

Original Article

CX3CR1 promoter methylation impairs immune cell migration and exacerbates bacterial pneumonia

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Abstract

Severe pneumonia remains a major contributor to infection-related deaths globally, often driven by inadequate pathogen clearance. Impaired immune cell trafficking to infected sites critically contributes to this outcome, yet the underlying regulatory mechanisms remain incompletely understood. Here, we analyze peripheral blood transcriptomic data from a public cohort of 184 pneumonia patients to identify prognosis-related genes, followed by prospective enrollment of 38 patients for integrated multi-omics profiling. Low CX3CR1 expression is associated with 28-day mortality and correlates with hypermethylation at the promoter CpG site cg00262061. Mechanistically, this methylation modification impedes RNA polymerase II recruitment to the CX3CR1 promoter, thereby suppressing transcription. CRISPR-dCas9-based targeted methylation or demethylation at cg00262061 in primary human CD14⁺ monocytes directly manipulates endogenous CX3CR1 expression, establishing causality. Notably, cg00262061 hypermethylation is detected exclusively in the CX3CR1⁻ subset of patient peripheral blood mononuclear cells, indicating selective epigenetic silencing. Functionally, this methylation or direct CX3CR1 knockdown markedly impairs monocyte and T cell migration. In a murine bacterial pneumonia model, systemic CX3CR1 inhibition reduces pulmonary immune cell infiltration, increases bacterial burden, exacerbates lung injury, and decreases survival. Competitive adoptive transfer confirms a cell-intrinsic migration defect of CX3CR1-deficient immune cells. These findings delineate a pathogenic pathway wherein promoter hypermethylation silences CX3CR1, compromises immune cell homing, and aggravates disease severity, offering a mechanistic basis for prognostic assessment and a conceptual framework for host-directed therapies, although clinical translation warrants further investigation.

Key words CX3CR1, DNA methylation, pneumonia, immune cell migration

Introduction

Pneumonia ranks among the most frequently encountered infectious diseases worldwide, carrying a significant clinical burden. Epidemiological data indicate that pneumonia accounts for 15%–45% of all hospital admissions [1]. Notably, severe pneumonia, characterized by rapid progression and poor prognosis, carries a mortality rate of 25%–45% [2] and remains a leading cause of in-hospital death. Deciphering the pivotal molecular mechanisms that determine pneumonia outcomes holds critical value for devising novel targeted treatments and enhancing patient survival.

A finely tuned immune response is essential during pneumonia development. Although timely immune cell recruitment is vital for eliminating pathogens, excessive and sustained inflammation may

inflict collateral tissue injury [3,4]. Although advances in anti-inflammatory therapies (e.g., corticosteroids) and supportive care have improved the management of the acute hyperinflammatory phase for many patients [5,6], a subset subsequently enters a state of immune dysfunction. This failure to clear pathogens and repair damaged tissue stems primarily from reduced trafficking of immune cells to infection sites. This defect is considered a pivotal factor contributing to mortality in severe pneumonia [7]. Therefore, deciphering the key mechanisms governing immune cell migration is a crucial direction for addressing current therapeutic limitations.

Epigenetic mechanisms, especially DNA methylation, govern gene transcription and participate in immune cell differentiation, inflammatory processes, and the establishment of immunotolerance

[8–10]. Emerging evidence suggests that the functional remodeling of immune cells post-infection may be dynamically regulated by epigenetic mechanisms. However, which specific methylation sites drive defective immune cell trafficking in pneumonia and their downstream targets have yet to be fully elucidated. Addressing this gap is essential for advancing our understanding of pneumonia immunopathology from an epigenetic perspective and for guiding the development of targeted therapeutic strategies.

Here, we sought to identify epigenetic determinants of pneumonia outcomes, with a focus on immune cell migratory capacity. Initial screening of public transcriptomic datasets revealed CX3CR1 as a prognostic marker for 28-day mortality, with markedly lower expression in non-survivors. Subsequently, by integrating multi-omics data from our patient cohort with molecular, cellular, and animal experiments, we investigated the epigenetic basis of CX3CR1 downregulation and its functional impact. We show that hypermethylation at select promoter CpG sites, notably cg00262061, critically suppresses *CX3CR1* transcription. This epigenetic modification hinders RNA polymerase II recruitment, downregulates CX3CR1 and subsequently impairs immune cell homing to the lungs, which compromises bacterial clearance and exacerbates disease severity. Our work uncovers a previously unrecognized pathway underlying immune dysfunction in pneumonia and points to the CX3CR1 epigenetic axis as a potential therapeutic target.

Materials and Methods

Clinical samples

This study was approved by the Research Ethics Committee of Jinan Central Hospital (approval No. SZR2024-008). Written informed consent was obtained from all participants or their legal guardians. We prospectively enrolled 38 patients diagnosed with pneumonia between January 2025 and December 2025. Diagnosis was made according to established guidelines for adult community-acquired pneumonia [1]. The inclusion criteria were as follows: age $\geq$ 18 years, meeting the diagnostic criteria for pneumonia, and completion of peripheral blood collection within 48 h of admission. Demographic and baseline clinical information was recorded for all patients.

Isolation, sorting, and culture of human primary immune cells

Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation using Polymorphprep (Serumwerk, Bernburg, Germany). CD14⁺ monocytes were enriched via 2-h adherence in RPMI-1640 (Gibco, Grand Island, USA) containing 10% fetal bovine serum (FBS; Procell, Wuhan, China). CD3⁺ T cells were positively selected using a commercial immunomagnetic bead kit (130-097-043; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Flow cytometry confirmed > 95% purity for both sorted populations.

Cells were cultured in RPMI-1640 medium supplemented with 10% FBS at 37°C in a 5% CO₂ incubator. For *in vitro* stimulation, cells were treated with 500 ng/mL LPS (Sigma-Aldrich, St Louis, USA) for 12 h. This concentration was selected based on cell viability assays (Supplementary Figure S1), which confirmed that 500 ng/mL LPS maintains > 90% cell viability in both CD14⁺ monocytes and CD3⁺ T cells under the experimental conditions. For functional assays, patient-derived cells were stratified into methylated or unmethylated groups based on the cg00262061 locus

methylation status.

DNA methylation microarray and transcriptomic microarray

Total DNA and total RNA were extracted in parallel from patient PBMCs using the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany). Genome-wide DNA methylation was profiled using the Infinium HumanMethylation450 BeadChip (Illumina, San Diego, USA). RNA expression was analyzed using the Arraystar Human mRNA Microarray V4.0 (Arraystar, Rockville, USA).

All microarray data underwent standardization and quality control, and gene expression matrix files were generated for subsequent analysis. External validation data were sourced from the GEO public database (GSE65682), which contains transcriptomic data and 28-day all-cause mortality information for 184 pneumonia patients, along with transcriptomic data from 42 healthy volunteers.

Mouse model of pneumonia

All animal procedures were approved by the Laboratory Animal Welfare and Ethics Committee of Jinan Central Hospital (approval No. JNCH2024-015) and were performed in compliance with laboratory animal care guidelines. Six- to eight-week-old, specific pathogen-free C57BL/6 mice (Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were used.

Based on our group's established protocols [11–13], a bacterial pneumonia model was induced via intratracheal instillation of *Klebsiella pneumoniae* (CMCC 46117; CMCC, Beijing, China; 6.0×10^8 CFU/kg) or *Staphylococcus aureus* (ATCC 12600; ATCC, Manassas, USA; 6.0×10^8 CFU/kg). Control (sham) animals were administered the same volume of sterile PBS as a vehicle. A total of 12 mice per group were used, with 6 mice allocated for survival analysis and the remaining 6 euthanized on day 5 post-infection for lung tissue collection and endpoint analysis.

Bronchoalveolar lavage fluid (BALF) collection

Following euthanasia, the trachea was cannulated and the lungs were lavaged three times with 0.8 mL of ice-cold sterile PBS per wash. The pooled BALF was centrifuged at 300 g for 10 min at 4°C. The supernatant was collected and stored at –80°C for subsequent cytokine measurement, and the cell pellet was resuspended in PBS containing 1% bovine serum albumin (BSA) for immediate flow cytometry analysis.

Lung histopathological analysis

Lung tissue sections were stained with H&E for histopathological evaluation. Under a light microscope (Olympus, Tokyo, Japan), two investigators blinded to the experimental groups performed semi-quantitative injury scoring (0–4 points) based on the following criteria: edema, inflammatory cell infiltration, hemorrhage, atelectasis, necrosis, and hyaline membrane formation. The sum of individual scores represented the total lung injury score.

Immunohistochemistry (IHC)

Lung tissue sections were deparaffinized, rehydrated, and subjected to antigen retrieval by heating in citrate buffer (pH 6.0) for 10 min. Endogenous peroxidase activity was blocked with 3% H₂O₂ for 10 min. Sections were then incubated with 5% normal goat serum for 30 min at room temperature to block non-specific binding.

Primary antibody (anti-pIRF3; Abcam, Cambridge, UK) was applied overnight at 4°C. After washing with PBS, sections were incubated with HRP-conjugated secondary antibody (Abcam) for 1 h at room temperature. Staining was visualized using diaminobenzidine (DAB) substrate (Zhongshan Golden Bridge, Beijing, China), and sections were counterstained with hematoxylin. Images were captured using a light microscope (Olympus), and integrated optical density (IOD) was quantified using ImageJ software (NIH, Bethesda, USA).

Assessment of bacterial burden

For CFU determination, lung homogenates or BALF were serially diluted, cultured on selective agar (sheep blood for *S. aureus*; Thermo Fisher Scientific, Waltham, USA; MacConkey for *K. pneumoniae*; Hopebio, Qingdao, China), and incubated at 37°C overnight. Visible colonies were enumerated and normalized to tissue weight or lavage volume.

Bacterial DNA was extracted from lung tissue or BALF using the QIAamp DNA Mini Kit (Qiagen). qPCR was performed on a LightCycler® 480 System (Roche, Basel, Switzerland) using SYBR Green Premix Ex Taq (Takara, Kusatsu, Japan) with specific primers targeting the *S. aureus* nuc gene and *K. pneumoniae* species-specific gene sequences ([Supplementary Table S1](#)). To address potential amplification of DNA from nonviable bacteria, we note that qPCR detects total bacterial DNA regardless of bacterial viability, whereas CFU assays specifically quantify viable, cultivable bacteria. In our active infection model, the strong correlation between CFU counts and qPCR-derived copy numbers (Pearson $r > 0.90$; [Supplementary Figure S2](#)) indicates that the majority of detected bacterial DNA originated from viable organisms. Discrepancies between the two methods, if observed, may reflect the presence of nonviable bacteria, antibiotic effects, or differential recovery efficiencies and should be interpreted accordingly.

