

RESEARCH ARTICLES

The content of docosahexaenoic acid in the suckling and the weaning diet beneficially modulates the ability of immune cells to response to stimuli[☆]

Caroline Richard, Erin D. Lewis, Susan Goruk, Catherine J. Field*

Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada, T6G 2E1

Received 29 February 2016; received in revised form 28 April 2016; accepted 26 May 2016

Abstract

The objective of the study was to isolate the effect of feeding a diet supplemented with docosahexaenoic acid (DHA) during the suckling and/or the weaning period on immune system development and function in offspring. Dams were randomized to one of two nutritionally adequate diets: control diet ($N=12$, 0% DHA) or DHA diet ($N=8$, 0.9% DHA). Diets were fed to dams throughout lactation, and then at weaning (21d), two pups per dam were randomly assigned to continue on the same diet as the dam or consume the other experimental diet for an additional 21d. At 6 weeks, splenocyte phenotypes and *ex vivo* cytokine production after stimulation with concanavalin A (ConA), lipopolysaccharide (LPS) or ovalbumin were assessed. Pups who received the control diet during both periods had the lowest production of IL-2 after ConA ($P<.05$ for interaction). Pups fed DHA during suckling had higher IL-10 production after all mitogens, regardless of the weaning diet ($P<.05$). Feeding DHA at weaning, regardless of the suckling diet, resulted in a lower production of IL-1 β and TNF- α in LPS-stimulated splenocytes and a higher proportion of total CD27⁺ cells (all $P<.03$). Our findings suggest that providing no DHA during critical periods of immune development resulted in a less efficient Th1 response upon challenge (IL-2 production). Feeding DHA during suckling had a programming effect on the ability of splenocytes to produce the regulatory cytokine IL-10. Feeding a DHA diet during weaning led to a lower TNF- α and IL-1 β response to a bacterial antigen. © 2016 Elsevier Inc. All rights reserved.

Keywords: Immunology; Lactation period; Weaning period; Offspring; Development; Programming

1. Introduction

T and B lymphocytes are key components of the acquired immune system that are important for the individual or animal to appropriately and effectively deal with environmental challenges [1]. Although there is considerable development of the immune system during pregnancy, the suckling period and the early weaning period are both critical stages where the acquired immune system matures [2]. T cells can be divided into T helper Th1 cells or Th2 cells according to the different cytokines they produce. The immaturity of the immune system at birth is characterized by a predominance of Th2 cells and maturation during infancy is associated with an increase proportion of Th1 cells as well as an increase in their ability to produce cytokines including interleukin IL-2, interferon IFN- γ and tumor necrosis factor TNF- α [3].

Studies have demonstrated that both the level of fat and the balance between omega-6 (n-6) and omega-3 (n-3) long-chain

polyunsaturated fatty acids (LCPUFA) in the diet modulate T cell function in different stages of the life cycle and after immune challenges [4,5]. More specifically, arachidonic acid (AA, n-6) and docosahexaenoic acid (DHA, n-3), which are found in significant amounts in breast milk, have both been suggested to have a beneficial effects on immune system development early in life [6]. Indeed, although the essentiality of AA for optimal growth is still under debate [7,8], providing adequate amount of AA early in life is hypothesized to be important for the immune system development as there is a rapid increase in the content of AA in thymocytes in the post natal period [9] that is a critical period for T cell development. Nutritional intervention studies consistently showed that infants fed formula supplemented with AA and DHA early in life have a reduce risk of developing allergic/atopic diseases (reviewed in Ref. [10]). Previous studies have also demonstrated some beneficial effects of fish oil supplementation either during pregnancy [11,12] or childhood [13] on the immune system development and/or function. The effect of diet during critical periods of development has profound implications on both the immediate biological response and the response later in life. However, this concept of nutritional programming has not been well established for immune function.

We have previously established [14] in our rodent model that (1) feeding a diet containing no DHA to lactating dams resulted in a breast milk fatty acid composition similar to what has been observed in human milk in the United States and Canada (0.24% DHA) and (2)

[☆] Sources of Funding: This study was supported by a Discovery grant from the Natural Sciences and Engineering Research Council of Canada. Authors report no conflict of interest in relation with this study.

* Corresponding author. Department of Agricultural, Food and Nutritional Science, University of Alberta, 4-126A Li Ka Shing Center for Health Research Innovation, Edmonton, Alberta, Canada, T6G 2E1. Tel.: +1-780-492-2597; fax: +1-780-492-2011.

E-mail address: Catherine.field@ualberta.ca (C.J. Field).

feeding a DHA diet (0.9% of total fatty acids as DHA) increased the DHA content of breast milk (1.09% DHA) to levels found in human milk from Japan and northern Canada ($\geq 0.8\%$ of DHA) where fish intakes or other sources of DHA are high [15] or of women taking fish oil supplements [16]. Using our previously established rodent model, we aimed at determining the optimal timing for providing an additional physiologically achievable amount of DHA early in life (*i.e.* suckling or weaning period) and its programming effect on the immune system. The objective of the current study was therefore to determine the effect of feeding a diet supplemented with DHA while containing an adequate amount of AA (so as to achieve the content in breast milk), during the suckling (intervention on the dam) and/or the weaning period on immune system development and function in offspring. Immune function was assessed by cytokine production by splenocytes stimulated with mitogens or ovalbumin (OVA), a dietary antigen.

2. Materials and methods

2.1. Animals and diets

All animal care and experimental protocols were conducted in accordance with the Canadian Council on Animal Care and approved by the University of Alberta Animal Ethics Committee. Primiparous Sprague–Dawley rats ($n=20$) were obtained from Charles River Laboratories (Montreal, Quebec, Canada) on day 14 of gestation and were individually housed in an environment that is controlled by temperature and humidity, with a 12/12-h reversed light cycle. Dams were fed standard rat chow (Lab diet 5001; PMI Nutrition International, Brentwood, MO, USA) throughout gestation and then randomized to one of two nutritionally adequate experimental diets 24–48 h prior to parturition: control diet (0.4% AA and 0% DHA of total fatty acids, $N=12$) or DHA diet (0.4% AA and 0.9% DHA of total fatty acids, $N=8$). The litters were culled to ensure 10 pups/dam and diets were fed *ad libitum* throughout lactation. Offspring were kept with their mothers until the end of the suckling period (3 weeks of age) where dams and two pups from each dam (pooled) were terminated. Data on 3-week-old pups have been previously published in Ref. [14]. Then, one pup from each dam (only females were kept) was randomly assigned to the control diet or the DHA diet in a crossover design and the diets were fed for an additional 3 weeks. At 6 weeks, pups were terminated. This study design allowed us to specifically investigate the impact of feeding a DHA diet during the suckling period but also during the weaning period and resulted in four different diet groups as presented in Fig. 1. The immune system development in rodents and humans has been shown to share many similarities and the suckling rat has been proposed as a good model for immunonutrition studies in early life [2,17].

