Epigenetic Regulation of Tolerance to Toll-Like Receptor Ligands in Alveolar Epithelial Cells

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Abstract

To protect the host against exuberant inflammation and injury responses, cells have the ability to become hyporesponsive or “tolerized” to repeated stimulation by microbial and nonmicrobial insults. The lung airspace is constantly exposed to a variety of exogenous and endogenous Toll-like receptor (TLR) ligands, yet the ability of alveolar epithelial cells (AECs) to be tolerized has yet to be examined. We hypothesize that type II AECs will develop a tolerance phenotype upon repeated TLR agonist exposure. To test this hypothesis, primary AECs isolated from the lungs of mice and a murine AEC cell line (MLE-12) were stimulated with either a vehicle control or a TLR ligand for 18 hours, washed, then restimulated with either vehicle or TLR ligand for an additional 6 hours. Tolerance was assessed by measurement of TLR ligand–stimulated chemokine production (monocyte chemoattractant protein [MCP]-1/CCL2, keratinocyte chemoattractant [KC]/CXCL1, and macrophage inflammatory protein [MIP]-2/CXCL2). Sequential treatment of primary AECs or MLE-12 cells with TLR agonists resulted in induction of either tolerance or cross-tolerance. The induction of tolerance was not due to expression of specific negative regulators of TLR signaling (interleukin-1 receptor associated kinase [IRAK]-M, Toll-interacting

protein [Tollip], single Ig IL-1–related receptor [SIGIRR], or suppressor of cytokine signaling [SOCS]), inhibitory microRNAs (miRs; specifically, miR-155 and miR146a), or secretion of inhibitory or regulatory soluble mediators (prostaglandin E₂, IL-10, transforming growth factor- β , or IFN- α / β). Moreover, inhibition of histone demethylation or DNA methylation did not prevent the development of tolerance. However, treatment of AECs with the histone deacetylase inhibitors trichostatin A or suberoylanilide hydrozamine resulted in reversal of the tolerance phenotype. These findings indicate a novel mechanism by which epigenetic modification regulates the induction of tolerance in AECs.

Keywords: Toll-like receptors; alveolar epithelial cells; endotoxin tolerance; epigenetics

Clinical Relevance

Previous work has shown the development of tolerance mechanisms to be important in regulating deleterious inflammatory responses. Our study is the first to show a role for epigenetic regulation of endotoxin tolerance in alveolar epithelial cells.

Inflammation is a strictly regulated biological response to harmful stimuli, such as infection or injury. Although inflammatory responses are necessary to begin the repair process, if left uncontrolled

they can lead to tissue damage or development of systemic disease. Therefore, counter-regulatory responses are necessary to dampen inflammation after the initial insult, thus protecting the host from further

injury (1). One response to inflammation is the induction of tolerance. The most common model of cellular tolerance is when cells are exposed to endotoxin, then become refractory to subsequent

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stimulation with LPS (2). Endotoxin tolerance occurs in a variety of different cell types and disease models. Mouse macrophages and human monocytes have been shown to undergo tolerance in response to repeated *in vivo* and *in vitro* stimulation with endotoxin (2). Other leukocytes, including T cells, dendritic cells, and neutrophils, as well as certain structural cells, also exhibit a tolerance phenotype upon sequential Toll-like receptor (TLR) stimulation (3). More recently, studies have identified an important role for tolerance of intestinal epithelial cells in regulating both homeostatic and inflammatory mechanisms in the gut (4).

TLRs are pattern-recognition receptors that play a fundamental role in sensing and responding to injury and inflammation. Virtually all cells express some combination of intracellular and extracellular TLRs, which allows cells to respond to a diverse repertoire of ligands. Initially, it was believed that TLRs evolved to respond to parasitic, bacterial, fungal, and viral pathogen-associated molecular patterns; however, more recent evidence indicates that endogenous molecules or damage-associated molecular patterns, such as hyaluronan and surfactant proteins, can also signal through TLRs (5). The end result of signaling through TLRs is the induction of a signal transduction event leading to the production of soluble mediators (1).

