Fish Oil Supplementation of Lactating Mothers Affects Cytokine Production in 2½-Year-Old Children

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ABSTRACT: n-3 PUFA influence immune functioning and may affect the cytokine phenotype during development. To examine whether maternal fish oil supplementation during lactation could modify later immune responses in children, 122 lactating Danish mothers with a fish intake below the population median were randomized to groups supplemented for the first 4 mon of lactation with 4.5 g/d of fish oil (equivalent to 1.5 g/d of n-3 long-chain PUFA) or olive oil. Fifty-three mothers with a fish intake in the highest quartile of the population were also included. The FA composition of erythrocyte membranes was measured at 4 mon and at 2½ yr. Plasma immunoglobulin E (IgE) levels and cytokine production in lipopolysaccharide-stimulated whole-blood cultures were determined at 2½ yr. Erythrocyte n-3 PUFA at 4 mon were higher in infants from the fish oil group compared with the olive oil group ($P < 0.001$) but were no longer different at 2½ yr. The median production of lipopolysaccharide-induced interferon γ (IFN-γ) in the fish oil group was fourfold higher than that in the olive oil group ($P = 0.034$), whereas interleukin-10 (IL-10) production was similar. The IFN-γ/IL-10 ratio was twofold higher in the fish oil group ($P = 0.019$) and was positively correlated with 20:5n-3/20:4n-6 in erythrocytes at 4 mon ($P = 0.050$). The percentages of atopic children and plasma IgE were not different in the two groups, but the study was not designed to look at atopy. Cytokine responses and erythrocyte FA composition in children of mothers with a high fish intake were intermediate in comparison with those in the randomized groups. Fish oil supplementation during lactation resulted in increased *in vitro* IFN-γ production in the children 2 yr after the supplementation was given, which may reflect a faster maturation of the immune system.

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Because of the immunosuppressive action of n-3 PUFA, there has been some concern about the safety of an increased intake of n-3 long-chain PUFA (LCPUFA) in infancy (1). However, it has also been hypothesized that an increased intake of n-3 PUFA may protect against atopy (2). There has been an increase in the prevalence of atopic diseases in the last decades, which could be due to environmental factors. Atopic sensitization occurs early in life and may therefore be specifically sensitive to environmental factors, e.g., diet, that are introduced in this period. It is therefore possible that some "nutritional programming" of the immune system may occur.

Infants are born with an immature immune system characterized by a polarization of T helper lymphocytes (Th) toward a pro-allergic Th2-type response. The capacity to induce protective Th1 immune responses is impaired in early childhood, and immune maturation in childhood is characterized by a Th1 polarization. Breast milk contains numerous components that may promote the development of the infant's immune system (3), including PUFA. The maternal diet is the most important determinant of infant PUFA accretion in membranes of breast-fed children (4). Thus, variations in maternal intake of PUFA may influence the maturation and polarization of the infant immune system.

Immune maturation occurs faster in breast-fed than in formula-fed infants and is enhanced by the addition of LCPUFA to infant formula (1,5). Fish oil (FO) supplementation of pregnant women has been shown to affect immune function in the neonate and atopic sensitization during early life (6,7). Some longitudinal studies found that a higher n-3 PUFA content in breast milk was associated with a decreased likelihood of atopy in infants (8–10), whereas another study found contrasting results (11). Supplementation with n-3 PUFA during lactation has been found to reduce the prevalence of wheezing during the first 18 mon of life (12). The effect of maternal FO supplementation during lactation on later immune function in the offspring has not been investigated.

We performed a randomized trial in which the n-3 LCPUFA intake of breast-fed infants was raised *via* FO supplementation to the mother during the first 4 mon of lactation. The trial was designed to investigate the effects on breast-milk FA composition, n-3 PUFA levels in infant erythrocytes (RBC), and development during the first year of life (13). The long-term effect on immune function was investigated at a follow-up visit, when the children were 2½ yr old. The aim of this study was to see whether maternal FO supplementation during lactation would affect later immune function, determined by the cytokine phenotype and assessed by the *in vitro* production of interferon γ (IFN-γ) and interleukin 10 (IL-10) and plasma immunoglobulin E (IgE). The study did not have the power to look at atopic sensitization.

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Abbreviations: 20:5n-3, eicosapentaenoic acid (individual fatty acids are named by the number of carbon atoms:number of double bonds followed by the position of the last double bond.); FO, fish oil; HFI, high fish intake; IFN-γ, interferon γ; IL, interleukin; IgE immunoglobulin E; LCPUFA, long-chain polyunsaturated fatty acid; LPS, lipopolysaccharide; OO, olive oil; PGE_2, prostaglandin E_2; RBC, erythrocytes; Th, T helper lymphocytes.

