

Conjugated linoleic acid ameliorates necrotizing enterocolitis by suppressing inflammatory responses and maintaining intestinal barrier integrity via the PPAR γ /NF κ B signaling pathway

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ARTICLE INFO

Keywords:

Neonatal necrotizing enterocolitis
Conjugated linoleic acid
PPAR γ
NF κ B
Metabolomics

ABSTRACT

Necrotizing enterocolitis (NEC) is a severe inflammatory gastrointestinal disorder affecting preterm infants. Through fecal metabolomics, we found linoleic acid compounds (LAs) reduced in NEC and negatively correlated with C-reactive protein and NEC Bell stage. Conjugated linoleic acid (CLA) is a special kind of LAs with multiple functions. This study aims to investigate the role and mechanism of CLA *in vivo* and *in vitro*. Experimental results in neonatal rats demonstrated that CLA significantly reduced mortality, weight loss, improved pathological scores, relieved inflammation and preserved intestinal cell homeostasis. *In vitro*, CLA promoted cell proliferation and reduced cell injury. RNA-sequencing analysis indicated that PPAR signaling pathway might be critical. Experiments showed that CLA facilitated PPAR γ /NF κ B signaling pathway by upregulating PPAR γ expression, inactivating phosphorylated NF κ B, and preventing translocation of NF κ B P65. This study underscores the therapeutic potential of CLA and provides new insight and direction for developing NEC interventions.

1. Introduction

Necrotizing enterocolitis (NEC) is a devastating inflammatory gastrointestinal disease in preterm neonates, lacking effective treatments (Altobelli, Angeletti, Verrotti, & Petrocelli, 2020). It affects about 5.48 % among infants with a birth weight <1500 g in China from 2015 to 2018 (Cao et al., 2022). NEC not only leads to high mortality rate up to 20–30 % (D. Zhou et al., 2022), but also gives rise to severe complications, including intestinal obstruction, stenosis, and an elevated risk of long-term neurodevelopmental delays (Neu & Walker, 2011). More adverse, the etiology of NEC is complex to completely understood and it's usually considered multifactorial (Koike et al., 2020).

Bacterial colonization and formula feeding are considered two predominant factors linked to NEC (Call et al., 2018). Existing studies imply that formula feeding infants have lower gut bacterial diversity and more likely to develop NEC than breastfed babies (Aziz, Prince, & Wang, 2022), further highlighting the importance of bacterial colonization. The gut microbiota influences host physiology indirectly through metabolites production, playing a crucial role in NEC development

(Krautkramer, Fan, & Backhed, 2021). For instance, butyric acid from *Firmicutes* attenuates NEC by alleviating inflammation and modulating microbiota (Sun et al., 2021). In addition, by secreting indole-3-lactic acid, *B. infantis* relieves NEC through the prevention of excessive inflammation in premature intestine (Meng et al., 2020).

Fecal metabolomics focuses on host, diet, and gut microbiota metabolic interactions, reveals changes in global metabolic state under disease conditions, making it a valuable tool for comprehensively exploring NEC (Marcobal et al., 2013). Thus, this study integrates metabolomic and clinical indicators from control (CTL) and NEC groups, identifying significantly reduced linoleic acid compounds (LAs) in NEC infants. LAs, with the molecular formula C₁₈H₃₂O₂, is a class of polyunsaturated essential fatty acid and has many health benefits (Marangoni et al., 2020; Mousavi et al., 2021). Conjugated linoleic acid (CLA) is a special kind of LAs, distinguished by its molecular structure that includes conjugated double bonds, setting it apart from ordinary linoleic acid (LA). This conjugated structure makes CLA more stable and augments its functions, so we selected CLA for subsequent experiments.

CLA is a “star molecule” with multiple bioactivities, like anti-cancer,

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reducing body fat, and modulating immune (J. H. Kim et al., 2016). In recent years, studies have shown that CLA modulates various intestinal diseases. Such as, CLA can alleviate dextran sulfate sodium-induced colitis (Borniquel, Jadert, & Lundberg, 2012; Chen et al., 2019; Moreira et al., 2018), and oral CLA can reduce cytokines and improve Crohn's disease symptoms (Bassaganya-Riera et al., 2012). However, much less is known for how CLA that improve symptoms of NEC. Extensive adult research on inflammatory bowel disease, with some similar pathologies, sheds light on less developed studies on NEC in neonates (Duci et al., 2023).

In present study, we conducted in vivo animal experiments and found that CLA significantly reduced mortality rates in NEC and preliminarily uncovered its effect on alleviate intestinal inflammatory responses and enhance gut barrier function. We also found that CLA promotes intestinal epithelial cell proliferation and mitigates cell damage. We further showed that CLA ameliorates NEC symptoms by activating the PPAR γ /NF κ B signaling pathway. These data suggest that CLA may be beneficial in the treatment of NEC.

2. Materials and methods

2.1. Materials

The commercial CLA was pure (95 %), and purchased from Macklin Biochemical Technology Co., LTD (Shanghai, China). The ELISA kits for inflammatory cytokines (IL-1 β , IL-6 and TNF- α) were purchased from Enzyme-linked Biotechnology Co., LTD (Shanghai, China). The primary antibodies include β -actin (Servicebio, Wuhan, China), ZO-1 (Proteintech, Wuhan, China), Occluding (Proteintech, Wuhan, China), IL-1 β (Wanlei, Shenyang, China), IL-6 (Wanlei, Shenyang, China), TNF- α (Proteintech, Wuhan, China), PPAR γ (Bioss, Beijing, China), NF κ B P-P65 (Sigma-Aldrich, St. Louis, MO, USA) and NF κ B P65 (Sigma-Aldrich, St. Louis, MO, USA). LPS, Phorbol 12-myristate 13-acetate (PMA) and GW9662 were obtained from MCE Company (Shanghai, China).

2.2. Metabolomics analysis

The collection of fecal samples has been approved by the Ethics Review Committee of Women's Hospital of Nanjing Medical University (2020KY068), with informed consent obtained from parents. The metabolomics analysis by LC-MS/MS was performed by Hangzhou Lian Chuan Biotechnology Co., Ltd and generally includes three processes.

2.2.1. Metabolite extraction

Extracting 20 μ l intestinal contents from each specimen (n = 9 for two groups) for mixing with 120 μ l prechill 50 % methanol buffer. The mixture was centrifugated at 4,000 g for 20 min after vortexed and then the supernatant was transferred for the next step. Also, a quality control (QC) sample was created by mix 10 μ l from each specimen.

2.2.2. LC-MS/MS analysis

Chromatographic separation was conducted utilizing using an ultra-performance liquid chromatography (UPLC) system (SCIEX, UK). The samples were reversed-phase separated by An ACQUITY UPLC T3 column (100 mm*2.1 mm, 1.8 μ m, Waters, UK) with a 0.4 mL/min-flow rate. The mobile phase consisted of solvent A (water, 0.1 % formic acid) and solvent B (Acetonitrile, 0.1 % formic acid). The solvent gradient was established as: 5 % B, 0–0.5 min; 5–100 % B, 0.5–7 min; 100 % B, 7–8 min; 100–5 % B, 8–8.1 min; and 5 % B, 8.1–10 min. a TripleTOF 5600 Plus high-resolution tandem mass spectrometer (SCIEX, Warrington, UK) with both positive and negative ion modes was used to analysis the molecules. The ion spray floating voltage was set at 5 kV (positive-ion mode) or –4.5 kV (negative-ion mode) and interface heater temperature was 650 $^{\circ}$ C. Survey scans were acquired every 150 ms and the MS data were acquired in the IDA.

2.2.3. Metabolomics data Processing

The raw LC-MS data was pretreated by XCMS and R software. Each ion was identified by the comprehensive information of retention time and m/z. Each peak's intensity was recorded and then generating a three-dimensional matrix containing arbitrarily assigned peak indices (retention time-m/z pairs), sample names (observations) and ion intensity information (variables). The metabolites were annotated by matching the exact molecular mass data (m/z) within KEGG and HMDB databases. Principal component analysis (PCA) was performed to detect outliers and batch effects using the pretreated dataset. Orthogonal partial least squares discriminant analysis (OPLS-DA) enhances model interpretability by isolating variance correlated with class distinctions to highlight disease-specific metabolic differences. Permutation testing assesses model reliability by comparing the accuracy of randomly shuffled labels to the original, verifying statistical significance.

