CCL3 aggravates intestinal tissue damage in NEC by promoting macrophage chemotaxis and M1 macrophage polarization.

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Abstract

Necrotizing enterocolitis (NEC) is a life-threatening inflammatory gastrointestinal disease in neonates, the mechanism of which is poorly elucidated. Intestinal mucosal barrier imbalance due to excessive inflammatory response is an important endogenous cause of NEC. Our study elucidates a novel mechanism of NEC development, in which CCL3 regulated the chemotaxis, polarization, and function of macrophages to promote NET progression. Our data show that CCL3 is highly expressed in the intestinal tissues of NEC patients and mice and induce macrophage infiltration. Transcriptome data from high-throughput sequencing showed that CCL3 strongly induced macrophages switch into a pro-inflammatory phenotype. Mechanistically, in vivo experiments confirmed that CCL3 induced M1 polarization of macrophages in NEC intestinal tissue, thereby aggravating inflammatory injury of intestinal tissue which can be reversed by anti-CCL3 treatment; in accordance, in vitro experiments showed that CCL3 could significantly enhance the expression of M1-related genes (e.g. iNOS, IRF5, CD86) in both peritoneal macrophages and bone marrow derived macrophages while inhibit the expression of M2-related genes (e.g. Arg-1, FIZZ1, YM1), which could also be reversed by anti-CCL3 treatment. Herein, our study has elucidated a novel mechanism of CCL3 involvement in the pathogenesis of NEC, in which the upregulated CCL3 expression exacerbated inflammatory intestinal damage via regulating macrophage chemotaxis and M1 phenotype polarization, suggesting that blocking CCL3 may be a potential strategy for effective intervention of NEC.

Introduction

Necrotizing enterocolitis (NEC) is a life-threatening illness in premature infants and is the leading cause of gastrointestinal related death in newborns[1]. In children with gestational age ≤33 weeks or birth weight ≤2500 g, the incidence of NEC is as high as 13%, with an average mortality of 20-30% and even 50% in those who require surgery[2]. In recent years, with the increased survival rate of low birth weight infants and premature infants, the incidence of NEC has also increased [3]. Although mild cases are curable, severe cases are usually accompanied with severe comorbidities like peritonitis, intestinal perforation, sepsis, multiple organ failure, leading to death[4]. And a high percentage of survivors suffer sequelae such as malabsorption[5], gastroxia gastroynsis[6], intestinal stricture[7], and short bowel syndrome[8].

The etiology of NEC is complex and poorly elucidated yet. Increasing clinical and animal experiments has provided evidence for various predisposing factors and mechanisms of NEC, including preterm birth, low birth weight[9], bacterial colonization[10], non-breastfeeding [11]and so on. The pathology of NEC is characterized by intestinal mucosal injury and diffuse or local intestinal necrosis due to multifactorial intestinal dyshomeostasis[1], that involves complex inflammatory responses and local immune disorders, in which cytokines, chemokines and related immune cells play important roles[12, 13]. Our current study indicated that CC motif chemokine ligand 3 (CCL3) is highly expressed in the intestinal lesions of NEC patients, mediates macrophage infiltration and induces M1-type polarization, thus participating in the occurrence and development of NEC. CCL3 is a pleiotropic chemokine, which plays an important role in a
variety of infectious diseases\cite{14, 15}, immune-related diseases \cite{16, 17} and oncology diseases\cite{18}. CCL3 can not only directly participate in immune regulation, but also works by recruiting certain types of immune cells and regulating their functions\cite{17}. The role and mechanism of CCL3 in NEC has been poorly understood. Our study provided the novel molecular pathological mechanism of CCL3 in driving NEC development, made an important supplement for the understanding of the pathological mechanism of NEC, and provided a new strategy for the treatment of NEC.

**Materials And Methods**

**Intestinal tissue specimen**

This study was approved by the Ethics Committee of Children's Hospital of Chongqing Medical University and was conducted in accordance with the principles of the Declaration of Helsinki. Clinical NEC is classified as stage I, II, or III based on the Bell's classification for evaluation of NEC severity. Neonates with severe NEC (Bell stage II or III) that met surgical criteria were performed necrotic bowel resection and enterostomy. The necrotic small intestine and the non-necrotic margins of surgical resection were collected for clinical study.

