



Nutritional Immunology

The Programming Effect of Plant-Based DHA, Along with Equivalent AA, on Immune System and Oral Tolerance Development in Six-Week Allergy Prone BALB/c Pups

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A B S T R A C T

Background: Docosahexaenoic acid (DHA) and arachidonic acid (AA) on oral tolerance (OT) development in allergy-prone infants is less known.

Objectives: We aim to determine the effects of early life DHA supplementation (1% of total fat, from novel canola oil), along with AA, on OT toward ovalbumin (ova, egg protein) in allergy-prone BALB/c pups at 6-wk.

Methods: Breastfeeding dams ($n \geq 10/\text{diet}$) were fed DHA+AA (1% DHA, 1% AA wt/wt of total fat) or control (0% DHA, 0% AA) suckling period diet (SPD) during which pups consumed dam's milk. At 3-wk, pups from each SPD group were assigned to either the control or DHA+AA weaning diet. For OT, pups from each diet group were either orally fed ova or placebo daily from 21–25 d. Systemic immunization to ova was induced through intraperitoneal injections before euthanizing 6-wk pups. Ova-specific immunoglobulin (ova-Ig) and splenocytes ex-vivo cytokine response to different stimuli were analyzed using a 3-factor analysis of variance.

Results: OT-induced suppression was seen in ova-stimulated splenocyte ex-vivo response, where ova-tolerized pups showed significantly lower total immunoglobulin (Ig)G, IgG1, interleukin (IL)-2 and IL-6 production than sucrose (placebo) pups. DHA+AA SPD was associated with 3 times lower plasma concentrations of ova-IgE ($P = 0.03$) than controls. DHA+AA weaning diet resulted in lower T helper type-2 cytokines (IL-4 and IL-6) with ova stimulation than controls, which may benefit OT. DHA+AA SPD resulted in significantly higher T cell cytokine response [IL-2, interferon-gamma, (IFN γ) and IL-1 β] to anti-CD3/CD28 stimulation than controls. The splenocytes stimulated with lipopolysaccharide produced lower inflammatory cytokines (IFN γ , tumor necrosis factor-alpha, IL-6, and C-X-C motif ligand 1), which may be because of lower CD11b+CD68+ splenocytes proportion in pups from DHA+AA SPD than control (all $P < 0.05$).

Conclusions: DHA and AA in early life may influence OT in allergy-prone BALB/c mouse offspring, as they effectively promote T helper type-1 immune responses.

Keywords: T helper type-2, infant immune system development, high-DHA canola oil

Introduction

The recent rise in the prevalence of food hypersensitivity has been associated with a higher ratio of dietary n-6 to n-3 PUFA [1–3]. This is mostly because of the increased consumption of vegetable oils which are high in n-6 PUFAs and low in n-3 PUFAs [3]. Further, plant-based oils are considered poor sources of dietary long-chain PUFA (LCPUFA), especially n-3 LCPUFA. The n-3 LCPUFAs are commonly obtained from oily fish, sea foods, and, more recently, from genetically modified single-cell

organisms and novel plant oils. However, because of the low intake of these foods, the n-3 LCPUFA status remains suboptimal and is considered to be a risk factor for food allergies [4–7]. Recently, a commercially available genetically modified canola has been shown to produce high DHA. The canola plant was genetically modified to introduce a set of desaturase and elongase enzymes involved in the n-3 and n-6 bioconversion pathways. The high concentration of DHA in these seeds is achieved by converting oleic acid to n-3 LCPUFAs such as EPA, DPA, and DHA [8]. Therefore, n-3 LCPUFA (DHA) intake from novel

Abbreviations: n, omega; OT, oral tolerance; ova, ovalbumin; SPD, suckling period diet; Th1, T helper type-1; Th2, T helper type-2; WD, weaning diet.

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high-DHA canola oil (Nuseed) can provide an efficient alternative to improve DHA status.

After birth, the infant's immune system undergoes a significant transition to become mature and more adult-like. First, the nonspecific responses by innate immunity transition to more specific and robust through the expansion of adaptive immunity (T cells and B cells) [9]. Second, the T helper cell (Th) type-2 (Th2)-biased immune responses (IL-4, IL-5, IL-13, IgG1, and IgE) transition to T helper type-1 (Th1) dominant responses (TNF α , IFN γ , IL-12, and IgG2a) [10]. Risk of developing food allergies during this period is also higher during this period [11]. However, the immune system during early life can generate a specific suppression, or ignorance, of cellular and humoral immune responses toward orally ingested food protein, defined as oral tolerance (OT) [12–14]; the failure to develop OT results in food allergies. Infants with prolonged Th2-biased immune responses promote the development of food hypersensitivity, mediated by antigen-specific IgE [15], and may hinder the development of OT during early life [14,16].

The first year after birth is considered a critical window of opportunity for infant immune system development [17]. The immune system adaptation obtained during this period can have a long-term programming effect that can be observed later in life [18,19]. Therefore, providing optimal nutrition is paramount during this period [17]. The supplementation of DHA (n-3 LCPUFA) and AA (n-6 LCPUFA) may be useful because of their immunomodulatory properties [20]. Depending on the dose and the balance of these 2 lipids, they can be either anti-inflammatory or inflammatory [21,22]. Therefore, studies that provide a balance of n-3 and n-6 LCPUFA may be crucial for immune system development. Past studies from our laboratory group using DHA and AA in pre-term and full-term infants have shown beneficial effects on immune system development [23,24]. Furthermore, similar findings have also been seen in studies evaluating a low-dose supplementation of DHA alongside AA in rodents with a healthy immune system [25] or allergy-prone immune system [26]. Therefore, it was hypothesized that the supplementation of a relatively higher dose of DHA alongside AA during early infancy in allergy-prone could promote the maturation of infant immune function and assist in preventing food hypersensitivity through OT development [27–29].

The study's overall objective was to determine the effects of DHA supplementation (1% of total fat), along with AA, during the suckling and weaning period on the development tolerance toward ovalbumin (ova) and maturation of the immune system. We used allergy-prone BALB/c mice in the study to model infants born with a genetic predisposition to developing atopic conditions such as food hypersensitivity and allergies. The primary outcomes measured were ex-vivo cytokine and antibody responses to different stimuli. Additionally, the main outcome evaluated for OT analysis was the ex-vivo production of cytokines and Ig by splenocytes stimulated with ova. We also analyzed plasma concentrations of ova-specific-Ig to evaluate in-vivo response to OT. The differences in immune cell phenotype were used to explain the observed changes in the immune cell functions. The hypothesis was that the DHA would promote beneficial changes in the immune function of BALB/c mouse offspring, resulting in the suppression of antigen-specific response to ova in-vivo and ex-vivo to induce OT.

Methods

Diet and study design

All the animal experiments and procedures were completed in compliance with the Canadian Council on Animal Care and were approved by the University of Alberta Animal Care and Use Committee. Timed pregnant BALB/c mice were obtained from Charles River Laboratories and housed in a temperature- and humidity-controlled environment on a 12 h–12 h reverse light cycle. Dams were acclimatized until 14 d of gestation, during which a standard rodent unpurified diet (Laboratory diet 5001; PMI Nutrition International) was fed. Dams were randomly assigned 5–7 d before parturition to consume 1 of the 2 suckling period diets (SPDs): DHA+AA SPD (1% DHA wt/wt of total fat and 1% AA wt/wt of total fat, $n = 10$) and control SPD (0% DHA or AA, $n = 12$) for 3-wk suckling period (Figure 1). Dams were housed in individual cages with their pups, during which pups consumed the dam's mammary gland milk. Following the suckling period, pups from each of the SPD groups were randomly assigned to 1 of 2 weaning diets (WD); DHA+AA WD (1% DHA wt/wt of total fat and 1% AA wt/wt of total fat, $n = 16$ pups) and control WD (0% DHA and AA, $n = 16$ pups), until pups were 6-wk-old. This resulted in 4 diet group combinations for the pups (i.e., control SPD to control WD, control SPD to DHA+AA WD, DHA+AA SPD to control WD, and DHA+AA SPD to DHA+AA WD). Note, after OT group allocation (described below), there were 8 group combinations formed, and each group consisted of $n = 8$ pups of 6-wk age. The 6-wk-old pups were considered the experimental unit for the current experiment.

All the experimental diets were isocaloric, isonitrogenous, and nutritionally complete. Food and water were provided ad libitum. The experimental diets were high-fat diets prepared with mixing nonfat powder diet (described previously [30]) with 20 % wt/wt oil mix (described in Supplemental Table 1). We used a combination of oils to attain a similar PUFA/SFA ratio and n-6/n-3 ratio in both experimental diets, such that the effects studied were primarily because of DHA and AA supplementation. The FA composition of the diet is now described in Supplemental Table 1 [31]. The DHA concentration of 1% in human milk is found in populations with lifelong fish intake, which also allows for maintaining the DHA status of the mother while improving the status of DHA in infants [32–34]. Therefore, the concentration of DHA in the diet was selected to model the upper end of concentrations found in human breast milk, and the concentration of AA in the maternal diet was selected to match DHA as per the current dietary recommendations for infant supplementation [35]. All the pups were killed 6 wk after birth using carbon dioxide asphyxiation and cervical dislocation. Blood was collected with a cardiac puncture, and the spleen was aseptically collected for further analysis.

