1 **Vitamin D3 alleviates intestinal injury in necrotizing enterocolitis rats and**

2 **lipopolysaccharide-induced inflammatory response in dendritic cells**

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Conflict of interest

There are no conflicts of interest to disclosure for all authors.

Informed consent

Ethical approval was granted by the Ethics Committee of Jiangxi Provincial People's Hospital (Approval NO. KT051). All participants provided informed consent in the format required by the relevant authorities and/or boards.

Vitamin D3 alleviates intestinal injury in necrotizing enterocolitis rats and lipopolysaccharide-induced inflammatory response in dendritic cells

Abstract

Background and aim: Necrotizing enterocolitis (NEC) is a serious condition which predominantly affects premature infants and involves an aberrant immune response and inflammatory cytokine release which results in intestinal epithelial damage. The current study investigated the immunoregulatory effects of vitamin D3 on the maturation and activation of dendritic cells (DCs) and the anti-inflammatory impact on the intestine in a neonatal rat model of NEC.

Materials and methods: Inflammatory damage to intestinal tissue was assessed via morphological changes and apoptosis and DC expression of co-stimulatory molecules, inflammatory and immunoregulatory factors by immune-histochemical staining, quantitative real-time PCR and immunofluorescence.

Results: The Fluorescein Isothiocyanate-Ovalbumin (FITC-OVA) uptake assay was

used to analyze DC endocytosis. Vitamin D3 administration attenuated intestinal damage and apoptosis, inhibiting Cluster of Differentiation 86 (CD86) and increasing Cluster of Differentiation 80 (CD80) expression. Lipopolysaccharide (LPS)-challenged Cluster of Differentiation 2.4 (DC2.4) cells in vitro showed up-regulated CD86, Tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β), inducible Nitric oxide synthase (iNOS) and Indoleamine 2,3-dioxygenase 1 (IDO-1) expression which were all reduced by vitamin D3, except for IDO-1. LPS inhibited CD80 expression, which was restored by vitamin D3 treatment, and the endocytic capacity was improved. Vitamin D3 ameliorated intestinal damage in neonatal NEC rats and exerted anti-inflammatory and immunomodulatory effects on DCs.

Conclusion: Vitamin D3 has the potential as a supplementary treatment for NEC patients.

Key words: Necrotizing enterocolitis, vitamin D_3, dendritic cells, inflammatory response

1. Introduction

Necrotizing enterocolitis (NEC) is an inflammatory intestinal disease with high morbidity and mortality, caused by the failure of premature neonates to adapt to enteral nutrition. NEC occurs in approximately 5% of infants admitted to neonatal intensive care units (NICUs), with an incidence of 9% of infants born at 22 to 29 weeks gestational age [1]. The vast majority (>90%) of NEC occurs in preterm infants, especially those who are of very low birthweight (<1500 g) (VLBW). The incidence of

1 NEC is inversely proportional to gestational age at birth (GA), and the time to onset of
2 NEC is longer in the more premature infant [2]. The mortality rate of NEC among
3 extremely premature infants was 30% [3]. NEC has multifactorial causes and is
4 associated with various prenatal and postnatal factors [4]. The disorder is characterized
5 by abdominal distension, vomiting, bloody stool and sepsis with intestinal wall and
6 portal vein pneumatosis visible on imaging [5, 6].
7 The etiopathogenesis of NEC remains unclear but an immature intestinal barrier, altered
8 microbiota, intestinal immaturity [7], enteral feeding with formula milk [8], bacterial
9 colonization, hypoxic-ischemic damage and an aberrant TLR4 (toll-like receptor
10 4)-mediated intestinal inflammatory response are contributory factors [9]. The excessive
11 inflammatory response may lead to intestinal mucosal edema, damage and intestinal
12 wall necrosis [10]. Treatment involves intestinal rest, abdominal decompression and
13 broad-spectrum antibiotics but peritoneal drainage, bowel resection and enterostomy
14 may be necessary in severe cases [11]. The therapeutic value of probiotics [12], stem
15 cell transplantation [13, 14], exosomes and targeted treatment of intestinal ischemia
16 with heparin-binding Epidermal Growth Factor-like (EGF-like) growth factor have all
17 been studied [15, 16].
18 Vitamin D_3 is a secosteroid hormone with roles in calcium homeostasis and bone
19 growth which promotes collagen matrix mineralization [17]. It is absorbed from the diet
20 or synthesized from 7-dehydrocholesterol on exposure of the skin to sunlight. Inactive
21 vitamin D_3 is hydroxylated by hepatic cytochrome P450 (CYP2R1), generating

1 25-hydroxyvitamin D (25(OH)D), which is converted into 1,25-dihydroxy vitamin D_3
2 (1,25$(OH)_2D_3$) by the hydroxylation of CYP27B1 [18, 19]. Active vitamin D_3 binds
3 the vitamin D receptor (VDR), consisting of ligand-binding and DNA-binding domains,
4 and causes transcriptional activation or repression mediated by the 1, 25$(OH)_2D_3$-VDR
5 complex [20]. Regulatory roles of vitamin D in cellular proliferation, differentiation,
6 apoptosis, angiogenesis and immunological responses have been reported and
7 deficiency associated with many diseases, including multiple sclerosis [21], Type I
8 diabetes [22], asthma [23] and autoimmune diseases [24]. The involvement of vitamin
9 D in immunoregulation, epithelial cell function and metabolic regulation has been
10 scrutinized but any immune regulatory effects in neonatal rats with NEC remain
11 uninvestigated. The impact of vitamin D_3 on intestinal damage in a rat model of
12 neonatal NEC and on dendritic cell (DC) maturation and activation were investigated in
13 the present study.

