

Toll-like receptor 2, 3, and 4 expression and function in human airway smooth muscle

Maria B. Sukkar, PhD,^a Shaoping Xie, PhD,^a Nadia M. Khorasani, BSc,^a Onn Min Kon, MD,^b Rex Stanbridge, MD,^b Razao Issa, PhD,^a and Kian Fan Chung, MD^a *London, United Kingdom*

Background: Host defense against microbial pathogens is elicited through the innate immune system by means of Toll-like receptors (TLRs). Airway smooth muscle cells (ASMCs) display proinflammatory and immunomodulatory functions. ASMCs might participate in airway inflammatory responses associated with innate immune activation.

Objectives: We determined the effects of cytokines, TLR ligands, and corticosteroids on TLR expression and function in human ASMCs.

Methods: Real-time PCR and flow cytometry were used to assess TLR mRNA and protein expression, respectively. ASMCs were stimulated with TLR ligands, and chemokine release was measured by means of ELISA.

Results: ASMCs expressed TLR1 to TLR10 mRNA, and TLR2 and TLR3 protein expression was demonstrated. TNF- α and double-stranded RNA (dsRNA; TLR3 ligand) were potent inducers of TLR2 and TLR3 mRNA expression, and both stimuli had additive or synergistic effects with IFN- γ on TLR2 and TLR4, but not TLR3, mRNA expression. Peptidoglycan (TLR2 ligand) and LPS (TLR4 ligand) weakly enhanced TLR2 mRNA expression. Peptidoglycan, dsRNA, and LPS induced IL-8 and eotaxin release, with dsRNA being most potent. dsRNA also modulated cytokine-induced chemokine release in a differential manner. Dexamethasone inhibited cytokine- and ligand-induced TLR2, TLR3, and TLR4 expression and chemokine release. However, dexamethasone potentiated TLR2 expression induced by combined IFN- γ and TNF- α stimulation.

Conclusion: Expression of TLR2, TLR3, and TLR4 is regulated by cytokines and TLR ligands, and their activation mediates chemokine release in ASMCs.

Clinical implications: Proinflammatory responses mediated by activation of pathogen-recognition receptors in ASMCs might contribute to infectious exacerbations of airway inflammatory conditions, such as asthma and chronic obstructive pulmonary disease. (*J Allergy Clin Immunol* 2006;118:641-8.)

Key words: Toll-like receptor, human airway smooth muscle, double-stranded RNA, peptidoglycan, lipopolysaccharide, IFN- γ , TNF- α , eotaxin, IL-8, corticosteroid

Toll-like receptors (TLRs) are a class of pathogen-recognition receptors that mediate recognition of pathogen-associated molecular patterns, which are conserved molecular structures found in microbes.¹ Infections of the respiratory tract by various pathogens, including bacteria, viruses, and fungi, are a frequent cause of disease exacerbation in inflammatory airway conditions, such as asthma and chronic obstructive pulmonary disease. Thus activation of TLRs in the airways might be a key event contributing to infectious exacerbations in airway inflammatory disease.^{2,3}

Currently, there are 11 known mammalian TLRs (TLR1-TLR11) that recognize distinct pathogen-associated molecular patterns found in bacteria, fungi, viruses, helminths, and protozoa. TLR2 and TLR4 recognize bacterial cell-wall components, such as peptidoglycan (PGN) and LPS, respectively, whereas TLR3 recognizes the viral replicative intermediate double-stranded RNA (dsRNA).¹ TLRs are also activated by endogenous ligands generated at sites of tissue injury or inflammation that represent “danger signals” to the host.⁴

TLRs signal through one or more of 4 adaptor proteins to activate downstream kinase cascades that lead to activation of nuclear factor κ B, mitogen-activated protein kinases, phosphoinositide 3-kinase, interferon-regulatory factor 3, and interferon-regulatory factor 7, leading to the production of proinflammatory cytokines, chemokines, type I interferons, hematopoietic factors, acute-phase proteins, and antimicrobial factors.⁵

Very little is known about the expression and function of TLRs in airway structural cells, such as airway smooth muscle cells (ASMCs). ASMCs are implicated in the orchestration of airway inflammatory responses by virtue of their capacity to express a diverse array of immunomodulatory molecules, including a number of cytokines, chemokines, cell adhesion molecules, and cell-surface immune receptors.^{6,7} In keeping with this immunologic capacity, TLR2 and TLR4 ligands amplify proinflammatory interactions between ASMCs and PBMCs by augmenting cytokine and chemokine production by these cells under coculture conditions.⁸ Furthermore, in mouse trachea TLR2, TLR3, and TLR4 ligands induce airway responsiveness to bradykinin.⁹

From ^athe Experimental Studies Group, National Heart and Lung Institute, Imperial College, and ^bSt Mary's Hospital.

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Reprint requests: Kian Fan Chung, MD, National Heart and Lung Institute, Imperial College, Dovehouse St, SW3 6LY, London, United Kingdom. E-mail: f.chung@imperial.ac.uk.

