Dietary (n-3) Polyunsaturated Fatty Acids Do Not Affect the In Vivo Development and Function of Listeria-Specific CD4+ and CD8+ Effector and Memory/Effector T Cells in Mice¹,²

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ABSTRACT We previously reported that in a mouse model, a diet high in (n-3) PUFA diminishes host survival following an infection from Listeria monocytogenes, a gram-positive bacterial pathogen. In this study we investigated the impact of (n-3) PUFA on the adaptive immune response to L. monocytogenes. BALB/c mice were fed experimental diets either devoid of or rich in (n-3) PUFA from fish oil for 4 wk and then infected with 10⁶ actA-deficient L. monocytogenes. At 7 and 35 d postchallenge, effector and memory/effector T cells in the spleen were enumerated by flow cytometry. Surprisingly, the number of Listeria-specific CD4⁺ and CD8⁺ effector and memory/effector T cells in the spleen was not affected by (n-3) PUFA. Also, the effector cells derived from mice fed either diet were equally capable of conferring protective immunity upon adoptive transfer to naive recipients. Despite our previous data, which demonstrated that (n-3) PUFA profoundly impaired host resistance to L. monocytogenes, pathogen-specific T cell responses were not substantially affected by dietary (n-3) PUFA.


KEY WORDS: (n-3) PUFA • fatty acid • infection • Listeria • T lymphocytes • mice

Listeria monocytogenes is a ubiquitous, gram-positive bacterium that causes sepsis and meningitis in immunocompromised patients and a serious maternal/fetal infection in pregnant women (1,2). With a mortality rate of 20–30%, listeriosis is the leading cause of death from all food-borne pathogens (3). Murine listeriosis has been used by many investigators as a model system to define the various factors and cells that are involved in the host response to intracellular pathogens (4,5). Cells of the innate immune system (e.g., macrophages, neutrophils, and natural killer cells) play a critical role in controlling bacterial growth during the initial stages of infection and direct the ensuing adaptive immune response (6,7). The adaptive immune response consists of pathogen-specific T lymphocytes that resolve acute infection and are capable of adoptively transferring protective immunity to a naive recipient (8). Long-term anti-listerial immunity is mediated by CD8⁺ memory T cells (9), which can be found in lymphoid and nonlymphoid tissues (10). This model provides an ideal framework for studies to determine how nutrition can affect innate and adaptive immune mechanisms associated with host resistance to infection from an intracellular pathogen.

(n-3) PUFA have anti-inflammatory and immunomodulatory activities (11–15). Among the most well-documented effects of (n-3) PUFA are reductions in pro-inflammatory eicosanoids and cytokine production as well as T cell proliferation. Whereas some individuals suffering from various autoimmune and chronic inflammatory diseases may benefit from such changes, diminished inflammatory responses in others may impair infectious disease resistance (16). In fact, feeding mice a diet rich in (n-3) PUFA from fish oils makes them highly susceptible to infection from L. monocytogenes, as evidenced by diminished survival and impaired bacterial clearance (17,18). During murine listeriosis, high (n-3) PUFA intake is associated with substantial reductions in major histocompatibility complex expression (MHC)³ (19), and IL-12 and IFNγ biosynthesis (20). These elements of the innate immune system are critical for host resistance against this pathogen (21,22). Given the strong symbiotic relation between innate and adaptive immune responses and the profound impairment of primary host resistance against L. monocytogenes, we hypothesized that dietary (n-3) PUFA would adversely affect the normal development of the adaptive immune response to this pathogen. Our previous investigation showed that, although high (n-3) PUFA intake was associated
with severe impairment of primary host resistance against *L. monocytogenes*, the ability of surviving mice to generate protective immunity appeared to be mostly intact (23). The present investigation sought to determine more directly the impact of dietary (n-3) PUFA on in vivo adaptive T cell responses to *L. monocytogenes*.

