are each able to induce both the local and systemic cytotoxic effects typical of flu (6–8).

Cells are armed with various latent mechanisms that are able to sense viral components and initiate intracellular signal transduction to respond rapidly to virus infections. Previously, the interferon (IFN)-inducible RNA-dependent protein kinase R was considered to be central in the interaction with dsRNA (5). However, cells from RNA-dependent protein kinase R-deficient mice still respond to polyinosinic-polycytidylic acid (poly(I-C)), a synthetic dsRNA analog, suggesting the existence of a more critical receptor involved in the sensing and signaling in response to this viral component. Alexopoulou *et al.* (9) demonstrated that dsRNA recognition relies on the Toll-like receptor (TLR) 3, a member of the conserved family of host innate immune receptors, essential for detecting pathogen-associated molecular patterns. The stimulation of TLR3 by dsRNA transduces signals to activate the transcription factors NF- κ B and interferon regulatory factor (IRF)/interferon-sensi-

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¹ The abbreviations used are: dsRNA, double-stranded RNA; DN, dominant-negative; ERK, extracellular signal-regulated kinase; IRF, interferon regulatory factor; ISRE, interferon-sensitive response element; MyD, myeloid differentiation; NS, nonstimulated; TLR, Toll-like receptor; poly(I-C), polyinosinic-polycytidylic acid; TIR, Toll-IL-1 receptor; TRIF, TIR domain containing adaptor inducing interferon- β ; PMA, phorbol 12-myristate 13-acetate; RANTES, regulated on activation normal T cell expressed and secreted; IL, interleukin; PI3K, phosphatidylinositol 3-kinase; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; PFU, plaque-forming units; LPS, lipopolysaccharide; FACS, fluorescence-activated cell sorter; RT, reverse transcription; FITC, fluorescein isothiocyanate; MAPK, mitogen-activated protein kinase; TNF, tumor necrosis factor; PBS, phosphate-buffered saline; CRE, cAMP-response element; JNK, c-Jun amino-terminal kinase.

tive response element (ISRE) via myeloid differentiation factor 88 (MyD88)-dependent and/or independent signaling pathways. The last of these involves a distinct adaptor molecule, namely the Toll-IL-1 receptor (TIR) domain containing adaptor-inducing interferon (IFN)- β (TRIF), also called the TIR domain containing adaptor molecule (TICAM)-1 (10, 11). This molecule elicits an anti-viral response, especially through the production of type I IFN.

In humans, TLR3 mRNA is detected in the lung, placenta, pancreas, liver, heart, and brain. It is expressed in dendritic cells (12) and in intestinal epithelial cells (13) but does not seem to be present in monocytes, lymphocytes, polymorphonuclear leukocytes, or natural killer cells (14). Most interestingly, although TLR3 expression *per se* was not reported, a study by Gern *et al.* (15) showed recently that viral dsRNA activates bronchial epithelial cells. Lung epithelial cells are the primary target and the principal host for influenza viruses, causing cytopathic effects to the respiratory tract as well as shedding of infective viral particles. These epithelial cells also play a key role in the initiation of innate and subsequently adaptive immune responses to the virus (16, 17).

Most surprisingly, very little information is available concerning the expression and localization of TLR3 in pulmonary epithelial cells. Moreover, the role of epithelial TLR3, its regulatory mechanisms, and the signaling pathways underlying the response to influenza A virus have not been investigated previously.

We first studied the activation of bronchial epithelial cells induced by either purified dsRNA or following influenza A virus infection. Next, we demonstrated that TLR3 is constitutively expressed in distinct human alveolar and bronchial epithelial cells, and we described its intracellular localization. Finally, we determined that TLR3 and its signaling-associated molecule TRIF play a key role in the immune response of respiratory epithelial cells to both dsRNA and influenza A virus.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—RPMI 1640, F-12K nutrient mixture (Kaighn's modification), antibiotics, glutamine, Hanks' balanced salt solution, and trypsin-EDTA were from Invitrogen. Fetal calf serum was from Hyclone (Logan, UT). Leupeptin, aprotinin, soybean trypsin inhibitor, phenylmethylsulfonyl fluoride, benzamide, paraformaldehyde, poly(I-C) acid, forskolin, and phorbol 12-myristate 13-acetate (PMA) were from Sigma. The p38 (SB203580) and ERK1/2 (PD98059) inhibitors were obtained from Calbiochem and Cell Signaling technology (Beverly, MA), respectively. The PI3K inhibitor, LY294002, was obtained from Cell Signaling technology. Horseradish peroxidase-conjugated secondary antibody was from Pierce. Anti-TLR3 antibodies used included the goat polyclonal antibody N-15, directed against an amino-terminal region of human TLR3 (Santa Cruz Biotechnology, Santa Cruz, CA), and the rabbit polyclonal antibody H-125, specifically raised against a peptide close to the transmembrane domain (amino acids 653–777) of TLR3 (Santa Cruz Biotechnology). The anti-human β -actin antibody was from Sigma, and the anti-NF- κ B-p65, anti-phospho-ERK1/2, and anti-phospho-JNK antibodies were from Santa Cruz Biotechnology. The anti-phospho-p38 was purchased from Cell Signaling. Fluorescein isothiocyanate (FITC)-labeled anti-goat and anti-rabbit antibodies were obtained from Dako and Rockland (Gilbertsville, PA). Anti-human-ICAM-1 antibody was from R&D Systems (Minneapolis, MN).

Cell and Culture Conditions—The human promonocytic cell line U-937, the human alveolar epithelial cell line A549, and the human bronchial epithelial cell line BEAS-2B were obtained from the American Type Cell Collection (Manassas, VA). The human tracheal epithelial cell lines CFT-2 and NT-1 were a kind gift from Dr. A. Paul (INSERM U402, Paris, France). CFT-2 was derived from primary tracheal epithelia homozygous for the common cystic fibrosis mutation Δ F508, and NT-1 was derived from normal primary tracheal epithelial cells (18). Cells were cultured as described previously (19).

Virus Preparation and Inactivation—Influenza A/Scotland/20/74(H3N2) virus was grown on Madin-Darby canine kidney cells in the presence of 2 μ g/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone-

treated trypsin. The supernatant was harvested on day 3 and clarified by centrifugation at 680 \times g for 15 min. Viral stocks were stored in aliquots at -80°C . Virus titers were determined by a standard plaque assay using Madin-Darby canine kidney cells. A noninfected cell culture was used for preparation of the control inoculum. UV light-inactivated virus was prepared by exposing stock virus solution (0.5 ml/6-cm Petri dish) to a 15-watt UV light at a distance of 20 cm for 15 min.

RT-PCR—Total RNA was extracted by using an RNeasy kit (Qiagen, Courtaboeuf, France). RT was performed using 0.5 μ g of total RNA extracted as described previously (19). PCR was performed using specific primers (Proligo, Evry, France) for human TLR3 (sense, 5'-AAA TTG GGC AAG AAC TCA CAG G-3'; and antisense, 5'-GTG TTT CCA GAG CCG TGC TAA-3'). As an internal control, we used primers for human β -actin (sense, 5'-AAG GAG AAG CTG TGC TAC GTC GC-3'; and antisense, 5'-AGA CAG CAC TGT GTT GGC GTA CA-3'). Amplifications were performed in a Peltier thermal cycler apparatus (MJ Research, Watertown, MA) using the Qbiotaq polymerase (Qbiogene, Illkirch, France). To detect TLR3, the thermocycling protocol was as follows: 95 $^{\circ}\text{C}$ for 3 min, 36 cycles of denaturation at 95 $^{\circ}\text{C}$ for 45 s, annealing at 56 $^{\circ}\text{C}$ for 45 s, and extension at 72 $^{\circ}\text{C}$ for 1 min. To detect β -actin, only 30 cycles were used, and the annealing temperature was 62 $^{\circ}\text{C}$. Amplification products were resolved on 1.5% agarose gel containing ethidium bromide. Band intensities on gels were recorded after amplification with an Ultra-Lum system (Ultra-Lum, Claremont, CA). Samples for each point were serially diluted to verify that PCR was performed in the linear phase of the amplification reaction (data not shown).

