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Research article

AKT/p65-dependent upregulation of CD64 by LPS drives pathogenesis and diagnostic potential in sepsis ☆

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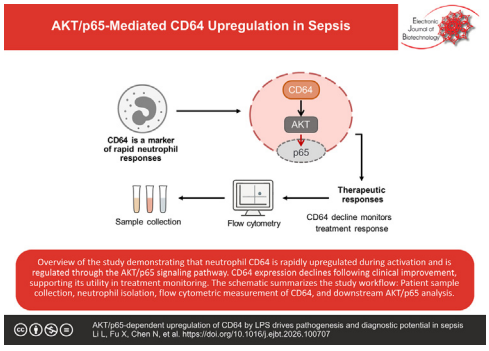
GRAPHICAL ABSTRACT

AKT/p65-dependent upregulation of CD64 by LPS drives pathogenesis and diagnostic potential in sepsis.



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ABSTRACT

Background: Sepsis is a life-threatening condition characterized by organ dysfunction caused by a dysregulated host response to infection. Despite improvements in clinical management, both incidence and mortality remain high. Identifying biomarkers with high sensitivity and specificity is critical for early diagnosis. CD64, an Fcγ receptor upregulated on neutrophils during infection, has emerged as a promising diagnostic indicator. This study aimed to evaluate the diagnostic and mechanistic role of CD64 in pediatric sepsis.

Results: A total of 200 children with sepsis and 166 healthy controls were enrolled. At a cutoff value of 0.165, the CD64 index achieved a diagnostic sensitivity of 87.0% and specificity of 92.8%, outperforming

Abbreviations: AUC, Area under the ROC curve; BCA, Bicinchoninic acid; BET, Bromodomain and extra-terminal; BRD4, Bromodomain-containing protein 4; ChIP, Chromatin immunoprecipitation; CRP, C-reactive protein; ERK, Extracellular Signal-Regulated Kinase; FBS, Fetal bovine serum; HDAC3, Histone Deacetylase 3; HIPK2, Homeodomain-Interacting Protein Kinase 2; HRP, Horseradish peroxidase; IFN-γ, Interferon-gamma; IL-1β, Interleukin-1β; IL-6, Interleukin-6; LPS, Lipopolysaccharide; MFI, Mean fluorescence intensity; Myc, Myelocytomatosis oncogene cellular homolog; NPV, Negative predictive value; PTX, Pentoxifylline; PMSF, Phenylmethylsulfonyl fluoride; PBS, Phosphate-buffered saline; p-AKT, Phosphorylated AKT; PMNs, Polymorphonuclear leukocytes; PVDF, Polyvinylidene difluoride; PPV, Positive predictive value; PCT, Procalcitonin; AKT, Protein Kinase B; PDTC, Pyrrolidine Dithiocarbamate; qRT-PCR, quantitative Real-Time Reverse Transcription Polymerase Chain Reaction; WB, Western blot; ROC, Receiver operating characteristic; RPMI, Roswell Park Memorial Institute Medium; SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; SDrats, Sprague-Dawley rats; SD, Standard Deviation; TBST, Tris-Buffered Saline with Tween 20.

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NF-κB
P65
Sepsis

conventional markers such as procalcitonin (PCT), C-reactive protein (CRP), interleukin-1β (IL-1β), and interleukin-6 (IL-6). Mechanistic assays demonstrated that lipopolysaccharide (LPS) stimulation induced CD64 expression through the Protein Kinase B (AKT) signaling pathway. Both LPS exposure and AKT overexpression promoted p65 nuclear translocation, and chromatin immunoprecipitation confirmed p65 binding to the CD64 promoter, thereby enhancing CD64 transcription.

Conclusions: CD64 exhibits superior diagnostic and prognostic performance compared to traditional inflammatory markers and serves as a reliable biomarker for pediatric sepsis. Mechanistically, CD64 upregulation is mediated by the AKT/p65 signaling axis. These findings provide a foundation for integrating CD64 into early diagnostic workflows and developing targeted therapeutic strategies in sepsis management.

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1. Introduction

Sepsis is a life-threatening syndrome defined by organ dysfunction resulting from a dysregulated host response to infection [1,2]. Owing to its complex pathophysiology, ongoing therapeutic challenges, and high mortality, it has become a major focus of public health concern [3]. While sepsis progression is often rapid and unpredictable, its pathogenesis is driven by well-characterized pathological processes—fundamentally reflecting the body's maladaptive reaction to infectious agents. Despite advances in anti-infective therapies and organ support technologies in modern clinical practice, the global mortality rate for pediatric septic shock and severe sepsis remains alarmingly high at 25% [3]. Beyond the human cost, sepsis is associated with substantial healthcare expenses and intensive medical resource consumption: these not only impose a heavy burden on healthcare systems but also exert a profound negative impact on patients' quality of life. Collectively, these challenges underscore the critical need for early identification and severity assessment of sepsis, highlighting the urgent demand for reliable biomarkers that can aid in diagnosis, guide therapy, and predict prognosis.

Recent studies have focused on improving sepsis diagnosis through integrative biomarker models. For example, Liu et al. [4] proposed the LIP score, a composite index combining lymphocyte count, international normalized ratio, and procalcitonin (PCT), to enhance diagnostic sensitivity and specificity in clinical practice. These advancements highlight the importance of developing novel immune-related markers, such as CD64, for accurate and rapid sepsis assessment.

CD64 (Fcγ receptor I), a transmembrane glycoprotein and high-affinity receptor for the Fc segment of IgG, acts as a critical mediator of immune responses [5,6]. It is constitutively expressed on macrophages, monocytes, and dendritic cells, while its expression on neutrophils is negligible under physiological conditions [7]. Notably, resting neutrophils display minimal CD64 levels; however, upon bacterial stimulation or endotoxin exposure, CD64 expression on these cells is rapidly upregulated within hours [8,9]. Upon ligand binding, this receptor triggers immune amplification cascades as early as 4–6 h post-activation. Conversely, when inflammatory stimuli are effectively controlled, CD64 levels decrease within 48 h and return to baseline within 7 d. Importantly, flow cytometry-based detection of CD64 in readily accessible peripheral blood samples offers a simple, accurate, and reproducible method for quantifying this receptor—facilitating its clinical application.

Furthermore, accumulating evidence suggests that transcriptional regulatory networks play a crucial role in modulating immune gene expression [10]. This offers a mechanistic framework for investigating the transcription-dependent regulation of CD64 expression in sepsis. The dynamic responsiveness of CD64 renders it particularly valuable for neonatal sepsis diagnosis, a setting where rapid biomarker detection is critical to improving outcomes. Clinical evidence has confirmed marked overexpression of CD64 on neutrophils during bacterial infections [10,11,12], further validating its diagnostic utility in sepsis [13]. Standardizing CD64 quantification as an "infection index" could significantly enhance clinical practice by enabling sensitive detection of early bacterial infections. Such standardization would support timely clinical interventions, ultimately contributing to improved management and prognosis for patients with sepsis.

2. Materials and methods

2.1. Patients

Children diagnosed with sepsis were identified in accordance with the International Pediatric Sepsis Consensus Guidelines to form the study cohort [14]. A total of 200 septic children and 166 healthy controls (aged 0–14 years) were enrolled, with a balanced gender distribution (roughly equal numbers of males and females). Written informed consent was obtained from the guardians of all participants (septic patients and healthy controls) prior to study initiation. The research protocol was approved by the Institutional Ethics Committee of the authors' affiliated hospital. For septic children, peripheral blood samples were collected within 6 h of hospital admission, followed by immediate quantification of the CD64 index and other infection-related biomarkers.