**MATERIALS AND METHODS**

**Mice and diets.** Specific pathogen-free, weaning female BALB/cAnNHsd mice were purchased (Harlan) and housed 4 per cage in the Animal Sciences Research Center. Mice had free access to autoclaved water and commercial mouse diet (Purina 5008 Rodent Chow, Purina Mills). After a 1-wk acclimation period, mice were randomly assigned to 1 of 2 experimental diet treatment groups. The experimental diets were nutritionally complete and were based on the semipurified AIN-93G diet (24), modified to contain 18% fat (wxw) while maintaining the nutrient-to-energy ratio of the original lower fat diet. The 2 diets were identical except for the major dietary fat source: lard [devoid of (n-3) PUFA] or menhaden fish oil [rich in (n-3) PUFA]. The fatty acid composition of the experimental diets was reported previously (23). Mice were fed experimental diets for 28 d prior to immunization or challenge. Care and treatment of mice were in accordance with federal guidelines and overseen by the Animal Care and Use Committee of the University of Missouri-Columbia.

**Bacteria.** Wild-type (10403S) and actA-deficient (DP-L3078) strains of *L. monocytogenes* were obtained as a generous gift from Daniel Portnoy, University of California-Berkeley, and were grown in tryptic soy broth at 37°C for 12 to 18 h prior to use. After 3 washes with sterile PBS (Life Technologies), the concentration of the bacteria was estimated via optical density and then diluted with PBS to the desired infectious dose. The actual dose administered in each experiment was confirmed by plate counts using blood agar plates. Bacterial challenges were given i.v. in a final volume of 0.2 mL PBS via the lateral tail vein. For primary challenge, 1 × 10⁸ colony-forming units (CFU) of an attenuated strain of *L. monocytogenes* (i.e., actA-deficient *L. monocytogenes*) was used. This mutant strain of *L. monocytogenes* is unable to express functional actA protein; thus it cannot polymerize an actin tail and spread intracellularly to adjacent cells (25). Infection with this organism elicited a strong adaptive immune response, as reported by others (26). One important benefit of using an attenuated strain was to avoid the mortality in the (n-3) PUFA-fed animals noted in our previous studies (23). Thus, in the present study we eliminated the potential effect of survivor bias in our investigation of the adaptive immune response. Adoptive transfer recipients were challenged with 5 × 10⁶ CFU of wild-type *L. monocytogenes* 10403S. For Listeria-specific IFNγ assays, antigen-presenting cells (APC) were infected with freshly thawed *L. monocytogenes* 10403S from stock frozen and stored at −70°C in 50% solution of PBS and glycerol (Sigma). Bacterial loads in the spleen and liver were determined by plating 10-fold serial dilutions of tissue homogenates on Listeria-supportive McBride agar plates (Difco, BD).

**Preparation of splenocytes.** Spleens were removed from mice fed lard or fish oil diets at the indicated times after primary infection with actA-deficient *L. monocytogenes*. Mice were anesthetized, spleens aseptically removed, and splenocytes isolated as described previously (27). For cytokine capture assays splenocytes were resuspended in HEPES-buffered RPMI medium (Gibco BRL Products, Invitrogen) containing 100 mL/L fetal bovine serum (U.S. Bio-Technologies) and 2 mmol/L L-glutamine (herein referred to as complete medium). Prior to their use in adoptive transfer studies, splenocytes were resuspended in sterile PBS.

