Endotoxin stabilizes protein arginine methyltransferase 4 (PRMT4) protein triggering death of lung epithelia

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Lung epithelial cell death is a prominent feature of acute lung injury and acute respiratory distress syndrome (ALI/ARDS), which results from severe pulmonary infection leading to respiratory failure. Multiple mechanisms are believed to contribute to the death of epithelia; however, limited data propose a role for epigenetic modifiers. In this study, we report that a chromatin modulator protein arginine N-methyltransferase 4/coactivator-associated arginine methyltransferase 1 (PRMT4/CARM1) is elevated in human lung tissues with pneumonia and in experimental lung injury models. Here PRMT4 is normally targeted for its degradation by an E3 ubiquitin ligase, SCF^{BXO9}, that interacts with PRMT4 via a phosphodegron to ubiquitinate the chromatin modulator at K228 leading to its proteasomal degradation. Bacterial-derived endotoxin reduced levels of SCF^{BXO9} thus increasing PRMT4 cellular concentrations linked to epithelial cell death. Elevated PRMT4 protein caused substantial epithelial cell death via caspase 3-mediated cell death signaling, and depletion of PRMT4 abolished LPS-mediated epithelial cell death both in cellular and murine injury models. These findings implicate a unique molecular interaction between SCF^{BXO9} and PRMT4 and its regulation by endotoxin that impacts the life span of lung epithelia, which may play a key role in the pathobiology of tissue injury observed during critical respiratory illness.

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INTRODUCTION

Acute lung injury and acute respiratory distress syndrome (ALI/ARDS) is a major public health concern with high mortality of approximately 30–40% [1–5]. Pathologically, ALI/ARDS is characterized by lung inflammatory cell infiltration, and alveolar-capillary leak of edema fluid into the airways leading to hypoxia and respiratory failure. Viral and bacterial lower-airway infection is a leading cause of ALI/ARDS, which contributes to approximately 40% of the total ALI/ARDS patients [6–9]. ALI/ARDS is also observed during pandemic coronavirus infection, caused by severe acute respiratory syndrome coronavirus 2 [10–13]. Among the mechanisms of the ALI/ARDS pathogenesis, lung epithelial cell death is a crucial signature event linked to loss of integrity of the lung lining leading to an alveolar-capillary leak with edema. Although many pathways have been described causing epithelial cell death [5, 14], there is limited investigation of unique molecular pathways involving epigenetic mechanisms. Molecular mechanistic studies are of particular importance in uncovering potential effective targets for therapeutic intervention in this illness.

Protein arginine methyltransferase 4 (PRMT4/CARM1) governs a range of life processes including gene transcription, proliferation, RNA splicing, development, nuclear factor (NF)-κB mediated inflammation, and p53-related signal transduction via its epigenetic and non-epigenetic functions [15–21]. PRMT4 is crucial in development, as knockout of PRMT4 in mice leads to neonatal death and developmental defects in respiratory system. In these mice, alveolar type II epithelial cells that are crucial for surfactant production and ion transport are smaller than normal but with increased numbers. Notably, depletion of PRMT4 blocks type II alveolar cell differentiation into type I cells, and no type I alveolar epithelial cells are generated in PRMT4 knockout mice lung [22]. Aberrant expression of PRMT4 proteins is identified in a number of neoplastic tissues that regulates many aspects of cancer, including carcinogenesis, metastasis, recurrence, and drug resistance [23, 24]. PRMT family members also regulate apoptosis as PRMT2 inhibits NF-κB signaling in mouse embryo fibroblasts [25]. PRMT4 expression induced by high-glucose loading triggers apoptosis of human retinal pigment epithelial cells via H3R17 di-methylation [26]. Recent studies reported that specific expression of PRMT4 in adipose tissue promotes lipolysis and increases serum triglycerides in type 1 diabetic mice [27]. Cardiac-specific expression of PRMT4 promotes apoptosis of cardiomyocytes in mouse models [28]. However, how bacterial pathogens regulate PRMT4 at the posttranslational level is not fully understood.

