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C646 modulates inflammatory response and antibacterial activity of macrophage

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ABSTRACT

C646 is a newly discovered competitive p300/CREB-binding protein-specific inhibitor. Previous studies have shown its potential antitumor activity, but the immunomodulatory function of C646 remains largely unknown. In this study, we investigated the effects of C646 in cytokine expression and antibacterial activity in mouse macrophages. Results showed that C646 significantly reduced LPS-induced pro-inflammatory cytokines, which relied on suppression of JNK, ERK1/2, and NF-κB p65 signaling pathways. In addition, the inhibitory effects were not associated with modulating the expression of CD14/TLR4/MD2 complex or antagonizing its binding ability to LPS. Furthermore, C646 also down-regulated the levels of FcγR III/II and CR3 on macrophage, impaired the phagocytic ability against *E. coli*, and blocked phagosome-lysosome fusion. Consistent with this, C646 inhibited macrophage-associated bactericidal ability. Collectively, these data indicated that C646 exhibited potent immunomodulatory effects on macrophage both in the production of pro-inflammatory cytokines and bacterial phagocytosis.

1. Introduction

The innate immunity plays a crucial role in triggering inflammation and host defense response, which is primarily mediated by phagocytic cells, such as macrophages, granulocytes and monocytes [1]. Studies have demonstrated that macrophages are not only the most typical initiators of local inflammation but also the principal components of tissue microenvironment [2,3]. Following infection or tissue injury, macrophages are usually activated and exhibit a mature phenotype, which is mainly depend on their pattern recognition receptors (PRRs) in sensing common bacterial constituents, foreign substances and dead cells. This recognition leads to quickly production of various pro-inflammatory mediators, such as TNF-α and IL-6, which participate in the activation of further antimicrobial mechanisms. In addition to triggering PRRs, macrophages could interaction with microorganisms through phagocytic receptors, which stimulate the engulfment and the destruction of pathogens in newly formed phagosomes. The fusion of lysosomes with phagosomes, which called "phagosome maturation", provides a strongly acidic microenvironment, usually killing ingested microbes [4]. Therefore, macrophage has been a potential target for treating inflammatory and infectious diseases.

Histone acetylation is a major posttranslational modification which is tightly regulated by histone acetyltransferases (HATs) and deacetylases (HDACs) [5]. Generally, acetylation by HATs is associated with gene activation, whereas deacetylation by HDACs is linked to transcription repression. Currently, > 20 HATs have been identified including p300/CBP, GCN5/PCAF, MYST and Rtt109. While the immunomodulatory effects of HDACs have been investigated extensively [6,7], few studies have been focused on that of HATs. Actually, studies have shown that aberrant forms of HATs are associated with various inflammation or infectious diseases [8–10]. Therefore, HATs may play an important role in immune response.

C646, a potent and selective p300/CBP histone acetyltransferase inhibitor, has been shown to have pleiotropic activity, including neuroprotective, anti-cancer and anti-epithelial-mesenchymal transition (anti-EMT) effects [11–13]. However, the immune-modulatory properties of C646 remain largely unclear. In this study, the effects of C646 on inflammatory cytokine production and antibacterial function of macrophage were investigated.

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2. Materials and methods

2.1. Reagents

C646 was obtained from Selleck (Houston, TX). LPS (*E. coli* O111:B4) was obtained from InvivoGen (San Diego, CA), FITC-LPS (*E.coli* O111:B4), penicillin and streptomycin were obtained from Sigma-Aldrich (St. Louis, MO). RPMI-1640 medium was obtained from Hyclone (Logan, UT), fetal bovine serum (FBS) was acquired from Atlanta Biologicals (Lawrenceville, GA). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo (Tokyo, Japan).

2.2. Primary cell cultures

Mouse bone marrow-derived macrophages (BMDMs) were prepared from C57BL/6 mice (6 to 8 weeks old, male) using methods described previously with few modifications [14]. In brief, bone marrow harvested from tibias and femurs was kept for 7 d in RPMI-1640 supplemented with 10% FBS, 1% streptomycin-penicillin, and 20 ng/ml M-CSF (R&D, USA). The purity of BMDMs was > 95%, as confirmed by flow cytometry analysis of F4/80 positive cells staining with an anti-F4/80 mAb (BioLegend, USA).

2.3. Cytotoxicity assay

The effect of C646 on BMDM viability was assessed using the CCK-8. The cells were seeded in 96-well plates at a density of 5×10^4 cells/well. BMDMs were cultured with different concentrations of C646 or DMSO for 24 h. Then, CCK-8 (20 μl/well) was added and incubated for 3–4 h. The absorbance was read at 450 nm using a microplate reader. The optical density (OD) of control cells was used as 100% viability, and the results of C646 or DMSO treatments were expressed as percentage of control.