2.3. Animal experiments

All animal experiments comply with the ARRIVE guidelines and are conducted in accordance with the National Research Council Guidelines for the Care and Use of Laboratory Animals. Nanjing Medical University's Research and Ethics Committee approved the animal study (IACUC-2303007). Neonatal SD rats aged 2 days were randomly divided into 3 groups (15 rats per group). Importantly, we used SD rats of both genders. CTL neonatal rats were dam-fed. The NEC experimental technique was made slight modifications following prior research for the NEC group. In summary, the rats were gavage fed with 50 μ l g – 1 body weight milk volume [15 g Similac 60/40 (Abbott Nutrition) mixed with 75 ml Esbilac Pet formula] and then exposure to hypoxia (5 % O $_2$, 95 % N $_2$, 10 min) 3 times a day for 4 days. On the 2nd and 3rd days, the rats were orally administered 4 μ g/g LPS for further NEC induction. In the NEC + CLA group, rats additional received 10 mg/kg/day CLA in their initial daily milk for 4 days. The weight and survival status of the rats were recorded daily. On the 4th day, the rats were sacrificed, harvested the ileum for pathologic analysis and subsequent experiments.

2.3.1. Histological analysis

Intestinal tissue 1 cm away from the distal colon was fixed in 4 % paraformaldehyde (PFA), embedded in paraffin after dehydrated with xylene and ethanol, subsequently cut into 5 μ m thick slices. These slices were stained by hematoxylin and eosin (H&E) for observation under a microscope (Zeiss, Germany).

2.3.2. Immunofluorescence and immunohistochemistry

Paraffin-embedded thick tissue sections were prepared as before for immunofluorescence (IF) and immunohistochemistry (IHC). In IF, the ileum was dewaxed and dehydrated. Next, 3 % BSA was used to blocking non-specific binding after antigen retrieval by heating through a microwave oven. Subsequently, specific primary antibody and fluorescein labeled secondary antibody were added in turn. Following, DAPI was add to stain the nucleus, then covered with microscope slide. The steps before adding secondary antibodies in IHC were identical to IF. But in IHC, the sections were stained with DAB to visualize positive signals, and cell nucleus were counterstained with hematoxylin after co-incubation with HRP labeled secondary antibodies.

2.4. Detection of inflammation level

Intestine samples preparation: A certain length of intestine was taken into a tissue tube with 300 μ l of prechilled PBS and homogenized. Subsequently, supernatant was collected after centrifugation (3000 rpm, 20 min). Cell samples preparation: The culture medium of cells was collected and centrifuged (3000 rpm, 20 min). to collect the supernatant. Levels of the inflammatory cytokines (IL-1 β , IL-6, and TNF- α) were measured according to the commercial ELISA kits. Concentrations of the cytokines were standardized based on the protein concentration of

intestinal or cells samples.

2.5. Cell assays

Rat intestinal epithelial cell (IEC-6) was purchased from ATCC (Manassas, VA, USA). Cells were cultured at 37 °C under 5 % CO₂ in a DMEM medium supplemented with fetal bovine serum (10 %, Gibco, USA) and penicillin/streptomycin (1 %, Gibco, USA). The cell culture medium was replaced every 2 days. NEC cell models were induced with 50 µg/ml LPS in serum-free medium (LPS group). IEC-6 cells were pre-treated with 100 µM CLA for 24 h before LPS stimulation (LPS + CLA group). In some experiments, GW9662, the antagonist specific for PPAR γ was used (LPS + CLA + GW9662 group).

2.5.1. Cell proliferation assay

The cell proliferation assay was carried out using a commercial testing kits (Beyotime, Shanghai, China) according to the instructions. Briefly, the IEC-6 cells were seeded into 12-well plates, and incubated with 10 µM EdU for 2 h, fixed in 4 % PFA for 15 min and 0.3 % Triton X-100 for another 15 min. Next, the cells were incubated with click reaction solution for 30 min (fluorescence labeling) and 1X Hoechst 33,342 for 10 min. A fluorescence microscope was used to record the images and following analyzed by ImageJ.

2.5.2. Cell death rate

Cell death rate was evaluated by Annexin V-FITC/PI Apoptosis Kit (APExBio, Houston, USA). After experimental manipulation, the cells were digested with EDTA-free trypsin. Next, the samples were PBS washed to prepare a single-cell suspension (5×10^5 cells/mL) and then light-shielded stained with Annexin V-FITC (5 µl) and PI (5 µl) for 30 min, followed by flow cytometry detection (Beckman, FL, USA).

2.5.3. Western blotting

Cells was lysed with RIPA buffer (Servicebio, Wuhan, China) and the protein concentration was detected by BCA Protein Assay (Thermo Fisher Scientific, USA). Following, 20 µg protein was add to the lane and separated by SDS-PAGE. Next, these proteins were transferred to PVDF membranes (Millipore, USA). Before incubated with specific primary antibodies overnight, the membranes were blocked with 5 % skim milk for 1 h. Subsequent day, the membranes were incubated with HRP-conjugated secondary antibodies (Servicebio, Wuhan, China) for 1 h. Finally, proteins were observed (luminescence detection reagent; Millipore, USA) and the grayscale of protein bands was analyzed by ImageJ.

2.5.4. Cell IF of NF κ B p65 translocation

Cells were fixed with 4 % PFA for 15 min, permeabilized in 0.3 % Triton X for 5 min and blocked with 5 % BSA for 10 min in room temperature. After incubated with NF κ B P65 primary antibody overnight and goat anti-rabbit second antibody (Servicebio, Wuhan, China) for 1 h, the cells were counterstained with DAPI for 5 min. NF κ B P65 translocation was visualized by a confocal microscope (Leica, Germany).

2.6. High-throughput RNA sequencing

The total RNA was extracted from the cells by MJzol Animal RNA Isolation Kit (Majorivd). Shanghai Biotechnology Corporation was entrusted with the sequencing process. In brief, double stranded cDNA was synthesized and performed terminal modifications after RNA quality control. Then RT-PCR was employed to increase the copies and enhance the signal strength. After the above steps to construct the cDNA library, the RNA-sequencing was performed fully according to the manufacturer's instructions (Illumina novaseq6000, USA). Removed the unqualified reads, the raw data was further analysed. Fold-change > 1.5 and false discovery rate (FDR) value < 0.05 was identified as significantly differential expressed genes (DEGs). GO and KEGG analyses were conducted using the DAVID platform, available at <https://david.abcc.ncifcrf.gov/>.

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2.7. Statistical analysis

The experimental data are shown as the mean \pm standard deviation. Two-tailed Student's *t*-test or Oneway ANOVA (LSD post-hoc test for multiple comparisons) were performed for statistical analysis using SPSS Statistics 26.0.0 (IBM, USA). Correlation among clinical metrics was calculated by Spearman's method and visualized as a heatmap using the R programming language (version 3.4.2). Other graphical visualization was accomplished using GraphPad Prism 8 software (GraphPad Software Inc., La Jolla, CA, USA). A *P* value < 0.05 indicated statistically significant.

2.8. The safety statement

CLA, a group of linoleic acid isomers found in humans and animals, has been approved as a food additive in many countries. Current toxicological research indicates that CLA is safe at standard dosages, with risks within acceptable limits.

3. Results

3.1. CLA potentially improves NEC: Insights from fecal metabolomics

Metabolomics analysis was performed on stool of 9 preterm infants with NEC and matched control group using LC-MS/MS. The PCA results presented in Fig. 1A reveal a clear separation trend between the CTL and NEC groups, with strong consistency within each group. Further validation by OPLS-DA analysis and permutation test (Figure S1) effectively illustrates the variability within the data, confirming the accuracy of the initial data analysis. Subsequently, a total of 9,042 differential metabolites were identified, with 4,650 metabolites upregulated in the CTL group (Fig. 1B). Notably, the LAs in the CTL group was 2.96 times than the NEC group (Fig. 1C). KEGG analysis identified the linoleic acid metabolism was one of the most significantly enriched pathways (Fig. 1D). Finally, LAs showed a negative correlation with CRP and NEC Bell stage in the infants (Fig. 1E), suggesting a potential key role in NEC. The molecular structures of the most common LAs are LA and CLA. Unlike LA, CLA features two double bonds separated by a single bond, a subtle distinction that alters its spatial configuration and enhances its biological functions (Fig. 1F).