The ubiquitin proteasome system degrades most cellular proteins in living cells and at times in coordination with the gene
transcriptional machinery. Ubiquitination proceeds in an enzymatic cascade, in which the final step involves an E3 ubiquitin ligase that recognizes a protein substrate to catalyze ligation of ubiquitin to the substrate. This process often results in substrate disposal within the proteasome. Among E3 ubiquitin ligases, a family of SCF (Skp1-Cul1-Fbox) E3 proteins have been linked to bacterial infection, antiviral immunity, and host defense [29–36]. Interestingly, the F-box protein FBXO9, a component of SCF{FBXO9} E3 ligase complex, was identified in a pooled RNAi screen as a labile protein degradation. PRMT4 is a labile protein degraded via the ubiquitin proteasomal machinery.

**RESULTS**

**Gram-negative bacterial-derived endotoxin increases PRMT4 protein expression**

We identified that *Escherichia coli*-derived LPS increases PRMT4 protein levels in lung alveolar epithelial type II-like MLE12 cells (Fig. 1A). LPS-mediated PRMT4 accumulation was observed in a concentration-dependent manner in the cells. We also observed that LPS increased PRMT4 cellular concentrations in human lung epithelial BEAS-2B cells (Fig. 1B) and in primary human small airway epithelial cells (HSAECs; Fig. 1C). To assess clinical relevance, we investigated PRMT4 protein levels in de-identified human lung samples from normal and infected patients were analyzed for PRMT4 and β-actin antibodies. For A–C, relative expression of PRMT4 was plotted in the below panels. D LPS is a proinflammatory mediator. EPRMT4 mRNA levels were determined by qRT-PCR in LPS-treated bronchial epithelial cells. Experiments n = 3. Statistical significance was indicated with asterisk **" between groups where P < 0.05 vs. control (0).

**SCF{FBXO9} E3 ubiquitin ligase targets PRMT4 in lung epithelial cells**

Protein ubiquitination is a cascade involving E1, E2, and E3 enzymes, in which an E3 ubiquitin ligase is substrate specific. By screening a library of SCF-E3 ubiquitin ligases, we identified that SCF{FBXO9} specifically degrades PRMT4 (Fig. 3A). Further, PRMT4
protein was degraded in an FBXO9 plasmid concentration-dependent manner (Fig. 3B, left panels). As a control, FBXO24, another F-box protein, did not degrade PRMT4 (Fig. 3B, right panels). IP studies showed that FBXO9 protein associates with PRMT4, as do the SCF complex components Skp1 and Cullin1 (components of SCF E3 ligase). Moreover, knockdown of FBXO9 with small hairpin RNA (shRNA) stabilized PRMT4 as compared with that of a scrambled shRNA control (Fig. 3D). Overall, these data indicate that PRMT4 is specifically targeted for its cellular disposal by the SCF FBXO9 ubiquitin proteasomal machinery.

**SCF<sub>FBXO9</sub> ubiquitinates PRMT4 at K228**

We next fine mapped the ubiquitination site(s) within PRMT4. We generated and expressed truncated PRMT4 constructs in cells to examine protein degradation of the truncations (Fig. 4A). Cellular expression of constructs harboring carboxyl terminal deletions resulted in PRMT4 accumulation suggesting that molecular signatures that mediate protein disposal are not localized within these fragments (Fig. 4B, left panels). We next examined NH₂-terminal deletions and observed that fragments 50–608 and 100–608 also resulted in PRMT4 accumulation after MG132 treatment. However, fragment 150–608 prevented PRMT4 accumulation (Fig. 4B, right panels), suggesting that aa100–150 contains a degradation element. We analyzed a series of lysine residues resided in the truncation mutant containing aa100–608. As compared with that of wild-type (WT) PRMT4 and other mutants, a K228A mutant was stable from proteasomal degradation (Fig. 4C, D). The K228A mutant was less ubiquitinated as compared with that of FBXO9 selectively degrades PRMT4 in BEAS-2B cells. Ectopically expressed FBXO9 plasmid degrades PRMT4 in a concentration-dependent manner not observed with FBXO24. Densitometric results are presented in the bottom panels. IP studies showing that PRMT4 interacts with Skp1 and Cullin 1, components of SCF E3 ligase. Knockdown of FBXO9 stabilizes PRMT4 using protein half-life studies with cellular exposure to cycloheximide (CHX) for various times to inhibit protein synthesis. The relative expression of PRMT4 was plotted in the right panel. \(^* P < 0.05\) vs. time 0. Experiments \(n = 3\).
compared with that of WT PRMT4 (Fig. 4E, left panels), which indicated that K228 is a ubiquitin acceptor site. In addition, mutation of K228 abrogated FBXO9-mediated PRMT4 degradation (Fig. 4F). These data suggest that K228 may be one authentic SCFFBXO9-catalyzed ubiquitin acceptor site.

