

RESEARCH ARTICLE

Effects of *n*-3 PUFA on the CD4⁺ type 2 helper T-cell-mediated immune responses in Fat-1 mice

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Scope: It has been suggested that *n*-3 PUFA can be used as a preventive or therapeutic strategy to control allergic asthma. But little is known about the exact mechanisms by which *n*-3 PUFA modulates it. Here, the effects of elevated *n*-3 PUFA on ovalbumin (OVA) induced airway inflammation were investigated using Fat-1 transgenic mice that can convert *n*-6 PUFA to *n*-3 PUFA endogenously.

Methods and results: First, we tested whether Fat-1 expression modulates CD4⁺ T-cell activation, proliferation, and differentiation in vitro and found that the Fat-1 expression attenuated all of these CD4⁺ T-cell responses by suppression of T-cell receptor mediated signaling and cytokine-mediated phosphorylation of STATs. When the Fat-1 mice were sensitized and challenged with the OVA, they showed a significant decrease in the recruitment of inflammatory cells into airway, the production of Th2 cytokines, eotaxin, and mucin in the lung, and the concentration of OVA-specific IgE in the serum. Furthermore, the differentiation of CD4⁺ T cells into Th2 was also decreased in the spleen of Fat-1 mice.

Conclusion: Our results showed that an elevated level of *n*-3 PUFA was effective in preventing allergic airway inflammation by modulating the activation and differentiation of CD4⁺ T cells in Fat-1 mice.

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1 Introduction

Allergic asthma is associated with a variety of immune cells and mediators such as cytokines and chemokines. Studies on allergic asthma have been conducted using human patients and animal experimental models, and these studies

have shown that CD4⁺ type 2 helper T cells (Th2 cells) produce inflammatory cytokines such as interleukin (IL)-4, IL-5, and IL-13 [1–3]. These inflammatory cytokines are involved in the activation of eosinophils and mast cells and the production of immunoglobulin (Ig) E, thus inducing the immunological mediators that are required to develop the airway hyperreactivity and remodeling. Th2 cytokines play a key role in the pathophysiology of allergic asthma; IL-4 promotes the production of IgE by B cells, IL-5 derives eosinophil differentiation/survival and recruitment to the lung, while IL-13 is thought to mediate the mucus secretion, airway remodeling, and hyperresponsiveness [4]. In addition, according to recent studies, IL-17-secreting Th17 cells enhance neutrophil

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Abbreviations: AHR, airway hyper-responsiveness; CFSE, carboxyfluorescein diacetate succinimidyl ester; OVA, ovalbumin; PUFA, polyunsaturated fatty acids; TCR, T-cell receptor; WT, wild-type

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infiltration and promote eosinophilic airway inflammation mediated by Th2 cells [5]. It can, therefore, be inferred that the modulation of Th2 and Th17 cells and their responses may offer a novel therapeutic approach to control allergic asthma.

Extensive studies demonstrated that dietary *n*-3 polyunsaturated fatty acids (PUFAs), including eicosapentaenoic acid and docosahexaenoic acid in particular, can have various effects on the immune and inflammatory responses [6]. Dietary consumption of *n*-3 PUFA is associated with a lower incidence of asthma and a preventive or therapeutic effect on asthma [7–9]. *n*-3 PUFA affects the inflammatory immune reactions by modulating CD4⁺ T-cell-mediated immune responses. Of note, *n*-3 PUFA suppresses the activation and proliferation of T cells and the differentiation of Th1 cells through an inhibition of the clonal expansion, IL-2 expression, and IL-2 receptor- α (IL-2R α , CD25) mRNA transcription [10]. It has been also demonstrated that *n*-3 PUFA modulates lipid rafts and the localization and activation of signaling proteins at the immunological synapse in mouse T cells [10–13]. A recent study has revealed that *n*-3 PUFA modulates the early Ca²⁺-mediated events following the interaction of T cells with antigen-presenting cells [14]. But it remains unclear whether *n*-3 PUFA modulates CD4⁺ T-cell differentiation and molecular mechanisms by which *n*-3 PUFA suppress CD4⁺ T-cell differentiation.

Mammals cannot produce *n*-3 PUFA from the major *n*-6 PUFA that is present in the diet due to a lack of desaturase. The eicosapentaenoic acid and/or docosahexaenoic acid should, therefore, be included in the diet to examine their physiological effects in an animal experimental model. The conversion from *n*-6 PUFA to *n*-3 PUFA can be mediated by *n*-3 fatty acid desaturase encoded by the Fat-1 gene in *Caenorhabditis elegans* [15]. In transgenic mice expressing the Fat-1 gene, there are constitutively higher levels of *n*-3 PUFA in the organs and tissues despite a lack of dietary *n*-3 PUFA. The Fat-1 mouse model would be valuable in examining the physiological role and molecular mechanisms of *n*-3 PUFA in vivo without dietary supplements.

Given the above background, we used the Fat-1 mice to examine the effects of abundant *n*-3 PUFA on Th2-cell-mediated immune responses in ovalbumin (OVA) induced allergic airway inflammation.

2 Materials and methods

2.1 Animals

The Fat-1 transgenic mice were housed in a laminar flow cabinet and maintained on AIN-93M diets containing 10% corn oil ad libitum (Supporting Information Table S1). Mice were aged 7–8 wk old at the start of each experiment. All experimental animals used in this study were maintained under the protocol approved by the Institutional Animal Care and Use Committee at Chonbuk National University (CBU 2011–0022).

2.2 CD4⁺ T-cell preparation

Single-cell suspensions were prepared from lymph nodes and spleens from two to three mice and CD4⁺ T cells were isolated by using Dynabeads FlowComp mouse CD4⁺ T-cell isolation kit (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. Purity of CD4⁺ T-cell populations was approximately 95%, as assessed by flow cytometry performed on Accuri (BD Biosciences, San Jose, CA, USA).

2.3 CD4⁺ T-cell proliferation assay

Anti-mouse CD3 monoclonal antibody (eBioscience, San Diego, CA, USA) at 1 μ g/mL was allowed to adhere in 96-well flat bottom plates at 4°C in 100 μ L DPBS. CD4⁺ T cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) according to the manufacturer's instructions (eBioscience). CFSE-labeled cells were cultured in uncoated or anti-CD3 antibody-coated 96-well plates with or without anti-mouse CD28 antibody (eBioscience). Plates were incubated for 72 h at 37°C in 5% CO₂ incubator and cell proliferation was evaluated by flow cytometry analysis performed on Accuri (BD Biosciences).

2.4 Western blot analysis for CD4⁺ T cells

To detect tyrosine phosphorylation, LCK, ZAP70, and NF- κ B activation upon T-cell stimulation, CD4⁺ T cells were stimulated with anti-CD3/CD28 antibodies for 2 or 15 min. To measure activation of STAT-1 or STAT-6 upon cytokine treatment, CD4⁺ T cells were stimulated with recombinant IL-12 (10 ng/mL) or IL-4 (10 ng/mL) for 2 or 15 min. Then, cell lysates were prepared and analyzed by Western blotting using anti-pTyr (4G10), anti-pp65, p65, I κ B- α , anti-LCK, anti-pLCK, anti-Zap70, anti-pZap70, STAT-1, pSTAT-1, STAT-6, pSTAT-6, and β -actin antibodies. All antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA), except anti-pTyr antibody (Millipore, Billerica, MA, USA). Recombinant mouse IL-12 and IL-4 were obtained from eBioscience.

2.5 CD4⁺ T-cell differentiation

CD4⁺ T cells were activated with plate-bound anti-CD3 antibody (1 μ g/mL) and soluble anti-CD28 antibody (2 μ g/mL). The cells were cultured under Th1 (5 ng/mL IL-12 and 10 μ g/mL anti-IL-4 antibody) or Th2 (10 ng/mL IL-4, 1 μ g/mL anti-IL-12, and 5 μ g/mL anti-IFN- γ antibody) polarization conditions in 2 mL RPMI complete media. Four days later, cells were stimulated with PMA and ionomycin in the presence of brefeldin A (eBioscience) for 4 h and subsequently stained for intracellular cytokines as described in Supporting Information data. All cytokines and recombinant proteins used are purchased from eBioscience.

2.6 Cell surface staining and intracellular cytokine staining for flow cytometry

Single-cell suspensions were stained with CD4-PECy5.5, CD25-PE, and CD69-FITC antibodies (eBioscience) for 30 min on ice and washed with an FACS buffer (2% FBS in PBS) three times. Then, cells were analyzed on Accuri (BD Biosciences). For intracellular cytokine detection, CD4⁺ T cells were cultured in triplicate at 1×10^6 cells per well in a 48-well plate and stimulated with plate-bound anti-CD3 and soluble anti-CD28 antibodies. After 48 h, cells were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μ M ionomycin in the presence of brefeldin A (eBioscience). After 4 h, cells were fixed using 4% paraformaldehyde solution. Cells were permeabilized with 1 $\times$ permeabilization solution (eBioscience) and then stained with fluorochrome-conjugated antibodies against cytokines. Stained cells were evaluated by flow cytometry analysis performed on Accuri (BD Biosciences). All antibodies and recombinant cytokines were purchased from eBioscience or Biolegend (San Diego, CA, USA).

