Involvement of nuclear factor erythroid 2-related factor 2 in neonatal intestinal interleukin-17D expression in hyperoxia

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Abstract. Interleukin 17D (IL-17D) plays an important role in host defense against inflammation and infection. In the present study, the role of nuclear factor erythroid 2-related factor 2 (Nrf2) in regulating the production of IL-17D was investigated under hyperoxia. For this purpose, neonatal rats were randomized into two groups; the model group was exposed to hyperoxia (80-85% O_2), while the control group was maintained under normoxic conditions (21% O_2). Small intestine tissue was collected on postnatal days 3, 7, 10 and 14. IL-17D expression was detected by immunofluorescence, immunohistochemistry and western blotting. The levels of Nrf2 and kelch-like ECH-associated protein 1 (keap1) were detected by immunohistochemistry and western blotting. Results showed that IL-17D expression in intestine epithelial cells increased steadily, reaching a peak on day 7, and decreased gradually on days 10 and 14 under hyperoxia. Nrf2 expression was consistent with IL-17D, and it was positively correlated with IL-17D. However, on postnatal days 10 and 14, the number of $CD4^+$ T cells and $CD19^+$ B cells expressing IL-17D was increased, and positive cells of the model group were significantly more than that of the control group. Keap1 levels were lower at the early stage. In conclusion, the expression levels of intestinal IL-17D and Nrf2 were altered simultaneously following neonatal rat development in hyperoxia, indicating that Nrf2 may be involved in regulating the expression of IL-17D in intestinal epithelial cells. Moreover, IL-17D in intestinal epithelial cells may play a unique immunological role during hyperoxia.

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Introduction

Oxygen supplementation is widely used in the treatment of acute and severe diseases suffered by newborns. However, high concentrations of oxygen can produce a large number of highly active superoxides, hydrogen peroxide and other toxic products (1) resulting in not only irreversible lung damage and pulmonary hypertension in adulthood, but also damage to other organs (2). Simultaneously, oxygen exposure may alter the developmental dynamics of the immune response and inhibit the expression of key genes involved in adaptive immunity (3).

The intestine is relatively immature in the first two weeks after birth, increasing the susceptibility of newborn animals to hyperoxia (4). A previous study found that neonatal hyperoxic exposure destroyed the intestinal barrier of neonatal rats, and in this case, oxidative stress stimulated the intestine to produce a variety of cytokines, indicating that oxidative stress is the main cause of intestinal injury in newborns (5).

The mammalian interleukin 17 (IL-17) family is considered one of the most ancient cytokine families, and is comprised of six cytokines, namely IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. Members of the family can directly or indirectly induce a variety of cytokines, chemokines, and inflammatory factors, as well as participate in both innate and adaptive immune responses. However, information regarding IL-17D is currently limited. Several studies have found that IL-17D has an important impact in inflammatory diseases, and host defense against bacterial infection of vertebrates such as poultry and teleosts (6-10). The expression of IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and other cytokines can be induced by IL-17D, similar to those of other family members (11). The difference, though, is that IL-17D is not only expressed on immune cells but also is preferentially expressed in skeletal muscle, brain, adipose, heart, lung, and pancreas (12). It was reported that IL-17D can regulate protective immunity during intracellular bacterial and viral infections (6). Furthermore, in tumors and in viral infections, IL-17D recruited natural killer (NK) cells via the chemokine (C-C motif) ligand 2 (CCL2). In this specific

process, nuclear factor erythroid 2-related factor 2 (Nrf2) is required for positively regulating IL-17D (13-16). Nrf2 is the primary responder to oxidative stress and is a sensitive factor of reactive oxygen species (ROS). This study aimed to investigate the changes in intestinal IL-17D during hyperoxia and to determine whether the Nrf2/kelch-like ECH-associated protein 1 (keap1) pathway is involved in regulating IL-17D.

Materials and methods

Animals. All Sprague-Dawley rats in this study were provided by the Animal Center of Shengjing Hospital of China Medical University. All surgical procedures were performed under anesthesia and all efforts were made to minimize animal suffering. All studies were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee of the China Medical University (no. 2018PS178K). All applicable institutional and/or national guidelines for the care and use of animals were followed.

Time-dated pregnant female Sprague-Dawley rats (42 female rats; 8 male mates; weight, 200-240 g; age,45-65 days) were housed in individual cages with free access to laboratory food and water, maintained on a 12:12-h light-dark cycle, and were allowed to deliver vaginally at term. Newborn rats were randomly divided into a model [exposure to hyperoxia (80-85% O_2) from day of birth], n≥10, and a control group [exposure to normoxia (21% O_2), n≥10, within 12 h of birth]. To avoid O_2 toxicity, maternal rats within the model and control groups were switched every 24 h.

Tissue harvest. At days 3, 7, 10 and 14 after the start of exposure to either hyperoxia (n≥10) or normoxia (n≥10) newborn rats from the model or control group were anesthetized with pentobarbital sodium (50 mg/kg; intraperitoneal) and sacrificed by cervical dislocation. The abdomen was opened through a midline incision, and the intestinal tract was carefully removed. The section of small intestine of each rat was flushed with saline to remove residual fecal contents. The middle 2 cm of the small intestine was excised and fixed with formalin, embedded in paraffin and sectioned using a microtome (Thermo Fisher Scientific, Inc.) at 3 μm for subsequent immunostaining. The remaining tissue was immediately stored at -80˚C to separate the protein at a later date.

Immunofluorescence

Single immunofluorescence. The tissue sections were deparaffinized in xylene and rehydrated in a gradient ethanol series. For antigen retrieval, the sections were microwaved in 0.01 M citrate buffer (pH 6.0) for 37 min, then incubated for 40 min at room temperature in normal goat serum (OriGene Technologies) and 3% H_2O_2 to block nonspecific antibody binding and endogenous peroxidase activity. Rabbit polyclonal anti-IL-17D (dilution 1:100, cat. no. bs-2612R, Bioss) was used for incubating the sections overnight at 4˚C, while phosphate-buffered saline (PBS) was used in place of the antibody for negative controls. The sections were then treated for 4 h at room temperature with fluorochrome-conjugated goat anti-rabbit IgG (dilution 1:100, cat. no. RS3611, Immunoway), followed by incubation with 4',6-diamidino-2-phenylindole (DAPI) incubation for nuclear staining. Immunofluorescence images were acquired using a confocal laser-scanning microscope (magnification, x400) (C1; Nikon, Tokyo, Japan).

