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Regulation of TLR2 Expression and Function in Human Airway Epithelial Cells

Tamene Melkamu · Diane Squillace · Hirohito Kita · Scott M. O'Grady

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Abstract Toll-like receptor (TLR1–6) mRNAs are expressed in normal human bronchial epithelial cells with higher basal levels of TLR3. TLR2 mRNA and plasma membrane protein expression was enhanced by pretreatment with Poly IC, a synthetic double-stranded RNA (dsRNA) known to activate TLR3. Poly IC also enhanced mRNA expression of adaptor molecules (MyD88 and TIRAP) and coreceptors (Dectin-1 and CD14) involved in TLR2 signaling. Additionally, mRNA expression of TLR3 and dsRNA-sensing proteins MDA5 and RIG-I increased following Poly IC treatment. In contrast, basal mRNA expression of TLR5 and TLR2 coreceptor CD36 was reduced by 77% and 62%, respectively. ELISA of apical and basolateral solutions from Poly IC-stimulated monolayers revealed significantly higher levels of IL-6 and GM-CSF compared with the TLR2 ligand PAM_3CSK_4. Pretreatment with anti-TLR2 blocking antibody inhibited the PAM_3CSK_4-induced increase in IL-6 secretion after Poly IC exposure. An increase in IL-6 secretion was also observed in cells stimulated with *Alternaria* extract after pretreatment with Poly IC. However, IL-6 secretion was not stimulated by zymosan or lipothechoic acid (LTA). These data demonstrated that upregulation of TLR2 following exposure to dsRNA enhances functional responses of the airway epithelium to certain (PAM_3CSK_4), but not all (zymosan, LTA) TLR2 ligands and that this is likely due to differences in coreceptor expression.

Keywords Toll-like receptor · Innate immunity · IL-6 · GM-CSF · *Alternaria alternata*

T. Melkamu
Department of Veterinary Population Medicine, University of Minnesota, St. Paul, MN 55108, USA

D. Squillace · H. Kita
Departments of Immunology and Medicine, Division of Allergic Diseases, Mayo Clinic College of Medicine, Rochester, MN 55905, USA

S. M. O'Grady (✉)
Departments of Integrative Biology and Physiology and Animal Science, University of Minnesota, 495 Animal Science/Veterinary Medicine Building, 1988 Fitch Ave, St. Paul, MN 55108, USA
e-mail: ograd001@umn.edu

Introduction

The airway epithelium, in addition to serving as a physical barrier against the entry of allergens and pathogens, orchestrates inflammatory and immune responses (Fan et al. 2003; Martin and Frevert 2005). The epithelium triggers these responses by recognizing pathogen-associated molecular patterns (PAMPS) through the sentinel action of Toll-like receptors (TLRs) (Bals and Hiemstra 2004; Hertz et al. 2003; Qureshi and Medzhitov 2003). TLRs are transmembrane proteins with an extracellular domain consisting of a 19–25 leucine-rich repeat (LRR) sequence that is involved in the recognition of a variety of pathogens. These receptors recognize and mediate intracellular signals for a wide range of microbial components: TLR1 (in association with TLR2) for tri-acyl lipopeptides, TLR2 for bacterial lipoproteins (BLPs) and peptidoglycans from gram-positive bacteria, TLR3 for double-stranded RNA (dsRNA) and its synthetic analogue polyinosinic polycytidylic acid (Poly IC), TLR5 for flagellin, TLR6 (in association with TLR2) for di-acyl lipopeptides, TLR7 and TLR8 for single-stranded RNA and TLR9 for nonmethylated CpG DNA. It is now believed that the combined activation of TLRs can result in complementary,

synergistic or antagonistic effects that modulate innate and adaptive immunity (Basu and Fenton 2004; Trinchieri and Sher 2007). Some TLRs function as homo- or heterodimers. The variety of ligands detected by TLR2, e.g., is enhanced by heterodimerization with TLR1 or TLR6, while TLR4 appears to form homomers (Lee et al. 2004). Important functional interactions of TLRs with non-TLR molecules are also well known, e.g., TLR4 with MD-2 and CD14 (Lee et al. 2004). TLR2 has been shown to functionally interact with structurally unrelated receptors like Dectin-1, CD14 and CD36 to recognize fungal products and gram-positive bacteria (Dillon et al. 2006; Takeda and Akira 2005). TLR2 has been shown to be selectively expressed at the apical surface of airway epithelial cells in response to challenge with *Pseudomonas aeruginosa* or *Staphylococcus aureus* in vitro (human cells) and in mice, indicating the importance of TLRs in protecting the airways from inhaled pathogens, particularly cystic fibrosis (CF) pathogens (Greene et al. 2005; Muir et al. 2004).

TLRs possess a conserved cytoplasmic region of approximately 200 amino acids, known as the Toll and interleukin-1 receptor (TIR) domain, which is crucial for receptor signaling (Akira 2003a; Beutler 2004). With the exception of TLR3, all TLRs share a common adaptor molecule, myeloid differentiation primary response gene 88 (MyD88), which also contains a TIR domain (Basu and Fenton 2004; Akira 2003b). In addition to MyD88, two major adaptor proteins exist: the TIR domain-containing protein (TIRAP, also named Mal), which is required for TLR2- and TLR4-dependent downstream signaling, and the TIR domain-containing adapter-inducing interferon-β (TRIF) or toll-like receptor adaptor molecule 1 (TICAM-1), which is essential for TLR3 and MyD88-independent TLR4 signaling (Basu and Fenton 2004; Akira 2003b).

Exacerbation of airway inflammatory disease is likely to involve simultaneous or subsequent activation of TLRs by multiple microbial pathogens (Fransson et al. 2005; Proud and Chow 2006; Schleimer 2004). For instance, sensitization to fungal antigens during asthmatic reactions is exacerbated by viral respiratory infections (Proud and Chow 2006; Beasley et al. 1988; Gern 2004; Groskreutz et al. 2006; Kauffman and van der Heide 2003; Mallia and Johnston 2006; Tan 2005). A synergistic effect between virus- and allergen-induced airway inflammation has been proposed (Murray et al. 2004), but no definitive molecular mechanism has been described to explain the interactions between viruses and allergens and their respective receptors. TLR2, TLR4 and TLR6 have been implicated in activation of epithelial cells by fungal allergens (Mallia and Johnston 2006). Upregulation of TLR2, TLR3 and TLR4 has been reported in the nasal mucosa of patients with allergic rhinitis, indicating a role for multiple TLRs in allergic airway inflammation (Fransson et al. 2005). More recently, hog confinement organic dust, the exact component of which has not been identified, was shown to upregulate TLR2 in vitro in human airway epithelial cells and in vivo in mouse lung (Bailey et al. 2008).

High levels of TLR3 mRNA expression have been reported in ex vivo nasal mucosal samples from healthy human subjects and challenge with rhinoviruses (RVs) increased mRNA expression of TLR2, TLR3, TLR4, TLR7 and TLR8 but reduced mRNA expression of TLR5 (Avila et al. 2005). In addition to TLR3, another host innate immune response to dsRNA has been recently described that involves cytoplasmic recognition by the RNA helicases; namely, retinoid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) (Meylan and Tschopp 2006). Previous studies in primary small airway epithelial cells and airway smooth muscle cells showed that activation of TLR3 using a synthetic dsRNA (Poly IC) increases mRNA expression of TLR2 and TLR3 and suppresses mRNA expression of TLR5, suggesting that viral infection can differentially alter the epithelial sensitivity and response to fungal allergens, bacteria and other viruses (Ritter et al. 2005; Sukkar et al. 2006). These results are suggestive of a collaborative interaction between multiple TLRs and various coreceptors to regulate the immune response of the airway epithelium. Thus, the objective of the present study was to investigate the effects of enhanced TLR2 expression induced by exposure to Poly IC on the innate immune response of human airway epithelial cells.