Cytokine qPCR

Lung RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, USA) and reverse transcribed using PrimeScript RT Reagent Kit (Takara). qPCR was performed with SYBR Green Premix Ex Taq (Takara) for *TNF*, *IL-6*, *CX3CR1*, and *IL-10*, normalized to *GAPDH*. All primer sequences are listed in [Supplementary Table S1](#).

AAV-mediated *in vivo* knockdown of CX3CR1

To achieve conditional knockdown of *CX3CR1* *in vivo*, mice received an intravenous tail vein injection of an adeno-associated virus (AAV9) carrying a CRISPR interference (CRISPRi) system targeting *CX3CR1* (AAV9-CRISPRi-CX3CR1, 1×10^{11} viral genomes [vg] per mouse). Control mice were injected with AAV9 carrying a non-targeting gRNA (AAV9-CRISPRi-Ctrl). Mice were euthanized 20 days post-injection. Lung tissues were collected, and blood was obtained via retro-orbital bleeding. Total protein was extracted from tissues and whole blood, and CX3CR1 expression levels were detected by western blot analysis to assess *in vivo* knockdown efficiency. The specific gRNA sequences used are detailed in [Supplementary Table S1](#).

Competitive adoptive transfer in NSG mice

Peripheral blood leukocytes (PBLs) were isolated from donor AAV-CRISPRi-CX3CR1 (KD) and AAV-CRISPRi-Ctrl (Ctrl) mice. KD PBLs were labeled with 5 μ M CFSE (Invitrogen), and Ctrl PBLs were

labeled with 5 μ M CellTrace Violet (Invitrogen) for 10 min at 37°C. After washing, equal numbers (2×10^6 cells each) were mixed and injected intravenously into NSG recipient mice (Jackson Laboratory, Bar Harbor, USA; $n = 6$ per group). After 24 h, BALF was collected, and the ratio of CFSE⁺ to Violet⁺ cells among CD45⁺ leukocytes was quantified by flow cytometry.

Cell viability assay

CD14⁺ monocytes were seeded in 96-well plates (1×10^4 cells/well) and treated with increasing concentrations of LPS (0–1000 ng/mL) for 24 h. CCK-8 solution (Dojindo, Kumamoto, Japan) was added and incubated for 2 h. Absorbance at 450 nm was measured using a microplate reader (Bio-Tek, Winooski, USA). Cell viability was calculated relative to untreated controls.

In vitro cell migration assays

Cell migratory capacity was assessed using Transwell chamber and scratch wound healing assays. Detailed protocols for Transwell and scratch wound healing assays are provided in the [Supplementary Materials](#).

Magneticactivated cell sorting of CX3CR1⁺ and CX3CR1[−] cells from human PBMCs

PBMCs were stained with FITC-conjugated anti-CX3CR1 antibody (BioLegend, San Diego, USA), followed by antiFITC microbeads (Miltenyi Biotec). CX3CR1⁺ and CX3CR1[−] fractions were separated using LS columns on a MACS separator (Miltenyi Biotec) according to the manufacturer's instructions. Purity was verified by flow cytometry (> 99%). Genomic DNA was extracted from each fraction, bisulfiteconverted, and subjected to MSP as described above.

CRISPR-dCas9-based targeted methylation and demethylation

Unmethylated or methylated CD14⁺ monocytes (5×10^5 cells) were transfected with a CRISPR-dCas9-DNMT3A or CRISPR-dCas9-TET1 system targeting the cg00262061 locus (plasmid design: pX458-dCas9-DNMT3A; Addgene, Watertown, USA; or FuwCas9Tet1C-D_{IM}; Addgene; with sgRNA sequence: 5'-GGTTTATAGAGCTAGAATAGC-3'). Transfection was performed using the Lonza Nucleofector Kit for human monocytes (Lonza, Basel, Switzerland). After 6–7 days, cells were harvested for MSP and western blot analysis.

Methylation-specific PCR (MSP)

MSP was employed to detect the methylation status of target CpG sites. Following bisulfite conversion of genomic DNA using the EZ DNA MethylationGold Kit (Zymo Research, Irvine, USA) (which converts unmethylated C to U while leaving methylated C unchanged), a single primer set specific for the methylated sequence was used for PCR amplification with 2 $\times$ Taq Plus PCR Master Mix (Vazyme, Nanjing, China) to assess methylation status (sequences of primers shown in [Supplementary Table S1](#)).

Bisulfite sequencing

Genomic DNA from peripheral blood leukocytes of methylated and unmethylated pneumonia patients was bisulfiteconverted using the EZ DNA MethylationGold Kit (Zymo Research). The region containing cg00262061 was amplified by PCR (primers: forward

5'-TGTTAGAAAGTGGATGGGGAGC-3', reverse 5'-CCTACTCATAA TAAAAAAATCCACCA-3'), and the amplicon was subcloned and inserted into the pMD19T vector (Takara). At least 10 clones per sample were sequenced to determine the methylation status.

Western blot analysis

Western blot analysis was performed using standard protocols. Protein signals were visualized using enhanced chemiluminescence (ECL) substrate (Millipore, Burlington, USA) and quantified with ImageJ. Antibody details are listed in [Supplementary Table S2](#).

Flow cytometry

Absolute quantification of CD45⁺ leukocytes in BALF was performed using flow cytometry with fluorescent counting beads (APC-Alexa Fluor 750; eBioscience, San Diego, USA). Collected BALF samples were centrifuged, and the cell pellet was stained with a fluorophore-conjugated anti-mouse CD45 antibody (eBioscience) at 4°C in the dark for 30 min. After staining, a known number of fluorescent counting beads (10,000 beads per test, according to the manufacturer's instructions) was thoroughly mixed with the cell suspension, and the sample was immediately acquired on a BD FACSCanto II flow cytometer. Populations of counting beads and CD45⁺ cells were gated according to their specific fluorescence signals. The absolute concentration of CD45⁺ cells was calculated using the following formula: (number of CD45⁺ cell events / number of counting bead events) × (total number of beads added / sample acquisition volume). Antibody information is provided in [Supplementary Table S2](#).

For analysis of immune cell subsets in BALF or cultured cells, single-cell suspensions were first blocked with anti-CD16/CD32 antibody (BD Biosciences) for 10 min at 4°C. Cells were then stained with fluorochrome-conjugated antibodies against surface markers (F4/80, CD11b, Ly6G, CD3, CD4, CD86, and CD206) for 30 min at 4°C in the dark. For intracellular staining of IFN- γ , IL-17A, and FoxP3, cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL; Sigma-Aldrich) and ionomycin (1 μ g/mL; Sigma-Aldrich) for 4 h in the presence of brefeldin A (10 μ g/mL; BioLegend). After surface staining, cells were fixed and permeabilized using the FoxP3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's protocol, followed by intracellular antibody staining. Data were acquired on a BD FACSCanto II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, USA).

DNA pull-down assay

To analyze transcription factor binding to the *CX3CR1* promoter, biotinylated DNA probes encompassing the cg00262061 site in both methylated and unmethylated states were synthesized. The probes were conjugated to streptavidin magnetic beads and then incubated with nuclear extracts at 4°C. After a series of washes to remove nonspecifically bound proteins, proteins bound to the DNA probes were eluted. The binding of the target protein (RNA polymerase II) was subsequently detected by western blot analysis.

Chromatin immunoprecipitation (ChIP)

ChIP was performed using antiRNA polymerase II antibody (Abcam) or control IgG (Abcam). Immunoprecipitated DNA was quantified by qPCR with primers targeting the *CX3CR1* promoter. Antibody details are provided in [Supplementary Table S2](#).

Dual-luciferase reporter assay

A dual-luciferase reporter assay was performed to evaluate the effect of cg00262061 methylation on *CX3CR1* promoter activity. The promoter region containing this site was cloned and inserted into pGL3-Basic (Promega, Madison, USA) to generate wild-type (WT-CX3CR1) and methylated (cg-CX3CR1, via *SssI* methyltransferase treatment; New England Biolabs, Ipswich, USA) reporter plasmids. These constructs were cotransfected with pRL-TK (Promega, Madison, USA) into CD3⁺ T cells or CD14⁺ monocytes. After 48 h, firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega), and relative promoter activity was calculated as the *Firefly/Renilla* ratio.

High-throughput data analysis

Bioinformatic analysis was conducted in two stages. Stage 1: based on the GEO public dataset (GSE65682), the empirical Bayes test within the limma R package was used to compare peripheral blood transcriptomic data between 184 pneumonia patients and 42 healthy volunteers. Differentially expressed genes (DEGs) were identified using thresholds of false discovery rate (FDR) < 0.05. Subsequently, univariate Cox proportional hazards regression analysis was performed on the identified DEGs to evaluate the association between their expression levels and patient 28-day mortality, screening for prognosis-related genes with a threshold of $P < 0.05$. Stage 2: DNA methylation microarray and transcriptomic data from our cohort of 38 pneumonia patients were integrated. For the prognosis-related genes identified in Stage 1, the methylation levels of their corresponding CpG sites and their gene expression values were extracted. Pearson correlation coefficients between methylation and expression were calculated. Methylation sites significantly correlated with target gene expression were screened using thresholds of $|r| > 0.6$ and $P < 0.05$ to reveal potential methylation regulatory mechanisms.

To address the risk of overfitting due to the limited sample size and to select the most relevant prognostic variables, we performed least absolute shrinkage and selection operator (LASSO) penalized Cox regression using the glmnet package in R (version 4.4.1). All candidate variables, including the four *CX3CR1* promoter CpG sites (cg00262061, cg25784617, cg13145644, and cg05046858), age, sex, pneumonia type, APACHE II score, leukocyte count, procalcitonin (PCT), C-reactive protein (CRP), pathogen identification, and prior antibiotic use, were included in the LASSO model. The optimal penalty parameter λ was selected by 5-fold cross-validation using the one-standard-error rule (λ_{1se}). The coefficient paths and cross-validation deviance were visualized. Calibration of the full Cox model (including all variables) was assessed by a calibration curve with bootstrap resampling (200 iterations) to evaluate the agreement between predicted and observed 28-day survival probabilities.