Double immunofluorescence. After routine deparaffinization, the sections were microwaved in 0.01 M citrate buffer. The sections were incubated for 40 min at room temperature in normal goat serum (OriGene Technologies) and 3% H_2O_2. A combination of two primary antibodies; rabbit polyclonal anti-IL-17D (dilution 1:100, cat. no. bs-2612R, Bioss) and mouse monoclonal anti-CD4 (dilution 1:100, cat. no. sc-2007, Santa Cruz Biotechnology, Inc.), or rabbit polyclonal anti-IL-17D (dilution 1:100, cat. no. bs-2612R, Bioss) and mouse monoclonal anti-CD19 (dilution 1:50, cat. no. sc-373897, Santa Cruz Biotechnology, Inc.) were used for incubating the sections overnight at 4˚C. The sections were then incubated with a mixture of two secondary antibodies, goat anti-rabbit IgG (dilution 1:100, cat. no. RS3611, Immunoway) and goat anti-mouse IgG (dilution 1:100, cat. no. RS3208, Immunoway) for 4 h at room temperature, followed by DAPI incubation for nuclear staining. Double immunofluorescence images were acquired using a confocal laser-scanning microscope (magnification, x400) (C1; Nikon).

Immunohistochemistry. The experiment was performed using the streptavidin peroxidase method (SPlink detection kit; OriGene Technologies) according to the manufacturer's instructions. Paraffin-embedded intestinal tissue sections were deparaffinized, rehydrated, blocked with 10% goat serum and treated with 3% H_2O_2. Then the sections were incubated overnight at 4˚C with IL-17D antibody (dilution 1:400, cat. no. bs-2612R, Bioss) or Nrf2 antibody (dilution 1:1,600, cat. no. ab 31163, Abcam), or keap1 antibody (dilution 1:1,600, cat. no. ab139729, Abcam). The sections were then incubated with biotin-labeled goat anti-rabbit IgG secondary antibody and streptavidin-horseradish peroxidase for 30 min at 37˚C. Finally, the sections were developed with 3,3'-diaminobenzidine and counterstained with hematoxylin. All immunostained sections were viewed using a digital camera Nikon Eclipse E800 (Olympus Corp.). Semiquantitative analysis of the optical density (OD) values of the positive staining in the villi of each section at x200 magnification was performed using ImageJ 6.0 (National Institutes of Health). The absorbance values of Nrf2, keap1 and IL-17D were compared via Prism Graph version 5.0 software (GraphPad Software, Inc.) after scanning.

Western blotting. Western blotting was performed using standard protocols. In brief, total proteins were extracted from small intestine tissue and mixed with loading buffer. Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyacrylamide difluoride (PVDF) membranes. After blocking with 5% skim milk for 2 h at room temperature, the membranes were incubated with anti-IL-17D (dilution 1:1,000, cat. no. bs-2612R, Bioss), anti-Nrf2 (dilution 1:1,000, cat. no. ab31163, Abcam), and anti-keap1 (dilution 1:1,000, cat. no. ab139729, Abcam), or anti-β-actin (dilution 1:2,000, cat. no. bs-0061R, Bioss) and left shaking gently overnight at 4˚C. The next day, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (dilution 1:5,000; cat. no. SA00001-2, Proteintech) for

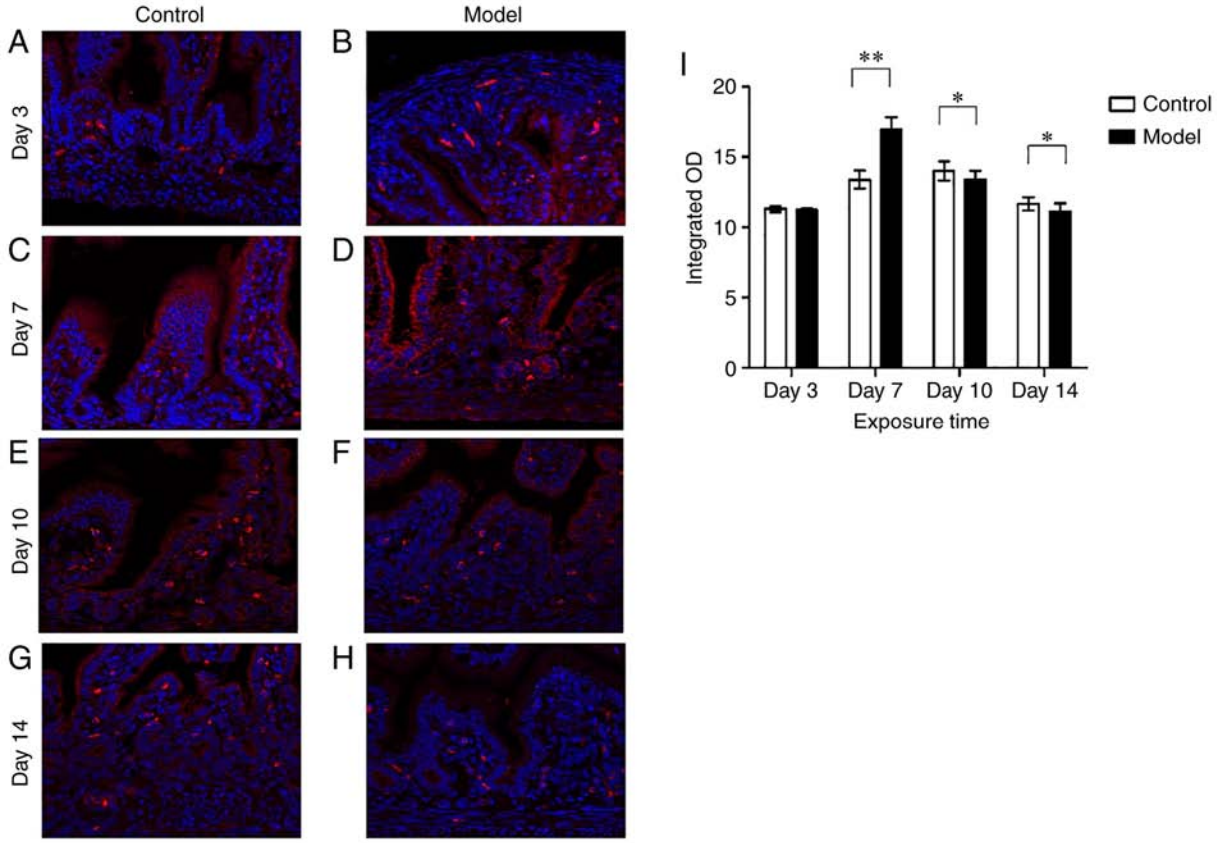

Figure 1. Intestinal IL-17D expression. Red fluorescence represents IL-17D. Intestinal IL-17D staining in the model group (A, C, E and G) on postnatal days 3, 7, 10 and 14, respectively. Intestinal IL-17D staining in the control group (B, D, F and H) on postnatal day 3, 7, 10 and 14, respectively. (I) Integrated optical density (OD) of IL-17D. Scale bars, 50 μm (n≥10 for each time point). *P<0.05 and **P<0.01. IL-17D, interleukin 17D.