**IFNγ production by cytokine secretion assay.** Pathogen-specific IFNγ production by CD4⁺ and CD8⁺ T cells was used as a marker to identify effector and memory effector T lymphocytes. J774A.1 cells, a murine H-2Kd macrophage cell line (ATCC TIB 67; American Type Cell Culture), were infected with *L. monocytogenes* and used as APCs to elicit antigen-specific IFNγ production. J774 cells maintained in complete RPMI 1640 medium without antibiotics were infected with *L. monocytogenes* 10403S at a multiplicity of infection of 10:1. After 25 min at 37°C, the infection was terminated by the addition of gentamycin (final concentration of 50 mg/L). APCs were incubated at 37°C for 4 h prior to the addition of splenocytes. At 7 or 35 d postchallenge with actA-deficient *L. monocytogenes*, effector or memory splenocyte preparations were isolated (respectively), and 5 × 10⁶ cells were added at a 10:1 ratio to infected and uninfected (control) APCs in 48-well plates. For nonspecific stimulation, cells were cultured with low-endotoxin, plate-bound anti-CD3e and soluble anti-CD28 (both from SouthernBiotech). Regardless of stimuli, cells were cocultured for 16 h at 37°C and then harvested for the cytokine secretion assay. Cells were transferred to 50-mL conical centrifuge tubes, washed twice in cold PBS with 0.5% bovine serum albumin and 2 mmol/L EDTA (Sigma), and labeled with anti-IFNγ capture reagent according to the manufacturer’s instructions (Milenyi). Tubes were filled with warm medium, fixed to a rotary mixer (DynaL), and incubated at 37°C under continuous rotation for 45 min. After this secretion period, cells were centrifuged, resuspended in cold PBS buffer, and labeled with phycoerythrin-conjugated anti-IFNγ detection Ab (Milenyi), rat anti-mouse CD4 (Clone CT-CD4) conjugated to fluorescein isothiocyanate, and rat anti-mouse CD8a (Clone CT-CD8a) conjugated to allophycocyanin (all from Caltag). Dead cells were excluded from flow cytometric analysis by the addition of propidium iodide at 0.5 mg/L (BD Pharmingen). Cells were analyzed on a FACSComp flow cytometer (BD Bioscience). Data collected on a minimum of 50,000 live cells per sample were analyzed using CellQuest software (version 3.1, BD Pharmingen).

**Adaptive transfer of immunity.** Donor cells were isolated from mice fed experimental diets for 4 wk and then adoptively transferred into recipients maintained on a commercial ration low in (n-3) PUFA diet (i.e., Purina 5008 Rodent Chow). PBS or crude effector lymphocytes (i.e., 5 × 10⁶ splenocytes isolated from mice 7 d postchallenge) were injected into a lateral tail vein of age- and sex-matched naive BALB/c mice. Within 1 h of cell transfer, all recipient mice received 5 × 10⁶ CFU of wild-type *L. monocytogenes* (10403S), a dose equivalent to the 50% lethal dose (LD₅₀) for a naive BALB/c mouse (28). After 36 h, bacterial loads in the spleen and liver were determined by plate counts.

**Statistical analysis.** Data were analyzed and graphed using GraphPad Prism, version 3.0a (GraphPad Software). The effect of dietary fat source on IFNγ secretion was analyzed by unpaired t test. The organ bacterial load data were log₂ transformed prior to analysis. The effects of dietary fat source and immunization on recipient bacterial load and other variables were analyzed using 2-way ANOVA with the Bonferroni post hoc test. P < 0.05 was considered significant.