2.7 Immunization and challenge in lung tissue

Mice were immunized i.p. with 20 μ g of OVA (grade V; Sigma-Aldrich, St Louis, MO, USA) plus 2.25 mg aluminum hydroxide adjuvant on day 0 and OVA alone without alum on day 14. The immunized mice were exposed to aerosolized OVA on days 28, 29, and 30. Aerosolization of OVA was performed using a chamber that was adapted for mice. Animals were exposed to OVA (3%) using an ultrasonic nebulizer (NE-U12, Omron; output 0.8 mL/min) for 30 min in a Plexiglas exposure chamber (24.5 $\times$ 40.5 $\times$ 15.0 cm). Control animals were neither immunized nor challenged with OVA.

2.8 Bronchoalveolar lavage (BAL)

BAL was performed at the time indicated after the last airway challenge. Mice were anesthetized and the trachea was cannulated while gently massaging the thorax. Lungs were lavaged with 0.8 mL of PBS. The BAL fluid (BALF) was collected and the number of cells in a 100 μ L aliquot was determined using a hemocytometer. The remaining sample was centrifuged, and the supernatant was stored at -70°C until cytokine assays. The pellet was resuspended in PBS and a cytospin preparation of BAL cells was stained with Diff-Quik (International Reagents, Kobe, Japan). The different cell types were enumerated based on their morphology and staining profile.

2.9 Cytokine assays

IL-13 (Invitrogen), IL-5, eotaxin (R&D Systems, Minneapolis, MN, USA), IL-2, IL-4, IL-17, IL-33, and IFN- γ (eBioscience)

in BALF or cell culture media and OVA-specific IgE (eBioscience) in serum were determined by ELISA.

2.10 Real-time RT-PCR analysis

The RNA was isolated from lungs or CD4⁺ T cells and real-time RT-PCR analysis was performed as described in Supporting Information Materials and methods Table 1.

2.11 Histological studies

Lungs were fixed with 10% formalin, and the tissues were embedded in paraffin. Fixed tissues were cut at a thickness of 4 μ m, placed on glass slides, which was followed by the deparaffinization. Sections were stained with H&E dye for a light microscopy. For the detection of mucin, tissues were stained with Periodic Acid-Schiff dye and counterstained with hemotoxylin.

2.12 Western blot analysis of lung tissues

Lung tissues were homogenized in T-PER mammalian protein extraction reagent containing protease and phosphatase inhibitors (Pierce, Rockford, IL, USA). The homogenates, containing 30 μ g of protein, were used for Western blot analysis as described in Western blot analysis for CD4⁺ T cells. Anti-GATA3 was purchased from Thermo Fisher Science (Rockford, IL, USA).

2.13 Ex vivo stimulation of splenocytes for cytokine assay

Spleens were collected from euthanized mice on the final day of asthma according to the experimental protocol. Single-cell suspensions were prepared and then cultured in RPMI 1640 medium containing 10% FCS and antibiotics, in the presence of 50 μ g/mL OVA. Cytokines in the supernatant were determined after 48 h by ELISA.

2.14 Ex vivo restimulation and intracellular cytokine detection

For intracellular cytokine detection, single-cell suspensions from spleen were cultured in triplicate at 1×10^6 cells/well in a 96-well U-bottom plate and stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μ M ionomycin in the presence of brefeldin A (eBioscience). After 4 h, cells were stained with CD4-PECy5.5 antibody, washed twice with PBS, and then fixed using 1% paraformaldehyde solution. Cells were permeabilized with 1 $\times$ permeabilization solution (eBioscience) and then stained with fluorochrome conjugated

Table 1. Sequences and accession numbers for primers (forward, FOR; reverse, REV) used in real-time RT-PCR

Gene	Sequences for primers	Accession number
IL-4	FOR: TCGGCATTTTGAACGAGGTC REV: GAAAAGCCCCGAAAGAGTCTC	NM.021283
IL-5	FOR: TCACCGAGCTCTGTTGACAA REV: CCACACTTCTCTTTTGGCG	NM.010558
IL-13	FOR: CGCAAGGCCCCCTACTAC REV: AAAGTGGGCTACTTCGATTTTGG	NM.008355
IL-33	FOR: CAAAGAAGTTTGCCCCATGT REV: AAGGCAAAGCACTCCACAGT	NM.133775
Eotaxin	FOR: AGAGGCTGAGATCCAAGCAG REV: CAGATCTCTTTGCCCAACCT	NM.011330
IFN- γ	FOR: TTGGCTTTGCAGCTCTTCCT REV: TGA CTGTGCCGTGGCAGTA	NM.008337
FOXP3	FOR: GAAACAGCACATTCCCAGAGTTC REV: ATGGCCCAGCGGATGAG	NM.054039
GATA-3	FOR: GGAAACTCCGTCAGGGCTA REV: AGAGATCCGTGCAGCAGAG	NM.008091
T-bet	FOR: TTCCCATTCCTGTCCTTCAC REV: CCACATCCACAAACATCCTG	AF_241242
GAPDH	FOR: CGTCCCGTAGACAAAATGGT REV: TTGATGGCAACAATCTCCAC	NM.008084

antibodies against cytokines in the presence of Fc ϵ III/II receptor. Stained cells were evaluated by flow cytometry analysis performed on Accuri (BD Biosciences). All antibodies and recombinant cytokines were purchased from eBioscience or Biolegend.

2.15 Statistical analysis

Data are expressed as mean $\pm$ SEM. Statistical comparisons were performed using one-way ANOVA followed by the Fisher test. The significance of differences between the groups was determined using Student's unpaired *t*-test. A value of *p* < 0.05 was considered statistically significant.

2.16 Additional methods

Detailed methods are provided in the Supporting Information Materials and methods.

3 Results

3.1 Fat-1 expression suppresses T-cell activation and proliferation in vitro

Previous studies have shown that dietary *n*-3 PUFA has inhibitory effects on T-cell activation and proliferation in

vitro [11, 12, 16, 17]. We, therefore, examined the effects of increased *n*-3 PUFA contents in CD4⁺ T cells via Fat-1 transgene expression on T-cell activation by analyzing CD69 and CD25 expression. CD4⁺ T cells were unstimulated or stimulated with anti-CD3 antibody alone or anti-CD3/CD28 antibodies together. As shown in Fig. 1 and Supporting Information Fig. 1, Fat-1 transgene expression significantly attenuated T-cell activation and inhibited the production of cytokines such as IL-2, IL-4, IL-13, IL-33, and IFN- γ as compared with the wild-type (WT) mice. Then, we evaluated T-cell proliferation by CFSE dilution assay and found that Fat-1 expression suppressed cell proliferation triggered by T-cell receptor (TCR) engagement and CD28 costimulation (Fig. 2). These results are in agreement with reduced T-cell growth factor (IL-2) production and its high affinity receptor (CD25) expression on the T-cell surface (Fig. 1).

3.2 Fat-1 expression attenuates T-cell activation pathway in CD4⁺ T cell

To examine the molecular mechanisms by which the T-cell activation and proliferation are attenuated, we compared the TCR-mediated tyrosine phosphorylation between the WT and Fat-1 transgenic CD4⁺ T cells. As shown in Fig. 3A, the overall degree of tyrosine phosphorylation triggered by anti-CD3/28 antibodies was lower in CD4⁺ T cells derived from Fat-1 mice and reduced phosphorylation of LCK and Zap70, TCR

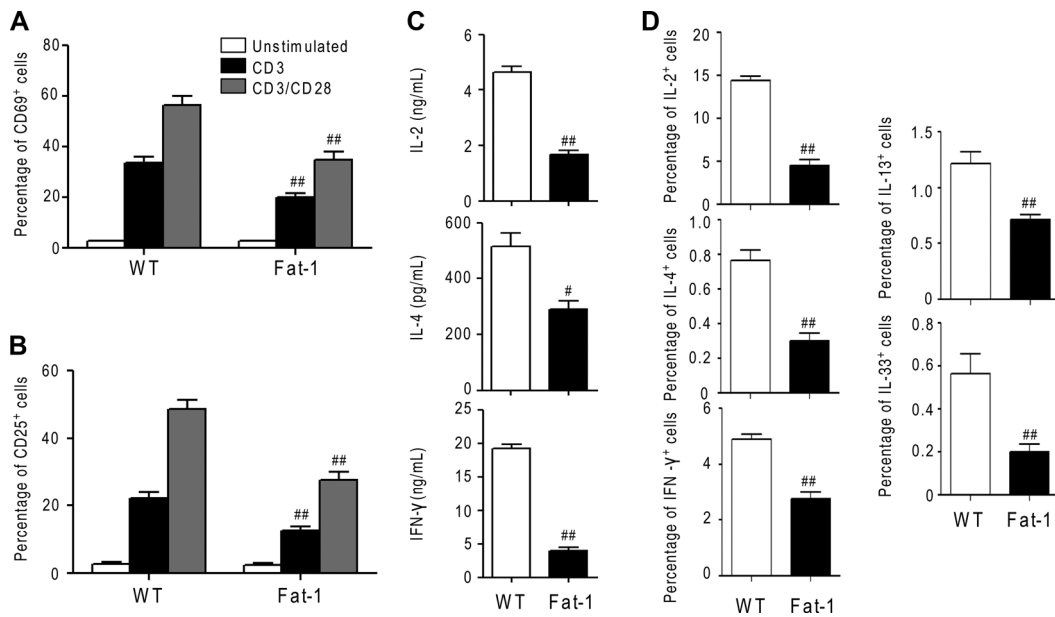


Figure 1. CD4⁺ T-cell activation and cytokine production in vitro. CD4⁺ T cells were activated with plate-bound anti-CD3 antibody with or without soluble anti-CD28 antibody for 24 h. T-cell activation was determined by flow cytometry analysis with anti-CD69 antibody (A) and anti-CD25 antibody (B) and percentage of the cells expressing each molecule was calculated. (C) IL-2, IL-4, and IFN- γ in the cell culture media were measured with ELISA. (D) After 2 days of culture, cells were stimulated with PMA and ionomycin plus brefeldin A for additional 4 h, and then intracellular cytokine content was evaluated. The percentages of cytokine-expressing cells are shown. Results of three independent experiments are expressed as mean $\pm$ SEM. [#] $p < 0.05$, ^{##} $p < 0.01$ versus WT.