2 h at room temperature, washed in 10 mM Tris/HCl, 150 mM NaCl, and 0.05% Tween-20, pH 7.5 TBST buffer three times, and developed using enhanced chemiluminescence reagents (NCM Biotech). Densitometry values were computed using ImageJ 6.0 (National Institutes of Health) and standardized to β-actin.

Statistical analysis. For each experiment, we tested at least 10 generations of each group. The data from all groups are reported as the means ± standard deviations. Statistical analysis was performed using SPSS 25.0 software (IBM Corp.). A P-value (P) of <0.05 was considered as indicative of a statistically significant difference. The statistical significance of the data was determined using two-way ANOVA with Bonferroni's post hoc test, and correlation analysis was performed via Pearson's test.

Results

IL-17D expression in rat intestinal epithelial cells under hyperoxia. The expression of IL-17D in small intestine tissues from both the control and model groups was analyzed by immunofluorescence staining. Red fluorescence represents IL-17D. As indicated in Fig. 1, IL-17D was expressed in intestinal epithelial cells of both the model group and the control group. In the control group, there was no change in IL-17D expression in a day-dependent manner. Compared with the control group, there was no difference in IL-17D expression in the model group on postnatal day 3 (Fig. 1A, B and I). On postnatal day 7, IL-17D expression in the intestinal epithelial cells in the model group was significantly increased compared to the control group (P<0.01) (Fig. 1C, D and I). On postnatal days 10 and 14, IL-17D expression of intestinal epithelial cells in the model and control groups was reduced compared to that of postnatal day 7, but IL-17D expression in the model group was significantly decreased when compared with the control group (P<0.05) (Fig. 1E-I). At the same time, IL-17D was expressed on the lamina propria cells in small intestines in both groups.

IL-17D expression in neonatal intestinal lamina propria T and B lymphocytes under hyperoxia. Glycoproteins CD4 and CD19 are selectively expressed on the surface of T and B lymphocytes, respectively. Green fluorescence represents CD4 or CD19, red fluorescence represents IL-17D, and co-expression of CD4 and IL-17D or CD19 and IL-17D appears as yellow or orange fluorescence. As shown in Figs. 2 and 3, IL-17D was expressed only on a small number of $CD4^{+}$ T cells and $CD19^{+}$ B cells in lamina propria on postnatal day 3.

In the model group the numbers of $CD4^{+}$ T cells which expressed IL-17D were higher than those in the control group (P<0.05) (Fig. 2A-D and I) on postnatal day 7, while the numbers of $CD19^{+}$ B cells which expressed IL-17D had no significant difference between the model and control group on postnatal day 7 (Fig. 3A-D and I). On days 10 and 14, the $CD4^{+}$ T cells and $CD19^{+}$ B cells which expressed IL-17D were

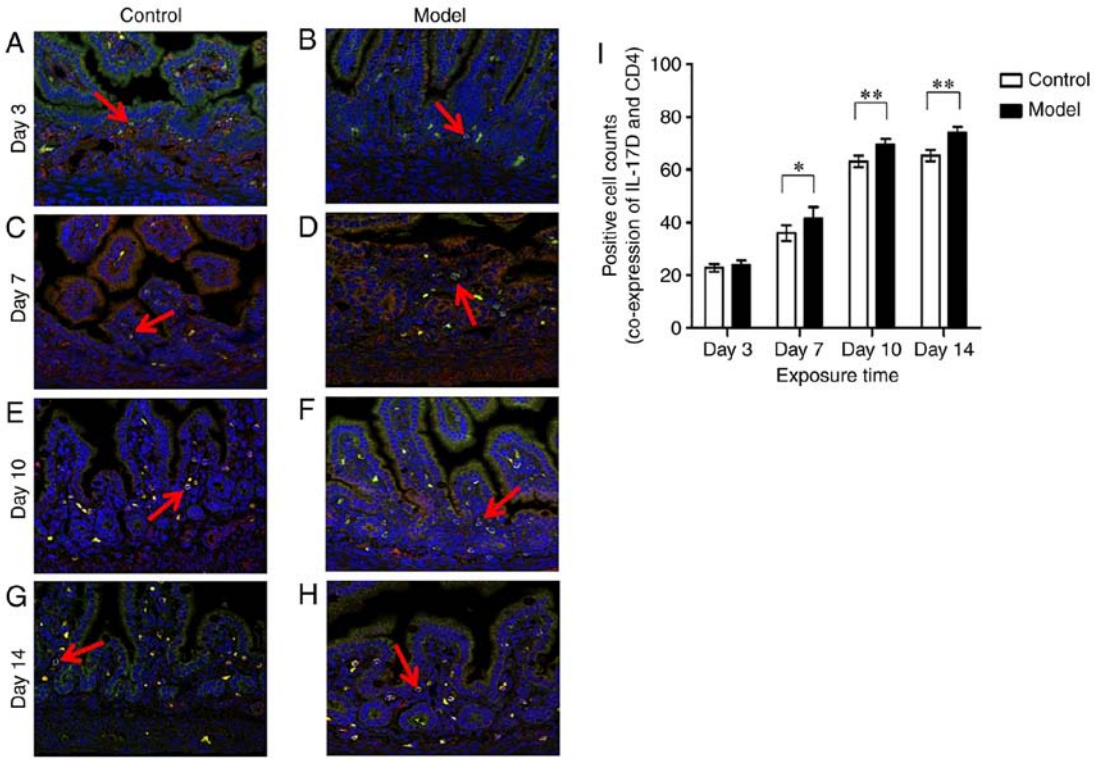

Figure 2. IL-17D expression in intestinal CD4$^+$T cells. Green fluorescence represents CD4, red fluorescence represents IL-17D and yellow fluorescence represents the co-expression of IL-17D and CD4 (red arrows indicate co-expression of IL-17D and CD4 in CD4$^+$ T cells). (A-D) Little co-expression of IL-17D and CD4 at postnatal days 3 and 7. (E-H) Greater co-expression of IL-17D and CD4 was observed at postnatal days 10 and 14. (I) Positive cell counts of co-expression of IL-17D and CD4. Scale bars, 50 μm (n≥10 for each time point). *P<0.05 and **P<0.01. IL-17D, interleukin 17D; CD4, cluster of differentiation 4.