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Abbreviations used

ASMC: Airway smooth muscle cell
dsRNA: Double-stranded RNA
NOD: Nucleotide-binding oligomerization domain
PGN: Peptidoglycan
TLR: Toll-like receptor

We hypothesized that human ASMCs possess functional TLRs that mediate proinflammatory responses in ASMCs. Infectious exacerbations of airway inflammatory conditions are likely to involve simultaneous activation of TLRs by microbial pathogens and inflammatory mediators. Thus we studied the effects of TLR ligands, together with the proinflammatory cytokines IL-1 β , TNF- α , and IFN- γ , on TLR expression and ligand-induced chemokine release.

METHODS**Materials**

Recombinant human cytokines were purchased from R&D systems (Abingdon, United Kingdom). A synthetic oligodeoxynucleotide that contains CpG motifs mimicking bacterial DNA (5'-ggGGGACGATCGTCgggggG-3'; the small letters indicate phosphorothioate linkages, the capital letters indicate phosphodiester linkages 3' of the base, and the bold letters indicate CpG dinucleotides) and primers for TLR1 through TLR10,¹⁰ glyceraldehyde-3-phosphate dehydrogenase, and 18S were purchased from Sigma Genosys (Suffolk, United Kingdom). Double-stranded polyinosine-polycytidylic acid (dsRNA) was purchased from Amersham Biosciences (Bucks, United Kingdom). Loxoribine, a guanosine analog, and flagellin isolated from *Salmonella thyphimurium* were purchased from InvivoGen (San Diego, Calif). PGN and zymosan A from *Saccharomyces cerevisiae* and LPS from *Escherichia coli*, serotype 0111:B4, were purchased from Sigma (Paisley, United Kingdom).

ASMC culture and stimulation

Human bronchial tissue was obtained from lungs of patients undergoing lung transplantation or surgical resection for carcinoma, and ASMCs were established in culture, as described previously.¹¹ Confluent ASMCs at passages 3 through 8 were serum deprived for 24 hours in Dulbecco's modified Eagle's medium supplemented with 4 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, 2.5 μ g/mL amphotericin B, 1% insulin-transferrin-selenium-X, 1:100 nonessential amino acids, and 0.1% BSA. After serum deprivation, cells were incubated in fresh serum-free supplemented Dulbecco's modified Eagle's medium containing recombinant human cytokines, TLR ligands, or dexamethasone, as indicated in individual experiments.

Real-time PCR

Total RNA was isolated from ASMCs by using the RNeasy Mini Kit (Qiagen, West Sussex, United Kingdom) and reverse transcribed with random hexamers and AMV reverse transcriptase (Promega, Southampton, United Kingdom). cDNA was amplified by means of quantitative real-time PCR (Rotor Gene 3000; Corbett Research, Sydney, Australia) with SYBR Green PCR Master Mix Reagent (Qiagen). Data from the reaction were analyzed by using the

complementary computer software (Corbett Research) with a standard curve. The standard curves were used to determine relative expression for the gene of interest by diluting a sample (eg, 1:1, 1:10, and 1:100) and giving the relative concentration to 1, 0.1, and 0.01 for each detection. Relative quantitations of gene expression were normalized to glyceraldehyde-3-phosphate dehydrogenase or 18s.

Flow cytometric analysis

To determine cell-surface TLR expression, 4×10^4 cells fixed in 3.2% formaldehyde were preincubated in 50 μ L of staining buffer (0.5% BSA and 0.1% sodium azide in PBS) containing 10% human serum and 30 μ g of human IgG (Sigma) for 30 minutes on ice to block Ig receptors. Phycoerythrin-conjugated anti-human TLR2 (clone TL2.1), TLR3 (clone TLR3.7), TLR4 (clone HTA125), or the relevant phycoerythrin-conjugated isotype control antibodies (IgG_{2a,k} or IgG_{1,k}; 0.75 μ g each; eBioscience, San Diego, Calif) were then added, and the cells were incubated for a further 60 minutes on ice. Saponin, 0.5%, was included in the staining buffer to determine intracellular TLR expression. A FACScan flow cytometer (Becton Dickinson Immunocytometry Systems [BIDS], Oxford, United Kingdom) was used, and typically 10,000 events were acquired in the viable cell region of the forward light scatter/side light scatter plots. Analysis was performed with CELLQuest software (BIDS). The fluorescence signal was calculated as the geometric mean fluorescence intensity of the gated ASMC population.

Chemokine ELISA assays

Measurement of IL-8 and eotaxin in culture supernatants was performed by using ELISA DuoSets (R&D Systems), according to the manufacturer's instructions.

Data analysis

Data are presented as means $\pm$ SEM. Comparison between groups was performed by using paired *t* tests. A *P* value of less than .05 was taken as statistically significant.

