Feeding a Bioactive Oil Enriched in Stearidonic Acid During Early Life Influences Immune System Maturation in Neonatal Sprague-Dawley Rats[1234]

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[1] Supplemental Tables 1 to 7 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/

[2] Abbreviations used: ALA, α-linolenic acid; ARA, arachidonic acid; DPA, docosapentaenoic acid; ETA, eicosatetraenoic acid; LA, linoleic acid; LCPUFA, long chain polyunsaturated fatty acid; n, omega; OT, oral tolerance; Ova, ovalbumin; PL, phospholipid; SDA, stearidonic acid; T_h, T helper

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Background: Long chain polyunsaturated fatty acids (LCPUFA) n-3 improve immune development and reduce atopic disease risk in infants. Stearidonic acid (SDA) may be a substrate for biosynthesis of n-3 LCPUFAs.

Objective: We aimed to determine the effect of feeding an SDA enriched diet during suckling and weaning on offspring immunity and ability to develop oral tolerance (OT).

Methods: Pregnant Sprague-Dawley rats were randomized to consume either SDA (3 g SDA/100 g fat) or control (no SDA) diet, 5d before parturition and through lactation (21d). For OT treatment, 10d pups were fed ovalbumin (Ova, 200 µL of 8 mg/mL) or placebo daily for 5d. At 21d pups (both sexes) were weaned to their respective maternal diet until 6-weeks of age or euthanized. Systemic immunization was induced using Ova (3-week) or Ova + adjuvant (6-week). The effect of suckling diet (3-week) or weaning diet (6-week) and OT treatment on immune function (main outcome) in spleen and blood was compared using two-way ANOVA.

Results: SDA enriched maternal diet resulted in higher plasma phospholipid eicosapentaenoic acid (EPA, 15x), docosapentaenoic acid (DPA, 3x) and docosahexaenoic acid (DHA, 0.8x) content in 3-week pups, accompanied by higher B cell function (plasma Ova-IgG1, 2x) (P<0.05). Splenocytes from these pups had more (23%) helper T (T_h) (CD3+CD4+) and activated (12%) T_h (CD4+CD28+) cells (P<0.02) than controls. At 6-weeks, SDA group had 30% more CD4+CD25+ splenocytes and when stimulated ex-vivo with lipopolysaccharide, produced less inflammatory IL-6 (50%) and TNF-α (30%) and more immunoregulatory IL-10 (45%) cytokines (P<0.05) than control group. The Ova-exposed group had less (30%) plasma Ova-IgG1 than placebo

group. Splenocytes and plasma phospholipids from 6-wk SDA group had more EPA (2x) and DPA (3.5x) ($P<0.05$), but not DHA than control group.

Conclusions: Feeding SDA, during lactation and weaning, altered immune responses in directions believed to be beneficial.

Keyword: nutritional immunology, stearidonic acid, immune system, eicosapentaenoic acid, lactation period, weaning period, ovalbumin, neonatal development, omega 3, mucosal tolerance

Introduction

The effect of diet during early stage development (critical window) has significant implications on both the immediate biological response of the young animal and response later in life. The ability of T cells to respond appropriately to immune challenges develops early in life and is influenced by the availability of dietary long chain polyunsaturated fatty acids (LCPUFA), omega-3 (n-3/ω-3) LCPUFA (reviewed by (1, 2)). Oral tolerance (OT), which is the ability to distinguish between harmful and harmless dietary antigens (3), also occurs early in life and failure to develop OT results in atopic diseases such as food allergies and asthma (4).

Our research has demonstrated that, in healthy animals and humans, feeding docosahexaenoic acid (DHA) and arachidonic acid (ARA) improves immune function and OT in animals and infants (1, 5-7). Clinical trials that supplemented n-3 LCPUFA to lactating mothers or infants at high risk of allergy have resulted in less atopic symptoms in some (8-10) but not all studies (11) (reviewed by (2)). It has been established that the dose-dependent incorporation of n-3 LCPUFA into plasma, RBC and lymphocytes occurs with a corresponding reduction in ARA, reviewed by (12). Studies have demonstrated that dietary intervention in formula fed infants (13) or suckled rodents (6) that provided ARA and DHA compared to one that did not, was found to benefit parameters indicative of immune development.

Desaturation of α-linolenic acid (ALA, 18:3 n-3) to stearidonic acid (SDA, 18:4 n-3) is mediated by the rate limiting ∆6 desaturase (**Figure 1**). We have previously shown that feeding an SDA-enriched diet to breast cancer tumor bearing nu/nu mice increased the content of eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA) but not

DHA in tumor phosphatidylcholine (major class of phospholipid in cell) (14). Providing SDA can bypass this rate-limiting step in the conversion to EPA, facilitating the bioconversion to EPA (Figure 1) (15). There is a growing body of literature suggesting that EPA (16) and DPA (17), also have effects on immune function in adults. However, they have not been extensively studied in infants. Increasing n3 LCPUFAs may be important for infant OT development as women with allergic disease have been found to have lower breastmilk concentration of EPA, DPA and DHA (18).

The objective of this study was to determine the immunomodulatory effects of feeding a bioactive oil enriched in SDA, as a source of n-3 LCPUFA, during suckling and weaning on immune system maturation and the development of OT. The secondary objective of the study was to evaluate the ability of SDA to increase the total phospholipid (PL) incorporation of n-3 LCPUFA in neonates. We hypothesized that feeding a diet enriched in SDA, through its conversion to EPA, DPA and other n-3 LCPUFA, would be beneficial for the immune system maturation of the young rat pups. Furthermore, these n-3 LCPUFA would also be beneficial for the development of OT.

Methods

Animal and diets

All animal care and experimental protocol were conducted in accordance with the Canadian Council of Animal Care and approved by the University of Alberta Animal Ethics Committee (AUP00000125). Primiparous Sprague-Dawley rats (*n*=22) were obtained from Charles River Laboratories on day 14 of gestation and were individually housed in a temperature- and humidity-controlled environment with a 12/12-hour reversed light cycle. The study design is illustrated in **Figure 2**. The experiments were

77 conducted in two blocks with *n*=6 and *n*=5 per diet group over two consecutive
78 summers, respectively. Pups were culled to have equal number of males and females
79 suckling with each dam. Dams were fed commercial standard rat chow (Lab diet 5001;
80 PMI Nutrition International) during acclimatization period and then, 5 days prior to
81 parturition, dams were randomly assigned to either nutritionally adequate diets; SDA
82 diet (3% of total fat as SDA, *n*=11) or control diet (0% SDA, *n*=11). The litter was culled
83 to 10 pups/dam (Figure 2) and diet was fed *ad libitum* during lactation and weaning.
84 Offspring were kept with their mothers until the end of the 3-week suckling period (day
85 21). At day 10, 4 pups from each dam were randomly assigned to OT treatment:
86 placebo (sucrose, *n*=2 pups/dam) or ovalbumin (Ova, *n*=2 pups/dam). At the end of the
87 3-week suckling period, tissues were collected from 1 pup/dam from each of the 4 diet-
88 OT treatment groups. One pup/dam from each group was weaned to their respective
89 maternal diet for an additional 3 week (weaning period). At 6-weeks of age all the pups
90 were euthanized by CO_2 asphyxiation and tissues were collected. When possible, male
91 and female pups were equally distributed between groups.
92 Both experimental diets were isocaloric and isonitrogenous (**Table 1)**. The semi-purified
93 basal diet has been previously described in detail (19). The added fat mixture (20 g /100
94 g diet) to rodent diet was a blend of lard, canola oil, vegetable oil and SDA enriched
95 flaxseed oil (14). All the main fatty acids were matched closely with the exception of
96 oleic acid, linoleic acid, ALA and SDA. Both diets met the essential fatty acid
97 requirements of the rodent and had similar PUFA/SFA ratio. Note, the diets differed in
98 the n-6 to n-3 ratio due to the difference in the content of SDA, ALA and linoleic acid
99 (LA), but the diets were matched for total PUFA content. Diets were prepared weekly

and stored at 4°C until fed; feed cups were replaced every 2-3 days to limit air exposure. Food intake and bodyweight were recorded every 2-3 days.

Ovalbumin administration and immunization

Mucosal OT to Ova (Sigma-Aldrich) was induced by repeated oral exposure during suckling period as previously described (6). Briefly, 200 µL Ova (8 mg/mL in 8% wt:vol sucrose solution) or placebo (8% wt:vol sucrose in water) solution were delivered orally via syringe once daily for 5 days to 2 pups/litter. To induce systemic immunization 3-week pups received an intraperitoneal (IP) injections of 10 µg Ova in 100 µL PBS 24 hours before euthanizing and 6-week pups received IP injections of 10 µg Ova in 100 µL PBS combined with an adjuvant (1:1, Imject Alum Adjuvant, Thermo-Scientific) 7 days before euthanizing (Figure 2).

Tissue collection and immune cell isolation

Immediately after euthanizing, blood was collected by cardiac puncture with a 5 mL syringe and stored in K2 EDTA containing tube. Within an hour of collection, whole blood was analysed on a hematology analyzer ADVIA 2120i (Siemens). Blood was then centrifuged (1734g X 10 minutes), and plasma removed and stored at -80°C until analysis. Spleen was collected aseptically, and immune cells were isolated as previously described (20). Isolated live immune cells were counted on a haemocytometer using trypan blue exclusion (Sigma) and diluted to $1.25x10^6$ cells/mL.

