

Original Article

FAM19A4 enhances neutrophil respiratory burst via p38 MAPK in lethal sepsis

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Abstract

Sepsis causes high mortality and resource strain, with neutrophil-derived reactive oxygen species (ROS) contributing to excessive inflammation. The secretory protein FAM19A4 modulates ROS release, but its role in sepsis is unclear. In this study, we find elevated FAM19A4 levels in septic patients and cecal ligation and puncture (CLP) mice, which correlate with increased mortality. *Fam19a4*^{−/−} mice subjected to CLP show significantly improved survival and attenuated multiorgan injury without impaired peritoneal bacterial clearance or altered circulating neutrophil counts. FAM19A4 deficiency reduces the cell counts of neutrophils (Ly6G⁺) and macrophages (F4/80⁺) in the lungs and liver, diminishes systemic ROS production tracked by bioluminescence, and decreases neutrophil extracellular trap (NET) formation in serum and lung tissue. *In vitro*, FAM19A4 enhances neutrophil phagocytosis and ROS generation but does not affect lipopolysaccharide-induced chemotaxis. Mechanistically, FAM19A4 drives neutrophil ROS release specifically through p38 MAPK signaling activation, as revealed by bulk RNA sequencing, western blot analysis, and treatment with p38 inhibitor SB203580. These results indicate that FAM19A4 is upregulated during sepsis and exacerbates outcomes by enhancing neutrophil ROS production via p38 MAPK, representing a promising therapeutic target for this condition.

Key words FAM19A4, respiratory burst, p38 MAPK, sepsis

Introduction

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection [1]. With an estimated incidence of 18.6 cases per 1000 hospitalizations and a mortality rate approaching 40% [2], sepsis imposes substantial long-term healthcare burdens on survivors while straining global medical resources [3,4]. The pathophysiology of sepsis involves hyperactivation of innate immunity, characterized by excessive cytokine production (“cytokine storms”), which drives cardiovascular collapse, multiorgan failure, and early mortality [5]. Despite progress, the key molecular drivers of this dysregulated response remain undefined, hampering the development of targeted diagnostics and therapies. Elucidating the mechanisms underlying

sepsis development represents a critical frontier for advancing the management of this condition.

Neutrophils are crucial mediators of dysregulated inflammation during the early innate immune phase of sepsis [6]. In particular, in sepsis-associated acute respiratory distress syndrome (ARDS), neutrophils, as first responders, are among the earliest and most abundant immune cells recruited to the lung [7]. While neutrophils are essential for microbial clearance, their pathological accumulation in vital organs triggers respiratory burst-derived reactive oxygen species (ROS) overproduction [8], and these oxygen radicals induce mitochondrial dysfunction and propagate cellular damage cascades [9]. Notably, activated neutrophils release DNA scaffolds decorated with histones, granule proteins, and antimicrobial

peptides, referred to as neutrophil extracellular traps (NETs) [10]. ROS generation is a critical driver of NET formation via NADPH oxidase and myeloperoxidase (MPO) [11]. Excessive NETosis exacerbates tissue injury through proinflammatory cascades and is correlated with sepsis-associated mortality rates [12,13]. As a core member of the stress-activated MAP kinase superfamily, p38 mitogen-activated protein kinase (p38 MAPK) undergoes phosphorylation-mediated activation at a conserved TGY motif within its kinase activation loop, and this process is initiated by proinflammatory cytokines and mitogens through sequential kinase cascades [14]. In response to proinflammatory stimuli and pathogen-associated molecular patterns, p38 MAPK potentiates neutrophil ROS generation by enhancing NADPH oxidase activation; critically, this pathway directly mediates sepsis-induced organ injury through ROS-induced tissue damage [15].

FAM19A4 (family with sequence similarity 19, member A4), also referred to as TAF4A, is a member of the TAF4A protein family [16]. Human FAM19A4 is widely expressed at low levels in various normal tissues and organs, whereas in mice, FAM19A4 is highly expressed only in the dorsal ganglia [17]. Lipopolysaccharide (LPS)-stimulated FAM19A4 enhances phagocytic capacity and ROS generation [18]. Furthermore, FAM19A4 directly modulates the *in vitro* inflammatory profile of macrophages from a mouse sunburn-like model of skin damage and promotes IL-10 production by dermal macrophages *in vivo* [19]. Given its immunomodulatory properties and association with myeloid cell function, FAM19A4 could be a critical regulator of sepsis pathophysiology; its impact on neutrophils (central effectors of innate immunity in sepsis) warrants particular investigation. In this study, we examined the organ-protective and prognostic benefits of FAM19A4 deficiency in sepsis and elucidated the mechanism in the context of ROS release.

Materials and Methods

Generation of *Fam19a4*-EGFP knock-in (KI) mice

To generate *Fam19a4*-EGFP KI mice (purchased from Shanghai Biomodel Organism Science & Technology Development Co., Ltd., Shanghai, China), the EGFP-WPRE-pA expression cassette was inserted through CRISPR/Cas9-mediated homologous recombination, and precisely 30 bases downstream of the *Fam19a4* gene start codon. *Cas9* mRNA and single-guide RNA (gRNA1, 5'-GGTGTCTGCTGTCACGCTGGC TGG-3'; gRNA2, 5'-AGTGGGTGCTGCTGTACGC TGG-3') were produced via *in vitro* transcription. A donor vector for homologous recombination containing a 4.1-kb 5' homology arm, the EGFP-WPRE-pA cassette, and a 2.8-kb 3' homology arm was constructed via In-Fusion cloning. Subsequently, *Cas9* mRNA, gRNA, and the donor vector were co-injected into fertilized C57BL/6 oocytes to produce F0 founder mice. The resulting chimeras were bred with wild-type (WT) C57BL/6 females to achieve germline transmission of the targeted allele. Genotyping was performed via quantitative PCR (qPCR). For all the experiments, age- and sex-matched littermates derived from the same breeding pairs were utilized, and all the animals were maintained on a pure C57BL/6 genetic background.

Cecal ligation and puncture (CLP)-induced polymicrobial sepsis model

Sepsis was induced in mice (20–22 g body weight) via a previously described CLP model [20]. Following skin disinfection with 0.3% chlorhexidine (Xi'an Fanghao Chemical Co., Ltd, Xi'an, China), a

midline laparotomy was performed under 2% isoflurane anesthesia (Abbott Laboratories, Chicago, USA) delivered in oxygen. The cecum was ligated at its distal 25% segment and subsequently punctured once with a 20-gauge needle. Postoperative resuscitation included the subcutaneous administration of 1 mL of sterile saline. Humane endpoints were enforced through CO₂ euthanasia for moribund animals or at 7 days (experiment termination). In the control group, the animals underwent anesthesia and laparotomy without cecal ligation or puncture.

Isolation of human neutrophils

Human neutrophils were isolated from healthy adult volunteer venous blood samples as described previously [21]. Venous blood collected in EDTA-coated vacutainers was diluted with 20 mL of PBS and layered with lymphocyte separation medium (8 mL, Shanghai Xinyu Biotechnology Co., Ltd., Shanghai, China), followed by centrifugation (700 g, 25 min; brake off). The RBC/neutrophil pellet was retained, resuspended in 20 mL of Hank's balanced salt solution (Sigma-Aldrich, St. Louis, USA) and 20 mL of 3% dextran / 0.9% NaCl, and then incubated for 30 min at 20°C. The neutrophil-rich upper layer was transferred and centrifuged (400 g, 8 min), followed by sequential osmotic lysis of the pellet by resuspension in 0.2% NaCl, followed by the addition of 1.6% NaCl, with centrifugation (400 g, 8 min) after each step. Finally, the pellets were resuspended in RPMI 1640 (Sigma-Aldrich) containing 14% fetal bovine serum (FBS; Beyotime, Shanghai, China). Neutrophil counts, viability ((assessed by trypan blue dye exclusion) were determined. Cell purity (> 99%) was confirmed by differential count of Giemsa-stained cytospin preparations.