SUBJECTS AND METHODS

The study hypothesis was tested in a parallel-group randomized trial. A diagram of the trial profile, with special focus on the present follow-up study, is shown in Figure 1. The details of the study design, recruitment procedure, and subjects, which have been reported elsewhere (13), are described briefly here.

During 1999, participants were selected from among pregnant women recruited for the Danish National Birth Cohort (14) based on their intake of n-3 LCPUFA. Women with a fish intake below the population median (<0.4 g n-3 LCPUFA/d) were recruited for the randomized intervention trial, and women with a fish intake in the upper quartile (>0.8 g n-3 LC-PUFA/d) as a high-fish-intake reference group (HFI group). The inclusion criteria were: an uncomplicated pregnancy, pre-pregnancy body mass index < 30 kg/m^2, no metabolic disorders, and the intention to breast-feed for at least 4 mon. Furthermore, the newborns had to be healthy, term, singleton infants with normal weight for gestation (15) and an Apgar score > 7, and the mothers were to begin taking the supplements within 2 wk after birth. One hundred twenty-two and 53 of the women with a low and high fish intake, respectively, fulfilled all criteria.

The protocols for the intervention trial and follow-up study were approved by the local scientific ethical committee (KF 01-300/98 and KF 01-183/01). Both parents of all participating children gave written consent to participate after the study had been explained to them orally as well as in writing.

Trial and supplements. After birth, women with a fish intake below the median were randomly allocated to daily supplementation for the first 4 mon of lactation with microencapsulated FO or olive oil (OO) given in müesli bars (Halo Foods Ltd., Tywyn Gwynedd, Wales, United Kingdom). The FO supplement (Dry n-3^{TM}; BASF Health and Nutrition A/S, Ballerup, Denmark) provided 1.5 g/d of n-3 LCPUFA (equivalent to 4.5 g/d of fish oil). As an alternative, the supplements were offered in homemade cookies or oil capsules (a gift from Lupe/ProNova Biocare, Lysaker, Norway). The overall self-reported compliance in both groups was, on average, 91% (range 67–100%, n = 64). Investigators and families were blinded to the randomization throughout the first year of life.

One hundred seven mothers complied with the criterion for exclusive breast-feeding for 4 mon. Mothers who did not fulfill this criterion were not excluded from the trial, but we estimated to which extent breast milk covered the energy needs of the infants from their intake of formula and complementary food. Breast milk was estimated to be the dominant source in all but 15 of the infants, most of whom were from the FO group [but the overall degree of breast-feeding did not differ between the two randomized groups (P = 0.059)]. The typical infant formulas on the Danish market at the time had an n-6/n-3 PUFA ratio of around 10 and contained no LCPUFA. One hundred mothers completed the intervention, and 50 mothers from the HFI group remained in the study for the initial 4-mon period. The biochemical effect of the intervention was assessed from the FA composition of breast milk and RBC from mothers and infants at the end of the intervention (13).

Follow-up study. When the children were 2½ yr old, all 150 families were invited to participate in the follow-up examination

