n-3 PUFAs enhance the frequency of murine B-cell subsets and restore the impairment of antibody production to a T-independent antigen in obesity

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Abstract The role of n-3 polyunsaturated fatty acids (PUFA) on in vivo B-cell immunity is unknown. We first investigated how n-3 PUFAs impacted in vivo B-cell phenotypes and antibody production in the absence and presence of antigen compared with a control diet. Lean mice consuming n-3 PUFAs for 4 weeks displayed increased percentage and frequency of splenic transitional 1 B cells. Upon stimulation with trinitrophenylated-lipopolysaccharide, n-3 PUFAs increased the number of splenic transitional 1/2, follicular, premarginal, and marginal zone B cells. n-3 PUFAs also increased surface, but not circulating, IgM. We next tested the effects of n-3 PUFAs in a model of obesity that is associated with suppressed humoral immunity. An obesogenic diet after ten weeks of feeding, relative to a lean control, had no effect on the frequency of B cells but lowered circulating IgM upon antigen stimulation. Administration of n-3 PUFAs to lean and obese mice increased the percentage and/or frequency of transitional 1 and marginal zone B cells. Furthermore, n-3 PUFAs in lean and obese mice increased circulating IgM relative to controls. Altogether, the data show n-3 PUFAs enhance B cell-mediated immunity in vivo, which has implications for immunocompromised populations, such as the obese.

Long-chain n-3 polyunsaturated fatty acids (PUFA) found in fish oil generally exert immunosuppressive effects and aid in the resolution of inflammation (1, 2). Preclinical studies show the immunomodulatory properties of n-3 PUFAs have clinical applications (3). For instance, n-3 PUFAs can suppress murine adipose inflammation, which has positive effects on the development of insulin resistance (4). In comparison, studies in humans remain inconclusive with some reports of potential benefits of n-3 PUFAs in prevention and/or treatment of rheumatoid arthritis (5).

One limitation in the clinical implementation of n-3 PUFAs for treating various disease symptoms is that the underlying cellular targets of the fatty acids remain uncertain. Marine n-3 PUFAs are increasingly accepted to suppress cell-mediated immunity in rodent models of health and disease (6). In contrast, there is a paucity of data on the immunomodulatory effects of n-3 PUFAs on the humoral arm of the immune system. In particular, B cells are key players in generating a humoral immune response. B cells also serve as antigen-presenting cells and express pattern recognition receptors to elicit responses in innate immunity (7, 8). B cells are broadly categorized as B1 cells, which are predominant in peritoneal cavities, and B2 cells, which are abundant in secondary lymphoid tissues, including the spleen and lymph nodes (9). B1 and B2 cells can be further categorized into various subsets, including, but not limited to, B1a, B1b, transitional, premarginal zone, marginal zone, follicular, regulatory B10, and newly identified GM-CSF-secreting innate response activator B cells (8–10). To date, no laboratory has tested the role of n-3 PUFAs in impacting the phenotype of differing B-cell subsets and antibody production to a T-independent antigen, particularly in vivo.

Supplementary key words humoral immunity • eicosapentaenoic acid • docosahexaenoic acid

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Abbreviations: DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TNP-LPS, trinitrophenylated-lipopolysaccharide.

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We previously reported that n-3 PUFA administration to C57BL/6 mice at high doses enhanced B-cell activation upon ex vivo stimulation with lipopolysaccharide (LPS), a T-independent antigen (11). While these findings challenged the dogma that n-3 PUFAs were immunosuppressive, we attributed these results to the use of a high dose of n-3 PUFAs (12). Subsequent studies in C57BL/6 and SMA Δ3/-/- mice consuming lower doses of n-3 PUFAs showed the same effect; that is, n-3 PUFAs exerted an immune-enhancing effect on ex vivo B-cell activation with LPS stimulation (13, 14). Therefore, the goal of this study was to test the hypothesis that n-3 PUFAs could exert an immune-enhancing effect on B cells upon in vivo stimulation with a T-independent antigen.

