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Membrane-Tethered MUC1 Mucin Is Phosphorylated by Epidermal Growth Factor Receptor in Airway Epithelial Cells and Associates with TLR5 To Inhibit Recruitment of MyD88

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MUC1 is a membrane-tethered mucin glycoprotein expressed on the apical surface of mucosal epithelial cells. Previous in vivo and in vitro studies established that MUC1 counterregulates airway inflammation by suppressing TLR signaling. In this article, we elucidate the mechanism by which MUC1 inhibits TLR5 signaling. Overexpression of MUC1 in HEK293 cells dramatically reduced Pseudomonas aeruginosa-stimulated IL-8 expression and decreased the activation of NF-κB and MAPK compared with cells not expressing MUC1. However, overexpression of MUC1 in HEK293 cells did not affect NF-κB or MAPK activation in response to TNF-α. Overexpression of MyD88 abrogated the ability of MUC1 to inhibit NF-κB activation, and MUC1 overexpression inhibited flagellin-induced association of TLR5/MyD88 compared with controls. The MUC1 cytoplasmic tail associated with TLR5 in all cells tested, including HEK293T cells, human lung adenocarcinoma cell line A549 cells, and human and mouse primary airway epithelial cells. Activation of epidermal growth factor receptor tyrosine kinase with TGF-α induced phosphorylation of the MUC1 cytoplasmic tail at the Y46EKV sequence and increased association of MUC1/TLR5. Finally, in vivo experiments demonstrated increased immunofluorescence colocalization of Muc1/TLR5 and Muc1/phosphotyrosine staining patterns in mouse airway epithelium and increased Muc1 tyrosine phosphorylation in mouse lung homogenates following P. aeruginosa infection. In conclusion, epidermal growth factor receptor tyrosine phosphorylates MUC1, leading to an increase in its association with TLR5, thereby competitively and reversibly inhibiting recruitment of MyD88 to TLR5 and downstream signaling events. This unique ability of MUC1 to control TLR5 signaling suggests its potential role in the pathogenesis of chronic inflammatory lung diseases. The Journal of Immunology, 2012, 188: 2014–2022.
NF-κB and MAPK pathways, resulting in the release of proinflammatory cytokines and chemokines.

In addition to NF-κB and MAPKs, TLR5 activates the PI3K pathway, a known mechanism for negative regulation of TLR5 signaling. However, our previous study demonstrated that although MUC1 activated the PI3K pathway in response to flagellin, a TLR5 agonist, PI3K was not responsible for MUC1-induced suppression of TLR5 signaling. In this study, evidence is presented that MUC1 expression disrupts TLR5–MyD88 interaction and downstream proinflammatory signaling and that epidermal growth factor receptor (EGFR) activation promotes this anti-inflammatory effect of MUC1 through tyrosine phosphorylation of the MUC1 CT.

Materials and Methods

Reagents

All reagents were purchased from Sigma (St. Louis, MO), unless otherwise stated. The sources of the Abs and reagents were phospho-p38 (Thr180 Tyr182) (3D7) rabbit monoclonal, phospho-p44/42 (Thr202/Tyr204) rabbit monoclonal, phosphoseryl phosphoryloy (P-Tyr-100) mouse monoclonal, EGFR rabbit monoclonal, AG1478 (Cell Signaling Technology, Beverly, MA), MyD88 (HFL296) rabbit polyclonal, MUC1 ectodomain (GP1.4) mouse monoclonal, normal rabbit IgG, normal mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA), TLR5 (IMG-664A) mouse monoclonal (Imgenex, San Diego, CA), HRP-conjugated goat anti-mouse IgG and anti-rabbit IgG (KPL, Gaithersburg, MD), TLR5 (IMG-664A) mouse monoclonal (Imgenex, San Diego, CA), TLR5 (IMG-664A) mouse monoclonal (Imgenex, San Diego, CA), TLR5 (IMG-664A) mouse monoclonal (Imgenex, San Diego, CA), and MyD88 (HFL296) rabbit polyclonal, MUC1 ectodomain (GP1.4) mouse monoclonal, rat, mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA), TLR5 (IMG-664A) mouse monoclonal (Imgenex, San Diego, CA), HRP-conjugated goat anti-mouse IgG and anti-rabbit IgG (KPL, Gaithersburg, MD), and FLAG Ab (10 μg/ml), anti-MUC1 ectodomain (GP1.4) Ab (30 μg/ml), anti-flagellin rabbit antiserum (Dr. Dan Wozniak, Wake Forest University, Rockford, IL) were added to precipitate immune complexes and the beads were separated by SDS-PAGE loading buffer. Immune complexes were separated by SDS-PAGE and subjected to Western blot analysis.