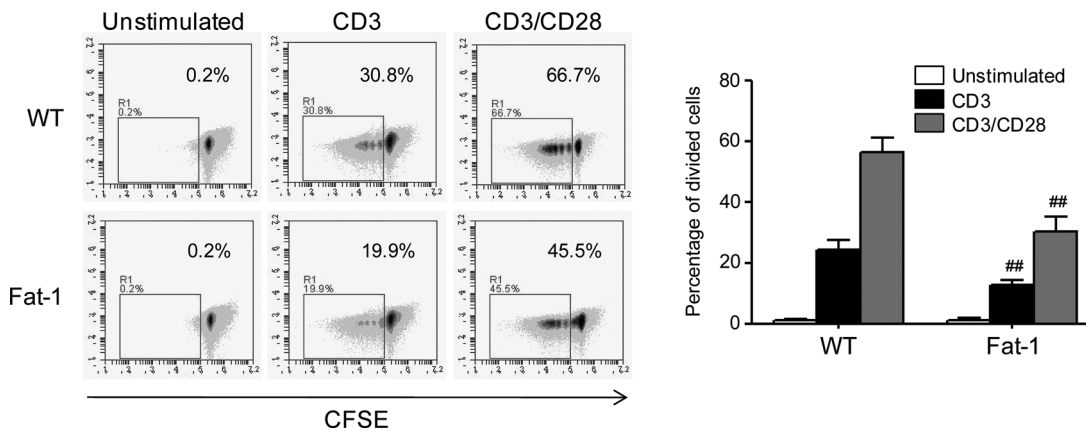


Figure 2. CD4⁺ T-cell proliferation. The level of CD4⁺ T-cell proliferation was determined by flow cytometry analysis with CFSE labeling. CFSE-labeled CD4⁺ T cells were stimulated by plate-bound anti-CD3 antibody with or without soluble anti-CD28 antibody for 4 days and percentage of divided cells were analyzed by flow cytometry. Results of three independent experiments are expressed as mean $\pm$ SEM. ^{##} $p < 0.01$ versus WT.

proximal signaling molecules, were detected in cells from Fat-1 mice (Fig. 3B). In addition, we specifically analyzed the NF- κ B pathway, one of key signal transduction pathways downstream of TCR. There was a complete inhibition of TCR-triggered phosphorylation of p65 subunit and I κ B- α

degradation in CD4⁺ T cells from Fat-1 mice (Fig. 3C). Since NF- κ B plays a central role in Th-cell activation, cytokine production and survival [18, 19], the defect in Fat-1 transgenic CD4⁺ T cells' response to TCR stimulation is related to the attenuated TCR signaling transduction.

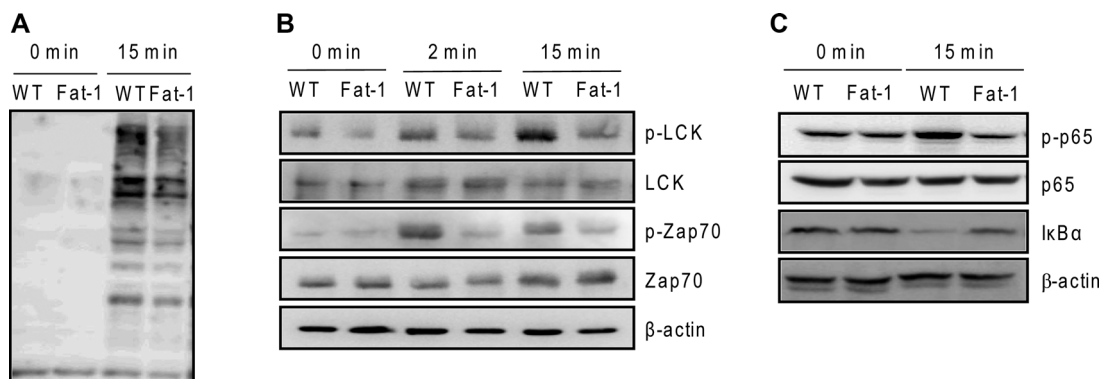


Figure 3. TCR-mediated signal transduction. CD4⁺ T cells isolated from WT or Fat-1 mice were stimulated with plate-bound anti-CD3 and soluble anti-CD28 for indicated periods. Cells lysates were immunoblotted with antibodies against pTyr (A), p-LCK, LCK, p-Zap70, and Zap70 (B) or p-p65, p65, and IκBα (C). Similar results were obtained from three independent experiments.

3.3 Fat-1 expression suppresses T-cell differentiation in vitro

To address whether Fat-1 expression affects CD4⁺ T-cell differentiation into Th1 or Th2, we cultured CD4⁺ T cells from WT and Fat-1 mice under Th1 or Th2 polarization condition for 4 days. IFN-γ and IL-4, hallmark cytokines of Th1 and Th2 differentiation, respectively, were measured by intracellular cytokine staining. CD4⁺ T-cell differentiation into either Th1 or Th2 cells were partially suppressed in CD4⁺ T cells from Fat-1 mice as compared with those of WT CD4⁺ T cells (Fig. 4A). Real-time RT-PCR analysis of Th1/Th2 cytokines also revealed that elevated *n*-3 PUFA in Fat-1 mice inhibited Th-cell differentiation (Supporting Information Fig. 2). Since STAT-1 and STAT-6 play an important role in Th-cell differentiation into Th1 and Th2, respectively, we evaluated the effect of Fat-1 expression on the activation of these signaling molecules. As shown in Fig. 4B, tyrosine phosphorylation of STAT-1 by IL-12 was slightly reduced, but STAT-6 activation triggered by IL-4 was markedly attenuated by elevated *n*-3 PUFA in Fat-1 mice. These results indicate that Fat-1 expression not only suppresses CD4⁺ T-cell activation/proliferation but also modulates CD4⁺ T-cell differentiation into Th1 and Th2.

3.4 Fat-1 mice are protected from OVA-induced allergic airway inflammation

Our in vitro experiments revealed that Fat-1 expression on CD4⁺ T cells introduced T-cell intrinsic defects in activation, proliferation, and differentiation. We, therefore, examined whether Fat-1 mice are less susceptible to CD4⁺ T-cell-mediated asthmatic injury. We used OVA-induced allergic airway inflammation as a murine model of human asthma (Supporting Information Fig. 3). Following OVA immunization and aerosol challenge, we assessed the lungs for inflammatory cell infiltration by analyzing cells in BALF.

While OVA inhalation significantly increased inflammatory cells in BALF from WT mice, there was a significant reduction in the total number of inflammatory cells in Fat-1 mice (Fig. 5A). We further analyzed the types of immune cells infiltrated into the lung by differential counts of BALF cytopsin preparations. The reduction in the total number of the inflammatory cells infiltrated in the lung was largely attributed to a remarkably lower number of eosinophils in Fat-1 mice. Cells in BALF from control mice were mostly macrophages, whereas over 95% cells in BALF from WT mice with OVA immunization and challenge were eosinophils and lymphocytes. BALF from Fat-1 mice contained a much lower percentage of eosinophils and lymphocytes but a higher percentage of macrophages as compared with that of WT mice. Consistently, histological examination of lung tissues revealed intense eosinophilic inflammation surrounding the bronchi and vessels in the lungs of WT mice while Fat-1 mice had almost no inflammation (Fig. 5B).

Since bronchiolar mucin accumulation is one of the important features of chronic asthmatic response in patients [20], we evaluated the amount of mucin produced from bronchial goblet cells. Hyperproliferation of mucus-secreting goblet cells was detected in asthmatic WT mice by Periodic Acid-Schiff staining (Supporting Information Fig. 4). However, airway epithelial mucin levels were significantly reduced in Fat-1 mice. Additionally, measurements of serum IgE revealed a markedly less OVA-specific IgE in Fat-1 mice as compared with WT mice (Fig. 5C). These results indicate that Fat-1 mice are protected from allergen-induced airway inflammation by suppressing eosinophil recruitment and IgE production.

