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

BO KE

CHAOYU LI

SONG LI

JINGJING YAN

LEKE SUN

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

Bo KE[1,2] , **Chaoyu LI[3,4]** , **Song LI[3,4]** , **Jingjing YAN[5]** , **Leke SUN[3,4*]**

[1]Department of Hematology, Jiangxi Provincial People's Hospital, The First Affiliated Hospital of Nanchang Medical College, Nanchang, China
[2]The Jiangxi Province Key Laboratory of Hematologic Diseases, Nanchang, China
[3]Department of Pediatrics, Jiangxi Provincial People's Hospital, The First Affiliated Hospital of Nanchang Medical College, Nanchang, China
[4]Jiangxi Medical College Nanchang University, China
[5]Department of Pediatrics, Nanchang People's Hospital, Nanchang, China

Background/aim: Necrotizing enterocolitis (NEC) is a serious condition that predominantly affects premature infants and involves an aberrant immune response and inflammatory cytokine release resulting 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 antiinflammatory impact on the intestines 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 costimulatory molecules, inflammatory factors, and immunoregulatory factors by immunohistochemical staining, quantitative real-time PCR, and immunofluorescence. The fluorescein isothiocyanate-ovalbumin (FITC-OVA) uptake assay was used to analyze DC endocytosis.

Results: Vitamin D₃ administration attenuated intestinal damage and apoptosis, inhibiting CD86 and increasing CD80 expression. Lipopolysaccharide (LPS)-challenged DC2.4 cells in vitro showed upregulated 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 endocytic capacity was improved. Vitamin D₃ ameliorated intestinal damage in neonatal rats with NEC and exerted antiinflammatory and immunomodulatory effects on DCs.

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

Key words: Necrotizing enterocolitis, vitamin D₃, 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, with an incidence of 9% among infants born at a gestational age of 22–29 weeks [1]. The vast majority (>90%) of NEC cases occur in preterm infants, especially those who are of very low birthweight of <1500 g. The incidence of NEC is inversely proportional to gestational age at birth, and the time to onset of NEC is longer in more premature infants [2]. The mortality rate of NEC among extremely premature infants was reported as 30% [3]. NEC has multifactorial causes and is associated with various prenatal and postnatal factors [4]. The disorder is characterized by abdominal distension, vomiting, bloody stool, and sepsis with intestinal wall and portal vein pneumatosis visible on imaging [5,6].

The etiopathogenesis of NEC remains unclear but an immature intestinal barrier, altered microbiota, intestinal immaturity [7], enteral feeding with formula milk [8], bacterial colonization, hypoxic-ischemic damage, and aberrant toll-like receptor 4 (TLR4)-mediated intestinal inflammatory response are contributing factors [9]. The excessive inflammatory response may lead to intestinal mucosal edema, damage, and intestinal wall necrosis [10]. Treatment involves intestinal rest, abdominal decompression, and broad-spectrum antibiotics, but peritoneal drainage, bowel resection, and enterostomy

---

* Correspondence: 13647910316@163.com

may be necessary in severe cases [11]. The therapeutic value of probiotics [12], stem cell transplantation [13,14], exosomes, and targeted treatment of intestinal ischemia with heparin-binding epidermal growth factor-like growth factor have all been studied [15,16].

Vitamin D_3 is a secosteroid hormone with roles in calcium homeostasis and bone growth that promotes collagen matrix mineralization [17]. It is absorbed from the diet or synthesized from 7-dehydrocholesterol upon exposure of the skin to sunlight. Inactive vitamin D_3 is hydroxylated by hepatic cytochrome P450 (CYP2R1), generating 25-hydroxyvitamin D (25(OH)D), which is converted into 1,25-dihydroxy vitamin D_3 (1,25(OH)$_2$D$_3$) by the hydroxylation of CYP27B1 [18,19]. Active vitamin D_3 binds the vitamin D receptor (VDR), consisting of ligand-binding and DNA-binding domains, and causes transcriptional activation or repression mediated by the 1,25(OH)$_2$D$_3$-VDR complex [20]. The regulatory roles of vitamin D in cellular proliferation, differentiation, apoptosis, angiogenesis, and immunological responses have been reported and deficiency is associated with many diseases, including multiple sclerosis [21], type I diabetes [22], asthma [23], and autoimmune diseases [24]. The involvement of vitamin D in immunoregulation, epithelial cell function, and metabolic regulation has been scrutinized, but immune regulatory effects in neonatal rats with NEC remain uninvestigated. The impacts of vitamin D_3 on intestinal damage in a rat model of neonatal NEC and on dendritic cell (DC) maturation and activation were investigated in the present study.

