

Dietary long chain n-3 polyunsaturated fatty acids prevent allergic sensitization to cow's milk protein in mice

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Summary

Background Cow's milk allergy is one of the most common food allergies in children and no treatment is available. Dietary lipid composition may affect the susceptibility to develop allergic disease.

Objective Assess whether dietary supplementation with long chain n-3 polyunsaturated fatty acids (n-3 LCPUFA) prevents the establishment of food allergy.

Methods Mice were fed a control or fish oil diet before and during oral sensitization with whey. Acute allergic skin response, serum immunoglobulins as well as dendritic cell (DC) and T cell subsets in mesenteric lymph nodes (MLN), spleen and/or small intestine were assessed.

Results The acute allergic skin response was reduced by more than 50% in sensitized mice fed the fish oil diet compared to the control diet. In addition, anti-whey-IgE and anti-whey-IgG1 levels were decreased in the fish oil group. Serum transfer confirmed that the Th2-type humoral response was suppressed since sera of fish oil fed sensitized mice had a diminished capacity to induce an allergic effector response in naïve recipient mice compared to control sera. Furthermore, the acute skin response was diminished upon passive sensitization in fish oil fed naïve recipient mice. In addition, the percentage of activated Th1 cells was reduced by fish oil in spleen and MLN of sham mice. The percentage of activated Th2 cells was reduced in both sham- and whey-sensitized mice. In contrast, whey-sensitized mice showed an increased percentage of CD11b+CD103+CD8 α - DC in MLN in association with enhanced FoxP3+ regulatory T cells (Treg) in spleen and intestine of fish oil fed whey-sensitized mice compared to sham mice.

Conclusions and Clinical Relevance Dietary n-3 LCPUFA largely prevented allergic sensitization in a murine model for cow's milk allergy by suppressing the humoral response, enhancing local intestinal and systemic Treg and reducing acute allergic symptoms, suggesting future applications for the primary prevention of food allergy.

Keywords acute allergic skin response, allergic sensitization, cow's milk allergy, dendritic cell, fish oil, immunoglobulins, long chain n-3 polyunsaturated fatty acids, mast cell, regulatory T cell, T cell subsets

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Introduction

Epidemiological research has demonstrated a rapid increase in allergic diseases. In developed countries, these affect between 15% and 30% of the population [1]. Allergic sensitization to food proteins occurs in about 6% of children and 3–4% of the adult population in westernized countries [2]. Cow's milk is introduced

in a child's diet early in life, making cow's milk allergy one of the most common food allergies in early childhood with a prevalence of 0.3–3.5% among children under five years of age [3, 4]. Major allergens characterized in cow's milk are distributed among the casein and whey fractions and include α _{s1}-casein and β -lactoglobulin respectively [5]. IgE-mediated allergy accounts for about 60% of the hypersensitivity reactions against

these proteins in milk [6]. Symptoms involve the skin, gastrointestinal and respiratory tract and even systemic anaphylaxis may occur [7]. There is no cure for food allergies so far, the only possibility is to avoid the culprit food allergen; however, this might affect children's growth and health [7]. Although approximately 90% of children outgrow the disease, most of them are predisposed to the development of other food or inhalant allergies [4, 8]. Therefore, there is a major interest in interventions to prevent or treat this condition.

The increase in prevalence of atopic disease is associated with a reduction in the consumption of long chain n-3 polyunsaturated fatty acids (n-3 LCPUFA) from fatty fish – generally considered anti-inflammatory – paralleled by an increase in the intake of margarine and vegetable oils containing high levels of n-6 PUFA [9–11]. Current developments in Japan provide additional evidence since the prevalence of atopic diseases is rising in parallel with a reduced ratio of n-3 to n-6 PUFA in the diet [12, 13]. Furthermore, it was observed that reduced n-3 LCPUFA serum levels were associated with allergic disease in Swedish adolescents [14]. In Finland, atopic disease prevalence is low in Eastern provinces where high percentages of n-3 LCPUFA, especially of EPA, and low percentages of n-6 PUFA are found in serum [9, 15, 16]. In addition, it has been demonstrated that increased fish oil consumption by pregnant and lactating woman can reduce the incidence of food allergy and IgE-associated eczema by more than 60% in children at risk [17].

Therefore, fish oil, rich in n-3 LCPUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), may have a role in the prevention of allergic diseases. In this study, it is assessed whether dietary supplementation with fish oil starting early in life, can reduce allergic sensitization in a murine model for orally induced cow's milk allergy.

Materials and Methods

Diets

Semi purified cow's milk protein-free AIN-93G-based diets were composed of either 10% soybean oil (control diet) or 4% soybean oil plus 6% tuna oil (fish oil diet) at Research Diet Services (Wijk bij Duurstede, the Netherlands) (Tables 1 and 2) [18]. The ratio n-3 : n-6 PUFA was 1 : 9.5 for the control diet while this ratio was reduced to 1 : 1 for the fish oil diet. Tuna oil was a kind gift from Bioriginal, Den Bommel, the Netherlands. The fat percentage of AIN-93G was adapted and extra fat was exchanged for cornstarch. The diets were stored at –20°C prior to use to prevent fatty acid oxidation.

Table 1. Dietary composition mouse chow (based on AIN-93G)

	Control diet (g/kg diet)	Fish oil diet (g/kg diet)
<i>Carbohydrates</i>		
Cornstarch	367.5	367.5
Dextrinized cornstarch	132.0	132.0
Sucrose	100.0	100.0
Cellulose	50.0	50.0
<i>Protein</i>		
Soya	200.0	200.0
Methionine	3.0	3.0
<i>Fat</i>		
Soybean oil	100.0	40.0
Tuna oil	0.0	60.0
<i>Others</i>		
Mineral mix AIN 93G	35.0	35.0
Vitamin mix AIN 93VX	10.0	10.0
Choline bitartrate	2.5	2.5
Tert-butylhydroquinone	0.014	0.014

Table 2. Fatty acid composition lipid source

Fatty acid	Soybean oil (%)	Tuna oil (%)
SFA	15.1	28.9
MUFA	24.9	22.8
PUFA	59.1	44.5
n-6 PUFA	53.1	5.5
C18 : 2 n-6 LA	53.1	1.3
C20 : 4 n-6 AA		1.8
C22 : 5 n-6		1.6
n-3 PUFA	5.6	38.5
C18 : 3 n-3 ALA	5.6	0.5
C20 : 5 n-3 EPA		7.0
C22 : 5 n-3 DPA		1.4
C22 : 6 n-3 DHA		27.8
Minor components	0.9	3.8

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, α -linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Oral sensitization and challenge of mice

Three-week-old specific pathogen free female C3H/He-OuJ mice ($n = 6$ /group) were purchased from Charles River Laboratories (L'Arbresle Cedex, France) and fed either the control or fish oil diet starting two weeks prior to the first sensitization and continued during the whole sensitization period until sacrifice (Fig. 1). The mice were housed in the animal facility at Utrecht University. Animal care and use were approved by and performed in accordance with the guidelines of the Animal Ethics Committee of Utrecht University (approval number: DEC2009.III.08.079).

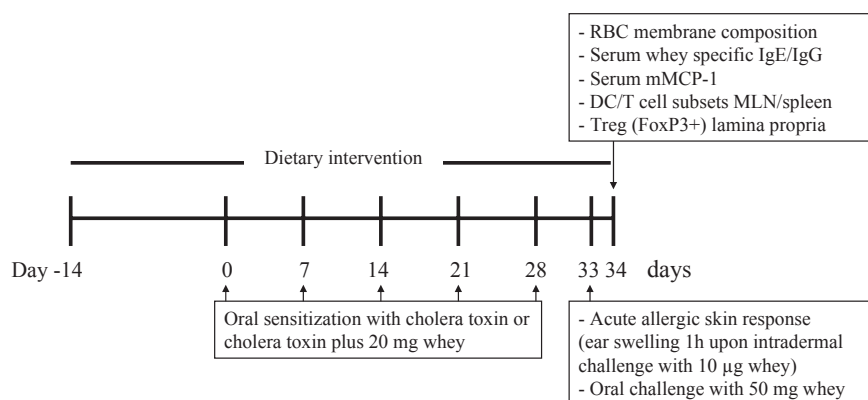


Fig 1. A schematic overview of the sensitization and challenge protocol and the parameters that are analysed. RBC, red blood cell; mMCP-1, mouse mast cell protease-1; DC, dendritic cell; MLN, mesenteric lymph nodes; Treg, regulatory T cell.