FAM19A4, IL-6, IL-10 and TNF- α measurement

Peripheral blood samples from mice and patients with sepsis were centrifuged (1800 g, 5 min) to collect serum for analysis. Bronchoalveolar lavage fluid (BALF) was obtained by clinicians following standardized protocols as follows: under local anesthesia, 100 mL of prewarmed (37°C) sterile saline was instilled into the right middle lobe or left lingula in 30–50 mL aliquots via electronic fiberoptic bronchoscopy; lavage fluid was aspirated under 50–100 mmHg negative pressure (minimum 40% recovery rate) and centrifuged (480 g, 10 min), and the supernatant was retained. The levels of cytokines (IL-6, IL-10, and TNF- α) in the serum and BALF were quantified via eBioscience ELISA kits (eBioscience, San Diego, USA), whereas the level of FAM19A4 was measured via MyBioSource ELISA kits (MyBioSource, Inc., San Diego, USA) per the manufacturers' protocols. Briefly, standards and samples were added to the antibody-precoated wells, followed by sequential incubation with a biotin-conjugated detection antibody and horseradish peroxidase (HRP)-conjugated avidin. All incubations were performed at 37°C; samples and the detection antibody were incubated for 60 min each, while the HRP-avidin complex and the 3,3',5,5'-tetramethylbenzidine (TMB) substrate were incubated for 30 min and 10 min, respectively. After terminating the reaction with sulfuric acid, the absorbance was measured at 450 nm. Sample concentrations were calculated by interpolating from the standard curve.

In situ hybridization

In situ hybridization was carried out with a digoxigenin-labeled probe (Roche Diagnostics GmbH, Mannheim, Germany), which

was hybridized for 20 h at 50°C. We then probed the liver and lung sections with a horseradish peroxidase (HRP)-labeled anti-digoxigenin antibody (Roche, Shanghai, China). Signal visualization was accomplished via a fluorescein/cy3/cy5 TSA plus amplification kit (Perkin Elmer Inc., Beijing, China).

The following oligonucleotides were used for probe synthesis via nested PCR:

Fam19a4-F1: 5'-TGCTCAGAAGTTCATAGCCAAA-3'

Fam19a4-R1: 5'-TAAAGGAACATTTGCAAGCTCA-3'

Fam19a4-F2: 5'-ATATGTGCAGTGTGG-3'

Fam19a4-R2+T7: 5'-TAATACGACTCACTATAGGGCAGCCAAGTTCAAAC-3'

Histopathology

For subsequent histopathological assessment, the tissues were subjected to fixation in 10% neutral-buffered formalin (Sigma-Aldrich) and subsequently processed through standardized protocols for paraffin embedding and sectioning. Serial 2- μ m sections were stained with hematoxylin & eosin (H&E; Sigma-Aldrich), anti-F4/80 (clone CI: A3-1; BMA Biomedicals, Augst, Switzerland), or anti-Ly6G (clone 1A8; BioLegend, San Diego, USA). H&E-stained sections were subjected to microscopic evaluation via semi-quantitative scoring as follows: renal tubular necrosis (0: no injury; 1: 0%–25%; 2: 25%–50%; 3: 50%–75%; 4: >75% affected tubules) [22], lung injury assessed via the Nishina score [23], and hepatic pathology graded via the Suzuki criteria [24]. For immunohistochemical staining, primary antibodies were used at a 1:100 dilution following antigen retrieval (BOND Epitope Retrieval kit; Leica, Beijing, China) with DAB visualization. The quantity of Ly6G⁺ and F4/80⁺ cells in kidney, liver, and lung tissues was evaluated via Definiens Tissue Studio software (Definiens, Inc., Munich, Germany). Representative regions were manually annotated to train artificial intelligence algorithms for target object identification (morphology/positivity-based) and background exclusion. Validated algorithms were subsequently applied for full-slide automated analysis, with diagnostic concordance confirmed by blinded histopathological review.

Quantitative PCR

Total RNA was isolated from liver, lung, kidney, and spleen tissues via RLT Plus buffer with β -mercaptoethanol homogenization (Qiagen, Hilden, Germany), followed by centrifugation (12,000 g, 3 min, 4°C). After genomic DNA removal via DNase I digestion on Omega Biotask columns, RNA was purified through RW1 buffer washes and eluted in RNase-free water, achieving spectrophotometric purity ($A_{260}/A_{280}=1.8\text{--}2.1$; $A_{260}/A_{230}>2.0$). Reverse transcription of 1 μ g of RNA via the PrimeScript RT Kit (Takara Bio Inc., Kusatsu, Japan) with random hexamer/oligo(dT) priming generated cDNA, which was subjected to quantitative PCR with PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Foster City, USA) on a QuantStudio 5 under optimized cycling conditions: UDG activation (50°C, 2 min), denaturation (95°C, 2 min), 40 cycles of 95°C / 15 s and 60°C / 1 min, followed by melt curve analysis. Relative gene expression was calculated via normalization to *Gapdh* via the $2^{-\Delta\Delta CT}$ method. The following primer sequences were used for amplification:

Fam19a4 forward: 5'-ATGAGGTCCCAAGGATGAGAGTCTG-3'

Fam19a4 reverse: 5'-CTCACAGGTCCCTTGCTTGATTTGGT-3'

Gapdh forward: 5'-GTGGCAAAGTGGAGATTGTTG-3'

Gapdh reverse: 5'-CGTTGAATTTGCCGTGAGTG-3'

Phagocytosis assay

Neutrophil phagocytic function was assessed via a pHrodo *E. coli* Bioparticles Phagocytosis Kit (Life Technologies, Carlsbad, USA). Neutrophils were pretreated with varying concentrations ((1 to 100 ng/mL) of the rmFAM19A4 protein (R&D Systems, Minneapolis, USA), either alone or in combination with LPS ((10 μ g/mL, Thermo Fisher Scientific, Waltham, USA)), followed by incubation with pHrodo *E. coli* bioparticles for 2 h at 37°C. Phagocytosis was quantified via either a TCS SP8 confocal microscope (Leica, Wetzlar, Germany) or by flow cytometry via a FACSVerse system (BD Biosciences, San Jose, USA) and BD FACSuite software. For flow cytometric analysis, the percentage of Ly6G⁺ cells exhibiting pHrodo *E. coli* positivity was determined on the basis of a gating strategy established from unstimulated versus bioparticle-treated cells. Fluorescence emission occurred following the internalization of particles into acidic phagosomal compartments. For microscopy-based quantification, three representative fields were imaged, and the average number of internalized pHrodo *E. coli* particles per neutrophil was calculated from ten randomly selected Ly6G⁺ cells.

Western blot analysis

Neutrophils were rinsed twice with ice-cold PBS. Cell lysis was carried out using RIPA buffer containing protease and phosphatase inhibitor cocktails (Applygen Technologies Inc., Beijing, China). The resulting lysates were mixed with 3 $\times$ SDS loading buffer, subjected to heat denaturation, and then loaded onto 8% SDS-PAGE gels. Proteins (25 μ g per lane) were separated electrophoretically with Tris-glycine running buffer (Bioss, Beijing, China) and subsequently transferred onto nitrocellulose membranes. The membranes were incubated sequentially with the following primary antibodies from Cell Signaling Technology (Danvers, USA): Phospho-p38 MAPK Rabbit mAb (Cat. No. #4511) at 1:1000, p38 MAPK Rabbit mAb (Cat. No. #8690) at 1:1000, Phospho-Erk1/2 Rabbit mAb (Cat. No. #4370) at 1:2000, Erk1/2 Rabbit mAb (Cat. No. #4695) at 1:1000, Phospho-JNK Mouse mAb (Cat. No. #9255) at 1:1000, JNK Rabbit mAb (Cat. No. #9252) at 1:1000, and GAPDH Rabbit mAb (Cat. No. #2118) at 1:1000. This was followed by incubation with HRP-conjugated anti-rabbit IgG (Cat. No. #7074) or anti-mouse IgG (Cat. No. #7076) secondary antibodies (Cell Signaling Technology) at a dilution of 1:5,000, as appropriate. Detection was performed with enhanced chemiluminescence (ECL) substrate (Millipore, Billerica, USA) via a FluorChem FC3 imaging system (ProteinSimple, Santa Clara, USA), and band intensities were quantified with AlphaView software (version 3.4.0). The levels of phosphorylated p38 were normalized to those of total p38 protein and are expressed as a percentage of the control values.

Bioluminescence imaging

The mice were anesthetized through sustained isoflurane (Abbott Laboratories, Chicago, USA) inhalation and oriented dorsally for image acquisition. Image capture was conducted on an IVIS 200 platform (Xenogen Corporation, Alameda, USA), which was set up for luminescence detection without an optical filter and with the aperture fixed at F/Stop 1. For every imaging session, L-012 was delivered intravenously at 25 μ g/g body weight in a solution of 20 μ g/ μ L in sterile PBS. The quantification of signals from user-defined regions (photon flux) was executed with Living Image

software (v.4.2, Xenogen Corporation) [25]. Statistical comparisons across time points were performed via two-way ANOVA supplemented with Bonferroni's post hoc analysis.