**RESULTS**

(n-3) PUFA effects on body weight, spleen size and cell yield, and nonspecific IFNγ production by splenic CD4⁺ and CD8⁺ T cells. (n-3) PUFA had no impact on the final body weight of these mice (Table 1). Mice allowed to recover from the primary infection continued to grow normally through 35 d postchallenge. Spleen weight at both 7 and 35 d postchallenge was substantially greater for mice fed (n-3) PUFA compared to those fed the lard diet. At 35 d following the initial infection, the splens of mice fed the lard-containing diet had returned nearly to normal size, whereas those from fish oil–fed mice remained substantially enlarged (Table 1). Total splenic cell yield was greater in (n-3) PUFA mice at 35 d postchallenge than in lard-fed mice at this time (*P < 0.05*). In contrast, the percentage of splenic CD8⁺ T lymphocytes that produced IFNγ in response to a noncognate stimulus (i.e., α-CD3/α-CD28) from mice either 7 or 35 d postchallenge with *L. monocytogenes* in contrast, the percentage of splenic CD8⁺ T lymphocytes that produced IFNγ in response to α-CD3/α-CD28 stimulation from mice 7 but not 35 d postchallenge was diminished by (n-3) PUFA (*P < 0.05*).
Effects in mice of dietary (n-3) fatty acids on body weight, spleen weight, splenic cell yield, and percentages of CD4⁺ and CD8⁺ T cells and of those that produce IFNγ in response to ex vivo stimulation with αCD3/αCD28 at 7 and 35 d following a primary infection with L. monocytogenes.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>7 d postchallenge</th>
<th>35 d postchallenge</th>
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<tr>
<td></td>
<td>Lard</td>
<td>Fish oil</td>
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<tr>
<td></td>
<td>Lard</td>
<td>Fish oil</td>
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<tr>
<td>Body, g</td>
<td>21.2 ± 0.2⁹</td>
<td>21.5 ± 0.4⁹</td>
</tr>
<tr>
<td>Spleen, g</td>
<td>0.25 ± 0.01⁹</td>
<td>0.30 ± 0.01⁹</td>
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<td>Spleen cell yield, (×10⁷)</td>
<td>9.9 ± 0.7⁹</td>
<td>8.3 ± 0.4⁹ab</td>
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<tr>
<td>CD4⁺ cells, %</td>
<td>18.0 ± 0.9</td>
<td>18.3 ± 1.2</td>
</tr>
<tr>
<td>CD8⁺ cells, %</td>
<td>8.9 ± 0.7</td>
<td>8.3 ± 0.7</td>
</tr>
<tr>
<td>CD4⁺ IFNγ⁺ cells, %</td>
<td>4.6 ± 0.4</td>
<td>3.8 ± 0.5</td>
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<tr>
<td>CD8⁺ IFNγ⁺ cells, %</td>
<td>2.1 ± 0.2</td>
<td>1.6 ± 0.2⁶</td>
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<td></td>
<td>28.9 ± 1.2⁹</td>
<td>27.1 ± 1.0⁹</td>
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<td>0.12 ± 0.01⁹c</td>
<td>0.23 ± 0.01⁹b</td>
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<td></td>
<td>4.2 ± 0.2c</td>
<td>7.8 ± 0.3⁹b</td>
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<td></td>
<td>21.4 ± 0.5</td>
<td>22.8 ± 1.1</td>
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<td>8.1 ± 0.3</td>
<td>7.9 ± 0.5</td>
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<td></td>
<td>5.6 ± 0.9</td>
<td>5.9 ± 1.2</td>
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<tr>
<td></td>
<td>2.0 ± 0.3</td>
<td>1.8 ± 0.4</td>
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1Values are means ± SEM (n = 15 mice per diet treatment group at 7 d and n = 22-26 per diet group at 35 d). Means within rows not sharing a superscript letter differ, P < 0.05 (2-way ANOVA). *Different from the lard mean at that time, P < 0.05 (unpaired t test).

(n-3) PUFA and adoptive transfer of immunity. We used adoptive transfer as a means to assess the in vivo functional activity of the Listeria-specific T cells generated during an adaptive immune response in an (n-3) PUFA-rich environment. To accomplish this, splenocytes were isolated from mice fed experimental diets and then adoptively transferred into naive recipients. The recipients were fed a low (n-3) PUFA diet (i.e., Purina 5008 Rodent Chow) both before and after the adoptive transfer. Bacterial loads in the spleen and liver were evaluated 36 h postchallenge. Compared to naive mice that did not receive immune cells, recipients of 7-d effector cells had a greater capacity (~1 log₁₀) to clear bacteria from both the spleen and the liver (Fig. 3, P < 0.001). However, bacterial clearance did not differ between dietary groups. The data

FIGURE 1 The impact of (n-3) PUFA on the frequency of Listeria-specific CD4⁺ and CD8⁺ effector T cells in mice 7 d following a primary challenge with L. monocytogenes. A 2-D dot plot of a representative spleen cell preparation from an individual mouse cocultured with uninfected (A, inset) or L. monocytogenes-infected APCs (A) and stained with αCD4 and αIFNγ antibodies is shown. Scattergrams show the impact of diet (n-3) PUFA on the frequency of Listeria-specific CD4⁺ (B) and CD8⁺ (C) effector T cells at 7 d postchallenge. The horizontal lines represent the mean within each diet group (n = 14 to 15).
shown in Figure 3 are from 1 of 2 independent experiments with similar results obtained in both studies.