3.2. CLA ameliorated the NEC rat model

A totally of 45 three-day old rats were used in the animal experiment. The experiment workflow was designed according to existing studies and spanning 4 days with hypoxia, hyperosmotic milk and LPS stimulation to establish the NEC animal model (Fig. 2A). The NEC group had a more lower survival rate (46.7 %), but the rats in NEC + CLA group maintained the high rate and even 80.0 % on the 4th day (Fig. 2B). Regarding the weight, dam-fed rats increased steadily during the 4 days, however, it decreased continuously on NEC and NEC + CLA groups. Interestingly, rats had a little higher weight after CLA intervention in the last day (Fig. 2C). As shown in Fig. 2D, the terminal ileum of NEC rats showed obvious edema, pneumatosis intestinalis and the appearance was dark red which indicates severe necrosis. Nevertheless, these adverse changes were reversed in NEC + CLA group (Fig. 2D). Further H&E staining and derived quantitative pathological scoring were used to assess the intestinal damage (Fig. 2E). We observed significant epithelial sloughing and damaged structure villus in NEC model which leads to higher NEC Scores, while gavaged with CLA helped preserve villus architecture, thus reducing the pathological scores (Fig. 2E). The enterocytic apoptosis was measured by TUNEL staining (green fluorescence). As shown in Fig. 2F, CLA supplement reverted the cell apoptosis, showing as weak FI. Taken together, CLA supplementation improved

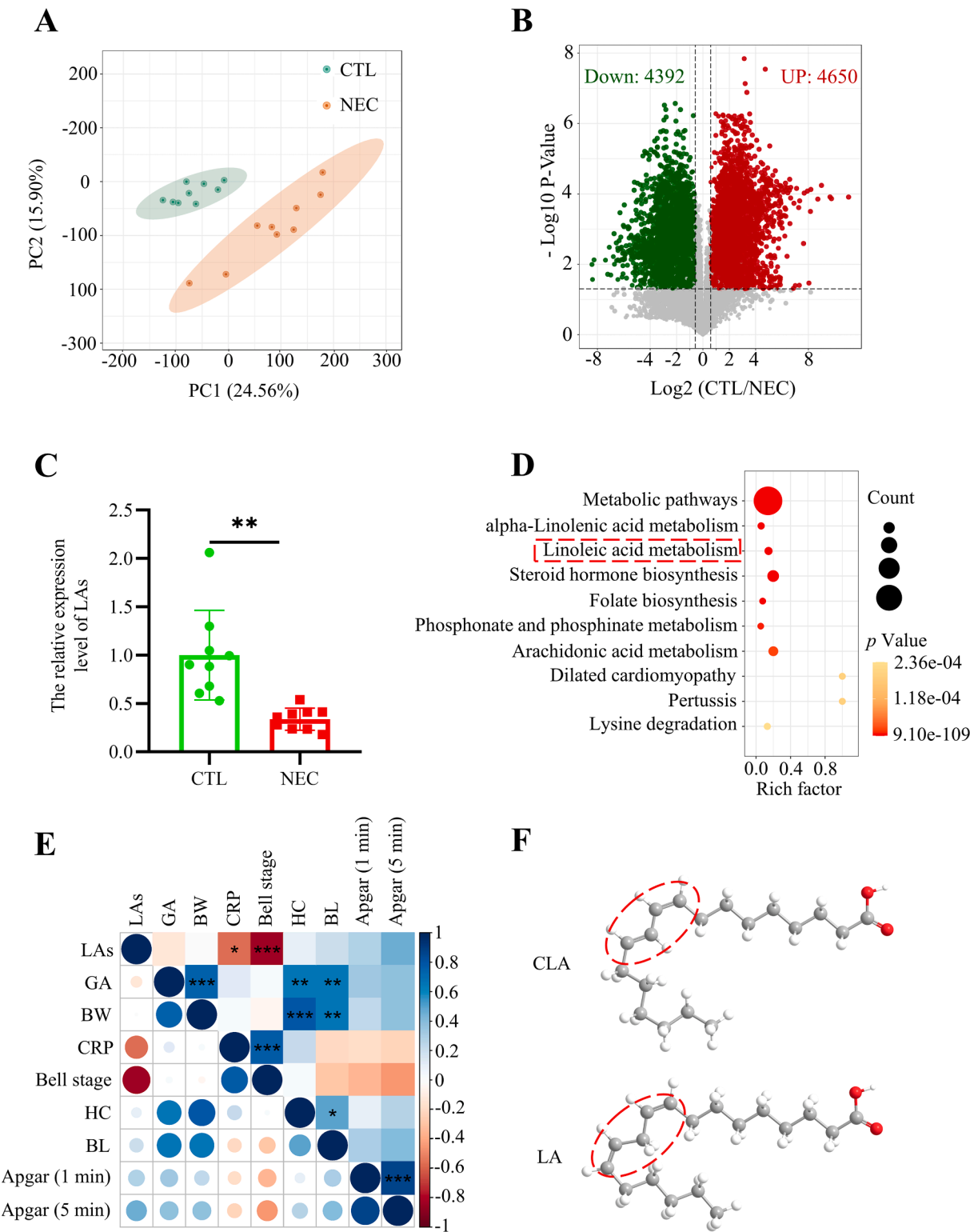


Fig. 1. Fecal metabolomics and correlation analysis uncovered CLA potentially improves NEC in preterm infants. (A) PCA analysis of the samples. (B) Volcano map showing the differential expressed genes. (C) Relative expression level of LAs across groups. (D) KEGG enrichment pathways identified with significantly enriched fecal metabolites (top 10). (E) The Spearman correlation heatmap of neonatal clinical metrics. LAs, linoleic acid compounds; GA, gestational age; BW, birth weight; CRP, C-reactive protein; HC, head circumference; BL, birth length. (F) The 3D Conformer of LA and CLA from the PubChem database. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

survival rates, attenuated the weight loss, reduced pathological scores, and mitigated intestinal cell apoptosis, thus ameliorating the NEC rat model.

3.3. CLA restored intestinal barrier related proteins and reduced inflammation in NEC animal model

To study the expression of three critical proteins for keeping

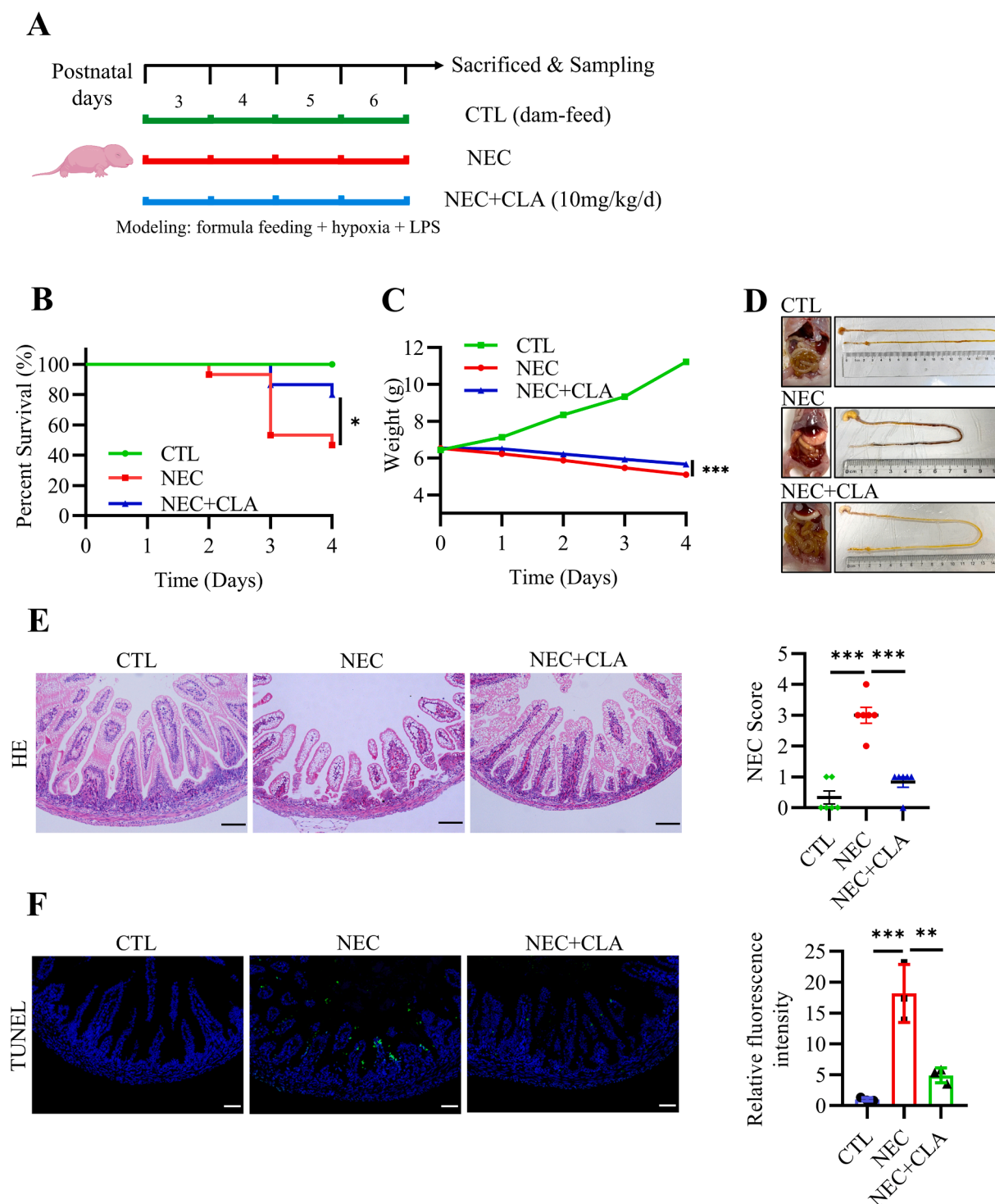


Fig. 2. CLA ameliorated the NEC animal model and alleviated enterocytic apoptosis. (A) Experimental design and detailed procedure. (B) Survival rate of rats within 4 days during intervention. (C) Weight change of rats during the experiment. (D) Morphological changes of the intestine in dam-fed (CTL), experimental NEC (NEC) and CLA at 10 mg/kg/d intervention (NEC + CLA) groups. (E) H&E staining of terminal ileum and the corresponding NEC Scores in three groups. (F) TUNEL staining results of intestinal tissue. All scale bar = 200 μ m. * P < 0.05, ** P < 0.01, *** P < 0.001.