3.5 Fat-1 expression reduces inflammatory cytokines and eotaxin in BALF and lung tissues

Since Fat-1 expression suppressed allergen-induced airway inflammation and mucin production in the airway, we next

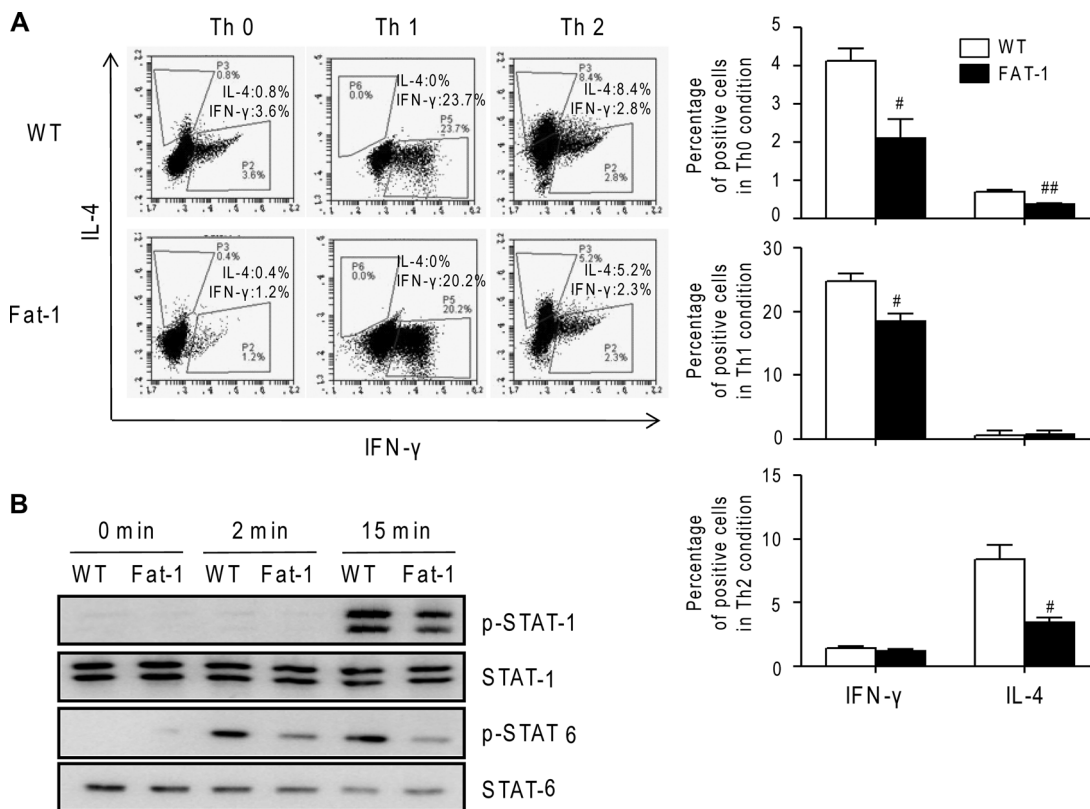


Figure 4. Differentiation of CD4⁺ T cells from Fat-1 transgenic mice under Th0-, Th1-, or Th2 polarization conditions. (A) CD4⁺ T cells from WT or Fat-1 mice were stimulated with plate-bound anti-CD3 antibody and soluble anti-CD28 antibody under Th0-, Th1-, or Th2 polarization condition for 4 days. The differentiated cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 4 h. Then, cells were stained for IL-4 and IFN- γ and analyzed by flow cytometry and the percentages of CD4⁺ cells expressing IFN- γ (Th1) and IL-4 (Th2) were expressed as mean $\pm$ SEM from three independent experiments. # p < 0.05, ## p < 0.01 versus WT. (B) CD4⁺ T cells isolated from WT or Fat-1 mice were stimulated with IL-12 (for STAT-1, 10 ng/mL) or IL-4 (for STAT-6, 10 ng/mL) for indicated periods. Cells lysates were immunoblotted with antibodies against p-STAT-1, STAT-1, p-STAT-6, or STAT-6. Similar results were obtained from three independent experiments.

examined the cytokines and eotaxin production in the BALF. Th2 cytokines such as IL-4, IL-5, IL-13, and IL-33 and IL-17 which is mainly produced by Th17 cells were significantly lower in BALF from Fat-1 mice as compared with WT mice (Fig. 6). Reduced Th2 response in Fat-1 mice was also confirmed by a remarkably lower expression of GATA3 in the lung tissues (Supporting Information Fig. 5). In addition, IFN- γ level was reduced in Fat-1 mice (Fig. 6). Real-time RT-PCR analysis proved similar results. That is, there was a significant decrease in the level of all the inflammatory cytokines in Fat-1 transgenic mice (Supporting Information Fig. 6). Moreover, the eosinophil-recruiting chemokine, eotaxin was downregulated at the protein level in the BALF and mRNA level in the lung tissues from Fat-1 mice (Fig. 6 and Supporting Information Fig. 6). Downregulation of eotaxin was correlated with reduced eosinophil numbers in BALF and eosinophil infiltration into lungs (Fig. 5). Collectively, these results indicate that enriched *n*-3 PUFA efficiently suppressed Th2 cytokines and eotaxin, leading to protection against OVA-induced airway inflammation.

3.6 Fat-1 expression suppresses CD4⁺ T-cell activation and differentiation in spleen

To evaluate the OVA-induced CD4⁺ T-cell response in WT and Fat-1 mice, we assessed CD4⁺ T-cell activation and cytokine production ex vivo. Splenic CD4⁺ T cells from Fat-1 mice were less activated and had a lower percentage of IL-2-producing cells as compared with CD4⁺ T cells from WT mice (Fig. 7A and B and Supporting Information Fig. 7A and B), indicating Fat-1 expression suppressed OVA-induced CD4⁺ T-cell activation in spleen. Moreover, CD4⁺ T-cell differentiation into Th1, Th2, or Th17 was also impaired in Fat-1 mice (Fig. 7C and Supporting Information Fig. 7C and D). Consistently, IL-4 production was significantly reduced in Fat-1 mice when splenocytes were restimulated with OVA ex vivo (Fig. 7D).

Then, we examined whether Fat-1 expression promotes CD4⁺ T cells differentiation into regulatory T cells (Tregs) in OVA-induced allergic airway inflammation condition. Splenocytes from WT and Fat-1 mice showed no difference in

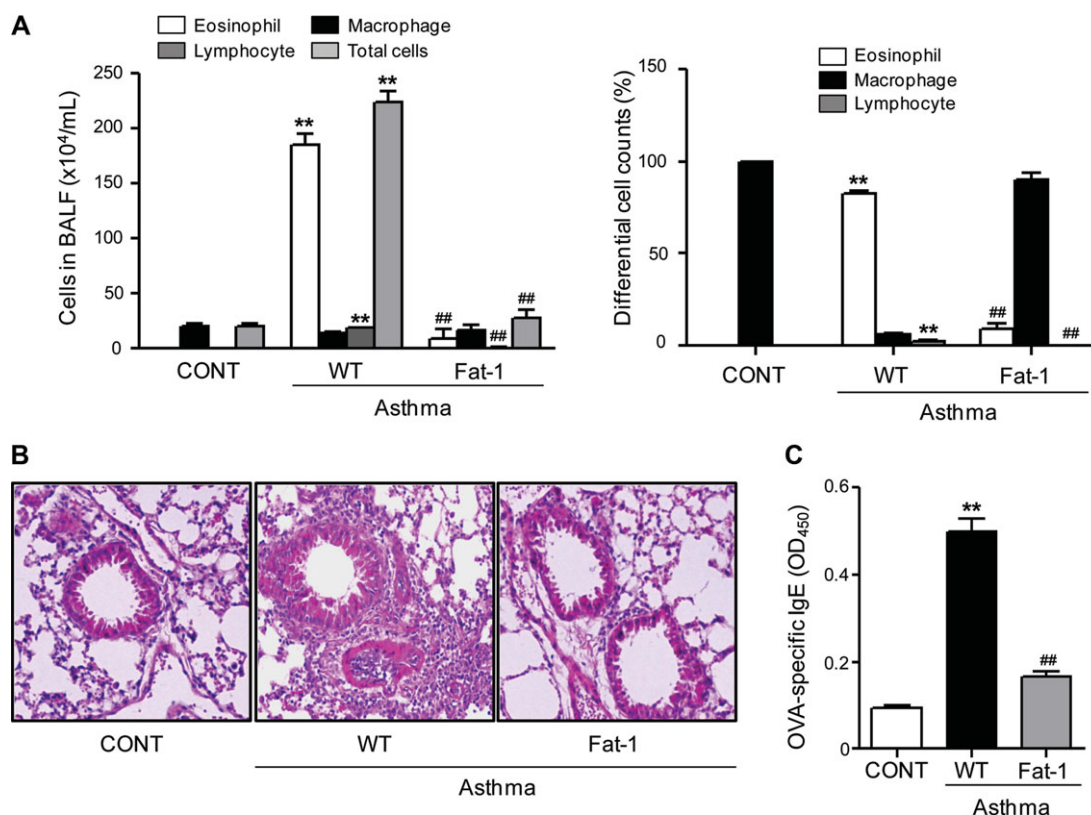


Figure 5. Differential cell counts in BALF, histological evaluation of lung inflammation, and OVA-specific IgE in serum following OVA challenge. (A) Differential cell counts in BALF were analyzed 48 h after the last OVA challenge. The absolute cell numbers of each cell type were counted. The proportions of macrophages, eosinophils, and lymphocytes in BALF were calculated based on differential cell counts. (B) Paraffin-embedded lung sections were prepared 48 h after the last OVA challenge and stained with H&E (100×) and representative photomicrographs of lung sections are shown. Data are representative of three independent experiments. (C) OVA-specific IgE levels in sera were determined using ELISA. Results from three independent experiments with four mice per group are given as mean $\pm$ SEM ($n = 12$). ** $p < 0.01$ versus untreated control; ## $p < 0.01$ versus WT.

the percentage of Tregs after OVA immunization and challenge, and there was also no significant difference in the Foxp3 mRNA level in the lung between the two groups (Supporting Information Fig. 6 and 8), suggesting that Fat-1 expression may not alter Tregs development and differentiation in Th2-mediated inflammation condition.