2.4. Measurement of pro-inflammatory cytokines

For TNF-α, MCP-1 and IL-6 measurements, BMDMs were pretreated with or without C646 for 2 h before LPS stimulation for another 24 h. The levels of pro-inflammatory cytokines in cell culture supernatants were analyzed by cytometric bead array (CBA) using flow cytometer. TNF-α, MCP-1 and IL-6 mRNA expression in BMDMs were assessed using real-time quantitative PCR as described previously. Briefly, total RNA was extracted by Trizol reagent (Invitrogen, USA) and transcribed using M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's instructions. Amplification of cDNA was conducted using LightCycler system (Roche Molecular Biochemicals, USA). The mRNA expression of the cytokines was normalized with the housekeeping gene actin. All primers used in this study were designed as follows: mouse TNF-α, forward, 5′-CCACCACGCTCTTCTGTCTAC-3′, reverse, 5′- AGGGTCTGGGCCATAGAACT-3′; mouse MCP-1, forward, 5′- CCTGCTGTTCACAGTTGCC-3′, reverse, 5′-ATTGGGATCATCTTGCT GGT-3′; mouse IL-6, forward, 5′-TAGTCCTTCCTACCCCAATTT-3′, reverse, 5′-TTGGTCCTTAGCCACTCCTTC-3′; mouse actin, forward, 5′-GAGGGAAATCGTGCGTGAC-3′, reverse, 5′- AGAAGGAAGGCTGGA AAA-3′.

2.5. Analysis of cell surface markers

BMDMs were cultured in 24-well plates (2×10^5 cells/well). The cells were pretreated with C646 or DMSO (as control) for 2 h, then collected and stained with anti-F4/80, anti-CD14, anti-TLR4/MD2, anti-CR3, anti-FcγR I, anti-FcγR III/II, and isotype control mAbs (all from BioLegend, USA) for 30 min at 4 °C. Cells were analyzed by flow cytometry after washing with phosphate buffered saline (PBS).

2.6. Analysis of LPS binding to BMDMs

The binding of FITC-LPS to BMDMs was monitored as described previously. In brief, BMDMs (5×10^5) were pretreated with C646 or DMSO (as control) for 2 h, then incubated with FITC-LPS for 1 h. The binding of FITC-LPS was detected by flow cytometry after washing twice with PBS,

2.7. Phagocytosis assay

Bacterial uptake includes bound bacteria and ingested bacteria, while phagocytosis includes only the ingested bacteria. As described previously [15], BMDMs were incubated with Alexa Fluor 488-*E. coli* (Invitrogen, USA) at a ratio of 1:20 (macrophage/bacteria) for 45 m, and then assessed by flow cytometry for bacterial uptake. After quenching the external fluorescence of the bound bacteria by 0.025% crystal violet (Sigma-Aldrich, USA), bacterial phagocytosis was further determined by flow cytometry.

2.8. Western blotting

Proteins were extracted in RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Roche Diagnostics, USA). Protein samples were quantified using Pierce BCA kit (Thermo Fisher Scientific, USA), 25–50 μg denatured protein was loaded on denaturing SDS-polyacrylamide gels, and then transferred to 0.45 μm PVDF membranes (Millipore, USA). Blots were blocked in 5% skim milk in Tris-buffered saline with Tween-20 (TBST) and probed with primary antibodies against ERK1/2, phospho-ERK1/2, JNK, phospho-JNK, p38, phospho-p38, p65, phospho-p65 (all from Cell Signaling Technology, USA), actin (Sigma, USA). After washing, the blots were incubated with secondary horseradish peroxidase (HRP)-conjugated antibodies. Blots were revealed with the chemiluminescence (ECL) western blotting system (GE Healthcare, USA).

2.9. Phagosome/lysosome fusion assay

As described previously [16], BMDMs were harvested and seeded into 35 mm glass bottom dish (Corning, USA) at 2×10^5 cells/dish. Culturing at 37 °C for 6 h, cells were incubated with 50 nM LysoTracker red (Invitrogen, USA) for 30 min and further co-cultured with Alexa Fluor 488-*E. coli* (macrophage/bacteria = 1:20) for 45 min. Washed five times vigorously in cold PBS, cells were fixed in 2% paraformaldehyde (Sigma-Aldrich). Hoechst 33342 (Molecular Probes) was used for staining cell nuclei. Fluorescent images were acquired using FV1000 confocal microscope (Olympus, Germany). Alexa Fluor 488-*E. coli* contained phagosomes and LysoTracker red labeled lysosomes were stained in green and red respectively, whereas phagosomes fused with lysosomes were stained in yellow due to the coexistence of Alexa Fluor 488 and LysoTracker red.

2.10. Intracellular bacterial killing assay

Bactericidal ability was detected in vitro as described previously [16]. Briefly, BMDMs were loaded with live *E. coli* (ATCC, USA) at 37 °C for 10 min (macrophage/bacteria = 1:20) and marked as time A. Whereas macrophages were incubated with bacteria for further 60 min and marked as time B. At each time point, macrophages were washed with PBS for three times and lysed in 0.5 ml lysis buffer for 5–10 min. The cell lysates were diluted serially and plated on agar (Sigma-Aldrich, USA). Bacterial CFU was counted after 24 h culturing at 37 °C. The following formula was used for calculating the bactericidal rate of macrophages: bactericidal rate (%) = (1 – CFU at time B/CFU at time A) × 100%.

