MUC1 Regulates Epithelial Inflammation and Apoptosis by Poly(I:C) through Inhibition of Toll/IL-1 Receptor-Domain-Containing Adapter-Inducing IFN- β (TRIF) Recruitment to Toll-like Receptor 3

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Abstract

MUC1/Muc1 (MUC1 in humans, Muc1 in animals) is a membrane-tethered mucin expressed by airway epithelial cells and plays an antiinflammatory role during airway bacterial infection. We previously demonstrated that MUC1/Muc1 is a negative regulator of Toll-like receptor (TLR) inflammatory signaling mediated through the myeloid differentiation primary response gene 88 (MyD88) adaptor protein. In the present study, we determined whether MUC1 regulates MyD88-independent TLR signaling mediated through the TLR3–Toll/IL-1 receptor-domain-containing adapter-inducing IFN- β (TRIF) pathway in response to poly(I:C). Compared with MUC1/Muc1-expressing controls, cells deficient in MUC1/Muc1 were more prone to poly(I:C)-induced apoptosis; had increased poly(I:C)-driven activation of caspase-3, caspase-8, IFN regulatory factor-3, and NF- κ B; and displayed heightened IFN- β gene expression. MUC1 overexpression by these cells had the opposite effects. Reciprocal coimmunoprecipitation experiments established constitutive TLR3/MUC1-CT (cytoplasmic tail) protein interaction in human embryonic kidney (HEK)293T cells overexpressing the two proteins and in lung epithelial cells expressing the endogenous proteins, the latter of which was confirmed by immunofluorescence colocalization of TLR3 with MUC1-CT. Coimmunoprecipitation

studies also revealed that MUC1 overexpression by HEK293T cells reduced poly(I:C)-induced TLR3/TRIF protein interaction. Finally, MUC1 overexpression had no effect on TRIF-dependent auto-activation of TLR3 signaling, suggesting that the site of action of the MUC1-CT in TLR3 signaling is not downstream of TRIF. These data indicate that MUC1-CT counter-regulates apoptotic and inflammatory responses of airway epithelial cell through constitutive association with TLR3, thereby inhibiting poly(I:C)-induced recruitment of TRIF to TLR3.

Keywords: MUC1; poly(I:C); Toll-like receptor 3; Toll/IL-1 receptor-domain-containing adapter-inducing IFN- β ; apoptosis

Clinical Relevance

Lung epithelium is the primary barrier during viral infection. This article describes how MUC1, an antiinflammatory molecule expressed by lung epithelium, controls inflammatory responses induced by an *in vitro* model of viral infection and suggests an important role of MUC1 in the pathogenesis of chronic inflammatory lung disease.

MUC1 (MUC1 in humans, Muc1 in animals) is a transmembrane glycoprotein expressed by the majority of mucosal epithelial cells and by hematopoietic cells (1). The MUC1 protein consists of two noncovalently associated polypeptide subunits, an NH₂-terminal extracellular MUC1 α subunit and a COOH-terminal MUC1 β subunit containing its cytoplasmic tail (MUC1-CT).

The MUC1 α subunit is composed almost entirely of a variable number of tandem repeats, whereas the MUC1 β subunit consists of a 58-amino acid (aa) extracellular juxtamembranous region, a 28-aa membrane spanning domain, and a highly conserved 72-aa CT with multiple sites of serine, threonine, and tyrosine phosphorylation (2). The MUC1 protein is

normally expressed in a variety of cell compartments, including the cell surface, nucleus, and endosomes (1, 2). The *MUC1* gene was originally cloned from epithelial-derived human breast and pancreatic cancer cells (3, 4), where it is overexpressed in a hypoglycosylated form (1). Aberrant MUC1 overexpression by these cells was postulated to contribute to oncogenesis by

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regulating gene transcription, stress-induced apoptosis, and necrosis through phosphorylation of its intracellular CT signaling domains (5).

A growing body of evidence indicates that the resolution of inflammation in the airway mucosal epithelia is regulated, in part, through increased MUC1 expression (2, 6). The first evidence came from *in vivo* studies that demonstrated that Muc1 knockout (KO) mice mount hyperinflammatory responses after intranasal instillation of *Pseudomonas aeruginosa* or its flagellin compared with their Muc1 wild-type (WT) littermates (7). Muc1 is up-regulated principally by the inflammatory cytokine TNF- α (8) to counter-regulate ongoing inflammation driven by a variety of respiratory pathogens in addition to *P. aeruginosa*, including respiratory syncytial virus (9) and *Haemophilus influenzae* (10). Our recent studies with cultured airway epithelial cells treated with *P. aeruginosa* revealed that the antiinflammatory activity of MUC1/Muc1 requires MUC1-CT phosphorylation by the receptor tyrosine kinase, epidermal growth factor receptor, which leads to the association of MUC1 with TLR5 to inhibit recruitment of the myeloid differentiation primary response gene 88 (MyD88) adaptor protein to the TLR5 Toll/IL-1 receptor (TIR) domain, thereby suppressing TLR5-dependent inflammation (11).