FIGURE 1. Effect of MUC1 expression on TLR5-dependent IL-8 promoter activity and activation of NF-κB, p38, and ERK1/2. HEK293-pcDNA3.1, HEK293–TLR5, and HEK293–TLR5/MUC1 cells (A–D) or Muc1−/− MEF cells transfected with pcDNA3.1 empty vector or MUC1 expression plasmid (E, F) were untreated (CON) or treated for 6 h with heat-inactivated PAK (1.0 × 10⁶ CFU/ml) or flagellin (1.0 μg/ml). Equal protein amounts of cell lysates were subjected to Western blotting with the indicated Abs (A, D, F) or luciferase assays for IL-8 promoter activity (B) or NF-κB reporter activity (C, E). Each bar represents the mean ± SEM (n = 3). The results are representative of three independent experiments. *p < 0.05 compared with untreated controls, †p > 0.05 compared with untreated controls.

Cells

Mouse embryonic fibroblast (MEF) cells were obtained from embryonic day 13 embryos and maintained in DMEM supplemented with 10% FBS (13). Primary mouse tracheal surface epithelial (MTSE) cells were prepared from 8–12-wk-old C57BL/6 mice and cultured as described (6). Normal human bronchial/tracheal epithelial (NHBE) cells were obtained from Lonza Walkersville (Walkersville, MD) and cultured at an air/liquid interface, according to the manufacturer’s protocol. A549 cells (CCL-185), a human lung epithelial cell line, as well as HEK293 cells (CRL-1573) and HEK293T cells (CRL-11268), human embryonic kidney cell lines, were purchased from American Tissue Culture Collection (Manassas, VA). A549 cells were cultured in Opti-MEM medium supplemented with 5% FBS and antibiotics. HEK293 cells were stably transfected with the pcDNA3.1 empty vector (HEK293-pcDNA3.1) cells or with a MUC1 cDNA cloned in pcDNA3.1 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). HEK293T cells were used for transient transfection. Both types of cells were subsequently transfected with FLAG-tagged human TLR5 cloned into the HindIII and Agel sites of the pDRed-Monomer-Hyg-N1 vector (Clontech Laboratories, Palo Alto, CA). Stable clones were isolated in the presence of hygromycin (100 μg/ml) and referred to as HEK293–TLR5 and HEK293–TLR5/MUC1 cells, respectively; they were cultured in DMEM containing penicillin (100 U/ml), streptomycin (100 μg/ml), hygromycin (100 μg/ml), and 10% FBS.

Western blot and immunoprecipitation analyses

Western blot analysis was performed as described (12). For direct immunoprecipitation (IP) analysis, the cells were extracted with lysis buffer (0.5 ml/100-mm dish), and equal amounts of protein were incubated with anti-FLAG Ab (10 μg/ml), anti-MUC1 CT (CT2) Ab (40 μl), anti-MUC1 ectodomain (GP1.4) Ab (30 μl), or isotype-matched normal IgG (Santa Cruz) at 4˚C for 16 h with continuous agitation. Protein G-agarose beads (Pierce, Rockford, IL) were added to precipitate immune complexes and the beads were washed five times at 4˚C with lysis buffer and eluted by boiling in SDS-PAGE loading buffer. Immune complexes were separated by SDS-PAGE and subjected to Western blot analysis.

bacteria at 60˚C for 30 min and stored at −80˚C. Pervanadate, a general protein tyrosine phosphatase inhibitor, was freshly prepared immediately before its use as a 100× stock solution by mixing 600 μM H₂O₂ and 0.2 μM Na₂VO₄ (11).