Immunoblotting—Epithelial cell extracts were prepared and solubilized as described previously (19). Aliquots (15 μ g of protein) were run on SDS-10% PAGE, and the proteins were then electrotransferred to a nitrocellulose membrane (Optitran BA-S 85, Schleicher and Schuell) and probed with specific antibodies, as specified in the figure legends. Bound antibodies were detected using ECL+ immunoblotting detection system (Amersham Biosciences), according to the manufacturer's instructions. Molecular masses were estimated from calibration standards included in each gel.

Flow Cytometry Analysis—Epithelial and monocytic cells were dispensed (1×10^6 cells/ml) into conical bottomed 96-well plastic plates (Nunc A/S, Roskilde, Denmark) and were centrifuged at 100 \times g at 4 $^{\circ}\text{C}$ for 10 min. Cells were washed with Hanks' balanced salt solution, 0.5% bovine serum albumin supplemented with 1 mM Ca^{2+} and Mg^{2+} , and a saturating concentration of anti-TLR3 antibodies (5 μ g/ml), anti-ICAM-1 antibody (1 μ g/ml), or nonimmune IgG as controls was then added, and the samples were incubated for 30 min at 4 $^{\circ}\text{C}$. Cells were washed and incubated for 30 min at 4 $^{\circ}\text{C}$ with the corresponding secondary FITC-conjugated antibody (5 μ g/ml). For intracellular staining, cells were fixed and permeabilized by incubation for 90 min on ice with a solution of PBS, 3.2% paraformaldehyde, 0.2% Tween 20; they were then incubated with an anti-TLR3 antibody (5 μ g/ml) and FITC-conjugated secondary antibodies (5 μ g/ml). FACScan flow cytometer (Immunocytometry Systems) was used for cytometric analysis.

NF- κ B Immunostaining—BEAS-2B epithelial cells were cultured on 22-mm glass cell culture coverslips (CML France, Nemours, France). Cells were washed three times with PBS and then fixed for 15 min in PBS, 3.2% paraformaldehyde. After washing with gentle shaking, cells were permeabilized for 5 min with 0.1% Triton X-100 and washed, as before, prior to incubation with an anti-NF- κ B-p65 antibody (0.4 μ g/ml) and a specific secondary antibody (5 μ g/ml). In control experiments, cells were incubated with nonimmune IgG as control isotypes. Finally, the cells were washed extensively with PBS, and the coverslips were mounted in fluorescence mounting medium. Fluorescence microscopy was performed with a 63 \times /1.4 oil objective lens on a confocal microscope (model LSM 510; Carl Zeiss France, Le Pecq, France), using laser excitation at 488 nm.

Epithelial Cell Transfection and Reporter Gene Studies—BEAS-2B cells were seeded at 5×10^4 on 24-well plates (Costar, New York) 96 h before transfection using FuGENE 6 transfection reagent (Roche Applied Science), according to the manufacturer's instructions. Each sample contained 200 ng of an NF- κ B-luciferase- (kindly provided by Dr. A. Israel, Pasteur Institute, Paris, France), an ISRE-luciferase-, or a CRE-luciferase-reporter plasmid (Clontech) and 500 ng of vector expressing a dominant-negative form of either MyD88 (DN-Myd88; a kind gift from Dr. M. Muzio, Mario Negri Institute, Milan, Italy) or TRIF (DN-TRIF). The TLR3 construct from which the TIR domain is deleted (pZERO-hTLR3) and encoding a nonfunctional TLR3 molecule were purchased from InvivoGen (San Diego, CA). The empty plasmids, pCDNA3 (Invitrogen) and pCMV (Clontech), were used as controls as appropriate. After 24 h, cells were left untreated or stimulated for 6 or 24 h at 37 $^{\circ}\text{C}$

with influenza A virus or 1 $\mu\text{g/ml}$ poly(I-C). Luciferase activity was measured in the cell lysates as described previously (19), using an EGNG Berthold luminometer. Results are expressed as relative luciferase units.

Cytokine Measurements—Human IL-8, IL-6, RANTES, and IFN- β concentrations in cell culture supernatants were determined using Duo-Set ELISA kits obtained from R&D Systems (Minneapolis, MN).

Statistical Analysis—Each point corresponds to the mean \pm S.D. of the indicated number of experiments. The statistical significance of differences between groups was tested using the unpaired Student's *t* test with a threshold of $p < 0.05$.

RESULTS

Comparison of the Activation of Bronchial Respiratory Cells by dsRNA and Influenza A Virus—dsRNA is known to accumulate within infected cells, and it has the required physical and biological properties needed to induce antiviral responses and pathological inflammatory processes (4, 5). Thus, it appears important to examine whether influenza A virus-induced activation of pulmonary epithelial cells shares any characteristics with that stimulated by a synthetic dsRNA such as poly(I-C). Fig. 1 reports a comparison of the effects from two inducers in the human bronchial epithelial cell line BEAS-2B. Poly(I-C) triggered a strong secretion of IL-8, in a concentration- (Fig. 1A) and time-dependent manner (Fig. 1B). The secretion started within 3 h and IL-8 accumulated in the culture medium up to 24 h (Fig. 1B). We also observed a time-dependent accumulation of IL-6 (Fig. 1C). RANTES was also induced by poly(I-C) but \sim 3 h after the start of production of the other cytokines. This suggests an autocrine/paracrine activation by another mediator; for example IFN- β might feed back on RANTES production (Fig. 1D). In that regard, IFN- β production peaked 6 h post-stimulation but was not detected at 24 h, suggesting a reprocessing of this cytokine by BEAS-2B cells (Fig. 1E). To exclude any stimulatory effect associated with contamination of poly(I-C) by bacterial endotoxin, experiments were also performed using poly(I-C) supplemented with 20 $\mu\text{g/ml}$ polymyxin B, a well characterized LPS inhibitor. Under these experimental conditions, IL-8 secretion by BEAS-2B cells was not modified (not shown).

When BEAS-2B cells were infected with influenza A virus, IL-8 secretion was clearly detected 24 h later and was dose-dependent (Fig. 1, G and H). RANTES and IL-6 secretion were also dose-dependent up to a maximum at 5×10^4 PFU/multiplicity of infection of 0.25 (Fig. 1, I and K). Cytokine secretion was delayed by 20 h with respect to that by poly(I-C)-stimulated BEAS-2B cells. This delay may be consistent with the time required to generate dsRNA within the infected cell, during the replication of the virus (Fig. 1, H and J, and data not shown).

Epithelial cells in the lung express ICAM-1, which is involved in the recruitment and the local accumulation of inflammatory cells through the binding to lymphocyte function-associated antigen (LFA)-1 (20). Previous studies have shown that pro-inflammatory mediators including LPS and TNF- α induce ICAM-1 expression on pulmonary epithelial cells (21). We confirmed these findings by flow cytometric analysis, and we extended them to dsRNA and influenza A virus by demonstrating a potent up-regulation of ICAM-1 expression, which was as strong as that obtained with TNF- α (Fig. 1F and not illustrated).