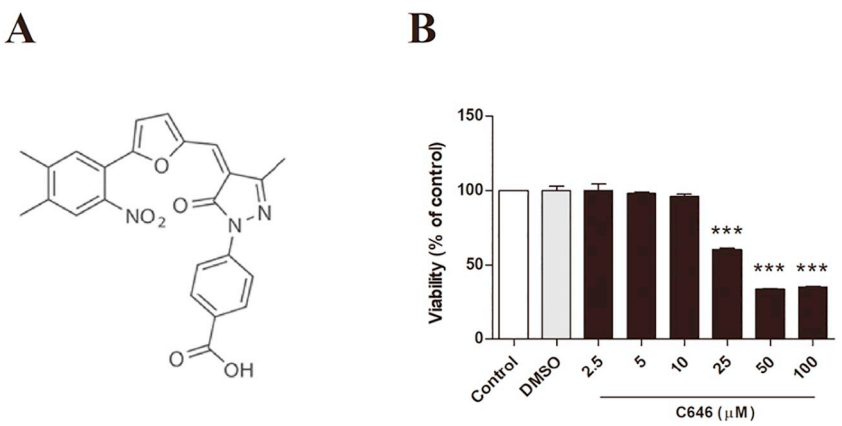

Fig. 1. The effect of C646 on BMDM viability. (A) Chemical structure of C646. (B) Effect of C646 on BMDM viability. BMDMs were treated with various concentrations of C646 or equal volume of DMSO (Con) for 24 h. Cell viability was determined by CCK-8 assay. The result expressed as mean ± SD of four independent experiments (n = 3). ***P < 0.001 compared to DMSO alone.

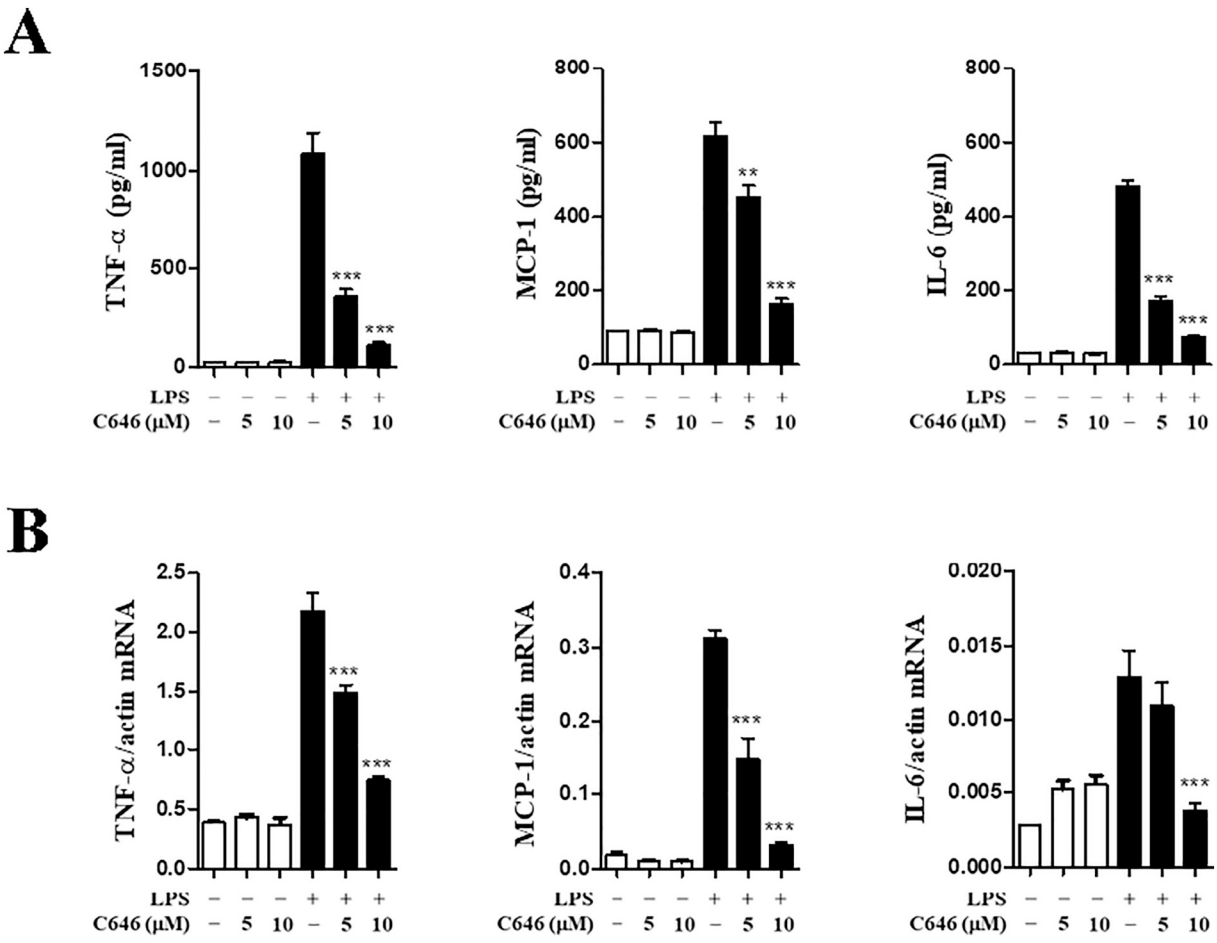

Fig. 2. C646 reduced production of pro-inflammatory cytokines in vitro. (A) BMDMs were treated with or without C646 (0, 5, 10 μM) for 2 h before LPS (10 ng/ml) for another 24 h. Production of TNF-α, MCP-1 and IL-6 in culture supernatants were tested by flow cytometry. (B) BMDMs were treated with or without C646 (0, 5, 10 μM) for 2 h before LPS (10 ng/ml) for another 6 h. The expression levels of TNF-α, MCP-1 and IL-6 mRNA were detected by real-time quantitative PCR. **P < 0.01, ***P < 0.001 compared to LPS alone. Data presented as mean ± SD of three independent experiments (n = 3).