2.2. Neutrophil isolation

Peripheral anticoagulated blood was collected and diluted 1:1 with sterile phosphate-buffered saline (PBS, pH 7.4) by gentle inversion. The diluted blood was carefully layered over an equal volume of lymphocyte separation medium (Ficoll-Paque Premium, GE Healthcare, USA) in a sterile centrifuge tube, ensuring a distinct interface between the two phases. Centrifugation was performed at 2500 ×g for 15 min at room temperature. After centrifugation, the buffy coat layer (containing mononuclear cells) was carefully aspirated and discarded. The remaining granulocyte-rich fraction was resuspended in 10-fold volume of erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM KHCO3, 0.1 mM EDTA, pH 7.4) and incubated for 20 min at 4°C to lyse red blood cells. Subsequently, centrifugation was conducted at 2000 ×g for 10 min at 4°C to pellet polymorphonuclear leukocytes (PMNs).

The crude PMN pellet was resuspended in sterile PBS and further purified via discontinuous Percoll density gradient centrifugation. Specifically, a two-layer Percoll system was prepared using 75% (v/v) and 60% (v/v) isotonic Percoll solutions (diluted with PBS containing 10% fetal bovine serum (FBS)). After centrifugation at 1200 ×g for 30 min at 4°C, the PMN-enriched fraction (concentrated at the 60–75% Percoll interface) was collected using a sterile Pasteur pipette. Finally, PMNs were washed three times with PBS to remove residual Percoll particles, and cell viability was verified via trypan blue exclusion (≥ 95% viability required for subsequent experiments).

2.3. Antibodies and Western blot (WB) analysis

The following primary antibodies were employed for WB analysis: anti-GAPDH (1:10,000; Proteintech, USA), anti-AKT (1:1000; Cell Signaling Technology, USA), anti-phosphorylated (p)-AKT (S473, 1:1000; Cell Signaling Technology, USA), anti-ERK (1:1000; Cell Signaling Technology, USA), anti-p-ERK (1:1000; Cell Signaling Technology, USA), anti-c-Myc (1:500; Santa Cruz Biotechnology, USA), anti-CD64 (1:1000; Bioss Antibodies, China), anti-p65 (1:500; Santa Cruz Biotechnology, USA), and anti-p-p65 (1:1000; ABclonal, China). Horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse secondary antibodies (both 1:10,000; Vicmed, China) were used for detection.

Cells were harvested and washed twice with cold PBS, followed by lysis in RIPA buffer (Vicmed, China) containing 1% phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, USA) to prevent proteolysis. The lysates were centrifuged at 14,000 ×g for 15 min at 4°C, and the supernatant containing total protein was collected. Protein concentration was determined using a bicinchoninic acid (BCA) assay kit (Pierce, USA) according to the manufacturer's instructions.

Equal amounts of protein were separated by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The membranes were blocked for 2 h at room temperature with 5% non-fat milk in Tris-Buffered Saline with Tween 20 (TBST) and then incubated overnight at 4°C with the indicated primary antibodies. After three washes with TBST, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The signals were detected using Chemistar™ High-sig ECL WB Substrate (Tanon, Shanghai, China).

2.4. Cell culture and transfection

Neutrophils were cultured in Roswell Park Memorial Institute Medium (RPMI) 1640 medium (Bio-Channel, China) supplemented with 10% FBS (Gibco, USA) and 1% penicillin–streptomycin (Vicmed, China) and maintained at 37°C in a 5% CO_2 humidified atmosphere.

For transient transfection, plasmids encoding Protein Kinase (AKT), p65, or an empty control vector were transfected into neutrophils using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. In brief, 2 μg of plasmid DNA was combined with Lipofectamine 2000 at 1:2.5 in RPMI 1640 medium and incubated for 20 min at room temperature to form complexes. The complexes were added to neutrophils seeded at 1×10^6 cells per well in 6-well plates.

2.5. Isolation of primary human neutrophils

Peripheral blood neutrophils were isolated using a standard Percoll density gradient method as previously described, which yields >95% purity with minimal activation. Fresh whole blood was layered onto a discontinuous Percoll gradient and centrifuged at 500 ×g for 30 min at room temperature without a brake. The neutrophil layer was collected, washed twice with PBS, and immediately assessed for purity and viability. Only preparations with ≥95% viability (trypan blue exclusion) were used for subsequent experiments.

2.6. Neutrophil culture conditions and LPS stimulation

Given the short lifespan of primary neutrophils *in vitro*, all experiments were performed within 24 h of isolation. Neutrophils were resuspended at 1×10^6 cells/mL in RPMI 1640 (Bio-Channel, China) complete medium supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 1% penicillin–streptomycin (Vicmed, China), 10 ng/mL Interferon-gamma (IFN-γ), 1 ng/mL GM-CSF, 30 ng/mL interleukin-1β (IL-1β), and 3 mM glucose, conditions known to maintain neutrophil functional viability *ex vivo*. For activation assays, cells were stimulated with lipopolysaccharide (LPS) (100 ng/mL; Sigma-Aldrich) for 60 min at 37°C in a 5% CO_2 incubator, a protocol widely used for rapid neutrophil priming.

2.7. Transfection of primary neutrophils

Primary human neutrophils were isolated by Percoll density gradient centrifugation and immediately subjected to transfection to minimize viability loss. Neutrophils ($1–2 \times 10^6$ cells) were resuspended in P3 Primary Cell Nucleofector™ Solution (P3 Kit, Lonza, Basel, Switzerland), mixed with siRNA (100 pmol) or plasmid DNA (1–2 μg), and electroporated using the 4D-Nucleofector™ System (Lonza) with program EO-100, which is optimized for terminally differentiated myeloid cells. Following electroporation, cells were gently transferred into pre-warmed RPMI-1640 medium supplemented with 10% FBS (Gibco, USA) and 1% penicillin/streptomycin.

To support short-term post-transfection viability, cultures were supplemented with IFN-γ (10 ng/mL), GM-CSF (1 ng/mL), and IL-1β (30 ng/mL), all purchased from PeproTech (Rocky Hill, NJ, USA). Cells were incubated at 37°C with 5% CO_2, and functional assays were performed 2–6 h post-transfection, during which exogenous gene expression is detectable and neutrophil viability remains ≥75%.

Cell viability was monitored using trypan blue exclusion (Sigma-Aldrich, Merck, St. Louis, MO, USA) and Annexin V–FITC/7-AAD Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA). Transfection efficiency was confirmed by flow cytometry or fluorescence microscopy for plasmid-based expression, and by quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR) or WB for siRNA-mediated knockdown.

2.8. Assessment of neutrophil viability

Neutrophil viability was monitored at three key stages: immediately after Percoll isolation, before stimulation/transfection, and following experimental treatments (6–24 h). Viability assessments were performed using both trypan blue exclusion for rapid screening and Annexin V/7-AAD flow cytometry for precise quantification of apoptotic changes. Across all experiments, neutrophil viability remained above 75% during the 6–24 h culture window, ensuring the reliability of downstream measurements.

2.9. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cultured cells and reverse-transcribed into cDNA. qRT-PCR was performed using SYBR Green PCR Master Mix (Thermo Fisher Scientific, USA). Gene expression was analyzed using the comparative threshold cycle ($2^{-\Delta\Delta}$CT)

method, with GAPDH as the endogenous control. All reactions were performed in triplicate. The primer sequences used were listed in Table 1. CD64-specific primers were commercially designed and synthesized by Sangon Biotech (Shanghai, China).