**Experimental NEC Model**

This experiment was reviewed and approved by the Animal Ethics Committee of Chongqing Medical University. 7-10 days’ old neonatal C57BL/6 mouse pups (male and female) were purchased from the Animal Center of Chongqing Medical University and fed in a neonatal incubator at 28°C. Mouse pups in the NEC group were fed with 20-30ul/g hypertonic formula milk by gavage every 4 hours for a total of 5 times/day. The formula milk contained Similac 60/40 (Abbott Laboratories, Saint-Laurent, Canada) and Esbilac (PetAg, Hampshire, Illinois). In addition, the pups were exposed to cold stimulation (4°C, 10min) and hypoxic environment (100% N2, 90 s) twice a day, and were fed with lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis) 5ug/g/d by gavage\cite{19}. In the NEC+CCL3 group, the pups were given NEC according to the above-mentioned protocol and injected with Recombinant Mouse CCL3/MIP-1 alpha Protein (R&D, USA) 50ng/ml. In the NEC+Anti-CCL3 group, the pups were treated according to the above-mentioned protocol. The pups were given NEC and intraperitoneally injected Mouse CCL3/MIP-1 alpha Antibody (R&D, USA) 50ng/ml. The pups in the healthy control group were breastfed by their mothers without any stress factors. Pups were excluded due to improper feeding or death within 24 hours after NEC induction. 96 hours after NEC induction, all surviving pups were euthanized, and ileum samples were collected for further analysis.

**RT-PCR**

Total RNA from intestinal tissue or cells was extracted using Trizol reagent (Invitrogen) and then reversely transcribed into cDNA using PrimeScript RT kit (RR037A, Takara, Japan) according to manufacturer's instructions. Real-time PCR (RT-PCR) was performed using SYBR Premix Ex Taq II kit (RR820A, Takara,
Japan) and Applied Biosystems 7500 fast real-time PCR system (ABI, Torrance, CA). GAPDH was used as an internal reference. Primer information can be found in Supplementary Table 1.

Table 1. Gene-Specific Oligonucleotide Primers Used for Q-PCR

| Gene     | Serial number | Primer sequence | product size(bp) |
|----------|---------------|-----------------|------------------|
| Human ARG1 | NM_000045     | Forward 5'- TGGACAGACTAGGAATTGGCA-3' Reverse 5' - CCAGTCCGTCACACATCAAACACT' | 102 |
| Mouse ARG1 | NM_007482     | Forward 5'- TTGGGTGGATGCTCACACTG-3' Reverse 5' - GTACACGATGTCTTTGGCAGA-3' | 166 |
| Human INOS | NM_000625     | Forward 5'- AGGGACAAGCCTACCCCTC-3' Reverse 5' - CTCATCTCCCGTCAGTTGGT-3' | 168 |
| Mouse INOS | NM_010927     | Forward 5'- GGAGTGACGGCAAACTGACT-3' Reverse 5' - TCGATGCAAACTGGGTAAC-3' | 127 |
| Human CD86 | NM_175862     | Forward 5'- CTGGCTCATCTATACACGGTTACC-3' Reverse 5' - GGAAACGTCGTACGTTCTGTG-3' | 133 |
| Mouse CD86 | NM_019388     | Forward 5'- CTGGACCTCTACGACTCAATG-3' Reverse 5' - AGTTGGCGATCACTGACAGTT-3' | 131 |
| Human FIZZ1 | NM_032579    | Forward 5'- CCGTCCCTTGGCCTCTCC-3' Reverse 5' - CTTTTGACACTAGCACAGA-3' | 173 |
| Mouse FIZZ1 | NM_020509    | Forward 5'- CCAATCCAGCTAATCTCCCTCC-3' Reverse 5' - ACCCAGTAGCAGTCCCA-3' | 108 |
| Mouse YM1 | NP_034022     | Forward 5'- CAGGTTGGCAATTCTTTGGA-3' Reverse 5' - GTCTTGCTCATGTTGTAAGTGA-3' | 197 |
| Mouse IRF5 | NM_012157     | Forward 5'-AGAGACAGGGAAGTACACTGAAG-3' Reverse 5' - TGGAGTTCAGGGCTTTGTTAAG-3' | 96 |

Primers were designed from the published sequences in the GenBank database under the indicated accession numbers. F forward primer, R reverse primer

ELISA Assay
ELISA kits for IL-1β, IL-10 and IFN-γ were purchased from BioLegend, Inc. and operate according to the manufacturer’s instructions.