The MyD88-independent TLR3 signaling pathway is mediated exclusively by the Toll/IL-1 receptor domain-containing adapter-inducing IFN- β (TRIF) protein (12, 13). Upon ligation of double-stranded (ds)RNA, various branches of the TLR3-TRIF signaling pathway lead to the activation of IFN regulatory factor (IRF)-3, NF- κ B, and other transcription factors that induce type I IFNs and related antiviral cytokines and stimulate apoptosis through caspase activation (14–17). We previously reported that peritoneal and alveolar macrophages from Muc1 KO mice secrete greater levels of TNF- α in response to polyinosinic:polycytidylic acid [poly(I:C)] compared with Muc1 WT macrophages (18). Conversely, MUC1 overexpression by human embryonic kidney (HEK)293T cells suppressed poly(I:C)-stimulated activation of NF- κ B. However, the mechanism through which MUC1 regulates this poly(I:C) response, presumably mediated through TLR3, was not elucidated. Therefore, the current study was undertaken to determine

the effects of MUC1 on poly(I:C)-induced, TLR3-dependent inflammatory and apoptotic signaling and to assess TLR3/MUC1-CT and TLR3/TRIF protein interactions in these cells.

Materials and Methods

Materials and Cell Culture

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. The antibodies (Abs) and reagents used were anti-caspase-8 Ab, anti-phospho-IRF-3 Ab, anti-phospho-I κ B α Ab, and anti-TRIF Ab (Cell Signaling Technology, Beverly, MA); anti-TLR3 Ab (Abcam, Cambridge, MA); anti-MUC1-CT Ab (CT-2) and MUC1-pcDNA3.1 expression plasmid (Dr. Sandra J. Gendler, Mayo Clinic, College of Medicine, Scottsdale, AZ); nonimmune rabbit and mouse IgGs (Santa Cruz Biotechnology, Santa Cruz, CA); HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG secondary Abs (KPL, Gaithersburg, MD); TLR3-FLAG and TRIF-FLAG epitope-tagged expression plasmids and IFN- β -luciferase (19) and IFN-stimulated response element (ISRE)-luciferase reporter constructs (Dr. Katherine Fitzgerald, University of Massachusetts Medical School, Worcester, MA); poly(I:C) (IMGENEX, San Diego, CA); and human alveolar adenocarcinoma A549 (CCL-185), human bronchial epithelial BEAS-2B (CRL-9609), and HEK293T (CRL-1573) cells (ATCC, Manassas, VA). A549 cells were cultured in Opti-MEM medium supplemented with 5% FBS and antibiotics. BEAS-2B cells were cultured in LHC-9 medium. HEK293T cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% FBS and antibiotics. All culture media and supplements were from Invitrogen (Carlsbad, CA). Primary mouse tracheal surface epithelial (mTSE) cells were isolated from 8- to 12-week-old C57BL/6 mice and cultured as described (7).

Manipulation of MUC1 Expression

Small interfering (si)RNAs and short hairpin (sh)RNAs were used to silence MUC1 expression. A549 cells were transiently transfected with a MUC1-targeting siRNA (Dharmacon, Waltham, MA) using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. Oligonucleotide sequences in the MUC1

siRNA have been described (20). BEAS-2B cells were stably infected with MUC1-targeting shRNA lentivirus particles (Santa Cruz Biotechnology). The MUC1 shRNA lentivirus constituted a pool of concentrated, transduction-ready viral particles containing three MUC1-specific constructs that encoded 19 to 25 nucleotide (plus hairpin) shRNAs. As negative controls, the cells were transfected or infected with nontargeting siRNAs or shRNAs. To overexpress MUC1, HEK293T cells were transiently transfected with a MUC1-pcDNA3.1 expression plasmid as described (11) or with pcDNA3.1 empty vector as a negative control.

Cytotoxicity Assay

mTSE, BEAS-2B, and A549 cells (5.0×10^3) were transfected/infected with MUC1 or control siRNAs/shRNAs, plated in 96-well flat-bottom plates, and incubated for 24 hours. The cells were treated for 24 hours at 37°C with 10, 100, 300, or 1,000 μ g/ml of poly(I:C) or with medium alone (control). The culture medium was replaced, and the cells were incubated for 2 to 4 hours with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The culture medium was removed, and the cells were incubated for 10 minutes at 37°C in DMSO (50 μ l/well). Formazan levels were determined by optical density at 570 nm.

Immunoblot and Coimmunoprecipitation Analyses

mTSE, A549, BEAS-2B, and HEK293T cells were extracted with the lysis buffer, and equal protein aliquots were separated by SDS-PAGE and subjected to immunoblot analysis as described (11). To control for protein loading and transfer, the blots were stripped and reprobed for β -tubulin. For coimmunoprecipitation assays, equal protein aliquots of cell lysates were incubated for 16 hours at 4°C with immunoprecipitating Ab (10 μ g) or with isotype-matched nonimmune IgG as described (11). Protein G-agarose beads (Pierce, Rockford, IL) were added. The beads were washed five times at 4°C with lysis buffer and eluted by boiling in SDS-PAGE loading buffer. Immunoprecipitated proteins were separated by SDS-PAGE and subjected to immunoblot analysis for the protein of interest. To control for protein loading and transfer, the blots

were stripped and reprobed with the immunoprecipitating Ab.