Previous work has shown that TLR expression by epithelial cells plays an integral role in the maintenance of host responses to injury and inflammation (6). Epithelial cell tolerance has been best characterized in the intestine, where the presence of a large community of commensal bacterial fails to induce an inflammatory response (7, 8). The lung has the largest surface exposure to the external environment of any organ, and, as a consequence, is continuously exposed to infectious and noninfectious insults. To maintain a sterile environment while protecting the delicate alveolar–capillary membrane, we hypothesize that alveolar epithelial cells (AECs), like intestinal epithelial cells, can develop a tolerized phenotype. To test this hypothesis, we exposed AECs (both primary and MLE-12) to sequential challenge with various TLR agonists and measured their ability to express neutrophil and monocyte/macrophage active chemokines. Our studies

have identified a novel mechanism of immune tolerance in structural cells of the lung.

Materials and Methods

Animals

Age- and sex-matched specific pathogen-free 6- to 8-week-old C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). In addition, interleukin-1 receptor associated kinase (IRAK)-M^{-/-} mice (9), single Ig IL-1-related receptor (SIGIRR)^{-/-} (10), and microRNA (miR)-155^{-/-} (Jackson laboratory) (11) mice (C57BL/6 background) were housed in specific pathogen-free conditions within the University of Michigan Animal Care Facility (Ann Arbor, MI). All animal experiments were performed in accordance with National Institutes of Health policies and were approved by the University Committee on Use and Care of Animals at the University of Michigan.

AEC Purification

Type II AECs were isolated from mice by the method developed by Corti and colleagues (12). We have previously shown that approximately 90% of the resulting cells are AECs by this method (13, 14).

Induction of Tolerance

Tolerance was induced in primary murine AECs or MLE-12 (murine AEC line; ATCC, Manassas, VA) by incubating cells with a selected TLR agonist for 18 hours, followed by a subsequent treatment with either the same agonist (tolerance) or a different TLR agonist (cross-tolerance) for an additional 6 hours. All results are shown at the 24-hour time point after initial stimulation.

ELISA Quantification

Keratinocyte chemoattractant (KC)/CXCL1, macrophage inflammatory protein (MIP)-2/CXCL2, monocyte chemoattractant protein (MCP)-1/CCL2, IL-10, active transforming growth factor (TGF)- β , and IL-10 (R&D Systems, Minneapolis, MN) levels and prostaglandin E₂ (PGE₂; Cayman Chemical, Ann Arbor, MI) were quantitated using specific ELISA. PGE₂ production was inhibited by incubation with indomethacin (5 μ M) for 30 minutes before the induction of tolerance.

Real-Time Quantitative RT-PCR

Total mRNA was isolated by trizol extraction (unless otherwise noted), and the measurement of gene expression was performed using the ABI Prism 7,000 Sequence Detection System (Applied Biosystems, Omaha, NE). Primer and probes were used according to the manufacturer's instructions (IDT DNA Technologies, Coralville, IA).

Targeted Small Interfering RNA Knockdown Assay

For inhibition of suppressor of cytokine signaling (SOCS)-1, primary AECs were transfected using lipofectamine siRNA max transfection agent with Smart-pool SOCS-1 small interfering RNA (siRNA) or the appropriate scrambled siRNA negative control (Thermo Scientific, Dharmacon, Lafayette, CO). Knockdown was assessed by determining SOCS-1 expression by RT-PCR in transfected cells after 48 hours.

Measurement of miR and mRNA Expression

Total RNA was extracted using the mirVana miRNA isolation kit (Ambion, Grand Island, NY) according to the manufacturer's instructions. The purity of the mRNA content was assessed, and analysis was performed on total mRNA extracts by two-step TaqMan RT-PCR protocol normalized to 18S, as previously described.

Epigenetic Modifiers

To inhibit epigenetic modifiers, AECs were cultured in the presence of vehicle control (DMSO) and trichostatin A (TSA; 500 nM), 5-aza (2.5 μ M), pargyline (3 μ M) or suberoylanilide hydroxamate (SAHA) (1 μ M) during the tolerance protocol.

Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation procedure was performed as previously published (15) using a modification of an assay kit (Upstate Biotechnology, Charlottesville, VA), according to the manufacturer's instructions. Briefly, DNA protein structure was then cross-linked and then immunoprecipitated with the antibody, anti-acetyl histone H4 (Upstate Biotechnology). Cross-linking was reversed, DNA was purified, and gene expression was determined by real-time RT-PCR.

Statistical Analysis

All data are expressed as mean ($\pm$ SEM) and were analyzed using Prism 5.0 statistical program (GraphPad Software, La Jolla, CA). A value of P less than 0.05 was considered significant.

