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Inhibitory effect of dietary n-3 polyunsaturated fatty acids to intestinal IL-15 expression is associated with reduction of TCRαβ+CD8α+ CD8β− intestinal intraepithelial lymphocytes

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Abstract

Intestinal intraepithelial lymphocytes (IELs) and their cytokines play an important role in the regulation of gut immune response and take part in gut immune barrier function. n-3 polyunsaturated fatty acid (PUFA) is an immunoregulator that has been shown to influence the process of gut inflammation. Interleukin (IL)-15 is a T-cell growth factor that has been shown to influence the differentiation of IEL. The aim of this study was to analyze the effects of dietary n-3 PUFA on IEL. IEL phenotype and cytokine (TNF-α, IFN-γ, IL-4, IL-10 and TGF-β1) profile were measured by FACS and real-time RT-PCR in healthy adult rats fed with fish oil diet for 90 days. Rats fed with corn oil diet served as controls. Intestinal IL-15 expression was measured by immunohistochemistry and real-time RT-PCR. The results demonstrated a decrease of intestinal IL-15 expression in the fish oil group. Associated with this deduction, n-3 PUFA significantly decreased the proportion of TCRαβ+CD8α+CD8β− cells and IEL-derived TNF-α, IFN-γ, IL-4 and IL-10. In conclusion, n-3 PUFA could inhibit intestinal mucosal expression of IL-15 and may influence phenotype and function of IEL through this mechanism.
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Keywords: Intestinal intraepithelial lymphocytes; n-3 PUFA; Rats; IL-15

1. Introduction

Interleukin (IL)-15 is a T-cell growth factor with a biological activity similar to IL-2 [1]. It stimulates differentiation of CD8 T cells and NK cells and, thus, has a role in lymphoid development and hemostasis [2]. It also regulates the development of intestinal intraepithelial lymphocytes (IELs) and is an immunoregulator in gut immune barrier function [3–5]. IELs are the innermost layer of gut-associated lymphoid tissue (GALT). They are primarily T cells with potent cytolytic and immunoregulatory capacities, which they use to sustain epithelial integrity [6]. This compartment of lymphocytes is distinct from their peripheral counterpart. According to their phenotypic and functional characteristics, IELs are primarily divided into two subpopulations. Type a includes TCRαβ+CD8αβ+ cells that primarily recognize antigens presented by conventional MHC Class I and II and are primed within the systemic circulation. Type b cells include TCRαβ+CD8αα+ IELs and TCRγδ+ IELs that respond to antigens not restricted by conventional MHC [6].

IELs are involved in various gut inflammatory processes. IEL-derived IL-10 showed a tendency to increase in treated celiac patients [7]. IELs from untreated celiac patients showed increased production of IFN-γ and TNF-α [8]. Sullivan et al. [9] reported that increased IEL-derived IL-4 and IL-10 expression may play a key role in successful engraftment of rat orthotopic small bowel transplantation with portal vein drainage.

n-3 polyunsaturated fatty acid (PUFA) has a variety of immunoregulatory effects [10]. It was shown to improve symptoms of rat colitis based on reduced morphological damage and plasma and colonic concentrations of inflammatory mediators [11–14]. In rat small bowel transplantation, n-3 fatty acids were shown to have an immunosuppressive effect on rat allogenic small intestinal transplantation based on histological findings of the grafts [15,16].

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We hypothesized that the potential effects of n-3 PUFA on IEL take part in its immunoregulatory function on various gut inflammation conditions. In this study, long-term effects of dietary n-3 PUFA on adult rat intestinal IELs were explored.

2. Materials and methods

2.1. Animals and diet

Male Lewis (RT-1[l]) rats (12 weeks old) were obtained from Vitalriver Company (Beijing, China). All animals were fed rat chow and water ad libitum for at least 7 days prior to the study to allow acclimatization to the laboratory. The protocol was approved by the Animal Research Committee of Nanjing University. All procedures were carried out in accordance with the *Principles of Laboratory Animal Care* (NIH Publication No. 85-23, revised 1985).