Materials and Methods

Materials

Poly IC, a synthetic analogue of viral dsRNA (a known activator of TLR3, RIG-I and MDA5) and *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2-*RS*)-propyl]-[*R*]-Cys-[*S*]-Ser-[*S*]-Lys_4 trihydrochloride (PAM_3CSK_4), a synthetic bacterial lipopeptide (activator of TLR2/TLR1 heterologue) were obtained from InvivoGen (San Diego, CA). Zymosan, a fungal product from *Saccahromyces cerevisiae*, and lipoteichoic acid (LTA), from *Staphylococcus aureus* (activators of TLR2/TLR6 heterologue), were purchased from Sigma-Aldrich (St. Louis, MO). Extract of *Alternaria alternata*, a fungus that can induce acute asthmatic episodes, was prepared from cultures of *A. alternata* following the protocol described by Inouei et al. (2005). All reagents were freshly prepared for each experiment.

Cell Culture

Normal human bronchial epithelial (NHBE) cells were obtained from Clonetics (Walkersville, MD). These cells

were life span-extended by transfection with the catalytic subunit of the human telomerase gene (hTERT) as previously described (Palmer et al. 2006). Low-passage (p10–p20), life span-extended NHBE cells grown in monolayer culture under air-interface conditions were used. The cells were cultured in medium supplemented with defined growth factors and retinoic acid (0.1 μg/l) contained within the SingleQuot kit of bronchial epithelial cell growth medium (BEGM) provided by Clonetics. Cells were maintained in humidified incubators at 37°C and 5% CO_2.

Experimental Conditions and Collection of Fluid Samples and Cell Lysates

NHBE cells were grown on transwell filters in medium containing BEGM for 7 days and then maintained under air-interface conditions for 3 days. To evaluate the functional consequence of TLR2 upregulation, confluent NHBE cells were pretreated with 10 μg/ml of Poly IC for 24 h in additive-free medium and then treated for a second 24-h period with TLR2 ligands, including PAM_3CSK_4, zymosan, LTA and *Alternaria* extract. Apical and basolateral fluids were then collected, snap-frozen in liquid nitrogen and stored at −80°C until use. Cells on filters were then washed with bronchial epithelial basal medium (BEBM) and treated with buffer RLT (Qiagen, Valencia, CA), used for lysis of cells for RNA isolation. For Western blot identification of TLR2 and CD36 proteins, cells were lysed with cell lysis buffer containing protease inhibitor cocktail (both from Sigma-Aldrich).

Quantitative RT-PCR

Total RNA was extracted from the lysed cells using RNeasy (Qiagen) and treated with DNase I (Qiagen) according to the manufacturer's instructions. Single-stranded cDNA was prepared with 500 or 1,000 ng of RNA with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random primers. Quantitative RT-PCR (QRT-PCR) was performed using the TaqMan method and an Applied Biosystems (Foster City, CA) 7500 sequence detection system. Primer sets for the target genes were developed using Primer 3 software or taken from published work, and all sequences developed for this study are given in Table 1. Aliquots of cDNA equivalent to 25 ng of total RNA were used for QRT-PCR, which were carried out using SYBER green detection protocols from Stratagene (Wilmington, DE). Real-time fluorescence was measured for 45 cycles, and mRNA levels between treatment and controls were evaluated by comparing the cycle threshold (C_T) of target genes. Quantification of mRNA expression levels was normalized to the median expression of GAPDH. Specificity and level of the different mRNA transcripts were assessed by analysis of melting point dissociation curves or by evaluating the bands of PCR products resolved on agarose gels.

Fluorescence-Activated Cell Sorting

NHBE cells were grown to 70–80% confluence and exposed to 10 μg/ml of Poly IC for 24 h. Poly IC-treated and untreated control monolayers were dissociated with EDTA (0.5 mM). The cells were subsequently washed with fluorescence-activated cell sorting (FACS) solution (1% bovine serum albumin [BSA], 5 mM NaN_3 in PBS) and centrifuged (1,000 × *g*). Pelleted cells were resuspended in FACS solution and their density adjusted to 10^6/100 μl by hemocytometer counting. Resuspended cells (100 μl) were then added to a FACS tube and incubated in the dark on ice for 2 h with 20 μl of phycoerythrin (PE)-labeled mouse anti-human TLR2 antibody (clone TLR2.1, 12-9922-71; eBioscience, San Diego, CA) or 5 μl of the corresponding PE-labeled mouse immunoglobulin G2a isotype control antibody (clone eBM2a, 12-4724-81; eBioscience) as recommended by the manufacturer. Unlabeled cells served as controls for autofluorescence. Labeled and unlabeled cells were then washed, centrifuged, resuspended in 500 μl FACS solution and analyzed using a FACS Diva caliber flow cytometer and BD CellQuest software (Becton-Dickinson, San Jose, CA). The mean fluorescence intensity (MFI) of cells labeled with PE-conjugated mouse anti-human TLR2 antibody was reported for 10,000 events for Poly IC-treated and untreated control cells.

Western Blotting

Monolayers treated for 24 h with Poly IC (10 μg/ml) or PAM_3CSK_4 (1 μg/ml) and untreated control samples were suspended in lysis buffer to which a cocktail of protease inhibitors was added (Sigma-Aldrich). The protein content of the samples was determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Up to 55 μg of protein were loaded onto 10% Tris-glycine gels and run for 150 min at 125 V. The proteins were then transferred onto a PVDF membrane (Bio-Rad, Hercules, CA) for 2 h at 25 V. Adequate transfer of proteins was confirmed by staining membranes with BLOT-FastStain (Chemicon, Temecula, CA). Subsequently, membranes were blocked in 5% Blotto nonfat dry milk in Tris buffer containing 1% Tween-20 for 1 h and probed overnight with corresponding primary antibodies (TLR2 [C-19, sc-8690] at a dilution of 1:100, CD36 [SMø, sc-7309] at 1:200 dilution and β-actin [C4, sc-47778] at 1:1,000 dilution; all purchased from Santa Cruz Biotechnology, Santa Cruz, CA). To determine the specificity of TLR2 labeling, an immunizing peptide blocking experiment was performed. Before proceeding

