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Respiratory Syncytial Virus Up-regulates TLR4 and Sensitizes Airway Epithelial Cells to Endotoxin*[S]

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Airway epithelial cells are unresponsive to endotoxin (lipopolysaccharide (LPS)) exposure under normal conditions. This study demonstrates that respiratory syncytial virus (RSV) infection results in increased sensitivity to this environmental exposure. Infection with RSV results in increased expression of Toll-like receptor (TLR) 4 mRNA, protein, and increased TLR4 membrane localization. This permits significantly enhanced LPS binding to the epithelial monolayer that is blocked by disruption of the Golgi. The increased TLR4 results in an LPS-induced inflammatory response as demonstrated by increased mitogen-activated protein (MAP) kinase activity, IL-8 production, and tumor necrosis factor α production. RSV infection also allowed for tumor necrosis factor α production subsequent to TLR4 cross-linking with an immobilized antibody. These data suggest that RSV infection sensitizes airway epithelium to a subsequent environmental exposure (LPS) by altered expression and membrane localization of TLR4. The increased interaction between airway epithelial cells and LPS has the potential to profoundly alter airway inflammation.

The ability of cells to respond to microbial motifs depends on expression of a family of Type I transmembrane receptors, Toll-like receptors (TLRs)[1] (1–9). Recent evidence in intestinal epithelial cells suggests that cells that are in constant contact with pathogenic microbes and other environmental exposures express some of the TLRs at very low levels (10–12). More particularly, these studies suggest that in intestinal epithelial cells, TLR4 is in low abundance, localized in the Golgi, and not present on the plasma membrane. The airway epithelium is another region that is in constant contact with multiple pathogen-related antigens and other environmental agents. These exposures, with the exception of significant pathogen load or an immunosuppressed host, do not normally elicit an immunological response. A recent study by Tsutsumi-Ishii and Nagaoka (13) suggests that the intestinal epithelial cell lack of TLR4 is also true of airway epithelial cells. They found no surface expression of TLR4 and a lack of LPS responsiveness (13).

The relative tolerance to foreign antigens that is demonstrated by normal airway epithelial cells is altered in people with asthma and after RSV infection. Asthma and RSV infection are characterized by non-specific responses to both infectious agents and environmental exposures that include heightened inflammatory responses and hyper-responsive airways. The factors that predispose a particular individual to developing asthma are for the most part unclear. One early exposure that has been linked to the subsequent development of asthma is a severe infection with RSV during the first year of life (14–16). The correlation is especially clear if the RSV infection results in hospitalization for bronchiolitis and other respiratory complications (15, 17).

RSV is found ubiquitously in the environment. Serious illness, however, is for the most part found only in very young children and immunosuppressed adults. It is an enveloped, non-segmented, negative-strand RNA virus and is a member of the family Paramyxoviridae. Entry into the host cell (primarily the respiratory epithelium) is by cell surface fusion. Eleven genes and their encoded proteins have been identified for RSV. These include the F or fusion protein, the G attachment protein, nucleocapsid proteins, a polymerase subunit, two N or non-structural proteins, and M2-2, a RNA regulatory protein (18). The F protein has been demonstrated to signal via TLR4 (19–21), and in a study by Haeberle *et al.* (19), TLR4 signaling in macrophages was found to be an important component of the early RSV response. RSV has not only been shown to predispose children to the later development of asthma, but the presence of asthma sensitizes to both subsequent viral infections and environmental responses (including LPS). The increased responsiveness of the asthmatic and RSV-infected airways suggests the possibility of alterations in inflammation-related receptor expression as sequelae to an asthma and RSV infection phenotype.

One ubiquitous environmental exposure that clearly triggers acute attacks in children with established asthma and after RSV infection is LPS (15, 22). Various studies have shown that

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplementary movies showing confocal *z* sections of control and RSV-infected HAE cells exposed to LPS-ALEXA488.

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[1] The abbreviations used are: TLR, Toll-like receptor; RSV, respiratory syncytial virus; LPS, lipopolysaccharide; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; TNF, tumor necrosis factor; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; HPRT, human hypoxanthine phosphoribosyltransferase; FACS, fluorescence-activated cell sorter; RT, reverse transcriptase; HAE, human airway epithelial cells; hTBE, human tracheobronchial epithelial cells.

Supplemental Material can be found at: http://www.jbc.org/cgi/content/full/M308093200/DC1

it is a component of household dusts, products of carbon combustion, grain dust, and plant-derived allergens (22). The effect of endotoxin on established asthma is clear. There is controversy, however, regarding the role of endotoxin in the development of asthma. Some studies suggest that early exposure to endotoxin protects children from subsequent development of asthma (23–27), whereas other observations provide little evidence for a protective effect of early endotoxin exposure (28). A recent study by Eisenbarth *et al.* (29) may explain, in part, these conflicting observations. They showed that low dose LPS exposure can function as an adjuvant for allergen-induced Th2 responses in a murine model of asthma (22). This adjuvant effect of LPS requires the presence of TLR4 as it does not occur in C3H/Hej mice, which have a non-functional TLR4. They also showed that this low dose effect of LPS (facilitation of Th2 responses) differs from high dose exposure that facilitates protective Th1 responses. These observations suggest a complicated role for LPS in the development of asthma that may depend on a number of interacting factors, including dose, genetics, and other environmental exposures.

In this study, we evaluated first the LPS responsiveness of a lung epithelial cell line and then the effect of RSV infection on TLR4 expression and membrane localization. The findings of the study include: RSV-induced TLR4 expression and membrane localization, increased binding of LPS to lung epithelial cells via a Golgi transport-dependent mechanism, and the conversion of lung epithelial cells from LPS-non-responsive to LPS-responsive cells. The basic observations (increased TLR4 and MD-2, as well as increased LPS binding) were confirmed in a number of other epithelial lines, including newly isolated human bronchotracheal epithelial cells. As a composite, these studies suggest that RSV infection alters epithelial environmental responsiveness via an effect on expression and function of the LPS receptor, TLR4.

EXPERIMENTAL PROCEDURES

Materials—Chemicals, including brefeldin A, were obtained from Sigma and Calbiochem. Protease inhibitors were obtained from Roche Applied Science. LPS was obtained from LIST Biologicals, Campbell, CA. Antibodies to TLR4 were obtained from AbCam, Cambridge, UK (extracellular domain) and Santa Cruz Biotechnology, Inc., Santa Cruz, CA (intracellular domain and TLR4/MD-2 complex (HTA-125)). Antibodies for use in the IL-8 and TNFα ELISAs were from R&D Systems, Minneapolis, MN. Nitrocellulose and ECL Plus were obtained from Amersham Biosciences. Antibody to phosphorylated ERK was obtained from Cell Signaling, Beverly MA. Other antibodies and developing antibodies were from Santa Cruz Biotechnology. ALEXA-conjugated reagents were obtained from Molecular Probes, Eugene, OR.

Epithelial Cell Culture and Viral Infection—A549 lung epithelial cells were obtained from American Type Culture Collection, CCL-185, Manassas, VA. Cells were maintained in 100-mm tissue culture flasks (Corning, Corning, NY) in minimal essential medium with 10% fetal calf serum. For infection, cells at ~80% confluency were treated with 1×10^6 $TCID_{50}$ of human RSV, strain A-2. Viral stocks were obtained from Advanced Biotechnologies Inc., Columbia, MD. The initial stock (1×10^9 $TCID_{50}$) was aliquoted and kept frozen at −135 °C. A fresh aliquot was thawed for each experiment. The virus was never refrozen.

