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ORIGINAL RESEARCH

The Role of Dectin-1-Akt-RNF146 Pathway in β-Glucan Induced Immune Trained State of Monocyte in Sepsis

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Background: Sepsis is regarded as a dysregulated immune response to infections. Recent study showed partially reversal of immunosuppression by trained immunity, which fosters an enhanced immune response towards a secondary challenge. However, the role of trained immunity in sepsis has not been fully understood.

Methods: We profiled the characteristics of peripheral blood mononuclear cells from septic patients using single-cell RNA sequencing (scRNA-seq) analyses. Murine double-hit models (pretreatment or post-treatment of β-glucan in septic mice) and murine monocyte/macrophage cell line RAW264.7 were used then.

Results: scRNA-seq revealed that Ring finger protein 146 (RNF146) and protein kinase B (Akt) were downregulated in the immunosuppression period of septic patients and were verified to be decreased in bone marrow and spleen monocytes from septic mice. While β-glucan pretreatment improved the immunosuppressed state in septic mice and increased dectin-1/Akt/RNF146 expressions in monocytes, along with the increased survival rate, inflammatory factors and aerobic glycolysis, indicating a change from immunosuppression to immune training. Moreover, RNF146 regulated dectin-1-Akt-mTOR signaling in the trained immune state of murine monocyte/macrophage RAW264.7 cell line and the expression of RNF146 was dependent on dectin-1-Akt activation. The inhibition of dectin-1 by its antagonist laminarin downregulated Akt-RNF146 signaling and partially reversed β-glucan induced trained immunity in septic mice.

Conclusion: RNF146 and Akt are downregulated in the immunosuppression period of sepsis, while increased after β-glucan pretreatment induced trained immunity in septic mice. Moreover, RNF146 regulates the immune trained state of monocyte through dectin-1-Akt-mTOR pathway, suggesting a possible target in reversal of immunosuppression in sepsis.

Keywords: RNF146, β-glucan, trained immunity, immunosuppression, sepsis

Introduction

Sepsis is a life-threatening syndrome, characterized by multiple organ failure resulting from a dysregulated host response to infection.[1–3] Despite rapid progresses made in the early diagnosis and treatment, the associated mortality of sepsis is still on the rise and can range anywhere from 30% to 50% depending on international registers.[4] Sepsis is regarded as a dysregulated complex immune response to infections, inducing defects in both innate and adaptive immune and eventually leading to prolonged and significant immunosuppression.[5–8] Specifically, blood leukocytes of septic patients have a diminished ability to release proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1,

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IL-6, and IL-12 following stimulation ex vivo.[9] Immunosuppression represents one extreme in the spectrum of innate immune memory and can be induced by high bacterial burden in vivo or lipopolysaccharide (LPS) exposure in vitro[9] and controlled by downregulated protein kinase B (Akt)-hypoxia-inducible factor 1 alpha (HIF-1α) pathway.[10] Immunosuppression leads to attenuated immune responses to secondary stimulus, so it is possible to target the immune system to promote host fitness and survival in patients with sepsis.[11]

Reversal of immunosuppression using innate immune "trainers" is a viable therapeutic strategy.[12] Trained immunity, defined by the boosted innate immune response after experiencing an initial stimulus, was first proposed by professor Netea in 2011 and is a powerful defense against infection, regardless of bacteria, fungi or virus.[9] Trained immunity can be induced by β-glucan or oxidized low-density lipoprotein (oxLDL), resulting in epigenetic reprogramming of myeloid cells, especially in monocytes and macrophages.[9] Boris et al reported that β-glucan was capable of reversing macrophage tolerance induced by ex vivo LPS exposure,[12] leading to a reversal inflammatory status[13] and antimicrobial activity.[14] As trained immunity can deliver nonspecific protection from re-infection, it may provide a new direction for preventing and treating immunosuppression in sepsis.[15] Therefore, inducers of trained immunity are of great interest as a treatment approach to reverse immunosuppression states in sepsis.

In this study, we aimed to investigate the immune suppressed state of sepsis and the role of trained immunity in sepsis-induced immunosuppression and the underlying mechanism. scRNA-seq analysis was conducted in septic patients, and then the potential role of trained immunity induced by β-glucan in sepsis-induced immunosuppression and the involvement of relevant pathways were investigated in murine models and murine monocyte/macrophage RAW264.7 cell lines.

Material and Methods

Patients

Our study was approved by the ethics committee of Zhongshan Hospital Fudan University (B2021-215R) and was registered at the Chinese Clinical Trial Registry (ChiCTR2100048148). Our study was complied with the Declaration of Helsinki. Patients who experienced sepsis induced by Gram-negative bacterial infection were recruited from the Intensive Care Unit (ICU) of Zhongshan Hospital, Fudan University, and all subjects were included from July to December 2021. Informed consent was obtained from the participants, or the person authorized by the participants. Patients with sepsis were required to fulfill the diagnostic criteria for sepsis-3.[16] Patients with immunosuppression were required to fulfil the following criteria: C-reactive protein (CRP) > 150 μg/dL; total lymphocyte count (LYM) < 0.8×109/L; serum albumin < 30 g/L; prealbumin level < 0.1 g/L; retinol-binding protein (RBP) < 1 mg/dL; creatinine height index (CHI) < 80%; weight loss > 10% or Body Mass Index < 18 during hospitalization. Exclusion criteria included: 1) immunodeficient persons and 2) those receiving immunomodulatory medications (including prednisone or steroid analogs, chemotherapy, or biological immunomodulators).

Single-Cell Preparation and Single-Cell RNA Statistical Analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from whole-blood samples by density-gradient centrifugation, as described previously.[17] Cells from each sample were labeled with sample tags (TotalSeqTM–C antibodies), following the manufacturer's protocol. The scRNA-seq and V(D)J libraries were generated using the 10× Genomics Chromium Controller Instrument and Chromium Single Cell 5′ library and gel bead kit, along with the V(D)J enrichment kit (10× Genomics, Pleasanton, CA). The scRNA-seq data analysis was performed by NovelBio Co., Ltd. with the NovelBrain Cloud Analysis Platform (www.novelbrain.com). In brief, we applied fastp[18] with the default parameters to filter the adaptor sequences and remove low-quality reads to achieve clean data. Then, feature-barcode matrices were obtained by aligning reads to the human genome (GRCh38 Ensemble: version 91) using CellRanger v3.1.0. The Seurat package (version: 3.1.4, https://satijalab.org/seurat/) was used for batch correction based on the expression table according to the UMI counts of each sample and percentage of mitochondria rate to obtain scaled data. Using the graph-based cluster method, we calculated marker genes using the FindAllMarkers function with the Wilcox rank sum test

algorithm under the following criteria: lnFC > 0.25; P-value < 0.05; min.pct > 0.1. All single-cell RNA sequencing data were deposited in the Gene Expression Omnibus (GEO: GSE217906).

Animals

Ten-week-old male C57BL/6 mice were purchased from animal experiment center of Fudan University, and maintained under pathogen-free conditions. All procedures were conducted in accordance with the guidelines of the Chinese council on animal care and we obtained approval from the animal review committee of Fudan University Shanghai Cancer Center (FUSCC-IACUC-S2023-0141). All surgeries were performed under anesthesia, and all efforts were made to minimize animal suffering.

Double-Hit Models

Two types of double-hit mouse models (pretreatment or post-treatment models) were used. Pretreatment double-hit model was constructed with β-1,3 (D) glucan (BG) (Invivo Gen, San Diego, USA) or 0.9% sodium chloride (NS) administration 7 days before cecal ligation and puncture (CLP) in mice. Specifically, mice received 2 mg/kg β-glucan or equal volume of NS intraperitoneally, and then CLP procedure was performed 7 days later (BG+CLP group, NS+CLP group). While post-treatment double-hit model was constructed with CLP procedure first, and 14 days later, 2mg/kg β-glucan or equal volume of NS was injected intraperitoneally (CLP+BG group, CLP+NS group), which is in line with previous study.[19] The Mouse Sepsis Score (MSS) and weight were measured each day. MSS assessed the severity of fecal-induced peritonitis concluding seven variables and 0–28 points from mild to severe.

Monocyte Harvesting

C57BL/6 mice were euthanized via cervical dislocation and spleen monocytes and bone marrow mononuclear cells (BMMC) were harvested by following manners. The spleen was dissected and weighed. For dissociation of splenic tissue, the spleen was passed through a 70 μm cell strainer. Mouse splenocytes were centrifuged and resuspended in red blood cell lysis buffer (ACK lysis buffer) (Servicebio, Wuhan, China) for red blood cell lysis. Bone marrow was extracted from femura and tibiae of mice as follows. Bones were prepared by removing all soft tissue without fracturing, followed by a decapping of the bone on both sides. Subsequently, bone marrow was flushed out with pre-warmed DMEM (HyClone, Thermo, Waltham, MA, USA) media. Then extracted mouse splenocytes and myeloid cells were cultured at 37°C in a 5% CO_2 incubator and were maintained in DMEM supplemented with 10% FBS (HyClone, Thermo, Waltham, MA, USA) and penicillin and streptomycin (P/S) (Gibco, Thermo, Waltham, MA, USA). Monocyte isolation was carried out by Moflo XDP Cell Sorter (Beckman Coulter, California, USA) according to the instructions provided by the manufacturer. Mouse splenocytes and myeloid cells extracted from mice were suspended respectively in FACS buffer (Phosphate Buffered Saline (PBS) with 1% BSA and 0.1% azide), and 1 μL/sample of Fc Block (BD Pharmingen, San Jose, CA, USA) was added to prevent nonspecific binding. The above suspended monocyte cell types were isolated using the following antibody combinations: PE anti-mouse Ly-6G (BioLegend, 127608, San Diego, CA, USA) and pacific blue anti-mouse CD11b (BioLegend, 101224, San Diego, CA, USA). The isolated mouse splenocytes and BMMC were then cultured in DMEM supplemented with 10% FBS and P/S at 37°C.