SCFFBXO9 recognizes a PRMT4 phosphodegron

As SCF E3 ubiquitin ligases specifically interact with their substrates, we next studied the FBXO9 docking site within PRMT4. We focused on the NH\textsubscript{2}-terminus because the above studies suggested that aa100–150 contains a degradation element, possibly an E3 ligase docking site. Results from in vitro pull-down assays showed that deletion of NH\textsubscript{2}-terminal 150 aa impairs PRMT4 and FBXO9 binding (Fig. 5A). We further fine mapped the docking site for FBXO9 within PRMT4 by pull-down assays (Fig. 5B, C). Pull-down results showed that amino acids after aa131 were critical for FBXO9 binding. We analyzed the sequence and identified a phosphorylation motif in this region: 132-TxxxS. To test whether this motif is a potential phosphodegron, we substituted T132 and S136 with a cysteine to mimic dephosphorylation or introduced an aspartic acid at T132 to mimic a phosphorylation site. Pull-down results suggest that a dephosphorylated T132 and phosphorylated S136 sites constituted an optimal FBXO9-binding motif constituting a phosphodegron for E3 SCF ligase interaction (Fig. 5D, E). Consistent with these observations, our recent published study showed that glycogen synthase kinase-3 beta (GSK-3\textbeta)‐mediated T132 phosphorylation stabilized PRMT4 [43]. We further tested the protein stability with these mutations (Fig. 5F, G). T132C and S136D mutants degraded faster than that of WT PRMT4. A S136C mutant displayed greater protein stability. T132D and T132AS136A double mutants completely stabilized the protein from degradation. However, a T132CS136D PRMT4 variant when expressed in cells degraded most rapidly (Fig. 5F, G). In all, these data indicate that SCFFBXO9 engages a phosphodegron 132-T\textsuperscript{dxxxS\textsuperscript{p}} to ubiquitinate PRMT4 at K228.

PRMT4 promotes caspase 3 activation and cell death in lung epithelial cells

We next assessed a potential pathophysiological role for elevated PRMT4 after bacterial infection. We tested whether actions of LPS on lung epithelial cell death are mediated by PRMT4 through its E3 ligase, SCFFBXO9. We studied cell death using activated caspase 3 as a marker in a variety of cell types including mouse type II MLE12 cells, human airway epithelial BEAS-2B cells, and human primary small airway epithelial cells after 8 h of LPS stimulation. As predicted, caspase 3 was activated in all of these cells, and its activation occurred in an LPS concentration-dependent manner (Fig. 6A–C, second upper panels). Notably, LPS downregulated FBXO9 protein variably in all the tested cells, suggesting a mechanism whereby LPS stabilizes PRMT4 in cells via depletion of SCFFBXO9 E3 ubiquitin ligase (Fig. 6A–C, second lower panels). To understand the potential role(s) of PRMT4 in caspase cleavage in lung epithelial cells, we ectopically expressed PRMT4 in BEAS-2B cells. Immunoblotting results showed that PRMT4 ectopic expression was sufficient to activate caspase 3, 8, and 9 in BEAS-2B cells.
We then depleted PRMT4 expression using small interfering RNA (siRNA) to further examine our findings. Immuno- blotting results showed that depletion of PRMT4 by siRNA reduced caspase 3, 8, and 9 activation to lower levels compared to the scrambled control (Fig. 6E). Ectopic expression of PRMT4 promoted LPS-induced caspase 3 activation (Fig. 6F) as well as epithelial cell death (Fig. 6G). In contrast, depletion of PRMT4 blocked LPS-mediated caspase 3 activation to baseline levels (Fig. 6H) and cell death of epithelia (Fig. 6I). Thus, these data suggest that LPS increases PRMT4 protein level possibly via downregulation of FBXO9, thereby facilitating the methyltransferase to trigger caspase 3 signaling in lung epithelial cells.