Other Epithelial Cells—All protocols were approved by the University of Iowa Institutional Review Board. Primary lung epithelial cells (HAE) were enzymatically isolated from bronchial epithelium of human donor lungs, as described previously (30). The cells were cultured on collagen-coated plastic dishes (type VI, human placental; catalog number C-7521, Sigma) in serum-free bronchial epithelial cell growth medium with supplements (catalog number CC3170, Clonetics/BioWhittaker) (31). HAE were used immediately after isolation (with no subculturing). Human tracheobronchial epithelial cells (hTBE) were obtained as described previously (32). Epithelial cells were isolated from tracheal and bronchial mucosa by enzymatic dissociation and cultured in Laboratory of Carcinogenesis-8e medium on plates coated with collagen/albumin for study up to passage 10. HeLa cells were obtained from ATCC (CCL-2) grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and 80 μg/ml gentamycin. All lines were infected with RSV in subconfluent conditions as described for A549 cells.

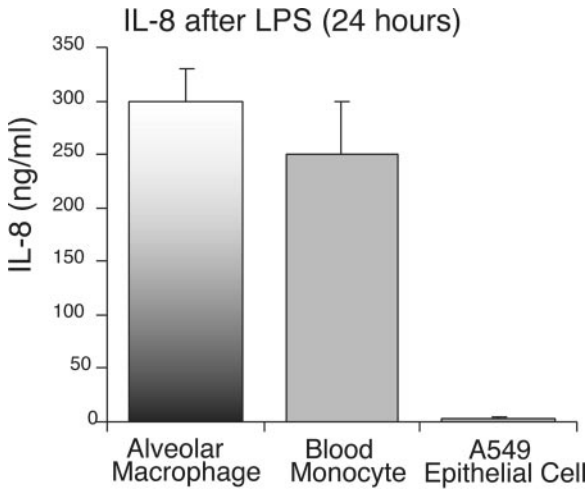

FIG. 1. **Airway epithelial cells do not produce IL-8 protein after LPS exposure.** Alveolar macrophages (10^6/ml), blood monocytes (10^6/ml), and A549 airway epithelial cells (confluent plate) were cultured with LPS (1 μg/ml) for 24 h. Supernatants were harvested, and IL-8 release was measured by ELISA. Each data point is the sum of three separate experiments.

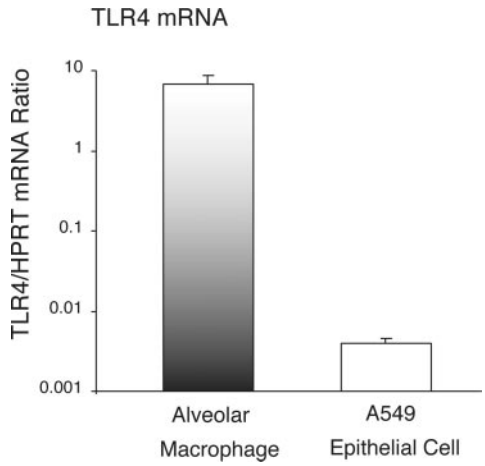

FIG. 2. **Airway epithelial cells produce very small amounts of TLR4 mRNA at baseline.** Alveolar macrophages shortly after isolation from the lungs of normal volunteers and A549 cells were lysed, and RNA was isolated. Equal amounts of mRNA (see "Experimental Procedures") were analyzed for TLR4 and HPRT mRNA by real-time RT-PCR. Comparison between cell types was done by determining the ratio of TLR4 mRNA to the housekeeping gene, *HPRT*, for both alveolar macrophages and A549 cells. The experiment was repeated three separate times.

Isolation of Human Alveolar Macrophages—Alveolar macrophages were obtained from bronchoalveolar lavage as described previously (33–36). Briefly, normal volunteers with a lifetime non-smoking history, no acute or chronic illness, and no current medications underwent bronchoalveolar lavage. Differential cell counts were determined using a Wright-Giemsa-stained cytocentrifuge preparation. All cell preparations had between 90 and 100% alveolar macrophages. This study was approved by the Committee for Investigations Involving Human Subjects at the University of Iowa.

Isolation of Human Blood Monocytes—180 ml of heparinized blood was obtained by venipuncture of the same volunteers who underwent bronchoscopy. Monocytes were then separated from the remaining mononuclear cells (T cells, NK cells, etc.) by removal of all non-monocytes with an antibody mixture, which cross-links the targeted cells to red blood cells before using a Ficoll-Hypaque gradient (Sigma) (RosetteSep mixture from StemCell Technologies. Monocyte purity was evaluated using Wright-Giemsa staining and was greater than 95%.

Isolation of Protein Extracts—Whole cell protein was obtained by lysing the cells on ice for 20 min in 500 μl of lysis buffer (0.05 M Tris, pH