2. Materials and methods

2.1. Animals
Thirty neonatal Sprague-Dawley rats were obtained from Jiangxi University of Chinese Medicine (Nanchang, China) and randomly divided into control, NEC, and vitamin D_3-treated groups. Animals were fed 0.1 mL of animal formula milk every 4 h via an orogastric tube adapted from a peripherally inserted central catheter. NEC and vitamin D_3-treated animals were subjected to hypoxia (100% nitrogen for 1 min) followed by hypothermia (4 °C for 10 min) twice a day beginning immediately after birth until the end of the experiment [25]. Vitamin D_3-treated rats were given daily vitamin D_3 at 2000 IU/kg body weight. At a dose of about 2000 IU/day, the calcium and phosphorus levels of the animals did not change during the follow-up period [26]. Animals were euthanized after 72 h. Ethical approval was granted by the Ethics Committee of Jiangxi Provincial People's Hospital (Approval No. KT051).

2.2. Reagents and antibodies
Esbilac animal formula milk was purchased from PetAg (Hampshire, IL, USA). Vitamin D drops were purchased from Sinopharm Xingsha Pharmaceuticals (Xiamen,

China). H&E staining regent (G1001), a TUNEL cell apoptosis detection kit (G1501), DAB (G1212), phosphate-buffered saline (PBS; G4202), and RPMI-1640 medium (G4531) were purchased from Servicebio (Wuhan, China). TRIeasy 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 D_3 (V8070), ovalbumin (OVA)-FITC (SF069), and DAPI (C0065) were purchased from Solarbio Life Sciences (Beijing, China). DC2.4 mouse DCs were 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 (St. Louis, MO, USA). Brefeldin A (M2294) was purchased from AbMole Bioscience (Houston, TX, USA). CD80 antibody, CD86 antibody, tumor necrosis factor-α (TNF-α) antibody, interleukin-1β (IL-1β) antibody, inducible nitric oxide synthase (iNOS) antibody, and indoleamine 2,3-dioxygenase 1 (IDO-1) antibody were purchased from Proteintech (Wuhan, China). Horseradish peroxidase (HRP)-conjugated anti-IgG antibody was purchased from MXB Biotechnologies (Fuzhou, China). Cy3-conjugated IgG secondary antibodies were purchased from ABclonal (Wuhan, China).

2.3. Cell culture
Mouse dendritic DC2.4 cells were grown in RPMI-1640 containing 10% fetal bovine serum at 37 °C in a 5% CO$_2$ incubator (Thermo, Rochester, NY, USA). Vitamin D_3 was dissolved in physiological saline and LPS in RPMI-1640 as a 100 mM stock solution and stored at –80 °C.

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

2.5. TUNEL assay
The FITC TUNEL cell apoptosis detection kit was used to measure rates of apoptosis. Briefly, paraffin-embedded samples were dewaxed and incubated with proteinase K at 37 °C for 30 min. An equilibration buffer was added to cover the sample and slides were incubated at room temperature for 10 min. TdT incubation buffer (recombinant TdT enzyme FITC-12-dUTP, labeling mix, and equilibration buffer = 1:5:50) was added at 37 °C for 1 h in the dark. Slides were washed with PBS and stained with antifluorescence-quenching sealing liquid containing DAPI before images were acquired with an inverted fluorescence microscope (Leica Biosystems, Wetzlar, Germany).

2.6. Immunohistochemistry

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

2.7. Immunofluorescence

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 treatment with 10 μg/mL LPS and Brefeldin A for 24 h. Cells were washed with 1X PBS buffer and fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were permeabilized with 0.5% Triton X-100 for 20 min and then blocked with 5% BSA for 30 min at room temperature. Antibodies raised against CD80, CD86, IDO-1, TNF-α, IL-1β, and 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 antifluorescence-quenching sealing liquid containing DAPI for 5 min for observation under an inverted fluorescence microscope. Mean fluorescence intensity was quantified using ImageJ 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 the TRIeasy 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 the $-2^{\Delta\Delta Ct}$ method 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 treatment 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 were expressed as mean ± 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 SD values, 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 chi-square test or Fisher's exact test (when the count was less than 5). Values of $p < 0.05$ were 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

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	CCATTGGGGGTCCTTTTTCTTCC

according to Barlow's protocol. Samples with an intact intestinal structure with a complete and 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. In contrast, severe intestinal necrosis with villus degeneration and edema, partial villus necrosis, disordered glands, edema of the lamina propria and submucosa, and thin and broken myometrium were seen in samples from the rats with NEC, which were given pathological scores of 3 or 4. However, neonatal NEC rats that had been treated with vitamin D_3 had only slight edema of the lamina propria and submucosa, with irregular villi and glands with slight intestinal lesions, and these samples received pathological scores of 0 or 1 (Table 2; Figure 1A). Thus, the pathological scores of the NEC group were higher than those of the controls and the vitamin D_3-treated animals. The vitamin D_3-treated group and the NEC group both received higher pathological scores than the control group ($p < 0.0001$; Figure 1B). TUNEL measurements showed that apoptosis of tissues in the NEC group was more significant than that in the control group and the samples from rats treated with vitamin D_3 (Figure 1C).

