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Protein Arginine Methyltransferase 4 (PRMT4) contributes to lymphopenia in experimental sepsis

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Abstract

Background—One hallmark of sepsis is reduced number of lymphocytes, termed lymphopenia, that occurs from decreased lymphocyte proliferation or increased cell death contributing to immune suppression. Histone modification enzymes regulate immunity by their epigenetic and non-epigenetic functions, however, the role of these enzymes in lymphopenia remains elusive.

Methods—We utilized molecular biological approaches to investigate the high expression and function of a chromatin modulator Protein Arginine N-methyltransferase 4/ Coactivator-Associated Arginine Methyltransferase 1 (PRMT4/ CARM1) in human samples from septic patients, cellular and animal septic models.

Results—We identified that PRMT4 is elevated systemically in septic patients and experimental sepsis. Gram-negative bacteria and their derived endotoxin LPS increased PRMT4 in B and T lymphocytes and THP-1 monocytes. Single-cell RNA sequencing results indicate an increase of PRMT4 gene expression in activated T-lymphocytes. Augmented PRMT4 is crucial for inducing lymphocyte apoptosis but not monocyte THP-1 cells. Ectopic expression of PRMT4 protein caused substantial lymphocyte death via caspase 3 mediated cell death signaling, and knockout

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Contributorship Statement: CZ conceived the science and designed the experiments. YL, XL, TL conducted immunoblotting analysis. YL and XL performed animal studies. KC conducted single cell RNA sequencing experiments. BJM, Xiaoyun L, YZ, SMN, and GDK provided human samples and conducted human related studies. SMN performed statistical analysis. JSL and RKM helped to develop the science and interpreted results. The manuscript was written by YL and CZ and edited by TN, YZ, BJM, GDK, SMN, JSL, and RKM. All authors read and approved the final manuscript.

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of PRMT4 abolished LPS mediated lymphocyte death. PRMT4 inhibition with a small molecule compound attenuated lymphocyte death in complementary models of sepsis.

Conclusions—These findings demonstrate a previously uncharacterized role of a key chromatin modulator in lymphocyte survival that may shed light on devising therapeutic modalities to lessen the severity of septic immunosuppression.

Keywords

lymphocyte; cell death; PRMT4; immunosuppression; bacterial infection; sepsis

Introduction

Increasing evidence has shown that an overwhelmed or hypo-immune response emerges as a critical factor contributing to the poor prognosis of patients with sepsis [1 2]. Immunosuppression can result from “immune paralysis” and is one sepsis hallmark that is increasingly appreciated and can also be observed in chronic infection and cancer [3 4]. Typical features of immunosuppression include a reduced population of immune cells, exhaustion and dysfunction of cell immunity, suppression of pro-inflammatory factor release, or an increase in anti-inflammatory factor release [5 6]. Immunosuppression is clinically manifested in patients by chronic or recurrent bacterial and viral infections, or acute infections with opportunistic pathogens leading to sepsis. Lymphopenia has emerged as a prominent feature in septic patients that is associated with a poor prognosis. Lymphocyte death has been observed in gram-negative bacteria derived lipopolysaccharide (LPS) induced injury in cellular or acute animal models [7-10]. Yet, the underlying molecular mechanism(s) of lymphopenia in sepsis remains to be defined.

Epigenetics govern DNA accessibility and concomitantly coordinates with transcription factors to control lymphocyte development, lineage differentiation, and maturation [11]. Epigenetic alterations occur in multi-organ failure, animal models of sepsis, and in critically ill patients [12-15]. Bacterial infection regulates the behavior of epigenetic enzymes to reprogram host defenses in inflammatory gene transcription, cell death and survival [16]. Histone H3K27 methylation has been previously implicated as an epigenetic mark in septic immunosuppression [17]. Epigenetic regulation is crucial to the pathogenesis of immunosuppression, and persistent epigenetic changes have been reported in exhausted T cells in chronic viral infection animal models [18]. Nevertheless, our understanding of the role of individual epigenetic enzymes in sepsis is limited as mechanisms are poorly described.

In this study, we identified that microbial factors (i.e., endotoxin) increase a type I protein arginine methyltransferase PRMT4 expression thereby inducing lymphocyte death and modulate host survival in experimental sepsis. The epigenetic enzyme PRMT4 regulates crucial life processes including gene transcription, proliferation, RNA splicing, and development [19-23]. Knockout of PRMT4 in mice leads to neonatal death and developmental defects in the respiratory system, reduced percentage of CD4-CD8 double negative T cells, and a block in thymocyte development in mice [24-26]. Our data show that bacterial pathogens increase PRMT4 in both B and T lymphocytes and monocytes. Further, we observe that PRMT4 triggers lymphocyte death and by screening putative small molecule inhibitors of

PRMT4, we identified that one compound, TP064, specifically attenuates lymphocyte cell death and protects mice after LPS lung injury and in polymicrobial sepsis.

Methods

Cell lines and reagents.

Human lymphoma Jurkat cells, SKW6.4 cells, THP-1 monocytes were purchased from ATCC. These cells and human primary pan-T cells (StemCell) were cultured with RPMI1640 (Gibco) containing 10% FBS. *Escherichia coli* was from ATCC. PRMT4 (Cat#:12495), cleaved caspase 3 (Cat#: 9661), and cleaved caspase 9 (Cat#: 7237) antibodies were from Cell Signaling (Danvers, MA). The lenti-PRMT4 shRNA was from Origene (Rockville, MD). β-actin (Cat#: A3853) antibody, bacterial LPS) from *E. coli* O111:B4 (Cat#: L4391, lot: 115M4090V) were from Sigma (Carlsbad, CA). TP064 (Cat#: 6008) was from Tocris Bioscience (Ellisville, MO). All other reagents were of the highest grade available commercially.

Cloning and Plasmid transfection.

PRMT4 were cloned into *pcDNA3.1D-His-V5-TOPO* plasmid using PCR-based approaches as previously described [16] . The accuracy of the insert was confirmed by DNA sequencing. The PRMT4 primers used in plasmid construction were forward primer: 5'-CACCATGGCAGCGGCGGCAGCG-3'; and reverse primer: 5'-CATCAGGATCCGGATGTCAAATG-3'. Plasmids were introduced into cells using electroporation executed with a nuclear transfection apparatus (Amaxa Biosystems, Gaithersburg, MD) in a preset program (X-001 for Jurkat cells), following the manufacturer's instructions as previously described [27].

Immunoblotting.

Immunoblotting was conducted as previously described [21]. Briefly, for immunoblotting, whole cell extracts (normalized to total protein concentration) were resolved by SDS-PAGE and transferred to *nitride cellulose* membranes by electroblotting. The membranes were blocked with 5% (w/v) non-fat milk in Tris-buffered saline and probed with a primary antibody as indicated (PRMT4 at 1:1000 dilution for 2 h incubation) and a secondary antibody at 1:5000 dilution. Membranes were developed by an enhanced chemiluminescence (ECL) system and images were acquired using a Biorad Chemi-Doc XRS+ system.

Human study and PRMT4 ELISA assays.

The University of Pittsburgh Institutional Review Board approved the parent Acute Lung Injury Registry and Biospecimen Repository (PRO10110387) and the exempt protocol to use deidentified samples and data for this study (MOD201800140). Written informed consent to participate in the parent registry was provided by all participants or their surrogates in accordance with the Declaration of Helsinki. Subjects with or without sepsis were selected from a cohort of mechanically ventilated patients at the University of Pittsburgh Medical Center (UPMC). Eligible patients were 18 years or older with acute respiratory failure requiring mechanical ventilation via endotracheal intubation with sepsis as defined by the presence or suspicion of infection and two or more systemic inflammatory

response syndrome (SIRS) criteria. Control patients were intubated and mechanically ventilated for airway protection without sepsis. Deidentified baseline clinical data were presented in table 1. Deidentified plasma from above samples were used for assay of PRMT4 expression using PRMT4 ELISA kit (Cat# MBS3244080, Mybiosource, SC, USA) as directed by the manufacturer.

Single cell RNA sequencing.

Mouse (strain C57BL/6J) CD4+ T cells were enriched by magnetic beads then activated with plate bound anti-CD3/CD28 overnight. Activated T cells were mixed with naïve unstimulated T cells and single-cell RNA-seq library was prepared by using the 10x Genomics Chromium Single Cell 3' Reagent kits, sequenced on an Illumina Novaseq (Illumina, CA, USA), and data was processed with Cell Ranger 5.0 then analyzed using Seurat R package. Gene expression was shown as two dimensional UMAP (Uniform Manifold Approximation and Projection) plots.

Mouse splenic lymphocyte isolation, flowcytometry, and annexin V apoptosis analysis.

Mouse (strain C57BL/6J) spleens were disrupted in PBS containing 2% FBS. Splenic lymphocytes were isolated using EasySep T cell or B cell Isolation Kits (Cat#: P19851, Cat#: 19854, Stemcell Technologies, Vancouver, Canada). Above isolated lymphocytes were stained with anti-CD4, CD8a or CD45R antibodies combined and mixed with Annexin V binding buffer. The samples were acquired on the LSRII flow cytometer (BD Biosciences, Ml, USA) and the data analyzed with Flowjo software (Tree Star, OR, USA). FITC Annexin V Apoptosis Detection Kit with Propidium Iodide (PI) (Cat#: 640914, BioLegend, CA, USA) was employed to detect apoptotic cell Jurkat cells. The samples were analyzed through BD Accuri C6 flow cytometer (BD Biosciences, MI, USA).

