Membrane-Tethered MUC1 Mucin Counter-Regulates the Phagocytic Activity of Macrophages

Kosuke Kato^{1,2*}, Reina Uchino^{2*}, Erik P. Lillehoj³, Kenneth Knox⁴, Yong Lin⁵, and K. Chul Kim^{1,2}

¹Department of Otolaryngology, University of Arizona College of Medicine, Tucson, Arizona; ²Center for Inflammation, Translational and Clinical Lung Research, Temple University School of Medicine, Philadelphia, Pennsylvania; ³Department of Pediatrics, University of Maryland School of Medicine, Baltimore, Maryland; ⁴Department of Medicine, University of Arizona College of Medicine, Tucson, Arizona; and ⁵Molecular Biology and Lung Cancer Program, Lovelace Respiratory Research Institute, Albuquerque, New Mexico

Abstract

MUC1 (MUC in human; Muc in animals) is a transmembrane mucin glycoprotein expressed in mucosal epithelial cells and hematopoietic cells. MUC1 is involved in the resolution of inflammation during airway *Pseudomonas aeruginosa* (Pa) infection by suppressing Toll-like receptor signaling in airway epithelial cells. Although alveolar macrophages are recognized as critical mediators of cell-mediated immunity against microorganisms inhaled into the airways, the role of MUC1 in regulating their response is unknown. The aims of this study were to determine whether macrophages express MUC1, and, if so, whether MUC1 expression might be associated with macrophage M0/M1/M2 differentiation or phagocytic activity. Human and mouse MUC1/Muc1 expression was drastically up-regulated in classically activated (M1) macrophages compared with nonactivated (M0) and alternatively activated (M2) macrophages. M1 polarization and Pa stimulation each increased MUC1 ectodomain shedding from the macrophage surface in a TNF- α -converting enzyme-dependent manner. MUC1/Muc1 deficiency in M0 macrophages increased adhesion and phagocytosis of Pa and *Escherichia coli* compared with MUC1/Muc1-expressing cells,

and attenuation of phagocytosis by MUC1 was augmented after polarization into M1 macrophages compared with M0 macrophages. Finally, MUC1/Muc1 deficiency in macrophages increased reactive oxygen species production and TNF- α release in response to Pa compared with MUC1/Muc1-sufficient cells. These results indicate that MUC1/Muc1 expression by macrophages is predominantly in the M1 subtype, and that MUC1/Muc1 expression in these cells decreases their phagocytic activity in an antiinflammatory manner.

Keywords: MUC1 mucin; macrophage; phagocytosis; inflammation; *Pseudomonas aeruginosa*

Clinical Relevance

MUC1 mucin plays an important role during the resolution phase of airway inflammation. However, its mechanistic study has been performed exclusively using airway epithelial cells. This article describes, for the first time, the role of MUC1 in macrophages in the context of airway inflammation.

Pathogens entering the airways are initially trapped by the airway surface liquid and removed by mucociliary clearance. Pathogens escaping this first line of defense are encountered and recognized by the

innate immune and scavenger receptors of underlying epithelial cells and macrophages (1). Alveolar macrophages (AMs) provide a crucial defense mechanism against bacterial pathogens

in the lower airways, and their phenotype and function often vary between the inflammatory state, the resolution of inflammation, and noninflammatory conditions (2). The M0/M1/M2 paradigm

(Received in original form May 28, 2015; accepted in final form September 15, 2015)

*These authors contributed equally to this project.

This work was supported by National Institutes of Health grants RO1 HL-047125 (K.C.K.) and RO1 ES-017328 (Y.L.).

Author Contributions: Conception and design: K. Kato, E.P.L., Y.L., and K.C.K.; analysis and interpretation: K. Kato, R.U., E.P.L., K. Knox, Y.L., and K.C.K.; drafting the manuscript: K. Kato, E.P.L., and K.C.K.

Correspondence and requests for reprints should be addressed to K. Chul Kim, Ph.D., 1656 East Mabel Street, MRB-419, University of Arizona College of Medicine, Tucson, AZ 85724. E-mail: kckim@oto.arizona.edu

Am J Respir Cell Mol Biol Vol 54, Iss 4, pp 515–523, Apr 2016

Copyright © 2016 by the American Thoracic Society

Originally Published in Press as DOI: 10.1165/rcmb.2015-0177OC on September 22, 2015

Internet address: www.atsjournals.org

of macrophage activation posits that foreign antigens stimulate a reprogramming of global gene expression resulting in the initial transition of resident M0 macrophages to an M1 phenotype expressing high levels of proinflammatory cytokines and mediating antimicrobial host defense. Later, during the resolution of inflammation, a conversion from M1 to an antiinflammatory M2 phenotype occurs after the phagocytosis of apoptotic neutrophils or in the presence of IL-4 (3, 4). M2 macrophages produce high levels of IL-10 and transforming growth factor- β to counter-regulate the activity of M1 macrophages, thereby contributing to the resolution of inflammation and promoting wound repair (5).