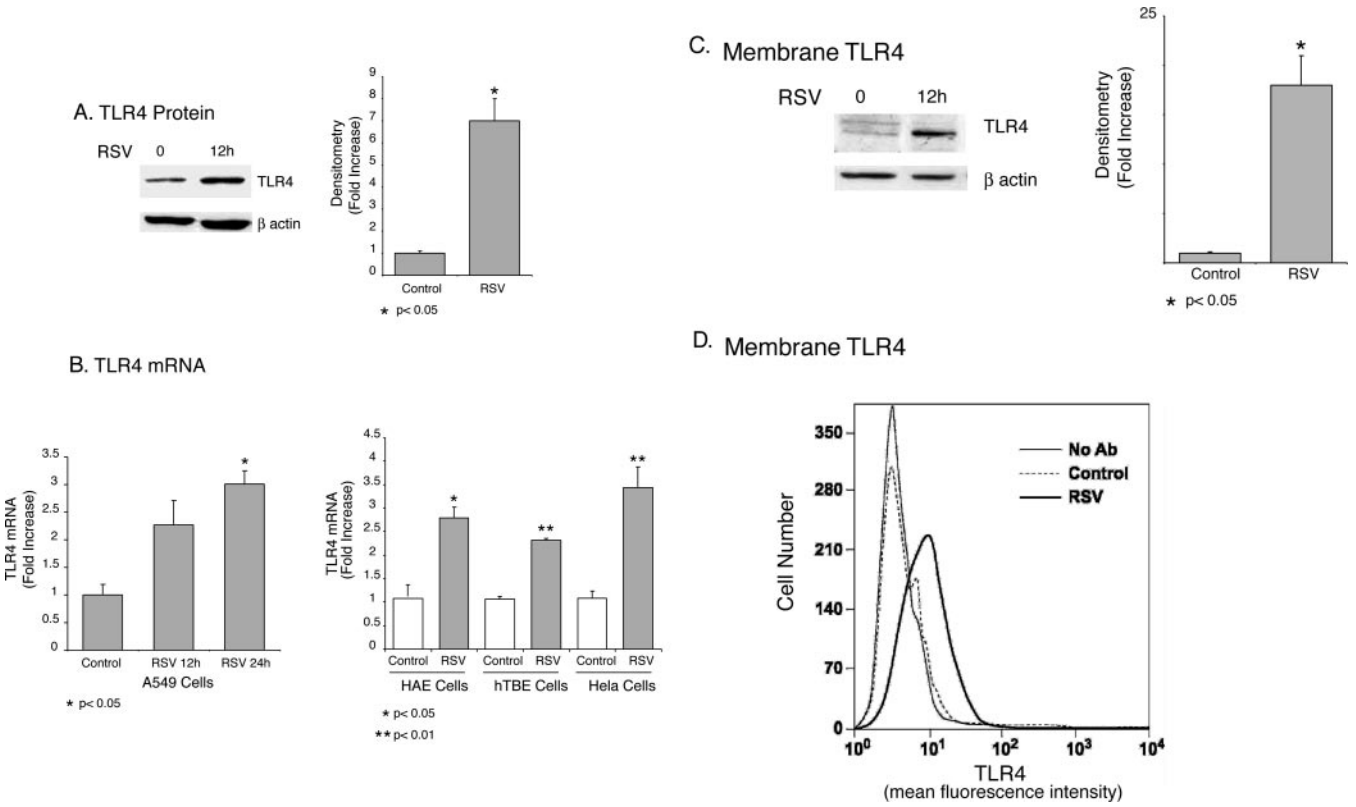

FIG. 3. **RSV infection induces TLR4 mRNA, protein, and membrane localization.** A549 cells were infected with 10^6 $TCID_{50}$ RSV for various times. *A*, Western analysis of whole cell proteins using an antibody to TLR4 (AbCam, AB1436). This blot is representative of four separate experiments. Densitometry is shown as -fold increase (experimental value/control value). *B*, mRNA levels, expressed as -fold increase (experimental value/control value) of four epithelial lines (A549 cells, HAE, hTBE, and HeLa cells). A549 cells were infected with 10^6 $TCID_{50}$ RSV for various times. The other lines were infected with an identical viral load and cultured for 16 h before harvesting mRNA for real-time RT-PCR. *C*, a Western blot of membrane proteins, isolated as described under "Experimental Procedures" (the densitometry is shown as described above and represents three separate experiments). For FACS analysis (*D*), cells were infected as described above, and at 24 h, cells were stained with TLR4 antibody followed by a phycoerythrin-conjugated developing antibody. This is representative of three experiments. *No Ab*, no antibody.

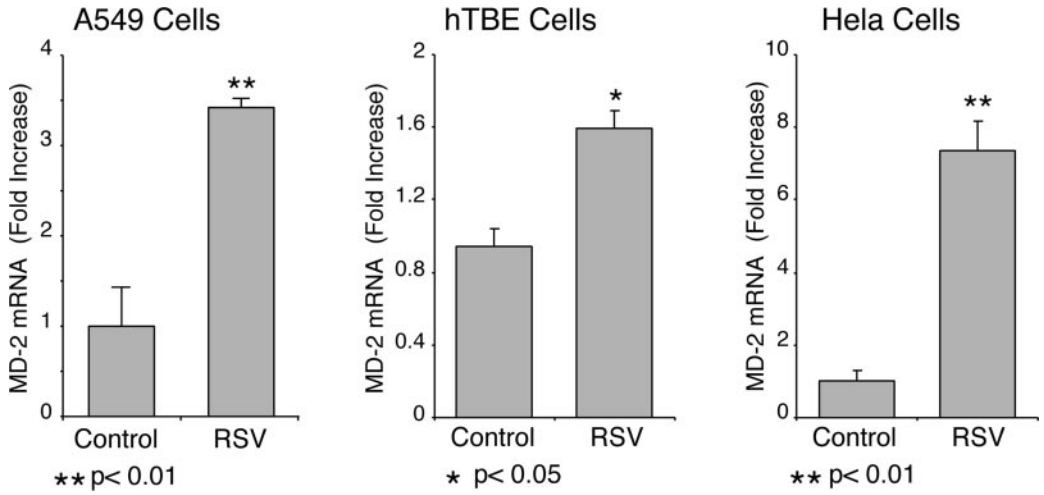

FIG. 4. **RSV infection increases MD-2 mRNA.** A549, hTBE, and HeLa cells were infected with 10^6 $TCID_{50}$ RSV for 24 (A549) or 16 (hTBE and HeLa) h. RNA was isolated, and MD-2 expression was quantified by real-time RT-PCR using primers and analysis procedures described under "Experimental Procedures." The data are a composite of three separate experiments.

7.4, 0.15 M NaCl, 1% Nonidet P-40, 1 protease minitab (Roche Applied Science)/10 ml and 1× phosphatase inhibitor mixture (catalog number 524625, Calbiochem). The lysates were then sonicated for 20 s and incubated at 4 °C for 30 min, and the insoluble fraction was removed by centrifugation at 15,000 × *g* for 10 min. Cytosol/membrane protein fractions were obtained by using an identical lysis buffer except for the lack of detergent. After sonication and an initial slow spin to remove the nuclei and debris, the lysates were spun at 55,000 rpm for 1 h to pellet the membranes. The supernatant was saved as cytosol, and the membrane pellet was resuspended in lysis buffer including detergent. After 30 min at 4 °C and another sonication, membrane debris was removed by a 14,000-rpm spin.

Western Analysis—Western analysis for the presence of particular proteins or for phosphorylated forms of proteins was performed as described previously (37–39). 50 μg of protein was mixed 1:1 with 2× sample buffer (20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.05% bromphenol blue, and 1.25 M Tris, pH 6.8) and loaded onto a 10% SDS-PAGE gel and run at 30 mA for 3 h. Cell proteins were transferred