Table 1 Primer sequences for QRT-PCR and characteristics of PCR products

Primer	Sequences (F and R, 5′ to 3′)	Accession number for designed primer or reference	mRNA size (bp)	Position	Product size (bp)	Melting temperature (°C)
TLR1	ATTCCGCAGTACTCCATTCCT	BC109093	2,605	2295–2450	156	55.9
	CTTTGCTTGCTCTGTCAGCTT					56.0
TLR2	GGGTTGAAGCACTGGACAAT	NM_003264	3,417	202–409	208	55.6
	TCCTGTTGTTGGACAGGTCA					56.2
TLR3	CCGTCTATTTGCCACACACTT	NM_003265	3,057	15–244	230	55.5
	TCATCGGGTACCTGAGTCAAC					56.2
TLR4	TGAGCAGTCGTGCTGGTATC	U88880	3,811	2443–2609	167	57.0
	CAGGGCTTTTCTGAGTCGTC					55.7
TLR5	CCTCATGACCATCCTCACAGT	BC109118	3,000	2297–2465	169	56.4
	ATTCTGCACCCATGTGAAGTC					55.4
TLR6	TGCCCATCTGTAAGGAATTTG	NM_006068	2,753	473–681	209	53.0
	TGGGTGAAAAACAAGGTGAAG					53.5
MyD88	GCACATGGGCACATACAGAC	NM_002468	2,826	2060–2298	239	56.4
	TGGGTCCTTTCCAGAGTTTG					54.7
TIRAP	AGAAGCCTAGAGGCCATTCTG	BC032474	2,151	1753–1944	192	56.4
	GTCCAAAGGTGAAGATGGTGA					54.8
TRIF	CGCCACTCCAACTTTCTGTAG	AB093555	2,139	995–1178	184	55.7
	TTCTGTTCCGATGATGATTCC					52.4
RIG-I	AGGAAAACTGGCCCAAAACT	Le Goffic et al. (2007)				55.0
	TTTCCCCTTTTGTCCTTGTG					53.2
MDA5	GTGCATGGAGGAGGAACTGT	Le Goffic et al. (2007)				57.1
	GTTATTCTCCATGCCCCAGA					54.4
IPS	GCAGCAGAAATGAGGAGACC	Le Goffic et al. (2007)				55.9
	AAAGGTGCCCTCGGACTTAT					56.1
CD14	CTGCAACTTCTCCGAACCTC	X13334	1,367	224–438	215	55.7
	CCAGTAGCTGAGCAGGAACC					57.6
CD36	TGGCTGTGTTTGGAGGTATTC	NM_001001548	2,338	345–542	198	55.1
	CTTGAATGTTGCTGCTGTTCA					54.0
Dectin-1	AGAACCACAGTCAACCCACAC	AY026769	744	269–467	199	57.4
	AGGAGATTAGAGCCCAGTTGC					56.7
IL-6	GCCCAGCTATGAACTCCTTCT	NM_000600	1,125	55–212	158	56.7
	TGAAGAGGTGAGTGGCTGTCT					57.9
GM-CSF	CACTGCTGCTGAGATGAATGA	E01817	767	150–360	211	55.1
	GATAATCTGGGTTGCACAGGA					54.7
RANTES	TTTCTACACCAGCAGCAAGTGC	Shi et al. (2003)				58.8
	CACACACTTGGCGGTTCCT					58.1
IFN-β	GATTCATCTAGCACTGGCTGG	Li et al. (1998)			186	55.4
	CTTCAGGTAATGCAGAATCC					50.8
GAPDH	ATGACATCAAGAAGGTGGTG	Lee et al. (2007)			177	52.4
	CATACCAGGAAATGAGCTTG					51.1

with the Western blot labeling protocol, TLR2 antibody was neutralized by incubation with a fivefold excess of peptide (sc-8690P, Santa Cruz Biotechnology) corresponding to the epitope recognized by the antibody for 2 h at room temperature. The neutralized TLR2 antibody was then used side by side with TLR2 antibody alone. After incubating with the respective secondary antibodies (donkey anti-goat IgG-HRP, sc-2020, 1:2,000; goat anti-mouse IgM-HRP, sc-2064, 1:2,000; and goat anti-mouse IgG-HRP, sc-2005, 1:2,000—also from Santa Cruz Biotechnology) for 1 h, chemiluminescent immunodetection was employed using a kit from Pierce. The signal was

visualized by exposure of membranes to HyBolt CL autoradiography film from Denville Scientific (Metuchen, NJ). Densitometry was performed using Image J software (available from NIH, Bethesda, MD). Densitometric results were expressed as the intensity of TLR2 or CD36 relative to β-actin, which served as the loading control.

Measurement of Cytokine/Chemokine Secretion

Triplicate samples of basolateral fluid harvested from each treatment condition were screened for several cytokines and chemokines using the Human Cytokine Antibody Array I (RayBiotech, Norcross, GA) following the manufacturer's instructions. Densitometry was performed on the images of the processed cytokine array membrane, and the data were expressed as a percentage of the positive control spots present on the membrane. The effect of Poly IC treatment on the expression of each of the proteins was determined by computing its percentage expression relative to the untreated controls. Cytokines and chemokines for which absolute quantitation was desired in the conditioned media were examined by specific ELISAs. Duoset kits for IL-6 (samples diluted at 1:20) and a Quantikine kit for GM-CSF (samples diluted at 1:10) (R&D Systems, Minneapolis, MN) were used for ELISAs.

Statistical Analysis

All data are reported as the mean ± SEM. Differences between groups were analyzed using an unpaired, two-tailed *t*-test or analysis of variance for multiple comparisons and considered to be significant at $P < 0.05$ (designated in the figures by * or °).

Results

Regulation of TLR, Coreceptor and Adaptor Molecule mRNA Expression in NHBE Cells

NHBE cells were analyzed for expression of TLR mRNAs by QRT-PCR. The analysis revealed expression of multiple TLR subtypes including TLRs 1–6, with TLR3 exhibiting the highest level of expression (Fig. 1a, b). NHBE cells also express MyD88, TIRAP and TRIF mRNA, which are critical for downstream TLR signaling. Treatment of NHBE cells with Poly IC for 24 h enhanced mRNA expression of TLR2, TLR3 and TLR4 but suppressed expression of TLR5 (Fig. 1c, d). Western blot data and densitometric analysis displayed in Fig. 2a show the effects of Poly IC and PAM_3CSK_4 stimulation (24 h) on TLR2 protein expression. The TLR2 antibody identified a protein of 90–100 kDa, consistent with the size of TLR2, that

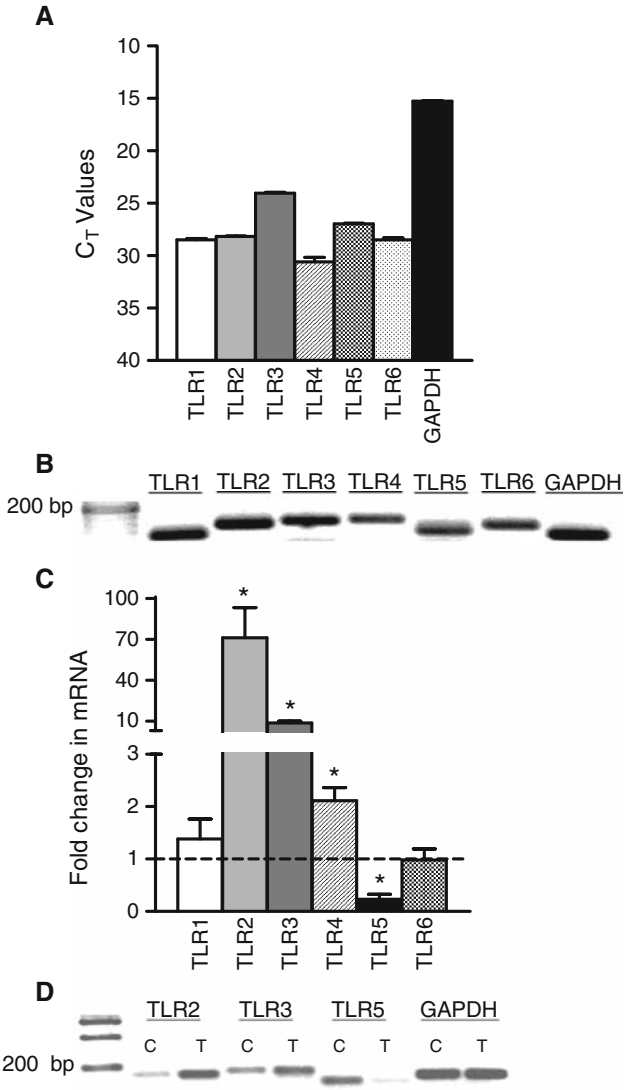


Fig. 1 Basal TLR mRNA expression in NHBE cells and response to Poly IC pretreatment. **a** QRT-PCR data showing the relative expression of TLRs under basal conditions ($n = 6$). **b** Amplified products at 45 cycles were resolved on a 2% agarose gel and the bands visualized by ethidium bromide staining. **c** Fold change in mRNA expression of Poly IC (10 μg/ml) for 24 h treated vs. untreated control monolayers showed that Poly IC stimulated expression of TLR2 and TLR3 and slightly increased TLR4 but suppressed TLR5 expression ($n \geq 6$). The difference in mRNA levels between Poly IC-treated and untreated cells was found to be statistically significant (*) for TLR2-5 but not for TLR1 or TLR6. **d** Peak differences in fluorescence intensity in mRNA expression between Poly IC (10 μg/ml) for 24 h treated (T) and untreated control (C) cells were revealed by comparing the QRT-PCR at 32 cycles and resolving the products on a 2% agarose gel stained with ethidium bromide. GAPDH was used as an internal control