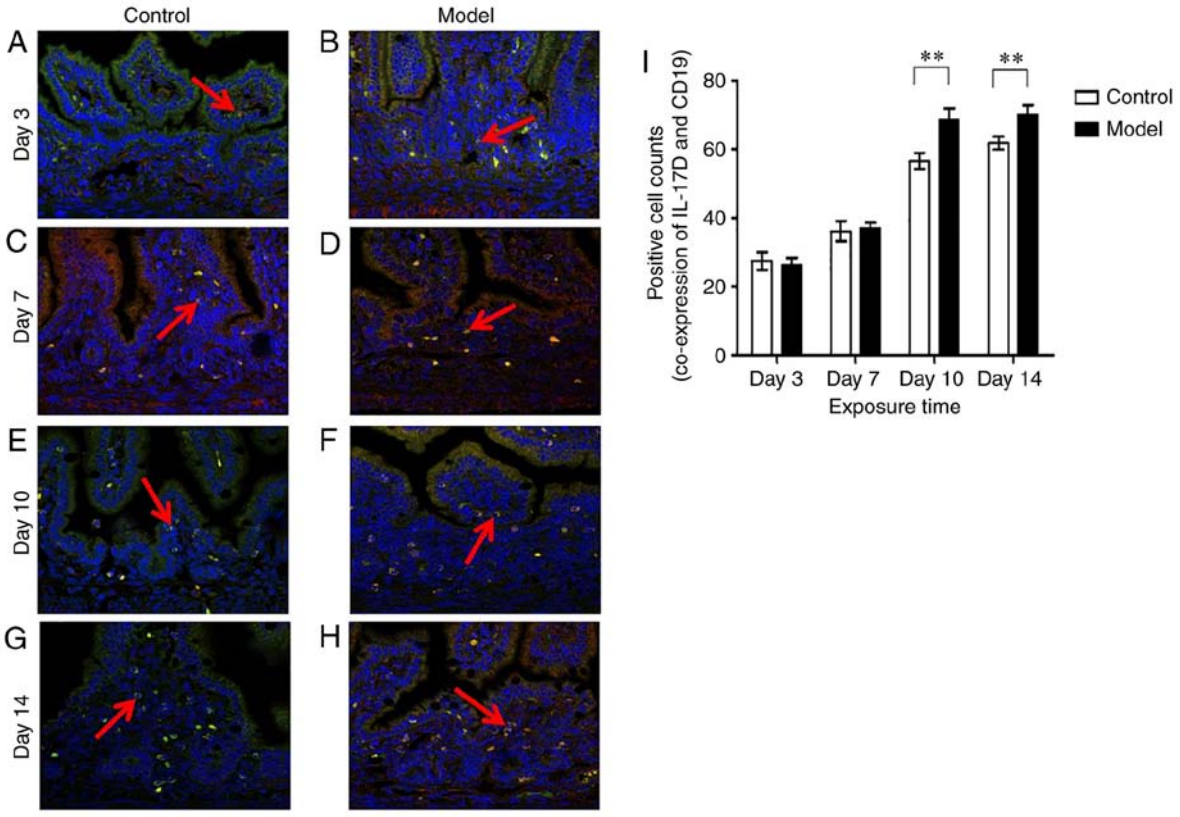

Figure 3. IL-17D expression in intestinal CD19$^+$B cells. Green fluorescence represents CD19, red fluorescence represents IL-17D and orange fluorescence represents the co-expression of IL-17D and CD19 (red arrows indicate co-expression of IL-17D and CD19 in CD19$^+$ B cells). (A-D) Little co-expression of IL-17D and CD19 at postnatal days 3 and 7. (E-H) Greater co-expression of IL-17D and CD19 was observed at postnatal days 10 and 14. (I) Positive cell counts of co-expression of IL-17D and CD19. Scale bars, 50 μm (n≥10 in each time point). **P<0.01. IL-17D, interleukin 17D; CD19, cluster of differentiation 19.