Quantification and visualization of NETs

The levels of circulating NET components, such as citrullinated histone 3 (CitH3), MPO, and DNA, were quantified via established CitH3-DNA and MPO-DNA complex ELISA according to a previously reported method [26]. Briefly, 96-well plates pre-coated with capture antibodies (anti-CitH3 or anti-MPO, Abcam, Beijing, China) were blocked, incubated with serum samples and subsequently incubated with peroxidase-conjugated anti-DNA detection antibodies. After tetramethylbenzidine substrate development, the absorbance was measured at 450 nm. NET levels are expressed as the fold change relative to control samples.

For NET visualization, lung tissue sections were subjected to immunostaining with specific primary antibodies and incubated overnight at 4°C. Afterward, the sections were treated with FITC-conjugated secondary antibodies (Abcam, Beijing, China) for 1 h at room temperature. The nuclei were labeled with DAPI-containing mounting medium (Agilent Technologies, Santa Clara, USA), and fluorescence images were obtained via microscopy. Both the DAPI and FITC signals from the same tissue regions were collected for colocalization analysis.

Determination of superoxide release

Extracellular ROS generation by neutrophils was assessed via the reduction of ferricytochrome c. Neutrophils (10^6 cells/mL) were incubated with 0.5 mg/mL ferricytochrome c (Sigma-Aldrich) and 1 mM Ca^{2+} at 37°C, pretreated with LPS (10 µg/mL, Thermo Fisher Scientific) for 3 min and cytochalasin B (1 µg/mL, Sigma-Aldrich) for 1 min, and then stimulated with varying concentrations of FAM19A4 (1, 10, and 100 ng/mL) for 5 min. The absorbance changes were monitored continuously at 550 nm via a U-3010 spectrophotometer (Hitachi, Tokyo, Japan).

Total ROS release measurement

ROS were measured via luminol-enhanced chemiluminescence. Neutrophil suspensions (1×10^6 cells/mL) were first incubated with 35 µM luminol (Sigma-Aldrich) and 5 U/mL HRP (Cell Signaling Technology) for 5 min at 37°C and then stimulated with LPS (10 µg/mL) for another 3 min. The cells were subsequently exposed to FAM19A4 at concentrations of 1, 10, or 100 ng/mL for 8 min. Real-time chemiluminescence signals were recorded on a Tecan Infinite F200 Pro multimode microplate reader (Tecan Group, Männedorf, Switzerland).

Intracellular ROS production assay

Intracellular ROS levels were quantified with a commercial assay kit utilizing the fluorescent indicator DCFH-DA (Sigma-Aldrich). Peripheral blood mononuclear cells (PBMCs) were obtained from CLP model mice via density gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). In brief, blood samples were diluted 1:1 with PBS, carefully layered onto the density gradient medium, and centrifuged at 400 g for 20 min at room temperature. The PBMC band was harvested, washed twice with PBS, and resuspended in RPMI-1640 medium at a density of 1×10^6 cells/mL. The cells were subsequently loaded with 10 µM DCFH-DA and incubated for 30 min at 37°C. After incubation, the

unincorporated dye was removed by washing, and the cells were stimulated with cytochalasin B (CB; 0.5 µg/mL) for 2 min to potentiate ROS generation. The reaction was stopped by adding ice-cold PBS supplemented with 2% FBS and 1 mM EDTA. The samples were promptly analyzed on a BD FACSCanto II flow cytometer using a 488 nm excitation laser and a 530/30 nm emission filter. A total of 10,000 viable cells, gated on forward and side scatter properties, were acquired per sample. The mean fluorescence intensity (MFI) of DCF-positive cells was determined via FlowJo software.

Bulk RNA sequencing

Total RNA was isolated from human neutrophils with TRIzol reagent (Cat. No. 15596026; Thermo Fisher Scientific). RNA purity was assessed via a NanoPhotometer spectrophotometer (IMPLEN, Munich, Germany), and RNA integrity was evaluated via a bioanalyzer (Agilent). The DNBSEQ platform was employed for RNA sequencing and subsequent bioinformatics processing. Differentially expressed genes (DEGs) were screened on the basis of relative expression and fold change thresholds ($\log_2$ (fold change) ≥ 0.5 and $P < 0.05$) and selected for further analysis. Volcano plots, heatmaps, and KEGG pathway enrichment data were constructed via the online tool suite at <http://www.omicstudio.cn/tools>. Volcano plots were generated from statistically significant DEGs. Intersecting gene sets were identified, and candidate sequences were chosen according to the KEGG enrichment results. Heatmaps were generated to display the expression patterns of the DEGs. The raw RNA sequencing data have been deposited in the Sequence Read Archive (SRA) at NCBI under accession number: PRJNA1155657 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA1155657>).

Ethics approval and consent to participate

The animal experiments were conducted in accordance with the principles outlined in the Animal Experimentation Ethics Committee Guide for the Care and Use of Laboratory Animals and were approved by the Animal Experimentation Ethics Committee of Peking University Peoples' Hospital (No. 2019PHE030). Experimental protocols involving human data and tissue were approved by the Institution of Human Subjects Committee at Peking University People's Hospital (No. 2019PHB191-01). Informed written consent was obtained from all the subjects.

Data and statistical analysis

Figures were prepared, and statistical analyses were performed with GraphPad Prism software. Histopathological evaluations were carried out under blinded conditions via Definiens Tissue Studio. The mice were assigned to experimental groups according to genotype and randomized within cohorts matched by sex and age. Group sizes were determined by empirical assessment of variability within each model or assay system, with the number of mice per group maximized to reduce both type I and type II errors. Data with a normal distribution are expressed as the mean $\pm$ SD, and non-normally distributed data are expressed as the median $\pm$ interquartile range (IQR). Since all datasets in this study passed normality testing, results are reported as the mean $\pm$ SD to reflect experimental variance. The significance of differences was assessed via the following tests: the non-parametric log-rank test for survival data; for parametric data, the two-tailed unpaired Student's *t* test was used for comparisons between two groups; and one-way ANOVA

followed by Tukey's multiple comparisons test was used for comparisons among more than two groups. All the parametric data were confirmed to be normally distributed by the Shapiro-Wilk test as described above. Significance notations are "ns" for not significant, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. For gene enrichment analysis, statistical significance was evaluated via the Q value, which represents the false discovery rate-adjusted P value.

Results

FAM19A4 expression is elevated in patients with sepsis and in mice subjected to CLP

Under physiological conditions, FAM19A4 expression in peripheral tissues (liver, lung, kidney, and intestine) is minimal, with higher levels detected in the central nervous system [16]. GEO profile data (GDS2856/GPL570 probe 242348_at; GDS2430/GPL97 probe 242348_at) indicate that LPS induces the upregulation of FAM19A4 in monocytes/macrophages. *In vitro*, LPS stimulation promotes FAM19A4 secretion from M1-polarized macrophages and monocytes [18]. To characterize FAM19A4 expression during sepsis hyperinflammation, we enrolled 40 patients meeting the Sepsis-3.0 criteria (≤ 7 days post-onset) and 10 healthy volunteers as the control group. Serum and BALF samples from patients and volunteers were analyzed via ELISA, which revealed significant FAM19A4 elevation in patients with sepsis compared with healthy volunteers (Figure 1A). Analysis of 28-day mortality revealed lower FAM19A4 concentrations in survivors than those in non-survivors (Figure 1B). FAM19A4 levels are correlated with both inflammatory response intensity and mortality risk, but they are almost undetectable in healthy volunteers. ELISA was used to quantify the levels of IL-6, TNF- α , and IL-10, which serve as reference biomarkers for systemic inflammation [25].

FAM19A4 is an evolutionarily conserved protein, and the amino acid sequences of human FAM19A4 and its murine homolog are 93% identical [16]. Analysis of murine FAM19A4 mirrored our clinical findings. Mice with CLP-induced sepsis presented markedly elevated serum FAM19A4 levels (Figure 1C). However, FAM19A4 expression in the peritoneal lavage fluid did not significantly differ among the groups (Supplementary Figure S2D). Tissue-specific analysis 24 h post-CLP revealed increased *Fam19a4* mRNA in the liver/lung compared with negligible baseline levels (Figure 1D). Analysis of *Fam19a4*-EGFP KI mice (Supplementary Figure S1A,B) facilitated spatial resolution of *Fam19a4* expression patterns. *In situ* hybridization confirmed CLP-induced *Fam19a4* upregulation in hepatic/pulmonary tissues compared with minimal renal expression (Figure 1E and Supplementary Figure S1C).