Mice were sensitized intragastrically (i.g.) using a blunt needle with 20 mg whey/0.5 mL PBS with 10 µg cholera toxin (CT) as an adjuvant (DMV International, Veghel, the Netherlands). Sham mice received CT (10 µg/0.5 mL PBS). Mice were sensitized once a week for 5 consecutive weeks as previously described by Schouten *et al.* [19]. One week after the last sensitization sham and whey-sensitized mice were challenged intradermally (i.d.) in the ear pinnae of both ears with 10 µg whey in 20 µL PBS to determine the acute allergic skin response (see below). The same day the mice were challenged i.g. with 50 mg whey/0.5 mL PBS and 18 h after the oral challenge blood samples were collected and centrifuged at 14 000 *g* for 15 min. Sera were stored at -70°C . Mice were killed by cervical dislocation and mesenteric lymph nodes (MLN), spleen and small intestine were obtained for flow cytometry or histological examination.

Fatty acid composition erythrocytes

Blood was collected in heparin tubes and after centrifugation plasma was removed. Erythrocytes were stored at -70°C until analysis. Erythrocyte lipids were extracted as described by Bligh and Dyer [20] and the membrane fatty acid composition was assessed using gas chromatography as previously described [21].

Acute allergic skin response

Ear thickness was measured in duplicate for each ear using a digital micrometer (Mitutoyo, Veenendaal, the Netherlands) at basal conditions prior to whey challenge and 1 h after i.d. challenge in the ear. Isoflurane was used for inhalational anaesthesia during measurements. The mean basal thickness per ear was subtracted from the mean thickness measured at 1 h after injection to express ear swelling as delta µm. Mean of left and right delta ear swelling were calculated for each mouse.

Plasma PGE₂ and serum immunoglobulins and mMCP-1

Concentrations of prostaglandin E₂ (PGE₂) in plasma and mouse mast cell protease-1 (mMCP-1) in serum were determined using commercially available ELISA kits (PGE₂: Oxford Biomedical Research, Oxford, USA; mMCP-1: Moredun Scientific Ltd., Penicuik, UK) according to the manufacturer's protocol.

Levels of whey-specific IgE, IgG1 and IgG2a were determined in serum by ELISA. Microtiter plates (Greiner, Alphen aan den Rijn, the Netherlands) were coated with 100 µL whey (20 µg/mL) in carbonate-bicarbonate buffer (0.05M, pH = 9.6) for 18 h at 4°C. Plates were washed and blocked for 1 h with 0.5% BSA in ELISA buffer (50 mM TRIS, 137 mM NaCl, 2 mM EDTA and 0.05% Tween20). Serum samples were incubated for 2 h at room temperature after which plates were washed and incubated with biotin-labelled rat anti-mouse IgE, IgG1 or IgG2a (1 µg/mL, BD Biosciences, Alphen aan den Rijn, the Netherlands) for 90 min at room temperature. After washing, the plates were incubated with streptavidin poly horseradish peroxidase (Sanquin, Amsterdam, the Netherlands) for 1 h, washed and developed with o-phenyldiamine (Sigma-Aldrich-Chemicals, Zwijndrecht, the Netherlands). The reaction was stopped with 4 M H₂SO₄ and absorbance was measured at 490 nm on a Benchmark microplate reader (Bio-rad, Veenendaal, the Netherlands). Results were expressed as arbitrary units (AU) composed using a titration curve of pooled sera from whey-alum i.p. immunized mice as positive reference serum serving as an internal standard.

Passive sensitization: transfer of dietary intervention serum

Sera obtained from sham and whey-sensitized mice fed the control or fish oil diet were transferred intravenously (i.v. 100 µL) using isoflurane anaesthesia to naïve recipient mice fed control diet ($n = 6/\text{group}$). Half an

hour after serum transfer the acute skin response was measured as described before, starting with measuring ear thickness at basal conditions prior to whey challenge followed by assessing ear swelling 1 h after i.d. challenge.

Passive sensitization: transfer of hyperimmune serum to fish oil fed recipients

Pooled ('hyperimmune') sera from whey-alum i.p. immunized mice were i.v. transferred (100 µL) to naïve mice fed the control diet or fish oil for two weeks prior to injection ($n = 6/\text{group}$). After half an hour, the acute skin response was measured as described before.

Dendritic cell (DC) and T cell subsets by flow cytometry

After the mice were killed, MLN were removed and after homogenization incubated with 0.2% collagenase IV and 2 kU/mL DNase for 30 min at 37°C, re-suspended and incubated for another 30 min. The enzymatic reaction was stopped by adding 0.5 mL FCS after which the cell suspension was filtered. In addition, single cell suspensions were prepared from mouse spleens. All cells were blocked for 20 min in PBS containing 1% BSA and 5% FCS. 5×10^5 Cells were plated per well and incubated for 30 min at 4°C with different antibodies (eBioscience, Breda, the Netherlands, unless otherwise stated) anti-CD11c-PerCpCy5.5 (1 : 50), anti-CD11b-PE (1 : 50), anti-CD8α-APC or FITC (1 : 100), anti-CD103-APC (1 : 100), anti-CD4-FITC or PerCpCy5.5 (1 : 100), anti-CD25-PE (1 : 100), anti-CD69-APC (1 : 50), anti-CXCR3-PE (1 : 50), anti-T1/ST2-FITC (1 : 50, MD Biosciences, St. Paul, MN, USA) and isotype controls were used. Cells were fixed using 0.5% paraformaldehyde, or permeabilized for intracellular staining with FoxP3-APC (1 : 50) using the Foxp3 staining buffer set (eBioscience) according to the manufacturer's protocol. Flow cytometry was performed using FACS Canto II (BD, Alphen aan den Rijn, the Netherlands) and analysed using FACSDiva software (BD).

Immunohistochemistry FoxP3+ cells

The small intestine (jejunum and proximal ileum) was carefully dissected, opened longitudinally and luminal contents were removed by gently washing in PBS. The intestine was placed with the mucosal side down and rolled from the distal to the proximal end. The Swiss rolls were fixed in formalin (10% v/v solution) and embedded in paraffin (Leica IG1150c; Leica Microsystems, Rijswijk, the Netherlands). Sections (5 µm) were cut using a microtome (Leica Microsystems) and

deparaffinized Swiss roll sections were stained for intracellular FoxP3 expression. In short, after dewaxing the sections were boiled in sodium citrate buffer (0.01 M) for 10 min. Then, sections were blocked with 5% rabbit serum (Dako, Heverlee, Belgium) in PBS with 1% BSA, followed by overnight incubation at 4°C with rat anti-mouse FoxP3 purified antibody (final concentration 2.5 µg/mL, eBioscience) or rat IgG2a isotype (2.5 µg/mL) as a control in 1% BSA. Then, slides were incubated for 30 min with 3% H₂O₂ in PBS. The primary antibody was detected with a biotinylated rabbit anti-rat antibody (2.5 µg/mL in 1% BSA, Dako) for 1 h at room temperature, followed by incubation with avidin biotin complex (ABC, Vector) in 1% BSA/PBS for 1 h. Colour was developed with 3,3'-diaminobenzidine (Sigma) and sections were counter-stained with haematoxylin, dehydrated and covered with Pertex mounting medium (Histolab, Göteborg, Sweden) and a cover glass. FoxP3+ cells were counted per villus-crypt unit. Per mouse 100 intact villus-crypt units were counted on a slide.

Statistics

All data were analysed using two-way ANOVA (factors diet and sensitization) or one-way ANOVA and post hoc Bonferroni test using GraphPad Prism software (version 5.0) and SPSS version 15 software. The hypothesis that the effect of sensitization would differ between the control and fish oil groups was tested by the interaction term between sensitization and diet in the two-way ANOVA. Therefore, in the legends the *P*-value for diet; sensitization and diet x sensitization interaction are reported. For serological data square root transformation was used before analysis to normalize data distribution. Data are presented as means ± SEM. For serological data, Tukey box-and-whisker plots were used, represented as median [25–75% percentiles] in text.

