Endotoxin responsiveness of human airway epithelia is limited by low expression of MD-2

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Jia, Hong Peng, Joel N. Kline, Andrea Penisten, Michael A. Apicella, Theresa L. Gioannini, Jerrold Weiss, and Paul B. McCray, Jr. Endotoxin responsiveness of human airway epithelia is limited by low expression of MD-2. Am J Physiol Lung Cell Mol Physiol 287: L428–L437, 2004. First published April 30, 2004; 10.1152/ajplung.00377.2003.—The expression of inducible antimicrobial peptides, including human β-defensin-2 (HBD-2) by epithelia, comprises a component of innate pulmonary defenses. We hypothesized that HBD-2 induction in airway epithelia is linked to pattern recognition receptors such as the Toll-like receptors (TLRs). We found that primary cultures of well-differentiated human airway epithelia express the mRNA for TLR-4, but little or no MD-2 mRNA, and display little HBD-2 expression in response to treatment with purified endotoxin ± LPS binding protein (LBP) and soluble CD14. Expression of endogenous MD-2 by transduction of airway epithelial cells with an adenoviral vector encoding MD-2 or extracellular addition of recombinant MD-2 both increased the responses of airway epithelia to endotoxin + LBP and scCD14 by >100-fold, as measured by NF-κB-luciferase activity and HBD-2 mRNA expression. MD-2 mRNA could be induced in airway epithelia by exposure of these cells to specific bacterial or host products (e.g., killed Haemophilus influenzae, the P6 outer membrane protein from H. influenzae, Neisseria meningitidis, and IFN-γ; AP-1, and NF-IL-6 response elements (24, 33, 53). These properties suggest that endotoxins derived from airborne gram-negative bacteria could also induce HBD-2 gene expression.

Sensitive cellular responses to many endotoxins require the concerted action of at least four host extracellular and cellular proteins: LPS binding protein (LBP), CD14, MD-2, and TLR-4 (49). We now show that in well-differentiated primary cultures of human airway epithelia, TLR-4 but little or no MD-2 is expressed, and these cells are relatively unresponsive to added endotoxin, even in the presence of LBP and CD14. However, the responsiveness of these cells to endotoxin is markedly amplified by either the endogenous expression or the exogenous addition of MD-2, indicating that the constitutively low levels of MD-2 expression in these cells at “rest” are important in maintaining their hyporesponsiveness to endotoxin. Changes in MD-2 expression in the airway epithelium and/or neighboring cells can be achieved by exposure of these cells to specific bacterial and host products and may thereby regulate airway responsiveness to endotoxin.

MATERIALS AND METHODS

Reagents

Phorbol myristate acetate (PMA), human recombinant IL-1β, TNF-α, and IFN-γ were obtained from Sigma (St. Louis, MO). Soluble CD14 (sCD14) and LBP were provided by Xoma (Berkeley, CA). Lipooligosaccharide (LOS) from nontypeable Haemophilus influenzae was isolated by a mini-phenol-water extraction procedure, as previously described (26). LOS was also isolated from Neisseria meningitidis and used as purified aggregates and as monomeric.
LOX-CD14 complexes as previously described (17, 19). P6 from nontypeable H. influenzae was a generous gift from Dr. Timothy F. Murphy (SUNY Buffalo, Buffalo, NY). Nontypeable Haemophilus influenzae (NTHi) strain 12 (14) was a kind gift of Dr. Dwight Look.

**Cell Culture**

Primary cultures of human airway epithelia were prepared from tracheal and bronchial tissues obtained from donated lungs not used for transplantation. Epithelia were isolated by enzymatic dispersion using well-established methods (30). Briefly, epithelial cells were dissociated and seeded onto collagen-coated, semipermeable membranes with a 0.4-μm pore size (Millicell-HA; surface area 0.6 cm²; Millipore, Bedford, MA). Human airway epithelial cultures were maintained in UltraPro G media at 37°C, 5% CO₂. Millicell inserts were placed into 24-well plastic cell culture plates (Costar, Cambridge, MA). Twenty-four hours after seeding, the mucosal medium was removed, and the cells were allowed to grow at the air-liquid interface as reported previously (30). Only unpassaged, well-differentiated cultures (~2 wk old) were used in these studies. The presence of tight junctions was confirmed by transepithelial resistance measurements using a voltohmmeter (World Precision Instruments, Sarasota, FL; resistance >500 Ω·cm²). For each experimental protocol, the interventions were performed on the indicated number of primary culture specimens from different donors. For each individual experiment, a matched, unstimulated sample from the same specimen served as the control. This study was approved by the Institutional Review Board of the University of Iowa.