4 Discussion

It is well known that *n*-3 PUFA-rich fish oil has a protective effect on inflammation-mediated diseases. In the current study, we examined whether enriched *n*-3 PUFA affects T-cell response and Th2-induced allergic airway inflammation using Fat-1 transgenic mice. Bilal et al. [8] have recently shown that Fat-1 transgenic mice are protected from allergic airway responses through an increased synthesis of protectin D1 and resolvin E1 in the lung. Our results provided further immunological mechanisms by which OVA-induced allergic airway inflammation is prevented by the suppression of the activation, proliferation, and differentiation of T cells and

their cytokine production because of an increase in the level of endogenous *n*-3 PUFA in mice.

In normal conditions, the immune system is not responsive to the nonharmful environmental factors such as pollens and dusts by immune tolerance, the functional unresponsiveness of T cells. Allergic asthma occurs with impairment of immune tolerance and the initiation of T-cell response to the nonharmful environmental factors and the production of cytokines [21]. Cytokines secreted by Th2 cells promote B cells to differentiate into plasma cells producing an enormous amount of the allergen-specific IgE. Then, IgE binds to the receptor on the surface of mast cells and thereby induces degranulation of mast cells by cross-linking with allergen. This leads to the release of a variety of molecules such as heparin, histamine, inflammatory cytokines (TNF- α , IL-1, and leukotriens), and chemokines, contributing to further recruiting the immune cells and thereby triggering the occurrence of allergic responses. It can, therefore, be inferred that it is one of the early interventional strategies to prevent or treat allergic asthma by suppressing the activation of CD4⁺ T cells. Recent research trends have focused on the Th2 cells

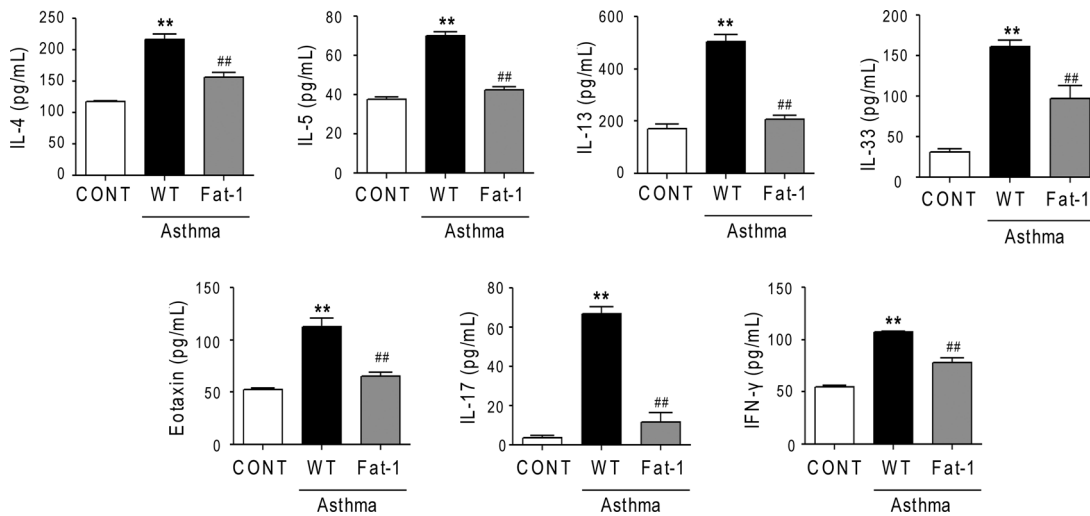


Figure 6. Assessment of proinflammatory cytokines in the BALF of OVA-sensitized mice. BALF were prepared 48 h after the last OVA challenge and various cytokines in BALF were measured by specific ELISA. Results of three independent experiments with four mice per group are expressed as mean $\pm$ SEM ($n = 12$). ** $p < 0.01$ versus untreated control; ## $p < 0.01$ versus WT.

and their products such as IL-4, IL-5, and IL-13 as a novel therapeutic target for asthma [3, 22].

Our results showed that CD4⁺ T cells from Fat-1 mice were not activated as effectively as those from WT mice. It was also shown that the degree of the expression of IL-2 and its receptor, CD25, was relatively lower in CD4⁺ T cells from Fat-1 mice. Presumably, a lower degree of the expression of IL-2 and CD25 might cause a decrease in the degree of the proliferation of CD4⁺ T cells from Fat-1 mice, which might be due to reduced TCR signal transduction as evidenced by lower levels of tyrosine phosphorylation of sig-

naling molecules (LCK and Zap70) and NF- κ B activation. It is also known that dietary *n*-3 PUFA alters the T-cell activation signaling pathways at multiple points as demonstrated by previous studies: lipid raft formation, immunological synapses formation, PTKs, PKC, NF- κ B, NFAT, MAPK, and calcium signaling [10, 11, 16]. These findings indicate that the Fat-1 expression may also target multiple signaling molecules during T-cell activation in addition to NF- κ B.

It has been suggested that *n*-3 PUFA exerts therapeutic and preventive effects on asthma although there is a controversy between the studies [7–9, 23]. A recent study has been

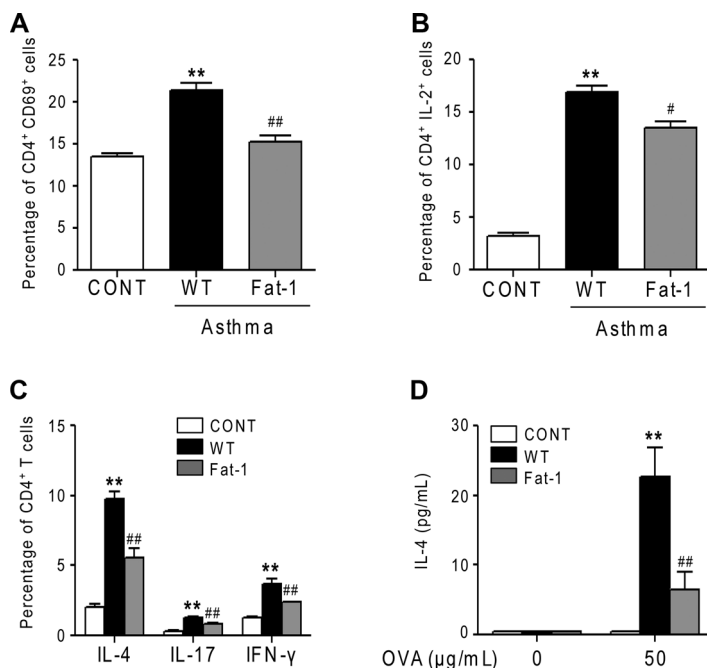


Figure 7. CD4⁺ T-cell activation and differentiation in spleen after OVA immunization and challenge. (A) Splenocytes taken from the mice were analyzed for CD69 expression on CD4⁺ T cells. Splenocytes were stained with anti-CD4 PE-Cy5.5 and anti-CD69-FITC antibodies and analyzed by flow cytometry. Percentage of the cells expressing each molecule was calculated. Splenocytes were restimulated for 4 h with PMA and ionomycin and brefeldin A and analyzed by flow cytometry for intracellular IL-2 (B) or IL-4, IL-17, and IFN- γ (C). The percentages of CD4⁺ cells expressing each cytokine are shown. (D) Splenocytes were restimulated for 48 h with 50 μ g/mL OVA and IL-4 levels in the culture media were determined by ELISA. Results of three independent experiments with four mice per group ($n = 12$) are expressed as mean $\pm$ SEM. ** $p < 0.01$ versus untreated control; # $p < 0.05$, ## $p < 0.01$ versus WT.