Previous studies have shown that production of inflammatory mediators in response to a viral infection can occur in the presence or absence of multiplication of the pathogen (22). We tested whether viral contact with the plasma membrane and/or viral penetration of the host cell was sufficient to trigger the inflammatory response. Bronchial epithelial cells were infected with UV-treated, and therefore nonreplicative, virus. Also,

BEAS-2B cells were treated with an intact virus in the presence of 1 $\mu\text{g/ml}$ amantadine, an influenza-specific inhibitor blocking the early release of the viral genome into the cytoplasm but not the endocytosis of the viral particle into the cell (1). Neither UV-treated virus nor virus in the presence of amantadine induced IL-8 release (Fig. 1L). Thus, components generated during viral replication are required for the inflammatory response. Similarly, there was no RANTES or IL-6 secretion in the absence of viral replication (data not shown). Hence, the inflammatory response to influenza A virus infection is replication-dependent and is not mediated solely by the initial virus-host cell interaction or by any artifact possibly present in the infectious inoculum.

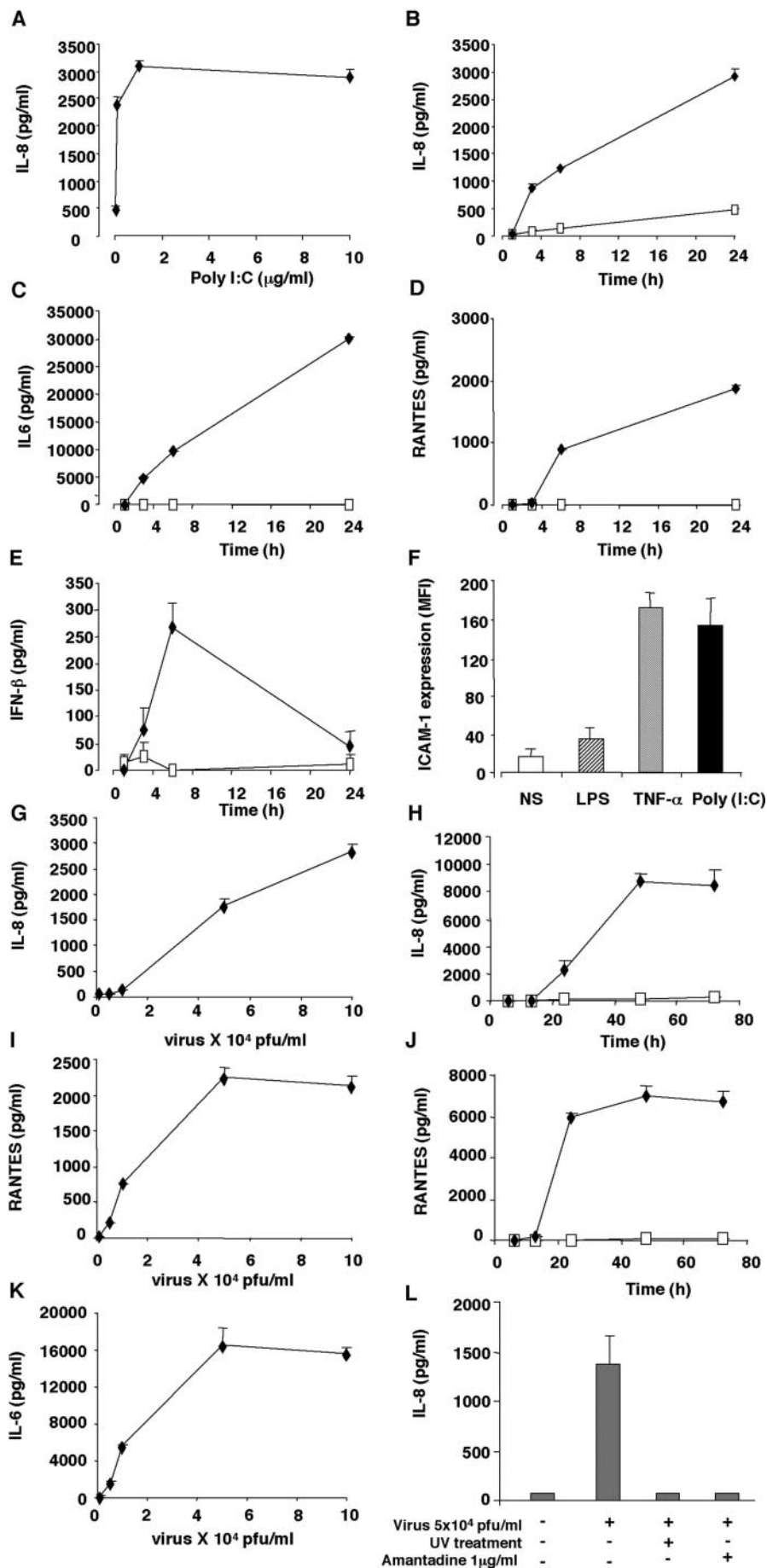
Expression and Localization of TLR3 in Pulmonary Epithelial Cells—Because the foregoing results suggested that dsRNA is required for the immunostimulatory activity of influenza A virus, we examined the expression of TLR3, a recently described dsRNA sensor, in pulmonary epithelial cells (9). We first used RT-PCR to test for the presence of TLR3 mRNA in unstimulated human respiratory epithelial cells. As shown in Fig. 2A, TLR3 mRNA was detected in PMA-differentiated U-937 cells and in both human alveolar (A549) and tracheobronchial (BEAS-2B, NT-1, and CFT-2) epithelial cells lines. RT-PCR analysis of β -actin mRNA confirmed the quality of all RNA preparations.

We then used two antibodies, N-15 and H-125, to test for TLR3 protein in human pulmonary epithelial cells. The specificity of these antibodies was confirmed by Western blotting with a recombinant TLR3-glycosylated peptide, consisting of amino acids 21–711 (not illustrated). Protein expression level of TLR3 was analyzed by flow cytometry in A549 and BEAS-2B cells (Fig. 2B). No TLR3 signal was detected at the cell surface of these respiratory cells (*left panels*), but abundant intracellular TLR3 was revealed using a mild fixation and permeabilization protocol (*middle and right panels*).

Epithelial TLR3 Expression Is Up-regulated by Poly(I-C) and Influenza A Virus—Next we examined whether different stimuli regulate the epithelial TLR3 mRNA. BEAS-2B cells were exposed to an optimal concentration of poly(I-C) (1 $\mu\text{g/ml}$), LPS (1 $\mu\text{g/ml}$), TNF- α (50 ng/ml), IL-1 β (20 ng/ml), or the potent protein kinase C activator PMA (15 nM) and to influenza A virus (5×10^4 PFU) for 24 h. Under these conditions, cell activation was fairly equipotent, as assessed by the measurement of IL-8 secretion induced by each stimulus (not shown). Expression of TLR3 mRNA was normalized to that of β -actin and is reported in Fig. 3B, *histogram bars*. This semi-quantitative densitometric measurement clearly shows that only influenza A virus infection and cell stimulation by the viral RNA mimetic component specifically up-regulated TLR3 mRNA; the other treatments had no significant effect (Fig. 3, A and B).