Both experimental diets were isocaloric and isonitrogenous, and the nutrient composition was identical differing only in the LCPUFA content (Table 1). The nonlipid nutrient composition of the experimental diets has been previously described [18]. The added fat mixture to the rodent diet was composed of flaxseed oil, sunflower oil, saturated canola oil, olive oil, a high AA oil and a DHA oil [both AA and DHA oils were provided by DSM (Nutritional Products, Columbia, MD, USA)] and each fatty acid was

Table 1

Fatty acid composition of the experimental control and DHA diet fed to pups during the suckling and the weaning period adapted from Ref. [14]

Fatty acid	Control diet	DHA diet
<i>g/100 g of total fatty acids</i>		
C14:0	0.1 \pm 0.0	0.4 \pm 0.0
C16:0	6.7 \pm 0.3	6.2 \pm 0.1
C16:1n-7	0.2 \pm 0.0	0.2 \pm 0.1
C18:0	38.8 \pm 1.2	40.6 \pm 0.2
C18:1n-9	29.0 \pm 1.7	24.8 \pm 0.3
C18:2n-6	21.2 \pm 0.5	21.6 \pm 0.0
C20:0	0.9 \pm 0.0	0.9 \pm 0.0
C18:3n-3 (ALA)	1.7 \pm 0.1	3.3 \pm 0.1
C20:3n-6	0.4 \pm 0.1	0.4 \pm 0.1
C20:4n-6 (AA)	0.4 \pm 0.0	0.4 \pm 0.0
C22:6n-3 (DHA)	0	0.9 \pm 0.1
Other fatty acids ^a	0.8	0.4
Total SFA	46.5 \pm 0.8	48.1 \pm 0.3
Total PUFA	23.6 \pm 0.6	26.6 \pm 0.1
Total n-6	21.9 \pm 0.5	22.3 \pm 0.1
Total n-3	1.6 \pm 0.1	4.2 \pm 0.1
Total MUFA	29.1 \pm 1.7	24.9 \pm 0.4
Ratio n-6/n-3	13.3	5.3
Ratio PUFA/SFA	0.5	0.6

Analysis by GLC of $n=2$ batches, mean \pm SEM; AA, arachidonic acid; ALA, α -linolenic acid; DHA, docosahexaenoic acid; MUFA, monounsaturated fatty acids; n, omega; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

^a Other fatty acids refer to fatty acids that contributed for less than 0.1% in the diet that included trace of 10:0, 12:0, 20:2n-6, 20:5n-3, 22:0, 22:4n-6 and 22:5n-3.

matched closely so the diet primarily differed only in the total n-3 content. Both diets met the essential fatty acid requirements of the rodent and had similar PUFA/SFA ratio. Diets were prepared weekly and stored at 4°C until fed; feed cups were replaced every 2–3 days to prevent oxidation. Dietary intake and body weight were recorded regularly throughout the intervention.

2.2. Tissue collection

At 6 weeks, pups were euthanized by CO₂ asphyxiation and subsequent cervical dislocation. Spleens were collected aseptically and immune cells were isolated (see below). Intestines were removed and the length recorded.

2.3. Immune cell isolation

Immune cells were isolated from spleens as previously described [19]. Briefly, single cell suspensions were obtained by disrupting tissue through a nylon mesh screen in sterile Krebs–Ringer Hepes buffer with bovine serum albumin (5 g/L) (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). Erythrocytes were lysed with ammonium

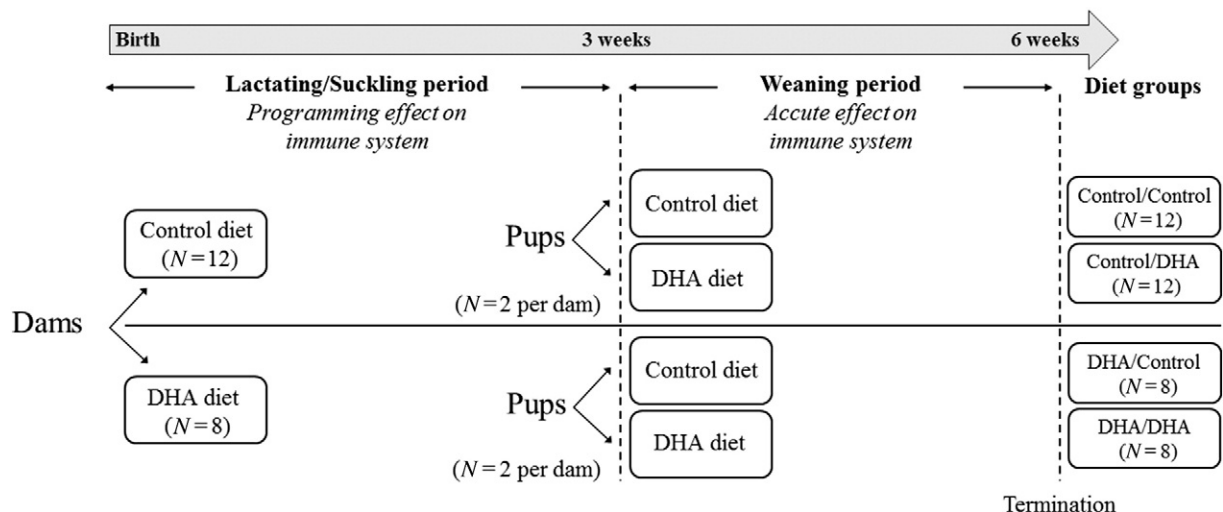


Fig. 1. Animal study design. Dams were randomly assigned to the control diet or the DHA diet for the duration of the lactating/suckling period. At 3 weeks, 2 pups from each dam were then randomly assigned to the control diet ($N=1$ per dam) or the DHA diet ($N=1$ per dam) in a crossover design and the diets were fed for an additional 3 weeks. At 6 weeks, pups were terminated and since the dams are the experimental unit in this study design the number of observation within each group is equal to the number of dams.

chloride lysis buffer (155 mM NH_4Cl , 0.1 mM EDTA and 10 mM KHCO_3 ; Fisher Scientific, Edmonton, Alberta, Canada). Cells were washed and resuspended in complete culture medium [RPMI 1640 medium supplemented with 5% (v/v) heat-inactivated fetal calf serum, 25 mM HEPES, 2.5 mM 2-mercaptoethanol and 1% antibiotic/antimycotic (pH 7.4); Invitrogen, Burlington, Ontario, Canada]. Cells were counted on a hemocytometer using trypan blue dye (Sigma) exclusion and diluted to 1.25×10^6 cells/ml.

2.4. Immune cell phenotype analysis

Immune cell subsets in freshly isolated immune cells were identified by direct immunofluorescence assay as previously described [20]. Briefly, immune cells (200,000) were incubated for 30 min at 4°C with prelabeled monoclonal antibodies applied in combination to quantify various immune cell phenotypes. Four-color flow cytometry allowed determination of the following surface molecule combinations for spleen: CD28/CD3/CD8/CD4, CD28/CD152/CD8/CD4, CD25/CD152/CD8/CD4, CD25/CD127/CD8/CD4, CD27/CD8/CD4, CD27/OX12/OX6/CD45RA, CD71/CD8/CD4, OX12/OX6/CD80, CD86/CD80/CD45RA, CD68/CD284/CD11b/c, OX62/CD25/OX6, CD161/OX62/CD3, IgG/IgM and IgA. All antibodies were purchased from eBiosciences (San Diego, CA, USA) or BD Biosciences (Mississauga, Ontario, Canada).

Cells were then washed and fixed in paraformaldehyde (10 g/L; Anachemia Science, Montreal, Quebec, Canada) in phosphate-buffered saline with sodium azide as the preservative. All samples were acquired within 72 h by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA, USA) according to the relative fluorescence intensity using Kaluza Software (Beckman Coulter, Mississauga, Ontario, Canada).