Mouse LPS-induced lung Injury and Cecal ligation and puncture (CLP) procedures.

LPS-induced lung Injury model was conducted as previously described [26]. C57BL/6J mice at the age of 10 weeks were used for the experiments. LPS (7 mg/kg) were intratracheally administrated and the mice were observed for 48 h. Polymicrobial sepsis was induced in mice by cecal ligation and puncture. Briefly, mice were anesthetized throughout the experiment. A 1 to 2 cm midline abdominal incision was performed. The cecum was exposed and ligated with a sterile silk suture 1 cm from the tip and double punctured with a 19-gauge needle. The cecum was gently squeezed to extrude a small amount of fecal material and was returned to the peritoneal cavity. The incision was closed with silk sutures. Mice were resuscitated with i.p. injection of 1 ml of pre-warmed 0.9% saline solution. Mice were then monitored every 12 hours for survival or euthanized at different time points for analysis of different parameters. All animal protocols and procedures were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC protocol #: IS00010084).

Lentivirus production.

Lentiviruses were generated by Lenti-X Packaging Single Shots VSV-G (Cat#: 631276, Clontech, CA, USA) according to the manufacturer’s procedures. Lentivirus-containing

supernatants were concentrated using Lenti-X Concentrator (Cat#: 631231). Concentrated lentiviruses were resuspended in PBS and titrated by Lenti-X GoStix (Cat#: 631280). The samples were aliquoted and stored at −80°C.

CRISPR/Cas9 PRMT4 Knock-out cell line.

Jurkat cells were co-transfected with PRMT4 CRISPR/Cas9 KO Plasmid (Cat#: sc-404087-KO-2) and HDR Plasmid (Cat#: sc-404087-HDR-2) using UltraCruz Transfection Reagent (Cat#: sc-395739). Stable KO cells were selected by puromycin following instructions from the CRSPR/Cas9 manufacturer. PRMT4 knockout was confirmed by immunoblotting.

Statistics.

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 and one-way analysis of variance (ANOVA). Multiple comparisons are conducted using 2-way ANOVA. For animal survival experiments, a sample size of 16 in each group has 80% power to detect a hazard ratio of at least 3 (i.e. overall survival of 0.81 in LPS+TP064 vs. 0.40 in LPS+PRMT4 group). This sample size has >90% power to detect an overall survival of 0.94 in LPS+shPRMT4 group vs. 0.56 in LPS group (type I error = 0.05, two sided test) (PASS 15, NCSS, LLC). Kaplan Meier estimates were used for survival analysis in mouse septic models. Meta-analysis among murine replication experiments was conducted with Random-effects REML model. Another approach was to combine two studies in one and adjust for the study effect (Stata). Values of p less than 0.05 were considered as significant.

Results

PRMT4 protein expression increases in human septic patients and in septic models.

T lymphocytes have been previously reported to respond to LPS, but the functional sequelae following LPS stimulation is not fully understood [28 29]. To understand whether LPS regulates PRMT4 in T lymphocytes, we first identified that *E. coli* derived LPS increased PRMT4 protein levels in human lymphoma Jurkat T cells (Fig. 1A), human lymphoma SKW6.4 B cells (Fig. 1B), and monocyte THP-1 cells (Fig. 1C). LPS stimulation increased PRMT4 expression in a concentration-dependent manner in these cells. In addition, live *E. coli* increased PRMT4 cellular concentrations in Jurkat cells (Fig. 1D). We analyzed PRMT4 in human peripheral blood leukocytes, in which approximately 1/3 of cells are lymphocytes and monocytes and observed a tendency in higher PRMT4 protein expression compared to that of non-septic diseased controls (Fig. 1E). However, PRMT4 protein levels were substantially higher in the plasma from septic patients as compared to those without sepsis (Fig. 1F). PRMT4 protein levels were remarkably higher in sera from polymicrobial infected mice as compared to that of untreated control mice (Fig. 1G). In LPS treated mice, PRMT4 was expressed in BALF (bronchoalveolar lavage fluid) agranular cells but less in granular cells (PRMT4 positive cells: 96.0% in agranular cells vs 7.7% in granular cells) (Fig. 1H, I). In addition, PRMT4 expression was comparable in CD4+ and CD8+ subpopulations (Fig.

1J). Hence, these data suggest that PRMT4 expression is induced in human peripheral blood leukocytes with sepsis and PRMT4 levels are increased during murine sepsis *in vivo*.

PRMT4 gene expression increases upon activation in CD4+ cells.

PRMT4 gene is expressed in B cells and monocytes and less so in naïve T cells (The Human Protein Atlas, https://www.proteinatlas.org/ENSG00000142453-CARM1/celltype). However, PRMT4 is necessary for T cell lineage development as reported in PRMT4−/− mice [25]. To address if pathogen may induce PRMT4 gene expression in T cells, we conducted single cell RNA sequencing (ScRNAseq) in CD4+ T cells with or without ligand activation. Mouse splenic CD4+ cells were activated with anti-CD3/CD28 overnight (Fig. 2A, B). Naïve T cell markers *Sell* and *IL7r* decreased in activated T cells, suggesting that T cell activation via CD3/CD28 was successful (Fig 2C, D). Anti-CD3/CD28 activation increased *PRMT4* gene expression in T cells (Fig. 2E). The expression of *IL2, IL2ra*, and *CD69* was increased in activated T cells as well (Fig. 2F-H). These observations indicate that PRMT4 is expressed in activated T cells.

LPS induced PRMT4 promotes caspase 3 activation in lymphocytes.

We next assessed a potential pathophysiological role for elevated PRMT4 after bacterial infection. PRMT4 triggers apoptosis of human retinal pigment epithelial cells via H3R17 dimethylation in high glucose treatment [30]. In addition, LPS induces lymphocyte death in cellular and acute animal models [7-10]. Thus, we tested if actions of LPS on lymphocyte death are mediated by PRMT4. As predicted, both caspase 3 and caspase 9 were activated in Jurkat cells and SKW6.4 cells, and its activation occurred in an LPS concentration dependent manner, suggesting that PRMT4-mediated Jurkat cell death is possibly via intrinsic apoptotic pathway (Fig. 3A-B, middle panels). Notably, LPS increased PRMT4 protein expression but without detection of cleaved caspase 3 and caspase 9 in THP-1 monocytes, suggesting a different mechanism whereby LPS stabilizes PRMT4 in cells with distinct outcomes between lymphocytes and monocytes (Fig. 3C). We observed similar results that LPS mediates caspase 3 activation in human peripheral blood pan-T cells and mouse splenic T cells (Fig. 3D, E). We then treated Jurkat cells with gut-derived live bacteria (bacteria overnight cultured in a LB plate from the fecal material of mouse cecum) and observed that PRMT4, cleaved caspase 3, and cleaved caspase 9 are upregulated (Fig. 3F). These data suggest that LPS increases PRMT4 in lymphocytes and monocytes, but caspase 3 activation appears to be cell-type specific.

We then observed that PRMT4 ectopic expression was sufficient to activate caspase 3 in Jurkat cells (Fig. 4A). Caspase 3 activation occurred in a PRMT4 plasmid concentration-dependent manner. We observed similar results in SKW6.4 cells (Fig.4B) but not in THP-1 monocytes (Fig. 4C). Furthermore, ectopic expression of PRMT4 promoted LPS-induced caspase 3 activation (Fig. 4D). Next, we generated PRMT4 knockout (KO) Jurkat cells using CRISPR/Cas9 to test our observations (Fig. 4E). KO of PRMT4 blocked LPS-mediated caspase 3 activation to baseline levels (Fig. 4F). Results from the isolated mouse splenic T-cells showed that expression of *PRMT4* promotes LPS-induced caspase 3 activation, and depletion of *PRMT4* by shRNA substantially blocks LPS-induced caspase 3 activation (Fig.

4G). Thus, these data suggest that LPS increases PRMT4 expression thereby facilitating the methyltransferase to trigger caspase 3 signaling in lymphocytes.

PRMT4 mediates lymphocyte death and PRMT4 depletion or chemical inhibition reduces lymphocyte cell death.