**Isolation and culture of mouse bone marrow derived macrophages (BMDM)**

Bone marrow cells were isolated from the femur and tibia of 16-18g male C57BL/6J mice and induced to differentiate into macrophages in the DMEM complete medium supplemented with 10ng/ml M-CSF (Peprotech, USA), 10% fetal bovine serum (Ausbian, Australia), 1% penicillin/streptomycin (Gibco, USA). The culture medium was refreshed on the day 3 and 5, and then on days 7, rCCL3/ anti-CCL3-treated cells were collected from the petri dish with a cell spatula. The cells were centrifuged at 500 RCF for 5 min to form a precipitate and then suspended in full DMEM medium for further use.

**Isolation and culture of mouse peritoneal macrophages (PMφ)**

Mice were intraperitoneally injected with 1ml sterile liquid paraffin and euthanized 3-5 days later. The mice were disinfected with 70% alcohol and their limbs were fixed on the operating board. The abdomens of the mice were massaged for several minutes. And then the skin to the left or right of the midline at the bottom of the peritoneum is cut lengthwise by surgical scissors to expose the clear peritoneum. 10ml precooled PBS containing EDTA was injected into the abdominal cavity of mice and collected in a 15mL centrifuge tube after several lavages. The cells were washed with PBS for 3 times and then cultured in a cell culture plate for 45-60 min. The cells were then washed with PBS for 2 times to discard the non-adherent cells, and the adherent cells were cultured in complete DMEM medium for further use.

**Isolation of leukocytes from mouse intestinal tissue**

After the 4th day of NEC mouse modeling, all surviving pups were euthanized, and intestinal tissue samples were collected. Bowel was dissected longitudinally and washed in PBS until PBS clarified to remove feces. Intestinal tissue was then cut into 0.5x0.5 cm segments and suspended in DMEM medium containing 5 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich), 2 mM 1,4-dithiothreitol (DTT; Sigma-Aldrich) and 1% FBS, and then shaken in a shaker (180RCF) and incubated (37°C) for 2x 20 min to separate the epithelial layer. the intestinal tissue without epithelial layer was minced and supplemented with 1 mg/ml (0.15 U/mg) collagenase D (Roche), 1% fetal bovine serum (FBS; Biological Industries) and 1000 U/ml DNase type I ( Worthington Biochemical Corporation) s DMEM was incubated in a shaker at 37 degrees (180RCF) for 30 minutes, and finally the supernatant containing the cells was filtered through a 70 mm filter (Falcon, Corning) to obtain a single cell solution.

**Flow Cytometry (FCM)**

The cell suspension was incubated with different fluorescent-labeled surface molecular antibodies and isotype control antibodies for 30 min, then the cells were washed and detected by flow cytometry and analyzed according to the product instructions. Antibody information is shown in Table 2

Table 2
### Antibody List

| Antibody       | SOURCE       | IDENTIFIER   |
|----------------|--------------|--------------|
| Anti-mouse CD11b | BD Horizon   | Cat#561690   |
| Anti-mouse Ly/6G | BD Horizon   | Cat#562060   |
| Anti-mouse F4/80 | BD Horizon   | Cat#565410   |
| Anti-mouse CD3  | BD Horizon   | Cat#565643   |
| Anti-mouse CD4  | BD Horizon   | Cat#743156   |
| Anti-mouse CD8  | eBioscience  | Cat#45-0081-82 |
| Anti-mouse CD86 | BD Horizon   | Cat#561963   |
| Anti-mouse CD206| eBioscience  | Cat#17-2061-82 |