intestinal barrier function, we use two experimental methods (IHC and IF) for each protein' evaluation. As for the intestinal proliferation-associated protein PCNA, IHC and IF showed a significantly reduction in rats suffered NEC modeling stimulation, and then CLA intake recovered its expression (Fig. 3A). Pups subjected to experimental NEC demonstrated downregulated tight junction proteins ZO-1 and Occludin, conversely NEC + CLA group manifested upregulated expression that

approach normal level (Fig. 3B-C). Quantification of IHC and IF for the three proteins shown in Figure S2. IL-1 β , IL-6 and TNF- α usually cause severe intestinal damage, thus worthing in-depth investigation. In the NEC group, levels of the three inflammation cytokines were significantly elevated. However, oral administration of CLA markedly decreased the production of these cytokines. In the NEC group, IL-1 β , IL-6 and TNF- α was 1.68, 1.14 and 2.21 times, respectively, compared with that of CTL

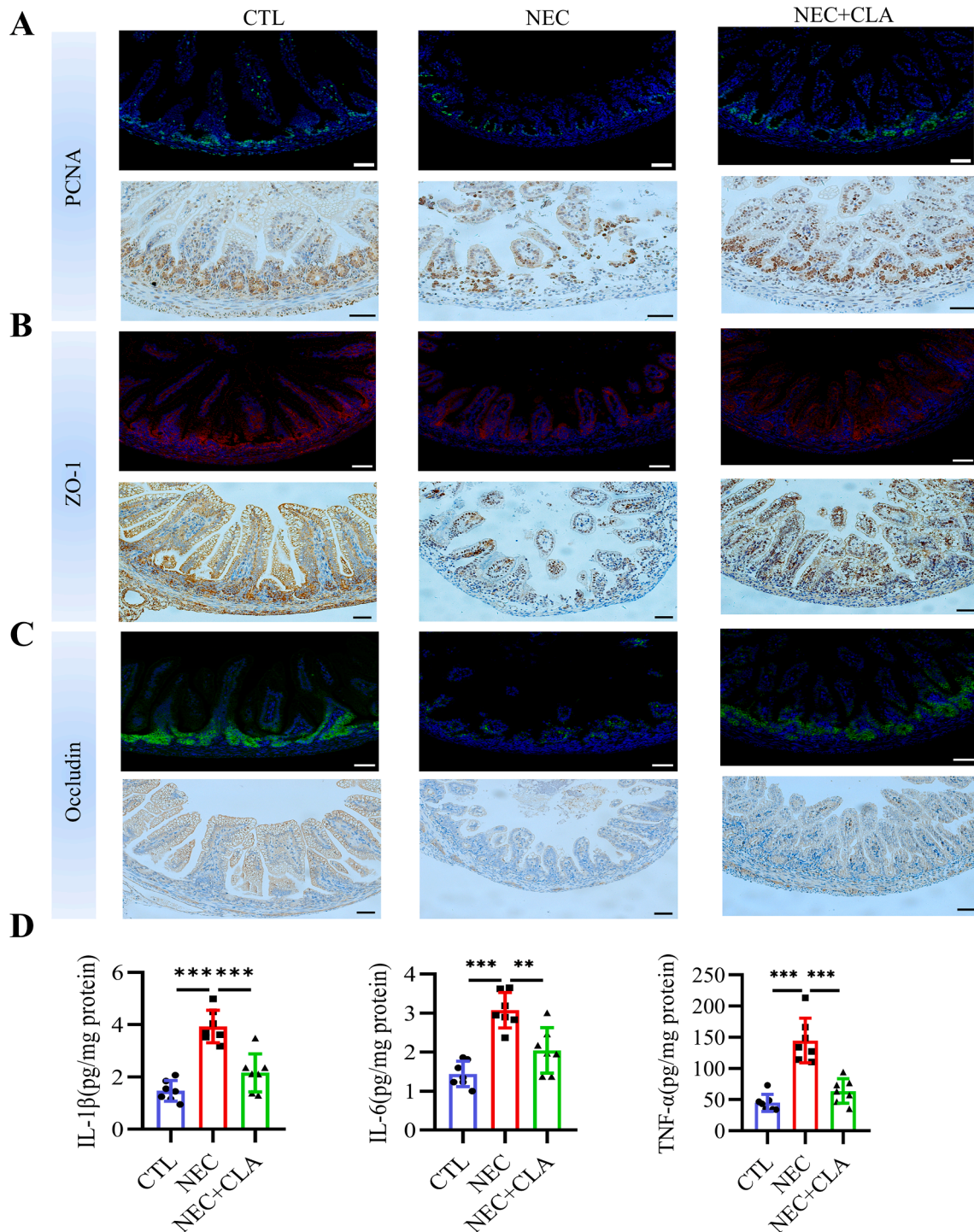


Fig. 3. Effects of CLA on intestinal cell proliferation, barrier proteins and inflammation level in rats. (A-C) Representative IF images of proliferative protein PCNA (A), tight junction proteins ZO-1 (B) and Occludin (C) in the ileum. (D) The protein concentrations of IL-1 β , IL-6, and TNF- α in three groups. IF, immunofluorescence. All scale bar = 200 μ m. ** $P < 0.01$, *** $P < 0.001$.

group. But the three cytokines were decreased by 54.85 %, 66.53 %, 44.13 %, respectively, while CLA was added (Fig. 3D).

3.4. CLA alleviated LPS-induced damage in IEC-6 cell model

Furthermore, we tried to test the protection effects of CLA in a NEC cell model of IEC-6. Cell proliferation was measured by EdU staining label. We found 33.41 % EdU + cells in the CTL group, however this rate decreased to only 4.73 % in the LPS group. And CLA helped partially

restore cell proliferation with 11.63 % EdU + cells (Fig. 4A). Fig. 4B presented the LPS group's cell death rate 6.29 times higher than the CTL groups. CLA mitigated this adverse effect by reducing the rate to half that observed in the LPS group. Additionally, barrier proteins ZO-1 and Occludin were sharply down-regulated by LPS. Nevertheless, the two proteins were significantly ameliorated after co-incubation with CLA (Fig. 4C). Western blot analysis was performed to assess the inflammatory cytokine levels within cells and semi quantitative was conducted (Fig. 4D). IL-1 β , IL-6 and TNF- α were 1.43, 1.47 and 2.00 times than CTL

