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Role of Primary Human Alveolar Epithelial Cells in Host Defense against *Francisella tularensis* Infection▽

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Francisella tularensis*, an intracellular pathogen, is highly virulent when inhaled. Alveolar epithelial type I (ATI) and type II (ATII) cells line the majority of the alveolar surface and respond to inhaled pathogenic bacteria via cytokine secretion. We hypothesized that these cells contribute to the lung innate immune response to *F. tularensis*. Results demonstrated that the live vaccine strain (LVS) contacted ATI and ATII cells by 2 h following intranasal inoculation of mice. In culture, primary human ATI or ATII cells, grown on transwell filters, were stimulated on the apical (AP) surface with virulent *F. tularensis* Schu 4 or LVS. Basolateral (BL) conditioned medium (CM), collected 6 and 24 h later, was added to the BL surfaces of transwell cultures of primary human pulmonary microvasculature endothelial cells (HPMEC) prior to the addition of polymorphonuclear leukocytes (PMNs) or dendritic cells (DCs) to the AP surface. HPMEC responded to S4- or LVS-stimulated ATII, but not ATI, CM as evidenced by PMN and DC migration. Analysis of the AP and BL ATII CM revealed that both *F. tularensis* strains induced various levels of a variety of cytokines via NF-κB activation. ATII cells pretreated with an NF-κB inhibitor prior to *F. tularensis* stimulation substantially decreased interleukin-8 secretion, which did not occur through Toll-like receptor 2, 2/6, 4, or 5 stimulation. These data indicate a crucial role for ATII cells in the innate immune response to *F. tularensis*.

The respiratory epithelium is a dynamic interface between the outside environment and the interior of the host (20, 56). Protection against respiratory infection is provided by the physical barrier formed by alveolar epithelial cells (AECs), which also are vital for maintaining lung homeostasis. AECs are abundant in number and line the pulmonary airways and alveoli. Alveolar type I (ATI) cells are the epithelial component of the thin air-blood barrier and comprise >95% of the alveolar surface area (57). Alveolar type II (ATII) cells cover approximately 4% of the mammalian alveolar surface but constitute 15% of all lung cells (8, 9, 20, 35, 57). ATII cells, and to a lesser extent ATI cells, have been shown to be important effector cells in many inflammatory responses and play a vital role in modulating lung innate immune responses via the production of cytokines such as interleukin-8 (IL-8), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), growth-related oncogene-α (GRO-α), regulated on activation normal T cell-expressed and -secreted protein (RANTES), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (6, 13, 36, 42, 53, 61). Recent studies have shown that AECs are capable of responding to infective agents via Toll-like receptors (TLRs) expressed on their cell surface (5, 33). TLRs are an important family of innate immune receptors that recognize a wide variety of conserved microbial products, including lipopolysaccharide (LPS), lipoprotein, and flagellin, which are collectively known as pathogen-associated molecular patterns (5, 33). Specific TLR recognition of microbial ligands often triggers the NF-κB signal transduction pathway (33), resulting in the secretion of cytokines (1).

In vivo, the physiological positioning of AECs relative to human pulmonary microvasculature endothelial cells (HPMEC) is such that the cytokines released from the basolateral (BL) surface of apically (AP) stimulated AECs signal the BL surface of HPMEC in order to promote the influx of immune cells from the periphery. MCP-1, produced by primary tumor necrosis factor alpha-stimulated ATII cells, induced transmigration of monocytes and lymphocytes across an endothelial cell monolayer (13, 36). Similarly, chemotactic mediators differentially expressed by ATII cells promote the influx of various immune cells, including alveolar macrophages (37, 38). In response to inhaled pathogens, immune cell migration to the lungs through HPMEC has been shown to be specific to the particular infecting organism (16). Recruitment of specific immune cells, including polymorphonuclear leukocytes (PMNs) and dendritic cells (DCs), is crucial for the development of an effective innate immune response to pulmonary injury caused by inhaled pathogens (16, 34).

Francisella tularensis is a small, gram-negative, facultative intracellular organism and is one of the deadliest inhaled pathogens known to humans (19). There are two major subspecies of *F. tularensis*. Type A strains induce aggressive pathologies, and if untreated, the resulting pulmonary tularemia can cause mortality in 30 to 60% of cases (11, 48). Schu 4 is one such virulent *F. tularensis* type A strain, originally isolated from a human case of tularemia (11). Infection with virulent *F.*

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tularensis can occur via multiple routes; however, as stated above, inhalation is the most life-threatening because as few as 10 organisms cause disease and can be detrimental to the host (14, 19, 32, 45, 55). Conversely, *F. tularensis* type B strains cause milder disease in humans than do type A strains. To date, the only known vaccine against tularemia is the live vaccine strain (LVS), which is derived from subspecies type B (1, 14, 18, 45, 48, 55). Because the basis for LVS attenuation is unknown, there is a risk for reversion to virulence (44), which has prevented its licensure for public use in the United States.

The hardy nature and high infectivity of this organism prompted the successful weaponization of *F. tularensis* type A strains by the governments of the United States, Japan, and Russia (22, 60) and has led to its categorization as a category A select agent. The select agent classification renewed interest in this organism to better understand the mechanism by which *F. tularensis* causes disease following inhalation and to develop better therapeutic strategies and vaccines. Analysis of the *F. tularensis* genome and comparison to other bacterial genomes may aid in identification of virulence determinants (40, 43). Despite this effort, virtually nothing is known about the contribution of the respiratory epithelium to the immune response against a pulmonary *F. tularensis* infection. Evidence obtained from other lung pathogens and inhaled particles, however, supports the hypothesis that lung epithelial cells play an important role in regulating the innate immune response (6, 8, 9, 13, 35–37, 42, 53, 59, 61).

It has become clear that, in an intact biological system, cellular communication between epithelial cells and professional immune cells is imperative for the stimulation of effective innate and acquired immune responses. Because respiratory epithelial cells are among the first types of cells to interact with inhaled foreign particles, understanding the contributions that pulmonary epithelial cells make during *F. tularensis* invasion of the airway is crucial to fully elucidating the pathogenic processes and may lead to novel therapeutic strategies against tularemia. Therefore, we tested the hypothesis that *F. tularensis* interacts with primary ATI and/or ATII cells, possibly through TLR stimulation, to induce the BL secretion of chemokines leading to leukocyte transendothelial migration into the lung. Our results showed that *F. tularensis*-stimulated ATII, but not ATI, cells secreted various cytokines that induced immune cell migration through the pulmonary endothelium. Further, the observed ATII cytokine secretion was dependent upon NF-κB activation but not stimulation of TLR2, TLR2/6, TLR4, or TLR5.

MATERIALS AND METHODS

Animal care and intranasal model. Female BALB/c mice (Jackson Laboratory, Bar Harbor, ME) weighing between 20 to 23 g were used in these studies. They were provided with food (Rodent Laboratory Chow 5001; Purina Mills, Inc., St. Louis, MO) and water ad libitum. All animals were acclimated for 1 week. The animals were housed in a facility approved by the American Association for the Accreditation of Laboratory Animal Care, and the experimental protocols were approved by the University of Texas Medical Branch (UTMB) Animal Care and Use Committee. In conducting the research described in this report, the investigators adhered to the Guide for the Care and Use of Laboratory Animals as set forth by the UTMB Committee on the Care and Use of Laboratory Animals.