2.5. Ex vivo cytokine secretion by mitogen-stimulated lymphocytes

Cytokine production by mitogen- or OVA-stimulated splenocytes was measured as previously described [21]. Briefly, the same number of immune cells (1.25×10^6 cells/ml) were cultured for 48 h without mitogen (unstimulated cells) or with concanavalin A (ConA, 5 $\mu\text{g}/\text{ml}$; MP Biomedicals, Montreal, Quebec, Canada), lipopolysaccharide (LPS, 100 $\mu\text{g}/\text{ml}$; Sigma) or OVA (100 $\mu\text{g}/\text{ml}$; Sigma). Cells were then centrifuged for 10 min at 1000 rpm and the supernatants were kept at -80°C . Commercial ELISA kits were used to measure the concentrations of IL-1 β , IL-2, IL-6, IL-10, TNF- α , TGF- β and IFN- γ according to the manufacturer's instructions and as described previously [21]. All detection limits 15.63–4000 pg/ml except for IFN- γ , 9.76–2500 pg/ml (R&D systems, Minneapolis, MN, USA). Concentrations were determined on a microplate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA) and all measurements were conducted in duplicate with CV < 10%.

2.6. Fatty acid analysis

In order to confirm that the change in dietary fat altered the pups' suckling diet and cells membrane phospholipids, the fatty acid composition of the pups' stomach content (representing breast milk) and the pups' splenocytes was determined. Briefly, total lipids from pups' stomach content were extracted using Folch ratio 4:1 chloroform:methanol (2:1) [22]. The samples were left overnight at 4°C; the bottom layer was removed and dried down under nitrogen. Lipid samples were then saponified with 1 M methanolic KOH for 1 h at 100°C. They were allowed to cool and then the samples were methylated with hexane and methanol BF_3 for an additional hour at 100°C. The upper hexane layer was removed and fatty acids were separated by gas chromatography. A modified Folch method was used to extract lipids from splenocytes as previously described [23]. Fatty acid methyl esters were prepared from the scraped silica bands of total phospholipid and separated by automated gas liquid chromatography (Agilent Technologies, Mississauga, Ontario, Canada) using a 100-m CP-Sil 88 fused capillary column (Varian Instruments, Mississauga, Ontario, Canada) as described previously [24]. Fatty acid composition is presented as percent of total fatty acids.

2.7. Statistical analysis

Data are reported as mean \pm standard error of the mean (SEM) unless indicated otherwise. Since this is two levels of treatment study and that dams are the experimental unit in this study design, data were analyzed using the PROC MIXED procedure for repeated measures in SAS (v9.4; Cary, NC, USA) with diets as the main effect (the dams suckling/lactating period diet, the pups weaning period diet and their interaction). In cases where heterogeneous variation was suspected, models assuming either heterogeneous or homogeneous variances were compared based on Akaike Information Criteria and the best fitting model was selected. Although the samples size were different for the control diet ($N=12$) and the DHA diet ($N=8$), the MIXED procedure allowed us to use all the data available for general trends while maximizing the study statistical power (i.e. $N=24$ for the control maternal diet, $N=16$ for the DHA maternal diet and $N=20$ for both the control and the DHA pups' diets comparison). However, this statistical analysis does not allowed us to compare each individual group and only significant interaction can be interpreted. Differences at P of .05 (two-sided) were considered significant.

3. Results

3.1. Growth parameters and phospholipids fatty acid composition of splenocytes

There was no significant effect of diet on final body weight, spleen weight, splenocytes isolated, liver weight or gut length (Table 2). Total phospholipids fatty acid composition of splenocytes in 6 weeks pups are reported in Table 3. Overall, there was no (programming) effect of the maternal suckling diet on splenocyte phospholipids composition. Splenocyte phospholipids from pups fed the DHA diet during the weaning period had a higher relative proportion of 18:2n-6, 20:3n-6, 20:5n-3 and 22:6n-3 and a lower proportion of 18:1n-9 compared with the control diet (all $P < .05$). Feeding a DHA diet at weaning also resulted in a higher proportion of total n-3 and a lower ratio of n-6/n-3 and lower proportion of total monounsaturated fatty acids in splenocytes compared with the control diet (all $P < .05$). The content of 20:4n-6 (AA) did not differ among groups.

3.2. Ex vivo cytokine production by mitogen-stimulated immune cells

Regardless of the weaning diet, pups fed the DHA diet during the suckling period had significantly higher IL-10 production by splenocytes after stimulation with ConA, LPS or OVA (all $P < .05$, Table 4). Providing a DHA diet to pups during the weaning period had no effect on the ability of cells to produce IL-10. There was a significant interaction between the maternal suckling diet and the weaning diet regarding the amount of IL-2 and TNF- α produced by splenocytes after stimulation with ConA (both $P < .05$ for interaction). Specifically, at 6 weeks, pups who received the control diet during both the suckling and the weaning period had the lowest IL-2 production after ConA stimulation, while those who received the DHA diet during the suckling period and then the control diet at weaning had the highest IL-2 production. Regarding the production of TNF- α by ConA-stimulated splenocytes, pups who were fed the DHA diet during the suckling period and then the control diet at weaning had the highest production of TNF- α . On the other hand, irrespective of the maternal suckling diet, pups who were fed the DHA diet during the weaning period had a significant lower production of IL-6 and TNF- α by ConA-stimulated splenocytes compared with those fed the control diet (both $P < .04$). Feeding a DHA diet during the weaning period also resulted in a lower production of IL-1 β and TNF- α by LPS-stimulated splenocytes compared with the control diet (both $P < .01$) while the maternal suckling diet had no effect. Production of IFN- γ by either ConA- or LPS-stimulated splenocytes was not affected by the suckling or the weaning diet.

3.3. Immune cell phenotypes in spleen

Overall, only small changes in the relative proportion of immune cells occurred with the different diet treatments. Although the total proportion of CD3+ T cells did not change, there were significant interactions between the suckling and the weaning diet and the proportion of helper T cells (CD3+CD4+) and helper T cells also expressing the costimulatory molecule CD28 (% of CD3+CD4+CD28+, both P for interaction $< .05$, Table 5). Specifically, pups who were fed the control diet during the suckling period and the DHA diet at weaning had the highest proportion of helper T cells expressing CD28, while pups who were fed the DHA during both periods had the lowest proportion. Feeding a DHA diet during both the suckling and the weaning period resulted in a higher proportion of total CD27+ cells compared with the control diet (both $P < .03$). There was also a significant interaction between the maternal suckling diet and the weaning diet and the proportion of CD27+OX12+ cells ($P = .036$ for interaction) for which the highest proportion was observed in pups fed the DHA diet during both suckling and weaning. Regardless of the

Table 2

Effect of the control and the DHA diet fed during the suckling and/or the weaning period on growth parameters

Variable	Control diet suckling period		DHA diet suckling period		<i>p</i> ^a	<i>p</i> ^b	<i>p</i> ^{inter}
	Control diet weaning period (N = 12)	DHA diet weaning period (N = 12)	Control diet weaning period (N = 8)	DHA diet weaning period (N = 7)			
Body weight (g)	162.0 ± 6.9	163.7 ± 8.0	155.6 ± 4.5	159.1 ± 7.9	.434	.709	.894
Spleen weight (g)	0.63 ± 0.05	0.63 ± 0.04	0.50 ± 0.02	0.58 ± 0.06	.137	.262	.207
Liver weight (g)	6.54 ± 0.38	6.44 ± 0.29	6.11 ± 0.22	6.54 ± 0.43	.695	.498	.307
Gut length (cm)	111.0 ± 1.9	111.5 ± 1.6	111.6 ± 1.9	111.9 ± 2.1	.790	.832	.942
Splenocytes (10 ⁶ /g spleen)	29.7 ± 2.9	24.9 ± 3.0	22.1 ± 1.9	23.1 ± 3.1	.116	.407	.316

Values are presented as mean ± SEM; DHA, docosahexaenoic acid.

p^{inter}, *P* interaction between the suckling and the weaning diet in the MIXED model on 6 weeks pups' outcome.^a *P* value from the main effect of the **suckling diet** in the MIXED model on 6 weeks pups' outcome.^b *P* value from the main effect of the **weaning diet** in the MIXED model on 6 weeks pups' outcome.

maternal suckling diet, feeding a DHA diet during the weaning period lead to a significantly lower proportion of CD86 + CD45RA + cells ($P < .001$) compared with the control diet. There was a significant interaction between the suckling and the weaning diet and the proportion of total cells expressing CD68 (macrophages) and CD284 for which pups who received the control diet during both the suckling and the weaning period had the highest proportion of these markers and pups who received the DHA diet during the suckling period and then the control diet at weaning had the lowest proportion of total expressing cells these two markers.