Statistical analysis

Data, excluding high-throughput results, are shown as the mean $\pm$ SEM. Data distribution normality was evaluated by the Shapiro-Wilk test. Comparisons between two groups were performed using an unpaired, two-tailed Student's *t* test. Comparisons among multiple groups were conducted via one-way analysis of variance. Survival analysis in mice was performed using the Kaplan-Meier method, and differences between groups were compared with the log-rank test. Statistical analyses for this study were primarily performed using R software (version 4.4.1). Statistical significance

was defined as $P < 0.05$.

Results

CX3CR1 downregulation is associated with poor prognosis in pneumonia patients

We first interrogated the public transcriptomic dataset GSE65682, which includes 184 pneumonia patients and 42 healthy controls (Figure 1A). Differential expression analysis yielded 168 genes meeting the thresholds ($FDR < 0.05$, $|\log_2FC| > 2$). Univariate Cox regression further showed that 12 genes, including CX3CR1, correlated significantly with 28-day mortality. Notably, lower CX3CR1 expression independently predicted higher mortality (HR = 0.13, 95% CI: 0.05–0.38; $P < 0.001$). The hazard ratios for the remaining 11 genes are shown in Figure 1B. As displayed in the expression heatmap of these 12 genes (Figure 1C), CX3CR1 expression was significantly reduced in pneumonia patients, particularly in nonsurvivors ($P < 0.001$).

CX3CR1 expression is inversely correlated with methylation at the cg00262061 locus

To investigate the epigenetic mechanism of CX3CR1 downregulation, we enrolled 38 pneumonia patients and collected peripheral blood within 48 h of admission to isolate PBMCs for analysis (patient details in Figure 1D). Using the Infinium HumanMethylation450K BeadChip and transcriptomic microarrays in parallel, we profiled genome-wide DNA methylation levels and gene expression in PBMCs. Pearson correlation analysis within the genomic regions of CX3CR1 and 11 other genes (thresholds: $|r| > 0.6$, $P < 0.05$) identified 6 CpG sites with strong correlations to their respective target gene expression levels (Figure 1E). Specifically, methylation levels at cg00262061, cg05046858, cg25784617, and cg13145644 showed a significant negative correlation with CX3CR1 mRNA expression, while sites cg23339092 and ch.20.1019750F correlated positively with LRRN3 expression. Given that DNA methylation typically represses transcription, we focused on the four sites negatively correlated with CX3CR1 for subsequent investigation (Figure 1F).

Further locus mapping confirmed that all four sites are located within the promoter region of the CX3CR1 gene (Supplementary Figure S3) at the following chromosomal positions on chr3: cg13145644 (39282787–39282836), cg25784617 (39282048–39282096), cg00262061 (39281236–39281285), and cg05046858 (39281155–39281204). These results suggest that hypermethylation at the promoter site cg00262061 may be critically involved in suppressing CX3CR1 expression.

To further explore the prognostic value of cg00262061 methylation while mitigating overfitting, we performed LASSO-Cox penalized regression including all candidate clinical and methylation variables. The coefficient path plot and cross-validation deviance plot (Supplementary Figure S4) showed that the optimal penalty retained only cg00262061 as a non-zero coefficient. However, bootstrap validation of the LASSO model yielded a C-index of 0.72 originally but dropped to a median of 0.54 after correction, indicating substantial overfitting. Calibration of the full Cox model (including all variables) showed acceptable agreement between predicted and observed 28-day survival probabilities, albeit with wide confidence intervals due to the small sample size (Supplementary Figure S5). Given this instability, we consider these prognostic findings exploratory, and we have refrained from

reporting the LASSO-derived hazard ratios in the main manuscript. The predictive value of cg00262061 methylation requires validation in larger, independent cohorts.

Hypermethylation at the cg00262061 locus is associated with poor prognosis in pneumonia

To validate the association between methylation at these four promoter sites (including cg00262061), CX3CR1 suppression, and patient outcome, we performed MSP on PBMC samples from the same 38 patients. Methylation at these four sites was detected in 7 patients (Figure 1G and Supplementary Figures S6–8). Survival analysis revealed that patients with methylation at these loci had a significantly higher 28-day all-cause mortality ($P < 0.001$), indicating a close link between this methylation event and adverse prognosis (Figure 1H). Expression comparison by methylation status analysis further demonstrated that patients with methylation at these sites had significantly lower CX3CR1 mRNA levels ($P < 0.001$), as shown in Figure 1I. Collectively, these findings imply that promoter methylation, notably at cg00262061, may contribute to unfavorable clinical outcomes in pneumonia by inhibiting CX3CR1 expression.

To assess whether the prognostic value of cg00262061 methylation is independent of clinical characteristics, we performed multivariate Cox regression analysis incorporating key variables, including age, sex, pneumonia type, disease severity (assessed by APACHE II score), laboratory parameters [leukocyte count, procalcitonin (PCT), C-reactive protein (CRP)], pathogen identification, and prior antibiotic use (within 3 months before admission). As shown in Supplementary Table S3, after adjusting for these potential confounders, cg00262061 methylation remained a significant independent predictor of 28-day mortality [hazard ratio (HR) = 20.1, 95% confidence interval (CI): 7.76–51.59, $P = 0.007$]. Similarly, low CX3CR1 expression retained its independent association with poor prognosis (HR = 0.17, 95% CI: 0.06–0.47, $P = 0.001$). These findings suggest that the epigenetic silencing of CX3CR1 provides prognostic information beyond traditional clinical risk factors, although validation in larger cohorts is warranted due to the modest sample size of the present study.

To address the potential quantitative limitation of MSP, we performed bisulfite sequencing of the cg00262061 region in peripheral blood leukocytes from representative methylated and unmethylated pneumonia patients. The sequencing results were fully concordant with the MSP data: the CpG site was methylated in methylated patients and unmethylated in unmethylated patients (Supplementary Figure S9), confirming the specificity and reliability of our MSP assay.

Methylation at the cg00262061 locus suppresses CX3CR1 transcription

To elucidate the direct transcriptional repression of CX3CR1 by promoter methylation at sites including cg00262061, we conducted mechanistic studies at the cellular level. CD3⁺ T cells and CD14⁺ monocytes were sorted and purified from patient PBMCs with or without methylation at these four sites (purity validation in Supplementary Figures S10,11). MSP and western blot analyses revealed that CX3CR1 expression was markedly lower in both cell types derived from patients with a methylated cg00262061 allele compared to those from the unmethylated group ($P < 0.001$; Figure 2A,B), indicating a close association between promoter methylation