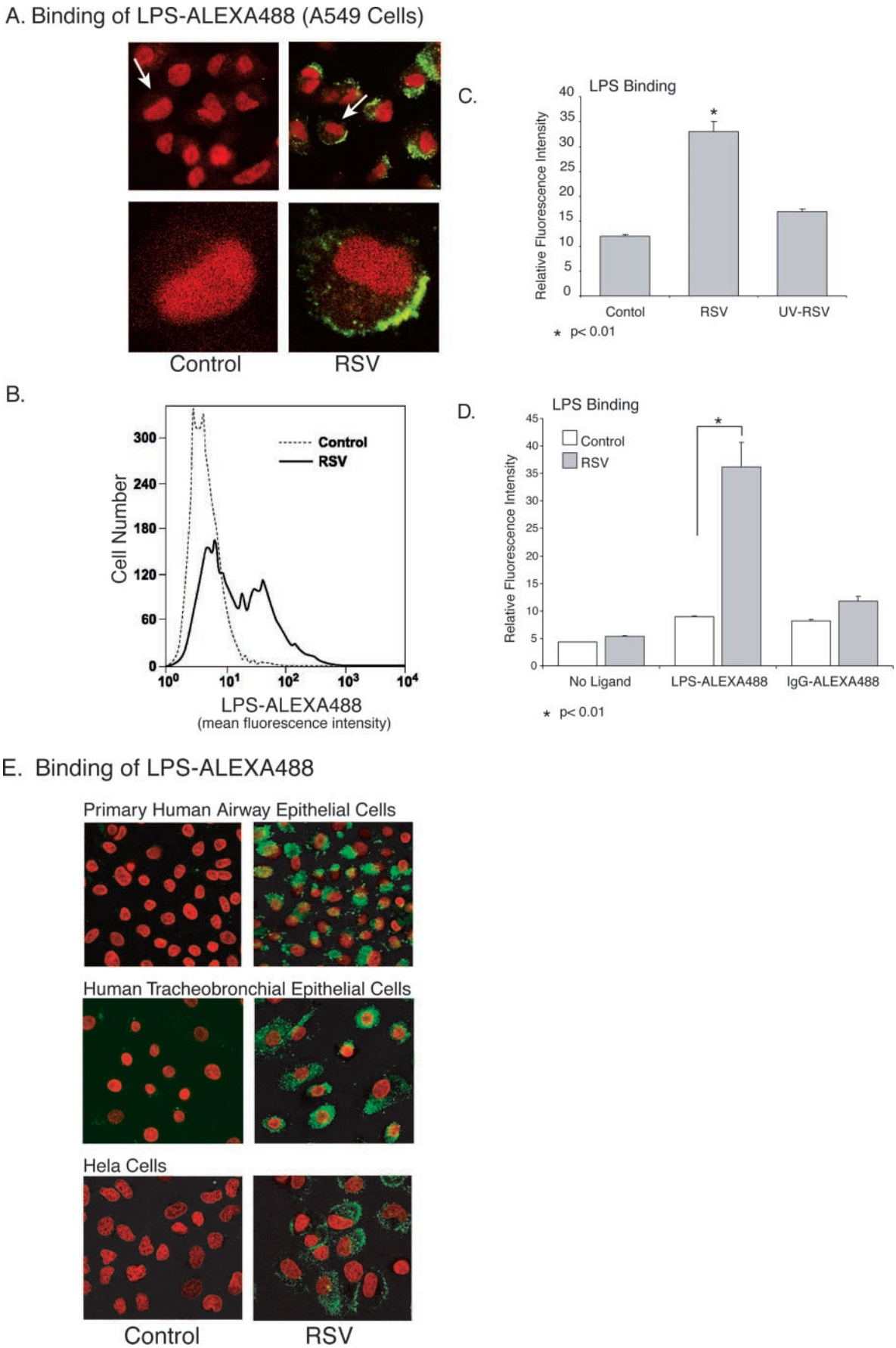

FIG. 5. **RSV infection increases LPS binding to airway epithelial cells.** A549 cells were infected with 10^6 $TCID_{50}$ RSV for 16 h on chambered culture slides. At the end of the incubation period, the cells were exposed to LPS conjugated to the fluorophore ALEXA488 (5 μg/ml) for 1 h at 37 °C. The chambers and media were then removed, the slides were cover-slipped using a fluorescent mounting medium, and LPS binding was analyzed by confocal microscopy (*A*). Bound and internalized LPS is shown by the *green* staining. This is representative of three separate

to nitrocellulose with a Bio-Rad semidry transfer system according to the manufacturer's instructions. Equal loading of the protein groups on the blots was evaluated using Ponceau S, a staining solution designed for staining proteins on nitrocellulose membranes. The nitrocellulose was then blocked with 5% milk in TTBS (Tris-buffered saline with 0.1% Tween 20) for 1 h, washed, and then incubated with the primary antibody at dilutions of 1:500–1:2000 overnight. The blots were washed four times with TTBS and incubated for 1 h with horseradish peroxidase-conjugated anti-IgG antibody (1:5000–1:20,000). Immunoreactive bands were developed using a chemiluminescent substrate, ECL Plus (Amersham Biosciences). An autoradiograph was obtained, with exposure times of 10 s to 2 min.

IL-8 and TNFα Release—A549 cells were infected with RSV with and without the addition of LPS (1 μg/ml) for 5–24 h. The culture supernatant was saved, and IL-8 or TNFα was measured by ELISA using the manufacturer's instructions (R&D Systems).

TLR4 Cross-linking—In these studies, A549 cells were infected with RSV for 16 h at 10^6 $TCID_{50}$. The cells were then subcultured, seeded onto 96-well plates (5×10^4/well) precoated with anti-TLR4 (HTA125) antibody or control antibody, and cultured for a further 24 h. Supernatants were harvested, and TNFα was measured. The antibody binding was performed as follows: sterile high protein binding polystyrene plates were coated overnight at 4 °C with 3.3 μg/ml anti-TLR4 (HTA125) or an isotype control (anti-mouse IgG, Class 2a) in PBS. The plates were washed four times with cold PBS before addition of the cells.

Isolation of RNA—Total RNA was isolated using the Absolutely RNA RT-PCR Miniprep kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. RNA was quantitated using RiboGreen kit (Molecular Probes). RNA samples were stored at −70 °C.

Real-time RT-PCR Detection of TLR4 and MD-2—1 μg of total RNA was reversed-transcribed to cDNA using RETROscript RT-PCR kit (Ambion, Austin, TX). The resulting cDNA was subjected to PCR in a Bio-Rad iCycler iQ system as follows: in a 0.2-ml PCR tube (Bio-Rad), 2 μl of cDNA was added to 48 μl of PCR reaction mixture containing 2 mM each dNTP (Invitrogen), 3.0 mM $MgCl_2$ (Invitrogen), 1:15,000 SYBR Green I DNA dye (Molecular Probes), 0.2 μM each of sense and antisense primers (IDT, Coralville, IA), and 2.5 units of Platimum TaqDNA (Invitrogen). Amplification and data collection were performed as described previously (35). Primers for human *TLR4*, *MD-2* and *HPRT* genes are as follows (5′ to 3′): TLR4, forward, ACA-ACC-TCC-CCT-TCT-CAA-CC; reverse, TGA-GAT-GTC-CAA-TGG-GGA-AG; MD-2, forward, TTC-CAC-CCT-GTT-TTC-TTC-CA; reverse, TCA-TCA-GAT-CCT-CGG-CAA-AT; HPRT, forward, TTG-GAA-AGG-GTG-TTT-ATT-CCT-C; reverse, TCC-CCT-GTT-GAC-TGG-TCA-TT.

Quantitation of mRNA—For each sample assayed, the threshold cycles (C_t) for reactions amplifying TLR4, MD-2, and HPRT were determined. The gene-specific C_t for each sample was corrected by subtracting the C_t for HPRT (ΔC_t). Untreated controls were chosen as the reference samples, and the ΔC_t values for all experimental samples were subtracted by the ΔC_t values for the control samples ($\Delta\Delta C_t$). Finally, sample mRNA abundance, relative to control mRNA abundance, was calculated by the formula $2^{-(\Delta\Delta Ct)}$. Validity of this approach was confirmed by using serial 10-fold dilutions of template containing experimental and *HPRT* genes. The amplification efficiencies for experimental and HPRT amplimers were found to be identical.

Confocal Microscopy—Subconfluent cultures of A549 cells in Lab-Tek® II chamber slides were infected with RSV. For LPS binding and uptake assays, the cells were incubated with ALEXA488-labeled LPS at the final concentration of 5 μg/ml for 1 h at 37 °C, washed several times with PBS, and fixed with 4% paraformaldehyde. Cell nuclei were counterstained with 7-aminoactinomycin D. Slide chambers were coverslipped using Vectashield® (Vector Laboratories, Burlingame, CA) and analyzed by using a Bio-Rad MRC-1024 confocal microscope at the University of Iowa Central Microscopy Research Facility. Ten random fields (512 × 512 pixels each) were collected for each experimental group. A representative picture is shown for each condition.