**Isolation of Macrophages from Bronchoalveolar Lavage Fluid**

Bronchoalveolar lavage fluid (BALF) was obtained from normal volunteers as previously reported (37). BALF was filtered through two layers of gauze and spun down at 3,500 rpm at 4°C for 5 min. The cell pellets were resuspended in 10 ml of sterile saline and quantified using a Coulter counter.

**Adenoviral Vector Constructs**

A commercially available NF-κB-Luc plasmid (Clontech Laboratories, Palo Alto, CA) was used as a template to generate a recombinant adenovirus vector (Ad-NF-κB-Luc). The fragment containing the firefly luciferase gene driven by four tandem copies of the NF-κB consensus sequence fused to a TATA-like promoter from herpes simplex virus thymidine kinase gene was released by KpnI and XbaI double digestion. The fragment was inserted into a promoterless adenoviral shuttle plasmid (pAd5mcspA), and Ad-NF-κB-Luc virus was generated by homologous recombination, as previously described, and stored in 10 mM Tris with 20% glycerol at 80°C for 1 min. The chain elongation, were conducted. PCR products were electrophoresed on a 2% agarose gel and visualized using ethidium bromide.

**Real-time quantitative PCR for detecting HBD-2 and MD-2.**

Real-time PCR was employed to detect HBD-2 and MD-2 mRNA in human airway epithelia and alveolar macrophages. One microgram of total RNA from each sample was reverse transcribed using random hexamer primers with SuperScript (GIBCO). First-strand cDNA was amplified by PCR. The primer set for HBD-2 consisted of forward: 5′-CTCGAATCTCGAGGTGTTGAGGAG-3′ and reverse: 5′-GTCAAGCTTACGGTCAACAAGGT-3′ and amplified a product of 166 bp. The primer set for CD14 consisted of forward: 5′-CTGCAAACCTCTCAGACAC-3′ and reverse: 5′-CCATAGCTGAGCGAAACCC-3′ and produced a cDNA fragment of 215 bp. The primer set for MD-2 consisted of forward: 5′-TGTAAAGCTTTGGAATTTTATTTA-3′ and reverse: 5′-TTTTAATAGGTTGTTGAAA-3′ and amplified a product of 508 bp. As a control for amplification, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified in each reaction using the following primers: forward: 5′-GTCATGTTGGAAGCTTAC-3′; reverse: 5′-AGGGGTTCACTATGGCACAATGC-3′. Each reaction contained ~1.25 pM of the primers, 3 mM Mg²⁺. After an initial denaturing step (95°C for 3 min), 35 cycles of denaturing (94°C for 30 s), annealing (60°C for 30 s), and extension (72°C for 30 s), followed by 5 min at 72°C for elongation, were conducted. PCR products were electrophoresed on a 2% agarose gel and visualized with ethidium bromide.

**Transduction of Human Airway Epithelia With Adenoviral Expression Vectors**

Primary cultures of well-differentiated human airway epithelia were prepared as described above. In vector transduction experiments, the cells were transduced either with Ad-NF-κB-Luc alone or with both Ad-NF-κB-Luc and Ad-MD-2-2 24 h after performing experimental interventions. The vectors were formulated in 5 mM EGTA and applied to the apical surface [50 multiplicities of infection (MOI)] of the cells for 2 h in a 100-μl volume, as described previously, to access receptors (55). The
vector solution was removed, and the cells were transferred to another multiwell culture dish with fresh culture medium. On the experimental day, LOS ± specific endotoxin-binding proteins (as indicated in the individual figure legends) were applied in a volume of 50 μl to the apical or basolateral side of the cells as noted. Control groups received PBS (negative control) or IL-1β (100 ng/ml, applied apically and basolaterally as positive control) (50). Eighteen to twenty-four hours later, the cells were disrupted in the 1× lysis buffer provided with the luciferase assay kit (Promega) to measure luciferase activity. In addition, some samples were prepared to isolate total RNA.