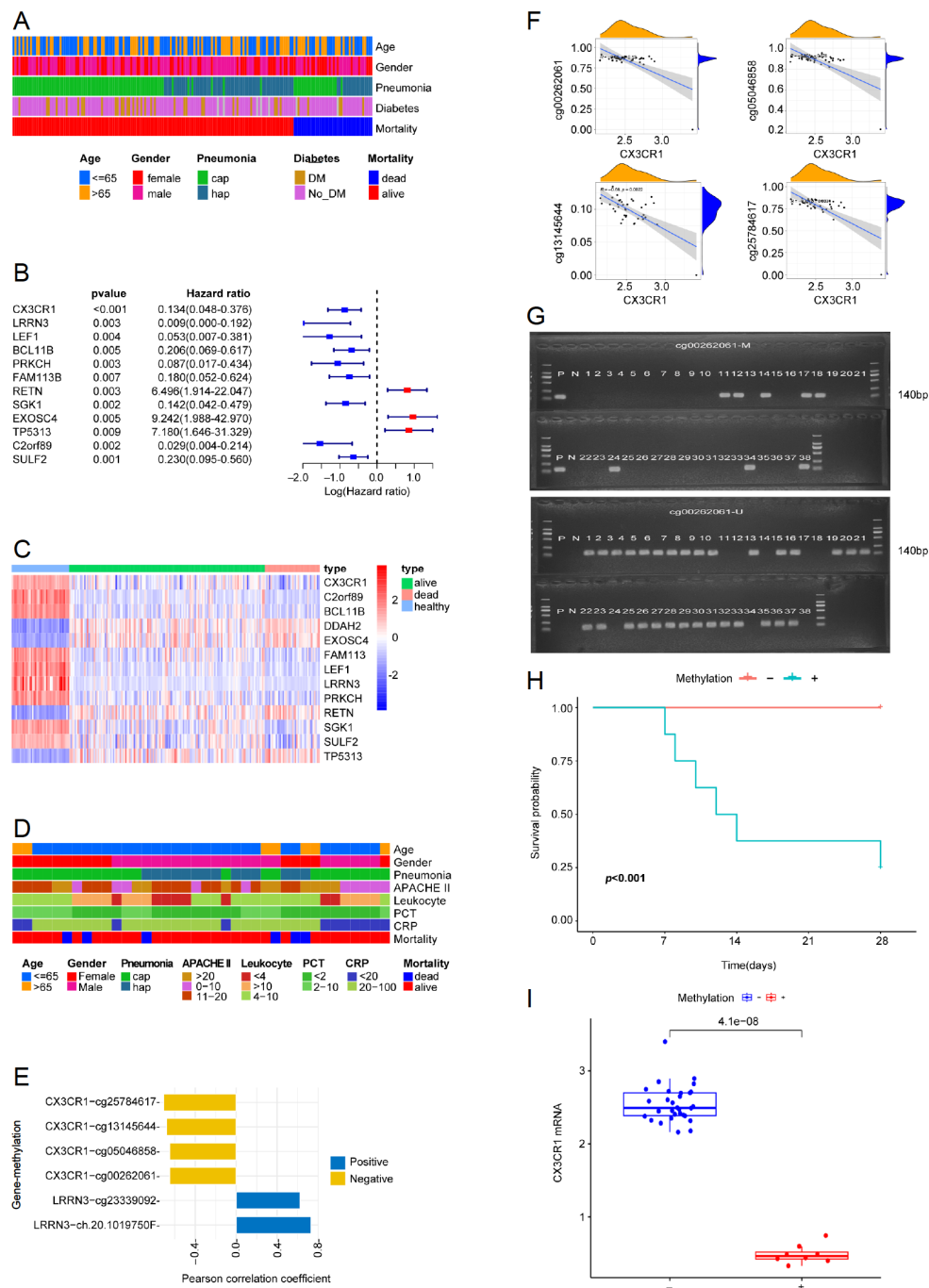


Figure 1. Clinical cohort analysis shows that reduced CX3CR1 expression and promoter methylation are associated with poor prognosis in pneumonia (A) Clinical characteristics of 184 pneumonia patients from the public GEO dataset (GSE65682). (B) Univariate Cox regression analysis identified 12 genes whose expression levels were significantly associated with 28-day all-cause mortality. Hazard ratios with 95% confidence intervals are shown. (C) Heatmap displaying the expression levels of the 12 prognosis-related genes in healthy volunteers ($n = 42$), pneumonia survivors ($n = 154$), and nonsurvivors ($n = 30$). (D) Baseline clinical characteristics of the 38 pneumonia patients prospectively enrolled in this study. Data include age, sex, severity scores, and laboratory parameters. (E) Correlation analysis between genome-wide DNA methylation and transcriptome data from 38 patients identified 6 CpG sites strongly correlated with their corresponding target gene expression ($|r| > 0.6$, $P < 0.05$). (F) Scatter plots showing the negative correlation between methylation β -values of four promoter CpG sites (cg00262061, cg05046858, cg25784617, and cg13145644) and CX3CR1 mRNA expression levels in PBMCs from 38 patients. Pearson correlation coefficients and P values are indicated. (G) Representative MSP analysis of the cg00262061 methylation status in PBMCs from 38 pneumonia patients. M: methylated; U: unmethylated. (H) Kaplan-Meier survival curves comparing 28-day cumulative survival between patients with ($n = 7$) and without ($n = 31$) cg00262061 methylation. The P value was calculated by the log-rank test. (I) Comparison of CX3CR1 mRNA expression levels between patients with ($n = 7$) and without ($n = 31$) cg00262061 methylation. Data are presented as the mean $\pm$ SEM. Statistical significance was determined by unpaired two-tailed Student's t test.

and CX3CR1 protein suppression.

To determine the optimal LPS concentration for *in vitro* stimulation without inducing significant cytotoxicity, we assessed the viability of CD14⁺ monocytes treated with increasing concentrations of LPS (0–1000 ng/mL) for 24 h using the CCK-8 assay. As shown in [Supplementary Figure S1](#), cell viability remained above 85% at concentrations up to 500 ng/mL but declined sharply at 1000 ng/mL (viability < 70%). These results indicate that 500 ng/mL LPS represents the optimal concentration for maximizing inflammatory stimulation while maintaining cell viability and was therefore selected for subsequent experiments.

A canonical mechanism by which DNA methylation represses transcription involves interfering with the recruitment of transcription machinery components, including RNAP II, to the promoter. To investigate this, we performed a DNA pull-down assay. The results showed that in both CD3⁺ T cells and CD14⁺ monocytes, the methylated cg00262061 DNA probe exhibited significantly reduced binding capacity for RNAP II compared to the unmethylated probe ([Figure 2C](#)). This finding was further supported by ChIP-qPCR using primers specifically targeting the *CX3CR1* promoter region adjacent to the cg00262061 site. The assay demonstrated markedly decreased RNAP II occupancy at this locus in cells carrying the methylated allele ([Figure 2D](#)).

Finally, we generated a luciferase reporter construct containing the *CX3CR1* promoter and subjected it to site-specific *in vitro* methylation at cg00262061 (cg-CX3CR1), with the unmethylated counterpart (WT-CX3CR1) as a control. Upon transfection into CD3⁺ T cells or CD14⁺ monocytes, the methylated promoter displayed significantly lower transcriptional activity than the unmethylated control ($P < 0.001$; [Figure 2E](#)). Together, these results demonstrate that methylation at the cg00262061 site is associated with reduced RNAP II occupancy and diminished *CX3CR1* promoter activity, leading to suppressed gene expression.

To evaluate the efficiency of CX3CR1 restoration in primary immune cells, we assessed CX3CR1 protein expression following transfection with the CRISPRa-CX3CR1 plasmid in CD14⁺ monocytes and CD3⁺ T cells. As shown in [Supplementary Figure S12](#), western blot analysis confirmed that CRISPRa-CX3CR1 transfection successfully restored CX3CR1 expression in both cell types compared to nontransfected controls. These results validate the functional efficacy of the CRISPRa-mediated overexpression system and provide the basis for subsequent rescue experiments assessing the role of CX3CR1 in immune cell migration.

Targeted methylation and demethylation at cg00262061 directly control CX3CR1 expression

To establish direct causality beyond correlation, we performed CRISPR-dCas9-based epigenetic editing in primary human CD14⁺ monocytes. In unmethylated CD14⁺ cells (cg00262061⁻), transfection with dCas9-DNMT3A targeting the cg00262061 locus induced site-specific methylation (MSP positive) and significantly reduced CX3CR1 protein expression compared to control cells. Conversely, in methylated CD14⁺ cells (cg00262061⁺), transfection with dCas9-TET1 targeting the same locus removed the methylation mark (MSP negative) and restored CX3CR1 expression ([Supplementary Figure S13](#)). These gain- and loss-of-methylation experiments provide direct causal evidence that methylation at this site is sufficient to suppress *CX3CR1* transcription in its native chromatin context.

CX3CR1 expression and cg00262061 methylation are selectively

enriched in the CX3CR1⁻ subset of methylated pneumonia patients

To determine whether cg00262061 methylation occurs uniformly across PBMCs or selectively within specific subsets, we first measured CX3CR1 surface expression on PBMCs from methylated pneumonia patients ($n = 6$), unmethylated pneumonia patients ($n = 6$), and healthy volunteers ($n = 6$) by flow cytometry. As shown in [Supplementary Figure S14](#), methylated patients exhibited a significantly lower percentage of CX3CR1⁺ cells ($4.81\% \pm 0.30\%$) than unmethylated patients ($15.20\% \pm 0.31\%$) and healthy controls ($24.56\% \pm 0.60\%$; both $P < 0.001$). We then magnetically sorted CX3CR1⁺ and CX3CR1⁻ cells from the same donors (purity > 99%; [Supplementary Figure S15A](#)) and performed MSP analysis targeting the cg00262061 locus. Remarkably, methylation was detected exclusively in the CX3CR1⁻ cells from methylated pneumonia patients, whereas no methylation was found in CX3CR1⁺ cells from the same patients or in either population from unmethylated patients or healthy controls ([Supplementary Figure S15B](#)). These data demonstrate that cg00262061 hypermethylation is not uniformly distributed across PBMCs but is selectively present in the CX3CR1⁻ subset and that the reduced CX3CR1⁺ cell frequency in methylated patients is associated with this methylation mark.

Methylation at the cg00262061 locus impairs immune cell migration via CX3CR1 downregulation

CX3CR1 is a chemokine receptor responsible for mediating the migration of immune cells into lung tissue. These cells not only exert bactericidal effects to reduce the bacterial load in pneumonia but also, in subsets such as M2 macrophages and regulatory T cells (Tregs), contribute to injury repair. Consequently, transcriptional repression of CX3CR1 represents a core mechanism linking cg00262061 methylation to impaired immune cell migration and, ultimately, poor prognosis.

To test this hypothesis, we evaluated the impact of cg00262061 methylation on the migratory capacity of CD14⁺ monocytes using both scratch wound healing and Transwell assays and on CD3⁺ T cells. Scratch assay results showed that the migration capacity of CD14⁺ monocytes with a methylated cg00262061 locus was significantly weakened under both basal conditions (PBS) and inflammatory stimulation (LPS 500 ng/mL for 24 h). This impaired migration was partially restored in rescue experiments where CX3CR1 expression was re-established ($P < 0.001$; [Figure 3A–C](#)). Transwell assays yielded corroborative results: both CD14⁺ monocytes and CD3⁺ T cells from the methylation group exhibited significantly reduced migratory capacity. This migration defect was reversed upon restoration of CX3CR1 expression ($P < 0.001$; [Figure 3D–F](#)). These collective findings indicate that methylation at the cg00262061 locus regulates immune cell migration function by suppressing CX3CR1 expression.

Inhibition of CX3CR1 expression exacerbates pathological injury and leads to poor prognosis in a murine pneumonia model

To establish a causal relationship between low CX3CR1 expression and adverse pneumonia outcomes *in vivo*, we employed a CRISPR interference (CRISPRi) system targeting *CX3CR1*. Mice received a tail vein injection of an adeno-associated virus carrying this system (AAV-CRISPRi-CX3CR1) to achieve specific transcriptional repression; control mice were injected with an AAV carrying a nontargeting gRNA (AAV-CRISPRi-Ctrl) ([Figure 4A](#)). Western blot