PRMT4 mediates lung epithelial cell death in experimental ALI

To test our hypothesis biologically, we assessed PRMT4 in an LPS lung injury mouse model. We overexpressed PRMT4 or knocked down PRMT4 using lentiviral constructs (1 × 10⁷ colony-forming units/mouse, intratracheal [i.t.] administration) in mice (C57BL/6J) for 14 days. LPS (5 mg/kg, i.t. in phosphate-buffered saline [PBS] buffer) was administrated (i.t.) into mice for 24 h. Results from histological studies showed that administration of LPS caused lung inflammation with cellular infiltration. Overexpression of PRMT4 enhanced lung inflammation and knockdown of PRMT4 or application of PRMT4 inhibitor TP064 (10 μg/kg) attenuated lung inflammation (Fig. 7A, upper panels). Results from terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling (TUNEL) staining showed that LPS caused cell death in lung tissues, in which over-expression of PRMT4 enhanced cell death and knockdown of PRMT4 or application of PRMT4 inhibitor attenuated cell death in the lung (Fig. 7A, lower panels, and Fig. 7B). Immunoblotting results from lung indicated that i.t. administration of LPS or lentiviral particles did modulate PRMT4 protein levels in lung tissues (Fig. 7C, D). A PRMT4-specific inhibitor TP064 partially protected lung epithelial cells from death. Overall, these data suggest that LPS-enhanced PRMT4 protein expression promotes lung epithelial cell death and targeting PRMT4 attenuates severity of cellular injury in an experimental mouse lung inflammation model.

DISCUSSION

Lung epithelial cell death is one of the mechanistic centerpieces of ALI/ARDS, a disorder resulting from severe pulmonary infection underscoring an unmet need to identify new molecular targets for therapeutic intervention. The fundamentally new findings in this study are that (i) PRMT4 protein is elevated in infected lung tissue of human subjects and lung epithelial cells exposed to bacterial endotoxin; (ii) SCFFBXO9, via unique molecular signatures, normally targets the labile protein PRMT4 for its cellular elimination, but the E3 ligase is decreased in cells by endotoxin resulting in stabilized PRMT4 that mediates lung epithelial cell death; and (iii) genetic depletion or chemical inhibition of PRMT4 attenuates lung epithelial cell death and protects mice after endotoxin-induced lung injury (Fig. 8). Dysregulation of PRMT4 has been reported in many pathophysiological settings. PRMT4 is aberrantly expressed in breast, prostate, and colorectal cancers that is associated with a poor prognosis by promoting tumor progress and cancer metastasis [24, 44–49]. Consistent with these observations, we identified that PRMT4 expression is increased in ALI. Here the proinflammatory PRMT4 protein was upregulated in cellular and mouse injury models and in human infected lung tissue samples. Increased PRMT4 protein was both required and sufficient to cause lung epithelial cell death after endotoxin exposure and this...
Fig. 6  Endotoxin reduces FBXO9 levels leading to PRMT4 accumulation and caspase 3 activation in lung epithelia. A–C MLE12 cells, BEAS-2B cells, and human primary small airway epithelial cells (HSAECs) were treated with LPS as indicated. Cell lysates were analyzed by PRMT4, cleaved caspase 3, FBXO9, and β-actin immunoblotting. Shown below is densitometric analysis of immunoblots.