Flow Cytometry

Flow cytometry was used to determine the levels of intermediate monocytes (Ly6CintCX3CR1hi) from the BMMC and splenocytes isolated from septic or control mice, in order to identify the inflammatory monocytes, which were also identified as monocyte subsets with trained immunity phenotype.[20,21] Isolated mouse splenocytes and BMMC were stained with PE anti-mouse CX3CR1 (BioLegend, 149005) and FITC anti-mouse Ly-6C (BioLegend, 128005) at 4°C for 30 min in the dark. Stained cells were analyzed by Cytomics FC 500 (Beckman Coulter, California, USA). Data were analyzed using FlowJo version 10.1 (Ashland, OR, USA).

Cytokine Measurements

Cytokine productions were assessed in supernatants of BMMC and spleen monocytes collected from septic or control mice using commercial Enzyme-Linked Immunosorbent Assay (ELISA) kits for mouse IL-10 (R&D systems, MN,

USA), IL-6 (R&D systems, MN, USA), IL-1β (R&D systems, MN, USA) and TNF-α (R&D systems, MN, USA), in accordance with the manufacturer's instructions.

Mitochondrial Respiration and Glycolysis Analysis

To analyze mitochondrial respiration and glycolysis, oxygen consumption rate (OCR, a measurement of oxidative phosphorylation) and extracellular acidification rate (ECAR, a measurement of glycolysis) were detected with Seahorse XF analyzer (Agilent Technologies, California, USA) in accordance with the manufacturer's instructions. Spare respiratory capacity = OCR between carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) and rotenone/antimycin A −OCR before oligomycin. Glycolytic reserve = ECAR between oligomycin and 2-deoxy-glucose (2-DG) −ECAR between glucose and oligomycin.

Cell Viability Assay

Isolated mouse splenocytes from septic or control mice were seeded in 96-well plate at densities of 5000 cells per well. A cell viability assay was conducted using the methyl tetrazolium (MTT) cell proliferation assay kit (R&D systems, MN, USA). 0.5 mg/mL of MTT was added to each well, and the plate was incubated at 37°C for 4 h. Then, the supernatant-containing MTT was removed, and 200 μL of dimethyl sulfoxide (DMSO) was added to each well. The plate was shaken thoroughly for 1 min on a shaker. The absorbance of the samples was measured at 570 nm by using a microplate reader (Bio-TEK Inc, Winooski, VT).

RNA Extraction, Quantitative Real-Time PCR (qPCR) and Western Blot Analysis

Total RNA was extracted using TRIzol (Invitrogen, Grand Island, NY, USA) and used to synthesize complementary DNA (cDNA) with PrimeScript RT reagent (TaKaRa) according to the manufacturer's instructions. qPCR was then performed using Hieff UNICON® qPCR TaqMan Probe Master Mix (Yeasen, Shanghai, China) in an ABI7500 Real-Time PCR system (Applied Biosystems). The primers used in the present study are shown in Supplementary Table 1. Protocol for Western blot analysis was shown in Supplementary Materials.

Reagents and Plasmids

Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA, USA). Anti-Ring finger protein 146 (RNF146) was purchased from Novus Biologicals (CO, USA). Anti-c-type lectin domain family 7 member A (dectin-1) mAbs was purchased from R&D systems (MN, USA). Anti-mammalian target of rapamycin (mTOR), anti-phosphorylated-mammalian target of rapamycin (p-mTOR), anti-Akt, anti-phosphorylated Akt (p-Akt) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mAbs were purchased from Proteintech (Chicago, IL, USA). Plasmid expressing pCDNA3.1-RNF146, siRNA RNF146, pCDNA3.1-dectin-1, and siRNA-dectin-1 (shown in Supplementary Table 2) was purchased from Genechem (Shanghai, China). We purchased plasmids expressing the constitutively active form of Akt (#10841, Addgene) and dominant negative form of Akt (#16243, Addgene) (shown in Supplementary Table 2).

Training State Model of RAW264.7 Cells

RAW264.7 cells (SIBS, Shanghai, China) were grown in DMEM with 10% FBS and penicillin and streptomycin and were incubated at 37°C with 5% CO_2. RAW264.7 cells in the logarithmic growth stage were collected for subculture and further experimentation. RAW264.7 cells (P3, $1.71*10^6$/mL) were treated with 100 ng/mL β-glucan for 24 h, and then washout and rested for 3 days in DMEM with 10% FBS followed by 100 ng/mL LPS stimulation for 24 h. The expressions of IL-1β, IL-6, TNF-α and IL-10 secreted by RAW264.7 cell were determined by ELISA (R&D systems, MN, USA) to confirm the training state.[22]

Immunofluorescence

RAW264.7 Cells were treated with 100 ng/mL β-glucan for 24 h, and then fixed with 4% paraformaldehyde in PBS, 2% bovine albumin (BSA) (Sigma-Aldrich), and 0.1% Triton X-100 (Sigma-Aldrich) for 1 h at room temperature. Then incubated with combinations of primary antibodies against RNF146, Akt (depending on the experiment) at 4°C

overnight. Then, the fixed samples were washed with PBS containing 0.1% Triton X-100 and incubated with corresponding secondary antibodies conjugated with fluorescent dye at room temperature for 1 h. Fluorescent images were obtained using a confocal microscope (LSM 710, Zeiss).

Co-Immunoprecipitation (Co-IP)

The lysates of RAW264.7 were extracted via immunoprecipitation (IP) lysis buffer, cultivated with the indicated antibodies and negative control IgG all night at 4°C, followed by incubation with secondary antibody Horseradish Peroxidase (HRP) Mouse Anti-Rabbit IgG light chain (Abbkine, A25022). After mixing with beads, the complex was rinsed in IP buffer for 3 times and boiled with SDS buffer, followed by elution and Western blotting.

Administration of Dectin-1 Receptor Antagonist in vivo

The dectin-1 receptor antagonist laminarin (LAM) (Sigma-Aldrich, L9634) was diluted to 10 mg/mL in vehicle (saline) according to the manufacturer's protocol. Then, LAM at 300 mg/kg/day or the same volume of NS was intraperitoneally injected once daily on the 7th day after CLP for the 2 subsequent days in double-hit mice model.

Statistical Analysis

We applied at least five samples for each experiment. Statistical evaluation was conducted using the GraphPad Prism software, version 9.5 (GraphPad, San Diego, CA, USA). Data were expressed as mean ± standard deviation (SD). For two-group comparisons, the unpaired two-tailed Student's *t* test was used. One-way analysis of variance (or Kruskal–Wallis for non-parametric data) was used to assess significant differences among more than two groups (ANOVA; more than two groups), followed by Bonferroni's multiple comparisons. Differences between survival curves were analyzed by the Log-Rank Mantel-Cox test. Differences of MSS and weight were analyzed by the Two-way ANOVA repeated measure, followed by Sidak's or Dunnett multiple comparisons. $P<0.05$ was considered significant.

Results

scRNA-Seq Revealed Changes in Monocyte from Septic Patients

We profiled the characteristics of PBMCs using scRNA-seq analyses from the patients in the immunosuppression period of sepsis (n=4), acute period of sepsis (n=2), and healthy individuals (n=3) (shown in Supplementary Table 3). The cell compositions of the groups were identified and immune signatures were profiled using scRNA-seq on the 10×Genomics 5′ sequencing platform (shown in Figure 1a). A total of 23 cell clusters were identified (shown in Figure 1b). The percentages of monocytes in the immunosuppression period of sepsis, acute period of sepsis, and healthy control groups were 11.7%, 18.2%, and 11.8% (shown in Figure 1c) separately. We specifically re-clustered monocytes and regrouped them into four distinct clusters including classical monocytes (CD14 and VCAN), monocyte-platelet aggregates (MPA) (PPBP and PF4), IL-1β+ monocytes (IL-1B), and macrophages (FCGR3A) (shown in Figure 1d). The expression of marker genes of each cluster is also shown in Figure 1d. The percentages of the four clusters varied significantly in the patients undergoing the immunosuppression period of sepsis (shown in Figure 1e), during which, the classical monocyte, which is the main cell subset of monocytes, decreased significantly. The percentages of classical monocytes in the immunosuppression period of sepsis, acute period of sepsis, and healthy control groups were 68.82%, 79.73%, and 79.02% (shown in Figure 1e), respectively. The proportions of MPA had a big increase only in the immunosuppression period of sepsis. These cells expressed CD14 (marker of monocytes), PPBP and PF (markers of platelets), and played crucial roles in the signal pathways of platelets degranulation, activation and coagulation (shown in Supplementary Figure 1).