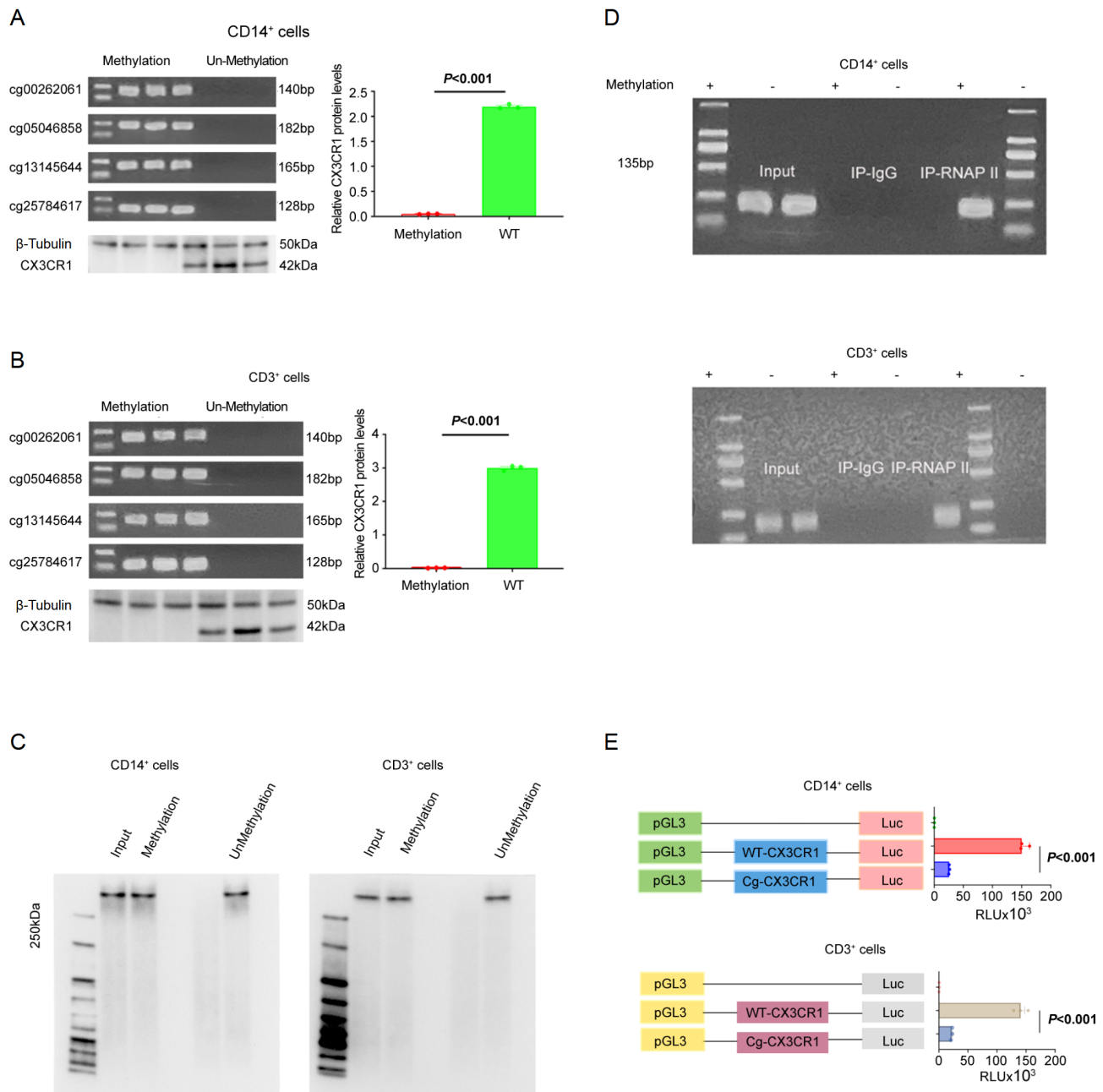


Figure 2. cg00262061 methylation directly suppresses CX3CR1 transcription in vitro (A) MSP validation of the cg00262061 methylation status in CD14⁺ monocytes isolated from methylation-positive (Me; $n = 3$) and methylation-negative (Un; $n = 3$) patients. Bottom: representative western blots and quantification of CX3CR1 protein levels in the same CD14⁺ monocyte samples. β -Tubulin served as a loading control. (B) Parallel MSP and western blot analysis performed in CD3⁺ T cells from the same patients (Me; $n = 3$ and Un; $n = 3$), showing the relationship between cg00262061 methylation status and CX3CR1 protein levels. (C) DNA pull-down assay analyzing RNAP II binding to biotinylated CX3CR1 promoter probes containing either methylated or unmethylated cg00262061. Probes were incubated with nuclear extracts from CD14⁺ monocytes (left) and CD3⁺ T cells (right). Bound RNAP II was detected by western blot analysis. Representative images from three independent experiments are shown. (D) ChIP assay measuring RNAP II enrichment at the CX3CR1 promoter region in CD14⁺ monocytes (left) and CD3⁺ T cells (right) from methylation-positive (Me) and methylation-negative (Un) patients. Enrichment was quantified by qPCR using primers targeting the CX3CR1 promoter region adjacent to cg00262061. Data are presented as the mean $\pm$ SEM from three independent experiments. (E) Dual-luciferase reporter assay comparing the transcriptional activity of methylated (cg-CX3CR1) versus unmethylated (WT-CX3CR1) CX3CR1 promoters. Reporter constructs were transfected into CD14⁺ monocytes (left) and CD3⁺ T cells (right). Firefly luciferase activity was normalized to Renilla luciferase activity. Data are presented as the mean $\pm$ SEM from three independent experiments performed in triplicate.

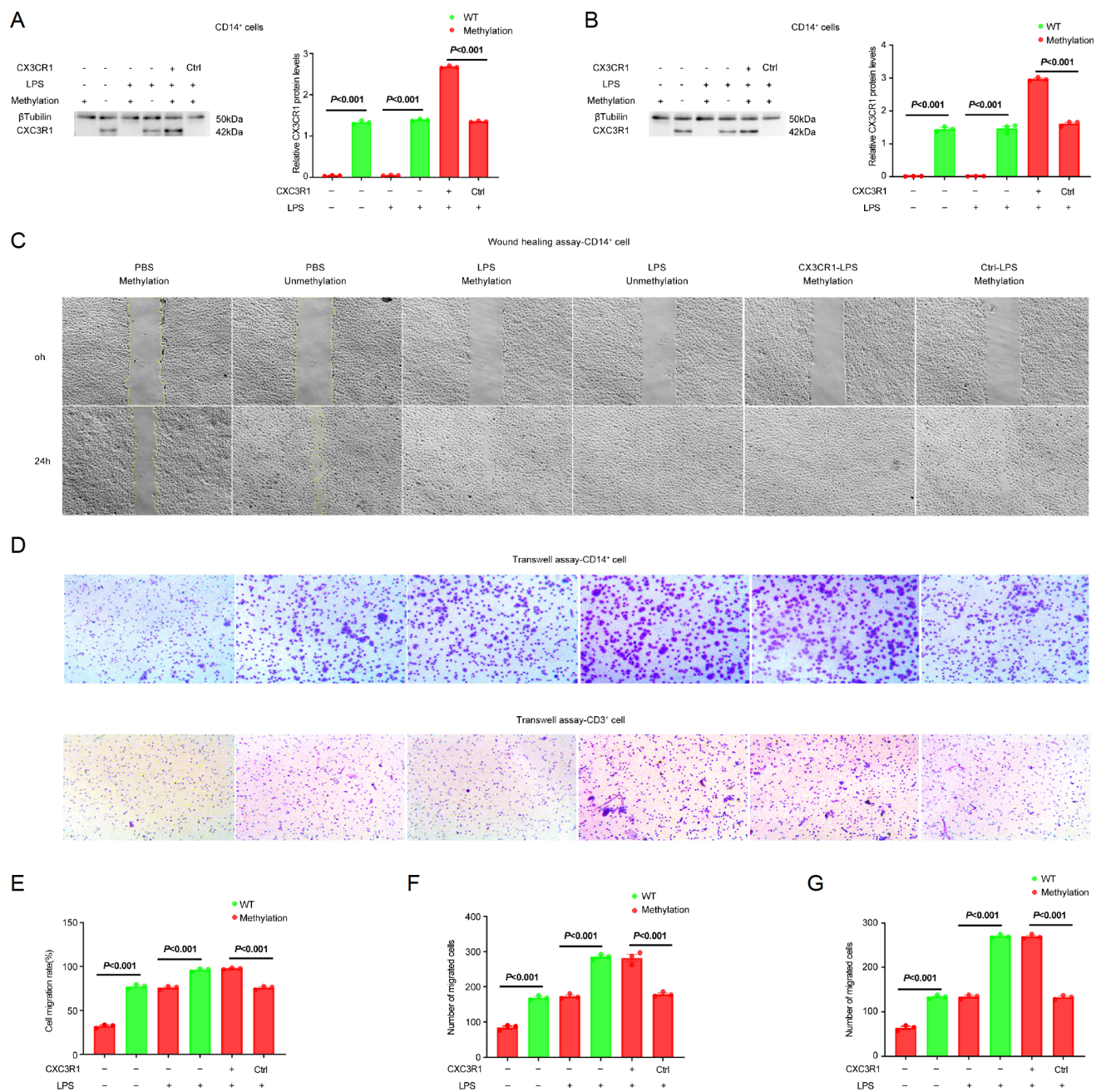


Figure 3. cg00262061 methylation impairs immune cell migration by downregulating CX3CR1 (A,B) Western blot analysis of CX3CR1 protein expression in CD14⁺ monocytes (A) and CD3⁺ T cells (B) isolated from methylation-positive (Me) and methylation-negative (Un) patients. Cells were treated with PBS (control) or 500 ng/mL LPS for 12 h. Right panels show CX3CR1 expression in methylation-positive cells following transfection with CX3CR1 CRISPRa plasmid (rescue) or control vector. β-Tubulin served as a loading control. Representative images from three independent experiments are shown. (C) Scratch wound healing assay assessing the migration capacity of CD14⁺ monocytes from methylation-positive and methylation-negative patients under PBS or LPS stimulation. The rescue group represents methylation-positive cells transfected with the CX3CR1 CRISPRa plasmid. Representative images were taken at 0 h and 24 h postscratch. Magnification: ×100. (D) Transwell migration assay evaluating the migration capacity of CD14⁺ monocytes (top) and CD3⁺ T cells (bottom) from methylation-positive and methylation-negative patients under PBS or LPS stimulation. The Rescue group represents methylation-positive cells transfected with the CX3CR1 CRISPRa plasmid. Representative images of migrated cells stained with crystal violet are shown. Magnification: ×100. (E) Quantitative analysis of the wound closure percentage from the scratch assays shown in (C). Data are presented as the mean ± SEM from three independent experiments. (F) Quantitative analysis of migrated CD14⁺ monocyte numbers from Transwell assays shown in (D). Data are presented as the mean ± SEM from three independent experiments. (G) Quantitative analysis of migrated CD3⁺ T-cell numbers from Transwell assays shown in (D). Data are presented as the mean ± SEM from three independent experiments.

analysis confirmed that AAV-CRISPRi-CX3CR1 treatment significantly reduced CX3CR1 protein levels in both peripheral blood and lung tissues of mice ($P < 0.001$; [Supplementary Figure S16](#)).

Subsequently, a mouse pneumonia model was established via intratracheal instillation of either *S. aureus* or *K. pneumoniae*. Lung tissues were harvested for analysis on day 5 postinfection. H&E staining of lung sections revealed that, compared to controls, mice with inhibited CX3CR1 exhibited more severe inflammatory infiltration, edema, and structural damage in response to infection with either pathogen ($P < 0.001$; [Figure 4B](#)). Survival analysis further demonstrated a significant decrease in the survival rate among CX3CR1-inhibited mice with pneumonia ($P < 0.05$; [Figure 4C](#)). These results confirm that normal CX3CR1 expression is crucial for mitigating pneumonia severity and improving survival prognosis.