Anesthetized (pentobarbital, 50 mg/kg) BALB/c mice ($n = 3$ mice/group) were suspended vertically by the upper incisors using commercial fishing line looped behind the upper incisors and connected to a support platform. Ten microliters of phosphate-buffered saline (PBS) containing 1×10^3 CFU of *F. tularensis* LVS was placed at the anterior of each naris, and the animals were allowed to inhale the bacteria. This high inoculation level was selected to enhance the likelihood of visualizing *F. tularensis* contacting AECs by transmission electron microscopy (TEM). Following intranasal administration, the animals were given 10 μl of sterile PBS/naris to ensure that the bacteria were washed into the lungs. The animals then were placed back in their cages and allowed to recover before being sacrificed by pentobarbital overdose at the indicated time points. The harvested lungs were fixed and sectioned for electron microscopy analysis.

Bacterial strains. Schu 4 was obtained from U.S. Army DPG, Life Sciences Division, Dugway, UT, and LVS (ATCC 29684) was obtained from Karen Elkins (CBER/FDA, Rockville, MD). The bacteria were stored at −80°C until use. An aliquot of each frozen culture was plated on cysteine heart agar (Difco Laboratories, Detroit, MI) plates for 2 days at 37°C with 5% CO_2. Bacterial colonies were expanded in 10 ml of modified Mueller-Hinton II broth supplemented with IsoVitaleX (Becton Dickinson, Cockeysville, MD) for 10 h (mid-logarithmic phase of growth) with shaking at 37°C. The bacteria were pelleted by centrifugation and then resuspended in 10 ml of sterile PBS. An aliquot of the bacteria was used to make a 1:100 dilution in 10% formalin, and then the bacteria were counted using a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA). The bacterial concentration then was adjusted to 1×10^9 CFU/ml using a Klett photoelectric densitometer (Scienceware, Pequannock, NJ), and the bacteria were diluted in sterile PBS to the desired concentrations. Bacterial 10-fold dilutions were plated on cysteine heart agar plates to confirm the experimental dosage.

TEM. Pieces of lung tissue (about 1 mm^3) were fixed in a mixture of 2.5% formaldehyde and 0.1% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) containing 0.03% trinitrophenol and 0.03% $CaCl_2$, washed in 0.1 M cacodylate buffer (pH 7.2), and postfixed in 1% OsO_4 in the same buffer. Each sample was stained en bloc with 1% uranyl acetate in 0.1 M maleate buffer, dehydrated in ethanol, and embedded in Poly/Bed 812 epoxy resin (Polysciences, Warrington, PA). Ultrathin sections, cut on an Ultracut S ultramicrotome (Reichert-Leica), were stained with 2% aqueous uranyl acetate and lead citrate and examined with a Philips 201 or CM 100 electron microscope at 60 kV.

Primary cell culture. Human primary AECs were purchased from ScienCell (San Diego, CA) as a cell culture containing >96% ATII cells obtained from fetal lung tissue. ATII cells have a distinct morphology, appearing as large, somewhat square cells with characteristic lamellar bodies that contain surfactant lipids (30). ATII cells were grown and maintained in bronchial epithelial medium (Clonetics Cell Systems-Cambrex) supplemented with 5% charcoal-stripped fetal bovine serum (FBS) (HyClone) and 10 ng/ml of keratinocyte growth factor (PeproTech). In the lung, ATII cells proliferate to provide daughter cells, some of which remain ATII cells and some of which differentiate to become ATI cells (30). Therefore, to drive ATII cells to differentiate into ATI cells, the frozen stock of ATII cells (as described above) was cultured in AEpiC medium (ScienCell) supplemented with epithelial growth serum (ScienCell), 20% FBS, and penicillin-streptomycin (ScienCell). ATII cells differentiate into ATI cells in 5 to 8 days. It is easy to discern ATI from ATII cells, as ATI cells are large flat cells (30, 46).

Primary HPMEC (Clonetics Cell Systems-Cambrex, Walkerville, Inc.) were grown and maintained in microvascular endothelial cell medium 2 (Clonetics) enhanced with the Bullet kit supplements provided by the manufacturer (Clonetics). For experimentation, ATI or ATII cells were seeded on type IV collagen-coated (Sigma, St. Louis, MO) 0.4-μm or 3.0-μm transwell filters (Corning Inc., Corning, NY) and grown to confluence. Cells grown on 3.0-μm transwell filters were used for immune cell migration studies. Before each experiment, the cells were washed twice with appropriate medium containing no serum or antibiotics. The serum-free medium used during experimentation with each cell type ensured no interference by serum proteins and did not adversely affect the health or metabolic activity of the cells as determined by MTT [3-(4,5-dimethylthylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.

PMN and DC purification and transmigration. PMN purification was performed as described previously (52). Briefly, anticoagulated venous blood was collected from normal volunteers aged 20 to 40 years with UTMB Institutional Review Board approval. PMNs were isolated by gradient separation with Monopoly resolving medium (MP Biomedicals, OH). Purified PMNs were resuspended in RPMI 1640 and 10% heat-inactivated FBS before use in migration experiments. DCs were isolated from monocytes purified from peripheral blood by negative selection using a magnetic column separation system (StemCell Technologies, Inc.) as described previously (3). Briefly, monocytes were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin and streptomycin, GM-CSF (100 ng/ml), and IL-4 (50 ng/ml) and seeded in 24-well tissue culture plates at 1×10^6 cells/ml. Nonadherent, homogenous

TABLE 1. Human TLR and GAPDH primer sets used for RT-PCR analyses

Gene	Primer sequence (5′→3′)	
	Forward	Reverse 5′–3′
TLR1	TGG TAT CTC AGG ATG GTG TGC	CAC CCA GAA AGA ATC GTG CCC
TLR2	GAA AAT GAT GTG GGC CTG GCT	GCA TCC CGC TCA CTG TAA GAA A
TLR3	TTC TCG ATT TGC AGC ATA ACA ACT	TGG ACT CCA AGT TAA GGA TGT GG
TLR4	TGG CAT GAA ACC CAG AGC TTT	AAC GGC AGC ATT TAG CAA GAA G
TLR5	GAC AAC GAG GAT CAT GGG AGA C	CCA TCA AAG GAG CAG GAA GGA A
TLR6	TAC TTG GAT CTG CCC TGG TAT CT	TGG AGG TTT CTT TGG AGT TCT TCT
TLR7	CAT TTG ACA GAA ATT CCT GGA GGT	GGG AGA TGT CTG GTA TGT GGT TA
TLR8	TGA GCA ACA CCC AGA TCA AAT ACA	TCA CAA GGC ACG CAT GGA AAT
TLR9	GCA ATG TCA CCA GCC TTT C	GTT CCA CTT GAG GTT GAG ATG
GAPDH	CAA CTA CAT GGT TTA CAT GTT C	CTC GCT CCT GGA AGA TG

immature DCs (characterized by high levels of CD1α and no CD83 expression via flow cytometry) were obtained after 7 days of culture. Cell counts were determined with a hemocytometer using trypan blue-stained aliquots. Viable cells were resuspended at the indicated concentrations in RPMI 1640 with 10% heat-inactivated FBS.