IL-8 but Not RANTES Secretion Triggered by dsRNA or Influenza A Virus Shares a Common Signaling Pathway—Virus infection of susceptible cells activates multiple signaling pathways, including dynamic protein phosphorylations, that orchestrate the induction of genes contributing to the innate immune response. The kinases involved include p38, extracellular signal-regulated kinase (ERK)1/2, and JNK. Little is known concerning the involvement of PI3K in virus-induced and/or TLR signaling. PI3K catalyzes the production of phosphatidylinositol 3,4,5-trisphosphate, which allows the recruitment of signaling proteins, including the serine-threonine kinase Akt (23). Therefore, we examined whether dsRNA activates p38, JNK, ERK1/2, and Akt in bronchial epithelial cells. Treatment with poly(I-C) strongly up-regulated phosphorylation of these signaling components. The maximum level of phosphorylation was between 15 and 60 min and declined

FIG. 1. Comparison of the activation of bronchial epithelial cells induced by dsRNA and influenza A virus. Monolayers of BEAS-2B cells were stimulated for 24 h with a series of concentrations of poly(I-C) (0.01, 0.1, 1, and 10 $\mu\text{g/ml}$ (A)) or with 1 $\mu\text{g/ml}$ poly(I-C) for various times (1, 3, 6, and 24 h; B-E). Supernatant fluids were tested for IL-8 (A and B), IL-6 (C), RANTES (D), and IFN- β (E) by ELISA. BEAS-2B cells were stimulated for 24 h or not stimulated (NS) with 1 $\mu\text{g/ml}$ LPS, 1 $\mu\text{g/ml}$ poly(I-C), or 20 ng/ml TNF- α , and ICAM-1 expression was assayed by FACS analysis (F). Results are expressed as mean fluorescence intensity (MFI). Monolayers of BEAS-2B cells were stimulated for 24 h by increasing concentrations of influenza A virus (0.5, 1, 5, and 10 $\times 10^4$ PFU/ml (G, I, and K)) or with 5 $\times 10^4$ PFU/ml for various times (6, 12, 24, 48, and 72 h (H and J)). Supernatant fluids were tested for IL-8 (G and H), RANTES (I and J), and IL-6 (K) by ELISA. L, BEAS-2B cells were stimulated or not for 24 h with 5 $\times 10^4$ PFU/ml of influenza A virus, of UV-treated influenza A virus, or of virus after cell pretreatment with 1 $\mu\text{g/ml}$ of amantadine. Supernatant fluids were tested for IL-8 by ELISA. Data are means \pm S.D. of triplicate determinations of a representative experiment performed three times. *Black diamonds* are poly(I-C)- or virus-treated cells, and *open boxes* represent non-treated samples.



A

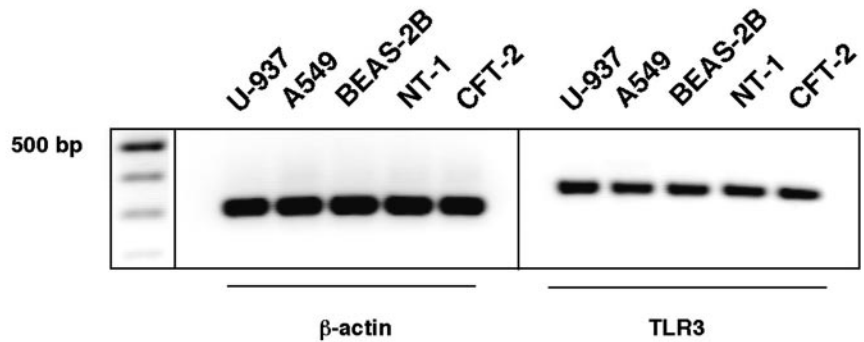


FIG. 2. Expression and localization of TLR3 in pulmonary epithelial cells. *A*, representative RT-PCR showing TLR3 expression in human alveolar (A549) and tracheobronchial (BEAS-2B, NT-1, and CFT-2) epithelial cell lines. The human macrophage cell line U-937, a known source of TLR3, was used as a positive control. *B*, representative FACS analysis of TLR3 expression in two different lung epithelial cell lines. A549 and BEAS-2B were permeabilized ((+)perm, right panels) or not (- perm, left panels) and immunostained with N-15 or H-125 as described under "Experimental Procedures." The light and dark line histograms represent the fluorescence signal obtained using isotype-control antibody and TLR3-specific antibody, respectively. Fluorescence tracings are representative of three independent experiments.

B

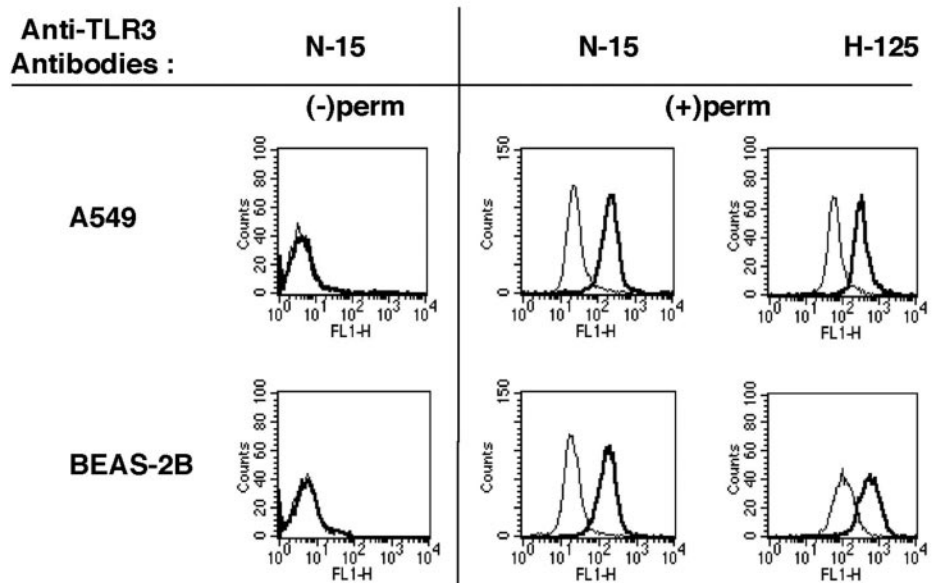
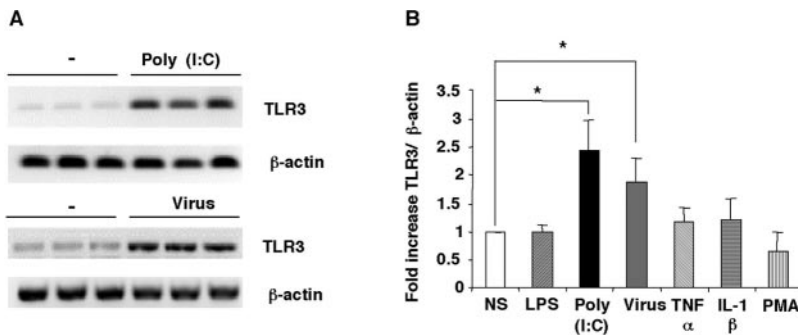


FIG. 3. TLR3 gene regulation by pro-inflammatory stimuli. BEAS-2B cells were stimulated or not stimulated (NS) with 1 μ g/ml LPS, 1 μ g/ml poly(I-C), 5×10^4 PFU/ml of influenza A virus, 20 ng/ml TNF- α , 50 ng/ml IL-1 β , or 15 nM PMA for 24 h. Total RNA was extracted, and TLR3 mRNA was analyzed by RT-PCR. *A*, representative result out of three is shown. *B*, histogram bars show the value for TLR3 normalized against that for β -actin and are the means \pm S.D. of three independent experiments performed in triplicate.