2.10. Flow cytometry

Cells were incubated in RPMI 1640 medium containing 10% FBS and 0.1% sodium azide for 30 min at 4°C to block non-specific binding. After washing, the cells were stained with FITC-conjugated anti-CD64 antibody (0.5 μg/mL) in DMEM supplemented with 10% FBS and 0.1% sodium azide for 30 min at 4°C.

Samples were acquired on a BD LSRFortessa flow cytometer (BD Biosciences, USA) and analyzed using FlowJo software version 10 (TreeStar, USA). Single cells were gated based on forward and side scatter properties, exclusion of dead cells (using fixable Aqua live/dead stain; Invitrogen), and specific surface staining. The following monoclonal antibody was used: anti-CD64.

All data were collected using CellQuest software on a FACScan instrument (Becton Dickinson), with a minimum of 10^5 events acquired per sample. Results are presented as histograms showing fluorescence intensity on a logarithmic scale.

2.11. Nuclear-cytoplasmic fractionation

The nuclear-cytoplasmic separation was performed using the Beyotime Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, P0027) according to the manufacturer's instructions. The collected cell pellet was resuspended in Cytoplasmic Protein Extraction Reagent A containing PMSF, vortexed vigorously, and incubated on ice for 15 min. Cytoplasmic Protein Extraction Reagent B was then added, followed by vortexing and incubation on ice for 1 min. The mixture was vortexed again and centrifuged at 16,000 g for 5 min at 4°C. The supernatant was collected as the cytoplasmic fraction. The remaining pellet was resuspended in Nuclear Protein Extraction Reagent containing PMSF, vortexed for 30 s, and incubated on ice for 30 min with intermittent shaking every 5 min. The mixture was then centrifuged at 16,000 g for 10 min at 4°C, and the supernatant was collected as the nuclear protein fraction.

2.12. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed using the ChIP Assay Kit (Beyotime, Shanghai, China) in accordance with the manufacturer's instructions. Briefly, cells were cross-linked with formaldehyde, lysed, and sonicated to shear chromatin. Immunoprecipitation was carried out with anti-p65 antibodies. After reversing cross-links, purified DNA was analyzed by qPCR.

2.13. Animal model

Sprague-Dawley rats (SD rats) (7 weeks old, 210–230 g) were obtained from a local animal center (Xuzhou, China). The animals were housed under a 12 h light/dark cycle in a temperature-controlled environment with free access to standard food and water. All procedures involving animals were conducted in accordance with international guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Ethics Committee.

Rats were randomly assigned to three experimental groups (n = 6 per group): (1) Control group; (2) LPS group: administered 15 mg/kg LPS; (3) LPS + pentoxifylline (PTX) group: administered 25 mg/kg pentoxifylline (PTX) along with LPS. The dosage of PTX was selected based on previous studies. Rats in the LPS + PTX group received intraperitoneal injections of PTX once daily for 7 d. Control and LPS groups received equal volumes of sterile PBS. One hour after the final PTX injection, rats in the LPS and LPS + PTX groups were injected intraperitoneally with LPS, while control rats received PBS. Six hours after the LPS challenge, all animals were anesthetized, and blood samples were collected via cardiac puncture for subsequent analysis.

Table 1
Primer sequences used in this study.

Primers	Primer sequences (5′ to 3′)
GAPDH-F	5′-TGCACCACCAACTGCTTAGC-3′
GAPDH-R	5′-AGCTCAGGGATGACCTTGCC-3′
AKT-F	5′-GAAGGACGGGAGCAGGC-3′
AKT-R	5′-AAGGTGCGTTCGATGACAGT-3′
P65-F	5′-ATGTGGAGATCATTGAGCAGC-3′
P65-R	5′-CCTGGTCCTGTGTAGCCATT-3′

2.14. CD64 index calculation and cutoff determination

CD64 expression on neutrophils was quantified using flow cytometry and expressed as a CD64 index. The CD64 index was calculated as the ratio of the mean fluorescence intensity (MFI) of CD64 on neutrophils to the MFI of the internal negative control population (lymphocytes) within the same **[Equation 1]**:

$$\text{CD64 index} = \frac{\text{MFICD64, neutrophils}}{\text{MFICD64, lymphocytes}} \quad (1)$$

A cutoff value of 0.165 was determined based on ROC curve analysis to distinguish sepsis patients from non-sepsis controls. This threshold corresponded to the optimal point on the ROC curve (Youden index), providing the best combined sensitivity and specificity. All CD64-related analyses in the Results section were performed using this cutoff.

2.15. Statistical analysis

All statistical analyses were performed using SPSS 17.0 and GraphPad Prism 8 (version 8.0.2). Data are presented as mean ± Standard Deviation (SD) from at least three independent experiments. Differences among groups were assessed by one-way analysis of variance (ANOVA), followed by the Newman-Keuls posthoc test. The assumptions of normality and homogeneity of variance were verified using the Shapiro–Wilk test and Levene's test, respectively. A p-value < 0.05 was considered statistically significant.

Receiver operating characteristic (ROC) curves were generated to determine the area under the curve (AUC), along with the sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) at the optimal cut-off point. ROC curve analyses were performed using GraphPad Prism 8.0.2 (GraphPad Software, USA), with validation by SPSS 17.0 (IBM, USA).

3. Results

3.1. CD64 outperforms other biomarkers in sepsis diagnosis and prognosis

Biomarkers play a critical role in the early diagnosis and prognostic evaluation of sepsis. Multiple authoritative studies have identified CD64 as a promising candidate biomarker for sepsis [15,16]. To further validate this finding, we analyzed clinical data from pediatric sepsis patients and healthy controls, and compared the diagnostic performance of CD64 with other commonly used infection-related biomarkers.

Over a two-year period, clinical data from 200 children with sepsis and 166 healthy children were collected and analyzed. ROC curves were generated for PCT, CRP, CD64, IL-1β, and IL-6. As shown in Fig. 1A, the area under the ROC curve (AUC) values were 0.776 (95% CI: 0.712–0.812) for PCT, 0.834 (95% CI: 0.789–0.878) for CRP, 0.902 (95% CI: 0.867–0.926) for CD64, 0.726 (95% CI: 0.672–0.779) for IL-1β, and 0.830 (95% CI: 0.787–0.874) for IL-6. CD64 exhibited the largest AUC among all biomarkers, indicating superior diagnostic efficiency. Detailed diagnostic metrics (sensitivity, specificity, PPV, and NPV) for all biomarkers are now summarized in Table 2 to improve clarity and readability, as recommended by the reviewer. To assess the prognostic value of biomarkers, we further analyzed the dynamic changes in the five infection-related indicators before and after treatment in 30 sepsis patients. As shown in Fig. 1B, no statistically significant differences were observed in PCT or IL-6 levels between the pre-treatment and post-treatment periods ($p > 0.05$). In contrast, significant reductions were detected in CD64, CRP, and IL-1β levels after treatment ($p < 0.05$), with CD64 showing the most consistent and clinically relevant changes.

3.2. LPS upregulates the expression of CD64

According to the Third International Consensus on Sepsis and Septic Shock [2], a large epidemiological study conducted across Asian countries and regions indicated that the pathogens responsible for sepsis primarily include bacteria, fungi, and viruses, with bacterial infections—especially those caused by *Gram-negative bacilli*—being the most prevalent [17]. It is important to emphasize that LPS, also known as endotoxin, constitutes a major structural component of the outer membrane of most *Gram-negative bacteria* and plays a central role in their pathogenicity. Although the involvement of LPS in the pathology of sepsis has been extensively documented, many mechanistic details remain incompletely elucidated. To address this, we sought to investigate the signaling mechanisms triggered by LPS in bacterial sepsis.