CX3CR1 downregulation increases bacterial burden by impairing immune cell recruitment to the lungs

To elucidate the potential mechanism by which CX3CR1 downregulation leads to poor prognosis *in vivo*, we assessed its impact on immune cell recruitment to the lungs and bacterial clearance capacity. First, we quantified the number of leukocytes (CD45⁺ cells) in BALF using flow cytometry. A significant reduction in CD45⁺ immune cell recruitment to infected lungs was observed upon CX3CR1 knockdown ($P < 0.001$; [Figure 4D](#)), corresponding to impaired migration due to CX3CR1 deficiency.

Next, we evaluated the bacterial burden in lung tissue using colony-forming unit (CFU) counts and qPCR targeting bacterial-specific genes. Mice with CX3CR1 knockdown harbored significantly greater lung bacterial burdens of either *S. aureus* or *K. pneumoniae* relative to control animals ($P < 0.001$; [Figure 4E,F](#)). Correlation analysis between the two quantification methods revealed a strong positive correlation between CFU counts and qPCR-derived bacterial DNA copy numbers ($R = 0.92$, $P = 2.6 \times 10^{-10}$; [Supplementary Figure S16](#)), confirming the consistency and reliability of these complementary approaches in assessing bacterial load.

These findings suggest that CX3CR1 downregulation compromises the host's bacterial clearance efficacy by weakening the migration of immune cells to the infection site, thereby leading to an increased bacterial burden that ultimately exacerbates the pathological progression and poor prognosis of pneumonia.

Adoptive transfer confirms a cell-intrinsic migration defect of CX3CR1-deficient immune cells

To directly determine whether the impaired pulmonary recruitment observed in systemic CX3CR1 knockdown mice is cell-autonomous, we performed competitive adoptive transfer experiments using NSG immunodeficient recipients. Peripheral blood leukocytes from AAV-CRISPRi-CX3CR1 (KD) mice were labeled with CFSE, while cells from AAV-CRISPRi-Ctrl (Ctrl) mice were labeled with CellTrace Violet. Equal numbers of labeled cells were mixed (1:1) and injected intravenously into NSG recipient mice. After 24 h, the ratio of KD to Ctrl cells recovered in BALF was significantly lower than the input ratio (KD/Ctrl ≈ 0.33 vs 1.0, $P < 0.01$; [Supplementary Figure S17](#)). These results demonstrate that CX3CR1-deficient immune cells have a cell-intrinsic migration defect, independent of any indirect effects from the lung microenvironment. This experiment, together with the systemic knockdown data, supports that the protective role of CX3CR1 in pneumonia is largely driven by its cell-autonomous

regulation of immune cell homing.

CX3CR1 expression is reduced in multiple immune cell subsets in the lungs of CX3CR1-knockdown mice

To directly assess the impact of systemic CX3CR1 knockdown on pulmonary immune cells, we analyzed CX3CR1 protein expression on distinct leukocyte populations in BALF from mice with bacterial pneumonia. As shown in [Figure 5](#), CX3CR1 was readily detectable on F4/80⁺ macrophages ([Figure 5A](#)), CD3⁺ T cells ([Figure 5B](#)), and CD11b⁺Ly6G⁺ neutrophils ([Figure 5C](#)) in control mice. However, in mice treated with AAV-CRISPRi-CX3CR1, CX3CR1 expression levels—quantified by mean fluorescence intensity (MFI)—were significantly reduced across all three cell types compared to AAV-CRISPRi-Ctrl controls (from $P < 0.05$ to $P < 0.001$). These results confirm that our systemic knockdown strategy effectively reduces CX3CR1 protein levels on key immune cell populations recruited to the infected airways, providing a mechanistic basis for the impaired leukocyte migration observed in [Figure 4D](#).

CX3CR1 knockdown impairs recruitment of multiple immune cell subsets to the lungs during bacterial pneumonia

We next assessed the impact of CX3CR1 deficiency on the recruitment of functionally distinct immune subsets. Notably, CX3CR1 knockdown led to a significant reduction in the proportions of both M1 (F4/80⁺CD86⁺) and M2 (F4/80⁺CD206⁺) macrophages in BALF ($P < 0.01$; [Figure 6A,B](#)). Furthermore, among CD4⁺ T cells, the proportions of Th1 (CD4⁺IFN- γ ⁺), Th17 (CD4⁺IL-17A⁺), and regulatory T cells (CD4⁺FoxP3⁺) were all markedly decreased following CX3CR1 knockdown ($P < 0.01$ to $P < 0.001$; [Figure 6C–E](#)).

We next examined the impact of CX3CR1 deficiency on the expression of key inflammatory cytokines in lung tissues by qPCR. Compared to Ctrl KD controls, CX3CR1 knockdown mice exhibited significantly lower mRNA levels of *TNF- α* , *IL-6*, and *IL-10* ($P < 0.05$ for all comparisons; [Supplementary Figure S18](#)).

Collectively, these results demonstrate that CX3CR1 is critical for the recruitment of multiple immune cell populations to the infected lung, and its deficiency broadly impairs pulmonary immune cell infiltration, thereby compromising the host's ability to control bacterial infection.

Discussion

Despite advances in treatment, severe pneumonia continues to carry high mortality, underscoring the need for deeper mechanistic understanding. Accumulating evidence points to defective immune cell trafficking as a central driver of poor pathogen control in pneumonia, but the upstream regulators remain elusive. Here, we link poor prognosis in pneumonia to reduced CX3CR1 expression, driven by promoter hypermethylation at cg00262061—an epigenetic event that silences this chemokine receptor. This regulatory pathway directly weakens the recruitment of immune cells to the lungs, thereby explaining, at both clinical and experimental levels, the intrinsic reason for failed bacterial clearance and aggravated pathological injury in a subset of patients. Our work provides a novel mechanistic perspective for understanding immune dysfunction in pneumonia and offers a potential basis for related prognostic assessment and targeted intervention.

Conventional clinical inflammatory indices, alongside specific inflammatory mediators such as IL-6 and suPAR, have been

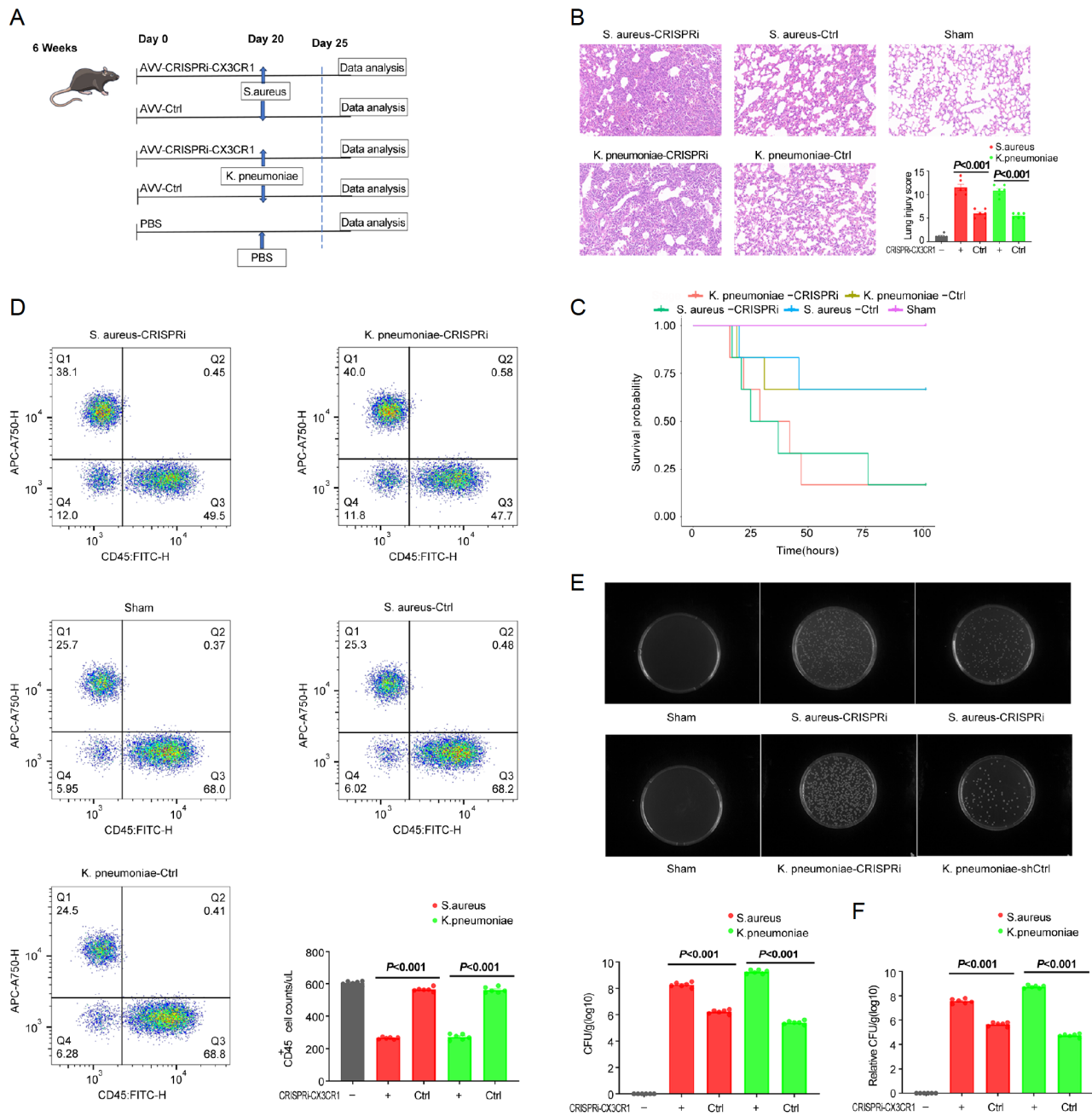


Figure 4. Inhibition of CX3CR1 *in vivo* aggravates bacterial burden and reduces survival in murine pneumonia (A) Experimental design schematic. C57BL/6 mice received a tail vein injection of AAV9-CRISPRi-Ctrl (control) or AAV9-CRISPRi-CX3CR1 (n = 12 per group). On day 20 postinjection, mice were intratracheally inoculated with 6.0×10^8 CFU/kg *S. aureus* or *K. pneumoniae*. Lung tissues and BALF were collected on day 5 postinfection for analysis (n = 6 per group for endpoint analysis). The remaining 6 mice per group were monitored for survival. (B) Representative H&E-stained lung sections from control and CX3CR1 knockdown mice on day 5 postinfection with *S. aureus* (left) or *K. pneumoniae* (right) infection (n = 6 per group). P values were calculated by the log-rank test. (C) Kaplan-Meier survival curves of control and CX3CR1 knockdown mice following *S. aureus* (left) or *K. pneumoniae* (right) infection (n = 6 per group). P values were calculated by the log-rank test. (D) Absolute numbers of CD45⁺ leukocytes in BALF quantified by flow cytometry using counting beads. Cells were stained with APC-Alexa Fluor 750-conjugated anti-CD45 antibody. Absolute counts were calculated as follows: (CD45⁺ events/bead events) $\times$ (total beads added/sample acquisition volume). Data are presented as the mean $\pm$ SEM (n = 6 per group). (E) Bacterial burden in lung homogenates assessed by CFU assay on day 5 postinfection with *S. aureus* (left) or *K. pneumoniae* (right). Data are presented as the mean $\pm$ SEM (n = 6 per group). (F) Bacterial DNA copy numbers in lung tissue quantified by qPCR using species-specific primers. Data are presented as the mean $\pm$ SEM (n = 6 per group).

