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Original Article

# The combination of ciprofloxacin and indomethacin suppresses the level of inflammatory cytokines secreted by macrophages *in vitro*

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ABSTRACT

*Purpose:* The combined use of antibiotics and anti-inflammatory medicine to manage bacterial endotoxin-induced inflammation following injuries or diseases is increasing. The cytokine level produced by macrophages plays an important role in this treatment course. Ciprofloxacin and indomethacin, two typical representatives of antibiotics and anti-inflammatory medicine, are cost-effective and has been reported to show satisfactory effect. The current study aims to investigate the effect of ciprofloxacin along with indomethacin on the secretion of inflammatory cytokines by macrophages *in vitro*.

*Methods:* Primary murine peritoneal macrophages and RAW 264.7 cells were administrated with lipopolysaccharide (LPS) for 24 h. The related optimal dose and time point of ciprofloxacin or indomethacin in response to macrophage inflammatory response inflammation were determined via macrophage secretion induced by LPS. Then, the effects of ciprofloxacin and indomethacin on the secretory functions and viability of various macrophages were determined by enzyme-linked immunosorbent assay and flow cytometry analysis, especially for the levels of interleukin (IL)-1β, IL-6, IL-10, and tumor necrosis factor (TNF)-α. The optimal dose and time course of ciprofloxacin affecting macrophage inflammatory response were determined by testing the maximum inhibitory effect of the drugs on pro-inflammatory factors at each concentration or time point.

*Results:* According to the levels of cytokines secreted by various macrophages ($1.2 \times 10^6$ cells/well) after administration of 1 μg/mL LPS, the optimal dose and usage timing for ciprofloxacin alone were 80 μg/mL and 24 h, respectively, and the optimal dose for indomethacin alone was 10 μg/mL. Compared with the LPS-stimulated group, the combination of ciprofloxacin and indomethacin reduced the levels of IL-1β ($p < 0.05$), IL-6 ($p < 0.05$), IL-10 ($p < 0.01$)), and TNF-α ($p < 0.01$). Furthermore, there was greater stability in the reduction of inflammatory factor levels in the combination group compared with those in which only ciprofloxacin or indomethacin was used.

*Conclusion:* The combination of ciprofloxacin and indomethacin suppressed the levels of inflammatory cytokines secreted by macrophages *in vitro*. This study illustrates the regulatory mechanism of drug combinations on innate immune cells that cause inflammatory reactions. In addition, it provides a new potential antibacterial and anti-inflammatory treatment pattern to prevent and cure various complications in the future.



## Introduction

Severe trauma and infection can lead to immune dysfunction and excessive inflammatory reactions, resulting in various complications such as multi-organ dysfunction syndrome, the mortality rate of which can reach as high as 70%.[1–3] Although antibiotics are widely applied against pathogenic microorganisms, they may trigger an excessive inflammatory reaction *in vivo* and accelerate the disease progression.[4,5] Therefore, the combined use of

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antibiotics and anti-inflammatory medicine may present an alternative to prevent and/or manage various complications following injuries and infections.[6]

Ciprofloxacin is a third-generation quinolone antibiotic with a broad antibacterial spectrum (e.g., *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*), fast absorption after oral administration and satisfactory safety, and thus has been widely used as an antibacterial treatment after the occurrence of various injuries and diseases.[7,8]

Indomethacin, an indoleacetic acid derivative, is a non-steroidal anti-inflammatory drugs (NSAIDs) that is inexpensive and effective, and is often used as an antipyretic and analgesic in the treatment of rheumatoid arthritis and other diseases.[9] Just as mentioned above, in the treatment of traumatic complications such as sepsis, we should pay attention to not only resisting bacteria but also attenuating excessive inflammatory response. Therefore, indomethacin along with suitable antibiotics (e.g. ciprofloxacin) maybe an available strategy to prevent and treat infections following severe trauma in clinic. Currently, many researchers have attempted to combine antibiotics with multiple drugs to improve the bactericidal and prognostic effects of antibiotics. However, few studies have reported the role of ciprofloxacin plus indomethacin in infection and inflammation.

As a type of innate immunocyte, macrophages are not only able to influence the immune homeostasis via phagocytosis, chemotaxis, and polarization, but also to secrete inflammatory cytokines to change the status of the inflammatory reaction.[10–12] Thus, macrophages act as a bridge between immunity and inflammation, and become potential cells for drug regulation.[13] Nevertheless, the regulation of combined drugs on the secretory function of macrophages remains unclear.

The body's immune system is complex. To simplify the regulatory factors, primary murine peritoneal macrophages and RAW264.7 cells were adopted as the research cell types used in this study. After the optimal dose and usage timing for ciprofloxacin or indomethacin alone had been determined, the regulation of ciprofloxacin along with indomethacin to determine their secretory function was further investigated *in vitro*. This study may provide an experimental foundation for illustrating the regulatory mechanism of drug combinations on the innate immune cells that cause an inflammatory reaction.

## Methods

### *Animals, cell lines, and reagents*

Eighty male C57BL/6 mice, 6–8 weeks old, 20–30 g each, were purchased from SPF (Beijing) Biotechnology Co., Ltd (Beijing, China). All mice were maintained in a specific pathogen-free facility under a 12-h light/dark cycle with free access to food and water. All animal procedures in this study were approved by the Laboratory Animal Welfare and Ethics Committee of Army Medical University (Third Military Medical University).

Mouse macrophage cell line RAW264.7 (ATCC-TIB-71TM) was purchased from the American Type Culture Collection (ATCC, USA). In addition, lipopolysaccharide (LPS, *Escherichia coli* 0111:B4) (L2880), ciprofloxacin·HCL (PHR1004), and indomethacin (I7378) were all purchased from Sigma-Aldrich, St. Louis, MO, USA.

### *Preparation of primary peritoneal macrophages and RAW264.7 cells*

Experiments were conducted using primary cultures of macrophages obtained from specific pathogen-free adult mice. After the mice were euthanized, 6 mL cold physiological saline (0.9% sodium chloride solution) was immediately injected into the abdominal cavity with a syringe. The abdomen was massaged with a cotton ball for 1–2 min, and a ventral incision was made. The peritoneal lavage fluid was gently extracted and transferred with a pipette to a sterile polypropylene centrifuge tube. Thereafter, the peritoneal lavage fluid was centrifuged at 300×*g* for 10 min, and its supernatant was discarded. Primary peritoneal macrophages in the precipitate were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (BISH1002, Biological Industries, Israel) containing 10% (vol/vol) fetal bovine serum, 10,000 units (U)/mL penicillin, and 10 mg/mL streptomycin at 37°C in a 5% $CO_2$ atmosphere. After 3 h, the non-adhesive cells were removed from the wells by three washes of phosphate-buffered saline.