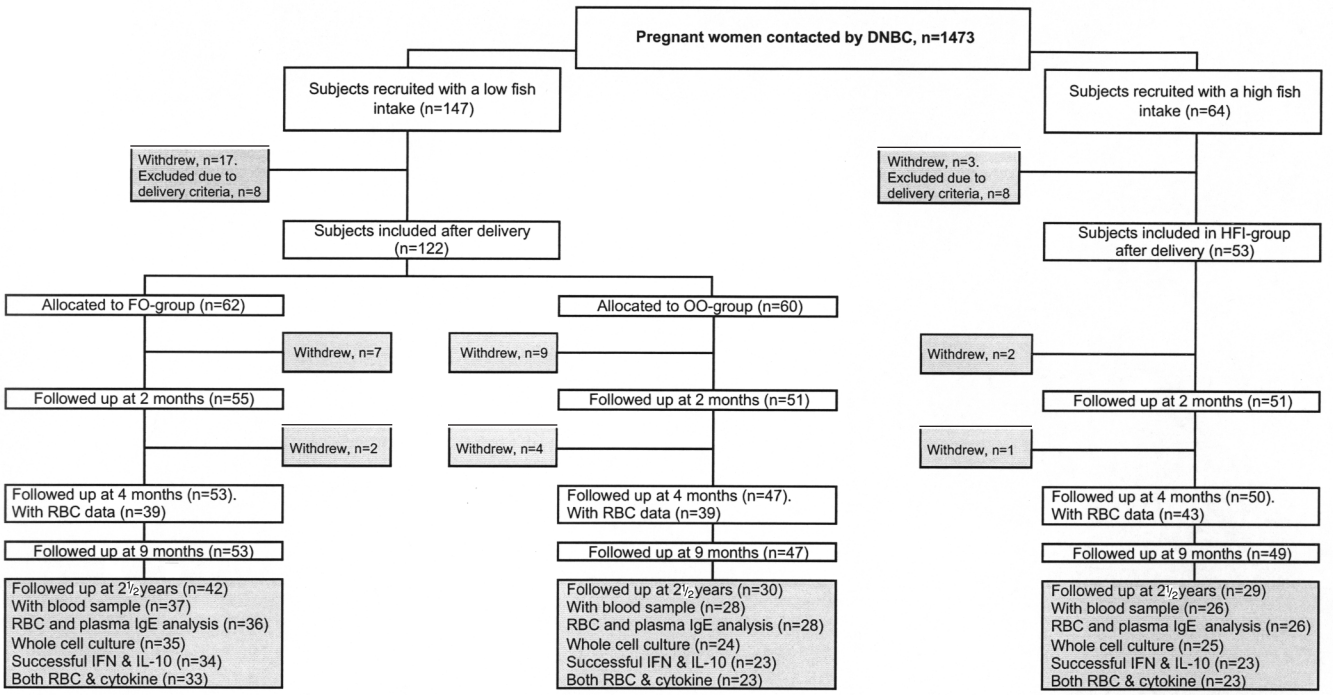

FIG. 1. Trial profile summarizing participant flow, number of randomization assignments, and follow-up examinations for all groups, with special focus on the assessment of immune function at 2½ years of age. DNBC, Danish National Birth Cohort; FO, fish oil; IFN, interferon; IL, interleukin; OO, olive oil; HFI-group, group of mothers with a high fish intake; RBC, red blood cell.

TABLE 1
Characteristics of the 91 Children in the Follow-up Study on Immune Function[a]

	HFI	Olive oil	Fish oil
n	26	28	37
Sex (% boys)	61.5	57.1	62.2
Parity (% with no siblings)	50	39	59
Birth weight (kg)	3.64 ± 0.08	3.56 ± 0.08	3.68 ± 0.07
Mean duration of breast-feeding (mon)	10.0 ± 0.9	9.9 ± 0.9	8.2 ± 0.8
Degree of breast-feeding (% 100 and <50)[b]	80.8 & 7.7	78.6 & 3.6	59.5 & 24.3
Age at follow-up (mon)	31.7 ± 0.2	31.9 ± 0.2	31.7 ± 0.1
Height at 2½ yr (cm)	93.4 ± 0.6	92.6 ± 0.6	92.8 ± 0.5
Weight at 2½ yr (kg)	14.0 ± 0.3	13.7 ± 0.2	14.2 ± 0.2
Family history of atopy (% with 1 and >1)[c]	36.0 & 20.0	42.9 & 21.4	33.3 & 16.7
Children with no signs, of eczema, wheezing, and food allergy (%)[d]	64, 20, 16, 4	64, 14, 21, 4	64, 14, 31, 9
Plasma IgE (titer-value)	8.7 (8.2––9.1)	8.8 (7.3––9.2)	8.9 (8.0––9.2)

[a]Data given as mean ± SE or percentage of all children of mothers with a habitual high fish intake (HFI) or mothers supplemented during lactation with olive oil or fish oil. There were no statistically significant differences between groups. IgE, immunoglobulin E.
[b]Children exclusively breast-fed in the first 4 mon of lactation and those estimated to have had less than 50% of their energy intake covered by breast milk.
[c]1 and >1 atopic family members (parents or siblings).
[d]Only children with an atopic diagnosis made by a physician.

at the Department of Human Nutrition, as previously described (16). The follow-up rates in the randomized groups and in the HFI group were 72 and 58%, respectively. At the time of the follow-up study, the children were healthy, i.e., they were not given continuous medication and did not suffer from chronic disease (except for some having allergies). The parents were interviewed about allergy diagnoses in the child, signs of allergic tendencies, and family history of allergy using questions that has been validated with respect to atopic dermatitis (17). Only allergic tendencies (itchy rash, wheezing, or food allergy) reported that had been verified by a doctor were used for categorization.

At the end of the follow-up examination, a 1-mL blood sample was collected from the children in ice-cold heparin-conditioned tubes by venipuncture. The study group in the present study consisted only of children with successful blood sampling; their characteristics are shown in Table 1.