RESULTS**TLR1 through TLR10 mRNA expression under basal conditions and after stimulation with cytokines and TLR ligands**

Using quantitative RT-PCR, we show that ASMCs express TLR1 through TLR10 mRNA under basal conditions. Threshold cycle numbers for TLR2, TLR3, and TLR6 were less than 25, whereas those for TLR1, TLR5, TLR7, TLR9, and TLR10 were equal to or less than 30. TLR4 and TLR8 both had threshold cycle numbers of less than 35 (Fig 1, A). Stimulation of ASMCs with TNF- α (10 ng/mL) for 24 hours increased TLR1 through TLR7 and TLR10 mRNA expression between 2- and 30-fold. IL-1 β (10 ng/mL) was a very weak inducer of TLR mRNA expression (Fig 1, B).

Stimulation with IL-1 β , TNF- α , and/or IFN- γ (10 ng/mL each) for 24 hours has differential effects on TLR2, TLR3, and TLR4 mRNA expression. IL-1 β increased TLR3 mRNA expression by approximately 4-fold but not TLR2 or TLR4 mRNA expression. IFN- γ and TNF- α increased the expression of all 3 receptors, with TNF- α being more effective than IFN- γ at the concentrations used. Combined IFN- γ and TNF- α had a marked synergistic effect on TLR2, an additive effect on TLR4, and,

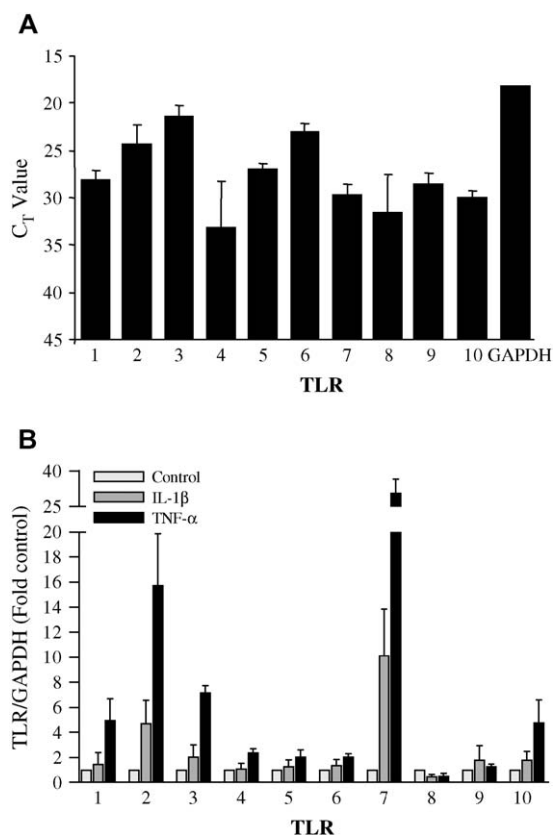


FIG 1. TLR mRNA expression in ASMCs. TLR1 through TLR10 mRNA expression under basal conditions (**A**) or after stimulation with IL-1 β or TNF- α (10 ng/mL) for 24 hours (**B**) is shown. Note that the scale for threshold cycle (C_T) is inverted, so that higher histogram bars represent higher levels of mRNA expression. Bars represent means $\pm$ SEM of 3 ASMC donors. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

in direct contrast, an inhibitory effect on TLR3 mRNA expression (Fig 2).

We also examined TLR2, TLR3, and TLR4 mRNA expression after a 24-hour stimulation period with TLR ligands. The TLR2 ligand PGN (10 μ g/mL) increased TLR2 mRNA expression by 4-fold but had no significant effect on TLR3 or TLR4 mRNA expression (Fig 3). Zymosan (100 μ g/mL), which is also a TLR2 ligand, did not have any consistent effect (Fig 3). The TLR3 ligand dsRNA (25 μ g/mL) significantly increased TLR2, TLR3, and TLR4 mRNA expression by 20-, 10-, and 5-fold, respectively (Fig 2). The TLR4 ligand LPS (10 μ g/mL) increased TLR2 mRNA expression by 4-fold and also had a 1.5-fold stimulatory effect on TLR3 mRNA. Interestingly, LPS had no effect on TLR4 mRNA expression (Fig 3). CpG-containing DNA (3 μ g/mL), a TLR9 ligand, had no effect on TLR2, TLR3, or TLR4 mRNA expression (Fig 3).

Other TLR ligands investigated, including the TLR5 ligand flagellin (10-100 ng/mL) and the TLR7 ligand loxoribine (100 μ M) had negligible effects on TLR2, TLR3, and TLR4 mRNA expression (data not shown).