First, whole blood from healthy children was co-cultured with Gram-negative *Escherichia coli* or Gram-positive *Staphylococcus aureus*. Flow cytometry was used to monitor changes in the CD64 index over a 12-h period, with measurements recorded at 0, 2, 4, 6, 8, 10, and 12 h. The results demonstrated that *E. coli* (Gram-negative) induced a more pronounced upregulation of CD64 expression compared to *S. aureus* (Gram-positive) (Fig. 2A). As LPS is a key virulence factor that triggers inflammatory cells to release pro-inflammatory cytokines, nitric oxide, and eicosanoids—leading to tissue inflammation, endothelial dysfunction, and altered angiogenesis [18,19] – we next stimulated neutrophils with LPS for 6 h and assessed CD64 fluorescence intensity by flow cytometry. The LPS-treated group exhibited significantly stronger CD64 fluorescence compared to the control group (Fig. 2B). To further validate the relationship between sepsis and CD64, we performed WB analysis to examine the effect of LPS on CD64 protein expression. As shown in Fig. 2C, neutrophils

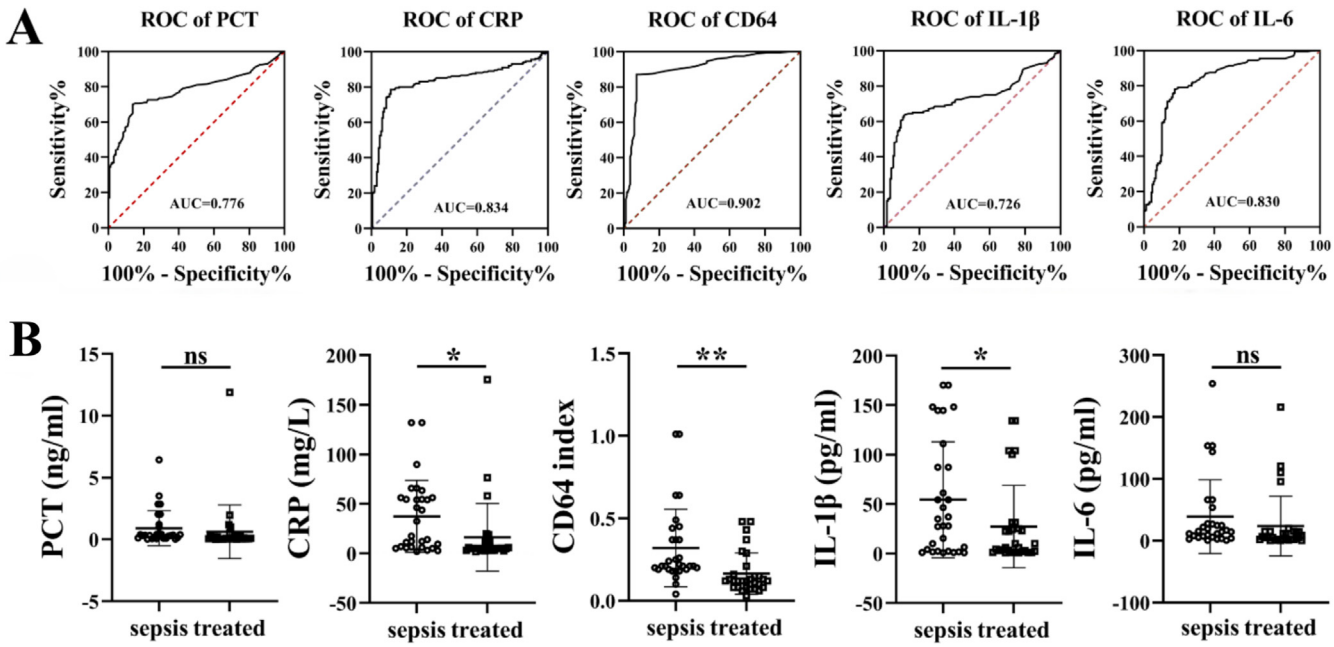

Fig. 1. CD64 exhibits superior diagnostic and prognostic value in patients with sepsis. (A) ROC curves of PCT, CRP, CD64, IL-1β, and IL-6 for sepsis diagnosis. CD64 showed the highest AUC (0.902). (B) Changes in CD64 expression before and after treatment in sepsis patients. CD64 levels were measured in paired peripheral blood samples collected from the same patient's pre-treatment and post-treatment. Data are presented as mean ± SD. Statistical comparisons were performed using a paired *t*-test. $p < 0.05$ was considered statistically significant. $^{*}p < 0.05$, $^{**}p < 0.01$. Circles or squares represent individual replicate data points for each experimental group.

Table 2
CD64 had better prognostic value compared to PCT, CRP, IL-1β, and IL-6.

	AUC (95% CI)	Cut-off	Sensitivity [%]	Specificity [%]	PPV [%]	NPV [%]
PCT [ng/ml]	0.776 (0.712–0.812)	0.265	70.0	86.1	85.0	68.9
CRP [mg/L]	0.834 (0.789–0.878)	11.000	78.5	89.2	89.2	77.4
CD64 index	0.902 (0.867–0.926)	0.165	87.0	92.8	93.5	85.6
IL-1β [pg/ml]	0.726 (0.672–0.779)	13.550	63.5	88.6	84.3	66.7
IL-6 [pg/ml]	0.830 (0.787–0.874)	5.850	78.0	82.5	83.2	76.1