P < 0.05 compared with untreated controls, †p > 0.05 compared with untreated controls.
Luciferase reporter assays for ELAM-1 and IL-8

An NF-κB-dependent ELAM-1–luciferase reporter plasmid (pELAM1-luc) was generated, as described elsewhere (14), by cloning a fragment (∼241 to ∼54 bp) of human E-selectin promoter into the pGL3 reporter plasmid (Promega, Madison, WI). Briefly, cells were transiently transfected with the pGL3-luciferase (15) or pELAM-1–luciferase reporter genes with a Renilla luciferase reporter construct, using Lipofectamine 2000, according to the manufacturer’s protocol. In addition, the cells were transfected with expression plasmids for TRAF6, IRAK1, or MyD88. The total amount of plasmid DNA was kept constant by addition of pcDNA3.1 for each transfection. At 24–36 h posttransfection, the cells were treated with appropriate stimuli for 6 h, and cell lysates were assayed for firefly and Renilla luciferase activities using the Dual-luciferase Reporter System (Promega) and an L-Max II luminometer (Molecular Devices, Sunnyvale, CA). Relative luciferase activity was determined by normalizing with Renilla luciferase activity.

Experimental P. aeruginosa lung infection

C57BL/6 Muc1−/− mice and Muc1+/+ littermates have been described (16). Mice were intranasally infected with 1.0 × 103 CFU PAK in a 40-μl suspension, as described (6). Left lung lobes were homogenized in 1.0 ml lysis buffer and subjected to IP, as described above. The remaining lobes were fixed with 10% paraformaldehyde and embedded in paraffin, and 5-μm sections were made. All procedures were approved by the Institutional Animal Care and Use Committee of Temple University.

Immunofluorescence

Paraffin sections on glass slides were deparaffinized in xylene, rehydrated, blocked in normal goat serum, and incubated overnight at 4°C with primary Abs (1:100 dilution). Nuclei were counterstained with DAPI. Coverslips were applied in antifade solution (Invitrogen); samples were visualized by laser scanning confocal microscopy (LSM 510; Zeiss, Stuttgart, Germany), and images were analyzed using LSM 510 software.

Statistical analysis

Differences between groups were assessed by comparing mean ± SEM values using the Student t test and were considered significant at p < 0.05.

Results

MUC1 expression attenuates TLR5-dependent IL-8 promoter activity and activation of NF-κB, p38, and ERK1/2

Airway epithelial cells sense P. aeruginosa infection through recognition of flagellin by TLR5 (17). Because MUC1 expression inhibits TLR5-driven NF-κB activation and IL-8 production by airway epithelia (6, 18), we sought to determine whether MUC1 suppresses TLR5 signaling and, if so, to elucidate the mechanism by building upon previous studies using the well-established HEK293 cell culture system (6, 11, 12, 18–20). Stable clones of HEK293-pcDNA3.1, HEK293-TLR5, and HEK293-TLR5/MUC1 cells were established in HEK293 cells not expressing MUC1 (Fig. 1A). P. aeruginosa–induced IL-8 promoter activity was significantly enhanced in HEK293-TLR5 cells compared with HEK293-pcDNA3.1 cells (Fig. 1B). TLR5-dependent IL-8 promoter activation was completely suppressed in HEK293-TLR5/MUC1 cells. TLR5 regulates IL-8 gene expression through NF-κB and MAPK pathways (21). Therefore, we asked whether MUC1 attenuates TLR5-dependent activation of NF-κB and/or MAPK. Treatment of HEK293-TLR5 cells with flagellin increased NF-κB–dependent reporter (ELAM-1) activity compared with untreated controls (Fig. 1C). However, NF-κB activity was completely suppressed in HEK293-TLR5/MUC1 cells. Similarly, treatment of HEK293-TLR5 cells with flagellin augmented the phosphorylation of p38 and ERK1/2 compared with controls, and both effects were drastically reduced in HEK293-TLR5/MUC1 cells (Fig. 1D).

MUC1 expression does not affect TNF-α/TNFR signaling

To determine whether inhibition of NF-κB and MAPK by MUC1 could be replicated in another innate immune ligand/receptor system, the effects of MUC1 expression on TNF-α/TNFR-mediated activation of NF-κB and MAPK were assessed. As before, flagellin-induced NF-κB activation was suppressed in HEK293-TLR5/MUC1 cells compared with HEK293-TLR5 cells (Fig. 2A). By contrast, TNF-α–induced NF-κB activities were equal in both cell types. (Note the different numerical scales on the two ordinate axes.) Combining the flagellin and TNF-α stimuli in HEK293-TLR5 cells produced an NF-κB activation response that was ∼2-fold greater than that achieved by the individual agonists. However, NF-κB activation in HEK293-TLR5/MUC1 cells in response to flagellin plus TNF-α was equal to that produced by TNF-α alone. Additionally, TNF-α–stimulated phosphorylations of p38 and ERK1/2 were not suppressed in HEK293-TLR5/MUC1 cells compared with HEK293-TLR5 cells (Fig. 2B). These combined data suggested that MUC1 attenuates activation of NF-κB and MAPK mediated by TLR5 but not by TNFR.