Animals were randomly assigned to one of the two experimental diets for 90 days. They were based on a nutritionally complete diet made with the addition of 70 g/kg fat containing the following (per kilogram): 400 g sucrose, 219.8 g cornstarch, 200 g casein, 60 g cellulose, 35 g mineral mix (AIN-76), 10 g vitamin mix (AIN-76), 3 g DL-methionine, 2 g choline bitartrate and 0.2 g TBHQ. The fish oil diet (FO group) contained 40 g/kg menhaden fish oil (Sigma, St. Louis, MO; EPA: 15.4%, DHA: 15.1%) and 30 g/kg corn oil (Sigma). The corn oil diet (CO group) contained 70 g/kg corn oil.

All animals were sacrificed after 90 days. IELs of whole small bowel were isolated and assigned for phenotypic and cytokine gene analysis. Intestinal samples were placed in 4% formaldehyde and embedded in paraffin for histopathological evaluation and immunohistochemical observation for IL-7 and IL-15.

2.2. Antibodies

Fluorescein isothiocyanate (FITC)-conjugated anti-CD3 monoclonal antibodies (mAbs); phycoerythrin (PE)-conjugated anti-CD4, anti-CD45, anti-NKR-P1A mAbs; and TC-conjugated anti-CD8α mAb were purchased from Caltag Laboratories (Burlingame, CA). FITC-conjugated anti-CD8β mAb was purchased from BD PharMingen (San Diego, CA). PE-conjugated anti-TCRαβ, anti-TCRγδ, anti-CD28 and anti-CTLA-4 mAbs were purchased from eBioscience (San Diego, CA). Appropriate isotype-matched control mAbs were used as negative controls. Goat polyclonal anti-IL-7 and anti-IL-15 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.3. Isolation of intestinal IELs

IELs were prepared from rat small intestine using previously described methods with minor modifications [17]. Briefly, the entire small intestine was gently flushed with cold RPMI 1640 to remove luminal contents and then incubated in ice-cold RPMI 1640 for 100 min to delaminate epithelial cells and IELs from the basement membrane. Detached cells were recovered by flushing the intestine five times with a total of 50 ml of HEPES-buffered HBSS medium containing 1 mM dithioerythritol (Sigma) and 10% fetal calf serum (FCS; Sijiqing, Hangzhou, China) at 37°C. Supernatants were pooled and mixed followed by a 10-min period of sedimentation. The sediments were discarded. The supernatants containing the IELs were washed twice with RPMI containing 10% FCS and separated on a discontinuous density gradient of 45% and 75% Percoll (Pharmacia, Piscataway, NJ). The gradient was centrifuged at 575×*g* for 30 min at room temperature. Viable lymphocytes at the interface were collected and extensively washed with phosphate-buffered saline (PBS). After quantifying with the use a hemocytometer, the purity of lymphocytes was measured by CD45 staining, which was always >96%.

2.4. Flow cytometric analysis

Freshly isolated IELs from individual rat were suspended in PBS containing 2% FCS at 10^6 cells/ml and stained in plastic tubes. Single-, two- and three-color flow cytometric analyses were performed as follows: Cells were incubated with a mixture of saturating concentrations of FITC-, PE- and/or TC-conjugated mAbs at 4°C in the dark for 20 min. Stained cells were washed with PBS containing 2% FCS and analyzed immediately using a FACSCalibur (Becton Dickinson, California). Lymphoid cells were identified by their forward and side light-scatter profiles and the detection of TCRαβ, TCRγδ or CD3 staining. Phenotypical results were expressed as the percentage of positive cells with respect to the total number of gated lymphocytes or with respect to a particular IEL subset (CD3+ cells). Anti-CD8α mAb was used to stain total CD8+ cells (both CD8αα and CD8αβ IEL populations).