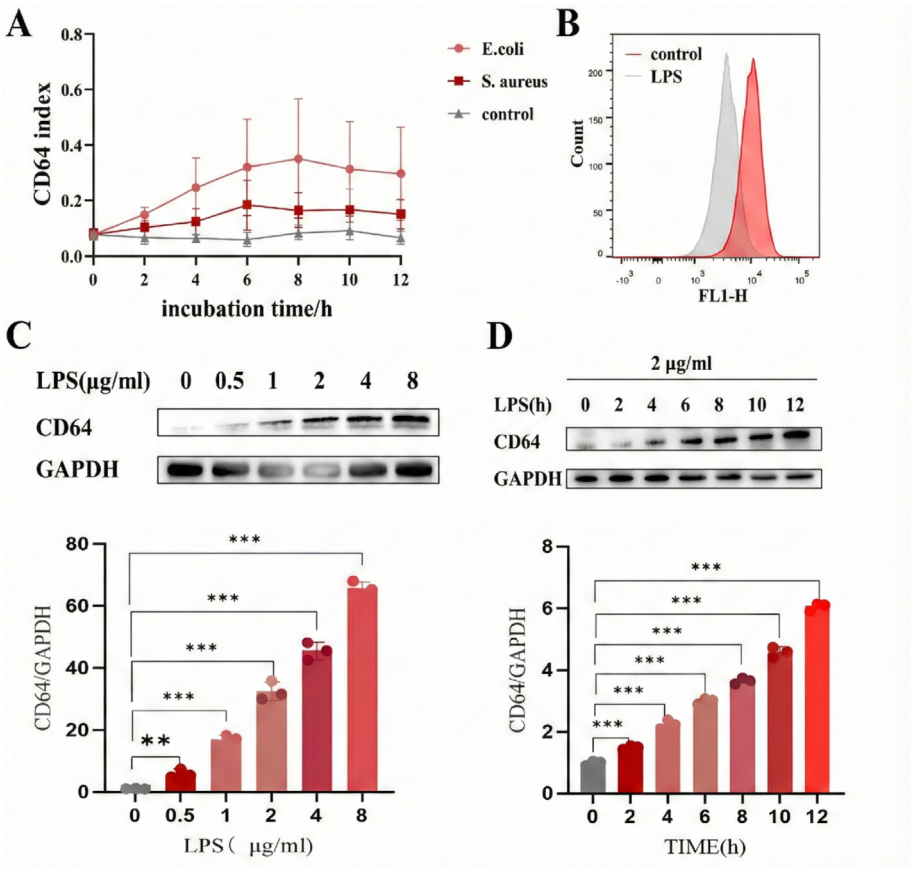

Fig. 2. LPS regulates CD64 expression in neutrophils. (A) Blood samples from healthy volunteers were stimulated with *E. coli* (*Gram-negative bacteria*) or *S. aureus* (*Gram-positive bacteria*), respectively. The expression of the surface biomarker CD64 (assessed as CD64 index) was analyzed by flow cytometry. (B) Neutrophils were stimulated with LPS for 6 h, and CD64 fluorescence intensity was determined by flow cytometry. (C) Neutrophils were incubated with LPS at different concentrations (0, 0.5, 1, 2, 4, or 8 μg/ml) for 6 h. CD64 expression levels were detected by WB analysis, as described in Section 2. (D) Neutrophils were treated with LPS for the indicated time periods, and CD64 expression was measured by WB analysis. ***$p < 0.001$; **$p < 0.01$. Circles represent individual replicate data points for each experimental group.

treated with increasing concentrations of LPS (0.5, 1, 2, 4, and 8 μg/mL) showed a dose-dependent increase in CD64 protein levels. Similarly, as illustrated in Fig. 2D, stimulation with 2.0 μg/mL LPS over a time course (2, 4, 6, 8, 10, and 12 h) resulted in a time-dependent upregulation of CD64.

3.3. LPS induces CD64 expression through the AKT pathway

The immune response in severe infections is governed by a limited set of mechanisms, and elucidating the signal transduction pathways involved at various stages of cellular activation could provide critical insights for therapeutic interventions aimed at inhibiting or blocking these signals. The molecular mechanism underlying CD64 upregulation in clinical sepsis remains poorly understood. A review of existing literature reveals that numerous receptors are activated in response to bacterial infections, raising the possibility of using receptor antagonists even prior to microbiological culture. Signaling pathway regulation is especially crucial during the early phase of infection [20].

LPS interacts with specific receptors on the surface of target cells—such as white blood cells and epithelial cells—triggering downstream signaling cascades that stimulate pro-inflammatory and immune responses, which may become dysregulated. To interrogate the upstream signaling pathways involved in LPS-induced CD64 upregulation, we first applied several pathway-specific inhibitors. Neutrophils were pretreated with JQ1, a selective bromodomain and extra-terminal (BET) inhibitor that disrupts Bromodomain-containing protein 4 (BRD4)-dependent transcriptional activation, including BRD4 – Myelocytomatosis oncogene cellular homolog (Myc) signaling, together with the Extracellular Signal-Regulated Kinase (ERK) inhibitor PD98059 and the AKT inhibitor wortmannin. Subsequent LPS stimulation revealed that only wortmannin markedly attenuated CD64 induction, whereas JQ1 and PD98059 had no appreciable effect (Fig. 3A–B). These findings indicate that BRD4–Myc–dependent transcription is not required for LPS-mediated CD64 upregulation, and further highlight AKT signaling as the principal upstream driver in this context. To further verify the role of the AKT pathway in LPS-mediated CD64 upregulation, an AKT overexpression plasmid was transfected into neutrophils. Overexpression of AKT resulted in increased CD64 protein levels and fluorescence intensity, as confirmed by WB, flow cytometry, and qPCR analyses (Fig. 3C–E). Additionally, neutrophils pre-treated with wortmannin before administration of SC79 (an AKT activator) showed markedly reduced CD64 expression and fluorescence intensity compared to the group treated with SC79 alone (Fig. 3F–H). These results indicate that SC79-induced CD64 expression can be attenuated by AKT pathway blockade. In summary, these findings demonstrate that LPS upregulates CD64 expression in neutrophils primarily through activation of the AKT signaling pathway.

3.4. AKT up-regulates CD64 expression via p65

Experimental results demonstrated that AKT enhances CD64 expression, whereas inhibition of AKT with wortmannin significantly reduced CD64 levels. However, the precise molecular mech-