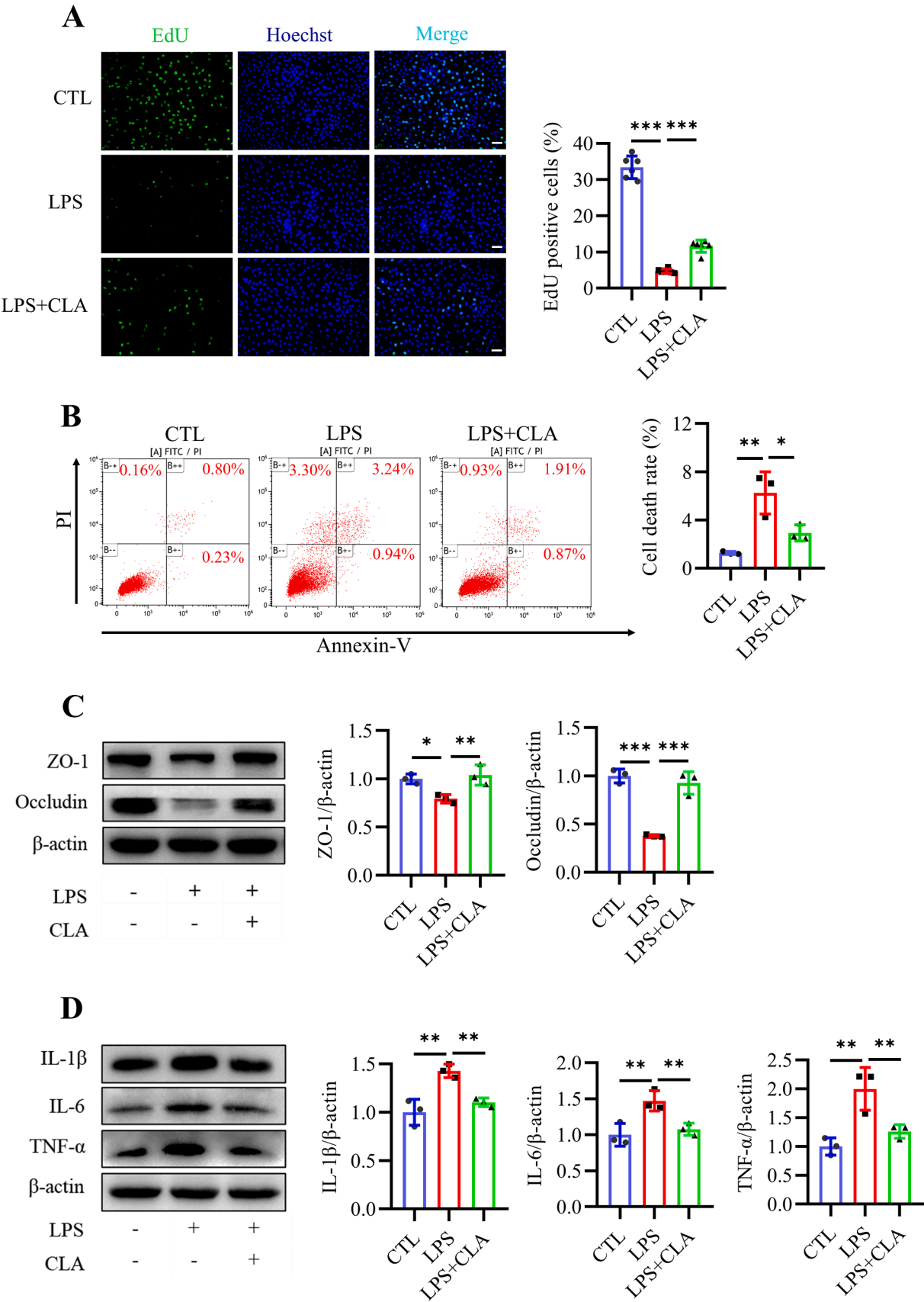


Fig. 4. The protective role of CLA in IEC-6 cell model. (A) Cell proliferation was evaluated by EdU staining label, scale bar = 50 μ m. (B) Flow cytometry analysis of cell death rates. (C) Western blot analysis showing the protein expression of tight junction proteins ZO-1 and Occludin. (D) Inflammatory cytokines (IL-1 β , IL-6 and TNF- α) of IEC-6 were tested by Western blot analysis. * P < 0.05, ** P < 0.01, *** P < 0.001.

after LPS stimulation. In contrast, with CLA supplementing, these cytokines were returned to 1.10, 1.08 and 1.26 times compared to CTL rats. Altogether, pretreated with CLA to IEC-6 cells promoted cell proliferation, reduced cell death, restored barrier proteins and lowered the inflammatory reaction.

3.5. CLA regulated PPAR γ signaling pathway to mitigate NEC

We utilized RNA- sequencing technology to investigate the

underlying mechanism. The results showed 471 DEGs between the LPS and LPS + CLA groups ($FC \geq 1.5$, $P < 0.05$) and were presented in the heat map (Fig. 5A). Between the screened DEGs, 242 were up-regulated, 229 were down-regulated in LPS + CLA group when compared to LPS group (Fig. 5B). GO analysis indicated DEGs are involved in various biological activities, including 2'-5'-oligoadenylate synthetase activity and CXCR3 chemokine receptor binding, etc (Figure S3). Next KEGG pathway analysis indicated these DEGs involved in several pathways. In the top 20 pathways, aside from metabolism-related ones like butanoate,

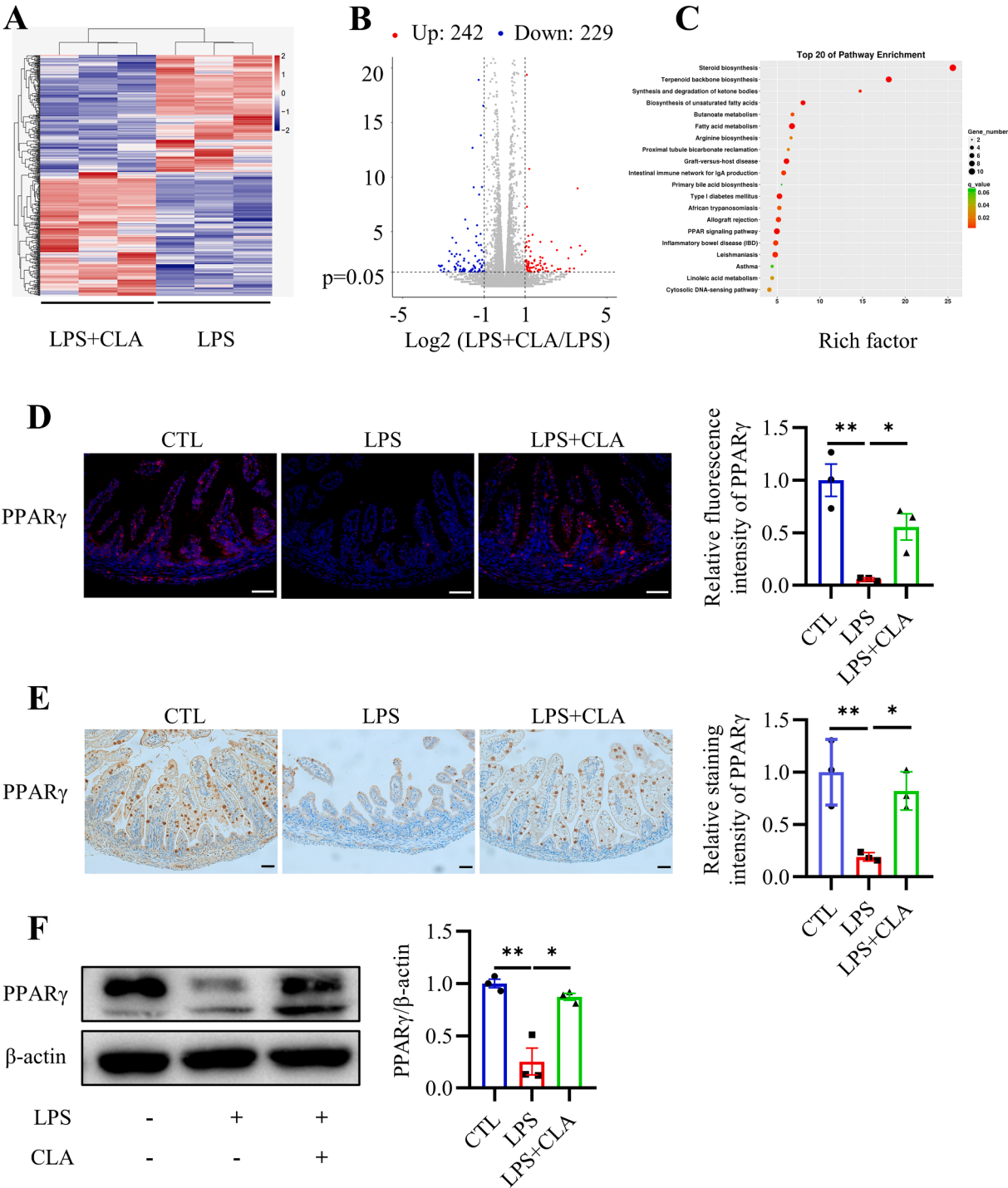


Fig. 5. Alterations of PPAR γ in NEC models. (A) Heat map of DEGs between LPS and LPS + CLA groups. (B) Volcano plot of up and down genes in the two groups. (C) KEGG enrichment pathways identified with significantly enriched DEGs (top 20). (D-E) IF and IHC analysis of PPAR γ in the distal ileum. (F) Western blot analysis showing the protein expression of PPAR γ in IEC-6. Figure (D-E) represent rats' indicators, others all from IEC-6. DEGs, differential expressed genes. IF, immunofluorescence; IHC, immunohistochemistry. All scale bar = 200 μ m. * $P < 0.05$, ** $P < 0.01$.

fatty acid, and linoleic acid metabolism, DEGs were also enriched in the inflammatory bowel disease pathway. Notably, the PPAR signaling pathway, a critical intracellular signaling mechanism regulating both metabolism and inflammation, drew our attention (Fig. 5C). PPAR γ , a subtype expressed in intestinal tissues, can be activated by natural unsaturated fatty acids to perform anti-inflammatory functions. Therefore, we validated the PPAR γ changes both in animal and cellular models. In LPS treated rats, as shown on IHC and IF, the PPAR γ was attenuated expression (Fig. 5D-E). However, CLA ameliorated this significantly. Western blot analysis of PPAR γ in IEC-6 further verified the lower expression in the LPS than CTL group, but CLA also restored the synthesis of PPAR γ (Fig. 5F).