Within 30 min after sampling, 30-μL aliquots of heparinized whole blood were cultured with lipopolysaccharide (LPS) for measurement of cytokine production. Within 1 h after sampling, RBC were separated from plasma and leukocytes by centrifugation and washed three times in physiological saline. The plasma samples were frozen at −80°C. The isolated, packed RBC were reconstituted 1:1 in physiological saline with 1 mM EDTA and 0.005% BHT (Sigma, St. Louis, MO) and kept at −80°C until the FA composition was determined (maximum storage time 8 mon).

Measurement of cytokine production by whole-blood cultures. Heparinized blood was diluted 1 in 7 with RPMI 1640 medium supplemented with 0.1% FCS and 30 IU/mL Na-heparin. Diluted whole-blood cultures were set up in 96-well culture plates, with 8 wells for each child. In 4 wells, LPS (from *Escherichia coli* O26:B6; Sigma) was added to give a final concentration of 1 μg/mL, and 4 wells with control cultures were the added medium alone. Cultures were incubated at 37°C in a 5% CO_2 atmosphere for 22.5 ± 1.5 h, the supernatant was carefully removed, and aliquots were frozen at −20°C.

Cytokine concentrations in the culture supernatants were measured by ELISA. IFN-γ, IL-12 p70, and IL-4 were measured with commercial kits (Duosets DY285, DY1270, and DY204; R&D Systems Europe, Abington, United Kingdom) in accordance with the manufacturer's instructions. The IL-10 concentration was determined with paired antibodies (BD Pharmingen, San Diego, CA) using anti-human IL-10 (clone JES3-19F1, 2 μg/mL) as the coating antibody and biotinylated anti-human IL-10 (clone JES3-12G8, 1 μg/mL) as the detector antibody. Cytokine concentrations were quantified relative to standard curves representing a range of dilutions of recombinant cytokine using a four-parameter curve-fit analysis (KinetiCalc software, version KC4 Rev 29; Bio-Tek Instruments). Limits of detection for these assays were 62 pg/mL (IFN-γ), 33 pg/mL (IL-10), 33 pg/mL (IL-12), and 31 pg/mL (IL-4). Nondetected values were set to 0.5× the limit of detection in calculating the ratios of IFN-γ to IL-10. IFN-γ IL-10, and IL-12 were determined in most of the children, whereas the IL-4 analysis was carried out only with the supernatants from 10 of the children. The were no correlations between the determined levels of cytokines in the supernatants and storage time (data not shown).

Measurement of IgE in plasma. IgE was measured in plasma from heparinized blood samples with paired antibodies (BD Pharmingen) in a sandwich ELISA. It was performed as described above, but with antibodies against human IgE. Anti-human IgE (clone G7-18) was used as the capture antibody at 2 μg/mL, and biotin-coupled anti-human IgE (clone G7-26) at 2 μg/mL was used as the detector antibody. Plasma from high-IgE producers (a kind gift from ALK-Abello, Hørsholm, Denmark) was used as a reference to control for plate-to-plate variation. Samples were serially diluted and antibody titers were expressed as $\log_2$ titers and defined as the dilution (four-parameter analysis, KinetiCalc software; Bio-Tek Instruments) of a blood sample leading to an absorbance at 0.2 above background.

RBC FA analysis. Thawed RBC from the heparin blood samples were hemolyzed in redistilled water, and the lipids were extracted by the procedure of Folch *et al.* (18). Lipids were methylated with BF_3 in methanolic NaOH (19), and the

resulting FAME were extracted and separated by GLC as previously described (13). All peaks from lauric acid (12:0) to docosahexaenoic acid (22:6n-3) were identified from the retention times of commercial standards (Nu-Chek-Prep Inc., Elysian, MN) as previously described (20). On average, 96.9 ± 0.1% (mean ± SE) of the chromatogram areas were identified (excluding BHT). The FA compositions of all RBC samples were determined in duplicate. Results are expressed as the area percentage of each FA relative to that of all FA peaks together.

Statistical analysis. The results are given as average ± SE for normally distributed variables (RBC FA composition) and as the median (10th and 90th percentiles) for other variables (all immune variables). RBC FA composition and immune responses in the two randomized groups and in atopic and nonatopic children were compared by Student's *t*-test and the Mann–Whitney U-test, respectively. The distribution of LPS-induced IL-10 production in the two randomized groups was performed by Levene's test for equal variances. Paired comparisons of the LPS stimulation in cytokine production were performed using a Wilcoxon signed ranks test. Associations between immune variables and between RBC FA composition and cytokine production were calculated by Kendall τ. A Pearson correlation analysis was used to analyze the correlation between RBC FA compositions in mothers and children. All data were analyzed using SPSS for Windows 11.0 (Chicago, IL).