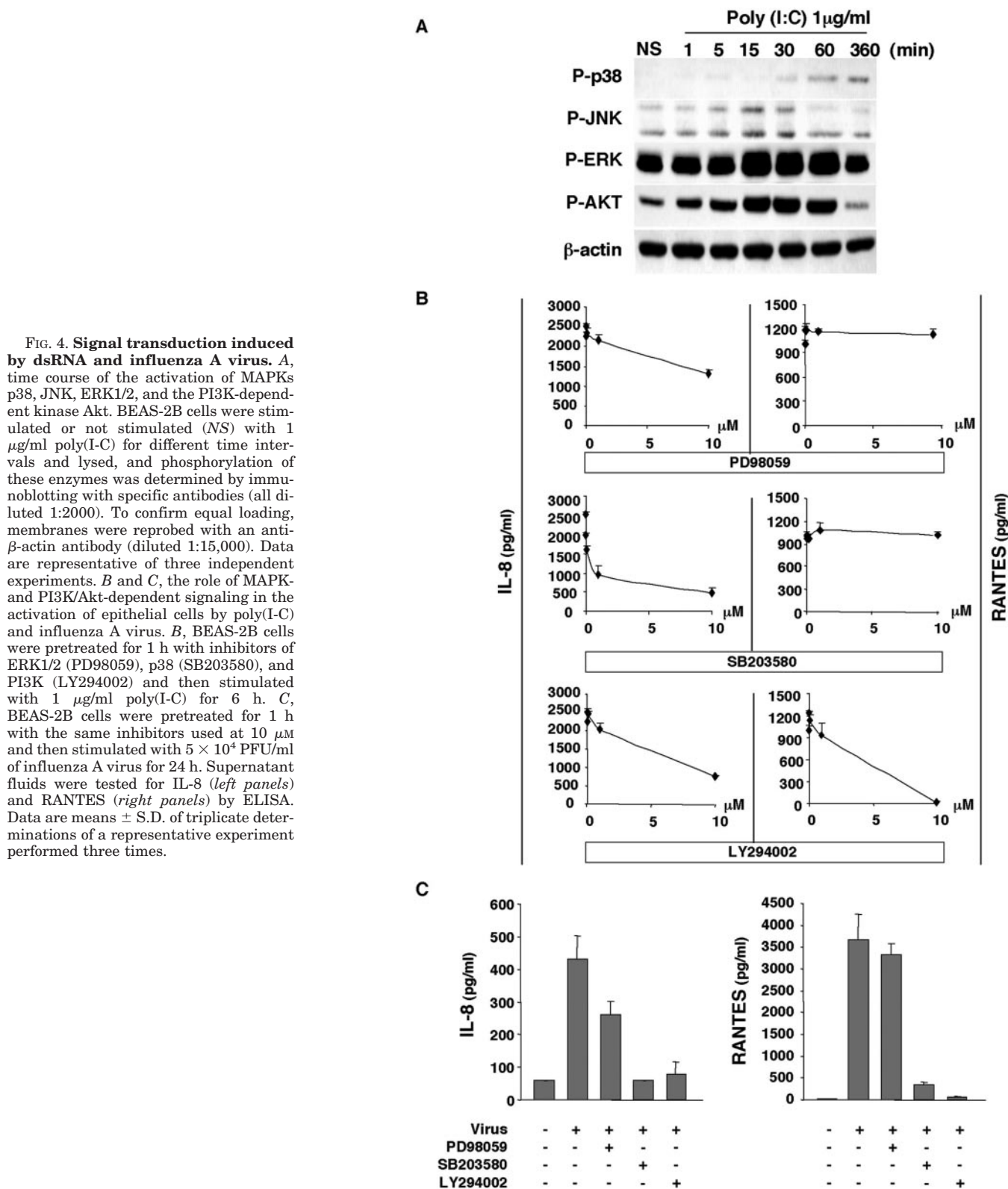


thereafter, except for p38 phosphorylation that increased until 360 min post-stimulation (Fig. 4A). To delineate specifically the role of these kinases in the production of epithelial cytokines, BEAS-2B cells were pretreated with the specific inhibitors PD98059 for ERK1/2, SB203580 for p38, and LY294002 for PI3K (Fig. 4B). None of these inhibitors caused cytotoxic effects on BEAS-2B cells at the concentrations used in these experiments. However, all inhibitors significantly reduced the dsRNA-induced production of IL-8 (Fig. 4B) and IL-6 (not illustrated). By contrast, only the PI3K/Akt pathway inhibitor reduced RANTES production.

When pulmonary epithelial cells were infected by influenza A virus in the presence of 10 μ M of the above inhibitors, IL-8

secretion was similar to that induced by synthetic dsRNA under the same experimental conditions: \approx 45% inhibition in the presence of PD98059, and a secretion almost abolished in the presence of SB203580 or LY294002. By contrast, RANTES release triggered by either stimulus does not exhibit a similar inhibitory pattern. Remarkably, although the p38 inhibitor did not affect dsRNA-induced RANTES secretion, it strongly inhibited RANTES secretion following influenza A virus infection (Fig. 4C).

The TLR3/TRIF Pathway Is Essential for dsRNA and Influenza A Virus-induced NF- κ B and IRF/ISRE Activation in Pulmonary Epithelial Cells—Activation of transcription factors is pivotal to many signal transduction pathways. For instance,



NF- κ B can be activated in response to many different stress conditions, including infection, inflammation, and tissue repair. IRFs consist of a growing family of related transcription proteins initially identified as regulators of the IFN- α/β gene promoters and the ISRE of various IFN-stimulated genes. Activators of the cyclic AMP-response element (CRE) contribute to diverse physiological processes, including the control of cellular metabolism and cell survival. We assessed the involvement of these regulatory signaling elements in the antiviral

immune response induced by dsRNA and influenza A virus. In that purpose, BEAS-2B cells were transfected with a set of vectors each of which contains a different *cis*-acting enhancer element (NF- κ B, ISRE, or CRE) upstream from a luciferase reporter gene. As shown in Fig. 5A, NF- κ B and IRF/ISRE were strongly activated upon dsRNA challenge in bronchial epithelial cells. In contrast, CRE was not activated under the same experimental conditions, even though forskolin (10 μ M), a potent activator of the cAMP signaling pathway, confirmed the