To explore the pathophysiological function of LPS-mediated PRMT4 elevation and subsequent caspase 3 activation, we measured lymphocyte apoptosis. We conducted Annexin V flow-cytometry to confirm the role of PRMT4 in LPS-mediated T cell death. Flow-cytometry results showed that the baseline levels of cell death were similar in PRMT4 KO Jurkat cells (8.19% and 8.03%) as compared to that of control cells (7.86%). LPS treatment substantially promoted cell death in non-targeted cells (26.65%) but not in PRMT4 KO cells (10.47% and 8.31%). Interestingly, PRMT4 overexpression promoted Jurkat cell death (19.75%) and augmented LPS-induced cell death (41.78%) (Fig. 5A, B). In mouse splenic T-cells, LPS did not affect cell survival in PRMT4 depletion but remarkably reduced cell survival in PRMT4 overexpression (Fig. 5C). Next, we assessed if a PRMT4 small molecule inhibitor(s) might suppress T cell death. We first screened commercially available PRMT4 inhibitors in LPS-treated Jurkat cells to test if the inhibitor(s) might inhibit caspase 3 activation. A selective and cell active PRMT4 inhibitor TP064 efficiently and most consistently inhibited caspase 3 activation at a concentration of ~ 40 nM (Fig. 5D). Further, experimental results suggest that TP064 can block LPS mediated caspase 3 activation at both 25 nM and 50 nM in mouse splenic T cells (Fig. 5E). These studies link PRMT4 to endotoxin-induced lymphocyte death and showcase the ability of a PRMT4 specific small molecule inhibitor to effectively antagonize endotoxin-induced lymphocyte death signaling.

PRMT4 mediates splenic lymphocyte death in experimental LPS injury mice model.

To test our hypothesis in animal models, we assessed PRMT4 in an LPS injury mouse model. We overexpressed PRMT4 or knocked down PRMT4 using lentiviral constructs (1×10^7 cfu/mouse, intratracheal [i.t.] administration) in mice (C57BL/6J) for 14 d. LPS (5 mg/kg, i.t. in PBS buffer) was then administrated (i.t.) into mice for 24 h. As i.t. administration of LPS induces systemic responses and disseminate to major organs such as the spleen, we observed that LPS induced substantial cell death in the lymphoid white pulp of spleen, which is rich in both T and B lymphocytes, by TUNEL assay (Fig. 6A, B). Further, overexpression of PRMT4 markedly enhanced LPS-induced lymphocyte death whereas silencing of *PRMT4* by shRNA significantly diminished cell death. TP064 partially protected splenic lymphocytes from death. We further analyzed splenocyte death by flow cytometry and identified that overexpression of PRMT4 enhanced cell death of CD4+ T cells (Fig. 6C, D). Further, depletion of PRMT4 using shRNA or inhibition of PRMT4 with small molecule decreased CD4+ cell death. We observed similar results in CD8a+ cells and CD45R+ cells (supplementary sFig. 1). In addition, results from survival studies indicated that *PRMT4* overexpressed mice showed a significantly lower survival rate. Depletion of PRMT4 by shRNA or application of TP064 remarkably improved mouse survival (Fig. 6E). We obtained similar results in an independent replication study (sFig. 2). Meta-analysis was conducted to analyze the reproductivity using these two sets of independent experiments (Fig. 6F, 6G, sTable 1). Meta-analysis using Random-effects REML model showed that overexpression of PRMT4 did not remarkably change the survival in experiments as

compared to that of LPS only (Fig. 6F). TP064 effectively promoted mouse survival in experiments ($p = 0.03$) (Fig. 6G). However, due to the insufficient sample size in shPRMT4 groups, we were unable to analyze the significance. We obtained similar results by combining the two experiments into one (sTable 1). These data suggest that LPS-enhanced PRMT4 protein expression promotes lymphocyte death and targeting PRMT4 improves survival in an LPS mouse lung injury model.

PRMT4 mediates splenic lymphocyte death in a polymicrobial sepsis model.

We next tested our hypothesis in a well characterized polymicrobial sepsis mouse model, Cecal ligation and puncture (CLP). Mice were subjected to lenti-PRMT4 overexpression and lenti-shRNA as above CLP procedure were followed to induce sepsis in animals for 48 h. One group of mice receiving lenti-vector only were given TP064 (0.2 μg/mouse) intravenously following CLP. Consistent with above observations, results from TUNEL staining of the splenic tissues showed that CLP was sufficient to cause splenic T cell death in the white pulp (Fig. 7A, B). Flow cytometric data confirmed these observations (Fig. 7C, D). Moreover, PRMT4 overexpression mediated splenic CD4+ T cell death in this model whereas silencing of PRMT4 by shRNA or administration of TP064 attenuated splenic T cell death. Consistent results were obtained in CD8a+ T cells and CD45R+ B cells (supplementary sFig. 3). Survival studies showed that lenti-PRMT4 overexpression decreased mouse survival, and depletion of PRMT4 by shRNA or application of the PRMT4 chemical inhibitor protected mice from death (Fig. 7E). We obtained similar results in an independent replication study (sFig. 4). Similar results were obtained from meta-analysis using these two sets of independent experiments (Fig. 7F-H, sTable 2). Meta-analysis using Random-effects REML model showed that overexpression of PRMT4 did not remarkably change the survival in experiments (Fig. 7F). Depletion of PRMT4 by shRNA significantly promoted survival ($p = 0.01$) (Fig. 7G). PRMT4 inhibitor TP064 effectively promoted mouse survival as well as compared with that of CLP only ($p = 0.03$) (Fig. 7H). We obtained similar results by combining the two experiments into one in meta-analysis (sTable 2). In summary, these results suggest that PRMT4 is a critical mediator of lymphocyte death in septic models.

Discussion

There is mounting evidence that a mechanistic centerpiece of sepsis is immunosuppression from loss of effector lymphocytes underscoring an unmet need to identify new molecular targets for therapeutic intervention [31]. The fundamental new findings in this study are that (i) PRMT4 protein is elevated in the plasma of human subjects with sepsis, preclinical models of sepsis, and lymphocytes exposed to bacterial endotoxin; (ii) LPS augments PRMT4 that mediates lymphocyte apoptosis; and (iii) chemical inhibition or genetic depletion of PRMT4 attenuates lymphocyte death and protects mice after endotoxin-induced lung injury and polymicrobial sepsis. PRMT4 is aberrantly expressed in breast, prostate, and colorectal cancers, and is associated with a poor prognosis by promoting tumor progress and cancer metastasis [32-35]. Consistent with these observations, we identified that PRMT4 expression is increased in sepsis. PRMT4 protein was upregulated in cellular and mouse septic models, human infected lung tissue samples, and peripheral blood leukocytes from

septic patients. Increased PRMT4 protein caused lymphocyte apoptosis and this finding may be one mechanism underlying the pathogenesis of septic immunosuppression, as apoptosis of lymphocytes is a key feature of sepsis-induced immunosuppression [36] and lymphopenia that develops during sepsis predicts early and late mortality [37].

Our studies uncover a unique role for PRMT4 in cell death linked to sepsis. Increased cell death has long been noticed as a key pathway in sepsis-induced immunosuppression but the factors leading to this increased cell death have not been fully defined [38 39]. Our findings here add a novel mechanism of immunosuppression at the epigenetic level: a pathogen-derived product induces cellular concentrations of a chromatin modulator, PRMT4, that in turn mediates lymphocyte death via caspase 3 related apoptosis, thus serving as a potential modulator in sepsis. Interestingly, PRMT4 appeared to cause cell death specifically at the white pulp of the spleen. The spleen white pulp is a lymphatic tissue where both B and T lymphocytes reside. Distinct from the matured lymphocytes in the red pulp, the lymphocytes in the white pulp are mostly naïve cells or cells in lineage differentiation and maturing. Considering CD4+ and CD8+ cells are less in PRMT4 knockout mice, our study suggests aberrant PRMT4 protein may play an important role in lineage differentiation for both B and T cells. Further studies are required to elucidate the molecular mechanisms whereby PRMT4 regulates apoptosis, epigenetically or via its methylation function driving post-translational modifications of key regulatory proteins that dictate cellular lifespan. Recent mass spectrometric studies uncovered more than 130 protein substrates modified by PRMT4 in breast cancer cell lines [40]. These targets might be suitable candidates for interrogation of post-translational modifications by PRMT4 associated with T-cell immunosuppression.

We observed higher plasma PRMT4 levels in septic patients compared to non-septic patients. Our results suggest that PRMT4 is highly expressed in leukocytes isolated from septic patients and PRMT4 is detectable in mouse sera and human plasma. Considering the fact that high levels of PRMT4 are cytotoxic, we speculate that PRMT4 in plasma of septic patients may originate from dying or dead cells. Peripheral blood leukocytes are classified into granulocytes and mononuclear agranulocytes, in which agranulocytes are composed of 35% in terms of total leukocytes. Agranulocytes are mononuclear that can be divided into lymphocytes, monocytes, and natural killer cells. We show that LPS increases PRMT4 expression in lymphocyte and monocytes. However, PRMT4 mediates caspase 3 cleavage in lymphocytes but not in monocytes. It remains to be determined if LPS promotes PRMT4 expression and activates caspase 3 in granulocytes. Interestingly, inhibition of PRMT4 by shRNA or with the small molecule inhibitor improved mice survival in both the LPS lung injury and CLP mouse models, suggesting that lymphocyte death may be a critical factor underlying the pathogenesis of endotoxin-mediated injury. We repeated the animal experiments as independent data and analyzed the data sets using meta-analysis approaches. Meta-analysis combined two studies together that may increase the power of the experiments, reach more accurate estimate of effect magnitude, and strengthen the conclusions from single study. Independent murine experiments may introduce heterogeneity, though the experimental individuals are with high consistence but less heterogeneity. However, meta-analysis is resources and time consuming, and requires experienced statistician to perform the analysis. In addition, not all studies provide adequate data for inclusion and analysis. Overall, pharmaceutical targeting of PRMT4 may be a

potential alternative therapeutic approach in septic patients with significantly low T cell counts and immunosuppression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability statement

All the data relevant to this study are included in the article. The original immunoblots are available upon request.