Ova administration and immunization protocol

To induce OT toward ova (food antigen derived from egg white protein; Sigma Aldrich), the 3-wk pups were orally fed ova (0.01 mg/g body weight) dissolved in sucrose water (8% wt/wt) using a syringe once a day from 21–25 d of age. For placebo control, half of the pups from all 4 diet groups were fed sucrose water (8% wt/wt) using a syringe once a day from 21–25 d. To induce systemic immune response toward ova, all the pups

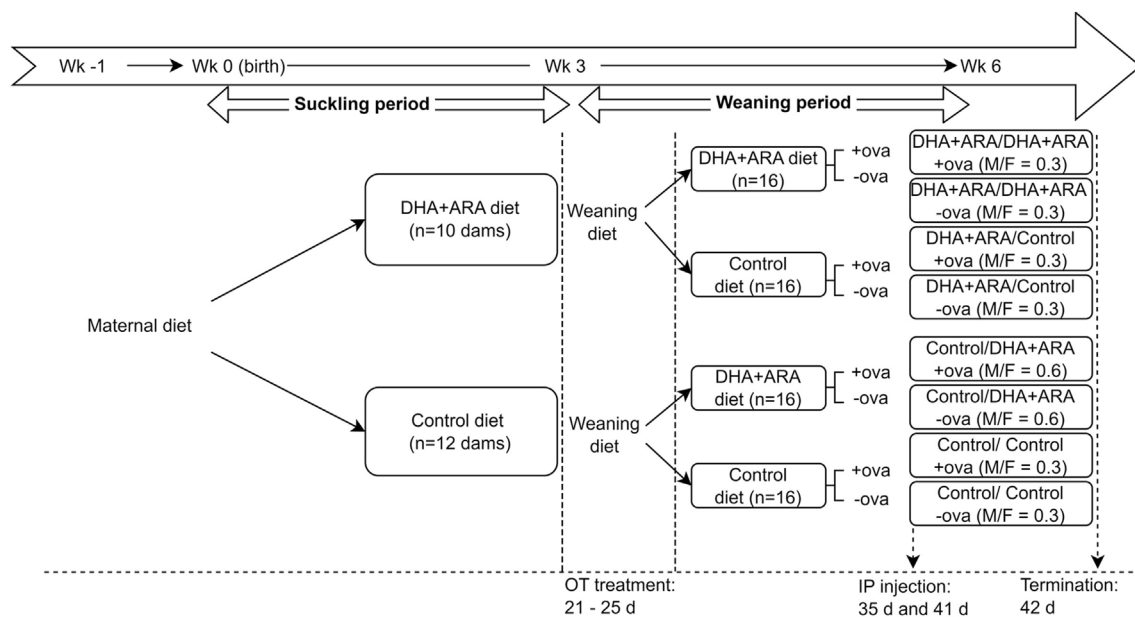


FIGURE 1. The study design shows BALB/c mice were randomly assigned to consume either DHA+AA maternal diet (1% DHA wt/wt of total fat and 1% AA wt/wt of total fat, $n = 10$ dams) or control maternal diet (no AA or DHA, $n = 12$ dams) beginning from 5–7 d pre-parturition to 3-wk suckling period when pups consumed their mother's breast milk. At 3 wk, pups from each maternal diet group were randomly allocated to either the DHA+AA diet (1% DHA wt/wt of total fat and 1% AA wt/wt of total fat, $n = 16$ pups) or the control diet (no AA or DHA, $n = 16$ pups) for addition 3-wk, this was referred to as weaning period. For inducing oral tolerance, 1-half of the pups from each weaning diet group were exposed to ovalbumin or sucrose placebo for 5 consecutive days starting from 21 d. The maternal diet, weaning diet, and oral tolerance interventions resulted in 8 groups with $n = 8$ pups/group. The 6-wk-old pups were considered the experimental unit for the current experiment. Intraperitoneal injections of ovalbumin and adjuvant were administered to all the pups to induce systemic immune response on 35 d and 41 d. All the pups were killed at 6-wk of age, and relevant tissues were collected. IP, intraperitoneal; M/F, male to female ratio; OT, oral tolerance; ova, ovalbumin.

received intraperitoneal injection of ova (10 μ g ova in 100 μ l PBS) with an adjuvant (1:1, Imject Alum Adjuvant; Thermo-Scientific) at 35 d (7 d before termination) and 41 d (24 h before termination, as a booster dose). At 6-wk (42 d), pups were killed, and blood was collected in K2 EDTA-containing tubes using a 2-mL syringe. 1 h after collection, blood tubes were centrifuged ($1734 \times g$; 10 min; 22°C), and plasma was collected and frozen at -80°C . The in-vivo analysis of OT was based on plasma concentrations of ova-specific IgE (ova-IgE) and ova-specific IgG (ova-IgG). Commercially available kits for ova-IgE and ova-IgG (MyBioSource, Inc.) were used with a detection of a range of 0.312–20 $\mu\text{g/mL}$ and 0.16–10 ng/mL, respectively. The ex-vivo analysis of OT was based on antibody response and cytokine response toward ova stimulation of splenocytes.

Immune cell isolation and ex-vivo cytokine response to mitogens and ova

The procedure to collect the spleen and isolate immune cells has been previously described [36]. Briefly, spleen tissue was cut into small pieces and pushed through nylon mesh to obtain a single-cell suspension. The RBCs in the cell suspension were lysed using ammonium chloride lysis buffer (155 mmol/L ammonium chloride, 0.1 mmol/L EDTA and 10 mmol/L potassium bicarbonate; Fisher Scientific). Cells were washed with 0.5% bovine serum albumin in Krebs-Ringer bicarbonate buffer and resuspended in complete cell culture medium (RPMI 1640 medium supplemented with 5% vol/vol fetal calf serum, 2.5 mmol/L 2-mercaptoethanol, and 1% antibiotic/antimycotic; Invitrogen). Cells were counted with the use of trypan blue dead

cell exclusion, and a single-cell suspension with a concentration of 1.25×10^6 cells/mL was prepared to be used for cell stimulation assay as described below.

The immune cell function was evaluated by cytokine production to ex-vivo stimulation with mitogens as described elsewhere [37]. We tested the splenocyte cytokine production function with no mitogen, LPS, phorbol-myristate-acetate, and ionomycin (PMAi), anti-CD3/anti-CD28, or ova. In brief, splenocytes were incubated with LPS (2 $\mu\text{L/mL}$; Sigma Aldrich Canada Ltd) in 5% carbon dioxide and 37°C to evaluate innate immune cells [macrophage and dendritic cells (DC) and B cells] or PMAi (2 $\mu\text{L/mL}$, cell stimulation cocktail; Thermo Fisher Scientific) in 5% carbon dioxide and 37°C to evaluate adaptive immune cells (lymphocyte). The immune cell response to the model food antigen was evaluated by stimulation with ova (200 $\mu\text{g/mL}$; Sigma) in 5% carbon dioxide and 37°C . For T cell stimulation, 12-well plates were coated with anti-CD3 antibody (2 $\mu\text{L/mL}$ prepared in PBS) overnight and washed with PBS prior to adding splenocytes (1.25×10^6 cells/mL) cell culture medium. Then anti-CD28 antibody (0.43 $\mu\text{L/mL}$; BD Biosciences) was added, and cells were cultured for 72 h in 5% carbon dioxide and 37°C .

The ex-vivo cytokine production was quantified using commercially available electrochemiluminescence kits (Pro-inflammatory Panel - 1, Meso Scale Discovery). The range of detection for measured cytokines is as follows; IFN γ , 0.0226–764 pg/mL; IL-10, 0.292–3540 pg/mL; IL-12, 4.95–32,500 pg/mL; IL-1 β , 0.0689–1530 pg/mL; IL-2, 0.204–2530 pg/mL; IL-4, 0.156–1790 pg/mL; IL-5, 0.0675–994 pg/mL; IL-6, 0.439–5240 pg/mL; chemokine C-X-C motif ligand 1 (CXCL-1, known as

keratinocyte chemoattractant/growth-regulated oncogene, KC/GRO], 0.265–2020 pg/mL and TNF α , 0.04–639 pg/mL. The ex-vivo concentrations of TGF β 1, 7.8–500 pg/mL, were quantified using commercial kits (LEGEND MAX; BioLegend). The ex-vivo concentrations of total IgG, IgG1 (Th2 type response), and IgG2a (Th1 type response) were quantified using commercial kits (Condrex, Inc.) with a 1.56–100 ng/mL detection range. Note, IgG2c was not measured. The dilution factor was calculated based on the estimated sample concentration for each cytokine/antibody ex-vivo response prior to analyzing samples.

Splenocyte immunofluorescence phenotyping

Splenocyte populations were identified by direct immunofluorescence (IF) assay as previously described [23,24]. Five to 7 fluorescent-labeled monoclonal antibodies (mAb) were used in combination to identify specific immune cell populations in splenocytes as follows CD28/CD25/CD4/CD152/CD8a/CD86/CD3, CD183/CD194/CD4/CD196/CD8a/CD3, CD45R/CD138/CD19/CD23/IgG/IgM, CD4/CD11c/CD19/CD103/MHC-II/CD86/CD8a, CD86/CD284/CD68/MHC-II/CD163/CD11b, CD27/CD45RB/CD4/CD49b/CD335/CD3/CD8a and latent-associated peptide/-FoxP3/CD4/CD25/CD3. All antibodies were purchased from Cedarlane and Thermo Fisher (developed by BD Biosciences or eBiosciences). Titration experiments were conducted to optimize the dilution factor for each mAb within a panel. The mAbs were selected to minimize spectral overlap. Further, compensation controls, isotype controls, and unstained controls were used to guide our gating strategy. Splenocytes from 3 different stimulation conditions, no stimulation (freshly isolated cells), LPS stimulation (collected after 12 h incubation with mitogen), or PMAi stimulation conduction (collected after 12 h incubation in mitogen) were used for IF assay. Briefly, splenocytes (200,000–500,000) were incubated for 30 min at 4°C with either of the monoclonal antibody combinations described earlier. Additionally, to identify T regulatory cells, the cell stained with latent-associated peptide/CD4/CD25/CD3 combination were fixed and permeabilized as per manufacturer's guideline (eBiosciences), followed by staining with anti-FoxP3 fluorescent-labeled antibody for 20 min and washed as per manufacturer's protocol. Cells stained with surface mAb were then washed with IF buffer (4% fetal calf serum in PBS stored and used at 4°C), and cells were fixed with 1% paraformaldehyde buffer (10 g/L, stored and used at 4°C; Thermo Fisher Scientific). The fluorescently labeled cells were acquired within 48 h by flow cytometry according to relative fluorescence intensity and analyzed using FlowJo™ v10.9 Software. Detailed gating strategy is provided in [Supplemental Figures 1–7](#).

