

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

CD25⁺ regulatory T cells transfer n-3 long chain polyunsaturated fatty acids-induced tolerance in mice allergic to cow's milk protein

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cow's milk allergy; fish oil; long-chain n-3 polyunsaturated fatty acids; regulatory T cell; tolerance.

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Abstract

Background: Recently, we have shown that dietary long-chain n-3 polyunsaturated fatty acids (n-3 LCPUFA) largely prevent allergic sensitization in a murine model for cow's milk allergy. The aim of this study was to assess the contribution of regulatory T cells (Treg) in the prevention of food allergy by n-3 LCPUFA.

Methods: C3H/HeOuJ female donor mice were fed a control or fish oil diet before and during oral sensitization with cow's milk protein whey. Acute allergic skin response (ASR), anaphylaxis, body temperature, serum immunoglobulins, and mouse mast cell protease-1 (mmcp-1) were assessed. Splenocytes of sham- or whey-sensitized donor mice fed either control or fish oil diet were adoptively transferred to naïve recipient mice. Recipient mice received a whole splenocyte suspension, splenocytes *ex vivo* depleted of CD25⁺ cells, or MACS-isolated CD4⁺ CD25⁺ Treg. Recipient mice were sham- or whey-sensitized and fed control diet.

Results: The ASR as well as whey-specific IgE and whey-specific IgG1 levels were reduced in whey-sensitized donor mice fed the fish oil diet as compared to the control diet. Splenocytes of control-diet-fed whey-sensitized donors transferred immunologic memory. By contrast, splenocytes of fish-oil-fed whey-sensitized – but not sham-sensitized – donors transferred tolerance to recipients as shown by a reduction in ASR and serum mmcp-1, and depletion of CD25⁺ Treg abrogated this. Transfer of CD25⁺ Treg confirmed the involvement of Treg in the suppression of allergic sensitization.

Conclusions: CD25⁺ Treg are crucial in whey allergy prevention by n-3 LCPUFA.

The current prevalence of allergic disease in developed countries is estimated to be 15–30% (1). Allergic sensitization to food proteins may occur early in life and affects 6% of children and 3–4% of adults (2). In food-allergic patients, harmless foods including egg, milk, nuts, and wheat can result in eczema, diarrhea, respiratory problems, or even systemic anaphylaxis. This is the result of an impaired development of tolerance or the breakdown of existing tolerance to food proteins (3, 4). Intact food-derived epitopes that escaped gastrointestinal degradation may be taken up in the Peyer's patches or lamina propria (LP) by dendritic cells (DCs). Presentation of

the antigen to naïve CD4⁺ T cells in the mesenteric lymph nodes (MLNs) will instruct regulatory T cells (Treg), which support oral tolerance induction (5). From the MLNs, tolerance can be transferred to the systemic compartment as Treg released in the blood home back to the LP, but also remain present systemically (6). Treg comprise several subsets including the naturally occurring CD4⁺ CD25⁺ Treg (nTreg) derived from the thymus and expressing the transcription factor forkhead box protein 3 (FoxP3) as well as peripheral antigen-induced CD4⁺ CD25⁺ Foxp3⁺ Treg (iTreg), interleukin (IL)-10- (Tr1) and TGF- β -producing (Th3) Treg (7–9).

Epidemiological data suggest that dietary components may affect oral tolerance induction. Recently, it has been shown that the long-chain n-3 polyunsaturated fatty acids (n-3 LCPUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), found in fish oil, may reduce the susceptibility to develop allergic disease. Daily dietary supplementation with n-3 LCPUFA during pregnancy and lactation reduced the incidence of food allergy and IgE-associated eczema in children at risk for atopy (10). Furthermore, dietary supplementation with fish oil largely reduced ovalbumin allergy in mice (11) and prevented oral sensitization to the cow's milk protein whey (12). Currently, the involvement of Treg in tolerance induction by n-3 LCPUFA is speculative and remains to be elucidated. In this study, we assessed whether Treg from whey-sensitized donor mice fed n-3 LCPUFA could adoptively transfer tolerance to whey-sensitized recipients.

Materials and methods

Oral sensitization donor mice

Animal use was performed in accordance with the guidelines of the Animal Ethics Committee of Utrecht University. Three-week-old specific pathogen-free female C3H/HeOuJ mice (Charles River Laboratories, L'Arbresle Cedex, France; $n = 6$ –12/group) were fed AIN-93G-based diets composed of either 10% soybean oil (control diet) or 4% soybean oil plus 6% tuna oil (fish oil diet) as described before (12). Tuna oil (38.5% n-3 PUFA) was a kind gift from Bioriginal (Den Bommel, the Netherlands) and contained 27.8% DHA and 7% EPA. 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. Mice were sensitized intragastrically (i.g.) using whey (DMV international, Veghel, the Netherlands) as described before (13, 14) (Fig. 1A). Donor mice received an intradermal (i.d) challenge in the ear pinnae with 10 μ g whey, and the day thereafter mice were killed.