References

1. Delano MJ, Ward PA. Sepsis-induced immune dysfunction: can immune therapies reduce mortality? J Clin Invest 2016;126(1):23–31. doi: 10.1172/JCI82224 [published Online First: 2016/01/05] [PubMed: 26727230]
2. Cohen J. The immunopathogenesis of sepsis. Nature 2002;420(6917):885–91. doi: 10.1038/nature01326 [published Online First: 2002/12/20] [PubMed: 12490963]
3. Venet F, Monneret G. Advances in the understanding and treatment of sepsis-induced immunosuppression. Nat Rev Nephrol 2018;14(2):121–37. doi: 10.1038/nrneph.2017.165 [published Online First: 2017/12/12] [PubMed: 29225343]
4. Hotchkiss RS, Monneret G, Payen D. Immunosuppression in sepsis: a novel understanding of the disorder and a new therapeutic approach. Lancet Infect Dis 2013;13(3):260–8. doi: 10.1016/S1473-3099(13)70001-X [published Online First: 2013/02/23] [PubMed: 23427891]
5. Sen DR, Kaminski J, Barnitz RA, et al. The epigenetic landscape of T cell exhaustion. Science 2016;354(6316):1165–69. doi: 10.1126/science.aae0491 [PubMed: 27789799]
6. Wang TS, Deng JC. Molecular and cellular aspects of sepsis-induced immunosuppression. Journal of molecular medicine 2008;86(5):495–506. doi: 10.1007/s00109-007-0300-4 [PubMed: 18259721]
7. Slama P, Kabourkova E, Sladek Z, et al. Effect of Lipopolysaccharide and Muramyl Dipeptide on Apoptosis of Bovine Mammary Gland Lymphocytes. Animals (Basel) 2020;10(6) doi: 10.3390/ani10060990 [published Online First: 2020/06/11]
8. Nielsen JS, Larsson A, Ledet T, et al. Rough-Form Lipopolysaccharide Increases Apoptosis in Human CD4(+) and CD8(+) T Lymphocytes. Scand J Immunol 2012;75(2):193–202. doi: 10.1111/j.1365-3083.2011.02613.x [published Online First: 2011/08/23] [PubMed: 21854408]
9. Tamayo E, Postigo J, Del Giudice G, et al. Involvement of the intrinsic and extrinsic cell-death pathways in the induction of apoptosis of mature lymphocytes by the Escherichia coli heat-labile enterotoxin. Eur J Immunol 2009;39(2):439–46. doi: 10.1002/eji.200838993 [published Online First: 2009/01/31] [PubMed: 19180465]
10. Nielsen JS, Larsson A, Brix-Christensen V, et al. Endotoxemia-induced lymphocyte apoptosis is augmented by a hyperinsulinemic-euglycemic clamp. Anesthesiology 2005; 102(4)768–73. doi: 10.1097/00000542-200504000-00012 [published Online First: 2005/03/26] [PubMed: 15791106]

11. Okada M, Hibino S, Someya K, et al. Regulation of regulatory T cells: epigenetics and plasticity. Adv Immunol 2014;124:249–73. doi: 10.1016/B978-0-12-800147-9.00008-X [PubMed: 25175778]
12. Kang SW, Shih PA, Mathew RO, et al. Renal kallikrein excretion and epigenetics in human acute kidney injury: expression, mechanisms and consequences. BMC nephrology 2011;12:27. doi: 10.1186/1471-2369-12-27 [PubMed: 21679467]
13. Carson WF, Cavassani KA, Dou Y, et al. Epigenetic regulation of immune cell functions during post-septic immunosuppression. Epigenetics 2011;6(3):273–83. [PubMed: 21048427]
14. Liu Y, Li H, Xiao T, et al. Epigenetics in immune-mediated pulmonary diseases. Clinical reviews in allergy & immunology 2013;45(3):314–30. doi: 10.1007/s12016-013-8398 [PubMed: 24242359]
15. Takebe M, Oishi H, Taguchi K, et al. Inhibition of histone deacetylases protects septic mice from lung and splenic apoptosis. J Surg Res 2014;187(2):559–70. doi: 10.1016/j.jss.2013.10.050 [PubMed: 24290430]
16. Zou C, Li J, Xiong S, et al. Mortality factor 4 like 1 protein mediates epithelial cell death in a mouse model of pneumonia. Sci Transl Med 2015;7(311):311ra171. doi: 10.1126/scitranslmed.aac7793
17. Das ND, Jung KH, Chai YG. The role of NF-kappaB and H3K27me3 demethylase, Jmjd3, on the anthrax lethal toxin tolerance of RAW 264.7 cells. PLoS One 2010;5(3):e9913. doi: 10.1371/journal.pone.0009913 [PubMed: 20360974]
18. Hotchkiss RS, Monneret G, Payen D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. Nature Reviews Immunology 2013;13(12):862.
19. Cheng D, Cote J, Shaaban S, et al. The arginine methyltransferase CARM1 regulates the coupling of transcription and mRNA processing. Molecular cell 2007;25(1):71–83. doi: 10.1016/j.molcel.2006.11.019 [PubMed: 17218272]
20. Covic M, Hassa PO, Saccani S, et al. Arginine methyltransferase CARM1 is a promoter-specific regulator of NF-kappaB-dependent gene expression. The EMBO journal 2005;24(1):85–96. doi: 10.1038/sj.emboj.7600500 [PubMed: 15616592]
21. Yadav N, Lee J, Kim J, et al. Specific protein methylation defects and gene expression perturbations in coactivator-associated arginine methyltransferase 1-deficient mice. Proc Natl Acad Sci U S A 2003;100(11):6464–8. doi: 10.1073/pnas.1232272100 [PubMed: 12756295]
22. Kleinschmidt MA, Streubel G, Samans B, et al. The protein arginine methyltransferases CARM1 and PRMT1 cooperate in gene regulation. Nucleic acids research 2008;36(10):3202–13. doi: 10.1093/nar/gkn166 [PubMed: 18413343]
23. Chen D, Ma H, Hong H, et al. Regulation of transcription by a protein methyltransferase. Science 1999;284(5423):2174–7. doi: 10.1126/science.284.5423.2174 [published Online First: 1999/06/26] [PubMed: 10381882]
24. O'Brien KB, Alberich-Jorda M, Yadav N, et al. CARM1 is required for proper control of proliferation and differentiation of pulmonary epithelial cells Development 2010;137(13):2147–56. doi: 10.1242/dev.037150 [PubMed: 20530543]
25. Kim J, Lee J, Yadav N, et al. Loss of CARM1 results in hypomethylation of thymocyte cyclic AMP-regulated phosphoprotein and deregulated early T cell development. J Biol Chem 2004;279(24):25339–44. doi: 10.1074/jbc.M402544200 [published Online First: 2004/04/21] [PubMed: 15096520]
26. Kim D, Lee J, Cheng D, et al. Enzymatic activity is required for the in vivo functions of CARM1. The Journal of biological chemistry 2010;285(2):1147–52. doi: 10.1074/jbc.M109.035865 [PubMed: 19897492]
27. Zou C, Synan MJ, Li J, et al. LPS impairs oxygen utilization in epithelia by triggering degradation of the mitochondrial enzyme Alcat1 J Cell Sci 2016;129(1):51–64. doi: 10.1242/jcs.176701 [published Online First: 2015/11/26] [PubMed: 26604221]
28. Gonzalez-Navajas JM, Fine S, Law J, et al. TLR4 signaling in effector CD4+ T cells regulates TCR activation and experimental colitis in mice. J Clin Invest 2010;120(2):570–81. doi: 10.1172/JCI40055 [published Online First: 2010/01/07] [PubMed: 20051628]