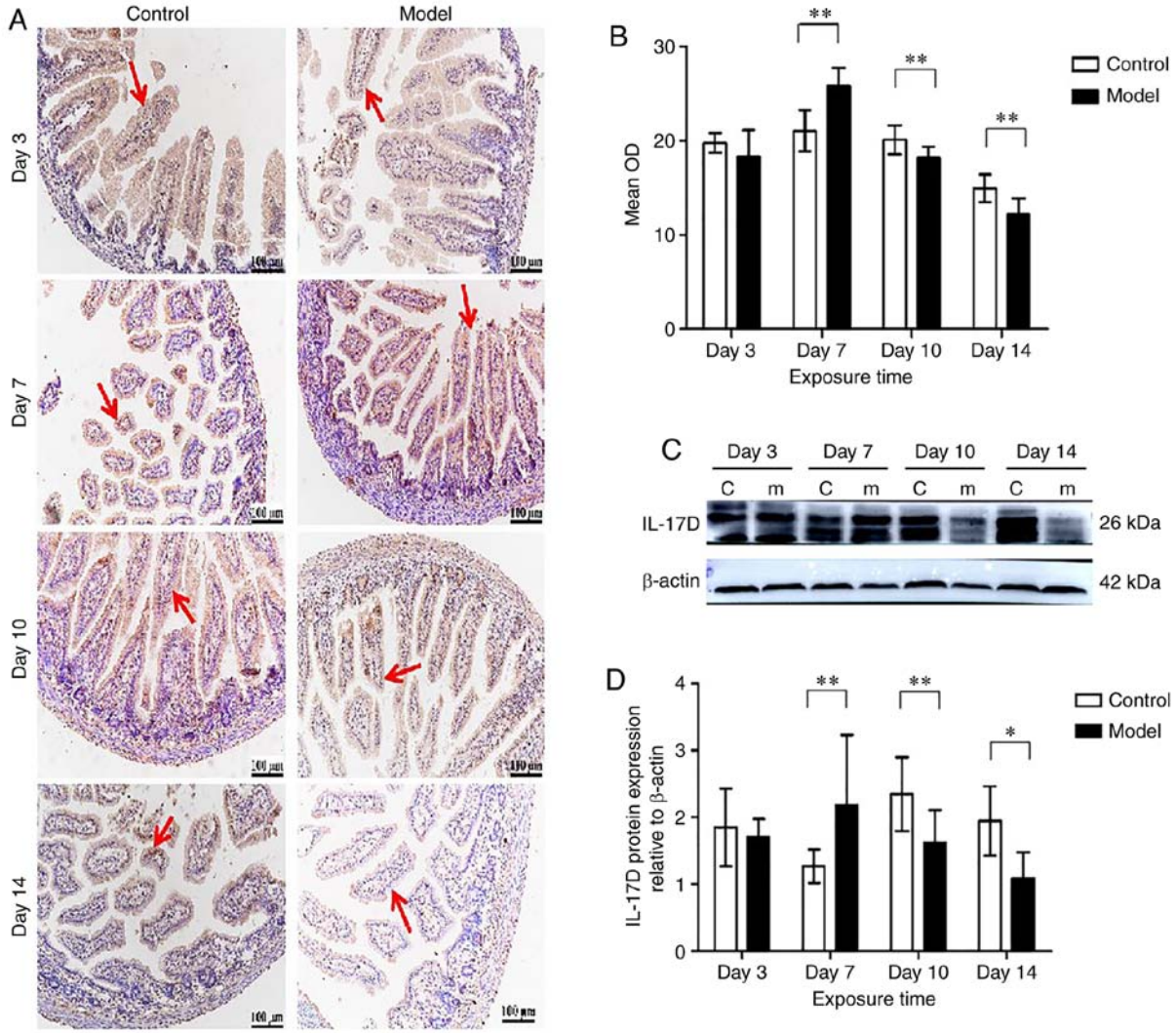

Figure 4. Intestinal IL-17D protein levels. (A) IL-17D was expressed in the cytoplasm of the cells (red arrows indicate IL-17D). (B) Mean optical density (OD) of IL-17D. (C) Intestinal IL-17D protein expression by western blotting. β-actin served as an internal control. c, control group; m, model group, kDa, kilodalton. (D) Densitometric analysis of IL-17D level normalized to β-actin. Scale bars, 100 μm (n≥10 in each time point). *P<0.05, **P<0.01. IL-17D, interleukin 17D.

significantly increased. In the model group, the numbers of CD4^{+} T cells and CD19^{+} B cells which expressed IL-17D were significantly higher than those in the control group (P<0.01) (Figs. 2E-I and 3E-I). This showed that the IL-17D expression on intestinal epithelial cells was exactly opposed to those of the intestinal lamina propria T and B lymphocytes. This demonstrated that the IL-17D of intestinal epithelial cells may play a unique immune role during hyperoxia.

IL-17D expression in neonatal intestines under hyperoxia. The immunohistochemistry results showed that at postnatal days 3, 7, 10 and 14, IL-17D protein was expressed in the cytoplasm of intestinal epithelial cells and intestinal lamina propria T and B cells (Fig. 4A). On postnatal day 3, there was no difference in the expression of IL-17D between the model group and the control group. IL-17D expression increased and peaked on day 7 in the model group compared with the control group (P<0.01), and then decreased gradually on days 10 and 14, remaining significantly lower than that of the control group (P<0.01) (Fig. 4B). Western blotting results showed clear and specific bands of IL-17D (26 kDa) (Fig. 4C). On days 3, 10 and 14 of hyperoxia exposure, IL-17D expression was lower than that of the control group, but increased and was significantly higher than that of the control group on day 7 (Fig. 4D). During hyperoxia exposure, IL-17D expression increased, and reached a peak on day 7 (P<0.01), and decreased gradually on days 10 and 14 (P<0.05).

Nrf2 expression in neonatal intestines in hyperoxia. As indicated in Fig. 5A, Nrf2 was expressed in the nucleus and the cytoplasm of intestinal epithelial cells and lamina propria cells. Compared with the control group, Nrf2 expression in the model group increased significantly, reaching a peak when exposed to hyperoxia for 7 days (P<0.01); however, Nrf2 expression was downregulated at days 10 and 14 (P<0.05) (Fig. 5B). Nrf2 protein levels of the small intestinal tissues were examined by western blotting (Fig. 5C). Compared with the control group, Nrf2 expression level in the model group was no different on day 3, but was significantly increased at day 7 (P<0.01). Subsequently, on days 10 and 14, Nrf2 protein levels in the model group were significantly downregulated compared with the control group (P<0.05) (Fig. 5D).