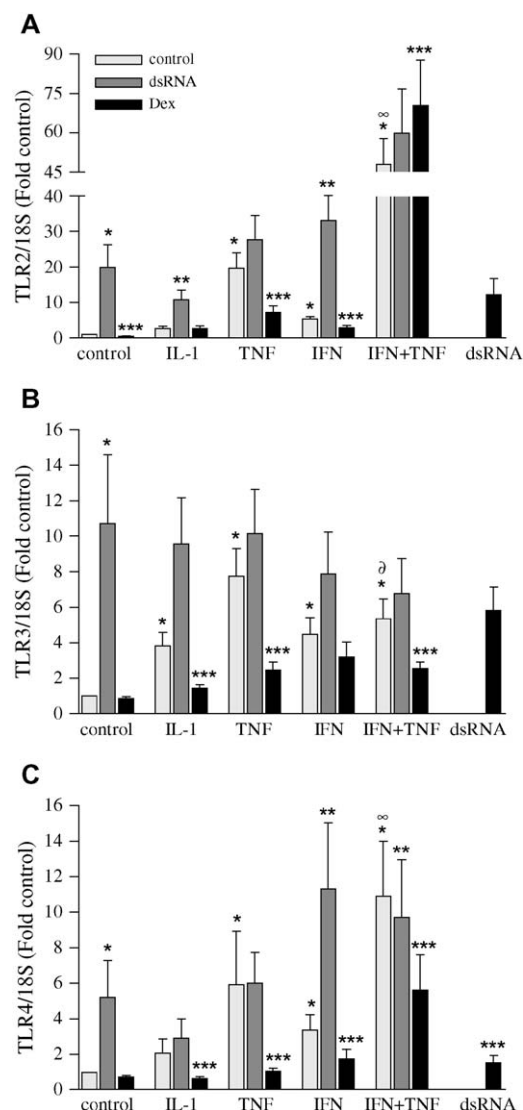


FIG 2. Effects of cytokines, dsRNA, and dexamethasone (Dex) on TLR2 (**A**), TLR3 (**B**), and TLR4 (**C**) mRNA. Cells were treated with cytokines (10 ng/mL each) or dsRNA (25 μ g/mL) $\pm$ dsRNA (25 μ g/mL) or dexamethasone (1 μ M) for 24 hours. Bars represent means $\pm$ SEM of 8 ASMC donors. * P < .05 versus unstimulated control; ** P < .05 versus dsRNA only; *** P < .05 versus stimulated cells in the absence of dexamethasone; ∞ P < .05 versus cells treated with IFN- γ or TNF- α only; ∂ P < .05 versus cells treated with TNF- α only.

Flow cytometric analysis of TLR2, TLR3, and TLR4 protein expression

Under basal conditions, there was negligible TLR2 protein expression. Stimulation with IFN- γ and TNF- α (10 ng/mL each) or dsRNA (25 μ g/mL) for 48 hours increased specific TLR2 staining in nonpermeabilized and permeabilized cells (Fig 4, A and C). Higher levels of specific staining in permeabilized versus nonpermeabilized cells (under either of the stimulation conditions used) indicate the presence of both surface and intracellular TLR2 expression. Under basal conditions, we observed

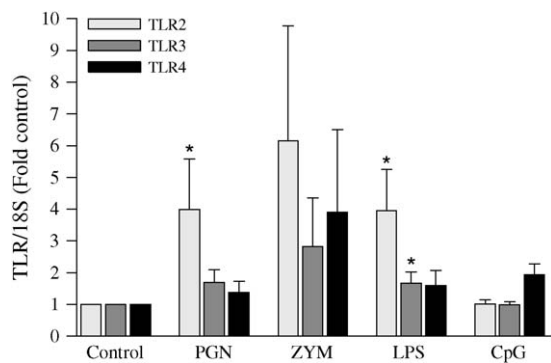


FIG 3. Effect of TLR ligands on TLR2, TLR3, and TLR4 mRNA. Cells were treated with PGN (10 μ g/mL), zymosan (ZYM; 100 μ g/mL), dsRNA (25 μ g/mL), LPS (10 μ g/mL), or CpG (3 μ g/mL) for 24 hours. Bars represent means $\pm$ SEM of 6 to 10 ASMC donors. * P < .05 versus control.

a 2.5-fold increase in mean fluorescence intensity of specific TLR3 staining in both nonpermeabilized and permeabilized cells. This pattern of staining suggests surface but not intracellular expression of TLR3 because there are no additional antibody targets on cell permeabilization. This is consistent with the fact that we observe increased TLR3 expression after cytokine stimulation in nonpermeabilized cells. dsRNA did not significantly increase surface TLR3 expression (Fig 4, B and C). TLR4 protein was not detectable in nonpermeabilized or permeabilized cells under basal or stimulated treatment conditions (data not shown).

Effect of TLR ligands on IL-8 and eotaxin release

Cells were treated with various TLR ligands or with IL-1 β (10 ng/mL) or TNF- α (10 ng/mL) for comparison, and IL-8 and eotaxin levels were measured in culture supernatants after 24 hours. Stimulation of cells with PGN (10 μ g/mL), dsRNA (25 μ g/mL), or LPS (10 μ g/mL) induced significant release of both chemokines (Fig 5). At low concentrations (0.1 or 1 μ g/mL), LPS had no significant effect on IL-8 or eotaxin release (data not shown). Stimulation of cells with higher concentrations of dsRNA (50 or 100 μ g/mL) did not further increase IL-8 or eotaxin release (data not shown). Other TLR ligands investigated, including the TLR2 ligand zymosan (100 μ g/mL), the TLR5 ligand flagellin (10-100 ng/mL), the TLR7 ligand loxoribine (100 μ M), and CpG-containing DNA (3-30 μ g/mL) did not induce significant IL-8 or eotaxin release (data not shown).