### Results

**CCL3 is highly expressed in NEC intestinal lesions and aggravates the intestinal damage**

To determine the expression of CCL3 in NEC, intestinal tissue from NEC patients undergoing surgery was homogenized for ELISA test. The results showed that the level of CCL3 in the homogenized necrotic intestinal tissue of NEC patients was significantly higher than that of the surgically excised, relatively non-necrotic adjacent sites, as shown in Fig.1A. Then, an NEC animal model was established for further study, and both ELISA results (Fig.1B) and immunohistochemical results (Fig.1C) showed that CCL3 expression in NEC mice was significantly higher than that in the control group. To characterize the role of CCL3 in NEC, mice in the intervention group were intraperitoneally injected with the recombinant CCL3 protein (rCCL3) or CCL3 neutralizing antibody (anti-CCL3) during modeling and maintained at an appropriate dose after modeling, while mice in the control group were intraperitoneally injected with PBS. Intestinal gross appearance (Fig.1D) and pathological results (Fig.1E) showed that the intestinal tissue damage in rCCL3 group was more severe than that in the control group, accompanied by severe intestinal flatulence and bleeding (Fig.1D), as well as necrotizing changes such as intestinal epithelial shedding and intestinal mucosal thinning (Fig.1E), all of which were significantly alleviated in the anti-CCL3 group. These results indicate that the increased expression of CCL3 is a harmful endogenous factor in the development of NEC.

**CCL3 promotes inflammation and macrophage recruitment in the intestinal tissues of NEC mice.**

Considering that CCL3 aggravates intestinal tissue damage during NEC, we further evaluated the effects of CCL3 on intestinal tissue inflammation and leukocyte infiltration. ELISA results showed that inflammatory cytokines IFN-γ and IL-1β were significantly increased in the rCCL3 treatment group, but significantly decreased in the anti-CCL3 treatment group (Fig.2A and B). The intestinal tissues of model
mice were then digested to prepare single-cell suspensions for the detection of leukocyte infiltration. FCM revealed a significant increase in CD11b^+F4/80^+ macrophages in the intestine of NEC mice injected with rCCL3, which was reversed by anti-CCL3 treatment (Fig. C and D). CCL3 can also promoted the recruitment of a small number of CD11b^+LY/6G^+ neutrophils (Fig. E and F), but has little effects on the accounts of lymphocytes (CD3, CD4, CD8) (Fig. G-K). Next, primary peritoneal macrophages (PMφ) were isolated and treated with rCCL3 or anti-CCL3, and culture supernatants were collected for the detection of inflammatory factors. IFN-γ and IL-1β levels in the supernatant of rCCL3 treatment group were significantly increased, while those in anti-CCL3 treatment group decreased (Fig. L and M), which was consistent with the results of in vivo experiments, further confirm the regulation of CCL3 on macrophages in NEC. These results suggest that CCL3 mediates macrophage chemotaxis and participates in NEC-related intestinal inflammation.

**Transcriptomics reveal the proinflammatory profile of CCL3-stimulated macrophages**

To further clarify the role of CCL3-activated macrophages in NEC, bone marrow derived macrophages (BMDM) were isolated and the spectral characteristics of rCCL3 and anti-CCL3-stimulated macrophages were evaluated by high-throughput sequencing. The volcano map of differentially expressed genes (DEGs) showed significant differences in gene expression between the CCL3-treated group, the anti-CCL3-treated group and control groups (Fig.3 A). To further analyze the DEGs results, Venn diagrams were used to describe the overlap of upregulation genes, and the results showed a higher rate of difference in DEGs between the control and rCCL3 groups than between the control and anti-CCL3 groups (Fig.3 B). Each small square on the heat map represents a DEGs, and all genes are listed in the supplementary material (Supplementary data 1). Importantly, cluster analysis revealed that CCL3 treatment has substantial impacts on the pro-inflammatory response of macrophages, as some inflammatory cytokines (NLRP3, TNF, IL-1B, IL-6, IL-12), chemokines (CCL5, CXCL10, CCL2, CCL7), and M1-type macrophage-related genes (iNOS, CD38, CD86) were highlighted in DEGs of rCCL3-treated macrophages (Fig.3 C), and the representative highlighted DEGs in rCCL3 group were summarized in Table 1. To further specify the functional regulation of CCL3 on macrophages, GO analysis was performed thereafter. DEGs were divided into three categories: biological process (BP), molecular function (MF) or cellular component (CC). Significant differences in GO analysis of DEGs in different groups were defined as \( P<0.05 \). Notably, functional annotations by GO analysis indicated that DEGs in rCCL3 pretreatment group were mainly associated with positive regulation of immune response, positive regulation of cytokine production and proliferation of leukocytes, etc. (Fig.3 F-H). To identify the relevant biological pathways involved in these effects, sequencing data were further analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Fig. 3D) and the Reactome Signaling Pathway database (Fig.3 E) according to the criteria of \( P<0.05 \). The data suggested that major pathways of DEGs after rCCL3 stimulation included chemokine signaling pathway, and cytokine-cytokine receptor interaction, et al. Based on the analysis of these data, we conclude here that macrophages treated with rCCL3 were highly expressing substantial pro-inflammatory factors associated with the M1 phenotype, whereas the anti-CCL3 group showed the opposite results.
Table 3 Partial representative DEGs highly expressed in rCCL3 group