Statistical analysis

All the parameters are presented as mean $\pm$ SEM unless stated otherwise. The sample size of $n = 8$ /group was determined to be sufficient to assess a difference of 20% or more in the primary outcome variable, ex-vivo cytokine production. Data analysis was conducted with PROC MIXED procedure 3-factor ANOVA (SAS version 9.4 software) and has been previously described [26,38]. The study followed a statistical model to evaluate 3 main independent variables (main effects); SPD, WD, and OT as well as their interactions (SPD $\times$ WD, SPD $\times$ OT, WD $\times$ OT, and SPD $\times$ WD $\times$ OT). The effect of pups' sex and the related interaction effect are not included in the current study and will be appropriately discussed with findings from subsequent studies.

When a main effect was found to be significant (in the absence of any statistically significant associated interactions), the means of groups within the main effects were reported for ease of understanding. When the main effect was found to be statistically significant along with significant interaction effect, then only the interaction effects were described, and post hoc analyses were conducted to compare group means associated with the interaction effect using Bonferroni adjustment for multiple comparisons. This adjustment is conservative in estimating P values to minimize type 1 errors (false positives), because of which some parameters may appear to be different but may not show statistical significance. Furthermore, limiting the statistical power to observe the difference between groups. For instance, when the P value for SPD $\times$ WD interaction was statistically significant, means from 4 diet groups were compared (control SPD and control WD, control SPD and DHA+AA WD, DHA+AA SPD and control WD, and DHA+AA SPD and DHA+AA WD). Note, prior to conducting inferential statistics, the assumptions were checked, and data was appropriately transformed to meet the assumption. Differences were assumed to be significant at $P \leq 0.05$.

Results

Anthropometric measurements of 6-wk-old pups

Bodyweight

The SPD and OT intervention effect or their interaction effect did not have an influence on the body weight of 6-wk pups. However, the pups fed DHA+AA WD had significantly high bodyweight than controls (19.1 ± 0.4 compared with 18.0 ± 0.3 ; $P = 0.02$).

Food intake

The SPD and OT intervention did not have an influence on the mean food intake between week 3 and week 6. However, a small but significant reduction in daily average food intake was observed in DHA+AA WD group pups compared to control WD (week-4: 2.8 ± 0.1 compared with 3.5 ± 0.2 ; $P < 0.001$, week-5: 5.0 ± 0.3 compared with 5.7 ± 0.4 ; $P = 0.01$, week-6: 7.3 ± 0.5 compared with 7.5 ± 0.6 ; $P = 0.03$).

Spleen weight and cell count

The weight of the spleen and splenocyte count showed no significant effect of SPD, WD, or OT intervention.

Plasma concentration of ova-IgE and ova-IgG in 6-wk pups

A significant SPD $\times$ OT interaction effect was observed for ova-IgE ([Figure 2A](#)). Post hoc analysis showed that feeding DHA+AA SPD resulted in lower concentrations of plasma ova-IgE than control SPD (7.6 ± 1.4 compared with 25.1 ± 2.9 ; $P = 0.01$) but only when pups were exposed to sucrose (placebo exposed) OT. In ova OT groups, ova-IgE showed no change in DHA+AA SPD-fed pups compared to control SPD ($P = 0.30$).

A significant OT effect was observed for ova-IgG ([Figure 2B](#)). Contrary to our hypothesis, pups exposed to ova showed higher ova-IgG concentration than sucrose-exposed groups (6.2 ± 0.4 compared with 5.4 ± 0.3 ; $P = 0.01$), irrespective of SPD or WD intervention.

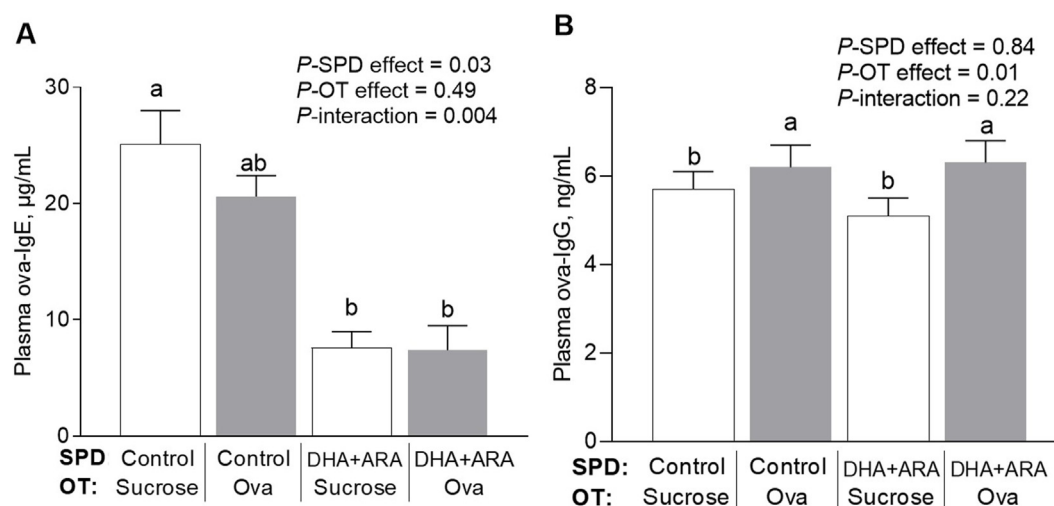


FIGURE 2. The effect of SPD and OT effects on in-vivo responses. The plasma concentration of ova-IgE (A) and ova-IgG (B). Statistical analysis was conducted using 3-factor ANOVA, and results from SPD × OT interactions are described here. Bars without a common letter differ significantly at $P < 0.05$. Values are presented in mean ± SEM. OT, oral tolerance; ova, ovalbumin; ova-IgG, ova-immunoglobulin G; SEM, standard error of the mean; SPD, suckling period diet.

Ex-vivo antibody response (IgG subtypes) by splenocytes with different stimuli

Ova stimulation

The ex-vivo production of total IgG and IgG1 by splenocytes after stimulation with ova was significantly lower in ova-exposed pups compared to the sucrose group (both $P < 0.001$),

irrespective of SPD or WD (shown in Figure 3A, B, respectively). The difference in IgG1 concentrations between ova and sucrose OT was greater in DHA+AA WD group pups (1.3 ± 0.3 compared with 27.2 ± 8.5 ; $P = 0.02$), compared to control WD group pups (1.0 ± 0.2 compared with 9.9 ± 3.4 ; $P = 0.02$) (Figure 3B). Stimulation of splenocytes with ova elicited higher IgG2a

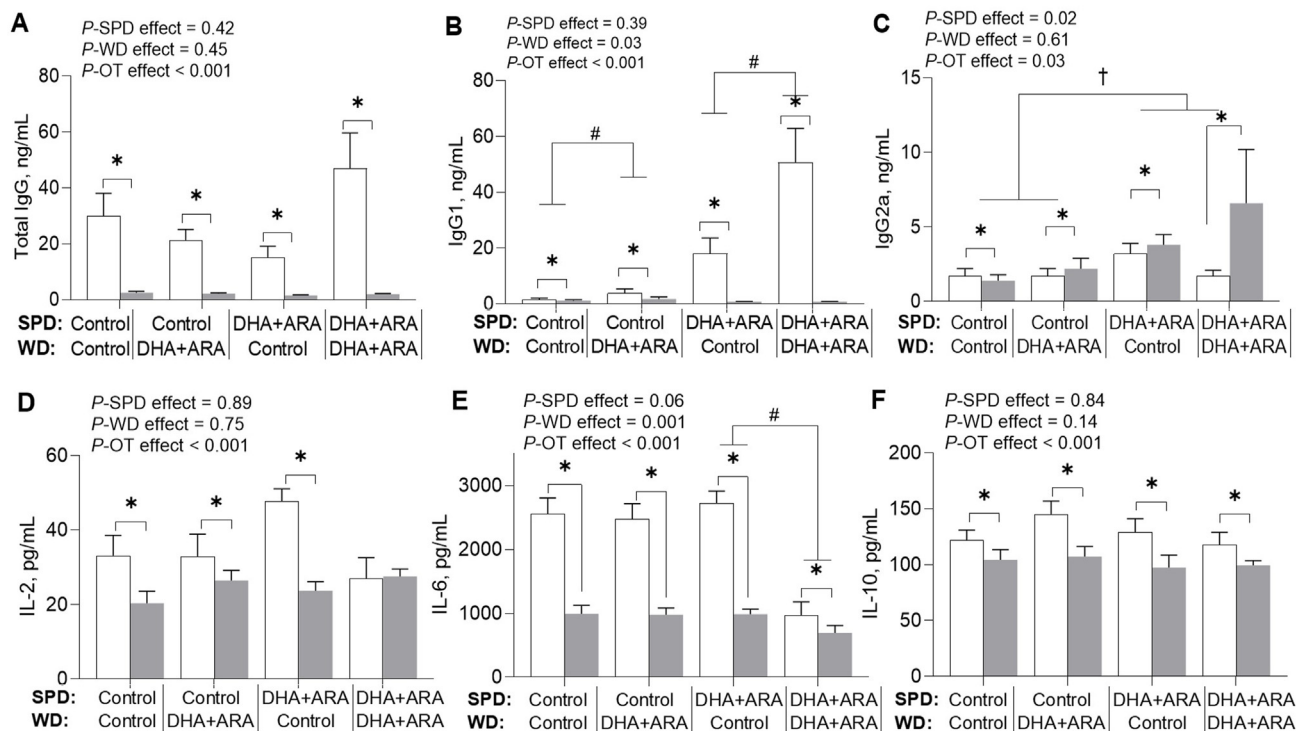


FIGURE 3. The SPD, WD, and OT effects on ex-vivo antibody and cytokine responses by splenocytes with different stimuli. Ex-vivo ova stimulated splenocyte concentrations of total IgG (A), IgG1 (B), and IgG2a (C). The production of IL-2 (D), IL-6 (E), and IL-10 (F) by splenocytes stimulated with ova. We reported a difference between a sucrose (white bars) and ova (gray bar) OT group (*) at $P < 0.05$. The difference between DHA+AA SPD and the control SPD group (†) is shown at $P < 0.05$. The difference between DHA+AA WD and the control WD group (#) is shown at $P < 0.05$. Values are presented as mean ± SEM. IgG, immunoglobulin G; IL, interleukin; OT, oral tolerance; ova, ovalbumin; SEM, standard error of the mean; SPD, suckling period diet; WD, weaning diet.