MUC1 interferes with flagellin-induced association of TLR5/MyD88

Because activation of NF-κB and MAPK by TLR5 and TNFR requires prior activation of TGF-β–activated kinase 1 (TAK1), and the signaling pathways leading to activation of TAK1 by these vectors increased NF-κB activation, as well as enhanced p38 and ERK1/2 phosphorylation, compared with untreated controls (Fig. 1E, 1F). However, all three of these effects were substantially reduced in Muc1−/− MEF cells overexpressing human MUC1 compared with Muc1−/− MEF-pcDNA3.1 cells.

FIGURE 2. Effect of MUC1 expression on TNF-α–induced activation of NF-κB, p38, and ERK1/2. HEK293-TLR5 and HEK293-TLR5/MUC1 cells were untreated or treated for 6 h with flagellin (1.0 μg/ml), TNF-α (100 ng/ml), or flagellin plus TNF-α. Equal protein amounts of cell lysates were subjected to luciferase assay to measure NF-κB–dependent reporter (A) and Western blotting with the indicated Abs (B). Each bar represents the mean ± SEM (n = 3). Note the different ordinate scales in A. The results are representative of three independent experiments. *p < 0.05 compared with untreated controls, †p > 0.05 compared with untreated controls.
receptors are distinct, the data presented above suggested that cross-talk between MUC1 and TLR5 signaling occurs upstream of TAK1. Therefore, experiments were performed to identify the possible site(s) between TLR5 and TAK1 that is/are suppressed by MUC1. Engagement of TLR5 by flagellin triggers the recruitment of MyD88 to the intracellular Toll/IL-1R (TIR) domain of TLR5. MyD88 subsequently binds to and activates IRAK1 (22), and activated IRAK1 phosphorylates TRAF6, thereby inducing Lys63-linked autoubiquitination and activation of TAK1 (23). Flagellin-treated HEK293-TLR5 and HEK293-TLR5/MUC1 cells exhibited equal flagellin/TLR5 binding (Fig. 3A), suggesting that MUC1 did not block binding of flagellin to its cognate receptor. Next, we determined whether MUC1-mediated suppression of TLR5 signaling was blocked following overexpression of TRAF6, IRAK1, or MyD88. Although overexpression of TRAF6 or IRAK1 did not reverse the inhibitory effect of MUC1 (Fig. 3B), MyD88 overexpression completely abrogated MUC1-dependent suppression of flagellin-induced NF-κB activation (Fig. 3B). Overexpression of

![FIGURE 3. Effect of MyD88, IRAK1, or TRAF6 overexpression on MUC1-mediated suppression of TLR5 signaling. A and C, HEK293-TLR5 and HEK293-TLR5/MUC1 cells were untreated or treated with flagellin (1.0 μg/ml) or heat-inactivated PAK (1.0 × 10^7 CFU/ml) for 30 min. Equal protein amounts of cell lysates were used for IP with anti-FLAG Ab (TLR5) or isotype-matched normal mouse IgG, and immunoprecipitated proteins were subjected to Western blotting with the indicated Abs. B, HEK293-TLR5 and HEK293-TLR5/MUC1 cells were transfected with TRAF6 (left panel), IRAK1 (middle panel), or MyD88 (right panel) expression plasmids. The cells were untreated or treated for 6 h with flagellin (1.0 μg/ml). Equal protein amounts of cell lysates were subjected to luciferase assay to measure NF-κB reporter activity. Each bar represents the mean ± SEM value (n = 3). The results are representative of three independent experiments. *p < 0.05.](http://www.jimmunol.org/)