3.2. Effect of vitamin D_3 on the expression of immune cell costimulatory molecules

Levels of CD80 (B7-1) and CD86 (B7-2) in DCs were analyzed by immunohistochemical techniques. CD80 was expressed at basal levels in control and NEC model intestinal tissues but was increased in the tissues obtained from vitamin D_3-treated animals. The reverse was true for CD86, which was highly expressed in the control and NEC groups but low in vitamin D_3-treated rats (Figure 2A). To provide further evidence of the effect of vitamin D_3 on the function of DCs, mouse dendritic DC2.4 cells were pretreated with vitamin D_3 before exposure to LPS, and CD80 was found to be downregulated by LPS treatment and restored by vitamin D_3 ($p < 0.01$; Figure 2B). The expression of CD86 was increased after LPS treatment and alleviated by vitamin D_3 ($p < 0.001$; Figure 2C).

3.3. mRNA expression

Expressions of mRNA corresponding to TNF-α, IL-1β, IDO-1, CD80, and CD86 were measured by qPCR. CD80 expression decreased after LPS treatment and recovered with vitamin D_3, while the opposite pattern was seen for CD86 ($p < 0.05$; Figures 3A and 3B). Expression levels of inflammatory factors TNF-α and IL-1β were increased in DC2.4 cells after LPS treatment ($p < 0.001$) and this effect was reversed by vitamin D_3 treatment ($p < 0.01$). LPS also induced the expression of IDO-1, an indicator of the tolerogenic DC phenotype, and this factor was further upregulated by vitamin D_3 ($p < 0.05$; Figures 3C–3E).

### 3.4. Effect of vitamin D_3 on LPS-induced maturation and activation of DC2.4 cells

Immunofluorescent measurements showed that the expression levels of TNF-α, IL-1β, and iNOS in DC2.4 cells increased after LPS treatment ($p < 0.0001$, $p < 0.05$, and $p < 0.01$) and these levels were reduced with vitamin D_3 ($p < 0.01$, $p < 0.05$, and $p < 0.01$) (Figures 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 of fetal alloantigens and influences the differentiation of regulatory T cell (Tregs). 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 that showed partial recovery with vitamin D_3. 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 nondigestible oligosaccharides

Table 2. Intestinal damage and pathological scores of the control groups, NEC groups, and vitamin D groups.

| Groups	Intestinal damage	Pathological score
Control groups	Intact intestinal structure with complete and continuous epithelium, neat arrangement of glands, and lamina propria and submucosa free of hyperemia and edema	0
NEC groups	Severe intestinal necrosis with villus degeneration and edema, partial villus necrosis, disordered glands, edema of the lamina propria and submucosa, and thin and broken myometrium	3 or 4
Vitamin D_3 groups	Only slight edema of the lamina propria and submucosa, irregular villi and glands with slight intestinal lesions	0 or 1
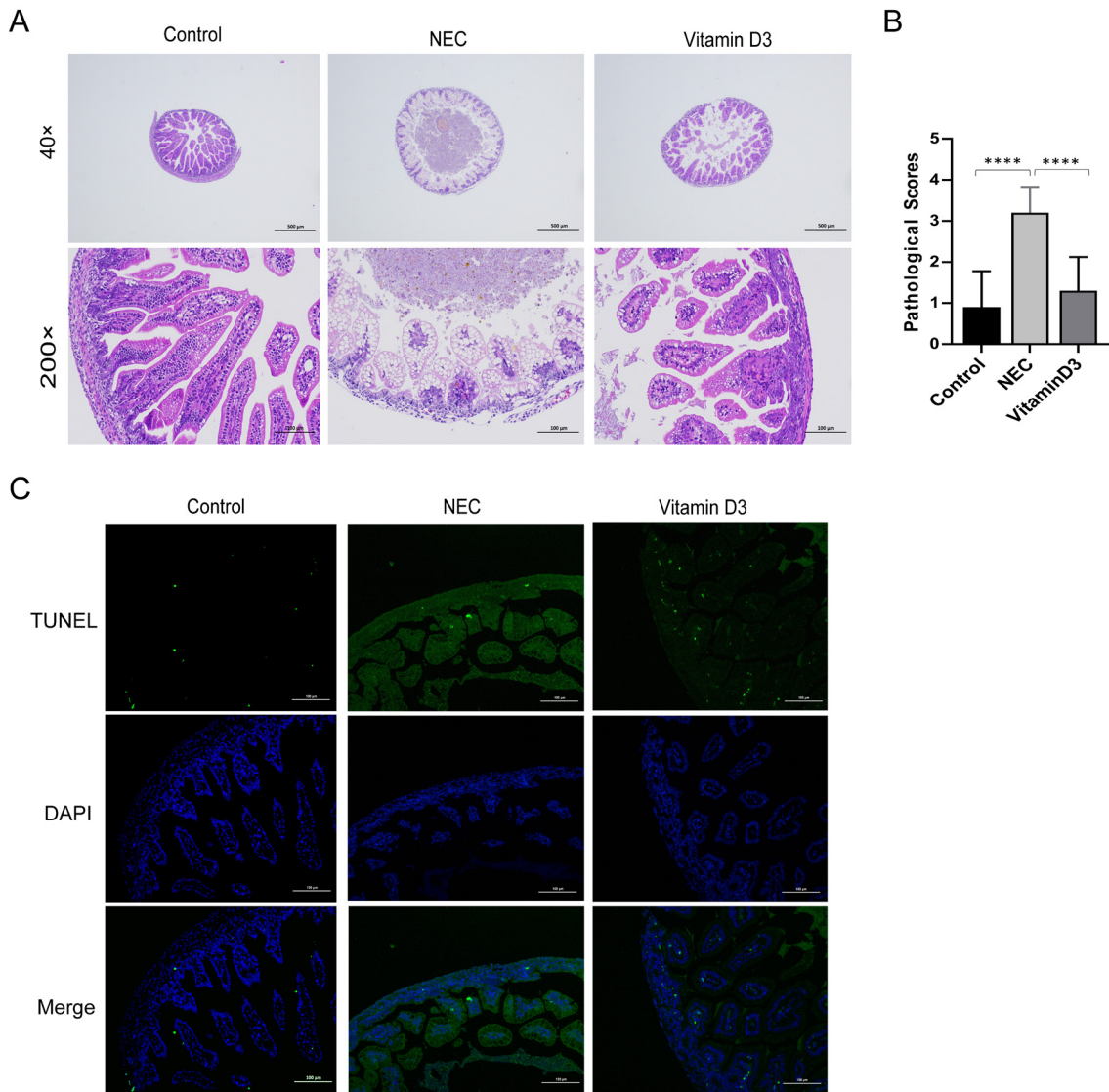
Figure 1. Effect of vitamin D_3 on intestinal damage in a neonatal rat model of NEC.

