TLR3 Activation Increases Chemokine Expression in Human Fetal Airway Smooth Muscle Cells

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Abstract

Viral infections, such as respiratory syncytial virus (RSV) and rhinovirus, adversely affect neonatal and pediatric populations resulting in significant lung morbidity, including acute asthma exacerbation. Studies in adults have demonstrated that human airway smooth muscle (ASM) cells modulate inflammation through their ability to secrete inflammatory cytokines and chemokines. The role of ASM in the developing airway during infection remains undefined. In our study, we used human fetal ASM cells as an in vitro model to examine the effect of toll-like receptor (TLR) agonists on chemokine secretion. We found that fetal ASM express multiple TLRs, including TLR3 and TLR4, which are implicated in the pathogenesis of RSV and rhinovirus infection. Cells were treated with TLR agonists, Poly (I:C) (TLR3 agonist) lipopolysaccharide (LPS; TLR4 agonist), or R848 (TLR7/8 agonist), and IL-8 and CCL5 secretion was evaluated. Interestingly, Poly (I:C) but neither LPS nor R848, increased IL-8 and CCL5 secretion. Examination of signaling pathways suggested that the Poly (I:C) effects in fetal ASM involve TLR and ERK signaling, in addition to another major inflammatory pathway, NFκB. Moreover, there are variations between fetal and adult ASM with respect to Poly (I:C) effects on signaling pathways. Pharmacological inhibition suggested that ERK pathways mediate Poly (I:C) effects. Overall, our data show that Poly (I:C) initiates activation of pro-inflammatory pathways in developing ASM, which may contribute to immune responses to infection and exacerbation of asthma.
Introduction

Clinical studies in pediatric populations indicate that early-life viral respiratory infections significantly contribute to development and exacerbation of wheezing and asthma (25, 27, 54, 61). A substantial burden of health care costs is associated with hospital admissions and emergency room visits for infection-induced wheezing during the first 3 years of life (54, 66). Virus-induced wheezing during the neonatal period is associated with a heightened risk for development of airway disease in childhood (27, 32). Emerging evidence suggests that approximately 80% of the causative agents of acute respiratory illness may be respiratory viruses (25, 30).

Respiratory syncytial virus (RSV), rhinovirus (RV), and influenza virus are strongly linked to the development of asthma and symptom exacerbations in young children (6, 67). Rhinovirus and RSV infect the lower airways. In fact, RSV is the most common cause of lower respiratory tract illness in infants (58). Although the airway epithelial cell is the principal host cell for most respiratory viruses (36), RSV and rhinovirus also infect airway smooth muscle (ASM) (23, 44).

The neonatal time is a particularly vulnerable period given the increased susceptibility to acquiring viral and bacterial infections (40). Certainly this susceptibility to infection and predisposition to the development of airway diseases such as asthma/wheezing is further enhanced by clinical situations such as preterm birth or exposure to chorioamnionitis in utero (5, 21). Also, in regards to in utero infection, the maternal-fetal interface is dynamic and viruses can traverse into the fetal lung (4, 6). Given the vulnerability to infections during the perinatal period there is a need to understand how infections impact the developing lung, particularly with respect to the airway.

Toll-like receptors (TLRs) are a group of pathogen recognition receptors that facilitate recognition of pathogen-associated molecular patterns in bacteria, viruses, and fungi (52, 63). Activation of TLRs on airway epithelium and ASM are pivotal events that facilitate airway inflammation during infection (14, 15, 22). For RSV and rhinovirus infection, stimulation of TLR3
and TLR4 mediates cytokine and chemokine production (31, 43, 65, 73). Prior studies have demonstrated that TLR3- and TLR4-deficient mice have altered immune response to RSV infection (35, 55, 56). Thus, examination of the effects of TLR3 and TLR4-mediated pathways would improve our understanding of viral induced airway inflammation.