Expressions of genes related to immune functional features were differentially affected in immunosuppressed and proinflammatory septic patients compared to healthy controls. Notably, RNF146, a novel Poly (ADP) ribosylation-directed ring finger E3 ubiquitin ligase and Akt were downregulated in the immunosuppression period of the sepsis group compared with acute sepsis group and healthy individuals. Akt plays an important role in macrophage-mediated innate immunity and is involved in regulating immune response during sepsis. Previous studies reported that activation of the

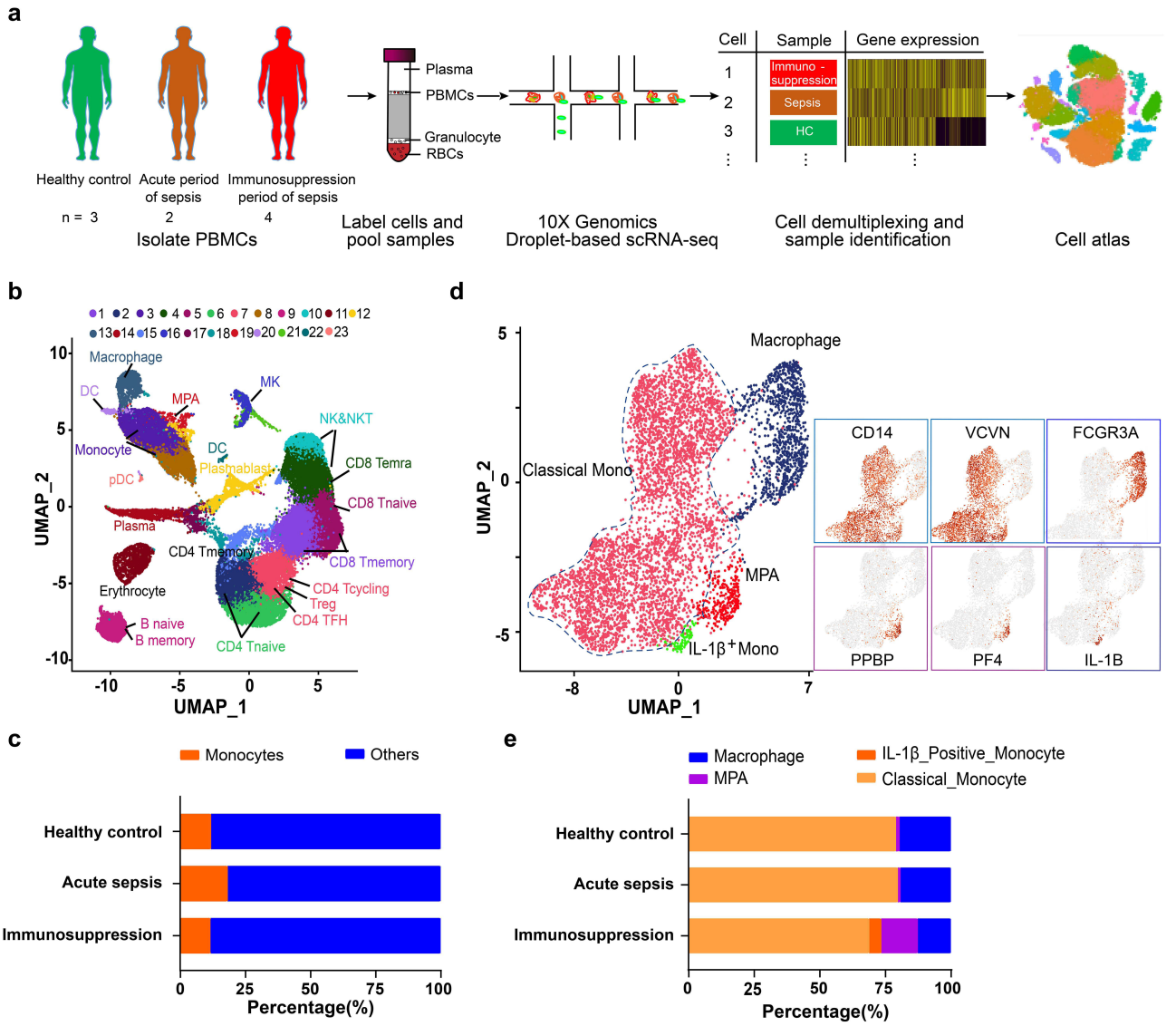

Figure 1 Single-cell RNA sequencing of septic patients. (**a**) Flow chart of blood sample processing for each group of subjects. PBMCs were labeled with cell hashing antibody and loaded on the single-cell RNA sequencing platform in droplets. According to the sequence of each barcoded antibody, the cells were demultiplexed and the polyploids were removed. (**b**) UMAP plot of 43,415 high-quality single cells obtained from all subjects combined. (**c**) Monocyte composition in different states. (**d**) UMAP plot and the expression of marker genes of the sub-clusters of monocytes in all subjects. (**e**) Cell composition of monocyte subsets in different states.
Abbreviations: PBMC, peripheral blood mononuclear cells; RBC, red blood cell; UMAP, Uniform manifold approximation and projection.

Akt signaling pathway promotes RNF146 expression in Parkinson's disease[23] and activated RNF146 enhances the glycolysis via the Akt signaling Pathway.[20] To determine the changes in expressions of RNF146 and Akt in the immunosuppression period of sepsis, we next detected the relative genes expressions in BMMC and spleen monocytes obtained from the CLP-induced immunosuppressed mouse.

Akt and RNF146 Expressions Were Down-Regulated in Bone Marrow and Spleen Monocytes from Septic Mice

First, we determined the CLP-induced immunosuppressed mouse model. We found that monocytes from CLP mouse model yielded reduced IL-1β, IL-6, TNF-α and increased IL-10 levels 14 and 21 days after CLP (shown in Supplementary Figure 2a and b). The CLP-induced immunosuppression in the mouse model was verified by testing the responsiveness of BMMC and spleen monocytes to LPS stimulation in vitro (shown in Figure 2a) and splenocyte apoptosis.[21] We found that BMMC exhibited reduced responsiveness to LPS stimulation in vitro, including decreased

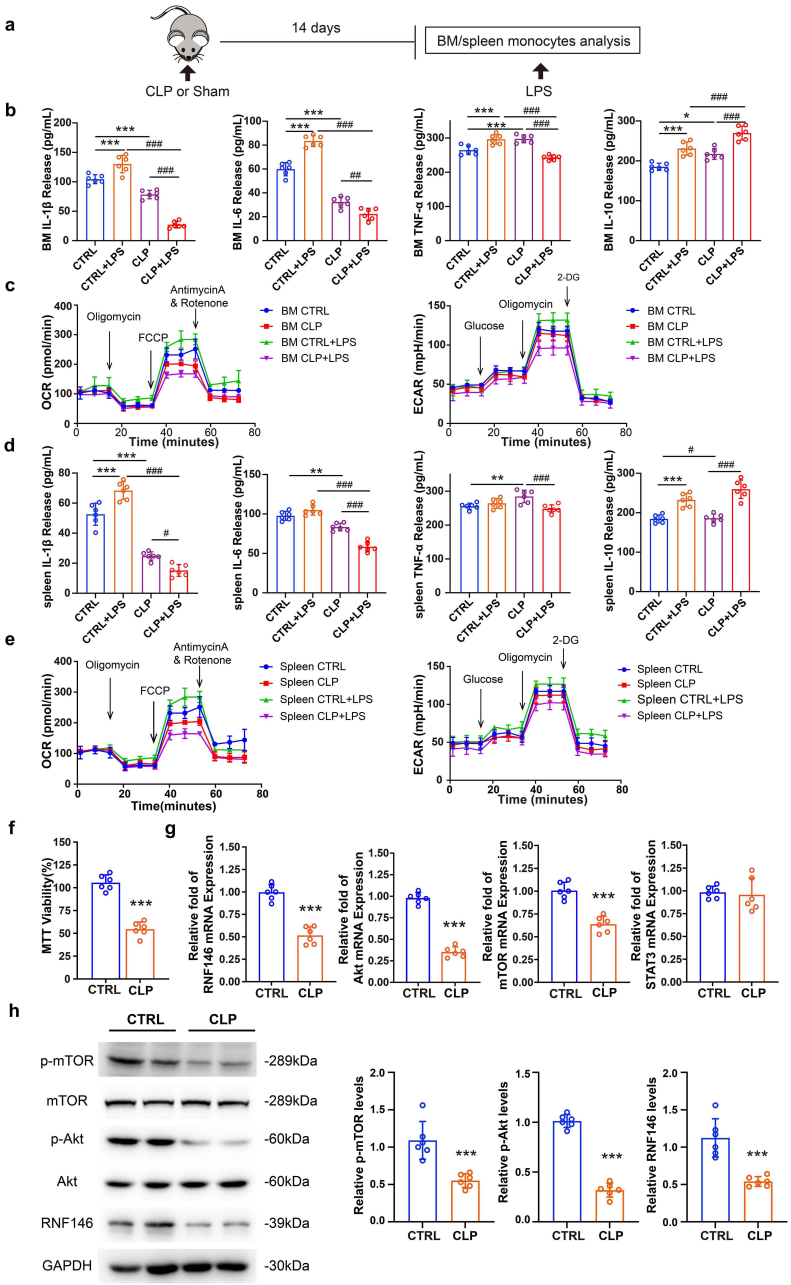

Figure 2 Akt and RNF146 expression were down-regulated in BMMCs and spleen monocytes from immunosuppressed septic mice. (**a**) C57BL/6 mice received CLP or sham surgery, and 14 days thereafter, monocytes were collected from BM/spleen and re-stimulated with LPS. (**b–d**) IL-1β, IL-6, TNF-α and IL-10 levels in BMMC (**b**) or spleen monocytes (d) from controls (CTRL) and septic mice (CLP) stimulated with LPS or not, were analyzed with ELISA (n=6 per group). (**c–e**) The Seahorse XF mitochondrial respiration analysis and glycolytic rate analysis (n=6 per group). All measured OCR and ECAR levels were normalized by total cellular protein, analyzed aerobic glycolysis of monocytes collected from BMMC (**c**) or spleen monocytes (**e**) from controls (CTRL) and septic mice (CLP) stimulated with LPS or not. (**f**) Cell viability was measured by the MTT assay in splenocytes from septic (CLP) or control mice (CTRL) (n=6 per group). (**g**) mRNA expressions of RNF146, Akt, mTOR and STAT3 in BMMC from septic (CLP) or control mice (CTRL) (n=6 per group). (**h**) mTOR, p-mTOR, Akt, p-Akt, RNF146 and GAPDH protein levels were detected in BMMC from septic (CLP) or control mice (CTRL) by Western blot (n=6 per group). The error bars are presented as mean ± SD; * P < 0.05, ** P < 0.01, *** P<0.001 compared with CTRL group. # P < 0.05, ## P < 0.01, ### P<0.001 compared with CLP+LPS group.