exhibited a 10-fold increase in expression following stimulation by Poly IC and a fivefold increase following stimulation with PAM_3CSK_4. TLR2 antibody specificity was evaluated by comparing labeling of the blocked TLR2 antibody with TLR2 antibody alone. The antibody bound to

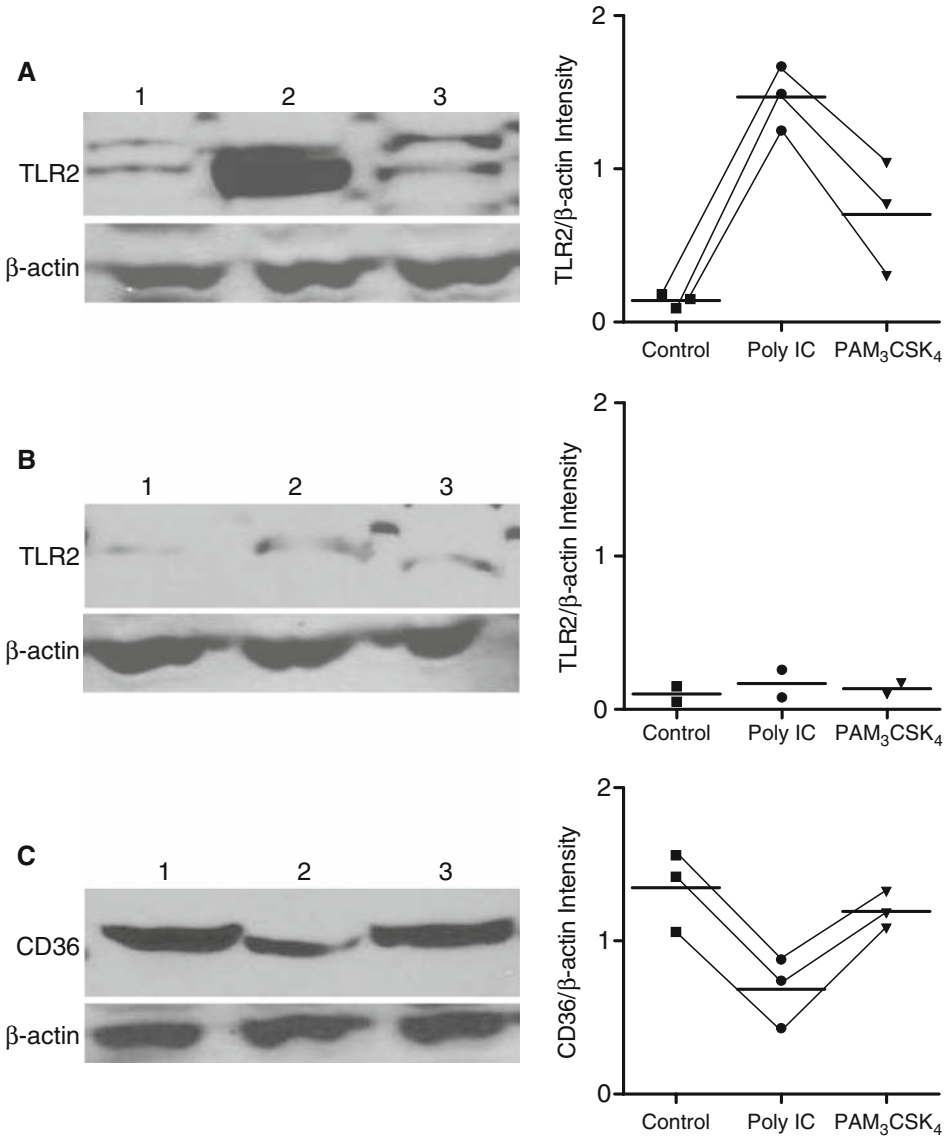


Fig. 2 Western blot analysis of TLR2 and CD36 protein expression. **a** TLR2 protein (~95 kDa) expression in NHBE cell lysates from untreated control monolayers (lane 1) and monolayers treated with 10 μg/ml Poly IC for 24 h (lane 2) or 1 μg/ml PAM_3CSK_4 for 24 h (lane 3). Blots were reprobed with anti-*β*-actin antibody to ensure equal protein loading. The anti-*β*-actin antibody identified a 43-kDa protein. Densitometric results were expressed as the TLR2/*β*-actin intensity ratio for each condition ($n = 3$). **b** Preabsorption control experiment showing TLR2 antibody labeling after treatment with antigenic peptide ($n = 2$); lanes 1–3 are the same treatment conditions as in **a**. **c** CD36 protein (~88 kDa) expression in NHBE cell lysates from untreated control monolayers (lane 1), Poly IC (10 μg/ml)-treated monolayers for 24 h (lane 2) and PAM_3CSK_4 (1 μg/ml)-treated monolayers for 24 h (lane 3). Densitometric results were expressed as the CD36/*β*-actin intensity ratio for each condition ($n = 3$)

the blocking peptide was no longer available to bind to the epitope associated with the TLR2 protein on the Western blot. TLR2 labeling was nearly absent from the Western blot obtained using the neutralized antibody (Fig. 2b). Poly IC treatment also increased plasma membrane expression of TLR2 protein (Fig. 3a, b), with a significant increase in MFI from 181.5 ± 24.2 to 420 ± 52.7 (Fig. 3c). In addition, Poly IC increased mRNA expression of MyD88 and TIRAP, adaptor proteins involved in TLR2 signaling, as well as TRIF, an adaptor protein involved in TLR3 signaling (Fig. 4a). In contrast, the TLR2 ligand PAM_3CSK_4 did not alter adaptor protein mRNA expression (Fig. 4a).

Treatment with Poly IC also enhanced mRNA expression of TLR coreceptors CD14 and Dectin-1 but reduced mRNA expression of CD36 (Fig. 4b), a coreceptor recently shown to be involved in immune responses to LTA in macrophages and BEAS-2B epithelial cells (Hoebe et al. 2005). Western blot and densitometric analyses indicated that the level of CD36 protein expression in Poly IC-treated cells decreased by 1.8- to 2-fold relative to untreated control and PAM_3CSK_4-treated cells (Fig. 2c). Poly IC also increased mRNA expression of dsRNA-sensing helicase proteins (RIG-I and MDA5) but not IPS, a downstream signaling molecule for these helicases (Fig. 4c). These results show that Poly IC (presumably by activating TLR3 or RIG-I and MDA5) differentially regulate mRNA expression of specific TLRs as wells as key coreceptors and adaptor molecules.