4. Discussion

We investigated for the first time the impact of feeding a DHA diet during two critical periods for female offspring immune system development, *i.e.* the suckling (*via* dietary intervention in the dams) and the weaning period, on the ability of immune cells to respond to different challenges. Feeding a DHA diet during the suckling and/or the weaning period did not significantly affect the growth of the offspring, and this is consistent with previous studies assessing the impact of infant formula enriched in DHA/AA [25,26]. We showed that providing more DHA in the suckling and the weaning diet beneficially modulated the ability of immune cells to respond to stimuli as summarized in Fig. 2. The sex difference in the response of DHA supplementation early in life should

be addressed in future experiments as we only studied female offspring in this study. On the other hand, there is evidence that maternal exposure to pharmacological doses of n-3 LCPUFA during gestation negatively alters the gut microbiota and some immune responses early in life [27,28] and not having assessed the impact of feeding physiologically achievable amount of DHA to lactating dams on the gut microbiota and the intestinal associated immune system is a limitation of the present study.

We have previously shown in 3-week-old suckled pups that feeding potentially physiologically achievable amount of DHA to lactating dams efficiently increased the n-3 content [mainly 18:3n-3 (ALA) and 22:6n-3 (DHA) but not EPA] of breast milk [14]. This demonstrates that, during the suckling period, pups from dams fed the DHA diet had a higher intake of ALA and DHA. The mixing of dietary oils in our study increased the content of ALA in the DHA diet (+ 1.6%). Although ALA can be metabolically converted into EPA and DHA that both modulate immune function [4], the conversion rate is rather low in human [29] and in rodent [30]. It is therefore unlikely that the small difference in the ALA content of the diet in our study is responsible for the effects of feeding the DHA diet during suckling. Indeed, we have previously reported that feeding these diets during suckling did not significantly affect the ALA ($0.46 \pm 0.01\%$ vs. $0.47 \pm 0.02\%$ w/w of total fatty acids) and EPA ($0.07 \pm 0.01\%$ vs. $0.10 \pm 0.01\%$ w/w of total fatty acids) composition of splenocyte phospholipids in suckled pups at 3

Table 3

Effect of the control and the DHA diet on fatty acid composition of splenocyte phospholipids of 6 weeks pups

Variable	Control diet suckling period		DHA diet suckling period		<i>p</i> ^a	<i>p</i> ^b	<i>p</i> ^{inter}
	Control diet weaning period (<i>N</i> = 6)	DHA diet weaning period (<i>N</i> = 6)	Control diet weaning period (<i>N</i> = 6)	DHA diet weaning period (<i>N</i> = 6)			
% of total fatty acid in splenocyte phospholipids							
C16:0	27.48 ± 0.67	27.61 ± 0.35	27.74 ± 0.81	26.79 ± 0.47	.647	.505	.379
C18:0	28.46 ± 1.02	30.02 ± 0.57	29.10 ± 0.90	29.04 ± 0.65	.873	.428	.379
C18:1n-9	10.46 ± 0.28	9.45 ± 0.18	10.03 ± 0.33	9.90 ± 0.16	.986	.032	.089
C18:2n-6	6.98 ± 0.41	8.02 ± 0.18	7.01 ± 0.39	8.11 ± 0.28	.849	.004	.929
C18:3n-3 (ALA)	0.73 ± 0.02	0.67 ± 0.02	0.72 ± 0.04	0.71 ± 0.05	.746	.361	.537
C20:2n-6	0.91 ± 0.07	0.87 ± 0.04	0.85 ± 0.06	0.93 ± 0.04	.999	.686	.219
C20:3n-6	0.98 ± 0.04	1.15 ± 0.03	1.07 ± 0.03	1.18 ± 0.02	.134	<.001	.430
C20:4n-6 (AA)	13.92 ± 0.98	11.83 ± 0.71	13.24 ± 1.04	13.04 ± 0.66	.774	.263	.314
C20:5n-3 (EPA)	0.05 ± 0.00	0.10 ± 0.00	0.06 ± 0.00	0.10 ± 0.01	.226	<.001	.417
C22:5n-3 (DPA)	0.22 ± 0.03	0.31 ± 0.02	0.21 ± 0.01	0.31 ± 0.02	.830	<.001	.676
C22:6n-3 (DHA)	0.45 ± 0.08	0.95 ± 0.07	0.51 ± 0.06	1.01 ± 0.09	.477	<.001	.752
Total SFA	58.00 ± 1.66	60.22 ± 0.81	59.14 ± 1.81	58.25 ± 1.01	.766	.637	.277
Total PUFA	24.47 ± 1.48	24.19 ± 0.77	23.93 ± 1.5	25.65 ± 0.91	.741	.554	.449
Total n-3	1.44 ± 0.11	2.04 ± 0.11	1.49 ± 0.11	2.13 ± 0.11	.526	<.001	.996
Total n-6	23.03 ± 1.37	22.15 ± 0.67	22.44 ± 1.39	23.52 ± 0.83	.762	.912	.420
Total MUFA	16.51 ± 0.31	14.57 ± 0.18	15.97 ± 0.47	14.98 ± 0.17	.835	<.001	.136

Values are presented as mean ± SEM; significant differences are indicated in boldface; AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; n, omega; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

p^{inter}, *P* interaction between the suckling and the weaning diet in the MIXED model on 6 weeks pups' outcome.^a *P* value from the main effect of the **suckling diet** in the MIXED model on 6 weeks pups' outcome.^b *P* value from the main effect of the **weaning diet** in the MIXED model on 6 weeks pups' outcome.