Previous studies have shown that human adult ASM cells express TLRs and secrete cytokines and chemokines in response to TLR agonists (63). Recent studies in human ASM reported that, in contrast to LPS, Poly (I:C) has a more robust response with respect to promoting cytokine and chemokine release including IL-6, IL-8, CCL11 (Eotaxin), and CCL5 (RANTES) (39). Our study focuses primarily on IL-8 and CCL5 as these inflammatory mediators have been implicated in the fetal inflammatory response. In a recent pediatric clinical study the presence of CCL5 in nasal epithelia was linked to increased risk of childhood asthma (3). While IL-8, a chemokine that regulates neutrophil chemotaxis, is increased in young children with RSV infection (60). Furthermore, TLR3 and TLR4 ligands increase allergen sensitization and enhance allergic airway disease in mice (51). However, it is difficult to directly extrapolate these results from adult human ASM cells, other cell types, or mice to the developing human airway, given that the environmental context in which airway development or neonatal infection occur is different.

A major limitation in the study of developing human airway has been the lack of human neonatal tissues or age-appropriate human in vitro or in vivo research models. Access to postnatal (neonatal/pediatric) tissues is limited, thus we utilize human fetal ASM to model the neonatal airway. We have previously reported a unique, non-immortalized, non-cancerous, human fetal ASM cell model (24) that offers significant advantages for mechanistic examination of signaling mechanisms in an age- and species-appropriate human tissue (albeit in vitro). Accordingly, in the present study, we investigated the effects of Poly (I:C) (TLR3 agonist, synthetic double-stranded RNA) and lipopolysaccharide (LPS; TLR4 agonist) on TLR expression, chemokine release, and activation of TLR, mitogen-activated protein kinase (MAPK)
and NFκB pathways in human fetal ASM cells. We hypothesized that the developing airway already possesses TLRs that can mediate inflammatory responses and that there is variation in the fetal and adult ASM response to inflammation. In this study, we found that TLR3 agonist, Poly (I:C), increases chemokine expression in human fetal ASM which represents an important means by which viral infections may contribute to pediatric airway disease.
Materials and Methods

Materials. Fetal bovine serum (FBS), Dulbecco’s Modified Eagle’s Medium F/12 (DMEM/F12), and additional cell culture supplies were obtained from Invitrogen (Carlsbad, CA). Remaining supplies and reagents were obtained from Sigma Aldrich (St. Louis, MO). The following ligands were utilized: LPS, (E. coli 055:B5, L4524; Sigma Aldrich, St. Louis, MO), Poly (I:C) (Invivogen, San Diego, CA), R848 (Invivogen, San Diego CA), and TNFα (R&D Systems, Minneapolis, MN). Primary antibodies were obtained from Cell Signaling Technology (Danvers, MA) unless otherwise stated.

Isolation of Human Airway Smooth Muscle Cells. Human fetal airway smooth muscle (fetal ASM) cells were enzymatically dissociated from fetal tracheobronchial tissue as previously described under protocols approved and considered exempt by the Mayo Institutional Review Board and Ethics Committees in the UK (24, 47). Fetal ASM cells were provided by Dr. Pandya from the University of Leicester, England and by Novogenix (Los Angeles, CA). Similarly, human adult ASM was dissociated from de-identified lung samples that became available through pneumenectomies or lobectomies. Our protocol was approved by the Mayo Clinic Institutional Review Board to collect non-infected lung specimens from surgical pathology at Saint Mary’s Hospital. Cells were isolated from 3rd-6th generation airway branches by removing ASM layer from adventitia and epithelium. The ASM layer was placed in Hank’s balanced salt solution (HBSS; Invitrogen). Cells were dissociated enzymatically by collagenase and papain, and then seeded in cell cultured flasks in DMEM/F-12 (Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin. Cells grown under standard conditions (95% air/5% CO₂) in a humidified incubator from passages 1-5 for adult ASM and 2-10 for fetal ASM of subculture were used for experiments. Media was phenol red free DMEM/F12 with 10% FBS. Cells were serum deprived in 0.5% FBS for a minimum of 24 h prior to experimental treatments.

For both fetal and adult human ASM cells, phenotype was confirmed from cell passages 1-5 for adult and 2-10 for fetal ASM with expression of smooth muscle markers as described in
previous studies (7, 24). In our prior investigations we identified that the expression pattern of smooth muscle actin, calponin and acetylcholine receptor (AchR) in fetal ASM cells was similar to that of adult human ASM cells (7, 24). This previous work supported the use of this in vitro ASM cell model for the remainder of this study.