D Overexpression of PRMT4 increases cleaved caspase 3, 8, and 9 baseline levels in BEAS-2B lung epithelial cells.

E Knockdown of PRMT4 in BEAS-2B epithelial cells with shRNA reduces cleaved caspase 3, 8, and 9 expression.

F, G Overexpression of PRMT4 enhances LPS-induced caspase 3 activation (F) and causes BEAS-2B lung epithelial cell death. Cell death is determined using LDH assay and the data are normalized with that from untreated control cells.

H, I Silencing of PRMT4 inhibits LPS-induced caspase 3 activation (H) and cell death (I) in BEAS-2B cells. *P < 0.05. Experiments n = 3.
A new molecular target of FBXO9 in its control of a protein arginine methyltransferase, PRMT4, that participates in ALI pathogenesis through control of lung epithelial cell viability. Endotoxin LPS decreases FBXO9 that impairs degradation of PRMT4 protein. Elevated PRMT4 activates both intrinsic and external apoptotic pathways to cause epithelial death. Before PRMT4 is targeted for ubiquitination and subsequent degradation, PRMT4 is recognized by the E3 ubiquitin ligase SCFFBXL2 through a phosphodegron. In this motif (132-TdxxxSp), degradation, PRMT4 is recognized by the E3 ubiquitin ligase before PRMT4 is targeted for ubiquitination and subsequent ubiquitination. Phosphodegrons have been widely cited in governing the stability of crucial molecules in the pathogenesis of diseases. In this regard, we previously identified that oxidative stress reduces PRMT4 levels via downregulation of a protein kinase GSK-3β. GSK-3β phosphorylates T132 that is crucial in regulating the affinity between the phosphodegron and the E3 ubiquitin ligase [43]. Interestingly, in the present study, we observed that LPS downregulates the E3 ubiquitin ligase component FBXO9 at the protein level to stabilize PRMT4 protein. Depletion of PRMT4 by shRNA or with a PRMT4 small-molecule inhibitor improved mouse lung cell viability in an LPS lung injury mouse model, suggesting that lung epithelial cell death mediated by this protein arginine methyltransferase may be a critical factor underlying the pathogenesis of endotoxin-mediated injury. Thus, it is reasonable to postulate that there exists a delicate balance between SCFFBXL2 and PRMT4 that, in part, governs the life span of epithelia during endotoxin stress. The results suggest a potential for pharmaceutical targeting of PRMT4 to reduce the severity of tissue injury in ALI/ARDS.

**MATERIALS AND METHODS**

**Cell lines and reagents**

Human lung epithelial BEAS-2B cells and mouse alveolar epithelial MLE12 cells were from ATCC, maintained with HITES medium (Gibco) containing 10% fetal bovine serum. Cells were maintained in a 37 °C incubator in the presence of 5% CO₂. Human primary small airway epithelial cells were from ATCC and cultured with airway cell basal medium (Cat#: ATCC PCS-300-040) accompanied with bronchial epithelial growth kit (Cat#: ATCC PCS-300-040). V5 antibody (Cat#: 37040) accompanied with bronchial epithelial growth kit (Cat#: ATCC PCS-300-040). V5 antibody (Cat#: 37040) and pcDNA3.1D-His-V5-TOPO cloning kit (Cat#: 880273) and E. coli Top10 competent cells (Cat#: C40006) were from Invitrogen (St. Louis, MO). PRMT4 (Cat#: 12495), cleaved caspase 3 (Cat#: 9661), cleaved caspase 8 (Cat#: 9748), and cleaved caspase 9 (Cat#: 9505) antibodies were from Cell Signaling (Danvers, MA); Ubiquitin (Cat#: sc-166553, lot: A2710) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). FBXO9 (Cat#: PAS-23474) antibody was from Thermo Fisher (Rockford, IL). The PRMT4 shRNA was from Origene (Rockville, MD). Cycloheximide (Cat#: ALX-380-269-G001, lot: 01061518) and Ubiquitin