Results

Increased DHA and EPA membrane content upon fish oil supplementation

Fish oil supplementation more than doubled DHA content of red blood cell membranes as compared to control diet fed mice in both sham and whey-sensitized mice ($P < 0.001$, Fig. 2a). Also, the percentage of EPA in the membranes did increase after fish oil treatment ($P < 0.001$, Fig. 2b). The incorporation of n-3 LCPUFA in the cell membrane occurred mainly at the expense of n-6 PUFA arachidonic acid (20 : 4n-6) ($P < 0.001$, Fig. 2c) and linoleic acid (18 : 2n-6)(whey-mice 16.3 ± 1.1 vs. $9.6 \pm 0.4\%$, $P < 0.001$) which were reduced in the erythrocyte membranes. In addition, the

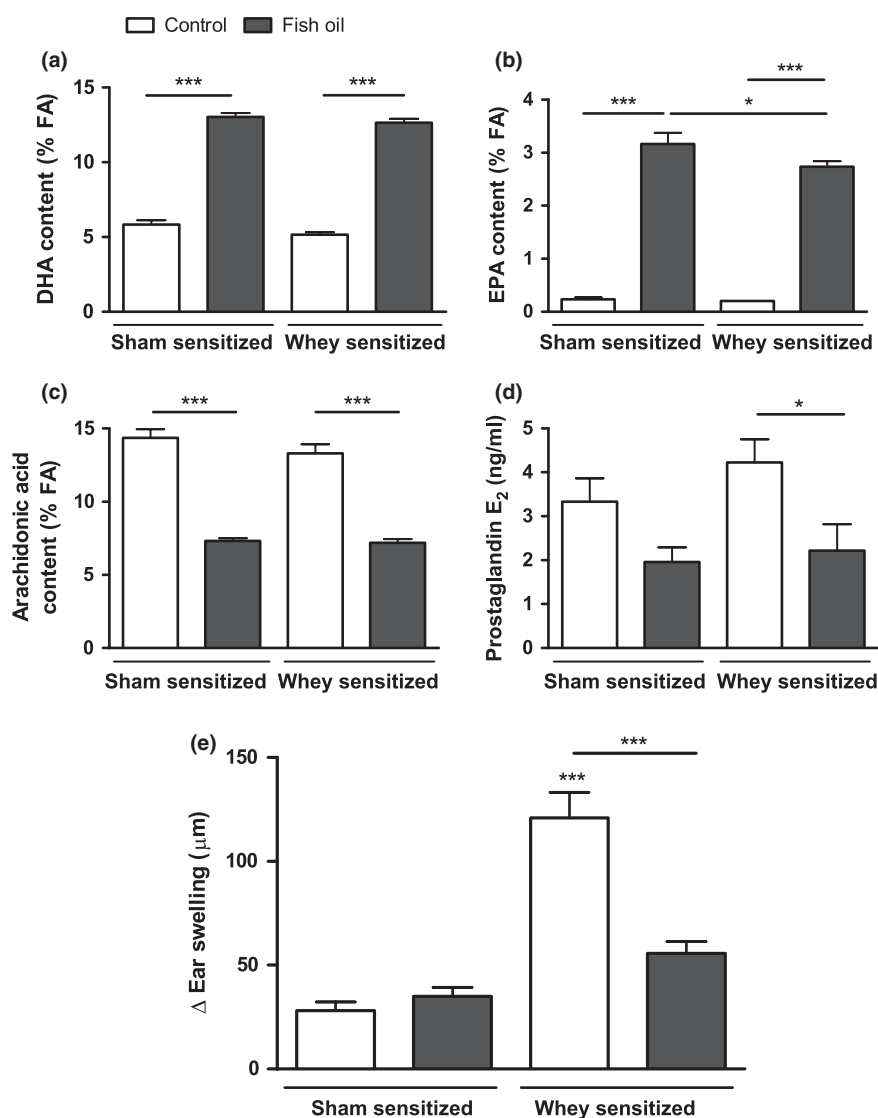


Fig. 2. N-3 polyunsaturated fatty acids (N-3 LCPUFA) content in erythrocyte membranes was enhanced in mice fed the fish oil diet as shown for (a) DHA (diet $P < 0.001$, sensitization $P < 0.05$, interaction NS) and (b) EPA (diet $P < 0.001$, sensitization $P = 0.06$, interaction $P = 0.11$). Incorporation occurred at the expense of n-6 PUFA such as (c) arachidonic acid (diet $P < 0.001$, sensitization NS, interaction NS). In addition, the arachidonic acid metabolite (d) prostaglandin E₂ was reduced in plasma of whey-mice fed fish oil (diet $P < 0.01$, sensitization NS, interaction NS). (e) The fish oil diet effectively reduced the whey-induced acute allergic skin response in whey-sensitized mice (diet $P < 0.01$, sensitization $P < 0.001$, interaction $P < 0.001$). Ear swelling (μm) was calculated as the increase in ear thickness induced by whey one hour after challenge. Data are presented as means $\pm$ SEM, $n = 6/\text{group}$. * $P < 0.05$, *** $P < 0.001$ two-way ANOVA followed by Bonferroni's multiple comparison test. NS, not significant.

concentration of PGE₂ was significantly reduced in whey-sensitized mice fed the fish oil diet as compared to control diet fed mice ($P < 0.05$, Fig. 2d).

Fish oil diet reduces acute allergic skin response in whey-sensitized mice

One hour after the dermal challenge the acute allergic skin response was measured to compare the effect of the control and fish oil diet on ear swelling. The delta ear swelling in the whey-sensitized mice fed control diet was significantly enhanced as compared to sham-

sensitized mice ($P < 0.001$, Fig. 2e). Whey-sensitized mice fed fish oil diet showed a more than 50% reduced acute allergic skin response ($P < 0.001$) compared to control diet fed whey-sensitized mice.

Reduced whey-specific IgE and IgG1 levels in whey-sensitized mice fed fish oil diet

Serum levels of whey-specific IgE were increased in mice orally sensitized with whey compared to sham mice ($P < 0.001$, Fig. 3a). The fish oil diet reduced whey-IgE levels in whey-sensitized mice compared to

the control fed mice ($P < 0.05$). Whey-specific IgG1 levels of whey-sensitized mice were enhanced compared to sham-sensitized mice ($P < 0.001$, Fig. 3b). Fish oil prevented the generation of Th2-type whey-IgG1 and levels were significantly reduced compared to control diet fed whey-sensitized mice ($P < 0.01$). In addition, Th1-type immunoglobulin IgG2a showed the same pattern. Sham-sensitized mice showed lower IgG2a levels than whey-sensitized mice fed control diet ($P < 0.05$, Fig. 3c), the fish oil diet reduced IgG2a in whey-sensitized mice compared to whey sensitized mice fed control diet ($P < 0.05$).

To assess mucosal mast cell degranulation, mMCP-1 was determined in serum 18 h after oral challenge with whey. Serum mMCP-1 concentrations of whey-sensitized mice fed control diet were enhanced when compared to sham-treated mice ($P < 0.05$, Fig. 3d). mMCP-1 was not enhanced in whey-sensitized mice fed fish oil diet when compared to sham mice. However, fish oil diet did not significantly reduce mMCP-1 in serum compared to control diet.

Passive transfer of sera from fish oil fed mice to naïve recipients confirms the humoral response to be suppressed

In recipient mice receiving sera from whey-sensitized donor mice fed the control diet a significant ear

swelling was induced as compared to recipients receiving sera from sham mice ($P < 0.001$, Fig. 4a). Sera of whey-sensitized donor mice fed the fish oil diet elicited a significantly lower whey-induced ear swelling response in recipient mice compared to serum of control diet fed whey-sensitized donor mice ($P < 0.001$).

Fish oil diet reduces acute allergic symptoms induced by passive sensitization using whey-hyperimmune sera

N-3 LCPUFA from fish oil diet possibly target the mast cell directly, which may affect mast cell phenotype or degranulation [22, 23]. To assess whether the fish oil diet itself is capable of suppressing acute allergic symptoms, hyperimmune serum – high in whey-specific immunoglobulins after whey-alum i.p. sensitization – was transferred to naïve recipients fed either the control or fish oil diet, after which the mice were challenged with whey in both ears. Transfer of whey-hyperimmune serum resulted in a significantly enhanced acute allergic skin response in recipient mice fed the control diet as compared to injection of naïve serum from non-sensitized mice ($P < 0.001$, Fig. 4b). Recipient mice fed the fish oil diet showed a reduced allergic skin response as compared to control diet fed recipients transferred with hyperimmune serum ($P < 0.001$).