Immune cell phenotype analysis

Isolated immune cells from spleen were identified by direct immunofluorescence assay as previously described (20). Four-color flow cytometry allowed determination of the following surface molecule combinations: Cluster of differentiation

(CD)3/CD25/CD4/CD8, CD28/CD152/CD4/CD8, CD4/CD25/FOXP3, OX62/CD25/OX6, CD68/CD284/CD11/CD45RA, OX12/OX6/CD80, CD27/OX12/OX6/CD45RA and CD71/CD8/CD4. All antibodies were purchased from eBiosciences or BD Biosciences. Note, the monoclonal antibody for detecting CD3+ cells bind to T cells in young rats at a lower than expected level, however, the addition of the identified CD4+ and CD8+ cells indicate an expected proportion of total T cells. CD28, co-stimulatory T cell marker, is expressed in all the T cells, which may be used to measure total T cells. Cells were then washed and fixed in paraformaldehyde (10g/L; Anachemia Science) in PBS. Within 72 hours of isolating and fixing the stained cells, immune cells were analyzed by flow-cytometry (FACSCalibur; Becton-Dickinson) according to the relative fluorescence intensity using Kaluza software (Beckman Coulter).

Ex vivo cytokine production by mitogen- or Ova-stimulated splenocytes and plasma Ova-specific Immunoglobulin G1 (IgG1) concentration.

Cytokine production by stimulated splenocytes was measured as previously described (21). Briefly, immune cells ($1.25x10^6$ cells/mL) were cultured for 48 hours with or without the mitogen lipopolysaccharide (LPS, a bacterial component that acts as a mitogen for immune cells,100 µg/mL, Sigma) or the food protein, Ova (150 µg/mL, Sigma). LPS stimulation was used to model an *in-vivo* bacterial challenge. Commercial ELISA kits were used to measure the concentration of interleukin (IL)-1β, IL-2, IL-6, IL-10, tumor Necrosis Factor-α (TNF-α), and interferon-γ (IFN-γ) (R&D Systems) in the stimulated supernatants, according to the manufacture's instructions. Detection limits for IL-1β, IL-2, TNF-α and IL-10 were 16-4000 pg/mL. IL-6 and IFN-γ had a detection limit of 125-8000 pg/mL. Ova specific IgG1 (Ova-IgG1) in the plasma was detected by ELISA kit

(Alpha Diagnostic International). Absorbance was read on a spectrophotometer and concentrations were calculated using a standard curve (SpectraMax 190, Molecular Devices). All measurements were conducted in duplicate with coefficient of variance <15%.

Fatty acid analysis

A modified Folch method was used to extract total lipids from cells and plasma as previously described (22). Fatty acid methyl esters were prepared from the PL band and they were separated and identified by automated gas liquid chromatography GLC7890A (Agilent Technologies) on a 100 m CP-SIL 88 fused capillary column (100 m x 0.25 mm Agilent) as described previously (23).

Statistical analysis

Data are reported as mean ± standard error of the mean (SEM) unless indicated otherwise. The study was powered to assess significant changes in immune function (as the primary outcome), and fatty acid changes (pups' plasma and splenocyte PL, as secondary outcomes). The sample size was based on previous study from our group to assess differences in ex-vivo cytokine production where n=6 per group was found to be sufficient to detect 20% (β-value) difference at significance level (α-value) of 5%. Data were analysed using the PROC MIXED procedure two-way analysis of variance (ANOVA) with diet and OT treatment as main effect in SAS (V.9.4 Cary, SAS Institute). The 2x2 study design allowed us to determine the effect of maternal diet (Control vs. SDA), OT treatment (Placebo vs. Ova) and the interaction between the two (diet × OT treatment). When the effect was found to be significant, we performed LSMEANS procedure to do post hoc statistical analysis comparing the individual 4 groups at either

3- or 6-week stage (control diet and placebo treatment, SDA diet and placebo treatment, control diet and Ova treatment, and SDA diet and Ova treatment). In the absence of significant OT treatment effect (for instance in complete blood cell count or fatty acid analysis), the two diet groups were compared using unpaired student's T-test and mean and SEM were reported with OT treatment group combined within a diet group. Differences at $P \leq 0.05$ (two sided) were considered significant.

Results

Growth and Hematologic parameters

There was no significant difference in weekly food intake (47.1 ± 2.9 g/week vs. 46.3 ± 2.7 g/week) nor final bodyweight (309 ± 9 g vs. 311 ± 10 g) between the dams fed control vs. SDA diet, respectively. At 3-weeks there was no effect of OT treatment on pups' bodyweight (data not shown). However, the pups weaned to SDA diet had a significantly higher bodyweight compared to pups weaned to control diet starting from week 4 through week 6 **(Figure 3)**. For 6-week pups, food intake did not differ between diet groups (24 ± 1.3 g/week vs. 25 ± 1.2 g/week). No significant differences were observed for complete blood cell count between diet groups or treatment groups for the dams or 6-week pups (data not shown). Although the white blood cell (WBC) count did not differ in the 3-week pups, the SDA group had a significantly higher lymphocyte proportion of WBC (reciprocally lower neutrophil) than the control diet pups ($P<0.01$, **Supplemental Table 1**).

Plasma ovalbumin-specific IgG1 (Ova-IgG1) concentrations

At 3-weeks, the SDA diet group had a significantly higher concentration of Ova-IgG1 in the plasma compared to the control group but there was no effect of OT treatment

(**Figure 4**). We also observed a significant interaction between diet and OT treatment effects, in which Ova-tolerized pups fed the SDA diet has plasma Ova-IgG1 levels significantly higher than Ova-tolerized pups fed the control diet (P=0.048, Figure 4A). At 6-weeks, there was no significant diet effect on the Ova-IgG1 plasma levels but there was an OT treatment effect in that Ova exposed animals had lower plasma Ova-specific IgG1 than the placebo group (Figure 4B, $P<0.03$).

Plasma phospholipid fatty acid composition in 3- and 6-weeks old pups

Total PL fatty acid composition of plasma collected from pups at 3-weeks and 6-weeks are reported in **Supplemental Table 2** and **Supplemental Table 3**, respectively. As there were no significant effects of OT treatment or any interaction only the diet effects are presented. At 3-weeks, pups from the SDA group had a significantly higher proportion of SDA, eicosatetraenoic acid (ETA), EPA, DPA & DHA compared to control group pups resulting in 1.7 times more total n-3 LCPUFA in plasma **(Figure 5A)**. Feeding the SDA diet decreased the total n-6 PUFA PL composition by 10% due to significantly lower proportion of all the n-6 LCPUFA, with the exception of ARA. The difference in total n-3 and n-6 PUFA resulted in a lower (almost 50%) n-6/n-3 LCPUFA ratio in SDA group compared to control.

At 6 weeks of age, SDA fed pups had significantly higher plasma PL content of total n-3 PUFA, due to more EPA and DPA but not DHA (**Figure 6A**). Similar to 3-weeks old pups, the SDA group had a significantly lower proportion of all n-6 fatty acids in plasma PL, but not ARA or linoleic acid, which did not differ between diet groups (Supplemental Table 3). For both 3-weeks and 6-weeks pups, the plasma PL proportion of total SFA

was higher and the proportion of monounsaturated fatty acid (MUFA) was lower for the SDA group.

Phospholipid fatty acid composition of spleen in 3- and 6-weeks old pups

At 3-weeks and 6-weeks, there were no OT treatment effects or interaction effects on PL fatty acid composition; diet effects are reported in **Supplemental Table 4** and **Supplemental Table 5** for 3- and 6-weeks pups respectively. In 3-weeks pups, the total PL fatty acid composition of the splenocytes from SDA group had significantly higher total n-3 fatty acids largely due to the significantly higher proportion of the n-3 LCPUFAs (ETA, EPA and DPA; **Figure 5B**). The relative proportion of DHA in PL did not differ between diet groups. Additionally, the total n-6 fatty acid was significantly lower in the SDA group compared to the control group which resulted in a significantly lower n-6/n-3 LCPUFA ratio in SDA group. No effect of the diet was observed on the total proportion of SFA, MUFA or PUFA to SFA ratio in 3-weeks pups. At 6-weeks, despite no differences in the total n-6 fatty acid composition (Supplemental Table 5), the total n-3 fatty acid composition of immune cell PL were significantly higher in pups that were weaned to the SDA diet, again due to a higher concentration of EPA and DPA (**Figure 6B**). This resulted in a lower n-6/n-3 LCPUFA ratio of spleen PL from rats fed the SDA diet when compared to control diet (Supplemental Table 5).

***Ex vivo* cytokine production by mitogen- and ovalbumin-stimulated immune cells**

Spleen

LPS stimulation: Ex-vivo cytokine (IL-1β, IL-6, IL-10, TNF-α and IFN-γ) production by LPS stimulated splenocytes from dams (data not shown) and 3-weeks pups (**Supplemental Table 6**) did not significantly differ with diet or OT treatment. However,

at 6-weeks, there was significantly lower IL-6 and TNF-α production and a significantly higher IL-10 production in stimulated splenocytes from SDA fed pups (**Figure 7A,** *P*<0.05). The production of IL-1β (inflammatory cytokine) did not differ between diet groups (Figure 7A). Additionally, OT treatment had a significant effect on IFN-γ production by splenocytes after ex-vivo stimulation with Ova. The Ova exposed group produced significantly less IFN-γ in comparison to the placebo group (**Supplemental Table 7, *P*=0.03**).