2.11. Statistical analysis

All data are represented as the mean ± SD. Statistical analysis was performed using the Mann-Whitney *U* test for multiple comparisons. P < 0.05 was considered statistically significant. All calculations were performed using GraphPad software (Prism, USA).

3. Results

3.1. Cytotoxicity of C646

BMDMs were treated with different concentrations of C646 (2.5–100 μM) for 24 h. Then, the cytotoxic effects of C646 on macrophages was evaluated by CCK-8 assay. Cells cultured in medium were set to 100%. Cells treated with different concentrations of C646 are showed as relative increase or decrease. Cell viability had been no changed in 10 μM C646, whereas significant cytotoxicity was displayed

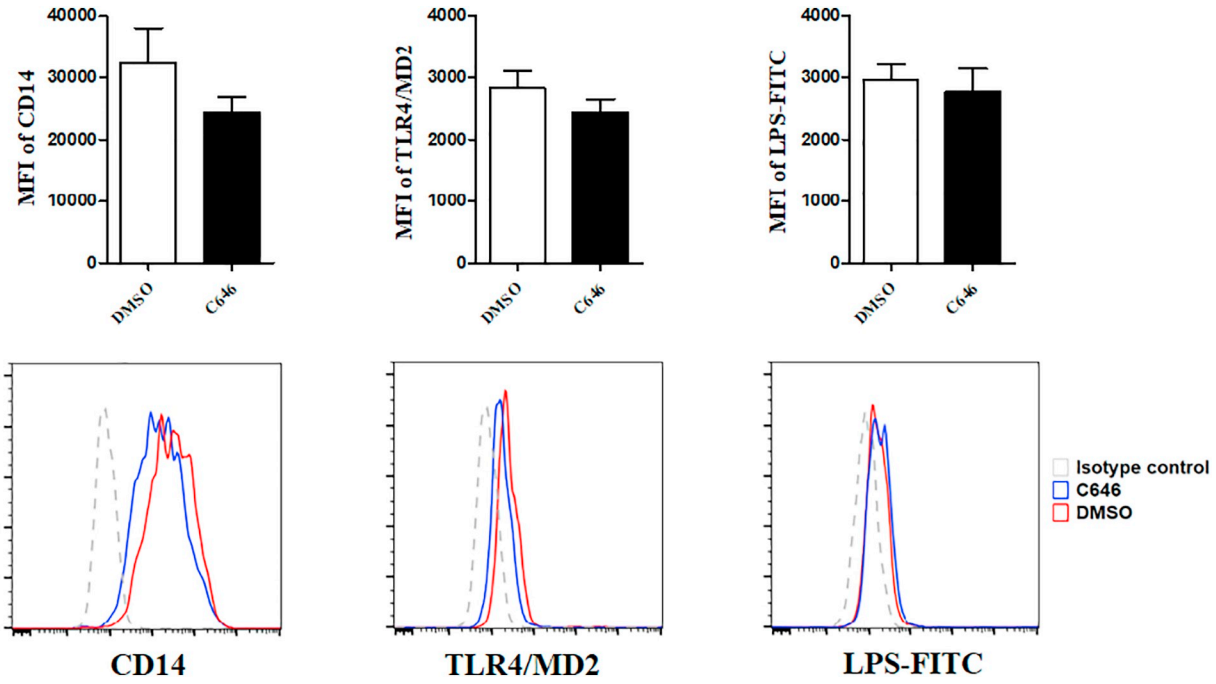

Fig. 3. The effects of C646 on CD14/TLR4/MD2 expression and LPS recognition. BMDMs were pretreated with C646 (10 μM) for 2 h. Surface expression of CD14 and TLR4/MD2 on BMDMs was assessed by flow cytometry. 1 μg/ml LPS-FITC was further incubated for another 1 h and assessed by flow cytometry. Data presented as mean ± SD of three or four independent experiments (n = 4).