RESULTS

FO supplementation had a pronounced effect on the FA composition of breast milk (13) and on that of RBC from infants in month 4 of lactation (Table 2). The ratio of 20:5n-3 to 20:4n-6 in the RBC of infants was significantly higher in the FO group than in the OO-group at the end of the 4-mon intervention period. The 91 children with follow-up blood samples at 2½ yr of age were similar to the children with no follow-up blood sample with respect to gestational age, weight, length at birth, degree of breast-feeding, dietary group, and RBC FA composition at 4 mon (data not shown). However, the follow-up group had significantly better compliance in the intervention trial (89 vs. 85%), and the follow-up rate for boys was higher (P = 0.020). At 2½ yr of age, no differences were apparent in RBC FA compositions between children from the two randomized groups (Table 2).

At 2½ yr of age, no group difference was observed in plasma IgE levels (Table 1). Cytokine production was not detectable in the supernatants of unstimulated cultures, except in a few children (Fig. 2). LPS induced a marked increase in IL-10 production to levels approximately 18 times above the detection limit (Fig. 2). Median LPS-induced IL-10 level did not differ between the FO and OO groups (P = 0.367), but the distribution of IL-10 in the two groups did (P = 0.003). LPS-induced IFN-γ production was significantly increased compared with the control in both the OO and FO groups (P = 0.008 and P < 0.001, respectively). LPS induced detectable levels of IFN-γ in 62% of the children in the FO group and 39% in the OO group (chi-square, P = 0.093). The median IFN-γ production was still undetectable in the OO group, but was 2.2 times above the limit of detection in the FO group and significantly higher than in the OO group (P = 0.034, Fig. 2). This difference was also significant when the child with detectable control levels of IFN-γ was excluded (P = 0.049), and there was a trend in the same direction when all children who were breast-fed <50% during the

TABLE 2
FA Composition of Erythrocytes at 4 mon and 2½ yr of Age from Offspring of Mothers Randomized to Groups Supplemented with Olive Oil or Fish Oil During the First 4 mon of Lactation or Mothers with a High Habitual Fish Intake (HFI)[a]

	4 mon			2 ½ yr		
	HFI	Olive oil	Fish oil	HFI	Olive oil	Fish oil
n	20	25	27	26	28	36
SFA	43.3 ± 0.7	42.4 ± 0.6	41.9 ± 0.4	41.2 ± 0.1	41.4 ± 0.2	41.7 ± 0.2[d]
MUFA	17.6 ± 0.5	17.9 ± 0.5	17.2 ± 0.5	16.7 ± 0.1	16.8 ± 0.2	16.8 ± 0.1
20:3n-9	0.05 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.005
18:2n-6	7.9 ± 0.2	8.6 ± 0.2[e]	8.6 ± 0.2[d]	10.2 ± 0.1	10.2 ± 0.2	10.5 ± 0.1
20:2n-6	0.29 ± 0.01	0.31 ± 0.01	0.30 ± 0.01	0.25 ± 0.01	0.24 ± 0.005	0.25 ± 0.01
20:3n-6	1.8 ± 0.1	1.9 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1
20:4n-6	15.1 ± 0.5	15.8 ± 0.5	14.8 ± 0.3	16.3 ± 0.2	16.2 ± 0.2	16.0 ± 0.2
22:4n-6	2.4 ± 0.1	2.8 ± 0.1[d]	2.4 ± 0.1[a]	2.9 ± 0.1	3.0 ± 0.1	3.0 ± 0.1
22:5n-6	0.41 ± 0.03	0.55 ± 0.03[e]	0.54 ± 0.02[f]	0.54 ± 0.02	0.58 ± 0.03	0.54 ± 0.02
n-6 PUFA	27.9 ± 0.7	29.9 ± 0.6[d]	28.4 ± 0.4[a]	31.9 ± 0.2	31.9 ± 0.3	31.9 ± 0.2
18:3n-3	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.15 ± 0.01
20:5n-3	0.95 ± 0.10	0.56 ± 0.06[e]	1.21 ± 0.12[c]	0.79 ± 0.04	0.75 ± 0.06[d]	0.78 ± 0.04
22:5n-3	2.0 ± 0.1	2.1 ± 0.1	1.9 ± 0.1	2.6 ± 0.1	2.5 ± 0.1	2.5 ± 0.1
22:6n-3	7.3 ± 0.4	6.3 ± 0.4	8.5 ± 0.5[c]	5.9 ± 0.2	5.5 ± 0.2	5.4 ± 0.2
n-3 PUFA	10.4 ± 0.6	9.0 ± 0.5	11.7 ± 0.6[b]	9.4 ± 0.2	9.0 ± 0.3	8.9 ± 0.2
n-3/n-6	2.8 ± 0.2	3.6 ± 0.2[d]	2.8 ± 0.3[a]	3.5 ± 0.1	3.7 ± 0.2	3.7 ± 0.1
20:5n-3/20:4n-6	0.063 ± 0.007	0.035 ± 0.003[f]	0.082 ± 0.009[c]	0.049 ± 0.003	0.047 ± 0.004	0.049 ± 0.003

