Inflammation, Vol. 42, No. 6, December 2019 (© 2019)
DOI: 10.1007/s10753-019-01056-4

ORIGINAL ARTICLE

Inhibition of Fibroblast Growth Factor Receptor by AZD4547 Protects Against Inflammation in Septic Mice

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Abstract— Sepsis is a life-threatening condition caused by the dysregulated host immune response to infection characterized by excessive secretion of inflammatory factors. AZD4547 is a selective inhibitor of fibroblast growth factor receptors that participates in the inflammatory response. The aim of this study was to investigate the inflammation-targeting effects and related molecular mechanisms of AZD4547 in sepsis using a cecal ligation and puncture model and RAW264.7 macrophages stimulated with lipopolysaccharide. AZD4547 improved the survival of CLP mice and exhibited a robust protective function against lung damage histologically. Pretreatment with AZD4547 significantly alleviated the expression of the pro-inflammatory factors IL-1β, IL-6, TNF-α, MMP9, and CXCL10 both *in vivo* and *in vitro*. In addition, AZD4547 suppressed the proliferative activity of macrophages in lung tissue and RAW264.7 macrophages. In addition, the LPS-induced phosphorylation of key proteins of NF-κB/MAPK/STAT3 pathways in RAW264.7 macrophages, such as p65, IκB-α, Erk1/2, JNK, and STAT3 proteins, could be inhibited by AZD4547 pretreatment. In conclusion, AZD4547 exerts a protective effect against excessive inflammatory damage in septic mice and may have the potential for use as an effective drug for the management of sepsis.

KEY WORDS: AZD4547; FGFR; Cytokine; Inflammation; Macrophage; Sepsis.

INTRODUCTION

An estimated 31.5 million and 19.4 million patients are diagnosed with sepsis and severe sepsis, respectively, with a potential 5.3 million deaths every year [1]. According to the latest international guidelines for the management of sepsis and septic shock (2016) of the Surviving Sepsis Campaign, sepsis is defined as a life-threatening condition caused by the dysregulated host response to infection [2]. An excessive inflammatory response contributes to major injury to organs, such as the lung, kidney, heart, and liver [3]. Therefore, specific treatment targeting inflammation represents a promising strategy for the management of sepsis.

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10753-019-01056-4) contains supplementary material, which is available to authorized users.

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Fibroblast growth factor receptor (FGFR), which includes FGFR1, FGFR2, FGFR3, and FGFR4, participates in a variety of cell activities by binding to members of the FGF family of proteins [4]. Fitzpatrick reported that the activation of FGFR1 resulted in amplification of the inflammatory response *via* immune cells [5]. Han and colleagues discovered that the expression of tumor necrosis factor alpha (TNF-α) in M0 macrophages was associated with FGF23–FGFR1 signaling [6]. These results suggest a connection between FGFR and the inflammatory response. AZD4547 is a new selective FGFR tyrosine kinase inhibitor that targets FGFR1, FGFR2, and FGFR3, with antitumor activity against FGFR-amplified tumors [7, 8]. Notably, AZD4547 has been proven both safe and biologically active in tumor treatment [4]. However, little is known about its inhibitory effects against the inflammatory response in sepsis.

In the present study, we reveal the robust inflammation-targeting effects of AZD4547 in sepsis model and investigated the potential molecular mechanism underlying this phenomenon.

METHODS AND MATERIALS

Experimental Animals

Male C57BL/6 mice (8–10 weeks old) were purchased from Shanghai SLAC Laboratory Animal Limited Liability Company (Shanghai, China). Mice were raised in a specific pathogen-free laboratory animal environment with a relative humidity of 55 ± 10%, ambient temperature of 23 ± 3 °C, and 12-h light–dark cycle. All animal experiments followed the requirements of the Institutional Animal Care and Use Committee of Wenzhou Medical University.

AZD4547 Preparation

For *in vivo* studies, AZD4547 (AbMole BioScience, Houston, TX, USA) was formulated in a 2.5% (*v*/*v*) solution of polyoxyethylenesorbitan monooleate (Tween-80) and 2.5% (*v*/*v*) solution of dimethyl sulfoxide (DMSO) in Phophate Buffered Solution (PBS). For *in vitro* studies, AZD4547 was prepared as a 1-mM stock solution in DMSO.

Cecal Ligation Puncture (CLP) Model Mice were divided into three groups randomly: Untreated control, CLP, and CLP + AZD4547. The CLP operation were described previously [9]. Untreated control mice were treated identically without ligating and puncturing cecum. AZD4547 (2.5 mg/kg) or dilution buffer was injected intraperitoneally 2 h before operation. The survival of mice was closely observed ($n = 12$ in each group) every 6 h within 4 days. For other set of experiments, mice were anesthetized 24 h post operation. Lung tissues (right lobes) were harvested, frozen immediately in liquid nitrogen, and stored at − 80 °C until analysis. The left lobes were processed in 4% paraformaldehyde for hematoxylin and eosin (H&E) and immunofluorescence staining.

Cell Culture

RAW264.7 macrophages (ATCC, Manassas, VA, USA) were cultured in DMEM (Gibco, Life Technologies, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin-streptomycin (PAN-Biotech, Aidenbach, Germany) at 37 °C in a 5% CO_2 incubator. Cells were divided into four groups: Buffer control group, AZD4547 group (1 μM for 2 h), lipopolysaccharide (LPS; from *Escherichia coli* O111:B4, Sigma, USA) group (1 μg/mL LPS for 6 h), and AZD4547 + LPS group (1 μM AZD4547 for 2 h, then LPS for 6 h).