2.5. Real-time RT-PCR analyses

Total RNA was extracted from 1.0×10^6/rat IEL or 100 mg ileum sample using Trizol reagent (Invitrogen Life Technologies, Gaithersburg, MD) according to the manufacturer's directions. RNA was frozen at −70°C until assayed. Quantification was measured via spectrophotometry at 260 nm absorption. Total RNA (3 μg) was treated with MMLV reverse transcriptase (Promega, Madison, WI), and 2.5 mM dNTP was used for reverse transcription according to the manufacturer's instructions. Quantitative real-time PCR analysis was performed on the Rotor-Gene 3000 real-time DNA analysis system (Corbett Research, Sydney, Australia) applying the real-time SYBR Green PCR technology according to the manufacturer's instructions. The reaction mixtures contained diluted cDNA, SYBR Green (Molecular Probes, Eugene, OR), 0.4 μM of each gene-specific primer and nuclease-free water to a final volume of 25 μl. The reaction conditions were 94°C for 4 min followed by 35 cycles at 94°C for 10 s, 60°C (for TGF-β1, TNF-α, IFN-γ, IL-4, IL-15 and β-actin) or 57°C (for IL-10) for 15 s and

72°C for 20 s. Test cDNA results were normalized to β-actin measured on the same plate. The primers were designed using the Primer 5.0 program (PREMIER Biosoft International, Silicon Valley, CA). The sequences of primers used in this study were as follows: TGF-β1, 5′-GCT GAA CCA AGG AGA CGG AAT A-3′ and 5′-ACC TCG ACG TTT GGG ACT GA-3′; TNF-α, 5′-CAG ACC CTC ACA CTC AGA TCA TC-3′ and 5′-TAT GAA ATG GCA AAT CGG CT-3′; IFN-γ, 5′-CTA CAC GCC GCG TCT TGG TTT-3′ and 5′-GCT GAT GGC CTG GTT GTC TTT-3′; IL-4, 5′-AAC AAG GAA CAC CAC GGA GAA-3′ and 5′-GTT CAG ACC GCT GAC ACC TCT-3′; IL-10, 5′-GTC CTT TCA CTT GCC CTC ATC-3′ and 5′-CAA ACT GGT CAC AGC TTT CGA-3′; β-actin, 5′-CCT CTA TGC CAA CAC AGT GC-3′ and 5′-GTA CTC CTG CTT GCT GAT CC-3′; IL-15, 5′-CTC CCT AAA ACA GAG GCC AAC T-3′ and 5′-CAC ATT CCT TGC AGC CAG AC-3′.

2.6. Immunohistochemistry

Samples from the small bowels were fixed in 10% buffered formalin. Immunohistochemical staining was performed using the immunoperoxidase technique. Paraffin sections (4 μm thick) were dewaxed in graded ethanol and rinsed twice with PBS. Endogenous peroxidase activity was blocked for 30 min at 37°C using a methanol/peroxide solution (0.03%). Nonspecific reactions were blocked by incubating the sections in a solution containing 1.5% normal goat serum in PBS. The sections were then incubated overnight at 4°C with a 1:200 dilution of goat polyclonal anti-IL-15 and 1:100 of goat polyclonal anti-IL-7 antibodies. The samples were then rinsed three times with PBS and incubated at room temperature for 1 h with the appropriate dilution of peroxidase-conjugated secondary antibodies. After three rinses with PBS, the sections were stained with 3,3-diaminobenzidine substrate (Sigma).

2.7. Statistical analysis

Results are expressed as means±S.D. Two-tailed Student's *t* test was used to determine the significance of differences. IL-15 protein expression by immunohistochemistry was graded blindly on a scale of 1–3 by one of the authors. The grade for each section was obtained, and comparisons between groups were made using the Mann–Whitney *U* test for unpaired data. All results were generated using SPSS statistical software (SPSS Inc., Chicago, IL). A P value <.05 was considered significant.

3. Results

3.1. n-3 PUFA altered the phenotype of IEL

Differences between IEL subpopulations in the CO group and the FO group were analyzed by FACS. Typical results are presented in Fig. 1. The mean number of each IEL subpopulation from five rats is summarized in Table 1. IELs were predominantly TCRαβ cells supplemented by small numbers of TCRγδ, NK and NK T cells. Proportions of CD8αα+TCRαβ+, CD28+/CD3+ and CD4+CD8+CD3+ IELs were markedly decreased in the FO group. CD8αβ+TCRαβ+ were markedly increased in the FO group. There were no significant differences in the proportions of TCRαβ+CD3+, TCRγδ+CD3+, NKR-P1A-+CD3+ and CTLA-4+CD3+ between the two groups.