Abbreviations: BM, bone marrow; CLP, cecal ligation and puncture; LPS, lipopolysaccharide; CTRL, controls; OCR, oxygen consumption rate; ECAR, extracellular acidification rate; FCCP, carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazone; 2-DG, 2-deoxy-glucose; IL, interleukin; TNF, tumor necrosis factor.

expression of pro-inflammatory cytokines (shown in Figure 2b) and decreased OCR and ECAR, indicating decreased spare respiratory capacity and decreased glycolytic reserve (P<0.001 and P<0.001 separately, shown in Figure 2c). Cytokine secretion and metabolism changes in spleen monocytes were found to be similar to those in BMMC (shown in

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Figure 2d and e). Moreover, CLP caused significant splenocyte cell viability impairment compared with the control (P<0.001, shown in Figure 2f), indicating CLP induces splenocyte apoptosis. The above changes suggested the immune suppressed state of monocytes in septic mice.

We next detected the mRNA levels of RNF146 and Akt pathway according to the results of scRNA-seq. Akt-mTOR pathway has been shown to regulate immune response during sepsis.[24] Moreover, the activation of the Akt-mTOR pathway is essential for the maintenance of immune homeostasis in sepsis.[15] Signal transducer and activator of transcription 3 (STAT3) regulates neutrophil release to help contain infection and limit inflammatory responses.[25] Hence, the mRNA levels of RNF146, Akt, mTOR, and STAT3 in BMMC from CLP-induced immune suppressed septic mice and control mice were detected. We found that RNF146, Akt and mTOR mRNA levels were significantly decreased in BMMC from immunosuppressed septic mice compared with control ones (P<0.001 for all comparisons, shown in Figure 2g). However, the mRNA level of STAT3 was not significantly different between the two groups (P=0.736, shown in Figure 2g). We then detected the expressions of RNF146, p-Akt, and p-mTOR in BMMC from CLP-induced immunosupressed septic mice using Western blot and found that the expressions of RNF146, p-Akt and p-mTOR were significantly downregulated (P=0.003, P<0.001 and P=0.001 separately, shown in Figure 2h). These results identified the down-regulation of RNF146 and Akt pathway in the immunosuppressed state of septic mice.

β-Glucan Pretreatment Improved the Immune Suppressed State in Septic Mice and Increased Dectin-1/Akt/RNF146 Expressions

Immunosuppression in sepsis was reported to partially reversed by β-glucan induced trained immunity.[12] To demonstrate the effect of trained immunity on the immune suppressed state in sepsis, we first developed a β-glucan induced trained immunity mouse model in accordance with previous research[26] (shown in Supplementary Figure 3a). We found increased levels of intermediate monocytes (Ly6CintCX3CR1hi), which is the trained immunity phenotype of monocyte,[27,28] increased levels of IL-1β, IL-6 and TNF-α, decreased IL-10, decreased OCR, and increased ECAR in mouse BMMCs and spleen monocytes, indicating the immune trained state of mouse model (shown in Supplementary Figure 3b–3f). Next, we used two types of double-hit mouse models to test the effect of trained immunity on immunosuppression in septic mice. We established the double-hit model in septic mice with β-glucan treatment before or after CLP (shown in Figure 3a). The β-glucan pretreatment group (BG+CLP group) showed a significantly higher survival rate than the control group (NS+CLP group) (P=0.018, shown in Figure 3b); while no difference was found in the β-glucan post-treatment group (CLP+BG group) and control group (P=0.542, shown in Figure 3c). Additionally, MSS, indicative of the severity in sepsis, was much lower in BG+CLP group than that in NS+CLP group (P<0.05 from the 10th day, shown in Figure 3b), and no difference was found in the CLP+BG group and CLP+NS group until the 27th day (P=0.023, shown in Figure 3c). Loss of body weight, attributed to ongoing catabolism in septic mice, was less in BG+CLP group than that in NS+CLP group (P<0.05 from the 14th day, shown in Figure 3b); while no difference was found in the CLP+BG group and CLP+NS group until the 25th day (P=0.024, shown in Figure 3c). Furthermore, spleen was found to be both larger and heavier in BG+CLP group than that in NS+CLP group (P<0.001, shown in Supplementary Figure 4a). Collectively, our data indicated that β-glucan pretreatment improves the survival rate of septic mice, and reduces the severity of sepsis. β-glucan pretreatment also resulted in enhanced inflammatory cytokines secretion, with increased levels of IL-1β, IL-6 and TNF-α release (P<0.001, P<0.001 and P=0.003 in BMMC supernatant; P<0.001, P<0.001 and P=0.003 in spleen monocytes supernatant, respectively), and deceased IL-10 release (P<0.001 in BMMC supernatant; P<0.001 in spleen monocytes supernatant, shown in Figure 3d and Supplementary Figure 4b). Furthermore, β-glucan pretreatment induced increased ECAR (increased glycolytic reserve in BMMC and spleen monocytes, P<0.001 and P=0.019 separately) and decreased OCR (decreased spare respiratory capacity in BMMC and spleen monocytes, P=0.001 and P<0.001 separately) in monocytes (shown in Figure 3e and Supplementary Figure 4c), indicating the enhanced glycolysis which is the characteristic metabolic change of trained immunity.

We then detected the expressions of RNF146 and Akt pathway in monocytes from β-glucan preteated septic mice. The dectin-1 was described as the specific β-glucan receptor expressed on myeloid cells, with crucial functions in immune responses including pro-inflammatory responses such as cytokine production, reactive oxygen species

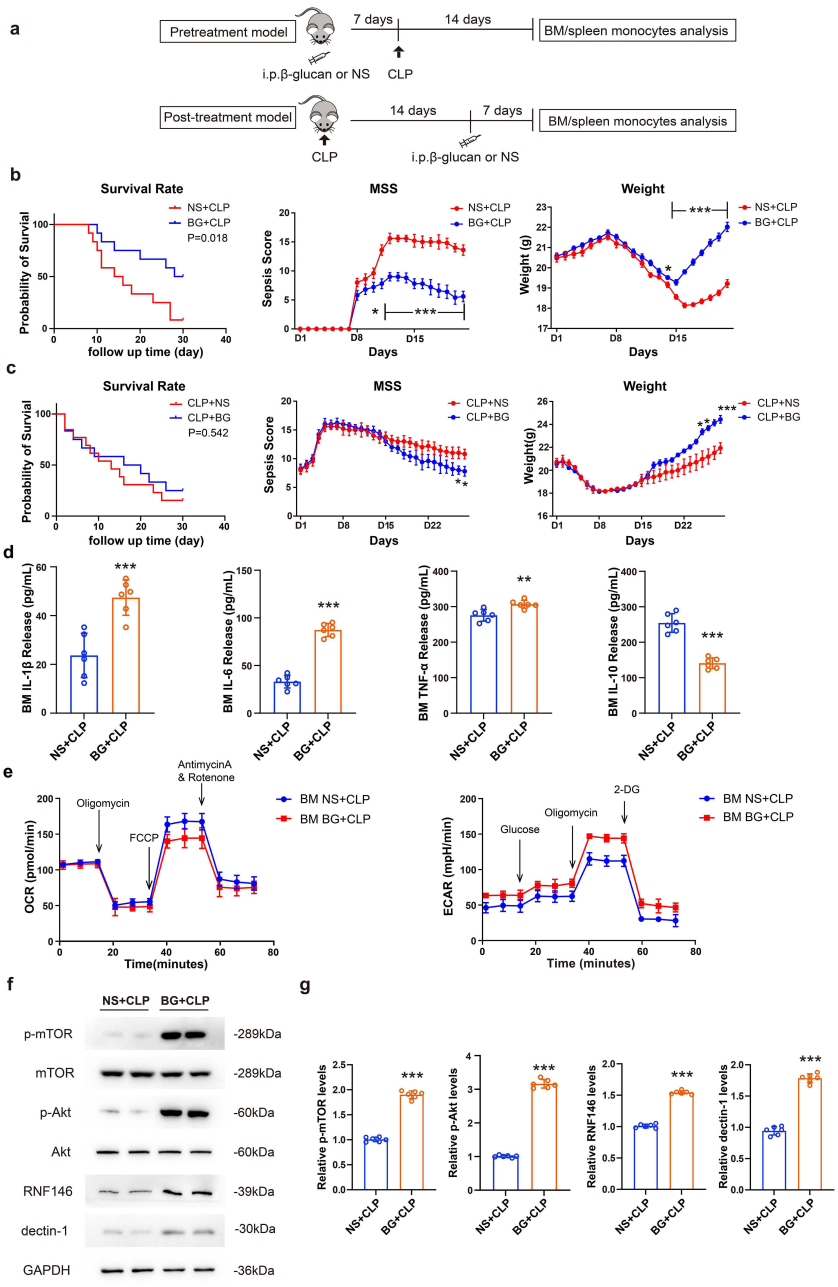

Figure 3 β-glucan pretreatment improved the immune suppressed state in septic mice and increased dectin-1/Akt/RNF146 expression. (**a**) Two types of double-hit mouse models (pretreatment or post-treatment models). Pretreatment model mice received β-glucan or NS intraperitoneally 7 days before CLP., BM or spleen monocytes were obtained for analysis 14 days later. Post-treatment model mice received CLP 14 days before β-glucan or NS intraperitoneal injection. BM or spleen monocytes were obtained for analysis 7 days later. (**b**) The survival rate, MSS and weight of β-glucan (BG+CLP) or NS pretreated septic mice (NS+CLP) (n=12 per group). MSS assessed the severity of fecal-induced peritonitis concluding seven variables and 0–28 points from mild to severe. * P < 0.05, *** P<0.001 compared with NS+CLP group. (**c**) The survival rate, MSS and weight of β-glucan (CLP+BG) or NS post-treated septic mice (CLP+NS) (n=12 per group). * P < 0.05, *** P<0.001 compared with CLP+NS group. (**d**) IL-1β, IL-6, TNF-α and IL-10 levels in BMMC from β-glucan (BG+CLP) or NS pretreated septic mice (NS+CLP) were analyzed with ELISA (n=6 per group). The error bars are presented as mean ± SD; ** P < 0.01, *** P<0.001 compared with NS+CLP group. (**e**) The Seahorse XF mitochondrial respiration analysis and glycolytic rate analysis (n=6 per group) in BMMC from β-glucan (BG+CLP) or NS pretreated septic mice (NS+CLP) were performed in accordance with the manufacturer's instructions, and normalized by total cellular protein. (**f** and **g**) mTOR, p-mTOR, Akt, p-Akt, RNF146, dectin-1 and GAPDH protein levels were detected in BMMC from β-glucan (BG+CLP) or NS pretreated septic mice (NS+CLP) by Western blot. The error bars are presented as mean ± SD; *** P<0.001 compared with NS+CLP group.

