Omega-3 Polyunsaturated Fatty Acids Impair In Vivo Interferon-γ Responsiveness via Diminished Receptor Signaling

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Background. A high intake of omega-3 polyunsaturated fatty acids (n-3 PUFAs) in mice causes impaired host resistance to *Listeria monocytogenes*. We wished to determine the role of interferon (IFN)–γ signaling in this increased disease susceptibility.

Methods. A feeding trial was conducted with mice unable to produce IFN-γ (IFN-γKO); we provided exogenous recombinant IFN-γ during *L. monocytogenes* challenge. The experimental diets were nutritionally complete and differed only in fat source: lard (devoid of n-3 PUFAs) or menhaden fish oil (rich in n-3 PUFAs).

Results. The administration of IFN-γ significantly enhanced bacterial clearance in IFN-γKO mice fed a diet devoid of n-3 PUFAs but had no effect in mice fed a diet rich in n-3 PUFAs. Ex vivo analysis of immune cells showed that n-3 PUFAs did not affect IFN-γ receptor expression on immune cells. However, on IFN-γ treatment, the phosphorylation of signal transducer and activator of transcription 1 was significantly reduced in peritoneal macrophages isolated from mice fed n-3 PUFAs.

Conclusions. These data suggest that diminished IFN-γ signaling in murine macrophages is one mechanism by which n-3 PUFAs impair host resistance to *L. monocytogenes*. To our knowledge, this is the first report of a nutrient affecting IFN-γ signaling and in vivo responsiveness to this cytokine.

Fish oils, which are rich in omega-3 polyunsaturated fatty acids (n-3 PUFAs), are reported to have anti-inflammatory and immunomodulatory activities [1–4]. Most of the biological activity of these fatty acids is thought to arise from their ability to antagonize the conversion of arachidonic acid to a family of lipid mediators, known as eicosanoids, and to diminish the production of proinflammatory cytokines [5, 6]. More recent findings have suggested that n-3 PUFAs may alter the function of immune cells at the level of gene transcription and cell-membrane dynamics [3]. The enrichment of membrane phospholipids with n-3 PUFAs appears to alter the stability and protein composition of lipid microdomains, which are known as lipid rafts [7, 8]. Interferon (IFN)–γ receptors are among the membrane proteins that are known to be concentrated in lipid rafts [9]. The present article provides, to our knowledge, the first evidence that n-3 PUFAs affect IFN-γ receptor function such that in vivo responsiveness to this cytokine is diminished. These data may help explain why mice fed diets rich in n-3 PUFAs are more susceptible to infection with *Listeria monocytogenes*.

Feeding mice a diet that contains n-3 PUFAs derived from fish oil reduces survival and bacterial clearance after an infectious challenge with *L. monocytogenes* [10–12]. *L. monocytogenes* is an intracellular, gram-positive bacteria that causes infectious disease in humans and animals [13]. Resistance to *L. monocytogenes* is highly dependent on the production of IFN-γ in the host [14, 15]. Mice with a targeted disruption of the IFN-γ or the IFN-γ receptor gene (IFN-γ−/− and IFN-γR-1−/− mice, respectively) are highly susceptible to listeriosis and show marked impairment in their ability to clear these bacteria [16, 17]. Mice fed diets enriched with n-
3 PUFAs have reduced IFN-γ production [18]. One objective of the present study was to access the impact of n-3 PUFA–mediated reduction of IFN-γ production on host resistance to L. monocytogenes. Therefore, IFN-γ−/− mice were used in a feeding trial. After these mice were fed either the control or the n-3 PUFA–rich diet for 4 weeks, they were challenged with L. monocytogenes. Half of the mice in each diet treatment group received daily injections of exogenous IFN-γ throughout the early course of infection. Bacterial clearance from the liver and spleen at the peak of the infection was assessed. It was hypothesized that impaired IFN-γ production played a central role in the increased susceptibility of mice fed fish oil to L. monocytogenes infection. Therefore, it was expected that the administration of exogenous IFN-γ would abrogate most, if not all, of the impairment in bacterial clearance that is typically observed in mice fed n-3 PUFAs.