MUC1 (MUC in human; Muc in animals) is a transmembrane mucin glycoprotein consisting of two polypeptide chains, an extracellular component (MUC1-EC) noncovalently associated with a cytoplasmic tail chain (MUC1-CT) (6). MUC1 was originally identified on the apical surfaces of most simple, secretory epithelia (6), and subsequently on a variety of hematopoietic cells, including T and B lymphocytes, dendritic cells, bone marrow mononuclear cells, and macrophages (7–11). In gastric epithelia, MUC1 contributes to the physicochemical barrier against infectious pathogens (12). On airway epithelial cells, MUC1-EC is an adhesion site for *Pseudomonas aeruginosa* (Pa) (13, 14), whereas MUC1-CT interacts with Toll-like receptors (TLRs) (15, 16). Binding of MUC1-CT to TLR5 competitively inhibits recruitment of MyD88 to the TLR5 cytosolic Toll/IL-1 receptor domain, thereby suppressing TLR5 signaling and downstream inflammatory responses (15). After experimental Pa lung infection, Muc1 null mice mount hyperinflammatory airway responses and exhibit enhanced bacterial clearance from the lungs compared with wild-type (WT) mice (17). During the early phases of bacterial lung infection, Muc1 levels in the lung are relatively low, permitting the development of an inflammatory response against the invading pathogen (18). Later, Muc1 levels are up-regulated by the proinflammatory cytokine, TNF- α (18, 19), to counter-regulate ongoing inflammation and prevent bystander tissue damage (20). The antiinflammatory properties of

MUC1/Muc1 have been demonstrated in response to a variety of respiratory pathogens in addition to Pa, including respiratory syncytial virus (21) and *Haemophilus influenzae* (22).

Although the antiinflammatory effect of Muc1 was clearly demonstrated during airway Pa infection in mice, these mechanistic studies were performed exclusively using cultured airway epithelial cells. Given that AMs play a critical role in the clearance of Pa from the lungs, we hypothesized that macrophages deficient in MUC1/Muc1 expression also exhibit hyperinflammatory responses to Pa. Here, we report that MUC1/Muc1 is expressed at much higher levels in M1 compared with M0 and M2 macrophages, and that MUC1/Muc1 deficiency in M0 or M1 macrophages is associated with increased phagocytosis of Pa or *Escherichia coli*, greater reactive oxygen species (ROS) production, and enhanced TNF- α release compared with MUC1/Muc1-expressing macrophages. These findings indicate that MUC1/Muc1 plays an important role in the macrophage response to pulmonary infection by Pa.

Materials and Methods

Cell Culture

All human subject research was performed using Institutional Review Board–approved protocols at the University of Arizona College of Medicine (Tucson, AZ) and Temple University School of Medicine (Philadelphia, PA). Primary human monocyte-derived macrophages (hMDMs) were isolated from normal human donors and treated with 10 ng/ml of human macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN) to induce macrophage differentiation (23). Human AMs (hAMs) were isolated by centrifugation of bronchoalveolar lavage fluid from healthy adult volunteers. Human monocytic cell line, THP-1 cells (ATCC, Manassas, VA) were uninfected or stably infected with MUC1-targeting or nontargeting small hairpin RNA (shRNA) lentivirus particles (Santa Cruz Biotechnology, Santa Cruz, CA). The MUC1 shRNA lentivirus constituted a pool of concentrated, transduction-ready viral particles containing three MUC1-specific constructs encoding 19- to 25-nucleotide (plus hairpin) shRNAs.

THP-1 cells were cultured for 3 days with 200 nM phorbol 12-myristate 13-acetate to induce macrophage differentiation (24). M1 human macrophages were prepared by cell culture for 24 or 48 hours with 100 ng/ml *E. coli* LPS (Sigma, St. Louis, MO) plus 20 ng/ml human IFN- γ (R&D Systems), as described previously (23). M2 THP-1 cells and hMDMs were prepared by cell culture with 20 ng/ml human IL-4 plus 20 ng/ml human IL-13 (R&D Systems), as previously described (23). Mouse macrophages were isolated from 6- to 12-week-old C57BL/6/J WT or Muc1 knockout (KO) mice. Bone marrow cells were harvested from the femurs and tibias, as previously described (25), and cultured for 7 days with 10 ng/ml murine macrophage colony-stimulating factor (R&D Systems) to induce mouse bone marrow-derived macrophage (mBMDM) differentiation. Mouse peritoneal macrophages (mPMs) were isolated from thioglycolate-treated mice and mouse AMs (mAMs) were isolated from lungs as previously described (25). Mouse macrophages were seeded at 1.0×10^5 cells/well in 96-well plates or at 2.5×10^6 cells/well in six-well plates in RPMI-1640 containing 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% FBS, and cultured for 3 days before differentiation into M1 cells with 100 ng/ml *E. coli* LPS (Sigma) plus 20 ng/ml murine IFN- γ (R&D Systems), or into M2 cells with 20 ng/ml murine IL-4 plus 20 ng/ml murine IL-13 (R&D Systems). All animal experiments were approved by the University of Arizona College of Medicine

Table 1. Primers and TaqMan FAM-Dye Labeled Probes Used for Quantitative RT-PCR

mRNA Target	Assay ID (Life Technologies)
CCR7	Hs01013469
CXCL9	Hs00171065
CXCL11	Hs04187682
MRC1	Hs00267207
MUC1	Hs00159357
GAPDH	Hs02758991
Mouse Muc1	Mm0044960
Mouse GAPDH	Mm99999915

Definition of abbreviations: CCR, CC chemokine receptor; CXCL, CC chemokine ligand; FAM, 6-carboxyfluorescein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MRC1, mannose receptor, C type 1.