3.6. CLA suppressed triggering of NF κ B pathway in IEC-6 cell model

Enhanced inflammatory responses are central to NEC pathology. Downstream regulation by PPAR γ encompasses various target genes, including those involved in inflammatory responses. Among them, NF κ B serves as a master regulator of inflammation, and it has been reported that NF κ B is activated in NEC. Therefore, IF was used to detect the activation of the NF κ B pathway. As illustrated in Fig. 6A, there was a notably higher fluorescence intensity observed in the IEC-6 cells following stimulation with LPS, compared to the CTL group. Intriguingly, pre-treatment with CLA appeared to effectively downregulate this response with weak fluorescence. Additionally, IEC-6 cells in the LPS

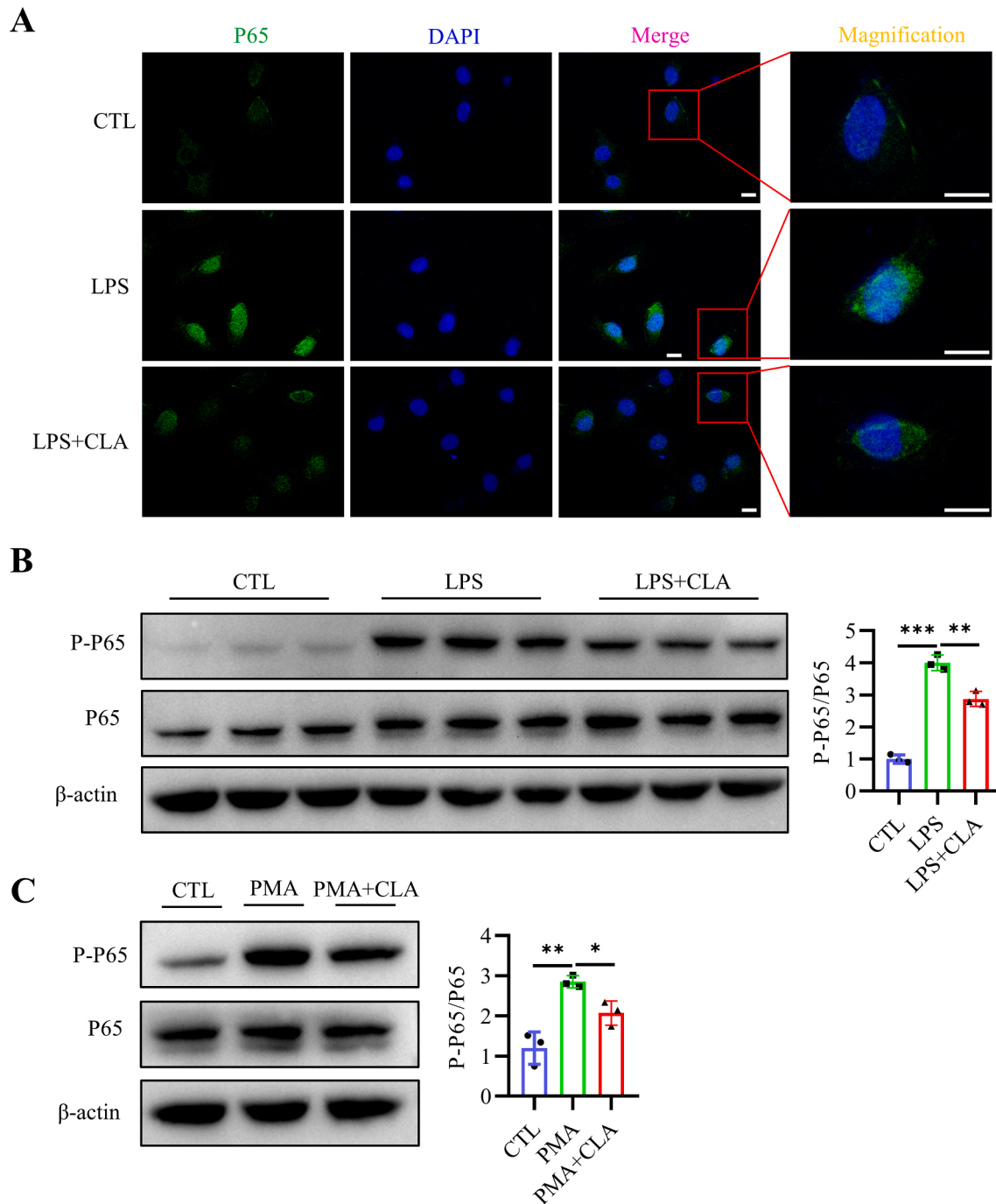


Fig. 6. CLA affect the nuclear translocation and phosphorylation of NF κ B P65. (A) IF graphs show the subunit of NF- κ B P65 in IEC-6, scale bar = 10 μ m. (B-C) Representative Western blotting images of NF- κ B P-P65 in LPS (B) or PMA (C) induced NEC cell models. IF, immunofluorescence. * P < 0.05, ** P < 0.01, *** P < 0.001.

group exhibited an elevated translocation rate of NF κ B P65 into nuclear, unlike the CTL group where NF κ B P65 predominantly remained in the cytoplasm. This observation underscored a distinct cellular response to stimuli in the IEC-6 cells. However, when pre-incubated with CLA, there was a noticeable shift in the localization of NF κ B P65 back to the cytoplasm, akin to the pattern observed in the LPS group. More intuitive protein semi-quantitative experiment was competed (Fig. 6B). In this WB experiment, the rate of NF κ B P-P65/P65 increasing highly after LPS-induced which was partial inhibited in CLA- exposed group. PMA was a kind of phorbol ester, it was the known and recognized activator of NF κ B pathway. To make the find more solid, we used it to specifically activate the pathway. As indicated in Fig. 6C, the rate of NF κ B P-P65/P65 was diminish in PMA + CLA group. In brief, CLA at least in part inhabited the activation of the NF κ B pathway that played a role in anti-inflammatory effect.

3.7. CLA inhibits NEC by preventing NF κ B pathway activation via PPAR γ activation

The subsequent experimental investigations were designed to rigorously validate the PPAR γ -dependent mechanistic model of CLA interaction, with a specific emphasis on elucidating its interplay with NF κ B pathway. As plainly illustrated in Fig. 7A, GW9662 inhibited PPAR γ protein expression, which was elevated in the LPS + CLA group. Accordingly, although the rate of NF κ B P-P65/P65 was lower in CLA than the LPS group, while the pretreated of 20 μ M GW9662 suppressed the rate. Next, three cellular phenotype alterations were assessed after GW9662 treatment. EdU staining label was performed again to test the cell proliferation (Fig. 7B). Similar to previous results, EdU + cell was lowest in the LPS group compared to CTL and LPS + CLA (11.36 % vs 42.10 % and 30.15 %). However, this effect of CLA was reversed by pretreated GW9662 which reduced to 8.95 % that approaching the LPS group. The flow cytometry detection of cell death rate also presented an approximate trend (Fig. 7C). In CTL and CLA groups, the death rate was low, 1.40 % and 3.94 % respectively. After LPS simulated, the death rate had peaked at 8.92 %, which was also high following the blockade with GW9662. ELISA was once again employed for the evaluation of inflammatory cytokines after adding the PPAR γ antagonist. The levels of IL-1 β , IL-6, and TNF- α exhibited similar patterns of changes across the four subgroups (Fig. 7D). When compared to CTL, cytokine levels in the NEC group were notably higher at 3.04, 3.40, and 1.64 times, respectively. With CLA incubation, these increases were more subdued at 2.20, 1.22, and 1.39 times. Conversely, adding GW9662, cytokines rose to 2.82, 2.21, and 1.62 times compared to CTL. Together, the beneficial effects of CLA on the NEC cellular model were weakened when a PPAR γ antagonist was added. This implied that PPAR γ activation was a crucial mechanism for CLA to exert its biological effects.

4. Discussion

NEC is a severe intestinal inflammatory and necrotic diseases affecting preterm infants. Although people have paid lots of efforts to overcome the multifactorial condition, its mortality rate remains high (Schnabl, Van Aerde, Thomson, & Clandinin, 2008), necessitating an urgency to alter the treatment approach. CLA was identified as bioactive molecule in improving NEC after fecal metabolites of NEC patients and additional analysis in this study. Subsequent experiments firmly conformed its effect to against NEC mainly by reducing pro-inflammatory cytokines, then sustaining the renewal of epithelium cells and restoring tight junction proteins.