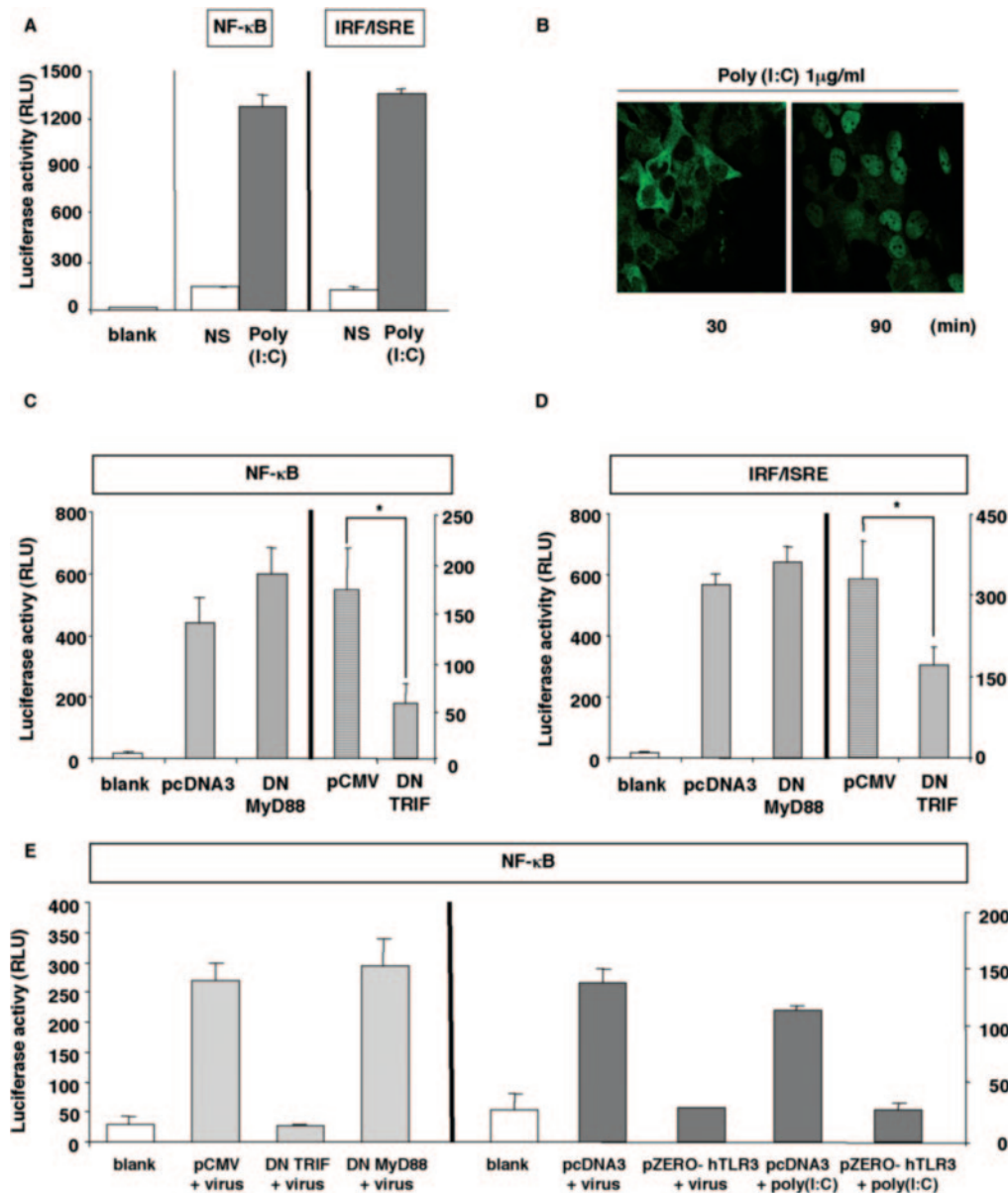


FIG. 5. Involvement of TLR3 and TRIF pathway in dsRNA and influenza A virus-induced NF- κ B and IRF/ISRE activation in pulmonary epithelial cells. *A*, BEAS-2B cells were transfected with 200 ng of an NF- κ B or IRF/ISRE luciferase reporter construct and then stimulated with 1 μ g/ml poly(I-C) for 6 h. Cell lysates were prepared and assayed for luciferase activity. Results are expressed as relative luciferase units (RLU) and are means \pm S.D. of triplicate determinations of a representative experiment performed three times. *B*, BEAS-2B cells were stimulated with 1 μ g/ml poly(I-C) for 30 and 90 min. The cells were then immunostained with an anti-NF- κ B antibody and examined by confocal microscopy as described under "Experimental Procedures." *C* and *D*, BEAS-2B cells were co-transfected with 200 ng of an NF- κ B- or IRF/ISRE luciferase reporter construct and with 500 ng of the expression plasmid encoding DN forms of MyD88 or TRIF, or with the corresponding control plasmids (pcDNA3 and pCMV). The transfected BEAS-2B cells were then stimulated with 1 μ g/ml poly(I-C) for 6 h, and cell lysates were prepared and assayed for luciferase activity. *E*, BEAS-2B cells were transfected with 200 ng of a NF- κ B luciferase reporter plasmid and with 500 ng of the plasmid encoding DN forms of MyD88, TRIF, or TLR3 (pZERO-hTLR3), or the respective control plasmids (pcDNA3 and pCMV). The transfected BEAS-2B cells were then stimulated with 5×10^4 PFU/ml influenza A virus or 1 μ g/ml poly(I-C) for 24 h, and cell lysates were prepared and assayed for luciferase activity.

CRE vector was functional (data not shown). NF- κ B activation was also confirmed by immunofluorescence staining (Fig. 5*B*); indeed, translocation of this transcription factor from the cytoplasm to the nucleus was clearly visible within 90 min of epithelial cell activation by poly(I-C).

We further investigated whether MyD88 and/or TRIF were involved in the NF- κ B and IRF/ISRE signaling pathways activated by dsRNA and influenza A virus, using luciferase reporter plasmids and either DN or control vectors. MyD88 is a protein harboring a carboxyl-terminal TIR domain and an amino-terminal death domain. The pathway triggered by the recruitment of MyD88 to the TIR domain of the TLRs has been

extensively studied (24). Once recruited, MyD88 interacts with the kinase IRAK4, via their respective death domain, culminating in the activation of various transcription factors crucial for the expression of pro-inflammatory genes. The vector expressing DN-MyD88 corresponds to a truncated protein (amino acid 152 to the carboxyl-terminal end) that contains the TIR region but not the death domain, and thus prevents a downstream signaling (25, 26). A similar approach was used to generate a truncated DN-TRIF that consists of the TIR domain only (10). dsRNA-mediated activations of both NF- κ B and IRF/ISRE in BEAS-2B cells transfected with 500 ng of the expression vector encoding DN-TRIF were ≈ 70 and $\approx 50\%$ lower,

TABLE I
Specificity of the expression plasmids encoding DN-MyD88 or DN-TRIF

BEAS-2B cells were co-transfected with 200 ng of an NF- κ B-luciferase reporter construct and with 500 ng of the plasmid encoding DN-MyD88 or DN-TRIF, or with the corresponding control plasmids (pcDNA3 and pCMV, respectively). The transfected BEAS-2B cells were then stimulated with either 20 ng/ml IL-1 β or 1 μ g/ml LPS for 6 h, and cell lysates were prepared and assayed for luciferase activity. Results are expressed as relative luciferase units (RLU) and are means \pm S.D. of triplicate determinations of a representative experiment performed two times.

Transfected plasmid	Stimulus	RLU	S.D.	Statistical significance
pCMV	Non-stimulated	99.0	± 19.1	$p > 0.05$
pCMV	IL-1 β	626.0	± 63.0	
DN-TRIF	IL-1 β	689.0	± 49.7	
pcDNA3	Non-stimulated	37.3	± 7.1	$p < 0.001$
pcDNA3	IL-1 β	390.0	± 25.9	
DN-MyD88	IL-1 β	216.3	± 11.6	
pcDNA3	LPS	121.7	± 5.5	$p < 0.001$
DN-MyD88	LPS	71.0	± 1.0	

respectively, than in control plasmid-transfected cells (Fig. 5, *C* and *D*). Influenza A virus also failed to activate NF- κ B in cells transfected with the vector encoding DN-TRIF (Fig. 5*E*, *left panel*). By contrast, transfection of BEAS-2B cells with a plasmid encoding DN-MyD88 did not alter dsRNA- or influenza A virus-mediated NF- κ B and/or IRF/ISRE activation (Fig. 5, *C-E*, *left panel*). We confirmed the specificity of these vectors by testing a negative control on DN-TRIF-transfected cells and positive controls on DN-MyD88-transfected cells. Thus, BEAS-2B cells were co-transfected with the NF- κ B-luciferase reporter construct and with the plasmid encoding DN-MyD88 or DN-TRIF or with the corresponding control plasmids (pcDNA3 and pCMV, respectively). The transfected BEAS-2B cells were then stimulated with either IL-1 β (20 ng/ml) or LPS (1 μ g/ml) for 6 h and further assayed for luciferase activity. Table I shows that IL-1 β - or LPS-induced NF- κ B activation, known to require MyD88 (19), was inhibited by prior transfection with the DN-MyD88 vector ($p < 0.001$). Conversely, IL-1 β -induced NF- κ B activation was not affected by the DN-TRIF plasmid, in comparison with the pCMV vector ($p > 0.05$).

By having demonstrated that TRIF-dependent signaling is involved in dsRNA- and influenza A virus-induced respiratory epithelial cell stimulation, we next verified the upstream role of TLR3 *per se* in sensing and cell activation by these viral entities. BEAS-2B cells were transfected with a plasmid (pZERO-hTLR3) encoding TLR3 from which the TIR domain had been deleted, to compete with the endogenous functional TLR3. Fig. 5*E* (*right panel*) shows that pZERO-hTLR3 abolished the cell responses to both dsRNA and influenza A virus. Overall, these data suggest a major role for the interaction between TLR3 and influenza A virus-derived dsRNA in the innate immune response of infected pulmonary epithelial cells.