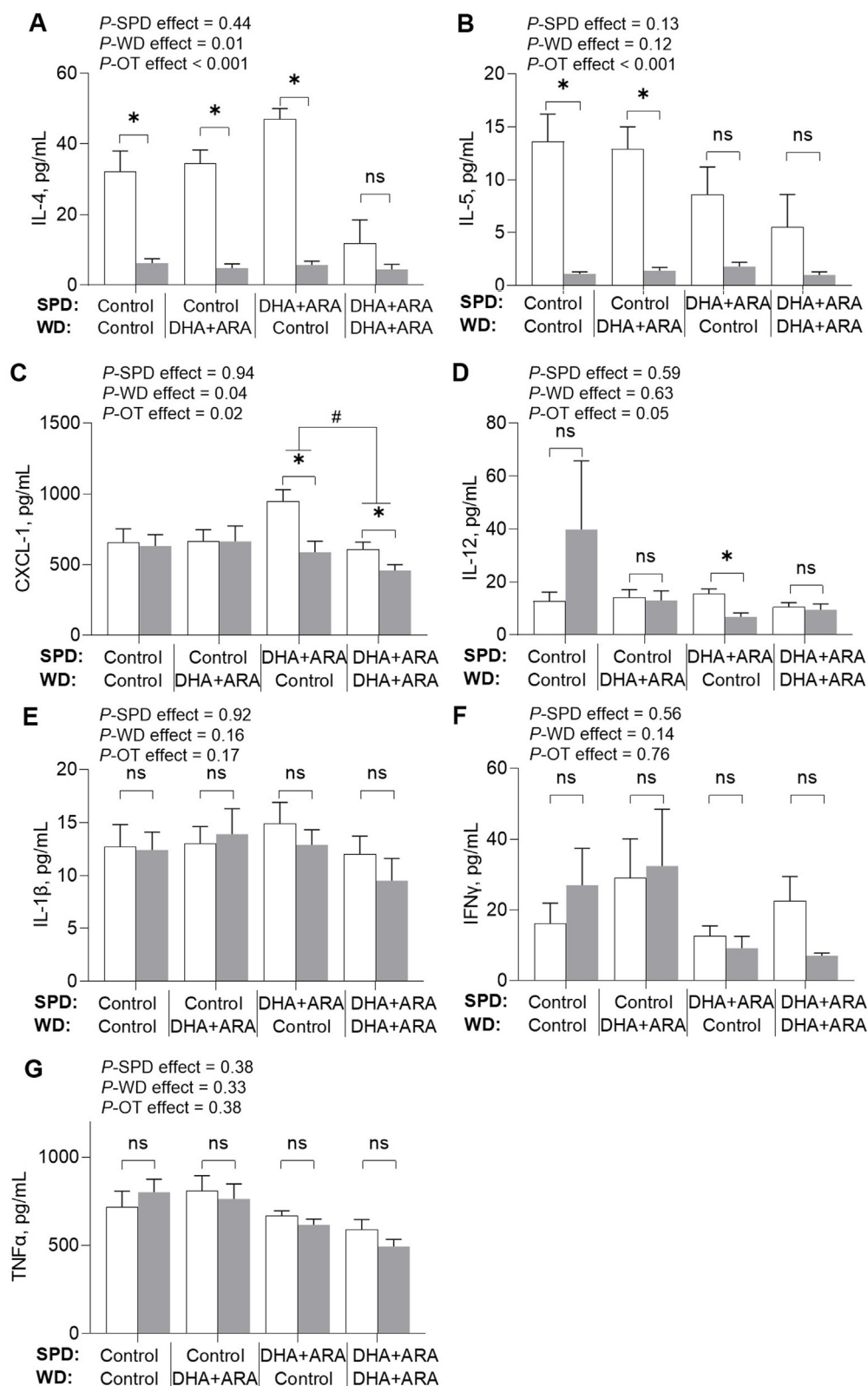


FIGURE 4. The SPD, WD, and OT effects on cytokine production by splenocytes stimulated with different stimuli. The production of IL-4 (A), IL-5 (B), CXCL-1 (C), IL-12 (D), IL-1 β (E), IFN γ , (F) and TNF α (G) by splenocytes stimulated with ova; Significant differences between sucrose (white bars) and ova (gray bar) OT group were reported (*) at $P < 0.05$. Significant differences between DHA+AA SPD and the control SPD group were reported (†) at $P < 0.05$. Significant differences between DHA+AA WD and the control WD group were reported (#) at $P < 0.05$. Values are presented as mean $\pm$ SEM. CXCL1, C-X-C motif ligand 1; IFN γ , interferon-gamma; IL, interleukin; ns, nonsignificant; OT, oral tolerance; ova, ovalbumin; SEM, standard error of the mean; SPD, suckling period diet; TNF α , tumor necrosis factor-alpha; WD, weaning diet.

concentrations (3.8 ± 0.9 compared with 1.8 ± 0.9 ; $P = 0.02$) in pups from DHA+AA SPD compared with control, whereas WD showed no significant effect (Figure 3C). Additionally, ova OT group pups showed higher IgG2a than the sucrose group (3.5 ± 1.0 compared with 2.1 ± 0.3 ; $P = 0.03$) (Figure 3C).

LPS stimulation

The ex-vivo production of total IgG and IgG1 by splenocytes after stimulation with LPS was significantly lower in ova-exposed pups compared to the sucrose group (both $P < 0.05$), irrespective of SPD or WD effect (shown in Supplemental Figure 8A, B, respectively). Note, the IgG1 also showed a significant SPD $\times$ WD $\times$ OT interaction ($P = 0.02$), but only relevant findings are shown in the figure. On the contrary, the IgG2a production with LPS stimulation showed no significant difference between OT group pups (4.2 ± 0.4 compared with 6.0 ± 1.7 , $P = 0.27$) (Supplemental Figure 8C).

PMAi stimulation

The ex-vivo production of total IgG and IgG1 by splenocytes after stimulation with PMAi was significantly lower in ova-exposed pups compared to the sucrose group (all $P < 0.001$) (shown in Supplemental Figure 8D, E). The ex-vivo production of IgG2a also showed a significant SPD effect where DHA+AA SPD-fed pups showed lower response compared to control SPD-fed pups (1.3 ± 0.2 compared with 3.0 ± 0.5 ; $P = 0.02$) (Supplemental Figure 8F). Additionally, ova OT group pups showed higher IgG2a concentrations than the sucrose group (Supplemental Figure 8F).

Ex-vivo cytokine production by splenocytes with different stimuli

Ova stimulation

The IL-2 production by ova-stimulated splenocyte showed a significant OT effect, where the ova-exposed OT group was lower than sucrose (24.5 ± 1.4 compared with 34.4 ± 2.9 ; $P < 0.001$) (shown in Figure 3D). Similarly, IL-6 production showed a significant OT effect, in which the ova OT group was lower than sucrose (912.9 ± 61.5 compared with 2165.5 ± 168 ; $P < 0.001$), irrespective of SPD or WD (Figure 3E). Additionally, a significant SPD $\times$ WD indicated that IL-6 production to ova stimulation ex-vivo was lower in pups from DHA+AA SPD + DHA+AA WD group compared to DHA+AA SPD + control WD (842.5 ± 126.1 compared with 1997 ± 282.2 ; $P = 0.03$) (Figure 3E). Finally, IL-10 production was also significantly lower in pups exposed to ova OT compared to the sucrose OT group (102.7 ± 4.1 compared with 128.4 ± 5.6 ; $P < 0.001$) irrespective of SPD or WD (Figure 3F).

The IL-4 production by splenocytes stimulated with ova showed a significant SPD $\times$ WD $\times$ OT interaction ($P = 0.03$, described in Figure 4A). The post hoc analysis indicated that the ova OT group was significantly lower than the sucrose OT group in all diet group combinations except DHA+AA SPD + DHA+AA WD. Thus, providing DHA+AA during SPD and WD lowered the IL-4 to ova stimulation, making the OT group difference nonsignificant (Figure 4A). The IL-5 production with ova stimulation showed a significant SPD $\times$ OT interaction effect, in which the ova OT group had lower IL-5 concentrations than the sucrose group but only when pups were fed control SPD (Figure 4B). This OT effect on IL-5 concentration was absent

when pups were fed DHA+AA SPD (Figure 4B). IL-1 β , IFN γ , and TNF α production by splenocytes stimulated with ova showed no significant differences because of SPD, WD, or OT (Figure 4E–G). However, CXCL-1 and IL-12 showed a significant SPD $\times$ OT effect in which ova-exposed OT group was lower for CXCL-1 and IL-12 than sucrose, but only when pups were fed DHA+AA SPD (Figure 4C, D). Note, the cytokine production by splenocytes in unstimulated conditions was found to be below detection in our preliminary analyses (data not shown).

LPS stimulation

For IL-4 and TGF β ex-vivo response to LPS stimulation, the ova OT group was lower than the sucrose OT group irrespective of SPD or WD intervention (Supplemental Figure 9A, B, respectively). A significant WD $\times$ OT interaction effect showed that IL-6 response to LPS was lower in the ova OT group than sucrose but only when pups were fed DHA+AA WD (3210.0 ± 388.8 compared with 4638.4 ± 505.5 ; $P = 0.006$, shown in Supplemental Figure 9C).

The LPS stimulation of splenocytes from pups from DHA+AA SPD elicited lower IFN γ , TNF α , CXCL-1 ($P = 0.07$), and TGF β compared to control SPD (Figure 5A–D). These SPD effects were observed independent of WD and OT intervention.

PMAi stimulation

The IL-2 production showed a marginal significant OT effect, in which the ova OT group was lower than sucrose (9169.2 ± 206.5 compared with 9312.6 ± 190.0 ; $P = 0.049$), irrespective of SPD or WD. The IFN γ production to PMAi stimulation showed a significant SPD $\times$ WD effect, but no post hoc differences were found. Further, the cytokines IL-10, IL-12, IL-1 β , IL-4, IL-5, IL-6, CXCL-1, and TNF α showed no significant differences because of SPD, WD, or OT intervention.