Abbreviations: BM, bone marrow; i.p., intraperitoneal injection; NS, 0.9% sodium chloride; CLP, cecal ligation and puncture; MSS, mouse sepsis score; IL, interleukin; TNF, tumor necrosis factor; OCR, oxygen consumption rate; ECAR, extracellular acidification rate; FCCP, carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazone; 2-DG, 2-deoxy-glucose; D, day.

generation and phagocytosis.[11] The induction of aerobic glycolysis through dectin-1-Akt pathway represents the metabolic cornerstone of trained immunity.[15] The activation of dectin-1 by β-glucan can induce the expression of p-Akt,[14,29] and trigger downstream Akt signaling pathway.[15] Therefore, we next examined the expressions of dectin-1, p-Akt and

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RNF146 in BMMC and spleen monocytes from β-glucan pretreated septic mice. Immunoblotting analysis revealed that the expressions of RNF146, p-mTOR, p-Akt and dectin-1 were upregulated in monocytes from septic mice with β-glucan pretreatment, which were also recognized as immune trained state in mice (shown in Figure 3f and g).

RNF146 Regulated Dectin-1-Akt-mTOR Signaling in the Trained Immune State of Murine Monocyte/Macrophage RAW264.7 Cell Line

RNF146 has been reported to be a key regulator in cell survival, energy metabolism, and immune function during both physical and pathological processes.[30,31] To further investigate the role of RNF146 in β-glucan induced trained immunity, murine monocyte/macrophage cell-line RAW264.7 was used. The immune-trained state of RAW264.7 was induced by β-glucan treatment for 24 h, and 3 days later, cells were treated with LPS for an additional 24 h (shown in Figure 4a). siRNA RNF146 was used to downregulate the expression of RNF146, and we found the decreased secretion of IL-1β, IL-6, TNF-α ($P=0.002$, $P=0.002$ and $P<0.001$, respectively) and increased secretion of IL-10 ($P=0.048$) in β-glucan pretreated RAW264.7 cells (shown in Figure 4b), while overexpression of RNF146 led to the opposite ($P=0.020$, $P=0.037$, $P<0.001$, and $P<0.001$ respectively) (shown in Figure 4c). Then OCR and ECAR analysis was performed and findings showed that knockdown of RNF146 resulted in decreased aerobic glycolysis activation (increased in spare respiratory capacity of OCR; decreased in glycolytic reserve of ECAR; $P=0.026$ and $P=0.011$, respectively, shown in Figure 4d and e), while the overexpression of RNF146 led to the opposite (decreased spare respiratory capacity of OCR; increased glycolytic reserve of ECAR, $P<0.001$ and $P=0.012$, respectively, shown in Figure 4d and e). Collectively, these results indicate that RNF146 regulates the trained immune state induced by β-glucan in monocyte/macrophage.

To further investigate the pathway, we transfected RAW264.7 with siRNA RNF146, which was treated with β-glucan and LPS, and found that knockdown of RNF146 restrained the activation of p-mTOR (shown in Figure 5a and b), while RNF146 overexpression enhanced the activation of p-mTOR (shown in Figure 5c and d). Interestingly, the expressions of p-Akt and dectin-1 increased after β-glucan and LPS treatment and were not affected by RNF146 (shown in Figure 5a–d). Furthermore, knockdown of dectin-1 using siRNA dectin-1 resulted in lower expressions of p-mTOR, p-Akt and RNF146 in β-glucan and LPS-treated RAW264.7, while dectin-1 overexpression enhanced the expressions of p-mTOR, p-Akt and RNF146 (shown in Figure 5e). We then knocked down the dectin-1 combined with the overexpression of RNF146 in RAW264.7, and found that p-mTOR increased after β-glucan and LPS treatment and the expression of p-Akt decreased, while the overexpression of dectin-1 and knockdown of RNF146 led to the opposite (shown in Figure 5f). Taken together, these results demonstrated that RNF146 might regulate the trained immune state of monocyte/macrophage via dectin-1-Akt-mTOR pathway.

Since RNF146 may serve as a regulator in the Akt pathway in trained immune state of RAW264.7, we then examined whether Akt activation alone is sufficient for RNF146 expression. To induce Akt activation, we generated a constitutively active form of Akt (Akt-CA) in RAW264.7 cells, with a dominant negative form of Akt (Akt-DN) serving as a negative control. The results showed that the forced activation of Akt through Akt-CA expression is sufficient to induce RNF146 expression ($P<0.001$) (shown in Figure 6a and b). Additionally, immunofluorescence analysis and co-immunoprecipitation (Co-IP) assay showed that Akt and RNF146 activated by β-glucan can be co-localized in RAW246.7 ($P<0.001$ and $P<0.001$, separately) (shown in Figure 6c and d), and RNF146 was immunoprecipitated (IP) by Akt (shown in Figure 6e), indicating an interaction between RNF146 and Akt. Together, these results strongly suggest that RNF146 serves as a downstream of decin-1 and Akt. RNF146 may function as a substrate of Akt and play a role in modulating trained immunity induced by β-glucan, depending on Akt activation.

Blockade of Dectin-1 Diminished Trained Immunity Induced by β-Glucan and Inhibited Akt-RNF146 Signaling in Mice

In order to further investigate the potential role of dectin-1-Akt-RNF146 pathway in trained immunity, the dectin-1 antagonist LAM was used in mice (shown in Figure 7a). After LAM treatment, the survival rate of β-glucan pretreatment mice (BG+CLP+LAM group) was not higher than the control (NS+CLP+NS group and NS+CLP+LAM group) any more ($P=0.634$ and $P=0.463$ separately) (shown in Figure 7b). And there was also no difference in MSS after 3 days of LAM injection ($P=0.256$ on the 18th day) and in weight after 2 days of LAM injection ($P=0.071$ on the 17th day) between the