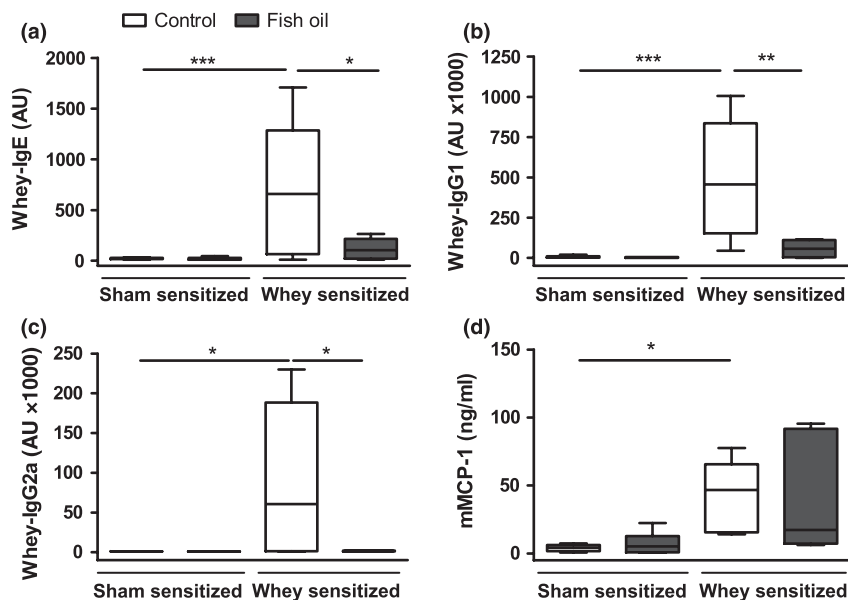


Fig 3. The effect of fish oil on the humoral response and mouse mast cell protease-1 (mMCP-1). Fish oil reduced whey-specific (a) IgE (diet $P < 0.05$, sensitization $P < 0.01$, interaction $P < 0.05$) (b) IgG1 (diet $P < 0.01$, sensitization $P < 0.01$, interaction $P < 0.01$) and (c) IgG2a (diet $P < 0.05$, sensitization $P < 0.05$, interaction $P < 0.05$) as compared to control diet fed whey-sensitized mice. (d) Serum mMCP-1 concentrations were increased in whey-sensitized mice fed control diet as compared to sham mice (diet NS, sensitization $P < 0.01$, interaction NS). Data are presented as box-and-whisker Tukey plots, $n = 6$ /group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ two-way ANOVA followed by Bonferroni's multiple comparison test. Data were square root transformed for analysis. NS, not significant.

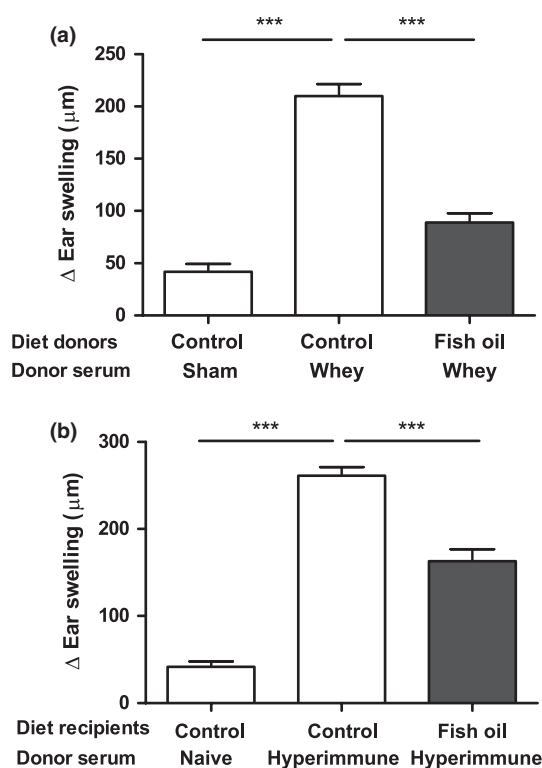


Fig 4. Effect of fish oil on the humoral and effector response (a) Passive transfer of sera of whey-sensitized mice fed the fish oil diet resulted in a reduced ear swelling upon i.d. whey challenge in comparison with recipient mice transferred with sera of sensitized mice fed control diet. (b) The acute allergic skin response was diminished in fish oil fed naïve recipient mice injected with whey-hyperimmune serum as compared to control diet fed recipients. Data are presented as means $\pm$ SEM, $n = 6$ /group. *** $P < 0.001$ one-way ANOVA followed by Bonferroni's multiple comparison test.

Fish oil diet alters the DC population and reduces percentages of Th1 and Th2 cells in MLN and spleen

MLN and splenocytes from whey-sensitized mice fed the control or fish oil diet were analysed for DC and T cell subsets. Th1 inducing CD11c+CD11b-CD8 α + lymphoid DC [24, 25] were reduced in MLN and spleen by the fish oil diet in sham-sensitized mice when compared to sham mice fed control diet ($P < 0.05$ for MLN; $P < 0.01$ for spleen, Fig. 5a). In contrast, the percentage of CD11c+CD11b-CD8 α - myeloid DC [24, 25] were enhanced by the fish oil diet in MLN of both sham and whey-sensitized mice ($P < 0.001$ for both sham and whey mice, Fig. 5a).

Total CD4+ cells are not enhanced by sensitization with whey and not reduced by the tuna oil diet (Figure S1). The fish oil diet reduced the percentage of activated Th1 cells (CD4+CD69+CXCR3+ [26–28]) in spleens of sham-sensitized mice by 50% ($P < 0.001$, Fig. 5b). Dietary fish oil supplementation also significantly reduced the percentage of splenic activated Th2 cells

(CD4+CD69+T1/ST2+ [29, 30]) in both sham- and whey- sensitized mice ($P < 0.001$ for sham; $P < 0.05$ for whey). Since the effect on the percentage of activated Th2 cells in spleen was more pronounced this resulted in an increased Th1/Th2 ratio in fish oil fed sham-sensitized mice as compared to sham mice fed control diet (3.3 ± 0.5 vs. 1.7 ± 0.1 , $P < 0.001$). Also, in MLN the percentage of activated Th1 but not activated Th2 cells was reduced by the fish oil diet in sham-sensitized mice ($P < 0.05$, Fig. 5b). This did not affect the Th1/Th2 ratio in MLN (data not shown). The fish oil diet exerted a similar tendency for percentages of total Th1 (CXCR3+ of CD4+) and total Th2 (T1/ST2+ of CD4+) cells in MLN and spleen but effects were less prominent (Figure S1).

Increase in FoxP3 positive cells in spleen and lamina propria of whey-sensitized mice fed fish oil diet

FoxP3+ regulatory T cell (Treg) inducing CD11c+CD11b+CD103+CD8 α - DC [31, 32] were increased in MLN of whey-sensitized mice fed fish oil as compared to fish oil fed sham mice ($P < 0.05$, Fig. 6a). The percentage of FoxP3+ Treg in MLN was low (data not shown). However, in spleen the percentage of FoxP3+ Treg in whey-sensitized mice fed the fish oil diet was enhanced as compared to sham mice fed fish oil ($P < 0.05$, Fig. 6b).

In addition to this systemic compartment an increased number FoxP3+ cells per villus-crypt unit was counted locally in the small intestine of whey-sensitized mice fed the fish oil diet as compared to sham-sensitized mice fed the fish oil diet ($P < 0.05$, Fig. 6c). Isotype control slides were all negative for FoxP3.

Discussion

Fish oil supplementation reduces allergic sensitization in a mouse model for cow's milk allergy since the acute allergic skin response in the fish oil fed mice was reduced by over 50% combined with low serum whey-IgE and whey-IgG1 levels. The current study implies that n-3 LCPUFA can suppress the susceptibility to develop allergic disease even when provided after birth.