present in breast milk may prevent NEC by inhibition of TLR4 through the Wnt/Notch pathway, contributing to epithelial maturation [29,30]. The release of inflammatory mediators by the damaged intestinal epithelium leads to the 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 D_3 on rats with NEC is exerted through alleviation of intestinal damage, manifested by improved intestinal integrity and

reduced numbers of apoptotic cells. Vitamin D_3 also altered the intestinal expression of CD80/CD86, in line with treatment of the DC2.4 cells with LPS and vitamin D_3. CD80/CD86 are B7 family ligands, largely restricted to DCs and B cells, and they share two receptors, CD28 and CTLA-4, which show competitive ligand binding and regulate T-cell activation and tolerance [34]. CD80 binds both receptors with a higher affinity than CD86, but CTLA-4, expressed on Treg cells, depletes CD80/CD86 by transendocytosis and induces IDO expression in antigen-presenting cells, leading to reduced CD28-mediated T cell activation [35,36].

The individual roles of CD80 and CD86 remain poorly characterized. The two proteins showed different expression changes in intestinal tissues, and altered DC2.4
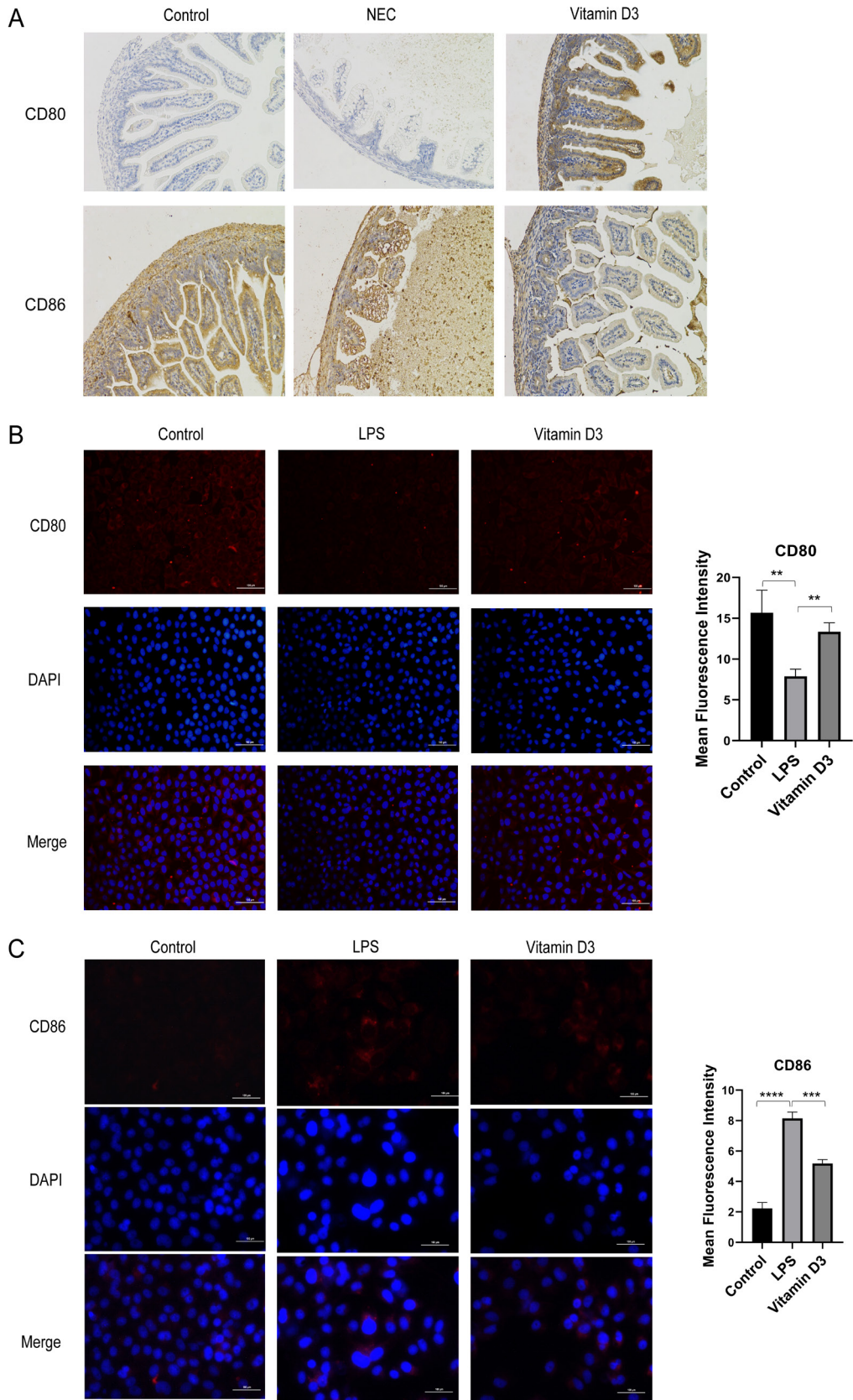
Figure 2. Effect of vitamin D$_3$ on expression of costimulatory molecules in immune cells.
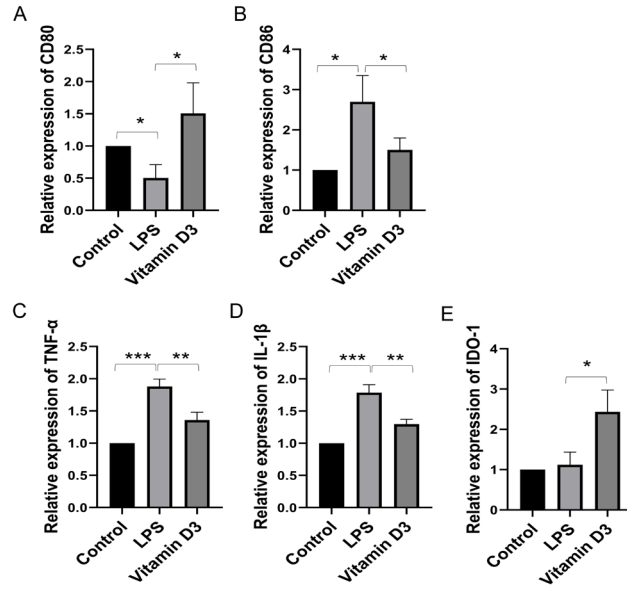
Figure 3. Effect of vitamin D$_3$ on the expression of inflammatory factors, costimulatory molecules, and immunoregulatory genes.
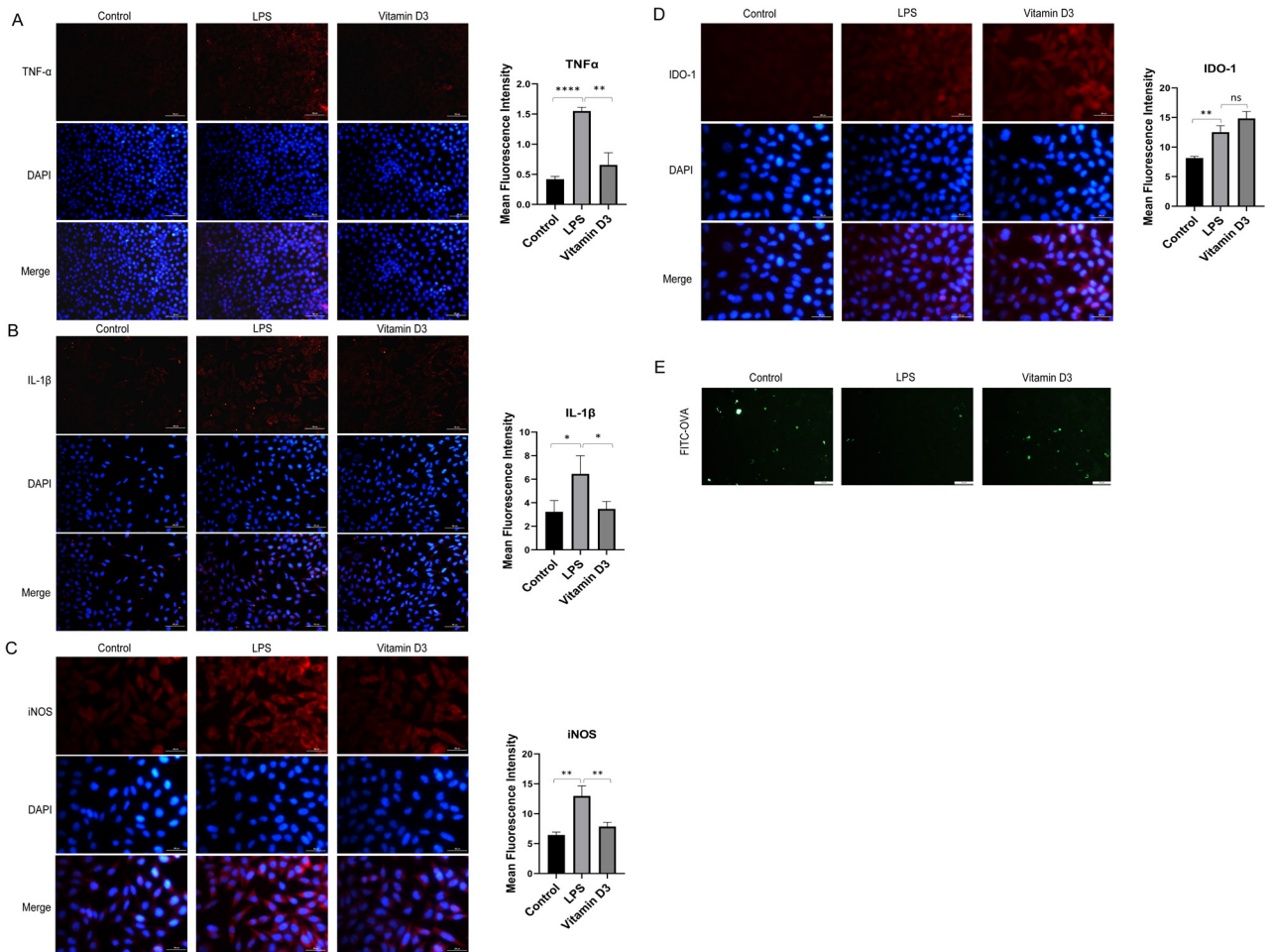
Figure 4. Effect of vitamin D$_3$ on the maturation of LPS-treated DC2.4 cells and expression of cytokines.