RAW264.7 cells were continuously passaged and cultured to reach approximately 70%–80% confluence in each well of a 24-well plate. All cells were incubated in RPMI-1640 medium containing 10% fetal bovine serum, 10,000 U/mL penicillin, and 10 mg/mL streptomycin at 37°C in a 5% $CO_2$ incubator.

### *Flow cytometry analysis*

The following antibodies were used to analyze the purity and viability of primary murine peritoneal macrophages and RAW264.7 cells: CD11b Percp-Cy5.5 (BioLegend, San Diego, CA, USA), F4/80 APC (BioLegend), F4/80 FITC (BioLegend), and 7AAD (BioLegend). Cells were collected after treatment, incubated for 20 min at 4°C, and then the reaction was terminated with phosphate-buffered saline. The macrophage ratio was measured by flow cytometry.

### *Grouping for drug administration*

Primary murine peritoneal macrophages and RAW264.7 cells were cultured ($1.2 \times 10^6$ cells/well, 1 mL medium/well) in 24-well plates. According to respective objects, the groups of peritoneal macrophages and RAW264.7 cells are shown in Tables 1–3.

In each drug administration group, the cells were treated with ciprofloxacin or indomethacin twice daily for the consideration of drug half-life. At 24 h after the initial drug administration, the supernatant in each well of different groups was collected for further measurements.

In this study, initial determinations were made for the optimal dose and time point for ciprofloxacin. The criteria for the above concepts indicated that with the use of the most optimal dose or time point, the strongest regulation by ciprofloxacin of the secretory function of various macrophages after LPS stimulation will occur. Once the optimal doses of ciprofloxacin and indomethacin were determined, a time-effect study of ciprofloxacin (optimal concentration) was carried out at 3 h, 6 h, 12 h, and 24 h (each time point each subgroup) to determine the optimal time point. Finally, three groups were selected in the study of drug combinations as follows: control (medium), LPS (1 μg/mL) only, and LPS (1 μg/mL) + ciprofloxacin (optimal concentration) + indomethacin (optimal concentration). Similar to the above approach, the supernatant in each well of different groups was collected for further measurement at the optimal time point.

### *Enzyme-linked immunosorbent assay*

The levels of interleukin (IL)-1β, IL-6, IL-10, and tumor necrosis factor (TNF)-α in the supernatants of all groups were measured via enzyme-linked immunosorbent assay (ELISA) kits (IL-1β from Animalunion Biotechnology, Shanghai, China; others from Boster, Wuhan, China) in accordance with the manufacturer's instructions, and the absorbance in each sample was measured at 450 nm by a microplate reader.

**Table 1**
Grouping for ciprofloxacin dose-effect study in RAW264.7 cells or primary peritoneal macrophages.

| Group | Medium (RPMI1640) | LPS (1 μg/mL) | CIP (10 μg/mL) | CIP (20 μg/mL) | CIP (40 μg/mL) | CIP (80 μg/mL) |
|---|---|---|---|---|---|---|
| Control | + | − | − | − | − | − |
| LPS only | + | + | − | − | − | − |
| LPS + CIP (10 μg/mL) | + | + | + | − | − | − |
| LPS + CIP (20 μg/mL) | + | + | − | + | − | − |
| LPS + CIP (40 μg/mL) | + | + | − | − | + | − |
| LPS + CIP (80 μg/mL) | + | + | − | − | − | + |

LPS: lipopolysaccharide; CIP: ciprofloxacin. +:Reagent added; -: No reagent added

**Table 2**
Grouping for indomethacin dose-effect study in RAW264.7 cells or primary peritoneal macrophages.

| Group | Medium (RPMI1640) | LPS (1 μg/mL) | IND (5 μg/mL) | IND (10 μg/mL) | IND (20 μg/mL) | IND (40 μg/mL) |
|---|---|---|---|---|---|---|
| Control | + | − | − | − | − | − |
| LPS only | + | + | − | − | − | − |
| LPS + IND (5 μg/mL) | + | + | + | − | − | − |
| LPS + IND (10 μg/mL) | + | + | − | + | − | − |
| LPS + IND (20 μg/mL) | + | + | − | − | + | − |
| LPS + IND (40 μg/mL) | + | + | − | − | − | + |

LPS: lipopolysaccharide; IND: Indomethacin. +: Reagent added; -: No reagent added.

**Table 3**
Grouping for two drug combinations study in RAW264.7 cells or primary peritoneal macrophages.

| Group | Medium (RPMI1640) | LPS (1 μg/mL) | IND (10 μg/mL) | CIP (80 μg/mL) |
|---|---|---|---|---|
| Control | + | − | − | − |
| LPS only | + | + | − | − |
| LPS + IND (10 μg/mL)+ CIP (80 μg/mL) | + | + | + | + |

LPS: lipopolysaccharide; CIP: ciprofloxacin; IND: indomethacin. +: Reagent added; -: No reagent added.

*Statistical analysis*

Data are presented as the mean ± standard deviation (SD) of triplicate experiments. Independent samples *t* tests and one-way analysis of variance (ANOVA) were performed using GraphPad Prism 8.0 software (GraphPad Software, Inc., Chicago, USA) and SPSS 20.0 software. A *p* value (two-tailed) less than 0.05 was considered statistically significant.

## Results

*Culture and identification of macrophages*

The morphology and ratio (purity) of RAW264.7 cells and primary peritoneal macrophages were observed via inverted phase-contrast microscope and flow cytometry analysis, respectively. The results showed that there was normal growth of RAW264.7 cells, which assumed a polygonal shape. Most cells were nearly confluent (Fig. 1A), and the ratio of RAW264.7 cells that expressed CD11b and F4/80 was 99.04% (Fig. 1B). Compared with the status of RAW264.7 cells, the primary peritoneal macrophages manifested similar growth with fewer cell numbers and lower confluence (Fig. 1C), and the population of CD11b + F4/80+ was 95.53% (Fig. 1D). Then, the various cells were grouped *in vitro* (Tables 1 and 2).