Adoptive transfer of splenocytes

For depletion of CD25⁺ Treg, splenocytes were *ex vivo* incubated with rat anti-CD25 monoclonal antibody PC61 as previously described (13). The mouse CD4⁺ CD25⁺ Regulatory T-cell Isolation kit (Miltenyi Biotec, Leiden, the Netherlands) was used for CD4⁺ CD25⁺ enrichment according to the manufacturer's instructions. The whole splenocyte suspension (cell count equal for control-diet- and fish-oil-fed mice), CD25⁺ Treg-depleted splenocytes (both 5×10^6 cells), or isolated CD4⁺ CD25⁺ Treg (1.2×10^5 cells; 85% was FoxP3⁺ as assessed by flow cytometry as previously described (12)) was intravenously injected in naïve recipient mice ($n = 6$ /group; Fig. 1A). Recipient mice were fed AIN-93G diet (15) and sensitized as described above. At day 33, they received an i.d challenge with 10 μ g whey and i.g. challenge with 50 mg whey, and at day 34, mice were killed.

Acute allergic skin response, anaphylaxis symptom score, and body temperature

The acute allergic skin response (ASR) was measured in duplicate for both ears 1 h after i.d. challenge with whey using a digital micrometer (Mitutoyo, Veenendaal, the Netherlands). Ear swelling was calculated by subtracting basal ear thickness from ear thickness after 1 h. To assess the severity of anaphylaxis symptoms 30 min after i.d. whey challenge, a validated anaphylaxis symptom scoring table was used (16). All mice were given an implantable electronic ID transponder (Plexx, Elst, the Netherlands) to measure individual changes in body temperature 1 h after i.d challenge.

Serum immunoglobulins and mmcp-1

Levels of whey-specific IgE, IgG1, and IgG2a were determined by ELISA in serum collected 18 h after oral challenge, as previously described (14). Serum mouse mast cell protease-1 (mmcp-1), reflecting mucosal mast cell degranulation, was determined using a commercially available ELISA kit (eBioscience, Vienna, Austria) according to the manufacturer's protocol.

Proliferation and cytokine release

Splenocytes (4×10^5 per well) were incubated in RPMI1640 supplemented with penicillin (100 U/ml), streptomycin (100 μ g/ml), and 10% FBS. Cells were stimulated for 48 h with 1 μ g/ml anti-CD3 plus 1 μ g/ml anti-CD28. Supernatants were harvested for cytokine measurements by ELISA (IL-10, TGF- β) according to the manufacturer's recommendations (eBioscience). T-cell proliferation was determined by measuring [³H]-thymidine incorporation.

Statistics

Data are presented as means $\pm$ SEM and were analyzed using two-way ANOVA and *post hoc* Bonferroni test using GraphPad Prism software. For anaphylaxis symptom score, medians are depicted, and Kruskal–Wallis test followed by Dunn's multiple comparison test was used. For serological data, Tukey's box-and-whisker plots are used, and square-root transformation was performed before analysis to normalize the data distribution. Spearman's correlation coefficients were calculated.

Results

Fish oil diet reduces whey-induced ASR and drop in body temperature in whey-sensitized donor mice

One hour after i.d. challenge, the ASR in whey-sensitized mice fed control diet was significantly enhanced as compared to sham-sensitized mice (Fig. 1B), and this was effectively reduced by the fish oil diet. Basal body temperature was approximately 37.5°C in all groups (data not shown). A decline to 33.5°C after challenge was observed in whey-sensitized mice