Caspase-3 Activity Assay

BEAS-2B cells infected with MUC1 shRNA or control shRNA lentiviruses were treated for 4 hours at 37°C with 10 µg/ml of poly(I:C) or medium control. The cells were washed, and caspase-3 activity of the cells was determined using the Caspase-Glo-3/7 luminescent assay kit (Promega, Madison, WI) according to the manufacturer's instructions. The caspase-3 activity of poly(I:C)-treated cells was normalized to that of the medium controls.

Transient Transfection and Luciferase Reporter Assay

A549 and BEAS-2B cells were silenced for MUC1 expression by RNA interference or were transfected with MUC1-pcDNA3.1 or TRIF-FLAG expression plasmids or with the respective controls and incubated for 24 hours. The cells were transiently transfected with IFN-β promoter-luciferase or ISRE-luciferase reporter constructs plus a *Renilla* luciferase internal control and cultured for an additional 24 hours. The cells were treated for 24 hours with 10 or 100 µg/ml of poly(I:C) or medium control, and relative luciferase activity was measured as described (7).

Colocalization

Immunofluorescence Microscopy

BEAS-2B cells were grown on 8-well glass slides (Nalge Nunc, Naperville, IL), washed five times with PBS, and fixed with 4% paraformaldehyde as described (11). The fixed cells were incubated for 30 minutes at room temperature with 2% BSA and 5% FCS in PBS and washed. The cells were incubated overnight at 4°C with hamster anti-MUC1-CT and rabbit anti-TLR3 primary Abs followed by Alexa Fluor 488-labeled goat anti-hamster and Alexa Fluor 594-labeled goat anti-rabbit secondary Abs for 1 hour at room temperature. The cells were counterstained and mounted with DAPI plus antifade reagent (Invitrogen) and visualized by laser scanning confocal microscopy using a Leica Sp5 microscope (Leica Microsystems, Buffalo Grove, IL) under a 63× oil objective.

Statistical Analysis

All values were expressed as means ± SEM. Differences between means were compared using the Student's *t* test and were considered significant at *P* < 0.05.

Results

MUC1 Expression Limits Poly(I:C)-Induced Cytotoxicity of Lung Epithelial Cells

Recent evidence suggests that the synthetic viral dsRNA analog poly(I:C) activates TLR3, leading to programmed cell death of human airway epithelial cells (16). Because MUC1 inhibits TLR signaling in airway epithelial cells (7, 10, 11), we asked whether manipulation of MUC1 expression regulates poly(I:C)-induced apoptosis. Primary mTSE cells isolated from Muc1 KO mice and their WT littermates were treated for 24 hours with 100 or 1,000 µg/ml of poly(I:C) or with medium control, and cell viability was measured by the MTT colorimetric assay. Poly(I:C)-induced cell death at both concentrations was greater in Muc1 KO mTSE cells compared with WT cells (Figure 1A). Similarly, silencing of MUC1 expression in BEAS-2B (Figure 1B) and A549 (Figure 1C) human lung epithelial cells by RNA interference increased poly(I:C)-stimulated cytotoxicity compared with MUC1-expressing cells. Because the effect of MUC1/Muc1 on poly(I:C)-induced apoptosis was consistent regardless of the types of airway epithelial cells tested, we decided to use epithelial cell lines for the mechanistic studies described below.

MUC1 Expression Attenuates Poly(I:C)-Induced Activation of Caspase-8 and Caspase-3

Upon ligation by poly(I:C), TLR3 associates with the adaptor protein TRIF to activate the Fas-associated protein with death domain (FADD)/caspase-8/caspase-3-dependent extrinsic apoptotic signaling pathway (16). Because MUC1 inhibits poly(I:C)-induced cytotoxicity (Figure 1), we explored the effect of manipulating MUC1 expression on poly(I:C)-induced activation of the apoptotic caspase-8 and caspase-3 proteins. After 4 hours of poly(I:C) treatment, BEAS-2B cells in which MUC1 expression was silenced exhibited increased cleaved caspase-8 levels (Figure 2A) and greater caspase-3 activity (Figure 2D) compared with MUC1-expressing, poly(I:C)-treated cells.

MUC1 Expression Inhibits Poly(I:C)-Induced IRF-3 and NF-κB Activation and IFN-β Gene Expression

To determine whether MUC1 counter-regulates poly(I:C)-induced inflammation,

we first examined the effect of MUC1 silencing on poly(I:C)-induced activation of downstream elements of the TLR3 signaling pathway, namely IRF-3 activation through its phosphorylation, NF-κB activation through phosphorylation of its inhibitor, IκBα, and IFN-β gene expression. Increased phospho-IRF-3 (Figure 2B) and phospho-IκBα (Figure 2C) levels were seen in BEAS-2B cells infected with MUC1 shRNA lentivirus after 2 hours of poly(I:C) treatment compared with control shRNA-infected cells. Increased IFN-β gene expression after 2 or 4 hours of poly(I:C) treatment was also seen in BEAS-2B cells in which MUC1 expression was silenced compared with cells infected with the control shRNA (Figure 2E). Similarly, in A549 cells, MUC1 silencing increased poly(I:C)-induced IFN-β gene expression compared with MUC1-expressing cells (Figure 2F), whereas MUC1 overexpression by these cells reduced poly(I:C)-induced IFN-β gene expression compared with empty vector controls (Figure 2G). These combined results suggest that MUC1 expression by lung epithelial cells attenuates poly(I:C)-induced activation of IRF-3 and NF-κB, thereby suppressing IFN-β production. Thus, in agreement with the role of MUC1 in poly(I:C)-induced apoptosis, the inhibitory effect of MUC1 on poly(I:C)-induced immune responses has been observed regardless of the epithelial cell types tested.