**Cell Treatments.** Fetal ASM cells were treated with control (sterile PBS), 10 ng/mL TNFα, 1 µg/mL LPS, or 1 µg/mL Poly (I:C) for 24 h, unless otherwise specified. Dose responses were performed to determine the optimum concentration of Poly (I:C) and R848 for all cell treatments. In some experiments, fetal ASM cells were pre-treated with 10 μM of ERK activation inhibitor peptide I (MEK1 inhibitor; Calbiochem; San Diego, CA), 10 μM p38 MAP kinase inhibitor (Calbiochem), 20 μM NFκB activation inhibitor III (SM-7368; EMD Millipore; Billerica, MA) or 20 μM TLR3 antagonist, CU CPT 4a (Tocris Bioscience; Minneapolis, MN) for 1 h prior to treatment with control (sterile PBS), 1 µg/mL Poly (I:C), LPS or R848 for 24 h. Nuclear extraction was performed after 2 h of cell treatment using the NE-PER® nuclear and cytoplasmic extraction kit per manufacturers protocol (ThermoScientific; Rockford, IL).

**Western Blot Analysis.** After 24 h of treatment, cells were harvested using standard techniques with cell lysis buffer containing protease inhibitors. Duration of cell treatment for examination of the MAP kinase pathway ranged from 5 minutes to 1 h. Lysate protein concentration was determined with a DC protein assay (Bio-Rad, Hercules, CA) and ~30 μg total protein was loaded onto either 10% or 4-15% gradient gels (Criterion Gel System; Bio-Rad, Hercules, CA). A Trans-Blot Turbo system was used to transfer proteins to nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked at room temperature with Odyssey Blocking buffer (Li-Cor Biosciences, Lincoln, NE) for 1 h. Membranes were incubated overnight at 4°C in 1 μg/mL of primary antibody of interest. Primary antibodies included: TLR2, TLR3, TLR4, TLR7, TLR9, phosphorylated and total extracellular signal-regulated kinase (ERK) (Santa Cruz Biotechnology, Dallas, TX), total ERK 1/2 (Santa Cruz Biotechnology), phosphorylated and total c-Jun N-terminal kinase (JNK) (Santa Cruz Biotechnology),...
phosphorylated and total p38, p50 (Santa Cruz Biotechnology), and p65 (Santa Cruz Biotechnology). GAPDH or TATA-box binding protein (TBP) (Abcam) were used to control for protein loading. Membranes were washed with TBS prior to incubation with infrared dye-conjugated secondary antibodies (LiCor Biosciences) for 60 minutes at room temperature. Membranes were imaged on a Li-Cor OdysseyXL system and densitometry quantified with Image Studio software. All blots were normalized to GAPDH unless otherwise specified.

**Real-Time PCR.** Following manufacturer’s protocol, total RNA was extracted from cells 6 h following cell treatments using an RNeasy Mini Kit (Qiagen). Standard techniques were used to synthesize and amplify cDNA. Real-time PCR was performed in triplicates per cDNA template using a Roche Light Cycler 480. mRNA expression was calculated by normalization of cycle threshold [C(t)] values of target gene to reference gene (GAPDH). The relative fold change was calculated by ΔΔCt method. Human primers used include: CCL5, CD14, GAPDH, IL-8, MD2, MyD88, TLR3, TLR4, and TRIF (Table 1).

**Enzyme Linked Immunosorbent Assay (ELISA).** Concentrated cell culture supernatants from cells treated with control (sterile PBS), 10 ng/mL TNFα, 1 µg/mL LPS, or increasing concentrations of either Poly (I:C) or R848 ranging from 0.01 – 1 µg/mL over 24 h were assayed for levels of CCL5 (RANTES) (range: 31.2-2000 pg/mL), and IL-8/CXCL8 (31.2-2000 pg/mL) using ELISA plates from R&D Systems (Minneapolis, MN). IL-8/CXCL8 and CCL5 ELISA plates were performed according to manufacturer instructions.

**MAP Kinase Inhibitor, NFκB Inhibitor, and TLR3 Antagonist Cell Treatments.** Concentrated cell culture supernatants from cells pre-treated for 1 hour with ERK inhibitor, p38 MAP kinase inhibitor, NFκB inhibitor or TLR3 antagonist prior to exposure with Poly (I:C) over 24 h were assayed for levels of CCL5 and IL-8/CXCL8 as per above.