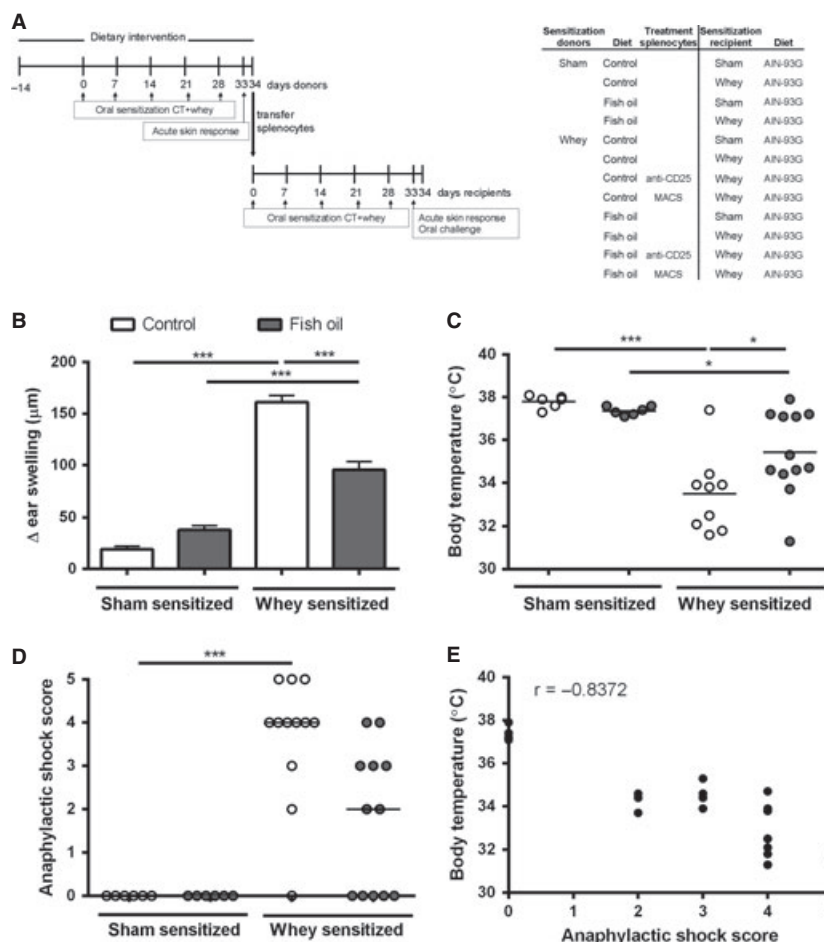


Figure 1 The allergic response in donor mice after an intradermal (i.d.) challenge with whey. (A) Schematic overview of the adoptive transfer protocol and experimental setup. Donor mice ($n = 6-12$ /group) were fed the control or fish oil diet, starting 2 weeks prior to the first sensitization and continued during the whole sensitization period until killing. Mice were sensitized intragastrically with 20 mg whey with 10 μ g cholera toxin (CT). Sham mice received CT alone. Mice were sensitized once a week for 5 consecutive weeks. At day 33, donors received an i.d. challenge with whey (10 μ g) in the ear, and at day 34, spleens were collected for transfer. Mice used for the measurement of serological parameters also received an oral challenge with 50 mg whey. Single-cell suspensions either treated or not treated with anti-CD25 antibody or MACS separation were transferred into naive recipients. Recipient mice were fed

AIN-93G diet and sham- or whey-sensitized. After the ear challenge, recipients also received an oral challenge (50 mg whey). At day 34, they were killed. Whey allergy was assessed in donor mice after an i.d. whey challenge by changes in (B) acute allergic skin response (C) body temperature, and (D) anaphylaxis symptom scores. The scoring was as follows: 0) no symptoms; 1) scratching nose and mouth; 2) swelling around eyes and mouth, piloerection, reduced activity, higher breathing rate; 3) shortness of breath, blue rash around mouth and tail, higher breathing rate; 4) no activity after stimulation, shivering, muscle contractions; and 5) death by shock. (E) Body temperature and anaphylaxis symptoms were negatively correlated ($P < 0.001$). Data are presented as means $\pm$ SEM, and median is shown for anaphylaxis symptom score, $n = 6-12$ /group. * $P < 0.05$, *** $P < 0.001$.

fed the control diet (Fig. 1C), while the fish oil diet reduced the drop in body temperature. Eleven of the twelve whey-sensitized control-diet-fed donors showed mild to severe symptoms, which were fatal for three mice (Fig. 1D). The fish oil diet tended to reduce anaphylaxis symptom scores, and in this group, all mice survived. Interestingly, in this group, four of eleven mice showed no signs at all. Body temperature showed a negative correlation with anaphylaxis symptoms (Fig. 1E).

Reduced whey-specific immunoglobulin levels and mmcp-1 in whey-sensitized donor mice fed fish oil diet

Serum levels of whey-specific IgE, IgG1, and IgG2a were increased in control-diet-fed mice sensitized with whey compared with sham-sensitized mice and reduced by the fish oil diet (Fig. 2A-C). In addition, fish-oil-diet-fed whey-sensitized mice showed lower serum mmcp-1 concentrations compared with control-diet-fed mice (Fig. 2D).