*Dose-effect of ciprofloxacin on the secretory function of macrophages*

To evaluate the dose-effect of different concentrations of ciprofloxacin (Table 1) on the secretory function of RAW264.7 cells and primary peritoneal macrophages, the levels of inflammatory cytokines in each group were measured by ELISA. Compared with those in the control group, the levels of IL-1β, IL-6, IL-10, and TNF-α were significantly increased in the LPS only group ($p < 0.01$) (Figs. 2 and 3). Moreover, in the presence of LPS, the IL-1β level was decreased by more than 50% when macrophages were subjected to 80 μg/mL of ciprofloxacin as compared to the LPS only group ($p < 0.01$) (Figs. 2A and 3A), while the levels of IL-6 and TNF-α also showed a downward trend (Figs. 2B, 2D, 3B, 3D). However, 80 μg/mL of ciprofloxacin increased the level of IL-10 in macrophages compared with the LPS only group, especially in RAW264.7 cells ($p < 0.0001$) (Figs. 2C and 3C). Therefore, the concentration of 80 μg/mL was determined as the optimal dose of ciprofloxacin, and it was applied for subsequent experiments.

*Time-effect of ciprofloxacin on the secretory function of macrophages*

To determine the optimal time point for ciprofloxacin, the various types of macrophages were treated with ciprofloxacin (80 μg/mL) in the presence of LPS (1 μg/mL) and then randomly divided into four sub groups with different time points: 3, 6, 12, and 24 h. At each time point, the supernatants of each group were collected to measure the levels of inflammatory cytokines by ELISA. Most of the dynamic changes in IL-1β, IL-6, IL-10, and TNF-α were similarly observed in RAW264.7 cells and primary peritoneal macrophages (Figs. 4 and 5).

Treatment with ciprofloxacin (80 μg/mL) elevated the level of IL-6 (Figs. 4B and 5B), which peaked at 24 h after the first administration of the drug ($p < 0.01$). The maximum level of TNF-α was measured at 12 h, and decreased to the base level at 24 h after the initial drug administration (Figs. 4D and 5D). In addition, in primary peritoneal macrophages, the level of IL-10 was elevated at 3 h and

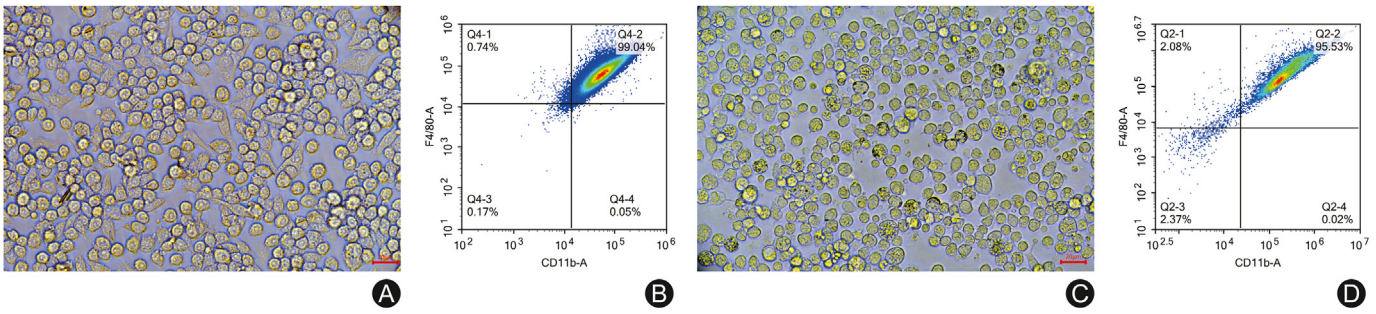


**Fig. 1.** Morphology and purity ratio of various macrophages. (A) RAW264.7 cells were cultured in RPMI-1640 medium (with 10% FBS, 10,000 U/mL penicillin, and 10 mg/mL streptomycin) under the conditions of 37°C and 5% $CO_2$. (B) RAW264.7 cells were stained with Percp-Cy5.5 CD11b and APC F4/80, and then the ratio of the population with $CD11b^+$ and $F4/80^+$ was evaluated by flow cytometry. (C, D) According to the above protocols, the cellular status and purity of primary peritoneal macrophages were also observed and analyzed.

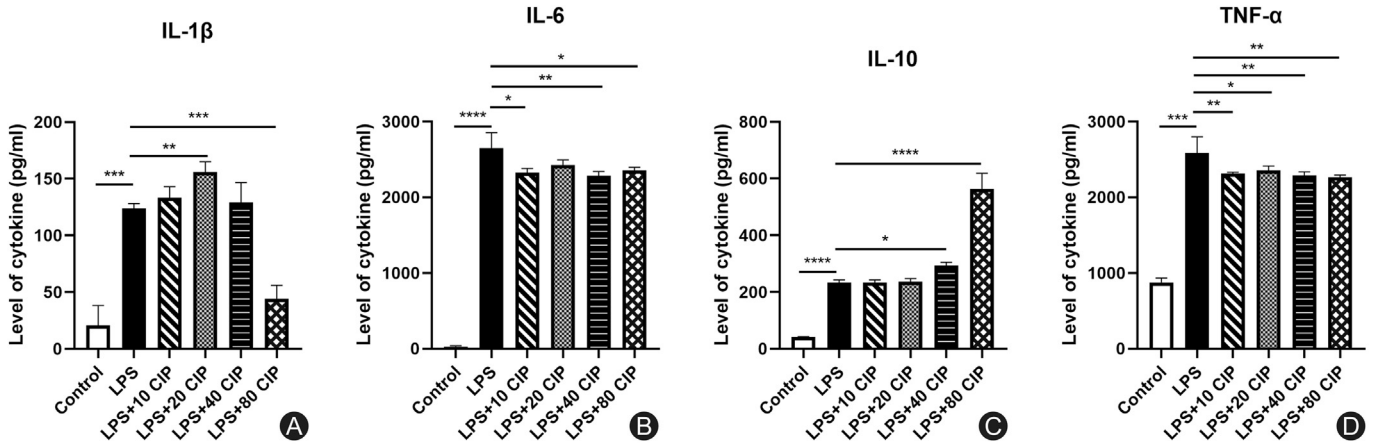


**Fig. 2.** RAW264.7 cells were subjected to different concentrations of ciprofloxacin. (A–D) In the absence or presence of 10, 20, 40, and 80 μg/mL ciprofloxacin, RAW264.7 cells were stimulated with medium only or LPS (1 μg/mL) for 24 h, and then ELISA was used to measure the levels of (A) IL-1β, (B) IL-6, (C) IL-10, and (D) TNF-α. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, using one-way ANOVA or independent samples $t$ tests. The data shown are representative of one of three separate experiments.
LPS: lipopolysaccharide; CIP: ciprofloxacin.