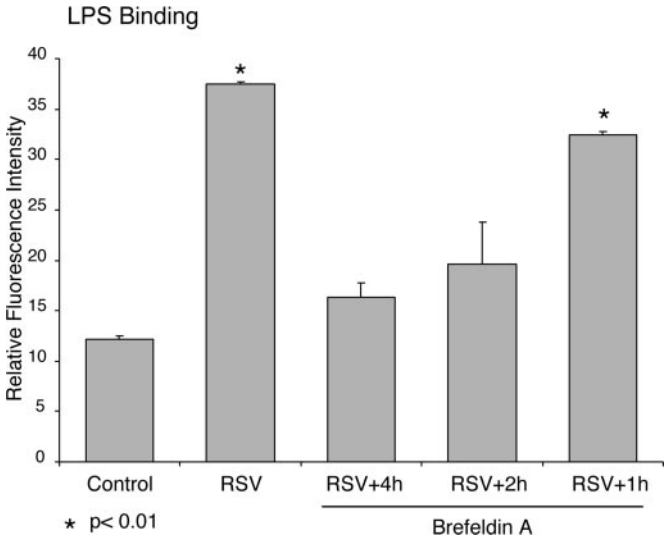

FIG. 6. **RSV-induced binding of LPS to airway epithelial cells requires intact Golgi function.** A549 cells were infected with 10^6 $TCID_{50}$ RSV for 16 h. During the final 4, 2, or 1 h, they were also exposed to brefeldin A (45). LPS binding (during a final 1-h incubation of 5 μg/ml LPS-ALEXA488) was evaluated by FACS analysis as described above. The data are a composite of three separate experiments.

Flow Cytometry (FACS) Analysis—A549 cells were grown to 80% confluence in 60-mm tissue culture dishes or 24-well tissue culture dishes, RSV was added, and the cells were incubated for an additional 12–24 h. For LPS binding, LPS-ALEXA488 (5 μg/ml) was incubated with the cells for the final hour. Cells were washed three times with PBS and then harvested by trypsinization. The cells were fixed in 1% paraformaldehyde for 15 min and stored in 70% ethanol at −20 °C until analysis. Analysis for LPS-ALEXA488 was done by flow cytometry, (FACScan™, BD Biosciences) using CELLQuest software (BD Biosciences). Staining for TLR4 was done by harvesting the cells by trypsinization following RSV infection and then staining first with a receptor-specific antibody and then with a secondary antibody conjugated to phycoerythrin or ALEXA488.

Statistical Analysis—Statistical analysis was performed on densitometry data, ELISA results, and real-time PCR data. Significance was determined by Student's *t* test.

RESULTS

Lung Epithelial Cells Are LPS-tolerant—We evaluated the ability of A549 cells to produce IL-8 after LPS exposure. As a comparison, human blood monocytes and human alveolar macrophages were treated with LPS. IL-8 is used in these studies as a marker of epithelial cell inflammation. It can be produced by monocytes, T cells, macrophages, neutrophils, endothelial cells, fibroblasts, and epithelial cells after exposure to cytokines, bacteria, bacterial products, and viruses (40). To evaluate LPS responsiveness, alveolar macrophages, blood monocytes, and A549 epithelial cells were all cultured with LPS (1 μg/ml) for 24 h. The supernatants were collected, and IL-8 production was analyzed. Fig. 1 demonstrates that both alveolar macrophages and blood monocytes produce large amounts (100-ng range) of IL-8. In contrast, A549 cells produced less than 10 ng/ml during the same time frame. This

experiments. In *B*, FACS analysis for the LPS fluorophore (ALEXA488) was performed after culture conditions identical to those outlined above. The chromatogram is representative of three separate experiments. All of the LPS-ALEX488 exposures occurred 16 h after RSV infection. Data are presented as the relative fluorescence intensity of three separate FACS analyses. *C* demonstrates that the increased LPS binding requires live RSV. RSV was treated as described previously or inactivated by UV irradiation (30 min at 9×10^5 μJ). Cells were then exposed to LPS-ALEXA488 (5 μg/ml) for 1 h, and binding was evaluated by FACS. In *D*, cells were infected as described for *A–C* and then treated for 1 h with 1) no fluorophore, 2) LPS-ALEXA488, or 3) IgG-ALEXA488. *E* demonstrates that RSV-induced LPS-ALEXA488 binding increases in three other epithelial cell cultures (HAE, hTBE, and HeLa (see "Experimental Procedures" for further descriptions of cells), including two human primary airway epithelial cell cultures. Cells were infected as described for A549 cells and exposed to LPS-ALEXA488 for 1 h, and confocal images were obtained. We have included supplementary data demonstrating confocal *z* sections of control and RSV-infected HAE cells exposed to LPS-ALEXA488 (supplemental material S1 (control) and S2 (RSV-exposed)).