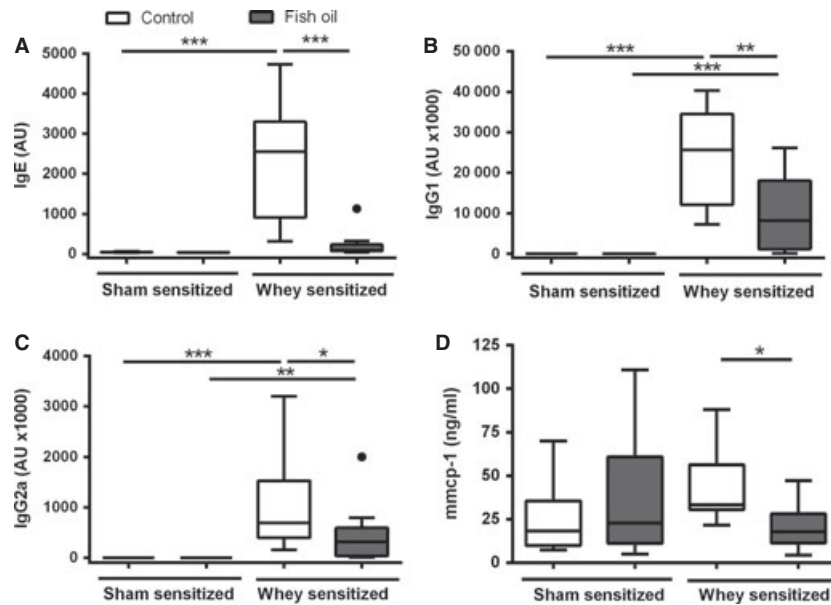


Figure 2 The humoral response in donor mice fed control fish oil diet after the oral challenge with whey. Fish oil reduced whey-specific (A) IgE (B) IgG1 (C) IgG2a, and (D) mouse mast cell protease-1

(mmcp-1) in serum as compared to control-diet-fed whey-sensitized mice. Data are presented as Tukey's box-and-whisker plots, $n = 6-12/\text{group}$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Cow's milk allergy is suppressed by adoptive transfer of splenocytes from whey-sensitized fish-oil-fed mice

To test whether splenocytes of fish-oil-fed donor mice were able to transfer n-3 LCPUFA-induced tolerance, an adoptive transfer experiment was performed. The ASR was enhanced in whey-sensitized as compared to sham-sensitized recipients transferred with splenocytes of sham-sensitized donors (Fig. 3A). Splenocyte transfer of control-diet-fed whey-sensitized donor mice as compared to sham-sensitized donor mice increased the ASR in whey-sensitized recipients. By contrast, splenocytes of fish-oil-fed whey-sensitized donors reduced the ASR in whey-sensitized recipients compared with control-diet-fed whey-sensitized donors and also compared with fish-oil-fed sham-sensitized donors, indicating an antigen-specific protective effect of fish oil. The body temperature of whey-sensitized recipients transferred with splenocytes from control-diet-fed whey-sensitized donors was significantly reduced as compared to their sham-sensitized counterparts (Fig. 3B). This effect was lost upon transfer of splenocytes of fish-oil-fed whey-sensitized donor mice. The serum mmcp-1 concentrations were in accordance with this (Fig. 3C), and there was a positive correlation between mmcp-1 and the ASR (Fig. 3D).

CD4⁺ CD25⁺ Treg are involved in transfer of tolerance

To study the contribution of CD25⁺ Treg in the allergy-preventive effects of fish oil, *ex vivo* CD25⁺ Treg-depleted splenocytes and MACS-isolated CD4⁺ CD25⁺ Treg were adoptively transferred. Recipients transferred with splenocytes of control-diet-fed whey-sensitized donors treated with anti-CD25 showed a slight reduction in ASR compared with

transfer of the total splenocytes (Fig. 4A). By contrast, recipients transferred with splenocytes of fish-oil-fed whey-sensitized donor mice depleted of CD25⁺ cells showed an increased ASR compared with recipients transferred with the whole splenocyte suspension. Transfer of isolated CD25⁺ Treg from control-diet- and fish-oil-diet-fed whey-sensitized donor mice reduced the ASR. Injection of CD25⁺ Treg-depleted cells from control-diet-fed whey-sensitized donors normalized body temperature in whey-sensitized recipients (Fig. 4B). Transfer of CD25⁺ Treg compared with whole splenocyte suspension from control-diet-fed whey-sensitized donor mice reduced mmcp-1, whereas mmcp-1 was already low in fish oil group (Fig. 4C).

Whey-specific IgE levels were significantly reduced in recipients transferred with CD25⁺ Treg from control-diet-fed whey-sensitized donor mice, when compared with the whole splenocyte suspension (Fig. 5A). Transfer of CD25⁺ Treg from both control-diet- and fish-oil-fed whey-sensitized donors completely abrogated whey-specific IgG1 production in recipient mice (Fig. 5B). The CD25⁺ cell-depleted population of control-diet-fed whey-sensitized donor mice induced whey-specific IgG2a levels in recipient mice compared with recipients transferred with the whole cell suspension, which were significantly reduced by the fish oil diet (Fig. 5C).

Enhanced IL-10 secretion in restimulated splenocytes isolated from recipients transferred with cells from fish-oil-diet-fed whey-sensitized donors

The proliferation upon anti-CD3/CD28 stimulation was enhanced in splenocytes isolated from recipients transferred with cells from fish-oil-fed whey-sensitized donors as compared to donor cells from the control-diet group (Fig. 6A).