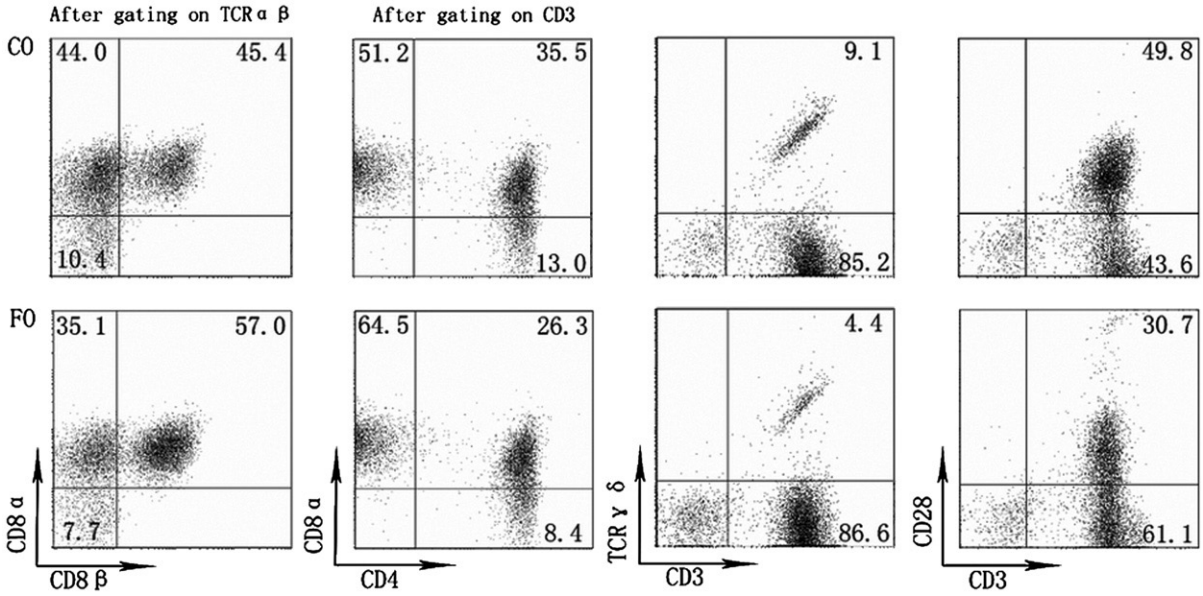

Fig. 1. Analysis of the IEL phenotype of the CO group and the FO group. Representative data from five independent experiments are shown. The cell surface markers used are indicated at the bottom left corners. Percentages of subsets are shown in the respective fractions. Groups are indicated on the left. The two leftmost panels were positively gated on TCRαβ and CD3, respectively.

Table 1

Cell population	CO group (%)	FO group (%)
CD4−CD8α+	60.5±6.2	65.3±5.6
CD4+CD8α+	29.6±4.6	23.4±3.6 *
CD4+CD8α−	9.4±2.4	10.5±2.1
TCRαβ+CD8α+CD8β− [a]	42.0±1.9	34.2±4.1 **
TCRαβ+CD8α+CD8β+ [a]	41.6±4.1	53.4±3.2 **
TCRαβ+	93.7±3.2	96.3±1.6
TCRγä+	7.8±3.2	5.4±1.6
NKR−P1A+CD3− [b]	6.0±1.2	6.3±0.3
CTLA-4+	7.6±4.1	4.2±2.1
CD28+	44.4±5.4	35.6±5.0 *

n=5 per group. Data are shown as the percentage of cells with respect to the total number of CD3+ cells.

[a] The values were calculated by multiplying the percentage of CD8α+CD8β− or CD8α+CD8β+ cells gating on TCRαβ+ and the percentage of TCRαβ+ cells gating on CD3+, given the fact that all TCRαβ+ cells are CD3+.

[b] The value was the ratio of the percentage of NKR−P1A+CD3− cells to that of CD3+ cells.

* P<.05 compared with the value of the CO group.

** P<.01 compared with the value of the CO group.

3.2. IEL mRNA expression of TNF-α, IFN-γ, IL-4 and IL-10 was significantly decreased in the FO group

n-3 PUFA significantly decreased the expression of mRNA for TNF-α, IFN-γ, IL-4 and IL-10, whereas the expression of mRNA for TGF-β1 was increased, although not significantly (Fig. 2). Fish oil diet induced a significant increase in the ratio of IFN-γ to IL-4 (8.31±1.73 and 15.98±3.09, respectively, P=.003).