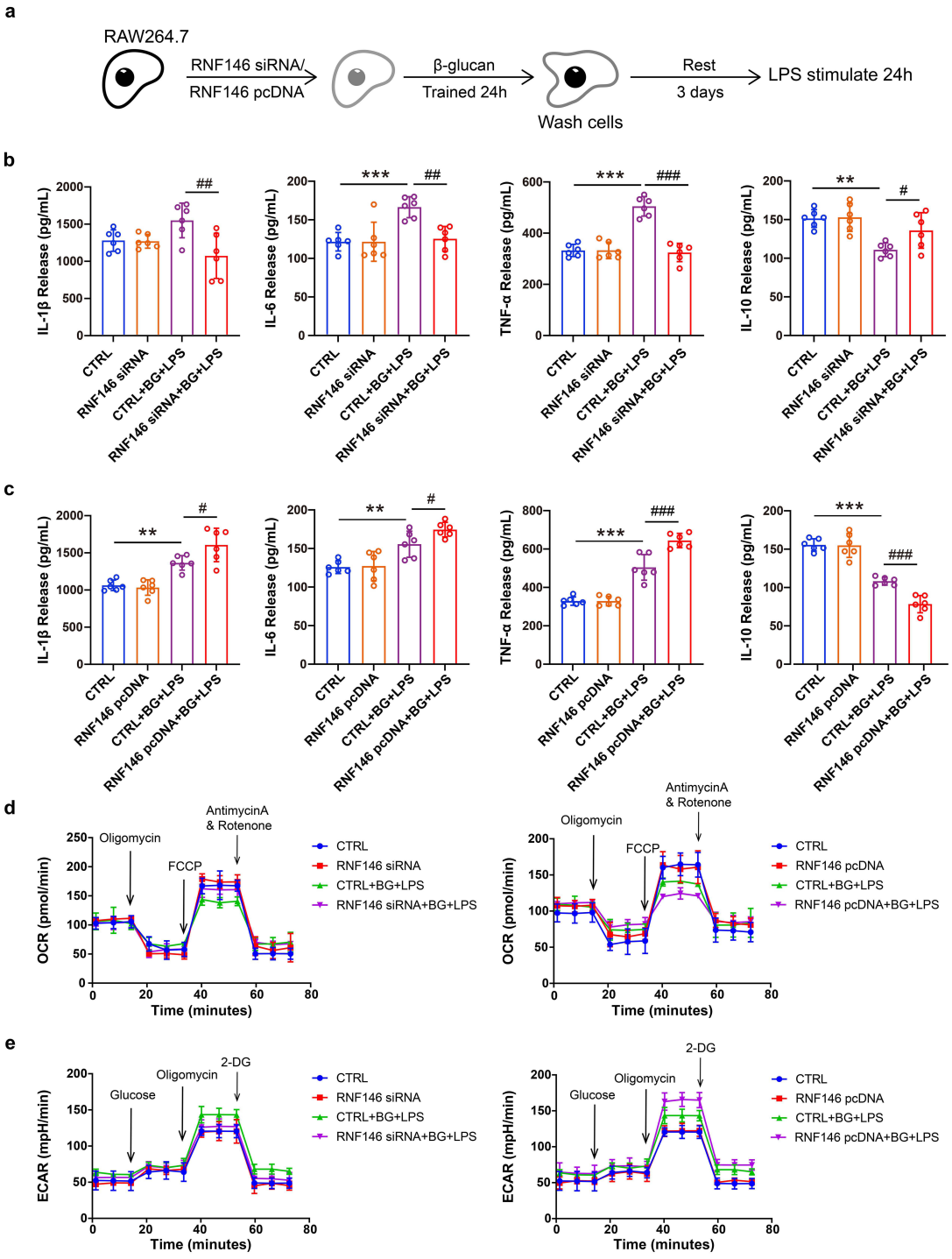

Figure 4 RNF146 regulated the release of inflammatory cytokines and cell metabolism in vitro under β-glucan pretreatment. (**a**) Schematic of in vitro RNF146 silenced or overexpressed immune trained experimental setup. RNF146 was silenced or overexpressed in the RAW264.7 cells by transient transfection with siRNA-mediated RNF146 (RNF146 siRNA) or pcDNA-RNF146 (RNF146 pcDNA). Transfected RAW264.7 cells were exposed to β-glucan (100ng/mL, 24 h), and 3 days later restimulated with LPS (100ng/mL, 24 h). (**b**) IL-1β, IL-6, TNF-α and IL-10 levels were analyzed with ELISA in control group (CTRL) and RAW264.7 transfected with RNF146 siRNA group (RNF146 siRNA) treated with BG and LPS or not (CTRL+BG+LPS, RNF146 siRNA+BG+LPS) (n=6 per group). (**c**) IL-1β, IL-6, TNF-α and IL-10 levels were analyzed with ELISA in control group (CTRL) and RAW264.7 transfected with RNF146 pcDNA group (RNF146 pcDNA) treated with BG and LPS or not (CTRL+BG+LPS, RNF146 pcDNA+BG +LPS) (n=6 per group). (**d**) The Seahorse XF mitochondrial respiration analysis in RNF146 silenced or overexpressed RAW264.7 treated with BG and LPS or not (n=6 per group) were performed in accordance with the manufacturer's instructions and normalized by total cellular protein. (**e**) The Seahorse XF glycolytic rate analysis in RNF146 silenced or overexpressed RAW264.7 treated with BG and LPS or not (n=6 per group) were performed in accordance with the manufacturer's instructions. CTRL group, RAW264.7 cells were transfected with negative control siRNA or control empty vector. The error bars are presented as mean ± SD; ** P < 0.01, *** P<0.001 compared with CTRL group; # P <0.05, ## P < 0.01, ### P<0.001 compared with CTRL+BG+LPS group.

Abbreviations: CTRL, control; OCR, oxygen consumption rate; ECAR, extracellular acidification rate; FCCP, carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazone; 2-DG, 2-deoxy-glucose; IL, interleukin; TNF, tumor necrosis factor.

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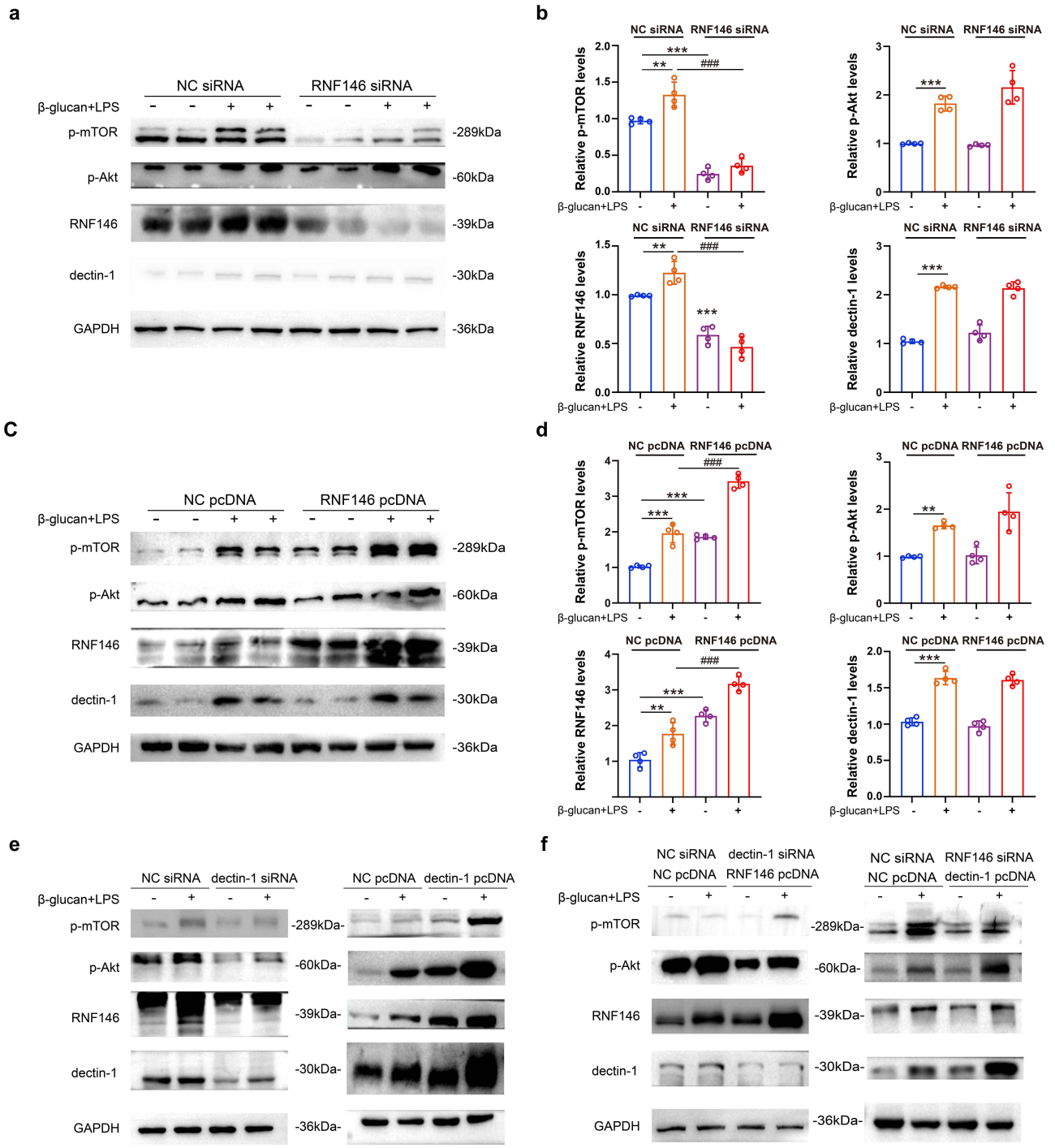

Figure 5 RNF146 regulated dectin-1-Akt-mTOR signaling in the trained immune state of murine monocyte/macrophage cell line RAW264.7. (**a** and **b**) RAW264.7 cells were transfected with RNF146 siRNA or negative control siRNA (NC siRNA) (n=4), and p-mTOR, p-Akt, RNF146, dectin-1 levels were detected under β-glucan+LPS treatment or not by Western blot. (**c** and **d**) RAW264.7 cells were transfected with pcDNA3.1-RNF146 (RNF146 pcDNA) or control empty vector (NC pcDNA) (n=4), and p-mTOR, p-Akt, RNF146, dectin-1 levels were detected under β-glucan+LPS treatment or not by Western blot. (**e**) Dectin-1 silenced or overexpressed RAW264.7 cells were constructed by transient transfection with siRNA-mediated decin-1 (dectin-1siRNA) or pcDNA-RNF146 (dectin-1pcDNA) (n=4). Control cells were transfected with negative control siRNA or control empty vector (NC siRNA or NC pcDNA) (n=4), and p-mTOR, p-Akt, RNF146, dectin-1 levels were detected under β-glucan+LPS treatment or not by Western blot. (**f**) RNF146 and decin-1 were silenced or overexpressed at the same time in RAW264.7 cells (dectin-1 siRNA RNF146 pcDNA or RNF146 siRNA dectin-1 pcDNA) (n=4). Control cells were transfected with negative control siRNA or control empty vector (NC siRNA NC pcDNA) (n=4), and p-mTOR, p-Akt, RNF146, dectin-1 levels were detected under β-glucan+LPS treatment or not by Western blot. All the transfected RAW264.7 cells were exposed to β-glucan (100ng/mL, 24 h) and LPS (100ng/mL, 24 h) restimulated 3 days after (+). Dectin-1, p-mTOR, RNF146, and p-Akt expressions were measured by Western blotting. The error bars are presented as mean ± SD; ** P < 0.01, *** P<0.001 compared with NC siRNA or NC pcDNA group; ### P<0.001 compared with NC siRNA+β-glucan+LPS or NC pcDNA+β-glucan+LPS group.

Abbreviations: LPS, lipopolysaccharide; CTRL, RAW264.7 cells transfected with negative control (NC) siRNA (or pcDNA) or control empty vector.