**Statistical Analysis.** Experiments were performed using cells from at least 3 different fetal ASM samples, with at least 3 repetitions per sample. Data were analyzed using Student’s t-test and one-way ANOVA or the nonparametric Kruskal-Wallis test when appropriate. Comparisons
between groups were made using Tukey \textit{post hoc} analysis. Values are expressed as mean ± standard error (SE) and statistical significance was established at p<0.05.
Results

Expression of Toll-like receptors and adaptor molecules in human fetal ASM

Similar to studies performed in human adult ASM (39), we found that human fetal ASM cells express TLR2, TLR3, TLR4, TLR7, and TLR9 as demonstrated via Western blot using human adult ASM as a positive control (Figure 1). Based on these findings and the relevance of TLR3 and TLR4 to pediatric lung infection, we investigated the effects of TLR3 and TLR4 agonists on fetal ASM. We performed real-time PCR for mRNA expression of TLR3 and TLR4. We found that Poly (I:C) and TNFα, but not LPS, significantly increased TLR3 mRNA expression 6 h following treatment (Figure 2A). TLR4 mRNA expression was not significantly altered by Poly (I:C), TNFα, or LPS (Figure 2B). However, protein expression of TLR3 and TLR4 were significantly increased by TNFα and Poly (I:C), compared to control cells (Figure 2C), suggesting differential regulation of these TLRs by inflammatory stimuli. TLR3 and TLR4 have associated adaptor molecules which are important for their signaling (38). We found that cell treatments with TNFα and Poly (I:C) resulted in a significant increase of MyD88 mRNA expression, but no effect was seen on MD2, TRIF, or CD14 (Figure 2D).

Effects of TLR3 and TLR4 agonists on chemokine secretion and expression

To determine optimal Poly (I:C) treatment concentration, human fetal ASM were exposed to control, and increasing concentrations of Poly (I:C) ranging from 0.01 – 1 µg/mL over a 24 h period to assess CCL5 and IL-8 secretion (Figures 3A and 3B). We found that the highest concentration of 1 µg/mL induced a significant increase in both CCL5 and IL-8 secretion as compared to the other exposures. We assessed the effects of increasing concentrations of TLR7/8 ligand, R848 (0.01 – 1 µg/mL), on CCL5 and IL-8 secretion respectively and found no differences in comparison to control at all concentrations with R848 cell treatments (Figures 3A and 3B).

Next, we examined the effects of Poly (I:C) and LPS on IL-8 and CCL5 mRNA expression and secretion. CCL5 and IL-8 mRNA levels were increased by Poly (I:C) and TNFα
Similarly, Poly (I:C) and TNFα both significantly increased CCL5 and IL-8 secretion (Figures 4C and 4D). Conversely, LPS did not increase IL-8 or CCL5 mRNA expression levels (Figures 4A and 4B). LPS did increase IL-8 secretion, but to a lesser extent than Poly (I:C) or TNFα (Figure 4D).

To determine if the effects of Poly (I:C) were mediated through TLR3, we utilized TLR3 antagonist, CU CPT 4a. TLR3 antagonist significantly reduced the ability of Poly (I:C) to increase both CCL5 and IL-8 secretion as measured by ELISA (Figures 5A and 5B).

**Mechanisms of Poly (I:C)-induced effects in chemokine expression**

The production of pro-inflammatory chemokines can be regulated by NFκB, in addition to MAP kinases (MAPK) p38, JNK, and ERK-mediated pathways (37, 49, 53). To evaluate potential mechanisms by which Poly (I:C) may be functioning, we examined phosphorylation of MAPKs. We first performed a timed treatment course where cells were treated with control or Poly (I:C) for 5 min, 10 min, 15 min, 30 min, or 60 min and assessed phosphorylation of ERK, p38, and JNK by Western blot (Figure 6A-C). Poly (I:C) significantly increased phosphorylation of ERK (Figure 6B) approximately 15 min following Poly (I:C) stimulation and p38 phosphorylation was increased at 5 min following Poly (I:C) stimulation (Figure 6A). Treatment with Poly (I:C) did not alter phosphorylation of JNK during the time course (Figure 6C).