[a]The values are given as area% of all FA in the chromatogram – mean ± SE. Individual FA are named by the number of carbon atoms:number of double bonds and the position of the last double bond. SFA, saturated FA; MUFA, monounsaturated FA. Statistical differences between groups are indicated by superscript letters: [a]P < 0.05, [b]P < 0.01, and [c]P < 0.001 compared with the olive oil group, and [d]P < 0.05, [e]P < 0.01, and [f]P < 0.001 compared with the HFI group.

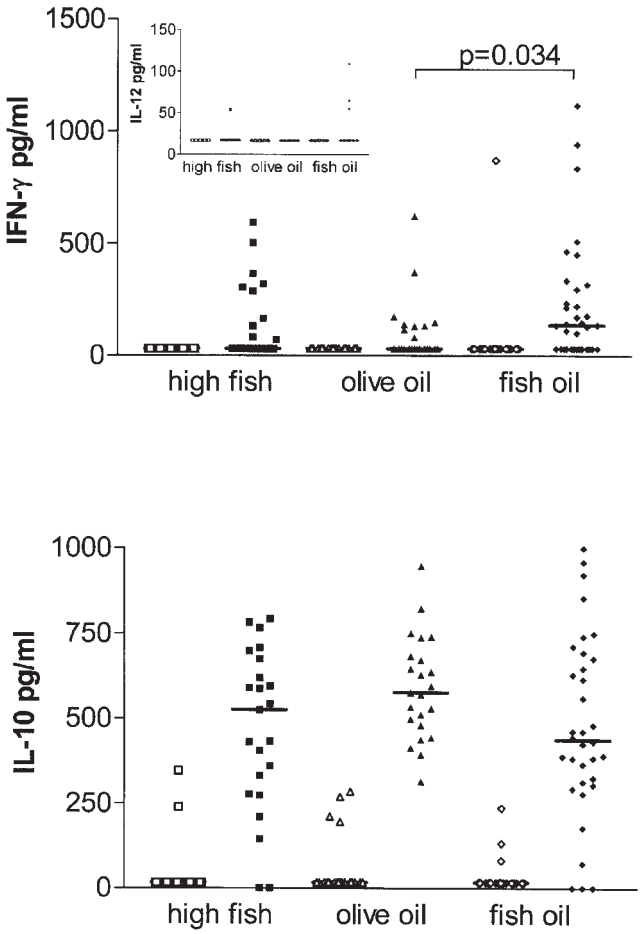

FIG. 2. *In vitro* lipopolysaccharide-stimulated cytokine production in $2\frac{1}{2}$-year-old children in the maternal dietary groups (high fish intake and olive oil- or fish oil-supplemented). Cytokine production (pg/mL) for each child is given as the mean of three to four values. Open and filled symbols give control and lipopolysaccharide-stimulated values, respectively. The solid lines indicate median values in each group (n = 23 in the high fish intake group, 23 in the olive oil group, and 34 in the fish oil group).

intervention were excluded from this analysis ($P = 0.063$). The HFI group was intermediate with respect to LPS-induced IFN-γ production, which was detectable in 44% of the children. LPS-induced IL-12 production was detectable in only three children from the FO group and one from the HFI group (Fig. 2, insert). LPS stimulation did not result in detectable levels of IL-4 (data not shown). The ratio of IFN-γ/IL-10 was significantly higher in the FO group compared with that in the OO group [being 0.27 (0.04–1.11) and 0.07 (0.04–0.56), respectively (P = 0.019)]. The ratio was also significantly different in the two randomized groups when those children who were breast-fed <50% during the intervention period were excluded from the analysis ($P = 0.033$, $n = 47$). The HFI group had an intermediate ratio (data not shown).