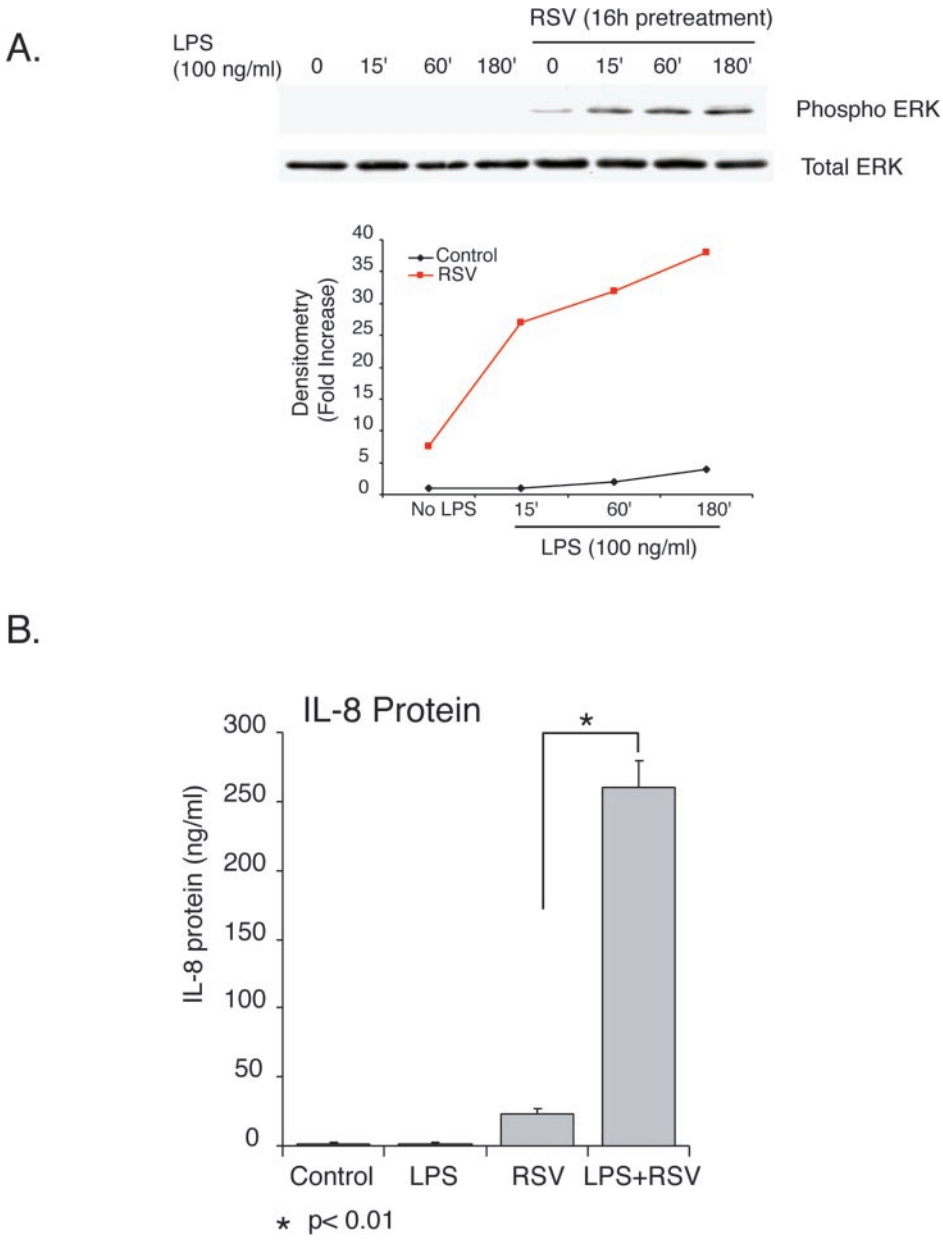

FIG. 7. **RSV infection induces LPS responsiveness in airway epithelial cells.** A549 cells were infected with 10^6 $TCID_{50}$ RSV for 16 h followed by a short term (ERK activity) or 24-h (protein) LPS exposure (1 μg/ml). *A*, a Western blot for activated ERK (phosphorylated (*Phospho*) threonine 202 and tyrosine 204) demonstrating ERK activation by LPS only after RSV infection. *B*, IL-8 protein release analyzed by ELISA. The data represent three separate experiments.

amount is not different from unstimulated cells.

Lung Epithelial Cells Produce Minimal Amounts of TLR4 mRNA—Because of the lack of LPS-induced IL-8 in A549 cells, we next compared the amounts of TLR4 mRNA in alveolar macrophages (LPS-sensitive cells) *versus* A549 cells. Unstimulated cells were isolated, and TLR4 mRNA levels were quantified using real-time RT-PCR. Fig. 2 shows the ratio of TLR4 mRNA to mRNA levels of the housekeeping gene, *HPRT*. HPRT levels in the two cell types are comparable. There is an approximate 1000-fold difference in TLR4 mRNA levels in alveolar macrophages as compared with A549 cells. This suggests the possibility that the lack of IL-8 produced following LPS exposure in A549 cells might be due to a lack of TLR4.

RSV Increases TLR4 Expression and Membrane Localization—To investigate the effect of RSV infection on TLR4 expression, A549 cells were infected with RSV for various times and harvested for RNA and whole cell protein. Fig. 3, *A* and *B*, demonstrate that RSV increases TLR4 mRNA and protein amounts (A549 cells). To determine that the RSV-induced increase in TLR4 was not specific to A549 cells, two primary cell lines (HAE and hTBE) and one unrelated epithelial cell line (HeLa) were infected with RSV for 16 h, and TLR4 mRNA analyzed by real-time RT-PCR. Fig. 3*B* demonstrates that, in newly isolated airway epithelial cells (HAE), in a non-transformed primary lung epithelial line (hTBE), and in an adenocarcinoma epithelial line (HeLa), RSV increases expression of TLR4 mRNA. To evaluate the effect of RSV on surface expression of TLR4, A549 cells were cultured in the presence of RSV for 16 h. Cytosol/membrane protein fractions were obtained, and Western analysis was performed for TLR4. RSV infection caused a significant increase in the amount of TLR4 present in the membrane fraction of lung epithelial cells (Fig. 3*C*). Surface expression of TLR4 was also evaluated using fluorescent antibodies and a fluorescent-activated cell sorter (FACS) in non-permeabilized cells and demonstrated the same increase in surface TLR4 (Fig. 3*D*).