Table 4

Effect of the control and the DHA diet fed during the suckling and/or the weaning period on *ex vivo* cytokine production by mitogen-stimulated splenocytes of 6-week-old pups

Variable	Control diet suckling period		DHA diet suckling period		<i>p</i> ^a	<i>p</i> ^b	<i>p</i> ^{inter}
	Control diet weaning period (<i>N</i> = 12)	DHA diet weaning period (<i>N</i> = 12)	Control diet weaning period (<i>N</i> = 8)	DHA diet weaning period (<i>N</i> = 7)			
ConA							
IL-2, pg/ml	9374 ± 1699	10,631 ± 2252	11,662 ± 2136	10,133 ± 1293	.833	.584	.046
IL-6, pg/ml	1069 ± 158	844 ± 152	1324 ± 167	1048 ± 123	.242	.034	.816
IL-10, pg/ml	937 ± 105	1017 ± 191	1450 ± 245	1518 ± 242	.045	.669	.940
IFN-γ, pg/ml	379 ± 105	406 ± 104	643 ± 76	551 ± 136	.246	.988	.380
TNF-α, pg/ml	161 ± 28	147 ± 31	254 ± 50	144 ± 27	.387	.002	.011
IFN-γ/IL-10	0.27 ± 0.11	0.44 ± 0.11	0.46 ± 0.06	0.44 ± 0.11	.502	.362	.275
LPS							
IL-1β, pg/ml	185 ± 30	135 ± 20	265 ± 33	175 ± 24	.089	.003	.296
IL-6, pg/ml	823 ± 181	654 ± 108	974 ± 95	767 ± 45	.455	.129	.846
IL-10, pg/ml	579 ± 98	574 ± 116	863 ± 133	1105 ± 129	.029	.130	.174
IFN-γ, pg/ml	59 ± 23	47 ± 13	85 ± 16	81 ± 18	.229	.513	.786
TNF-α, pg/ml	738 ± 117	572 ± 79	915 ± 131	610 ± 64	.444	.005	.331
IFN-γ/IL-10	0.11 ± 0.03	0.07 ± 0.01	0.11 ± 0.02	0.07 ± 0.01	.990	.205	.977
OVA							
IL-2, pg/ml	14 ± 4	19 ± 8	22 ± 10	21 ± 5	.546	.864	.585
IL-6, pg/ml	685 ± 161	825 ± 222	647 ± 75	1041 ± 145	.580	.101	.428
IL-10, pg/ml	446 ± 68	442 ± 70	658 ± 65	704 ± 125	.025	.979	.672
TGF-β, pg/ml	1693 ± 94	1518 ± 241	1739 ± 158	1722 ± 258	.701	.564	.834

Values are presented as mean ± SEM; significant differences are indicated in boldface; ConA, concanavalin A; DHA, docosahexaenoic acid; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; OVA, ovalbumin; TGF, transforming growth factor, TNF, tumor necrosis factor.

p^{inter}, *P* interaction between the suckling and the weaning diet in the MIXED model on 6 weeks pups' outcome.

^a *P* value from the main effect of the **suckling diet** in the MIXED model on 6 weeks pups' outcome.

^b *P* value from the main effect of the **weaning diet** in the MIXED model on 6 weeks pups' outcome.

weeks despite a higher content of ALA (+1.4%) in the dams' breast milk [14]. However, feeding the DHA diet, used in this study, to lactating dams led to a higher proportion of DHA (0.64 ± 0.06% vs. 1.50 ± 0.18% w/w of total fatty acids) in splenocyte phospholipids of suckled pups at 3 weeks [14]. The ALA content of splenocyte phospholipids in offspring at 6 weeks did not differ between the control and the DHA diet (Table 2); however, feeding the DHA diet at weaning (different from only at suckling) resulted not only in a higher DHA (2-fold) but also a higher EPA (2-fold) and DPA (1.5-fold) composition in the splenocyte membranes of the 6-week-old pups. As EPA and DPA are the endogenous end products of ALA [29], one cannot rule out the possibility that a small portion of the increased proportion of DHA in splenocyte phospholipids is coming from the higher amount of ALA in the DHA diet.

4.1. Effect of the suckling diet and programming effect on the immune system

We demonstrated for the first time that, regardless of the weaning diet, feeding dams a DHA diet during the suckling period consistently led to a higher production of IL-10 by splenocytes stimulated with ConA (T cell mitogen), LPS (mitogen that stimulates both B cells and macrophages) and OVA (dietary antigen) later in life in offspring even when a diet devoid in DHA was provided at weaning. IL-10 is an important regulatory Th2 cytokine thought to be involved in the establishment of oral tolerance (a state of none responsiveness to a dietary antigen) by preventing an exaggerated inflammatory response to antigens [31–33]. Moreover, a prospective study has reported that a lower production of IL-10 early in life (at 1 month) was associated with

Table 5

Splenocyte phenotypes of 6-week-old pups fed the control and the DHA diet during the suckling and/or the weaning period

Variable	Control diet suckling period		DHA diet suckling period		<i>p</i> ^a	<i>p</i> ^b	<i>p</i> ^{inter}
	Control diet weaning period (<i>N</i> = 6)	DHA diet weaning period (<i>N</i> = 6)	Control diet weaning period (<i>N</i> = 6)	DHA diet weaning period (<i>N</i> = 6)			
% of total cells							
Total CD3 +	37.6 ± 1.5	39.8 ± 1.6	38.5 ± 1.4	39.5 ± 1.1	.793	.248	.623
CD3 + CD4 + (helper T cells)	20.5 ± 1.5	22.8 ± 1.9	21.8 ± 1.0	20.2 ± 0.7	.953	.498	.017
CD3 + CD8 + (cytotoxic T cells)	12.7 ± 1.5	13.4 ± 1.6	11.8 ± 1.5	11.5 ± 1.7	.291	.931	.921
% of CD3 + that also express CD4 + CD28 +	61.1 ± 2.5	63.7 ± 3.9	62.8 ± 2.8	57.2 ± 1.4	.827	.754	.041
CD3 + CD4 + CD25 + FoxP3 + (Treg)	8.2 ± 0.6	7.5 ± 0.4	8.1 ± 1.0	8.1 ± 1.3	.970	.890	.620
Total CD27 +	41.7 ± 1.2	44.5 ± 1.6	44.7 ± 1.2	49.1 ± 1.1	.022	.006	.364
CD27 + OX12 +	10.7 ± 0.8	10.5 ± 1.0	10.8 ± 0.8	13.4 ± 0.8	.121	.065	.036
Total CD68 + (macrophages)	10.9 ± 0.7	10.1 ± 0.7	9.4 ± 0.6	10.1 ± 0.1	.269	.678	.043
Total CD284 +	10.7 ± 0.7	9.9 ± 0.7	9.3 ± 0.6	10.0 ± 0.1	.339	.678	.034
CD86 + CD45RA +	20.8 ± 1.3	18.8 ± 1.1	22.1 ± 1.1	19.9 ± 0.9	.466	<.001	.134
CD68 + CD284 +	6.4 ± 0.7	4.8 ± 0.7	4.2 ± 0.5	4.8 ± 0.6	.199	.395	.064

Values are presented as mean ± SEM; significant differences are indicated in boldface; values are a proportion of the total gated cells as determined by immunofluorescence. CD, cluster of differentiation; DHA, docosahexaenoic acid; no significant differences were observed in any other phenotypes among groups (mean ± SEM, *n* = 24): total CD25 + : 8.5 ± 0.5%; CD4 + CD25 + : 4.9 ± 0.4%; total CD28 + : 47.9 ± 1.7%; total CD152 + : 6.8 ± 0.4%; total CD45RA + : 45.3 ± 2.0%; CD11 + CD284 + : 4.1 ± 0.5%; OX6 + OX62 + (dendritic cells): 9.2 ± 0.6%.

p^{inter}, *P* interaction between the suckling and the weaning diet in the MIXED model on 6 weeks pups' outcome.

^a *P* value from the main effect of the **suckling diet** in the MIXED model on 6 weeks pups' outcome.

^b *P* value from the main effect of the **weaning diet** in the MIXED model on 6 weeks pups' outcome.