14 **2. Materials and methods**

15 **2.1. Animals**

16 Thirty neonatal Sprague-Dawley rats were obtained from Jiangxi University of Chinese
17 Medicine (Nanchang, China) and randomly divided into control, NEC and vitamin
18 D_3-treated groups and fed 0.1 mL animal formula milk every 4h via orogastric tube
19 adapted from a peripherally inserted central catheter. NEC and vitamin D_3-treated
20 animals were subjected to hypoxia (100% nitrogen for 1 min) followed by hypothermia
21 (4°C for 10 min) two times per day beginning immediately after birth until the end of

the experiment [25]. Vitamin D_3-treated rats were given 2000 IU/Kg body weight vitamin D_3 daily. At a dose of around 2000 IU/day, the calcium and phosphorus levels did not change during the follow-up period [26]. Animals were euthanized after 72 hr. Ethical approval was granted by the Ethics Committee of Jiangxi Provincial People's Hospital (Approval NO. KT051).

2.2. Reagents and antibodies

Animal formula milk was purchased from PetAg® ESBILAC (Hampshire, IL). Vitamin D drops were purchased from Sinopharm Xingsha Pharmaceuticals (Xiamen, China). H&E staining regent (G1001), TUNEL cell apoptosis detection Kit (G1501), DAB (G1212), phosphate-buffered saline (PBS, G4202) and RPMI-1640 medium (G4531) were purchased from Servicebio (Wuhan, China). TRleasy™ Total RNA Extraction Reagent (10606ES60), Hifair® III 1st strand cDNA Synthesis SuperMix (11139ES60) and Hieff® qPCR SYBR Green Master Mix (11201ES08) were purchased from Yeasen (Shanghai, China). Vitamin D3 (V8070), ovalbumin (OVA)-FITC (SF069) and DAPI (C0065) were purchased from SolarbioLife Sciences (BeiJing, China). Mouse dendritic cell DC2.4 was purchased from Fenghui Biotechnology (ChangSha, China). Fetal bovine serum (FSP500) was purchased from ExCell Bio (ShangHai, China). LPS (*Escherichia coli* O55:B5) was purchased from Sigma-Aldrich (MO, U.S.A.). Brefeldin A (M2294) was purchased from Abmole Bioscience (Houston, U.S.A.). CD80 antibody, CD86 antibody, Tumor necrosis factor-α (TNF-α) antibody, Interleukin-1β (IL-1β) antibody and inducible Nitric oxide synthase (iNOS) antibody and IDO-1 antibody were

1 purchased from Proteintech® (WuHan, China). Horseradish peroxidase
2 (HRP)-conjugated anti-IgG antibody was purchased from MXB Biotechnologies
3 (Fuzhou, China). Cy3-conjugated IgG secondary antibodies were purchased from
4 ABclonal® (Wuhan, China).

5 **2.3. Cell culture**

6 Mouse dendritic DC2.4 cells were grown in RPMI-1640 containing 10% fetal bovine
7 serum at 37°C in a 5% CO_2 incubator (Thermo, NY, USA). Vitamin D_3 was dissolved
8 in physiological saline and LPS in RPMI-1640 as a 100 mM stock solution and stored at
9 -80°C.

10 **2.4. Hematoxylin and eosin (H&E) staining**

11 Intestinal specimens were dissected and fixed with 10% formalin solution for 24 h,
12 embedded in paraffin and sliced into 3–5 μm sections for H&E staining. Stained
13 sections were examined by a blinded observer under an optical microscope and
14 morphological changes were graded as normal, mild or moderate, according to Barlow's
15 protocol [27].

16 **2.5. TUNEL assay**

17 A fluorescein (FITC) TUNEL cell apoptosis detection kit was used to measure rates of
18 apoptosis. Briefly, paraffin-embedded samples were dewaxed, and incubated with
19 Proteinase K at 37℃ for 30 min., An equilibration buffer was added to cover the sample
20 and slides were incubated at room temperature for 10 minutes. TdT incubation buffer
21 (Recombinant TdT enzyme: FITC-12-dUTP Labeling Mix: Equilibration Buffer=1: 5 :

1 50) was added at 37°C for 1 hour in the dark. Slides were washed with PBS and stained
2 with anti-fluorescence quenching sealing liquid containing DAPI before images were
3 acquired by an inverted fluorescence microscope (Leica Biosystems, Wetzlar,
4 Germany).