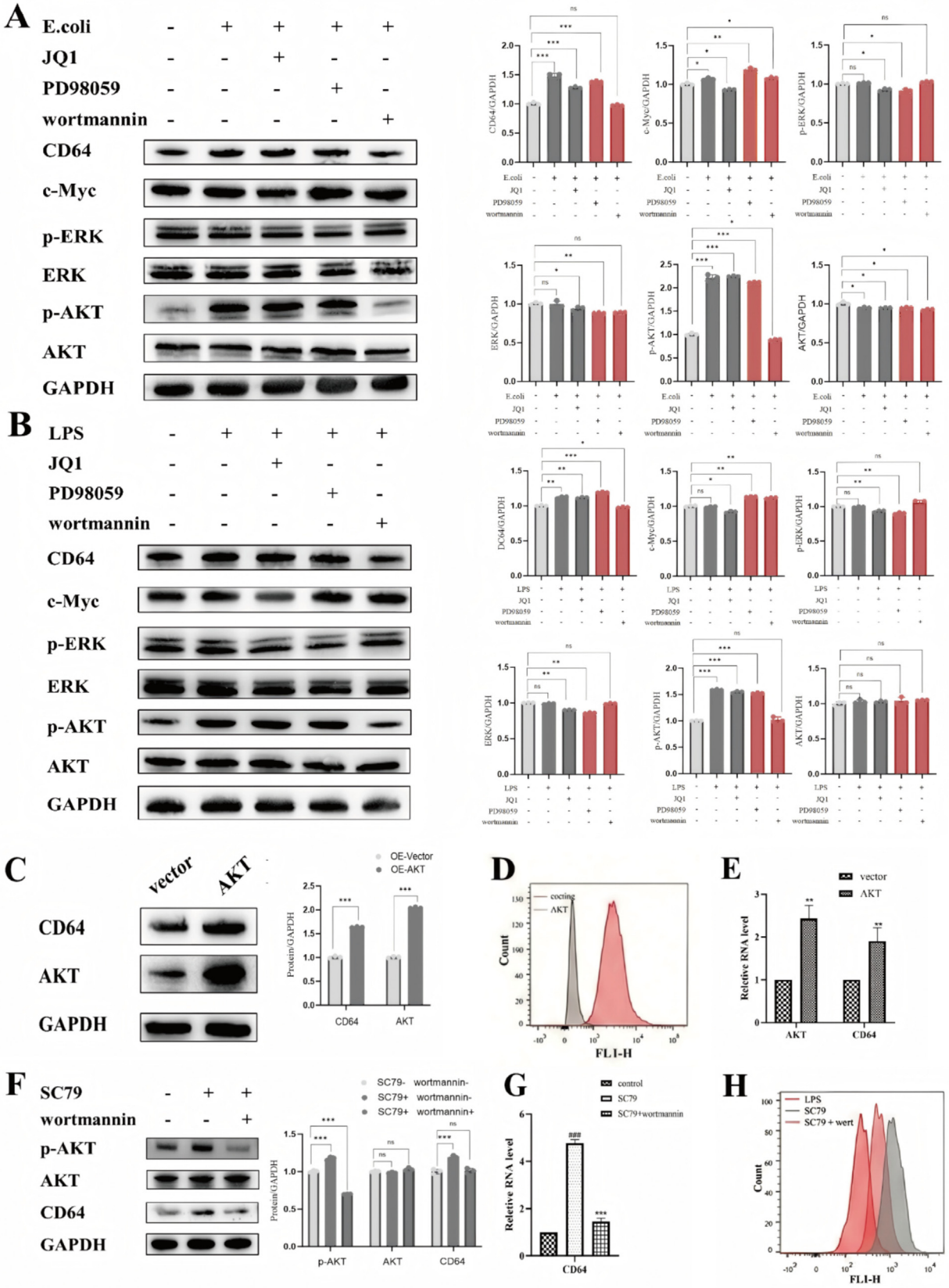

Fig. 3. The AKT signaling pathway is involved in LPS-induced CD64 expression. (A) Neutrophils were pretreated with the indicated inhibitors (JQ1, PD98059, or wortmannin) prior to activation with *E. coli* for the specified time periods. The expression levels of CD64 and c-Myc, as well as the phosphorylation levels of extracellular signal-regulated kinase 1/2 (Erk-1/2, detected as pErk-1/2) and AKT (detected as p-AKT), were analyzed by WB, as described in Section 2. (B) Neutrophils were pretreated with the indicated inhibitors before stimulation with LPS for the specified time periods. The expression of CD64 and c-Myc, and phosphorylation of Erk-1/2 and AKT, were detected by WB analysis. (C–E) WB analysis, flow cytometry, and qPCR were used to detect CD64 expression in neutrophils with AKT overexpression. (F–H) Neutrophils were cultured with SC79 (an AKT activator) in the presence or absence of wortmannin (an AKT inhibitor) for 24 h. CD64 expression was analyzed by WB, flow cytometry, and qPCR. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$. Circles represent individual replicate data points for each experimental group.

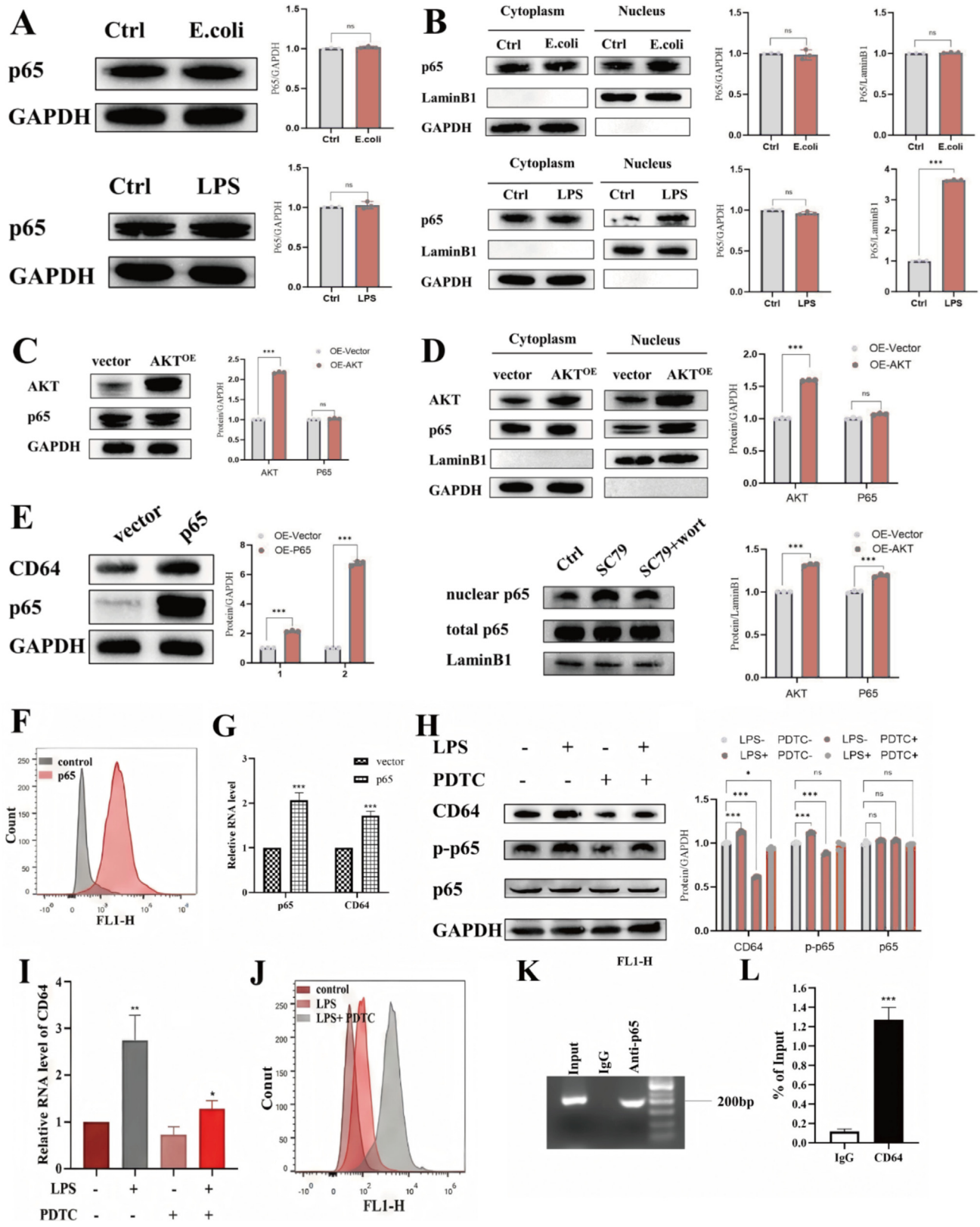

Fig. 4. P65 regulates CD64 expression in neutrophils. (A) WB analysis of p65 expression in neutrophils stimulated with *E. coli* or LPS. (B) The cellular distribution of p65 in neutrophils stimulated with *E. coli* or LPS was assessed by WB. (C) WB analysis of p65 expression in neutrophils with AKT overexpression. (D) The cellular distribution of p65 was determined by WB in neutrophils transfected with an AKT-overexpressing plasmid or treated with SC79 (AKT activator) and wortmannin (AKT inhibitor). (E–G) WB analysis, flow cytometry, and qPCR were performed to detect CD64 expression in neutrophils with NF-κB p65 overexpression. (H–J) Neutrophils were stimulated with LPS in the presence or absence of PDTC (an NF-κB inhibitor). CD64 expression was analyzed by WB, qPCR, and flow cytometry. (K–L) p65 binds to the CD64 promoter and activates CD64 transcription. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