FAM19A4 deficiency reduces mortality and attenuates organ injury severity in mice subjected to CLP

FAM19A4 modulates innate immune cell function during inflammatory responses [18]. To investigate the role of FAM19A4 in sepsis, *Fam19a4*^{-/-} mice and WT littermate controls were subjected to CLP-induced sepsis. Complementary intervention involved the intraperitoneal administration of recombinant FAM19A4 protein to WT mice at 1 and 3 h post-CLP to simulate hyperexpression. Compared with WT controls, genetic ablation of FAM19A4 significantly improved 7-day survival rates and attenuated weight loss. Exogenous FAM19A4 supplementation did not affect survival outcomes or weight trajectories (Figure 2A,B).

Liver, lung, and kidney tissue damage was assessed in mice at

24 h post-CLP by H&E staining and light microscopy (Figure 2C), with histopathological scoring of the damage (Figure 2D). Mice subjected to CLP presented marked pathological alterations across multiple organs, including focal necrosis in liver lobules, increased hepatocyte destruction, and exacerbated renal tubular epithelial cell damage. In contrast, compared with WT mice, *Fam19a4*^{-/-} mice subjected to CLP presented attenuated histological changes. To validate these findings, recombinant FAM19A4 protein was administered intraperitoneally to *Fam19a4*^{-/-} mice at 1 and 3 h post-CLP, which restored severe pathological damage and elevated mortality rates. Transmission electron microscopy of liver and lung tissues 24 h post-CLP revealed vacuolar degeneration and rupture of intracellular mitochondria, the endoplasmic reticulum, and other organelles in WT mice, whereas *Fam19a4*^{-/-} mice presented relatively mild ultrastructural lesions (Supplementary Figure S2A). These findings suggest that FAM19A4 deficiency may mitigate sepsis severity and enhance clinical outcomes.

To investigate whether FAM19A4 directly modulates neutrophil function, thereby influencing the recruitment of these cells to primary migration sites and the subsequent control of the microbial load, we analyzed changes in peripheral blood neutrophil counts and the peritoneal bacterial burden in mice after manipulation of FAM19A4 expression. Compared with WT mice, *Fam19a4*^{-/-} mice presented no significant increase in peritoneal bacterial colony counts; however, intraperitoneal recombinant FAM19A4 (rmFAM19A4) protein administration significantly reduced peritoneal bacterial colonization in both WT and *Fam19a4*^{-/-} mice (Supplementary Figure S2B). No significant differences in peripheral blood neutrophil counts were detected among the groups (Supplementary Figure S2C).

FAM19A4 deficiency decreases neutrophil and macrophage numbers in organs and reduces ROS and NET formation in mice subjected to CLP

Activation of the innate immune system during the early phase of sepsis is critical for bacterial clearance; however, the accumulation of inflammatory cells, such as neutrophils and macrophages, in non-infected distant organs, along with their activation and release of cytotoxic substances, significantly contributes to organ damage and dysfunction [26]. Liver and lung tissues were harvested from *Fam19a4*^{-/-} mice 24 h post-CLP. To quantify the number of cells, immunohistochemistry was performed using Ly6G⁺-labeled neutrophils and F4/80⁺-labeled macrophages. Neutrophil and macrophage numbers were significantly lower in liver and lung tissues from *Fam19a4*^{-/-} mice than in those from WT mice (Figure 3A,B).

To investigate the effect of FAM19A4 on ROS levels in sepsis, L-012 (a luminol analog) was used to quantify systemic ROS production in mice subjected to CLP. At 24 h post-CLP, the luminescence intensity was >8 -fold greater in WT mice than in control mice, with pronounced abdominal signal localization. This response was markedly attenuated in *Fam19a4*^{-/-} mice. Furthermore, intraperitoneal administration of the rmFAM19A4 protein to WT mice at 1 and 3 h post-CLP (followed by L-012 labeling) resulted in increased ROS release compared with that in WT+CLP mice and restored peritoneal ROS levels in *Fam19a4*^{-/-}+CLP mice (Figure 3C,D).

ROS contribute critically to NET formation [27]. Dysregulated NET formation may cause cellular damage in adjacent tissues, thereby contributing to acute organ injury [12]. We next measured the extent of NET formation in both serum and organ tissue samples

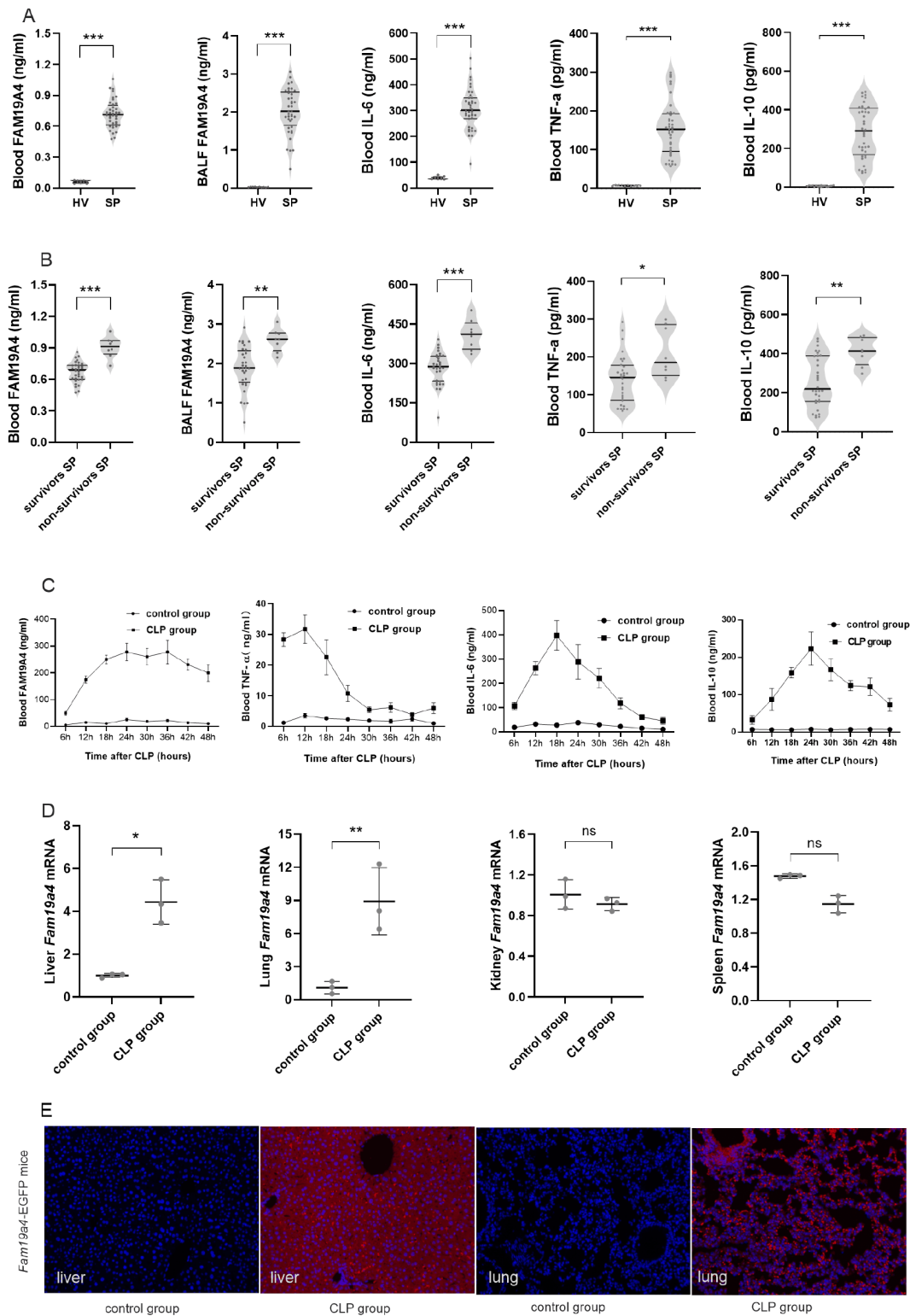


Figure 1. FAM19A4 expression is elevated in patients with sepsis and in mice subjected to CLP (A–B) FAM19A4 levels in serum and BALF samples from septic patients. The serum levels of IL-6, TNF- α , and IL-10 were used as reference markers of the inflammatory response. (A) Patients with sepsis (SP, $n = 40$) vs. healthy volunteers (HV, $n = 10$); (B) Survivor SP ($n = 32$) vs. non-survivor SP ($n = 8$). (C) FAM19A4, IL-6, TNF- α and IL-10 levels in mouse serum 48 h after CLP. (D) *Fam19a4* expression in the liver, lung, kidney and spleen of mice 24 h after CLP. (E) *Fam19a4*-EGFP knock-in (KI) mice were used for CLP modeling. *Fam19a4* expression in the liver and lung was detected via *in situ* hybridization (200 $\times$). Blue dots, cell nuclei; red dots, *Fam19a4*. The results from one representative experiment out of three independent replicates are shown. The data in A–D are presented as the mean $\pm$ SD and were analyzed via unpaired Student's *t* tests. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