Effects of Poly IC on Cytokine and Chemokine mRNA Expression

Poly IC increased the secretion of several proinflammatory cytokines and chemokines from monolayers under air-

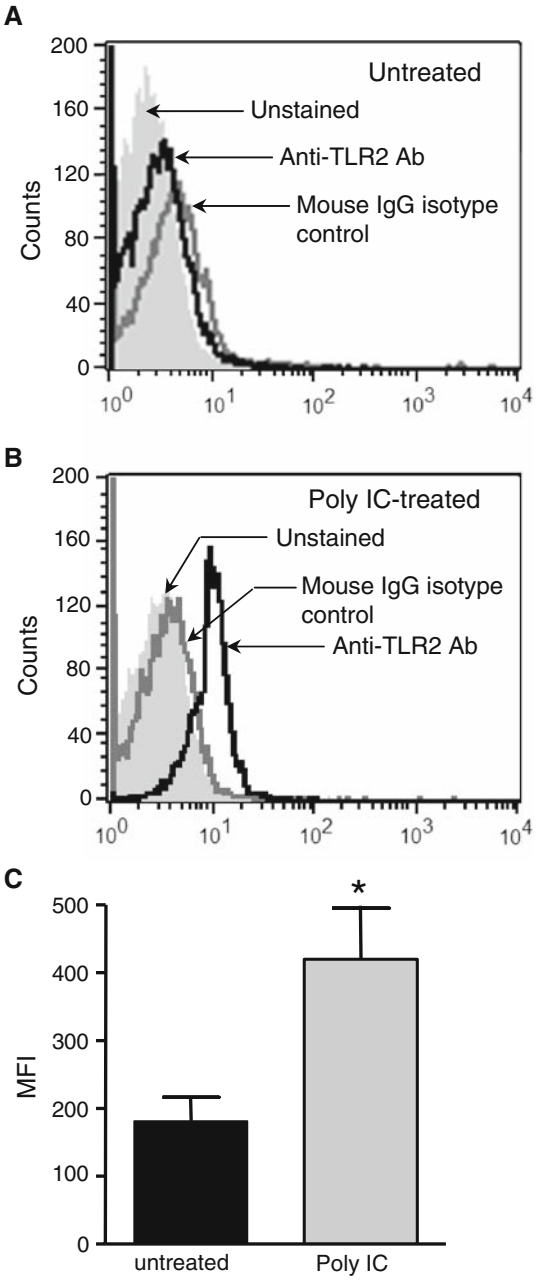


Fig. 3 Effects of Poly IC on plasma membrane expression of TLR2 protein. **a** Plasma membrane expression of TLR2 protein was determined by FACS performed on unstimulated control cells. Gray histogram, untreated control cells; open histogram with black solid line, PE-conjugated anti-human TLR2 antibody; open histogram with gray solid line, PE-conjugated mouse IgG2aK isotype control antibody. **b** FACS was performed as described above after treatment with 10 μg/ml of Poly IC for 24 h. **c** Bar graph showing statistically significant increase (*) in MFI in cells treated with Poly IC (10 μg/ml) for 24 h compared to untreated control cells. Graph represents the mean of four independent experiments

interface conditions (Fig. 5). Secretion of IL-6, GM-CSF and RANTES was stimulated to the greatest degree. Induction of IL-6 and GM-CSF mRNA expression following stimulation with Poly IC was time-dependent (Fig. 6a). Moreover, IL-6 mRNA levels were profoundly enhanced within 3 h following stimulation and reached a plateau that was ~50-fold higher than baseline after 24 h. On the other hand, induction of GM-CSF mRNA synthesis was more gradual, achieving a similar maximum fold increase as IL-6 after 24 h. A comparison of mRNA expression for IL-6, GM-CSF, RANTES and IFN-β, a cytokine typically associated with viral infection, after Poly IC treatment for 24 h is shown in Fig. 6b. Stimulation with PAM_3CSK_4 for 24 h produced only a modest increase in mRNA expression of IL-6 and GM-CSF but did not affect RANTES or IFN-β. However, expression of IL-6 mRNA was augmented by 40-fold in response to PAM_3CSK_4 when cells were pretreated for 24 h with Poly IC, which was shown to upregulate mRNA expression of TLR2 (Fig. 6b).

Apical Poly IC Exposure Enhances Secretion of IL-6 and GM-CSF Across the Apical Membrane

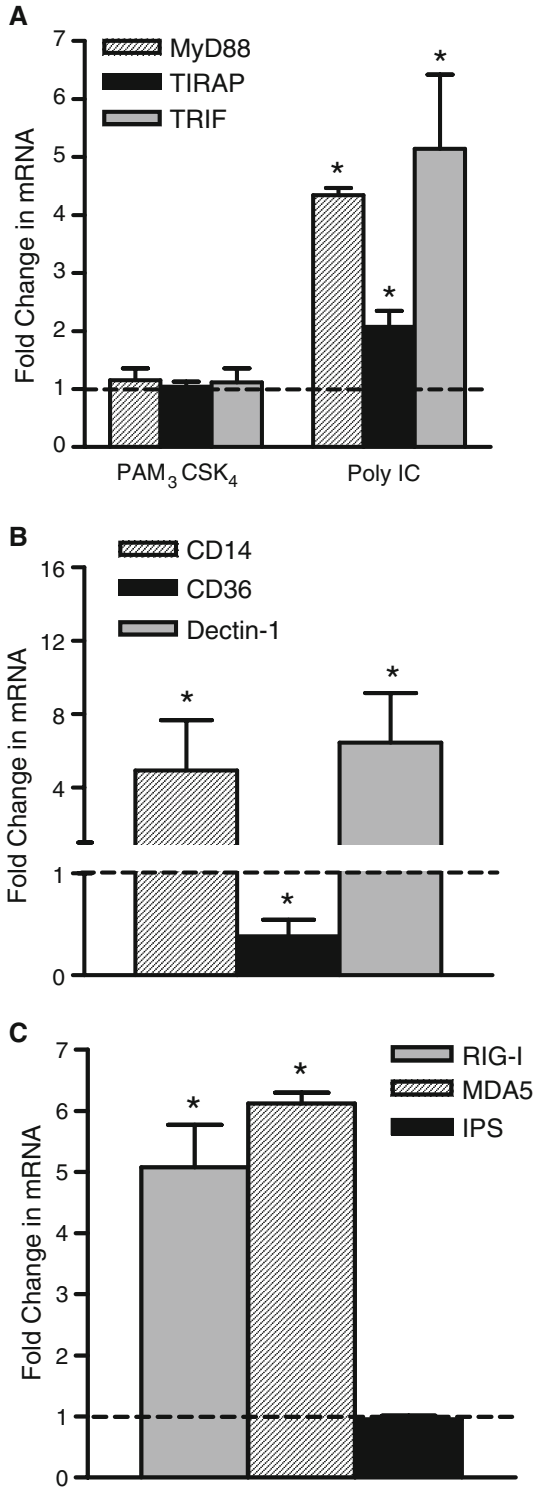
In contrast to PAM_3CSK_4, apical exposure of monolayers to Poly IC induced robust, polarized secretion of IL-6 mainly across the apical membrane (Fig. 7a). The effect of Poly IC on GM-CSF secretion was lower than that observed for IL-6, although a greater amount of secretion was directed into the apical solution, similar to IL-6 (Fig. 7b). Stimulation of monolayers with PAM_3CSK_4 following 24-h pretreatment with Poly IC resulted in significant enhancement of IL-6 secretion into both the apical and basolateral solutions (Fig. 7a). Similarly, an increase in apical and basolateral secretion of GM-CSF was also measured in response to PAM_3CSK_4 following apical exposure to Poly IC. These results demonstrate significant directional secretion of IL-6 secretion across the apical membrane of NHBE monolayers following apical Poly IC exposure that is significantly enhanced by PAM_3CSK_4.