Results

Sequential LPS Stimulation Induces Tolerance in Primary AECs

To determine whether AECs could be tolerized to TLR ligands, primary AECs

were harvested and tolerance was induced by sequential LPS treatments, as described in the MATERIALS AND METHODS section.

The induction of tolerance was assessed by measuring production of chemokines by both mRNA expression (Figures 1A, 1C, and 1E) and protein production (Figures 1B, 1D, and 1F). We observed increased expression and production of MCP-1/CCL2 (Figures 1A and 1B), MIP-2/CXCL2 (Figures 1C and 1D) and KC/CXCL1 (Figures 1E and 1F) after 18 hours of LPS stimulation. Importantly, subsequent stimulation with LPS considerably reduced

protein and mRNA expression of all of the chemokines measured (Figure 1). These data show that AECs, like other cells, undergo endotoxin tolerance.

AECs Undergo Tolerance and Cross-Tolerance When Sequentially Stimulated with Various TLR Ligands

Although LPS is a major inducer of tolerance, there are other TLR ligands that have also been shown to induce this phenomenon (16, 17). Previous work has shown that primary AECs express multiple TLRs (18–20). Thus, to

Figure 1. Endotoxin tolerance observed in alveolar epithelial cells (AECs). Primary AECs were harvested from the lungs of wild-type (WT) mice. After 3 days on fibronectin-coated plates, AECs were stimulated with either vehicle control or LPS (100 ng/ml) for 18 hours, then washed and stimulated with either vehicle control or LPS (100 ng/ml) for an additional 6 hours. In each set of experiments, there were three groups: unstimulated (vehicle control at both time points); stimulated (LPS at 6 hours only); and tolerized (LPS at 18 hours and 6 hours). The expression of specifically monocyte chemoattractant protein (MCP)-1/chemokine (C-C motif) ligand 2 (CCL2) (A and B), macrophage inflammatory protein (MIP)-2/chemokine (C-X-C motif) ligand 2 (CXCL2) (C and D), and keratinocyte chemoattractant (KC)/CXCL1 (E and F) was measured by real-time RT-PCR (A, C, and E) or specific ELISA (B, D, and F); $n = 4$ samples/group repeated three times. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ when compared with unstimulated control sample. Data are expressed as mean ($\pm$ SEM). trmt, treatment.

Figure 2. Tolerance in AECs can be induced a variety of Toll-like receptor (TLR) ligands. Primary AECs were harvested from the lungs of WT mice. After 3 days on fibronectin-coated plates, AECs were stimulated with either vehicle control or Pam3Cys-Ser-(Lys)4 (Pam3CSK) (TLR1/2 ligand, 100 ng/ml), polyinosinic:polycytidylic acid (PolyI:C) (TLR3 ligand, 50 ng/ml), or flagellin (Flag) (TLR5 ligand, 200 ng/ml) for 18 hours, then cells were washed and stimulated with either vehicle control or the indicated ligand. The expression of KC/CXCL1 was measured by real-time RT-PCR (A and C) and specific ELISA (B and D); $n = 4$ samples/group repeated twice. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ when compared with unstimulated control sample. Data are expressed as mean ($\pm$ SEM).

determine whether tolerance could be induced by other TLR ligands, AECs were stimulated with ligands that activate several extracellular myeloid differentiation primary response gene 88 (MyD88)-dependent TLRs, including Pam3Cys-Ser-(Lys)4 trihydrochloride (Pam3CSK) (ligand for TLR1/2) and flagellin (ligand for TLR5), as well as an intracellular TIR-domain-containing adaptor protein-inducing IFN- β (TRIF)-dependent, MyD88-independent, TLR3 agonist, PolyI:C. Stimulation of primary AECs with these TLR ligands

resulted in an increase in KC/CXCL1 protein production (Figure 2A) and mRNA expression (Figure 2B). Repeated stimulation with the same TLR ligands resulted in a significantly blunted expression of both KC/CXCL1 mRNA and protein. The ability of AECs to be cross-tolerized was assessed by first stimulating cells with LPS for 18 hours and then subsequently stimulating with PolyI:C for an additional 6 hours. Sequential treatment with LPS, followed by PolyI:C, resulted in reduced expression of both KC/CXCL1 protein (Figure 2C) and

mRNA (Figure 2D), indicative of cross-tolerance. These data demonstrate that the induction of tolerance in AECs is not specific for LPS, but can be induced by other extracellular and intracellular TLR ligands.