In recent human trials, fish oil supplementation was able to lower the risk of allergic disease. Since allergic diseases are determined early in life, or even *in utero*, these trials were performed during pregnancy. Daily supplementation with n-3 LCPUFA during late pregnancy has been shown to reduce the risk of food allergy, including milk allergy and IgE-associated eczema in children at risk of atopic disease [17, 33–36]. Furthermore, fewer severe allergic symptoms have been reported in fish oil treated infants [33]. Results from our study suggest the possibility for primary prevention

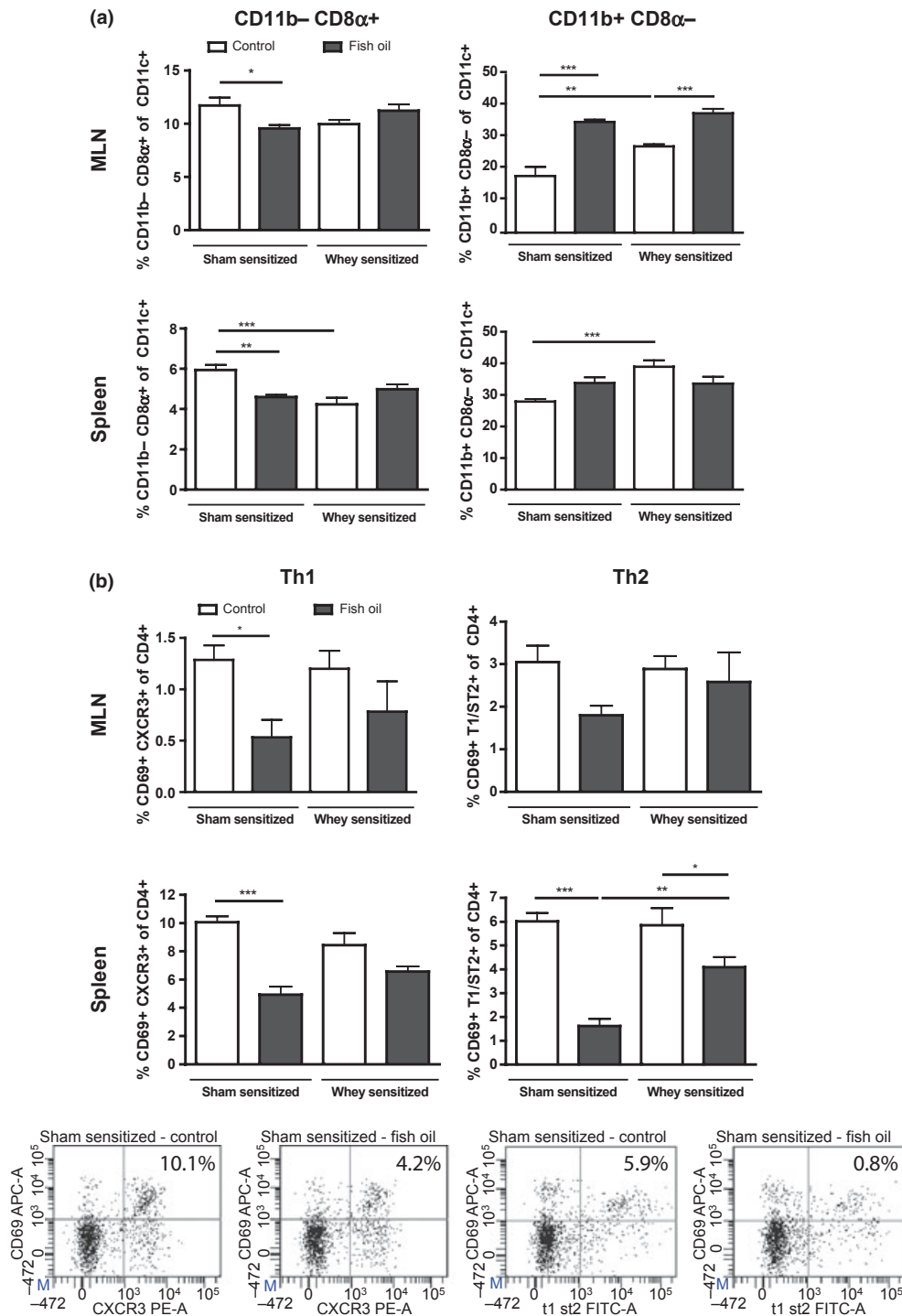


Fig 5. Effect of the fish oil diet on dendritic cell (DC) and T cell subsets in mesenteric lymph nodes (MLN) and spleen by flow cytometry. (a) Percentage of CD11b-CD8α⁺ lymphoid DC was reduced in MLN (diet NS, sensitization NS, interaction $P < 0.01$) and spleen (diet NS, sensitization $P < 0.05$, interaction $P < 0.001$) of fish oil fed sham mice while % CD11b⁺ CD8α⁻ myeloid DC was enhanced by the fish oil diet in MLN (diet $P < 0.001$, sensitization $P < 0.01$, interaction $P = 0.06$) but not spleen (diet NS, sensitization $P < 0.01$, interaction $P < 0.01$) (b) Percentage of activated Th1 cells (CD4⁺CD69⁺CXCR3⁺) was reduced by the fish oil diet in MLN (diet $P < 0.05$, sensitization NS, interaction NS) and spleen (diet $P < 0.001$, sensitization NS, interaction $P < 0.05$) of sham mice. Percentage of splenic activated Th2 cells (CD4⁺CD69⁺T1/ST2⁺) was reduced in both sham- and whey-sensitized mice ($P < 0.001$, sensitization $P < 0.05$, interaction $P < 0.05$). Th2 cells in MLN were not significantly altered (diet $P = 0.10$, sensitization NS, interaction NS). Representative FACS plot are shown for splenic Th1 and Th2 cells in sham-sensitized mice. Data are presented as means $\pm$ SEM, $n = 6$ /group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ two-way ANOVA followed by Bonferroni's multiple comparison test. NS, not significant.

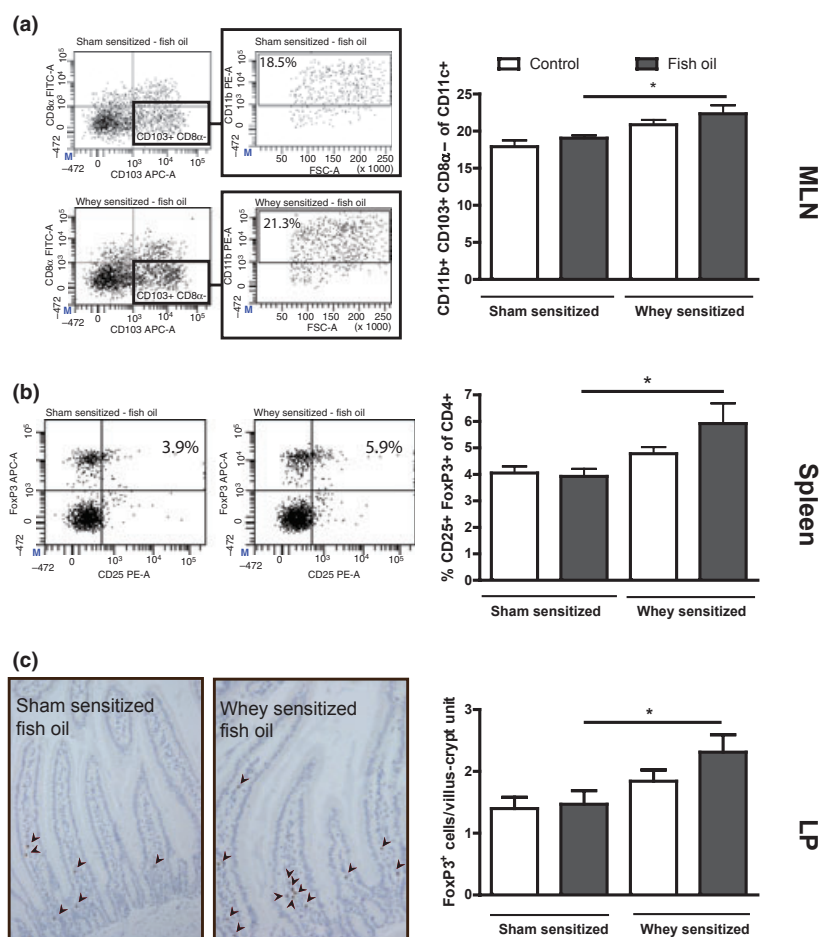


Fig 6. Effect of the fish oil diet on CD103⁺ dendritic cells (DC) and FoxP3⁺ regulatory T cells (Treg) (a) Percentage of CD11b⁺CD103⁺CD8 α ⁻ DC was enhanced in mesenteric lymph nodes (MLN) of whey-sensitized mice fed the fish oil diet when compared to sham mice (diet $P = 0.07$, sensitization $P < 0.01$, interaction NS). Representative FACS plots are shown. Furthermore, the percentage of FoxP3⁺ Treg was enhanced in whey-sensitized mice fed the fish oil diet in (b) spleen (flow cytometry, diet NS, sensitization $P < 0.01$, interaction NS) and (c) lamina propria (LP) of small intestine (diet NS, sensitization $P < 0.01$, interaction NS; immunohistochemical staining for FoxP3 (arrows); magnification 200 $\times$; typical example shown). Data are presented as means $\pm$ SEM, $n = 6$ /group. * $P < 0.05$ two-way ANOVA followed by Bonferroni's multiple comparison test. NS, not significant.

of allergic sensitization when providing fish oil starting early in life after birth. The Childhood Asthma Prevention Study (CAPS) reported that fish oil supplementation starting after birth did not prevent the onset of atopy at age of 5 in high risk children [37, 38]. However, the study population with a family history of asthma may be less susceptible to interventions due to genetic setting. In addition, the dosage aimed at may have been insufficient to prevent the onset of atopy in this critical period. Furthermore, the suboptimal adherence to oil capsules (51% for fish oil group) suggests that the average ingested n-3:n-6 fatty acid ratio was lower than the 1 : 5 aimed at for the fish oil group. Therefore, the fatty acid ratio of 1 : 7 achieved in plasma by fish oil is considered suboptimal in this study. Another post-natal intervention study supplemented newborns from birth to 6 months of age with

650 mg fish oil and reported lowered allergen-specific Th2 responses [39].