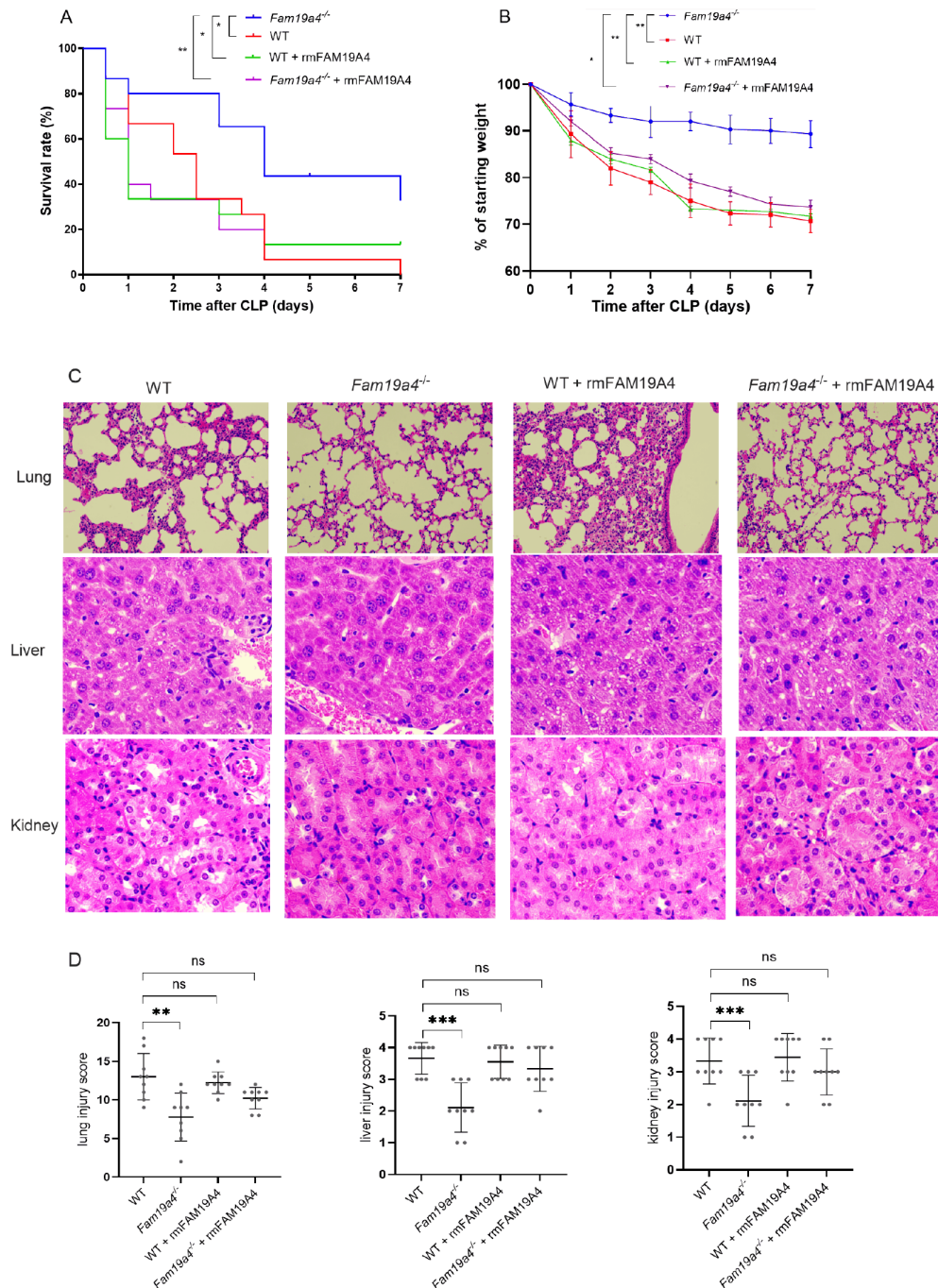


Figure 2. FAM19A4 deficiency reduces mortality and attenuates organ injury severity in mice subjected to CLP (A) Survival rate and (B) body weight change (relative to initial weight) were monitored over time after CLP. (C) Representative H&E-stained sections of lung, liver, and kidney tissues 24 h after CLP (200 $\times$). (D) Pathological injury scores of the lungs, liver, and kidney for each group. Experimental groups: WT: WT mice subjected to CLP; *Fam19a4*^{-/-}: *Fam19a4*^{-/-} mice subjected to CLP; WT+rmFAM19A4: WT mice with CLP receiving intraperitoneal injection of rmFAM19A4 (100 μ g/mouse) at 1 and 3 h after CLP; *Fam19a4*^{-/-}+rmFAM19A4: *Fam19a4*^{-/-} mice receiving rmFAM19A4 as above; $n = 3-6$ per group. The data in B and D are presented as the mean $\pm$ SD. The image in (C) is representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; log-rank test (A), two-way analysis of variance (ANOVA) (B), or Student's t test (D).

obtained from mice subjected to CLP using CitH3-DNA and MPO-DNA complexes as key markers of circulating NETs. Exogenous administration of FAM19A4 by intraperitoneal injection led to substantial increases in the serum levels of CitH3-DNA and MPO-DNA. In contrast, these biomarkers were significantly decreased in

Fam19a4^{-/-} mice (Figure 3E). Next, we conducted immunofluorescence staining to assess the role of FAM19A4 in NET formation within the lungs. Significantly fewer NETs (CitH3⁺ structures) were observed in *Fam19a4*^{-/-} mice than in WT mice at 24 h post-CLP (Figure 3F). Collectively, these data demonstrate that FAM19A4