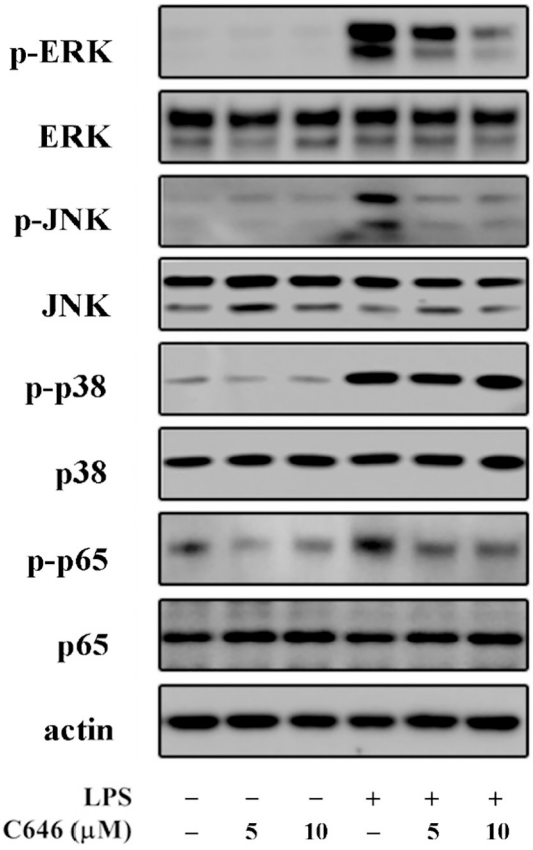

Fig. 4. C646 inhibited LPS-induced activation of MAPKs and NF-κB in BMDMs. Cells were treated with or without C646 (0, 5, 10 μM) for 2 h before LPS (10 ng/ml) for another 30 min. Levels of phosphor-ERK, phosphor-JNK, phosphor-p38 and phosphor-p65 were evaluated by western blot. Data are representative of three experiments (n = 6).

in higher concentrations of C646 (≥25 μM) (Fig. 1B). So 10 μM was used as the maximum concentration of C646 in subsequent experiments.

3.2. C646 suppresses LPS-stimulated cytokines production

LPS induced macrophages activation and elevated expression of pro-inflammatory cytokines. The effect of C646 on LPS-induced cytokine production was first examined. As shown in Fig. 2A, the levels of TNF-α, MCP-1 and IL-6 in BMDM supernatants were significantly inhibited by C646 in a dose-dependent manner. However, C646 did not alter the production of these cytokines without LPS stimulation. Moreover, the mRNA levels of TNF-α, MCP-1 and IL-6 were also abrogated by C646 (at 5 and 10 μM) in BMDMs stimulated by LPS (Fig. 2B). While no effect was found on TNF-α or MCP-1 mRNA levels, the pretreatment with C646 alone induced a slight increase of IL-6 mRNA.

3.3. C646 does not alter LPS recognition

Studies have proved that LPS receptor consists of TLR4, MD-2 and CD14. As LPS recognition by macrophages is the initial step in LPS-induced cytokine expression, the levels of TLR4/MD-2 and CD14 on BMDMs pretreated with or without C646 were first identified. In Fig. 3, it is shown that C646 reduced the levels of TLR4/MD-2 and CD14 slightly, but no significant difference was found between C646 and DMSO treated BMDMs. Next, the effect of C646 on LPS binding was further assessed. Similarly, the binding of LPS to BMDMs was unchanged after treatment with C646 (Fig. 3). Therefore, it is suggested that the inhibitory effect of C646 is not associated with the expression levels of CD14/TLR4/MD-2 complex or their ability to bind LPS.

3.4. Anti-inflammatory effect of C646 by modulating LPS-induced TLR4 signaling

To further clarify the mechanism underlying the anti-inflammatory effects of C646, LPS/TLR4 intracellular signaling pathways were next studied. As shown in Fig. 4, LPS (10 ng/ml) induced significant