3.3. Histology

To further determine the mechanism by which n-3 PUFA influences the differentiation of IEL, we examined the expression of IL-7 and IL-15 on intestinal tissue by immunohistochemistry. The expression of IL-7 and IL-15 is moderately expressed on mucosa in both groups. n-3 PUFA significantly decreased the expression of IL-15 while IL-7 remained unchanged (Figs. 3 and 4).

3.4. Ileum mRNA expression of IL-15 was significantly decreased in the FO group

n-3 PUFA significantly decreased the expression of mRNA for IL-15 (Fig. 5).

4. Discussion

In this study, we found that long-term fish oil diet resulted in a parallel decrease of CD4+CD8α+ IEL cells and TCRαβ+CD8α+CD8β− IEL cells (from 29.6% to 23.4% and from 42.0% to 34.2%, respectively). This result was supported by the view that majority of CD4+CD8α+ double-positive IELs express CD8α/α but not CD8α/β [18–20]. It is a logical assumption that they belong to the same population, which is CD4+CD8α+CD8β−TCRαβ+. As the majority subset of rat type b IEL, TCRαβ+CD8α+CD8β− and CD4+CD8+ double-positive cells play an important role in the IEL regulatory function [6,21–23].

As the innermost layer of GALT, IEL is continuously stimulated by environmental antigens even in physiological conditions. Considering that any culturing process will influence the cytokine expression profile, we measured the mRNA expression of freshly isolated IEL. Our results demonstrated that rat IELs spontaneously secrete comparable levels of TGF-β1, TNF-α, IFN-γ, IL-4 and IL-10. Cytokines secreted by T lymphocytes play an important role in the regulation of gut immune responses and in the pathogenesis of intestinal inflammation [24,25]. The relationship between IEL-secreted cytokines and mucosal immunity was explored in various pathological conditions. Reconstitution of severe combined immunodeficiency mice recipients with intraepithelial TCRαβ+CD8α+CD8β− T cells prevents colitis and does so in an IL-10-dependent fashion [26]. Leon et al. [7] found a tendency toward IL-10 production in association with asymptomatic situations in celiac disease. Under conditions of total parenteral nutrition, villous atrophy and increased IEC apoptosis were associated with declined IEL expression of IL-10 and increased expression of IL-4, TGF-β1, TNF-α and IFN-γ [27].

The balance between pro- and anti-inflammatory cytokines has been suggested to be of importance in various inflammatory disorders [28–32]. Here, we used the ratio of IFN-γ to IL-4 as a marker of inflammatory balance, which showed a significant increase, and thus, n-3 PUFA feeding decreases both proinflammatory effect and anti-inflammatory effect to a proinflammatory direction. The literature concerning effects of n-3 PUFA on inflammation is controversial. It is overall believed to have anti-inflammatory properties [33,34]. However, the proinflammatory effect

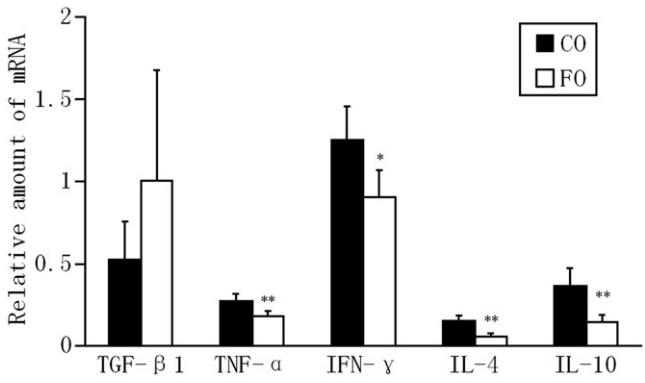

Fig. 2. Real-time PCR analysis of cytokine expression in intestinal IELs. RNA was prepared from the IEL of the FO group and the CO group and subjected to reverse transcription followed by real-time PCR, as described in Section 2. Results are expressed as the ratio of the number of copies of cytokine genes to the number of copies of the β-actin gene multiplied by 10^{-1}. Each bar represents the results of the mean of four animals in the CO group and five animals in the FO group. *P<.05, **P<.01 compared to the CO group, as determined by two-tailed Student's t test.