The MLE-12 Murine Epithelial Cell Line Is Also Tolerized to Endotoxin and TLR Ligands

Although previous work has shown that the isolation of primary AECs resulted in a relatively pure population ($\sim 90\%$ AECs) (13), it is possible that the contaminating cells, such as macrophages, contribute to the observed phenotype. To verify that tolerance phenotype occurs in pure populations of AECs, we used a murine AEC cell line (MLE-12 cells). As shown in primary AECs, stimulation of MLE-12 cells with LPS induced a significant increase in KC/CXCL1 mRNA expression (Figure 3A) and protein production (Figure 3B). Subsequent stimulation with LPS resulted in the induction of tolerance, as indicated by decreased KC/CXCL1 expression. Similarly, sequential stimulation with flagellin (TLR5 ligand) or Pam3CSK (TLR1/2 ligand) also resulted in a tolerance phenotype (Figure 3C). These findings corroborate primary AEC data, and confirm that AECs, rather than contaminating leukocyte populations, were responsible for the LPS tolerance phenotype observed.

Negative TLR Regulators Do Not Mediate Tolerance in AECs

The induction of negative regulators of TLRs is important in mediating endotoxin tolerance in macrophages and intestinal epithelial cells (21–24). To determine whether negative regulators of TLRs could also promote endotoxin tolerance in AECs, mRNA expression of IRAK-M, Toll-interacting protein (Tollip), and SIGIRR were measured in tolerized AECs. Treatment of primary AECs with LPS resulted in a 2.5-fold increase in IRAK-M mRNA, but not SIGIRR or Tollip, as compared with resting cells (data not shown). No superinduction of IRAK-M, SIGIRR, or Tollip was noted after sequential LPS stimulation. To further assess the potential contribution of these negative TLR inhibitors to tolerance in AECs, AECs were harvested from either IRAK-M or SIGIRR knockout mice, and mRNA expression of MIP-2/CXCL2 was assessed

Figure 3. Tolerance can be induced in murine AEC line. MLE-12 cells were cultured, and then tolerance was induced using LPS (TLR4 ligand, 100 ng/ml), flagellin (TLR5 ligand, 200 ng/ml), or Pam3CSK (TLR1/2 ligand, 100 ng/ml). Expression of KC/CXCL1 was measured by real-time RT-PCR (A and C) and specific ELISA (B); $n = 4$ samples/group repeated twice. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ when compared with unstimulated control sample. Data are expressed as mean ($\pm$ SEM).

after sequential LPS stimulation (Figure 4A). In AECs isolated from both wild-type (WT) and mutant mice (IRAK-M^{-/-} and SIGIRR^{-/-}), we observed a significant increase in the expression of MIP-2/CXCL2 after 18 hours of LPS stimulation, and reduced expression of MIP-2/CXCL2 after secondary LPS challenge (Figure 4A). These data suggest that IRAK-M and SIGIRR do not mediate tolerance in primary AECs.

miRs are important regulators of TLR-dependent signaling cascades (25). We examined the role of two miRs, miR-146a and miR-155 (26, 27), that have been shown to mediate inhibitory phenotypes. To determine whether miR-146a and miR-155 had a role in regulating endotoxin tolerance in AECs, we measured their expression after sequential LPS challenge. As compared with resting AECs, we observed no induction of miR-146a in response to primary or secondary stimulation with LPS (data not shown). There was a trend toward elevated miR-155 expression after the initial LPS challenge and a statistically significant increase in miR-155 after secondary LPS stimulation

(data not shown). To further define the contribution of miR-155, AECs were isolated from WT and miR-155^{-/-} mice and sequentially challenged with LPS. Primary LPS stimulation resulted in a significant increase in the production of KC/CXCL1 mRNA (Figure 4B) and protein (Figure 4C) expression in both WT and miR-155^{-/-} AECs. In addition, secondary LPS stimulation similarly reduced KC/CXCL1 expression equally in both WT and mutant AECs (Figures 4B and 4C).