DISCUSSION

Influenza A virus causes pulmonary inflammation and exacerbates chronic lung diseases, because of an infiltration of inflammatory cells and an increased airway hyper-responsiveness. Bronchial epithelial cells play an important role in the pathogenesis of this viral infection (27). However, although many of the molecular events in influenza A virus replication have been described, the underlying mechanisms by which virus-epithelium interaction triggers the inflammation process have yet to be fully characterized. The discovery of TLR3 as a key receptor for dsRNA led us to investigate the contribution of this receptor to the activation of pulmonary epithelial cells by dsRNA and influenza A virus. We show the following: (i) that TLR3 is constitutively expressed in respiratory epithelial cells in an intracellular compartment; (ii) that TLR3 expression is up-regulated either by influenza A virus or by purified dsRNA but not by other major inflammatory mediators; (iii) that TLR3 plays a central role in the immune response of bronchial epithelial cells triggered by these stimuli; and (iv) that influenza A

virus and dsRNA induce epithelial cell activation through MAPK, PI3K/Akt signaling, and TRIF- but not MyD88-dependent activation of the transcription components NF- κ B and IRF/ISRE.

It is unclear how much true dsRNA, *i.e.* full duplexes between positive and negative sense RNA, is present in infected cells upon replication of influenza A and other single-stranded RNA viruses. Nevertheless, RT-PCR experiments and binding of anti-helical dsRNA antibodies to viroplasm from whole cell extracts suggest that true dsRNA accumulates within virus-infected cells (5). Also, previous studies reported that as little as one molecule *per* cell can have profound effects on cellular physiology (5). Thus, dsRNA is likely the most immunostimulatory entity of influenza A virus, eliciting epithelial antiviral and inflammatory responses, as suggested by the results of the experiments using amantadine- and UV-treated virus. Moreover, we found that dsRNA can substitute for the virus in terms of secretion of the cytokines IL-8, IL-6, RANTES, and IFN- β that may promote leukocyte infiltration. Of note is that the kinetics of cytokine release differ between the two stimuli, and this interval may be consistent with the time required for the virus replication process to generate dsRNA (28).

Our findings suggest that human lung epithelial cells express TLR3 only in an intracellular compartment. The same is true for immature human dendritic cells subsets (29). The life cycle of a virus includes entry into the target cell by endocytosis and transport of the viral genome either to the cytoplasm or to the nucleus where it is transcribed and amplified (30). Thus, the intracellular localization of TLR3 is rather consistent with its sensing role of viral replicative elements. Nonetheless, part of our epithelial cell activation experiments were performed using exogenously added poly(I-C), which is reminiscent of viral dsRNA leaking from dying cells, that may act directly on neighboring cells (31). Consequently, for exogenous dsRNA to encounter TLR3, it must penetrate the cell. In that regard, internalization was found to be essential for poly(I-C) to induce interferon activity or cell toxicity in murine LM cells (32). Hence, exogenous dsRNA is presumably internalized in pulmonary epithelium after cell surface recognition that may involve proper binding structures, including scavenger receptors as it has been demonstrated in macrophages (4).

Previous studies of the regulation of TLR expression report diverse findings depending on the stimulus and the cell type or tissue considered (33). In addition, Heinz *et al.* (34) reported a species-specific regulation of the *TLR3* gene. Therefore, we examined whether various inflammatory mediators regulate the expression of *TLR3* in human respiratory epithelial cells. Of the mediators we tested, only influenza A virus and dsRNA up-regulated *TLR3* expression, suggesting that the signaling pathways controlling the induction of this gene are restricted. An attenuated strain of the measles virus also stimulates the expres-

sion of *TLR3* through a type I IFN-dependent and an ISRE-dependent regulatory mechanism (35). We are currently investigating whether the modulation of *TLR3* expression by influenza A virus involves similar regulatory pathways. Our findings also suggest that to some extent dsRNA and influenza A virus use different signaling mechanisms to induce inflammatory epithelial responses; dsRNA triggers IL-8 secretion via at least the signal-transducing molecules ERK, p38, and PI3K/Akt, whereas RANTES release appears to be mainly dependent on a PI3K/Akt activation. These results are in agreement with recent work showing p38-dependent production of IL-8 but not of RANTES in lung epithelial cells following dsRNA treatment (15). Also, the role of the PI3K-Akt pathway in *TLR3*-mediated gene induction was robustly supported by Sarkar *et al.* (36) in a very recent study. Most interestingly, we found that in influenza A-infected epithelial cells, RANTES production required both PI3K/Akt and p38 MAPK activation. These results implicate PI3K/Akt and p38 signaling pathways rather than ERK1/2 signaling pathways in the epithelial immune response to dsRNA and influenza A virus infection. The effect of the p38 inhibitor on the virus but not the synthetic dsRNA-induced RANTES secretion suggests, unsurprisingly, that the molecular interactions governing influenza A infection are more complex than those induced by dsRNA alone. Presumably, in addition to dsRNA, other viral entities such as virion-associated proteins may contribute to virus-induced signal transduction (37). Generally, IL-8 gene regulation is considered to be highly dependent on NF- κ B activation, whereas RANTES expression required the combination of both NF- κ B and IRF (38, 39). Thus, our study can be used to suggest a model of how dsRNA activates human respiratory epithelial cells. After either a receptor-mediated transfer of exogenous dsRNA across the plasma membrane or a viral replication-dependent accumulation within the cell, dsRNA encounters intracellular *TLR3*, triggering a downstream signaling. This process involves at least the *TLR3* adaptor TRIF but not MyD88. The kinases p38, JNK, ERK1/2, MAPK, and PI3K/Akt are important mediators of this cell activation process, activating the transcription components NF- κ B and/or IRF/ISRE to regulate selectively the expression of various inflammatory mediators, including IL-8 and RANTES.

Remarkably, a recent work claimed that *TLR3* signaling pathways do not appear to influence significantly the generation of effective host responses in murine models of infection to four different viruses (lymphocytic choriomeningitis virus, vesicular stomatitis virus, murine cytomegalovirus, and reovirus (40)). However, our study clearly indicates that human pulmonary mucosa does interact with viral products and elicits inflammatory and/or anti-viral immune responses through *TLR3* signaling. These contradictory results may be explained either by the virus itself and/or the species of the *TLR3* considered. In that regard, it is of note that different *TLR3* expression patterns have been reported in mice and humans, a phenomenon also observed for other TLR family members. Differences between species include the presence of TLR transcripts in different cell types and dissimilar regulation of transcription following cellular activation (33). Therefore, it cannot be excluded that differences in cell types and the patterns of basal or induced expression of *TLR3* influence the immune responses in the two species. Moreover, *TLR7* and *TLR8* were recently characterized as new sensors for viral nucleic acids with single-stranded RNA being their ligands, and innate antiviral responses following influenza A virus infection of mouse dendritic cells have been demonstrated to be *TLR7*-dependent (41–43). In fact, genetic complementation experiments with human *TLR7* and *TLR8* suggest that *TLR8*, not *TLR7*, is responsible for the recognition of single-stranded RNA in humans (41). Moreover, *TLR7* and *TLR8* are not expressed in human bron-

chial epithelial cells (44), and our experiments using a vector encoding a dominant-negative form of MyD88 rule out a role for those receptors in the activation of influenza A-infected human epithelial cells. Indeed, it was established that MyD88 is essential for the signaling downstream from *TLR7* and *TLR8* (41–43, 45).

Influenza A virus infections impose a considerable socioeconomic burden upon society, despite annual vaccination campaigns. Therefore, the present work on the detailed function of *TLR3* in human respiratory cells may help to elucidate the pathogenesis of influenza A infection and thereby contribute to the design of new molecules to prevent the excessive host inflammatory response produced by this virus.