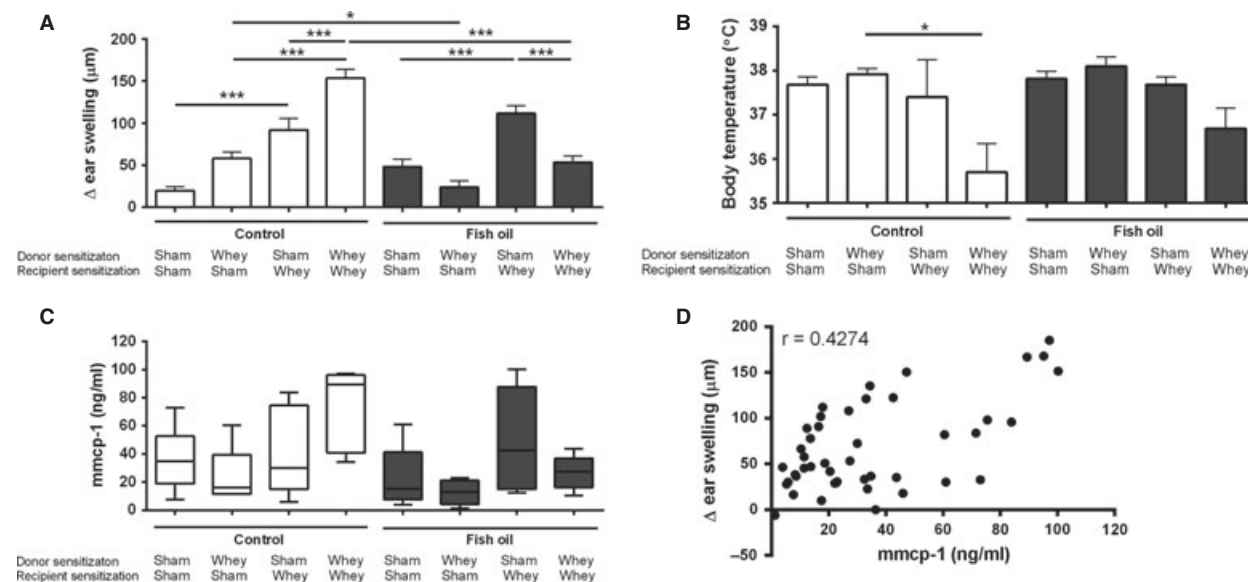


Figure 3 Adoptive transfer of splenocytes from control-diet- or fish-oil-diet-fed donors to sham- or whey-sensitized recipient mice fed AIN-93G diet. After an intradermal challenge, the (A) acute allergic skin response and (B) body temperature were assessed. (C) mmcp-1

was assessed in serum after an oral challenge. (D) mmcp-1 showed a positive correlation with the acute allergic skin response ($P < 0.01$). Data are presented as means $\pm$ SEM or Tukey's box-and-whisker plots, $n = 6$ /group. * $P < 0.05$, *** $P < 0.001$.

Accordingly, the concentration of IL-10 was significantly increased in supernatants of this group (Fig. 6B), while TGF- β was not enhanced (Fig. 6C).

Discussion

In the current study, a mouse model for orally induced cow's milk allergy was used to investigate the contribution of CD25⁺ Treg in fish-oil-induced tolerance to whey. Adoptive transfer of whole splenocyte suspensions compared with *ex vivo* CD25⁺ Treg-depleted splenocytes or isolated CD4⁺ CD25⁺ Treg populations confirmed that CD25⁺ Treg are required for the protective effects of fish oil on the induction of cow's milk allergy in mice.

In this study, fish oil supplementation after birth effectively prevented allergic sensitization to whey in mice, as demonstrated before (12). Furthermore, it has been shown that postnatal n-3 LCPUFA supplementation during the first 6 months of life reduced allergen-specific Th2 responses in humans (17). However, it is unknown how n-3 LCPUFA exactly may exert their effects. Recently, we showed that fish oil was able to reduce the percentage of activated Th1 and Th2 cells in spleen and/or MLN of sham-sensitized mice while reducing the percentage of Th2 cells in the spleen of whey-sensitized mice (12). Although we cannot exclude the possibility that the fish oil diet may have affected the mucosal toxicity of cholera toxin, reduced sensitization upon dietary fish oil supplementation has been described to occur in the absence of the use of cholera toxin as well (11, 18). Furthermore, the fish oil diet enhanced the number of FoxP3⁺ cells locally in the LP of the jejunum as well as the

percentage of FoxP3⁺ Treg systemically in the spleen in this mouse model for whey allergy, while these cells were not affected in the sham-sensitized mice treated with cholera toxin (12). Also other studies show n-3 LCPUFA to support Treg function; however, results are complex and sometimes contradictory. Iwami et al. suggested EPA to induce Treg via peroxisome proliferator-activated receptor- γ (PPAR γ) (19). Furthermore, EPA-fed mice demonstrated increased FoxP3⁺ cells as well as PPAR γ expression in transplanted hearts. *In vitro* EPA enhanced proliferation of Treg, whereas a PPAR γ antagonist abrogated this (20). On the other hand, DHA was reported to induce the expression of Treg markers FoxP3, cytotoxic T-lymphocyte-associated antigen 4, and/or transforming growth factor (TGF)- β , while Treg suppressive function was diminished (21, 22). While we focused on T-cell subsets assessed by flow cytometry in the previous study, it was now further investigated whether the CD25⁺ Treg are functionally involved in the tolerance induction instructed by the fish oil diet.