Ovalbumin (dietary antigen) stimulation: There was no effect of diet on the cytokine response (IL-1β, IL-2, IL-6, IL-10, TNF-α) of splenocytes to the ex-vivo Ova challenge in the 3-weeks pups (Supplemental Table 6). However, IL-10 showed significant interaction effect between diet and OT treatment, with the Ova exposed SDA group pups producing significantly less than Ova exposed control group pups but this was not different from placebo exposed pups irrespective of the diet group**.** At 6-weeks, pups from the SDA group produced significantly less IL-6 and TNF-α after incubation with Ova (**Figure 7B,** *P*<0.05). A significant treatment effect was seen with IL-10 production, in which pups exposed to Ova produced less IL-10 than the placebo exposed group irrespective of diet (Figure 7B, *P*<0.05).

Effect of diet and OT treatment on immune cell phenotype

Spleen

There was no effect of diet on the proportion of different immune cell types in the spleen of dams (data not shown). At 3-weeks, splenocytes from the SDA group pups had a significantly higher proportion of T_h cells (CD3+CD4+), a significantly lower proportion of natural killer cells (NK) (CD3-CD161+) and macrophages (CD11+) compared to pups

fed the control diet (**Table 2,** $P<0.01$). There were no OT treatment or interaction effects for any immune cell phenotype in spleen from 3-weeks pups. In splenocytes from 6-weeks old pups, there was a significantly lower proportion of CD28+ cells in Ova exposed pups compared to placebo group (**Table 3**, $P<0.05$). In the pups that were fed the SDA diet, the proportion of CD4+CD25+ and total CD25+ cells was higher compared to the control diet group (Table 3, $P<0.05$).

Discussion

We investigated the effect of feeding a diet containing a plant oil enriched in stearidonic acid (SDA, 18:4 n-3) on the immune system development and the induction of OT to a food antigen (Ova) in neonatal rats. We examined the suckling diet effect in 3-week old pups and suckling + weaning diet effect in 6-week old pups. Feeding SDA to dams resulted in a higher n-3 LCPUFA status (EPA, DPA and DHA in plasma, and EPA and DPA, but not DHA in splenocytes) in 3-weeks old pups. These increases in n-3 LCPUFA may have contributed to better overall growth, early maturation of adaptive immunity (markers of immune system development) and an improved *in-vivo* humoral response (Ova-IgG1) to dietary food antigens. More importantly, higher total PL content of EPA and DPA in splenocytes of 6-weeks old pups was associated with a better inflammation resolving response when challenged *ex-vivo* with bacterial antigen, characterized by lower inflammatory cytokines and a higher immunoregulatory cytokine, IL-10. Lastly, increasing EPA+DPA, unlike DHA, had no beneficial effect on the development of OT to dietary food antigen (Ova).

Feeding 3% of total fat as SDA to lactating dams resulted in significantly higher EPA, DPA as well as DHA in the total plasma PL of the suckled pups. This suggests

283 that dietary SDA is a substrate for EPA, DPA and DHA biosynthesis in the dam and this
284 likely gets transferred into breast milk. Previous studies in rodents (24) and infants (25,
285 26) have demonstrated that higher DHA content in breast milk increases infants' plasma
286 DHA status which may have contributed to the higher growth rate in the SDA offspring
287 in the current study. Additional DHA during suckling has been shown to be beneficial for
288 growth parameters: birthweight in infants (27, 28) and infant development (of brain (29),
289 neural tissue (31), immune function (30), and respiratory system (31)).

290 In the current study, we confirmed that feeding SDA to dams, led to a more
291 mature immune cell phenotype in pups at the end of suckling. More specifically, the
292 spleen contained more adaptive immune cells (T_h cells) and less innate immune cells
293 (NK cells and macrophages); a pattern that more closely resembles adult rat splenocyte
294 composition (32). Consistent with this, SDA supplementation in the human diet (1 g/day
295 SDA for 12 weeks) resulted in a lower NK cell proportion in the peripheral blood
296 mononuclear cell (PBMC) population (33), another indicator of maturation. Despite the
297 changes in immune cell phenotypes, there was no significant effect of the SDA diet on
298 the splenocytes' *ex-vivo* response to LPS, a mitogen that stimulate B cells, dendritic
299 cells and macrophages. The comparison between the effect of increasing DHA (with
300 DHA supplementation) and EPA+DPA (with SDA supplementation) on immune cell
301 function of 3 weeks old suckled pup suggests a different effect of these LCPUFA on
302 immune development.

303 At 3-weeks, splenocyte PLs from SDA pups had a higher EPA and DPA content
304 and this might facilitate adaptive immune cell maturation (16, 34). Consistent with this,
305 feeding the SDA diet led to signs of early maturation of the humoral response (B cell)

characterised by a higher in-vivo plasma concentration of Ova-IgG1, after an IP challenge with only Ova. B cell maturation begins in the bone marrow and involves isotype switching from primary Igs (IgM and IgA) to secondary Igs (IgG, IgD and IgE) in the periphery, resulting in enhanced antigen-specific humoral immunity (Ova-IgG1). The greater Ova-IgG1 level without any corresponding increase in the proportion of total B cell (total OX12+ and CD45+ cells) in spleen may suggest a higher antigen-specific adaptive immunity. N-3 LCPUFA have been previously reported to affect B cell function (35, 36), therefore a higher EPA and DPA in splenocyte PL may favor B cell maturation. Consistent with the current study, a fish-oil supplementation study that increased total n-3 LCPUFA (including EPA, DPA but also DHA) was shown to improve immunoglobulin production when challenged (35). In a previous study, supplementing the maternal diet with DHA, there was no effect of increasing DHA (without any changes in EPA or DPA) in splenocyte phospholipids on plasma IgG concentration (37). This suggests that EPA+DPA and DHA may have different effects on B cell function in the young animals, similar to what has been reported in adults (36). Although, the clinical significance of higher antigen-specific IgG1 levels in plasma are unknown, low concentrations of plasma IgG1 and total IgG are associated with a high rate of respiratory infections (38). Further studies are required to understand the functional importance of altering neonatal status of n-3 LCPUFA on plasma immunoglobulin concentrations.

At 6-weeks, Ova or LPS stimulated splenocytes from the SDA group produced less pro-inflammatory cytokines (IL-6 and TNF-α). They also produced a higher immunoregulatory cytokine response (IL-10) but only with LPS stimulation. Splenocytes from SDA fed pups had a greater proportion of total CD25+ (activation marker) and

CD4+CD25+ (activated T_h cells) cells. Taken together, these observations suggest that the higher splenocyte content of EPA and DPA supports a more anti-inflammatory response (by increasing IL-10) in neonatal rat splenocytes after stimulation. Similarly, a study feeding a higher SDA diet (10% w/w of total fat), reported significantly lower ex-vivo TNF-α production by whole blood stimulated with LPS and a trend towards a lower TNF-α production by splenocytes when stimulated with LPS (39). However, in this same study, the total lipid composition of splenocytes was higher in DPA and DHA in pups fed SDA diet, but there was also a significantly reduced ARA content in these splenocytes (39). Therefore, the anti-inflammatory effect might have been due to both an increase in DPA and DHA, and a decrease in ARA composition of splenocytes. Similar anti-inflammatory effects were also seen in PBMC from a human study supplementing 9.7mL per day of 0, 5, 10 or 17% SDA (40). This study reported a linear dose related increase in plasma and PBMC total lipid composition of EPA (with no change in DHA), which was associated with a significantly higher production of IL-10 with LPS stimulation of PBMC (40). In comparison, an earlier study that supplemented dam and pup diet with DHA reported an increase in DHA (but not ARA) was associated with a lower pro-inflammatory cytokine response (IL-1β and IL-6) by LPS stimulated splenocytes from 6-weeks pups (41). It is well established that the n-3 LCPUFA have anti-inflammatory effect on immune response (42), and our results suggest that a dietary intervention that increases EPA+DPA has an anti-inflammatory effect similar to that observed in previous studies where diets were supplemented with DHA (41). Further studies directly comparing the effect of dietary intervention that increases EPA+DPA and/or DHA are

needed to determine specific mechanisms of the different n-3 LCPUFA on immune function.

Our previous findings suggest that increasing DHA in cells thought DHA supplementation, lowers inflammatory cytokine (IL-1β and IL-6) production after mitogen stimulation (6), while the current study suggests that increasing EPA+DPA may have a regulatory effect through increasing IL-10 production. It does not appear that a reduction in ARA content of tissues is required for the anti-inflammatory effects of these n-3 LCPUFA. Results from the current study and others (33, 40) support the hypothesis that SDA is an efficacious substrate for EPA or DPA biosynthesis. Additionally, others have shown at higher SDA doses, it may be a precursor for DHA (39).

OT was induced in pups previously exposed to Ova in both diet groups at 6-weeks. Upon systemic immunization, pups from the Ova OT treatment group had a significantly lower plasma concentration of Ova-IgG1 ($P<0.03$) compared to the placebo exposed group. This is further supported by results from *in-vitro* analysis. Consistent with other studies (41, 43), we showed that there was a reduction in IL-10 production by splenocytes stimulated with Ova in the Ova exposed OT group compared to the placebo exposed group. This is particularly important as IL-10 enhances the IgE mediated mast cell response in allergic reactions to food antigens (Ova) (44), therefore a lower IL-10 response to Ova would support OT induction. Additionally, there was a lower proportion of CD28+ cells in the splenocyte population from the Ova exposed OT group (in both diets) compared to placebo group. This finding may indicate the suppression of antigen specific T cells in the development of OT (45) in our model using a multiple low-dose exposure to a food antigen. The interaction of the B7 molecule of dendritic cells with

either the co-stimulatory CD28 or co-inhibitory cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) of naïve T cells determines T cell response (46). Providing Ova (orally) early in life may have led to less CD28+ cells in spleen compared to placebo group allowing B7 to interact more with CTLA-4 on T cells, thus promoting tolerance by inducing the antigen-specific T cell to undergo clonal suppression (47). It is important to note that there was no beneficial effect at 6-weeks of feeding the SDA weaning diet on OT induction (plasma Ova-IgG1 levels), despite increased EPA+DPA in splenocytes. This is contrary to our previous experiments where we fed DHA and found it beneficially affected the OT induction but also increased the DHA content of splenocyte PLs (41).