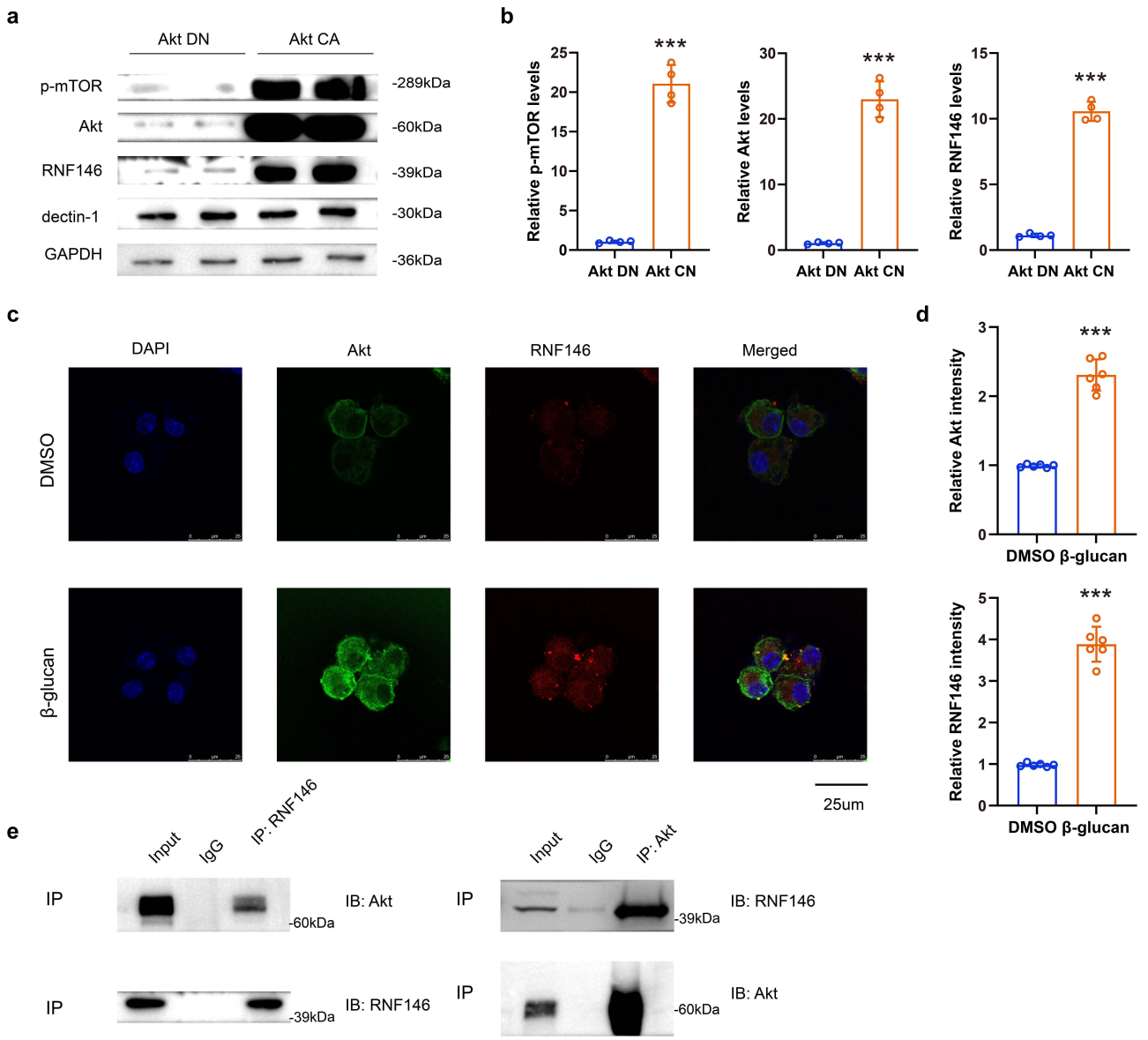

Figure 6 The role of RNF146 in Akt pathway in RAW264.7 pretreated by β-glucan. (**a** and **b**) Western blotting analysis of p-mTOR, Akt, dectin-1, and RNF146 in RAW264.7 cells transfected with Akt-CA or Akt-DN as negative control (n=4). (**c** and **d**) immunofluorescence imaging and quantitative analysis of RNF146 and Akt abundance in RAW 264.7 cells treated with β-glucan (100 ng/mL, 24 h) or control (DMSO). Scale bar=25 μM. n=6 per group. The error bars represent mean ± SEM; (**e**) Co-immunoprecipitation (Co-IP) assay of the Akt and RNF146 enrichments in the precipitated products of anti-RNF146 and anti-Akt. Akt DN: RAW264.7 cells transfected with plasmids expressing the dominant negative form of Akt (Akt-DN) as negative control; Akt CA: RAW264.7 cells transfected with plasmids expressing the constitutively active form of Akt (Akt-CA). All experiments were conducted in triplicates. *** P < 0.001 by unpaired two-tailed student's *t* test.

BG+CLP+LAM group and the NS+CLP+NS group (shown in Figure 7b). But the MSS after CLP in the NS+CLP+LAM group was significantly higher than that in the BG+CLP+LAM group except on the 18th day (P=0.092 on the 18th day, P<0.05 for other comparisons) (shown in Figure 7b). Besides, after the LAM injection, the MSS was much higher and the loss of weight was more in BG+CLP+LAM group than BG+CLP+NS group (P<0.01 for all comparisons) (shown in Figure 7b). In comparison to the BG+CLP+NS group, decreased levels of intermediate monocytes (Ly6CintCX3CR1hi) were observed from the BG+CLP+LAM group, indicating that blockade of dectin-1 reduces the inflammatory phenotype of monocyte (P<0.05) (shown in Figure 7c). LAM treatment significantly decreased IL-1β, IL-6, TNF-α release and increased IL-10 release from BMMC and decreased p-mTOR, p-Akt and RNF146 expression in BMMC from mice in BG+CLP+LAM group compared with BG+CLP+NS group (P<0.001 for all comparisons) (shown in Figure 7d–f). Taken together, these results indicated that inhibition of dectin-1 diminishes the trained immunity induced by β-glucan in septic

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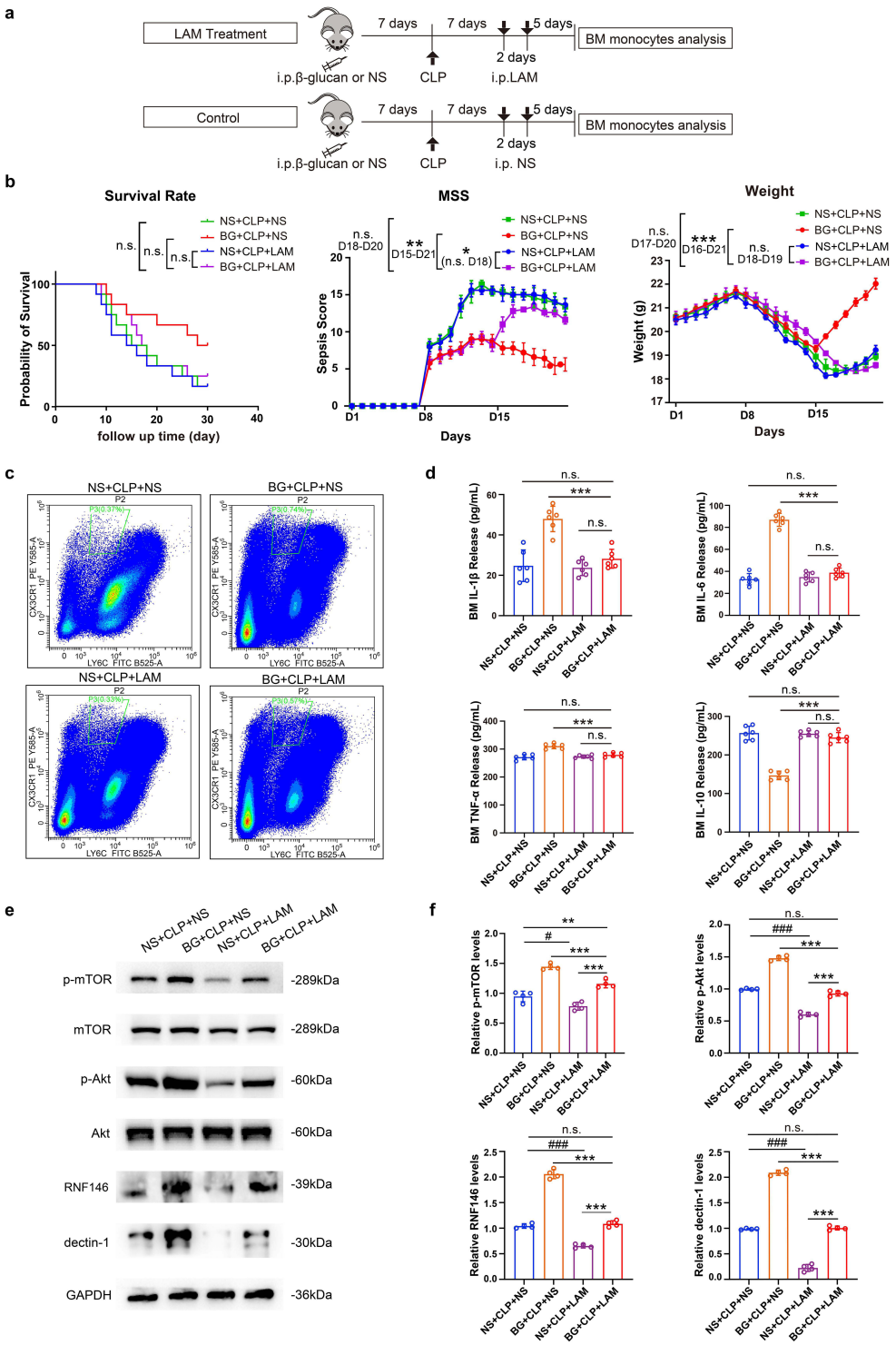

Figure 7 Blockade of dectin-1 diminishes trained immunity activation and inhibits Akt-RNF146 signaling. (**a**) C57BL/6 mice received β-glucan or NS intraperitoneally, CLP surgery performed 7 days later, and then 300 mg/kg/day dectin-1 receptor antagonist LAM or same volume of NS injected intraperitoneally once daily on the 7th day after CLP for the 2 subsequent days. BM monocytes were extracted for analysis 5 days later. (**b**) The survival rate, MSS and weight of each group (n=12 per group). n. s., no significance, * P<0.05, ** P<0.01, *** P<0.001. (**c**) Representative gating for Ly6CintCX3CR1hi intermediate monocytes (n=6). (**d**) IL-1β, IL-6, TNF-α and IL-10 were analyzed with ELISA (n=6 per group). NS+CLP+NS group and BG+CLP+NS group: C57BL/6 mice received NS or β-glucan intraperitoneally, CLP surgery performed 7 days later, and then NS injected intraperitoneally once daily on the 7th day after CLP for the 2 subsequent days as control; NS+CLP+LAM group and BG+CLP+LAM group: C57BL/6 mice received NS or β-glucan intraperitoneally, CLP surgery performed 7 days later, and then 300 mg/kg/day LAM injected intraperitoneally once daily on the 7th day after CLP for the 2 subsequent days. The error bars are presented as mean ± SD; *** P<0.001, n. s., no significance compared with BG+CLP+LAM group. (**e** and **f**) mTOR, p-mTOR, Akt, p-Akt, RNF146, dectin-1 and GAPDH protein levels were detected in monocytes collected from bone marrow of the mice by Western blotting. The error bars are presented as mean ± SD; # P<0.05, ### P<0.001 compared with NS+CLP+NS; ** P<0.05, *** P<0.001, n. s, no significance compared with BG+CLP+LAM group.