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FIGURE 4. Association of MUC1 and TLR5 in HEK293T cells. HEK293T cells were untransfected or transiently transfected with expression plasmids for TLR5-FLAG and/or full-length MUC1 (A) or MUC1 ΔCT (B). At 24 h posttransfection, equal protein amounts of cell lysates were used for IP with isotype-matched normal mouse IgG, anti-FLAG (TLR5-FLAG), anti-CT2 (full-length MUC1), or anti-MUC1 ectodomain (GP1.4) (MUC1 ΔCT). Immunoprecipitated proteins were subjected to Western blotting with the indicated Abs. Protein expression levels of TLR5 and MUC1 were verified in the same lysates used for IP. The results are representative of two or three independent experiments.
TRAF6, IRAK1, and MyD88 following transient transfection was confirmed by Western blotting (data not shown). Finally, treatment of HEK293-TLR5 cells with flagellin or heat-inactivated P. aeruginosa increased association of TLR5/MUC1 compared with untreated cells (Fig. 3C). In contrast, such association was not observed in HEK293-TLR5/MUC1 cells. Taken together, these results suggested that MUC1 overexpression abrogates flagellin-stimulated recruitment of MyD88 to TLR5, thus inhibiting NF-κB activation; however, this inhibitory effect is fully reversible upon MyD88 overexpression.

**MUC1 associates with TLR5**

Two possible mechanisms were considered for MUC1’s ability to reversibly block the interaction between MyD88 and TLR5: binding of MUC1 to MyD88 and/or binding of MUC1 to TLR5. Reciprocal coimmunoprecipitation studies revealed that MUC1 constitutively forms a protein complex with TLR5, but not with MyD88 (data not shown), in lysates of HEK293T cells transiently transfected with MUC1 and TLR5 (Fig. 4A). Deletion of the MUC1 CT domain (MUC1 ΔCT) almost completely abated its association with TLR5 (Fig. 4B), suggesting that the MUC1 CT domain is necessary to interact with TLR5. Constitutive interaction between endogenously expressed MUC1 and TLR5 was also demonstrated in lysates of A549 cells, as well as in primary MTSE cells and NHBE cells (Fig. 5A). In addition, association of MUC1/TLR5 in NHBE cells was increased 5-fold over basal levels following treatment with P. aeruginosa (Fig. 5B). In summary, these results indicated that MUC1 binds to TLR5 through its CT domain.

**Activation of EGFR induces MUC1 CT tyrosine phosphorylation and increases association of MUC1/TLR5**

Tyrosine residues in the MUC1 CT are targets of cytosolic and receptor protein kinases, and these phosphorytases serve as docking sites for oncoproteins (24). For example, EGFR phosphorylates the MUC1 CT at a Y46EKV sequence that, upon phosphorylation, serves as a binding site for the kinase (25). Therefore, we investigated constitutive and EGFR-induced CT tyrosine phosphorylation and the role of phosphorylation in MUC1/TLR5 association. Treatment of A549 cells with pervanadate, a broad-spectrum tyrosine phosphatase inhibitor, increased constitutive MUC1 CT tyrosine phosphorylation and augmented MUC1/TLR5 association compared with untreated cells (Fig. 6A). To investigate the possible role of the Y46 residue in MUC1 CT phosphorylation and association of MUC1/TLR5, HEK293T cells were cotransfected with TLR5 and a CD8/MUC1 chimeric protein (26): either one with the CD8 EC and transmembrane domains and the MUC1 CT (CD8/MUC1-1 WT) or CD8/MUC1-Y46F, in which Tyr-46 residue was mutated to phenylalanine (11, 26) (Fig. 6B). Pervanadate treatment increased both constitutive tyrosine phosphorylation of CD8/MUC1-1 WT (Fig. 6C, lower panel) and association of CD8/MUC1-1 WT and TLR5 (Fig. 6C, upper panel) compared with untreated cells. However, CD8/MUC1-Y46F exhibited significant decreases in both constitutive tyrosine phosphorylation and association with TLR5 compared with CD8/MUC1-1 WT (Fig. 6C). Next, we determined whether Y46 is important for the suppressive effect of MUC1 on PAK-induced phosphorylation.