Suppressor of cytokine signaling (SOCS) proteins are important negative regulators of the TLR signaling pathway, and have been shown to play a role in the induction of tolerance in macrophages (28, 29). The expression of suppressor of cytokine signaling 1 was measured in LPS-tolerized AECs, and was found to significantly increase after secondary LPS (Figure 5A). To determine the role of SOCS1 in mediating tolerance in AECs, expression of SOCS1 was inhibited using siRNA constructs. There was an approximately 60% reduction in SOCS1 expression in siRNA-treated AECs compared with control cells (data not

shown). Expression of MIP-2/CXCL2 was measured from SOCS1 siRNA and control-treated cells, and there was no difference in the tolerance phenotype between these two groups, as shown by expression of MIP-2/CXCL2 mRNA expression (Figure 5B) and protein production (Figure 5C). Although a complete knockdown of SOCS1 was not achieved, these data suggest that SOCS1 does not regulate the tolerance phenotype in AECs.

Secreted Inhibitory Mediators Do Not Regulate Tolerance Phenotype in AECs

Previous work has shown that AECs are major producers of the lipid mediator, PGE₂, which has been shown to dampen a variety of immune responses (30). To determine whether PGE₂ could mediate endotoxin tolerance, PGE₂ was measured in AECs after sequential LPS challenges. LPS induced a significant increase in PGE₂ after 18 hours, and subsequent LPS stimulation resulted in a further increase in PGE₂ (Baseline PGE: 4,599 $\pm$ 97 pg/ml, LPS stimulation: 6,427 $\pm$ 172 pg/ml, LPS tolerance: 7,252 $\pm$ 513 pg/ml). PGE₂ production was inhibited by treated cells with indomethacin; however, this did not alter the development of endotoxin tolerance (data not shown).

Cytokines, specifically, anti-inflammatory cellular mediators, have also been shown to regulate the tolerance phenotype in certain cell types. To determine whether a secreted immunoregulatory cytokine might mediate tolerance in our model, we measured the production expression of IL-10, IFN- α/β , and TGF- β after primary and secondary LPS challenge. There was no significant induction of IL-10 or active TGF- β after primary or secondary LPS stimulation (data not shown). There was a significant increase in the expression of IFN- α after primary LPS treatment (4.3-fold), which remained elevated after sequential LPS challenge (data not shown). However, incubation of AECs with anti-murine IFN- α/β antibody did not alter the development of the endotoxin tolerance phenotype in these cells.

Role of Epigenetics in Regulating Endotoxin Tolerance in AECs

Regulation of gene expression in tolerance has been shown to be controlled by epigenetic changes to histones binding to tolerizable genes or methylation of CpG

Figure 4. Negative TLR regulators do not induce tolerance in AECs. AECs were harvested from the lungs of WT, IL-1 receptor associated kinase (IRAK)-M^{-/-}, single Ig IL-1-related receptor (SIGIRR)^{-/-}, and microRNA (miR)-155^{-/-} mice, and, after 3 days on fibronectin-coated plates, endotoxin tolerance was induced. Expression of MIP-2/CXCL2 (A) and KC/CXCL1 (B and C) was measured by real-time RT-PCR (A and B) or specific ELISA (C); $n = 4$ samples/group repeated twice. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ when compared with unstimulated WT control sample. Data are expressed as mean ($\pm$ SEM).

islands within promoters of inflammatory genes (31). To determine whether AEC tolerance was under epigenetic control, AECs were treated with selected inhibitors of epigenetic machinery, and the expression of KC/CXCL1 was measured by mRNA expression (Figure 6) and protein production (data not shown). Treatment of AECs with 5-aza (an inhibitor of DNA methylation) or pargyline (a histone demethylase inhibitor) did not block the development of tolerance, as assessed by KC/CXCL1 mRNA or protein expression

(Figure 6A). In addition, secondary LPS stimulation did not alter the methylation pattern of 26 different CpG islands assessed within the KC/CXCL1 promoter (data not shown).

Acetylation of certain histones, including histone 4, results in disruption of histone:DNA binding, opening of the DNA, and activation of gene transcription. Enhanced histone deacetylase activity has been observed in endotoxin-tolerized macrophages and macrophages isolated from septic mice *ex vivo* (15, 31), resulting