**DISCUSSION**

During a primary infection with *L. monocytogenes* there is a symbiotic relation between the innate and adaptive immune responses (7). In light of the severe impairment of host resistance, substantial reduction in certain innate immune cell functions (i.e., MHC expression and IL-12 biosynthesis), and the ability of (n-3) PUFA to reduce in vitro lymphocyte proliferation, we hypothesized that high dietary (n-3) PUFA intake would impair the development of pathogen-specific effector and memory T cells. The generation of *Listeria*-specific CD4$^+$ and CD8$^+$ effector/memory T cells in the spleen was not adversely affected by (n-3) PUFA intake. Further, the ability of Ag-experienced cells to confer protective immunity to naive recipients was not adversely affected by (n-3) PUFA intake. These data suggest that the effects of (n-3) PUFA on the host immune response to *L. monocytogenes* infection occur primarily at the level of the innate, and to a much lesser extent the adaptive, immune system.

We assessed pathogen-specific T lymphocytes using ex vivo IFN$\gamma$ production as a marker for effector and memory/effector T cells (29–31). Previously, we reported that (n-3) PUFA feeding had no effect on ex vivo, *Listeria*-specific IFN$\gamma$ production by splenocytes (23). However, the stimulation of splenocytes in bulk culture does not permit the contribution of different lymphocyte subsets to be distinguished. In this report, we used flow cytometry to measure *Listeria*-specific IFN$\gamma$ production by CD4$^+$ and CD8$^+$ effector and memory/effector T cells isolated at 7 and 35 d postchallenge, respectively. Our new results indicate that high (n-3) PUFA intake prior to and during a primary infection in mice with *L. monocytogenes* does not alter the frequency or absolute number of *Listeria*-specific CD4$^+$ and CD8$^+$ effector T cells in the spleen 7 d postchallenge. In contrast to the antigen-driven response, non-specific IFN$\gamma$ production by CD8$^+$ T cells stimulated by αCD3/αCD28 was diminished by (n-3) PUFA intake (25%, $P < 0.05$). Stimuli-dependent modulation of T cell proliferation by (n-3) PUFA has been reported previously (32,33). Caution should be exercised when interpreting data from (n-3) PUFA studies that use different T cell stimuli.

In contrast to effector T cells, *Listeria*-specific memory T cell development and/or survival appeared to be modestly affected by (n-3) PUFA. The exact mechanisms involved in memory cell differentiation are poorly understood, but memory cells are believed to derive from antigen-specific effector CD4$^+$ and CD8$^+$ T lymphocytes that survive growth factor–induced clonal contraction (34,35). The number of memory T cells generated is closely correlated with the extent of effector cell proliferation during the primary response (36). At 35 d postchallenge, mice fed the high (n-3) PUFA diet were found to have a 30–50% lower frequency of *Listeria*-specific CD4$^+$ and CD8$^+$ memory/effector T cells in the spleen. However, the absolute number of *Listeria*-specific CD4$^+$ and CD8$^+$ memory/effector T cells per spleen at 35 d postchallenge did not differ between diet treatment groups. This apparent paradox can be explained in part because of the impact that consuming a (n-3) PUFA-rich diet has on spleen size and cellularity following a primary infection with *L. monocytogenes*.