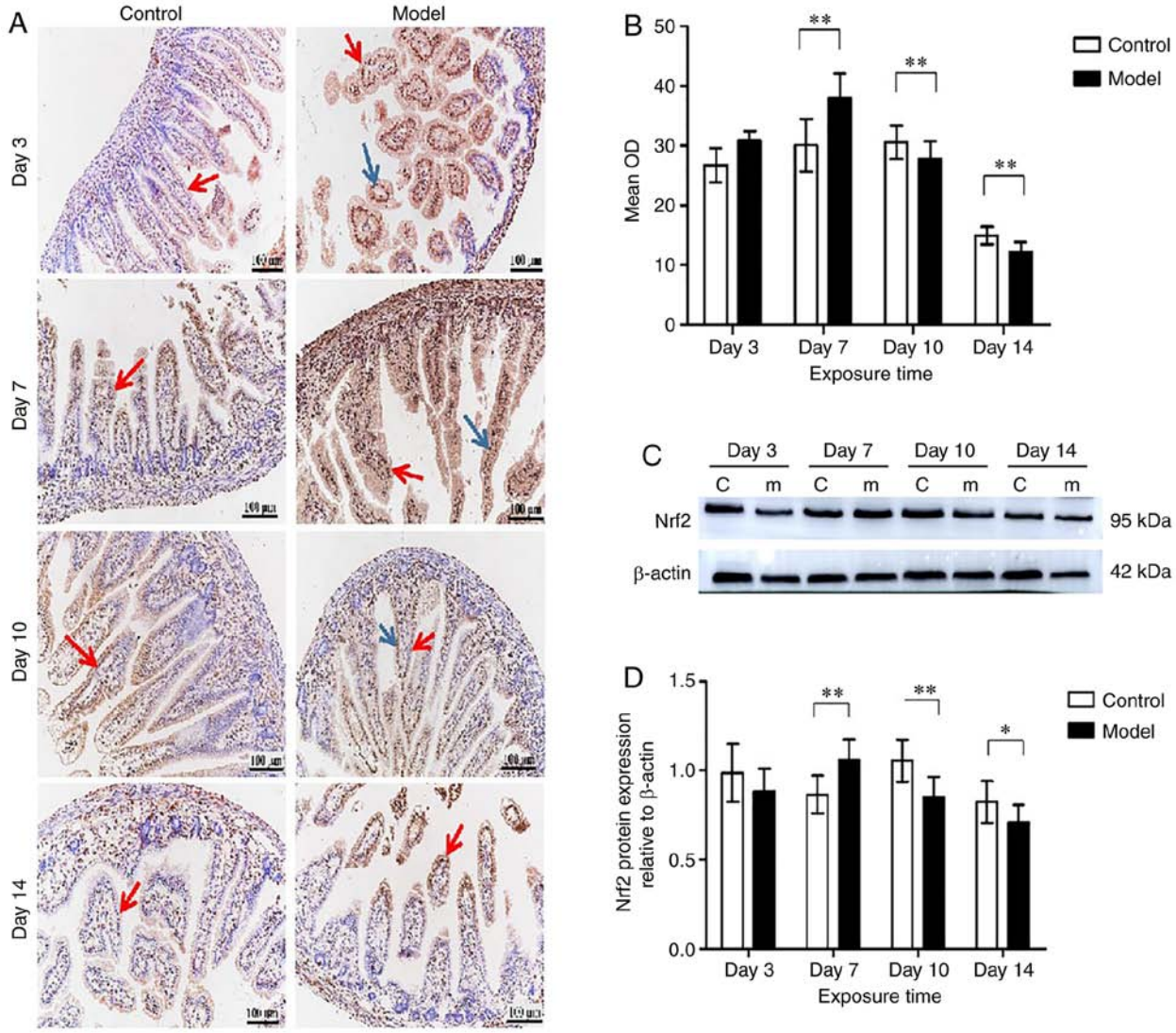

Figure 5. Nrf2 expression in the intestines. (A) Nrf2 was expressed in the nucleus and cytoplasm of cells (red arrows indicate Nrf2 in the cytoplasm, blue arrows indicate Nrf2 in the nucleus.). (B) Mean optical density (OD) of Nrf2. (C) Western blotting for Nrf2. β-actin served as an internal control. c, control group; m, model group, kDa, kilodalton. (D) Densitometric analysis of Nrf2 level normalized to β-actin Scale bars, 100 μm (n≥10 in each time point). *P<0.05, **P<0.01. Nrf2, nuclear factor erythroid 2-related factor 2.

Keap1 expression in neonatal intestines under hyperoxia. Keap1 was expressed in the cytoplasm of intestinal epithelial cells and lamina propria cells (Fig. 6A). Keap1 protein expression was downregulated after hyperoxia exposure at day 3 (P<0.05) and was decreased significantly at day 7 (P<0.01) under hyperoxia. When hyperoxia exposure was prolonged to 14 days, there was no difference between the model and the control group in keap1 expression (Fig. 6B). In the model group, keap1 expression was no different in a day-dependent manner. Keap1 protein levels in total small intestine tissues were significantly downregulated in the model group at days 3 and 7 compared with the control group, as demonstrated by western blot analysis (P<0.01) (Fig. 6C and D).

Correlation between IL-17D expression and Nrf2 or keap1 expression in neonatal intestines. To confirm the correlation between IL-17D protein expression and Nrf2, a correlation analysis was performed. Protein expression of IL-17D was significantly positively correlated with Nrf2 (r=0.501, P<0.01) (Fig. 7), while IL-17D showed a poor correlation with keap1 protein (data not shown).

Discussion

In the interleukin (IL)-17 family, IL-17A plays an important role in host defense against bacterial and fungal infections, and IL-17F is mainly involved in mucosal host defense (17). Another family member, IL-17B, is widely expressed in various tissues and has the potential to affect tumor progression, whereas IL-17E enhances T helper cell 2 (Th2) immune responses by inducing Th2 cytokines, contributing to the host defense against nematodes and allergic disorders (18,19). IL-17C promotes Th17 cell responses via the IL-17 receptor E, which is critical in preventing intestinal pathogen infection and the pathogenesis of a variety of autoimmune diseases, such as psoriasis, inflammatory bowel disease, and multiple sclerosis (20-23). However, little research has been conducted into investigating the expression and potential role of IL-17D.