Figure 1. Up-regulation of MUC1 by proinflammatory stimuli is conserved among various lineages of macrophages. (A) Primary human monocyte-derived macrophages (hMDMs) were treated for 24 or 48 hours with LPS plus IFN- γ to polarize into M1 or M2 macrophages or with IL-4 plus IL-13 to polarize into M2 macrophages. Total RNA was isolated and subjected to quantitative RT-PCR (qRT-PCR) for the M1 markers, chemokine C-X-C motif, ligand 9 (CXCL9), CXCL11, and CC chemokine receptor 7 (CCR7), or the M2 marker, mannose receptor, C type 1 (MRC1). Error bars represent mean $\pm$ SEM mRNA levels normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels and expressed as fold increase over M0 macrophage mRNA normalized levels ($n = 3$). *Increased normalized CXCL9, CXCL11, or CCR7 mRNA levels of M1 macrophages compared with M0 or M2 macrophages, or increased normalized MRC1 mRNA levels of M2 macrophages compared with M0 or M1 macrophages ($P < 0.05$). (B) hMDMs were untreated (M0) or polarized into M1 or M2 macrophages, and MUC1 mRNA levels were quantified by qRT-PCR. (C) hMDMs were untreated (M0) or polarized into M1 macrophages and whole-cell lysates were processed for MUC1-cytoplasmic tail chain (MUC1-CT) immunoblotting. To control for protein loading and transfer, the blots were stripped and reprobed for β -actin. Molecular weight is indicated on the left (kD). (D) Densitometric analysis of the blots in (C). Error bars

Figure 2. *Pa* induces MUC1-EC shedding from macrophages. (A) THP-MUC1 small hairpin RNA (shRNA) cells or THP-control shRNA cells were treated for 4 or 24 hours with PBS control or 1.0×10^8 CFU/ml of heat-killed PAK, and cell culture supernatants were processed for MUC1-EC levels by ELISA. Error bars represent mean $\pm$ SEM shed MUC1-EC levels ($n = 4$). *Increased shed MUC1-EC levels in supernatants of PAK-treated cells compared with PBS controls at $P < 0.05$. (B) hAMs were untreated (M0) or treated for 24 hours to polarize into M1 or M2 macrophages, and cell culture supernatants were processed for MUC1-EC levels by ELISA. *Increased MUC1 levels in supernatants of M1 macrophages compared with M0 or M2 cells at $P < 0.05$. (C) THP-1 cells were pretreated for 30 minutes with DMSO vehicle alone or 100 μ M TNF- α protease inhibitor (TAPI)-1. The cells were washed, treated for 4 hours with PBS control or 1×10^8 CFU/ml heat-killed PAK, and culture supernatants processed for MUC1-EC levels by ELISA. **Decreased MUC1-EC levels in supernatants of PAK plus TAPI-treated macrophages compared with PAK plus DMSO-treated cells ($P < 0.05$). The results are representative of three independent experiments.

and Temple University School of Medicine Institutional Animal Care and Use Committees.

Quantitative RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) and converted to cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). Pretreatment with TaqMan Universal Master Mix II and the AmpErase Uracil N-Glycosylase Kit (Applied Biosystems) was performed to minimize carryover contamination, after which the cDNA was amplified using the StepOnePlus Real-Time PCR system (Applied Biosystems) with primers for human or mouse MUC1/Muc1, human CXCL9, CXCL11, CCR7, or mannose receptor, C type 1 (MRC1), and human or mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 1) (Life Technologies, Carlsbad, CA).

MUC1 Immunoblotting

hMDMs were extracted with PBS (pH 7.2) containing 1.0% Triton X-100, 1.0% sodium deoxycholate, and 1.0% protease inhibitor cocktail (Sigma), as previously described (19). Equal protein aliquots were resolved by SDS-PAGE and subjected to immunoblot analysis using anti-MUC1-EC (GP1.4) antibody (Pierce, Rockford, IL) or anti-MUC1-CT (Ab2) antibody (kindly provided by Dr. Sandra J. Gendler, Mayo Clinic College of Medicine, Scottsdale, AZ). To control for protein loading and transfer, the blots were stripped and reprobed for β -actin or glyceraldehyde 3-phosphate dehydrogenase.

Pa

Pa strain K is a nonmucoid, piliated, and motile strain. *Pa* expressing green fluorescent protein was kindly provided by

Dr. Joanna B. Goldberg (University of Virginia, Charlottesville, VA) (14). Bacteria were cultured overnight in Luria-Bertani (LB) broth supplemented with 60 μ g/ml carbenicillin, washed with PBS, and cell concentrations determined spectrophotometrically using an OD₆₀₀ of $0.5 \approx 5.0 \times 10^8$ cells (14).