Thirty-six percent of the children were reported to have a diagnosis of eczema, wheezing, or food allergy, and these children were equally distributed between the three groups (Table 1). Total plasma IgE was not significantly higher in children with a diagnosis of eczema, wheezing, or food allergy compared with nondiagnosed children, although there was a trend in that direction ($P = 0.071$). LPS induced detectable levels of IFN-γ in 37% of the whole-blood cultures from diagnosed children and 58% from nondiagnosed children ($P = 0.084$). There was no significant difference between atopic and nonatopic children with respect to LPS-stimulated production of IFN-γ ($P = 0.378$), IL-10 ($P = 0.782$), or IFN-γ/IL-10 ($P = 0.435$). Also, there was no significant association between plasma IgE and LPS-induced IFN-γ or IL-10 production ($r = 0.011$, $P = 0.895$ and $r = 0.142$, $P = 0.075$, respectively). No differences were observed in the FA composition of RBC from atopic and nonatopic children at either 4 mon or $2\frac{1}{2}$ yr of age (data not shown).

In children from the randomized groups, LPS-induced IFN-γ production and the ratio of IFN-γ to IL-10 correlated with the ratio of 20:5n-3 to 20:4n-6 in infant RBC ($r = 0.220$ and $r = 0.205$, respectively; $P = 0.050$ and $n = 44$ for both). The correlations between LPS-induced IFN-γ production and IFN-γ/IL-10 and maternal RBC 20:5n-3/20:4n-6 were more prominent, probably due to the higher power ($n = 56$; $r = 0.249$, P = 0.011 and $r = 0.222$, $P = 0.016$, respectively). IFN-γ/IL-10 was also correlated with the levels of 22:6n-3 in the RBC of mothers at the end of the intervention and of the randomized children at $2\frac{1}{2}$ yr of age (data not shown). However, these correlations were weaker (lower r-values and less significant), and the RBC 22:6n-3 of both mothers and $2\frac{1}{2}$-yr-old children was associated with RBC 20:5n-3/20:4n-6 at the end of the intervention (data not shown). No correlation was observed between LPS-induced IL-10 production and any PUFA in the RBC of the mother, infant, or child.

DISCUSSION

FO is known to be immunosuppressive and to suppress the production of inflammatory cytokines (2). Because of such immunomodulatory actions of n-3 PUFA, several FO intervention studies have been conducted in atopic patients. Atopy is characterized by increased B-lymphocyte IgE-production, which is regulated by cytokines produced by Th cells according to the Th1/Th2 hypothesis (21). FO-supplementation studies show conflicting results regarding the effect on Th1/Th2 cytokines and tend to show a limited efficacy once the allergic immune responses are established (2). However, atopic sensitization occurs early in life; therefore, perinatal n-3 PUFA exposure may affect immune maturation in infants and the risk of subsequent disease. Several studies indicate that the development of allergies may be associated with breast-milk PUFA (8–10). FO-supplementation of lactating atopic mothers has been found to reduce the prevalence of wheezing in the child (12). To our knowledge, no other studies have assessed the potential immunomodulatory function of FO supplementation in breast-feeding mothers.

Maturation of the immune system during infancy is characterized by an increase in IFN-γ production and a shift from Th2 to Th1 polarization in the immune response (1). In the present

study, maternal FO supplementation increased LPS-induced IFN-γ production. Furthermore, IL-12 was found only in children of FO-supplemented mothers or mothers with a high fish intake, which strengthens the IFN-γ result. No effect of FO was observed on IL-10 production, although this had a significantly broader distribution, but the IFN-γ to IL-10 ratio was higher after maternal FO supplementation during lactation. Our data indicate that an increased n-3 LCPUFA intake early in life gives rise to faster immune maturation and is able to polarize the immune response later in childhood toward Th1. These results are in agreement with other observations. Field *et al.* (5) observed a slower maturation of the immune response in formula-fed infants compared with breast-fed infants, which disappeared with the addition of LCPUFA (22:6n-3 and 20:4n-6) to the formula. Maternal FO supplementation during pregnancy has been shown to reduce levels of IL-13 in cord plasma (6) and to reduce the allergen-induced production of IL-10 (7).