29. Zanin-Zhorov A, Tal-Lapidot G, Cahalon L, et al. Cutting edge: T cells respond to lipopolysaccharide innately via TLR4 signaling. J Immunol 2007;179(1):41–4. doi: 10.4049/jimmunol.179.1.41 [published Online First: 2007/06/21] [PubMed: 17579019]
30. Kim D-i, Park M-j, Lim S-k, et al. High-glucose-induced CARM1 expression regulates apoptosis of human retinal pigment epithelial cells via histone 3 arginine 17 dimethylation: role in diabetic retinopathy. Archives of biochemistry and biophysics 2014;560:36–43. [PubMed: 25072916]
31. Deutschman CS, Tracey KJ. Sepsis: current dogma and new perspectives. Immunity 2014;40(4):463–75. doi: 10.1016/j.immuni.2014.04.001 [published Online First: 2014/04/22] [PubMed: 24745331]
32. Hong H, Kao C, Jeng MH, et al. Aberrant expression of CARM1, a transcriptional coactivator of androgen receptor, in the development of prostate carcinoma and androgen-independent status. Cancer 2004;101(1):83–9. doi: 10.1002/cncr.20327 [published Online First: 2004/06/29] [PubMed: 15221992]
33. Kim YR, Lee BK, Park RY, et al. Differential CARM1 expression in prostate and colorectal cancers. BMC Cancer 2010;10:197. doi: 10.1186/1471-2407-10-197 [published Online First: 2010/05/14] [PubMed: 20462455]
34. Cheng H, Qin Y, Fan H, et al. Overexpression of CARM1 in breast cancer is correlated with poorly characterized clinicopathologic parameters and molecular subtypes. Diagn Pathol 2013;8:129. doi: 10.1186/1746-1596-8-129 [published Online First: 2013/08/07] [PubMed: 23915145]
35. Habashy HO, Rakha EA, Ellis IO, et al. The oestrogen receptor coactivator CARM1 has an oncogenic effect and is associated with poor prognosis in breast cancer. Breast Cancer Res Treat 2013;140(2):307–16. doi: 10.1007/s10549-013-2614-y [published Online First: 2013/07/28] [PubMed: 23887673]
36. McDunn JE, Hotchkiss RS. Leukocyte phenotyping to stratify septic shock patients. Crit Care 2009;13(2):127. doi: 10.1186/cc7748 [published Online First: 2009/05/15] [PubMed: 19439023]
37. Drewry AM, Samra N, Skrupky LP, et al. Persistent lymphopenia after diagnosis of sepsis predicts mortality. Shock 2014;42(5):383–91. doi: 10.1097/SHK.0000000000000234 [published Online First: 2014/07/23] [PubMed: 25051284]
38. Pinheiro da Silva F, Nizet V. Cell death during sepsis: integration of disintegration in the inflammatory response to overwhelming infection. Apoptosis 2009;14(4):509–21. doi: 10.1007/s10495-009-0320-3 [published Online First: 2009/02/10] [PubMed: 19199035]
39. Bantel H, Schulze-Osthoff K. Cell death in sepsis: a matter of how, when, and where. Crit Care 2009;13(4):173. doi: 10.1186/cc7966 [published Online First: 2009/08/15] [PubMed: 19678906]
40. Shishkova E, Zeng H, Liu F, et al. Global mapping of CARM1 substrates defines enzyme specificity and substrate recognition. Nature communications 2017;8:15571.

Key messages

What is already known on this topic?

Lymphopenia is observed in septic patients with poor prognosis, however, our understanding on the mediators of lymphocyte death is limited.

What this study adds?

PRMT4 is increased in cellular and animal sepsis models and in peripheral blood leukocytes from patients with sepsis. Elevated PRMT4 protein causes caspase 3 activation and leads to lymphocyte death. Genetic depletion or chemical inhibition of PRMT4 improves mouse survival and prevents lymphocytes from death in animal sepsis models.

How this study might affect research, practice or policy?

This manuscript reveals a novel pathway: a chromatin modulator PRMT4 as a crucial cell death mediator that causes lymphocyte death in sepsis.

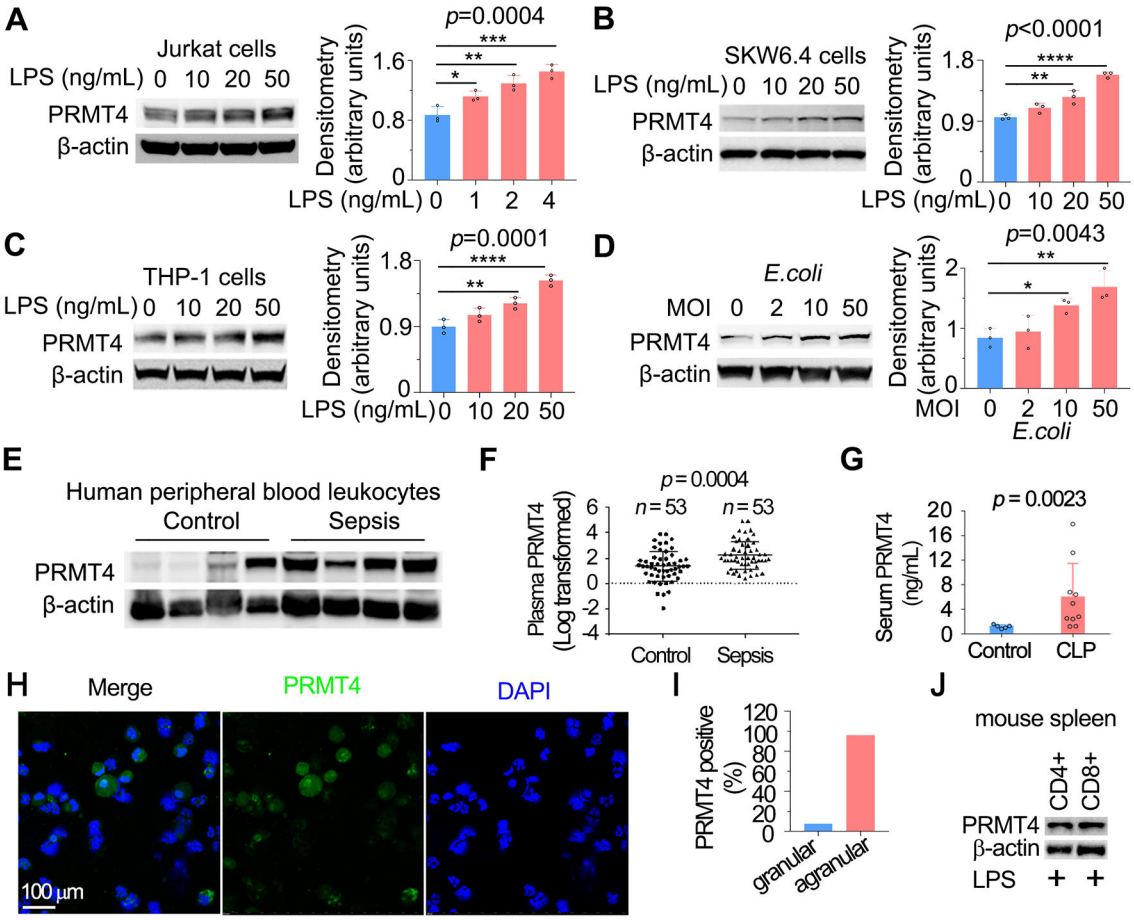

Fig. 1. LPS and *E. coli* increases PRMT4 protein expression in lymphocytes in vitro, and PRMT4 is increased in experimental septic models.

A, B, C. Jurkat cells(A), SKW6.4 cells (B) and THP-1 cells (C) were treated with LPS as indicated, cell lysates were immunoblotted with PRMT4 and β-actin antibodies. Independent experiments n=3. D. Jurkat cells were treated with live *E. coli* as indicated, cell lysates were immunoblotted with PRMT4 and β-actin antibodies. Densitometry was plotted in the lower panel. Independent experiments n=3. E. Lysates of peripheral blood leukocytes from deidentified human samples with or without sepsis were immunoblotting analyzed with PRMT4 and β-actin. F. PRMT4 protein levels were determined by ELISA from blood plasma from septic patients (n=53) and non-septic control patients (n=53). Lines indicate the median and IQR, Mann-Whitney U test, p=0.0004. G. CLP procedures were subjected to C57BL/6J mice for 48 h, mice sera were collected from untreated controls (n=5) and polymicrobial infected mice (n=10) for PRMT4 ELISA analysis. H, I. Leukocytes isolated from BALF in LPS treated mouse were immunofluorescent stained with PRMT4 antibody. PRMT4 expression were visualized using confocal microscopy, the nuclei were stained by DAPI (4',6-diamidino-2-phenylindole) (H). Total cells were counted and positively stained granular and agranular cells were presented as percentage (I). A total of 300 granulocytes and 100 agranulocytes were counted. J. Isolated CD4+ and CD8+ cells from LPS treated mouse were lysed and immunoblotting analyzed with PRMT4 antibody.

Independent experiments n=3. “*” denotes p=0.05-0.01, “**” denotes p=0.01-0.001, “***” denotes p=0.001-0.0001, “****” denotes p<0.0001. Scale bar=100 μm.

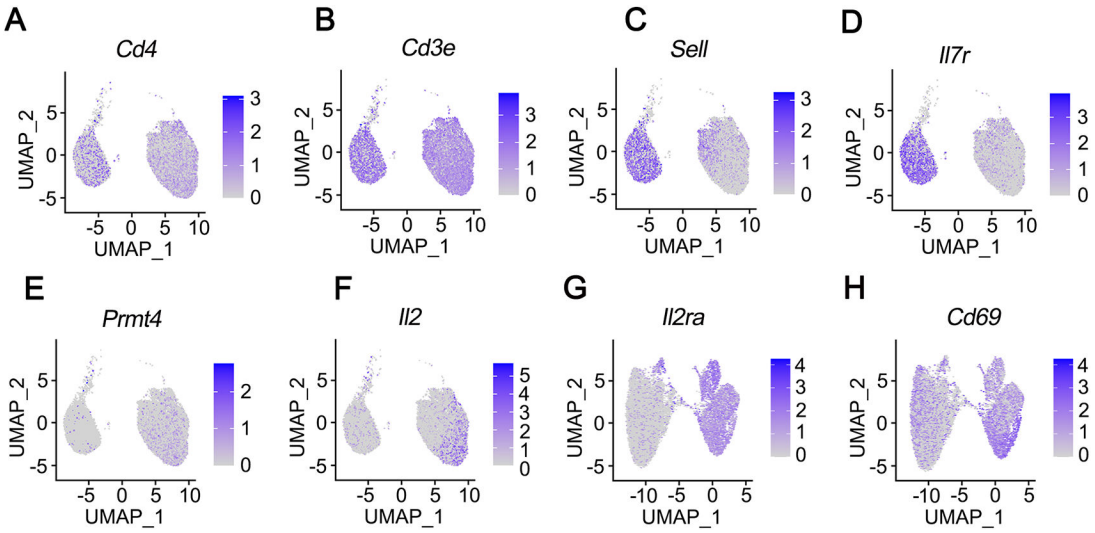

Fig. 2. PRMT4 gene expression increases upon activation in CD4+ T cells.