For the transmigration of immune cells, HPMEC were seeded on the AP surface of 3.0-μm transwell filters and grown to confluence. Because HPMEC and AECs are oriented in vivo with their BL surfaces in contact, the bacterium-free BL conditioned medium (CM) from Schu 4- or LVS-stimulated ATI and ATII cells was placed on the BL surface of HPMEC for 2 h. Unstimulated HPMEC were used as negative controls. To model the influx of immune cells from the lumen of the bloodstream, 5×10^5 freshly isolated PMNs or DCs were added to the AP surface of the BL-stimulated HPMEC cells. Migration was allowed to proceed for 4 h, and then AP and BL media were collected to count migrating cells by light microscope. The immune cells that had migrated to the BL chamber and adhered to the bottom of the well also were enumerated to ensure that all immune cells that migrated were accounted for. Five fields at ×25 were counted and averaged per sample.

Collection of *Francisella*-stimulated ATI and ATII cell CM and cytokine analysis. To maintain physiological accuracy, LVS or Schu 4 (multiplicity of infection [MOI] of approximately 175:1, 100:1, 50:1, 25:1, or 10:1) was placed in the AP chambers and incubated at 37°C with 5% CO_2. Additional ATII cells were pretreated with 40 μM of parthenolide (PTL) (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA), an NF-κB inhibitor, prior to the addition of LVS or Schu 4 as described above. The AP and BL CM then were collected at 6 or 24 h postinfection, filtered through a 0.22-μm filter, and stored at −80°C until analyzed. Unstimulated cells were used as negative controls. Cytokines in the AP and BL media were quantified via a multiplexed sandwich enzyme-linked immunosorbent assay (ELISA) array (SearchLight proteome array; Pierce, Woburn, MA) specifically designed for these experiments to measure IL-8, GRO-α, IL-8, MIP-1α, MCP-1, and GM-CSF. Additionally, IL-8 alone was measured in the media of designated samples by ELISA (Pierce/Endogen, Rockford, IL).

HEK293 cells expressing TLRs. Human embryonic kidney (HEK) 293 cells genetically manipulated to express TLR2, TLR2/6, TLR4, or TLR5 (Invivogen, San Diego, CA) were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) (Cellgro Mediatech Inc., Herndon, VA) supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mg/ml basticidin, and 2 mM glutamine. To analyze activation of the transcription factor NF-κB, each of HEK293 cell lines was transfected and then plated in a 96-well format using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Briefly, 1×10^4 cells were seeded in 100 μl medium in each well of 96-well ViewPlates (Perkin-Elmer Life Sciences Inc., Boston MA). The cells were 60 to 75% confluent at the time of transfection with 2.5 μg/1×10^6 cells of the firefly luciferase (NF-κB) reporter plasmid pNiFty2-Luc (Invivogen). The day after transfection, the existing medium was replaced with 100 μl of DMEM containing no antibiotics, followed by addition of Schu 4 or LVS at an MOI of 100:1, 50:1, or 25:1. Commercial TLR agonists (Invivogen) served as positive controls and included 10 ng/ml *S. enterica* serovar Typhimurium lipoprotein (TLR2 and -2/6), 1×10^8/ml heat-killed *Listeria monocytogenes* (TLR2 and -2/6), 10 ng/ml LPS (TLR4), and 10 ng/ml flagellin (TLR5). TLR agonist concentrations were used as per the manufacturer's instructions. Medium alone and HEK293 cells with no agonist were used as negative controls. The cells were incubated with live *F. tularensis* for 6 h, and then the medium was aspirated, replaced with fresh DMEM without antibiotics, and incubated overnight at 37°C and 5% CO_2. Samples were analyzed for luciferase activity by The SuperLight luciferase reporter gene assay (BioAssay Systems, Hayward, CA) as described by the manufacturer and measured using a SpectraMax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA). The data were expressed as relative luciferase activity.

Quantitative RT-PCR analysis of TLR expression. Primary AECs (1×10^5) were lysed for RNA extraction using the RNeasy spin column kit as per the manufacturer's instructions (QIAGEN, Valencia, CA). Briefly, RNA was eluted in 60 μl of total organic carbon-free water. Synthesis of cDNA was performed on 30 μl of RNA in a reaction volume of 50 μl using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Complementary DNA representing 1,000 cells was evaluated in duplicate by quantitative real-time PCR (RT-PCR) on an iCycler outfitted with real-time optics (Bio-Rad). Data were analyzed using the associated software (Bio-Rad) and are presented as expression level/1,000 cells. The human TLR primer sets (IDT, Coralville, IA) were designed using Beacon Designer software version 3.0 (Premiere Biosoft, Palo Alto, CA) (Table 1). Standard curves for extrapolation of the starting quantity of specific TLR cDNA were generated from 10-fold serial dilutions of quantified cloned amplimers. PCR efficiencies were in the range of 90 to 110%. Correlation coefficients were 0.990 to 1.00. The housekeeping GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene was run in parallel to normalize the variation in cell counts, cell viability, extraction efficiency, and cDNA quality. Controls included RNA alone and PCR without template.

Statistical analyses. Where applicable, numerical results are reported as means ± standard errors of the means (SEM) from three or four independent experiments. Analysis of numerical data was done by one-way analysis of variance (ANOVA) followed by Tukey's test, using Prism software (Graph Pad, San Diego, CA). Differences were considered statistically significant when the P value was <0.05.

RESULTS

***F. tularensis* LVS interacts with alveolar epithelial cells in vivo.** To determine if *F. tularensis* interacts with AECs in vivo, BALB/c mice were infected intranasally with 1×10^3 LVS organisms. Mouse lungs were harvested at selected time points postinfection, fixed, and analyzed by TEM. The resulting images showed that within 2 h postinfection, LVS localized to the airways and interacted with large flat cells morphologically identifiable as ATI cells (Fig. 1A and B). Similarly, at 2 h following infection, LVS was found in contact with ATII cells (Fig. 1C), which have distinct morphological traits such as intracellular lamellar bodies and AP microvilli. Additionally, we also examined mouse lungs at 4, 8, and 24 h postinfection and found a large number of LVS organisms that were phagocytosed by macrophages and neutrophils, but we did not find LVS inside ATI or ATII cells at any time point (data not shown). These findings were reproduced in lung sections from additional mice, supporting further investigation of the interaction between *F. tularensis* and primary human AECs.

BL CM from *Francisella*-stimulated human AECs promotes immune cell transendothelial migration. Based on our mouse

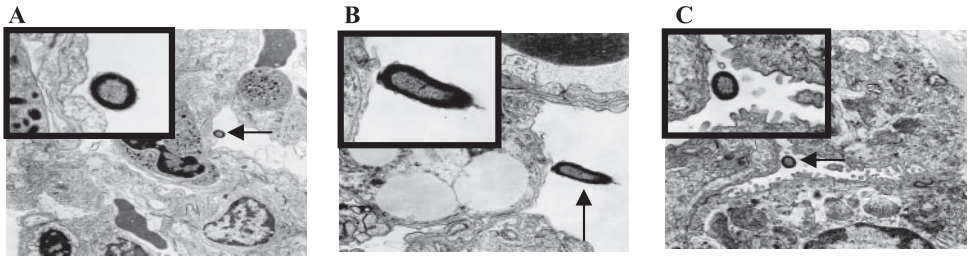

FIG. 1. LVS interacts with AECs in vivo. BALB/c mice ($n = 3$/group) were intranasally infected with 1×10^3 CFU/ml of LVS. The lungs were harvested, sectioned, and fixed for TEM analysis. LVS (arrows) was found in contact with both ATI cells (flat cells) (A and B) and ATII cells (large, square cells with AP microvilli) (C) at 2 h postinfection. Panels show enlargements of the TEMs in the upper left corners. Magnification, ×31,500.