MUC1-CT Associates with TLR3 in Human Lung Epithelial Cells

Under basal conditions and in response to *P. aeruginosa* bacteria, the MUC1-CT associates with TLR5 (11). Therefore, we queried whether the MUC1-CT might similarly interact with TLR3 and, if so, whether poly(I:C) increases TLR3/MUC1-CT association. HEK293T cells, engineered to be highly transfectable, were examined in reciprocal coimmunoprecipitation assays after transfection with MUC1-pcDNA3.1 and TLR3-FLAG expression plasmids. Immunoprecipitation of cell lysates with anti-FLAG Ab coprecipitated MUC1-CT, and immunoprecipitation with anti-MUC1-CT Ab coprecipitated TLR3-FLAG (Figure 3A). Identical results were seen in BEAS-2B (Figure 3B) and A549 (Figure 3C) cells endogenously expressing the MUC1-CT and TLR3 proteins. However, treatment of the lung epithelial cells with 10 or 100 µg/ml of poly(I:C) for 1 or 24 hours

Figure 1. MUC1 deficiency augments poly(I:C)-induced cytotoxicity in lung epithelial cells. Primary mouse tracheal surface epithelial (mTSE) cells isolated from (A) MUC1 wild-type (WT) and knockout (KO) mice, (B) BEAS-2B cells infected with control or MUC1 short hairpin (sh)RNAs, and (C) A549 cells transfected with control or MUC1 small interfering (si)RNAs were treated for 24 hours with the indicated concentrations of poly(I:C). Cell viability was determined by MTT assay, and % viability was determined relative to medium controls. Each point represents the mean $\pm$ SEM value ($n = 4$). *Significantly reduced cell viability of MUC1-expressing versus MUC1-deficient cells at $P < 0.05$. MUC1-CT expression of the various cells was determined by immunoblotting (right panels). The results are representative of two independent experiments.

had no effect on the level of TLR3/MUC1-CT coimmunoprecipitation (data not shown). Further, poly(I:C) treatment of A549 cells did not affect MUC1-CT expression levels (Figure 3D). Laser scanning confocal microscopy of permeabilized BEAS-2B cells revealed colocalized intracellular TLR3/MUC1-CT immunostaining that, again, was not affected by poly(I:C) treatment compared with medium controls (Figure 4). Together, these results suggest that MUC1 and TLR3 are physically associated with each other in unstimulated cells and that activation of TLR3 with poly(I:C) treatment has no effect on the level of MUC1 expression or on the degree of the association.

MUC1 Expression Impedes Poly(I:C)-Induced Recruitment of TRIF to TLR3

Recently, we showed that MUC1-CT association with TLR5 inhibits recruitment of MyD88 to the TLR5 TIR domain in response to *P. aeruginosa* bacteria (11). Because the MUC1-CT also associates with TLR3 (Figures 4 and 5), we asked whether TLR3/MUC1-CT association interferes with TRIF recruitment to TLR3. HEK293T cells were studied in TLR3/TRIF coimmunoprecipitation assays after transfection with TLR3-FLAG with or without MUC1 overexpression. In the absence of MUC1 expression, poly(I:C) treatment for 1 hour increased TLR3/TRIF association (Figure 5A). However, poly(I:C)-induced

TLR3/TRIF interaction in the presence of MUC1 overexpression was reduced to a level equal to that seen in the absence of poly(I:C). Because TRIF overexpression reportedly auto-activates IFN- β and ISRE gene expression in the absence of poly(I:C) (16), we next examined the effect of TRIF overexpression on TLR3 ligand-independent TRIF signaling. TRIF overexpression by HEK293T cells in the absence of MUC1 increased constitutive IFN- β and ISRE gene expression in a dose-dependent manner compared with empty vector controls (Figure 5B). Further, cotransfection of MUC1 with TRIF did not affect auto-activation of either reporter gene, suggesting that the MUC1-CT does not inhibit TLR3 signaling downstream of TRIF. Taken together, the results in Figures 3–5 suggest that MUC1 constitutively interacts with TLR3, thereby blocking poly(I:C)-induced recruitment of TRIF to TLR3.

Discussion

Our previous study demonstrated that MUC1 expression suppresses TLR5-induced inflammation in lung epithelial cells in response to *P. aeruginosa* bacteria through its ability to interact with TLR5 and competitively inhibit recruitment of MyD88 to the TLR5 cytoplasmic TIR domain (11). The current report extends the MUC1 inhibitory effect to the TLR3-TRIF pathway, a MyD88-independent TLR signaling pathway, where MUC1 counter-regulates inflammatory signaling and apoptosis in response to poly(I:C). Down-regulation of MUC1 expression in human lung epithelial cells by RNA interference resulted in greater poly(I:C)-induced activation of caspase-8, caspase-3, IRF-3, and NF- κ B and increased IFN- β gene expression compared with MUC1-expressing cells. Coimmunoprecipitation and confocal immunofluorescence colocalization studies revealed that TLR3 and MUC1-CT constitutively form a protein complex in lung epithelia that was not affected by poly(I:C). Finally, MUC1 overexpression abrogated the poly(I:C)-driven recruitment of TRIF to TLR3 but did not influence IFN- β or ISRE gene expression induced by TRIF overexpression. These findings unveil the underlying molecular mechanism through which MUC1 suppresses ligand-induced, TLR3-dependent apoptosis and inflammation by lung epithelial cells (Figure 6).