5 **2.6. Immunohistochemistry**

6 Paraffin-embedded samples (3-5 μm) were dewaxed and rehydrated before the addition
7 of ethylene diamine tetra-acetic acid (EDTA) antigen retrieval solution and microwave
8 antigen retrieval of aldehyde-fixed paraffin-embedded sections. H_2O_2 and 4% bovine
9 serum albumin were used to block the endogenous peroxidase and nonspecific antigens
10 and sections were incubated with rabbit anti-*CD80* and mouse anti-*CD86* antibodies
11 (1:500 dilution) at 4°C overnight, followed by washing and incubation with horseradish
12 peroxidase (HRP)-conjugated anti-IgG (1:500 dilution) at room temperature for 30
13 minutes. Sections were stained with diaminobenzidine (DAB) and counterstained with
14 hematoxylin solution for dehydration and mounting.

15 **2.7. Immunofluorescence**

16 DC2.4 cells were seeded into a 12-well plate at a density of 1×10^5 cells/mL and
17 pretreated with 10 nM vitamin D_3 for 12 hrs followed by treatment with 10 μg/mL LPS
18 and Brefeldin A for 24 hrs. Cells were washed with 1×PBS buffer and fixed with 4%
19 paraformaldehyde for 15 min at room temperature. The cells were permeabilized with
20 0.5% Triton X-100 for 20 min and then blocked with 5% BSA for 30 min at room
21 temperature. Antibodies raised against *CD80*, *CD86*, indolamine 2,3-dioxygenase 1

(*IDO-1*), tumor necrosis factor-α (*TNF-α*), interleukin-1β (*IL-1β*) and inducible nitric oxide synthase (iNOS) were added to cells for incubation at 4°C overnight, after which cells were washed and incubated with Cy3-conjugated IgG secondary antibodies for 1 h in the dark. Cells were washed and stained with anti-fluorescence quenching sealing liquid containing DAPI for 5 min for observation under an inverted fluorescence microscope. Mean fluorescence intensity was quantified by Image J software.

2.8. Quantitative real-time PCR assay

DC2.4 cells were seeded into 12-well plates at a density of 1×10^5 cells/mL and pretreated with 10 nM vitamin D_3 for 12 h followed by 10 μg/mL LPS for 24 h. Total RNA was extracted using TRleasy™ Total RNA Extraction Reagent and cDNA was synthesized using Hifair® III 1st strand cDNA Synthesis SuperMix. Real-time PCR was performed using Hieff® qPCR SYBR Green Master Mix and gene-specific primers designed by Primer 3 software (Table 1), on a TL988 Real-Time PCR system (TIANLONG, Xi'an, China). Relative gene expression was calculated using -2^ΔΔCt with β-actin as the internal reference.

2.9. FITC-OVA uptake assay

DC2.4 cells were seeded into 12-well plates at a density of 1×10^5 cells/mL and pretreated with 10 nM vitamin D_3 for 12 h followed by being treated with 10 μg/mL LPS for 24 h and incubated with 2 μg/ml ovalbumin (OVA)-FITC at 37 °C for 30 min. Cells were washed and endocytosis was observed and photographed under an inverted fluorescence microscope.

2.10. Statistical analysis

Data are expressed as means ± standard deviation (SD) and Graphpad Prism 9 software was used for statistical analysis. For normally distributed continuous variables, we summarized the data as mean and standard deviation, and comparisons between groups were performed using Student's t-test and ANOVA. For continuous data exhibiting skewed distribution, differences in the data were tested using the Mann–Whitney U test or Kruskal–Wallis test. Categorical data were summarized as counts with relative frequencies as percentages. Differences in the groups were analyzed using the χ^2 test or Fisher's exact test (when count less than 5). A value of $p<0.05$ was considered statistically significant.

3. Results

3.1. Effect of vitamin D_3 on intestinal damage in a neonatal rat model of NEC

Oral vitamin D_3 was given to rats with NEC and intestinal specimens were taken for H&E staining and graded according to the method of Barlow B Clear, intact intestinal structure with complete, continuous epithelium, a neat arrangement of glands and lamina propria and submucosa that were free of hyperemia and edema were seen in control specimens and given a pathological score of 0. By contrast, severe intestinal necrosis with villus degeneration and edema, partial villus necrosis, disordered glands, edema of the lamina propria and submucosa and a thinning and broken myometrium were seen in the rats with NEC and given pathological scores of 3-4. However, neonatal NEC rats that had been treated with vitamin D_3 had only slight edema of the lamina

1 propria and submucosa, irregular villi and glands with slight intestinal lesions and
2 received pathological scores of 0-1 (Table 2, Figure 1A). Thus, pathological scores of
3 the NEC group were higher than those of controls or vitamin D3-treated animals. The
4 vitamin D3-treated group and NEC group received higher pathological scores than
5 controls ($p < 0.0001$; Figure 1B). TUNEL measurements showed that the apoptosis of
6 tissues in the NEC group was higher than that in the control group and samples treated
7 with vitamin D3. (Figure 1C).