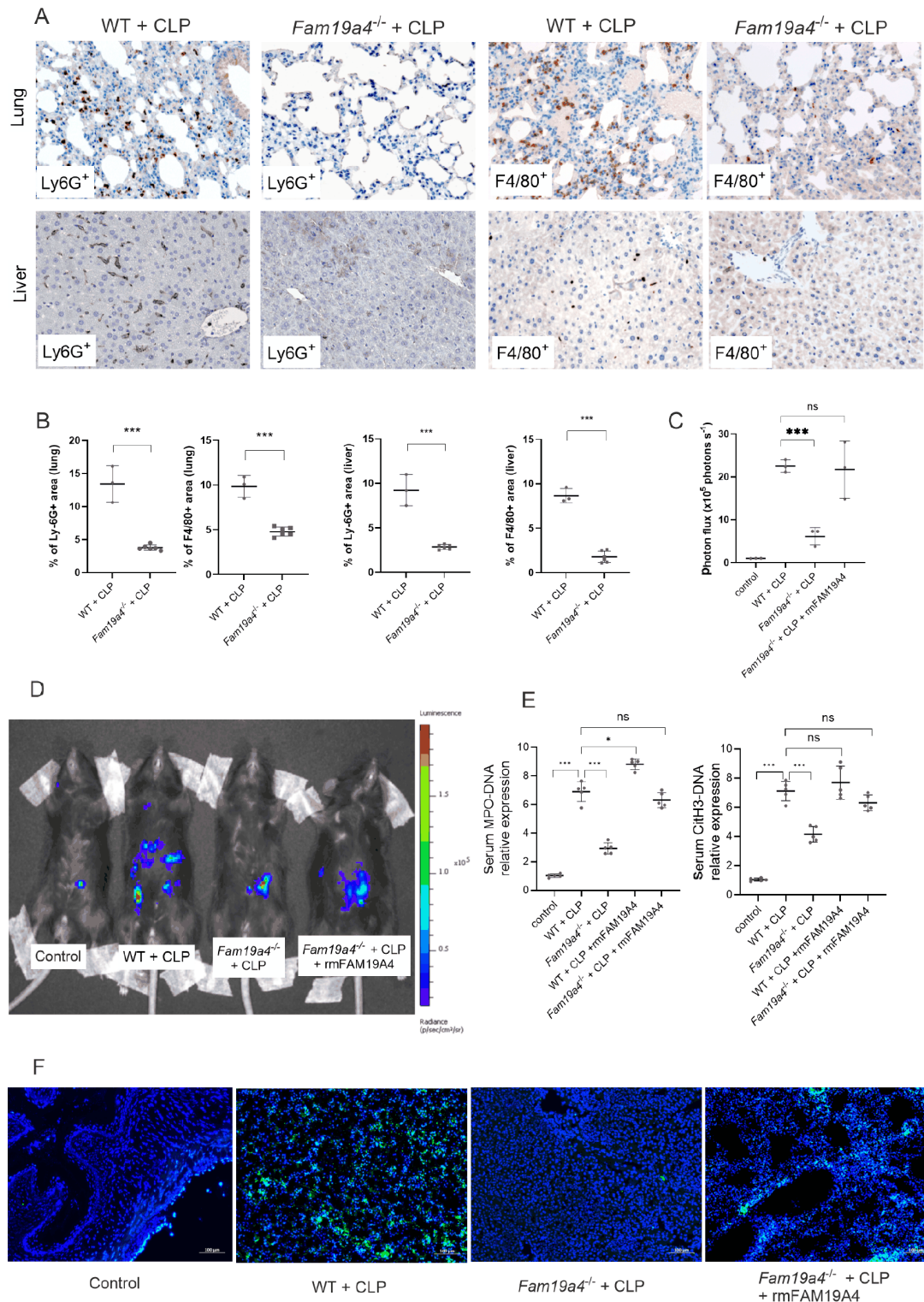


Figure 3. FAM19A4 deficiency decreases neutrophil and macrophage numbers in organs and reduces ROS and NET formation in mice subjected to CLP (A) Representative immunohistochemical images of lung and liver sections stained for neutrophils (Ly-6G) and macrophages (F4/80) from wild-type (WT) and *Fam19a4*^{-/-} mice 24 h after CLP (200×). (B) Quantification of the percentages of Ly6G⁺ and F4/80⁺ areas in tissues (WT, *n* = 3; *Fam19a4*^{-/-}, *n* = 6). (C) Average luminescence intensity in mice injected with L-012 24 h after CLP (*n* = 3 per group). (D) Representative bioluminescence images of ROS production. The pseudocolors represent photons/s/cm²/sr. (E) Circulating NET biomarkers (MPO-DNA and CitH3-DNA complexes) were measured via ELISA. (F) Immunofluorescence imaging of NETs (CitH3, green) in lung tissue 24 h after CLP. Scale bar, 100 μm. The data in B, C, and E are presented as the mean ± SD; **P* < 0.05, ***P* < 0.01, ****P* < 0.001; Student's *t* test (B, C) or two-way ANOVA (E). The images in A, D, and F are from one representative experiment out of three independent replicates.

deficiency attenuates NET formation in the serum and lung tissue of mice subjected to CLP.

FAM19A4 enhances neutrophil phagocytosis and ROS release *in vitro*

Neutrophils are immune cells that are primarily responsible for ROS release during the early phase of sepsis [28]. To directly assess the functional impact of FAM19A4, LPS-pre-stimulated human neutrophils were treated with rmFAM19A4 *in vitro*, after which chemotaxis, phagocytosis, and ROS release were evaluated. FAM19A4 had no significant chemotactic effect on neutrophils, regardless of LPS pre-stimulation (Supplementary Figure S3). Confocal microscopy revealed that rmFAM19A4 enhanced the phagocytic capacity of neutrophils toward *E. coli*, with a more pronounced effect observed in LPS-pre-stimulated cells (Figure 4A–D). However, blocking formyl peptide receptor 1 (FPR1) did not influence the ability of rmFAM19A4 to promote phagocytic function (Supplementary Figure S4).

Neutrophils remain quiescent in peripheral blood, but bacterial components can prime their transition to an activation-ready state [29]. Differential ROS production across neutrophil activation states may contribute to dysregulated inflammatory responses in sepsis [30]. Neutrophils were treated with graded concentrations of FAM19A4 in experimental groups, including LPS- and PBS-prestimulated groups. Extracellular superoxide anion ($O_2^{\cdot-}$) generation was quantified via a superoxide dismutase-inhibitable ferricytochrome C reductase assay, while total ROS levels were measured via luminol-enhanced chemiluminescence. In resting neutrophils (without LPS induction), low FAM19A4 concentrations had a minimal effect on ROS release, whereas high concentrations significantly induced ROS production. Furthermore, LPS-prestimulated neutrophils exhibited increased ROS release, even at low FAM19A4 concentrations (Figure 4D,E).

p38 MAPK signaling is critical for FAM19A4-induced ROS in neutrophils

To elucidate the intracellular signaling pathways mediating FAM19A4 activity in human peripheral neutrophils, we performed bulk RNA sequencing (RNA-seq) to identify alterations in the transcription levels of FAM19A4 target genes. The transcriptome profiles of PBS-treated controls and neutrophils exposed to graded concentrations of FAM19A4 for 2 h were compared. The differential gene expression profile, including both upregulated and downregulated transcripts in FAM19A4-treated neutrophils versus controls, is presented in Figure 5A. KEGG pathway analysis revealed marked enrichment of the MAPK signaling pathway following FAM19A4 exposure (Figure 5B), with heatmap visualization highlighting the 20 most significantly regulated transcription factors (Figure 5C). Pseudogenes and unclassified genes were systematically excluded from the analysis.

Guided by these findings and literature, we prioritized three MAPK subpathways (ERK1/2, p38 MAPK, and JNK) for functional validation. Using human peripheral neutrophils, we assessed signaling pathway activation after FAM19A4 stimulation via western blot analysis. This approach revealed a significant increase in p38 phosphorylation following FAM19A4 treatment (Figure 5D and Supplementary Figure S5). To establish functional causality, we compared FAM19A4-treated neutrophils with those co-treated with the specific p38 inhibitor SB 203580 [30]. Compared with no

treatment, pharmacological inhibition of p38 substantially and significantly attenuated FAM19A4-induced ROS production (Figure 5E,F). We also intraperitoneally injected SB203580 to inhibit p38 MAPK function in the CLP model in WT mice, and ROS release in the PBMCs of these mice was significantly reduced (Figure 5G). Collectively, these data indicate that p38 MAPK signaling constitutes a key mechanistic pathway through which FAM19A4 potentiates neutrophil ROS release.

Moreover, clustering analysis further revealed an increase in Src kinase expression following FAM19A4 treatment (Figure 5C). According to a recent report [31], ICAM-1 facilitates the release of ROS and NETs under septic conditions. Upon binding to LFA-1 (CD11a/CD18), ICAM-1 activates Src kinase and downstream signaling mediators, leading to p38 MAPK activation. To determine whether FAM19A4-induced phagocytosis is mediated through the ICAM-1 pathway, a cell-permeable penetrating-conjugated ICAM-1 C-terminal peptide (RQIKIWFQNRRMKWKKQRKIKKYRLQQAQ; Peptide 2.0) and a scrambled control peptide (RQIKIWFQNRRMKWKKVDDSDDFESVSV; Peptide 2.0) were synthesized via previously established methods [32]. These peptides were applied to whole blood samples to inhibit downstream ICAM-1 intracellular signaling. No significant difference in phagocytosis was observed between the two groups (data not shown), suggesting that ICAM-1 is not involved in FAM19A4-mediated neutrophil phagocytosis.

Discussion

In this study, we revealed that the secretory protein FAM19A4, whose level is elevated during the early innate immune response phase of sepsis, contributes to the injury of remote organs, such as the liver and lung, and is associated with poor prognosis. Under physiological conditions, FAM19A4 is negligibly expressed in most organs except those of the nervous system [16]. Recent studies have revealed its roles in inflammatory responses [19,31,33]. Wang *et al.* [18] reported that FAM19A4 is a macrophage-derived secretory protein that induces ROS production, and we hypothesized that it could play a critical role in acute infectious inflammation. Data from both clinical samples and CLP-induced mouse models confirmed that FAM19A4 is elevated in early sepsis, paralleling the increase in other cytokines, such as IL-6, TNF- α , and IL-10. This temporal association suggests that FAM19A4 may contribute to the disruption of early immune homeostasis in sepsis.