Recent studies have reported the importance of microbe to the gut health, it impacts largely in immune maturation, metabolism and disease evolution in early life (Xiao, Wang, Zheng, Li, & Zhao, 2021). However, these studies did not delve into the deeper molecular mechanisms, as they primarily concentrated on the general imbalances between microbial communities and diseases (Murphy, Ross, Ryan,

Dempsey, & Stanton, 2021; Raba, O'Sullivan, & Miletin, 2021). Microbial metabolites, key in disease progression, mediate distant organ communication (Cheng, Ho, & Chang, 2022). In fact, intestinal microbiota secretes majority of metabolites apart from host. So, focusing on protective roles through these metabolites is an effective research strategy. For instance, short-chain fatty acids (SCFAs) are investigated extensively (Dalile, Van Oudenhove, Vervliet, & Verbeke, 2019; C. H. Kim, 2021a, 2023b). SCFAs are mainly produced by phylum Firmicutes from dietary fiber, maintaining intestinal homeostasis by strengthening gut barrier, supplying energy and counting inflammation (Parada Venegas et al., 2019). Also, SCFAs have been recognized as effective metabolites to against NEC (Yu et al., 2023). In light of these findings, we performed a metabolomics analysis of CTL and NEC fecal samples to detect their discrepancy in metabolites.

Our results showed LAs was obviously reduce in NEC infants than CTL. Then the correlation analysis of LAs with clinically indicators further implied its potential bioactive characteristics. LAs primarily consist of linoleic acid and its common isomer, CLA, both of which are octadecadienoic acids differing only in the position of their double bonds, rendering them indistinguishable by mass spectrometry. However, CLA was identified in the primary mass spectrometry. And its unique conjugated double bonds confer more anti-inflammatory bioactive functions than linoleic acid, as reported in the literature (Shokryzadan et al., 2017). This suggesting that CLA was more likely the molecule exerting anti-NEC effects in this study. Additionally, CLA primarily originate from intestinal microbiota (Yang et al., 2017), may naturally synergize with host physiological processes, potentially reflecting the intrinsic link between the microbiome and NEC. In fact, various microorganisms' microbiota in the intestine produces linoleic acid isomerase (LAI), an enzyme that converts LA to CLA. The efficiency of CLA production depends on the species and strains of the microorganisms (Salsinha, Pimentel, Fontes, Gomes, & Rodriguez-Alcala, 2018). In newborns, CLA can synthesized by colonized gut microbiota, such as *Lactobacillus reuteri*, *L. acidophilus*, *Bifidobacterium breve*, and *B. longum* (Yang et al., 2017). Research shows that intestinal flora composition changes in babies with NEC, the abundance of *Lactobacillus reuteri* and *Bifidobacterium breve* was significantly decreased, but the opportunistic bacteria (*E. coli*, *Enterococcus faecalis*, etc.) was increased (Berkhout, Niemarkt, de Boer, Benninga, & de Meij, 2018). The NEC microflora changes may accompany alterations in the LAI-producing flora, resulting in the reduction of protective CLA in NEC infants. Thus, CLA could play a crucial role in the pathogenesis of NEC, highlighting the need for thoroughly research.

In the context of NEC, pro-inflammatory cytokines have been identified as key pathophysiological agents (Frost, Jilling, & Caplan, 2008). IL-1 β has been confirmed with increasing the neutrophil population, phagocyte activation and lymphocyte costimulation in the inflammation, it presents in intestine of NEC (Markel et al., 2006). IL-6 levels were evaluated by a report in 309 preterm infants, revealing its associated with increased risk NEC (Goepfert et al., 2004). Moreover, another study demonstrated elevated IL-6 levels in NEC patients (Cakir et al., 2020). TNF- α was also increased in NEC and it was well studied in the development of the disease previously. This could be attributed to TNF- α instigated matrix metalloproteinases pathway activation, inducing intestinal necrosis and subsequent tissue damage in NEC (Markel et al., 2006). In our study, the three pro-inflammatory cytokines were all enhanced in the NEC group both in vivo or vitro models, simulating the onset of inflammation. Notably, after intervention of CLA, the cytokines IL-1, IL-6 and TNF- α were all decreased. This suggests that CLA exerted anti-inflammation effects against NEC. In addition, pro-inflammatory cytokines may increase intestinal permeability by direct immune activating and disrupting the intestinal tight junction barrier (Kaminsky, Al-Sadi, & Ma, 2021). The intestinal barrier guards against external toxins and pathogens. Tight junction proteins like OCC and ZO-1 are critical structural components to maintain gut barrier integrity (Otani & Furuse, 2020). Generally, NEC as an emergency gastrointestinal disease occurs

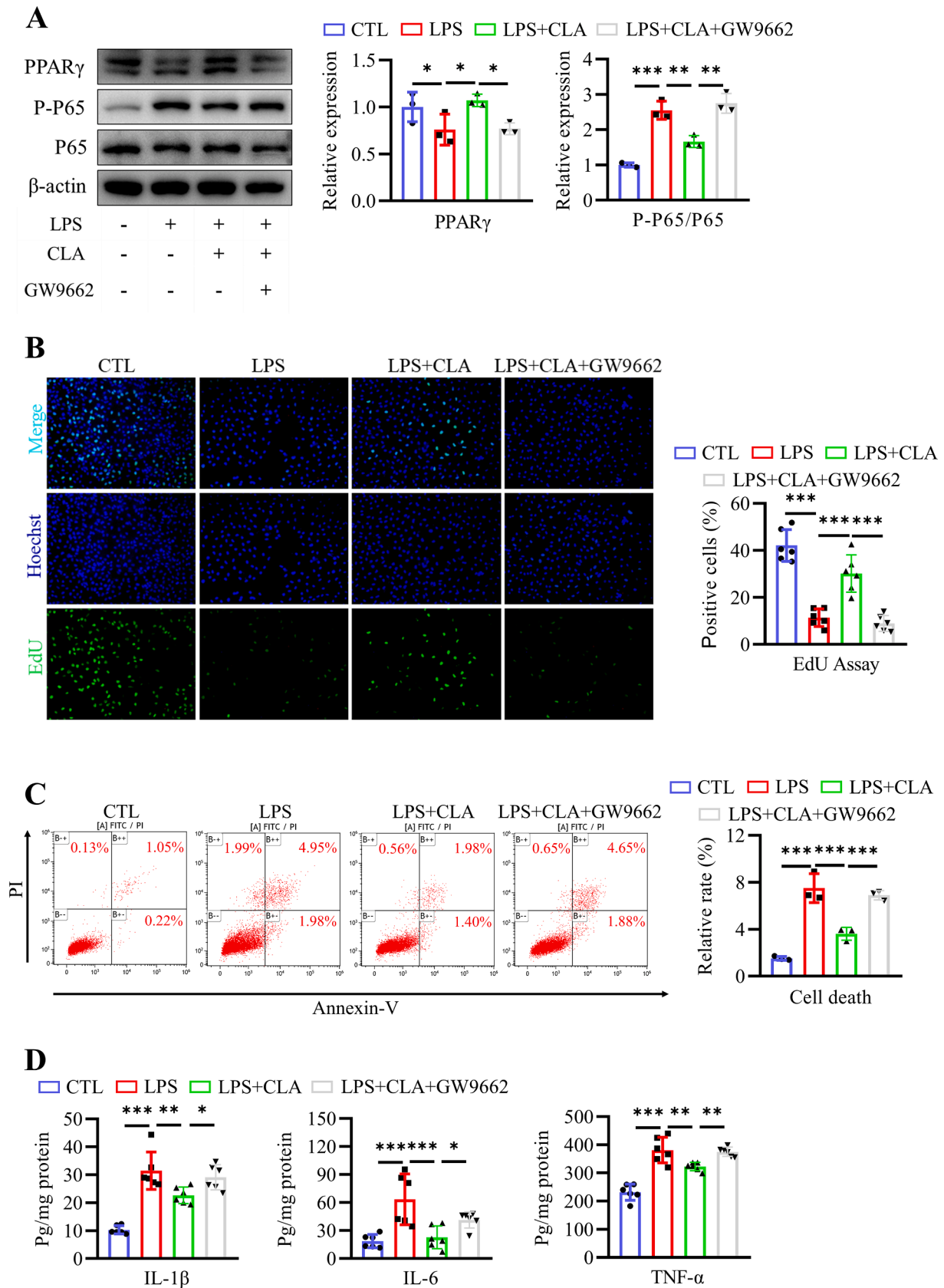


Fig. 7. Intervention of GW9662 in LPS induced IEC-6 cell model. (A) Expression of PPAR γ and NF κ B P-P65 proteins in LPS stimulated IEC-6. (B) EdU-labeled cell proliferation was assessed in the four groups, including one with GW9662 intervention, scale bar = 50 μ m (C) Cell death rates were re-evaluated by flow after antagonist GW9662 was added. (D) The protein concentrations of IL-1 β , IL-6, and TNF- α in cell supernatant of the four groups. * P < 0.05, ** P < 0.01, *** P < 0.001.

commonly in preterm infant, it was largely due to immature intestinal barrier function (Lin & Stoll, 2006). The impaired and even destructed of the neonatal intestinal barrier is an vital step leads to the development of NEC, which involves the deficiency of tight junctions proteins (Wei, Meng, Li, Dang, & Wu, 2023). In the study, through multiple experimental techniques, we have observed the barrier disruption in NEC. However, treating with CLA restores barrier proteins OCC and ZO-1 apparently. Chen et al. demonstrated that CLA improved concentration of ZO-1, occludin, and claudin-3 in Dss-induced colitis, suggesting a similar result (Chen et al., 2019). Moreover, Ren et al. reconfirmed the role of CLA in the protection of tight junction proteins in the same disease model (Ren et al., 2020).