Furthermore, the second objective of the present study was to determine whether the dietary consumption of n-3 PUFAs alters IFN-γ receptor signaling in murine immune cells. It has been reported elsewhere [19] that total cellular, as well as surface, expression of IFN-γR-1 on peritoneal macrophages and splenocytes was diminished in mice in response to a diet high in n-3 PUFAs. An early and critical step involved in signal transduction after IFN-γR-1 ligation is the phosphorylation of the protein known as signal transducer of activator of transcription 1 (STAT1) [20]. Therefore, peritoneal macrophages were isolated from IFN-γ knockout (KO) mice fed either the control or the high n-3 PUFA diets and were incubated with recombinant (r) IFN-γ, and then Western-blot analysis of phosphorylated STAT1 was performed. The hypothesis was that immune cells from the mice fed large amounts of n-3 PUFAs would show diminished STAT1 phosphorylation, consistent with the reduction in IFN-γR-1 expression.

**MATERIALS AND METHODS**

**Mice and experimental diets.** Specific pathogen–free, weanling female BALB/cAnN selections (wild-type [wt]) and IFN-γ−/− mice on a BALB/c background (IFN-γKO) were purchased (Harlan) and housed, 4 mice/cage, in the Animal Sciences Research Center. The room was maintained on a 12-h light/dark cycle at 23°C and with 40%–50% relative humidity. Mice had free access to autoclaved water and commercial rodent diet (Purina Mills). After a 1-week acclimation period, mice were randomly assigned to 1 of 2 experimental dietary treatment groups. The experimental diets were nutritionally complete and were based on the semipurified AIN-93G diet [21], modified to contain 18% fat (wt/wt) while maintaining the nutrient:calorie ratio of the original lower-fat diet. We chose this level of fat to mimic the average level of fat in the current diet of the US population and to provide a greater level of n-3 PUFA enrichment from a widely used fish oil source.

The 2 experimental diets were identical, except that the major dietary fat sources used were lard or refined menhaden fish oil (gift from Omega Protein). A small amount of corn oil was added to the fish oil (125 g/kg), so that it would match the essential fatty-acid content of lard (i.e., ∼10% linoleic acid). The ingredients and fatty-acid composition of the experimental diets have been reported elsewhere [11]. Unless noted, diet ingredients were purchased from ICN Biomedicals. Both fat sources were stabilized against auto-oxidation by the addition of a synthetic antioxidant (i.e., 0.02 g tertiary-butylhydroquinone/100 g fat). The care and treatment of mice were in accordance with federal guidelines and were overseen by the Animal Care and Use Committee of the University of Missouri, Columbia.

**Bacterial infection.** Mice were challenged with a rapidly growing culture of L. monocytogenes strain EGD (a gift from Charles J. Czuprynski, University of Wisconsin–Madison) that was grown overnight in tryptic soy broth (Difco BD). The culture was washed in PBS, and the concentration was estimated by measurement of the optical density at 595 nm. The bacterial suspension was diluted in PBS to obtain the desired infectious dose of 1.4 × 10⁴ cfu/mouse. The actual dose administered in each experiment was confirmed by plate counts by use of blood agar. Bacterial challenges were administered intravenously (iv) in a final volume of 0.2 mL of PBS via the lateral tail vein.

**Exogenous IFN-γ administration.** After challenge, each IFN-γKO mouse was administered, daily, 0.2-mL iv injections of PBS or 1 µg of murine rIFN-γ (~1 × 10⁶ U/mg, endotoxin <0.1 ng/µg of cytokine by use of the limulus amebocyte lysate method; ebioscience).