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 proinflammatory phenotype in a previous study [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 restored the endocytic capacity. These in vitro results indicate that vitamin D_3 regulated IDO-1 and CD80/CD86 expression to influence DC maturation.

There are some limitations to the current study. The mechanism by which vitamin D_3 regulates CD80/CD86 and IDO-1 expression remains unclear and it has not been established whether the observed attenuation of intestinal damage in NEC rats was dependent on altered CD80/CD86 and IDO-1 expression. Further study of vitamin D_3 function in intestinal epithelial cell lines and animal models in vivo is required.

In conclusion, vitamin D_3 has been demonstrated to influence DC maturation and activation, probably through an effect on CD80/CD86 and IDO-1 expression. Vitamin D_3 decreased CD86 expression and increased that of CD80 and IDO-1, maintaining immature and inactive DC status. Further study of the mechanisms underlying vitamin D_3 regulation of immune cell maturation, differentiation, and responses may have significance for disease prevention and adjuvant treatment.

Data availability

The corresponding author can provide the data used to support this study upon request.

Conflict of interest

There are no conflicts of interest to disclose 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.

References

1. Duchon J, Barbian ME, Denning PW. Necrotizing enterocolitis. Clinics in Perinatology 2021; 48 (2): 229-250. https://doi.org/10.1016/j.clp.2021.03.002

2. Dominguez KM, Moss RL. Necrotizing enterocolitis. Clinics in Perinatology 2012; 39 (2): 387-401. https://doi.org/10.1016/j.clp.2012.04.011

3. Wen SW, Smith G, Yang Q, Walker M. Epidemiology of preterm birth and neonatal outcome. Seminars in Fetal and Neonatal Medicine 2004; 9 (6): 429-435. https://doi.org/10.1016/j.siny.2004.04.002

4. Kaplina A, Kononova S, Zaikova E, Pervunina T, Petrova N et al. Necrotizing enterocolitis: the role of hypoxia, gut microbiome, and microbial metabolites. International Journal of Molecular Sciences 2023; 24 (3): 2471. https://doi.org/10.3390/ijms24032471

5. Esposito F, Mamone R, Di Serafino M, Mercogliano C, Vitale V et al. Diagnostic imaging features of necrotizing enterocolitis: a narrative review. Quantitative Imaging in Medicine and Surgery 2017; 7 (3): 336-344. https://doi.org/10.21037/qims.2017.03.01

6. Hu SF, Liu HB, Hao YY. Portal vein gas combined with pneumatosis intestinalis and emphysematous cystitis: a case report and literature review. World Journal of Clinical Cases 2022; 10 (25): 8945-8953. https://doi.org/10.12998/wjcc.v10.i25.8945

7. Stone JR, Wilkins LR. Acute mesenteric ischemia. Techniques in Vascular and Interventional Radiology 2015; 18 (1): 24-30. https://doi.org/10.1053/j.tvir.2014.12.004

8. Kastenberg ZJ, Sylvester KG. The surgical management of necrotizing enterocolitis. Clinics in Perinatology 2013; 40 (1): 135-148. https://doi.org/10.1016/j.clp.2012.12.011