In the current study, the induction of functional Treg by the fish oil diet was confirmed using an adoptive transfer approach with the spleen as donor organ. Treg originating from the MLN confer local intestinal tolerance for antigens delivered via the gastrointestinal tract to systemic tolerance, which can be confirmed by transfer of splenocytes (5, 13, 23). Splenocytes from whey-sensitized donor mice fed the control diet transferred allergy to recipient mice. In contrast, splenocytes of fish-oil-fed whey-sensitized donor mice did not transfer an immunologic memory response, but rather tolerance to recipients. Transfer of cells from sham-sensitized donors fed fish oil did not protect recipient mice, indicating the

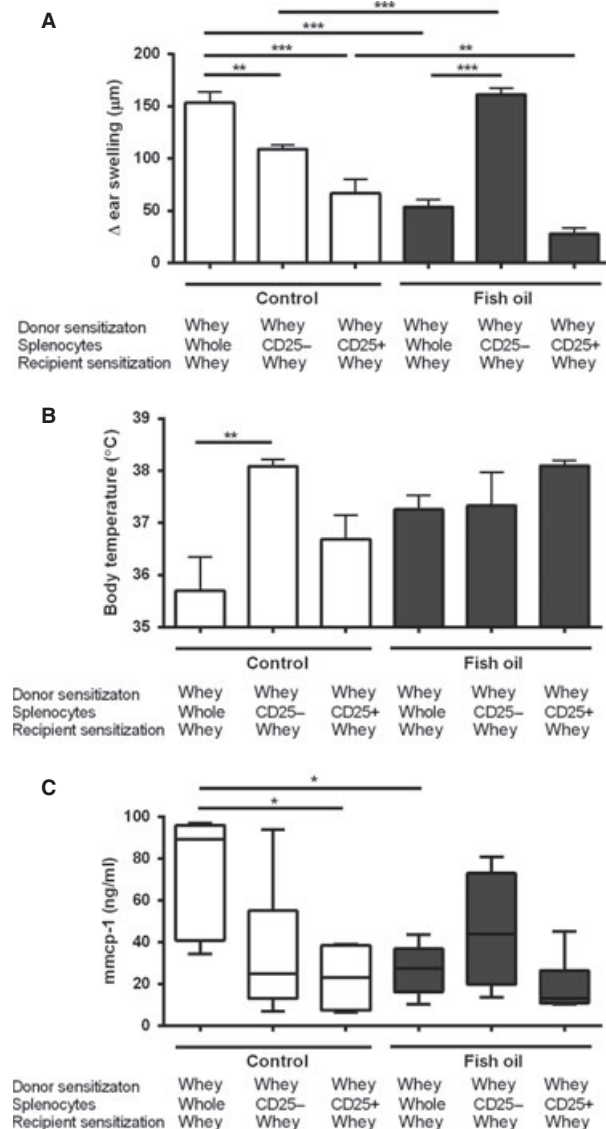


Figure 4 The effect of CD25⁺ Treg-depleted and CD25⁺ Treg populations from control-diet- or fish-oil-diet-fed donors on allergic parameters in whey-sensitized recipient mice fed AIN-93G diet. After an intradermal challenge, the (A) acute allergic skin response and (B) body temperature were assessed. (C) mmcp-1 was assessed in serum after an oral challenge. CD25⁻ splenocytes represent the *ex vivo* CD25⁺ Treg-depleted splenocyte population; CD25⁺ splenocytes represent the MACS-isolated CD25⁺ Treg population. Data are presented as means $\pm$ SEM or Tukey's box-and-whisker plots, $n = 6$ /group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

involvement of an antigen-specific Treg population contributing to whey allergy prevention by fish oil. Whey-specific immunoglobulin levels were not reduced by transfer of splenocytes isolated from whey-sensitized donors fed fish oil compared with control diet. Yamashita et al. reported high ovalbumin-specific serum IgE in recipient mice after transfer of MLN cells from ovalbumin-tolerant mice (24). However, the ASR and serum mmcp-1 were reduced by transfer of cells