in reduced LPS responsiveness. To assess whether changes in histone acetylation might mediate tolerance in AECs, we measured acetylation of histone 4 binding to the KC promoter by chromatin immunoprecipitation analysis. As shown in Figure 6B, primary stimulation with LPS resulted in a substantial increase in H4 acetylation (~ 25 -fold). Interestingly, there was a marked decrease in histone 4 after secondary LPS stimulation, suggesting increased histone deacetylase activity in tolerized AECs. To further elucidate the contribution of histone deacetylase activity to AEC tolerance, cells were treated with LPS sequentially in the presence or absence of TSA (a histone deacetylase inhibitor) or vehicle. As shown in Figure 6A, treatment with TSA, as compared with vehicle, resulted in a significant increase in LPS-induced KC/CXCL1 mRNA expression. Importantly, the expression of KC/CXCL1 mRNA expression was maintained in TSA-treated cells after secondary LPS challenge, indicating restoration of LPS responsiveness. Due to the potential off-target effects of TSA treatment on cells, tolerance was also induced in AECs in the presence or absence of SAHA, another histone deacetylase (HDAC) inhibitor. As shown in Figures 6C and 6D, treatment with SAHA similarly resulted in the reversal of the tolerance phenotype, as determined by MCP-1/CCL2 protein (Figure 6C) or mRNA (Figure 6D) expression. Taken together, our data indicate that LPS tolerance in AECs is predominantly mediated by enhanced histone deacetylase activity.

Discussion

Our findings indicate that AECs, like other cells of both hematopoietic and nonhematopoietic origin, can be tolerized and cross-tolerized to various TLR ligands. We explored multiple cellular and molecular mechanisms responsible for inducing tolerance in other cell types. Although various molecules, including IRAK-M (10, 23), Tollip (32), SIGIRR (22), miR-155 (33), and SOCS1 (28), have been shown to regulate endotoxin tolerance in macrophages and structural cells (i.e., biliary epithelial cells), this was not the case in AECs. In addition, target-secreted immunomodulatory cytokines did not contribute to the tolerance phenotype

Figure 5. Increased suppressor of cytokine signaling 1 (SOCS1) expression in tolerized AECs does not regulate production of chemokines. AECs were harvested from the lungs of WT mice. After 3 days on fibronectin-coated plates, endotoxin tolerance was induced and expression of SOCS1 was measured by real-time RT-PCR (A). Primary AECs were transfected with SOCS1 siRNA (100 nM) or scrambled control siRNA (100 nM) for 48 hours, before measuring the expression of MIP-2/CXCL2 by real-time RT-PCR (B) or ELISA (C); $n = 4$ samples/group repeated twice. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ when compared with unstimulated control sample. Data are expressed as mean ($\pm$ SEM). shRNA, short hairpin RNA; siRNA, small interfering RNA.

observed. Finally, we investigated the role of epigenetic modifiers in regulating tolerance in our AECs. Previous work has shown that a variety of different epigenetic modifiers can regulate tolerance phenotypes (15, 31). We found that the activity of histone deacetylases is a primary mechanism mediating the tolerance response to stimulation with TLR ligands in primary AECs.

The acquisition of immune tolerance as a means to modulate deleterious

inflammatory responses was first described in 1946, based on the observation that rabbits injected daily with typhoid vaccine eventually become afebrile (34). The phenomenon of endotoxin tolerance was later described in the 1960s, when it was observed that animals had reduced mortality when rechallenged with a lethal dose of LPS (reviewed in Ref. 35). Tolerance, manifest by reduced inflammatory cytokine expression, has also been demonstrated in normal healthy

volunteers administered LPS intravenously (36) and in various cells *in vitro*, including macrophages, dendritic cells, and, more recently, epithelial cells. Studies involving both animals and humans have shown that cross-tolerance to multiple TLR ligands also occurs (37). Moreover, the concept of immune tolerance has been expanded to include cancer, autoimmunity, and developmental stages (e.g., the neonatal period).

The majority of studies examining endotoxin tolerance have focused primarily on circulating monocytes, tissue macrophages, and other cells of hematopoietic origin. However, there is limited research examining the role of tolerance in structural cells. Previous work has shown that TLR expression of epithelial cells plays an important role in the maintenance of stable microbial colonization of neonates (38). Intestinal epithelial cells lose LPS responsiveness immediately after birth due to a post-transcriptional down-regulation of IRAK1 (39). These cells also undergo endotoxin tolerance in response to various proinflammatory stimuli (TNF- α , IL-6, and IL-1 β), an effect that is not dependent on TLR4/myeloid differentiation protein (MD)-2 signaling complex (40) or the expression of A20, a negative regulator of TLR signaling (41). Instead, it has been suggested that intestinal epithelial cells decrease TLR surface expression and up-regulate Tollip expression, resulting in reduced response to bacterial products (42). In contrast, biliary epithelial cells are susceptible to tolerance induced by LPS, but not PolyI:C, and tolerance in these cells is mediated by the TLR signaling inhibitor, IRAK-M (43). In our study, we found that AEC tolerance or cross-tolerance did not require MyD88, as PolyI:C was equally capable of inducing tolerance, as was TLR agonists that can signal in an entirely MyD88-dependent fashion (flagellin) or agonists that can signal in either an MyD88- or TRIF-dependent fashion.