Macrophage Phagocytosis Assay

Heat-killed and glutaraldehyde-fixed green fluorescent protein-*Pa* or FITC-labeled *E. coli* (Life Technologies) were incubated for 2 hours (for THP-1 cells, mBMDMs, and mPMs) or 6 hours (for hAMs) at various bacteria: macrophage ratios in 96-well plates. The cells were washed, incubated for 1 minute with 0.4% trypan blue to quench extracellular fluorescence, and washed again, and intracellular fluorescence intensity was quantified by fluorometry.

Figure 1. (Continued). represent mean MUC1-CT signal normalized to β -actin signal and expressed as fold increase over M0 macrophage MUC1-CT normalized levels. (E) Human alveolar macrophages (hAMs), human monocytic cell line (THP-1) cells, and mouse bone marrow-derived macrophages (mBMDMs) were untreated (M0) or polarized into M1 or M2 macrophages, and MUC1 mRNA levels were quantified by qRT-PCR. * $P < 0.05$ compared with M0, M1, or M2 macrophages, respectively. (F) hAMs were untreated (M0) or polarized into M1 or M2 macrophages, and whole-cell lysates were processed for MUC1 extracellular component (MUC1-EC) immunoblotting. (G) hAMs were untreated (0 hour) or treated for 24 or 48 hours with 1.0×10^8 CFU/ml of heat-killed *Pseudomonas aeruginosa* (*Pa*) strain K (PAK), and whole-cell lysates were processed for MUC1-EC immunoblotting. The results are representative of three independent experiments.

Figure 3. Inhibition of MUC1 expression enhances phagocytic activity of human macrophages. (A) THP-MUC1 shRNA cells or THP-control shRNA cells were untreated (M0) or treated for 24 hours to polarize into M1 or M2 macrophages and whole cell lysates processed for MUC1-CT immunoblotting. To control for protein loading and transfer, the blots were stripped and reprobed for GAPDH. (B) Densitometric analysis of the blots in A. Vertical bars represent mean MUC1-CT signal normalized to GAPDH signal and expressed as fold increase over M0 macrophage MUC-CT normalized levels. (C and D) Heat-killed and glutaraldehyde-fixed green fluorescent protein (GFP)-Pa or FITC-*Escherichia coli* (*E. coli*) were incubated for 2 hours at the indicated bacteria:macrophage ratios with (C) M0 or (D) M1 THP-control shRNA or THP-MUC1 shRNA cells. The cells were washed, incubated for 1 minute with 0.4% Trypan blue to quench extracellular fluorescence, washed, and intracellular fluorescence intensity quantified by fluorometry. Error bars represent mean $\pm$ SEM fluorescence units ($n = 4$). *Increased intracellular fluorescence of THP-MUC1 shRNA cells compared with THP-control shRNA cells at $P < 0.05$. The results are representative of three independent experiments.

MUC1 ELISA

The human CA15-3/MUC1 ELISA kit (Life Technologies) was used to quantify MUC1-EC levels in macrophage culture supernatants according to the manufacturer's protocol. MUC1-EC levels were expressed (mU/ml) based on the MUC1 protein standard included in the

kit. In selected experiments, THP-1 cells were pretreated for 30 minutes with 100 μ M of the TNF- α converting enzyme (TACE) inhibitor, TNF- α protease inhibitor I-1 (TAPI-1), or with the DMSO vehicle control, and washed before treatment with 1.0×10^8 CFU/ml of heat-killed Pa strain K.

ROS Assay

THP-1 cells and mPMs were cultured in 96-well plates, washed, and incubated for 1 hour with 5.0 μ M 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) (Life Technologies), a cell-permeable indicator of ROS. Baseline fluorescent intensity was quantified (0 h), after which 1.0×10^8 CFU/ml of heat-killed Pa was added and fluorescence intensity was quantified by fluorometry at 1-hour intervals.

Statistical Analysis

All values are expressed as means ($\pm$ SEM). Differences between means were compared using ANOVA and were considered significant at a P value less than 0.05.

Results

MUC1/Muc1 Expression Is Up-regulated in M1 and Pa-Stimulated Macrophages

AMs play a critical role in homeostasis in the lungs by promoting innate immune responses against pathogens as well as skewing inflammation toward resolution (2). To elucidate the pattern of MUC1 expression in pro/antiinflammatory macrophages, we employed the M0/M1/M2 paradigm in which foreign antigens and proinflammatory cytokines trigger transition of resident macrophages (M0) to a proinflammatory phenotype (M1), while antiinflammatory cytokines trigger transition to an antiinflammatory phenotype (M2). M0 macrophages were treated either with LPS and IFN- γ to differentiate into M1 macrophages, or with IL-4 and IL-13 to differentiate into M2 macrophages. Differentiation of hMDMs into M1 or M2 macrophages was verified by measuring CXCL9, CXCL11, and CCR7 (M1) or MRC1 (M2) expression (Figure 1A). MUC1 mRNA and MUC1-EC and MUC1-CT proteins were expressed at greater levels in M1 macrophages compared with M0 and M2 macrophages derived from hMDM, hAMs, the human macrophage cell line, THP-1, and mBMDMs (Figures 1B–1F). In addition, a significant increase in MUC1-EC protein was seen in hAMs stimulated for 48 hours with Pa compared with unstimulated cells (Figure 1G). Together, these results indicate that MUC1/Muc1 levels are greater in M1 and Pa-stimulated pulmonary and