| Gene ID       | Gene name | Log2FC  | P-Value  |
|--------------|-----------|---------|----------|
| ENSMUSG00000027398 | Il1b      | 8.625474027 | <10^-5   |
| ENSMUSG00000020826 | Nos2      | 8.393199099 | <10^-5   |
| ENSMUSG00000029084 | CD38      | 7.463980027 | <10^-5   |
| ENSMUSG00000035042 | Ccl5      | 6.738212218 | <10^-5   |
| ENSMUSG00000024401 | Tnf       | 4.370028163 | <10^-5   |
| ENSMUSG00000025498 | Irf7      | 3.148264233 | <10^-5   |
| ENSMUSG00000032691 | Nlrp3     | 3.120521887 | <10^-5   |
| ENSMUSG00000051439 | Cd14      | 2.477026117 | <10^-5   |
| ENSMUSG00000018459 | Slc13a3   | 2.311923179 | 2.02E-74 |
| ENSMUSG00000022901 | Cd86      | 1.275628056 | 1.45E-10 |

Table 3 Partial representative data and detailed data can be obtained from the authors for reasonable reasons.

**CCL3 mediates M1 macrophage polarization in intestinal tissue of NEC.**

Since RNA-Seq data have identified that CCL3 regulate macrophage into a pro-inflammatory phenocyte, we then sought to examine the expression of different phenotypes of macrophage-related genes in intestinal tissues of patients and NEC mice. Compared with the control group, the expression of iNOS and CD86, two representative marker of M1 macrophages, was higher in the necrotic intestinal tissue of NEC patients, while the expression of M2-related genes Arg-1 was lower (Fig. 4 A-C). In NEC mouse model, the expression of M1 macrophage-related genes iNOS, CD86 and IRF5 was also increased in rCCL3-treated mice compared with the control group, consistent with that of human results (Fig. 4 D-G). On the contrary, the expression of M2 macrophage-related genes Arg1, FIZZ1 and YM1 decreased in rCCL3-treated mice compared with the control group (Fig. 4 H-J). Unsurprisingly, the anti-CCL3 group showed the opposite results to the rCCL3 group (Fig. 4 E-J). Then, intestinal tissue from mice in different treatment groups was digested to prepare single-cell suspensions for the detection of macrophage phenotype. FCM results showed a significant increase in F4/80+CD86+ cells in the intestine of NEC mice treated with rCCL3, which was not observed in the anti-CCL3 group (Fig. 4 K-L). Together, these data suggest that CCL3 promotes polarization of M1-type macrophages in the intestinal tissues of NEC patients and mice.

**CCL3 promotes M1 macrophage polarization in PMφ and BMDM in vitro.**

We then sought to verify the regulatin of CCL3 on macrophage phenotype by using peritoneal macrophages (PMφ) and BMDMs in vitro. FCM was used to detect the phenotype of PMφ treated with
rCCL3 and anti-CCL3. The results showed that the proportion of F4/80+CD86+ cells in rCCL3-treated cells increased, while the proportion of F4/80+CD206+ cells decreased; The opposite was observed in the anti-CCL3 treated cells. In addition, in GM-CSF and rIL-4 stimulated BMDMs, the proportion of F4/80+CD86+ cells was also increased with CCL3 treatment, while no significant difference was observed in the proportion of F4/80+CD206+ cells with/without CCL3 treatment. Intriguingly, both M1-related genes and M2-related genes were highly expressed in M2 macrophages after CCL3 treatment, indicating that CCL3 promoted the transition from M2 to M1 macrophages. Furthermore, we assessed the change of M1 macrophage-related genes and M2 macrophage-related genes, and found that PMφ and BMDM in the CCL3-treated group highly expressed M1 macrophage-related genes, but low expressed M2 macrophage-related genes, which was highly consistent with the results of the in vivo experiment. These evidences supported that CCL3 can promote the pro-inflammatory phenotype of macrophages, which may be closely related to the aggravation of NEC related intestinal tissue injury. Therefore, blockade of CCL3 may be a potential novel immunotherapy strategy to reduce NEC-related intestinal tissue damage.