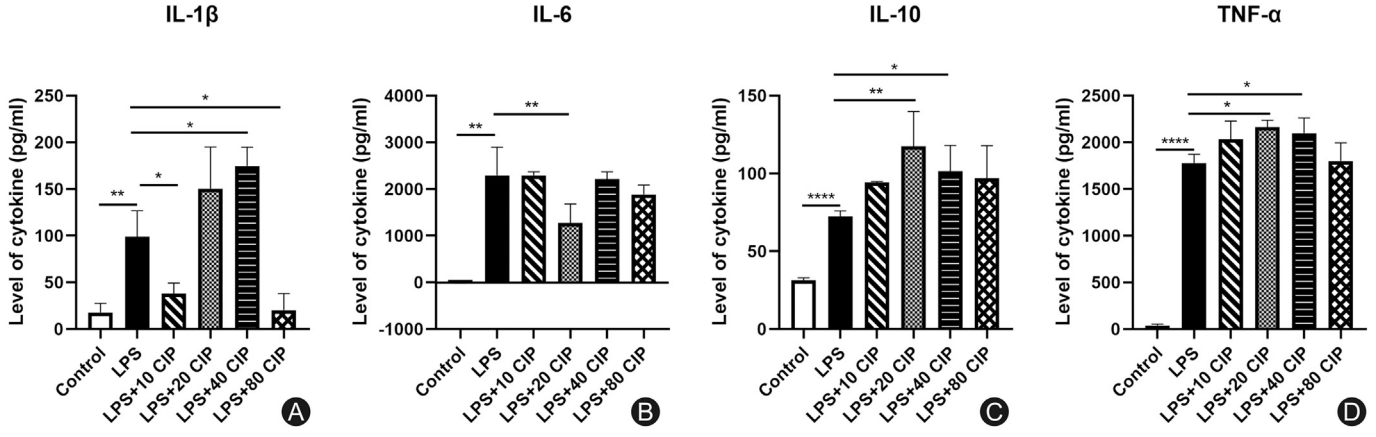


**Fig. 3.** Primary peritoneal macrophages were subjected to different concentrations of ciprofloxacin. (A–D) In the absence or presence of 10, 20, 40, and 80 μg/mL ciprofloxacin, primary peritoneal macrophages were stimulated with medium only or LPS (1 μg/mL) for 24 h, and then ELISA was used to measure the levels of (A) IL-1β, (B) IL-6, (C) IL-10, and (D) TNF-α. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, using one-way ANOVA or independent samples $t$ tests. The data shown are representative of one of three separate experiments.
LPS: lipopolysaccharide; CIP: ciprofloxacin.

reached the maximum level at 24 h after the initial drug administration ($p < 0.01$) (Fig. 5C), and IL-1β levels started to decrease after 12 h ($p < 0.01$) (Fig. 4A). Nevertheless, in RAW264.7 cells, the level of IL-10 gradually decreased at 6 h after the first treatment of ciprofloxacin ($p < 0.0001$) (Fig. 4C), and IL-1β levels showed a gradual decrease (Fig. 5A).

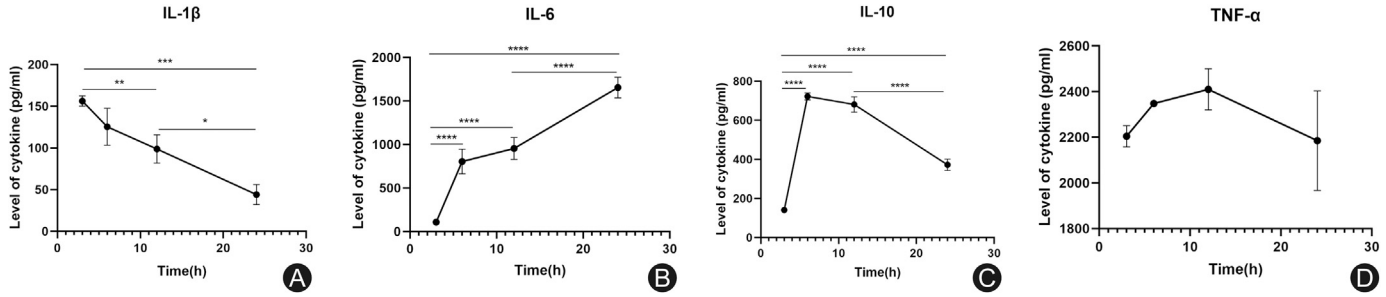


**Fig. 4.** Inflammatory cytokine levels for RAW264.7 cells at 3, 6, 12, and 24 h after ciprofloxacin (80 μg/mL) treatment and lipopolysaccharide (1 μg/mL) stimulation. The levels of (A) IL-1β, (B) IL-6, (C) IL-10, and (D) TNF-α were measured in the supernatant of RAW264.7 cells at various time points by ELISA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, using one-way ANOVA or independent samples *t*-test. The data shown represent one of three separate experiments.

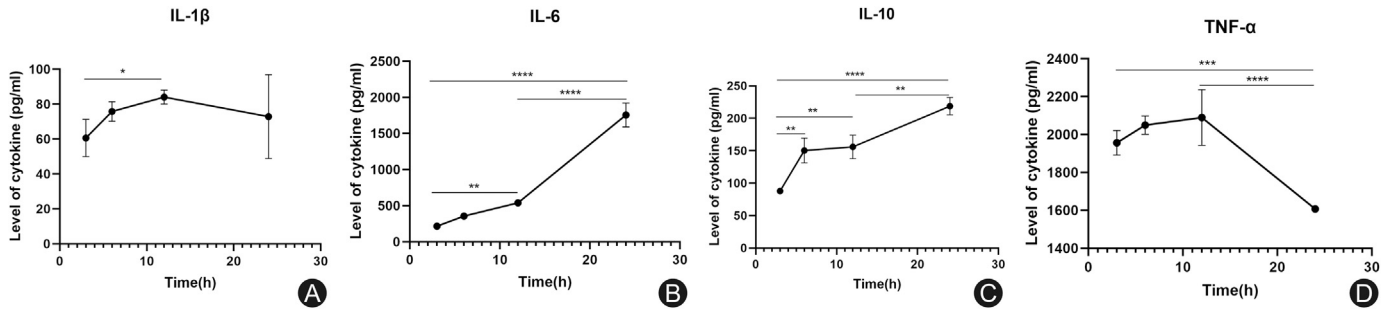


**Fig. 5.** Inflammatory cytokine levels for primary peritoneal macrophages at 3, 6, 12, and 24 h after ciprofloxacin (80 μg/mL) treatment and lipopolysaccharide (1 μg/mL) stimulation. The levels of (A) IL-1β, (B) IL-6, (C) IL-10, and (D) TNF-α were measured in the supernatant of primary peritoneal macrophages at various time points by ELISA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, using one-way ANOVA or independent-samples *t*-test. The data shown represent one of three separate experiments.