**Bacterial clearance.** Viable L. monocytogenes cells in the spleen and liver were counted 3 days after challenge by platting serial 10-fold dilutions of organ homogenates in PBS on McBrine Listeria-supportive agar plates (Presque Isle Cultures). Bacteria load data are expressed as the number of colony-forming units per organ.

**IFN-γR-1 flow cytometry.** Splenocytes were obtained from uninfected wt or IFN-γKO mice. Lymphocytes were isolated by density-gradient centrifugation over Histopaque-1077 (Sigma). Macrophages were harvested from the peritoneum of IFN-γKO mice 4 days after the peritoneal injection of 1 mL of a sterile 3% thioglycollate broth. Cells were washed and suspended in PBS that contained 2% fetal bovine serum (FBS) and 0.1% NaN₃. Staining was performed by use of biotinylated rat anti–mouse IFN-γR-1 or biotinylated rat anti-mouse anti–KLH IgG2a (both from BD Pharamingen), followed by detection with streptavidin-conjugated SensiLight P-3L (Martek). Flow cytometry was performed by use of a BD FACS Vantage (Becton Dickinson), and data were analyzed by use of CellQuest software (version 3.3; Becton Dickinson) at the Cell and Immunology Core facility at the University of Missouri, Columbia.
Western-blot analysis of STAT1 phosphorylation. Resident peritoneal macrophages were harvested and suspended in HEPES-buffered RPMI 1640 medium (Gibco BRL Products, Invitrogen) that contained 40 mL/L FBS, 2 mmol/L l-glutamine, 50,000 U/L penicillin, and 50 mg/L streptomycin. Cells were plated in 6-well plates at $2 \times 10^6$ cells/well. After 2 h of incubation at $37^\circ C$, nonadherent cells were washed away with PBS, and adherent cells were incubated for 30 min with fresh medium and 0 or 20 U/mL rIFN-γ. Cells were washed with PBS and then were lysed by the addition of 0.4 mL of sample buffer (62.5 mmol/L Tris, 10% vol/vol glycerol, 2% wt/vol SDS, and $5 \mu$L/mL protease inhibitor mixture; Sigma). Cell lysates were harvested, sonicated, and heated; the amount of protein present was determined by use of a detergent-compatible BCA kit (Bio-Rad Laboratories). The protein concentration in the samples was normalized, and the samples were separated by SDS-PAGE on a 4%–20% gradient gel at 100 V for 50 min. Proteins were transferred to a nitrocellulose membrane and probed with anti-STAT1 or β-actin, according to manufacturer’s instructions. Detection was performed with an antirabbit–specific secondary antibody conjugated to horseradish peroxidase. Bands were visualized by chemiluminescence using Phototope-HRP Western Blot Detection Kit (Pierce). The quantification of protein bands was determined by use of Kodak 1D Image Analysis Software (Eastman Kodak).

Statistical analysis. The main effects of dietary fat source and exogenous IFN-γ treatment were compared by use of 2-way analysis of variance and the post hoc mean test with Bonferroni’s correction (GraphPad Software). Organ bacteria counts were log$_{10}$ transformed before analysis. Receptor expression and STAT1 phosphorylation data were analyzed by use of an unpaired $t$ test in the context of the dietary fat treatment only.

RESULTS

Body and organ weights. Before the start of feeding experimental diets and before the bacterial challenge, body weights between diet treatment groups were not significantly different (data not shown). Body, spleen, and liver weights measured 3 days after challenge are shown in table 1. The body weight of lard-fed mice that received rIFN-γ was significantly greater than that of other groups of mice ($P < .01$). Spleen weight as a percentage of final body weight was significantly greater in mice fed the diet rich in n-3 PUFAs than in those fed the lard diet but was not affected by rIFN-γ treatment. Similar to spleen weight, the relative liver weight was not significantly affected by rIFN-γ treatment. Mice fed the fish-oil diet and treated with exogenous rIFN-γ had significantly larger livers than mice fed the lard diet, regardless of rIFN-γ treatment.