Although AECs have not been carefully studied in regard to their ability to be tolerized to endotoxin or other TLR agonists *in vitro*, previous studies have suggested that LPS might protect lung epithelial cells from subsequent challenge from other pathogen-associated molecular patterns or damage-associated molecular patterns. For example, pretreatment of rats with a low

Figure 6. Epigenetic regulation of tolerance phenotype in AECs. AECs were harvested from the lungs of WT mice. After 3 days on fibronectin-coated plates, endotoxin tolerance was induced in the presence of vehicle control (DMSO) and trichostatin A (TSA; 500 nM), 5-aza (2.5 μ M), or pargyline (3 μ M). Expression of KC/CXCL1 was measured by real-time RT-PCR (A). (B) Expression of DNA binding to the KC/CXCL1 after chromatin immunoprecipitation assay using antibodies specific against histone acetylation (histone 4 [ACh4]). Data are shown as relative increase based on control unstimulated AECs. (C and D) Primary AECs were cultured in the presence of vehicle control (DMSO) or suberoylanilide hydroxamate (SAHA; 1 μ M), and expression of MCP-1/CCL2 was measured by specific ELISA (C) or real-time RT-PCR; $n = 4$ samples/group repeated twice. ** $P < 0.01$ and *** $P < 0.001$ when compared with unstimulated control sample. Data are expressed as mean ($\pm$ SEM). NS, not significant.

dose of endotoxin before exposure to high oxygen concentration was shown to protect AECs from undergoing cell death in a rat model of hyperoxia (44). Primary human type II AECs express both mRNA and protein for functional TLR2 and TLR4 (45). Since that discovery, several studies have

explored the role of TLRs on structural cells and how they might function to protect the lung against infectious and noninfectious stimuli (46). For example, exposure of lung epithelial cells to environmental particulates resulted in down-regulation of TLR4, but TLR2 expression, which was

associated with reduced responsiveness to LPS in both lung macrophages and epithelial cells (47). Given the diverse group of TLR agonists that induce tolerance in AECs, selective down-regulation of TLRs is unlikely to account for tolerance observed in our study.

As first described by Foster and colleagues (31), epigenetic remodeling of chromatin is a key mechanism regulating downstream TLR signaling in response to specific stimuli. This is particularly true in endotoxin-tolerant macrophages, as both enhanced histone deacetylase and histone demethylase activity is observed and contributes to endotoxin hyporesponsiveness. It should be noted that epigenetics can include both inheritable and noninheritable gene changes in gene activity (48). We have previously shown that epigenetic mechanisms contribute to lung macrophage hyporesponsiveness observed in septic mice, almost certainly contributing to the immune suppression that occurs in both experimental and clinical sepsis (49). Expression of

MCP-1/CCL2 in macrophages is also regulated by epigenetic histone modification (50), and KC/CXCL1 and MIP-2/CXCL2 expression in normal human bronchial epithelial cells is regulated by histone post-translational modifications (51). In our study, we found that histone deacetylase activity, but not demethylase activity, constitutively regulates LPS-induced KC/CXCL1 expression and contributes to the LPS tolerance response observed. We do not know which specific HDAC is responsible for the changes observed, as both SAHA and TSA broadly inhibit both class I and class II HDACs. However, we have previously found that, of the class I HDACs, experimental abdominal sepsis selectively up-regulates HDAC2 (16).

In summary, our study demonstrates an important role for epigenetic regulation of endotoxin tolerance in AECs. Currently, there is considerable interest in the development of small-molecule drugs that can target epigenetic mechanisms in the lung (52); however, understanding the effects of these drugs on specific cell populations has been lacking. These findings provide additional rationale for targeting of enzymes and proteins associated with epigenetic machinery that may prove to be important in the regulation of TLR signaling in the lung airspace in response to endogenous or exogenous stimuli. ■

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

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