Enzyme-Linked Immunosorbent Assay (ELISA)

Twenty-four hours after operation, whole blood was collected without anticoagulant and incubated for 30 min at room temperature. Serum was obtained by centrifugation at 6000 ×*g* for 15 min and stored at − 80 °C. RAW264.7 cell supernatants were collected and stored at − 80 °C until analysis. ELISA was used to determine the concentrations of cytokines and chemokines including interleukin-1β (IL-1β), IL-6, TNF-α, and C-X-C motif chemokine ligand 10 (CXCL10) in the serum and supernatant according to the manufacturer's instructions (Multi Science, China).

Cell Counting Kit-8 (CCK-8) Assay

The CCK-8 assay (MedChem Express, Shanghai, China) was used to determine the effects of AZD4547 on the propagation of RAW264.7 macrophages *in vitro*. RAW264.7 cells (5×10^3 cells/well) were seeded onto a 96-well plate and received the indicated treatments described above. The supernatant was replaced with fresh medium after 24 h. Next, CCK-8 solution (10 μL/well) was added and incubated at 37 °C for 2 h. The optical density at 450 nm was measured using a microplate reader (Molecular Devices, San Jose, CA, USA).

RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted from lungs and RAW264.7 macrophages using Trizol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from 2 μg of RNA using the GoScript Reverse Transcription System Kit (Promega, Madison, WI, USA) following the manufacturer's instructions. Real-time quantitative PCR (qPCR) was carried out using SYBR Green (Roche Diagnostics, Mannheim, Germany) on a LightCycler (Roche Diagnostics, Risch-Rotkreuz, Switzerland) to detect gene expression. The primer sequences are listed in Table 1. The qPCR products of FGFR1, FGFR2, FGFR3, and FGFR4 were subjected to agarose gel electrophoresis as previously described. The results were normalized to GAPDH.

Western Blot Analyses

Lung tissues and RAW264.7 macrophages were lysed with RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing 1% PMSF and 1% protein phosphatase inhibitor (P1260; Applygen, Beijing, China). Lysates containing 40–60 μg of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to PVDF membranes (EDM Millipore, Billerica, MA, USA). After blocking with 5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween 20 for 1.5 h at room temperature, specific proteins were detected with the respective primary antibody and horseradish peroxidase (HRP)-conjugated secondary antibody. WesternBright ECL (Advansta, Inc., San Jose, CA, USA) was used for visualization, and the densities of the immunoreactive bands were analyzed using ImageJ software (NIH, Bethesda, MD, USA). The following primary antibodies were used: mouse anti-β-actin (Cell Signaling Technology, Danvers, MA, USA), anti-IL-1β (Cell Signaling Technology), rabbit anti-IL-6 (Proteintech, Wuhan, China), anti-TNF-α (Cell Signaling Technology), anti-matrix metalloproteinase 9 (MMP9; Proteintech), anti-p65 (Cell Signaling Technology), anti-phospho-p65 (p-p65; Cell Signaling Technology), anti-IκB-α (Cell Signaling Technology), anti-phospho-IκB-α (p- IκB-α; Cell Signaling Technology), anti-Erk1/2 (Cell Signaling Technology), anti-phospho-Erk1/2 (p-Erk1/2; Cell Signaling Technology), anti-phospho-cJun N-terminal kinase (p-JNK; Cell Signaling Technology), anti-STAT3 (Cell Signaling Technology), and anti-phospho-STAT3 (p-STAT3; Cell Signaling Technology). The following secondary antibodies were used: goat anti-rabbit HRP-conjugated polyclonal antibody (Bio-Rad, Hercules, CA, USA) and goat anti-rabbit HRP-conjugated polyclonal antibody (Bio-Rad).

Histological and Pathological Scoring of Lung Injury

For H&E staining, lung tissues were harvested and processed with a series of steps, including fixation in 4% paraformaldehyde, dehydration, embedding, and sectioning at 4-μm thickness. H&E staining was performed to assess the severity of lung injury according to the following four categories: interstitial inflammation, neutrophil infiltration, congestion, and edema. Lung injury was scored on a 0–4-point scale as follows: no injury = 0; injury in 25% of the field = 1; injury in 50% of the field = 2; injury in 75% of the field = 3; and injury throughout the field = 4. Ten microscopic fields from each slide were analyzed and the total scores were averaged to evaluate the severity of lung injury [10]. The analyses of lung histology were performed in a blinded manner.

Table 1. Primer Sequences for PCR

Gene	Forward primer	Reverse primer
GAPDH	GCACAGTCAAGGCCGAGAAT	GCCTTCTCCATGGTGGTGAA
IL-1β	TGCCACCTTTTGACAGTGATG	TGATACTGCCTGCCTGAAGC
IL-6	TGATGGATGCTACCAAACTGGA	TGTGACTCCAGCTTATCTCTTGG
TNF-α	ACCCTCACACTCACAAACCA	ACCCTGAGCCATAATCCCCT
MMP9	GGACCCGAAGCGGACATTG	CGTCGTCGAAATGGGCATCT
CXCL10	CCAAGTGCTGCCGCTCATTTTC	TCCCTATGGCCCTCATTCTCA
FGFR1	TGAAGATCGCAGACTTTGGC	TGGTACAGTTACTGGGCTTGTC
FGFR2	TAAATACGGGCCTGATGGG	GCAAAAGATGACTGTCACCACC
FGFR3	GAAGAATGGCAAAGAATTCCG	CCTCTAGCTCCTTGTCGGTGG
FGFR4	GCTGCTGGCCGGGGTGTATC	CCGAGCACCAACCTGTCCCG