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REFERENCES

- Ludwig, S., Planz, O., Pleschka, S., and Wolff, T. (2003) *Trends Mol. Med.* **9**, 46–52
- Julkunen, I., Melen, K., Nyqvist, M., Pirhonen, J., Sareneva, T., and Matikainen, S. (2000) *Vaccine* **19**, Suppl. 1, 32–37
- Kiso, M., Mitamura, K., Sakai-Tagawa, Y., Shiraishi, K., Kawakami, C., Kimura, K., Hayden, F. G., Sugaya, N., and Kawaoka, Y. (2004) *Lancet* **364**, 759–765
- Majde, J. A. (2000) *J. Interferon Cytokine Res.* **20**, 259–272
- Jacobs, B. L., and Langland, J. O. (1996) *Virology* **219**, 339–349
- Kimura-Takeuchi, M., Majde, J. A., Toth, L. A., and Krueger, J. M. (1992) *J. Infect. Dis.* **166**, 1266–1275
- Majde, J. A., Brown, R. K., Jones, M. W., Dieffenbach, C. W., Maitra, N., Krueger, J. M., Cady, A. B., Smitka, C. W., and Maassab, H. F. (1991) *Microb. Pathog.* **10**, 105–115
- Fang, J., Bredow, S., Taishi, P., Majde, J. A., and Krueger, J. M. (1999) *J. Med. Virol.* **57**, 198–203
- Alexopoulou, L., Holt, A. C., Medzhitov, R., and Flavell, R. A. (2001) *Nature* **413**, 732–738
- Yamamoto, M., Sato, S., Mori, K., Hoshino, K., Takeuchi, O., Takeda, K., and Akira, S. (2002) *J. Immunol.* **169**, 6668–6672
- Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T., and Seya, T. (2003) *Nat. Immun.* **4**, 161–167
- Muzio, M., Bosisio, D., Polentarutti, N., D'Amico, G., Stoppacciaro, A., Mancinelli, R., van't Veer, C., Penton-Rol, G., Ruco, L. P., Allavena, P., and Mantovani, A. (2000) *J. Immunol.* **164**, 5998–6004
- Cario, E., and Podolsky, D. K. (2000) *Infect. Immun.* **68**, 7010–7017
- Matsumoto, M., Funami, K., Oshiumi, H., and Seya, T. (2004) *Microbiol. Immunol.* **48**, 147–154
- Gern, J. E., French, D. A., Grindle, K. A., Brockman-Schneider, R. A., Konno, S., and Busse, W. W. (2003) *Am. J. Respir. Cell Mol. Biol.* **28**, 731–737
- Reynolds, H. Y. (2002) *Curr. Opin. Pulm. Med.* **8**, 154–165
- Message, S. D., and Johnston, S. L. (2004) *J. Leukocyte Biol.* **75**, 5–17
- Mergey, M., Lemnaouar, M., Veissiere, D., Perricaudet, M., Gruenert, D. C., Picard, J., Capeau, J., Brahimi-Horn, M. C., and Paul, A. (1995) *Am. J. Physiol.* **269**, L855–L864
- Guillot, L., Medjane, S., Le-Barillec, K., Balloy, V., Danel, C., Chignard, M., and Si-Tahar, M. (2004) *J. Biol. Chem.* **279**, 2712–2718
- Beck-Schimmer, B., Schimmer, R. C., and Pasch, T. (2004) *News Physiol. Sci.* **19**, 129–132
- Fakler, C. R., Wu, B., McMicken, H. W., Geske, R. S., and Welty, S. E. (2000) *Inflamm. Res.* **49**, 63–72
- Geiss, G. K., An, M. C., Bumgarner, R. E., Hammersmark, E., Cunningham, D., and Katze, M. G. (2001) *J. Virol.* **75**, 4321–4331
- Franke, T. F., Kaplan, D. R., Cantley, L. C., and Toker, A. (1997) *Science* **275**, 665–668
- McGettrick, A. F., and O'Neill, L. A. (2004) *Mol. Immunol.* **41**, 577–582
- Muzio, M., Ni, J., Feng, P., and Dixit, V. M. (1997) *Science* **278**, 1612–1615
- Zhang, F. X., Kirschning, C. J., Mancinelli, R., Xu, X. P., Jin, Y., Faure, E., Mantovani, A., Rothe, M., Muzio, M., and Arditi, M. (1999) *J. Biol. Chem.* **274**, 7611–7614
- Matsukura, S., Kokubu, F., Noda, H., Tokunaga, H., and Adachi, M. (1996) *J. Allergy Clin. Immunol.* **98**, 1080–1087
- Lamb, R., and Krug, R. (2001) in *Fields Virology*, (Knipe, D. M., and Howley, P. M., eds) Vol. 1, pp. 1487–1531, Lippincott-Raven Publishers, Philadelphia
- Matsumoto, M., Funami, K., Tanabe, M., Oshiumi, H., Shingai, M., Seto, Y., Yamamoto, A., and Seya, T. (2003) *J. Immunol.* **171**, 3154–3162
- Smith, A. E., and Helenius, A. (2004) *Science* **304**, 237–242
- Majde, J. A., Guha-Thakurta, N., Chen, Z., Bredow, S., and Krueger, J. M. (1998) *Arch. Virol.* **143**, 2371–2380
- Milhaud, P. G., Silhol, M., Salehzada, T., and Lebleu, B. (1987) *J. Gen. Virol.* **68**, 1125–1134
- Rehli, M. (2002) *Trends Immunol.* **23**, 375–378
- Heinz, S., Haehnel, V., Karaghiosoff, M., Schwarzfischer, L., Muller, M., Krause, S. W., and Rehli, M. (2003) *J. Biol. Chem.* **278**, 21502–21509

35. Tanabe, M., Kurita-Taniguchi, M., Takeuchi, K., Takeda, M., Ayata, M., Ogura, H., Matsumoto, M., and Seya, T. (2003) *Biochem. Biophys. Res. Commun.* **311**, 39–48
36. Sarkar, S. N., Peters, K. L., Elco, C. P., Sakamoto, S., Pal, S., and Sen, G. C. (2004) *Nat. Struct. Mol. Biol.* **11**, 1060–1067
37. Rassa, J. C., and Ross, S. R. (2003) *Microbes Infect.* **5**, 961–968
38. Ieki, K., Matsukura, S., Kokubu, F., Kimura, T., Kuga, H., Kawaguchi, M., Odaka, M., Suzuki, S., Watanabe, S., Takeuchi, H., Schleimer, R. P., and Adachi, M. (2004) *Clin. Exp. Allergy* **34**, 745–752
39. Hoffmann, E., Dittrich-Breiholz, O., Holtmann, H., and Kracht, M. (2002) *J. Leukocyte Biol.* **72**, 847–855
40. Edelmann, K. H., Richardson-Burns, S., Alexopoulou, L., Tyler, K. L., Flavell, R. A., and Oldstone, M. B. (2004) *Virology* **322**, 231–238
41. Boehme, K. W., and Compton, T. (2004) *J. Virol.* **78**, 7867–7873
42. Diebold, S. S., Kaisho, T., Hemmi, H., Akira, S., and Reis e Sousa, C. (2004) *Science* **303**, 1529–1531
43. Lund, J. M., Alexopoulou, L., Sato, A., Karow, M., Adams, N. C., Gale, N. W., Iwasaki, A., and Flavell, R. A. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 5598–5603
44. Homma, T., Kato, A., Hashimoto, N., Batchelor, J., Yoshikawa, M., Imai, S., Wakiguchi, H., Saito, H., and Matsumoto, K. (2004) *Am. J. Respir. Cell Mol. Biol.*
45. Hemmi, H., Kaisho, T., Takeuchi, O., Sato, S., Sanjo, H., Hoshino, K., Horiuchi, T., Tomizawa, H., Takeda, K., and Akira, S. (2002) *Nat. Immun.* **3**, 196–200