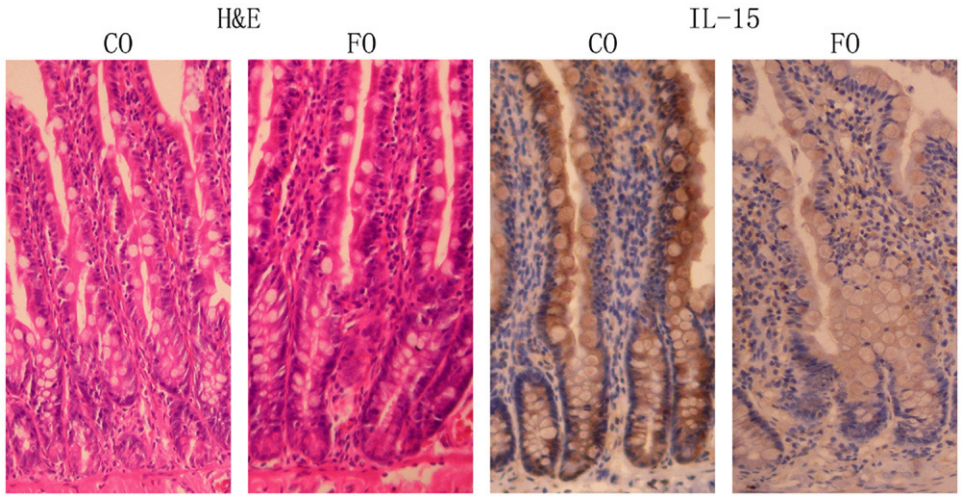

Fig. 3. Histological changes in the ileum of both groups. The first two panels show H&E staining, while the last two panels show immunohistochemical staining of IL-15. Groups are indicated at the top of each panel.

of n-3 PUFA is also documented in various conditions. Holm et al. [31] found that in heart transplant recipients treated with long-term n-3 fatty acids, plasma levels of TNF-α increased and those of IL-10 decreased, resulting in a rise in TNF-α/IL-10 ratio. This result was coincident with that of Petursdottir et al. [35] who found that fish oil increased TNF-α expression and decreased IL-10 expression by peritoneal macrophages after ex vivo stimulation with lipopolysaccharide. In our study, we also found an increased expression of TGF-β1, although not significantly (P=.22). This could be attributed to the immunosuppressive effect of n-3 PUFA. TGF-β1 is known as an anti-inflammatory cytokine [36,37]. Previous studies have shown that low plasma n-3 PUFA level is associated with high TGF-β expression in a normal population [36]. This up-regulatory mechanism takes part in the protective effect of fish oil to mice experiencing autoimmune disease [38]. The significance of our study is that it provides a certain cytokine expression profile of rat IEL in physiological conditions. From our results, it is difficult to get an absolute conclusion of proinflammatory or anti-inflammatory property. This may depend on the amount of n-3 PUFA administered or on the time-dependent cytokine expression alteration.

As potential lymphoid growth factors, IL-7 and IL-15 could help in immune recovery [1,39]. They were shown to

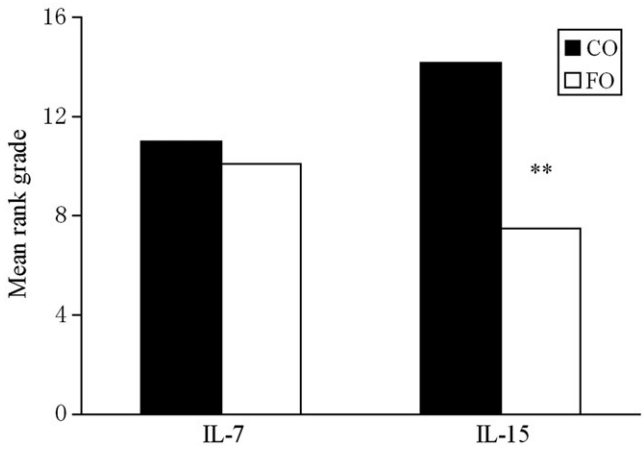

Fig. 4. IL-7 and IL-15 expression in the epithelium of the intestines in the two groups. The protein expression was graded blindly on a scale of 1–3 by one of the authors. The grade for each section was obtained, and comparisons between groups were made. Each bar represents the results of the mean rank grade of 9 animals in the CO group and 11 animals in the FO group. **P<.01 compared to the CO group, as determined the Mann–Whitney U test for unpaired data.