The n-3:n-6 PUFA ratio of the diets in our study should be seen in the view of the current human Western diet with a ratio of 1 : 10–20 vs. the diet of Inuit's, a fish consuming population with low prevalence of allergic disease consuming a 2.5 : 1 ratio [40–42]. Inuit plasma phospholipids have a 1 : 1 n-3:n-6 PUFA ratio [43]. Here, the fatty acid ratio of erythrocytes was reduced from about 1 : 5 in the control group to 1 : 1 in the fish oil fed mice. In human pregnancy studies, supplementing with 4–4.5 gram of fish oil daily to prevent allergic outcomes in offspring, ratios about 1 : 1.5 were shown to be effective [33, 34].

IgE and IgG are known to sensitize mast cells and basophils and immunoglobulin levels correlate positively with the intensity of the acute allergic skin

response [44]. Whey-specific IgE and IgG1 levels were reduced by the fish oil diet in our study, in association with a reduced acute allergic skin response. The suppressed Th2-type humoral response was further confirmed using serum transfer suggesting that n-3 LCPUFA have suppressed the sensitization to whey.

Studies report that arachidonic acid metabolite PGE₂ promotes IgE production by B cells [45]. Substitution of arachidonic acid by n-3 LCPUFA did reduce plasma PGE₂ concentrations and may indirectly have suppressed immunoglobulin generation. Furthermore, IL-4 and IL-13 induce IgE isotype switching and production of these cytokines by Th2 cells as well as mast cells may be reduced by n-3 LCPUFA [23, 46, 47]. In addition, DHA was found to reduce IgE production by human B cells via the IL-4 pathway or via an effect on CD40 [48]. Animal studies up to now are inconclusive about the effect of n-3 LCPUFA on IgE production [49]. Mice fed 1% soybean oil plus 10% fish oil tended to have enhanced OVA-specific IgE levels in serum as compared to mice fed 1% soybean oil plus 10% sunflower oil [50]. Since sunflower oil is very low in ALA, the requirements for an adequate amount of ALA as recommended by Reeves et al. are not met in the diet, which may explain the discrepancy with our results [18]. Like in our study, Matos et al. reported in addition to reduced OVA-specific IgE, also reduced OVA-IgG1 levels using a 7% fish oil diet [51]. Furthermore, dietary intervention with n-3 LCPUFA during pregnancy showed a tendency to reduce IgE antibody levels in infants at risk which is in line with our observations [17].

Our data indicate that n-3 LCPUFA not only target the humoral response but also may directly target the effector response. To further investigate this, naïve mice were fed the fish oil diet prior to passive sensitization with hyperimmune serum high in whey-IgE. This indicates that dietary fish oil modulates the mast cell response resulting in less acute allergic symptoms upon mast cell degranulation. The effects of LCPUFA on mast cell function have not been fully addressed in literature and many mechanisms may be involved. However, it has been proven that n-3 LCPUFA alter the mast cell phenotype and reduce the formation of eicosanoids of the 2/4 series which are causatively involved in inducing oedema and allergic symptoms [23].

Besides dampening the humoral and effector response, this study shows n-3 LCPUFA to affect DC and T cells. The fish oil diet was found to reduce the percentage of CD11c+CD11b-CD8 α + conventional lymphoid DC which are IL-12 producing and therefore Th1 polarizing [24, 25]. This corresponds well with our observation that the percentage of Th1 cells is diminished in spleen and MLN of sham-sensitized

mice. On the other hand, conventional myeloid DC (CD11c+CD11b+CD8 α -), which may induce tolerogenic or Th2 responses [24, 25], were enhanced by the fish oil diet in MLN. Since the diet reduced the development of Th2 cells, especially in the spleen, this may imply that this subset represents a tolerogenic phenotype in the current setting. Also, in a recent study EPA and DHA were found to suppress IL-12 secretion by LPS-stimulated DC while regulatory IL-10 production was enhanced [52]. As the percentage of Th2 cells was reduced most prominent in our study, the Th1/Th2 ratio was increased in the sham group fed the fish oil diet which implicates skewing away from the Th2 phenotype. Suppression of both Th1 and Th2 cell subsets relate to the findings of Dunstan et al. reporting that neonatal mononuclear cells secreted less Th1 and Th2 cytokines in response to allergen stimulation after fish oil consumption [33]. Another study showed a reduced IL-4 production by human peripheral blood mononuclear cells after n-3 LCPUFA consumption, enhancing the Th1/Th2 cytokine ratio [48]. Furthermore, supplementation with high doses of fish oil (3–6 g/day) reduced IL-2 and IFN- γ production [53, 54]. Although 90% of Th1 cells express CXCR3, we cannot exclude that other CD4+ cells also express this marker and are included in the analysis [27]. However, serological data confirm these findings. In our study, the effect of fish oil on Th1 and Th2 cells was shown most pronounced for sham-sensitized mice. We hypothesize that this effect was dampened in whey-sensitized mice due to the continuous boost of the immune response by the repeated sensitizations while for sham mice the challenge is the first contact with whey. Typically, the suppression of Th1 and Th2 cell subsets in both sham and whey-sensitized mice was most pronounced when the early activation marker CD69 was taken into account [55]. This suggests that apart from a minor population of whey-specific T cells also aspecific or bystander T cell activation was caused by whey. Intrinsic capacities of the whey protein fraction may contribute to this [56, 57]. Since percentages of both activated Th1 and Th2 cells were reduced by the fish oil diet in sham mice this suggests a general suppression of immune activation by the fish oil diet.

Besides Th1 and Th2 subsets dietary n-3 LCPUFA were observed to enhance Treg in whey-sensitized mice fed the fish oil diet both systemically in the spleen as well as locally in the small intestine. FoxP3+ Treg play an important role in modulating the immune response and oral tolerance induction [58, 59]. CD103+ cells migrate from lamina propria to the MLN and induce CD4+FoxP3+ Treg via a retinoic acid dependent mechanism [24, 32, 60]. In particular, the CD11b+CD103+CD8 α - DC subset is known to express retinal dehydrogenase isoform 2 which catalyses the conversion of

retinal to retinoic acid [31, 32]. This subset was enhanced in the MLN of fish oil fed whey-sensitized mice. FoxP3+ Treg generated in the MLN, transfer tolerance systemically and Treg home to the gut lamina propria to maintain homeostasis [59]. The fish oil diet was found to enhance FoxP3+ Treg in spleen and lamina propria of whey-sensitized mice. There is not much known about the effects of PUFA on Treg, but it has been suggested that n-3 LCPUFA support Treg function [61]. Kleemann *et al.* reported increased regulatory IL-10 mRNA expression in Peyer's patches of fish oil-fed BB rats [62], whereas Yaqoob *et al.* supplemented mice with fish oil and found IL-10 production by cultured spleen cells to be reduced [63]. Treg may reduce B cell and mast cell activation whereas they enhance oral tolerance induction, this may contribute to the reduced sensitization to whey observed [58].

In this study, we demonstrated the effect of dietary fish oil supplementation starting early in life, in a

mouse model for cow's milk allergy. Fish oil reduced the generation of a whey-specific humoral response by B cells, leading to attenuated production of Th2-type immunoglobulins and reduced acute allergic symptoms. Besides the suppression of allergic sensitization, the fish oil diet also directly reduced the allergic effector response. Furthermore, the fish oil diet increased Treg in both spleen and small intestine thus enhancing tolerizing capacities of the adaptive immune system. Together this implicates beneficial effects of fish oil supplementation in early life for the primary prevention of atopic diseases such as food allergy.