**Human Embryonic Kidney Cell Activation Assay**

Human embryonic kidney (HEK) cells ± TLR-4 were obtained from Dr. Jesse Chow (Eisai Research Institute, Andover, MA) and were cultured as described (18, 57). For cell activation assays, cells from Dr. Jesse Chow (Eisai Research Institute, Andover, MA) and were cultured as described (18, 57). For cell activation assays, cells were grown to confluence in 48-well plates. Epithelia were washed and supplemented with 0.1% human serum albumin before use in bioassays.

**Results**

**Stimulation of Airway Epithelia With Proinflammatory Products**

Several agents were applied to human airway epithelia to investigate the regulation of MD-2 expression. The human MD-2 cDNA was first subcloned into pGEM-T easy vector for transformation of *Escherichia coli* JM109 and amplification. The DNA was then isolated, linearized, and inserted into pBAC11 (using Nco- and XhoI-sensitive restriction sites) for transfection into insect cells. A vector encoding a six-histidine ("poly-HIS") extension of the COOH terminus was used. The DNA encoding MD-2 was sequenced in both directions to confirm the fidelity of the product. Sf9 cells were used for production and multiplication of virus containing pBAC11 plasmids. To maximize recombinant protein production, High Five (Invitrogen) cells were inoculated with recombinant virus in serum-free medium, incubated for 24–48 h, and culture medium was then collected for analysis. The presence of MD-2-(HIS)6 was determined by SDS-PAGE and immunoblots of the culture medium using an anti-His4 Mab (Qiagen). The culture medium was dialyzed against sterile Hanks' balanced salt solution and 0.1% human serum albumin with the supplements indicated (see Fig. 3). Activation of HEK cells was assessed by measuring accumulation of extracellular IL-8 by ELISA as previously described (10).

**Production of Recombinant MD-2 Protein**

Recombinant MD-2 protein (rMD-2) was produced in baculovirus for application to airway epithelia. The human MD-2 cDNA was first subcloned into pGEM-T easy vector for transformation of *Escherichia coli* JM109 and amplification. The DNA was then isolated, linearized, and inserted into pBAC11 (using Nco- and XhoI-sensitive restriction sites) for transfection into insect cells. A vector encoding a six-histidine ("poly-HIS") extension of the COOH terminus was used. The DNA encoding MD-2 was sequenced in both directions to confirm the fidelity of the product. Sf9 cells were used for production and multiplication of virus containing pBAC11 plasmids. To maximize recombinant protein production, High Five (Invitrogen) cells were inoculated with recombinant virus in serum-free medium, incubated for 24–48 h, and culture medium was then collected for analysis. The presence of MD-2-(HIS)6 was determined by SDS-PAGE and immunoblots of the culture medium using an anti-His4 Mab (Qiagen). The culture medium was dialyzed against sterile Hanks' balanced salt solution buffered with 10 mM HEPES, pH 7.4, and supplemented with 0.1% human serum albumin before use in bioassays.

**Statistical Analysis**

Unless otherwise noted, all numerical data are presented as means ± SE. Statistical analysis was performed using a two-tailed, unpaired Student’s t-test using Microsoft Excel software.

**RESULTS**

**Human Airway Epithelia Are Hyporesponsive to Applied Endotoxin**

NTHi is a common commensal, and sometimes a pathogen, of the respiratory tract (6, 32, 51). We asked whether endotoxin (LOS) from NTHi increased HBD-2 expression after application to the apical surface of human airway epithelia. As shown in Fig. 1, A and B, after the apical application of LOS in the presence of sCD14 and LBP, there was little or no change in the HBD-2 mRNA abundance. In contrast, IL-1β stimulated a large increase in HBD-2 expression (>10,000-fold), consistent with our previous studies (50).