Organ bacterial loads. Three days after challenge, the number of $L$. monocytogenes in the liver and spleen were enumerated by plate counts (figure 1). In mice given PBS, the fish-oil diet was associated with a 100-fold greater bacterial load in the liver and a 10-fold greater load in the spleen than those in mice fed lard ($P < .001$). Compared with those given PBS, mice fed lard that were given rIFN-γ displayed an enhanced capacity to control bacterial growth in organs, as displayed by $>10$-fold fewer bacteria in both the liver and spleen ($P < .001$). However, mice fed fish oil that were given rIFN-γ had bacterial loads in organs that were not different from those of mice fed fish oil that received only PBS.

IFN-γR expression. The surface expression of IFN-γR-1 was determined by flow cytometry and was found to be expressed ubiquitously on the cell types analyzed (figure 2). High intake of n-3 PUFAs did not affect the expression of IFN-γR-1 on either splenocytes or thioglycollate-elicited peritoneal macrophages isolated from IFN-γKO mice (table 2). Compared with splenocytes derived from wt mice, splenocytes from IFN-γKO mice fed the experimental diets displayed significantly higher levels of IFN-γR-1 expression (data not shown). IFN-γR-1 expression on peritoneal macrophages was relatively high, compared with that on splenocytes, but, as with splenocytes, a high intake of n-3 PUFAs had no effect on the level of IFN-γR-1 expression.

STAT1 phosphorylation. Figure 3A shows a representative immunoblot from a pair of mice within each dietary treatment group. Quantitative image analysis of these data, as well as data from another pair of mice within each diet treatment group, are shown in figure 3B. The total cellular STAT1 content did

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<td>PBS</td>
<td>rIFN-γ</td>
</tr>
<tr>
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<td>Body</td>
<td>$22.1 \pm 1.2$ (7)*</td>
<td>$26.5 \pm 1.2$ (9)*</td>
</tr>
<tr>
<td>Spleen</td>
<td>$1.10 \pm 0.05* $</td>
<td>$1.21 \pm 0.07*$</td>
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<tr>
<td>Liver</td>
<td>$6.36 \pm 0.38*$</td>
<td>$5.79 \pm 0.08*$</td>
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**NOTE.** Data are mean ± SE (no. of animals per group). Organ weights are % of body weight. Within each row, means not sharing a superscript letter are significantly different ($P < .05$, 2-way analysis of variance with post hoc mean tests).

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not differ between mice fed the control and n-3 PUFA–rich diets (data not shown).

**DISCUSSION**

Previous studies have indicated that, compared with mice fed the control diet, mice fed a diet rich in n-3 PUFAs have significantly lower (~50%) levels of circulating IFN-γ 24 h after challenge with *L. monocytogenes* [18]. Because this cytokine plays such a critical role in host resistance to *L. monocytogenes*, such an effect may explain why mice fed fish oil show reduced clearance and survival after challenge with this pathogen. In the present study, IFN-γ-KO mice were used, in conjunction with the administration of murine rIFN-γ, to equalize IFN-γ exposure across both diet treatment groups throughout the early stages of such an infection. As expected, the daily administration of rIFN-γ to IFN-γ-KO mice fed the control diet significantly enhanced their resistance to *L. monocytogenes*, as noted by greater clearance of the bacteria from the liver and spleen. In contrast, IFN-γ-KO mice fed the n-3 PUFA–rich diet did not respond to the administration of exogenous rIFN-γ. These data clearly show, for the first time, that n-3 PUFAs impair IFN-γ responsiveness in vivo.