### *Dose-effect of indomethacin on the secretory function of macrophages*

According to the above description designed in Table 2, the levels of inflammatory cytokines in the control group, LPS (1 μg/mL) only, LPS (1 μg/mL) + indomethacin (5 μg/mL), LPS (1 μg/mL) + indomethacin (10 μg/mL), LPS (1 μg/mL) + indomethacin (20 μg/mL), and LPS (1 μg/mL) + indomethacin (40 μg/mL) groups were measured by ELISA. Compared with that in the LPS only group, the indomethacin (10 μg/mL) + LPS (1 μg/mL) group exhibited a decrease in the levels of IL-6, IL-10, and TNF-α, especially in RAW264.7 cells ($p < 0.01$) (Figs. 6B, 6C, 6D) for the levels of the above three cytokines and primary peritoneal macrophages for the levels of IL-1β, IL-6, and IL-10 ($p < 0.01$) (Figs. 7A, 7B, 7C). On the contrary, in RAW264.7 cells, the level of IL-1β was enhanced in the LPS (1 μg/mL) + indomethacin (10 μg/mL) group under the same conditions (Fig. 6A), and a similar tendency was shown in primary peritoneal macrophages for the level of TNF-α (Fig. 7D). In the current study, treatment with indomethacin (10 μg/mL) resulted in a corresponding stable and significant effect compared with that in the other groups. Accordingly, this concentration was determined as the optimal dose of indomethacin for the following experiments.

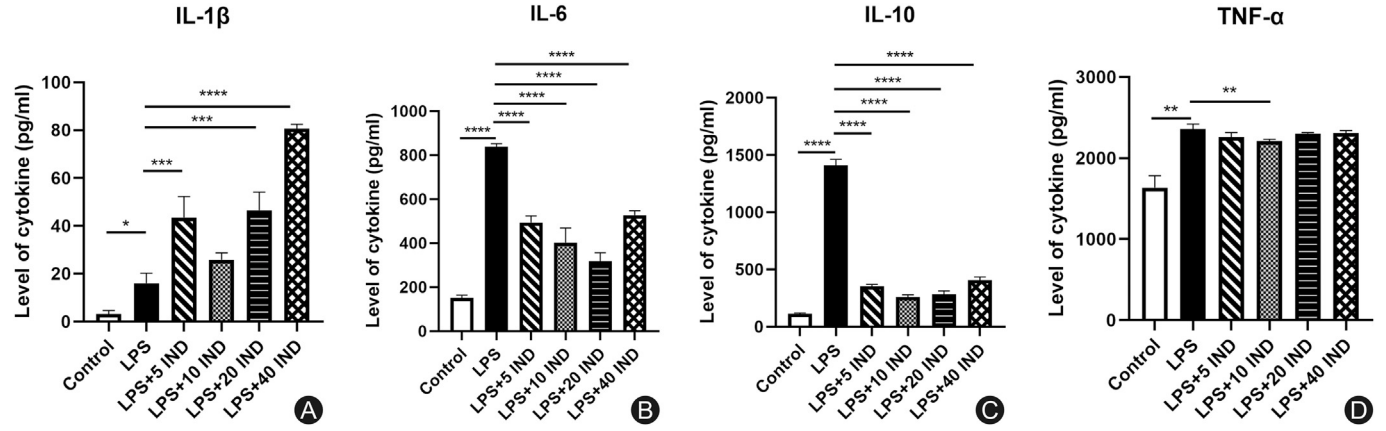


**Fig. 6.** RAW264.7 cells were subjected to different concentrations of indomethacin. (A–D) In the absence or presence of 5, 10, 20, and 40 μg/mL indomethacin, RAW264.7 cells were stimulated with medium only or LPS (1 μg/mL) for 24 h, and then ELISA was performed to measure the levels of (A) IL-1β, (B) IL-6, (C) IL-10, and (D) TNF-α. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, using one-way ANOVA or independent samples *t*-test. The data shown are representative of one of three separate experiments.
LPS: lipopolysaccharide; CIP: ciprofloxacin.

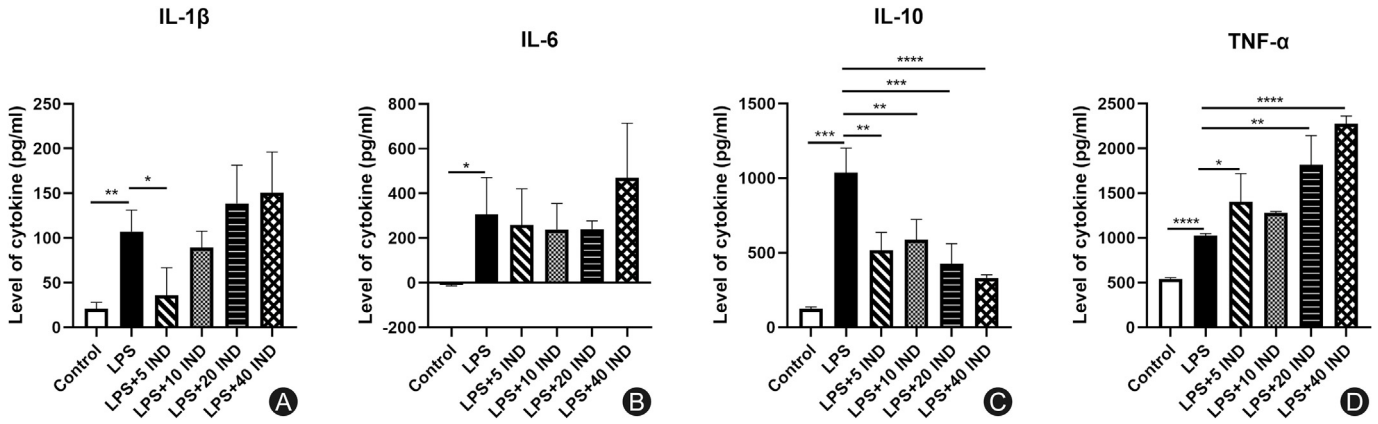


**Fig. 7.** Primary peritoneal macrophages were subjected to different concentrations of indomethacin. (A–D) In the absence or presence of 5, 10, 20, and 40 μg/mL indomethacin, primary peritoneal macrophages were stimulated with medium only or LPS (1 μg/mL) for 24 h, and then ELISA was performed to measure the levels of (A) IL-1β, (B) IL-6, (C) IL-10, and (D) TNF-α. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, using one-way ANOVA or independent samples *t*-test. The data shown are representative of one of three separate experiments.
LPS: lipopolysaccharide; CIP: ciprofloxacin.