Our study demonstrated that genetic ablation of FAM19A4 improved survival and ameliorated multi-organ damage in a CLP-induced sepsis model. Although FAM19A4 deficiency did not affect peripheral neutrophil counts or direct neutrophil chemotaxis *in vitro*, it significantly reduced neutrophil and macrophage counts in remote organs (liver and lung), suppressed systemic ROS production, and inhibited NET formation. Neutrophil-derived ROS represent one of the primary mechanisms for bacterial clearance. Accordingly, the intraperitoneal administration of rmFAM19A4 to CLP mice, thereby increasing its local concentration, significantly reduced the bacterial load in the peritoneal cavity. Notably, however, endogenous FAM19A4 expression was not markedly elevated in the peritoneal lavage fluid of CLP mice, and its genetic deletion did not compromise bacterial clearance at the primary infection site. These results suggest that FAM19A4 primarily exacerbates detrimental aspects of neutrophil activity in sepsis, such as excessive ROS and NET-mediated tissue injury, rather than

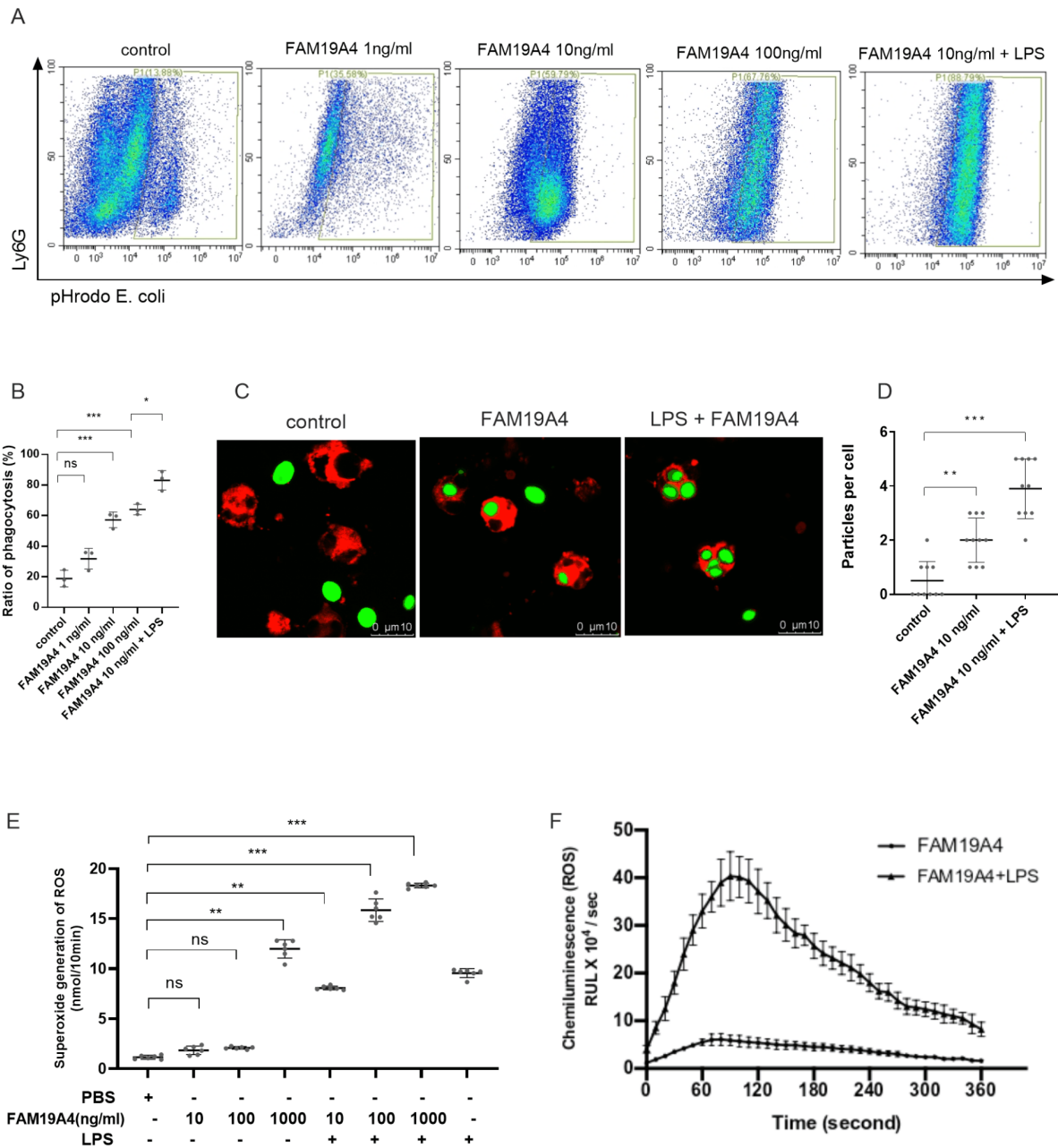


Figure 4. FAM19A4 enhances neutrophil phagocytosis and ROS release *in vitro* (A, B) Neutrophils were treated with different concentrations of the FAM19A4 protein, with or without LPS, and then incubated with pHrodo *E. coli* bioparticles. Phagocytosis was analyzed by flow cytometry. (A) Representative flow cytometry dot plots from three independent experiments. (B) Quantification of phagocytosis from pooled flow cytometry data. (C,D) Phagocytosis of pHrodo *E. coli* particles was observed via confocal microscopy. (C) Representative images. (D) Quantification of the number of phagocytosed particles per neutrophil. (E) Superoxide release from neutrophils (1×10^6 cells/mL), pre-incubated with PBS or LPS and then stimulated with FAM19A4 (1 to 100 ng/mL), was measured by ferricytochrome c reduction. (F) Total ROS production was detected via the luminol-peroxidase system in neutrophils treated with LPS or PBS, followed by DMSO or FAM19A4 (100 ng/mL). Data in B, D, E, and F are presented as the mean $\pm$ SD; ** $P < 0.01$, *** $P < 0.001$; two-way ANOVA (B) or Student's *t* test (D, E, and F).

impairing their essential antibacterial role [10]. The spatial heterogeneity in FAM19A4 expression, together with its specific impact on ROS and NET generation in distant organs, highlights the therapeutic potential of targeting FAM19A4 to reduce tissue damage without weakening host defense.

The organ-specific neutrophil patterns likely arise from complex inflammatory networks involving both infiltrating and resident immune cells and may also reflect the contextual regulation of

FAM19A4 signaling within different tissue microenvironments. Overall, FAM19A4 modulates neutrophil effector functions rather than recruitment. Future studies using single-cell sequencing could further clarify neutrophil subpopulations and their functional specialization in FAM19A4-mediated organ protection, particularly given emerging insights into resident neutrophil heterogeneity in sepsis pathophysiology.