GADPH glyceraldehyde-3-phosphate dehydrogenase, *IL-1β* interleukin-1β, *IL-6* interleukin-6, *TNF-α* tumor necrosis factor alpha, *MMP9* matrix metalloproteinase 9, *CXCL10* C-X-C motif chemokine ligand 10, *FGFR* fibroblast growth factor receptor

For tissue immunofluorescence staining, the pretreated lung sections were blocked with 5% bovine serum albumin in phosphate-buffered saline containing 0.1% Tween 20 for 30 min and incubated overnight at 4 °C with primary antibodies against tissue macrophage marker F4/80 (Santa Cruz, CA, USA), Ki67 (Abcam, Cambridge, UK), and MMP9 (Proteintech). The sections were then incubated for 1 h at room temperature with FITC (Abcam) or cyt5 (Abcam), and nuclei were stained with DAPI (Cell Signaling Technology) for 5 min. Images were obtained with a laser scanning confocal microscope (Leica, Wetzlar, Germany) in three random areas.

Statistical Analysis

SPSS ver. 24.0 statistical software (IBM Corp., Armonk, NY, USA) was used for data analysis. Artworks were created by using GraphPad Prism 7.0 (GraphPad Software, USA). All data are presented as the mean ± standard error of the mean (SEM). The statistical evaluation of the data was performed using one-way ANOVA (Dunnett's t test). A value of $p < 0.05$ was considered statistically significant.

RESULTS

AZD4547 Improves Survival and Alleviates Systemic Inflammation in Septic Mice

To investigate the effect of AZD4547 on CLP mice, survival analysis was performed. The overall 96 h survival rate of CLP group was 8.3% (1/12), while it was 66.7% (8/12) in the CLP+AZD4547 group. Log-rank analysis indicated that CLP mice pre-treated with AZD4547 had significantly improved survival compared with CLP group (Fig. 1a; $p < 0.01$).

An overwhelming widespread inflammatory response is one of the main characteristics of sepsis. To explore whether AZD4547 alleviated systemic inflammation, ELISA was used to determine the concentrations of serum inflammatory factors. Serum inflammatory-associated cytokines and chemokines, such as IL-1β, IL-6, TNF-α, and CXCL10, were significantly higher in the CLP group than the untreated control group (Fig. 1b). As expected, pretreatment with AZD4547 significantly decreased the concentrations of these inflammatory factors induced by CLP operation. Our results suggest that AZD4547 protects septic mice from the CLP-induced systemic inflammatory response.

AZD4547 Alleviates Acute Lung Injury and Pulmonary Inflammation in Septic Mice

We further explored the role of AZD4547 on CLP-induced acute lung injury. Lung tissues were harvested and subjected to histologic and morphometric analyses. H&E staining showed that the CLP group had more severe lung injury based on the highest semi-quantitative pathological scores, whereas the untreated control and AZD4547+CLP groups showed the opposite results (Fig. 2a; $p < 0.01$and $p < 0.01$, respectively).

Next, we compared the expression of inflammatory markers in lung tissues among the three groups. As expected, both untreated control mice and AZD4547-pretreated CLP mice showed significantly lower mRNA expression of IL-1β, IL-6, TNF-α, CXCL10, and MMP9 than CLP alone mice (Fig. 2b), And the overexpressions of IL-1β, IL-6, and MMP9 induced by CLP operation were significantly decreased by the pretreatment of AZD4547 (Fig. 2c). Besides, immunofluorescence staining showed that MMP9 were also more abundant in the CLP group than the AZD4547+CLP and untreated control groups, indicating that AZD4547 decreased the numbers of MMP9 positive cells in lung tissues (Fig. 3a).

Macrophages are the major initiator cells of inflammatory process and play a critical role in the activation of the inflammatory cascade. Immunofluorescence staining of lung sections was performed to investigate whether AZD4547 alleviated the infiltrative and proliferative activities of macrophages. F4/80 is a specific biomarker of macrophages and Ki67 is a cellular marker of proliferation [11]. CLP operation led to excessive accumulation of macrophages in lung tissue. In contrast, only a lower number of F4/80+ and Ki67+ cells were detected in the lung tissues of CLP mice pretreated with AZD4547 (Fig. 3b).

AZD4547 Inhibits RAW264.7 Macrophages from Secreting LPS-Induced Pro-inflammatory Factors

Macrophages (RAW264.7) were divided into four groups: neither LPS nor AZD4547 (control), AZD4547 alone, LPS alone, and LPS pretreated with AZD4547 (AZD4547+LPS). RAW264.7 macrophages stimulated with LPS were cultured in the presence of AZD4547 at concentrations of 0.1, 0.2, 0.5, and 1 μM. Western blot analysis revealed that AZD4547, at a concentration of 1 μM, exhibited the best attenuation effect against the inflammatory response in LPS-stimulated RAW264.7 cells (Fig. 4a).