Feeding mice diets enriched with n-3 PUFAs creates a condition that looks remarkably like a macrophage-selective unresponsiveness to IFN-γ. Dighe et al. [22] generated a series of transgenic mice that selectively expressed a dominant-negative form of the IFN-γ receptor α-chain (DN–IFN-γR-1) to study the cell-specific function of this cytokine. Mice expressing DN–IFN-γR-1 in macrophages have impaired in vitro nitric oxide (NO) production in response to costimulation with IFN-γ and lipopolysaccharide (LPS). Also reported in that study was that an intraperitoneal challenge with 5 × 10⁷ cfu of *L. monocytogenes* killed 3 of 9 DN–IFN-γR-1 mice, whereas no wt littermates died. Furthermore, bacterial clearance from the spleen and liver was impaired, such that DN mice had ~100-fold higher numbers of bacteria in these organs than did wt littermates by day 5 after challenge. As was described previously, feeding mice a diet rich in n-3 PUFAs affects host resistance to this pathogen very much like the immune defects observed in these transgenic mice expressing the DN–IFN-γR-1 in macrophages.

We believe that impaired macrophage activation via reduced responsiveness to IFN-γ is 1 underlying mechanism by which n-3 PUFAs diminish host resistance to *L. monocytogenes*. Macrophages are among the primary cells initially infected by *L. monocytogenes*, which escapes the phagosome and replicates in the cytosol [23]. Phagocytes become nonpermissive to intracellular bacteria growth after becoming activated by IFN-γ, which allows these cells to play a critical role in clearing this

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**Figure 1.** Impact of high intake of omega-3 polyunsaturated fatty acids on liver (A) and spleen (B) bacteria loads in interferon (IFN–γ) knockout (KO) mice during infection with *Listeria monocytogenes* with and without exogenous recombinant (r) IFN–γ administration. IFN–γ KO mice were fed a lard or fish oil diet for 4 weeks before intravenous challenge with 10⁶ cfu of *Listeria monocytogenes* strain EGD. Mice were injected daily with either 1 mg rIFN–γ or PBS (for negative controls), beginning at the time of challenge. Bacteria in the liver and spleen were enumerated 3 days after challenge. Data were log₁₀ transformed before statistical analysis. Geometric means (horizontal bars) not sharing a letter are significantly different ( ), as determined by 2-way analysis of variance P < .01 with Bonferroni post hoc mean tests. cfu, colony-forming units.

**Figure 2.** Representative histogram of interferon (IFN–γ) receptor (R-1) surface expression (filled line) on splenocytes isolated from IFN–γ knockout (KO) mice. Also shown are histograms for isotype control antibody (thick line, unshaded) and streptavidin conjugate only (dotted line). Splenocytes isolated from IFN–γ-KO mice were stained with biotinylated anti-mouse rat IgG2a IFN–γR-1 or biotinylated rat IgG2a anti-KLH, washed, then incubated with streptavidin-conjugated SensiLight P-3L. Flow cytometry was performed by use of a BD FACS Vantage.
pathogen from host tissues [24]. NO production in high concentrations appears to be both necessary and sufficient for the killing of intracellular pathogens such as *L. monocytogenes* [25]. However, optimal macrophage activation and inducible NO synthase expression requires simultaneous treatment with IFN-γ and a Toll-like receptor (TLR) agonist, such as LPS [26].