Figure 4. Muc1 deficiency enhances phagocytic activity of mouse macrophages. Heat-killed and glutaraldehyde-fixed GFP-*Pa* or FITC-*E. coli* were incubated for 2 hours (A and B) or 6 hours (C) at the indicated bacteria:macrophage ratios with (A) mBMDMs, (B) mouse peritoneal macrophages (mPMs), or (C) mouse AMs (mAMs) from wild-type (WT) mice or Muc1 knockout (KO) mice. The cells were washed, incubated for 1 minute with 0.4% Trypan blue to quench extracellular fluorescence, washed, and intracellular fluorescence intensity was quantified by fluorometry. Error bars represent mean $\pm$ SEM fluorescence units ($n = 4$). *Increased intracellular fluorescence of Muc1 KO cells compared with WT cells at $P < 0.05$. (D) In the absence of 0.4% Trypan blue treatment, extracellular FITC-*E. coli* was collected in the postincubation cell culture supernatants of mBMDMs (left) or mPMs (right) and quantified by fluorography. **Decreased extracellular fluorescence of Muc1 KO cells compared with WT cells ($P < 0.05$). The results are representative of three (A, B, and D) or one (C) experiment(s).

extrapulmonary human and mouse macrophages compared with M0 or M2 macrophages.

Pa Induces MUC1-EC Shedding from Macrophages

MUC1/Muc1 is expressed on the cell surface of epithelial cells and its ectodomain serves as an adhesion site for *Pa* (13, 14). Because *Pa* lung infection in mice was accompanied by increased shed Muc1-EC levels in bronchoalveolar lavage fluid (18), we hypothesized that MUC1-EC would be released from macrophages after incubation with *Pa*. MUC1-EC levels were rapidly increased within 4 hours in culture supernatants of M0 THP-1 cells after incubation with *Pa* compared with cells incubated with medium control (Figure 2A). In hAMs, MUC1-EC shedding into culture supernatants was increased after M1 polarization compared with M0 and M2 cells (Figure 2B). Previously, it was reported that *Pa* up-regulates TACE expression by human airway epithelial cells (26), and that the MUC1-EC is shed from the surface of human uterine epithelial cells by the metalloprotease, TACE (27). We asked whether TACE might mediate MUC1-EC shedding from macrophages. Treatment of M0 THP-1 cells with *Pa* in the presence of the TACE inhibitor, TAPI-1, dramatically attenuated MUC1-EC shedding compared with the DMSO vehicle control (Figure 2C). These results indicate that *Pa* and proinflammatory stimuli mediate MUC1-EC shedding from macrophages in a TACE-dependent manner.

MUC1/Muc1 Deficiency Enhances Bacterial Phagocytosis by Macrophages

Because a major function of macrophages is phagocytosis, we sought to determine whether their phagocytic activity is affected by the presence or absence of MUC1. MUC1 knockdown in THP-1 cells by transduction of lentivirus containing a MUC1-targeting small hairpin RNA (THP-MUC1 shRNA cells) completely suppressed basal and inducible MUC1-CT protein levels compared with cells transduced with a scrambled shRNA (THP-control shRNA cells) (Figures 3A and 3B). In bacterial phagocytosis assays, MUC1 knockdown in M0 THP-1 cells was associated with 1.4-fold greater intracellular *Pa* at a 150:1 (*Pa*:THP-1)

Figure 5. MUC1/Muc1 deficiency enhances bacterial adhesion to macrophages. (A) THP-control shRNA cells or THP-MUC1 shRNA cells, or (B) mBMDMs, from WT mice or Muc1 KO mice were incubated for 2 hours with FITC-*E. coli* in the presence of 2.0 μ M cytochalasin B. The cells were washed and cell-associated fluorescence intensity was quantified by fluorometry. Error bars represent mean $\pm$ SEM fluorescence units ($n = 4$). *Increased cell-associated fluorescence of MUC1/Muc1-deficient cells compared with MUC1/Muc1-expressing cells ($P < 0.05$). The results are representative of three independent experiments.

ratio compared with MUC1-expressing cells (Figure 3C, left). In M1 THP-1 cells, MUC1 knockdown was associated with 2.5-fold greater intracellular Pa at a 300:1 ratio compared with MUC1-expressing cells (Figure 3D, left). An identical trend was seen with THP-1 cells and *E. coli* (Figures 3C and 3D, right). Similarly, M0 mBMDMs, mAMs, and mPMs from Muc1 KO mice exhibited greater phagocytic activity for Pa and *E. coli* compared with the corresponding macrophages from Muc1 WT mice (Figures 4A–4C). Reciprocally, fewer extracellular bacteria were seen in the postincubation cell culture supernatants of Muc1 KO mBMDMs and mPMs compared with Muc1 WT macrophages (Figure 4D). Collectively, these results indicate an inverse correlation between MUC1/Muc1 expression levels and bacterial phagocytosis by M0 and M1 human and mouse macrophages.