Figure 2. Manipulation of MUC1 expression alters poly(I:C)-induced activation of apoptotic and inflammatory signaling cascades. (A–C) BEAS-2B cells infected with control or MUC1 shRNA lentiviruses were treated for the indicated times with 10 $\mu\text{g/ml}$ of poly(I:C) or medium control. Cell lysates were processed for immunoblotting with cleaved caspase-8 (A), phospho-IRF-3 (B), or phospho-I κ B α (C) antibodies (Abs). To control for protein loading and transfer, the blots were stripped and reprobed for β -tubulin. Densitometric analysis of band intensities normalized to β -tubulin in the same lane is displayed above each blot. (D) BEAS-2B cells infected with control or MUC1 shRNA lentiviruses were treated for 4 hours with 100 $\mu\text{g/ml}$ of poly(I:C) or medium control. Caspase-3 activity was quantified, and activity of poly(I:C)-treated cells was normalized to that of medium controls. (E) BEAS-2B cells infected with control or MUC1 shRNA lentiviruses were transfected with an IFN- β promoter-luciferase reporter plasmid plus *Renilla* luciferase plasmid as an internal control and treated for the indicated times with 10 $\mu\text{g/ml}$ poly(I:C) or medium control. Firefly luciferase activity driven by the IFN- β promoter was normalized to *Renilla* luciferase activity, and normalized luciferase activity of poly(I:C)-treated cells was expressed relative to that of medium control-treated cells. (F, G) A549 cells were cotransfected with IFN- β promoter-luciferase reporter and *Renilla* luciferase plasmids plus MUC1 or control siRNAs (F) or luciferase plasmids plus MUC1-pcDNA3.1 (pMUC1) or pcDNA3.1 empty vector (G). The cells were treated for 16 hours with 10 or 100 $\mu\text{g/ml}$ poly(I:C) or medium control, and normalized luciferase activity of poly(I:C) treated cells was expressed relative to that of medium control-treated cells. In (D–G), each bar or point represents the mean $\pm$ SEM value ($n = 4$). *Significantly reduced luciferase activity of MUC1-expressing versus MUC1-deficient cells (F) or MUC1-overexpressing versus MUC1 normal cells (G) at $P < 0.05$. †Not significant. The results are representative of two independent experiments.

dsRNA, particularly its synthetic analog poly(I:C), induces apoptosis through engagement of TLR3 (16, 19, 21). *In vitro* knockdown of TLR3 expression diminished

the cytotoxic effects of poly(I:C) (15), whereas up-regulation of TLR3 had the opposite effect (19). KO mice lacking TLR3 or TRIF were completely protected from

poly(I:C)-induced programmed cell death, and this apoptotic response in WT littermates was independent of non-TLR3 signaling pathways (21). Poly(I:C)-induced

Figure 3. Association of MUC-CT with Toll-like receptor (TLR)3. (A) Human embryonic kidney (HEK)293T cells were cotransfected with expression plasmids encoding for TLR3-FLAG and MUC1. The cells were lysed, and the lysates were immunoprecipitated with nonimmune IgG or with anti-FLAG or anti-MUC1-CT Abs. The IgG and anti-FLAG Ab precipitates were processed for anti-MUC1-CT immunoblotting, and the IgG and anti-MUC1-CT Ab precipitates were processed for anti-FLAG immunoblotting. To control for protein expression, whole cell lysates (WCL) were processed for immunoblotting with anti-FLAG, anti-MUC1-CT, and anti-β-tubulin Abs. (B, C) BEAS-2B and A549 cells were lysed, and the lysates were immunoprecipitated with nonimmune IgG or anti-MUC1-CT Abs. The precipitates were processed for anti-TLR3 immunoblotting. To control for protein loading and transfer, the blots were stripped and reprobed with anti-MUC1-CT Abs. (D) A549 cells were treated for the indicated times with 100 μg/ml poly(I:C) or medium control. Cell lysates were processed for immunoblotting with anti-MUC1-CT or anti-β-tubulin Abs. The results are representative of three independent experiments.

apoptosis is mediated through TRIF recruitment to the TLR3 cytoplasmic TIR domain and subsequent activation of a FADD/caspase-8/caspase-3 pathway (16). Based on the results presented in this report, we suggest that the MUC1-CT interferes with TLR3-dependent apoptosis through its ability to block TLR3/TRIF protein interaction. This conclusion is not incompatible with prior studies demonstrating that aberrant overexpression of MUC1 by cancer cells promotes their growth and survival through interaction of the MUC1-CT with cytosolic and receptor kinases, including phosphatidylinositol 3-kinase (PI3K), c-Src, and epidermal growth factor receptor, and constitutive activation of the Akt and mitogen-activated protein kinase signaling cascades (22). Raina and colleagues (23) demonstrated that sustained inhibition of MUC1 expression by the MUC1-CT mimetic peptide GO-203, in non-small lung cancer cells, blocked activation of the PI3K-Akt-mTOR pathway, increased ROS levels, and induced programmed cell death. Whether MUC1-CT mimetic peptides in combination with poly(I:C) might further promote apoptosis of normal or cancer cells remains to be tested.