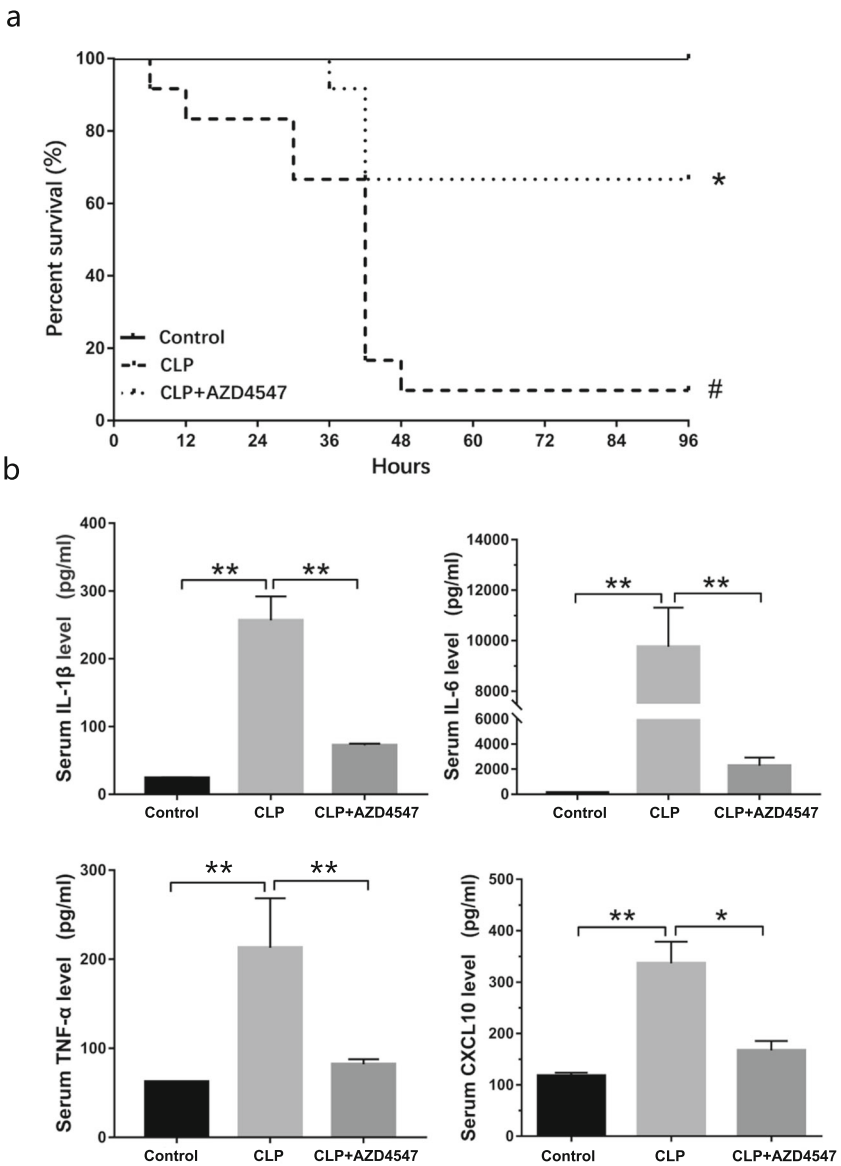

Fig. 1. AZD4547 improves survival and alleviates systemic inflammation in septic mice. **a** Mice were divided randomly into three groups—untreated control group ($n = 12$), CLP group ($n = 12$), and CLP+AZD4547 group ($n = 12$), and were processed accordingly. Survival was monitored and the differences were analyzed by using log-rank (Mantel-cox) test. Pound sign indicated the difference between untreated control group and CLP group (#, $p < 0.05$); asterisk indicated the difference between CLP group and CLP+AZD4547 group (*, $p < 0.05$). **b** ELISA were used to determine the concentrations of serum IL-1 β, IL-6, TNF-α, and CXCL10. ($n = 5$ in each group). Values were shown as mean±SEM ($n = 3$ or 5 in each group), *$p < 0.05$, **$p < 0.01$.

Total mRNA and protein were extracted to analyze the expression of pro-inflammatory factors. Pretreatment with AZD4547 significantly decreased the mRNA levels of IL-1β, IL-6, TNF-α, CXCL10, and MMP9 induced by LPS stimulation (Fig. 4b). Moreover, decreased protein expressions of IL-1β, TNF-α, and MMP9 was observed in the AZD4547+LPS group compared with the LPS alone group (Fig. 4c).

ELISA was used to determine the concentrations of pro-inflammatory factors in the supernatant. Compared with the control group, the release of IL-1β, IL-6, and TNF-α, which participate in the acute phase of the inflammatory response, was dramatically increased in the LPS alone group. However, pretreatment with AZD4547 alleviated the expression of these pro-inflammatory factors (Fig. 4d). Besides, CCK-8 results suggested that the