8 **3.2. Effect of vitamin D_3 on expression of immune cell co-stimulatory molecules**

9 Levels of *CD80* (B7-1) and *CD86* (B7-2) in DCs were analyzed by
10 immunohistochemical techniques. *CD80* was expressed at basal levels in control and
11 NEC model intestinal tissue but was increased in tissue from vitamin D_3-treated animals.
12 The reverse was true for *CD86* which was highly expressed in controls and NEC but
13 low in vitamin D_3-treated rats (Figure 2A). To provide further evidence to confirm the
14 effect of vitamin D_3 on the function of dendritic cells, mouse dendritic cells DC2.4 were
15 pre-treated-with-vitamin D_3 before exposure to LPS and *CD80* found to be
16 down-regulated by LPS treatment and restored by vitamin D_3 ($p < 0.01$; Figure 2B). The
17 expression of *CD86* was increased after LPS treatment and alleviated by vitamin D_3 (p
18 < 0.001; Figure 2C).

19 **3.3. mRNA expression**

20 Expression of mRNA corresponding to TNF-α, IL-1β, IDO-1, CD80 and CD86 were
21 measured by qPCR. CD80 expression decreased after LPS treatment and recovered with

vitamin D_3 and the opposite pattern was seen for CD86 ($p < 0.05$; Figure 3A-3B). Expression of the inflammatory factors, TNF-α and IL-1β, were increased in DC2.4 after LPS treatment ($p<0.001$) and this effect was reversed by vitamin D_3 treatment ($p<0.01$). LPS also induced expression of IDO-1, an indicator of the tolerogenic DC phenotype, and this factor was further up-regulated by vitamin D_3 ($p < 0.05$; Figure 3C-3E).

3.4. Effect of Vitamin D3 on LPS-induced maturation and activation of DC2.4 cells

Immunofluorescent measurements showed that expression of TNF-α, IL-1β and iNOS in DC2.4 cells increased after LPS treatment ($p < 0.0001$, $p<0.05$, $p<0.01$) and levels were reduced with vitamin D_3 ($p<0.01$, $p<0.05$, $p<0.01$) (Figure 4A-4C). Thus, vitamin D_3 appears to inhibit the LPS-induced inflammatory response in DC cells. DC cells induce tolerogenic antigen-presenting cells in addition to controlling infection and promoting inflammation. *IDO-1* mediates maternal tolerance toward fetal alloantigens and influences regulatory T cell (Tregs) differentiation. *IDO-1* expression increased with LPS ($p<0.01$), an effect that was especially pronounced in the vitamin D_3-treated group (Figure 4D). FITC-OVA uptake assays showed a reduced capacity of soluble antigen uptake by DC2.4 cells after LPS treatment, a reduction which showed partial recovery with vitamin D3. Thus, vitamin D_3 appears to preserve the immature state of DCs (Figure 4E).

4. Discussion

Excessive inflammatory response to luminal microbial stimuli has been observed in NEC animal models and clinical cases and may aggravate intestinal barrier damage [28]. Increased TLR4 expression has been reported in NEC animal models and resected intestines from infants and it is acknowledged that non-digestible oligosaccharides present in breast milk may prevent NEC by inhibition of TLR4 through the Wnt/Notch pathway, contributing to the epithelial maturation [29, 30]. The release of inflammatory mediators by damaged intestinal epithelium leads to infiltration of leukocytes, including neutrophils, macrophages and DCs. The interaction of pathogen-associated molecular patterns with surface receptors of DCs embedded in the epithelium causes DC maturation and DC-mediated differentiation of naive T cells [31]. Probiotics are considered to promote the generation of tolerogenic DCs which influence Treg proliferation and differentiation and promote immune homeostasis [32, 33]. Our study found that the effect of vitamin D3 on NEC rats is to alleviate intestinal damage, manifested by improving intestinal integrity and reducing the number of apoptotic cells. Vitamin D_3 also altered intestinal expression of CD80/CD86, in agreement with treatment of the DC2.4 cell line with LPS and vitamin D_3. CD80/CD86 are B7 family ligands, largely restricted to DCs and B cells and share two receptors, *CD28* and CTLA-4, which show competitive ligand binding and regulate T cell activation and toleranc [34]. CD80 binds both receptors with a higher affinity than *CD86* but CTLA-4, expressed on Treg cells, depletes CD80/CD86 by trans-endocytosis and induces IDO expression in antigen-presenting cells, leading to reduced CD28-mediated T cell

activation [35, 36].

Individual roles of CD80 and CD86 remain poorly characterized. The two proteins showed different expression changes in intestinal tissues and altered DC2.4 cell phenotypes were consistent with those in intestinal tissue. Vitamin D_3 inhibited the expression of LPS-induced inflammatory factors and increased that of IDO-1 in DC2.4 cells. A decrease in CD86 expression is thought to suppress DC maturation, prolonging allograft survival in heart-transplanted mice and relieving RSV-induced asthma [37, 38] IDO is a tryptophan-catabolizing enzyme that is predominantly expressed in innate immune cells, such as macrophages and DCs, where it catabolizes and depletes the tryptophan required for T-cell proliferation [39]. IDO gene transcription is regulated by inflammatory mediators and interferon (IFN)-sensitive elements are present in the promoter regions of both mouse and human IDO genes [40]. LPS and inflammatory cytokines enhance IDO expression synergistically with IFN-γ *in vitro* and soluble CTLA4-immunoglobulin binds CD80/CD86 to induce IDO expression [41, 42]. IDO is considered to regulate DC maturation and antigen-presenting cell-mediated T-cell suppression. IDO inhibited CD86 expression and upregulated DC PD-L1 and IDO-deficient DCs promoted the differentiation of T cells towards a pro-inflammatory phenotype during previous work [43]. LPS increased IDO-1 expression and decreased endocytosis in the DC2.4 cells of the current study and vitamin D_3 further upregulated IDO-1 and rescued the endocytic capacity. These in vitro results indicate that vitamin D_3 regulated IDO-1 and CD80/CD86 expression to influence DC maturation.