We wondered if the striking lack of responsiveness of airway epithelia to endotoxin alone reflected a polar distribution of receptors for endotoxin on the basolateral surface of airway epithelia. To address this possibility, NTHi LOS was applied to either the apical or basolateral surface of airway epithelia, and HBD-2 expression was quantified by real-time PCR. As shown in Fig. 1B, neither the apical nor the basolateral application of LOS in the presence of sCD14 and LBP...
caused significant induction of HBD-2 mRNA expression. In contrast, IL-β application to either or both sides of the epithelium again induced robust HBD-2 expression. From these experiments, we conclude that human airway epithelia are relatively hyporesponsive to endotoxin.

**MD-2 Expression in Human Airway Epithelia Enhances Endotoxin Responsiveness**

Pattern recognition receptors from the family of TLRs play a central role in the recognition of bacterial products, including endotoxin from gram-negative bacteria (39). TLR-4 is a key receptor for recognition and signaling in response to endotoxin (40), and optimal responses require presentation of the bacterial product in the presence of LBP, CD14, and MD-2 (2, 18, 49). We screened human airway epithelia for the expression of TLR-4, CD14, and MD-2 mRNAs using RT-PCR. Alveolar macrophages served as a positive control. Figure 2 demonstrates that airway epithelia express the mRNAs for TLR-4 and CD14. However, although macrophages demonstrated an MD-2 signal, no significant MD-2 transcripts were detected in airway epithelia following 35 cycles of PCR.

The apparent absence of MD-2 expression in primary cultures of human airway epithelia suggests that the failure of endotoxin to induce HBD-2 expression in these cells reflects a lack of available MD-2 and a resultant inability to form an optimal complex for TLR-4-dependent signaling. To address this hypothesis, we attempted to circumvent the deficiency of MD-2 in airway epithelia by using an adenoviral vector containing the human MD-2 cDNA. To demonstrate that the Ad-MD-2 construct directed expression of functional MD-2 protein, the vector was first used to transduce parental HEK-293 cells. Conditioned medium recovered from the transduced cells was then assayed for the presence of active MD-2 by measuring activation of HEK-293 cells ± TLR-4 by adding LOS:sCD14 (the bioactive product of LBP:sCD14 treatment of LOS Refs. 17–19). As shown in Fig. 3, conditioned medium from Ad-MD-2-transduced cells produced a dose-dependent augmentation of IL-8 release by HEK/TLR-4 cells, but not the parental (TLR-4) cells, consistent with the functional expression of MD-2 by the vector.

Transduction of the human airway epithelial cells with Ad-MD-2, but not with the control Ad-vector, markedly increased the cellular levels of MD-2 mRNA (data not shown). Importantly, the Ad-MD-2-complemented epithelia exhibited markedly enhanced endotoxin responsiveness following apical application of either purified LOS (17) + LBP and sCD14 (Fig. 4A) or purified LOS:sCD14 aggregates (19) (Fig. 4C).

LOS-induced increases in HBD-2 mRNA were paralleled by increased secretion of HBD-2 peptide (data not shown) and increased NF-κB luciferase activity (Fig. 4B) in MD-2-complemented cells. In contrast, basolateral application of LOS to Ad-MD-2-complemented epithelia failed to elicit significant NF-κB signaling or induction of HBD-2 expression (n = 2, data not shown). These results indicate that the expression of MD-2 in airway epithelia confers endotoxin (LOS) responsiveness. Conversely, in the absence of MD-2, surface epithelia are relatively unresponsive to apical or basolateral endotoxin stimulation.
Extracellular Complementation With rMD-2 Protein Enhances Endotoxin Signaling in Human Airway Epithelia

Genetically manipulated cells, such as HEK/TLR-4⁺ cells, have been used to show that endogenous (co)expression of MD-2 or addition of secreted MD-2 to TLR-4⁺/MD-2⁻ cells confers increased endotoxin responsiveness. The primary cultures of human airway epithelia provide a more natural setting to test the effect of exogenous addition of rMD-2 on cellular responsiveness to endotoxin (i.e., LOS:sCD14). As shown in Fig. 4D, addition of conditioned insect cell culture medium containing rMD-2 increased the response of the human airway epithelial cultures to LOS:sCD14 by ≥100-fold. Control-conditioned medium, by contrast, had no effect (data not shown).