CD4 + cells were isolated from the spleen of a mouse (strain C57BL/6J). The mixture of naïve unstimulated T cells and CD4 T cells activated with anti- CD3/CD28 comprising a total of 10,000 cells each were applied to Single-cell RNA-seq. UMAP (uniform manifold approximation and projection) plots as two dimensional were used to plot the expression of *CD4* specific genes *CD4* (A) and *CD3e* (B), naïve T cell specific genes *Sell* (C) and *IL7r* (D), CD4+ cell activation increased *PRMT4* (E), *IL2* (F), *IL2ra* (G), as well as *CD69* (H) gene expression.

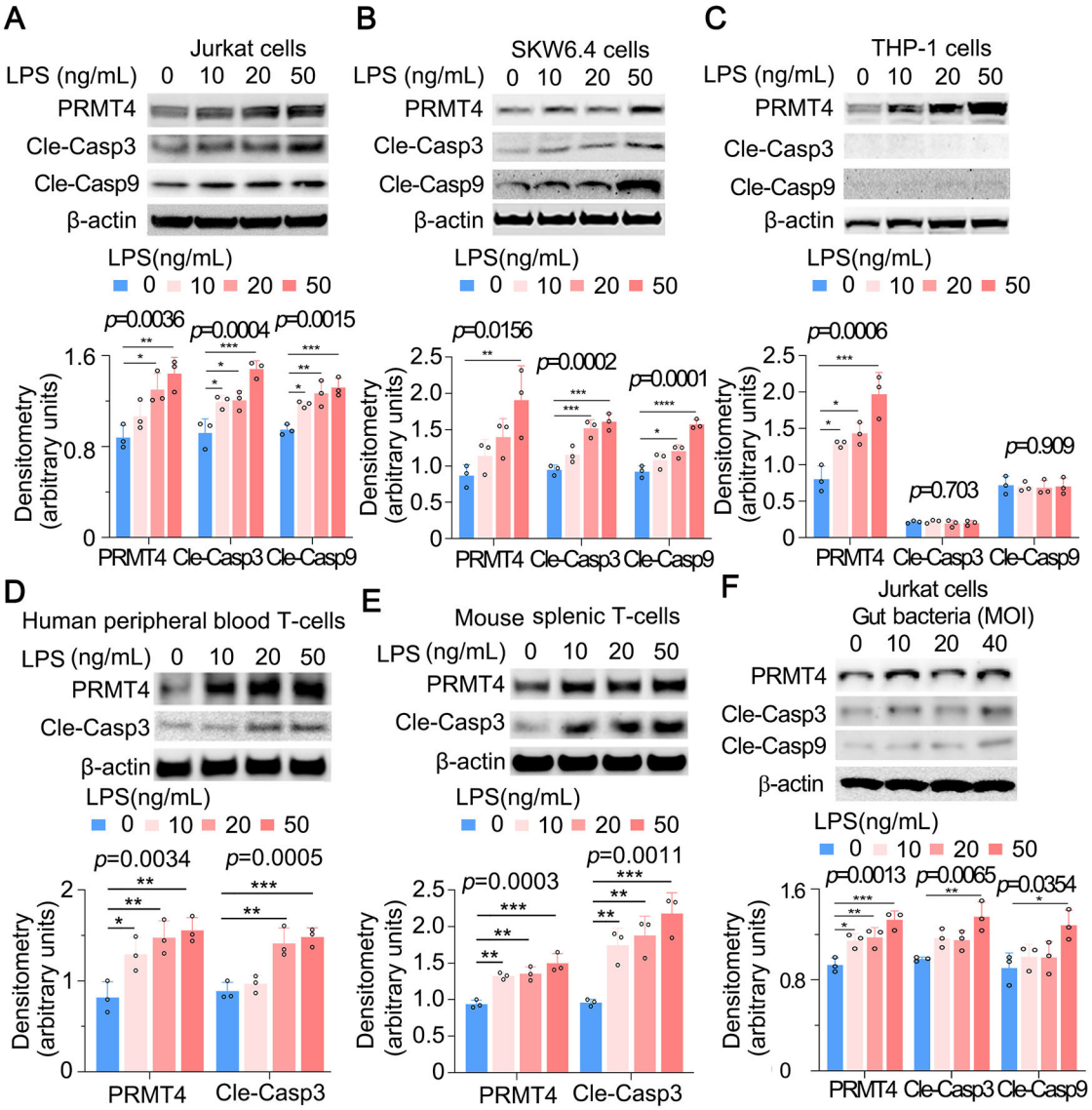

Fig. 3. LPS increases PRMT4 expression and activates caspase 3 in lymphocytes.

A-C. Jurkat cells (A), SKW6.4 cells (B) and THP-1 cells (C) were treated with LPS as indicated. Cell lysates were subjected to immunoblotting for PRMT4, cleaved caspase 3, cleaved caspase 9, and β-actin. The densitometric results were plotted in the lower panels. Independent experiments n=3. D, E. Primary mouse splenic lymphocytes (D) and human peripheral blood T cells (E) were treated with LPS as indicated. Cell lysates were analyzed by PRMT4, cleaved caspase 3 and β-actin immunoblotting. The plotted data were shown in the lower panels. Independent experiments n=3. F. The fecal material from mouse cecum was cultured in a LB plate overnight. Jurkat cells were treated with above gut-derived live bacteria for 2 h. Cell lysates were immunoblotting analyzed with PRMT4, cleaved caspase 3, cleaved caspase 9, and β-actin. The plotted data were shown in the lower panel. Independent experiments n=3. "*" denotes p=0.05-0.01, "**" denotes p=0.01-0.001, "***" denotes p=0.001-0.0001.

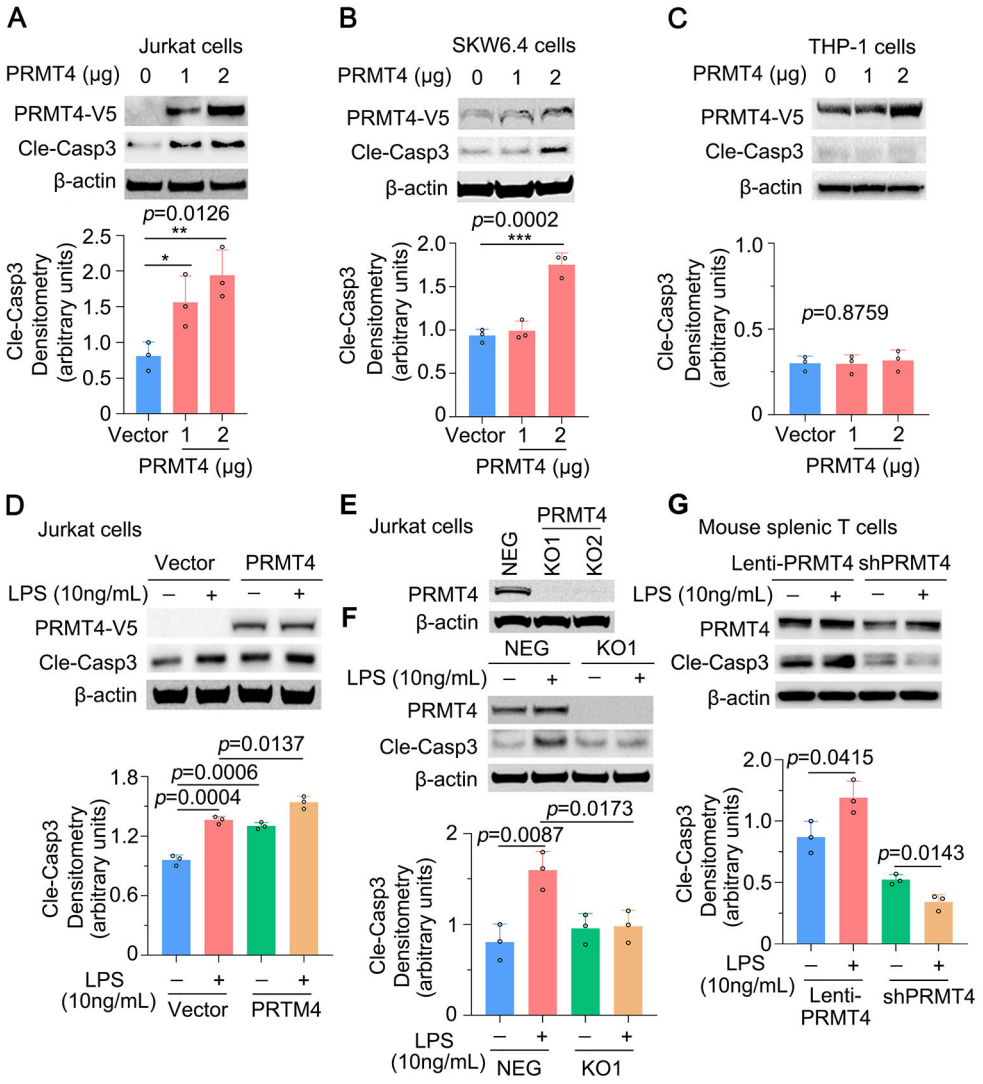

Fig. 4. Caspase 3 activation is PRMT4 dependent in lymphocytes.