MUC1/Muc1 Deficiency Enhances Bacterial Adhesion to Macrophages

Because bacterial adhesion to macrophages is prerequisite to their phagocytosis (28), we asked whether MUC1/Muc1 deficiency enhances Pa adhesion to macrophages. MUC1/Muc1-expressing and nonexpressing THP-1 and mPMs were pretreated with cytochalasin B to inhibit phagocytosis before incubation with Pa,

and bacterial adhesion to the cells was quantified. MUC1 silencing in M0 THP-1 cells was associated with 2.9-fold greater Pa adhesion compared with MUC1-expressing cells (Figure 5A). M0 mPMs from Muc1 KO mice had 1.8-fold greater bacterial adhesion compared with cells from Muc1 WT mice (Figure 5B). These results suggest that MUC1/Muc1 expression attenuates bacterial adhesion to macrophages.

MUC1/Muc1 Deficiency Enhances ROS Production and TNF- α Release by Macrophages

Ingestion of pathogens by macrophages is followed by formation of the phagosome and production of ROS (29). Therefore, we investigated whether augmented bacterial phagocytosis in MUC1/Muc1-deficient macrophages is associated with increased ROS production. M0 mPMs from Muc1 KO mice and M0 THP-MUC1 shRNA cells exhibited greater ROS production after Pa treatment compared with M0 mPMs from WT mice or M0 THP-control cells (Figures 6A and 6B). Moreover, M0 THP-MUC1 shRNA cells had increased TNF- α release in response to Pa compared with M0 THP-control shRNA cells (Figure 6C).

In summary, the collective results of this study indicate that: (1) MUC1/Muc1 is expressed in human and mouse

macrophages; (2) MUC1/Muc1 expression is greater in M1 macrophages compared with M0 or M2 cells; (3) Pa stimulation increases MUC1-EC shedding by human macrophages in a TACE-dependent manner; and (4) MUC1/Muc1 expression by M0 macrophages decreases bacterial adhesion and phagocytosis, and reduces Pa-stimulated ROS production and TNF- α release compared with MUC1/Muc1-expressing cells, all of which contribute to an antiinflammatory effect during Pa bacterial lung infection.

Discussion

This report documents increased MUC1/Muc1 expression in M1 macrophages compared with M0 and M2 cells, both in human and mouse cell culture systems and in pulmonary and extrapulmonary macrophages. Both M1 polarization and Pa stimulation promoted MUC1-EC shedding from the macrophages in a TACE-dependent manner. MUC1/Muc1 deficiency in M0 and M1 macrophages increased adhesion and phagocytosis of Pa and *E. coli* compared with MUC1/Muc1-expressing cells. MUC1/Muc1 deficiency in M0 macrophages also increased ROS production and TNF- α release in response to Pa compared with MUC1/Muc1-sufficient cells. These results indicate that MUC1/Muc1 regulates macrophage function in the airways during Pa lung infection.

Increased expression of MUC1 by hAMs has been shown to be associated with the histopathogenesis of some lung diseases (11, 30). KL-6, a complex carbohydrate antigen of human MUC1, was elevated in AMs and regenerating type 2 pneumocytes in patients with idiopathic interstitial pneumonia (30). A causal effect of cigarette smoke on increased expression of MUC1/Muc1 by mAMs *in vivo* and human macrophage cell lines was reported (11). The results of the current study are consistent with previous *in vivo* data demonstrating that Pa lung infection up-regulated mouse Muc1 levels in lung tissue and increased shed Muc1-EC levels in bronchoalveolar lavage fluid (18). In addition, we observed that MUC1 deficiency is associated with greater bacterial phagocytosis by M0 and M1 macrophages compared with MUC1-expressing cells. Attenuation of phagocytic activity by MUC1 was

Figure 6. MUC1/Muc1 deficiency enhances reactive oxygen species production by macrophages. (A) mPMs from WT mice or Muc1 KO mice or (B) THP-control shRNA cells or THP-MUC1 shRNA cells were pretreated for 1 hour with 5.0 μ M of 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate. The cells were washed and treated for the indicated times with 1.0×10^8 CFU/ml of heat-killed Pa and fluorescence intensity was quantified by fluorometry. Data points represent mean $\pm$ SEM fluorescence units ($n = 4$). *Increased fluorescence intensity of MUC1/Muc1-deficient cells compared with MUC1/Muc1-expressing cells ($P < 0.05$). (C) THP-control shRNA cells or THP-MUC1 shRNA cells were treated for the indicated times with PBS control or 1.0×10^8 CFU/ml of heat-killed PAK, and TNF- α levels in cell culture supernatants were quantified by ELISA. Data points represent mean $\pm$ SEM TNF- α levels ($n = 4$). *Increased TNF- α release from PAK-treated THP-MUC1 shRNA cells compared with PAK-treated THP-control shRNA cells ($P < 0.05$). The results are representative of three independent experiments.