There are limitations in the current study. First, the study was conducted in two blocks. Although we used a sensitive statistical model to control for effects of random errors on the independent variable (diet), it was not entirely possible to correct for some block differences and this also prevented us from reporting data from two blocks combined in some cases. Second, we calculated the sample size based on previous studies that have been conducted to study the diet effect on neonatal development in females. This prevents us from reporting on sex effect on the results. It has been shown that the immune system develops differently in males and females (48) and there is a difference in their ability to elongate and desaturate n-3 PUFA (49, 50), which may change their risk to develop allergies (breach in OT) (51). Finally, this study used healthy rodents. Further investigation could include employing a more allergy sensitive model to determine if the changes in maturation observed in the current study impact the development of food allergy.

In conclusion, feeding SDA led to the *in vivo* biosynthesis of EPA and DPA but not DHA while maintaining tissue concentrations of ARA. At 3-weeks, increasing n-3 LCPUFA led to better growth and immune system maturation. At 6-weeks, EPA+DPA had an anti-inflammatory effect to stimulation but unlike DHA supplementation studies did not alter the ability to develop OT.

Acknowledgments: We thank Peter Blenis, who helped with the statistical analysis; Nicole Coursen for technical assistance; and undergraduate students Emily Wage and Reid Steele who were involved in data collection and analysis throughout the project. The authors' responsibilities were as follows–CJF, CR and GC designed the study; DP, MN and SG conducted research and analyzed data; DP performed the statistical analysis and wrote the manuscript; and CJF had primary responsibility for final content. All authors read and approved the final manuscript.

409 Table 1: Lipid composition of the experimental diets fed to Sprague-Dawley rat dams
410 and weaning pups as determined by gas-liquid chromatography[1]

Fatty acids	Control diet, g/100 g total fatty acids	SDA diet, g/100 g total fatty acids
14:0	1.2 ± 0.0	1.3 ± 0.0
16:0	22.0 ± 0.0	25.0 ± 0.2
16:1	1.9 ± 0.0	1.9 ± 0.0
18:0	12.0 ± 0.3	14.0 ± 0.1
18:1 n-9 (Oleic acid)	41.0 ± 0.1	35.0 ± 0.0
18:2 n-6 (LA)	17.0 ± 0.3	13.0 ± 0.1
18:3 n-3 (ALA)	1.9 ± 0.1	3.6 ± 0.0
18:4 n-3 (SDA)	0.0 ± 0.0	2.7 ± 0.0
20:0	0.1 ± 0.0	1.5 ± 0.0
20:3 n-6	0.3 ± 0.0	0.2 ± 0.0
Total SFA	36.0 ± 0.3	42.0 ± 0.0
Total PUFA	19.0 ± 0.5	20.0 ± 0.0
Ratio PUFA/SFA	0.5 ± 0.0	0.5 ± 0.0
Total n-6	17.0 ± 0.3	13.0 ± 0.1
Total n-3	2.0 ± 0.1	6.3 ± 0.1
Ratio n-6/n-3	8.8 ± 0.5	2.1 ± 0.0

411 [1]Values are presented in mean ± SEM (*n*=3). Fatty acids below 0.1 g/ 100 g of total fat
412 are not included. LA, linoleic acid; SDA, stearidonic acid; ALA, α-linolenic acid; n,
413 omega; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

414

Table 2: Effect of the diet on the immune cell phenotype isolated from the spleens of 3-week old Sprague Dawley rat pups[1]

	Control diet (*n*=6), % of total splenocytes	**SDA diet (*n*=6), % of total splenocytes**	***P*-Diet**
[2] Total CD3+	15.7 ± 0.7	17.7 ± 1.0	0.14
CD3+CD4+ (helper T cells)	7.7 ± 0.4	9.5 ± 0.6	0.02
CD3+CD8+ (cytotoxic T cells)	7.2 ± 0.4	7.8 ± 0.5	0.38
[3] Total CD28+ (T cell co-receptor)	49.7 ± 1.6	45.5 ± 2.8	0.21
CD4+CD28+ (mature helper T cells)	15.8 ± 0.6	17.7 ± 0.5	0.02
CD8+CD28+ (mature cytotoxic T cells)	10.8 ± 0.5	10.6 ± 0.8	0.82
Total CD25+	3.1 ± 0.2	3.4 ± 0.2	0.46
CD4+CD25+	3.1 ± 0.2	3.2 ± 0.4	0.91
CD8+CD25+	0.6 ± 0.1	0.8 ± 0.1	0.16
Total OX12+ (B cells)	17.4 ± 1.3	17.9 ± 1.1	0.8
Total OX6+ (antigen presenting cells)	41.5 ± 0.7	41.2 ± 1.0	0.86
Total OX62+ (dendritic cells)	6.0 ± 0.4	4.9 ± 0.5	0.09
Total CD161+	10.1 ± 0.7	8.1 ± 0.6	0.02
CD3-CD161+ (natural killer cells)	6.6 ± 0.4	4.8 ± 0.3	0.005
Total CD11+ (macrophages)	15.7 ± 0.5	12.6 ± 0.5	<0.001

[1] Values are presented as mean ± SEM; values are reported as percent of the total gated splenocytes (which includes in addition to lymphocytes, monocytes, macrophages, dendritic cells, etc.) as determined by immunofluorescence.

[2] Monoclonal antibody for CD3 (1F4, ThermoFisher Scientific) stains low in naïve T cells from young pups and it does not stain γδ T-cell. This may have contributed to the lower CD3+ lymphocytes than what is previously reported (32).

[3] CD28, co-stimulatory T cell marker, is expressed in all the T cells, which may be used to determine Total T cells

Samples form the first block of experiments were used for the analysis.

CD, cluster of differentiation; SDA, stearidonic acid.

Table 3: Effect of diet and oral tolerance treatment on immune cell phenotype isolated from the spleen of 6-week old Sprague Dawley rat pups[1]

	Control diet, % of total splenocytes		**SDA diet, % of total splenocytes**		***P*-Diet**	***P*-Treatment**	***P*-Interaction**
	Placebo (*n*=11)	Ova (*n*=11)	Placebo (*n*=10)	Ova (*n*=10)			
[2]Total CD3+	29.6 ± 1.5	29.9 ± 1.1	30.0 ± 1.5	29.2 ± 2.0	0.87	0.68	0.33
CD3+CD4+ (helper T cells)	16.0 ± 0.8	15.7 ± 0.5	15.9 ± 1.0	15.2 ± 1.0	0.70	0.15	0.59
CD3+CD8+ (CTL)	12.8 ± 0.8	12.6 ± 0.6	12.0 ± 0.7	12.6 ± 1.0	0.69	0.72	0.57
CD3+CD4+CD25+FOXP3+ (regulatory T cells)	1.8 ± 0.2	1.9 ± 0.2	2.1 ± 0.3	1.9 ± 0.3	0.66	0.99	0.33
[3]Total CD28+ (T cell co-receptor)	42.1 ± 1.4[a]	41.2 ± 1.3[b]	40.9 ± 1.0[a]	38.5 ± 1.8[b]	0.49	0.04	0.33
CD4+CD28+ (mature helper T cells)	17.6 ± 0.8	17.6 ± 0.7	18.1 ± 0.9	16.9 ± 1.0	0.85	0.09	0.09
CD8+CD28+ (mature cytotoxic T cells)	13.9 ± 0.8	13.6 ± 0.6	13.7 ± 1.0	12.9 ± 0.7	0.86	0.55	0.95
Total CD25+	4.6 ± 0.3 [a]	4.6 ± 0.2 [a]	5.6 ± 0.5 [b]	5.3 ± 0.4 [b]	0.05	0.53	0.60
CD4+CD25+	1.9 ± 0.2 [a]	1.8 ± 0.2 [a]	2.6 ± 0.2 [b]	2.2 ± 0.2 [b]	<0.001	0.07	0.10
CD8+CD25+	0.8 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.24	0.11	0.29
Total OX6+ (APC)	39.7 ± 2.4	40.4 ± 2.4	40.3 ± 2.6	38.3 ± 2.5	0.29	0.39	0.08
Total OX12+ (B cells)	36.7 ± 1.6	37.2 ± 1.9	37.3 ± 1.8	35.5 ± 1.9	0.54	0.64	0.44
Total OX62+ (dendritic cells)	4.3 ± 0.2	4.0 ± 0.3	4.6 ± 0.2	4.9 ± 0.3	0.38	0.97	0.35
Total CD161+	13.1 ± 1.5	12.6 ± 1.2	13.2 ± 1.7	12.3 ± 1.6	0.73	0.10	0.67
CD3-CD161+ (natural killer cells)	8.8 ± 1.2	7.9 ± 0.8	8.5 ± 1.3	7.4 ± 1.0	0.29	0.39	0.08
Total CD11+ (macrophages)	10.5 ± 0.7	11.1 ± 0.9	10.1 ± 1	9.9 ± 0.8	0.15	0.68	0.41

[1] Values are presented as mean ± SEM; values are reported as percent of the total gated splenocytes (which includes in addition to lymphocytes, monocytes, macrophages, dendritic cells, etc.) as determined by immunofluorescence.