data, we tested the hypothesis that inhaled *F. tularensis* would interact with human AECs and elicit biological responses. To test this hypothesis, analysis of CM from AECs exposed to *F. tularensis* was performed to determine if the CM contained factors that induced immune cell migration through the lung endothelium. ATI and ATII cells, grown to confluence on transwell filters, were stimulated AP with Schu 4 or LVS (MOI, 175:1). At 6 and 24 h poststimulation, CM was collected from both the AP and BL chambers. However, to mimic the in vivo physiological stimulation of endothelium by AECs, the bacterium-free ATI or ATII BL CM was added to the BL surface of confluent HPMEC monolayers grown on 3.0-μm transwell filters. Purified human DCs or PMNs then were placed on the AP surface of HPMEC. After 4 h, immune cell migration was quantified, and the results showed that HPMEC exposed to CM from unstimulated ATI or ATII CM promoted only minimal DC migration (Fig. 2A). However, the CM from 6- and 24-h Schu 4- and LVS-stimulated ATII cells elicited significantly higher DC migration through HPMEC than did the unstimulated controls or HPMEC exposed to Schu 4- or LVS-stimulated ATI CM ($P < 0.05$) (Fig. 2A). There was slightly less, although still elevated, transendothelial DC migration in cultures treated with the 24-h Schu 4- or LVS-stimulated ATII CM than in those treated with the 6-h CM (Fig. 2A). Interestingly, there was no difference in DC migration between Schu 4-stimulated ATI or ATII CM compared to LVS-stimulated ATI or ATII CM.

PMN transendothelial migration was significantly higher for the 6-h Schu 4- or LVS-stimulated ATII cell CM than for the Schu 4- or LVS-stimulated ATI CM ($P < 0.05$) (Fig. 2B). Further, there was significantly higher PMN migration with the 6-h Schu 4- or LVS-stimulated ATII CM than with the corresponding 24-h ATII CM ($P < 0.05$) (Fig. 2B). As predicted, HPMEC exposed to CM from unstimulated ATI or ATII cells promoted minimal PMN migration (Fig. 2B). Although the 6- and 24-h Schu 4-stimulated ATI CM did not induce DC migration, the 6-h Schu 4-stimulated ATI CM did provoke significant PMN migration compared to that found with HPMEC exposed to LVS-stimulated ATI CM and unstimulated controls ($P < 0.05$) (Fig. 2B). Collectively, these data suggested that ATII cells, much more so than ATI cells, were important participants in the fight against *F. tularensis* via the secretion of crucial soluble factors that triggered the transendothelial migration of PMNs and DCs through HPMEC.

***F. tularensis* induces differential cytokine secretion from the AP and BL surfaces of ATII cells.** The bacterium-free ATII CM likely contained soluble factors that elicited the observed immune cell migration. A selected panel of known chemoat-

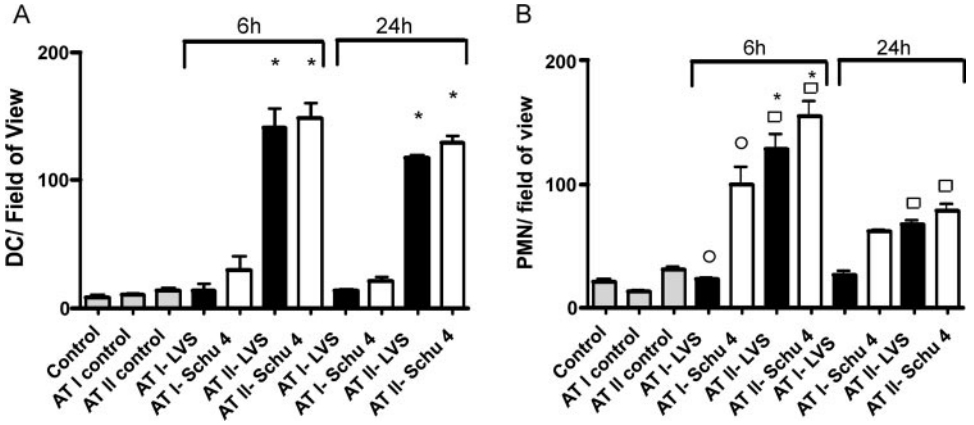

FIG. 2. Immune cell migration through HPMEC. DC and PMN migration through HPMEC cells in response to BL Schu 4- or LVS-stimulated AEC CM was measured. (A) DC migration was greatest toward both Schu 4- and LVS-stimulated AT II cell CM at 6 and 24 h. Neither the Schu 4- nor LVS-stimulated ATI CM stimulated DC migration. (B) PMN migration was greatest toward Schu 4- and LVS-stimulated 6-h ATII CM. PMN migration was also significant in response to the 6-h Schu 4-stimulated ATI CM. The 6-h ATII CM was a greater inducer of PMN migration than the 24-h ATII CM. Experiments were performed three times in duplicate to ensure reproducibility. $P < 0.05$ by ANOVA followed by Tukey's test; data are represented as means ± SEM. ∗, ATI versus ATII CM; ○, 6-h versus 24-h supernatant time point; □, Schu 4 versus LVS stimulant.