Anti-CD3/anti-CD28 stimulation

The ex-vivo production of IL-2 and IFN γ was significantly higher in pups fed DHA+AA SPD compared to control (12481.3 ± 119.0 compared with 11429.7 ± 42.5 ; $P < 0.001$ and 4036.1 ± 65.1 compared with 3007.7 ± 56.5 ; $P < 0.001$, respectively), irrespective of WD and OT interventions (Figure 5E, F). Similarly, IL-1 β was also significantly higher in the DHA+AA SPD group than control (12.1 ± 0.6 compared with 8.7 ± 0.4 ; $P < 0.001$) (Figure 5G). There was a significant interaction between SPD, WD, and OT, suggesting the difference in IL-1 β associated with SPD changes was dependent on WD and OT intervention (for specific differences, see Figure 5G). The stimulation of splenocytes with anti-CD3/anti-CD28 resulted in significantly lower IL-4 and IL-6 when pups were fed DHA+AA WD compared to control (363.0 ± 24.5 compared with 471.4 ± 33.5 ; $P = 0.01$ and 2615.9 ± 254.2 compared with 2956.2 ± 205.9 ; $P = 0.04$, respectively), irrespective of SPD or OT intervention (not shown in the figure). Finally, a significant OT effect on IL-5 and CXCL-1 has observed in which ova-exposed OT group pups were lower than sucrose OT group pups, respectively 162.4 ± 17.2 compared with 198.0 ± 10.2 ; $P = 0.02$ and 88.7 ± 7.9 compared with 129.0 ± 13.8 ; $P = 0.03$ (Figure 5H, I, respectively). These OT effects were observed independent of SPD or WD.

Overall, the effects of supplementing SPD and WD on ex-vivo cytokine production by splenocytes to different stimuli are provided in Supplemental Table 2.

Immune cell phenotype of unstimulated splenocytes

In summary, [Table 1](#) provides phenotype differences associated with SPD (irrespective of WD or OT), [Table 2](#) provides phenotype differences associated with SPD $\times$ OT interaction, and [Table 3](#) provides phenotype differences associated with WD (irrespective of SPD or OT). Overall, some changes in the relative proportion of immune cells were observed between different SPD, WD, and OT groups. These changes are described below. In splenocytes, the proportion of total CD3⁺ cells, CD3⁺ CD4⁺ (Th) cells, and CD3⁺ CD8⁺ (cytotoxic T) cells did not differ significantly between SPD group pups. However, some specific T cell phenotypes were found to be significantly lower in splenocytes from pups fed DHA+AA SPD compared to control SPD. These phenotypes include CD3⁺ CD45R⁺ (naïve T cells), CD3⁺ CD4⁺ CD25⁺ (CD25⁺ Th cells), CD3⁺ CD4⁺ CD152⁺ (cytotoxic T-lymphocyte associated protein-4 Th cells), and CD3⁺ CD8⁺ CD152⁺ (cytotoxic T-lymphocyte associated protein-4 cytotoxic T cell) ([Table 1](#)). A 3-way SPD $\times$ WD $\times$ OT interaction was observed for some T cell phenotypes (see [Figure 6](#)). The post hoc analysis showed that when pups did not receive DHA+AA in SPD and/or WD, the ova OT group had significantly higher total CD3⁺, CD3⁺ CD45RB⁺, CD3⁺ CD4⁺ CD27⁺, and CD3⁺ CD8⁺ CD27⁺ proportion in splenocytes than sucrose OT group pups ([Figure 6A–D](#)). In other words, when pups received DHA+AA during SPD and/or WD, these differences between OT groups were absent.

The splenocyte of pups from DHA+AA SPD groups was associated with a lower proportion of CD11c⁺ cells (total DC and some macrophage) and CD3⁺ CD49b⁺ CD27⁺ (CD27⁺ NK cells) compared to control SPD (both $P < 0.05$) ([Table 1](#)). Additionally, a significant SPD $\times$ OT effect was also observed for CD11c⁺ CD8a⁺ CD4⁺ [enriched for conventional DC type-1 (cDC1)] cell proportion, in which the ova group was significantly higher than sucrose OT group but only when pups were fed control SPD (1.1 ± 0.06 compared with 0.9 ± 0.05 ; $P = 0.002$) ([Table 2](#)).

Some phenotypic changes were also observed for the B cell in splenocytes. The proportion of IgM⁺ on CD45R⁺ CD19⁺ CD138⁺ (IgM plasma cells) was found to be significantly lower in pups fed DHA+AA SPD compared with control SPD ([Table 1](#)). The DHA+AA WD group pups' splenocytes showed a higher proportion of CD19⁺ IgM⁺ (primary B cell) than control WD, irrespective of SPD and OT ([Table 3](#)). The CD19⁺ IgG⁺ (secondary B cell) proportion showed no significant difference because of the WD intervention. Overall, the effects of supplementing SPD and WD on the phenotype of splenocytes without stimulation are provided in [Supplemental Table 3](#).

Immune cell phenotype of LPS-stimulated splenocytes

Stimulating splenocytes with LPS can activate antigen-presenting cells (macrophages, DC, and B cells) through toll-like receptor-4 (identified by CD284). Thus, we have reported phenotypic changes in the splenocytes associated with these cell types (see [Table 1](#)). In the splenocytes stimulated with LPS, the proportion of CD3⁺ CD49b⁺ (NKT cells), CD3⁺ CD49b⁺ CD27⁺ (CD27⁺ NKT cells) was significantly lower in DHA+AA SPD compared to control SPD. However, the proportion of CD3⁺ CD49b⁺ CD335⁺ (CD335⁺ NKT cells) splenocytes after LPS stimulation was higher in the DHA+AA SPD group compared to

control SPD ([Table 1](#)). The CD3⁺ CD49b⁺ (NK cells) showed no difference because of the SPD, WD, or OT intervention. The proportion of total CD11c⁺ cells (DC), CD11b⁺ CD68⁺ (macrophages), and CD11b⁺ CD68⁺ CD163⁺ (M2 macrophage) in LPS-stimulated splenocytes were significantly lower in pups from DHA+AA SPD group compared to control SPD, irrespective of WD and OT interventions ([Table 1](#)). The proportion of CD11b⁺ MHC-II⁺ cells was found to be higher with DHA+AA SPD compared with control SPD (45.9 ± 1.9 compared with 31.8 ± 1.6 ; $P = 0.001$).

The splenocyte stimulation with LPS resulted in significant changes in the phenotype of B cells because of SPD and WD. First, the DHA+AA SPD resulted in higher CD19⁺ CD23⁺ (follicular B cells) and CD19⁺ CD45R⁺ CD138⁺ (enriched for plasma cells) compared to control SPD ([Table 1](#)). Next, pups from DHA+AA WD showed that the splenocytes after LPS stimulation resulted in a significantly higher proportion of CD19⁺ IgG⁺ (secondary B cells) than pups from control WD ([Table 3](#)). Furthermore, LPS stimulation of splenocytes also showed a higher proportion of total MHC-II cells and CD19⁺ MHC-II⁺ in DHA+AA WD group pups compared to control WD ([Table 3](#)). Overall, the effects of supplementing SPD and WD on the phenotype of splenocytes with LPS stimulation are provided in [Supplemental Table 4](#).

Immune cell phenotype of PMAi stimulated splenocytes

The SPD significantly affected the phenotype of splenocytes after stimulation with PMAi. Only the relevant changes are described here. A significant SPD $\times$ OT interaction was observed in the proportion of total CD3⁺, CD3⁺ CD4⁺ CD25⁺, and CD3⁺ CD8⁺ CD25⁺ splenocytes were found to be significantly lower in pups fed DHA+AA SPD and ova OT group compared to the control SPD and ova OT group ([Table 2](#)). The splenocyte proportion of CD196⁺ CD194⁺ on CD3⁺ CD4⁺ (enriched for Th2) was lower in the DHA+AA SPD group compared to control SPD (5.5 ± 0.4 compared with 12.4 ± 0.7 ; $P < 0.001$) ([Table 2](#)).

In the pups from the DHA+AA SPD group, we observed a significantly lower proportion of CD3⁺ CD49b⁺ (NKT cell) and CD3⁺ CD49b⁺ CD27⁺ (CD27⁺ NKT cell) compared to control SPD ([Table 2](#)). However, the proportion of CD3⁺ CD49b⁺ CD335⁺ (CD335⁺ NKT cells; 0.86 ± 0.05 compared with 0.55 ± 0.03) splenocytes after PMAi stimulation was higher in the DHA+AA SPD group compared to control SPD (0.55 ± 0.03 compared with 0.86 ± 0.05 ; $P = 0.02$). The WD and OT intervention did not change these phenotypes. The proportion of CD3⁺ CD4⁺ CD28⁺ splenocytes was found to be significantly higher in the ova OT group than sucrose, irrespective of SPD or WD (14.3 ± 1.1 compared with 12.8 ± 1.1 ; $P = 0.028$). Whereas the splenocytes from ova OT group pups' splenocytes after PMAi stimulation resulted in a significantly lower proportion of CD3⁺ CD152⁺ (3.89 ± 0.01 compared with 4.12 ± 0.01 ; $P = 0.003$) and CD152⁺ on CD3⁺ CD4⁺ (8.8 ± 0.6 compared with 9.8 ± 0.8 ; $P = 0.021$). The DHA+AA SPD group showed significantly lower CD19⁺ IgM⁺ (primary B cell, 12.3 ± 0.4 compared with 20.2 ± 0.8 ; $P = 0.001$) compared to the control SPD group pups splenocytes after PMAi stimulation ([Table 2](#)). Lastly, pups from the DHA+AA WD group showed higher CD19⁺ CD23⁺ (enriched for follicular B cell, 60.6 ± 1.2 compared with 55.9 ± 1.5 ; $P = 0.006$) and lower proportion of CD19⁺ CD23⁺ (enriched