The findings presented above indicate that MD-2 expression is the principal limiting factor for responsiveness of human airway epithelia to endotoxin. The need for MD-2 could reflect its role either in TLR-4 trafficking, posttranslational modifications and surface expression (42), and/or in endotoxin recognition and delivery to TLR-4 (18). To test the latter possibility more directly, we examined the responsiveness of airway epithelial to purified LOS:MD-2 complex. Figure 5 shows that the apical application of 2 ng/ml (400 pM) of LOS:MD-2 produced significant activation of resting epithelia, but not of cells induced by adenoviral transduction, to express MD-2 along with TLR-4. This contrasts with effects of LOS:sCD14 that potently activated cells transduced with Ad-MD-2, but not resting cells (Fig. 4C). Because cell activation by LOS:MD-2 requires expression by cells of TLR-4 without MD-2 (18), these findings confirm that the well-differentiated primary cultures of airway epithelia express and produce TLR-4 without MD-2.

Expression of MD-2 in Airway Epithelia Is Regulated by Proinflammatory Stimuli

The demonstration that expression (or addition) of MD-2 could markedly increase the responsiveness of the airway epithelia to endotoxin prompted a search for more natural conditions in which endogenous MD-2 expression might be upregulated. We therefore examined the effects of specific host or bacterial products on cellular MD-2 levels. As shown in Fig. 6A, real-time PCR demonstrated marked increases in steady-state MD-2 mRNA levels after exposure of the apical surface of the cultured airway epithelium to heat-killed NTHi, the NTHi outer membrane protein P6, or the combination of TNF-α and IFN-γ. The levels of MD-2 mRNA attained under these conditions were similar to that induced by PMA but were still significantly less than MD-2 mRNA levels in human alveolar macrophages. These findings demonstrate that, in
response to specific stimuli, levels of MD-2 transcript can be upregulated in human airway epithelia. To address whether the upregulation of MD-2 expression following stimulation with proinflammatory agents results in enhanced responsiveness to LOS:CD14 aggregates, we measured HBD-2 mRNA induced by LOS:sCD14 before and after treatment of the cells with NTHi (to upregulate MD-2 mRNA). We observed a greater cellular response when LOS:CD14 was added after pretreatment of cells with NTHi (Fig. 6B). However, because of the magnitude of the cellular response to NTHi alone and the variability of the overall response between individual cell cultures (donors), the heightened response observed when LOS:sCD14 was added to NTHi-pretreated cells was not statistically significant.

**DISCUSSION**

The present studies support and extend previous findings (7, 11, 21) indicating that, under resting conditions, human airway epithelia are hyporesponsive to endotoxin stimulation. Our observations were obtained using LOS from two different gram-negative bacterial species and several different presentations of LOS, including LOS:sCD14, which is active at picograms/milliliters toward highly endotoxin-responsive cells (13, 44). Although airway epithelial cells are generally hyporesponsive to endotoxin (7, 11, 21), these cells are not generally more responsive to all stimuli, as shown by their robust responses to proinflammatory cytokines including IL-1β.