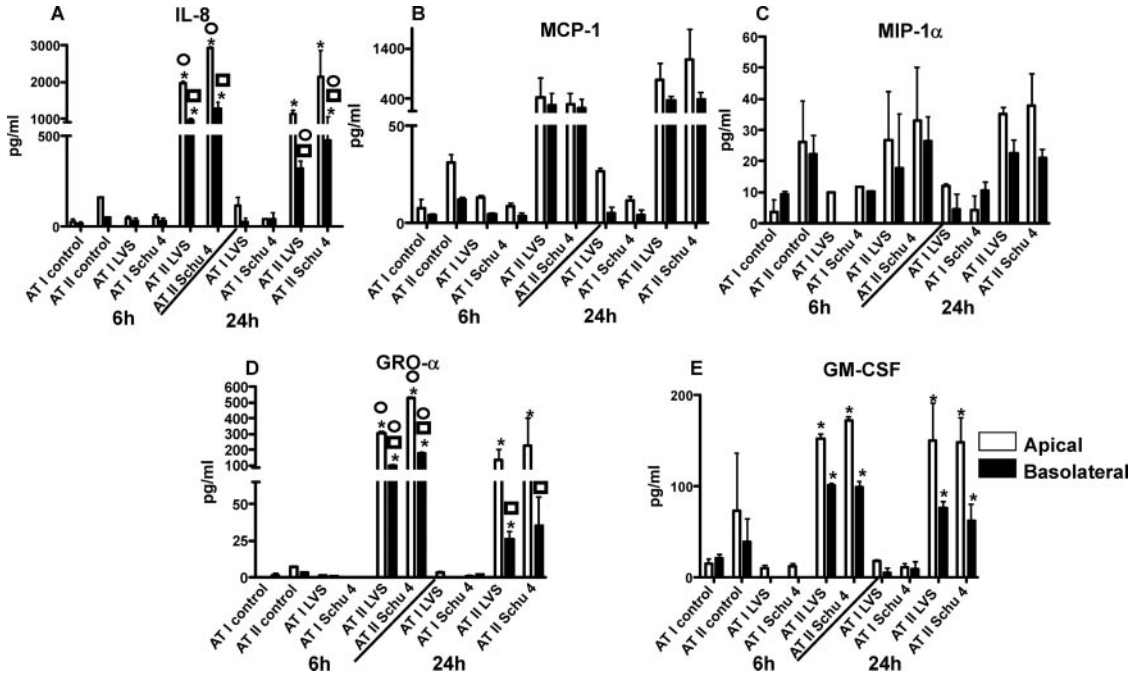

FIG. 3. Cytokine production by Schu 4- or LVS-stimulated AECs. AP and BL CM were collected from Schu 4- or LVS-stimulated ATI or ATII cell transwell filters at 6 and 24 h and analyzed for cytokine secretion by a multiplexed proteome array. At 6 and 24 h, the AP and BL CM of Schu 4- and LVS-stimulated ATII cells were significantly higher secretors overall of IL-8 (A), GRO-α (D), and GM-CSF (E) than was *F. tularensis*-stimulated ATI CM (*, $P < 0.05$). The BL, but not AP, IL-8 and GRO-α levels secreted by ATII cells significantly declined by 24 h (□, $P < 0.05$). The 6-h Schu 4-stimulated AP ATII supernatant and the 24-h BL ATII CM elicited significantly higher levels of IL-8 than LVS-stimulated AT II cells (○, $P < 0.05$). The 6-h Schu 4-stimulated AP and BL ATII CM had significantly higher levels of GRO-α than did LVS-stimulated ATII cells (○, $P < 0.05$). Data are representative of duplicate samples from four experiments. $P < 0.05$ by ANOVA followed by Tukey's test; data are represented as means ± SEM. Statistical symbols: *, ATI versus ATII levels; □, 6-h versus 24-h time point; ○, Schu 4 versus LVS stimulant.

tractant factors were quantified by a multiplexed protein assay for the AP and BL *F. tularensis*-stimulated ATI and ATII CM. Levels of GM-CSF, IL-8, GRO-α, MCP-1, and MIP-1α were assayed in 6- and 24-h CM. Unstimulated AECs served as negative controls. The results of this quantitative kinetic analysis allowed us to establish the average cytokine induction from the AP stimulation of primary human AECs by *F. tularensis* that should correlate with human inhalation of *F. tularensis*. A number of comparisons were made with these data, as follows.

(i) ATI versus ATII cytokine secretion. *F. tularensis*-stimulated ATII cells secreted higher cytokine levels from both their AP and BL surfaces than did *F. tularensis*-stimulated ATI cells. Specifically, the results showed that Schu 4- and LVS-stimulated ATII cells secreted higher AP and BL IL-8, MCP-1, MIP-1α, GM-CSF, and GRO-α levels than did *F. tularensis*-stimulated ATI cells at 6 h and 24 h (Fig. 3), with IL-8, GRO-α, and GM-CSF levels statistically higher in *F. tularensis*-stimulated ATII CM than in *F. tularensis*-stimulated ATI CM ($P < 0.05$) (Fig. 3). Another observation revealed by these results is that overall the AP surface of *F. tularensis*-stimulated ATII cells secreted higher cytokine levels than did the BL surface, with the greatest differences observed for IL-8, GRO-α, and GM-CSF (Fig. 3A, D, and E).

(ii) Cytokines secreted at 6 versus 24 h. BL, but not AP, IL-8 and GRO-α levels secreted by ATII cells significantly dropped by 24 h following Schu 4 or LVS stimulation ($P < 0.05$) (Fig. 3A and D). Levels of MCP-1, MIP-1α, and GM-CSF, however, remained generally elevated at both 6 and 24 h after Schu 4 or LVS stimulation of ATII cells.

(iii) Schu 4 versus LVS stimulation. Although Schu 4 induced a visible increase in cytokine secretion compared to LVS, the MCP-1, MIP-1α, and GM-CSF levels (Fig. 3B, C, and E) were statistically indistinguishable. However, the 6-h Schu 4-stimulated AP ATII supernatant and the 24-h Schu 4-stimulated BL ATII CM had significantly higher IL-8 levels than did the LVS-stimulated ATII cells ($P < 0.05$) (Fig. 3A). Further, the 6-h Schu 4-stimulated AP and BL ATII cell CM had significantly higher levels of GRO-α than the LVS-stimulated ATII cells ($P < 0.05$) (Fig. 3D). These data suggested that ATII cells responded to *F. tularensis* via cytokine secretion that correlated with the elevated PMN and DC transendothelial migration (Fig. 2) in response to ATII BL CM relative to the ATII CM.

Further, to determine if the pulmonary endothelium contributed to the cytokine secretion that promoted immune cell migration, HPMEC, grown on a transwell filter, were stimulated with 6- or 24-h *F. tularensis*-stimulated ATI or ATII BL CM or live Schu 4 or LVS (MOI, 175:1). HPMEC AP and BL CM were collected at 6 and 24 h, and then the CM was analyzed for inflammatory cytokine production. To distinguish the contribution of HPMEC cytokine secretion from the *F. tularensis*-stimulated AEC CM, the *F. tularensis*-stimulated AEC cytokine values were quantified and subtracted from the corresponding CM combining HPMEC and *F. tularensis*-stimulated AEC CM. The results showed that HPMEC stimulated