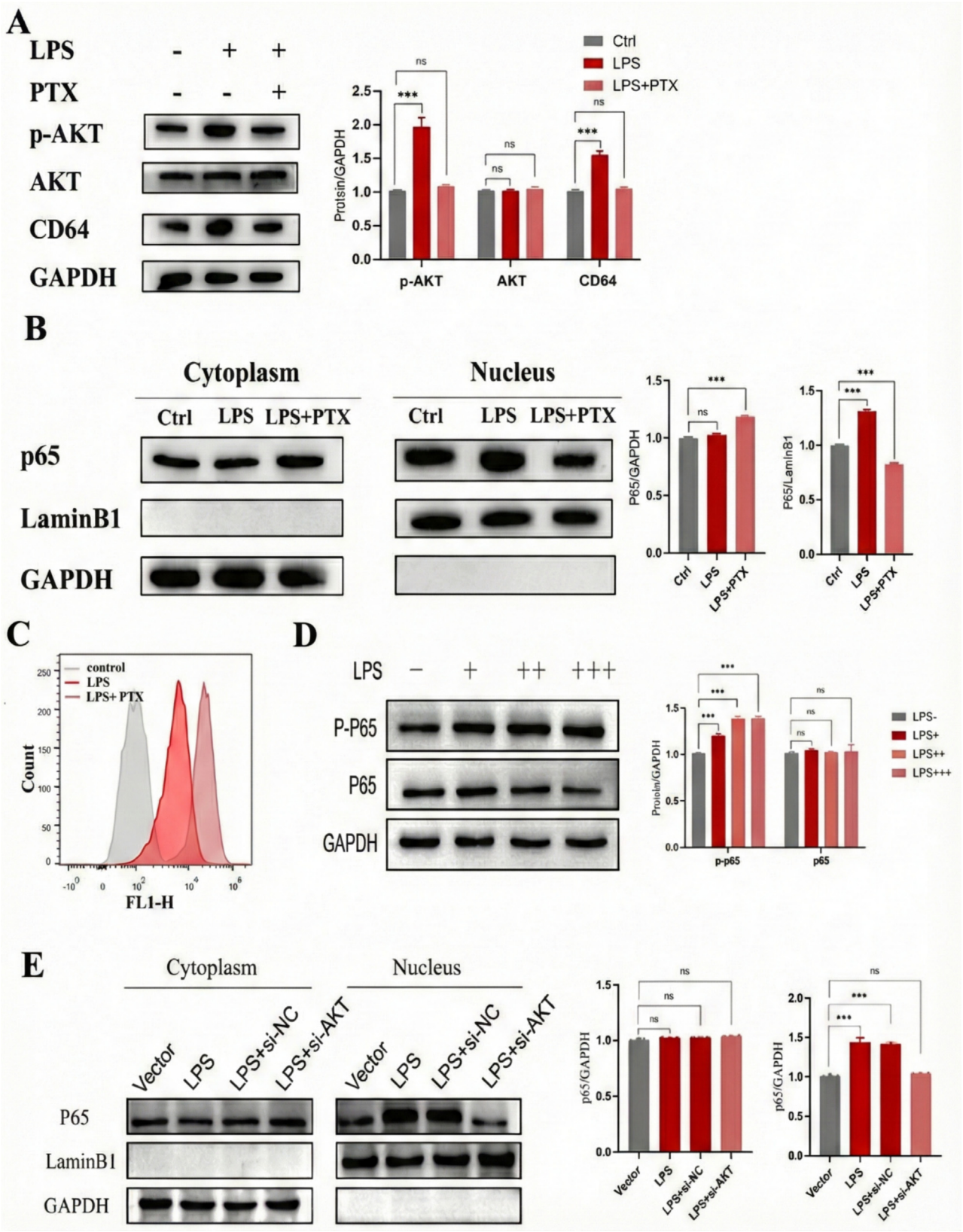

Fig. 5. *In vivo* experiments confirm that LPS regulates CD64 expression via the AKT signaling pathway. (A) For *in vivo* validation, neutrophils were isolated from rats, and CD64 expression and AKT phosphorylation (p-AKT) were detected by WB. (B) PTX (a G protein inhibitor) inhibited the nuclear translocation of p65 in neutrophils. Additionally, GAPDH amplification was not detected, as only nuclear protein fractions were analyzed in this experiment, which do not contain cytoplasmic GAPDH expression. (C) Neutrophils were isolated from mice, and CD64 fluorescence intensity was measured by flow cytometry. (D) WB of p-P65/total P65 in cells treated with varying LPS concentrations (–: 0 concentration, +: 50 ng/ml, + +: 100 ng/ml, +++: 150 ng/ml); GAPDH as control. Right histogram: band intensity normalized to GAPDH. Results: p-P65 increased significantly with LPS (***$p < 0.001$); total P65 showed no difference (ns). (E) Cytoplasmic-nuclear fractionation of cells treated with Vector/LPS/LPS + si-NC/LPS + si-AKT; WB for P65 (GAPDH: cytoplasmic control; LaminB1: nuclear control). Right histograms: P65 intensity (normalized to controls). Results: LPS promoted P65 nuclear translocation (***$p < 0.001$); AKT knockdown reversed this; cytoplasmic P65 showed no difference (ns). Circles represent individual replicate data points for each experimental group.

anism remained unclear. In mammals, the NF-κB transcription factor family comprises five members, among which p65 (RelA) is one of the most extensively studied and plays a central role in numerous signaling pathways, particularly in inflammatory responses across multiple organs. For instance, losartan has been shown to modulate macrophage polarization via the TLR4/NF-κB p65/MAPK axis to alleviate sepsis-induced cardiomyopathy [21]. Similarly, butorphanol promotes M2 macrophage polarization through NF-κB to reduce sepsis-related lung injury in mouse models [22]. Additionally, phosphorylation of Histone Deacetylase 3 (HDAC3) by Homeodomain-Interacting Protein Kinase 2 (HIPK2) enhances NF-κB acetylation and ameliorates sepsis-associated colitis [23]. Given the critical function of p65 in mediating cellular responses to external stimuli—especially in inflammation and immunity—we investigated whether NF-κB is involved in CD64 expression during sepsis.

Since the cell wall of *Gram-negative bacteria* contains multiple components such as phospholipids, proteins, peptidoglycan, and LPS—each acting through distinct mechanisms—we specifically examined whether LPS contributes to CD64 expression. WB analysis revealed that neither *E. coli* nor LPS stimulation altered total p65 protein levels in neutrophils (Fig. 4A). However, nuclear-cytoplasmic fractionation experiments showed a marked increase in nuclear translocation of p65 upon stimulation with either *E. coli* or LPS (Fig. 4B). To determine whether AKT regulates p65 activation, we overexpressed AKT in neutrophils and again observed no change in total p65 expression (Fig. 4C). Subsequent subcellular fractionation, however, demonstrated that AKT overexpression promoted p65 nuclear accumulation. Furthermore, when neutrophils were pretreated with wortmannin (an AKT inhibitor) before SC79 (an AKT activator) stimulation, nuclear p65 levels were lower than in cells treated with SC79 alone (Fig. 4D). These results indicate that AKT facilitates p65 nuclear translocation. As a core component of inflammatory signaling, nuclear p65 induces pro-inflammatory cytokine production [24]. However, whether p65 directly regulates CD64 expression had not been previously established. To investigate this, we overexpressed p65 in neutrophils and observed a corresponding increase in CD64 protein levels via WB (Fig. 4E). Consistent with this, both qPCR and flow cytometry confirmed elevated CD64 mRNA expression and surface fluorescence intensity (Fig. 4F–G). It is well established that phosphorylation and nuclear translocation of p65 are essential for NF-κB activation in the canonical pathway. Using the NF-κB inhibitor Homeodomain-Interacting Protein Kinase 2 (PDTC), we found that LPS-induced activation of p65 and upregulation of CD64 were substantially suppressed (Fig. 4H–J), indicating that PDTC reverses both p65 activation and CD64 expression triggered by LPS. ChIP assays confirmed that activated p65 directly binds the CD64 promoter (Fig. 4K), a result further validated by ChIP-qPCR (Fig. 4L). Collectively, these findings demonstrate that AKT upregulates CD64 expression via p65. As a nuclear transcription factor, p65 likely promotes CD64 expression by binding to its promoter region.

3.5. In vivo validation of the molecular mechanism of CD64 in sepsis

To further investigate the signaling mechanisms involving CD64 in sepsis, we established a septic rat model via intraperitoneal injection of LPS (15 mg/kg). PTX (25 mg/kg) was administered intraperitoneally as an interventional treatment [25]. Peripheral blood samples were collected for Western blot and flow cytometry analyses.