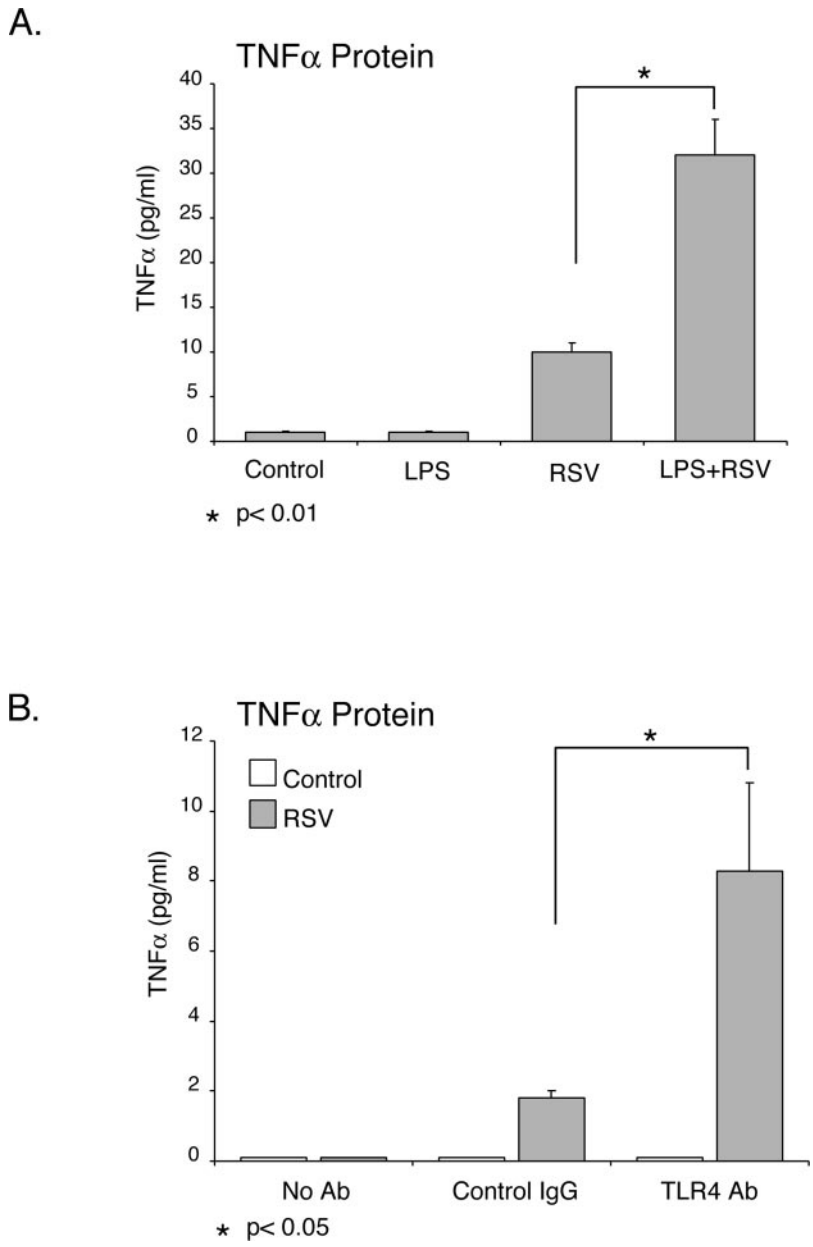

FIG. 8. **LPS and TLR4 cross-linking induce TNFα after RSV infection.** In *A*, A549 cells were infected with 10^6 $TCID_{50}$ RSV for 16 h followed by a 24-h LPS exposure (1 μg/ml). TNFα production was measured in the supernatants by ELISA. The data are a composite of three separate experiments. In *B*, A549 cells were infected as described above, except that after the 16-h infection time, they were not exposed to LPS. Instead, the cells were subcultured and placed on a 96-well plate coated with either anti-TLR4 (eBiosciences, HTA-125) or an isotype control IgG. (Plates were coated by an overnight 4 °C incubation at 3.3 μg/ml followed by vigorous washing with PBS.) Cells were cultured on the antibody-coated plates for 24 h, and supernatants were harvested for TNFα measurements. The data are a composite of three separate experiments.

In considering reasons for the altered localization, the link between MD-2 and TLR4 membrane colocalization is a good candidate. MD-2 is a small, secreted glycoprotein that binds to the extracellular portion of TLR4. A recent study by Nagai *et al.* (41) used an MD-2 knockout animal to link MD-2 to membrane localization of TLR4. They showed that without MD-2, TLR4 remained in the Golgi. A549 cells were infected with RSV for 24 h, and then mRNA was isolated and MD-2 levels were determined by real-time RT-PCR. As with TLR4, we also analyzed mRNA from other epithelial lines (a non-transformed primary human lung epithelial line (hTBE) and HeLa cells). In these two cell lines, we found that RSV infection increased MD-2 mRNA levels. Fig. 4 demonstrates that RSV infection causes a significant increase in MD-2 mRNA. As a composite, these data suggest that RSV infection increases both the TLR4 amount and membrane localization in both transformed and primary lung epithelial cell lines

RSV Increases LPS/Epithelial Cell Interactions—As shown in Fig. 1, A549 cells normally respond very poorly to LPS exposure. The effect of RSV-induced changes in TLR4 amounts and membrane localization on LPS epithelial interactions was investigated using LPS bound to the fluorophore, ALEXA488. In these experiments, A549 cells were cultured with RSV for varying lengths of time and then exposed to LPS-ALEXA488 for 1 h. The cultures were then washed, and LPS binding was evaluated by FACS and confocal microscopy. Fig. 5*A* demonstrates that at baseline, there is very little interaction between LPS and lung epithelial cells. This is consistent with the low physiological response to LPS. In contrast, after RSV infection, LPS binds avidly to the epithelial monolayer. To quantitate the confocal observation, the same cultures were evaluated by FACS analysis. Fig. 5*B* demonstrates that RSV infection causes a significant increase in LPS binding to the epithelial monolayer. Fig. 5, *C* and *D*, demonstrate FACS quantitation of a number of experimental controls. The controls demonstrate first that LPS binding to the RSV-infected monolayer requires live virus as a UV-inactivated virus (30 min at 9×10^5 μJ) had no effect (Fig. 5*C*). Secondly, the increased LPS binding was not due to a non-specific effect on the binding of ALEXA488 to the monolayer (Fig. 5*D*). To determine that the RSV-induced increase in LPS binding was not specific to A549 cells, two primary cell lines (HAE and hTBE) and one unrelated epithelial

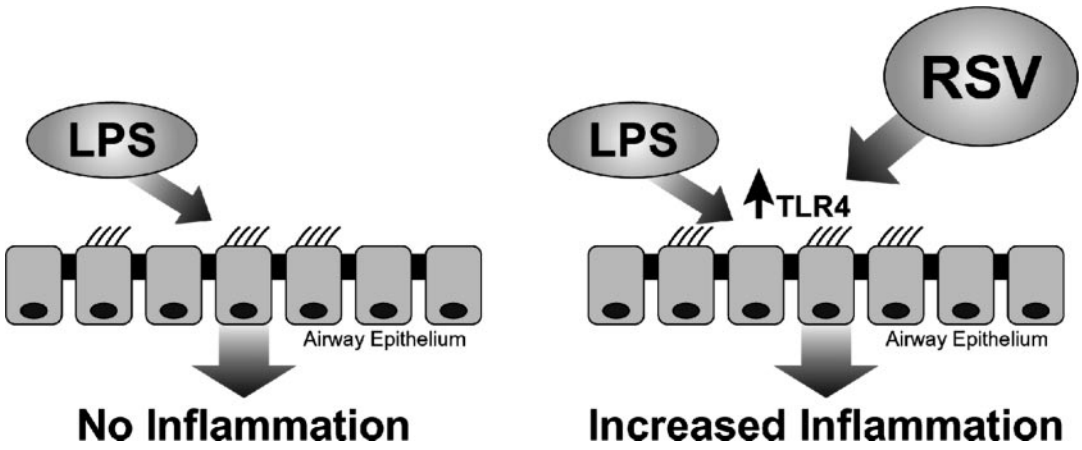

FIG. 9. **Infection of airway epithelial cells with RSV increases TLR4 amounts and membrane expression.** This results in conversion of airway epithelial cells from an LPS-tolerant phenotype to an LPS-responsive phenotype.

cell line (HeLa) were infected with RSV for 16 h, and LPS-ALEXA488 binding was analyzed by confocal microscopy. Fig. 5*E* demonstrates that in newly isolated airway epithelial cells (HAE), in a non-transformed primary lung epithelial line (hTBE), and in an adenocarcinoma epithelial line (HeLa), infection with RSV increases binding of LPS to an epithelial monolayer (also see supplemental data of *z* sectioning of control and RSV-infected HAE cells exposed to LPS-ALEXA488, Fig. 5E, supplemental material S1 and S2). Images were also obtained of primary HAE RSV-infected cells exposed to IgG-conjugated ALEXA488 as a control. There was no increased fluorescence with the control fluorophore (IgG-ALEXA488) after RSV infection (data not shown). These data demonstrate that RSV infection increases the binding of LPS to a variety of epithelial cell lines (primary HAE and hTBE, A549, and HeLa cells).

RSV-induced LPS Binding Requires Intact Golgi Function—Without MD-2, TLR4 remains localized to the Golgi (41). To evaluate the effect of Golgi function on epithelial cell/LPS interactions, we utilized the Golgi blocker, brefeldin A. The fungal metabolite, brefeldin A, inhibits the transport of proteins from the Golgi by inhibiting the activity of BIG1 and BIG2, the guanine-nucleotide exchange proteins for ADP-ribosylation factors. This results in a specific and reversible block of translocation of proteins from the endoplasmic reticulum to the Golgi apparatus without affecting endocytosis or lysosome function (42). To analyze the role of Golgi transport in RSV-induced LPS binding, Golgi transport in RSV-infected cells was blocked 4, 2, and 1 h before the addition of LPS conjugated to ALEXA488. Blocking protein transport from the Golgi after RSV infection inhibited LPS binding (Fig. 6). This is consistent with a requirement for a brefeldin A-sensitive transport mechanism in the increased binding of LPS to epithelial cells following RSV infection. As a composite, these studies demonstrate that RSV infection of airway epithelial cells results in a significant increase in LPS interactions with epithelial cells.