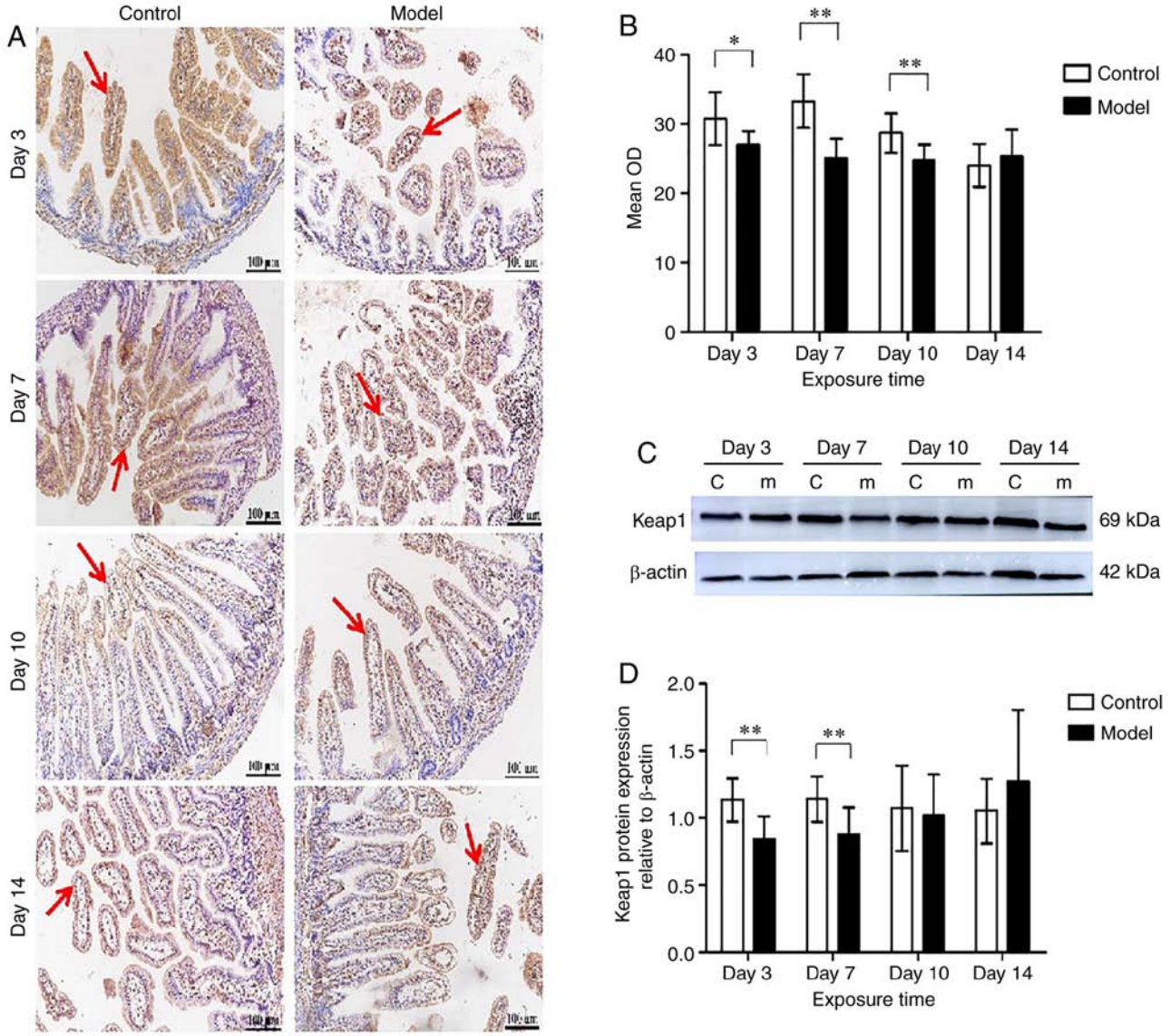

Figure 6. Keap1 expression in the intestine. (A) Keap1 was expressed in the cytoplasm of cells (red arrows indicate keap1). (B) Mean optical density (OD) of keap1. (C) Intestinal keap1 protein expression by western blotting. β-actin served as an internal control. c, control group; m, model group, kDa, kilodalton. (D) Densitometric analysis of keap1 level normalized to β-actin. Scale bars, 100 μm (n≥10 in each time point). *P<0.05, **P<0.01. Keap1, kelch-like ECH-associated protein 1.

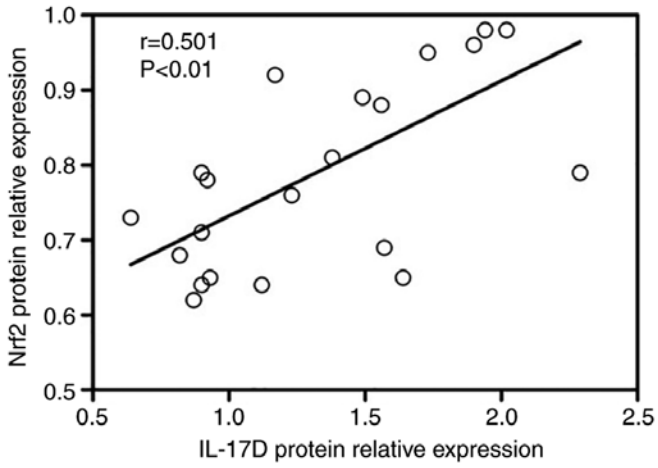

Figure 7. Correlation analyses between IL-17D and Nrf2 in neonatal intestine. r=0.501, P<0.01. IL-17D, interleukin 17D; Nrf2, nuclear factor erythroid 2-related factor 2.

Currently, it has only been reported that IL-17D can regulate the production of cytokines and inhibit hematopoiesis *in vitro* (24,25). In addition to this, it has been reported that expression of IL-17D changes during the development of certain diseases: For example, IL-17D expression is decreased in psoriatic skin (26), but increased in rheumatoid nodules (27). It appears that the main role of tumor-expressed IL-17D is to initiate antitumor immunity and stimulate the infiltration of natural killer (NK) cells into the tumor microenvironment (28). Recent studies have found that IL-17D inhibits bacterial phagocytosis in macrophages by mediating downregulation of NF-κB activation in macrophages, which increases mortality in patients with sepsis (29). IL-17D is expressed in a variety of tissues and is expressed in only resting $CD4^{+}$ T and $CD19^{+}$ B cells in immune cells (18). It is well known that the intestinal epithelial cells include differentiated cells of various lineages such as absorptive intestinal cells, goblet cells, enteroendocrine cells, Paneth cells, tufted cells and microfold cells (M cells),

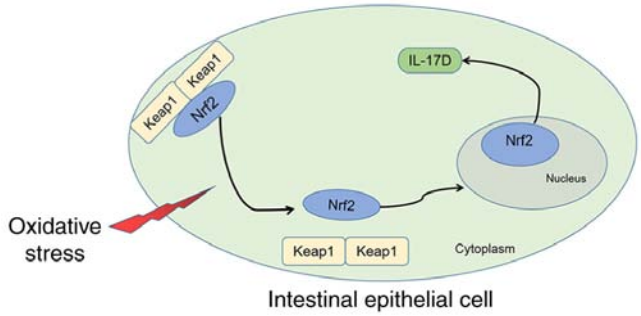

Figure 8. Under homeostatic conditions Nrf2 is kept inactive being bound to its endogenous inhibitor, keap1. Oxidative stress causes Nrf2 to detach from keap1 and translocate to the nucleus, inducing IL-17D expression. IL-17D, interleukin 17D; Nrf2, nuclear factor erythroid 2-related factor 2; Keap1, kelch-like ECH-associated protein 1.