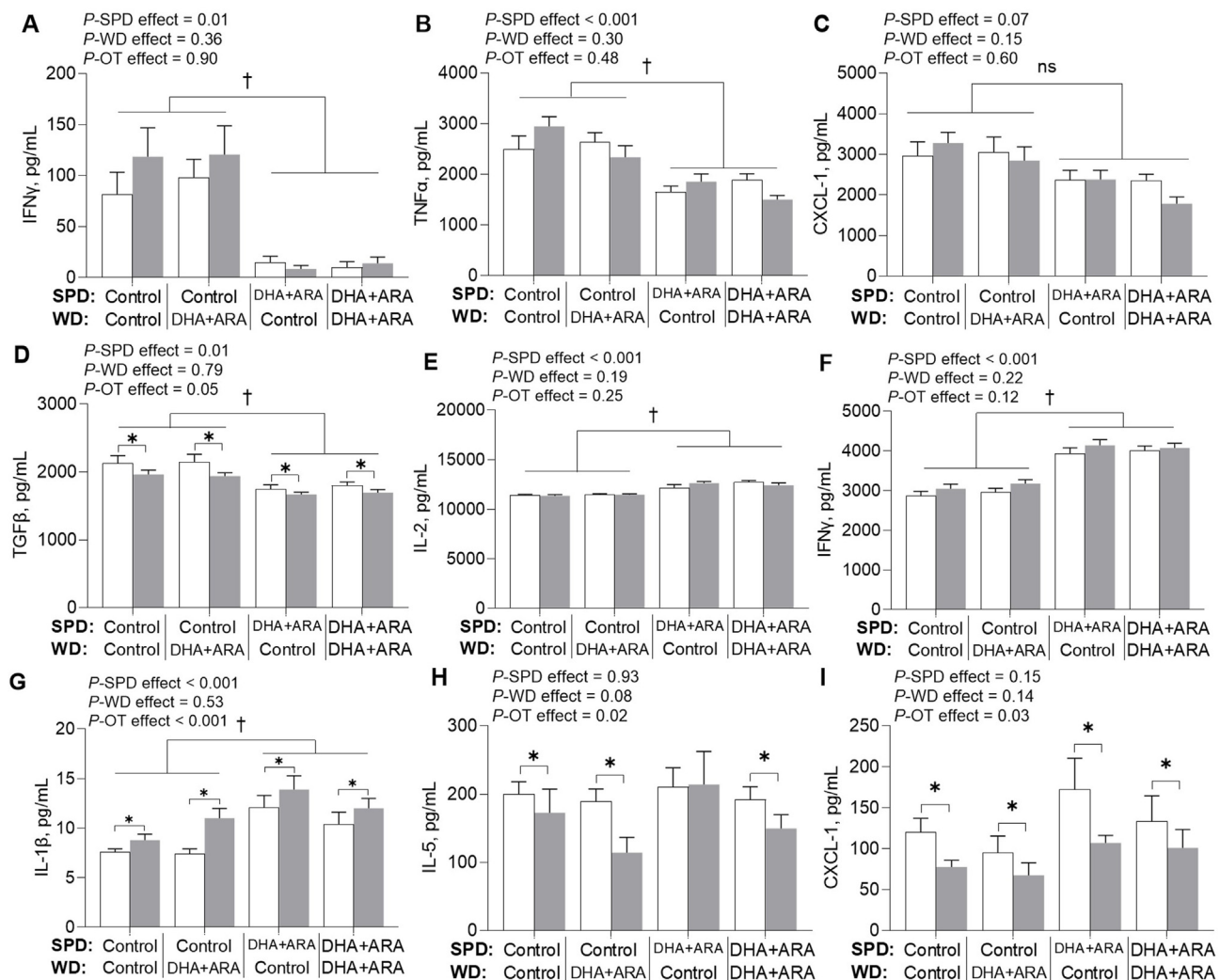


FIGURE 5. The SPD, WD, and OT effects on ex-vivo cytokine response by splenocytes with different stimuli. The production of IFN γ (A), TNF α (B), CXCL-1 (C), and TGF β (D) by splenocytes stimulated with LPS, and IL-2 (E), IFN γ (F), IL-1 β (G), IL-5 (H) and CXCL-1 (I) by splenocytes stimulated with anti-CD3/anti-CD28. The statistical analysis was conducted using 3-factor ANOVA. Significant differences between sucrose (white bars) OT and ova (gray bar) OT group were reported (*) at $P < 0.05$. Significant difference between DHA+AA SPD and the control SPD group was reported (†) at $P < 0.05$. CXCL1, C-X-C motif ligand 1; IFN γ , interferon-gamma; IL, interleukin; ns, nonsignificant; OT, oral tolerance; ova, ovalbumin; SPD, suckling period diet; TGF β , transforming growth factor-beta; TNF α , tumor necrosis factor-alpha; WD, weaning diet.

for marginal B cell, 39.4 ± 1.2 compared with 44.1 ± 1.5 ; $P = 0.008$) (Table 3). Similarly, CD19+ MHC-II+ was significantly higher in pups from DHA+AA WD groups (49.1 ± 0.7 compared with 46.1 ± 0.8 ; $P = 0.05$) compared to control WD. Overall, the effects of supplementing SPD and WD on the phenotype of splenocytes with PMAi stimulation are provided in Supplemental Table 5.

Discussion

We evaluated the effect of plant-based DHA from novel canola oil, along with the same amount of AA, on the immune system and OT development in Th2-skewed BALB/c mouse pups (summarized in Supplemental Table 6). As expected, the DHA composition of splenocytes was higher in the DHA+AA group compared to the control (5.8 ± 0.2 compared with 3.5 ± 0.2 ; $P < 0.001$), whereas the AA composition showed no significant difference (13.9 ± 0.3 compared with 12.3 ± 0.5 ; $P = 0.22$).

OT development and immune responses associated with ova stimulation in DHA+AA fed 6-wk pups

We investigated the DHA+AA supplementation effect on OT development in 6-wk pups through repetitive exposure to orally fed low-dose of ova between 21–25 d. The in-vivo response toward ova did not show a significant OT effect based on the plasma concentrations of ova-IgG and ova-IgE in a 6-wk BALB/c mouse (Figure 2). Although diet did not show a significant change in the in-vivo OT outcomes (plasma ova-IgE or ova-IgG), there was a significant reduction in in-vivo ova-IgE plasma concentrations associated with DHA+AA SPD compared to control [39,40]. These findings differ from previous studies from our group, where we showed LCPUFAs induced in-vivo suppression of plasma ova-specific-Ig's response [26,41]. Nevertheless, the splenocytes showed ex-vivo suppression of immune responses toward ova (Figure 3). For instance, in ova-tolerized pups, ova-stimulated splenocytes resulted in a nearly 10-fold reduction in IgG1 (associated with Th2 response) [42]

TABLE 1

The effect of suckling period diet on immune cell phenotype of splenocytes with or without stimulation

Suckling period diet		Control diet	DHA+AA diet	P value
Phenotype, % splenocyte	Cell types ¹			
Unstimulated condition				
CD3+ CD45RB+	Naïve T cells	23.6 ± 0.8	20.4 ± 0.6	0.003
CD25+ CD3+ CD4+	CD25+ Th	3.8 ± 0.2	3.3 ± 0.12	0.159
CD152+ CD3+ CD4+	CTLA-4+ Th	2.3 ± 0.1	1.0 ± 0.1	0.03
CD152+ CD3+ CD8+	CTLA-4+ CTL	0.6 ± 0.03	0.3 ± 0.02	0.002
CD3+ CD4+ CD183+ CD194-	Th1 cells	5.8 ± 0.2	5.0 ± 0.2	0.23
Total CD19+	Total B cells	52.3 ± 0.9	53.4 ± 0.5	0.59
CD19+ CD23+	Follicular B cells	37.2 ± 0.9	38.1 ± 0.6	0.65
CD19- CD45R+ CD138+	Plasma total cells	3.5 ± 0.3	2.0 ± 0.1	0.21
IgM+ on CD19- CD45R+ CD138+	IgM+ plasma cells	60.4 ± 1.8	40.5 ± 1.6	0.001
CD3+ CD49b+	NKT cells	6.1 ± 0.6	4.6 ± 0.6	0.43
CD3+ CD49b+ CD27+	CD27+ NKT cells	4.9 ± 0.2	3.5 ± 0.1	0.18
CD3- CD49b+	NK cells	10.7 ± 0.5	8.5 ± 0.3	0.19
CD3- CD49b+ CD27+	CD27+ NK cells	3.5 ± 0.1	2.6 ± 0.1	0.007
Total CD11c+	DC	16.0 ± 0.5	10.7 ± 0.3	0.002
CD11b+ CD68+	Macrophages	12.9 ± 0.5	9.1 ± 0.2	0.02
CD11b+ CD68+ CD163+	M2 type macrophage	3.9 ± 0.2	3.4 ± 0.2	0.87
LPS stimulation				
Total CD19+	Total B cells	52.9 ± 0.8	55.6 ± 0.8	0.96
CD19+ CD23+	Follicular B cells	29.8 ± 0.8	35.2 ± 0.9	0.01
CD19- CD45R+ CD138+	Plasma total cells	4.9 ± 0.2	7.2 ± 0.3	0.006
IgM+ on CD19- CD45R+ CD138+	IgM+ plasma cells	49.7 ± 1.6	47.1 ± 1.2	0.57
CD3+ CD49b+	NKT cells	12.7 ± 0.01	6.6 ± 0.01	0.005
CD3+ CD49b+ CD27+	CD27+ NKT cells	10.2 ± 0.5	4.1 ± 0.1	0.003
CD3+ CD49b+ CD335+	CD335+ on NKT cells	2.9 ± 0.2	4.2 ± 0.3	0.03
CD3- CD49b+	NK cells	9.4 ± 0.1	8.9 ± 0.1	0.45
Total CD11c+	DC	15.8 ± 0.6	9.2 ± 0.2	0.009
CD11b+ CD68+	Macrophages	12.5 ± 0.5	8.4 ± 0.3	0.02
CD11b+ CD68+ CD163+	M2 type macrophage	10.1 ± 0.5	6.5 ± 0.2	0.007

CTL, cytotoxic T cell; CTLA-4, cytotoxic T-lymphocyte associated protein-4; DC, dendritic cells; IgM, immunoglobulin-A; LPS, lipopolysaccharide; M2; NK, natural killer cells; NKT, natural killer T cells; Th, T helper cell.