*Influence of ciprofloxacin + indomethacin on the secretory function of macrophages*

Based on the optimal concentrations and time points determined in the above experiments, various macrophages under LPS (1 μg/mL) stimulation were treated with ciprofloxacin (80 μg/mL) and indomethacin (10 μg/mL). Then, the supernatant was collected for assay at 24 h after the first administration of the drugs. Compared with that in the LPS only group, the results showed an obvious suppressive tendency of the combination of ciprofloxacin + indomethacin on the levels of IL-1β, IL-6, IL-10, and TNF-α ($p < 0.05$) (Fig. 8).

*Effect of ciprofloxacin plus indomethacin on macrophage viability*

To confirm the effect of the drug combination on the cellular viability of various macrophages, we used F4/80 and 7AAD to co-label the cells treated with/without ciprofloxacin and indomethacin. Flow cytometric analysis showed that for RAW264.7 cells, there was a small increase in 7AAD values in the ciprofloxacin + indomethacin (Fig. 9A) group compared to the control group (Fig. 9B), but the difference was not statistically significant ($p > 0.05$) (Fig. 9C). Similarly, there were no significant differences between the two groups in primary peritoneal macrophages ($p > 0.05$) (Figs. 10A and B), although the 7AAD values were slightly lower in the drug combination group compared with that in the control group (Fig. 10C).

## Discussion

The inflammatory reaction is a protective response of the body against external pathogenic factors, while an excessive inflammatory reaction will cause an imbalance in homeostasis, apoptosis, and immunosuppression, and even result in septic shock and organ dysfunction.[14–16] Trauma and infection are two important factors that can induce an excessive inflammatory reaction and several serious complications. For example, in the process of multi-organ dysfunction syndrome, bacterial endotoxin becomes the key trigger in the "storm" effect of a systemic inflammatory reaction. Although antibiotics have been widely used for killing bacteria and preventing the invasion of pathogenic microorganisms to treat many complications mediated by infection, on the contrary, their side effects are ignored to some extent.[17] In fact, along with the process of antibiotic administration, the endotoxin, exotoxin, bacterial DNA, and cell wall components released by dead bacteria can trigger an excessive inflammatory reaction that can aggravate the condition of patients. Therefore, a viable clinical strategy would be to select drugs that not only resist bacteria but also suppress excessive inflammation.[18–20]

The application of antibiotics in clinical practice has become one of the indispensable methods for medical treatment, especially in the field of trauma rescue.[21] Currently, appropriate antibiotics are selected for prevention and treatment of infection, including traumatic complications.[22,23] Antibiotics negatively affect the structure of pathogenic microorganisms and interfere with their metabolic processes, such as inhibiting the synthesis of nucleic acid and protein, altering cellular membrane permeability, and interfering with energy metabolic systems.[24,25] Ciprofloxacin is a third-generation quinolone antibacterial drug that is the most widely used fluoroquinolone antibiotic in the world. Although its antibacterial spectrum is similar to that of norfloxacin, the antibacterial activity of ciprofloxacin is 2–10 times stronger than that of norfloxacin. In fact, it is the strongest *in vitro* antibacterial activity in this class of drugs.[26] Furthermore, a previous study showed that cytotoxicity had not been detected when ciprofloxacin was used to treat macrophages.[27] Nonetheless, Fan et al.[28] indicated that ciprofloxacin promoted the polarization of $CD86^{+}CD206^{-}$ macrophages and induced cell apoptosis.

NSAIDs confer antipyretic and analgesic effects, and most of them have anti-inflammatory and anti-rheumatic effects.[29] These drugs include aspirin, acetaminophen, indomethacin, naproxen, nabumetone, diclofenac, and ibuprofen. NSAIDs are mainly used to treat inflammation, mild to moderate pain, fever, cancer, and neurological disorders.[30] NSAIDs inhibit cyclooxygenase in the metabolism of arachidonic acid and decrease prostaglandin synthesis.[31,32] Indomethacin, a typical representative of NSAIDs, is one of the most potent prostaglandin synthase inhibitors. Indomethacin also inhibits phosphatidic acid $A_2$ and phosphatidic acid C, and reduces granulocyte migration and lymphocyte proliferation. Furthermore, its anti-inflammatory effect is 10–40 times stronger than that of aspirin, with a significant analgesic effect on inflammatory pain.[33–35] However, the clinical application of indomethacin for its anti-inflammatory effects remains controversial because it may have potential adverse effects on the gastrointestinal tract, central nervous system, and hematopoietic system, as well as provoke potential allergic reactions, in the case of overdose.[36] In