Hyporesponsiveness to endotoxin is a common characteristic of epithelial cells lining mucosal surfaces that are repeatedly exposed to gram-negative bacteria or cell-free (sterile) forms of endotoxin. The molecular basis of endotoxin hyporesponsiveness is generally unknown. Because sensitive responses to endotoxin generally depend on TLR-4-dependent signaling (49), hyporesponsiveness likely represents the functional deficiency of one or more elements of pathway(s) leading to and resulting from TLR-4 activation. TLR-4 is a membrane protein containing repeats of a leucine-rich motif in the extracellular portion of the protein and a cytoplasmic domain homologous to the intracellular domain of the human IL-1 receptor (40). The IL-1 responsiveness of airway epithelia indicates that the overlapping intracellular signaling pathways for activated TLR and IL-1 receptors (8, 25, 28) are present and functionally intact in human airway epithelia, including those important in NF-κB-regulated HBD-2 expression. Even though we and others (4, 7) have demonstrated that airway epithelia express MD-2, also termed lymphocyte antigen 96, was first identified by Shimazu and colleagues (49, also see Ref. 57) as a molecule that conferred TLR-4-dependent responses to minute amounts of endotoxin. Cells that are TLR-4+/MD-2⁺ are virtually unresponsive to endotoxin (42, 49). In the current study, we observed that human airway epithelia exhibit endo-

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toxin responses consistent with a TLR-4+/MD-2− phenotype. The complementation of this deficiency of MD-2 via endogenous expression or extracellular addition was sufficient to convert human airway epithelia from endotoxin hyporesponsive to highly endotoxin responsive without changing basal NF-κB-regulated HBD-2 expression of these cells or those stimulated by treatment with an unrelated agonist (e.g., IL-1β). These data support the notion that the hyporesponsiveness of resting airway epithelia is due specifically to the absence of MD-2 expression. Our findings confirm and extend earlier observations of Becker and coworkers (7), who detected expression of TLRs 1–6 and CD14 in differentiated human airway epithelia. Although they reported that P. aeruginosa LPS activated CD14-dependent NF-κB signaling, the increase in HBD-2 mRNA they observed in response to P. aeruginosa LPS was no greater (~3-fold) than what we found with added LOS in the absence of MD-2 complementation; i.e., very modest when contrasted to the induction of HBD-2 expression caused by IL-1β or by endotoxin in the presence of MD-2. In contrast to our findings in primary cultures, Guillot and colleagues (21) recently reported that human alveolar and airway epithelial cell lines express detectable levels of MD-2 as well as TLR-4 mRNA by RT-PCR. However, even in those cell lines, levels of MD-2 (protein) may be limiting, since little or no TLR-4 is detected on the cell surface. Our prejudice is that the well-differentiated primary cultures of human airway epithelia better mimic the phenotype of these cells in vivo and therefore provide an important model for studies of the regulation of MD-2 expression in future studies. Additional methods of investigating MD-2 expression, such as in situ hybridization, will be required in future studies using more complex cell culture and animal models to test this prediction more rigorously.

Similar to the findings in airway epithelia, cell lines derived from intestinal epithelia respond robustly to IL-1β stimulation but not to purified, protein-free LPS with NF-κB signaling (2). However, published data on intestinal epithelia indicate both diminished TLR-4 expression as well as absent MD-2 (2). Accordingly, complementation of both TLR-4 and MD-2 in intestinal epithelia is needed for significant activation of NF-κB and IL-8 reporter genes in response to endotoxin. These data suggest that airway and intestinal epithelia, both derivatives of the endodermal foregut, have evolved pattern recognition receptor profiles that minimize stimulation by endotoxin that is frequently encountered at these mucosal surfaces. Whether or not the apparent differences in the molecular basis of endotoxin hyporesponsiveness observed in these studies vs. our findings reflect differences between airway and intestinal epithelial cells or differences between well-differentiated primary cultures and cell lines requires further study. The ability of the airway to mobilize protective TLR-4-dependent responses without the need for MD-2 in response to specific products of gram-positive bacterial airway pathogens (e.g., pneumococci) (34) is consistent with the apparently greater
retention of TLR-4 expression by airway vs. intestinal epithelia.