We sought to compare and contrast mechanisms of Poly (I:C)-induced effects in chemokine expression between human fetal and adult ASM. Similar to experiments conducted in fetal ASM, we examined NFκB, as well as phosphorylation of MAPK p38, JNK, and ERK in adult ASM. We performed a timed treatment course where adult ASM cells were treated with control or Poly (I:C) for 5 min, 10 min, and 15 min (Figure 7A-C). Poly (I:C) did not have an effect on ERK (Figure 7B), but significantly increased phosphorylation of p38 and JNK approximately 10-15 minutes following Poly (I:C) stimulation (Figures 7A and 7C).

After nuclear extraction, we also assessed the effect of the various treatments for a 2 h duration on nuclear translocation of the NFκB subunit p50 and p65 in human fetal ASM.
Treatment with TNFα and Poly (I:C) both increased p50 and p65 nuclear levels (Figure 8A).

Likewise, in nuclear fractions we assessed the effect of the TNFα and Poly (I:C) treatments over a 2 h duration on nuclear translocation of the NFκB subunits p50 and p65 in human adult ASM. Similar to findings in the human fetal ASM, treatment with both TNFα and Poly (I:C) in human adult ASM induced p50 and p65 nuclear translocation (Figure 8B).

**MAPK and NFκB inhibitor treatments and effects in chemokine expression**

In the fetal airway smooth muscle cells, we pharmacologically inhibited ERK activation, p38 kinase activity, and NFκB to determine if these pathways contribute to CCL5 and IL-8 secretion upon treatment with Poly (I:C). Fetal ASM cells were pre-treated with MEK1 inhibitor (inhibits ERK activation), p38 inhibitor, or NFκB inhibitor for 1 h followed by treatment with control (sterile PBS) or Poly (I:C) for 24 hours. Treatment with the MEK1 inhibitor and NFκB inhibitor attenuated Poly (I:C)-induced CCL5 and IL-8 secretion, while the p38 inhibitor did not have a significant effect on either chemokine (Figures 9A and 9B).
Airway inflammation triggered by viral pathogens is an important contributor to the development of wheezing and asthma in the pediatric population. Recent epidemiologic studies reveal that severe viral lower respiratory tract infection during the neonatal period are associated with increased risk of developing asthma in children (26, 59). Premature infants are even more susceptible to acquiring viral infections. RSV, a RNA virus, is frequently detected in preterm neonates making it relevant to study. A recent prospective study indicated that RSV infections in premature infants resulted in increased health care utilization and costs in the first two years of life (18). The presence of airway inflammation induced by viral infections could result in deleterious long-term effects on airway structure and function, including bronchoconstriction, enhanced airway contractility, increased cell proliferation, and remodeling (6, 48). These changes may translate clinically into the development of asthma and wheezing disorders (6, 48).

Using human fetal ASM cells as a model for the neonatal airway, we evaluated the role of Poly (I:C) and its receptor, TLR3, in addition to other pro-inflammatory mediators, LPS and TNFα, on chemokine secretion. We compared mechanisms by which Poly (I:C) induced effects in chemokine expression between human fetal and adult ASM through examination of the MAPK and NFκB pathways. We acknowledge that there are limitations in the use of fetal vs. neonatal ASM cells. Prior studies in neonatal ASM by Fayon et al. showed greater cell proliferation in neonatal ASM than adult ASM. Our group observed similar findings in fetal ASM, suggesting there may be similarities between fetal and neonatal ASM (20, 24).

Viral infections instigate asthma exacerbations through activation of TLRs which promote airway inflammation (14). Airway smooth muscle contributes to pro-inflammatory responses due to its ability to augment expression of inflammatory mediators such as cytokines and chemokines (14). Toll-like receptor mRNA expression has been characterized in a prior study showing that human adult ASM expressed TLR1 through TLR10 (63). Human ASM
exposure to rhinovirus or RSV results in production of a variety of cytokines including IL-1β, IL-6, IL-8, IL-11, IFNγ, and TNFα and enhances expression of TLRs (8, 14, 15, 42, 46). However, there is a paucity of data on the expression of TLRs and chemokine secretion in the developing ASM. Analogous to human adult ASM, we found that fetal ASM express TLRs, which are known to mediate pulmonary inflammatory responses. With respect to TLR3 activation, Poly (I:C) stimulates the production of multiple chemokines, including CCL11 (45). Similarly, our studies demonstrate that Poly (I:C) stimulates IL-8 and CCL5 production in human fetal ASM. Importantly, the effects of Poly (I:C) were significantly reduced by a TLR3 antagonist.