Interaction of dsRNA and proinflammatory cytokines on TLR expression and function

We determined whether simultaneous activation of ASMCs with dsRNA and proinflammatory cytokines has additive or synergistic effects on TLR expression and chemokine release. TLR2 and TLR4 mRNA expression was regulated similarly. IL-1 β attenuated dsRNA-induced TLR2 mRNA expression and also tended to suppress dsRNA-induced TLR4 mRNA expression. In contrast,

combined stimulation with dsRNA and IFN- γ leads to a synergistic augmentation of TLR2 and TLR4 mRNA expression. Stimulation with both IFN- γ and TNF- α in the presence of dsRNA, however, did not further potentiate the level of TLR2 or TLR4 mRNA expression induced by this cytokine combination alone. TNF- α did not modulate dsRNA-induced TLR2 or TLR4 mRNA expression (Fig 2, A and C). The effects of combined cytokine-dsRNA stimulation on TLR3 mRNA expression were in clear contrast to those observed for TLR2 and TLR4. IL-1 β and TNF- α had no modulatory effects, whereas IFN- γ , either alone or in combination with TNF- α , tended to suppress dsRNA-induced TLR3 mRNA expression (Fig 2, B).

Stimulation with dsRNA in the presence of IL-1 β or TNF- α synergistically potentiated IL-8 release. In direct contrast, however, dsRNA had a significant inhibitory effect on IL-1 β - and TNF- α -induced eotaxin release. IFN- γ alone did not induce IL-8 or eotaxin release. IFN- γ , however, did have a small inhibitory effect on dsRNA-induced eotaxin release and also tended to suppress dsRNA-induced IL-8 release (Fig 6).

Effects of corticosteroids on TLR2, TLR3, and TLR4 expression and function

Cells were pretreated with dexamethasone (1 μ M) for 2 hours before stimulation with cytokines (10 ng/mL each) or dsRNA (25 μ g/mL) for 24 hours. The effects of dexamethasone treatment on TLR2 mRNA expression were stimulus dependent. Dexamethasone inhibited TNF- α - or IFN- γ -induced TLR2 mRNA expression but potentiated TLR2 mRNA expression in the presence of combined IFN- γ and TNF- α stimulation (Fig 2, A). In contrast, dexamethasone inhibited TLR3 and TLR4 mRNA expression under all cytokine stimulation conditions examined (Fig 2, B and C). The capacity for dsRNA to upregulate TLR2, TLR3, and TLR4 mRNA expression was generally suppressed in the presence of dexamethasone, but significant inhibition was only observed with respect to TLR4 mRNA (Fig 2).

We also examined the effect of dexamethasone on TLR2 and TLR3 protein expression induced by combined IFN- γ and TNF- α (10 ng/mL each) or by dsRNA (25 μ g/mL) stimulation for 48 hours. Consistent with gene expression data, we observed stimulatory and inhibitory effects of dexamethasone on TLR2 protein induced by IFN- γ and TNF- α and dsRNA, respectively (Fig 4, A). Also consistent with gene expression data, dexamethasone inhibited cytokine-induced TLR3 protein (Fig 4, B).

Dexamethasone inhibited both IL-8 and eotaxin release induced by PGN (10 μ g/mL), LPS (10 μ g/mL), and dsRNA (25 μ g/mL). Dexamethasone also inhibited IL-1 β - or TNF- α -induced IL-8 and eotaxin release, as has been previously shown (Fig 5).

DISCUSSION

We have shown that human ASMCs express TLR1 through TLR10 mRNA in the basal state and that, with the