augmented to a greater effect in M1 THP-1 cells, where MUC1 expression and shedding are more prominent, compared with M0 THP-1 cells. During the host response to bacterial lung infection, we presume that the relatively low levels of MUC1 expression in resting M0 macrophages allow efficient engulfment of pathogens during the initial stage of infection and inflammation. Subsequently, during the resolution phase, when MUC1 expression is increased in M1 macrophages, phagocytic activity is diminished. This presumption seems to be in line with our previous *in vivo* observation that Muc1 KO mice exhibit enhanced airway inflammation and bacterial clearance in an

acute infection model (18), but failed to control inflammation in a chronic infection model (20).

Whereas reprogramming and polarization of macrophages are highly context dependent in other studies (31), our results showed that hMDMs, hAMs, THP-1 cells, mBMDMs, and mPMs all express and/or shed MUC1. This suggests that the regulation and pathophysiological properties of MUC1 are conserved among different macrophage lineages. On the other hand, in airway epithelial cells, TNF- α is the key regulator of MUC1/Muc1 levels (18, 19). However, TNF receptor KO mice exhibited some degree of Muc1 up-regulation during Pa infection (18),

suggesting a mechanism autonomous from TNF- α and/or epithelial cells. The current observations that increased MUC1 expression accompanies macrophage transition from an M0 to an M1 phenotype might explain TNF- α -independent up-regulation of Muc1 levels in the lung during Pa infection.

Membrane-tethered mucins exhibit a diversity of functions in different epithelial tissues in the context of mucosal barrier function, the host response to infection, innate immunity, and pathogen clearance. MUC1 in gastric epithelial cells acted as a releasable decoy receptor against *Helicobacter pylori* that limited bacterial infection (32) and attenuated *H. pylori*-driven proinflammatory cytokine release (33, 34). In other studies, whereas both MUC1 and MUC16 were expressed in corneal epithelial cells, only MUC16 provided a barrier to bacterial adherence (35). Our unpublished results revealed that MUC1 gene expression in M1-polarized hMDMs was highest among the membrane-bound mucins examined (MUC4, MUC16, MUC18, MUC20, MUC21, and MUC22). These data suggest functional heterogeneity of MUC1 depending on the tissue of expression and/or coexpression of other membrane-tethered mucins.

In summary, the current studies demonstrate that MUC1/Muc1 expression is up-regulated in classically activated M1 macrophages compared with M0 and M2 cells. MUC1/Muc1 deficiency increases bacterial adhesion, phagocytic activity, ROS production, and TNF- α release by human and mouse macrophages. To the best of our knowledge, this is the first study to identify a subset of MUC1/Muc1-expressing macrophages, and to demonstrate that MUC1/Muc1 regulates the function of these cells. Future studies will be necessary to further define the molecular mechanisms through which this membrane-bound mucin controls macrophage activity. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Dr. Hui Gao and Alicia Lo (Temple University School of Medicine) and Alec Hanss and Nicole Morgan (University of Arizona) for technical assistance, and Dr. Thomas Rogers (Temple University) for providing human blood monocytes.