In our study we noted that compared to mice fed an (n-3) PUFA-free diet, fish oil–fed mice have heavier spleens with more immune cells in them at 35 d postchallenge. Splenomegaly associated with fish oil feeding in mice has been reported previously (19,37). This increase in spleen size is associated with increased cellularity, with little or no gross effect on the proportion of cell types found. In contrast, fish oil feeding of rats substantially increased splenic red pulp as well as macrophages and B cell numbers (38). In the context of diet manipulation of host infectious disease resistance, the absolute number of effector and effector/memory cells may have more...
physiologic relevance than relative abundance (i.e., percentage of cells). However, a limitation of the present work is our reliance on the spleen as a sole source of immune cells for T cell analysis. After resolution of a L. monocytogenes infection, the spleen contains only a small percentage of the memory T cells generated in vivo, with large numbers residing in the liver, lungs, and lamina propria of the small intestine (10). Thus it is possible that dietary (n-3) PUFA affected T cell responses in one of these other compartments, but not in the spleen. In the future, we hope to investigate the impact of (n-3) PUFA on T cell development throughout the body.

Our use of whole animals to study nutrient modulation of T cell development and function in vivo is one of the major strengths of this study. The immune response to infection involves a complex network of host cells and humoral factors, which cannot be readily reproduced in vitro (39). During in vivo immune responses participating cells change over time due to cell migration as well as through activation/differentiation processes. Inflammation and infection alter immune cell trafficking through the expression of various adhesion molecules on the surface of vascular endothelial cells (40). The expression of adhesion molecules on endothelial cells as well as on lymphocytes can be affected by dietary (n-3) PUFA (41–43). Such changes resulted in reduced adherence between these 2 cell populations in vitro. Feeding rats a diet rich in (n-3) PUFA from fish oil diminished neutrophil trafficking to the lungs following an in vivo endotoxin challenge (44). Although these data are intriguing, little is known about whether, and how, diet (n-3) PUFA intake might affect T cell trafficking in vivo.

It has been over 40 years since lymphocytes were first shown to be indispensable for immunity to L. monocytogenes (45,46). The protective effect is associated mostly with the cytolytic activity of CD8+ T cells, because cells from IFNγ-knockout mice can transfer protective immunity (47), whereas cells from perforin-knockout mice cannot (48). We used this well-characterized and biologically relevant approach to assess the impact of (n-3) PUFA on the generation of in vivo cytotoxic T lymphocyte (CTL) activity. Effector spleen cells were generated in vivo under conditions that were either rich or poor in (n-3) PUFA. These effector T cells were adoptively transferred to naive recipients fed a control diet [i.e., low in (n-3) PUFA] just prior to a challenge with a high dose of wild-type L. monocytogenes. As expected, substantial protection was afforded to mice receiving effector cells. However, there was no indication that the presence of a (n-3) PUFA-rich environment during effector T cell development adversely affected the in vivo cytolytic function of these cells in recipient mice. These results are consistent with our previous findings that (n-3) PUFA have little to no impact on the generation of immunological protection within the same host (23). Similarly, we previously reported that ex vivo, anti-viral CTL activity in the spleen 7 days following a vaccinia virus challenge was similar in mice fed a diet rich in (n-3) or (n-6) PUFA from fish oil and corn oil, respectively (49). In contrast, others have reported that dietary (n-3) PUFA diminish ex vivo anti-viral CTL (50). Feeding mice a diet containing (n-3) PUFA from fish oil decreased specific lysis of influenza-infected target cells by 50%. Further, addition of unsaturated, but not saturated, free fatty acids to the culture medium of a CTL clone decreased CTL degranulation dose-dependently as measured by esterase release (51). The underlying reason(s) for these discrepancies is unclear at the present time. However, our data suggest that caution should be exercised in the interpretation of in vitro fatty acid immune modulation studies.

In summary, to our knowledge this is the first report to directly investigate the potential impact of (n-3) PUFA on the in vivo proliferation and differentiation of naive CD8+ T cells. Our data provide strong evidence that, despite severely impairing primary host resistance against L. monocytogenes, dietary (n-3) PUFA do not substantially affect the in vivo adaptive immune response. Rather, the diminished host resistance that accompanies high (n-3) PUFA intake in mice must primarily be a consequence of changes in the innate immune system. Future studies will focus on identifying the specific defects in innate immune cell function that are both necessary and sufficient to cause the (n-3) PUFA-mediated reduction in host defense against this common food-borne pathogen.

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LITERATURE CITED