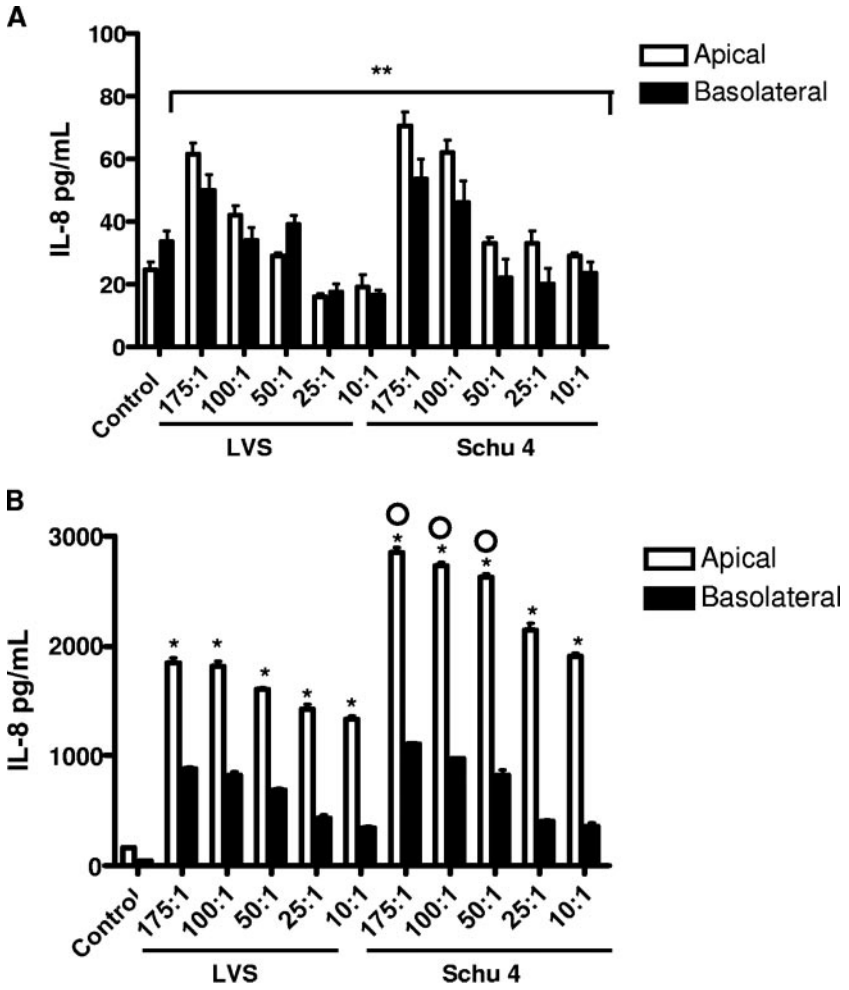

FIG. 4. *F. tularensis*-stimulated AECs secrete IL-8 in a dose-dependent manner. ATI (A) and ATII (B) cells were stimulated with Schu 4 or LVS at an MOI of 175:1, 100:1, 50:1, 25:1, or 10:1. Medium was collected at 6 h to quantitate IL-8 levels via ELISA. The results showed that both cell types secreted IL-8 in a dose-dependent manner; however, ATI cells (A) secreted significantly less AP and BL IL-8 overall than ATII cells (B) (**, $P < 0.001$). Note the *y* axis in panel A compared to panel B to appreciate that IL-8 induction from ATI cells is minimal at best compared to that from unstimulated controls and ATII cells. Schu 4- and LVS-stimulated ATII cells (B) secreted significantly higher levels IL-8 from their AP surface compared to BL IL-8 secretion (*, $P < 0.05$). Further, AP CM from Schu 4-stimulated ATII cells showed significantly higher IL-8 secretion compared to LVS-stimulated ATII cells, being significantly higher at MOIs of 175:1, 100:1, and 50:1 (○, $P < 0.01$). Data are representative of duplicate samples from three experiments and are represented as means ± SEM. *P* values were determined by ANOVA and Tukey's test.

with *F. tularensis*-stimulated AEC CM or live *F. tularensis* did not induce the chemokines (data not shown) secreted by *F. tularensis*-stimulated AECs as shown in Fig. 3. These data demonstrate both the ability of ATII cells to respond to *F. tularensis* and their role in the initiation of the early inflammatory response.

***F. tularensis* induces IL-8 secretion from AECs in a dose-dependent manner.** It is known that fewer than 20 organisms of inhaled *F. tularensis* can be detrimental to the host if left untreated (14, 19, 32). Therefore, we stimulated polarized ATI or ATII cells with Schu 4 or LVS at an MOI of 175:1, 100:1, 50:1, 25:1, or 10:1 for 6 h and then measured IL-8 secretion in the AP and BL CM. These samples were quantified for IL-8, because it was the most abundant cytokine secreted by both cell types (Fig. 3A) and it is a potent chemoattractant for immune cells such as neutrophils (23). Even though the results showed that ATI cells secreted significantly lower levels of IL-8 at all tested *F. tularensis* MOIs than did ATII cells ($P < 0.01$) (Fig. 4A and B), IL-8 secretion from both cell types was dose dependent (Fig. 4A and B). Schu 4-stimulated ATII cells secreted higher IL-8 levels from their AP surface than did LVS-stimulated ATII cells, with significance observed using MOIs of 175:1, 100:1, and 50:1 ($P < 0.05$) (Fig. 4B). At MOIs of 25:1 and 10:1, *F. tularensis* still stimulated production of high IL-8 levels from ATII cells stimulated with Schu 4 or LVS, with AP IL-8 secretion significantly higher than that of the BL ($P < 0.05$) (Fig. 4B). These data demonstrated that *F. tularensis* induced IL-8 secretion from AECs, specifically ATII cells, in a dose-dependent manner.

ATI and ATII cells express specific TLRs, but NF-κB activation appears to be independent of TLR stimulation in *F. tularensis*-stimulated ATII cells. Because NF-κB signal transduction is a main pathway for cytokine induction, we investigated whether NF-κB activation was responsible for cytokine