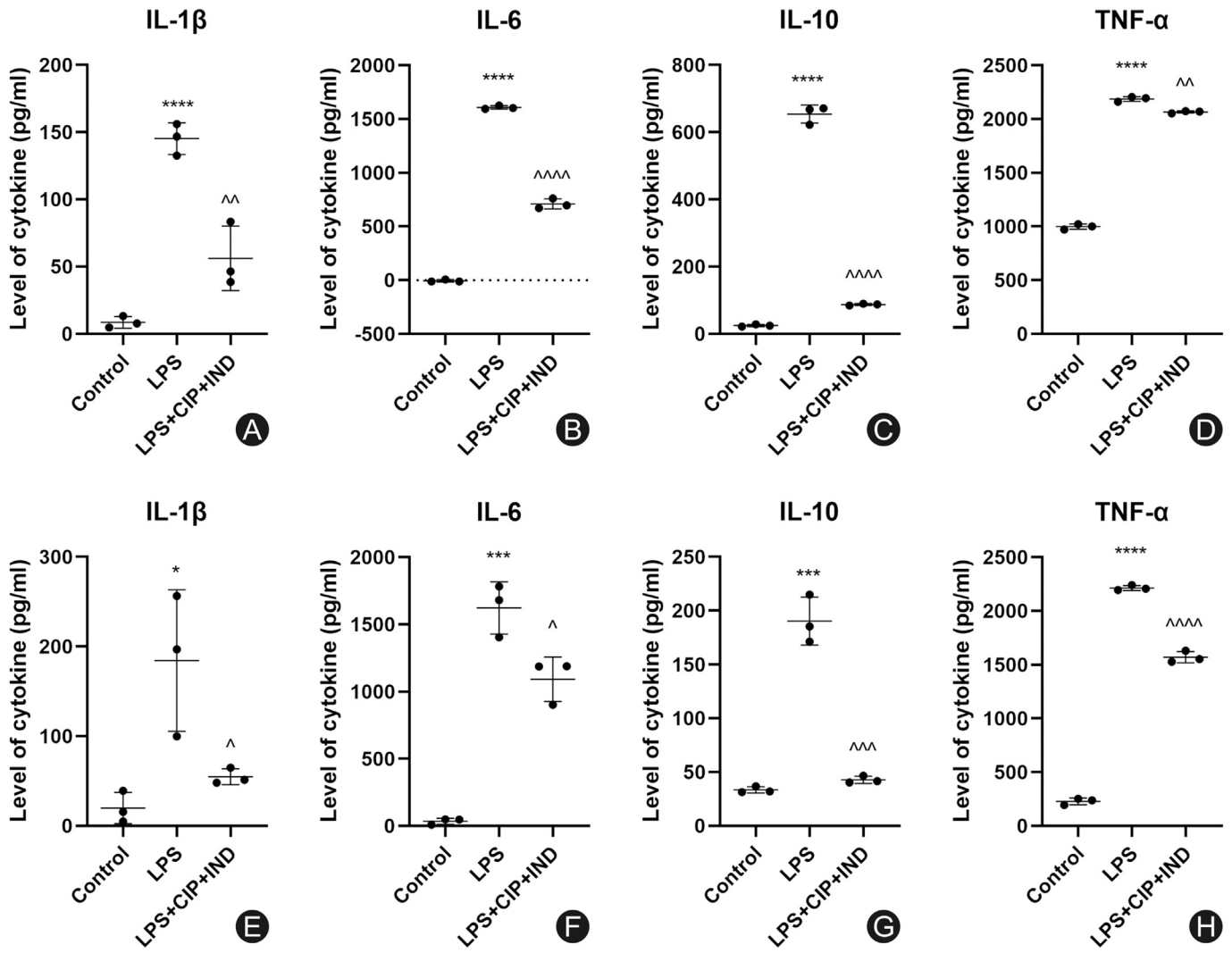


**Fig. 8.** Various macrophages were subjected to combined drug administration. (A–D) In the absence or presence of 80 μg/mL ciprofloxacin and 10 μg/mL indomethacin, RAW264.7 cells were stimulated with medium only or LPS (1 μg/mL) for 24 h, and then ELISA was performed to measure the levels of (A) IL-1β, (B) IL-6, (C) IL-10, and (D) TNF-α. (E–H) The same process was carried out in primary peritoneal macrophages. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, *vs.* control; ^$p < 0.05$, ^^$p < 0.01$, ^^^$p < 0.001$, ^^^^$p < 0.0001$, *vs.* LPS only, using one-way ANOVA or independent-samples *t*-test. The data shown are representative of one of three separate experiments. Indomethacin: IND. Ciprofloxacin: CIP.

addition, Chae[37] reported that indomethacin significantly decreased the levels of interferon (IFN)-γ and IL-6 secreted by macrophages, which were results similar to what we obtained. Moreover, Navas et al.[38] also showed that indomethacin conferred no cytotoxicity on cell viability *in vitro*.

Thus far, the combination of antibiotics and NSAIDs has become one of the novel practical strategies in the treatment of various complications.[39] There is a significant synergistic role for some of them in the control of bacterial infection and inflammatory reaction.[40–42] For instance, NSAIDs reduced the production of pro-inflammatory mediators in multi-drug-resistant bacterial infections and decreased the antibiotic resistance to some degree.[43] In addition, early application of NSAIDs combined with the appropriate systemic antibiotic treatment further attenuated infection-induced articular cartilage damage and improved the prognosis of wounded patients.[44] Moderate doses of NSAIDs played a positive role in the treatment of severe trauma, and when used in combination with antibiotics, significant synergistic effects were observed, and they also assisted in the delay or even prevention of the development of severe complications from trauma.[43,44] All of the above results imply that it is necessary for us to further explore the effect of drug combinations such as ciprofloxacin and indomethacin on the functions of target immunocytes.

Macrophages are an important immunocyte type in innate immunity that participates in host defense to resist microbial infection and maintain tissue homeostasis.[10,45,46] Activated macrophages bridge immunological response and inflammation reaction through strong phagocytosis, rapid chemotaxis, convertible polarization, and proper secretion.[11,47] Among these functions, the levels of inflammatory cytokines secreted by macrophages may directly influence the immunological balance and prognosis of patients.[12] Thus, it is beneficial for potential targeted cellular therapy to further explore the role of drug combinations on the secretory function of various macrophages.[13] Up to now, there have been no similar studies in immunological regulation via macrophages.

In the traditional view, ciprofloxacin is a bactericide that inhibits DNA synthesis and replication by acting on the A subunit of bacterial DNA helicase. However, it also acted in an anti-inflammatory capacity via inhibiting the levels of cytokines to some extent in this study. RAW264.7 cells and primary murine peritoneal macrophages were adopted as the experimental materials, and the