Additionally, overexpression pro-inflammatory cytokines further damage to the intestinal barrier by increasing cell apoptosis and decreasing cell proliferation (Miyasaka et al., 2013). Intestinal epithelial cell renewal involves cell death (including apoptosis) and proliferation, which maintain normal barrier function by removing damaged or aged cells and replenishing new ones. However, in experimental NEC, increased cell shedding and death due to amplified apoptosis leading to bacterial translocation and excessive activation of the host immune system (Y. Zhou et al., 2017). Concurrently, activation of the AKT-GSK3 β pathway inhibits cell proliferation, impairing intestinal repair in NEC (Sodhi et al., 2010). Our study found that CLA intervention reduced cell loss and promotes cell proliferation, thereby maintaining normal intestinal cell homeostasis and countering NEC progression. These results indicated that intestinal inflammatory cytokines may be the critical indicators of NEC onset, which was the key point of CLA exerting its function. CLA restored barrier protein expression, and maintained normal intestinal cell renewal by inhibiting inflammatory responses, thus ameliorating NEC.

To further explore the underlying mechanisms, we performed RNA-sequencing analysis. The analysis revealed enrichment of DEGs in the PPAR signaling pathway, which contains three receptor subtypes: PPAR α , β/δ , and γ (Janani & Ranjitha Kumari, 2015). PPAR γ belongs to nuclear receptor family, and involved in inflammation, adipogenesis and lipid metabolism (Ahmadian et al., 2013). Studies have confirmed that activation of PPAR γ improves gut inflammation in different disease models. For example, Paris polyphylla extract mitigated colitis in mice by modulating PPAR γ mediated Treg/Th17 balance (He et al., 2023). Another study suggested that Ghrelin alleviate ulcerative colitis in aged mice through the PPAR γ pathway, adjusting colonic cell metabolism (Muthyala, Chapkin, Wu, & Wu, 2022). Additionally, early research had indicated that PPAR γ agonists offer protective effects in NEC (Baregamian, Mourot, Ballard, Evers, & Chung, 2009). Also, Corsini et al observed pioglitazone (PPAR γ agonist) was effective in reducing severity and inflammatory processes of NEC (Corsini et al., 2017). Therefore, we hypothesized that PPAR γ was the molecular basis mediating the NEC protective effect. Accordingly, we evaluated the expression of PPAR γ using various experimental approaches. The results consistently showed a reduction in PPAR γ expression in the NEC model group, which was restored following CLA intervention. This suggests that CLA functions through the activation of PPAR γ receptors.

Prior reports have suggested that PPAR γ negatively regulates NF κ B signaling pathway. PPAR γ , by binding with NF κ B, reduced inflammatory cytokines, which NF κ B directly regulated (Li et al., 2019). NF κ B activation occurs following the degradation of its inhibitors. This leads to the nuclear translocation of the NF κ B P65 subunit, which subsequently regulates the expression of various inflammatory cytokine genes. Consequently, IF further intuitively showed CLA inhibited nuclear translocation which can reduce the inflammatory responses. And the relative expression levels of NF κ B phospho-P65 was assessed to further evaluate the activation NF κ B signaling pathway (Ma et al., 2019). In the present study, it was increased significantly after LPS or Phorbol 12-myristate 13-acetate (known NF κ B activator) stimulation. However, CLA could partially decrease its expression. Thus, it is concluded that CLA can indirectly inhibit the NF κ B signaling pathway downstream of

PPAR γ , thereby exerting a protective effect. To make the results more solid, specific antagonists GW9662 was used to inhabit the CLA-mediated PPAR γ activity. The protein of PPAR γ was reduced after GW9662 treatment, followed by increased NF κ B phospho-p65 rate. As the results indicated, CLA reduced cell death, increased cell proliferation and attenuated the inflammatory responses, but GW9662 reversed these phenotypic changes. In general, CLA played a protect role in NEC, at least in part, through activating PPAR γ /NF κ B signaling pathway.

CLA exhibited multiple biological functions, notably its potential in improving NEC, yet its safety for human consumption also warrants consideration. CLA is naturally abundant, well absorbed and has a high bioavailability, making it easy for humans to ingest and incorporate into body tissues and blood. Clinical studies robustly affirm CLA's safety, with no adverse effects observed in adults consuming up to 6 g of CLA for one year, or 3.4 g for two years (J. H. Kim et al., 2016). In fact, CLA was recognized as a safe food ingredient in the United States, Europe, and China between 2008 and 2010 (Badawy et al., 2023; J. H. Kim et al., 2016). Furthermore, animal studies have demonstrated minimal toxicity even at high doses of 2000 mg/kg (Anadon et al., 2010; Badawy et al., 2023). These suggest that our animal dose fell within safe boundaries and are promisingly translatable to oral consumption in preterm infants. Nonetheless, some studies highlight CLA has potential health risks, such as affecting glucose homeostasis, hepatic steatosis, oxidative stress and milk-fat depression. Despite the concerns, these risks are generally not considered to pose significant long-term health effects (Badawy et al., 2023; J. H. Kim et al., 2016). Despite cumulative evidence from regulatory agencies and clinical studies supports its safety as a food additive at conventional doses, addressing the stability issues remains crucial. CLA is chemically unstable and susceptibility to oxidative degradation. Therefore, to optimize stability, bioavailability, and palatability for neonatal applications, antioxidants may be incorporated, or modificate CLA via glycerol or methyl esterification. And it can be added to formula milk as an additive. Also, early lipid emulsions provide preterm infants with high energy and essential fatty acids by parenteral nutrition (Chan, Rostas, Rogers, Martin, & Calkins, 2023). So it' another way for CLA intervention. Additionally, as the existing clinical studies on CLA were based on adults; however, preterm infants differ significantly in physiology, requiring a more cautious approach to CLA usage.

In conclusion, this direct and detailed study is an initial investigation into the effects of CLA on NEC. Our research indicates that CLA ameliorated experimental NEC by exerting anti-inflammatory effects and restoring the gut barrier integrity. Importantly, it appears that the CLA-activated PPAR γ /NF κ B signaling pathway may contribute to the protective effects against experimental NEC. Furthermore, oral CLA is safe with high bioavailability, offering significant potential for clinical application in preterm infants. In brief, the current study unveils fresh perspectives in evolving therapies for NEC.

Disclosure

There are no prior publications or submissions with any overlapping information, including studies and patients.

Ethical statement

- Research involving humans:** Ethical approval for the involvement of human subjects in this study was granted by Women's Hospital of Nanjing Medical University Research Ethics Committee, Reference number 2020KY068.
- Research involving animals:** All animal experiments comply with the ARRIVE guidelines and are conducted in accordance with the National Research Council Guidelines for the Care and Use of Laboratory Animals. Nanjing Medical University's Research and Ethics Committee approved the animal study (IACUC-2303007).
- Submission declaration and verification:** This article has not been published previously, that it is not under consideration for

publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copy-right-holder.

Authors contributions

Chengyao Jiang: Conceived and designed the research, performed the experiments, revised the manuscript, analyzed data and writing-original draft. Fan Zhang and Min Zhang: Analyzed data, experiment execution and methodology. Xiangyun Yan, Yanjie Chen, Qinlei Yu and Wenjuan Chen: Assisted in sample collection and experimental operations. Xiaohui Chen: Supervised the research and writing-manuscript revision. Shushu Li: Provided project supervision and writing-manuscript editing. Shuping Han: Conceptualized the research, administered the project, supervised the research, and writing-edited the manuscript.

CRediT authorship contribution statement

Chengyao Jiang: Writing – original draft, Methodology, Conceptualization. **Fan Zhang:** Writing – original draft, Methodology, Formal analysis. **Min Zhang:** Methodology, Formal analysis. **Xiangyun Yan:** Methodology, Formal analysis. **Yanjie Chen:** Methodology, Data curation. **Qinlei Yu:** Methodology. **Wenjuan Chen:** Methodology, Funding acquisition, Formal analysis. **Xiaohui Chen:** Validation, Funding acquisition, Conceptualization. **Shushu Li:** Writing – review & editing, Project administration, Funding acquisition. **Shuping Han:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Funding

This work was supported by grants from the National Natural Science Foundation of China (grant no. 82271744), the Natural Science Foundation of Jiangsu Province (BK20221182), Nanjing Medical Science and Technological Development Program (grant no. JQX23008, YKK22149) and Jiangsu Provincial Health Commission Medical Research Project (grant no. H2023012).

Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jff.2024.106581>.

Data availability

Data will be made available on request.

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