**FIGURE 6.** Phosphorylation of the MUC1 CT at Y46 by EGFR increases MUC1/TLR5 association. A and C–H, A549 cells or HEK293T cells, transiently transfected with expression plasmids for TLR5 plus CD8/MUC1-1 WT or CD8/MUC1-Y46F, were untreated or treated for 30 min with pervanadate (0.2 μM), AG1478 (100 nM), or TGF-α (100 ng/ml). Equal amounts of cell lysates were used for IP with anti-CT2, anti-CD8 (CD8/MUC1), or anti-FLAG (TLR5-FLAG), and immunoprecipitated proteins were subjected to Western blotting with the indicated Abs. B, Schematic illustration of the CD8/MUC1 chimeric protein. D, For ELAM-1–luciferase reporter assay, cells were untreated or treated with PAK and assayed as in Fig. 1. Luciferase activity of pcDNA3.1 transfecants treated with PAK was set as 100%. Each bar represents the mean ± SEM (n = 4). Transfection efficiency of CD8/MUC1-1 WT and CD8/MUC1-Y46F was verified by Western blotting. The results are representative of three independent experiments. *p < 0.01.
NF-κB activation, as shown in Fig. 1. Overexpression of CD8/MUC1-WT and CD8/MUC1-Y46F decreased PAK-induced ELAM-1 activity by 65% and 25%, respectively, compared with pcDNA3.1 control, suggesting that Y46 of MUC1 CT plays an important role in suppressing PAK-induced TLR5 signaling (Fig. 6D). Even in the absence of pervanadate, treatment of A549 cells with an EGFR agonist, TGF-α, increased tyrosine phosphorylation of the MUC1 CT and enhanced association of MUC1/TLR5, as well as MUC1/EGFR (Fig. 6E). Identical results were observed using epidermal growth factor as the stimulus (data not shown). Similarly, TGF-α stimulated tyrosine phosphorylation of CD8/MUC1-WT (Fig. 6F, lower panel) and increased association of CD8/MUC1-WT with TLR5 (Fig. 6F, upper panel) compared with untreated cells. However, TGF-α treatment failed to increase association of CD8/MUC1-Y46F with TLR5 (Fig. 6G). In addition, treatment of CD8/MUC1-WT–transfected cells with a selective EGFR inhibitor, AG1478, abrogated TGF-α–induced association of CD8/MUC1-WT/TLR5 (Fig. 6H). In conclusion, these data suggested that EGFR-dependent Y46 phosphorylation of the MUC1 CT increases association of MUC1 CT with TLR5, hence presumably enhances MUC1-mediated TLR5 suppression.

**Immunofluorescence colocalization of Muc1 and TLR5 and tyrosine phosphorylation of the Muc1 CT in vivo**

P. aeruginosa infection of mice was shown to activate EGFR in airway epithelium (27). Therefore, we asked whether P. aeruginosa airway infection of mice stimulates tyrosine phosphorylation of the Muc1 CT in vivo and increases Muc1/TLR5 interaction. Mice were infected intranasally with P. aeruginosa, and lung homogenates were subjected to Muc1 CT IP and phosphotyrosine immunoblot analysis at 0, 4, and 24 h postinfection. Airway infection was confirmed by increased inflammatory cell infiltration into the lungs at 4 and 24 h (data not shown). Muc1 CT tyrosine phosphorylation was increased at 24 h postinfection compared with 0 and 4 h (Fig. 7A). Identical results were observed in the reciprocal approach when lung homogenates were subjected to phosphotyrosine IP, followed by Muc1 CT immunoblotting (Fig. 7B). As a negative control, a 25-kDa tyrosine-phosphorylated CT protein band was not detected in the lungs of P. aeruginosa–infected Muc1−/− mice (Fig. 7C). Finally, laser-scanning confocal immunofluorescence microscopy was used to assess the Muc1, TLR5, and phosphotyrosine staining patterns in lungs of uninfected and P. aeruginosa–infected mice. In uninfected animals, no evidence of Muc1/TLR5 or Muc1/phosphotyrosine colocalization was observed (Fig. 8). However, epithelial cell apical colocalization of the Muc1/TLR5 and Muc1/phosphotyrosine immunostaining patterns was seen in P. aeruginosa–infected mice at 24 h postinfection (Fig. 8).

**Discussion**

MUC1/Muc1 expression counterregulates airway inflammation during P. aeruginosa infection (6, 8), and this anti-inflammatory activity is attributed to its ability to suppress TLR5 signaling (6, 12, 18). However, the mechanism of cross-talk between TLR5 and MUC1/Muc1 is unknown. In this article, we demonstrated the suppressive effect of MUC1 expression on TLR5–dependent IL-8 promoter activity (Fig. 1B), as well as NF-κB and MAPK activation (Figs. 1C–F), which correlated with reduced TLR5/MyD88 association (Fig. 3C) and increased MUC1/TLR5 association (Figs. 4–6). NF-κB inhibition was completely reversed upon MyD88 overexpression (Fig. 3B). Increased MUC1 CT phosphorylation, likely at the Y46EKV site, and greater MUC1/TLR5 association were associated with TGF-α–dependent EGFR activation (Fig. 6). In vivo experiments established increased Muc1 CT tyrosine phosphorylation in mouse lung homogenates following P. aeruginosa infection (Fig. 7) and greater Muc1/TLR5 and Muc1/phosphotyrosine immunofluorescence colocalization in infected mouse airway epithelium (Fig. 8). Together, these results suggested a mechanism whereby EGFR tyrosine-phosphorylates the MUC1 CT, thus increasing its association with TLR5 and competitively and reversibly inhibiting recruitment of MyD88 to TLR5 and subsequent proinflammatory signal transduction.