Conflicts of interest

None of the authors has any financial or personal conflict of interest to report. This study was financially supported by a grant from the Nutricia Research Foundation.

References

- Pascual CY, Crespo JF, Perez PG, Esteban MM. Food allergy and intolerance in children and adolescents, an update. *Eur J Clin Nutr* 2000; 54(Suppl 1):S75–8.
- Wang J, Sampson HA. Food allergy. *J Clin Invest* 2011; 121:827–35.
- Rona RJ, Keil T, Summers C *et al.* The prevalence of food allergy: a meta-analysis. *J Allergy Clin Immunol* 2007; 120:638–46.
- Venter C, Arshad SH. Epidemiology of food allergy. *Pediatr Clin North Am* 2011; 58:327–49.
- Fiocchi A, Brozek J, Schunemann H *et al.* World Allergy Organization (WAO) Diagnosis and Rationale for Action against Cow's Milk Allergy (DRACMA) Guidelines. *Pediatr Allergy Immunol* 2010; 21(Suppl 21): 1–125.
- Kattan JD, Cocco RR, Jarvinen KM. Milk and soy allergy. *Pediatr Clin North Am* 2011; 58:407–26.
- Burks AW, Tang M, Sicherer S *et al.* ICON: food allergy. *J Allergy Clin Immunol* 2012; 129:906–20.
- Rhodes HL, Sporik R, Thomas P, Holgate ST, Cogswell JJ. Early life risk factors for adult asthma: a birth cohort study of subjects at risk. *J Allergy Clin Immunol* 2001; 108:720–5.
- Black PN, Sharpe S. Dietary fat and asthma: is there a connection? *Eur Respir J* 1997; 10:6–12.
- Blumer N, Renz H. Consumption of omega3-fatty acids during perinatal life: role in immuno-modulation and allergy prevention. *J Perinat Med* 2007; 35(Suppl 1):S12–8.
- Kankaanpää P, Sutas Y, Salminen S, Lichtenstein A, Isolauri E. Dietary fatty acids and allergy. *Ann Med* 1999; 31:282–7.
- Nakagomi T, Itaya H, Tominaga T, Yamaki M, Hisamatsu S, Nakagomi O. Is atopy increasing? *Lancet* 1994; 343:121–2.
- Sugano M, Hirahara F. Polyunsaturated fatty acids in the food chain in Japan. *Am J Clin Nutr* 2000; 71:S189–S96.
- Yu G, Björkstén B. Polyunsaturated fatty acids in school children in relation to allergy and serum IgE levels. *Pediatr Allergy Immunol* 1998; 9:133–8.
- Poysa L, Korppi M, Pietikainen M, Remes K, Juntunen-Backman K. Asthma, allergic rhinitis and atopic eczema in Finnish children and adolescents. *Allergy* 1991; 46:161–5.
- Moilanen T, Nikkari T, Viikari J *et al.* Atherosclerosis precursors in Finnish children and adolescents. V. Fatty acid composition of serum cholesteryl esters: regional differences in Finland. *Acta Paediatr Scand Suppl* 1985; 318:111–7.
- Furuhjelm C, Warstedt K, Larsson J *et al.* Fish oil supplementation in pregnancy and lactation may decrease the risk of infant allergy. *Acta Paediatr* 2009; 98:1461–7.
- Reeves PG, Nielsen FH, Fahey GC Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* 1993; 123:1939–51.
- Schouten B, van Esch BC, Hofman GA *et al.* Oligosaccharide-induced whey-specific CD25(+) regulatory T-cells are involved in the suppression of cow milk allergy in mice. *J Nutr* 2010; 140:835–41.
- Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959; 37:911–7.
- Levant B, Ozias MK, Carlson SE. Diet (n-3) polyunsaturated fatty acid content and parity affect liver and erythrocyte phospholipid fatty acid composition in female rats. *J Nutr* 2007; 137:2425–30.
- van den Elsen L, Garssen J, Willemsen L. Long chain N-3 polyunsaturated fatty acids in the prevention of allergic and cardiovascular disease. *Curr Pharm Des* 2012; 18:2375–92.
- van den Elsen LW, Nusse Y, Balvers M *et al.* n-3 Long-chain PUFA reduce allergy-related mediator release by human mast cells in vitro via inhibition of reactive oxygen species. *Br J Nutr* 2012; 1–11. <http://dx.doi.org/10.1017/S0007114512003959>.

- 24 Coombes JL, Powrie F. Dendritic cells in intestinal immune regulation. *Nat Rev Immunol* 2008; 8:435–46.
- 25 Johansson C, Kelsall BL. Phenotype and function of intestinal dendritic cells. *Semin Immunol* 2005; 17:284–94.
- 26 Groom JR, Luster AD. CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunol Cell Biol* 2011; 89:207–15.
- 27 Kim CH, Nagata K, Butcher EC. Dendritic cells support sequential reprogramming of chemoattractant receptor profiles during naive to effector T cell differentiation. *J Immunol* 2003; 171:152–8.
- 28 Sallusto F, Lenig D, Mackay CR, Lanzavecchia A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J Exp Med* 1998; 187:875–83.
- 29 Xu D, Chan WL, Leung BP *et al.* Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *J Exp Med* 1998; 187:787–94.
- 30 Lohning M, Stroehmann A, Coyle AJ *et al.* T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. *Proc Natl Acad Sci U S A* 1998; 95:6930–5.
- 31 Fujimoto K, Karuppuhamy T, Takemura N *et al.* A new subset of CD103 + CD8alpha+ dendritic cells in the small intestine expresses TLR3, TLR7, and TLR9 and induces Th1 response and CTL activity. *J Immunol* 2011; 186:6287–95.
- 32 Uematsu S, Fujimoto K, Jang MH *et al.* Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. *Nat Immunol* 2008; 9:769–76.
- 33 Dunstan JA, Mori TA, Barden A *et al.* Fish oil supplementation in pregnancy modifies neonatal allergen-specific immune responses and clinical outcomes in infants at high risk of atopy: a randomized, controlled trial. *J Allergy Clin Immunol* 2003; 112:1178–84.
- 34 Furuhjelm C, Warstedt K, Fageras M *et al.* Allergic disease in infants up to 2 years of age in relation to plasma omega-3 fatty acids and maternal fish oil supplementation in pregnancy and lactation. *Pediatr Allergy Immunol* 2011; 22:505–14.
- 35 Palmer DJ, Sullivan T, Gold MS *et al.* Effect of n-3 long chain polyunsaturated fatty acid supplementation in pregnancy on infants' allergies in first year of life: randomised controlled trial. *BMJ* 2012; 344:e184.
- 36 Klemens CM, Berman DR, Mozurkewich EL. The effect of perinatal omega-3 fatty acid supplementation on inflammatory markers and allergic diseases: a systematic review. *BJOG* 2011; 118:916–25.
- 37 Marks GB, Mhrshahi S, Kemp AS *et al.* Prevention of asthma during the first 5 years of life: a randomized controlled trial. *J Allergy Clin Immunol* 2006; 118:53–61.
- 38 Mhrshahi S, Peat JK, Webb K *et al.* The childhood asthma prevention study (CAPS): design and research protocol of a randomized trial for the primary prevention of asthma. *Control Clin Trials* 2001; 22:333–54.
- 39 D'Vaz N, Meldrum SJ, Dunstan JA *et al.* Fish oil supplementation in early infancy modulates developing infant immune responses. *Clin Exp Allergy* 2012; 42:1206–16.
- 40 Simopoulos AP. Importance of the omega-6/omega-3 balance in health and disease: evolutionary aspects of diet. *World Rev Nutr Diet* 2012; 102:10–21.
- 41 Krause TG, Koch A, Poulsen LK, Kristensen B, Olsen OR, Melbye M. Atopic sensitization among children in an arctic environment. *Clin Exp Allergy* 2002; 32:367–72.
- 42 Bang HO, Dyerberg J, Sinclair HM. The composition of the Eskimo food in north western Greenland. *Am J Clin Nutr* 1980; 33:2657–61.
- 43 Dyerberg J, Bang HO, Hjorne N. Fatty acid composition of the plasma lipids in Greenland Eskimos. *Am J Clin Nutr* 1975; 28:958–66.
- 44 Schouten B, van Esch BC, Hofman GA, van den Elsen LW, Willemsen LE, Garssen J. Acute allergic skin reactions and intestinal contractility changes in mice orally sensitized against casein or whey. *Int Arch Allergy Immunol* 2008; 147:125–34.
- 45 Kaur K, Harris SG, Padilla J, Graf BA, Phipps RP. Prostaglandin E2 as a modulator of lymphocyte mediated inflammatory and humoral responses. *Adv Exp Med Biol* 1999; 469:409–12.
- 46 Dunstan JA, Mori TA, Barden A *et al.* Maternal fish oil supplementation in pregnancy reduces interleukin-13 levels in cord blood of infants at high risk of atopy. *Clin Exp Allergy* 2003; 33:442–8.
- 47 Mizota T, Fujita-Kambara C, Matsuya N *et al.* Effect of dietary fatty acid composition on Th1/Th2 polarization in lymphocytes. *JPEN J Parenter Enteral Nutr* 2009; 33:390–6.
- 48 Weise C, Hilt K, Milovanovic M, Ernst D, Ruhl R, Worm M. Inhibition of IgE production by docosahexaenoic acid is mediated by direct interference with STAT6 and NFkappaB pathway in human B cells. *J Nutr Biochem* 2011; 22:269–75.
- 49 Calder PC. N-3 polyunsaturated fatty acids and immune cell function. *Adv Enzyme Regul* 1997; 37:197–237.
- 50 Johansson S, Lonnqvist A, Ostman S, Sandberg AS, Wold AE. Long-chain polyunsaturated fatty acids are consumed during allergic inflammation and affect T helper type 1 (Th1)- and Th2-mediated hypersensitivity differently. *Clin Exp Immunol* 2010; 160:411–9.
- 51 de Matos OG, Amaral SS, Pereira da Silva PE *et al.* Dietary supplementation with omega-3-PUFA-rich fish oil reduces signs of food allergy in ovalbumin-sensitized mice. *Clin Dev Immunol* 2012; 236564 [Epub ahead of print].
- 52 Draper E, Reynolds CM, Canavan M, Mills KH, Loscher CE, Roche HM. Omega-3 fatty acids attenuate dendritic cell function via NF-kappaB independent of PPARgamma. *J Nutr Biochem* 2011; 22:784–90.
- 53 Gallai V, Sarchielli P, Trequattrini A *et al.* Cytokine secretion and eicosanoid production in the peripheral blood mononuclear cells of MS patients undergoing dietary supplementation with n-3 polyunsaturated fatty acids. *J Neuroimmunol* 1995; 56:143–53.
- 54 Meydani SN, Endres S, Woods MM *et al.* Oral (n-3) fatty acid supplementation suppresses cytokine production and lymphocyte proliferation: comparison between young and older women. *J Nutr* 1991; 121:547–55.
- 55 Shinoda K, Tokoyoda K, Hanazawa A *et al.* Type II membrane protein CD69 regulates the formation of resting T-