Effects of Poly IC on Epithelial Responsiveness to TLR2 Ligands

The effect of known TLR2 ligands on apical IL-6 secretion was measured after pretreatment with Poly IC for 24 h. Both PAM_3CSK_4 and *Alternaria* extract produced a significant increase in secretion compared to their effects in the absence of Poly IC (Fig. 8a). In contrast, zymosan and LTA did not produce significant changes in IL-6 secretion alone, and no significant increase was detected when monolayers were pretreated with Poly IC (data not shown).

PAM_3CSK_4-Mediated Increase in IL-6 Secretion was Dependent on TLR2 Activation

To verify that the increase in IL-6 secretion evoked by PAM_3CSK_4 was dependent on binding to TLR2,



◀ **Fig. 4** Effects Poly IC on the expression of adaptor proteins, non-TLR receptors and coreceptors involved in TLR signaling. **a** Comparison of Poly IC-treated vs. untreated cells showing that Poly IC, but not the TLR2 ligand PAM_3CSK_4, increases mRNA expression of MyD88 and TIRAP, adaptor proteins involved in downstream TLR2 signaling. Poly IC also increased mRNA expression of TRIF, an adaptor protein involved in TLR3 signaling ($n = 3$). **b** Treatment with Poly IC (24 h) also increased mRNA expression of TLR coreceptors CD14 and Dectin-1 but reduced mRNA expression of CD36 ($n = 6$). **c** Exposure to Poly IC (24 h) enhanced mRNA expression of dsRNA sensing helicase proteins RIG-I and MDA5 but not IPS ($n = 3$). GAPDH was used as an internal control (*significantly different compared to untreated control)

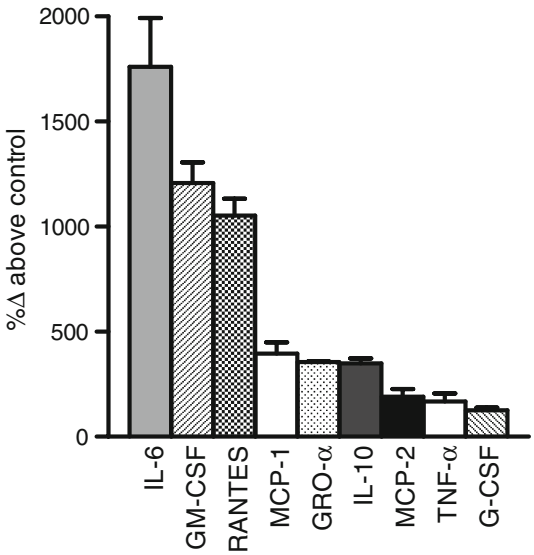


Fig. 5 Effects of Poly IC treatment on cytokine and chemokine secretion. Basolateral fluid was collected following apical exposure to 25 μg/ml Poly IC for 24 h. The fluid was analyzed for protein levels of 23 different cytokines and chemokines using cytokine array kits described in "Materials and Methods." Results for all cytokines and chemokines that exhibited ≥200% induction over untreated controls are reported. GM-CSF, granulocyte monocyte colony stimulating factor; RANTES, regulated upon activation, normal T cell expressed and secreted; MCP, monocyte chemotactic protein; GRO growth-regulated oncogene; TNF, tumor necrosis factor; and G-CSF, granulocyte colony-stimulating factor

monolayers were pretreated with TLR2 blocking antibody during the time period they were exposed to PAM_3CSK_4. The results (Fig. 8b) show that monolayers simultaneously treated with TLR2 blocking antibody failed to exhibit a significant increase in IL-6 secretion in response to PAM_3CSK_4, demonstrating that the TLR2 blocking antibody was effective at inhibiting the PAM_3CSK_4 response after Poly IC treatment. In contrast, pretreatment with TLR2 blocking antibody failed to block the increase in IL-6 secretion evoked by *Alternaria* extract following Poly IC exposure, suggesting that TLR2 was not responsible for the increase in responsiveness to *Alternaria* (data not shown).

Discussion

In this study TLR mRNAs that detect viruses (TLR3), bacteria (TLR1, TLR2, TLR4–6) and fungal allergens

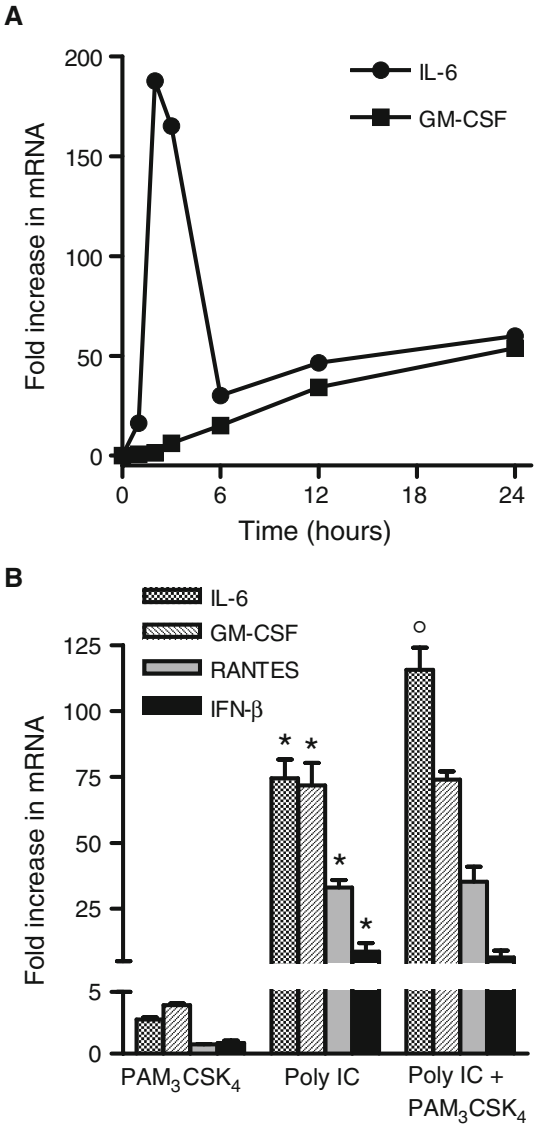


Fig. 6 Effects of TLR2 activation on mRNA expression of selected cytokines/chemokines after pretreatment of NHBE cells with Poly IC. **a** Time course showing mRNA expression of IL-6 and GM-CSF following exposure to 10 μg/ml Poly IC ($n = 3$). **b** Enhanced mRNA expression of proinflammatory cytokines (IL-6) and chemotactic chemokines (GM-CSF, RANTES) and the antiviral cytokine (IFN-β) was observed in cells treated with 10 μg/ml Poly IC for 24 h in contrast to 1 μg/ml of PAM_3CSK_4, which produced a small increase in mRNA expression of IL-6 and GM-CSF but did not affect RANTES or IFN-β compared to untreated cells ($n = 3$). *Significantly different compared to untreated control. The increase in mRNA expression between Poly IC-treated (48 h) and Poly IC (48 h) + PAM_3CSK_4 (24 h)-treated cells was significant for IL-6 (°$n = 3$). GAPDH was used as an internal control