Abbreviations: LAM, laminarin; BM, bone marrow; i.p., intraperitoneal injection; NS, 0.9% sodium chloride; CLP, cecal ligation and puncture; MSS, mouse sepsis score; IL, interleukin; TNF, tumor necrosis factor; D, day.

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mice and the expressions of p-Akt and RNF146 in BMMC are decreased, providing evidence for the potential involvement of dectin-1-Akt-RNF146 signaling in trained immunity.

Discussion

In this study, our scRNA-seq results showed that RNF146 and Akt were downregulated in the immunosuppression period of septic patients, while β-glucan-induced trained immunity increased the expressions of RNF146 and Akt in monocytes from immune suppressed septic mice. We discovered a previously unknown role of RNF146 in regulating immune trained state of monocyte/macrophage via dectin-1-Akt-mTOR pathway. Moreover, β-glucan-induced trained immunity reduced the severity of sepsis and improved the survival rate of septic mice.

In sepsis, CLP-induced immunosuppression of innate immune cells is known to contribute to immune paralysis and metabolic reprogramming,[7] which places the individual at greater risk of opportunistic infections.[32,33] Previous animal studies approached the concept of "post-septic immunosuppression" using double-hit models, and those studies analyzed the susceptibility to a second stimulus from days to months after the insult.[34–37] In this study, we demonstrated CLP can be done after 7 days pretreatment with β-glucan to construct a double-hit mouse model, which was in accordance with previous research.[37]

Sepsis has long been recognized for suppressive influence on hematopoiesis, resulting in myelosuppression.[33,38,] In the past decade, emerging evidence has shown long-term adaptation[9,35] of the innate immune system through epigenetic and metabolic programming of myeloid cells,[36] resulting in hyper-responsiveness upon re-stimulation in these cells. Meanwhile, there is growing evidence that sepsis induces a trained immunity phenotype in BMMCs after the initial insult via functional reprogramming, which refines the current hypothesis of a persisting global post-septic immunosuppression on the cellular level.[39,40] In this study, we found an enhanced inflammatory response and increased aerobic glycolysis in isolated bone marrow and spleen monocytes from β-glucan pretreated, but not post-treated septic mice, which was in accordance with the characteristics of immune-trained monocytes. β-glucan induced trained immunity is similar to the clinical Bacillus Calmette Guerin (BCG) vaccine injection. Our study suggested that β-glucan pretreatment can improve the immunosuppressed state of sepsis and reprogram the metabolism of monocytes. The suppression period of sepsis is usually in the late period of sepsis and may last for a period of time depending on the patient's condition. Therefore, β-glucan-like treatment may be effective for septic patients with immunosuppression and can be applied in high-risk patients. The effectiveness of β-glucan as immunoadjuvants in sepsis was observed in the patients with Sars-Cov-2 infection.[41] While there are different chemical compositions of β-glucan and the processing for extraction and purification varies based on their functional properties, the biological response-modifying actions of its effect on septic immunosuppression need further study.[42,43] Thus, it is necessary to explore the effect of β-glucan-induced trained immunity on immunosuppression during sepsis and the potential mechanisms.

Induction of trained immunity is mediated by activation of immune and metabolic pathways.[44] The glycolytic phenotype is linked to the immune response.[45] Metabolism is a guiding force for trained immunity.[46] Aerobic glycolysis in the trained monocytes might induce pro-inflammatory cytokine production.[15] For example, glycolysis is necessary for IL-1β expression in inflammatory diseases and promotes inflammatory activation. Activated glycolytic phenotype HIF-1α regulates inflammatory gene IL-1β induction.[15,45,47] Recent research also highlights the physiological role of cytokines in the immune-metabolic interplay. IL-1β was reported to be activated to allocate glucose to immune cells, and TNF is activated from lipolysis and induces insulin resistance.[47] However, the hydrolysis of glycogen was also reported to produce several products which inhibited or reduced production and secretion of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α in inflammatory diseases.[48] Therefore, when discussing the immune training effect of β-glucan, the observed changes in cytokines in this study are due to glycolysis or immune training itself, which deserves further study.

Changes in the inflammatory capacity of monocytes are associated with altered metabolic processes.[7] A key part of metabolic rewiring of innate immune cells is the switch from oxidative phosphorylation to aerobic glycolysis.[49,50] The axis involving dectin-1 (known as a β-glucan receptor and widely expressed on the surface of the myeloid lineage[51]), Akt and mTOR are involved in this metabolic shift,[15] and play a crucial role in reducing basal respiration rate, increasing glucose consumption, and higher lactate production. Transcriptional upregulation of genes involved in glycolysis as well

as genes involved in mTOR signaling, a key regulator of glucose metabolism, are responsible for the enhanced glycolytic activity.[15,52] In our study, β-glucan pretreatment induced aerobic glycolysis in BMMC and led to the increased expression of p-mTOR, p-Akt and RNF146, indicating the involvement of Akt/mTOR pathway and RNF146 in the process. Our scRNA-seq results also suggested the potential role of RNF146 and Akt pathway in the immunosuppression period of septic patients. Xu et al previously reported that upregulation of RNF146 reduced oxidative stress-induced cell death by inhibiting parthanatos in traumatic brain injury.[53] Additionally, studies have found that RNF146 results in the ubiquitination and degradation of phosphatase and tensin homolog which is recognized as a protein substrate of RNF146, to activate the Akt/mTOR pathway and promote tumor cell proliferation and glycolysis.[54] Though the relationship of RNF146 and mitochondrial dysfunction and redox imbalance in immune cells has been extensively studied in sepsis, little is known about the function of RNF146 in trained immunity. In this study, RNF146 silencing largely eliminated the role of β-glucan induced training immunity, further confirming the role of RNF146 in the regulation of trained immunity in sepsis. Moreover, our results suggested that the effect of RNF146 in immune trained monocytes/macrophages was carried out through dectin-1-Akt-mTOR activation, and overexpression of RNF146 in RAW264.7 can fine-tune the dectin-1-Akt pathway. All the above results suggested that RNF146 may act as a promising target in sepsis-induced immunosuppression and β-glucan pretreatment induced trained immunity may prevent immunosuppressed state of sepsis.

Our scRNA-seq results also showed that MPA, which is usually regarded as a sensitive measure of platelets activation and the bridge between thrombosis and inflammation,[55,56] had an obvious increase in the immunosuppression period of septic patients compared with healthy controls. Recent researches have associated MPA with thrombotic disorders, especially cardiovascular diseases.[57–59] Thus, the increase of MPA may be an indicator of hypercoagulable state in immunosuppression state of septic patients. According to the previous researches,[60,61] the higher MPA levels are related to higher septic mortality.[60] Further studies are needed to identify the role of MPA in sepsis. OxLDL induced trained immunity was reported to acquire inflammatory phenotype of monocyte and result in increased cardiometabolic risk such as atherosclerosis.[62] Indeed, epigenetic reprogramming of monocytes induces a long-lasting pro-atherosclerotic phenotype, characterized by increased cytokine and chemokine release, such as IL-1β, which was reported to play a critical role in atherogenesis.[63] In Anette's study, the levels of proinflammatory cytokines IL-1β and IL-6 in oxLDL-induced atherogenesis were two or three times higher than control group,[63] while IL-1β and IL-6 levels in β-glucan induced trained immunity model in this study did not exceed twice that of the control group. This suggests a lower risk of atherosclerosis in our model. Furthermore, the survival rate was higher and MSS was lower in β-glucan pretreatment septic mice, suggesting the protective effect of β-glucan in our model. Ganesh et al reported a case that nichi glucan food supplement, which is a 1.3–1,6 β-glucan derived from the black yeast, decreased LDL levels, very low-density lipoprotein levels and triglycerides to the normal range and increased high-density lipoprotein levels in a subject with nondiabetic dyslipidemia on medication with rosuvastatin,[64] which also suggesting a possible protective effect against cardiovascular risk of β-glucan. Overall, the only "pre-treatment" beneficial role of β-glucan implies preventive/prophylactic treatment in high-risk septic patients, therefore future study needs to focus on safety in the stimulating trained immunity in sepsis.

Conclusion

This study indicates that β-glucan pretreatment can improve the immunosuppressed state of sepsis, reprogram the metabolism of monocytes and increase the survival rate of septic mice. For the first time, our study demonstrated that E3-ubiquitin ligase RNF146 interacted with dectin-1-Akt pathway in immune trained monocyte/macrophage. The inhibition of dectin-1 by LAM downregulated Akt-RNF146 signaling and partially reversed the β-glucan induced trained immunity in septic mice. These results will help understand the mechanism of trained immunity in sepsis and provide new targets for treating immunosuppression in sepsis.

Data Sharing Statement

The data that support the findings of our study are not publicly available due to their containing information that could compromise the privacy of research participants but are available from the corresponding author JZ upon reasonable request.

Statement of Ethics

The animal procedure, in accordance with the guidelines of the Chinese council on animal care, was approved by the animal review committee of Fudan University Shanghai Cancer Center (FUSCC-IACUC-S2023-0141). Our study was approved by the ethics committee of Zhongshan Hospital Fudan University (B2021-215R) and was registered at the Chinese Clinical Trial Registry (ChiCTR2100048148).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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