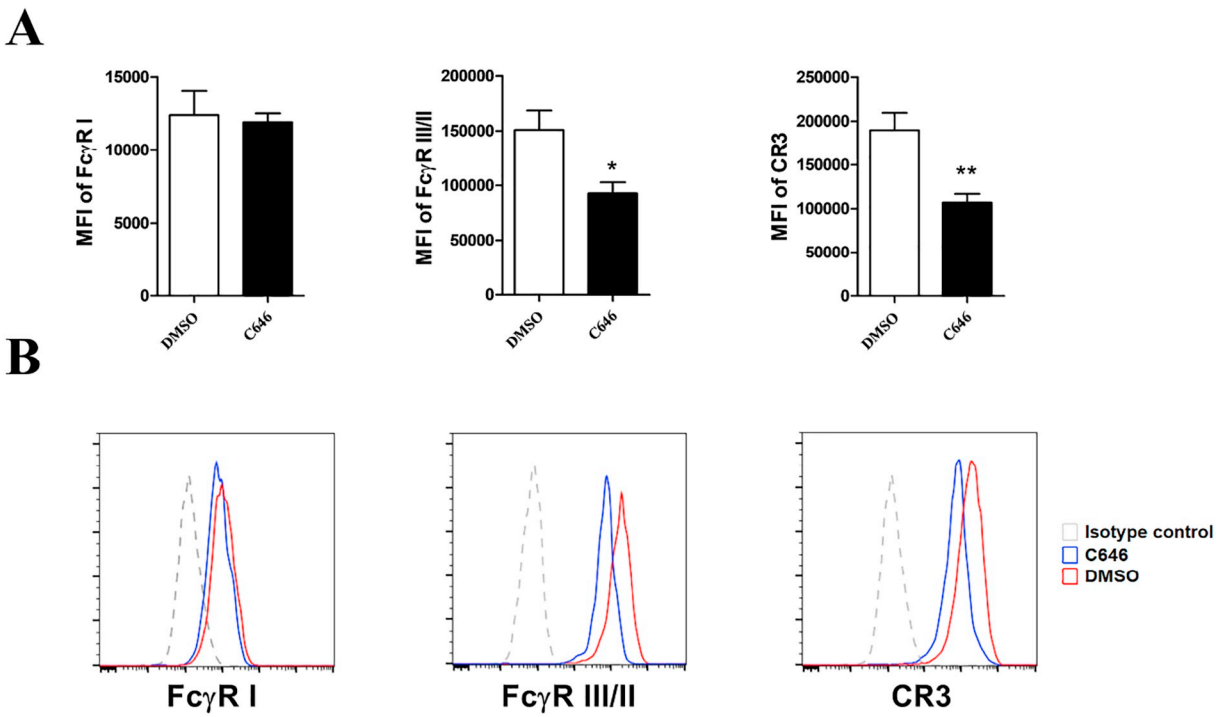

Fig. 5. C646 reduced the expression of phagocytic receptors FcγR III/II and CR3 on BMDMs. The effects of C646 on FcγR I, FcγR III/II and CR3 expression on BMDMs was assessed by flow cytometry. Data presented as mean ± SD of three or four independent experiments (n = 4). **P < 0.01, ***P < 0.001 compared to DMSO alone.

phosphorylation of ERK, JNK, p38 and p65. However, 2 h pretreatment of BMDMs with C646 significantly reduced the phosphorylation of ERK, JNK and p65 in a dose-dependent manner, although total ERK, JNK and p65 was unchanged by C646. Meanwhile, the levels of total and phosphorylated p38 remained almost unaltered by treatment of C646. The results showed above indicated that C646 inhibited cytokines production via reduction of MAPKs and NF-κB signaling pathways.

3.5. The effect of C646 on macrophage-mediated antibacterial activity

Macrophage-mediated antibacterial response is characterized by bacterial phagocytosis and intracellular killing. Phagocytic receptors play a crucial role in the phagocytic process, including both bacterial uptake and ingestion. The effect of C646 on phagocytic receptors, including Fcγ receptor (FcγR) III/II, FcγR I and complement receptor 3 (CR3), was first examined by flow cytometry. A significantly diminished expression of FcγR III/II and CR3, but not FcγR I, was found in cells treated by C646 (Fig. 5).

We next monitored whether C646 affected phagocytic activity of macrophage. As shown in Fig. 6A, confocal microscopy analysis confirmed that, compared to DMSO group, fewer Alexa Fluor 488-*E. coli* were localized in cytoplasm of BMDMs treated by C646. In addition, both uptake and ingestion of *E. coli* by C646-treated BMDMs were significantly less than those of DMSO-treated cells (Fig. 6B).

Phagosome maturation is critical for phagocytosis-mediated intracellular killing, which is characterized by phagosome-lysosome fusion. The extent of phagosome maturation was subsequently measured in C646-pretreated BMDMs. In accordance to the results described above, a substantially reduced co-localization of Alexa Fluor 488-*E. coli* and LysoTracker red labeled lysosomes in BMDMs pretreated by C646 (Fig. 7). Moreover, C646 also diminished bactericidal ability of macrophage. When exposed to live *E. coli* in vitro, intracellular bacterial killing of macrophages was suppressed by C646 (Fig. 8). These results showed that C646 significantly impaired macrophage-mediated antibacterial activity.

4. Discussion

Due to the emergence of drug-resistant bacteria, new strategies to develop novel anti-inflammatory and anti-infective drugs are needed. Here, we evaluated the value of targeting HAT activity for anti-bacterial drug development. C646 is a potent and selective inhibitor of p300/CBP histone acetyltransferases, which has shown as potential anti-tumor agent in gastric, cervical, and lung cancer [17–19]. It was reported that C646 inhibited the viability and proliferation of cancer cells. However, the effect on immune cell is not well elucidated. Macrophages are the primary effector cells of innate immune response in protecting against pathogens. So, in this study, the effect of C646 on macrophage was evaluated for the inflammatory response and phagocytosis.

We found that C646 suppressed the secretion of TNF-α, MCP-1 and IL-6 in LPS stimulated BMDMs. Meanwhile, similar inhibition of these cytokines was observed in the mRNA levels, which implies transcriptional rather than translational regulation was influenced by C646. Studies have demonstrated that TLR4/MD-2/CD14 complex is essential for LPS recognition. LPS extracted from bacterial membranes is transferred to CD14 and TLR4/MD-2, which then promote the dimerization of TLR4/MD-2. The conformational changes of TLR4 induced a recruitment of intracellular adaptor proteins, which then activated downstream signaling pathways. As a result, the genes of pro-inflammatory cytokines and chemokines were transcribed. In the study, C646 have no significant effect on CD14 and TLR4/MD-2. The LPS binding ability was unaltered by C646 either. It is suggested the suppression of cytokine production by C646 without blocking LPS recognition or reducing the expression of CD14 and TLR4/MD-2.