Flagellin binding to the TLR5 ectodomain induces receptor homodimerization, resulting in a protein conformational change in its CT domain and allowing recruitment of the MyD88 adapter protein (22). The data presented in this study indicated that MUC1 mediates its anti-inflammatory effects at the level of the TLR5 intracellular domain and are entirely consistent with the previous publication demonstrating that transfection of RAW264.7 cells with MUC1 ΔCT, but not MUC1 ΔEC, abolished the ability of MUC1 to inhibit TLR-driven TNF-α production (18). This effect was achieved not only with the MyD88–dependent TLR2, TLR4, TLR7, and TLR9, but also with TLR3, which signals through TRIF rather than MyD88. Given that all TLRs and many adaptor proteins share the conserved TIR domain mediating homo- and heterotypic interactions, it seems very likely that MUC1 suppresses TLR signaling by associating with receptor TIR domains, thus acting as a steric hindrance/decoy receptor and excluding recruitment of MyD88 and TRIF to their respective receptors. Interestingly, the MUC1 CT contains an amino acid sequence (R17DTYHP) that is homologous to the consensus RDXΦΦΦΦΦG motif (where Φ represents a hydrophobic residue, and X represents any residue) of the TIR domain and that was shown to be responsible for homeric interaction between TLR2/4 and MyD88 (28). However, the possible role of the MUC1 CT “TIR domain-like” sequence in TLR signal transduction remains speculative.
EGFR regulates innate immune responses in the airways, including mucin secretion by goblet cells, and chemokine production and proliferation by epithelial cells (29). As with all EGFR ligands, TGF-α is synthesized in a latent form as a membrane-tethered precursor protein on the surface of airway epithelial cells. Proteolytic cleavage of pro-TGF-α by TNF-α converting enzyme precedes EGFR activation and proinflammatory signaling (30). Like TGF-α, TNF-α converting enzyme is initially synthesized in an inactive form that is activated by a variety of diverse stimuli, including airway bacterial pathogens (31), cigarette smoke (32), and reactive oxygen species (33), the last of which are upregulated by TLRs and dual oxidase (33). Based on the current results, a second function can now be ascribed to activated EGFR apart from proinflammatory signaling: tyrosine phosphorylation of the MUC1 CT and MUC1/TLR5 protein interaction. Of note, airway epithelial cell expression levels of both MUC1 and EGFR are increased by a common proinflammatory cytokine, TNF-α (8, 29, 34, 35). It is tempting to speculate that simultaneous upregulation of MUC1 and EGFR in the vicinity of an ongoing inflammatory response facilitates sequential steps of EGFR-mediated tyrosine phosphorylation of MUC1, MUC1/TLR5 interaction, and counterregulation of airway inflammation.

In addition to its anti-inflammatory properties mediated through its CT, a growing body of evidence suggests that the MUC1 EC regions contribute to the pathogenesis of microorganisms that colonize and infect mucosal surfaces (36). MUC1/Muc1 is an EC adhesion site for *P. aeruginosa* on airway epithelia (19, 37, 38), as well as for *Escherichia coli* and *Salmonella enterica* on intestinal epithelia (39, 40). *Helicobacter pylori* binds to the MUC1 ectodomain on gastric epithelial cells, and MUC1 ectodomain shedding acts as a releasable decoy to block infection by this pathogen (36, 41). McAuley et al. (42) demonstrated that Muc1−/− mice are more susceptible to infection by gastrointestinal *Campylobacter jejuni* compared with Muc1+/+ littermates. Increased bacterial colonization by *C. jejuni* was accompanied by severe epithelial damage and exaggerated penetration through the intestinal barrier, eventually resulting in systemic infection. As originally proposed by Gendler (43), the heterodimeric nature of the MUC1 protein may provide a mechanism for rapid ectodomain shedding that concurrently signals to the cell interior, through its CT, the presence of an invading pathogen. Although Muc1 acts as a decoy receptor for invading bacteria in the intestinal tract, it concurrently plays an anti-inflammatory role in the respiratory tract by a discrete mechanism not involving its ectodomain. These multidimensional effects may relate to the functional difference between the two organs, with the former constituting an impenetrable barrier against commensal bacteria and the latter dealing with the timely resolution of inflammation to maintain its vital function of gas exchange.