**Fig. 7** Inhibition of PRMT4 suppresses cell death in a LPS lung injury model. A–D PRMT4 was silenced or overexpressed by i.t. administered lentiviral constructs for 14 days. Mice were then given (i.t.) LPS with or without a PRMT4 inhibitor as indicated for 24 h (n = 8). Lung tissues were stained with hematoxylin–eosin (H&E) (A, upper panels) and TUNEL staining (A, lower panels). B TUNEL-positive cells in lung tissues were quantitated. C PRMT4 overexpression and knockdown in lung tissues from A were analyzed by immunoblotting. The data from C were quantitated and plotted in D (n= 3). *P< 0.05 vs. scrambled control. Scale bar = 100 μm.

Apoptosis was quantitated and plotted in

**Cell Death and Disease** (2021) 12:828

Y. Lai et al.
aldehyde (Cat#: BML-UW8450-0050, lot: 07021447) were from Enzo Life Sciences (Farmingdale, NY). β-Actin (Cat#: A3853) antibody and bacterial lipopolysaccharide (LPS) from E. coli 0111:B4 (Cat#: L4391, lot: 115M4090V) were from Sigma (Carlsbad, CA). MG132 (Cat#: F1105, lot: F11052079) was from UBPlBio (Aurora, CO). TP064 (Cat#: 6008) was from Tocris Bioscience (Ellisville, MO). QuikChange II XL site-directed mutagenesis kits (Cat#: 200522) were from Agilent Technologies (Santa Clara, CA). TnT Quick Coupled Transcription/Translation Systems (Cat#: L1170) were from Promega (Madison, WI). Immunoblotting and co-IP were conducted as previously described [55]. Immunoblotting and co-IP were conducted as previously described [55]. Brieﬂy, one million cells in 100 µL of transfection buffer (20 mM Hepes in PBS buffer) were mixed with 3 µg of plasmids (including expression and shRNA constructs). After electroporation, the cells were cultured with 2 mL of electroporation medium in 6-well plates for 24 h for further analysis.

Cloning and mutagenesis
V5-tagged PRMT4 truncations were cloned into pcDNA3.1D-His-V5-TOPO plasmid using PCR-based approaches as previously described [55]. Mutagenesis was introduced by using a QuickChange II XL site-directed mutagenesis kit according to the manufacturer’s instructions. The accuracy of the mutagenesis was conﬁrmed by sequencing. The primers used in the construction of PRMT4 truncations and site-directed mutagenesis are listed in Table 1.

Plasmid transfection
All plasmids were introduced into cells using electroporation executed with a nuclear transfection apparatus (Amaxa Biosystems, Gaithersburg, MD) with a preset program (T-013 for MLE12 and BEAS-2B cells), following the manufacturer’s instructions as previously described [55]. Brieﬂy, one million cells in 100 µL of transfection buffer (20 mM Hepes in PBS buffer) were mixed with 3 µg of plasmids (including expression and shRNA constructs). After electroporation, the cells were cultured with 2 mL of electroporation medium in 6-well plates for 24 h for further analysis.

Immunoblotting and co-IP
Immunoblotting and co-IP were conducted as previously described [55]. Brieﬂy, for immunoblotting, whole-cell extracts (normalized to total protein concentration) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to membranes by electroblotting. The membranes were blocked with 5% (w/v) non-fat milk in Tris-buffered saline (TBS) – polyacrylamide gel electrophoresis and transferred to membranes by electroblotting. The membranes were blocked with 5% (w/v) non-fat milk in Tris-buffered saline and probed with primary antibodies as indicated. Membranes were developed by an enhanced chemiluminescence (ECL) system. For IP, 1 mg of cell lysates (in PBS with 0.5% Tween 20 plus protease inhibitors) were incubated with speciﬁc primary antibodies for 2 h at room temperature. The mixture was added with 35 µL of protein A/G agarose beads for an additional 2 h at room temperature. The precipitated complex was washed for three times with 0.5% Tween 20 in PBS and analyzed by immunoblotting with ECL system.