identified to be correlated with pneumonia severity and prognosis [14,15]. However, these molecules largely reflect the systemic

inflammatory response. The specific prognostic value of the chemokine and receptor family, which directly regulates the

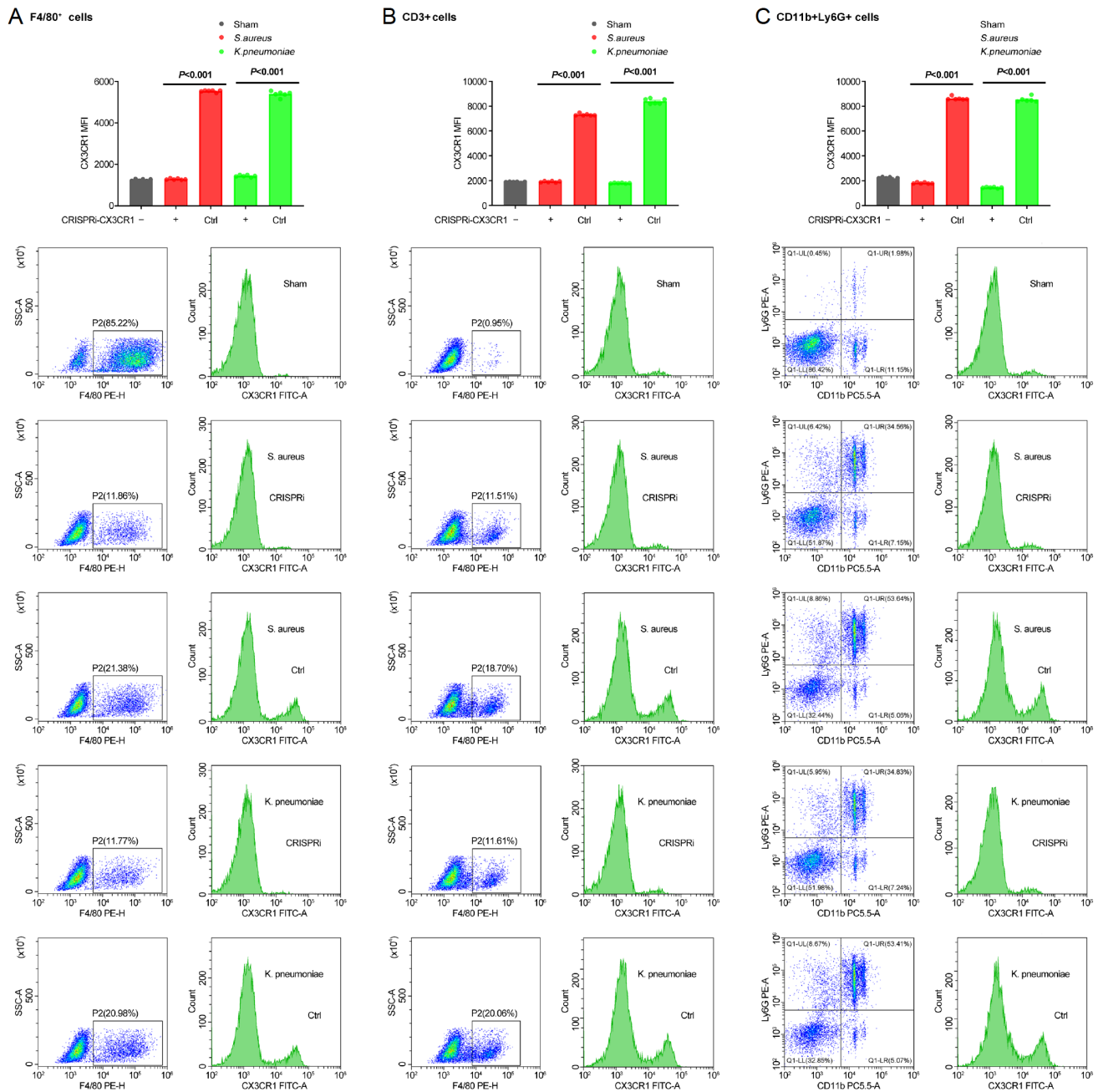


Figure 5. CX3CR1 expression on macrophage, T cell, and neutrophil subsets in BALF from mice with bacterial pneumonia Mice were injected with AAV-CRISPRi-Ctrl or AAV-CRISPRi-CX3CR1 and subsequently infected with *S. aureus* or *K. pneumoniae* as described in Figure 4. On day 5 postinfection, BALF cells were collected and analyzed by flow cytometry. (A–C) CX3CR1 expression was assessed on (A) macrophages (F4/80⁺ cells), (B) T cells (CD3⁺ cells), and (C) neutrophils (CD11b⁺Ly6G⁺ cells). Representative histograms (left panels) and quantitative analysis of mean fluorescence intensity (MFI) (right panels) are shown. Data are presented as the mean $\pm$ SEM ($n = 6$ mice per group). Statistical significance was determined by one-way ANOVA with Tukey's *post hoc* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the corresponding AAV-CRISPRi-Ctrl group infected with the same pathogen.

directional migration of immune cells to sites of infection, has not been fully elucidated in pneumonia. Through large-cohort analysis, this study is the first to identify low the expression of the chemokine receptor CX3CR1 as an independent risk factor for 28-day mortality in pneumonia patients, with its expression in peripheral blood being

significantly lower in nonsurvivors than in survivors. This finding shifts the focus of prognostic assessment from “level of inflammation” to the more fundamental functional aspect of “immune cell migratory capacity”. As the receptor for its ligand CX3CL1 (Fractalkine), CX3CR1 plays a central role in mediating the

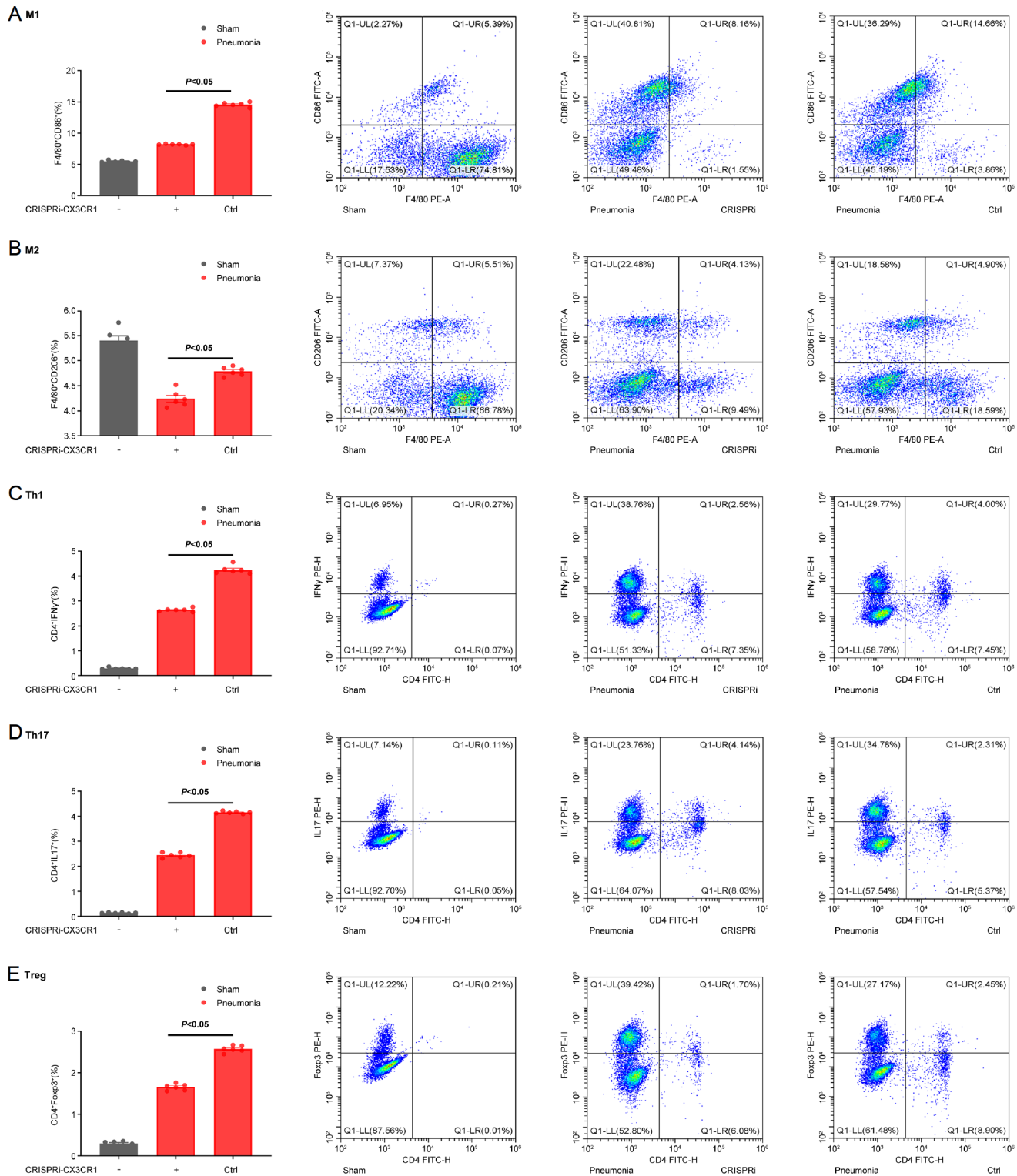


Figure 6. Proportions of macrophage and CD4⁺ T-cell subsets in BALF from mice with bacterial pneumonia Mice were injected with AAV-CRISPRi-Ctrl or AAV-CRISPRi-CX3CR1 and subsequently infected intratracheally to establish bacterial pneumonia as described in Figure 4. On day 5 postinfection, BALF cells were collected and analyzed by flow cytometry. (A–E) The percentages of (A) M1 macrophages (F4/80⁺CD86⁺), (B) M2 macrophages (F4/80⁺CD206⁺), (C) Th1 cells (CD4⁺IFN- γ ⁺), (D) Th17 cells (CD4⁺IL-17A⁺), and (E) Treg cells (CD4⁺Foxp3⁺) among total BALF cells are shown. Data are presented as the mean $\pm$ SEM ($n = 6$ mice per group). Statistical significance was determined by unpaired Student's t test.

migration of monocytes and lymphocytes into tissues, and its signaling axis is crucial for maintaining pulmonary immune homeostasis [16]. Cross-validation in two independent cohorts (the public database GSE65682 and our prospective institutional cohort) consistently confirmed the strong association between CX3CR1 expression and patient survival, significantly enhancing the reliability of this discovery. Consequently, the CX3CR1 expression level holds promise as a novel prognostic biomarker. Its value lies in its potential to warn of underlying impaired immune cell “homing” function, thereby identifying patients who, despite receiving conventional anti-infective therapy, remain at high risk due to inadequate immune clearance.