How might expression of MD-2 in the airway increase epithelial responsiveness to endotoxin? On the basis of current understanding of MD-2 actions, mobilization of MD-2 in the airway could have at least two roles: 1) combining, when endogenously expressed, with endogenously expressed TLR-4 to increase surface expression of TLR-4 (MD-2) (43), and 2) participating directly in endotoxin recognition by extracting endotoxin from monomeric endotoxin:CD14 complexes either before or after interaction of MD-2 with TLR-4 (18) (Fig. 7). TLR-4 alone has no apparent ability to engage the endotoxin:MD-2 complex (18). The ability of soluble, extracellular MD-2 plus endotoxin:CD14 (Fig. 4D) and preformed endotoxin:MD-2 complex (Fig. 5) to activate resting human airway epithelia implies that sufficient TLR-4 is produced by these cells to respond sensitively to endotoxin when endotoxin is presented as a complex with MD-2. No mechanism for TLR-independent interactions of endotoxin:MD-2 complex has been described (18). Therefore, we believe that the ability of picomolar amounts of LOS:MD-2 to activate resting human airway epithelia suggests that there is sufficient apical surface expression of TLR-4 (even without endogenous MD-2) to interact directly with extracellular endotoxin:MD-2 and produce potent cell activation. This could be biologically important because it would permit TLR-4 from one cell to engage MD-2 or endotoxin:MD-2 derived from MD-2 secreted by a neighboring cell. This potency of endotoxin:MD-2:TLR-4 interactions may explain how small amounts of surface TLR-4 may be sufficient for robust cell activation yet insufficient for recognition by fluorescent or neutralizing antibodies (21).

Our results suggest that the regulation of MD-2 expression may be a key determinant of airway epithelial responses to endotoxin. Under resting conditions, low MD-2 expression in airway epithelia renders cells poorly responsive to endotoxin, whereas upregulation of MD-2 alone can greatly enhance cellular responses to endotoxin. As shown in Fig. 6, a variety of stimuli may induce MD-2 mRNA expression in airway epithelia, perhaps by more than one receptor-mediated pathway. Under what circumstances might there be sufficient mobilization of MD-2 to permit the airway epithelium to efficiently respond to endotoxin via TLR-4? MD-2 can be secreted by epithelia or mononuclear cells, and the application of secreted MD-2 enhances TLR-4 signaling in MD-2-deficient cells (18, 54). There may be stimuli (e.g., cytokines, bacterial products) that induce MD-2 expression in airway epithelia to levels sufficient to enhance endotoxin responsiveness. In intestinal epithelia, expression of MD-2 is regulated by cytokine signals, including TNF-α and IFN-γ (1, 2). Stimulated pulmonary macrophages might also secrete sufficient MD-2 to enhance TLR-4 signaling in epithelia. In either case, production of MD-2 by epithelia or exogenous provision of MD-2 from neighboring cells could complement the airway cells for enhanced TLR-4 signaling in response to endotoxin. However, because TLR-4-dependent cell activation by endotoxin requires simultaneous interaction of MD-2 with endotoxin and TLR-4 (Ref. 18; Fig. 7), excessive production of MD-2 could blunt cellular responsiveness to endotoxin by promoting conversion of extracellular endotoxin:CD14 complex to endotoxin:MD-2 while presaturating TLR-4 with MD-2, leaving no cellular targets (i.e., free TLR-4) for the extracellular endotoxin:MD-2 complex (Figs. 5 and 7). Thus the regulation of MD-2 expression in the airways (e.g., airway epithelia and/or pulmonary macrophages) may permit both up- and downregulation of endotoxin responsiveness in the airway.

One advantage of such a hierarchy of responses is that it may help to minimize the frequency of epithelial-induced inflammatory signals from endotoxin. Ambient air contains bacteria and endotoxin (41), and the aerosolized concentrations of endotoxin can increase dramatically in some agricultural and industrial environments from <10 to >1,000 endotoxin units/m3 (12). Under normal conditions, the low expression of MD-2 in epithelia may serve to dampen endotoxin responsiveness to common environmental exposures and thereby avoid unwanted states of chronic inflammation in the face of frequent encounters with environmental endotoxin and other bacterial cell wall components. Conversely, upregulation of MD-2 expression may be important to enhance host defense responses to invading gram-negative bacteria. However, in certain disease states, such as cystic fibrosis or asthma, enhanced expression of MD-2 could lead to exaggerated endotoxin responsiveness with pathological consequences. Future studies will be designed to better define the cells in the airway responsible for MD-2 production and the conditions that regulate its expression.

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REFERENCES