Real-time PCR
Total RNA from cells was isolated with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and reverse-transcribed by using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s manual instructions. Single-stranded cDNA was then amplified by RT-PCR with speciﬁc primers of PRMT4 and GAPDH. RT-PCR was performed on an ABI Prism 7000 thermocycler (Applied Biosystems, Thermo-Fisher Scientiﬁc, Waltham, MA, USA) with SYBR Green PCR Master Mix (Roche, Basel, Switzerland). For each experiment, samples (n = 3) were run in triplicate. Relative gene expression was calculated using the comparative CT method.

In vitro pull-down assay
We conducted in vitro binding assays to identify the FBXO9-binding domain within PRMT4. V5-tagged PRMT4 truncated or site-directed mutant proteins were in vitro expressed using a TnT coupled reticulocyte system. Endogenous FBXO9 protein was obtained by FBXO9 IP from MLE12 cell lysate. FBXO9-puriﬁed protein A/G agarose beads were incubated with a variety of PRMT4 truncations or mutants for 2 h. The beads were washed extensively with 0.5% Tween 20 in PBS and analyzed by V5 and FBXO9 immunoblotting.

Cell death detection
Lactate dehydrogenase (LDH) assay kit (Takara, Japan) was used to detect the epithelial cell death according to the manufacturer’s instructions. Absorbance of the samples at wavelength of 490 and 620 nm (background) were measured using a SpectraMax M2 microplate reader (Molecular Devices, CA, USA). The LDH activity was determined by subtracting the optical density (OD) at 620 nm absorbance value from the OD at 490 nm. The fold change of epithelial cell death was indicated with the relative LDH activity comparing to the control group and plotted in a bar graph.

Lentivirus production
Lentiviruses were generated by Lent-X Packaging Single Shots VSVG-G (Cat#: 631276, Clontech, CA) according to the manufacturer’s procedures. Lentivirus-containing supernatants were concentrated using LenticoX Concentrator (Cat#: 631231). Concentrated lentiviruses were resuspended in PBS and titrated by Lent-X GoStix (Cat#: 631280). The samples were aliquoted and stored at −80°C.

Mouse LPS-induced lung injury procedures
All animal protocols and procedures were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC protocol #: I500100084). LPS-induced lung injury model was conducted as previously described. Brieﬂy, C57BL/6J mice at the age of 10 weeks were anesthetized by injecting intraperitoneally a solution of 1:1 ketamine (100 mg/kg) and xylazine (20 mg/kg) throughout the experiment. LPS (7 mg/kg) were i.t. administrated. The mice were observed for 48 h and sacriﬁced. Lung tissues were collected and applied for hematoxylin–eosin staining, TUNEL assay, and western blotting.