- helper memory. *Proc Natl Acad Sci U S A* 2012; **109**:7409–14.
- 56 Jensen GS, Patel D, Benson KF. A novel extract from bovine colostrum whey supports innate immune functions. II. Rapid changes in cellular immune function in humans. *Prev Med* 2012; **54**(Suppl):S124–9.
- 57 Di Genova G, Savelyeva N, Suchacki A, Thirdborough SM, Stevenson FK. Bystander stimulation of activated CD4 + T cells of unrelated specificity following a booster vaccination with tetanus toxoid. *Eur J Immunol* 2010; **40**:976–85.
- 58 Akdis CA, Akdis M. Mechanisms and treatment of allergic disease in the big picture of regulatory T cells. *J Allergy Clin Immunol* 2009; **123**:735–46, quiz 47–8.
- 59 Ding Y, Xu J, Bromberg JS. Regulatory T cell migration during an immune response. *Trends Immunol* 2012; **33**:174–80.
- 60 del Rio ML, Bernhardt G, Rodriguez-Barbosa JJ, Forster R. Development and functional specialization of CD103 + dendritic cells. *Immunol Rev* 2010; **234**:268–81.
- 61 Calder PC, Krauss-Etschmann S, de Jong EC *et al*. Early nutrition and immunity - progress and perspectives. *Br J Nutr* 2006; **96**:774–90.
- 62 Kleemann R, Scott FW, Worz-Pagenstert U, Nimal Ratnayake WM, Kolb H. Impact of dietary fat on Th1/Th2 cytokine gene expression in the pancreas and gut of diabetes-prone BB rats. *J Autoimmun* 1998; **11**:97–103.
- 63 Yaqoob P, Calder PC. The effects of dietary lipid manipulation on the production of murine T cell-derived cytokines. *Cytokine* 1995; **7**:548–53.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effect of the fish oil diet on total Th1 and Th2 cell subsets in mesenteric lymph nodes (MLN) and spleen by flow cytometry. The percentage of CD4+ cells in MLN (diet NS, sensitization NS, interaction NS) and spleen (diet $P < 0.05$, sensitization NS, interaction $P = 0.09$ for spleen) were not reduced by the fish oil diet. The percentage of total Th1 cells (CD4+CXCR3+)

was reduced by the fish oil diet in spleen of sham mice (diet $P < 0.01$, sensitization NS, interaction NS). Percentages of Th1 cells in MLN showed a similar tendency (diet $P < 0.05$, sensitization NS, interaction NS) as well as the percentage of total Th2 cells (CD4+T1/ST2+) in spleen ($P < 0.01$, sensitization NS, interaction NS) and MLN (diet NS, sensitization NS, interaction NS). Data are presented as means $\pm$ SEM, $n = 6$ /group. $*P < 0.05$ two-way ANOVA followed by Bonferroni's multiple comparison test. NS = not significant.

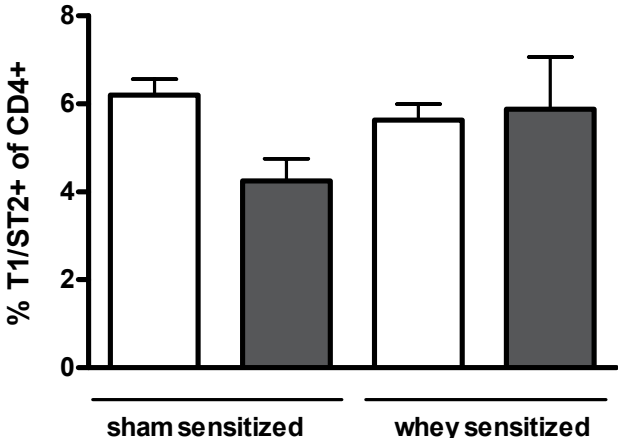
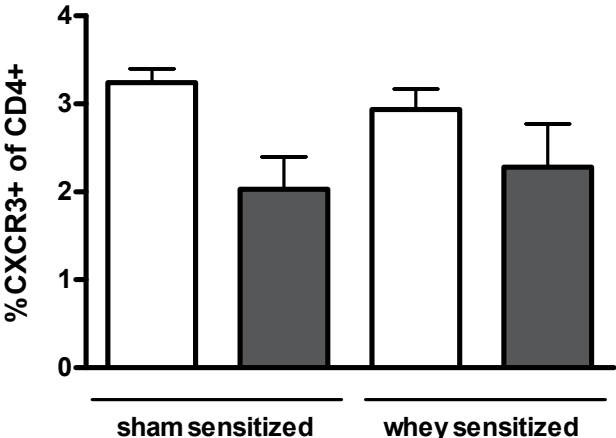
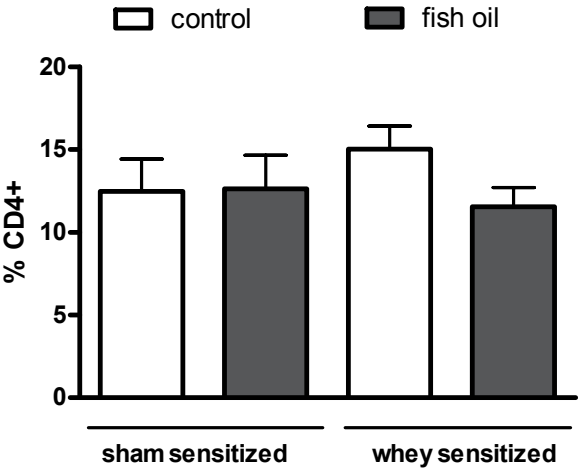
Supplementary Figure 1

CD4+

Th1

Th2

MLN



Spleen