References

- Basu S, Fenton MJ. Toll-like receptors: function and roles in lung disease. *Am J Physiol Lung Cell Mol Physiol* 2004;286:L887–L892.
- Aggarwal NR, King LS, D'Alessio FR. Diverse macrophage populations mediate acute lung inflammation and resolution. *Am J Physiol Lung Cell Mol Physiol* 2014;306:L709–L725.
- Taylor EL, Rossi AG, Dransfield I, Hart SP. Analysis of neutrophil apoptosis. *Methods Mol Biol* 2007;412:177–200.
- Johnston LK, Rims CR, Gill SE, McGuire JK, Manicone AM. Pulmonary macrophage subpopulations in the induction and resolution of acute lung injury. *Am J Respir Cell Mol Biol* 2012;47:417–426.
- Cao Q, Wang Y, Zheng D, Sun Y, Wang Y, Lee VW, Zheng G, Tan TK, Ince J, Alexander SI, et al. IL-10/TGF-beta-modified macrophages induce regulatory T cells and protect against adriamycin nephrosis. *J Am Soc Nephrol* 2010;21:933–942.
- Hattup CL, Gendler SJ. Structure and function of the cell surface (tethered) mucins. *Annu Rev Physiol* 2008;70:431–457.
- Agrawal B, Krantz MJ, Parker J, Longenecker BM. Expression of MUC1 mucin on activated human T cells: implications for a role of MUC1 in normal immune regulation. *Cancer Res* 1998;58:4079–4081.
- Treon SP, Mollick JA, Urashima M, Teoh G, Chauhan D, Ogata A, Raje N, Hilgers JH, Nadler L, Belch AR, et al. Muc-1 core protein is expressed on multiple myeloma cells and is induced by dexamethasone. *Blood* 1999;93:1287–1298.
- Brugger W, Bühring HJ, Grünebach F, Vogel W, Kaul S, Müller R, Brümendorf TH, Ziegler BL, Rappold I, Brossart P, et al. Expression of MUC-1 epitopes on normal bone marrow: implications for the detection of micrometastatic tumor cells. *J Clin Oncol* 1999;17:1535–1544.
- Wykes M, MacDonald KP, Tran M, Quin RJ, Xing PX, Gendler SJ, Hart DN, McGuckin MA. MUC1 epithelial mucin (CD227) is expressed by activated dendritic cells. *J Leukoc Biol* 2002;72:692–701.
- Xu X, Padilla MT, Li B, Wells A, Kato K, Tellez C, Belinsky SA, Kim KC, Lin Y. MUC1 in macrophage: contributions to cigarette smoke-induced lung cancer. *Cancer Res* 2014;74:460–470.
- McGuckin MA, Lindén SK, Sutton P, Florin TH. Mucin dynamics and enteric pathogens. *Nat Rev Microbiol* 2011;9:265–278.
- Lillehoj EP, Hyun SW, Kim BT, Zhang XG, Lee DI, Rowland S, Kim KC. Muc1 mucins on the cell surface are adhesion sites for *Pseudomonas aeruginosa*. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L181–L187.
- 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.
- 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.
- Kato K, Lillehoj EP, Kim KC. MUC1 regulates epithelial inflammation and apoptosis by PolyI:C through inhibition of Toll/IL-1 receptor domain-containing adapter-inducing IFN- β (TRIF) recruitment to Toll-like receptor 3. *Am J Respir Cell Mol Biol* 2014;51:446–454.
- Lu W, Hisatsune A, Koga T, Kato K, Kuwahara I, Lillehoj EP, Chen W, Cross AS, Gendler SJ, Gwirtz 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.
- Koga T, Kuwahara I, Lillehoj EP, Lu W, Miyata T, Isohama Y, Kim KC. TNF-alpha 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.
- 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.
- 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.
- Kyo Y, Kato K, Park YS, Gajhate S, Umehara T, Lillehoj EP, Suzuki H, Kim KC. Antiinflammatory role of MUC1 mucin during infection with nontypeable *Haemophilus influenzae*. *Am J Respir Cell Mol Biol* 2012;46:149–156.
- Martinez FO, Gordon S, Locati M, Mantovani A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol* 2006;177:7303–7311.
- Daigneault M, Preston JA, Marriott HM, Whyte MK, Dockrell DH. The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *PLoS One* 2010;5:e8668.
- Zhang X, Goncalves R, Mosser DM. The isolation and characterization of murine macrophages. *Curr Protoc Immunol* 2008;Chapter 14:Unit 14.1.
- Gómez MI, Sokol SH, Muir AB, Soong G, Bastien J, Prince AS. Bacterial induction of TNF-alpha converting enzyme expression and IL-6 receptor alpha shedding regulates airway inflammatory signaling. *J Immunol* 2005;175:1930–1936.
- Thathiah A, Blobel CP, Carson DD. Tumor necrosis factor-alpha converting enzyme/ADAM 17 mediates MUC1 shedding. *J Biol Chem* 2003;278:3386–3394.
- Aderem A, Underhill DM. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol* 1999;17:593–623.
- Forman HJ, Torres M. Reactive oxygen species and cell signaling: respiratory burst in macrophage signaling. *Am J Respir Crit Care Med* 2002;166:S4–S8.
- Kohno N, Kyoizumi S, Awaya Y, Fukuhara H, Yamakido M, Akiyama M. New serum indicator of interstitial pneumonitis activity: sialylated carbohydrate antigen KL-6. *Chest* 1989;96:68–73.
- Xue J, Schmidt SV, Sander J, Draffehn A, Krebs W, Quester I, De Nardo D, Gohel TD, Emde M, Schmidleithner L, et al. Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity* 2014;40:274–288.
- Lindén SK, Sheng YH, Every AL, Miles KM, Skoog EC, Florin TH, Sutton P, McGuckin MA. MUC1 limits *Helicobacter pylori* infection both by steric hindrance and by acting as a releasable decoy. *PLoS Pathog* 2009;5:e1000617.
- Guang W, Ding H, Czinn SJ, Kim KC, Blanchard TG, Lillehoj EP. Muc1 cell surface mucin attenuates epithelial inflammation in response to a common mucosal pathogen. *J Biol Chem* 2010;285:20547–20557.
- Sheng YH, Triyana S, Wang R, Das I, Gerloff K, Florin TH, Sutton P, McGuckin MA. MUC1 and MUC13 differentially regulate epithelial inflammation in response to inflammatory and infectious stimuli. *Mucosal Immunol* 2013;6:557–568.
- Gipson IK, Spurr-Michaud S, Tisdale A, Menon BB. Comparison of the transmembrane mucins MUC1 and MUC16 in epithelial barrier function. *PLoS One* 2014;9:e100393.