It has been shown that the lung epithelial cell line A549 is poorly responsive to poly(I:C) in the range of 10 to 100 μg/ml

due to low expression of endogenous TLR3, whereas pretreatment with antiviral IFNs induced up-regulation of TLR3 and conferred antiviral immune

response in these cells (24). Consistent with this previous study, the lung epithelial cells used in this study required a high dose of poly(I:C) (100–1,000 μg/ml)

Figure 4. Colocalization of MUC1-CT and TLR3. BEAS-2B cells were cultured in 8-well chambers, incubated for 0.5 hours with 100 μg/ml poly(I:C) or medium control, fixed with 4% paraformaldehyde, and permeabilized. The cells were stained with anti-MUC1-CT Ab plus Alexa Fluor 488-conjugated goat anti-hamster IgG secondary Ab and anti-TLR3 Ab plus Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary Ab. The cells were visualized by confocal immunofluorescence microscopy for (A) MUC1-CT (green) and (B) TLR3 (red). Colocalization of MUC1-CT with TLR3 is indicated by yellow staining of the merged images (arrows). The results are representative of three independent experiments.

Figure 5. MUC1 overexpression inhibits poly(I:C)-induced recruitment of Toll/IL-1 receptor domain-containing adapter-inducing IFN- β (TRIF) to TLR3. (A) HEK293T cells were cotransfected with an expression plasmid encoding for TLR3-FLAG plus MUC1-pcDNA3.1 or pcDNA3.1 empty vector and cultured for 24 hours. The cells were treated for 1 hour with 100 μ g/ml poly(I:C) or medium control and lysed. Equal protein aliquots of cell lysates were immunoprecipitated with nonimmune IgG or anti-TRIF Ab. The IgG and anti-TRIF Ab precipitates were processed for anti-FLAG immunoblotting. To control for protein loading and transfer, the blots were stripped and reprobed with anti-TRIF Abs. Densitometric analysis of the anti-FLAG Ab band intensities normalized to anti-TRIF band intensities in the same lane are shown below the blots. To control for protein expression, WCL were processed for immunoblotting with anti-FLAG, anti-TRIF, anti-MUC1-CT, and anti- β -tubulin Abs. (B) HEK293T cells were cotransfected with MUC1-pcDNA3.1 (pMUC1) or pcDNA3.1 empty vector plus 0, 0.1, or 1.0 μ g of a TRIF expression plasmid (pTRIF) and cultured for 16 hours. The cells were washed and transfected with IFN- β promoter-luciferase reporter plasmid (upper panel) or IFN-stimulated response element (ISRE)-luciferase reporter plasmid (lower panel), each with *Renilla* luciferase plasmid as an internal control, and incubated for 16 hours. Firefly luciferase activities driven by the IFN- β promoter or ISRE were normalized to *Renilla* luciferase activity. Each bar represents the mean $\pm$ SEM value ($n = 4$). † Not significant. The results are representative of three independent experiments.

to induce apoptosis. It is conceivable that high levels of IFNs produced by immune cells during viral infection might up-regulate TLR3 in lung epithelial cells, resulting in sensitizing to cytotoxicity of dsRNA.

The antiinflammatory property of MUC1/Muc1 in the lung was initially revealed by studies demonstrating that Muc1 KO mice had increased levels of TNF- α and keratinocyte chemoattractant (murine IL-8) in bronchoalveolar lavage fluid after *in vivo* challenge with *P. aeruginosa* bacteria or its flagellin compared with Muc1 WT littermates (7). Because bacterial flagellin is an established ligand for TLR5 (25), subsequent studies were directed at dissecting the relationship between MUC1 and TLR5 in the airway response to flagellin. Using HEK293T cells, which do not express MUC1, Lu and

colleagues (7) showed that cotransfection with MUC1 and TLR5 expression plasmids abrogated flagellin-induced IL-8 production compared with cells transfected for TLR5 expression alone. Cotransfection with TLR5 plus a MUC1-CT deletion mutant lacking the 72-aa intracellular domain restored flagellin-induced IL-8 production to a level that was equal to that of cells expressing TLR5 alone, thus localizing the antiinflammatory activity of MUC1 to its cytoplasmic region. In more recent studies, the MUC1-CT and TLR5 were shown to constitutively form a protein complex in lung epithelial cells, and this interaction was increased after treatment with *P. aeruginosa*, leading to decreased TLR5/MyD88 association and diminished inflammation (11). The current studies demonstrate that the MUC1-CT interacts with TLR3, leading to reduced TLR3/TRIF

association. Unlike the TLR5/MyD88 system, however, TLR3/MUC1-CT interaction was not increased by poly(I:C) ligand engagement.