1 There are some limitations in the current study. The mechanism by which vitamin D_3
2 regulates CD80/CD86 and IDO-1 expression remains unclear and it has not been
3 established whether the observed attenuation of intestinal damage in NEC rats was
4 dependent on altered *CD80*/*CD86* and *IDO-1* expression. Further study of vitamin D_3
5 function in intestinal epithelial cell lines and animal models in vivo is required.
6 In conclusion, vitamin D_3 has been demonstrated to influence DC maturation and
7 activation, probably through an effect on CD80/CD86 and IDO-1 expression. Vitamin
8 D_3 decreased CD86 expression and increased that of CD80 and IDO-1, maintaining
9 immature and inactive DC status. Further study of the mechanisms underlying vitamin
10 D_3 regulation of immune cell maturation, differentiation and responses is suggested to
11 have significance for disease prevention and adjuvant treatment.
12 **Data availability**
13 The relevant author may provide you with the information used to support this study
14 upon request.
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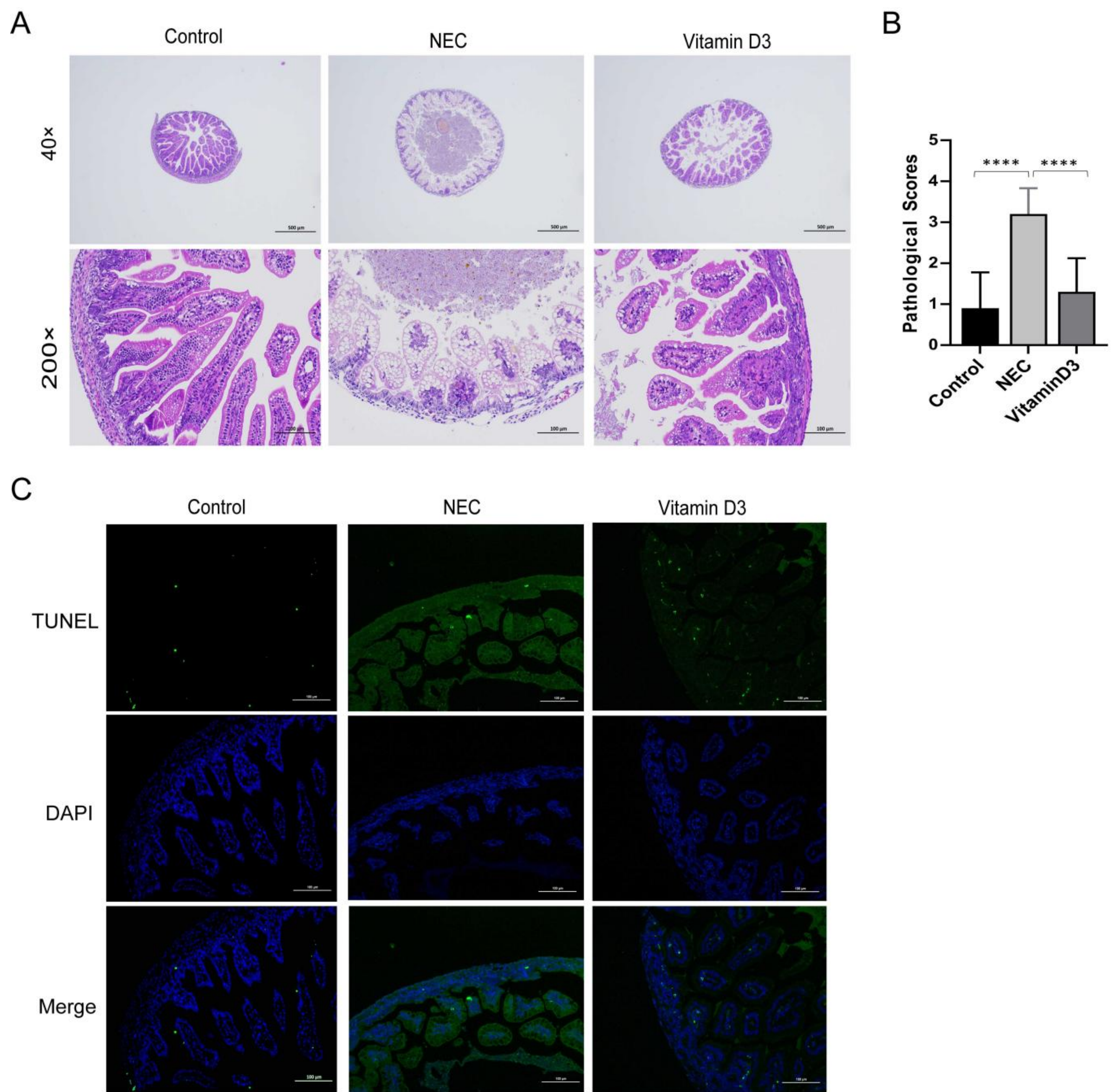