9. Hackam DJ, Sodhi CP. Toll-like receptor-mediated intestinal inflammatory imbalance in the pathogenesis of necrotizing enterocolitis. Cellular and Molecular Gastroenterology and Hepatology 2018; 6 (2): 229-238. https://doi.org/10.1016/j.jcmgh.2018.04.001

10. Cai J, Sun L, Gonzalez FJ. Gut microbiota-derived bile acids in intestinal immunity, inflammation, and tumorigenesis. Cell Host & Microbe 2022; 30 (3): 289-300. https://doi.org/10.1016/j.chom.2022.02.004

11. Ergenekon E, Tayman C, Özkan H. Turkish Neonatal Society necrotizing enterocolitis diagnosis, treatment and prevention guidelines. Turkish Journal of Pediatrics 2021; 56 (5): 513-524. https://doi.org/10.5152/TurkArchPediatr.2021.21164

12. Tegegne BA, Kebede B. Probiotics, their prophylactic and therapeutic applications in human health development: a review of the literature. Heliyon 2022; 8 (6): e09725. https://doi.org/10.1016/j.heliyon.2022.e09725

13. Zeng R, Wang J, Zhuo Z, Luo Y, Sha W et al. Stem cells and exosomes: promising candidates for necrotizing enterocolitis therapy. Stem Cell Research & Therapy 2021; 12 (1): 323. https://doi.org/10.1186/s13287-021-02389-4

14. Andermann TM, Rezvani A, Bhatt AS. Microbiota manipulation with prebiotics and probiotics in patients undergoing stem cell transplantation. Current Hematologic Malignancy Reports 2016; 11 (1): 19-28. https://doi.org/10.1007/s11899-016-0302-9

15. McCulloh CJ, Olson JK, Wang Y, Zhou Y, Tengberg NH et al. Treatment of experimental necrotizing enterocolitis with stem cell-derived exosomes. Journal of Pediatric Surgery 2018; 53 (6): 1215-1220. https://doi.org/10.1016/j.jpedsurg.2018.02.086

16. Dao DT, Anez-Bustillos L, Adam RM, Puder M, Bielenberg DR. Heparin-binding epidermal growth factor-like growth factor as a critical mediator of tissue repair and regeneration. American Journal of Pathology 2018; 188 (11): 2446-2456. https://doi.org/10.1016/j.ajpath.2018.07.016

17. Ding F, Nie X, Li X, He Y, Li G. Data mining: biological and temporal factors associated with blood parathyroid hormone, vitamin D, and calcium concentrations in the Southwestern Chinese population. Clinical Biochemistry 2021; 90: 50-57. https://doi.org/10.1016/j.clinbiochem.2021.01.014

19. Meyer MB, Pike JW. Mechanistic homeostasis of vitamin D metabolism in the kidney through reciprocal modulation of *Cyp27b1* and *Cyp24a1* expression. Journal of Steroid Biochemistry and Molecular Biology 2020; 196: 105500. https://doi.org/10.1016/j.jsbmb.2019.105500

20. Mutchie TR, Yu OB, Di Milo ES, Arnold LA. Alternative binding sites at the vitamin D receptor and their ligands. Molecular and Cellular Endocrinology 2019; 485: 1-8. https://doi.org/10.1016/j.mce.2019.01.011

21. McLaughlin L, Clarke L, Khalilidehkordi E, Butzkueven H, Taylor B et al. Vitamin D for the treatment of multiple sclerosis: a meta-analysis. Journal of Neurology 2018; 265 (12): 2893-2905. https://doi.org/10.1007/s00415-018-9074-6

22. Daskalopoulou M, Pylli M, Giannakou K. Vitamin D deficiency as a possible cause of type 1 diabetes in children and adolescents up to 15 years old: a systematic review. Review of Diabetic Studies 2022; 18 (2): 58-67. https://doi.org/10.1900/RDS.2022.18.58

23. Frieri M, Valluri A. Vitamin D deficiency as a risk factor for allergic disorders and immune mechanisms. Allergy and Asthma Proceedings 2011; 32 (6): 438-444. https://doi.org/10.2500/aap.2011.32.3485

24. Murdaca G, Tonacci A, Negrini S, Greco M, Borro M et al. Emerging role of vitamin D in autoimmune diseases: an update on evidence and therapeutic implications. Autoimmunity Reviews 2019; 18 (9): 102350. https://doi.org/10.1016/j.autrev.2019.102350

25. Ares GJ, McElroy SJ, Hunter CJ. The science and necessity of using animal models in the study of necrotizing enterocolitis. Seminars in Pediatric Surgery 2018; 27 (1): 29-33. https://doi.org/10.1053/j.sempedsurg.2017.11.006

26. Kojecky V, Matous J, Kianicka B, Dite P, Zadorova Z et al. Vitamin D levels in IBD: a randomised trial of weight-based versus fixed dose vitamin D supplementation. Scandinavian Journal of Gastroenterology 2020; 55 (6): 671-676. https://doi.org/10.1080/00365521.2020.1774921