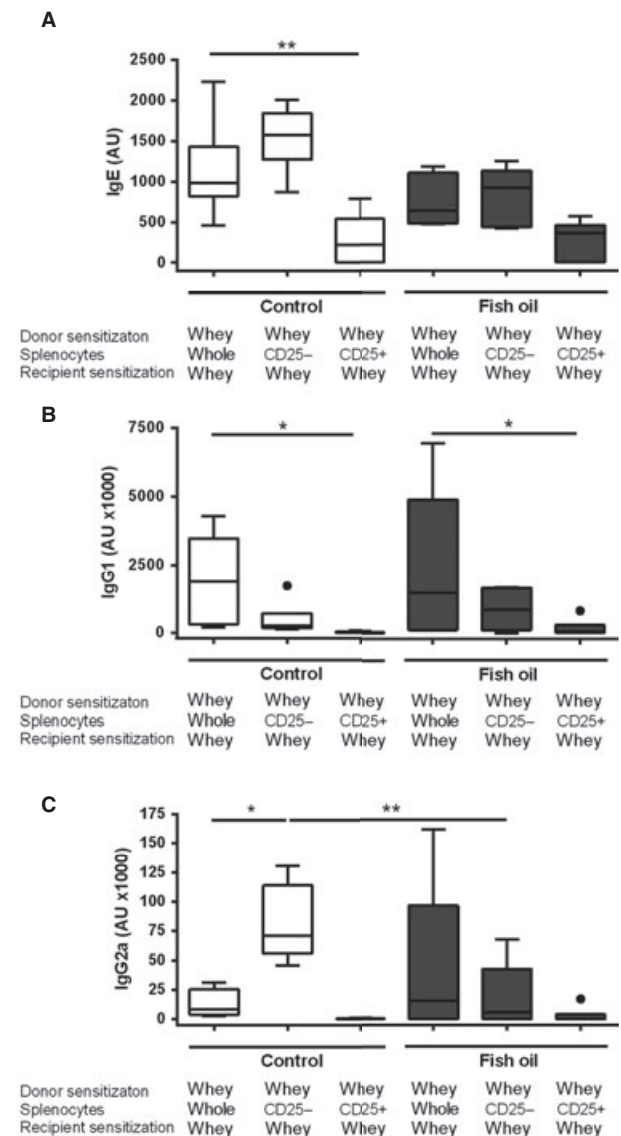


Figure 5 The effect of CD25⁺ Treg-depleted and CD25⁺ Treg populations from control-diet- or fish-oil-diet-fed donors on immunoglobulin levels in whey-sensitized recipient mice fed AIN-93G diet. (A) IgE (B) IgG1, and (C) IgG2a in serum after an oral challenge. CD25⁻ splenocytes represent the *ex vivo* CD25⁺ Treg-depleted splenocyte population; CD25⁺ splenocytes represent the MACS-isolated CD25⁺ Treg population. Data are presented as Tukey's box-and-whisker plots, $n = 6$ /group. * $P < 0.05$, ** $P < 0.01$.

of fish-oil-fed whey-sensitized donors, indicating suppression of mast cell degranulation in recipient mice. CD25⁺ Treg were shown to inhibit IgE-mediated mast cell degranulation via cell-cell contact (25–27). Furthermore, IL-10 was shown to reduce mast cell degranulation and cytokine release (28, 29). Hence, CD25⁺ donor splenocytes may have suppressed the capacity of mast cells to degranulate. Indeed, only *ex vivo*-stimulated splenocytes from recipients transferred with cells from fish-oil-fed whey-sensitized donors