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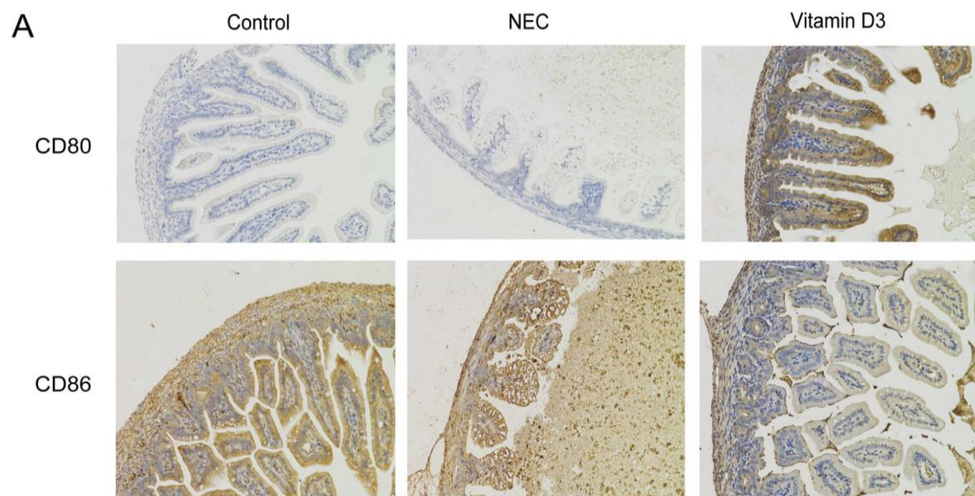
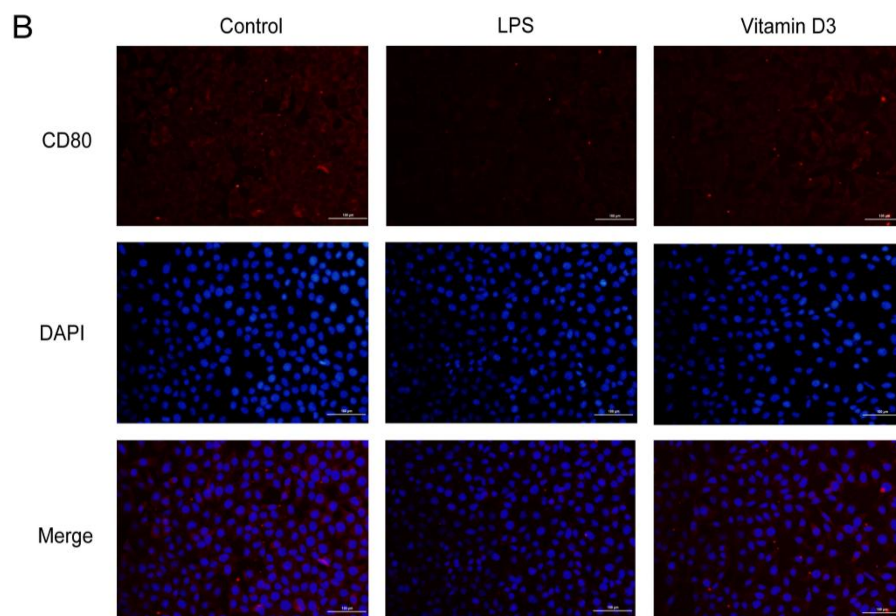
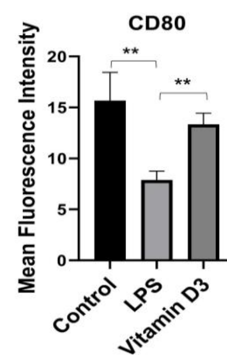
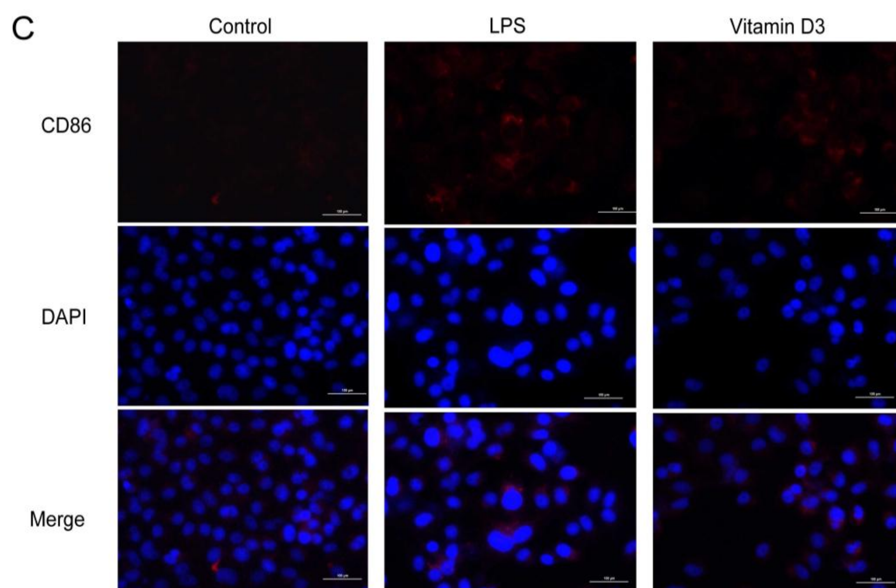
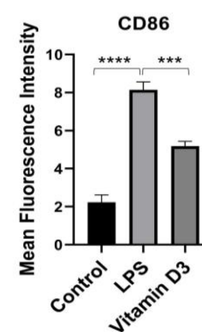
2 **Figure 1. Effect of vitamin D on intestinal damage in a neonatal rat model of NEC.**