TUNEL assay
Mouse lung tissues were ﬁxed in 10% formalin for 48 h and then embedded in parafﬁn and sectioned. Slides were deparafﬁnized and
| Primers | Sequences |
|---------|-----------|
| **Truncation** | |
| PRMT4F1 | CACCATGGCAGCGCGGCGAGCG |
| PRMT4F2 | CACCATGACGCGCGGACAGCAG |
| PRMT4F3 | CACCATGACCTGGGCGCTGAAC |
| PRMT4F4 | CACCATGTTCACGTTCTGACG |
| PRMT4F5 | CACCATGTTTTTTGCTTGCTAAGC |
| PRMT4F6 | CACCATGAAATGGACATATAC |
| PRMT4F7 | CACCATGGAACCTGTCATGAG |
| PRMT4R1 | ACTCCCATAGTGGAGGGGTTT |
| PRMT4R2 | GAGATGGGTTGATAGTACG |
| PRMT4R3 | TGGTTCCCATATTTCCTAGG |
| PRMT4R4 | GTCATGACTGCTCTTCCG |
| PRMT4R5 | TATGGGACGCAAAGAAGCAAC |
| PRMT4R6 | CATCAAGGTCGAGTTCAATG |
| DN131f | CACCATGACCTGGGCGCTGTTTG |
| DN132f | CACCATGAAATGGACATATAC |
| DN133f | CACCATGGAACCTGTCATGAG |
| DN134f | CACCATGACCTGGGCGCTGTTTG |
| DN135f | CACCATGAAATGGACATATAC |
| DN136f | CACCATGAAATGGACATATAC |
| DN137f | CACCATGAAATGGACATATAC |
| DN138f | CACCATGAAATGGACATATAC |
| DN139f | CACCATGAAATGGACATATAC |
| DN140f | CACCATGAAATGGACATATAC |
| **Mutagenesis** | |
| T132Cf | CCTGTCGGGCGCCGACTGGAGGACAGGCTCTGTG |
| T132Cr | CACAGGACGCTCCAGGACAGGCTCTGTG |
| T132Df | CCTGTCGGGCGCCGACTGGAGGACAGGCTCTGTG |
| T132Dr | CACAGGACGCTCCAGGACAGGCTCTGTG |
| S136Cf | CCAGACTGGAGGAGGACAGGCTCTGTG |
| S136Cr | CCGCTCACTGAACACACAGGACAGGCTCTGTG |
| S136Df | CCAGACTGGAGGAGGACAGGCTCTGTG |
| S136Dr | CCGCTCACTGAACACACAGGACAGGCTCTGTG |
| T132AS136Af | CCAGACTGGAGGAGGACAGGCTCTGTG |
| T132AS136Ar | CCGCTCACTGAACACACAGGACAGGCTCTGTG |
| T132CS136Df | CCGCTCACTGAACACACAGGACAGGCTCTGTG |
| T132CS136Dr | CCGCTCACTGAACACACAGGACAGGCTCTGTG |
| K463Af | GACACGAGCGCGCTCCGGAGTCTAGG |
| K463Ar | CCACTGGAGGTACCACGAGGCTCTGTG |
| K471Af | GTACCCTGAGGTACCACGAGGCTCTGTG |
| K471Ar | CCACTGGAGGTACCACGAGGCTCTGTG |
| K310Af | CATGAGGAGGTACCACGAGGCTCTGTG |
| K310Ar | CATGAGGAGGTACCACGAGGCTCTGTG |
| K228Af | GAGACGAGGTACCACGAGGCTCTGTG |
| K228Ar | CTGAGGAGGTACCACGAGGCTCTGTG |
| K242Af | CGTGAGGAGGTACCACGAGGCTCTGTG |
| K242Ar | GAGACGAGGTACCACGAGGCTCTGTG |

The orientation of all primers is in 5’ to 3’ direction.
permeabilized with 0.1% Triton X-100 in citrate buffer (0.1%, pH 6.0). TUNEL staining was performed on these slides using an In Situ Cell Death Detection Kit (Sigma-Aldrich, Cat#: 11648795910) according to the manufacturer’s instructions. The slides were visualized by a confocal microscope (NIKON A1 Spectral Confocal, Japan) with excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm.

**Statistics**

The data represent the mean ± standard deviation in the graphs depicting the error bars or as specifically indicated. Prism 7 (GraphPad software, San Diego, CA) was used to determine statistical significance. Comparisons between groups were made using unpaired, two-tailed Student’s t test (two groups) and one-way analysis of variance with post hoc Tukey honestly significant difference or Bonferroni and Holm multiple comparisons. Kaplan-Meier estimate was used for survival analysis in mouse septic models. P < 0.05 indicates statistical significance.

**DATA AVAILABILITY**

The data that supporting the finding of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

CZ conceived science and designed the experiments. YL, XL, and TL conducted immunoblotting analysis. YL and XL performed animal studies. BJM, YZ, SMN, and GDK provided human samples. JSL and RKM helped to develop the science and interpreted the results. The manuscript was written by YL and CZ and edited by KC.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This paper includes the use of deidentified human lung samples. All animal protocols and procedures were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC protocol #: IS00010084).

ADDITIONAL INFORMATION

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