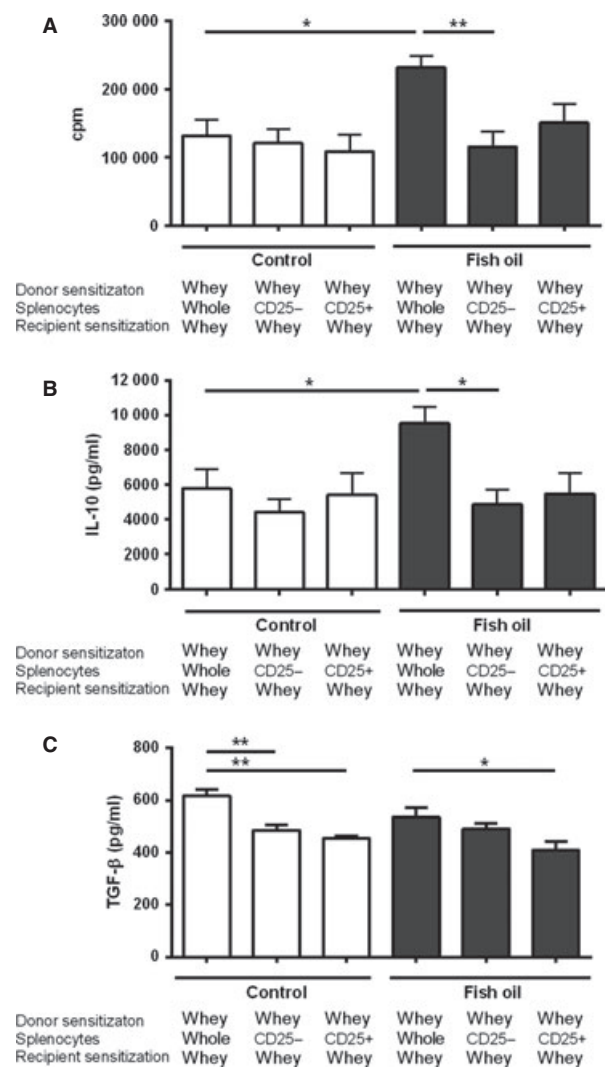


Figure 6 Proliferation and IL-10 secretion by *ex vivo* anti-CD3/CD28-stimulated (48 h) splenocytes from whey-sensitized orally challenged recipient mice fed AIN-93G diet. (A) Tritiated thymidine incorporation and concentration of (B) IL-10 and (C) TGF- β in culture supernatants. CD25⁻ splenocytes represent the *ex vivo* CD25⁺ Treg-depleted splenocyte population; CD25⁺ splenocytes represent the MACS-isolated CD25⁺ Treg population. Data are presented as means $\pm$ SEM, $n = 3$ –6/group. * $P < 0.05$, ** $P < 0.01$. cpm = counts per minute.

showed increased IL-10 secretion, which was in association with proliferation. On the other hand, regulatory TGF- β , which has also been shown to suppress mast cell function (30), was not enhanced in these supernatants pointing toward a more pronounced effect of the fish oil diet on CD25⁺ IL-10-producing cells.

We hypothesized the CD25⁺ Treg to be a potential candidate mediating the fish oil-induced oral tolerance to whey. The importance of CD25⁺ Treg in oral tolerance induction to cow's milk was demonstrated in several studies. The percentage of circulating CD25⁺ Treg was enhanced, and *in vitro*

proliferation of PBMC was reduced in tolerant compared with patients allergic to cow's milk (31). Shreffler et al. (32) reported enhanced Treg proliferation in heated-milk-tolerant vs heated-milk-allergic individuals, while depletion of CD25⁺ cells increased effector T-cell proliferation. Similar results confirming Treg involvement were found by Sletten et al. (33). In mice, iTreg also contribute to oral tolerance induction and transfer of tolerance (13, 34). Indeed, *ex vivo* Treg depletion with anti-CD25 antibody prior to transfer completely abrogated the transfer of tolerance in this study. Humoral factors were not enhanced; therefore, loss of CD25⁺ Treg or suppressive mediator secretion by these cells (e.g., IL-10) may have resulted in the development of the ASR. The reduced ASR and abrogation of temperature drop in recipients transferred with CD25⁺ Treg-depleted cells from control-diet-fed whey-sensitized donors probably were the result of a partial deletion of effector T cells, while the increase in IgG2a possibly was caused by a disbalanced Th response as a result of the depletion of both Treg and effector cells.

To confirm the contribution of Treg in the tolerizing effect of the fish oil diet, CD4⁺ CD25⁺ Treg of whey-sensitized donors were isolated and transferred. The CD25⁺ -enriched cell population (85% of these cells were FoxP3⁺) of both control-diet- and fish-oil-fed donors showed suppressive effects on the ASR as well as serologic parameters. As still CD25⁺ FoxP3⁺ cells were present in the population transferred, we cannot exclude the possible contribution of this cell population to the protective effect of the fish oil diet. However, transfer with CD25⁺ Treg was capable of completely preventing sensitization in recipient mice. Yamashita et al. (24) also reported reduced ovalbumin-specific IgE in recipients when transferring isolated CD25⁺ Treg independent from tolerance induction in donors. Transfer of whole splenocyte suspension from control-diet-fed whey-sensitized donors containing this functional CD25⁺ Treg population did not suppress whey allergy; hence, this population of Treg probably was unable to overrule the immunologic memory present in the whole cell suspension.

In the present study, it was shown that the protective effect of fish oil on allergic sensitization could be adoptively transferred using splenocytes. The whey-specific ASR of recipients transferred with donor cells of whey-sensitized mice fed the fish oil diet was reduced, which correlated with a reduction in mmcp-1. *Ex vivo* CD25⁺ Treg depletion prevented the transfer of tolerance from fish-oil-fed donor to control-diet-fed recipient mice. These results imply functional Treg to be involved in the prevention of cow's milk allergy induced by fish oil. Therefore, fish oil supplementation early in life may support tolerance induction and herewith prevent the development of food allergy.

Author contributions

L.v.d.E. and L.W. designed the study with input from L.M., B.v.E., and J.G.; L.v.d.E. performed the experiments together with L.M. and B.v.E. G.H. assisted with sensitizing and dissecting the mice. L.B. provided anti-CD25, anti-CD3, and

anti-CD28 antibodies. L.W. and J.G. provided supervision during the study. L.v.d.E. analyzed the data and drafted the manuscript with input from L.W. All authors contributed to and approved the final version of the manuscript.

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Conflicts of interest

None of the authors has any financial or personal conflicts of interest to report.

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