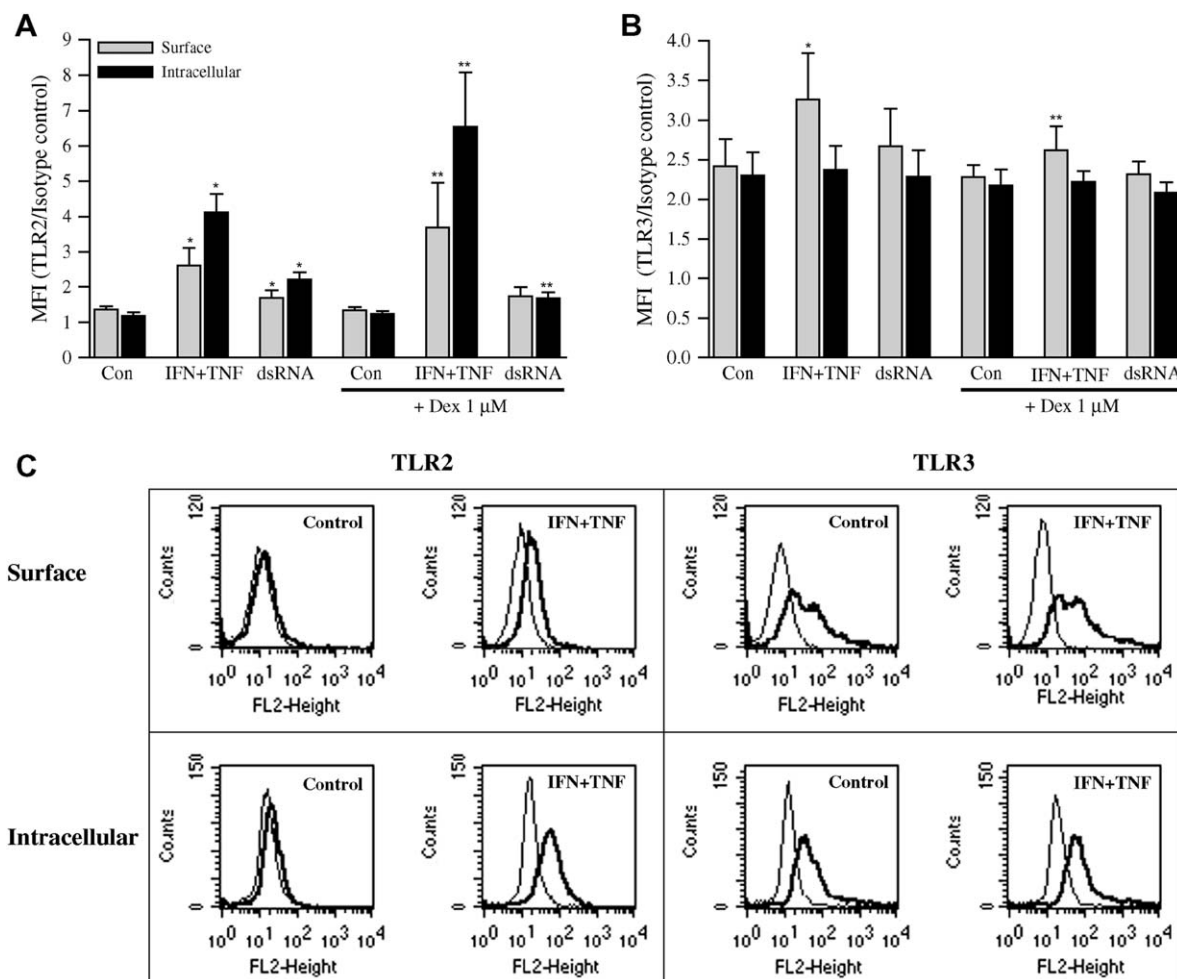


FIG 4. Flow cytometric analysis of TLR2 (**A**) and TLR3 (**B**) expression in ASMCs. Cells were treated with IFN- γ and TNF- α (10 ng/mL each) or dsRNA (25 μ g/mL) $\pm$ dexamethasone (Dex) for 48 hours. Bars represent means $\pm$ SEM of 7 to 9 ASMC donors. **C**, Representative histograms: thin lines represent cells stained with isotype control antibodies, and bold lines represent cells stained with TLR2 or TLR3 antibodies. * P < .05 versus appropriate control (Con); ** P < .05 versus appropriate control in the absence of dexamethasone. MFI, Mean fluorescence intensity.

exception of TLR8 and TLR9, their expression is increased by TNF- α . In more detailed studies we demonstrate that IFN- γ , TNF- α , and the TLR3 ligand dsRNA have significant regulatory effects on TLR2, TLR3, and TLR4 expression. Stimulation of ASMCs with TLR2, TLR3, or TLR4 ligands leads to IL-8 and eotaxin release, and dsRNA differentially regulates chemokine release, causing inhibition or potentiation of cytokine-induced eotaxin and IL-8 release, respectively. Finally, we show that TLR expression and function is regulated by corticosteroids. Overall, dexamethasone has a suppressive effect on cytokine- and ligand-induced TLR2, TLR3, and TLR4 expression and chemokine release. However, dexamethasone increased TLR2 expression induced by combined IFN- γ and TNF- α stimulation.

Basal expression of TLR4 mRNA was relatively low in comparison with that of other TLRs, and stimulated levels were comparatively lower than those observed for TLR2

and TLR3. The lack of detection of TLR4 protein by means of flow cytometry might be due to the lower expression of TLR4. TLR2 and TLR3 protein expression was readily detectable, but there was no concordance in the regulation of TLR3 mRNA and protein by cytokines and dsRNA. This might indicate posttranslational control of TLR3, which warrants further investigation.