27. Likhithaswamy HR, Madhushankari GS, Selvamani M, Kokila G, Mohan Kumar KP et al. Comparison of staining adequacy between tissues stored in formalin and paraffin embedded blocks for prolonged duration. Journal of Oral and Maxillofacial Pathology 2020; 24 (3): 586. https://doi.org/10.4103/jomfp.JOMFP_49_20

28. Hackam DJ, Sodhi CP. Toll-like receptor-mediated intestinal inflammatory imbalance in the pathogenesis of necrotizing enterocolitis. Cellular and Molecular Gastroenterology and Hepatology 2018; 6 (2): 229-238. https://doi.org/10.1016/j.jcmgh.2018.04.001

29. Sodhi CP, Wipf P, Yamaguchi Y, Fulton WB, Kovler M et al. The human milk oligosaccharides 2'-fucosyllactose and 6'-sialyllactose protect against the development of necrotizing enterocolitis by inhibiting toll-like receptor 4 signaling. Pediatric Research 2021; 89 (1): 91-101. https://doi.org/10.1038/s41390-020-0852-3

30. de Jong JCW, Ijssennagger N, van Mil SWC. Breast milk nutrients driving intestinal epithelial layer maturation via Wnt and Notch signaling: implications for necrotizing enterocolitis. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 2021; 1867 (11): 166229. https://doi.org/10.1016/j.bbadis.2021.166229

31. Peron G, de Lima Thomaz L, Camargo da Rosa L, Thomé R, Cardoso Verinaud LM. Modulation of dendritic cell by pathogen antigens: where do we stand? Immunology Letters 2018; 196: 91-102. https://doi.org/10.1016/j.imlet.2018.02.001

32. Liu Y, Tran DQ, Rhoads JM. Probiotics in disease prevention and treatment. Journal of Clinical Pharmacology 2018; 58 (Suppl. 10): S164-S179. https://doi.org/10.1002/jcph.1121

33. Baradaran Ghavami S, Asadzadeh Aghdaei H, Sorrentino D, Shahrokh S, Farmani M et al. Probiotic-induced tolerogenic dendritic cells: a novel therapy for inflammatory bowel disease? International Journal of Molecular Sciences 2021; 22 (15): 8274. https://doi.org/10.3390/ijms22158274

34. Lorenzetti R, Janowska I, Smulski CR, Frede N, Henneberger N et al. Abatacept modulates CD80 and CD86 expression and memory formation in human B-cells. Journal of Autoimmunity 2019; 101: 145-152. https://doi.org/10.1016/j.jaut.2019.04.016

35. Gu P, Gao JF, D'Souza CA, Kowalczyk A, Chou KY et al. Trogocytosis of CD80 and CD86 by induced regulatory T cells. Cellular and Molecular Immunology 2012; 9 (2): 136-146. https://doi.org/10.1038/cmi.2011.62

36. Kim GR, Choi JM. Current understanding of cytotoxic T lymphocyte antigen-4 (CTLA-4) signaling in T-cell biology and disease therapy. Molecules and Cells 2022; 45 (8): 513-521. https://doi.org/10.14348/molcells.2022.2056

37. Xiong A, Wang J, Mao XL, Jiang Y, Fan Y. MiR-199a-3p modulates the function of dendritic cells involved in transplantation tolerance by targeting CD86. HLA 2019; 94 (6): 493-503. https://doi.org/10.1111/tan.13677

38. Sun S, Yao M, Yuan L, Qiao J. Long-chain non-coding RNA n337374 relieves symptoms of respiratory syncytial virus-induced asthma by inhibiting dendritic cell maturation via the CD86 and the ERK pathway. Allergologia et Immunopathologia 2021; 49 (3): 100-107. https://doi.org/10.15586/aei.v49i3.85

39. Zhou Q, Shi Y, Chen C, Wu F, Chen Z. A narrative review of the roles of indoleamine 2,3-dioxygenase and tryptophan-2,3-dioxygenase in liver diseases. Annals of Translational Medicine 2021; 9 (2): 174. https://doi.org/10.21037/atm-20-3594

40. Salminen A. Role of indoleamine 2,3-dioxygenase 1 (IDO1) and kynurenine pathway in the regulation of the aging process. Ageing Research Reviews 2022; 75: 101573. https://doi.org/10.1016/j.arr.2022.101573

41. Meireson A, Devos M, Brochez L. IDO expression in cancer: different compartment, different functionality? Frontiers in Immunology 2020; 11: 531491. https://doi.org/10.3389/fimmu.2020.531491

42. Zander R, Schauder D, Xin G, Nguyen C, Wu X et al. CD4^{+} T cell help is required for the formation of a cytolytic CD8^{+} T cell subset that protects against chronic infection and cancer. Immunity 2019; 51 (6): 1028-1042. https://doi.org/10.1016/j.immuni.2019.10.009

43. Lacorcia M, Bhattacharjee S, Laubhahn K, Alhamdan F, Ram M et al. Fetomaternal immune cross talk modifies T-cell priming through sustained changes to DC function. Journal of Allergy and Clinical Immunology 2021; 148 (3): 843-857. https://doi.org/10.1016/j.jaci.2021.02.031