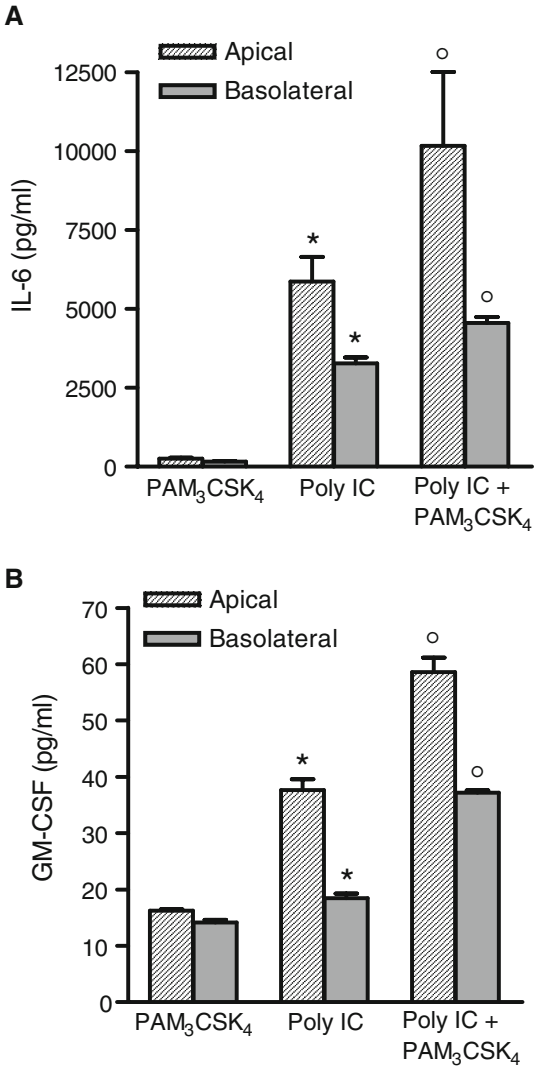


Fig. 7 Effects of Poly IC pretreatment on sensitivity to PAM_3CSK_4. **a** Apical exposure to Poly IC (10 μg/ml) induced release of IL-6 into the apical and basolateral fluid that was significantly greater than that produced by PAM_3CSK_4 alone (*). A significantly greater amount of IL-6 was secreted into the apical solution compared to the basolateral solution ($n = 6$). Pretreatment with Poly IC for 24 h followed by PAM_3CSK_4 for 24 h in the presence of Poly IC resulted in a significant increase in apical and basolateral IL-6 secretion compared to Poly IC treatment alone (°). **b** Apical Poly IC treatment also increased GM-CSF secretion into the apical and basolateral fluid relative to PAM_3CSK_4 (*), with a greater amount of secretion directed into the apical solution relative to the basolateral solution. The secretion of GM-CSF into the apical and basolateral fluids was significantly enhanced by pretreating cells with 10 μg/ml of Poly IC for 24 h before stimulating them with 1 μg/ml of PAM_3CSK_4 for 24 h in the presence of Poly IC (°)

(TLR1, TLR2, TLR6) were shown to be expressed by NHBE cells. Stimulation with Poly IC increased TLR2, TLR3 and TLR4 but reduced TLR5 mRNA expression, consistent with earlier studies using primary small airway epithelial cells (Ritter et al. 2005) and airway smooth muscle cells (Sukkar et al. 2006). Although these previous studies demonstrated that exposure of airway epithelial cells and smooth muscle cells to dsRNA results in a dramatic (> 50-fold) increase in TLR2 mRNA, effects on protein expression were not determined. In the present

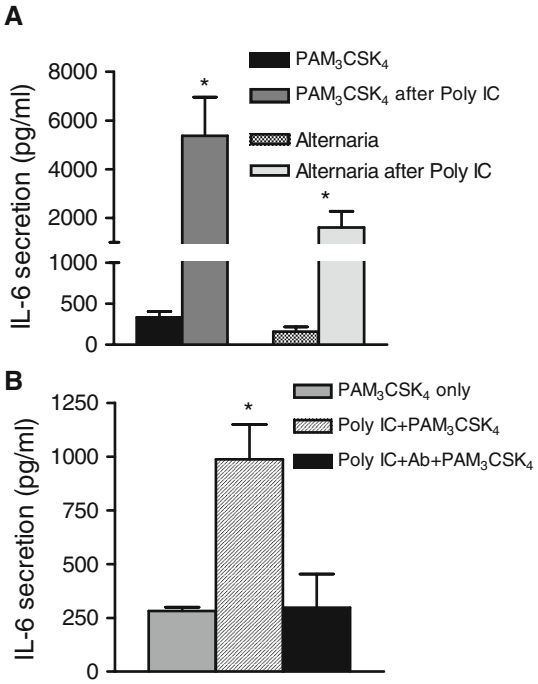


Fig. 8 Apical IL-6 secretion in response to specific TLR2 ligands following pretreatment with Poly IC. **a** Secretion of IL-6 into the apical fluid was compared in monolayers treated with PAM_3CSK_4 (1 μg/ml) and *A. alternata* extract (100 μg/ml) in the absence and presence of Poly IC (10 μg/ml). Polarized monolayers were incubated with apical Poly IC for 24 h, followed by the specified ligand for another 24 h. Basal secretion was determined by subtracting the level of IL-6 measured in untreated monolayers from the amount measured in TLR2 ligand treated monolayers. Secretion induced by each TLR2 ligand after Poly IC treatment was determined by subtracting the level of IL-6 secretion measured in Poly IC-treated monolayers from the amount measured in Poly IC + TLR2 ligand-exposed monolayers. A significant difference in IL-6 secretion was observed between monolayers treated with PAM_3CSK_4 alone and cells pretreated with Poly IC and then PAM_3CSK_4 ($*n = 6$). Similarly, IL-6 secretion was significantly greater in monolayers pretreated with Poly IC followed by *Alternaria* extract compared to monolayers that were treated with *Alternaria* extract alone ($*n = 11$). **b** The increase in apical IL-6 secretion induced by PAM_3CSK_4 after Poly IC pretreatment was inhibited by TLR2 blocking antibody. Monolayers were apically treated with Poly IC for 24 h, then treated with anti-TLR2 antibody at 20 μg/ml for 2 h before and during incubation with PAM_3CSK_4. A significant difference in IL-6 secretion was observed between monolayers treated with PAM_3CSK_4 and those pretreated with Poly IC followed by treatment with PAM_3CSK_4 ($*n = 4$). Monolayers treated with TLR2 blocking antibody exhibited no significant increase in IL-6 secretion in response to Poly IC + PAM_3CSK_4 compared to cells treated with PAM_3CSK_4 alone, without TLR2 blocking antibody ($n = 4$)

study, Western blot analysis revealed a 10-fold increase in the expression of TLR2 after treatment with Poly IC, whereas treatment with a TLR2-specific agonist (PAM_3CSK_4) resulted in a fivefold increase. In addition, FACS analysis indicated that Poly IC exposure enhanced plasma membrane expression of TLR2 protein by 2.3-fold. The results also showed that mRNA expression of adaptor proteins MyD88 and TIRAP, which are involved in TLR2 signaling, increased following exposure to Poly IC. These results and those of a previous study suggested that detection of dsRNA may lead to an overall sensitization of the airway epithelium to TLR2 activating ligands (LeVine et al. 2001). Additionally, RIG-I and MDA5 mRNA expression increased in response to Poly IC treatment, suggesting that detection of viral RNA may also involve these RNA helicases in addition to TLR3.