conducted in Fat-1 mice, thus demonstrating that the airway inflammation and hyperresponsiveness are reduced with an elevated level of *n*-3 PUFA in the lung tissue in an OVA-induced asthma model [8]. Our results also showed that allergic airway inflammation was suppressed in the Fat-1 mice and suggests that Fat-1 expression affected the activation and differentiation of CD4⁺ T cells, which has previously not been examined in an animal experimental model of asthma using Fat-1 mice. Cytokine assay was performed in the BALF and lung tissue, which showed that the increased level of *n*-3 PUFA in Fat-1 mice might suppress Th1, Th2, and Th17 differentiation. The defect in Th-cell differentiation in Fat-1 mice was also demonstrated by in vitro polarization experiments. This result can be related with lower levels of T-cell activation, CD25 expression, and IL-2 production in CD4⁺ T cells with Fat-1 expression. In addition, decreased tyrosine phosphorylation of STAT-1 and STAT-6 upon IL-12 and IL-4 treatment, respectively provide evidence that Fat-1 expression in CD4⁺ T cells may interfere with Th1-/Th2-cell differentiation through downregulating IL-12- and IL-4-mediated signaling pathways [24]. Th2 cytokines play a key role in the development of allergic asthma. Its lower level in the lung as well as a lower fraction of Th2 cells in the spleen of Fat-1 mice after OVA immunization and challenge can explain the protective effect of Fat-1 expression on airway inflammation. The effect of *n*-3 PUFA on T-cell differentiation is contradictory between the studies. It has shown that dietary *n*-3 PUFA shifts Th1 phenotype to Th2 phenotype by suppression of Th1 development [12, 17, 25]. It is well known that Th1 response is one of the pathogenic factors that are responsible for the occurrence of autoimmune and inflammatory diseases. It is, therefore, presumed that there would be a protective effect of *n*-3 PUFA on the autoimmune and inflammatory diseases when it enhances the Th2 response. Inconsistent with previous reports about dietary *n*-3 PUFA, we and Bilal et al. [8] demonstrated that the Th2 response was not enhanced but suppressed with an elevated level of endogenous *n*-3 PUFA. Presumably, this might be due to not only the difference in an experimental model of the disease regarding whether it is designed based on the Th1 response mediated inflammation or Th2 one but also that in the source of PUFA regarding whether it is dietary or endogenously converted from Fat-1 transgene. The Fat-1 mice have a higher ratio of *n*-3 to *n*-6 PUFA, being approximately 1, although it is almost impossible to achieve this ideal ratio from the dietary *n*-3 PUFA [15]. It is also known that IL-17-producing Th17 cells, as well as Th2 cells, play a role in the pathogenesis of asthma [5, 26]. Our results showed that the degree of Th17-cell response was decreased with the expression of Fat-1, which may also contribute to reduced airway inflammation in the lung. Other studies have also shown that the Fat-1 expression has an inhibitory effect on the differentiation of Th17 cells [27, 28]. Taken together, our results indicate that the allergic immune response might be attenuated by an elevated level of endogenous *n*-3 PUFA through a modulation of the T-cell differentiation.

Tregs are characterized by the expression of lineage-specific transcription factor, Foxp3, and they perform an immunosuppressive function and play a pivotal role in the occurrence of immune-mediated disorders such as type 1 diabetes, multiple sclerosis, rheumatoid arthritis, and asthma [29, 30]. Recent studies have shown that *n*-3 PUFA promotes the development of Tregs in mice, leading to the suppression of inflammatory disorders [31, 32]. We, therefore, examined whether the fraction of Tregs is increased after the induction of allergic airway inflammation in the Fat-1 mice. This showed, however, that an increased level of *n*-3 PUFA in the Fat-1 mice did not affect the expression of Foxp3 mRNA in the lung tissue and the development of CD4⁺Foxp3⁺ T cells in the spleen. These findings indicate not only that the Fat-1 expression is not effective in inducing the production of Tregs but also that Tregs may not be associated with the effects of the Fat-1 expression in preventing the occurrence of OVA-induced allergic inflammation in the lung.

To summarize, our results showed that the Fat-1 transgene expression was effective in inhibiting the occurrence of OVA-induced airway inflammation by suppressing the CD4⁺ T-cell-mediated immune responses at an elevated level of *n*-3 PUFA in mice. In conclusion, our results indicate that the dietary supplementation of *n*-3 PUFA may modulate uncontrolled Th2-driven allergic inflammatory response, having beneficial effects on the prevention and treatment of allergic asthma.

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The authors have declared no conflict of interest.

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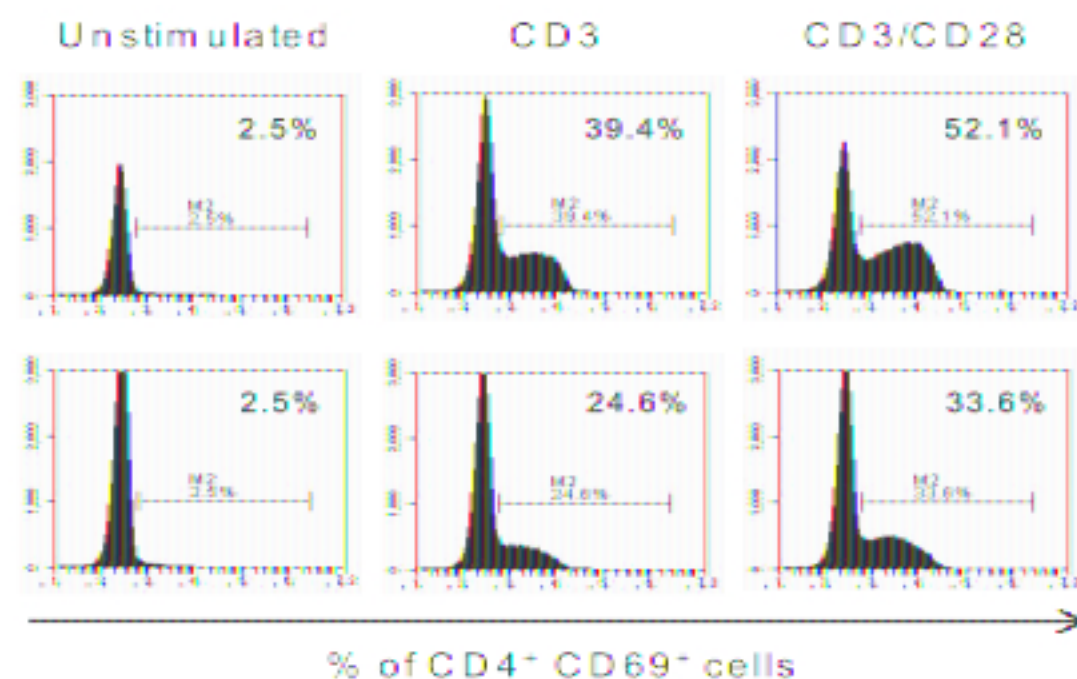
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A