RSV Infection Increases LPS-induced Signaling, IL-8 Production, and TNFα Production—We next determined whether the increase in LPS binding resulted in an increased cellular response to LPS. LPS exposure in macrophages induces inflammatory mediator production through the activation of a number of signaling cascades. One pathway linked to the production of TNFα and IL-8 is the ERK MAP kinase pathway. We have shown previously that ERK activity is required for RSV-induced IL-8 production in airway epithelial cells (43) and that ERK activity is required for LPS-induced TNFα (44). To examine RSV-induced alterations in LPS responsiveness, previously RSV-infected cells were treated with LPS, and ERK MAP kinase signaling was analyzed (Fig. 7*A*). The data show that RSV infection allows for LPS-induced ERK activity, consistent with the increased membrane expression of TLR4 shown in Fig. 3. To evaluate the effect of RSV infection on LPS-induced IL-8 responses, lung epithelial cells were infected with RSV for 16 h and then treated with LPS for 24 h (protein). Fig. 7*B* demonstrates that following RSV infection, LPS induces significant amounts of IL-8 protein, far greater than the IL-8 produced by RSV alone. To evaluate the effect of RSV infection on LPS-induced TNFα responses and to link that observation to the RSV-induced TLR4, lung epithelial cells were infected with RSV for 16 h and then either treated with LPS or subcultured onto a 96-well plate containing immobilized TLR4 antibody to the extracellular domain of TLR4 or an isotype control antibody (45). Fig. 8*A* demonstrates that following RSV infection, LPS induces significant amounts of TNFα protein. Fig. 8*B* demonstrates that cross-linking the RSV-induced TLR4 with immobilized antibody resulted in TNFα production. As a composite, these data clearly demonstrate that infection of airway epithelial cells with RSV increases LPS responsiveness by increasing membrane expression of TLR4.

DISCUSSION

Airway epithelial cells are constantly exposed to inhaled LPS from a variety of environmental sources. Under normal conditions, the epithelial cell response to this exposure is severely dampened. Clinically, this tolerance appears altered after RSV infection and in asthma, conditions that are characterized by airway hyper-responsiveness. RSV infection has been both clinically and experimentally linked to the development of asthma. It was our hypothesis that RSV infection (consistent with the clinical data) would increase receptors on airway epithelial cells that bind inhaled environmental agents. We used inhaled LPS as a paradigm for these studies. We found that RSV infection increased TLR4 expression and membrane localization on airway epithelial cells (A549 cells, primary human airway epithelial cells (HAE and hTBE) and HeLa cells). The increased membrane TLR4 led to increased binding of LPS to airway epithelium that was blocked by Golgi disruption. The increased LPS binding led to LPS-induced MAP kinase activation and inflammatory cytokine production. Cross-linking the RSV-induced TLR4 mimicked the production of TNFα by LPS after RSV infection. These studies suggest that RSV infection can alter lung epithelial responses (both primary tracheobronchial and transformed cell lines) to some inhaled environmental agents by up-regulating TLR4 (Fig. 9).

A recent study by Suzuki *et al.* (46) found that, in intestinal epithelial cells, interferon γ can increase LPS responsiveness in

an LPS-hyporesponsive cell line. In two cell lines (HT-29 and Colo205), the authors found that TLR4 was present in the cytoplasm but required interferon γ-induced increases in MD-2 and LPS uptake for a response. In other intestinal epithelial lines (HCT-116 and Caco-2), they found that interferon γ did not up-regulate LPS responses. In a fifth epithelial cell line, they found constitutive LPS responsiveness. The data reinforce our feeling that LPS hyporesponsiveness is specific to some epithelial cells, especially those in frequent contact with environmental stimuli. The study by Suzuki *et al.* (46) showing epithelial cell hyporesponsiveness and up-regulation of LPS responses after interferon γ is not inconsistent with our observation that viral infections can up-regulate LPS responsiveness in pulmonary epithelial cells. Both studies (ours and those of Suziki *et al.*) suggest that epithelial cells that are in frequent contact with environmental stimuli (intestine and lung) are hyporesponsive to LPS at baseline and need a priming event to induce LPS responsiveness.

A recent study by Tsutsumi-Ishii and Nagaoka (13) found a complete lack of TLR4 expression in A549 cells. This is consistent with the lack of LPS responsiveness we find. Our data varied slightly from theirs in that we found low levels of TLR4 expression, but both observations suggest a state of LPS hyporesponsiveness in normal airway epithelial cells. This effect is likely accentuated by an apparent lack of membrane localization. The study by Abreu *et al.* (11) found that in intestinal epithelial cells, increased expression of MD-2 was required to bring TLR4 to the membrane and confer LPS responses. RSV, in our study, increased the expression of and membrane localization of TLR4. The increased membrane localization of TLR4 likely was mediated by RSV induction of MD-2, which also functions as a co-receptor with TLR4 for LPS. Consistent with the membrane localization of TLR4, we observed increased binding and cellular responses to LPS after RSV infection. These observations suggest that the hyper-responsiveness of airway to LPS and other environmental exposures after RSV infection may be due to increased expression of receptors that bind these inhaled agents.

A study by Latz *et al.* (45) used a TLR4-YFP construct to study TLR4 trafficking and signaling localization. They found that TLR4 shuttled between the Golgi and the plasma membrane. Of interest, they found that uptake of LPS in the absence of TLR4 was not sufficient to generate an NFκB response. Especially relevant to our finding that RSV increases both membrane expression of TLR4 and LPS responses, they found that although TLR4 rapidly shuttles between the Golgi and the plasma membrane, it is from the plasma membrane that signaling to NFκB is initiated. It is interesting to speculate that in airway epithelial cells, the lack of MD-2 prevents TLR4 remaining on the membrane long enough to initiate an inflammatory response.

There is no previous description of the induction of TLR4 by RSV. There are, however, three studies showing that RSV can signal via TLR4. The initial study by Kurt-Jones *et al.* (21) showed that the RSV fusion protein signaled via TLR4. This study was done in human mononuclear studies and did not examine epithelial cells. A study in TLR4-deficient mouse models demonstrated a role for TLR4 in viral clearance (20). Finally, a study by Haeberle *et al.* (19) found that lung induction of NFκB by RSV depended on TLR4. It is interesting to speculate that RSV up-regulation of TLR4 in epithelial cells might lead not only to increased LPS responsiveness but also to increased RSV responsiveness in the airway. Our study, however, focuses on the role of RSV in increasing LPS/TLR4 epithelial cell responses and not on the possible role of TLR4 in RSV signaling.

LPS is the most bioactive component of the cell membrane of Gram-negative bacteria. It is also a ubiquitous contaminant of environmental exposures such as grain dust, house dust, and air pollution particles (47–51). For this reason, the reactivity of airway epithelium, especially as it relates to LPS, can have a profound effect on lung inflammatory responses. The data presented here show that RSV infection of lung epithelial cells (both primary and transformed cell lines) results in increased expression and membrane localization of TLR4. This leads to increased binding of LPS and the induction of an LPS-dependent inflammatory response. This change has the potential to profoundly alter airway inflammation.

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