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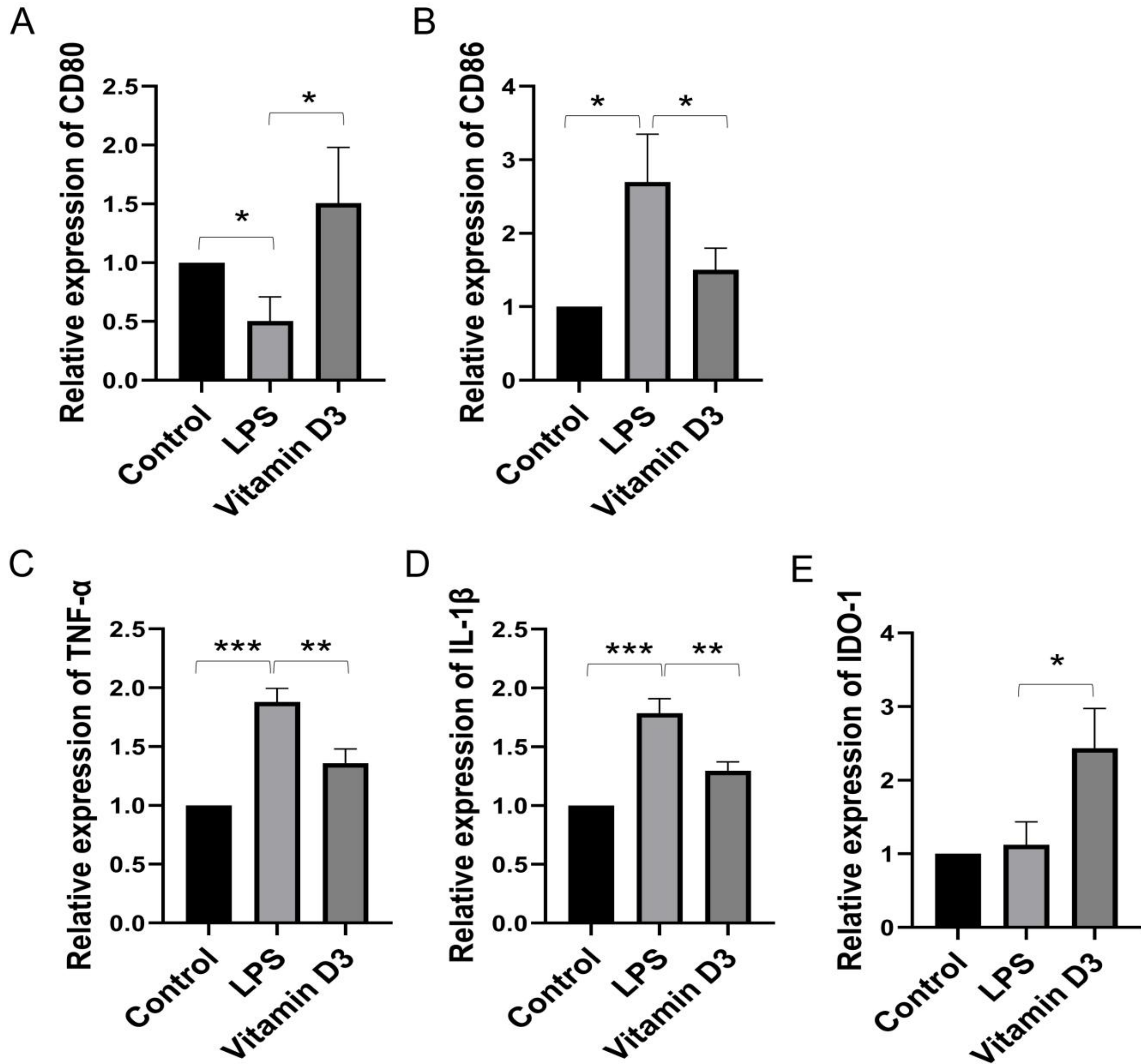
1 **Figure 2. Effect of vitamin D_3 on expression of co-stimulatory molecules in immune cells.**



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3 **Figure 3. Effect of vitamin D_3 on the expression of inflammatory factor, costimulatory**