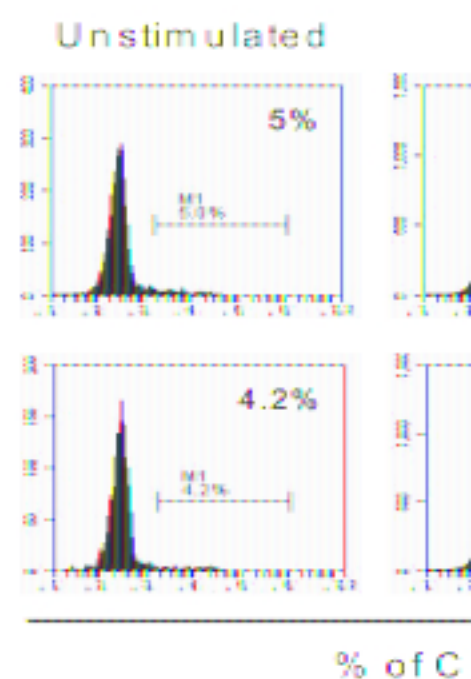
WT

Fat-1

**B**

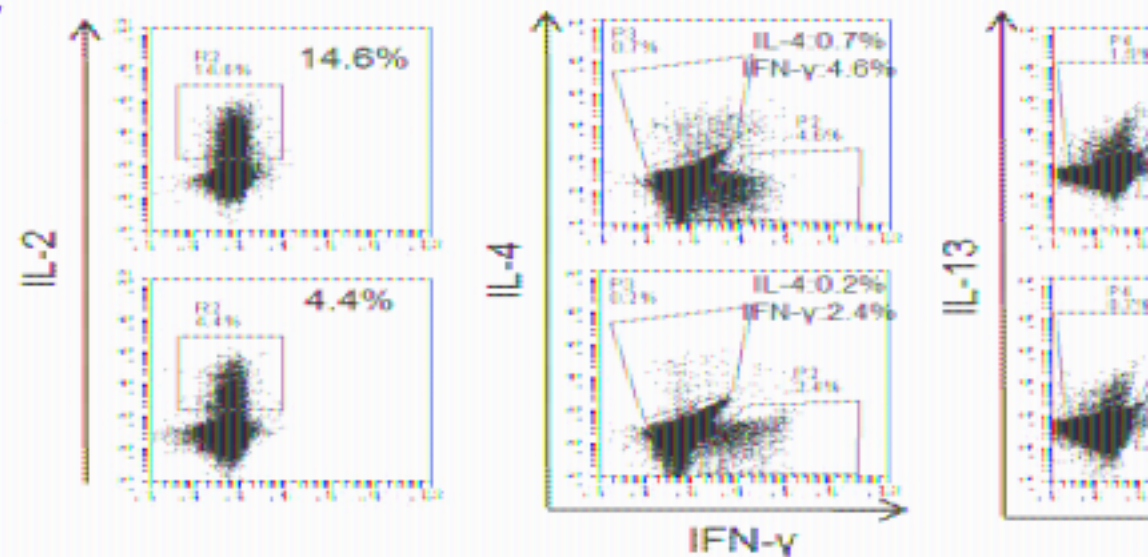
WT

Fat-1

**C**

WT

Fat-1

**Figure S1**

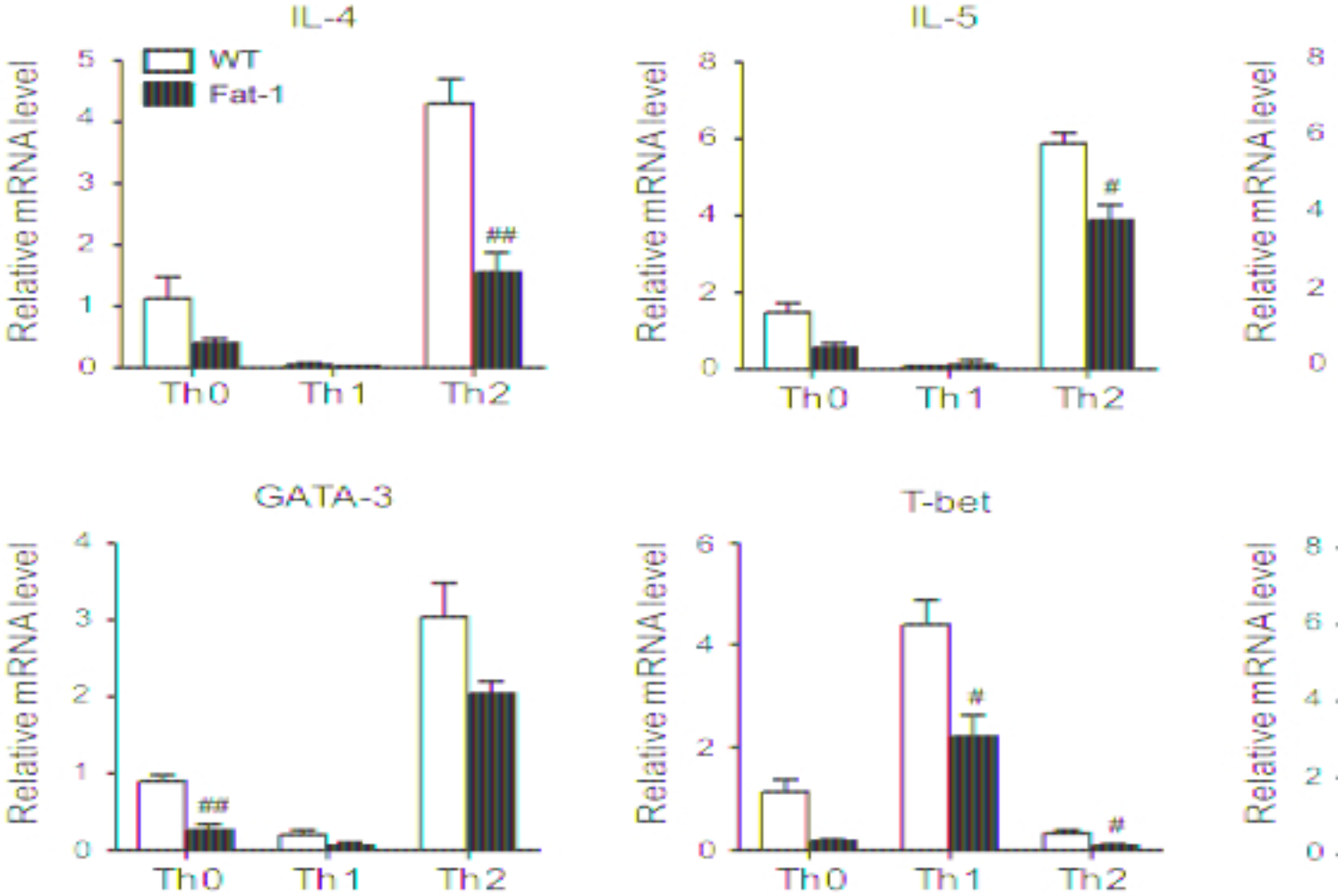


Figure S2

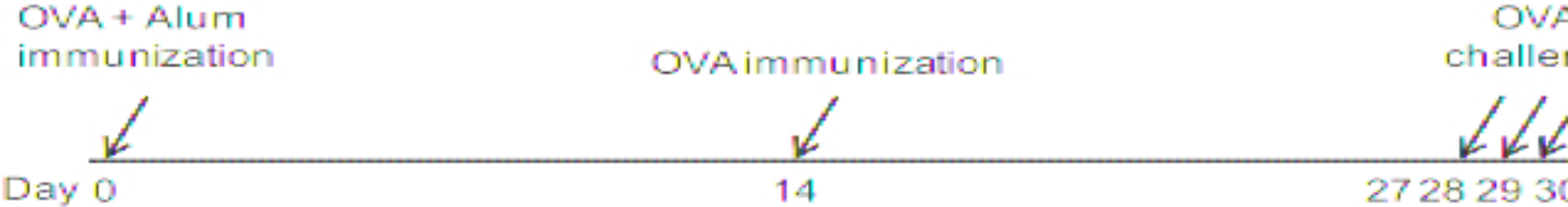
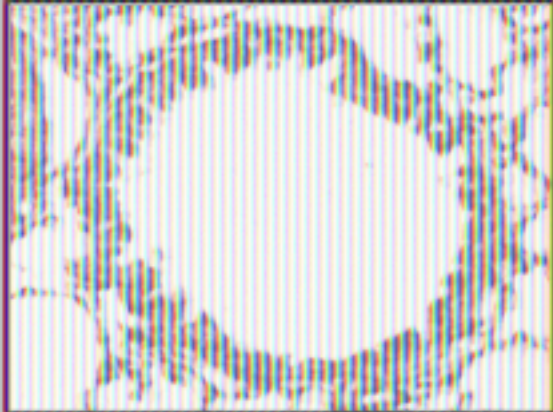
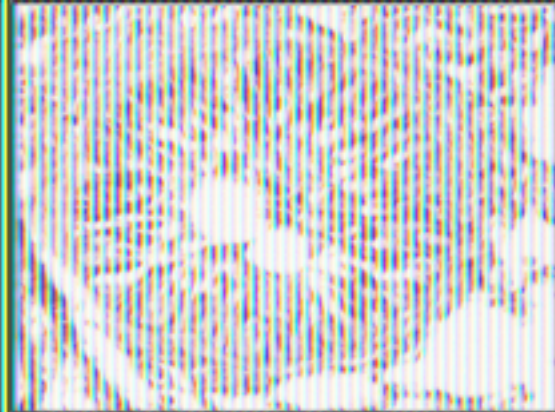


