# *n*-3 PUFA fail to affect *in vivo*, antigen-driven CD8^{+}T-cell proliferation in the spleen of naïve mice

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One of the most frequently reported immunomodulatory actions of *n*-3 PUFA is their ability to diminish *in vitro* lymphocyte proliferation. The purpose of this study was to determine if *n*-3 PUFA intake affects the kinetics or magnitude of the antigen-driven expansion of CD8^{+}T-lymphocytes *in vivo*. In this study we utilized a well-characterized model of T-cell immunity (i.e. infection with the intracellular bacterium, *Listeria monocytogenes*). Weanling BALB/c mice were fed one of two experimental diets that differed solely in fat source. Our control diet contained lard (180 g/kg) and was devoid of long-chain *n*-3 PUFA. The experimental diet contained 150 g/kg menhaden fish oil and 30 g/kg corn oil, thus providing approximately 8 % of energy from long-chain *n*-3 PUFA. After 4 weeks, mice were infected intravenously with $10^6$ colony-forming units of actA-deficient *L. monocytogenes*. Clonal expansion of antigen-specific CD8^{+}T-cells in the spleen was measured at 5, 7, 9 and 14 d post-challenge using a class I MHC tetramer loaded with the immunodominant peptide from this pathogen (i.e. $K^d$:$LLO_{91-99}$). We report that feeding mice a diet rich in *n*-3 fatty acids did not significantly impact either the kinetics or magnitude of *in vivo*, antigen-driven expansion of CD8^{+}T-cells. Furthermore, contraction of this T-cell population was not affected by *n*-3 PUFA treatment. To our knowledge this is the first time MHC tetramers have been used to investigate the influence of *n*-3 PUFA on *in vivo* CD8^{+}T-cell proliferation.

*n*-**3 PUFA: CD8^{+}T-cell: Proliferation: MHC tetramer: *Listeria*: Mice**

Clonal expansion of naïve T-cells is an essential process in the development of an adaptive immune response (Lanzavecchia & Sallusto, 2005). T-lymphocytes mediate both regulatory and effector functions primarily through the expansion and differentiation of CD4^{+} and CD8^{+}T-cell subpopulations. It is widely believed that one of the important immunomodulatory activities of long-chain *n*-3 PUFA is their ability to diminish T-lymphocyte proliferation (Harbige, 1998; Calder *et al.* 2002). In some reports, however, *n*-3 PUFA fail to reduce T-cell proliferation (Kew *et al.* 2003; Wallace *et al.* 2003) and under some circumstances actually increase it (Byleveld *et al.* 2000; Arrington *et al.* 2001). The majority of studies examining the impact of *n*-3 PUFA on T-cell proliferation have used immune cells stimulated *in vitro* with polyclonal activators (e.g. concanavalin A, lipopolysaccharide, anti-CD3, phytohaemaglutinin, phorbol esters and calcium ionophore). Such *in vitro* assays have proven invaluable in elucidating the various signalling pathways and steps associated with T-cell activation and differentiation. However, it is unclear the extent to which these *in vitro* approaches replicate antigen-driven responses of T-cells *in vivo* (Lyons, 2000; Jenkins *et al.* 2001; Zinkernagel, 2002). Recently, we measured the impact of dietary *n*-3 PUFA on *in vivo* CD4^{+}T-cell proliferation (Anderson & Fritsche, 2004). Using a transgenic mouse model in conjunction with an adoptive transfer protocol, we reported that *in vivo*, antigen-specific CD4^{+}T-cell proliferation was not affected by *n*-3 PUFA from fish oil. In contrast, mice fed a diet rich in *n*-6 PUFA from corn oil showed significantly higher *in vivo* clonal expansion (20 %, $P<0.05$) compared to mice fed an identical diet, except that the fat source was lard, rich in saturated fatty acids and MUFA. When we initiated this investigation, there had been no studies examining the effects of *n*-3 PUFA on *in vivo* CD8^{+}T-lymphocyte proliferation.