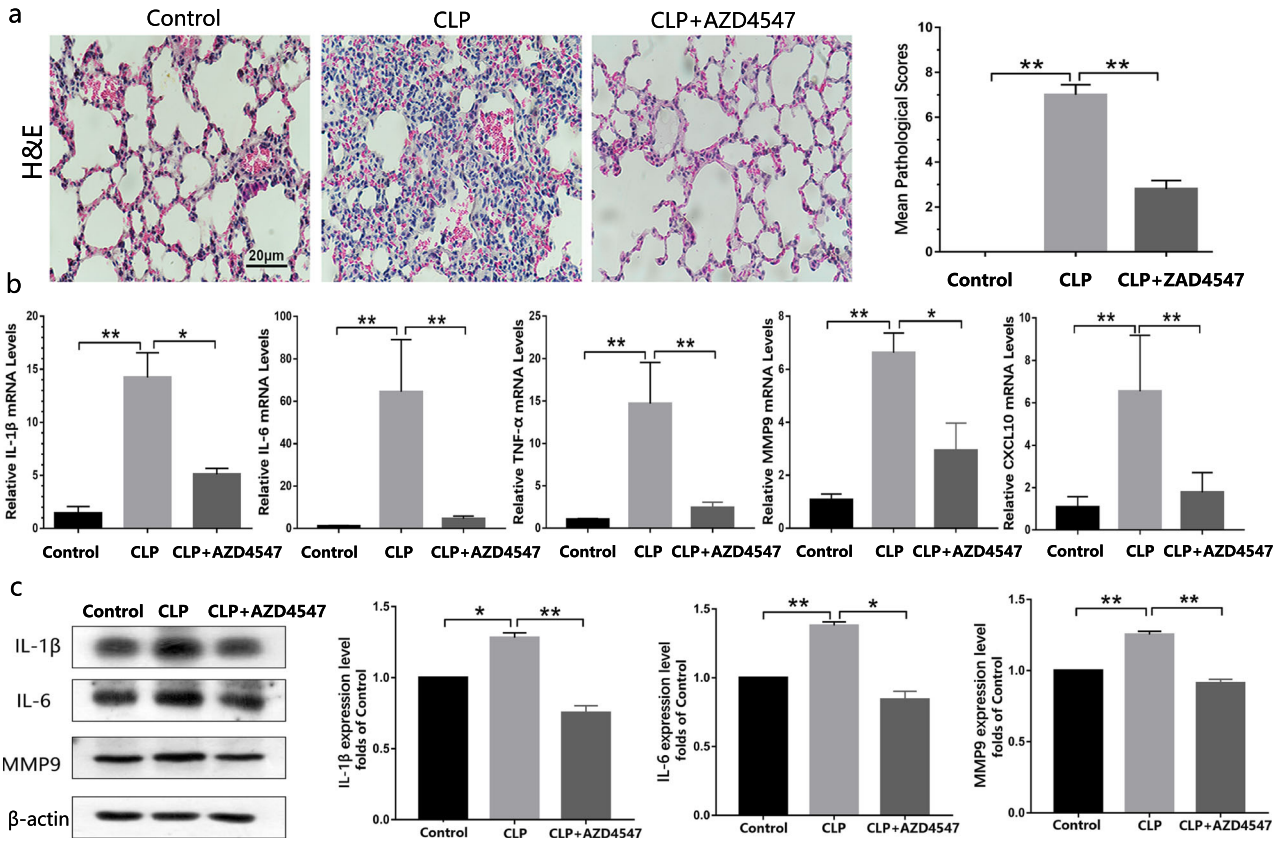

Fig. 2. AZD4547 alleviates acute lung injury and pulmonary inflammation in septic mice. Twenty-four-hour after operation, lung tissues were collected to assess the inflammatory response. **a** Lung tissues of untreated control, CLP, and CLP+AZD4547 groups were stained with H&E. A representative result was shown at × 400 magnification (left). Semi-quantitative scores of lung injury were calculated based on H&E staining ($n = 5$ in each group, right). **b** Relative mRNA levels of IL-1β, IL-6, TNF-α, MMP9, and CXCL10 in lung tissues ($n = 3$ in each group). **c** Western blot results of IL-1β, IL-6, and MMP9 in lung tissues (left). Relative protein expression levels of IL-1β, IL-6, and MMP9 normalized to β-actin (right). Values were shown as mean±SEM, $*p < 0.05$, $**p < 0.01$.

proliferative activity of RAW264.7 macrophages could be inhibited by AZD4547 pretreatment (Fig. 4e).

AZD4547 Inhibits the Acute Inflammatory Response *via* the NF-κB, MAPK, and STAT3 Signaling Pathways

In vivo and *in vitro* experiments demonstrated that AZD4547 alleviated the septic inflammatory response. NF-κB, MAPK, and STAT3 signaling pathways play a key role in regulating the inflammatory response. They interact with each other to adjust the production of cytokines such as IL-1β, IL-6, and TNF-α [12, 13]. Therefore, we investigated whether AZD4547 inhibited the acute inflammatory response *via* these signaling pathways.

Regarding the NF-κB pathway, phosphorylated p65 and IκB-α were more abundant in the LPS-stimulated group than the control group. More importantly, phosphorylation of two key proteins involved in the NF-κB pathway was inhibited by pretreatment with AZD4547 (Fig. 5a). Regarding the MAPK pathway, pretreatment of RAW264.7 macrophages with AZD4547 significantly reduced the phosphorylation levels of Erk1/2 and JNK induced by LPS stimulation, indicating that AZD4547 inhibited the activation of the MAPK signaling pathway (Fig. 5b). In addition to the NF-κB and MAPK pathways, LPS stimulation activated the STAT3 pathway, which was inhibited by pretreatment with AZD4547 (Fig. 5c). These results suggest that