TNF- α is a key regulator of MUC1 expression during lung inflammation (8, 26). For example, whereas Muc1 protein levels in mouse lungs were relatively low before infection with *P. aeruginosa*, Muc1 levels were increased after bacterial infection, reaching maximum levels at 2 days which remained even at 7 days (8). However, TNF receptor KO mice failed to up-regulate Muc1 expression after *P. aeruginosa* infection. Further, Muc1 and TNF receptor KO mice were unable to resolve *P. aeruginosa*-induced airway inflammation in this experimental model of bacterial lung infection. Of relevance to the current report, we propose that TNF- α is produced at an early stage after infection

Figure 6. Schematic representation of the negative regulation of TLR3 and TLR5 signaling by MUC1. TLR3 expressed in endosomes by airway epithelial cells senses viral-derived dsRNA and triggers TRIF-dependent signaling pathways to induce inflammation and apoptosis. TLR5 expressed on the surface of these same cells senses bacterial flagellin and triggers a myeloid differentiation primary response gene 88 (MyD88)-dependent signaling pathway to induce inflammation. During this early stage of inflammation, MUC1 expression is relatively low. TNF- α produced during the ongoing inflammatory response up-regulates MUC1 expression. The TLR3 and TLR5 signaling pathways are subsequently inhibited through interaction of the MUC1 CT with the respective cytoplasmic domains of TLR3 and TLR5.

with bacterial or viral airway pathogens after engagement of host innate immune receptors, such as TLRs, with their cognate pathogen-associated molecular pattern molecules, including bacterial flagellin (for TLR5) and viral dsRNA (for TLR3). Increased TNF- α up-regulates MUC1 expression with the MUC1-CT binding to the cytoplasmic domains of these TLRs to inhibit recruitment of their respective adaptor proteins (MyD88 and TRIF), thereby attenuating the airway inflammatory response (Figure 6). Thus, dysfunction of MUC1 by the failure to up-regulate MUC1 at the end of inflammation or by mutation of the MUC1 CT, which is

essential for its antiinflammatory activity (7, 11), might be responsible for the pathogenesis of chronic inflammatory lung diseases such as chronic obstructive pulmonary disease (COPD). We are planning to test this hypothesis using *in vivo* models of COPD, such as cigarette smoking in mice.

In summary, this report provides the first description of the molecular mechanism for the antiinflammatory effect of MUC1 during TLR3 activation, which may be of clinical significance for human airway infectious diseases. For example, a variety of airway viral pathogens, including rhinovirus, influenza virus, and

respiratory syncytial virus, are important causes of exacerbations in patients with COPD (27). However, the underlying mechanism accounting for the increased susceptibility of patients with COPD to viral infections remains poorly understood. Kang and colleagues (28) demonstrated that parenchymal and airway inflammation, as well as apoptosis, induced by dsRNA were increased in mice exposed to cigarette smoke compared with those exposed to smoke-free air, suggesting that dsRNA may play a pivotal role in the pathogenesis of virus-associated COPD exacerbations. MUC1/Muc1 is expressed throughout the entire luminal surface of airway epithelia and by type II pneumocytes of the lung parenchyma, where it is strategically positioned to counter-regulate local inflammatory responses to infectious and injurious stimuli (29). Because Muc1 levels are increased in the airways of mice exposed to cigarette smoke (K. Kato, unpublished observations), it is possible that MUC1/Muc1 might play an important role in controlling lung inflammation and epithelial cell apoptosis during viral infection, particularly in conjunction with exposure to tobacco smoke. Ongoing studies in our laboratory are directed toward testing this hypothesis.

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References

- Hatrup CL, Gendler SJ. Structure and function of the cell surface (tethered) mucins. *Annu Rev Physiol* 2008;70:431–457.
- Kim KC, Lillehoj EP. MUC1 mucin: a peacemaker in the lung. *Am J Respir Cell Mol Biol* 2008;39:644–647.
- Gendler SJ, Lancaster CA, Taylor-Papadimitriou J, Duhig T, Peat N, Burchell J, Pemberton L, Lalani EN, Wilson D. Molecular cloning and expression of human tumor-associated polymorphic epithelial mucin. *J Biol Chem* 1990;265:15286–15293.
- Lan MS, Batra SK, Qi WN, Metzgar RS, Hollingsworth MA. Cloning and sequencing of a human pancreatic tumor mucin cDNA. *J Biol Chem* 1990;265:15294–15299.
- Kufe DW. Functional targeting of the MUC1 oncogene in human cancers. *Cancer Biol Ther* 2009;8:1197–1203.
- Lillehoj EP, Kato K, Lu W, Kim KC. Cellular and molecular biology of airway mucins. *Int Rev Cell Mol Biol* 2013;303:139–202.
- Lu W, Hisatsune A, Koga T, Kato K, Kuwahara I, Lillehoj EP, Chen W, Cross AS, Gendler SJ, Gewirtz AT, et al. Cutting edge: enhanced pulmonary clearance of *Pseudomonas aeruginosa* by Muc1 knockout mice. *J Immunol* 2006;176:3890–3894.
- Choi S, Park YS, Koga T, Treloar A, Kim KC. TNF- α is a key regulator of MUC1, an anti-inflammatory molecule, during airway *Pseudomonas aeruginosa* infection. *Am J Respir Cell Mol Biol* 2011;44:255–260.
- Li Y, Dinwiddie DL, Harrod KS, Jiang Y, Kim KC. Anti-inflammatory effect of MUC1 during respiratory syncytial virus infection of lung epithelial cells *in vitro*. *Am J Physiol Lung Cell Mol Physiol* 2010;298:L558–L563.