[2] Monoclonal antibody for CD3 (1F4, ThermoFisher Scientific) stains low in naïve T cells from young pups and it does not stain γδ T-cell. This may have contributed to the lower CD3+ lymphocytes than what is previously reported (32).

[3] CD28, co-stimulatory T cell marker, is expressed in all the T cells, which may be used to determine Total T cells

Outliers were excluded from the analysis resulting in $n<11$ per diet group. P represents the main effect of diet or tolerance treatment in the mixed model on 6-week pups. Within a row, means without a common superscript letter are significantly different, $P<0.05$. CD, cluster of differentiation; SDA, stearidonic acid; APC, antigen presenting cell

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Figure 1: Fatty acid bioconversion pathways. Precursor n-3 and n-6 PUFAs compete for desaturase and elongase in the biosynthesis of LCPUFA. N, omega; LCPUFA, long chain polyunsaturated fatty acid, ALA, α-linolenic acid; SDA, stearidonic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; GLA; γ-linoleic acid; dihomo-γ-linolenic acid; ARA, arachidonic acid

Figure 2: Animal study design. Sprague-Dawley rat dams were randomly assigned to either control (*n*=11) or SDA (*n*=11) diet 5 days prior to parturition and continued on the same diet through suckling period (3 weeks). After birth, litter was culled to have equal number of males and females per dam. For OT treatment; 4 pups/dam were randomized to either ovalbumin (Ova, *n*=2 pups/dam) or placebo (sucrose, *n*=2 pups/dam) for 5 consecutive days between day 10 and 15. Twenty-four hours prior to euthanization, 3-week old pups, received IP injection to induce systemic immunization. Pups were euthanized from each of the 4 diet-OT treatment groups (*n*=1 pup/dam) and tissues were collected at 3-weeks (suckling period) for analysis. The remaining pups (*n*=1 pup/dam) were weaned to the same diets as their mother for an additional 3 weeks. IP injection, Ova with an adjuvant (alum), was administered 7 days (at week 5) prior to euthanizing to induce systemic immunization in 6-week old pups. The dams are considered the experimental unit therefore sample size of each group is equal to the number of dams (*n*=11). The experiments were conducted in two blocks with *n*=6 and *n*=5 per diet group over two consecutive summers, respectively. SDA, stearidonic acid; Ova, ovalbumin; OT, oral tolerance; IP, intraperitoneal

Figure 3: Effect of diet on the bodyweight of Sprague-Dawley rat pups from birth to 6 weeks. Values are mean ± SEM; *n*=11 for each diet. Significant diet effect on pups' bodyweight is indicated by * different from Control diet, *P*<0.05. There was no effect of oral tolerance treatment on body weight therefore treatment groups within each diet groups were combined. SDA, stearidonic acid

Figure 4: Plasma Ova-specific IgG1 levels measured in Sprague-Dawley rat pups at (A) 3-weeks and (B) 6-weeks. Ovalbumin OT (Ova, *n*=6) treatment groups are depicted by solid colored (grey) bars and placebo OT treatment groups (sucrose, *n*=6) are depicted by open bars. Diet X OT treatment indicates the interaction between the main diet effect and OT treatment effect. Labeled means without a common letter differ, *P*<0.05 based on post hoc analysis. Groups marked by * differ from placebo OT treatment group, *P*<0.05. *P* values for the main effect of the diet, OT treatment and interaction between main effects were calculated using 2-way ANOVA (MIXED procedure, SAS). Samples form the first block of experiments were used for the analysis. Ova, ovalbumin; SDA, stearidonic acid; OT, oral tolerance Ova-IgG1, ovalbumin specific immunoglobulinG1

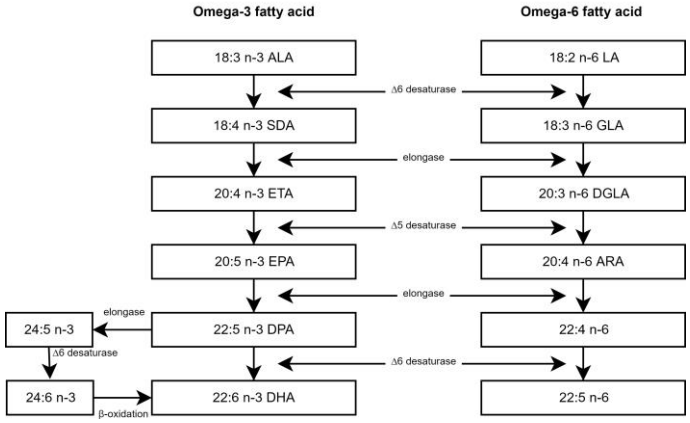
Figure 5: Effect of diet on the 3-week old Sprague-Dawley rat pups' (A) plasma and (B) spleen total phospholipid fatty acid composition. There was no significant effect of OT treatment nor a significant interaction effect so the treatment groups within each diet

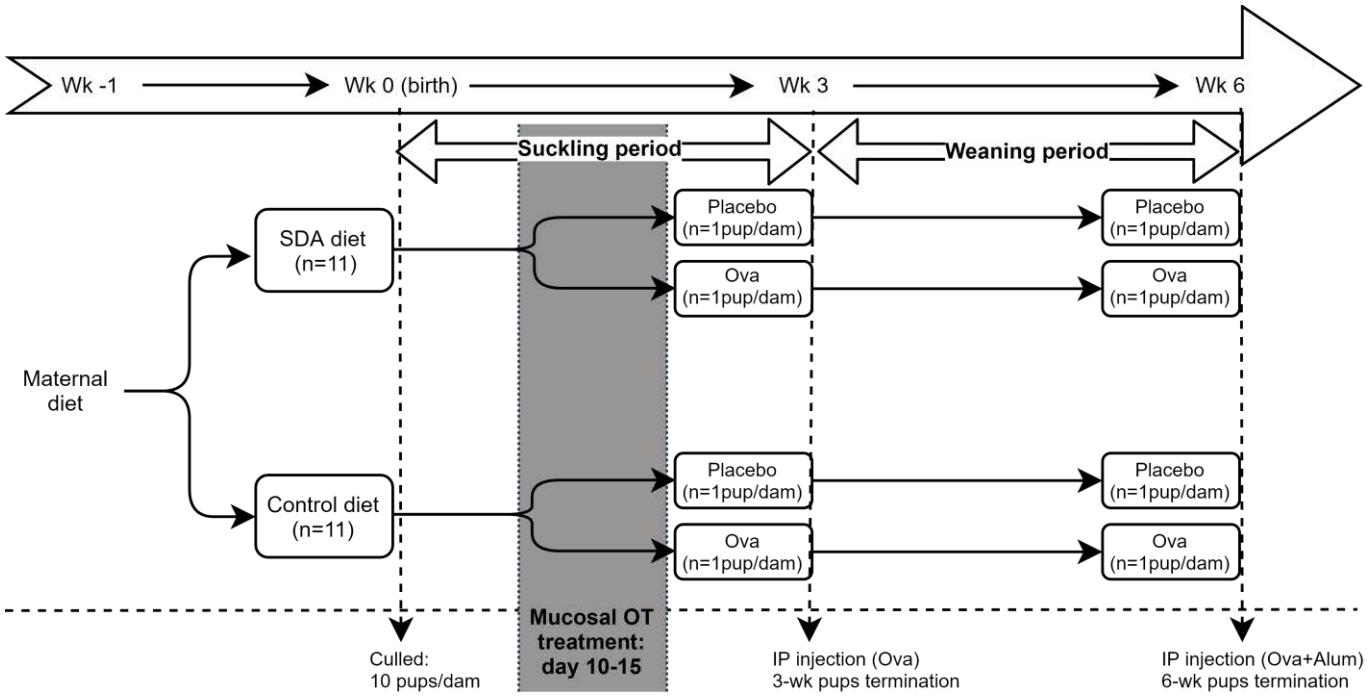
were combined. Significant diet effect was calculated by unpaired student's t-test, * differ from Control diet, $P<0.05$. Outliers were excluded from the analysis resulting in $n<11$ per diet group. ALA, α-linolenic acid; SDA, stearidonic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; n, omega

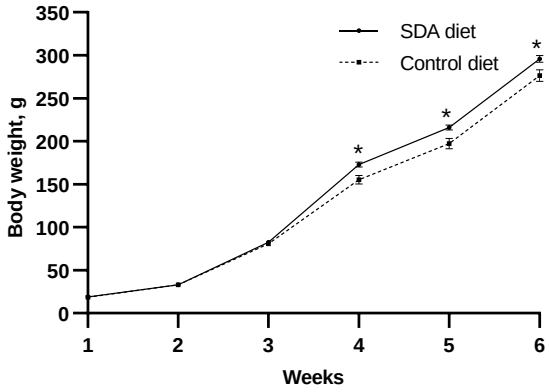
Figure 6: Effect of diet on the 6-week old Sprague-Dawley rat pups' (A) plasma and (B) spleen total phospholipid fatty acid composition. There was no significant effect of OT treatment nor a significant interaction effect so the treatment groups within each diet were combined. Significant diet effect was calculated by unpaired student's t-test, * differ from Control diet, $P<0.05$. Outliers were excluded from the analysis resulting in $n<11$ per diet group. ALA, α-linolenic acid; SDA, stearidonic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; n, omega