We first tested the impact of n-3 PUFAs in the absence and presence of antigen stimulation on murine B-cell subsets in the bone marrow and spleen, in addition to surface and circulating antibody levels. We then tested the potential immune-enhancing properties of n-3 PUFAs in a model of diet-induced obesity. The rationale for studying B-cell phenotypes and antibody production with mice consuming high-fat diets was that obese individuals respond poorly to vaccinations and infections (15–18). Supporting studies in rodents show obesity is associated with impaired antibody production upon viral infection, although the impact of obesity on B-cell phenotypes is far less known (18, 19). Therefore, this clinical population could benefit as n-3 PUFAs can boost humoral immunity. Our data reveal that n-3 PUFAs, upon antigen stimulation, enhance the frequency of select B-cell phenotypes, accompanied by an increase in surface and/or circulating IgM antibody production in lean and obese mice relative to controls.

MATERIALS AND METHODS

Mice and diets

Male C57BL/6 mice were fed a purified control low-fat diet enriched in soybean oil (Harlan-Teklad) or a diet enriched in menhaden fish oil as previously described for four weeks (19, 20, 21). Briefly, 1.3% of the total kilocalories from the fish oil was from docosahexaenoic acid (DHA) and 2% from eicosapentaenoic acid (EPA). For diet-induced obesity studies, mice were fed for 10 weeks the control and low-fat n-3 PUFA diets described above in addition to a high-fat diet and a high-fat diet + fish oil providing 1.3% and 2% of total kilocalories from DHA and EPA, respectively. The dose of n-3 PUFAs corresponded to the equivalent of pharmacological levels in humans (~4–5 g of n-3 PUFAs per day), which is used in clinical trials and in the treatment of elevated triglycerides (22, 23). Both of the high-fat diets were 45% of total kilocalories from fat, which is an adequate representation of high-fat consumption in humans in the Western population (23). The composition of the diets is shown in Table 1. Mice were euthanized via CO2 inhalation followed by cervical dislocation. All of the experiments with mice fulfilled the guidelines established by the East Carolina University Brody School of Medicine for euthanasia and humane treatment.

Shotgun lipidomics

Cells lyophilized in PBS were first subjected to monophasic chloroform extraction, then the extracts were dried under a stream of nitrogen, washed twice using 10 mM ammonium bicarbonate, dried completely under a stream of nitrogen prior to dissolving in 250 μl of 4:2:1 isopropanol/methanol/chloroform, and stored at −80°C. Prior to mass spectrometry analysis, 10 μl of each extract and 2 μl of 10 mM phosphatidylcholine PC(14:0/14:0) internal standard were placed into a Whatman Multichem 96-well plate (Sigma Aldrich, St. Louis, MO) and dried under nitrogen, then subjected to sequential derivatization of aminophospholipids with 13C5-C5,SS-dimethylthiobutanoylhydroxysuccinimide ester (13C5-DMBNHS) and plasmalogen containing lipids with iodine and methanol, as previously described (24, 25). After washing twice with 10 mM ammonium bicarbonate and dried, samples were resuspended in 40 μl 4:2:1 isopropanol/methanol/chloroform + 20 mM ammonium formate and introduced to a high-resolution/accurate mass Thermo LTQ Orbitrap Velos (Thermo Scientific, San Jose, CA) mass spectrometer using an Advion Nanomate Triversa (Advion, Ithaca, NY) nano electrospray source. Mass spectra (MS) were acquired for 2 min at 100,000 mass resolving power in positive ionization mode. Identification and relative quantification of individual lipid ions (i.e., assignment of the head group identity and acyl/alkyl chain linkage type and total carbons/double bonds) from the MS spectra was performed using LIMSA (26), as previously described (24). Higher-energy collisional dissociation (HCD)-MS/MS of polyunsaturated lipid ions containing at least 4 degrees of unsaturation were acquired for 30 s, each in negative ionization mode at 100,000 mass resolving power. The relative fatty acid content within individual PC and phosphatidylethanolamine (PE) polyunsaturated lipids was then assigned using the relative abundances of the fatty acid anions formed by HCD-MS/MS.