Stimulation with Poly IC resulted in increased secretion of several proinflammatory cytokines including IL-6, GM-CSF, G-CSF, TNF-α (an anti-inflammatory cytokine), IL-10 and inflammatory chemokines including RANTES, MCP-1, MCP-2 and GRO-α. These signaling molecules have been previously shown to be involved in airway inflammation by enhancing immune cell activation and recruitment (Bishop and Lloyd 2003; Gern et al. 2003; Gonzalo et al. 1998; Lukacs et al. 1996; Yamashita et al. 2005). NHBE cells also exhibited a significant increase in mRNA expression of IFN-β in response to Poly IC stimulation, which is a well-established antiviral response that occurs following activation of TLR3 (Meylan and Tschopp 2006; Hewson et al. 2005; Kumar et al. 2006; Matsukura et al. 2006). The effect of Poly IC on IL-6 and GM-CSF secretion was investigated further using polarized monolayers under apical air-interface conditions instead of cells grown on standard tissue culture plates. Apical stimulation with Poly IC resulted in preferential secretion across the apical membrane. Polarized secretion of cytokines and chemokines has been reported for several epithelial cell types, but the directionality of secretion varies depending on the stimulus or surface of exposure. Earlier studies with mouse and human endometrial epithelial cells showed that IL-6 and GM-CSF were secreted into the apical fluid by as much as four- to fivefold over basolateral secretion (Fahey et al. 2005; Jacobs et al. 1992). Similarly, experiments with primary human nasal epithelial cells under air-interface conditions and alveolar epithelial cell monolayers have shown that exposure of the apical surface to particulates, in the case of nasal epithelial cells, or *Francisella tularensis*, in the case of alveolar epithelial cells, evokes IL-6 and GM-CSF secretion across the apical membrane (Auger et al. 2006; Gentry et al. 2007). In contrast, experiments using IEC-6 cells have demonstrated basolateral secretion of IL-6 in response to IL-1β stimulation and nearly equal amounts of apical and basolateral secretion following stimulation with TNF-α (Mascarenhas et al. 1996). Results of the present study suggest that apically directed IL-6 and GM-CSF secretion would most likely activate and recruit immune cells already present in the airway lumen to the site of viral infection or epithelial damage. Given the large surface area of the epithelium and a relatively dispersed population of immune cells, greater amounts of IL-6 and

GM-CSF secretion may partially compensate for the limitations of diffusion.

In order to investigate the functional consequences of TLR2 and associated coreceptor upregulation on the epithelial immune response to various TLR2 ligands, monolayers were first pretreated with Poly IC for a period of 24 h to enhance (TLR2, Dectin-1 and CD14) or reduce (CD36) receptor expression. Subsequent stimulation with PAM_3CSK_4 produced an increase in apical IL-6 secretion compared to the level evoked from monolayers that were not treated with Poly IC. Enhanced IL-6 secretion was inhibited in monolayers that were simultaneously treated with anti-TLR2 blocking antibody, indicating that the effect of PAM_3CSK_4 was dependent on binding to TLR2. Previous studies have shown that tri-acyl lipopeptides such as PAM_3CSK_4 stimulate TLR1–TLR2 heterodimers, ultimately activating nuclear factor kappa B (NF-κB)-dependent cytokine/chemokine expression and secretion (Takeuchi et al. 2002; Triantafilou et al. 2006). The increase in both IL-6 mRNA and protein secretion observed in NHBE cells was consistent with this interpretation. Moreover, increasing the level of TLR2 expression appears to be necessary for enhancing the responsiveness of airway epithelial cells to tri-acyl lipopeptides.

Although apical IL-6 secretion evoked by *Alternaria* extract was also enhanced by pretreatment with Poly IC, it was not inhibited by simultaneous treatment with TLR2 blocking antibody. *A. alternata* is a common saprophyte that is found in the soil and often on decaying vegetation in many locations worldwide (Fung et al. 2000). Inhaled *Alternaria* spores can cause respiratory distress and airway hyperresponsiveness, particularly in asthma patients; and individuals sensitized to *Alternaria* are three times more likely to develop asthma (Kauffman and van der Heide 2003). *A. alternata* was recently shown to constitutively express heat shock protein 60 (Hsp60) (Buzina et al. 2008). Hsp60 possesses intrinsic immunostimulatory activity mediated by TLR2 and TLR4 signaling pathways, leading to the activation of NF-κB and mitogen-activated protein kinases (Vabulas et al. 2001), implicating the potential importance of this airborne mold in airway inflammation. The results of the present study suggest that exposure to dsRNA associated with viral infection may predispose some individuals to sensitization to *Alternaria*, but upregulation of TLR2 expression does not appear to be responsible for the effect. Therefore, Poly IC may regulate the expression of another unidentified receptor or signaling pathway that mediates the increase in responsiveness to ligands associated with *Alternaria*.

Stimulation of monolayers with zymosan, a cell wall preparation extracted from yeast (*Saccharomyces cerevisiae*), did not stimulate IL-6 secretion in either control or Poly IC-treated monolayers. Previous studies with macrophages and dendritic cells showed that TLR2 and Dectin-1 function collaboratively in the detection and response to fungal cell wall components, including zymosan (Gantner et al. 2003; Underhill 2003). Dectin-1 is known to activate Src and Syk kinases, triggering phagocytosis and production of reactive oxygen species (ROS) (Goodridge and Underhill 2008). In addition, Dectin-1 is known to enhance cytokine secretion synergistically with TLR2 by activating NF-κB through stimulation of CARD9, although it is not clear whether Dectin-1 activation in the absence of TLR stimulation is sufficient to induce NF-κB-mediated transcription (Gross et al. 2006). Our results are consistent with a previous study using human bronchial epithelial cells (BEAS-2B cells), which also failed to show activation in response to zymosan (Mayer et al. 2007). BEAS-2B cells do not appear to express Dectin-1 receptors, which would explain the lack of activation. Although Poly IC exposure increased Dectin-1 mRNA expression, levels were much lower compared to CD14 (basal mRNA expression of Dectin-1 [$C_T = 31.7$] was approximately 1,000-fold lower than expression of CD14 [$C_T = 21.8$]). It is worth noting, however, that failure to detect stimulation of cytokine secretion does not necessarily mean that NHBE cells do not respond to zymosan. Unlike macrophages, Dectin-1 signaling through CARD9 may not occur in airway epithelial cells; but activation of Src/Syc kinase-dependent signaling, perhaps leading to ROS production or phagocytosis, may have taken place but was not measured in the present study.

Experiments with LTA also failed to show stimulation of IL-6 secretion in either control or Poly IC-treated monolayers. This result was again consistent with data from BEAS-2B cells (Mayer et al. 2007). However, when BEAS-2B cells were transfected with TLR2 and CD36 as a means to increase protein expression for both receptors, cytokine secretion occurred in response to LTA and the gram-positive bacterium *Staphylococcus aureus*. It was concluded from the results with BEAS-2B cells that hyporesponsiveness to gram-positive bacteria observed in airway epithelial cells was due to low expression of TLR2 and CD36 and that expression of both receptors was necessary to observe responses from the complete set of TLR2 ligands. Results from the present study support the idea that upregulation of both TLR2 and CD36 is necessary for airway epithelial cells to respond to LTA since Poly IC was shown to decrease CD36 mRNA expression by 62% and protein expression by approximately 1.8- to 2.0-fold. Based on these findings, it appears that reduced expression of CD36 in monolayers exposed to Poly IC may have contributed to the lack of enhanced responsiveness to LTA, in spite of elevated TLR2 expression.

In summary, experiments with NHBE cells demonstrated that exposure to synthetic dsRNA as a means to

activate TLR3 and perhaps basally expressed RIG-I and MDA5 resulted in upregulation of TLR2 and certain co-receptors that conferred increased responsiveness to triacyl lipopeptides and to constituents present in extracts from *A. alternata.* Failure to detect LTA correlated with decreased mRNA expression of CD36, providing support for the contention that upregulation of TLR2 and CD36 receptor expression is necessary for increasing responsiveness of the airway epithelium to LTA and gram-positive bacteria.

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