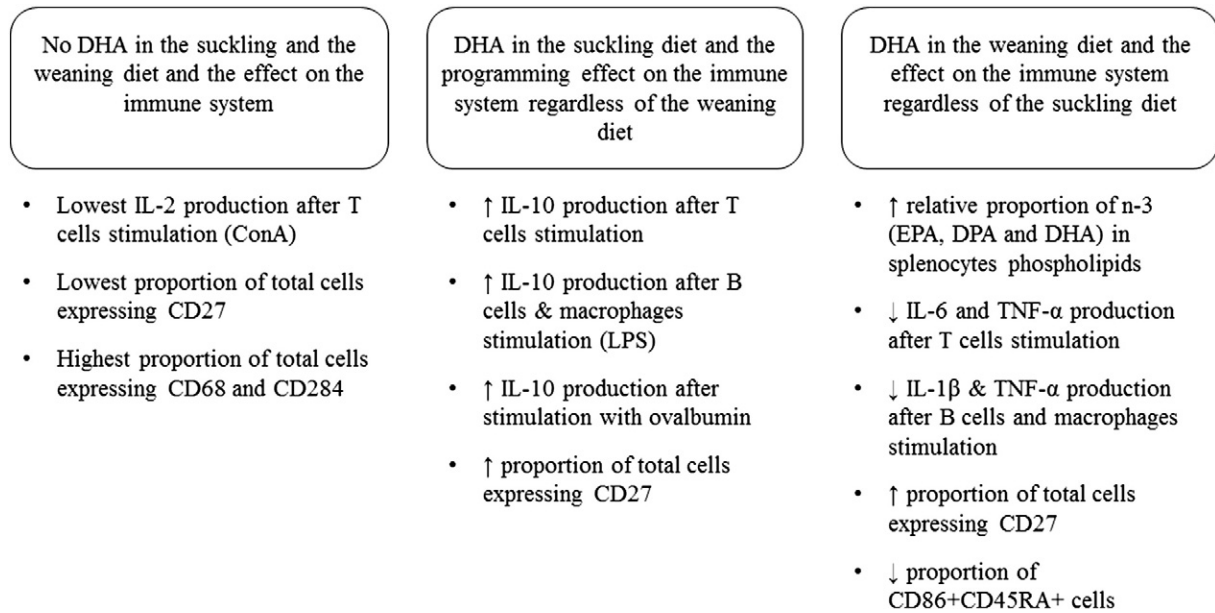


Fig. 2. Summary of the significant effects on immunity in pups who received no DHA, DHA in the suckling diet or DHA in the weaning diet. DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; IL, interleukin; LPS, lipopolysaccharide; n-3, omega-3; TNF, tumor necrosis factor;

the risk of developing atopic dermatitis at 1 year [34]. Our results are consistent with previous studies showing that providing a fish oil supplement to children for 12 weeks [13] or a dietary supplement containing AA and DHA to children for 7 months [35] resulted in higher IL-10 production by mononuclear cells after stimulation with B cell mitogens (pokeweed mitogen and LPS). Altogether, our findings highlight the importance of supplementing the maternal diet in DHA during the suckling period, which has a programming effect on the ability of immune cells to produce the regulatory cytokine IL-10. Although we did not assess any *in vivo* markers of allergy in this study (*i.e.* plasma OVA-specific immunoglobulins), the higher IL-10 production after *ex vivo* OVA stimulation (food protein) might explain to some extent the lower risk of allergic/atopic diseases in infants who consume diets higher in DHA (reviewed in Ref. [10]). Future studies are however needed to determine the programming effect of feeding DHA early in life on the establishment of oral tolerance.

The immaturity of the immune system at birth is characterized by a predominant Th2 response and maturation during infancy is associated with a shift to a more predominant Th1 response including IL-2, IFN- γ and TNF- α production [3]. IL-2 is a cytokine known to induce T cells proliferation and the ability to produce IL-2 after stimulation is an indicator of the maturation of the acquired immune system [17]. We showed that pups who did not receive additional DHA throughout the study (suckling and weaning) had the lowest IL-2 production by ConA-stimulated splenocytes, the lowest proportion of total immune cells expressing the memory marker (CD27) and the highest proportion of macrophages (CD68) and immune cells expressing the Toll-like receptor 4 (TLR4 (CD284)). When LPS binds to the TLR4, it triggers the activation of the innate and acquired immune system leading to inflammatory cytokine production toward a Th1 response [36]. In our study, pups who received the control diet during both the suckling and the weaning period had the lowest Th1 response after ConA stimulation (lowest IL-2 production) despite having the highest proportion of macrophages expressing the TLR4. This suggests that feeding additional DHA early in life enhances the maturation of the immune system toward a better Th1 response. This is further supported by the fact that pups who received the DHA diet at suckling and then the control diet at weaning had the highest Th1 response (TNF- α and IL2). We interpret these results as providing more DHA during suckling enhance the Th1 response whereas providing

a DHA diet at weaning had the expected anti-inflammatory effect (but not immunosuppressive by negatively impacting the IL-2 response). Therefore, pups who received DHA at suckling and then the control diet at weaning had the carry-over of the higher Th1 response that was not counterbalanced by feeding DHA in the weaning diet. Although we did not measure the Th2 response (IL-4, IL-5 and IL-13) in our study, feeding a high DHA diet during the suckling and the weaning period to OVA-sensitized BALB/c mice led to a lower production of IL-4 and IL-13 by splenocytes stimulated with OVA compared to the control diet containing no DHA [37]. Consequently, it is also possible that providing adequate amount of DHA early in life lowers the Th2 response.

4.2. Effect of DHA in the weaning diet on the immune system

We demonstrated that the weaning diet had a significant effect on both T and B cells cytokines response after stimulation, and this is regardless of the suckling diet, suggesting that feeding DHA at weaning modulates immune function. Pups fed the DHA diet at weaning consistently had a lower production of TNF- α after T cell (ConA) and B cell (LPS) stimulation, a cytokine that can often functions as proinflammatory. Pups fed the DHA diet at weaning also had a lower production of IL-6 and IL-1 β by ConA- and LPS-stimulated splenocytes, respectively. These results are consistent with the well-documented antiinflammatory effects of n-3 (EPA and DHA) fatty acids on the immune system. It has been demonstrated in rodents [38,39] and in humans [40,41] that a higher intake of EPA and/or DHA increases the n-3 content of immune cell membranes that, in turn, modulates several signaling pathways and thereby altering their function [42]. This is also consistent with our data showing an increase in the n-3 content (EPA, DPA and DHA) of splenocytes phospholipids when pups were fed the DHA diet during the weaning period. Incorporation of DHA into cell membranes leads to the generation of antiinflammatory lipid mediators implicated in the resolution of inflammation such as resolvins D1 and protectins D1, which have been shown to inhibit the production of IL-1 β and TNF- α [43]. Cell culture studies [44,45], animal studies [46,47] and human study [48] have also demonstrated that EPA/DHA or fish oil can inhibit the production of IL-1 β , IL-6 and TNF- α through the inhibition of NF- κ B by LPS-stimulated monocyte/macrophages. Despite the overall anti-inflammatory effect of feeding a

DHA diet at weaning, our results should not be interpreted as reduced immune function, since there was no difference in the ability of cells to proliferate upon challenge (no change in IL-2 with the weaning diet). Rather, our results suggest that providing a DHA diet during weaning led to a more effective immune response or possibly enhanced the ability to resolve the inflammation after stimulation, which is consistent with the action of lipids mediator derived from DHA.