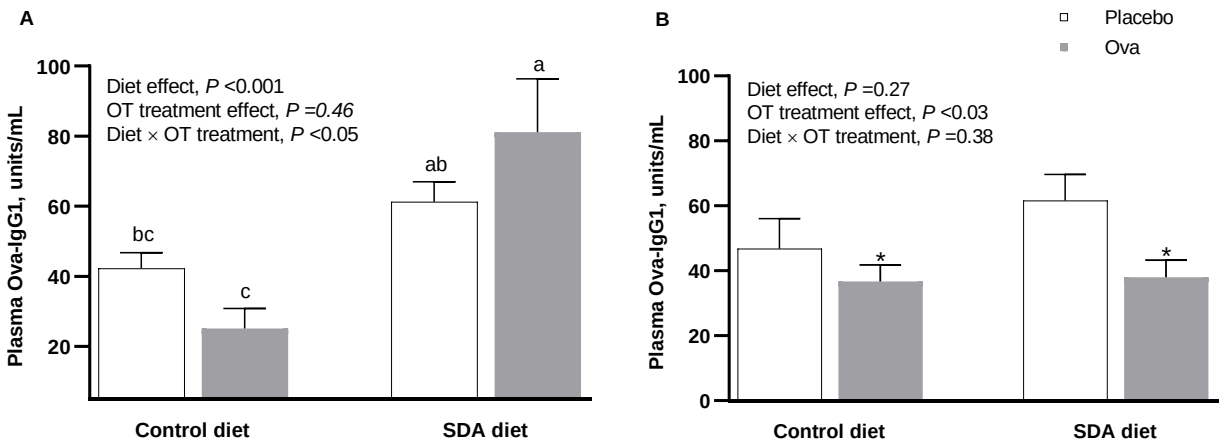
Figure 7: The effect of diet on *ex-vivo* cytokine production upon (A) LPS and (B) Ova stimulation response by splenocytes from 6-week old Sprague-Dawley pups. No major OT treatment effect was observed in LPS stimulated splenocyte cytokine production, therefore the means from Ova and placebo groups are combined for (A). Significant diet effect was calculated by unpaired student's t-test, * differ from Control diet, $P<0.05$. For Ova stimulated cytokine production, as there was OT treatment effect, each diet group shows means from Ova and placebo treatment group separately. Labeled means

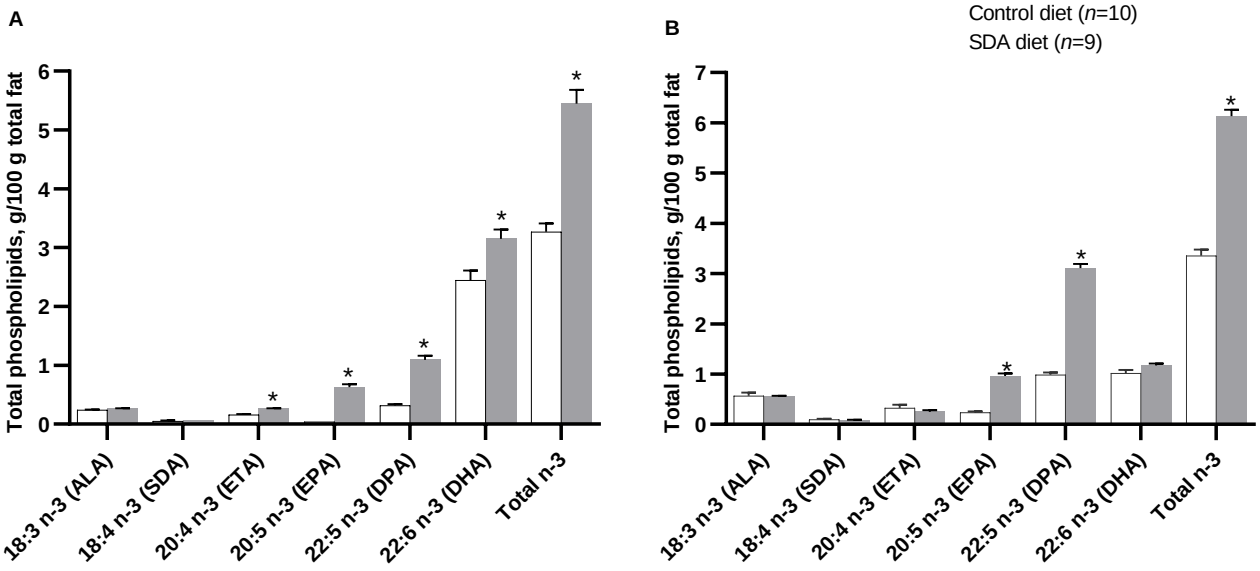
without a common letter differ, $P<0.05$ based on post hoc analysis in 2-way ANOVA. Samples form the first block of experiments were used for the analysis. IL, interleukin; TNF-α, tumor necrosis factor-alpha; LPS, Lipopolysaccharide; Ova, Ovalbumin

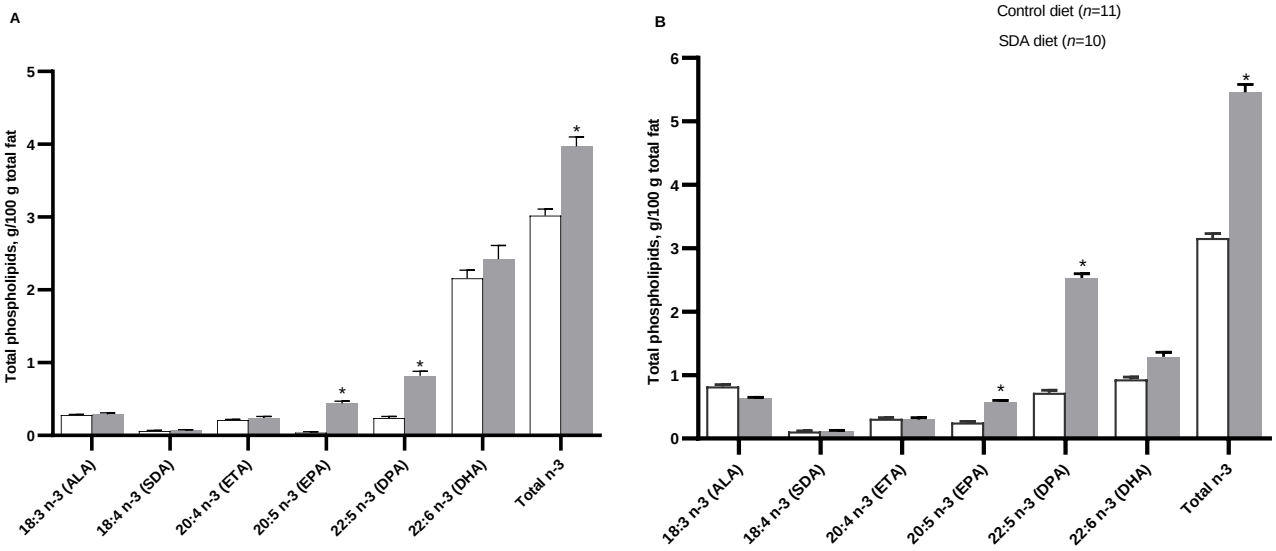

Omega-3 fatty acid
Omega-6 fatty acid
18:3 n-3 ALA
18:2 n-6 LA
Δ6 desaturase
18:4 n-3 SDA
18:3 n-6 GLA
elongase
20:4 n-3 ETA
20:3 n-6 DGLA
Δ5 desaturase
20:5 n-3 EPA
20:4 n-6 ARA
elongase
elongase
24:5 n-3
22:5 n-3 DPA
22:4 n-6
Δ6 desaturase
Δ6 desaturase
24:6 n-3
22:6 n-3 DHA
22:5 n-6
β-oxidation

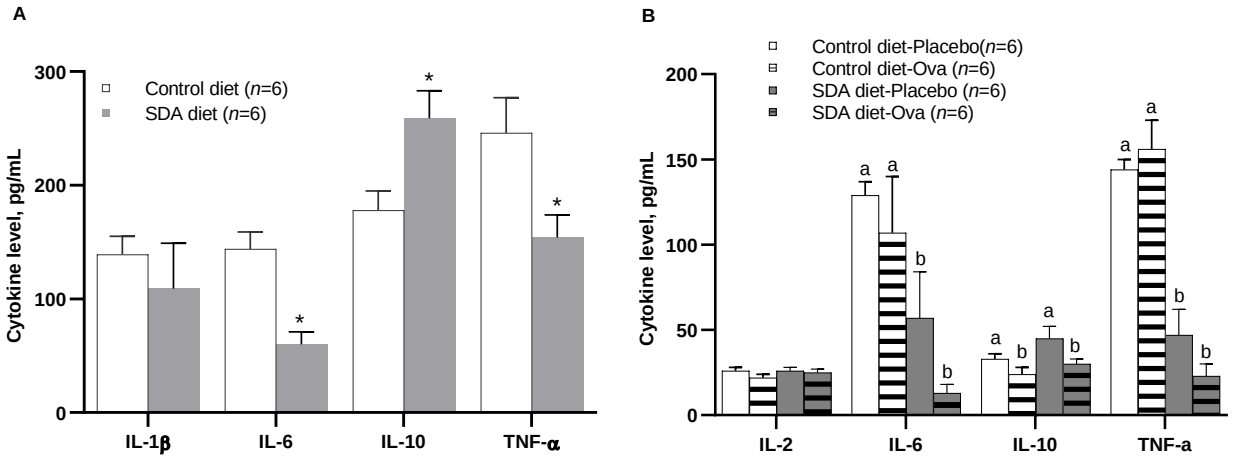

Wk -1
Wk 0 (birth)
Wk 3
Wk 6
Suckling period
Weaning period
Maternal diet
SDA diet (n=11)
Control diet (n=11)
Placebo (n=1pup/dam)
Ova (n=1pup/dam)
Placebo (n=1pup/dam)
Ova (n=1pup/dam)
Placebo (n=1pup/dam)
Ova (n=1pup/dam)
Placebo (n=1pup/dam)
Ova (n=1pup/dam)
Mucosal OT treatment: day 10-15
Culled: 10 pups/dam
IP injection (Ova) 3-wk pups termination
IP injection (Ova+Alum) 6-wk pups termination