**Discussion**

NEC has emerged as one of the most destructive diseases occurring in neonatal intensive care[23]. Progress in our understanding of the pathophysiology, prevention and treatment of NEC has been hampered for many reasons[4]. Included among these is the fact that the true underlying causes of intestinal barrier imbalance are complex and poorly elucidated. In the current study, we revealed that CCL3 was a harmful endogenous factor in the development and progression of NEC, as CCL3 promoted macrophage infiltration and strongly induced the inflammatory phenotype (M1) during NEC, thus enhancing the expression of inflammatory factors in intestinal tissue, which in turn aggravates the inflammatory intestinal injury. Blocking CCL3 effectively reduced NEC-related intestinal tissue injury.

As a pleiotropic chemokine, CCL3 plays an important role in a variety of diseases by regulating inflammation and immunity, including chemotaxis of various leukocytes to inflammatory sites and direct regulation of inflammatory responses, etc.[17]. By binding to receptors on immune cells, CCL3 promotes the production of inflammatory cytokines, and overactive of CCL3 has a direct detrimental effect on the prognosis of the disease[16, 24].

In this study, RNA-Seq data showed that CCL3 induced extensive inflammatory gene expression in macrophages. In vivo and in vitro experiments confirmed that CCL3 directly induced M1 macrophages polarization, thereby leading to increased expression of inflammatory factors, which is closely related to NEC-mediated intestinal tissue injury. Macrophages are essential immune cells in the innate immune system, and their polarization imbalance can lead to intestinal barrier dysfunction, intestinal microbial environment imbalance, and increased levels of pro-inflammatory cytokines[25]. Our study elucidated the pathogenic role of CCL3-mediated hyperpolarization of M1 macrophages in the production of inflammatory cytokines and impairment of intestinal barrier function. Since blocking CCL3 can significantly reduce NEC-related intestinal injury, such as severe intestinal edema, intestinal villus
shedding, intestinal wall thinning, and intestinal tissue necrosis. Our study provides a potential new approach for clinical intervention of NEC.

Although our study sheds light on the underlying mechanism by which CCL3 aggravates NEC-related intestinal injury, there are still some problems worth discussing. For example, it is still unclear why CCL3 is overexpressed in the lesion site of NEC patients and the underlying regulatory mechanisms of CCL3 production. Although macrophages predominate in CCL3-mediated leukocyte infiltration of intestinal tissue, however, the target cells of CCL3 may not be limited to macrophage. As neutrophils are also recruited by CCL3 in our study, the regulation of CCL3 on neutrophils is also worthy of further discussion.

In Fig. 5C and D, M2 macrophages in the rCCL3-treated group were strongly inverse polarized into M1 macrophages, represented by a significant increase in the proportion of F4/80+CD86+ cells. However, no significant reduction in the proportion of M2 macrophages was observed in the rCCL3-treated group, one possible explanation maybe due to the increased proportion of dual-yang cells expressing both M1 and M2 markers after rCCL3 treatment. M2 Mφ treated with rCCL3 subsequently expressed M1-type markers, which may be an important process in the transformation of M2 macrophages to M1-type induced by CCL3.

In conclusion, our study elucidates that CCL3, as a harmful endogenous factor of NEC, exacerbates NEC-mediated inflammatory intestinal injury by regulating macrophage chemotaxis and polarization of inflammatory phenotypes. Blockade of CCL3 with anti-CCL3 can effectively reduce NE-related intestinal tissue, which provides a new idea for the clinical treatment of NEC.

**Abbreviations**

Arg-1
Arginase 1
BMDM
bone marrow derived macrophages
CCL3
CC motif chemokine ligand 3
DEGs
differentially expressed genes
FBS
fetal bovine serum
FCM
Flow Cytometry
FIZZ1
found in inflammatory zone 1
iNOS Inducible Nitric-Oxide Synthase
IRF5
interferon regulatory factor 5
Mφ
macrophages
NEC
Necrotizing enterocolitis
RNA-seq
RNA sequencing
RT-PCR
real-time PCR
YM1
chitinase-like protein 3, Chil3.