Figure S3



CONT



WT



Asthma

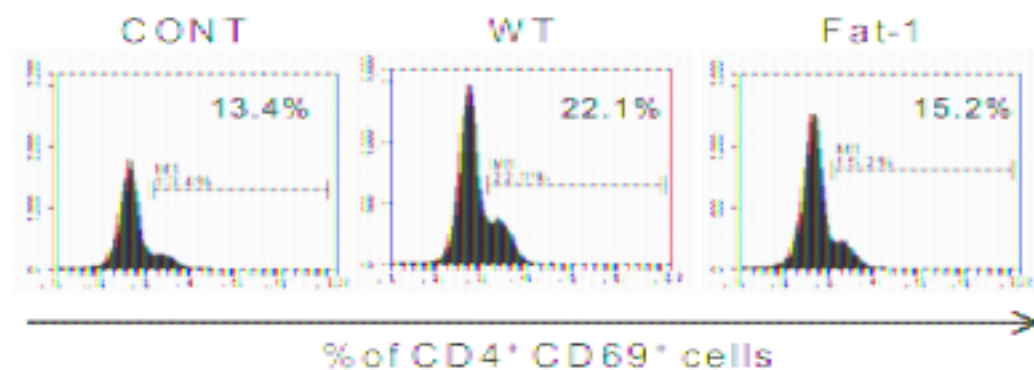
Figure S4



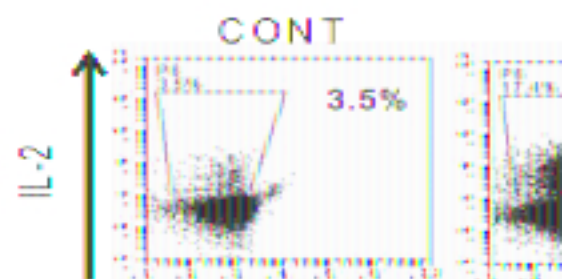
Figure S5

A

Asthma

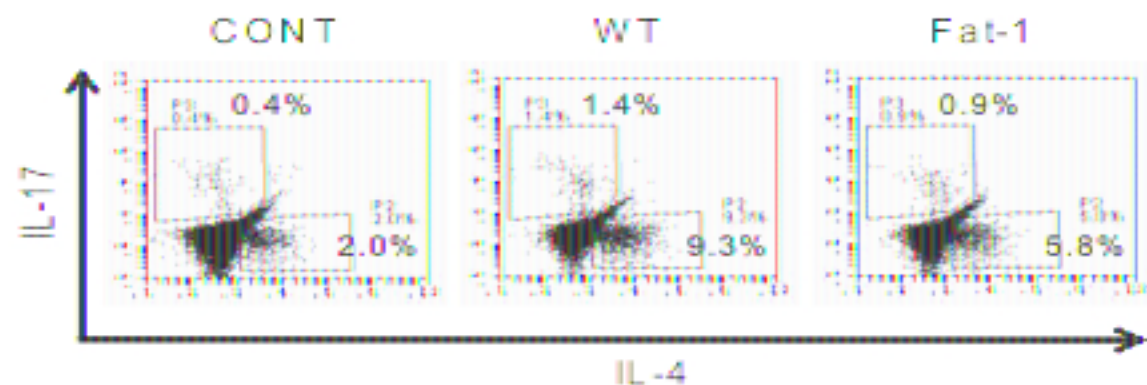


B



C

Asthma



D

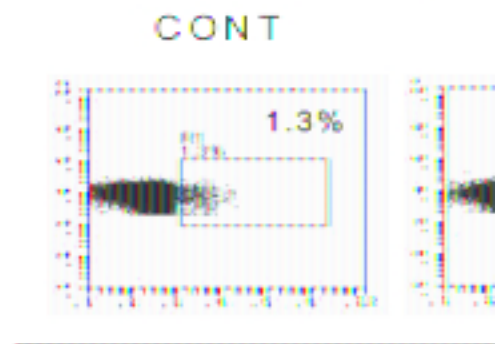
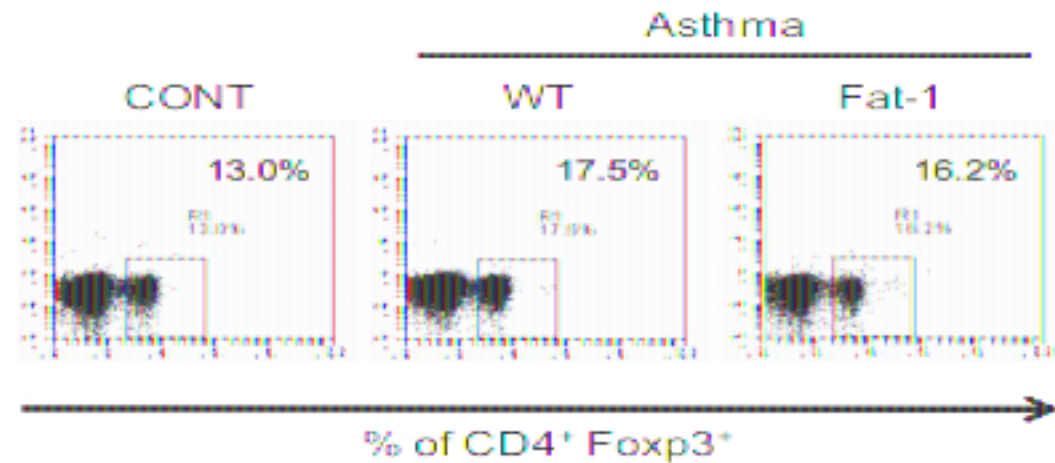
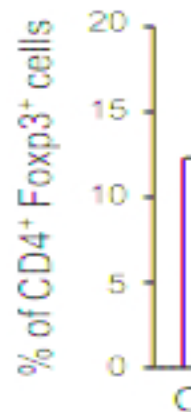


Figure S7

A**B****Figure S8**

Effects of n-3 PUFA on the CD4⁺ type 2 helper T-cell mediated immune responses in the Fat-1 mice

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Supporting Information Materials and Methods

Real-time RT-PCR analysis

The RNA was isolated from lungs or CD4⁺ T cells using Trizol (Invitrogen) and was precipitated with isopropanol and dissolved in DEPC-treated distilled water. Total RNA (2 µg) was treated with RNase-free DNase (Invitrogen), and first-strand cDNA was generated using a random hexamer primer with a first-strand cDNA synthesis kit (Applied Biosystems, Foster City, CA USA). Specific primers for each gene were designed using primer express software (Applied Biosynthesis). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an invariant control. Specific primers for each gene (Table 1) were designed using primer express software (Applied Biosystems). Real-time RT-PCR mixtures consisted of 10 ng reverse transcribed total RNA, 167 nmol/l forward and 2× PCR master mixture in a final volume of 10 µl. Reactions were carried out in 384-well plates using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems).

Treg cells analysis in spleen

Single cell suspensions were prepared from the spleen and analyzed for CD4⁺ Foxp3⁺ expression using PerCPcy5.5-labeled anti-CD4 and FITC-labeled anti-Foxp3 (eBioscience) according to the manufacturer's instructions. Briefly, splenocytes (1×10^6) were stained for CD4 expression and then incubated with 1 mL of fixation/permeabilization solution at 4°C for 30 min. The cells were washed with 2 mL of permeabilization buffer and stained with anti-Foxp3 antibodies for 30 min on ice. Finally, the cells were washed with permeabilization buffer, resuspended in flow cytometry buffer (PBS with 2% FBS), and analyzed by flow cytometry.

Supporting Information Figure Legends

Figure S1. CD4⁺ T cell activation and cytokine production. CD4⁺ T cells were purified and stimulated with anti-CD3 antibody alone or with anti-CD3/CD28 antibodies. Cells were culture for 24 h and stained with anti-CD69 antibody (A) or anti-CD25 antibody (B), and analyzed by flow cytometry. (C) Cells were culture for 48 h and stained with anti-IL-2, IL-4, IL-13, IL-33 and IFN- γ antibodies and analyzed by flow cytometry. The representative flow cytometry plots are shown.

Figure S2. Real-time RT-PCR analysis of Th1 or Th2 cytokines and transcription factors in CD4⁺ T cells differentiated under Th1 or Th2 polarization condition. Total RNA was extracted from CD4⁺ T cells cultured for 3 days under Th0-, Th1- or Th2-differentiation condition. Then mRNA levels of indicated cytokines and transcription factors were analyzed by real-time RT-PCR. Results of three independent experiments are expressed as mean $\pm$ SEM. [#] $p < 0.05$, ^{##} $p < 0.01$ vs. WT.

Figure S3. Schematic diagram of the experimental protocol. Mice were immunized intraperitoneally with 20 µg of OVA plus 2.25 mg aluminum hydroxide adjuvant on day 0 and OVA alone without alum on day 14. The immunized mice were exposed to aerosolized OVA on days 28, 29 and 30. Mice were euthanized and peripheral blood, BALF, lung tissues and spleen were prepared for analysis.

Figure S4. Histological assessment of mucin production. Periodic Acid Schiff (PAS) staining was performed on sectioned lung tissues to visualize mucin production and mucin is stained as a pink to red-stained material within the airway. Representative slides are shown of sections from 5 mice per se.

Figure S5. Protein expression of GATA3 in lung tissues. Lung tissues from mice were homogenized in lysis buffer and subjected to Western blot analysis. Similar results were obtained from at least three independent experiments.

Figure S6. Inflammatory gene expression in OVA-induced allergic airway inflammation. Lung tissues were collected 48 h after the last OVA challenge and homogenized. The mRNA levels in lung extracts were measured by real-time RT-PCR. Results of three independent experiments with 4 mice/group are expressed as mean±SEM (n=12). ^{**}*p*<0.01 vs. untreated control; ^{##}*p*<0.01 vs. saline.

Figure S7. Representative data of flow cytometry analysis for CD4⁺CD69⁺ T cells (A), IL-2 producing CD4⁺ T cells (B), IL-4 or IL-17 producing CD4⁺ T cells (C), and IFN-γ producing CD4⁺ T cells (D) in spleen. Representative data of at least three experiments with similar results is shown.

Figure S8. Tregs in splenocytes. Splenocytes from OVA immunized and challenged mice were stained with anti-CD4 and anti-Foxp3 antibodies and analyzed by flow cytometry. (A) Representative flow cytometry data from three independent experiments with 5 mice/group is shown. (B) Average percentage of Tregs in spleen. Results of three independent experiments with 4 mice/group are expressed as mean $\pm$ SEM (n=12).

Table S1. Composition of the diet

Ingredients	gm	kcal
Casein	14	56
Corn starch	43.56	174
Maltodextrin	12.5	50
Sucrose	10	40
Cellulose	5	0
AIN-93 mineral mix	3.5	0
AIN-93 vitamin mix	1	40
L-Cystine	0.18	0.72
Choline bitartrate	0.25	0
tert-Butylhydroquinone	0.01	0
Corn oil	10	90
Total	100	451

All diet ingredients were purchased from Research Diet (New Brunswick, NJ, USA). Diets were prepared and stored in aliquots at -20°C.