FAM19A4 induces dose-dependent release of ROS from neutro-

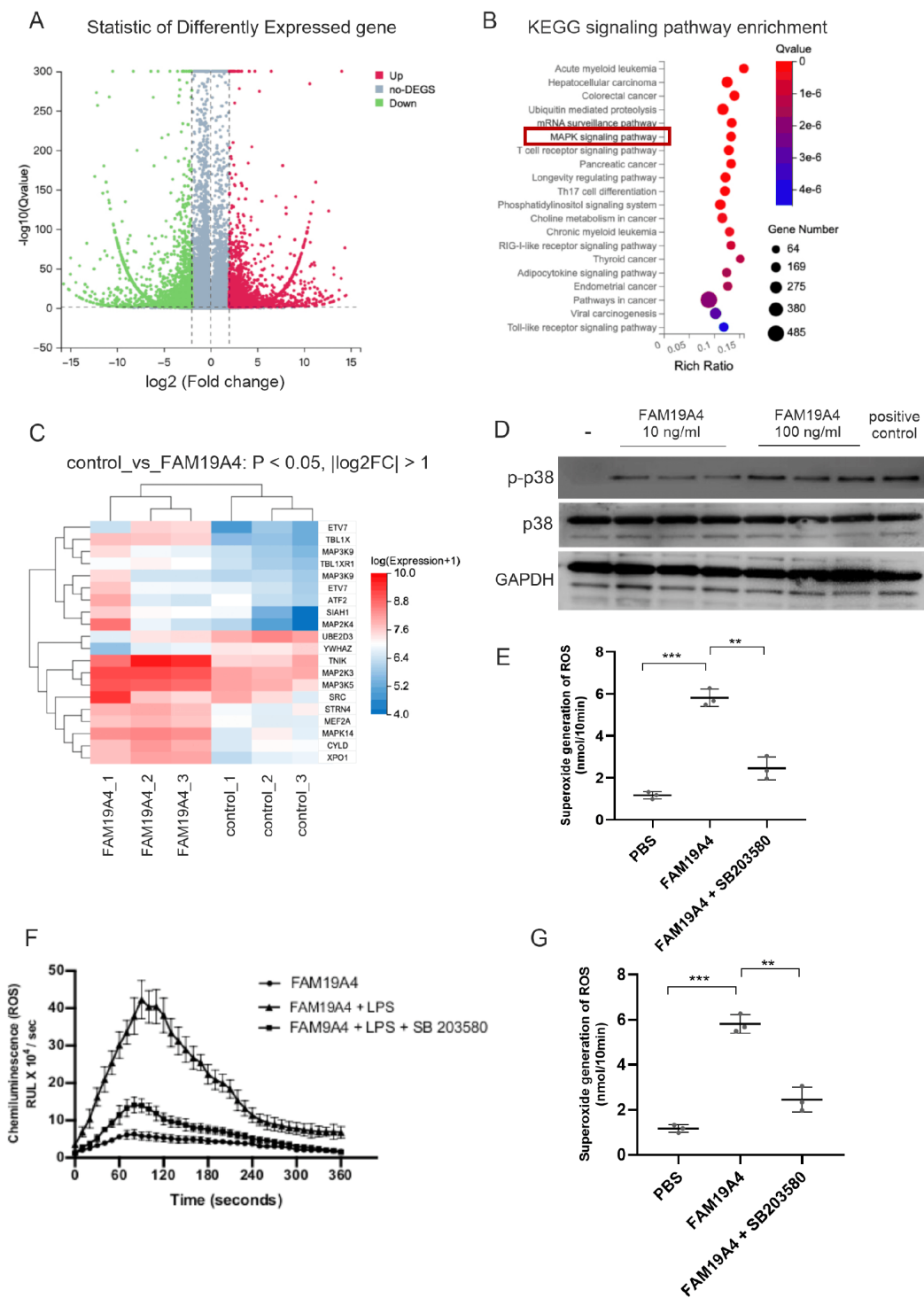


Figure 5. p38 MAPK signaling is critical for FAM19A4-induced ROS in neutrophils (A) Volcano plot of differentially expressed genes in neutrophils treated with rmFAM19A4 (100 ng/mL, 2 h) versus the PBS control. Red and green dots indicate up- and down-regulated genes, respectively. (B) KEGG enrichment analysis. The bubble size reflects the number of genes per pathway; the color indicates the Q value. (C) Heatmap of DEGs clustered by expression level (red: higher). (D) Western blots of p-p38 in isolated neutrophils stimulated with rmFAM19A4 (10–100 ng/mL); GAPDH served as a loading control. The image is representative of three independent experiments. (E) Superoxide release detected by ferricytochrome c reduction after pre-treatment with PBS or SB203580 (10 μ M). (F) Total ROS levels were measured via a luminol-peroxidase system in neutrophils pre-treated with PBS, SB203580, or LPS. (G) Intracellular ROS levels (DCFH-DA MFI) in PBMCs from mice under four conditions: WT control, WT+CLP, Fam19a4^{-/-}+CLP, and WT+CLP+SB203580. The data in E–G are presented as the mean $\pm$ SD; $n = 3$ –5; ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (Student's t test).

phils: high concentrations directly trigger ROS production, whereas subthreshold levels synergistically increase LPS-primed ROS generation through increased NADPH oxidase activation [6,29,34]. This priming phenomenon aligns with the presence of primed neutrophil subpopulations in septic patients [35]. Thus, sepsis-induced elevation of FAM19A4 in the blood and organs likely exacerbates oxidative stress in pre-activated neutrophils. We also observed increased neutrophil extracellular trap (NET) formation, collectively promoting multiple organ dysfunction. ROS are key inducers of NETosis, in which PAD4 mediates chromatin decondensation via histone citrullination. Since most cytokines influence NET formation via PAD4, we hypothesize that FAM19A4 may also affect NETosis in a PAD4-dependent manner, warranting further mechanistic study.

The p38 MAPK pathway was identified as a key mediator of FAM19A4-induced ROS release, which is consistent with its established role in balancing antimicrobial responses and tissue injury in sepsis [36,37]. However, given the complex network-based regulatory mechanisms inherent to sepsis and in light of our results and those of previous studies, we suggest that the effects of FAM19A4 involve multifaceted network regulation beyond single-pathway modulation. Our bulk RNA-seq data revealed increased expression of Src kinase. Previous studies reported that FAM19A4 may promote macrophage chemotaxis and phagocytosis via FPR1 [18] and that activated FPR1 can directly and atypically activate Src kinase, thereby promoting neutrophil phagocytosis and ROS release. However, our data revealed that rmFAM19A4 enhanced neutrophil phagocytosis independently of FPR1 and had no effect on neutrophil chemotaxis. Consistent with these findings, Hoeffel *et al.* [19] reported that FAM19A4 had no chemotactic effects on mouse bone marrow-derived or peritoneal macrophages *in vitro*, suggesting that it regulates macrophage inflammatory profiles in an FPR1-independent manner.

Additionally, p38 MAPK acts as a downstream signaling component of ICAM-1, which activates Src kinase and subsequently triggers p38 MAPK activation. Nevertheless, prior studies have indicated that ICAM-1 intracellular signaling is not essential for *Staphylococcus aureus* phagocytosis or ROS generation in human neutrophils [32]. We also found that the FAM19A4-induced increase in phagocytosis is not dependent on ICAM-1. Furthermore, ICAM-1 signaling is not critical for these neutrophil functions. More in-depth studies are warranted to elucidate the molecular mechanisms by which FAM19A4 plays a regulatory role in sepsis.

We observed that FAM19A4 selectively activated p38 MAPK without significantly affecting the JNK or ERK pathway. This selectivity is likely due to differences in upstream activation mechanisms, receptor preferences, and feedback regulation among MAPK family members. p38 MAPK is highly sensitive to stress and inflammatory stimuli (such as cytokines and ROS) and is primarily activated through the MKK3/MKK6 cascade [38,39]. In contrast, JNK and ERK are more responsive to growth factors and differentiation signals, respectively [40,41]. Structural features of p38, such as the TGY motif within its TxY activation loop, may facilitate its preferential phosphorylation by Src family kinases, which is consistent with our observation of upregulated Src expression [42]. Thus, the functional phenotype induced by FAM19A4 may reflect the assembly of specific receptor-signal complexes that precisely regulate the p38 pathway to coordinate ROS release and NET formation in neutrophils. The exact under-

lying mechanisms require further investigation.

In clinical practice, sepsis patients present at various stages due to differences in the interval between symptom onset and hospital admission. This study focused on early-phase sepsis, restricting enrollment to patients with a confirmed diagnosis within 7 days post-onset sepsis on the basis of retrospective medical history. Consequently, an ideal dynamic blood sampling protocol (e.g., days 1, 3, 7, 14 and 28) was not feasible. The clinical cohort was relatively small (40 patients and 10 controls), and the clinical heterogeneity among sepsis patients may limit the generalizability of the relationship between FAM19A4 levels and clinical outcomes. Future studies should include larger populations to validate these findings and further explore clinical correlations.

Our results demonstrate that the p38 MAPK inhibitor SB203580 can inhibit FAM19A4-induced ROS production, suggesting its potential therapeutic utility in sepsis. However, it has been reported that the effective dose of SB203580 is close to its toxic level in models of other diseases, indicating a narrow therapeutic window [43]. Moreover, the p38 MAPK pathway can be activated by multiple cytokines and membrane proteins in sepsis and represents a common node in the networked immune regulation of sepsis [44]. The overall consequences of SB203580-induced inhibition within this complex regulatory network remain unclear. Therefore, further studies are warranted to evaluate the therapeutic potential of SB203580.

Our findings establish FAM19A4 as a key mediator in sepsis pathogenesis. Its effects appear to be closely integrated within the broader cytokine network, suggesting a complex role in the inflammatory cascade. Further studies—directly comparing FAM19A4 with other inflammatory mediators and utilizing multi-cytokine blockade models—are warranted to elucidate its unique versus synergistic functions and to evaluate its potential as a therapeutic target in sepsis.

In summary, this study revealed that elevated levels of the cytokine FAM19A4 exacerbate organ damage in sepsis, while its deficiency reduces mortality and tissue injury by diminishing neutrophil numbers in remote organs, thereby suppressing ROS formation. Mechanistically, FAM19A4 directly enhances neutrophil ROS production via p38 MAPK signaling without affecting chemotaxis or bacterial clearance. These findings identify FAM19A4 as a therapeutic target and advance the understanding of dysregulated immune networks in sepsis.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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