Declarations

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Author contributions

Z.S., and D.C. conceived and designed the experiments. X.Y., W.L., Y.L. and H.L. performed the experiments. W.L. and Y.Y. collected and analyzed the data. Z.S. and X.Y. wrote of the manuscript. All authors had reviewed and approved the final submitted and published versions.

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This study was approved by the Ethics Committee of Children's Hospital of Chongqing Medical University and the Animal Ethics Committee of Chongqing Medical University.

Competing interests

The authors declare that they have no competing interests.

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**Supplementary**

Supplementary Data 1 is not available with this version

**Figures**
Figure 1

The expression and role of CCL3 in NEC. (A) expression of CCL3 in intestinal tissue of patients, detected by ELISA kit (N=5-8). (B) expression of CCL3 in intestinal tissue of mice, detected by ELISA kit (N=10-15). (C) expression of CCL3 in intestinal tissue of patients, detected by immune-histochemistry (DAB chromogenic, Scale bar=50 μm). (D) Macroscopic view of mouse intestinal tissue from Control, NEC, NEC+rCCL3, NEC+anti-CCL3 groups. (E) Representative images of H&E-stained sections from Control, NEC, NEC+rCCL3, NEC+anti-CCL3 groups (Scale Bar =100μm). Data are expressed as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 when compared between groups.
Figure 2

The chemotaxis and inflammatory regulation of CCL3 in NEC. (A-B) IL-1β and IFN-γ expression in the intestinal lysates of NEC mice treated with rCCL3 or anti-CCL3. (n=3-5) (C-K) FCM analysis was performed to evaluate the chemotactic effects of rCCL3 (50ng/ml) and anti-CCL3 (50ng/ml) on CD11b^+ F4/80^+ cells, CD11b^+ LY/6G^+ cells and T cells (CD3, CD4, CD8). (L-M) Levels of IL-1β and IFN-γ in the supernatant of macrophage treated with rCCL3 or anti-CCL3. All data are expressed as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 when compared between groups.
Gene expression profiles of BMDMs treated with rCCL3 and anti-CCL3. (A) Volcanic map analysis showed differences in gene expression between rCCL3 group, anti-CCL3 group and control group, detected by RNA-Seq (n=3 per group). (B) Venn diagram analysis showing upregulation of DEGs in rCCL3 vs. control group (yellow) and anti-CCL3 vs. control group (purple). (C) Stratified clustering and heat maps showing RNA-Seq data of DEGs in BMDMs pretreated with rCCL3 or anti-CCL3. (D-E) DEGs data analysis in rCCL3-stimulated macrophages based on KEGG and Reactome databases. (F-H) GO analysis was used to
classify the biological processes, molecular functions, or cellular components involved in DEGs after rCCL3 and anti-CCI3 stimulation.

**Figure 4**

CCL3 mediates M1 macrophage polarization in NEC. (A-C) RT-PCR analysis of the relative mRNA expression of M1 and M2 macrophage-associated genes in the intestines of NEC neonates \(2^{-\Delta\Delta CT}\). (D) Expression of iNOS (DAB staining) in the sections of the intestinal tissue in mouse with or without NEC. (E-J) RT-PCR analysis of the mRNA expression of M1 and M2 macrophage-associated genes in the
intestines of Ctrl, NEC, NEC+rCCL3 (50ng/ml), NEC+anti-CCL3 (50ng/ml) groups. (K-L) FCM analysis was performed to evaluate the effects of rCCL3 and anti-CCL3 on macrophages polarization in the intestines of Ctrl, NEC, NEC+rCCL3, NEC+anti-CCL3 groups. *p < 0.05, **p < 0.01, ***p < 0.001 when compared between groups.

Figure 5

The role of CCL3 in inducing M1 Mφ polarization. (A-B) Flow cytometry analysis was performed to evaluate the effects of rCCL3 (10ng/ml) and anti-CCL3 (10ng/ml) on PMφ polarization. (C-D) Flow
cytometry analysis was performed to evaluate the effects of rCCL3 (10ng/ml) on BMDMs polarization. (E-N) RT-PCR analysis of M1 and M2 macrophage-related genes expression in PMφ and BMDMs treated with rCCL3/anti-CCL3. Data are expressed as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 when compared between groups.