CCL5 and IL-8 are important chemokines for airway inflammation during infection and asthma (33, 69). Chemokines not only mediate cell recruitment to areas of inflammation, but also function to mediate the production of cytokines (13, 62). IL-8 acts primarily as a mediator for neutrophil chemotaxis, whereas, CCL5 (RANTES) functions as a potent chemoattractant for eosinophils (13, 62). In a study examining mature asthmatic human airway smooth muscle cells, exposure to rhinovirus induced IL-8 release (46). Additionally, in primary culture of adult human bronchial smooth muscle cells Poly (I:C) enhanced CCL5 production (45). Consistent with previous studies, our data in developing ASM demonstrate that exposure to Poly (I:C) results in increased secretion of IL-8 and CCL5. These data suggest that activation of TLRs in the developing ASM may contribute to inflammatory cell recruitment during infection.

Although we found that fetal ASM express TLR4, the effects of LPS on chemokine expression were less pronounced than Poly (I:C). LPS did not instigate significant effects on IL-8 and CCL5 mRNA expression, and IL-8 secretion following LPS stimulation was significantly less than with Poly (I:C). Our data are consistent with a prior investigation in human ASM demonstrating that Poly (I:C) stimulates chemokine secretion, while LPS had minimal effects on chemokine production (39). We speculated that limited responses to LPS in ASM could be attributed to lack of expression of key TLR4 adaptor molecules, MD2, CD14, and MyD88. Our data show that fetal ASM express MD2, CD14, and MyD88. Interestingly, Poly (I:C) and TNFα
increased MyD88 expression, suggesting potential modulation of TLR4 by TLR3- and TNFR-mediated signaling. We speculate that there may be intrinsic differences in the TLR4 pathway that limit the effects of LPS in ASM in regard to chemokine production. Certainly, additional studies will be needed to further interrogate TLR signaling pathway in developing ASM.

TNFα is a potent pro-inflammatory cytokine that has multiple effects during airway inflammation (11). Similar to TLR3 and TLR4 signaling, TNFα activates multiple signaling pathways including MAPK and NFκB (16). Treatment with TNFα increased TLR3 and TLR4 mRNA and protein expression in fetal ASM and also increased IL-8 and CCL5 production. The data suggest that TNFα may potentiate the effects of TLR ligands through enhancement of TLR expression (10, 38).

Pro-inflammatory cytokines and TLR ligands activate the MAPK and NFκB pathways and through these pathways regulate key processes during airway inflammation such as airway remodeling resulting in cell proliferation and extracellular matrix deposition (17). We found that Poly (I:C) stimulated phosphorylation of ERK and p38, and activated NFκB (p50 and p65) in human fetal ASM. Similar to recent findings in human bronchial epithelial cells showing increased phosphorylation of ERK with Poly (I:C) exposure (64). Another study in murine ASM found that TLR3 stimulation with Poly (I:C) increased NFκB and JNK signaling pathways (2). We speculate that possible differences between species (human and murine) ASM could explain variations between observations with respect to how we did not observe activation of JNK by Poly (I:C) in human fetal ASM. In our studies, pre-treatment with an ERK inhibitor, but not p38 inhibitor, blunted IL-8 and CCL5 secretion stimulated by Poly (I:C). A previous study similarly found that utilization of an ERK inhibitor resulted in a reduction in IL-1β induced CCL11 (Eotaxin) production in adult human ASM (71). Our experiments in human fetal ASM with specific inhibitors of MAPKs suggest that the Poly (I:C) induced expression of chemokines in human fetal ASM is mediated, at least in part, through the ERK pathway.
Our data suggest variations in mechanisms between human adult and fetal ASM. We observe that the phosphorylation of ERK is upregulated by Poly (I:C) in fetal ASM, but this is not seen in the adult ASM. Moreover, phosphorylation of JNK is not increased by Poly (I:C) in fetal ASM, but this is observed in adult ASM. NFκB mediated signaling is integral to asthmatic inflammation (9, 19). Infection with RSV and rhinovirus, RNA type viruses, have been shown to induce activation of NFκB in adult human nasal epithelial cells, adult human airway epithelium, and adult human airway smooth muscle; however there is a paucity of data specifically focused on the developing airway (12, 41, 46). Consistent with prior studies, in the human adult ASM we observed a significant increase in the nuclear translocation of NFκB subunits p50 and p65 with inflammation triggered by TLR3 agonist, Poly (I:C), and TNFα (1, 7, 28, 71, 72). Secretion of CCL5 and IL-8 was reduced after pre-treatment with NFkB inhibitor in the presence of Poly (I:C). NFκB has been shown to play an important role in pro-inflammatory cytokine production and hypercontractility in human ASM (50, 57).