| Ingredients                  | Control | Low Fat | Low Fat + n-3 PUFA | High Fat | High Fat + n-3 PUFA |
|------------------------------|---------|---------|--------------------|----------|--------------------|
| Casein                       | 185.0   | 185.0   | 205.0              | 205.0    |
| L-cystine                    | 2.5     | 2.5     | 3.0                | 3.0      |
| Corn starch                  | 369.98  | 369.98  | 98.94              | 98.94    |
| Maltodextrin                 | 140.0   | 140.0   | 140.0              | 140.0    |
| Sucrose                      | 150.0   | 150.0   | 225.0              | 225.0    |
| Cellulose (fiber)            | 50.0    | 50.0    | 24.0               | 24.0     |
| Soybean oil                  | 50.0    | 50.0    | 15.0               | 15.0     |
| Anhydrous milk fat           | 0.0     | 0.0     | 225.0              | 160.0    |
| Fish oil                     | 0.0     | 50.0    | 0.0                | 65.0     |
| Mineral mix, AIN-93M-MX      | 35.0    | 35.0    | 44.0               | 44.0     |
| Vitamin mix, AIN-93-VX       | 15.0    | 15.0    | 19.0               | 19.0     |
| Choline bitartrate           | 2.5     | 2.5     | 3.0                | 3.0      |
| TBHQ, antioxidant            | 0.02    | 0.02    | 0.06               | 0.06     |

Values are grams per kilogram.
In vivo injections and serum collection

Mice were fed the control and experimental diets for three or nine weeks, and then injected intraperitoneally with saline or 1 µg trinitrophenylated-lipopolysaccharide (TNP-LPS, Biosearch Technologies). The dose of TNP-LPS was selected to elicit a mild immune response (27, 28). Mice continued to consume the diets for an additional seven days when blood was collected using a capiject tube (Fisher) coated with a serum separator. The blood was centrifuged at 1,300 rpm for 15 min at room temperature, and the serum was collected and frozen for subsequent analyses.

Isolation of splenic B cells

B220+ B cells were purified from splenocytes with a negative-selection microbead kit (Miltenyi Biotec) as previously described (13, 20). Splenic B cells (0.5 × 10^6) were transferred to a 96-well plate and stained with a combination of B220-FTTC (Miltenyi Biotec), IgM-PE (Southern Biotech), CD40-PerCP/Cy5.5 (Biolegend), IgD-APC (Biolegend), CD21-APC/Cy7 (Biolegend), MHC class II-PE (Bio X Cell), and CD19-PerCP/Cy5.5 (Biolegend) in 1× PBS supplemented with 0.1% BSA for 20 min on ice. The major splenic B-cell populations analyzed were IgM^+IgD^+CD21^- (transitional 1), IgM^+IgD^-CD21^+ (transitional 2/follicular), IgM^+IgD^-CD21^- (premarginal zone), and IgM^+IgD^-CD21^- (marginal zone) (9, 29–31).

Isolation of bone marrow cells

Bone marrow was obtained from the mouse tibia and femur. Each leg was removed from the mouse, degloved, and placed in RPMI 1640 1× media (Mediatech) supplemented with 5% heat-inactivated defined fetal bovine serum (FBS) (Hyclone), 2 mM L-glutamine, and 1% penicillin/streptomycin. Muscle tissue was inactivated defatted inactivated defatted defatted inactivated defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatted defatt
n-3 PUFAs exerted differential effects on the percentage of bone marrow B cells in the absence and presence of antigen

We next tested the effects of n-3 PUFAs on the percentage of B cells in the bone marrow (Fig. 2A and supplementary Fig. II-B). Compared with the control diet, n-3 PUFAs decreased the percentage of naïve B220<sup>hi</sup>IgM<sup>-</sup> cells by 22% and mature B220<sup>hi</sup>IgM<sup>+</sup> cells by 13%, but they had no effect on pre/pro B220<sup>lo</sup>IgM<sup>-</sup> B cells (Fig. 2A). Upon antigen stimulation, n-3 PUFAs decreased the percentage of naïve B220<sup>hi</sup>IgM<sup>-</sup> cells by 24% with no effect on pre/pro B220<sup>lo</sup>IgM<sup>-</sup> or mature B220<sup>hi</sup>IgM<sup>+</sup> cells (Fig. 2B).

n-3 PUFAs increased surface IgM

Flow cytometry analysis of the MFI showed that surface IgM, IgD, and CD19 expression were unchanged with n-3 PUFA intervention (Fig. 3A). Upon antigen stimulation, surface IgM (Fig. 3B) was elevated by 13%, and CD19 was decreased by 9% on splenic B cells with n-3 PUFAs. IgD surface expression was not influenced by n-3 PUFAs (Fig. 3B).