Results showed that CD64 protein expression was significantly elevated in LPS-induced septic rats compared with the control group. In contrast, PTX treatment markedly reduced both CD64 and phosphorylated AKT (p-AKT) levels relative to the LPS group (Fig. 5A). Moreover, nuclear p65 expression was lower in PTX-treated septic rats than in the untreated sepsis model. GAPDH amplification was not detected, as only nuclear protein fractions were analyzed in this experiment, which do not contain cytoplasmic GAPDH expression (Fig. 5B). Consistent with these findings, flow cytometry revealed a pronounced increase in CD64 fluorescence intensity in the LPS group, which was attenuated by PTX administration (Fig. 5C).

4. Discussion

Sepsis represents a major global health burden and is recognized as a priority by the World Health Organization due to its substantial impact on mortality [26]. This life-threatening condition poses significant challenges in clinical management, affecting not only adults but also pediatric populations. In 2018, an estimated 3 million newborns and infants were affected by sepsis worldwide, with mortality rates ranging from 11% to 19% [27]. In addition to triggering a systemic inflammatory response, sepsis can lead to multi-organ dysfunction, affecting the heart, kidneys, and respiratory system. It can also lead to long-term neurodevelopmental consequences, many of which are challenging to quantify. Given the rapid progression and high fatality rate of pediatric sepsis, timely intervention is crucial. While early treatment often involves empirical antibiotic use, which carries risks such as overtreatment, inappropriate drug selection, and potential antimicrobial resistance, delaying therapy in suspected cases is not an option [28]. There is therefore an urgent need for biomarkers that can facilitate early diagnosis shortly after the onset of inflammation.

To date, nearly 200 biomarkers associated with sepsis have been investigated, reflecting the complexity of its pathophysiology [20]. While numerous biomarkers have been proposed for diagnosis and prognosis, most have not been adopted into routine clinical practice due to limited impact on patient outcomes [29]. Consequently, current efforts focus on identifying biomarkers with high diagnostic accuracy, sensitivity, and specificity in early stages of sepsis. This study aims to evaluate the utility of the CD64 index as a biomarker for diagnosing sepsis in children.

We performed statistical analyses on commonly used clinical infection indicators using data from 200 children with sepsis and 166 healthy controls. ROC curve analysis demonstrated that CD64 had the largest AUC compared to PCT, CRP, IL-1β, and IL-6. At a cutoff value of 0.165, the CD64 index exhibited 87.0% sensitivity, 92.8% specificity, a PPV of 93.5%, and a NPV of 85.6%, outperforming all other markers compared. Furthermore, analysis of 30 patients pre- and post-treatment confirmed that CD64 retains its diagnostic and evaluative utility throughout clinical management. These findings affirm that CD64 offers superior diagnostic and prognostic value relative to conventional biomarkers and represents a promising candidate for early sepsis detection—consistent with previous reports [30]. Nevertheless, further validation through larger, multi-center studies is warranted.

We selected CD64 as the primary biomarker in this study not only due to its early detectability during inflammatory infection but also because of its capacity to reliably differentiate between bacterial and viral infections. Previous research has demonstrated that CD64 levels are significantly elevated in febrile children with infections compared to non-infected individuals, and furthermore, that its expression is higher in bacterial infections than in viral cases [31]. Upon infection, neutrophil surface CD64 is markedly up-regulated. CD64 also offers several advantages for clinical testing: its expression is specifically induced upon neutrophil activation by bacterial infection, remains stable across age groups, and can be reliably measured after room temperature storage for over thirty hours. Additionally, CD64 detection is rapid (requiring less than 1 h) and requires only a small blood sample (less than

100 μL) [32], making it particularly suitable for sepsis patients, from whom only limited blood volumes can often be obtained. These characteristics support the practical use of CD64 as a diagnostic indicator in clinical settings, enabling clinicians to initiate earlier and more targeted treatments. In summary, we conclude that CD64 shows strong potential for diagnosing sepsis and may contribute to improved clinical accuracy.

Although numerous studies have reported on the clinical diagnostic value of CD64 in sepsis, its regulatory mechanisms remain poorly understood. Our study focused on elucidating the molecular mechanisms underlying CD64 up-regulation in sepsis. We observed that CD64 expression significantly increased within 4 to 8 h after LPS stimulation, corroborating previous findings on its value in early sepsis diagnosis [33]. Further investigation revealed that CD64 expression in LPS-induced sepsis is both time- and dose-dependent. To explore the potential molecular mechanisms involved, and based on established knowledge of inflammatory signaling, we applied inhibitors of the AKT signaling pathway. As anticipated, AKT signaling was closely associated with the up-regulation of CD64. Subsequently, to identify additional factors that may have future relevance in combined detection strategies, we investigated p65—a key transcription factor in the NF-κB pathway whose activation promotes nuclear translocation and target gene transcription. Through a series of experiments, we confirmed that AKT facilitates nuclear translocation of p65 via the classical NF-κB pathway, and that nuclear p65 in turn binds to the CD64 promoter to enhance its transcription.

The promising diagnostic performance of CD64, particularly its high specificity and early detectability, supports its potential integration into routine clinical workflows. In practical terms, the CD64 index could aid in several critical aspects of sepsis management: early suspicion and detection in high-risk pediatric patients, guidance for initiating or withholding antibiotics, and monitoring of treatment response. Its rapid turnaround time and low blood volume requirement further enhance its suitability for pediatric and critical care settings [34]. Looking ahead, the development of a multi-biomarker panel represents a logical and promising direction for improving sepsis diagnosis. Given the distinct kinetic and diagnostic profiles of various biomarkers—CD64 rising early with high specificity, PCT increasing steadily in systemic bacterial infections, and CRP serving as a general inflammation marker—their combination is likely to capture a more comprehensive picture of the host response. For instance, a panel combining CD64 with PCT could potentially enhance the differentiation of bacterial sepsis from non-infectious inflammation or viral syndromes, possibly achieving an AUC value surpassing that of any single marker. Similarly, the integration of CD64 with CRP might improve sensitivity in early time windows when CRP levels are still increasing. Future studies should systematically evaluate the diagnostic gain from such combinations, including the use of statistical models to weigh each biomarker's contribution, to construct an optimal, clinically viable sepsis signature.

However, an ideal biomarker for sepsis diagnosis should exhibit both high specificity and sensitivity, allowing it to play a central role in early detection, risk stratification, therapeutic guidance, and prognostic evaluation. Relying on a single indicator is generally insufficient for comprehensive disease diagnosis. The combined use of multiple infection markers has the potential to significantly enhance the accuracy of clinical diagnosis for infectious diseases, though such integrated approaches have yet to be widely adopted in clinical practice. Accordingly, to improve diagnostic performance for sepsis, several studies suggest that CD64 should be used in conjunction with other established parameters, such as CRP. Therefore, subsequent research could focus on evaluating CD64 in combination with other infection-related indicators to improve diagnostic sensitivity and specificity.

In light of this, CD64 represents a valuable and reliable marker for early diagnosis and prognosis in sepsis patients. More comprehensive studies are warranted to fully elucidate its clinical utility. Future research should aim to integrate clinical symptoms, disease assessment indicators, other rapid diagnostic markers, as well as the timing of antibiotic initiation and cessation, to facilitate deeper mechanistic and translational exploration.

CRediT authorship contribution statement

Lin Li: Writing – original draft, Methodology. **Xiaoqing Fu:** Validation, Data curation. **Nayun Chen:** Methodology. **Daihua Fang:** Supervision, Project administration.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary material

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Data availability

All data generated or analyzed during this study are included in this published article.

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