4 **molecule and immune regulatory genes.**

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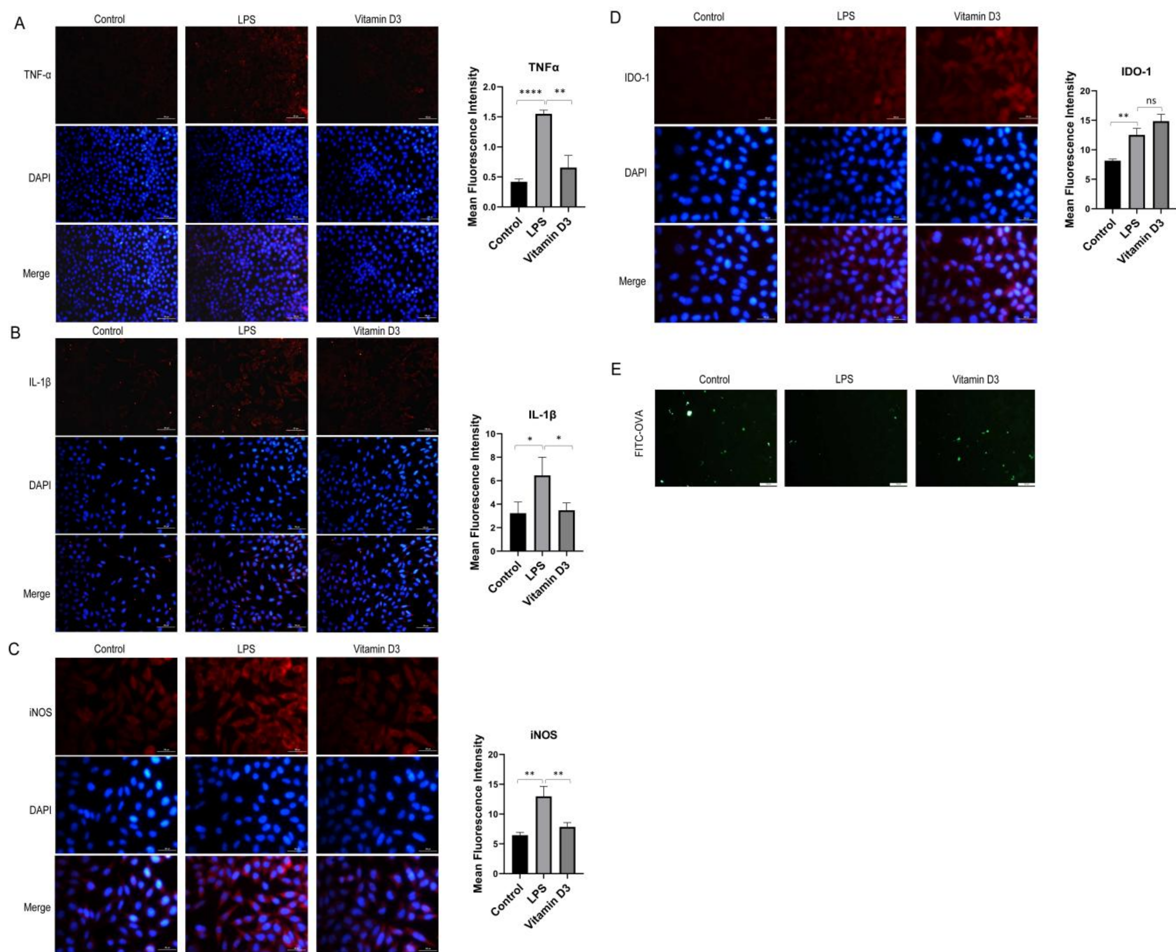

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2 **Figure 4. Effect of vitamin D₃ on the maturation of LPS-treated DC2.4 cells and**

3 **expression of cytokines**

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1 **Table 1. Primers for qRT-PCR**

Primer	Sequence
Actin forward	GGTACCACCATGTACCCAGG
Actin reverse	AGGGTGTAAAACGCAGCTC
CD80 forward	CGTCGTCATCGTTGTCATCATC
CD80 reverse	AAGGAAGACGGTCTGTTCAGC
CD86 forward	TCCTCCTTGTGATGCTGCTC
CD86 reverse	CTGCATTTGGTTTTGCTGAAGC
TNF-α forward	GACACCATGAGCACAGAAAGC
TNF-α reverse	TTGGTGGTTTGCTACGACGT
IL-1β forward	GCACTACAGGCTCCGAGATGA
IL-1β reverse	TTGTTGGTTGATATTCTGTCCATTG
IDO-1 forward	CATCACCATGGCGTATGTGTG
IDO-1 forward	CCATTGGGGTCCTTTTTCTTCC

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1 **Table 2. Intestinal damage and pathological score of the control group, NEC group,**

2 **and vitamin D group**

Group	Intestinal damage	Pathological score
Controls group	intact intestinal structure with complete, continuous epithelium, a neat arrangement of glands and lamina propria and submucosa that were free of hyperemia and edema	0
NEC group	severe intestinal necrosis with villus degeneration and edema, partial villus necrosis, disordered glands, edema of the lamina propria and submucosa and a thinning and broken myometrium	3-4
Vitamin D3 group	only slight edema of the lamina propria and submucosa, irregular villi and glands with slight intestinal lesions	0-1

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