It is well established that impaired migration of immune cells to infection sites during pneumonia pathogenesis is closely associated with poor outcomes [16,17]. However, the current understanding of this functional phenotype largely remains at the observational level. The precise upstream molecular regulatory mechanisms, particularly epigenetic regulation, are not fully understood. While studies suggest that epigenetic modifications play a key role in immune cell programming [18–22] and that DNA methylation can directly regulate CX3CR1 expression in T cells and affect their migration, our study’s innovation extends beyond validating this association. By performing systematic molecular interaction assays in two key primary immune cell types—CD14⁺ monocytes and CD3⁺ T cells—derived from pneumonia patients, we delineated a mechanistic cascade linking cg00262061 hypermethylation to CX3CR1 transcriptional suppression. DNA pull-down, chromatin immunoprecipitation, and luciferase reporter assays consistently demonstrated that methylation at this site is associated with reduced RNAP II occupancy at the promoter and diminished transcriptional activity. These findings are consistent with impaired RNAP II recruitment rather than direct physical blockade. Thus, our work moves beyond phenomenological observation by functionally implicating a specific DNA methylation event as a key epigenetic switch associated with defective immune cell homing in pneumonia patients. This provides mechanistic insight into pneumonia-related immune dysregulation from an epigenetic perspective and highlights a potential axis for future therapeutic exploration.

This study systematically reveals the central role of the CX3CR1-cg00262061 axis in the immunopathology of pneumonia. Our work not only establishes the dysregulation of a crucial chemokine receptor as an independent prognostic factor but also, by elucidating its upstream epigenetic suppression mechanism, offers a fresh molecular perspective for understanding the phenomenon of “immune paralysis”, particularly in severe pneumonia. From a translational perspective, this discovery provides a mechanistic basis for potential prognostic applications. For instance, detecting CX3CR1 expression or cg00262061 methylation status in peripheral blood mononuclear cells may aid in identifying patients at higher risk of immune dysfunction and poor outcomes, enabling more refined risk stratification. Therapeutically, this epigenetic axis could represent a conceptual target; however, the feasibility of such interventions remains to be established. Important challenges include the lack of evidence regarding the reversibility of this specific methylation mark *in vivo*, as well as safety concerns associated with systemic demethylation (e.g., off-target effects) or CX3CR1 agonism (e.g., potential exacerbation of inflammatory injury due to enhanced leukocyte recruitment). Further studies are needed to determine whether targeted modulation of this pathway

is achievable and beneficial in the clinical setting.

How CX3CR1 promoter hypermethylation is established in a subset of pneumonia patients remains unknown. We speculate that infection-driven inflammation may contribute to this epigenetic modification. Severe bacterial infection and the accompanying inflammatory response could create a microenvironment that favors *de novo* methylation at specific genomic loci, including the CX3CR1 promoter. However, the exact upstream signals and molecular mediators remain to be identified. Future studies are needed to determine whether inflammatory cytokines, pathogen components, or other host factors actively induce this repressive methylation mark.

In addition to DNA methylation, other epigenetic mechanisms, such as histone acetylation and methylation, may also contribute to CX3CR1 transcriptional regulation [23]. For instance, permissive histone marks (e.g., H3K4me3) at the promoter region are associated with active transcription, whereas repressive marks (e.g., H3K27me3, H3K9me3) can promote gene silencing [24–26]. Emerging evidence suggests that crosstalk between DNA methylation and histone modifications is critical for immune cell function and inflammatory responses. Whether such epigenetic interplay affects CX3CR1 expression and disease severity in bacterial pneumonia remains to be explored in future studies.

While our study focused on CX3CR1 as the effector molecule, its cognate ligand CX3CL1 (fractalkine) is equally critical for immune cell trafficking. The CX3CL1-CX3CR1 axis has attracted interest as a therapeutic target in inflammatory diseases, but the optimal intervention strategy remains unclear. Targeting the receptor (CX3CR1) with small-molecule antagonists or blocking antibodies could directly inhibit immune cell migration but may cause broad immunosuppression due to the wide expression of CX3CR1 on multiple immune subsets (monocytes, T cells, and NK cells). Conversely, targeting the ligand (CX3CL1)—for example, by blocking its cleavage from the membrane-bound form or by neutralizing soluble CX3CL1—might offer more nuanced modulation, preserving CX3CR1-dependent homeostatic signals while reducing pathological recruitment. However, the functional redundancy of chemokine systems and the incomplete understanding of CX3CL1 proteolysis in pneumonia pose major challenges. Therefore, from a translational perspective, further preclinical studies are needed to compare the efficacy and safety of CX3CR1-targeted versus CX3CL1-targeted interventions in relevant pneumonia models. Our current findings provide a mechanistic rationale for such future investigations.

Several limitations of this study should be acknowledged. First, the small sample size of our prospective cohort ($n = 38$, with only 7 methylation-positive cases) limits the statistical power and stability of our prognostic analyses. The high hazard ratio (HR = 20.1) observed in multivariate analysis is likely inflated, and LASSO-Cox bootstrap validation revealed substantial overfitting (original C-index 0.72 vs bootstrap median 0.54). Therefore, these clinical findings are exploratory, and the predictive value of cg00262061 methylation requires validation in larger, independent cohorts. Second, while our *in vivo* experiments establish a causal link between low CX3CR1 expression and poor outcomes in bacterial pneumonia, the use of systemic AAV9-CRISPRi delivery results in CX3CR1 knockdown across multiple tissues and cell types. This approach limits our ability to distinguish whether the observed phenotype is primarily driven by impaired peripheral immune cell

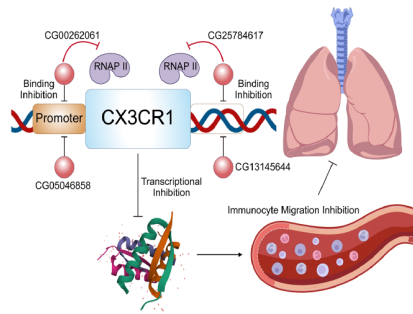


Figure 7. Schematic diagram of the proposed mechanism Under normal conditions, the chemokine receptor CX3CR1 mediates the migration of immune cells from the circulation to infected lung tissue, facilitating pathogen clearance. This study revealed that aberrant hypermethylation occurs at specific sites (e.g., cg00262061) within the *CX3CR1* promoter in a subset of pneumonia patients. This epigenetic modification impedes the binding of RNA polymerase II to the promoter, thereby suppressing *CX3CR1* transcription. The down-regulation of CX3CR1 impairs the recruitment of immune cells to the lungs and reduces pathogen clearance efficiency, ultimately leading to increased bacterial load, aggravated lung injury, and poor patient prognosis.

migration, alterations in the pulmonary microenvironment, or a combination of both. It is worth noting that CX3CR1 is predominantly expressed on immune cells (monocytes/macrophages, NK cells, and certain T-cell subsets), with minimal or undetectable expression on lung endothelial or epithelial cells under physiological or inflammatory conditions [25,26]. Moreover, our competitive adoptive transfer experiment demonstrated that *CX3CR1*-deficient immune cells have a cell-intrinsic migration defect, as they were significantly outcompeted by control cells when co-injected into wild-type recipients. This result strongly supports that impaired recruitment is largely driven by immune cell-autonomous effects rather than by indirect alterations of the lung microenvironment. Nevertheless, our model should be interpreted as demonstrating the consequence of systemic CX3CR1 suppression rather than cell-type-specific inhibition. Accordingly, we have revised the manuscript to consistently describe the intervention as “systemic inhibition” or “global *CX3CR1* knockdown”. Future studies employing cell-type-specific conditional knockout models (e.g., LysM-Cre for myeloid cells, CD4-Cre for T cells) will be essential to precisely delineate the cellular compartment responsible for the protective effects of CX3CR1 during pneumonia.

In summary, our results integrate clinical and mechanistic evidence to demonstrate that promoter hypermethylation at the cg00262061 site is associated with *CX3CR1* silencing, defective immune cell migration, and adverse outcomes in pneumonia. This epigenetic modification is linked to suppressed gene transcription, impaired immune cell migration, and reduced pulmonary bacterial clearance (Figure 7). Our work delineates a previously unrecognized pathway underlying immune dysregulation in pneumonia, spanning epigenetic regulation, gene expression, cellular function, and clinical prognosis. These findings offer a mechanistic basis for prognostic assessment and highlight a potential avenue for future investigation into targeted therapeutic strategies.

Supplementary Data

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

Data Availability

The high-throughput transcriptome data for the pneumonia patient cohort analyzed in this study are publicly available in the GEO repository under accession number GSE65682. DNA methylation data are available in the [Supplementary Materials](#). All other data supporting the findings of this study, including raw data and original images, are available from the corresponding author upon reasonable request.

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Author Contributions

S.Z.: conceptualization, funding acquisition, investigation, writing—original draft, writing—review & editing. Y.J., Q.C., and Y.Z.: investigation, methodology, validation, formal analysis. X.J.: discussion. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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