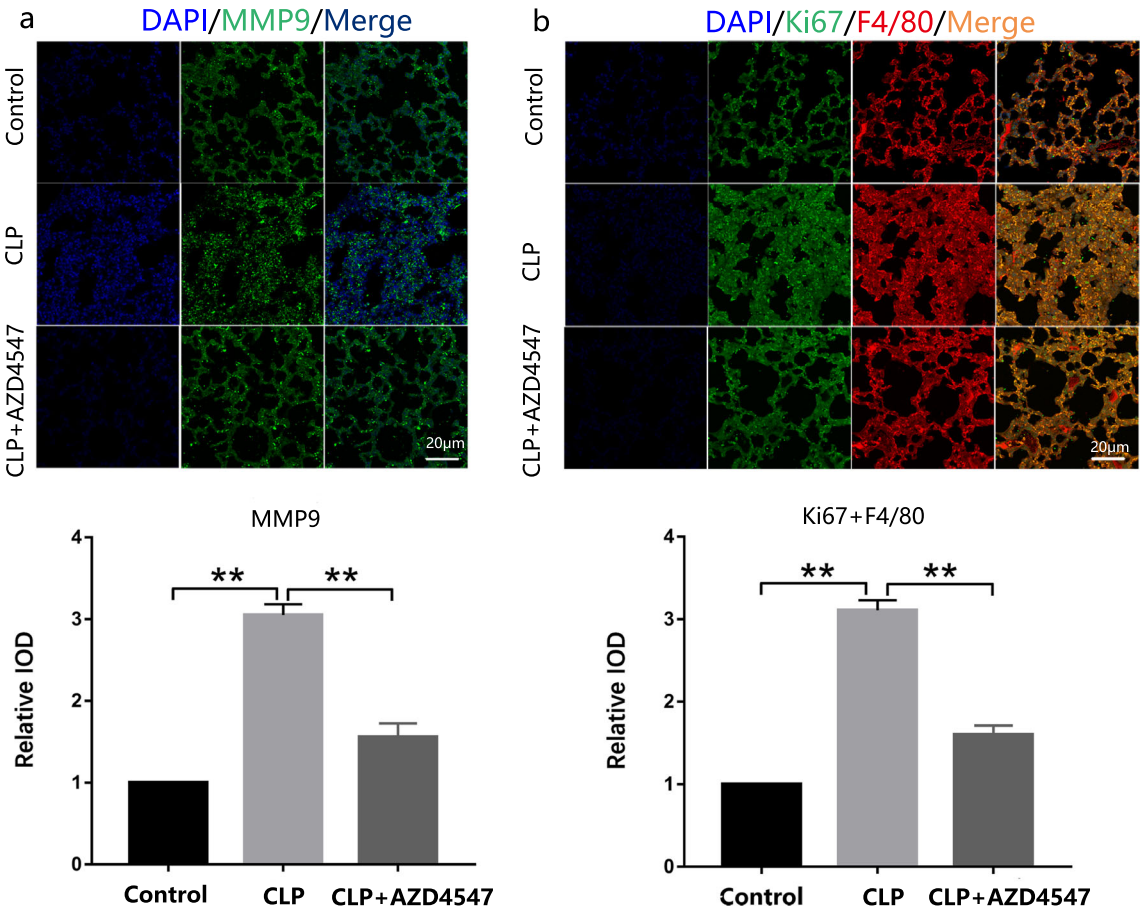

Fig. 3. AZD4547 decrease the expression of MMP9 in lung tissue and the propagation of pulmonary macrophage in septic mice. Twenty-four-hour after operation, lung tissues were collected and subjected to immunofluorescent staining. **a** the expression of MMP9 (green) in lung tissue was evaluated by immunofluorescence under a confocal microscope (× 400, above). **b** Immunofluorescent staining of F4/80 (red) and Ki67 (green) in lung tissues. Representative images are shown above (× 400). Nuclei were stained with DAPI (blue). Comparisons of relative IOD (integrated optical density) are shown below ($n = 5$ per group). IOD values are shown as mean±SEM, * $p < 0.05$, ** $p < 0.01$.

AZD4547 may exert anti-inflammatory effects by acting on the NF-κB, MAPK, and STAT3 signaling pathways.

DISCUSSION

Severe sepsis and septic shock remain the major cause of morbidity and mortality in intensive care units, affecting millions of people worldwide, and is increasing in incidence [14]. Although a variety of strategies have been developed, there are no available specific therapeutic agents for the management of sepsis. The anti-inflammatory drug ulinastatin has been used in the clinical treatment of sepsis, and has been proven to lower the death rate [15]. The exploration of effective drugs that target the inflammatory response is of great significance to the management of sepsis. In this study, the inhibitor AZD4547 was revealed as a potent candidate to protect septic mice from an overwhelming inflammatory response and the potential mechanism was elucidated *via in vivo* and *in vitro* experiments.

AZD4547 is a selective FGFR tyrosine kinase inhibitor targeting FGFR1, FGFR2, and FGFR3 [4]. We detected the expressions of FGFR1/2/3/4 in lung tissues of mice and in RAW264.7 macrophages by using agarose gel electrophoresis. FGFR1, FGFR2, and FGFR4 are expressed in RAW264.7 cells (Supplementary.1a), and FGFR 1/2/3/4 are all expressed in lung tissues (Supplementary.1b), supporting their use as attractive targets for AZD4547