We also showed that feeding a DHA diet during both the suckling period and the weaning period led to a higher proportion of mature B cells. Indeed, there was a higher proportion of total cells expressing CD27 that was mainly attributable to the subset of cells expressing both CD27 and OX12 (a specific marker of B cells) when pups were fed the DHA diet during both time periods. The immune system at birth is considered naïve due to low antigen exposure *in utero* and is characterized by a low proportion of memory B and T cells [2,49]. CD27 is a marker of memory [50] and, although also expressed on a variety of other cell types, the highest proportion of CD27 + OX12 + cells suggests an enhanced maturation of the B cell population when additional DHA is provided during the suckling and weaning period. Regardless of the suckling diet, pups fed DHA at weaning had a lower proportion of cells expressing both CD86 and CD45RA. In rodents, CD45RA is a B cell marker whereas CD86 is expressed on antigen-presenting cells including B cells and provides a costimulatory signal for T cell activation. In humans, B cells expressing CD86 have been shown to modulate T cell cytokine production toward a Th1 response [51]. Indeed, a positive correlation between the proportion of CD19 + CD86 + B cells and the production of IL-1 β , IL-6 and TNF- α by mononuclear leukocytes after T cell stimulation has been reported in human [52]. The lower proportion of CD86 + CD45RA + cells observed in our study is therefore consistent with the lower proinflammatory cytokine production after ConA and LPS stimulation when pups were fed the DHA diet at weaning. Overall, our data suggest a beneficial effect of DHA supplementation on B cells' maturation and activation during two critical stages of immune function development, the suckling and the weaning period, with a higher proportion of memory B cells.

5. Conclusions

Our findings suggest that providing more DHA during the suckling period (through breast milk) favors the maturation of the immune system toward a more efficient Th1 response (IL-2 and TNF- α production). Feeding a DHA diet at suckling appears to also have a programming effect on the ability of the offspring's splenocytes to produce the regulatory cytokine IL-10 that may contribute to the lower risk for allergic/atopic diseases reported in infants fed diets higher in DHA early in life. Providing a diet enriched in DHA at weaning generally led to an antiinflammatory effect with a reduction in the production of proinflammatory cytokines after T and B cell stimulation (without altering IL-2 production) along with a higher proportion of mature B cells that suggests a beneficial effect of supplementing DHA in the weaning diet. Overall, our results suggest that both the suckling and the weaning periods are important developmental periods during which increasing DHA intake modulates immune function in female rodent.

Disclosures

Authors report no conflict of interest in relation with this study.

Authors' contributions

C. J. Field has designed and obtained funding for this study. S. Goruk and E. Lewis conducted the animal study and were responsible for the laboratory analysis. C. Richard performed the statistical analyses,

analyzed the data and wrote the manuscript, which was reviewed critically by all authors.

Acknowledgements

The authors would like to acknowledge the technical assistance of Nicole Coursen and Marnie Newell. We express our gratitude to Peter Blenis who helped with the statistical analysis. We also thank the undergraduate students, Dev Dutta, Michael Lee and David Ma, who were involved in data collection and analysis throughout the project. C.R. is a recipient of postdoctoral fellow scholarships from Canadian Institutes of Health Research, Fonds de Recherche en Santé du Québec and Izaak Walton Killam Memorial Postdoctoral Fellowships. E.D.L. is a recipient of a Natural Sciences and Engineering Research Council Doctoral Postgraduate Scholarship and Izaak Walton Killam Memorial Scholarship.

References

- [1] Garside P, Mowat AM. Oral tolerance. *Semin Immunol* 2001;13:177–85.
- [2] Perez-Cano FJ, Castellote C, Marin-Gallen S, Gonzalez-Castro A, Franch A, Castell M. Phenotypic and functional characteristics of rat spleen lymphocytes during suckling. *Dev Comp Immunol* 2007;31:1264–77.
- [3] Wilson CB, Kollmann TR. Induction of antigen-specific immunity in human neonates and infants. *Nestle Nutr Workshop Ser Clin Perform Programme* 2008; 61:183–95.
- [4] Gottrand F. Long-chain polyunsaturated fatty acids influence the immune system of infants. *J Nutr* 2008;138:1807S–12S.
- [5] Sijben JW, Calder PC. Differential immunomodulation with long-chain n-3 PUFA in health and chronic disease. *Proc Nutr Soc* 2007;66:237–59.
- [6] Field CJ. The immunological components of human milk and their effect on immune development in infants. *J Nutr* 2005;135:1–4.
- [7] Crawford MA, Wang Y, Forsyth S, Brenna JT. The European food safety authority recommendation for polyunsaturated fatty acid composition of infant formula overrules breast milk, puts infants at risk, and should be revised. *Prostaglandins Leukot Essent Fatty Acids* 2015;102:1–3.
- [8] Lauritzen L, Fewtrell M, Agostoni C. Dietary arachidonic acid in perinatal nutrition: a commentary. *Pediatr Res* 2015;77:263–9.
- [9] Harbige LS. Fatty acids, the immune response, and autoimmunity: a question of n-6 essentiality and the balance between n-6 and n-3. *Lipids* 2003;38:323–41.
- [10] Richard C, Lewis ED, Field CJ. Evidence for the essentiality of arachidonic and docosahexaenoic acid in the postnatal maternal and infant diet for the development of the infant's immune system early in life. *Appl Physiol Nutr Metab* 2016;41:461–75.
- [11] Dunstan JA, Mori TA, Barden A, Beilin LJ, Taylor AL, Holt PG, et al. Fish oil supplementation in pregnancy modifies neonatal allergen-specific immune responses and clinical outcomes in infants at high risk of atopy: a randomized, controlled trial. *J Allergy Clin Immunol* 2003;112:1178–84.
- [12] Klemens CM, Berman DR, Mozurkewich EL. The effect of perinatal omega-3 fatty acid supplementation on inflammatory markers and allergic diseases: a systematic review. *BJOG* 2011;118:916–25.
- [13] Vaisman N, Zaruk Y, Shirazi I, Kaysar N, Barak V. The effect of fish oil supplementation on cytokine production in children. *Eur Cytokine Netw* 2005;16:194–8.
- [14] Richard C, Lewis ED, Goruk S, Field CJ. The content of docosahexaenoic acid in the maternal diet differentially affects the immune response in lactating dams and suckled offspring. *Eur J Nutr* 2015 [Epub ahead of print].
- [15] Yuhas R, Pramuk K, Lien EL. Human milk fatty acid composition from nine countries varies most in DHA. *Lipids* 2006;41:851–8.
- [16] van Goor SA, Dijk-Brouwer DA, Hadders-Algra M, Doornbos B, Erwich JJ, Schaafsma A, et al. Human milk arachidonic acid and docosahexaenoic acid contents increase following supplementation during pregnancy and lactation. *Prostaglandins Leukot Essent Fatty Acids* 2009;80:65–9.
- [17] Perez-Cano FJ, Franch A, Castellote C, Castell M. The suckling rat as a model for immunonutrition studies in early life. *Clin Dev Immunol* 2012;2012:1–16.
- [18] Ruth MR, Proctor SD, Field CJ. Feeding long-chain n-3 polyunsaturated fatty acids to obese leptin receptor-deficient JCR:LA-cp rats modifies immune function and lipid-raft fatty acid composition. *Br J Nutr* 2009;101:1341–50.
- [19] Field CJ, Wu G, Metroz-Dayer MD, Montambault M, Marliss EB. Lactate production is the major metabolic fate of glucose in splenocytes and is altered in spontaneously diabetic BB rats. *Biochem J* 1990;272:445–52.
- [20] Field CJ, Thomson CA, Van Aerde JE, Parrott A, Euler A, Lien E, et al. Lower proportion of CD45RO+ cells and deficient interleukin-10 production by formula-fed infants, compared with human-fed, is corrected with supplementation of long-chain polyunsaturated fatty acids. *J Pediatr Gastroenterol Nutr* 2000;31:291–9.
- [21] Blewett HJ, Gerding CA, Ruth MR, Proctor SD, Field CJ. Vaccenic acid favourably alters immune function in obese JCR:LA-cp rats. *Br J Nutr* 2009;102:526–36.
- [22] Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 1957;226:497–509.