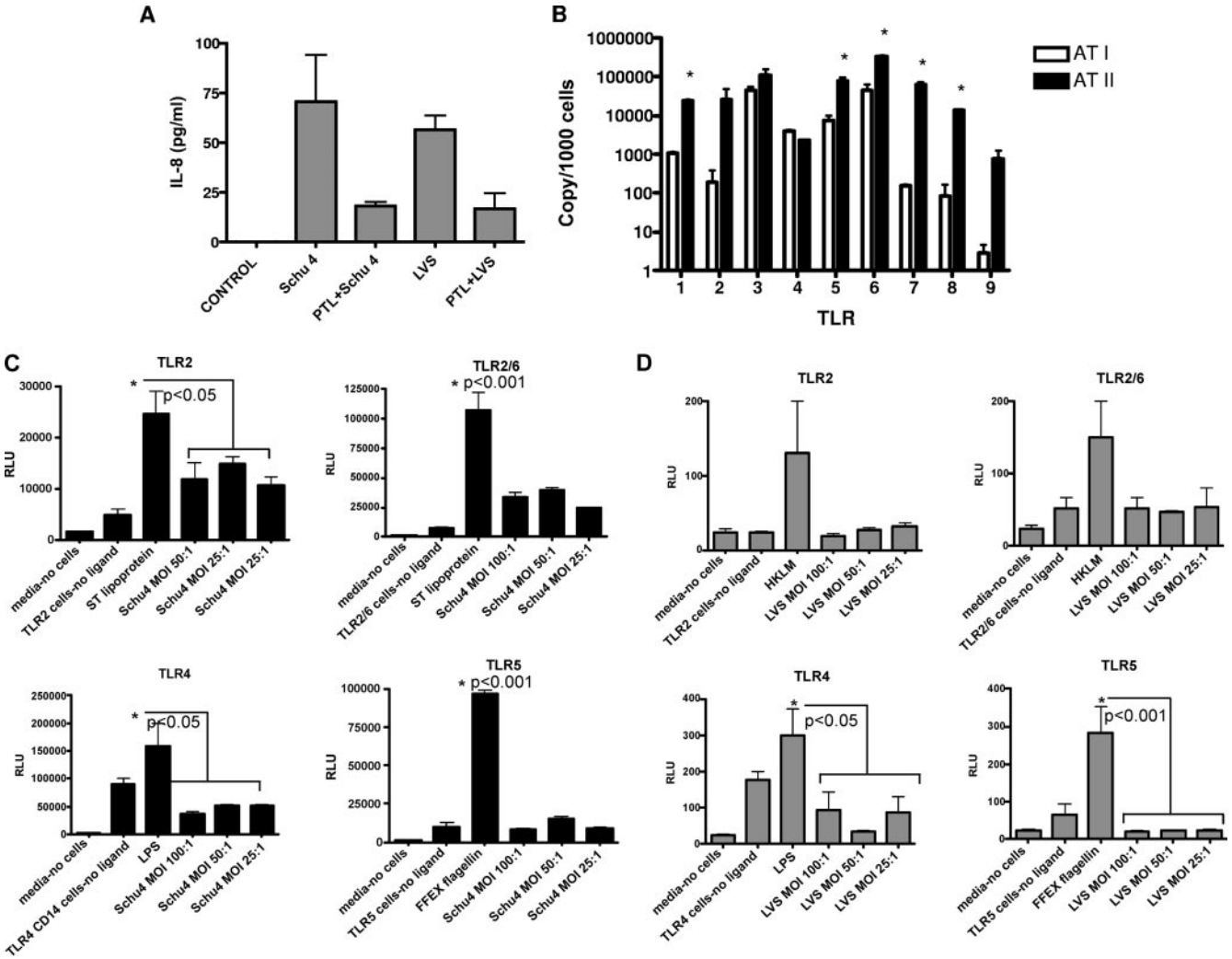

FIG. 5. Cytokine secretion by *Francisella*-stimulated ATII cells is NF-κB dependent but TLR independent. (A) ATII cells were pretreated with 40 μM of the NF-κB inhibitor PTL for 1 h prior to the addition of Schu 4 or LVS (MOI of 175:1). Medium was collected at 6 h and analyzed by ELISA for IL-8 levels. PTL substantially decreased IL-8 secretion in Schu 4- and LVS-stimulated AT II cells. (B) Quantitative RT-PCR was performed to determine the expression levels of ATI and ATII TLRs. ATII cells expressed significantly higher levels of TLR1, -5, -6, -7, and -8. Experiments were performed in triplicate with duplicate samples per repeat, and data are represented as means ± SEM. *, $P < 0.05$ by ANOVA followed by Tukey's test. (C and D) To determine if *F. tularensis* stimulated various TLRs, HEK293 cells expressing TLR2, -2/6, -4, or -5 and transfected with the firefly luciferase plasmid pNiFty2-Luc were stimulated with Schu 4 or LVS at an MOI of 100:1, 50:1, or 25:1. Medium without cells and unstimulated cells alone served as negative controls. The TLR agonist served as a positive control. The cells were incubated with live bacteria for 6 h, and then the medium was aspirated and replaced with fresh DMEM without antibiotics and incubated overnight at 37°C and 5% CO_2. The TLR agonists remained with the cells for an overnight incubation. The following day, samples were analyzed to determine luciferase activity. Data are expressed as relative luciferase activity (RLU). Neither Schu 4 (C) nor LVS (D) stimulated any of the tested TLRs. Schu 4 (C) induced elevated, but not significant, levels of luciferase via TLR2 compared to unstimulated controls. The TLR agonists *S. enterica* serovar Typhimurium (ST) lipoprotein (TLR2 and -2/6), heat-killed *Listeria monocytogenes* (TLR2 and -2/6), LPS (TLR4), and flagellin (TLR5) stimulated their designated TLR better than either *F. tularensis* strain. Three experiments were performed using triplicate samples, and data are represented as means ± SEM. *, $P < 0.05$ or $P < 0.01$ by ANOVA followed by Tukey's test.

elevation in *F. tularensis*-stimulated ATII cells. Because ATII cells responded significantly better to *F. tularensis* than did ATI cells, ATII cultures were pretreated with 40 μM of PTL (an NF-κB inhibitor) for 1 h prior to the addition of Schu 4 or LVS (MOI of 175:1). At 6 h poststimulation, supernatants were collected for IL-8 quantification. The results showed that PTL pretreatment substantially reduced IL-8 secretion from Schu 4- and LVS-stimulated ATII cells compared to vehicle-pretreated ATII cells stimulated with *F. tularensis* (Fig. 5A).

Because the inflammatory cytokines examined in this study are frequently produced as a result of TLR stimulation triggering NF-κB translocation, TLR expression was quantified for primary human ATI and ATII cells. Quantitative RT-PCR indicated that TLR1 to -9 were expressed differentially by ATI and AT II cells. ATII cells expressed significantly higher levels of TLR1, -5, -6, -7, and -8 than AT I cells ($P < 0.05$) (Fig. 5B). Further, ATII cells expressed higher levels, though this was not significant, of TLR2, -3, and -9 (Fig. 5B). Interestingly, TLR4, the receptor for bacterial LPS, was the only TLR expressed at higher levels in ATI cells. The results also indicated that only

a subpopulation of cells in the heterogeneous ATI cultures expressed TLR2, -7, -8, and -9. Based on the ATII TLR expression levels, it appeared that the ATI cultures may have contained approximately 1% (10 in 1,000) of the ATII cells.

To determine if *F. tularensis* stimulated one or more selected TLRs, HEK293 cells expressing TLR2, TLR2/6, TLR4, or TLR5 were transfected with a luciferase reporter plasmid driven by an NF-κB responsive promoter. After transfection, the cells were distributed evenly to 96-well plates and were exposed to Schu 4 or LVS at an MOI of 100:1, 50:1, or 25:1. AT 6 h poststimulation, the bacteria were removed and the cells were incubated overnight. Supernatants were collected and analyzed for activation of the NF-κB-dependent luciferase reporter gene. As expected, maximal luciferase levels, indicating NF-κB activation, were detected when purified TLR agonists were applied to cultures expressing the respective TLR (Fig. 5C and D). Interestingly, neither Schu 4 (Fig. 5C) nor LVS (Fig. 5D), at any dose, induced NF-κB activation via TLR2/6, TLR4, or TLR5 compared to agonist-stimulated cells. However, Schu 4, but not LVS, did induce noteworthy, but not significant, NF-κB activation via TLR2 compared to unstimulated controls (Fig. 5C). These data suggest that NF-κB activation in *F. tularensis*-stimulated ATII cells is independent of TLR2, TLR2/6, TLR4, or TLR5 stimulation.

DISCUSSION

Because of the potential for airborne bioweaponization, there is a great need to understand the interaction of *F. tularensis* with resident lung cell populations. Understanding the contribution that AECs make to the induction of the innate immune response following exposure to inhaled *F. tularensis* is crucial to elucidating the pathogenic and host response processes. For the first time, our data show that *F. tularensis* interacts with AECs both in vivo and in vitro and that human primary ATII cells vigorously respond to *F. tularensis* Schu 4 and LVS via cytokine secretion that subsequently leads to immune cell migration through HPMEC. Mechanistically, the cytokines secreted by *F. tularensis*-stimulated ATII cells required NF-κB activation, but induction was independent of TLR2, -4, -5, and -6 stimulation.

Many reports have suggested that soluble factors available in the inflammatory milieu can determine the pattern of immune cell influx (10, 27, 29, 38). In an in vivo situation, cytokines released from the BL surface of stimulated AEC signal the BL surface of HPMEC to recruit immune cells from the bloodstream into the site of infection. Our study utilized transwell filters of human primary alveolar lung cells and human primary endothelial cells to create an ex vivo model that best represents the in vivo physiological positioning of these cell types. The migration of PMNs into the lung is important for controlling tularemia in mice, as PMN depletion in mice infected with LVS caused lethality, even with a sublethal dose (49, 54). These findings indicated that PMNs are central to the response against a *F. tularensis* infection. More recent studies have shown that DCs also play an important role in the pathogenesis of *F. tularensis* (3). Our data showed that CM from Schu 4- or LVS-stimulated ATII cells elicited significant PMN and DC migration through lung endothelial cells. The migration of DCs and PMNs supports our hypothesis that ATII cells secrete soluble factors, specifically cytokines, in response to *F. tularensis* that signal surrounding cells to recruit immune cells to the area of infection.