Murine listeriosis is a well-characterized model for studying *in vivo* CD8^{+}T-lymphocyte expansion and differentiation. Host immunity to infection with *Listeria monocytogenes* consists of both innate and adaptive immune responses. Cells of the innate immune system (i.e. macrophages, neutrophils and natural killer cells) play a critical role controlling bacterial growth during the initial stages of infection, and direct the ensuing adaptive immune response (Czuprynski & Haak-Frendscho, 1997; Unanue, 1997). The adaptive immune response consists principally of pathogen-specific

CD8$^+$T-lymphocytes that resolve acute infection, and provide long-term anti-listerial immunity as memory T-cells (Bishop & Hinrichs, 1987; Kaufmann, 1988). The immunodominant epitope (LLO$_{91-99}$) against which *Listeria*-specific CD8$^+$ effector and memory cells are generated is derived from the virulence factor, listeriolysin O (McKay & Lu, 1991; Pamer *et al.* 1991). Antigen-specific lymphocytes can be identified and enumerated by flow cytometry using fluorochrome-conjugated class I MHC tetramers that present LLO$_{91-99}$. In light of previous reports linking high *n*-3 PUFA intake with reduced survival of mice during a primary *Listeria* infection (Fritsche *et al.* 1997; de Pablo *et al.* 2000), and the tendency for *n*-3 PUFA to decrease T-lymphocyte proliferation, we hypothesized that high *n*-3 PUFA intake would be associated with reduced *in vivo* CD8$^+$T-cell proliferation.

## Materials and methods

### Mice and diets

Specific pathogen-free, weanling female BALB/cAnNHsd mice were purchased (Harlan, Indianapolis, IN, USA) and housed four per cage in the Animal Sciences Research Center at the University of Missouri-Columbia. The room was maintained on a 12:12 h light–dark cycle at 23°C and 40–50 % relative humidity. Mice had free access to autoclaved water and commercial rodent diet (5008; Purina Mills, St Louis, MO, USA). After a 1-week acclimation period, mice were randomly assigned to one of two experimental diet treatment groups. The experimental diets were nutritionally complete and were based on the semi-purified AIN-93G diet (Reeves *et al.* 1993) modified to contain 18 % fat (w/w) while maintaining the nutrient-to-calorie ratio of the original lower fat diet. The two diets were identical except for the major dietary fat source: lard or menhaden fish oil (a generous gift from Omega Protein, Reedville, VA, USA). A small amount of corn oil was added to the fish oil (125 g/kg) to match the essential fatty acid content of lard (approximately 10 % linoleic acid). The fatty acid composition of the experimental diets has been reported previously (Irons *et al.* 2003). The fish oil diet contained *n*-3 PUFA, from eicosapentaenoic acid and docosahexaenoic acid, at the level of 27·9 g per 100 g total fatty acids. Fats were stabilized against auto-oxidation by a synthetic antioxidant, tertiary-butylhydroquinone (0·2 g/kg). Mice were provided with fresh diet daily, and food bowls were changed at least twice weekly. Mice were fed experimental diets for at least 28 d prior to 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

The actA-deficient (DP-L3078) strain of *L. monocytogenes* was obtained as a generous gift from Daniel Portnoy, University of California-Berkeley, and was grown in tryptic soya broth at 37°C for 12–18 h prior to use. After three washes with sterile PBS (Life Technologies, Grand Island, NY, USA), the concentration of the bacteria was estimated via optical density, then diluted with PBS to the desired infectious dose. The actual dose administered in each experiment was confirmed by plate counts using blood agar. Bacterial challenges of $1 \times 10^6$ colony-forming units of *actA*-deficient *L. monocytogenes* were given in a final volume of 0·2 ml PBS via the lateral tail vein. 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, Franklin Lakes, NJ, USA).

### Preparation of splenocytes

Spleens were removed from mice consuming lard or fish oil diets at the indicated times after primary infection with *actA*-deficient *L. monocytogenes*. To accomplish this, mice were anaesthetized with an intramuscular injection of ketamine (200 mg/kg) and xylazine (16 mg/kg), and then humanely killed by exsanguination. Spleens were aseptically removed, placed in sterile PBS and forced through a sterile tissue sieve into a single-cell suspension. Erythrocytes were removed by density gradient centrifugation using Histopaque-1077 (Sigma, St Louis, MO, USA) as described previously (McGuire *et al.* 1997). Immune cells were enumerated electronically with a Coulter Counter, model ZM (Beckman Coulter, Fullerton, CA, USA).