¹ The combinations of surface markers are suggestive of described specific immune cell subtypes. Note, alternative combinations of markers are also possible.

production compared to non-tolerized pups. Whereas, Th1 type response showed higher production of IgG2a by pups from the ova OT group compared to sucrose-exposed pups. Together, a lower Th2 and higher Th1 response toward ova suggest that the OT induction in Th2 skewed BALB/c model may involve Th2-specific suppression [43–45]. OT-induced suppression of immune response was also observed in ex-vivo cytokine production by ova-stimulated splenocytes. Ova-tolerized pups showed lower ex-vivo IL-2 (a surrogate proliferation marker), IL-6, and IL-10 production than the sucrose OT group (Figure 3). IL-2 acts as a growth factor and promotes lymphocyte proliferation through CD25 (IL-2 receptor), whereas IL-10 and IL-6 are involved in antibody production by B cells [46]. The reduction of IL-2 showed no significant change in the splenocyte proportion of T cells or B cells. However, a lower IL-6 and IL-10 may have contributed toward lower B cell IgG1 production by ova-tolerized pups. Since elevated IL-6 is observed in food hypersensitivity, this finding may support OT development [47]. Previous reports using allergy-prone Brown Norway (Th2-biased) model supported a lower IL-6 production by splenocytes stimulated with ova in tolerized pups (ova exposed) [26]; however, similar findings were not seen with Sprague Dawley (Th1-biased) rat offspring [25]. Other studies with allergy-prone mouse models also observed a reduction in the expression of IL-6, and the elevation of IL-10 was associated with OT [48].

The anti-inflammatory properties of DHA and immunosuppression associated with OT are known to suppress inflammatory cytokine response [49]. However, the current study showed no differences in IL-1 β , IFN γ , and TNF α production by splenocytes stimulated with ova from ova-tolerized groups or DHA+AA supplemented group. Thus, suggesting the development of OT led to lower Th2 (IgG1, IL-6, and IL-10) responses without affecting inflammatory cytokine responses to ova stimulation.

Programming effect of suckling period DHA+AA supplementation on T cell and innate immune cell responses

Early life nutrition can have a programming effect on the immune system of infants, particularly during the suckling period when infants consume their mother's breast milk [50]. This period also corresponds with developing a robust adaptive immune system (B and T cells) and switching the Th2 dominant infant immune response to more balanced Th1/Th2 responses [51]. In the current study, providing DHA+AA in SPD showed a significant increase in pro-inflammatory cytokines (IL-2, IL-1 β , and IFN γ) without affecting IL-4, IL-5, IL-6, and IL-10 cytokine response by anti-CD3/anti-CD28 stimulated splenocytes. These outcomes were observed despite a small decrease in the proportion of CD25+ Th cells involved in pro-inflammatory response (characterizes activation phenotype) associated with

TABLE 2

The suckling period diet and oral tolerance interaction effect on immune cell phenotype of splenocyte with or without stimulation

Suckling period diet		Control diet	Control diet	DHA+AA diet	DHA+AA diet	SPD × OT
OT group		Sucrose	Ova	Sucrose	Ova	P value
Phenotype, % splenocytes	Cell type ¹					
Unstimulated						
CD3+ CD49b+	NKT cells	5.8 ± 0.6	6.0 ± 0.6	4.6 ± 0.6	4.7 ± 0.6	0.83
CD3+ CD49b+ CD27+	CD27+ NKT	4.8 ± 0.3	5.1 ± 0.3	3.4 ± 0.1	3.5 ± 0.2	0.46
CD3- CD49b+	NK cells	10.8 ± 0.01	10.5 ± 0.01	8.4 ± 0.03	8.5 ± 0.03	0.62
CD3- CD49b+ CD27+	CD27+ NK cells	3.6 ± 0.2	3.5 ± 0.2	2.7 ± 0.1	2.6 ± 0.1	0.79
Total CD11c+	DC	16.2 ± 0.7	15.8 ± 0.6	10.7 ± 0.4	10.7 ± 0.4	0.94
CD11c+ CD4+ MHC-II+	pDC	5.4 ± 0.3	5.7 ± 0.3	3.5 ± 0.1	3.4 ± 0.1	0.22
CD11c+ CD8a+ CD4- ²	cDC1	0.9 ± 0.05 b	1.1 ± 0.06 a	0.6 ± 0.04 c	0.5 ± 0.04 c	0.002
CD11b+ CD68+	Macrophage	13.2 ± 1.1	12.2 ± 1.1	8.7 ± 1.1	9.7 ± 1.1	0.07
PMAi stimulation						
Total CD3+	Total T cells	35.4 ± 1.5 b	31.8 ± 1.5 a	23.2 ± 1.6 a	25.3 ± 1.6 a	0.006
CD3+ CD49b+	NKT cells	8.2 ± 1.1 †	10.3 ± 1.1 †	6.6 ± 1.1	5.9 ± 1.1	0.007
CD3+ CD49b+ CD27+	CD27+ NKT	9.65 ± 0.79 †	8.7 ± 0.66 †	4.71 ± 0.19	4.31 ± 0.15	0.33
CD3- CD49b+	NK cells	11.9 ± 0.01	10.5 ± 0.01	9.1 ± 0.01	9.2 ± 0.01	0.20
CD3- CD49b+ CD27+	CD27+ NK cells	4.2 ± 0.2	3.5 ± 0.2	3.6 ± 0.2	3.5 ± 0.2	0.14
CD3+ CD4+ CD183+ CD196-	Th1	4.3 ± 0.5	3.6 ± 0.5	2.2 ± 0.28	2.6 ± 0.2	0.02
CD3+ CD4+ CD194+ CD196-	Th2	13.3 ± 1.1 †	11.5 ± 0.8 †	6.1 ± 0.6	4.9 ± 0.5	0.15
CD3+ CD4+ CD25+	CD25+ Th	6.9 ± 0.9 b	9.9 ± 0.9 a	4.4 ± 0.6 b	4.2 ± 0.5 b	0.04
CD3+ CD8+ CD25+	CD25+ CTL	1.8 ± 0.2 b	2.7 ± 0.2 a	1.2 ± 0.1 b	1.1 ± 0.2 b	0.01
Total CD19+	Total B cells	45.6 ± 2.2	48.4 ± 2.4	40.0 ± 1.3	38.5 ± 1.1	0.63
CD19+ CD23+	Follicular B cells	27.3 ± 5.3	30.2 ± 5.2	21.9 ± 5.4	20.9 ± 5.4	0.04
CD19+ CD23-	Marginal B cells	19.4 ± 0	17.5 ± 0.8	16.2 ± 0.8	16.8 ± 0.8	0.04
CD19+ IgM+	IgM+ B cells	20.1 ± 1.2 †	20.3 ± 1.1 †	12.3 ± 0.6	12.4 ± 0.5	0.59
CD19+ IgG+	IgG+ B cells	26.1 ± 1.3	29.6 ± 1.7	27.9 ± 1.3	27.1 ± 1.2	0.30

cDC1, conventional DC type-1; CTL, cytotoxic T-lymphocyte; NKT, natural killer T cells; OT, oral tolerance; ova, ovalbumin; pDC, plasmacytoid DC; PMAi, phorbol-myristate-acetate and ionomycin; SPD, suckling period diet; Th1, T helper type-1; Th2, T helper type-2.

¹ The combinations of surface markers are suggestive of described specific immune cell subtypes. Note, alternative combinations of markers are also possible.

² In a row, means with uncommon letters or symbols are significantly different according to post hoc analysis with multiple groups comparison-adjusted.

DHA+AA SPD. Therefore, this can be considered a more efficient T cell response [52,53].

Additionally, providing DHA+AA during the suckling period may support a reduction in pro-inflammatory responses by

splenocytes upon stimulation with LPS, a bacterial component that can activate innate immune cells [54,55]. DHA+AA during the suckling period led to lower production of IFN γ , TNF α , IL-6, and CXCL-1 toward LPS compared to control group pups. This

TABLE 3

The effect of weaning diet on immune cell ex phenotype of splenocyte with or without stimulation

Weaning diet		Control diet	DHA+AA diet	P value
Phenotype, % splenocytes	Cell type ¹			
Unstimulated				
Total MHC-II+	I-A ^d /I-E ^d APC	49.9 ± 0.7	52.0 ± 0.6	0.06
Total CD19+	Total B cells	52.3 ± 0.8	53.4 ± 0.6	0.09
CD19+ MHC-II+	I-A ^d /I-E ^d B cells	47.5 ± 0.9	47.7 ± 0.8	0.94
CD19+ IgM+	Primary B cells	22.0 ± 0.7	24.2 ± 0.5	0.01
CD19+ IgG+	Secondary B cells	41.2 ± 1.0	42.4 ± 0.7	0.27
CD19+ CD23+	Follicular B cells	36.6 ± 0.8	38.7 ± 0.6	0.11
CD19+ CD23-	Marginal B cells	15.7 ± 0.8	14.6 ± 0.6	0.12
LPS stimulation				
Total MHC-II+	I-A ^d /I-E ^d APC	53.3 ± 2.6	60.3 ± 2.7	0.01
Total CD19+	Total B cells	53.7 ± 0.9	54.9 ± 0.8	0.87
CD19+ MHC-II+	I-A ^d /I-E ^d B cells	42.7 ± 2.8	45.5 ± 2.8	0.04
CD19+ IgM+	Primary B cells	23.6 ± 0.7	22.4 ± 0.9	0.07
CD19+ IgG+	Secondary B cells	38.4 ± 1.1	41.0 ± 1.0	0.03
CD19+ CD23+	Follicular B cells	29.2 ± 3.5	32.8 ± 3.6	0.05
CD19+ CD23-	Marginal B cells	22.4 ± 0.9	20.3 ± 0.9	0.35

APC, antigen presenting cells; OT, oral tolerance; ova, ovalbumin.

¹ The combinations of surface markers are suggestive of described specific immune cell subtypes. Note, alternative combinations of markers are also possible.