In vitro cytokine production in whole-blood cultures of the children in the present study was on the limit of detection, which is a general problem in studies of young children (6,22). We were able to detect only IL-12 in a few of the children, although this may have been improved if we had primed the cells with IFN-γ. Instead, we measured the concentration of IFN-γ, as we had demonstrated that IFN-γ production in stimulated whole-blood could be inhibited by the addition of anti-IL-12 antibodies (Jensen, M., Christensen, H.R., and Frøkiær, H., unpublished manuscript). Moreover, we used only one toll-like receptor ligand to stimulate cytokine production; hence, we explored only a small potential of the immune response, and LPS is primarily a Th1 polarizing agent. We were not able to detect IL-4 in LPS-stimulated whole-blood samples and therefore used IL-10, which was detectable in almost all samples, as a Th2 marker. However, IL-10 is secreted by a wide variety of cell types, including Th cells, monocytes, macrophages, dendritic cells, and mast cells. IL-10 possesses potent immunesuppressive functions and is currently believed to drive the differentiation of regulatory T cells. However, we found that IL-10 tended to correlate positively with plasma IgE, which, although not significant, may indicate that IL-10 in young children may be a marker of Th2 response. The LPS-induced responses in IL-10 and IFN-γ production were variable. This variability could be due to differences in cell counts of mononuclear cells between samples, as we did not assess this. However, it is not likely to give rise to a bias between the groups that could explain the observations. Unfortunately, the power of the study was not sufficient to show any difference in the number of high and low responders between the groups, although there was a trend. To overcome the differences in response level, we focused on the ratio between the production of IFN-γ and IL-10. In the study we used OO as a control supplement. However, OO also has been suggested to have some immunomodulatory actions and anti-inflammatory effects, and many of the effects observed for FO are also shown for OO (23). Accordingly, the difference between the FO group and the control group may have been larger if we had used a more neutral control oil.

A fast immune maturation and Th1 polarization could result in better immune functioning and a decreased risk of atopic sensitization. The study was not powered to look at atopic sensitization, and no differences in atopy and plasma IgE were observed between the groups. The children were not selected to have a high risk of atopy, but the prevalence of self-reported eczema, wheezing, or food allergy was high. Furthermore, the study is limited by a low rate of follow-up (around 70%). Plasma IgE is a very crude measure of atopy and does not give an indication of allergen sensitization. Allergen-specific immune responses provide information on cytokine production by allergen-reactive cells influenced by prior antigen exposure, whereas cytokine production after polyclonal stimulation, as in the present study, examines the underlying predisposition of cytokine production. This is especially important in light of the findings that both Th1 and Th2 allergen-induced cytokine responses are increased in children with atopic disease concomitant with a reduced polyclonal Th1 response (22). No association was found between *in vitro* cytokine production and plasma IgE levels; also, there was no association between IgE and eczema, wheezing, or food allergy, although both associations tended to be positive. A weak association may not be surprising considering the self-reported diagnosis and the low power of the study with respect to atopy. Supplementation with n-3 PUFA during pregnancy and in the first year of life has been shown to reduce the prevalence of self-reported wheeze symptoms, which were not associated with a decrease in serum IgE or atopy (12). Allergic diseases may be related to a dysfunction of various IL-10-producing regulatory cells, and allergen-specific IL-10 is known to be down-regulated in atopic children (24). Low levels of IL-12 in cord blood have been associated with later IgE sensitization (25), and high-risk children who developed clinical allergy tended to have lower neonatal Th1 responses (26,27). It has been suggested that a relative absence of IL-12 favors the development of a default Th2 cytokine profile (28). Maturation of antigen-presenting cells, and thus increased IL-12 production, is a key rate-limiting step in the postnatal development of Th1 function.

The present data show that n-3 LCPUFA intake in early infancy appears to be able to increase the polyclonal Th1 response. The FO-induced changes in RBC FA composition during the intervention were no longer evident at $2\frac{1}{2}$ yr of age. RBC were used as a proxy for the FA composition of body tissue membranes in general. Therefore, the effect of FO supplements on immune function during lactation does not appear to be mediated *via* membrane n-3 LCPUFA in whole-blood cultures. We speculate that a long-term effect may be caused by a shift in immune polarization during infancy, which, because of a mutual inhibition between the Th1 and Th2 responses, is maintained or even attenuated throughout early childhood. The mechanism behind the immune-modulating effect of n-3 PUFA could be mediated through changes in prostaglandin E_2 (PGE_2) synthesis (2), but other mechanisms cannot be excluded. PGE_2 enhances Th2 differentiation and suppresses the differentiation of Th1 cells. *In vitro* studies have shown that all PGE subtypes equipotently inhibit Th1 cytokine production (29,30), but they

may influence the ratio of Th1 to Th2 cytokines and T cell proliferation differently (29). We found an association between IFN-γ/IL-10 and RBC 20:5n-3/20:4n-6 in RBC, thus suggesting that n-3 PUFA may exert the effect on cytokine production by competition in eicosanoid synthesis and action.

In summary, we found an increased LPS-induced *in vitro* IFN-γ production and higher IFN-γ to IL-10 ratio in $2^1/_2$-year-old children after maternal FO supplementation in lactation. These results indicate that early n-3 PUFA intake may result in faster maturation of the immune system and/or a TH1 polarization. An important finding of this present study is the observation that the immunomodulating effect is present 2 yr after the supplements were given.

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