### Tetramer staining and flow cytometry

Antigen-specific CD8$^+$T-cell populations were detected by concurrent staining with rat anti-mouse CD62L (L-selectin) conjugated to phycoerythrin, and an allophycocyanin-conjugated tetrameric MHC complex loaded with the immunodominant peptide of *Listeria*, listeriolysin O$_{91-99}$ (i.e. GYKDG-NEYI; Pamer *et al.* 1991), CD62L staining was used in conjunction with tetramer staining to distinguish activated cells (CD62L$^{Lo}$) from cells that are not activated (CD62L$^{Hi}$). Prior to tetramer analysis, $0·5-1 \times 10^8$ splenocytes were positively enriched for CD8$^+$T-cells using anti-CD8α microbeads (clone 53–6·7) and autoMACS columns according to the manufacturer's instructions (Miltenyi, Auburn, CA, USA). Aliquots of $1 \times 10^6$ CD8$^+$T-cells were resuspended in PBS with 1 % bovine serum albumin (BSA) and 0·2 % NaN$_3$, blocked with heat-inactivated mouse serum, and incubated with the following antibodies on ice for 15 min: rat anti-mouse CD8α-FITC, rat IgG2a-FITC, rat anti-mouse CD62L-PE, rat IgG2a-PE (all from Caltag, Burlingame, CA, USA), and K$^d$:LLO$_{91-99}$-APC (obtained from NIH Tetramer Facility). Cells were washed, fixed with 2 % paraformaldehyde and analysed on a FACSvantage flow cytometer (BD Bioscience, San Jose, CA, USA). Light scatter was used to exclude dead cells, and data were collected on a minimum of 50 000 live cells. Cells displaying autofluorescence in a PE *v*. FL3 dot plot were also excluded from analysis. CD8$^+$ cells were gated to determine the percentage of CD62L$^{Lo}$ K$^d$:LLO$_{91-99}$-positive cells. Splenocytes from uninfected mice, stained with tetrameric MHC complex were routinely used in our studies to set the horizontal threshold for detecting LLO$_{91-99}$-specific cells. Data were analysed using CellQuest software (version 3.1, BD Pharmingen, San Diego, CA, USA).

*Statistical analysis*

Data were analysed and graphed using GraphPad Prism version 3.0a (GraphPad Software, San Diego, CA, USA). The organ bacterial load data were $log_{10}$ transformed prior to analysis. The main effect of dietary fat source on bacterial load and tetramer-positive cell frequency was analysed by one-way ANOVA and unpaired *t* test, respectively. $P < 0.05$ was considered to be statistically significant.

## Results

### n-*3 PUFA and antigen load during primary infection*

Infection with the actA-deficient strain did not cause mortality in the *n*-3 PUFA-fed mice during primary infection (data not shown). *Listeria* enumerated in the spleen and liver of mice at 1, 2 and 3 d post-challenge showed that the number of bacteria in both organs declined over time (Fig. 1). At 3 d post-challenge, the *n*-3 PUFA diet was associated with 10-fold greater numbers of bacteria in the spleen and liver ($P < 0.001$). The present findings are similar to what we observed during infection with wild-type *L. monocytogenes* (Irons *et al.* 2003), namely consumption of the *n*-3 PUFA diet is associated with reduced bacterial clearance, and greater antigen load at 3 d post-challenge in naïve BALB/c mice. However, regardless of diet treatment, the absolute levels of bacteria at 3 d post-challenge was several orders of magnitude lower in mice challenged with the actA-deficient *v.* wild-type *L. monocytogenes* (data not shown).
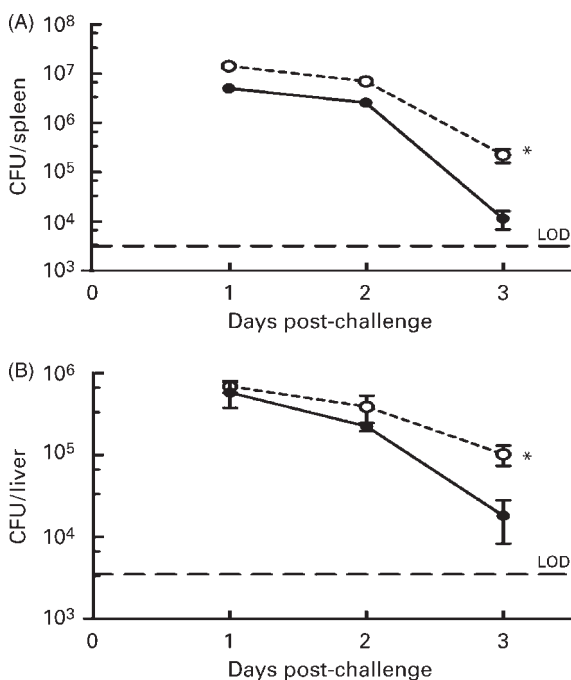