In our study we found that fetal ASM express not only TLR3 and TLR4, which were the focus of this paper, but other toll-like receptors including TLR2, TLR7, and TLR9. TLR2 prompts immune response through recognition of bacteria, TLR7 is activated by viral single-stranded RNA, and TLR9 responds to unmethylated CpG DNA present in bacterial and viral DNA (29, 43). In addition to TLRs, other receptors such as, RIG-1 and MDA5, are also involved in responses to viral infection (70). Recent studies demonstrated a role for RIG-1 and MDA5 in interferon production by ASM upon rhinovirus infection (8). Future experiments examining the roles of other TLRs and RNA virus sensing receptors in the developing airway would improve our understanding of the significance of these receptors in the developing ASM.

Inflammation is a central feature of infection which can promote asthmatic exacerbations and contribute to altered airway structure and function. Pulmonary infection, triggered by viral pathogens, is prevalent in the neonatal population and represents an important contributor to the development of asthma and wheezing. The present studies suggest that Poly (I:C), TLR3
agonist, can enhance the inflammatory response in the developing airway through up-regulation of CCL5 and IL-8 through involvement of mechanisms related to the TLR3 receptor, in addition to the MAPK and NFκB signaling pathways. Although there are limitations in using TLR agonists as opposed to a live virus, it does offer the opportunity to study mechanisms as there is less concern about variation in virulence or viral load. Prior in vitro work suggests that rhinovirus epithelial infection decreases β2-adrenoceptor function on ASM (68). In addition, another study shows that rhinovirus infection induces airway remodeling through increased deposition of extracellular matrix proteins (34). Future studies will be needed to examine the effects of live RSV or rhinovirus on fetal ASM.

Our investigations demonstrate that fetal ASM cells respond to stimulation with Poly(I:C). Clinically, these findings suggest that fetal ASM contributes to inflammatory responses in the fetal lung during antenatal infection, which is relevant to postnatal lung development in preterm infants and possibly more long-term in childhood. Mechanistically, it appears that there are variations demonstrated between human fetal and adult ASM which may have important implications on the development of potential treatments to offer more specific, targeted therapy for the developing airway. Further studies aiming to identify therapeutic measures to target TLR signaling and to examine ways to down-regulate inflammation in the developing airway will be important for clinical advances in the treatment of neonatal airway disease.
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Toll-like receptor, Inflammation, Lung, Development


Figure Legends

Figure 1. Expression of TLRs in fetal ASM. Expression of TLR2, 3, 4, 7, and 9 was detected in fetal ASM by Western blot. Fetal ASM were treated with media only (control) for 24 h and TLR2, 3, 4, 7, and 9 expression relative to GAPDH was evaluated. Adult ASM treated with media only was used as a positive control. Data are presented as mean ± SEM, n= 3-4 samples.

Figure 2. Effect of TLR agonists on TLR3 and TLR4 and adaptor molecule expression. Cells were treated with media only (control), TNFα, LPS, or Poly (I:C) for 6 or 24 h. (A) TNFα and Poly (I:C) increased TLR3 mRNA expression after 6 h. (B) No differences were observed in TLR4 mRNA expression after 6 h when cells were exposed to either TNFα or Poly (I:C). (C) TNFα and Poly (I:C) increased TLR3 and TLR4 protein expression after 24 h. (D) There was a notable increase in MyD88 TLR adaptor molecule mRNA expression with cells exposed to TNFα and Poly (I:C) treatments, but no changes in MD2, TRIF, or CD14. Data are presented as mean ± SEM, n= 3-6 samples, p<0.05. * indicates significant difference from control.