- [23] Field CJ, Ryan EA, Thomson AB, Clandinin MT. Dietary fat and the diabetic state alter insulin binding and the fatty acyl composition of the adipocyte plasma membrane. *Biochem J* 1988;253:417–24.
- [24] Cruz-Hernandez C, Deng Z, Zhou J, Hill AR, Yurawecz MP, Delmonte P, et al. Methods for analysis of conjugated linoleic acids and trans-18:1 isomers in dairy fats by using a combination of gas chromatography, silver-ion thin-layer chromatography/gas chromatography, and silver-ion liquid chromatography. *J AOAC Int* 2004;87:545–62.
- [25] Field CJ, Van Aerde JE, Goruk S, Clandinin MT. Effect of feeding a formula supplemented with long-chain polyunsaturated fatty acids for 14 weeks improves the *ex vivo* response to a mitogen and reduces the response to a soy protein in infants at low risk for allergy. *J Pediatr Gastroenterol Nutr* 2010;50:661–9.
- [26] Field CJ, Van Aerde JE, Robinson LE, Clandinin MT. Effect of providing a formula supplemented with long-chain polyunsaturated fatty acids on immunity in full-term neonates. *Br J Nutr* 2008;99:91–9.
- [27] Gibson DL, Gill SK, Brown K, Tasnim N, Ghosh S, Innis S, et al. Maternal exposure to fish oil primes offspring to harbor intestinal pathobionts associated with altered immune cell balance. *Gut Microbes* 2015;6:24–32.
- [28] Myles IA, Pincus NB, Fontecilla NM, Datta SK. Effects of parental omega-3 fatty acid intake on offspring microbiome and immunity. *PLoS One* 2014;9:1–7.
- [29] Arterburn LM, Hall EB, Oken H. Distribution, interconversion, and dose response of n-3 fatty acids in humans. *Am J Clin Nutr* 2006;83:1467S–76S.
- [30] Igarashi M, Ma K, Chang L, Bell JM, Rapoport SI, DeMar Jr JC. Low liver conversion rate of alpha-linolenic to docosahexaenoic acid in awake rats on a high-docosahexaenoate-containing diet. *J Lipid Res* 2006;47:1812–22.
- [31] Pabst O, Mowat AM. Oral tolerance to food protein. *Mucosal Immunol* 2012;5:232–9.
- [32] de Waal MR, Yssel H, Roncarolo MG, Spits H, de Vries JE. Interleukin-10. *Curr Opin Immunol* 1992;4:314–20.
- [33] Howard M, O'Garra A. Biological properties of interleukin 10. *Immunol Today* 1992;13:198–200.
- [34] Belderbos ME, Knol EF, Houben ML, van Bleek GM, Wilbrink B, Kimpfen JL, et al. Low neonatal toll-like receptor 4-mediated interleukin-10 production is associated with subsequent atopic dermatitis. *Clin Exp Allergy* 2012;42:66–75.
- [35] Mazurak VC, Lien V, Field CJ, Goruk SD, Pramuk K, Clandinin MT. Long-chain polyunsaturated fat supplementation in children with low docosahexaenoic acid intakes alters immune phenotypes compared with placebo. *J Pediatr Gastroenterol Nutr* 2008;46:570–9.
- [36] Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006;124:783–801.
- [37] MacLean E, Madsen N, Vliagoftis H, Field C, Cameron L. N-3 fatty acids inhibit transcription of human IL-13: implications for development of T helper type 2 immune responses. *Br J Nutr* 2013;109:990–1000.
- [38] Fritsche K. Important differences exist in the dose–response relationship between diet and immune cell fatty acids in humans and rodents. *Lipids* 2007;42:961–79.
- [39] Wallace FA, Neely SJ, Miles EA, Calder PC. Dietary fats affect macrophage-mediated cytotoxicity towards tumour cells. *Immunol Cell Biol* 2000;78:40–8.
- [40] Kew S, Mesa MD, Tricon S, Buckley R, Minihane AM, Yaqoob P. Effects of oils rich in eicosapentaenoic and docosahexaenoic acids on immune cell composition and function in healthy humans. *Am J Clin Nutr* 2004;79:674–81.
- [41] Yaqoob P, Pala HS, Cortina-Borja M, Newsholme EA, Calder PC. Encapsulated fish oil enriched in alpha-tocopherol alters plasma phospholipid and mononuclear cell fatty acid compositions but not mononuclear cell functions. *Eur J Clin Invest* 2000;30:260–74.
- [42] Calder PC. The relationship between the fatty acid composition of immune cells and their function. *Prostaglandins Leukot Essent Fatty Acids* 2008;79:101–8.
- [43] Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol* 2008;8:349–61.
- [44] Babcock TA, Novak T, Ong E, Jho DH, Helton WS, Espot NJ. Modulation of lipopolysaccharide-stimulated macrophage tumor necrosis factor-alpha production by omega-3 fatty acid is associated with differential cyclooxygenase-2 protein expression and is independent of interleukin-10. *J Surg Res* 2002;107:135–9.
- [45] Khalfoun B, Thibault F, Watier H, Bardos P, Lebranchu Y. Docosahexaenoic and eicosapentaenoic acids inhibit in vitro human endothelial cell production of interleukin-6. *Adv Exp Med Biol* 1997;400B:589–97.
- [46] Renier G, Skamene E, DeSanctis J, Radzioch D. Dietary n-3 polyunsaturated fatty acids prevent the development of atherosclerotic lesions in mice. Modulation of macrophage secretory activities. *Arterioscler Thromb* 1993;13:1515–24.
- [47] Yaqoob P, Calder P. Effects of dietary lipid manipulation upon inflammatory mediator production by murine macrophages. *Cell Immunol* 1995;163:120–8.
- [48] Capo X, Martorell M, Llompert I, Sureda A, Tur JA, Pons A. Docosahexaenoic acid diet supplementation attenuates the peripheral mononuclear cell inflammatory response to exercise following LPS activation. *Cytokine* 2014;69:155–64.
- [49] Duchamp M, Sterlin D, Diabate A, Uring-Lambert B, Guerin-El Khourouj V, Le Mauff B, et al. B-cell subpopulations in children: national reference values. *Immun Inflamm Dis* 2014;2:131–40.
- [50] Agematsu K. Molecules involved in characteristics of naive/memory B cells. *Nihon Rinsho Meneki Gakkai Kaishi* 2004;27:309–14.
- [51] Lemoine S, Morva A, Youinou P, Jamin C. Human T cells induce their own regulation through activation of B cells. *J Autoimmun* 2011;36:228–38.
- [52] Mantani PT, Ljungcrantz I, Andersson L, Alm R, Hedblad B, Björkbacka H, et al. Circulating CD40+ and CD86+ B cell subsets demonstrate opposing associations with risk of stroke. *Arterioscler Thromb Vasc Biol* 2014;34:211–8.