SDA diet
Control diet
Body weight, g
350
300
250
200
150
100
50
0
1
2
3
4
5
6
Weeks
*
*
*


A
Plasma Ova-IgG1, units/mL
100
80
60
40
20
Diet effect, P <0.001
OT treatment effect, P =0.46
Diet × OT treatment, P <0.05
bc
c
ab
a
Control diet
SDA diet
B
Placebo
Ova
Plasma Ova-IgG1, units/mL
100
80
60
40
20
0
Diet effect, P =0.27
OT treatment effect, P <0.03
Diet × OT treatment, P =0.38
*
*
Control diet
SDA diet


A
B
Control diet (n=10)
SDA diet (n=9)
Total phospholipids, g/100 g total fat
0
1
2
3
4
5
6
7
*
18:3 n-3 (ALA)
18:4 n-3 (SDA)
20:4 n-3 (ETA)
20:5 n-3 (EPA)
22:5 n-3 (DPA)
22:6 n-3 (DHA)
Total n-3


A
B
Control diet (n=11)
SDA diet (n=10)
Total phospholipids, g/100 g total fat
0
1
2
3
4
5
6
*
18:3 n-3 (ALA)
18:4 n-3 (SDA)
20:4 n-3 (ETA)
20:5 n-3 (EPA)
22:5 n-3 (DPA)
22:6 n-3 (DHA)
Total n-3


A
Control diet (n=6)
SDA diet (n=6)
Cytokine level, pg/mL
300
200
100
0
IL-1β
IL-6
IL-10
TNF-α
*
B
Control diet-Placebo(n=6)
Control diet-Ova (n=6)
SDA diet-Placebo (n=6)
SDA diet-Ova (n=6)
Cytokine level, pg/mL
200
150
100
50
0
IL-2
IL-6
IL-10
TNF-a
a
b

Online Supplementary Material

Supplemental Table 1: Effect of diet and oral tolerance treatment on hematologic parameters of 3-week old Sprague Dawley rat pups[1]

Type of cells in blood	Control Diet (*n*=11)	SDA Diet (*n*=8)	*P*-Diet
WBC, 10^9 cells/L	6.0 ± 0.5	5.5 ± 0.3	0.60
Lymphocytes, % of WBC	80.6 ± 1.4	84.3 ± 0.9	0.03
Monocyte, % of WBC	3.7 ± 0.4	2.6 ± 0.2	0.69
Basophils, % of WBC	0.8 ± 0.1	0.5 ±0.1	0.32
Neutrophil, % of WBC	0.7 ± 0.2	0.6 ± 0.1	0.01

[1]Values are presented in mean ± SEM. *P* represents the probability for the main effect of diet on 3-week pups. As there was no significant effect of treatment nor a significant interaction the diet/treatment groups have been combined. Abbreviation; WBC, white blood cells, SDA, stearidonic acid

Supplementary Data

Supplemental Table 2: Effect of diet on the total plasma phospholipid fatty acid composition of 3-week old Sprague-Dawley rat pups measured using gas-liquid chromatography[1]

Fatty acid, g/100g total fat	**Control (*n*=11)**	**SDA (*n*=9)**	***P*-Diet**
16:0	21.07 ± 0.27	24.02 ± 0.67	0.002
18:0	28.45 ± 0.49	28.56 ± 0.57	0.84
18:1	6.23 ± 0.22	5.48 ± 0.13	0.005
18:2 n-6 (LA)	19.86 ± 0.87	16.17 ± 0.79	0.01
18:3 n-3 (ALA)	0.24 ± 0.01	0.26 ± 0.01	0.39
18:4 n-3 (SDA)	0.05 ± 0.01	0.07 ± 0.01	0.01
20:2 n-6	0.31 ± 0.01	0.28 ± 0.01	0.03
20:3 n-6	1.11 ± 0.04	1.57 ± 0.06	<0.001
20:4 n-6 (ARA)	15.00 ± 0.73	14.79 ± 0.70	0.91
20:4 n-3 (ETA)	0.16 ± 0.01	0.26 ± 0.01	<0.001
20:5 n-3 (EPA)	0.04 ± 0.01	0.63 ± 0.05	<0.001
22:4 n-6	0.23 ± 0.01	0.11 ± 0.01	<0.001
22:5 n-6	0.82 ± 0.09	0.09 ± 0.01	<0.001
22:5 n-3 (DPA)	0.32 ± 0.02	1.09 ± 0.07	<0.001
22:6 n-3 (DHA)	2.45 ± 0.16	3.15 ± 0.16	0.009
Total SFA	50.53 ± 0.37	53.72 ± 0.50	<0.001
Total MUFA	8.83 ± 0.28	7.64 ± 0.16	<0.001
Total PUFA	40.64 ± 0.25	38.64 ± 0.56	0.007
Total n-6	37.37 ± 0.24	33.19 ± 0.46	<0.001
Total n-3	3.27 ± 0.14	5.45 ± 0.23	<0.001
Ratio PUFA/SFA	0.18 ± 0.01	0.14 ± 0.01	0.003
Ratio n-6/n-3 LCPUFA	11.62 ± 0.54	6.18 ± 0.26	<0.001

[1]Values are presented in mean ± SEM. As there was no significant effect of treatment nor a significant interaction within diet, treatment groups have been combined. *P*-Diet represents the probability for the main effect of maternal diet on 3-week pups. Abbreviations; LA, linoleic acid; SDA, stearidonic acid; ARA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; ETA, eicosatetraenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; MUFA, monounsaturated fatty acids; n, omega; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

Supplementary Data

Supplemental Table 3: Effect of diet on the total plasma phospholipid fatty acid composition of 6-week old Sprague Dawley rat pups measured using gas-liquid chromatography[1]

Fatty acid, g/100 g total fat	Control (*n*=11)	SDA (*n*=10)	*P*-Diet
16:0	20.59 ± 0.31	21.85 ± 0.30	0.008
18:0	29.06 ± 0.36	29.87 ± 0.50	0.27
18:1	6.61 ± 0.19	5.62 ± 0.15	<0.001
18:2 n-6 (LA)	18.37 ± 0.48	17 ± 0.45	0.08
18:3 n-3 (ALA)	0.28 ± 0.01	0.29 ± 0.02	0.84
18:4 n-3 (SDA)	0.06 ± 0.01	0.07 ± 0.01	0.23
20:2 n-6	0.33 ± 0.01	0.33 ± 0.02	0.78
20:3 n-6	1.18 ± 0.05	1.67 ± 0.05	<0.001
20:4 n-6 (ARA)	16.11 ± 0.38	15.16 ± 0.59	0.17
20:4 n-3 (ETA)	0.21 ± 0.01	0.24 ± 0.02	0.14
20:5 n-3 (EPA)	0.04 ± 0.01	0.44 ± 0.03	<0.001
22:4 n-6	0.25 ± 0.01	0.13 ± 0.01	<0.001
22:5 n-6	0.33 ± 0.04	0.10 ± 0.02	<0.001
22:5 n-3 (DPA)	0.24 ± 0.02	0.82 ± 0.06	<0.001
22:6 n-3 (DHA)	2.16 ± 0.11	2.42 ± 0.19	0.47
Total SFA	50.78 ± 0.34	52.97 ± 0.69	0.02
Total MUFA	9.58 ± 0.23	8.13 ± 0.24	<0.001
Total PUFA	39.64 ± 0.33	38.90 ± 0.76	0.41
Total n-6	36.62 ± 0.32	34.63 ± 0.62	0.02
Total n-3	3.02 ± 0.09	3.97 ± 0.13	<0.001
Ratio PUFA/SFA	0.19 ± 0.01	0.15 ± 0.01	<0.001
Ratio n-6/n-3 LCPUFA	12.24 ± 0.39	8.31 ± 0.35	<0.001

[1]Values are presented in mean ± SEM. As there was no significant effect of treatment nor a significant interaction within diet, treatment groups have been combined. *P*-Diet represents the probability for the main effect of weaning diet on 6-week pups. Abbreviations; LA, linoleic acid; SDA, stearidonic acid; ARA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; ETA, eicosatetraenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; MUFA, monounsaturated fatty acids; n, omega; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

Supplementary Data

Supplemental Table 4: Effect of diet on the fatty acid composition of total phospholipids in spleen of 3-week old Sprague Dawley rat pups measured using gas-liquid chromatography[1]