TLRs are not completely restricted in their expression patterns and show different expression patterns in different cell types. We provide evidence for both intracellular and cell-surface expression of TLR2, which is consistent with evidence of both surface and intracellular expression in other structural cell types.¹²⁻¹⁵ TLR3 is commonly expressed as an intracellular receptor^{16,17}; however, more recently, cell-surface expression has been demonstrated in several cell types, including fibroblasts,¹⁸ microglia and astrocytes,¹⁹ $\gamma\delta$ T cells,²⁰ and various epithelial cell types, including primary human tracheobronchial epithelial

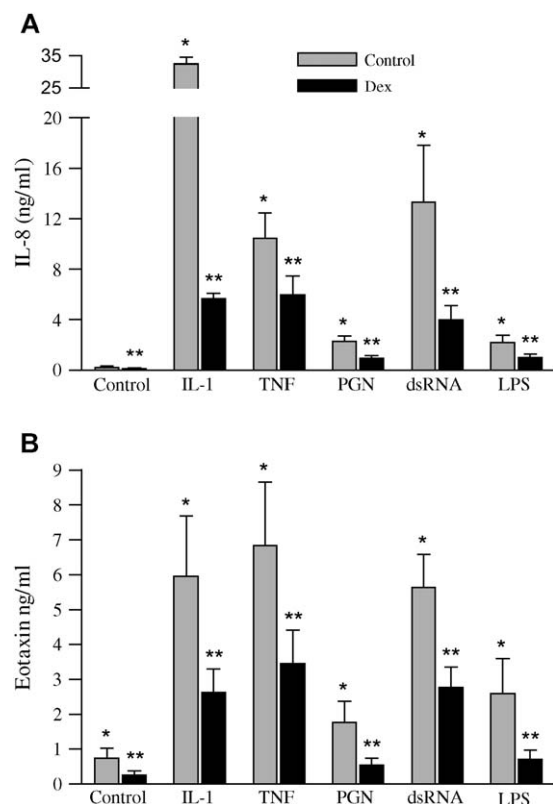


FIG 5. Effect of cytokines and TLR ligands on IL-8 (A) and eotaxin (B) release. Cells were treated with IL-1 β (10 ng/mL), TNF- α (10 ng/mL), PGN (10 μ g/mL), dsRNA (25 μ g/mL), or LPS (10 μ g/mL) $\pm$ dexamethasone (Dex; 1 μ M) for 24 hours. Bars represent means $\pm$ SEM of 6 to 8 ASMC donors. * P < .05 versus unstimulated cells. ** P < .05 versus cells in the absence of dexamethasone.

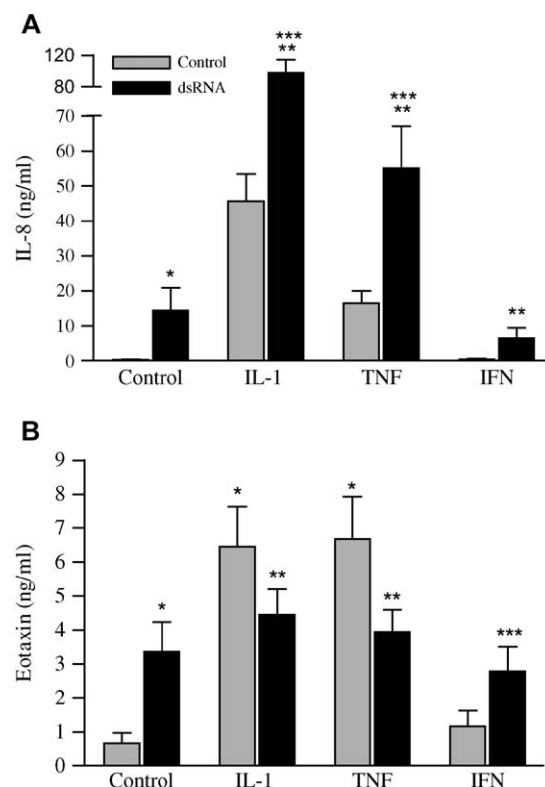


FIG 6. Effects of dsRNA on cytokine-induced IL-8 (A) and eotaxin (B) release. Cells were treated with IL-1 β (10 ng/mL), TNF- α (10 ng/mL) or IFN- γ (10 ng/mL) $\pm$ dsRNA (25 μ g/mL) for 24 hours. Bars represent means $\pm$ SEM of 6 to 8 ASMC donors. * P < .05 versus unstimulated cells; ** P < .05 versus stimulated cells in the absence of dsRNA; *** P < .05 versus cells stimulated with dsRNA only.

cells, as well as airway and intestinal epithelial cell lines.^{10,21,22} Our finding that ASMCs express surface but not intracellular TLR3 is intriguing, and the functional relevance of this expression pattern should be an important area of further investigation.

We evaluated several TLR ligands for their capacity to induce TLR2, TLR3, and TLR4 expression and to stimulate IL-8 and eotaxin release in ASMCs. Ligands that recognize TLR5, TLR7, and TLR9 had negligible effects on TLR mRNA expression and chemokine release. At the specific concentrations of TLR ligands examined, dsRNA was most potent with respect to induction of TLR2, TLR3, and TLR4 mRNA expression and chemokine release. The time course of TLR2, TLR3, and TLR4 mRNA induction by dsRNA was similar, indicating that induction of one TLR did not lead to the subsequent induction of another TLR (M. B. Sukkar, unpublished observation).