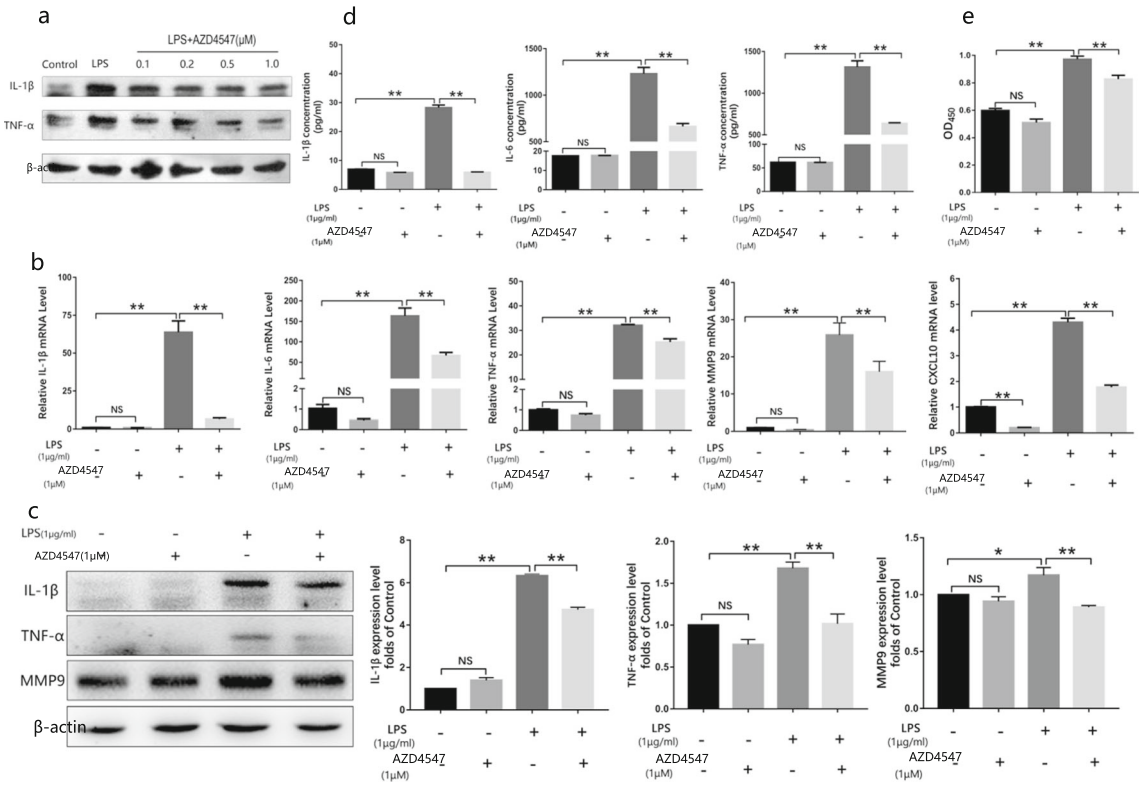

Fig. 4. AZD4547 inhibits RAW264.7 macrophages from secreting LPS-induced pro-inflammatory factors. **a** Western blot results of the IL-1β and TNF-α expression in LPS-stimulated RAW264.7 macrophages pretreated with AZD4547 at concentrations of 0.1, 0.2, 0.5, and 1 μM. **b** Effects of AZD4547 on the relative mRNA levels of IL-1β, IL-6, TNF-α, MMP9, and CXCL10 in LPS-induced RAW264.7 macrophages. **c** Western blot results of IL-1β, TNF-α, and MMP9 protein levels in RAW264.7 macrophages (left). Relative protein expression levels of IL-1β, TNF-α, and MMP9 normalized to β-actin (right). **d** Concentrations of IL-1β, IL-6, and TNF-α in the supernatant determined by ELISA. **e** Proliferative activity of RAW264.7 macrophages in the four groups evaluated by the CCK-8 assay. Values are shown as mean ± SEM, $*p < 0.05$, $**p < 0.01$. NS, not significant.

therapy. These results suggest that the application of AZD4547 to protect septic mice is theoretically feasible.

Macrophages are the most important cells in the innate immune system, given their wide distribution from peripheral blood to various tissues and organs, such as the lung and liver [16]. Additionally, macrophages are major innate immunity cells that produce pro-inflammatory cytokines, leading to an excessive inflammatory response resulting in tissue injury, fatal multi-organ dysfunction, and even death [17, 18]. Excessive accumulation and subsequent activation of macrophages during sepsis may directly affect the outcome of sepsis. Our *in vivo* experiments showed that CLP operation elicited higher density of macrophages, whereas AZD4547 pretreatment significantly decreased the accumulation of macrophages with reduced proliferative activity. Notably, the accumulation of macrophage in lung tissues of CLP mice could be resulted from local macrophage proliferation and recruitment from the surrounding tissues [19]. Whether AZD4547 could block tissue recruitment that contributes to the accumulation of macrophages needs to be further explored. Moreover, *in vitro* experiments demonstrated that AZD4547 inhibited the proliferative activity of RAW264.7 macrophages. In addition, pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, were down-regulated by pretreatment with AZD4547. These results suggest that AZD4547 attenuates the inflammatory response in septic mice by inhibiting the proliferation of macrophages and decreasing cytokine production.

As described above, AZD4547 not only down-regulated the expressions of IL-1β, IL-6, and TNF-α both *in vivo* and *in vitro*, but also decreased the expressions of CXCL10 and MMP9 proteins. CXCL10, which belongs to the CXC chemokine family, was proven a valid biomarker for sepsis [20]. It is also related to physiological dysfunction and organ injury in acute inflammation syndrome.