10. Kyo Y, Kato K, Park YS, Gajhate S, Umehara T, Lillehoj EP, Suzaki H, Kim KC. Anti-inflammatory role of MUC1 mucin during nontypeable *Haemophilus influenzae* infection. *Am J Respir Cell Mol Biol* 2012;46:149–156.
11. Kato K, Lillehoj EP, Park YS, Umehara T, Hoffman NE, Madesh M, Kim KC. Membrane-tethered MUC1 mucin is phosphorylated by epidermal growth factor receptor in airway epithelial cells and associates with TLR5 to inhibit recruitment of MyD88. *J Immunol* 2012;188:2014–2022.
12. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, *et al.* Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway. *Science* 2003;301:640–643.
13. Zhang SY, Herman M, Ciancanelli MJ, Pérez de Diego R, Sancho-Shimizu V, Abel L, Casanova JL. TLR3 immunity to infection in mice and humans. *Curr Opin Immunol* 2013;25:19–33.
14. Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, Akira S. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN- β promoter in the Toll-like receptor signaling. *J Immunol* 2002;169:6668–6672.
15. Numata T, Araya J, Fujii S, Hara H, Takasaka N, Kojima J, Minagawa S, Yumino Y, Kawaishi M, Hirano J, *et al.* Insulin-dependent phosphatidylinositol 3-kinase/AKT and ERK signaling pathways inhibit TLR3-mediated human bronchial epithelial cell apoptosis. *J Immunol* 2011;187:510–519.
16. Han KJ, Su X, Xu LG, Bin LH, Zhang J, Shu HB. Mechanisms of the TRIF-induced interferon-stimulated response element and NF- κ B activation and apoptosis pathways. *J Biol Chem* 2004;279:15652–15661.
17. Guillot L, Le Goffic R, Bloch S, Escriou N, Akira S, Chignard M, Si-Tahar M. Involvement of Toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J Biol Chem* 2005;280:5571–5580.
18. Ueno K, Koga T, Kato K, Golenbock DT, Gendler SJ, Kai H, Kim KC. MUC1 mucin is a negative regulator of Toll-like receptor signaling. *Am J Respir Cell Mol Biol* 2008;38:263–268.
19. Taura M, Fukuda R, Suico MA, Eguma A, Koga T, Shuto T, Sato T, Morino Koga S, Kai H. TLR3 induction by anticancer drugs potentiates polyI:C-induced tumor cell apoptosis. *Cancer Sci* 2010;101:1610–1617.
20. Kato K, Lillehoj EP, Kai H, Kim KC. MUC1 expression by human airway epithelial cells mediates *Pseudomonas aeruginosa* adhesion. *Front Biosci (Elite Ed)* 2010;2:68–77.
21. McAllister CS, Lakhdari O, Pineton de Chambrun G, Gareau MG, Broquet A, Lee GH, Shenouda S, Eckmann L, Kagnoff MF. TLR3, TRIF, and caspase 8 determine double-stranded RNA-induced epithelial cell death and survival *in vivo*. *J Immunol* 2013;190:418–427.
22. Kufe DW. Mucins in cancer: function, prognosis and therapy. *Nat Rev Cancer* 2009;9:874–885.
23. Raina D, Kosugi M, Ahmad R, Panthamoorthy G, Rajabi H, Alam M, Shimamura T, Shapiro GI, Supko J, Kharbanda S, *et al.* Dependence on the MUC1-C oncoprotein in non-small cell lung cancer cells. *Mol Cancer Ther* 2011;10:806–816.
24. Tissari J, Sirén J, Meri S, Julkunen I, Matikainen S. IFN- α enhances TLR3-mediated antiviral cytokine expression in human endothelial and epithelial cells by up-regulating TLR3 expression. *J Immunol* 2005;174:4289–4294.
25. Zhang J, Xu K, Ambati B, Yu FS. Toll-like receptor 5-mediated corneal epithelial inflammatory responses to *Pseudomonas aeruginosa* flagellin. *Invest Ophthalmol Vis Sci* 2003;44:4247–4254.
26. Koga T, Kuwahara I, Lillehoj EP, Lu W, Miyata T, Isohama Y, Kim KC. TNF- α induces MUC1 gene transcription in lung epithelial cells: its signaling pathway and biological implication. *Am J Physiol Lung Cell Mol Physiol* 2007;293:L693–L701.
27. Yoshida T, Tudor RM. Pathobiology of cigarette smoke-induced chronic obstructive pulmonary disease. *Physiol Rev* 2007;87:1047–1082.
28. Kang MJ, Lee CG, Lee JY, Dela Cruz CS, Chen ZJ, Enelow R, Elias JA. Cigarette smoke selectively enhances viral PAMP- and virus-induced pulmonary innate immune and remodeling responses in mice. *J Clin Invest* 2008;118:2771–2784.
29. Umehara T, Kato K, Park YS, Lillehoj EP, Kawauchi H, Kim KC. Prevention of lung injury by Muc1 mucin in a mouse model of repetitive *Pseudomonas aeruginosa* infection. *Inflamm Res* 2012;61:1013–1020.