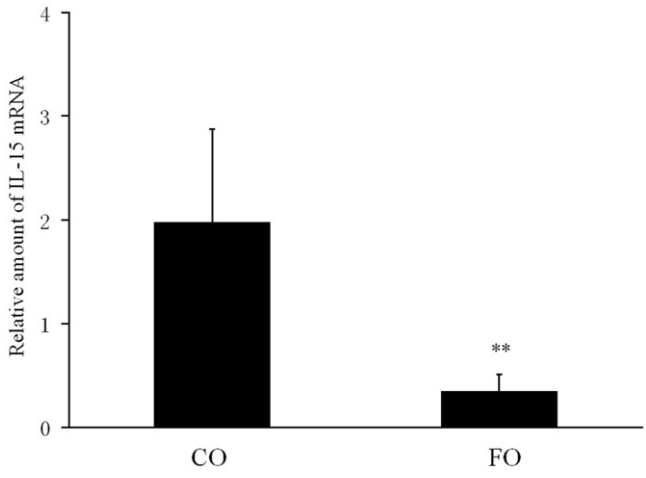

Fig. 5. Real-time PCR analysis of IL-15 expression in the ileum sample. RNA was prepared from the samples of the FO group and the CO group and subjected to reverse transcription followed by real-time PCR, as described in Section 2. Results are expressed as the ratio of the number of copies of the IL-15 gene to the number of copies of the β-actin gene multiplied by 10^{-1}. Each bar represents the results of the mean of five animals in each group. **P<0.01 compared to the CO group, as determined by two-tailed Student's t test.

exhibit an immune-stimulating capacity closely related to various conditions including cancer, HIV infection and other inflammatory processes [40–43]. In mucosal immunity, these two ILs are thought to be the regulatory factor to IEL differentiation by IEC [3–5]. Kaneko et al. [4] reported that IEC-derived IL-15 could promote the development of TCRαβ+CD8α+CD8β− IEL. Yu et al. [44] demonstrated that the introduction of IL-15 transgene into MyD88(−/−) mice partly restored the numbers of TCRαβ+ CD8α+CD8β− and TCRγδ+ IELs, which were reduced because of the lack of MyD88 and IEC-derived IL-15. Our data support these results. TCRαβ+CD8α+CD8β− cells significantly decreased in the FO group. TCRγδ+ also decreased, although not significantly (P=.179). It could be concluded that n-3 PUFA influences the IEL phenotype and cytokine gene profile possibly by decreasing the expression of mucosal IL-15. A previous in vitro experiment [45] showed that immunosuppressants like cyclosporine (CsA), rapamycin and dexamethasone could not inhibit the production of IL-15 by kidney tubular epithelial cells. An in vivo study in our laboratory also showed that long-term CsA had no effects on the production of IL-15 or IL-7 by rat intestinal mucosa (unpublished data). IL-15 was shown to take part in IL-2-independent clinical rejection [46]. Li et al. [47] demonstrated that there was a strong correlation between acute renal graft rejection and IL-15 expression. The inhibition of mucosal IL-15 production strongly suggests the immunosuppressive function of n-3 PUFA on intestinal mucosa.

In summary, we found that dietary n-3 PUFA could down-regulate CD4+CD8+ regulatory cells and CD28+ cells in rat intestinal IEL. This is associated with the down-regulation of intestinal IL-15 expression. Decreased expression of IEL-derived TNF-α, IFN-γ, IL-4 and IL-10 could also be observed. Inhibition of IL-15 makes n-3 PUFA attractive in immunosuppressive therapy. Considering the extremely conflicting data in different physiological and pathological conditions, further experiments using special pathological models would be beneficial.

Acknowledgments

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