Circulating levels of serum TNP-LPS-specific IgM were not modified in response to n-3 PUFAs relative to the control diet (Fig. 3C).

n-3 PUFAs enhanced transitional and marginal zone B-cell subsets in lean and obese mice upon long-term n-3 PUFA intervention

The next goal of this study was to determine whether n-3 PUFAs could exert immune enhancing effects in the context of diet-induced obesity. To promote obesity, mice were fed various diets for 10 weeks. This promoted a 22% weight gain in mice fed a high-fat diet relative to the control diet (supplementary Fig. III). Incorporation of n-3 PUFAs into the high-fat diet also resulted in significant weight gain by 30% relative to the control (supplementary Fig. III). Furthermore, Echo-MRI measurements on a few animals revealed an increase in the percentage of fat mass of mice consuming the high-fat and high-fat + fish oil diets compared with the low-fat control and low-fat n-3 PUFA diets (data not shown). To be consistent with our aforementioned
The number of splenocytes was not increased with any of the experimental diets (Fig. 4A). The high-fat diet + n-3 PUFAs increased the number of B cells by 47% relative to the control diet (Fig. 4A). Phenotype analysis revealed that the n-3 PUFA and the high-fat diet + n-3 PUFAs diets increased the percentage of IgM+IgD+CD21+ (transitional 1) B cells by 43% and 57%, respectively (Fig. 4B). The high-fat diet also increased the percentage of transitional 1 B cells by 33% (Fig. 4B). The high-fat + n-3 PUFA diet lowered the percentage of IgM+IgD−CD21− (premarginal zone) cells by 10% (Fig. 4B) relative to the control. The frequency of the IgM+IgD−CD21− (transitional 1) and IgM+IgD−CD21− (marginal zone) B cells was elevated by 69% and 55%, respectively, with the high-fat + n-3 PUFA diet relative to the control diet (Fig. 4C). When analyzing the percentage of bone marrow populations with n-3 PUFA intervention in the context of obesity, we observed no change in the percentage of pre/pro, naïve, or mature B cells (Fig. 5).

**DISCUSSION**

The purpose of this study was to determine the functional role of n-3 PUFAs on in vivo B-cell phenotypes and antibody production. The field has focused heavily on the impact of dietary n-3 fatty acids on cell-mediated immunity (6). This study opens a new avenue of research by demonstrating that n-3 PUFAs can target B cells in vivo and specifically improve murine antibody production to a T-independent
n-3 fatty acids enhance IgM levels

Antigen in the context of an obesogenic diet. B cells have recently emerged as major players in the etiology of several diseases (10, 32, 33). Thus, it is vital to determine how long-chain n-3 PUFA regulate B-cell phenotypes and thereby their function, given that individuals are increasingly consuming marine n-3 PUFA as over-the-counter or prescription supplements.

Implications of enhancing transitional and marginal zone B cells

A significant advancement from this study is that n-3 PUFA increased the frequency of transitional 1 B cells in the absence of antigen. Transitional 1 B cells are either eliminated through negative selection in response to strong B-cell receptor signals or with appropriate signals will progress to transitional 2 B cells and ultimately become mature follicular B cells (34). Since the data show that n-3 PUFA are promoting the formation of this subset in the absence of antigen, these cells may be poised to become T2 or mature B cells, which could have potential benefits in boosting B-cell-mediated responses to various T-independent and T-dependent antigens that are of relevance for humans. However, increased T1 cells with long-chain n-3 PUFA could also be detrimental for some diseases. For instance, lupus erythematosus and Sjögren’s syndrome are characterized by an increased number of transitional B cells (35). Consumption of long-chain n-3 PUFA for these select individuals could exacerbate their disease.

Upon antigen stimulation, we measured an increase in the number of all B-cell subsets. Select studies with CD23 as a marker showed that the majority of cells in the IgM+IgD+CD21− (transitional 1), IgM+IgD+CD21+ (transitional 2/follicular), IgM+IgD−CD21− (pre-marginal zone), and IgM−IgD−CD21− (marginal zone) subsets upon antigen stimulation. Data are from six independent experiments. Letters that do not match indicate statistical significance (P < 0.05).