Potent effects of dsRNA on chemokine release have been reported in other structural cells.^{10,23,24} The TLR2 ligand PGN and the TLR4 ligand LPS weakly stimulated TLR2 mRNA expression and induced comparable levels of IL-8 and eotaxin release. Others have previously shown LPS to be a weak stimulator of IL-8 release in human ASMCs,⁸ whereas stimulatory effects of PGN on

chemokine release have been demonstrated in synovial fibroblasts.^{25,26} Zymosan is a cell-wall component derived from yeast that also activates TLR2. Unlike PGN, zymosan had no consistent effect on TLR2 mRNA expression and did not stimulate chemokine release. In epithelial cells of the genitourinary tract, zymosan induces production of IL-8 at 10 μ g/mL, a 10-fold lower concentration than used here, and thus it is unlikely that the lack of effect was due to a suboptimal concentration of zymosan.^{15,27} Lack of a functional response to zymosan is in agreement with the results of another study, in which Pam3CSK4, a synthetic TLR2 agonist, had no effect on IL-8 release in human ASMCs.⁸

The response to PGN we observed might be explained by its activation of nucleotide-binding oligomerization domain 1 (NOD1) and NOD2, which are intracellular pathogen-recognition receptors that recognize distinct peptide motifs of the PGN molecule.²⁸ TLR2 was initially characterized as the receptor for PGN, but it has since been shown that TLR2 activation by commercial PGN is mediated by copurified lipoproteins and lipoteichoic acids.²⁹ Furthermore, a greater induction of cytokine production by commercial PGN compared with purified PGN was due to synergistic interactions between NOD2 and TLR2.³⁰ A

similar mechanism is likely to underlie the chemokine production we observed in response to commercial PGN.

We show that TNF- α and IFN- γ are important regulators of ASMC TLR expression. In a recent study Morris et al⁸ demonstrated TLR2 and TLR4 mRNA expression in ASMCs after stimulation with IL-1 β or TNF- α . In their study IL-1 β and TNF- α had comparable effects on TLR2 mRNA, whereas IL-1 β was more effective than TNF- α in stimulating TLR4 mRNA expression. This is in direct contrast to our findings, in which TNF- α was consistently more potent than IL-1 β in stimulating TLR2 and TLR4 mRNA expression. The findings of Morris et al reflect data obtained from a single ASMC donor purchased commercially, whereas our findings are representative of at least 15 known ASMC donors.

A complex and differential interplay between dsRNA and proinflammatory cytokines on TLR expression and chemokine release is also demonstrated. Of particular interest is the interaction between dsRNA and IFN- γ because this was associated with a synergistic enhancement in TLR2 and TLR4 expression, suggesting a mechanism for amplification of TLR-mediated inflammatory responses in ASMCs. dsRNA also had potent modulatory effects on IL-1 β - and TNF- α -stimulated chemokine release. Increased IL-8 production with concomitant inhibition of eotaxin production presents a scenario that favors neutrophilic over eosinophilic inflammation and is consistent with the fact that respiratory viral infections are associated with increased neutrophil infiltration of the airways.³

Apart from their inhibitory effects on inflammatory gene expression, other therapeutic benefits of glucocorticoids might be derived from their ability to spare or even enhance innate immune responses and thereby prevent susceptibility to pathogenic microorganisms that can trigger disease exacerbation.³¹ Glucocorticoids can increase TLR2 expression in airway epithelial cells, either directly or by acting in synergy with proinflammatory cytokines, such as IL-1 β , TNF- α , and IFN- γ .³²⁻³⁴ We observed dexamethasone-mediated potentiation of TLR2 mRNA and protein expression induced by IFN- γ and TNF- α . Interestingly, dexamethasone inhibited PGN-induced chemokine production, suggesting inhibition of TLR2 functional responses. In addition, dexamethasone had a general inhibitory effect on TLR3 and TLR4 mRNA expression and chemokine production induced by their respective ligands. Thus on balance, corticosteroids appear to have an overall suppressive effect on TLR responses in ASMCs. Whether this further aggravates infectious exacerbations of airway inflammation is an important area of future investigation.

Our studies were performed in ASMCs isolated from the lungs of patients undergoing surgery for carcinoma. Although cells were isolated from "macroscopically normal" areas of lung that are clearly away from cancerous tissue, we cannot assume that ASMCs from otherwise healthy subjects would show a similar pattern of TLR expression and activation. It is therefore important that these studies are performed in ASMCs obtained from

healthy individuals. Nevertheless, the demonstration that ASMCs can express almost the entire repertoire of TLRs, combined with evidence of a functional response to TLR ligands, particularly TLR2 and TLR3, supports an immunomodulatory role for ASMCs in airway inflammatory responses. It remains to be seen whether TLR activation in ASMCs is linked to further pathology or has a protective effect.

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