A, B. Over-expression of *PRMT4* increased cleaved caspase 3 baseline levels in Jurkat cells (A) and SKW6.4 cells (B). Relative expression of cleaved caspase 3 were plotted in the lower panel. C. PRMT4 overexpression does not activate caspase3 in THP-1 cells. D. Ectopic expression of PRMT4 enhances LPS-induced caspase 3 activation in Jurkat cells. E. Knockout of PRMT4 in Jurkat cells with the CRISPR-Cas9 technique. NEG: negative. F. KO of PRMT4 limits LPS-induced caspase 3 activation. G. Lentiviral expression of PRMT4 enhances LPS-mediated caspase 3 activation and depletion of PRMT4 by lenti-shPRMT4 reduces cleaved caspase 3 in mouse splenic lymphocytes. Independent experiments n=3. "*" denotes p=0.05-0.01, "**" denotes p=0.01-0.001, "***" denotes p=0.001-0.0001.

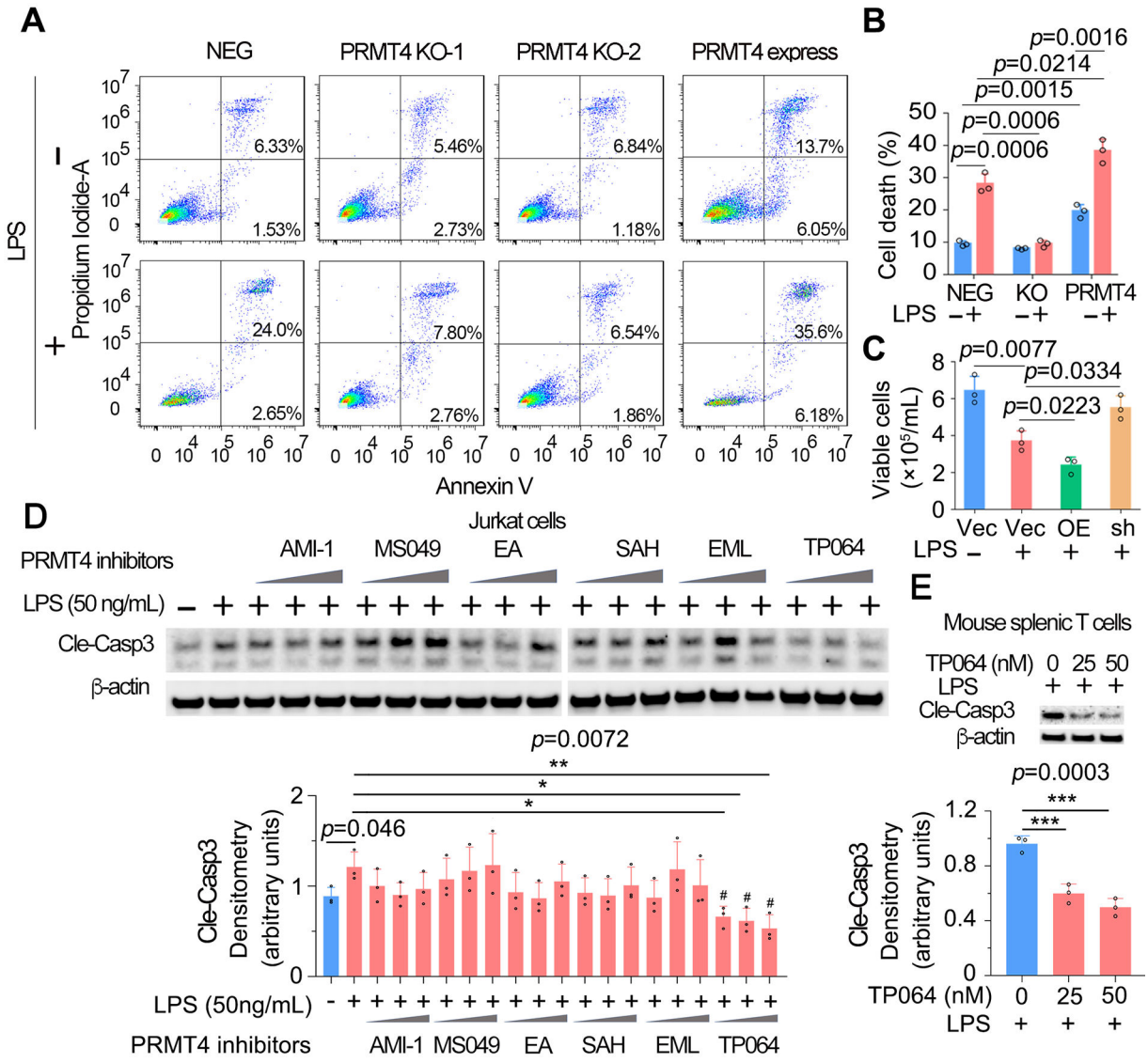

Fig. 5. High protein level of PRMT4 causes lymphocyte death.

A, B. FACS analysis of apoptosis in PRMT4 KO or overexpressed Jurkat cells with or without LPS treatment. Data of A were quantitated in B. C. Lenti-*PRMT4* or shRNA particles were delivered intratracheally into the mouse. Mouse splenic T cells were isolated and treated with LPS for 18 h, viable cells were counted. “Vec” denotes vector; “OE” denotes PRMT4 overexpression; “sh” denotes PRMT4 shRNA. D. Jurkat cells were treated with LPS and a range of PRMT4 inhibitors as indicated for 3 h. Cell lysates were analyzed for cleaved caspase 3. Relative expression of cleaved caspase 3 in each group were plotted in the lower panel. Independent experiments n=3. E. Isolated mouse splenic T cells were treated with LPS and TP064, cleaved caspase 3 was immunoblotting analyzed and plotted in the lower panel. Independent experiments n=3. “*” denotes p=0.05-0.01, “**” denotes p=0.01-0.001, “***” denotes p=0.001-0.0001.