which fold to form crypts and villous structures (30). In this study, we observed that IL-17D was expressed on intestinal epithelial cells of small intestinal villi. Compared with the control group, IL-17D expression in intestinal epithelial cells during hyperoxia increased and reached a peak during early postnatal stages. However, along with neonatal growth and development, in the later stages of hyperoxia, IL-17D was lower in intestinal epithelial cells. Prior research has shown that in hyperoxia the apoptotic rate of intestinal epithelial cells is significantly increased, and that increased expression of tumor necrosis factor α (TNFα) induces the production of intracellular reactive oxygen species (ROS) (31), which may be the cause of the above results. At the same time, IL-17D was also expressed in intestinal lamina propria lymphocytes (such as $CD4^+$ T cells and $CD19^+$ B cells), and IL-17D expression in intestinal lamina propria T and B lymphocyte cells was exactly opposed to those of intestinal epithelial cells. In the later stages of hyperoxia, the numbers of $CD4^+$ T cells and $CD19^+$ B cells expressing IL-17D were significantly increased. These results seemed to indicate that IL-17D acts through different mechanisms in different cells. At present, the research on IL-17D mainly focuses on its role in antitumor and viral infections, but there is not enough clinical data to confirm its affect in intestinal epithelial cells in the hyperoxia response. Therefore, it is important to further explore the potential mechanism of IL-17D in hyperoxia treatment in the future.

The gastrointestinal tract represents the largest mucosal membrane surface in the body and is one of the most complex human organs. Intestinal epithelial cells not only constitute the first line of defense for the intestine, but also constantly pass signal information between the gut lumen and immune cells (32). Although hyperoxia is an indispensable treatment in clinical critical care for various neonatal diseases, the neonatal small intestine is highly sensitive to oxygen (30). The structure of intestinal villi in neonatal rats was destroyed in the hyperoxic environment, which may have affected the barrier function of their intestines, making them susceptible to bacterial insult (33). Culturing of intestinal epithelial cells *in vitro* showed that hyperoxia could inhibit cell growth, and destroy intestinal epithelial cells, which would promote the invasion of intestines by bacteria (33). Hyperoxemia caused intestinal damage and predisposed premature neonates to necrotizing enterocolitis (5). Lee *et al* found that IL-17D expression in hepatocytes decreased during listeria monocytogenes expressing ovalbumin (LM-OVA) infection, which indicated that IL-17D could be inhibited during LM-OVA infection (34). In the present study, after exposure to hyperoxia, the expression level of intestinal total IL-17D in the model group reached a peak during the early postnatal stages, and subsequently decreased, indicating that IL-17D expression is repressed in the later postnatal stages. This indicates that inflammation occurred during hyperoxia. This is also likely as the hyperoxic exposure destroyed the intestinal epithelial cells, the apoptotic rate of the intestinal epithelial cells increased, and thus decreased the level of IL-17D expressed by intestinal epithelial cells. Although the number of $CD4^+$ T cells and $CD19^+$ B cells which expressed IL-17D increased on days 10 and 14 during hyperoxia exposure, low proportion of $CD4^+$ T cells and $CD19^+$ B cells compared to the intestinal epithelial cells was observed, and the intestinal total IL-17D was lower. Thus it is necessary to explore the different mechanisms of IL-17D in different cells under hyperoxia in the future.

The transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2), is a highly conserved basic leucine zipper (bZip) belonging to the cap-n-Collar (CNC) regulatory protein family, that plays a central role in oxidative stress. The *Nrf2* gene is expressed in most cell types and activates a wide range of cellular defense processes, thereby enhancing the overall capacity of cells to detoxify and eliminate harmful substances (35). Suzuki *et al* found that Nrf2 contains hundreds of target genes (36). It has been reported that Nrf2 gene-knockout mice develop inflammation-related diseases such as autoimmune diseases, indicating that Nrf2 may be an important endogenous protective factor for autoimmune supervision (37). The adaptor subunit of Cullin 3-based E3 ubiquitin ligase, keap1, is the negative regulator of Nrf2 (38). Under basal conditions, keap1 binds to Nrf2 in the cytoplasm to form a dimer, which keeps the cells in a stable state (Fig. 8). When keap1 and Nrf2 dissociate under oxidative stress conditions, Nrf2 then translocates into the nucleus and binds to the small Maf protein to form a heterodimer, which binds to the antioxidant response element (ARE) in the gene promoter, exerting antioxidant capacity accordingly (39,40). At present, the application of this antioxidant system protection mechanism in many treatments has entered the preclinical research stage, including multiple sclerosis, psoriasis and retinal vascular disease (41). The present study demonstrated that keap1 levels were lower at the early stage, but the change in keap1 was not obvious in the later stage. In contrast, expression of Nrf2 in the model group reached a peak in the early stages and decreased in the later stages compared with the control group. On days 10 and 14, the expression of Nrf2 in total small intestines in the model group was also reduced, which was consistent with IL-17D expression. In tumor and viral infection, Nrf2 involvement in the expression of IL-17D has been extensively reported (14-16). This study showed that Nrf2 was consistent with IL-17D expression in the small intestine under hyperoxia and was positively correlated with IL-17D. Based on these findings, we can deduce that intestinal IL-17D expression may be modulated by Nrf2 to exert biological effects on the intestines (Fig. 8). As IL-17D can induce the expression of inflammatory cytokines (24,25), and in hyperoxia TNFα is increased (31), it can be concluded that,

in the early stage of hyperoxia, IL-17D expressed by intestinal epithelial cells aggravates intestinal inflammation by inducing the production of several inflammatory cytokines. However, IL-17D expressed by intestinal immune cells can also exert protective effects on the intestinal epithelium by controlling inflammation and mediating immune responses. These effects and underlying mechanisms need further investigation.

In conclusion, the expression of intestinal IL-17D and Nrf2 were simultaneously altered following neonatal development under hyperoxia, indicating that Nrf2 may be involved in regulating the expression of IL-17D in intestinal epithelial cells. Moreover, IL-17D in intestinal epithelial cells may play a unique immunological role during hyperoxia. Although the use of Nrf2 agonists can reduce the inflammatory response caused by hyperoxia (42), the specific process of IL-17D regulated by Nrf2 under hyperoxia and the mechanism of action of IL-17D remain unclear. Therefore, further research is required to clarify the induction of inflammatory factors by IL-17D in intestinal epithelial cells under hyperoxia and whether Nrf2 is necessary to regulate IL-17D in this process.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XL and QC acquired, analyzed, and interpreted the experimental data. DZ acquired the data. SS drafted the work and revised it critically for important intellectual content. DL conceptualized and designed the study, and gave the final approval of the submitted manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The animal studies were ethically approved by the Institutional Animal Care and Use Committee of the China Medical University (no. 2018PS178K) for animal experimentation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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