Cytokine production is an early event in the innate host response that alerts the immune system to an insult (31). Previous studies have shown that ATI and ATII cells respond to lung insults by secretion of cytokines such as GM-CSF, MIP-1α, MCP-1, and GRO-α that are important for immune cell chemotaxis (13, 37, 42, 51, 58, 59). Although ATII cells are not found in confluent monolayers but are intermittently dispersed among ATI cells in vivo, it was important to examine the effects of *F. tularensis* on individual cell populations of the lung to better understand its pathogenesis and to determine the response of host cells during an *F. tularensis* infection of the lung. We found that, even at an MOI of 10:1, ATII cells, but not ATI cells or HPMEC, responded to Schu 4 or LVS with an up-regulation of cytokine secretion from their AP and BL surfaces. However, the lack of ATI cell and HPMEC reaction to *F. tularensis* via cytokine secretion does not exclude the possibility that they respond to *F. tularensis* by alternate, undefined pathways. It has been shown that LVS induced human umbilical vein endothelial cells to express adhesion molecules and to secrete the chemokine CXCL8, also known as IL-8 (15). Therefore, further studies evaluating the interaction of *F. tularensis* with ATI cells and HPMEC are warranted. Additionally, our data showed that neither Schu 4 nor LVS was found inside ATI or ATII cells at 6 or 24 h (data not shown). Thus, the internalization of *F. tularensis* was not necessary to induce AEC cytokine secretion. Conversely, Hall et al. (17) reported that ATII cells internalized LVS at 6 h postinfection, followed by LVS replication inside the cells at 24 h. The discrepancies between these studies may be due to the differences between primary human ATII cells grown in a polarized fashion, as utilized in our studies, and the transformed, nonpolarized A549 cells used by Hall et al. (17). Comparative expression profiling of primary and immortalized cells has shown changes in immortalized cell lines, including cell cycle regulatory proteins (47), gene expression in response to specific stimuli (4), and deficiencies in cytokine secretion (24, 26), compared to their primary counterpart. These studies show that the changes incurred by the cells as a result of immortalization affect how they interact with bacteria compared to their primary cells and thus also may affect bacterial internalization.

The increased secretion of chemoattractants by *F. tularensis*-stimulated ATII cells correlated with the observation that this CM induced DC and PMN transendothelial migration compared to *F. tularensis*-stimulated ATI cell CM. Schu 4-stimulated ATII cells did secrete significantly higher levels of the potent chemoattractants IL-8, and GRO-α at 6 h than did LVS-stimulated ATII cells. PMN migration also was highest in response to the 6-h Schu 4-stimulated ATII cell CM exposure. Minimal differences in the induced levels of the other tested cytokines were found following Schu 4 and LVS stimulation of ATII cytokine secretion and immune cell migration. Further, IL-8 secretion from *F. tularensis*-stimulated AECs, though significantly higher in ATII cells, was induced in a dose-dependent manner, and cells continued to secrete IL-8 even at an MOI of 10:1. Although the lung mucosal surfaces signify the first line of defense against inhaled *F. tularensis*, these mucosal immune responses are virtually unknown. Our data show that ATII cells offer a similar level of defense against Schu 4 and

LVS. Why we found few significant differences between Schu 4 and LVS with respect to their ability to induce immune cell migration and ATII cytokine secretion remains under investigation by our laboratory.

ATII cells are located at the interface between the alveolar airspace and the lung interstitium, which is a prime location for regulating the recruitment of immune cells by secreting cytokines in response to inflammatory stimuli (9, 39, 46, 61). Several studies have reported that upon bacterial stimulation, cytokine secretion by ATII cells was significantly up-regulated and resulted in the induction of immune cell migration (37, 38, 42, 46, 59). While ATII cells constitute only 15% of all lung cells, their strategic positioning in the lung allows them to respond more readily to inhaled particles, including invasive pathogenic bacteria such as *F. tularensis*. Similarly, we showed that ATII cells were capable of responding to low doses of *F. tularensis* via cytokine secretion. Conversely, the lack of response by ATI cells to *F. tularensis* may be related to the fact that they have been described as "inert" cells mainly providing a physical barrier function in the lung (8, 9, 30). The hyporesponsiveness of ATI cells to *F. tularensis* may be explained by the fact that they line >95% of the lung. A vigorous ATI cell response to inhaled particles could result in constitutive and/or damaging levels of inflammation. An estimated 10^{10} infective agents or foreign antigenic particles per day come into contact with the alveolar space (5×10^8 alveoli, with a surface area of approximately 100 m^2), yet they do not typically generate serious danger to the host (9). Interestingly, although ATI cells secreted minimal levels of the evaluated cytokines in response to *F. tularensis*, the Schu 4-stimulated ATI CM induced elevated levels of PMN, but not DC, transendothelial migration. This observation was one of the clearer differences between virulent Schu 4 and LVS that we observed in this study. This may be due to the production of a cytokine(s) or soluble factor(s) secreted by Schu 4-stimulated ATI cells that was not examined in our study. Moreover, because ATI cells maintain lung homoeostasis, including fluid transport, it is possible that ATI cells are responding to *F. tularensis* through other mechanisms besides cytokine secretion.

NF-κB activation often is responsible for the production of inflammatory cytokines in bacterially stimulated host cells. Recently, ATII cells have been shown to interact and respond to *Chlamydophila pneumoniae* via NF-κB activation (58). Similarly, pretreatment with an NF-κB inhibitor substantially reduced IL-8 secretion in ATII cells. The activation of the NF-κB pathway and subsequent cytokine production are often the result of bacteria stimulating one or more TLRs (1, 12, 33, 58). The quantification by RT-PCR of cDNA from ATI and ATII cells showed that they expressed varying mRNA levels of TLR1 to -9. Recent studies have shown that primary ATII cells express functional TLR2 and TLR4 and vigorously respond to LPS and lipoteichoic acids via cytokine production (2). However, the role of TLRs expressed by ATI cells has yet to be determined. Our study showed that neither *F. tularensis* strain stimulated TLR2/6, TLR4, or TLR5 expressed on HEK293 cells, although Schu 4, but not LVS, induced notable, but not significant, NF-κB activation via TLR2-expressing HEK293 cells compared to unstimulated controls. The role of TLRs, especially TLR2, in tularemia pathogenesis is not clear. Some studies have shown that LVS elicited inflammatory responses from mice via TLR2 (21, 25, 28), where mice deficient in TLR2 were more susceptible to infection by LVS, as demonstrated by a higher bacterial burden and increased mortality (28). It is not clear why we did not observe TLR2 stimulation by LVS. Conversely, another study showed no crucial role for TLR2 during an *F. tularensis* infection. That study reported that TLR2-, TLR4-, or TLR9-deficient mice survived a sublethal dose of LVS, compared to mice lacking a universal adaptor protein known as myeloid differentiation factor 88, which is used by TLRs to activate NF-κB, which survived only a short time following LVS infection (7). Together these studies demonstrate the need to further delineate the role that TLRs play in *F. tularensis* infections.

In conclusion, we showed that *F. tularensis* interacts with AECs in vivo and induces an inflammatory cascade from human primary ATII cells that results in immune cell migration. These findings are the first to demonstrate *Francisella*'s interaction with AECs via cytokine secretion and a potentially important role for ATII cells in the fight against tularemia. Further, our current studies provide a clearer picture of *Francisella* pathogenesis and host innate mucosal defense strategies that are utilized to fight the infection and will potentially identify novel avenues for targeted therapeutic and vaccine development.

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