After LPS binding to TLR4/MD-2/CD14 complex, several adaptor proteins are recruited, finally resulting in the activation of NF-κB and MAPK to induce expression of pro-inflammatory cytokines [20,21]. Our previous studies have identified a deficiency in TLR signaling caused reduced production of cytokines upon LPS stimulation [22,23]. As C646 is cell-permeable, we next investigated the effect of C646 on TLR4 signaling pathways. The results showed that C646 suppressed LPS-

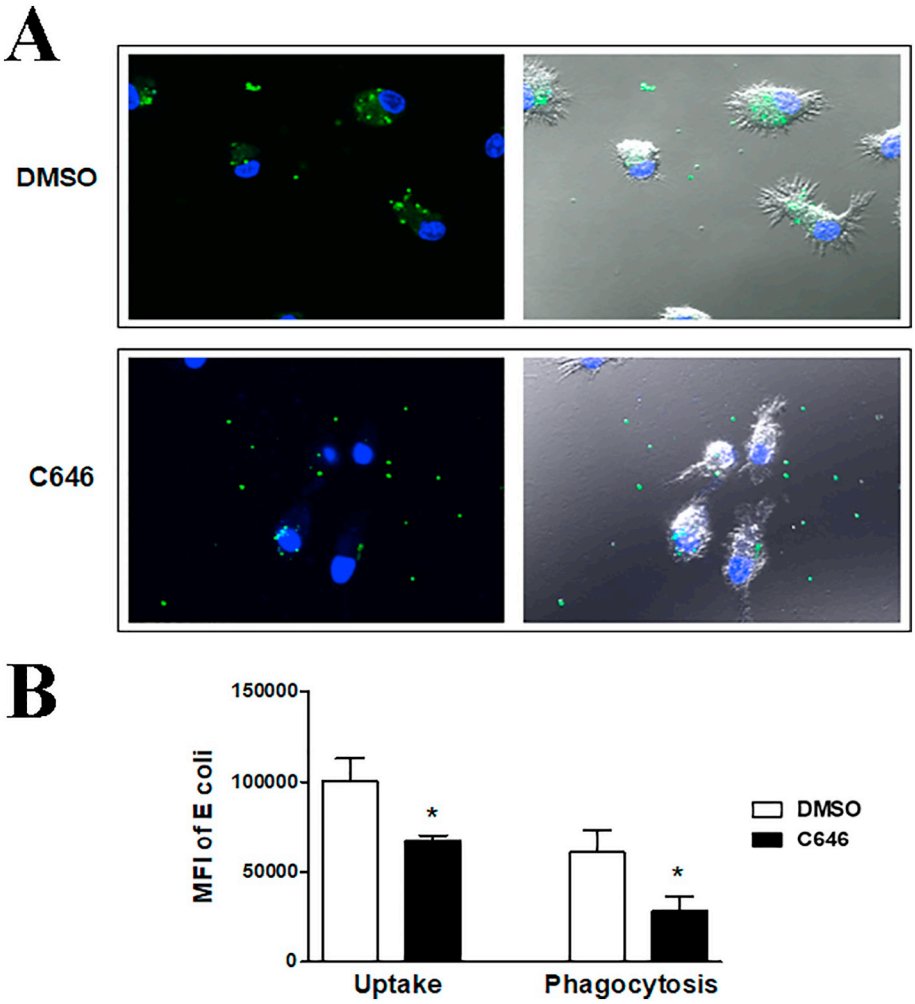

Fig. 6. C646 attenuated macrophage-mediated bacterial uptake and phagocytosis. (A) BMDMs were incubated with Alexa Fluor 488-*E. coli* for 45 min, Fluorescent images were acquired using confocal microscope (original magnification = ×20). (B) Bacterial uptake and phagocytosis were assessed by flow cytometry and compared between DMSO and C646 groups. Data presented as mean ± SD of three independent experiments (n = 4). *P < 0.05 compared to DMSO alone.