In 1989, Somers et al. [27] reported that n-3 PUFAs significantly diminished macrophage responsiveness to in vitro treatment with LPS and IFN-γ. At low levels of IFN-γ (2–10 U/mL), macrophages from mice fed fish oil expressed significantly lower in vitro tumoricidal activity, compared with the same cells derived from mice fed diets rich in n-6 PUFAs or devoid of PUFAs (i.e., safflower oil and hydrogenated coconut oil, respectively). These observations are entirely consistent with our in vivo data. Interestingly, the adverse effect of n-3 PUFAs could be overcome in their system by increasing the concentration of IFN-γ to 25 U/mL, a concentration 5-fold above that required to maximize the tumoricidal response of macrophages from mice fed control diets. In our in vivo studies, we administered, daily, a single dose of rIFN-γ to the IFN-γKO mice; thus, we do not know whether higher levels of rIFN-γ might have overcome the n-3 PUFA–mediated impairment of host resistance to *L. monocytogenes*. The macrophage hyporesponsiveness to IFN-γ observed by Somers et al. [27] was not present in the absence of LPS, a TLR4 agonist. Interestingly, a recent study that used a mouse macrophage cell line (i.e., RAW 264.7) demonstrated that in vitro treatment with docosahexaenoic acid (an n-3 PUFA in fish oil) diminished TLR2- and TLR4-mediated signaling [28]. TLR2 has been shown to play an important role in host defense against *L. monocytogenes* [29]. Thus, it is possible that the impaired host resistance observed in mice fed n-3 PUFAs is a consequence of diminished signaling through both TLR and IFN-γ receptors. Indeed, impaired IFN-γ signaling is not the only mechanism by which n-3 PUFAs impair host resistance to *L. monocytogenes*, because, even in the absence of rIFN-γ, the n-3 PUFA–rich diet impaired bacterial clearance.

Ligation of the IFN-γ receptor results in the activation of the receptor-associated tyrosine kinases, Janus kinase 1 (JAK1) and JAK2 [20]. Subsequently, this leads to the phosphorylation and activation of STAT1. STAT1 mediates many of the immune and proinflammatory effects of IFN-γ. Several pathogens, such as *Mycobacterium* and cytomegalovirus, impair IFN-γ signaling as part of their survival strategy [30–32]. Among the mechanisms used are (1) reduced phosphorylation of IFN-γR1, JAK1, JAK2, and STAT1; (2) interference with the interaction between STAT1 and its transcriptional coactivators CBP and p300; and (3) depletion of JAK activity via a proteosome-dependent process. It is our contention that dietary n-3 PUFAs affect ≥1 aspects of this signal transduction pathway, leading to impaired macrophage activation and diminished host resistance to pathogens that depend heavily on macrophage responses.

In the present study, it has been demonstrated that a high intake of n-3 PUFAs was associated with diminished ex vivo STAT1 phosphorylation after IFN-γ treatment of peritoneal macrophages and splenocytes from IFN-γKO mice. This re-

### Table 2. No effect of dietary intake of omega-3 polyunsaturated fatty acids on surface interferon (IFN)–γ receptor expression by immune cells from IFN-γ knockout mice.

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<th>Cell type</th>
<th>Lard diet</th>
<th>Fish-oil diet</th>
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<tr>
<td>Splenocytes</td>
<td>48.3 ± 3.2</td>
<td>47.3 ± 1.9</td>
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<tr>
<td>Macrophages a</td>
<td>413.8 ± 45.9</td>
<td>408.0 ± 19.0</td>
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**NOTE.** Data are mean fluorescence intensity ± SE.  
* Peritoneal macrophages were collected 4 days after the administration of thioglycollate.
duction in STAT1 phosphorylation did not appear to be a consequence of diet-induced alterations in IFN-γ receptor expression. The results of flow-cytometric analysis indicated that surface expression of IFN-γR-1 on immune cells isolated from IFN-γKO mice was not affected by dietary n-3 PUFAs. These data are in direct contrast to our previous findings in wt mice [19]. One possible explanation for this discrepancy is that, in the IFN-γKO mice, the normal feedback regulation of IFN-γR-1 expression may be dysfunctional or is less sensitive to the effects of n-3 PUFAs. In fact, we did note a substantially greater level of IFN-γR-1 expression on immune cells from the IFN-γKO mice, compared with the same cells isolated from wt mice (data not shown). Regardless, the data presented here support our hypothesis that n-3 PUFAs diminish not only early IFN-γ production but also IFN-γ signaling. Additional studies will be needed to delineate which specific steps in this signaling pathway are affected by n-3 PUFAs, as well as the underlying biochemical mechanism(s) involved.

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References