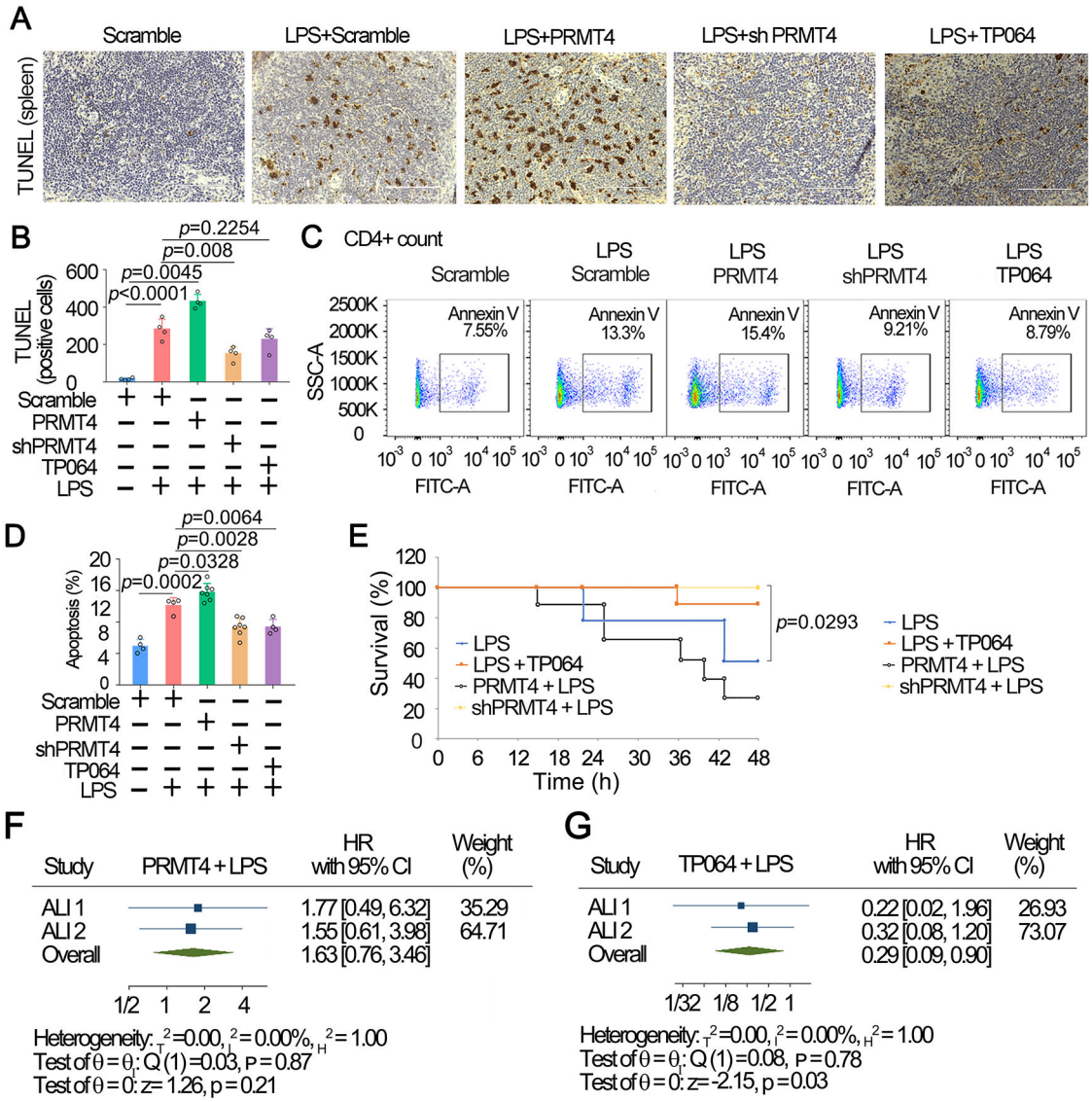

Fig. 6. Inhibition of PRMT4 suppresses splenic lymphocyte death in an LPS challenged mouse model.

A. PRMT4 was knocked down or overexpressed by i.t. administrated lentiviral constructs for 14 d. LPS or PRMT4 inhibitor were given (i.t.) as indicated for 24 h (n=8). Spleen tissues were stained with TUNEL. B. TUNEL positive cells in spleen tissues were quantitated. C, D. CD4 positive lymphocytes were isolated from splenic tissues in above PRMT4 knockdown or overexpression experiments (A) and analyzed with flow cytometry. CD4 was used as a T cell marker. % of apoptosis were quantitated in D (n=3). E. Survival studies were conducted in the LPS lung injury model, mice were observed for 48 h (n = 10). F,G. Two stage meta-analysis was conducted using two independent sets of murine data using LPS only group as reference: PRMT4 +LPS (F) and TP064 + LPS (G). The data of shPRMT4 group was not shown due to the hazard ratio (HR) was not computable. Two independent experiments were conducted [n = 26, (10, 16)]. Scale bar=100 μm.

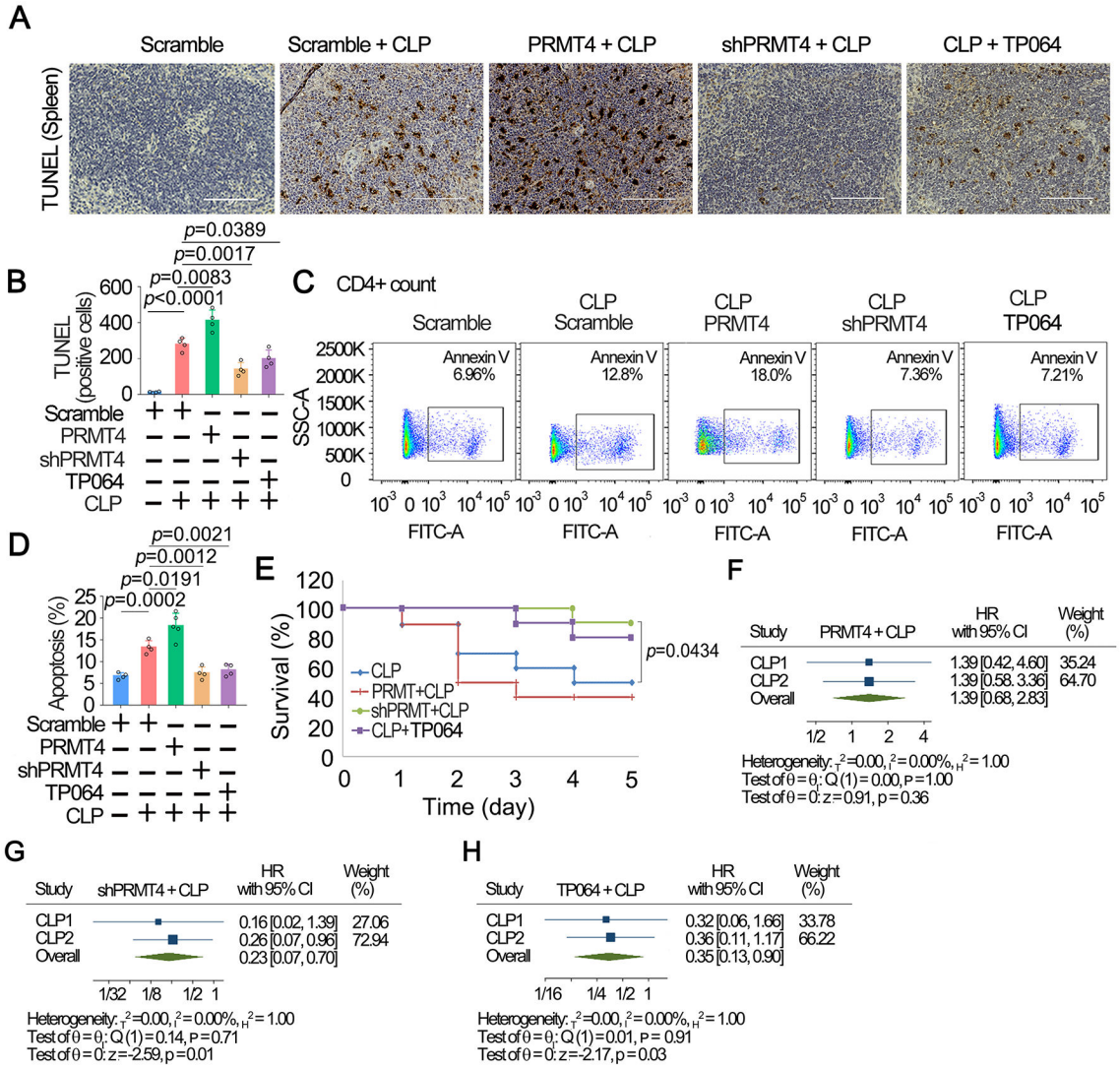

Fig. 7. Inhibition of PRMT4 suppresses splenic lymphocyte death in a polymicrobial sepsis model.

A. CLP was performed in PRMT4 knocked down or overexpressed mice (n=8). TP064 (0.2 μg/mouse) was administrated i.v. in one group for 48 h. Spleen tissues were stained with TUNEL. B. TUNEL positive cells in spleen tissues. C, D. Isolated splenic CD4+ T cells were analyzed by flow cytometry (C). CD4 was used as a T cell marker. The data from C were plotted in D. E. Survival studies were conducted in the CLP model, and mice were observed for 5 days (n = 16). F-H. Meta-analysis was conducted among two independent sets of murine data using CLP only as reference group: PRMT4 + CLP (F), shPRMT4 + CLP (G), and TP064 + CLP (H). Two independent experiments were conducted [n = 26, (10, 16)]. Scale bar=100 μm.

Table 1 –

Patient Characteristics

N	Control 53	Sepsis 53
PRMT4, mean (SD, ng/mL)	7.8 (11.0)	18.4 (28.6)
Demographics		
Age, mean (SD)	55(15.0)	55 (17.5)
Male, n (%)	31 (58.5)	24 (45.3)
BMI, mean (SD)	28.8 (9.1)	30.9 (9.4)
Comorbidities, n (%)		
Diabetes	16 (30.2)	22 (41.5)
COPD	6 (11.3)	11 (20.7)
Immunosuppression	9 (17)	9 (17)
Chronic Cardiac Failure	3 (5.7)	7 (13.2)
Chronic Kidney Disease	6 (11.3)	5 (9.4)
Pulmonary Fibrosis	0 (0)	2 (3.8)
Active Neoplasm	3 (5.7)	3 (5.7)
Chronic Liver Disease	9 (17)	6 (11.3)
Alcohol Use	13 (24.5)	8 (15.1)
Severity of Illness		
SOFA score[1], mean (SD)	5.2 (2.4)	7.4 (2.6)
PaO2:FiO2 ratio, mean (SD)	224 (152)	196 (88)
Shock, n (%)	10 (18.9)	34 (64.2)
Acute Kidney Injury, n (%)	22 (41.5)	40 (75.5)
ICU LOS, days, mean (SD)	8.5 (9.4)	13.4 (9.8)
30-day mortality, n (%)	6 (11.3)	12 (22.6)
90-day mortality, n (%)	7 (13.2)	13 (24.5)

BMI - body mass index, COPD - chronic obstructive lung disease, SOFA - sequential organ failure assessment, PaO2 - arterial partial pressure of oxygen, FiO2 - fraction of inspired oxygen, ICU - intensive care unit, LOS - length of stay.