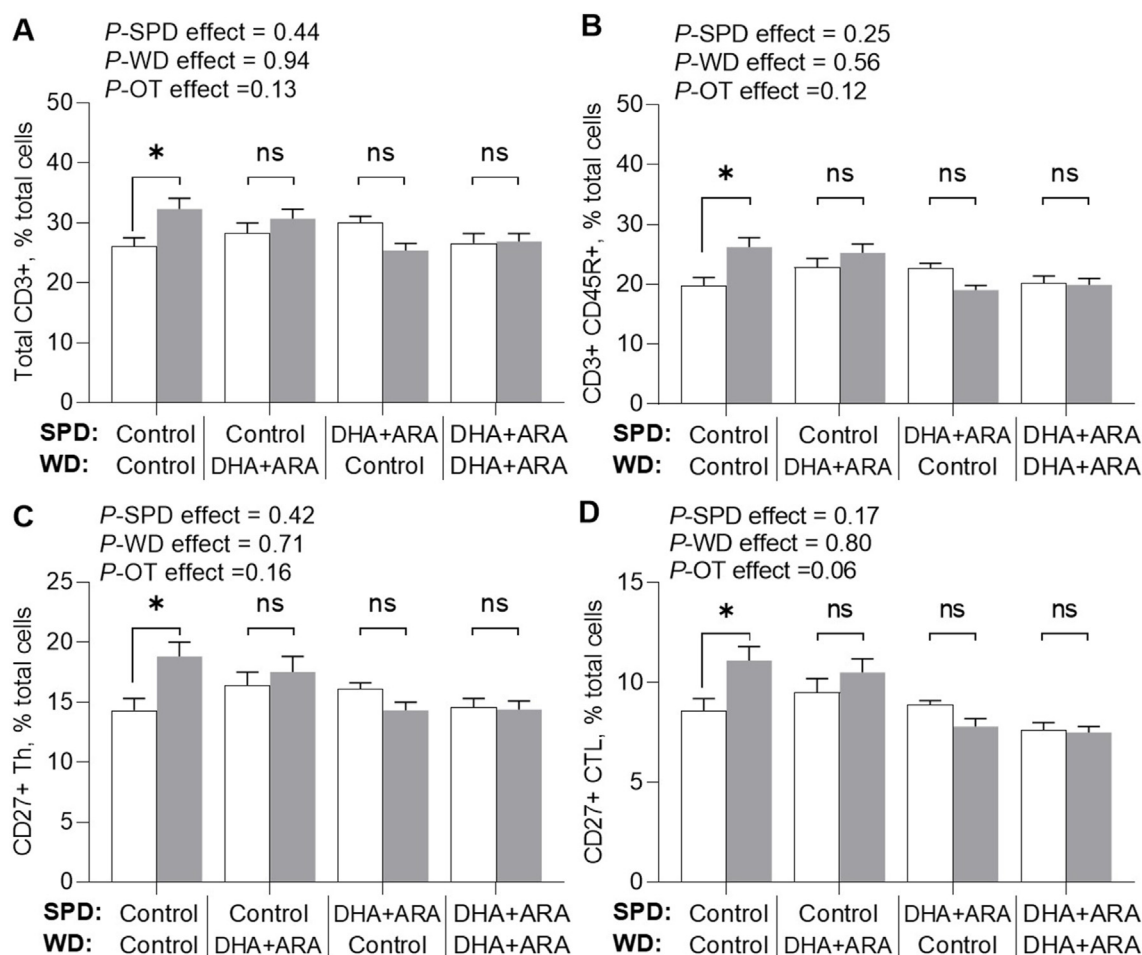


FIGURE 6. The SPD $\times$ WD $\times$ OT interaction effect on the T cell phenotypes in unstimulated splenocytes, total CD3+ (A), CD3+ CD45R+ (B), CD3+ CD4+ CD27+ (C), and CD3+ CD8+ CD27+ (D). The statistical analysis was conducted using 3-factor ANOVA. Significant differences between sucrose (white bars) OT and ova (gray bar) OT group were reported (*) at $P < 0.05$. Values are presented as means $\pm$ SEMs. CTL, cytotoxic T cell; ns, nonsignificant; OT, oral tolerance; ova, ovalbumin; SEM, standard error of the mean; SPD, suckling period diet; SPD, suckling period diet; Th, T helper; WD, weaning diet.

may be because of the lower proportion of CD11b+ CD68+ macrophages, CD11c+ DC, and CD3- CD49b+ CD27+ NK cells. Immune cells with toll-like receptor-4 (receptor for LPS), macrophages, DC, NK cells, and some B cells can be activated with LPS stimulation. However, macrophages are the key producers of inflammatory cytokines in the spleen, and their polarization toward M1 or M2 phenotype can modulate their function [56]. In the presence of Th1 cytokines (IFN γ and TNF α), the naïve macrophages polarize toward the M1 phenotype, which is involved in producing strong inflammatory responses and recruitment of cytotoxicity-producing NK cells and Th1 cells [56]. Furthermore, the phenotype of LPS-stimulated splenocytes also showed some changes in DHA+AA SPD that may contribute to the suppression of pro-inflammatory response. This includes a lower proportion of CD3+ CD49b+ NKT cells, CD27+ NKT cells, and CD163+ macrophage (associated with M1 phenotype) in LPS-stimulated splenocytes with DHA+AA SPD supplementation. Similar anti-inflammatory properties of DHA (and n-3 PUFA) have also been observed in past studies from our group and others using allergy-prone and healthy models [25,26,40,57–59].

DHA+AA in the weaning period modulated B cell phenotype and induced lower Th2 cytokines

The DHA+AA supplementation of WD resulted in lower IL-6 production by splenocytes upon stimulation with ova, anti-CD3/anti-CD28, and LPS (only in ova-tolerized OT group). Additionally, providing DHA+AA during suckling, followed by DHA+AA in WD, results in lower IL-4 by ova-stimulated splenocytes from ova-exposed pups compared to other diet combinations (Figure 4A). DHA+AA WD-associated decrease in IL-6 production toward ova may be beneficial for OT development since elevated IL-6 has been observed in atopic conditions, including food allergies [46]. Food, allergen-specific IgE production, is induced by IL-4 and IL-13 and can be enhanced by IL-6 resulting in mast cell activation and anaphylactic response [15]. Therefore, DHA+AA supplementation in the weaning period may induce changes in cytokine production toward ova stimulation that can be considered beneficial for OT response. Past studies from our laboratory suggested the beneficial effect of supplementation on OT (lower ova-specific Ig) may be because of the decrease in IL-6 and increase in IL-10 associated with DHA [25,26,49]. The current study found no differences in the OT

outcomes (in-vivo and ex-vivo) because of the WD supplementation, although there were significant phenotypic changes in B cells from splenocytes. In the DHA+AA WD group pups, the proportion of LPS-stimulated splenocytes showed higher CD19+ CD23+ follicular B cells and CD19+ IgG+ B cells than control [60]. Note, the total proportion of CD19+ B cells was not different in unstimulated or LPS-stimulated splenocytes between WD groups. Additionally, the proportion of CD19+ MHC-II+ B cells was also higher in splenocytes stimulated with LPS or PMAI. The expression of MHC-II on B cells may be important for providing a costimulatory signal, akin to antigen-presenting cells, in the activation of CD4+ T cells, which in turn promotes B cells' Ig production function [61]. Although we did not observe any Ig functional changes associated with DHA+AA supplementation of WD, the higher proportion of B cells in 6-wk pups can be associated with the maturation of adaptive immunity.

The study has some limitations. The current study used a previously developed and validated protocol of OT induction in Brown Norway and Sprague Dawley rat models. This may have resulted in a nonsignificant difference in in-vivo OT outcomes. Therefore, future studies should validate ova tolerization and immunization protocols to measure the significance of the in-vivo responses. Nevertheless, we induced OT toward ova (suppressing ex-vivo antibody response with ova stimulation). Secondly, the use of multiple surface mAb in combination to identify specific immune cell subtypes is common in immunology literature. However, we recognize the limitation of using surface mAb in identifying immune cell types and suggest the use of functional changes to support immune cell phenotype data. For instance, the current study uses CD183+ markers on Th cells and CD194+ CD196- on Th cells to identify Th1 and Th2 cells. This should be supported by Th1 and Th2-specific cytokines, such as IFN γ and IL-4, respectively. Additionally, the use of transcription factors can also provide further evidence to support the changes observed in immune cell phenotype. The development of OT is initiated in the gut; therefore, future studies should evaluate immune cells from mesenteric lymph nodes and Peyer's patches. Last, the current study explored the functional and phenotypic changes in the immune system and did not study the mechanism of actions involving OT or immune system development. Future studies should explore the mechanism of action behind the observed changes associated with DHA+AA supplementation.

In conclusion, this study provides evidence supporting the beneficial effects of DHA (from novel canola oil), alongside AA, on immune system development using Th2 dominant BALB/c mouse. The development of OT toward ova suggests specific suppression of IgG responses toward ex-vivo ova stimulation. Supplementing DHA+AA during suckling may support in-vivo OT outcomes (lower plasma ova-IgE concentrations). The programming effect of DHA+AA in the suckling period resulted in better T cell response (IL-2 and IFN γ to anti-CD3/anti-CD28 stimulation) to enhance select Th1-specific responses. Furthermore, DHA+AA SPD promoted a lower inflammatory response toward LPS stimulation, supporting the anti-inflammatory properties of DHA. Lastly, providing DHA+AA during the weaning period is associated with lower Th2 response (IL-4 and IL-6) toward ova stimulation and a higher proportion of B cells with class-switched IgG+ cells and antigen-presenting marker (MHC-II+) cells, which may support adaptive immune system maturation in 6-wk pups. Overall, our findings suggest that the

supplementation of plant-based DHA, along with AA, can induce some Th1-specific responses and reduce the Th2 response, which could influence the development of OT and the immune system of allergy-prone BALB/c pups.

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Author disclosures

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Author contribution

The authors' responsibilities were as follows– DP, SG, CR, and CJF: designed the study; DP, SG, and JM: conducted the research and analyzed data; DP: performed the statistical analysis and drafted the manuscript with the supervision of CJF and CR; CJF: have the main responsibility for the final draft and all authors: read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://doi.org/10.1016/j.tjn.2023.06.002>.

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