Figure 3. Effects of increasing concentrations of Poly (I:C) and R848 on CCL5 and IL-8 secretion. Fetal ASM were treated with media only (control), Poly (I:C) or R848 0.01, 0.1, 0.5, 1 µg/mL respectively for 24 h. Poly (I:C), but not R848 increased CCL5 and IL-8 secretion in a dose response manner and treatment of 1 µg/mL resulted in the most significant increase in CCL5 (A) and IL-8 (B) secretion. Data are presented as mean ± SEM, n= 3-4 samples, p<0.05. * indicates significant difference from control.

Figure 4. Effects of Poly (I:C) on CCL5 and IL-8 expression and secretion. Fetal ASM were treated with media only (control), TNFα, LPS, or Poly (I:C) for 6 or 24 h. TNFα and Poly (I:C), but not LPS, increased (A) CCL5 and (B) IL-8 mRNA expression. (C) TNFα and Poly (I:C), but not LPS increased CCL5 secretion. (D) TNFα, Poly (I:C), and LPS all significantly increased IL-8
secretion. Data are presented as mean ± SEM, n= 3-5 samples, p<0.05. * indicates significant difference from control.

**Figure 5.** Effect of a TLR3 antagonist on Poly (I:C)-induced CCL5 and IL-8 expression. Fetal ASM were pre-treated with a TLR3 antagonist, CU CPT 4a, for 1 hour and subsequently treated with media only (control) or Poly (I:C) for 24 h. Pre-treatment with TLR3 antagonist significantly decreased (A) CCL5 and (B) IL-8 secretion induced by Poly (I:C). Data are presented as mean ± SEM, n=4 samples, p<0.05. * indicates significant difference from control. † indicates significant difference between Poly (I:C) and TLR3 Antagonist+Poly (I:C) treatments.

**Figure 6.** Activation of ERK and p38 phosphorylation by Poly (I:C). Fetal ASM were treated with media only (control) or Poly (I:C) for 0-60 min. Poly (I:C) increased phosphorylation of (A) p38 and (B) ERK, but not (C) JNK. Data are presented as mean ± SEM, n=4 samples, p<0.05. * indicates significant difference from control.

**Figure 7.** Activation of ERK and p38 phosphorylation by Poly (I:C). Adult ASM were treated with media only (control) or Poly (I:C) for 0-15 min. Poly (I:C) increased phosphorylation of (A) p38 and (B) but not ERK, and also increased phosphorylation of (C) JNK. Data are presented as mean ± SEM, n=5 samples, p<0.05. * indicates significant difference from control.

**Figure 8.** Nuclear translocation of the p50 and p65 NFκB subunits. (A) In human fetal ASM and in (B) human adult ASM p50, p65 and TATA-box binding protein (TBP) in nuclear extracts, in addition to p50, p65, and GAPDH in cytoplasm extracts were respectively measured by Western blot showing that TNFα and Poly (I:C) promoted nuclear translocation of the p50 and p65 NFκB subunits in both human fetal and adult ASM. There was no global increase in either p50 or p65.
expression in the cytoplasm fraction. Data are presented as mean ± SEM, n=4-7 samples, p<0.05. * indicates significant difference from control.

**Figure 9.** Effect of ERK, p38, and NFkB inhibition on Poly (I:C)-induced CCL5 and IL-8 expression. Fetal ASM were pre-treated with ERK, p38 or NFkB inhibitor for 1 hour and subsequently treated with media only (control) or Poly (I:C) for 24 h. (A) Pre-treatment with ERK and NFkB inhibitors, but not p38 inhibitor decreased CCL5 secretion induced by Poly (I:C). (B) Pre-treatment with ERK and NFkB inhibitors, but not p38 inhibitor attenuated IL-8 secretion induced by Poly (I:C). Data are presented as mean ± SEM, n=5-6 samples, p<0.05. * indicates significant difference from control. † indicates significant difference of ERK inhibitor or NFkB inhibitor compared to Poly (I:C) only.
Table 1. Human primers used for Quantitative real-time PCR.

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<tr>
<th>Gene</th>
<th>Forward Primer</th>
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Toll-like receptor, Inflammation, Lung, Development