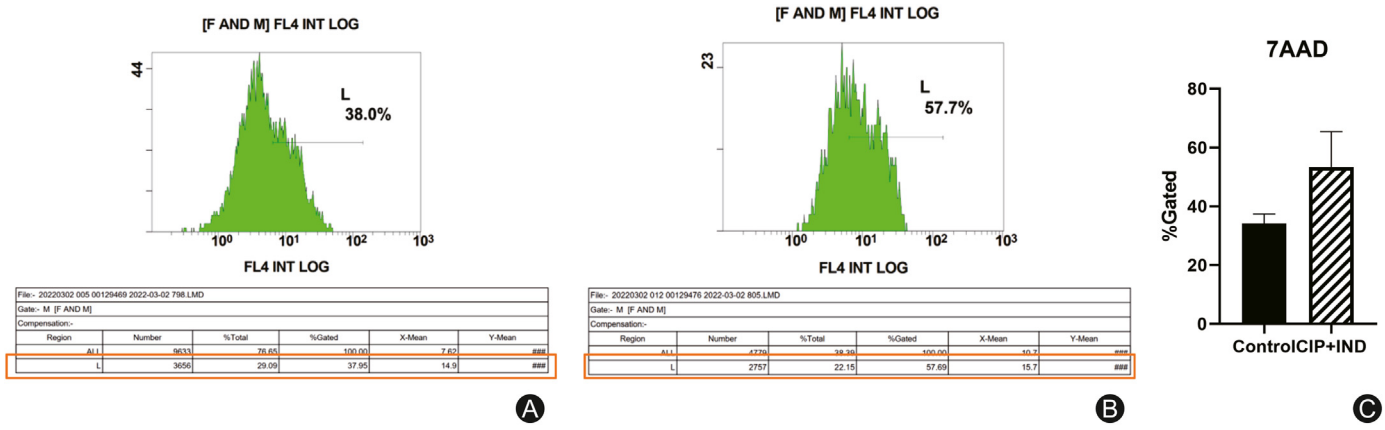


**Fig. 9.** Viability of RAW264.7 cells after combined drug administration. In the absence or presence of 80 μg/mL ciprofloxacin and 10 μg/mL indomethacin, 24 h after the first dosing of the cells, RAW264.7 cells in each group were labeled with F4/80. Apoptosis in control group cells (A) and CIP + IND group cells (B) were analyzed by flow cytometry and finally statistical analysis (C) was performed. Data shown were representative of one of three separate experiments.
CIP: ciprofloxacin; IND: indomethacin.

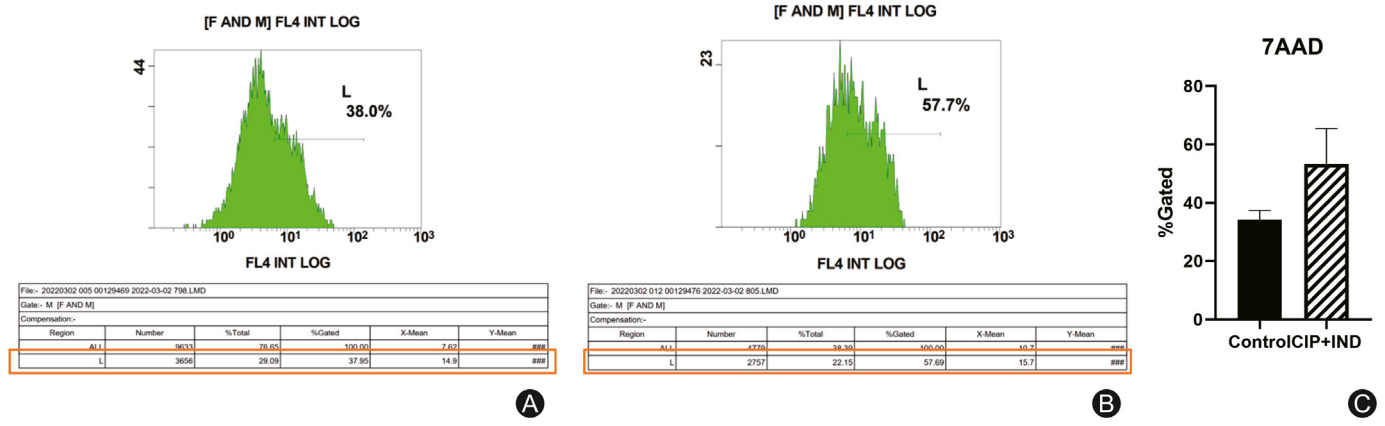


**Fig. 10.** Viability of primary peritoneal macrophages after combined drug administration. In the absence or presence of ciprofloxacin (80 μg/mL) and indomethacin (10 μg/mL), 24 h after the first dosing of the cells, primary peritoneal macrophages in each group were labeled with F4/80. Apoptosis in (A) control group cells and (B) CIP + IND group cells was analyzed by flow cytometry, and finally, statistical analysis (C) was performed. The data shown are representative of one of three separate experiments.
CIP: ciprofloxacin; IND: indomethacin.

optimal dose of ciprofloxacin/indomethacin and optimal time point for ciprofloxacin were determined for each. Then, ciprofloxacin (80 μg/mL) and indomethacin (10 μg/mL) were administered to various macrophages under LPS (1 μg/mL) stimulation. At 24 h after the initial drug administration, the levels of inflammatory cytokines were measured by ELISA. Because this study aims to explore the interventional role of indomethacin on ciprofloxacin administration rather than that of ciprofloxacin, we observed the dose-effect of indomethacin only and the dose/time effects of ciprofloxacin. Compared with the LPS-stimulated group, ciprofloxacin along with indomethacin decreased the levels of IL-1β, IL-6 ($p < 0.05$), IL-10 ($p < 0.01$), and TNF-α ($p < 0.01$). Meanwhile, these results were more stable than those obtained where only ciprofloxacin or indomethacin was used. They imply that the above drug combination can significantly attenuate an excessive inflammatory reaction mediated by various macrophages. In addition, although several reports showed that ciprofloxacin or indomethacin alone did not lead to macrophage apoptosis, there was no additional obvious evidence of adverse effects from this drug combination that was found prior to the study. In this study, the effect of ciprofloxacin along with indomethacin on cellular death was investigated, and the results also showed that the drug combination did not influence the viability of the studied cells. Combined with the corresponding results *in vivo* (data not shown), these drugs may be widely applied in the future for treating bacterial endotoxin–associated inflammatory mediators due to their broad antibacterial spectrum, stable anti-inflammatory activity, and low cost.

A limitation of the current study is that the experiments were performed only *in vitro*. Moreover, the investigation focused on the role of drug combinations in regulating the secretory function of macrophages and did not address other related mechanism. Although the research *in vitro* may simplify the influencing factors, and several animal experiments *in vivo* were performed (data not shown), it will still be necessary to expand the experimental modes and further explore related molecular mechanisms in subsequent studies. This study serves as a satisfactory starting point that clarifies the participation of ciprofloxacin plus indomethacin in the secretory functions of various macrophages *in vitro*.

This study initially revealed that ciprofloxacin combined with indomethacin may significantly downregulate the levels of IL-1β, IL-6, IL-10, and TNF-α secreted by RAW264.7 cells and primary murine peritoneal macrophages after LPS stimulation. Furthermore, the drug combination resulted in no degradation of cellular

viability of various macrophages. Therefore, it may provide a novel potential strategy for treating bacterial endotoxin–induced inflammation to prevent various serious complications mediated by infection.

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## Ethical statement

This study was approved by Laboratory Animal Welfare and Ethics Committee of Army Medical University (Third Military Medical University).

## Declaration of competing interests

The authors declare that they have no conflict of competing interest.

## Author contributions

Ke Liu performed the experiments and drafted the manuscript. Jing Yu, Yu Xia and Lei-ting Zhang participated in the experiments. Sui-Yan Li provided suggestions and guided the study. Jun Yan designed the experiments, performed data management, revised the manuscript and support the study. All authors read and approved the final manuscript.

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