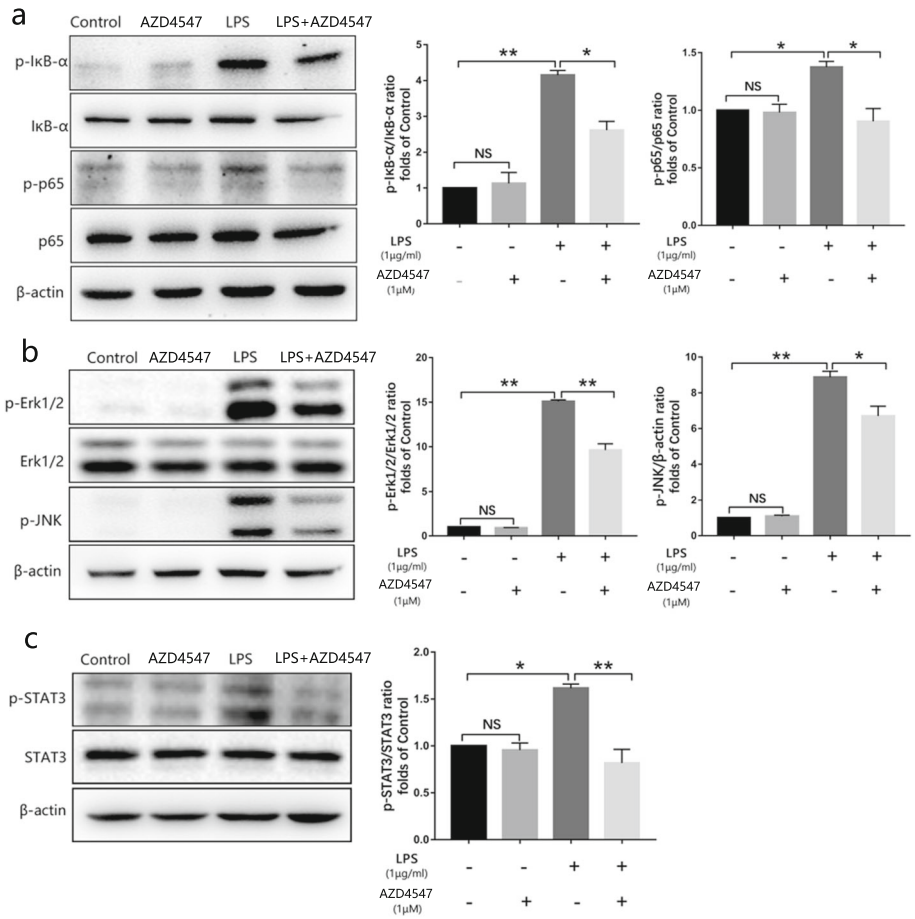

Fig. 5. Assessment of three potential signaling pathways that participate in the inflammatory response *via* Western blot. **a** Effects of AZD4547 on LPS-induced phosphorylation of p65 and IκB-α in RAW264.7 macrophages stimulated by LPS. **b** The expression of Erk1/2, p-Erk1/2, and p-JNK proteins in LPS-treated RAW264.7 macrophages. **c** AZD4547 inhibited the activation of STAT3 in LPS-stimulated RAW264.7 macrophages. The expression of STAT3 and p-STAT3 proteins were detected by western blot. The data are present as mean±SEM, $*p < 0.05$, $**p < 0.01$. NS, not significant.

CXCL10 knockout resulted in lower concentrations of IL-6 in the plasma and improved the survival rate of CLP mice [21]. Moreover, MMP9 is an independent predictor of 28-day mortality in septic patients [22]. The inhibition of MMP9 attenuated septic lung injury by regulating the platelet-dependent infiltration of neutrophils and tissue damage [23]. In the present study, the expressions of CXCL10 and MMP9 in CLP mice and LPS-stimulated RAW264.7 macrophages were significantly higher than control groups, which could be down-regulated by pre-treatment with AZD4547.

NF-κB, MAPK, and STAT3 signaling pathways are closely related to the secretion of various inflammatory mediators during sepsis. The NF-κB signaling cascade is a key initiator of hyperinflammation in sepsis [24]. In resting cells, NF-kB dimers interact with inhibitory proteins of the IκB family (IκB-α) in the cytoplasm. The phosphorylation of IκB-α protein results in the ubiquitination and degradation of IκB-α proteins from NF-κB. Next, activated NF-κB is translocated into the nucleus, where it binds to specific DNA sequences and promotes the transcription of target genes that together regulate the inflammatory response [25, 26]. Erk1/2 and JNK proteins are members of the MAPK pathway, which is also sensitive to extracellular stimulation, such as bacterial products, cytokines, and chemokines. The Erk1/2 protein has an important influence on macrophages, regulating cytokine production *via* both transcriptional and post-transcriptional mechanisms. Additionally, JNK is a crucial mediator involved in the activation of proinflammatory cytokines [27, 28]. In conjunction with NF-κB and MAPK, the STAT pathway is critical for persistent inflammation in

many conditions, such as infection and tumorigenesis [29]. The activation of STAT3 is related to the production of various pro-inflammatory molecules [30, 31], and the inhibition of STAT3 activity attenuates inflammation in LPS-induced acute lung injury [29]. In this study, we demonstrated that pretreatment with AZD4547 significantly inhibited the phosphorylation of key proteins of NF-κB, MAPK, and STAT3 pathways in RAW264.7.

In conclusion, we first report that AZD4547 could improve the survival of CLP mice and exhibit a robust protective function against septic lung injury. These positive effects may be mediated by alleviating inflammatory cytokines and inhibiting the proliferative and secretory functions of macrophages. Whether AZD4547 protects other organs from excessive inflammatory damage in sepsis and whether other FGFR inhibitors exhibit similar positive functions remain to be explored.

ACKNOWLEDGMENTS

All animal experiments were approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University.

FUNDING INFORMATION

This work was supported by the National Natural Science Foundation of China (81671968) and the Medical Innovation Discipline of Zhejiang Province (Critical Care Medicine, Y2015).

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