Fatty acid, g/100 g total fat	Control (*n*=10)	SDA (*n*=9)	*P*-Diet
16:0	27.95 ± 0.46	28.51 ± 0.29	0.37
18:0	19.58 ± 0.31	20.14 ± 0.28	0.15
18:1	13.16 ± 0.19	12.25 ± 0.15	0.26
18:2 n-6 (LA)	7.66 ± 0.2	7.26 ± 0.16	0.17
18:3 n-3 (ALA)	0.57 ± 0.06	0.55 ± 0.02	0.054
18:4 n-3 (SDA)	0.10 ± 0.01	0.08 ± 0.01	0.10
20:2 n-6	0.71 ± 0.02	0.58 ± 0.01	<0.001
20:3 n-6	1.70 ± 0.05	2.28 ± 0.06	<0.001
20:4 n-6 (ARA)	16.30 ± 0.49	14.76 ± 0.5	0.86
20:4 n-3 (ETA)	0.33 ± 0.06	0.25 ± 0.03	0.13
20:5 n-3 (EPA)	0.24 ± 0.02	0.96 ± 0.05	0.002
22:4 n-6	0.66 ± 0.04	0.17 ± 0.02	0.98
22:5 n-6	0.45 ± 0.06	0.36 ± 0.04	0.31
22:5 n-3 (DPA)	0.99 ± 0.04	3.11 ± 0.08	0.04
22:6 n-3 (DHA)	1.02 ± 0.06	1.17 ± 0.04	0.44
Total SFA	50 ± 0.51	51.44 ± 0.46	0.27
Total MUFA	18.74 ± 0.26	16.32 ± 0.25	0.13
Total PUFA	31.04 ± 0.46	31.97 ± 0.49	0.76
Total n-6	27.68 ± 0.45	25.84 ± 0.37	0.003
Total n-3	3.36 ± 0.12	6.13 ± 0.13	0.004
Ratio PUFA/SFA	0.62 ± 0.02	0.62 ± 0.01	0.51
Ratio n-6/n-3 LCPUFA	8.40 ± 0.28	4.23 ± 0.05	<0.001

[1]Values are presented in mean ± SEM. As there was no significant effect of treatment nor a significant interaction within diet, treatment groups have been combined. *P*-Diet represents the probability for the main effect of maternal diet on 3-week pups. Abbreviations; LA, linoleic acid; SDA, stearidonic acid; ARA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; ETA, eicosatetraenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; MUFA, monounsaturated fatty acids; n, omega; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

Supplementary Data

Supplemental Table 5: Effect of diet on the fatty acid composition of total phospholipids in spleen of 6-week old Sprague Dawley rat pups measured using gas-liquid chromatography[1]

Fatty acid, g/100 g total fat	Control (*n*=11)	SDA (*n*=10)	*P*-Diet
16:0	29.48 ± 0.69	30.66 ± 0.54	0.23
18:0	19.20 ± 0.33	19.25 ± 0.26	0.73
18:1	12.11 ± 0.19	12.04 ± 0.21	0.81
18:2 n-6 (LA)	6.49 ± 0.25	5.91 ± 0.23	0.43
18:3 n-3 (ALA)	0.82 ± 0.03	0.63 ± 0.02	0.40
18:4 n-3 (SDA)	0.11 ± 0.01	0.12 ± 0.01	0.76
20:2 n-6	0.74 ± 0.02	0.55 ± 0.02	0.09
20:3 n-6	1.81 ± 0.07	2.24 ± 0.05	<0.001
20:4 n-6 (ARA)	15.28 ± 0.58	13.54 ± 0.44	0.99
20:4 n-3 (ETA)	0.31 ± 0.02	0.30 ± 0.03	0.61
20:5 n-3 (EPA)	0.25 ± 0.02	0.58 ± 0.02	<0.001
22:4 n-6	0.55 ± 0.02	0.16 ± 0.02	<0.001
22:5 n-6	0.64 ± 0.05	0.63 ± 0.05	0.68
22:5 n-3 (DPA)	0.72 ± 0.04	2.53 ± 0.07	<0.001
22:6 n-3 (DHA)	0.93 ± 0.04	1.29 ± 0.07	0.07
Total SFA	51.54 ± 0.66	53.06 ± 0.65	0.39
Total MUFA	19.36 ± 0.28	18.24 ± 0.35	0.88
Total PUFA	28.76 ± 0.75	28.36 ± 0.65	0.74
Total n-6	25.60 ± 0.73	22.90 ± 0.62	0.93
Total n-3	3.16 ± 0.07	5.46 ± 0.12	<0.001
Ratio PUFA/SFA	0.56 ± 0.02	0.54 ± 0.02	0.42
Ratio n-6/n-3 LCPUFA	8.18 ± 0.25	4.23 ± 0.14	<0.001

[1]Values are presented in mean ± SEM. As there was no significant effect of treatment nor a significant interaction within diet, treatment groups have been combined. *P*-Diet represents the probability for the main effect of weaning diet on 6-week pups. Abbreviations; LA, linoleic acid; SDA, stearidonic acid; ARA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; ETA, eicosatetraenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; MUFA, monounsaturated fatty acids; n, omega; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

Supplementary Data

Supplemental Table 6: Effect of diet and oral tolerance treatment on *ex-vivo* cytokine production by stimulated splenocytes from 3-week old Sprague Dawley rat pups[1]

	Control diet		**SDA diet**				
	Placebo (*n*=5)	**Ova (*n*=5)**	**Placebo (*n*=4)**	**Ova (*n*=4)**	***P*-Diet[2]**	***P*-Treatment[3]**	***P*-Interaction[4]**
LPS							
IL-1β, pg/mL	228 ± 49	208 ± 46	208 ± 70	273 ± 41	0.69	0.69	0.46
IL-6, pg/mL	467 ± 182	397 ± 147	386 ± 166	352 ± 172	0.72	0.76	0.91
IL-10, pg/mL	228 ± 18	188 ± 45	205 ± 38	125 ± 44	0.27	0.13	0.59
TNF-α, pg/mL	589 ± 169	549 ± 152	515 ± 153	496 ± 166	0.70	0.86	0.95
Ova							
IL-1β, pg/mL	88 ± 15	179 ± 91	79 ± 31	69 ± 24	0.33	0.5	0.4
IL-2, pg/mL	8 ± 1	11 ± 2	5 ± 2	4 ± 3	0.09	0.56	0.32
IL-6, pg/mL	384 ± 108	354 ± 137	248 ± 90	399 ± 145	0.72	0.63	0.48
IL-10, pg/mL	39 ± 12[a]	95 ± 4[b]	48 ± 12[a]	39 ± 5[a]	0.12	0.12	0.04
TNF-α, pg/mL	459 ± 124	416 ± 127	395 ± 104	423 ± 92	0.81	0.95	0.76

[1]Values are presented in mean ± SEM. Labeled means in a row without a common superscript letter differ, P < 0.05.

[2]*P*-Diet represents the probability for the main effect of maternal diet on 3-week pups

[3]*P*-Treatment represents the probability for the main effect of OT treatment on 3-week pups

[4]*P*-Interaction represents the probability for the interaction of the main effects on 3-week pups

Samples from only the first block of experiments were used for analysis resulting in *n* of less than 11 per diet group. Abbreviations; IL, interleukin; TNF-α, tumor necrosis factor-alpha; IFN-γ, Interferon-gamma; LPS, Lipopolysaccharide; OT, oral tolerance; Ova, ovalbumin

Supplementary Data

Supplemental Table 7: Effect of diet and oral tolerance treatment on *ex-vivo* cytokine production by stimulated splenocytes from 6-week old Sprague Dawley rat pups[1]

	Control diet		**SDA diet**				
	Placebo (*n*=6)	**Ova (*n*=6)**	**Placebo (*n*=6)**	**Ova (*n*=6)**	***P*-Diet[2]**	***P*-Treatment[3]**	***P*-Interaction[4]**
LPS							
IL-1β, pg/mL	140 ± 16	139 ± 31	154 ± 91	69 ± 21	0.49	0.29	0.30
IL-6, pg/mL	148 ± 24 [a]	140 ± 19 [a]	79 ± 19 [b]	42 ± 9 [b]	0.001	0.28	0.48
IL-10, pg/mL	188 ± 26 [a]	168 ± 24 [a]	292 ± 35 [b]	227 ± 28 [b]	0.01	0.16	0.44
TNF-α, pg/mL	205 ± 24 [a]	287 ± 54 [a]	154 ± 43 [b]	155 ± 8 [b]	0.03	0.29	0.31
IFN-γ, pg/mL	6 ± 4 [a]	4 ± 2 [b]	16 ± 4 [a]	4 ± 1 [b]	0.07	0.03	0.13
Ova							
IL-2, pg/mL	26 ± 2	22 ± 2	26 ± 2	25 ± 2	0.61	0.35	0.45
IL-6, pg/mL	129 ± 8 [a]	107 ± 33 [a]	57 ± 27 [b]	13 ± 5 [b]	0.002	0.17	0.63
IL-10, pg/mL	33 ± 3 [a]	24 ± 4 [b]	45 ± 7 [a]	30 ± 3 [b]	0.06	0.01	0.52
TNF-α, pg/mL	144 ± 6 [a]	156 ± 17 [a]	47 ± 15 [a]	23 ± 7 [a]	<0.001	0.65	0.19

[1]Values are presented in mean ± SEM. Labeled means in a row without a common superscript letter differ, P < 0.05.

[2]*P*-Diet represents the probability for the main effect of weaning diet on 6-week pups

[3]*P*-Treatment represents the probability for the main effect of OT treatment on 6-week pups

[4]*P*-Interaction represents the probability for the interaction of the main effects on 6-week pups

Samples from only the first block of experiments were used for analysis resulting in *n* of less than 11 per diet group. Abbreviations; IL, interleukin; TNF-α, tumor necrosis factor-alpha; IFN-γ, Interferon-gamma; LPS, Lipopolysaccharide; OT, oral tolerance; Ova, ovalbumin