It is also apparent that the MUC1 CT exhibits functional activities apart from its ability to directly block TLR signaling. Ahmad et al. (44, 45) demonstrated that the MUC1 C-terminal subunit promotes TNF-α–induced activation of NF-κB in human breast cancer cells. In contrast, we reported that the MUC1 CT binds to the IKKγ subunit to inhibit *H. pylori*-dependent NF-κB activation in a human gastric cancer cell line (46). It is unknown whether IKK/NF-κB are recruited to the inner leaflet of the membrane and whether this interaction can be disrupted by TLR signaling. It is also possible that IKK/NF-κB signaling is disrupted by other means such as PKC activation.

**FIGURE 8.** Colocalization of Muc1 and TLR5 in mouse airway epithelium in vivo. Paraffin-embedded mouse lung sections from uninfected (Control) and PAK intranasally infected Muc1+/+ mice were processed for immunostaining with anti-CT2 and anti-TLR5 (A) or anti-phosphotyrosine (B) Abs, followed by incubation with fluorescein-conjugated secondary Abs and DAPI to counterstain nuclei. Scale bars, 20 μm. Arrowheads indicate areas of colocalization.

**FIGURE 9.** Schematic illustration of the proposed mechanism through which MUC1 negatively regulates TLR5 signaling. Step 1, TLR5 on epithelial cells senses bacteria-derived flagellin. Step 2, Activated TLR5 triggers MyD88-dependent signaling to induce the release of inflammatory mediators, which results in recruitment of leukocytes into the site of infection to clear the bacteria. Step 3, Inflammatory products, such as neutrophil elastase and TNF-α, upregulate MUC1 expression. Step 4, EGFR activated by TLRs or other means phosphorylates the MUC1 CT at Y46. Step 5, MUC1 binds to TLR5, which interferes with the recruitment of MyD88 to TLR5.
plasma membrane by the MUC1 CT or whether the membrane-bound CT is released into the cytoplasm to interact with IKK/NF-kB. Nonetheless, apically polarized MUC1 presumably favors its ability to interact with TLR5 and, hence, selectively suppresses TLR5-induced NF-kB activity per se. Interestingly, exposure of polarized airway epithelial cells to cigarette smoke redistributed apical MUC1 into the cytosol, suggesting that exogenous insults can affect the subcellular localization of MUC1 and, by implication, its functional properties in the lung (47). Elucidation of the factors controlling MUC1 cellular and functional heterogeneity deserves further investigation.

In conclusion, we propose the following sequence of events during airway P. aeruginosa infection in the context of the anti-inflammatory role of MUC1/Muc1 (Fig. 9). Host defense against the pathogen is mediated primarily by mucociliary clearance and phagocytosis. During the early stage of infection, TLR5 on respiratory epithelial cells (and, perhaps, resident macrophages) senses P. aeruginosa through its interaction with flagellin (step 1) and triggers MyD88-dependent signaling (step 2) to induce inflammatory mediators that result in recruitment of leukocytes into the site of infection to clear the bacteria. The inflammatory products generated during this process, such as neutrophil elastase and TNF-α (8, 34, 48) (step 3), upregulate MUC1/Muc1 expression during the late stage of infection following clearance of the pathogen from the airways. Activation of EGFR by TLRs and/or alternative mechanisms stimulates phosphorylation of the MUC1 CT at Y46 (step 4), leading to MUC1/TLR5 interaction (step 5), and TNF-α induces epithelial proinflammatory gene expression. Of MUC1 products, such as MUC5AC mucin overproduction via tumor necrosis factor-alpha-converting enzyme, helps the host defend against P. aeruginosa infection via recognition of flagellin by Toll-like receptor 5 (TLR5) that activates the extracellular signal-regulated kinase.

**References**


**Disclosures**

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**References**