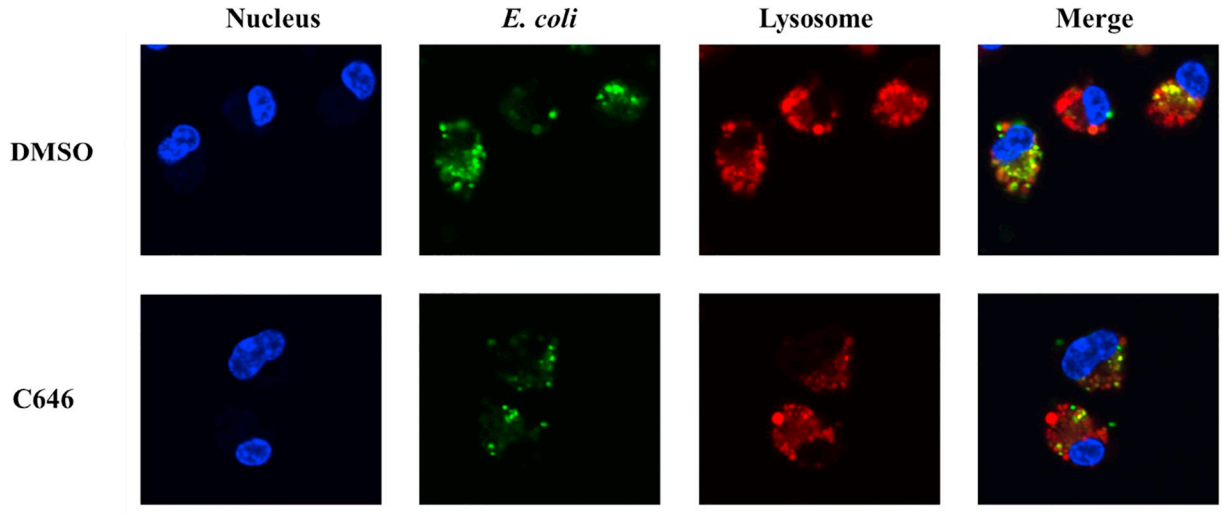

Fig. 7. C646 impaired phagosome/lysosome fusion in BMDMs. BMDMs were loaded with LysoTracker red (50 nM) for 30 min and further incubated with Alexa Fluor 488-*E. coli* for 45 min. Cell nuclei were stained with Hochest 33342. Fluorescent images were acquired using confocal microscope (original magnification = ×40). Results shown represent one experiment from a total of four separate experiments performed (n = 4). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

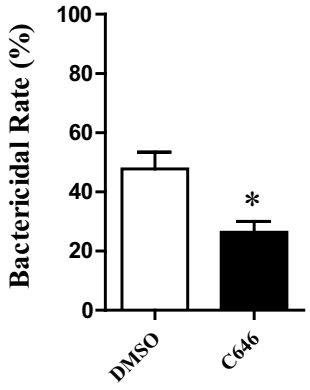

Fig. 8. C646 diminished in vitro bactericidal activity of BMDMs. Intracellular bacterial killing by BMDMs, as represented by the bactericidal rate (%), was determined at 10 and 60 min after macrophages were co-cultured with live *E. coli*. Data are shown as mean ± SD of three independent experiments (n = 5). *P < 0.05 compared to DMSO alone.

induced phosphorylation of p65, ERK and JNK. These results suggested that the anti-inflammatory effects of C646 is involved with inhibition of TLR4 signaling. Our results are supported by the previous study of van den Bosch et al. [24], who described reduced pro-inflammatory gene expression and NF-kB activity in macrophage cell line RAW264.7 treated with C646. Here, we further demonstrated C646 suppressed the LPS-stimulated production of pro-inflammatory cytokines (TNF-α, IL-6, and MCP-1) in both protein and mRNA levels, but without affecting the LPS receptors expression (CD14/TLR4/MD-2) and LPS recognition in macrophage. Additionally, we found C646 not only inhibited phosphorylation of NF-κB p65, but also reduced ERK and JNK activation.

There are several studies implicating C646 in regulating macrophage polarization. However, the results have been slightly ambiguous. Jian Ji et al. found the expression of Arg1 and Ym1 in M2 macrophage was reduced by C646 [25], but in another study C646 conversely unregulated Ym1 in IL-4-stimulated mouse BMDMs [26]. Regardless of the deviating effects of C646 on M2 macrophage, our present study and van den Bosch T' work will help to produce a more conclusive result – that is C646 impaired the pro-inflammatory function of macrophage under LPS stimulation.

Phagocytosis is a central function for macrophage, which can be divided into recognition, internalization, phagosome formation and maturation. Ingestion of pathogenic microbes and subsequent phagosome maturation are critical events in macrophage antimicrobial responses, which is mediated by various phagocytic receptors. Our data showed that both phagocytic receptors CR3 and FcγR II/III on BMDM were decreased by C646. As a result, the downregulated CR3 and FcγR may cause a defect in antibacterial response. When exposed to Alexa Fluor® 488-*E. coli*, BMDM treated by C646 displayed reduced bacterial uptake and phagocytosis of *E. coli*. It is also revealed impaired phagolysosome fusion, as exhibited by the impaired co-localization of Alexa Fluor® 488-*E. coli* with LysoTraker red-labeled lysosomes in C646 treated BMDMs. These data indicated that C646 inhibit the phagocytic properties of macrophages, including bacterial uptake, internalization and intracellular killing. Previous studies have demonstrated that NF-κB not only participates in inflammation, but also involved in the mechanism of phagocytosis [27]. Phagocytic stimuli have been reported to induce NF-κB activation, and inhibition of NF-κB significantly block macrophage phagocytosis. Therefore, it is suggested that the inhibitory effects of C646 on phagocytosis rely on NF-κB pathway. In addition, it remains unclear whether NF-κB inhibition is accounted for the decrease of CR3 and FcγR III/II levels. Further investigations are needed to clarify the exact molecular mechanism. Moreover, the suppression of macrophage phagocytosis may hamper the elimination of pathogens. So the destructive potential of C646 on host immune response should also be carefully investigated in future studies.

Collectively, our results showed that C646 had an inhibitory effect on inflammatory responses and phagocytosis, delineated new insights into the immunomodulatory function of C646, which may highlight the role of p300/CBP in epigenetic regulation of macrophage function. Our data may also assist to determine the therapeutic potential of C646 in inflammatory and infectious diseases.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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