The role of n-3 PUFA in enhancing IgM in lean and obese mice

B cells from mice fed n-3 PUFA for 4 weeks displayed increased surface but not circulating IgM levels. The lack of an increase in circulating IgM levels could be due to several reasons. We assayed IgM levels a week after immunization and perhaps n-3 PUFA were increasing IgM levels at other time points after immunization. The rationale for selecting one week after immunization was that TNP specific IgM levels are highest at this time point (30). Furthermore, n-3 PUFA may be better at promoting immune enhancing effects in response to secondary immunizations. We did not conduct secondary immunizations since TNP-LPS does not promote a robust secondary response.
One concern may be that administration of our high-fat diet did not completely model obesity, which is a multi-faceted disease that manifests in response to dietary, genetic, and/or environmental factors (44). We defined obesity in the context of excess nutrition leading to increased fat mass, which was ensured via Echo-MRI and total body weight measurements relative to a lean control diet. Nevertheless, future studies will need to address the extent of the obesity phenotype including adipocyte hyperplasia, the degree of adipose inflammation and possible impairment in insulin sensitivity. This will aid in determining whether the high-fat diet itself or obesity is driving the reduction in IgM levels.

The reduction in IgM levels in response to the high-fat diet were consistent with a recent study by Winer et.al (33). In their study, they report a reduction in IgM levels from obese mice that consumed a high-fat diet for ~8 weeks in the absence of antigen stimulation (33). In addition, the decrement in IgM levels were shown to be a ramification of increased class-switched mature B cells resulting in elevated IgG2 autoantibodies. In our study we did not measure class-switched autoantibodies. Interestingly, B-cell phenotypes from the obesity model in our study remained unchanged compared with the control with the exception of an increased percentage of transitional 1 B cells. This aspect of our study did not agree with the study by Winer et. al., which showed a decrease in the percentage of transition 1 B cells in the spleen but an increase in the adipose (33). The differences between studies could be due to the levels of fat used and the duration of administration of the high-fat diets. It is possible that phenotypic changes were occurring in the adipose and even other B-cell subsets, including B1a and B1b B cells, with the high-fat diets in our model system, which we aim to investigate in the future.

The n-3 PUFA diet restored antibody production in the context of obesity compared with the high-fat diet alone and elevated IgM levels in lean mice relative to lean controls. These data raise the exciting possibility that n-3 PUFAs may have potential clinical applications not only in suppressing inflammation, as many studies suggest, but also in boosting immunity by targeting B cells (6). It is interesting to note that the elevation in IgM in the lean condition was time dependent (i.e., we measured an effect at 10 weeks but only a trend at 4 weeks of feeding). Similarly, we observed a decrease in IgD surface expression subsequent to the 10-week feeding period that was not observed at 4 weeks. We also observed a reduction in naïve and mature B cells in the absence of antigen stimulation but only a reduction in naïve B cells in bone marrow after 4 weeks of n-3 PUFA feeding. The effects on the bone marrow were abolished after 10 weeks of feeding. Thus, these finding suggest that the effects of n-3 PUFAs are time and antigen dependent. In the case of the bone marrow, n-3 PUFAs could be targeting B-cell receptor signaling and/or factors such as IL-7 that regulate B-cell development (45). Alternatively, n-3 PUFAs may be targeting the recirculating pool of B cells in the bone marrow.

One possible explanation of increased circulating IgM could be due to decreased CD19 surface expression, a
marker shown to regulate B-cell development (46). Indeed, we observed decreased CD19 expression in the bone marrow as well (data not shown). Tsitsikov et al. reported that decreased CD19 signaling resulted in increased circulating IgM, suggesting that the observed decrease in surface expression may be correlated with increased circulating antibody (47).

Finally, enhanced IgM production with n-3 PUFAs may have clinical benefits for atherosclerosis. Natural IgM antibodies have been shown to bind oxidized low-density lipoprotein and protect against atherosclerosis (48, 49). n-3 PUFAs could be developed as adjuvant therapy to boost IgM levels. Therefore, it will be of utility to test the impact of n-3 PUFAs on B1a-derived IgM antibodies to determine whether similar effects are measured as observed in this study.

In summary, we show that n-3 PUFAs increased the frequency of B cells upon short-term intervention and, in the context of high-fat feeding, upon antigen stimulation. Moreover, n-3 PUFAs increased IgM levels in lean mice and rescued the decrement in antibody production of obese mice. These findings open the possibility of testing various n-3 PUFAs for boosting B-cell-mediated immune responses. This may have significant beneficial effects for select clinical populations, such as the obese.

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