**Fig. 1.** Recovery of *Listeria* in the spleen (A) and liver (B) during the first 3 d post-challenge in mice consuming the experimental diets either devoid of (●, lard) or rich in long-chain *n*-3 PUFA (○, fish oil). The number of bacteria per organ was determined by plate counts. For details of procedures, see p. 839. Values are means with their standard errors depicted by vertical bars (*n* 6). Data were analysed using one-way ANOVA (*$P < 0.001$), and are representative of two independent experiments. CFU, colony-forming units; LOD, limit of detection.

### n-*3 PUFA and antigen-specific CD8+T-cell kinetics*

At 5, 7, 9 and 14 d post-challenge, the number of CD8+T-lymphocytes specific for $K^d$:$LLO_{91-99}$ were enumerated by tetramer staining. CD62L expression was used to distinguish between naïve (CD62L$^{high}$) and activated (CD62L$^{low}$) phenotypes. Representative dual-parameter flow cytometric dot plots of CD8+ cells are shown in Fig. 2. The numbers in the upper quadrants represent the percentage of antigen-specific cells out of the total splenic CD8+T-cell population. Combined data from five individual experiments showed that $LLO_{91-99}$-specific CD8+T-cells were not detectable in naïve mice, but expansion of these cells peaked 7 d post-challenge in both lard- and fish oil-fed mice, before undergoing rapid contraction (Fig. 3). There were no differences in the expansion or contraction of $LLO_{91-99}$-specific CD8+T-cells as a result of dietary fat source.

## Discussion

The expansion and differentiation of CD8+T-cells is critical for host defence against viral and intracellular bacterial infections. *n*-3 PUFA have been shown to decrease lymphocyte proliferation *in vitro*, but their effects on the proliferation of CD8+T-cells *in vivo* has not been studied directly. We used MHC class I tetramer staining to conduct the first *in vivo* kinetic analysis of the impact of dietary *n*-3 PUFA on proliferation of CD8+T-cells. While in the present study we focused solely on the CD8+T-cell response to the immunodominant antigen of *Listeria* (i.e. $LLO_{91-99}$), we believe these results are representative of all CD8+T-cells responding to this infectious challenge. Busch *et al.* (2000) compared the CD8+T-cell response to the two immunodominant peptides of *Listeria*, $LLO_{91-99}$ and $p60_{217-225}$. They report that the expansion kinetics of H2-$K^d$-restricted *Listeria*-specific T-cell populations specific for two different peptides were similar during primary *L. monocytogenes* infections. The frequency of CD8+T-cells specific for each peptide peaked at the same time (i.e. 7–8 d after primary infection). These authors went on to state that, 'The synchronous *in vivo* expansion of T-cells specific for the different *L. monocytogenes* epitopes is surprising because these peptides are present in vastly different amounts and have dramatically different stabilities'.

The kinetics and magnitude of the pathogen-specific CD8+T-cell response that we observed in the present study were consistent with what has been published by others (Busch *et al.* 1998). More importantly, our results clearly demonstrate that the *in vivo* expansion of antigen-specific CD8+T-cells in the spleen appears to be unaffected by *n*-3 PUFA. Our data suggest that the diminished host resistance to this pathogen associated with *n*-3 PUFA intake, as reported by us (Fritsche *et al.* 1997; Irons *et al.* 2003) and others (Puertollano *et al.* 2001), may not be a consequence of impaired CD8+T-cell expansion.

Data on the impact of *n*-3 PUFA on lymphocyte proliferation in response to antigen-specific activation are quite limited. Delayed-type hypersensitivity (DTH) tests have been widely used as an *in vivo* measure of antigen-specific, cell-mediated immune response. Consumption of *n*-3 PUFA from fish oil can reduce DTH in man (Meydani *et al.* 1993) and mice (Fowler *et al.* 1993), but in another human study *n*-3
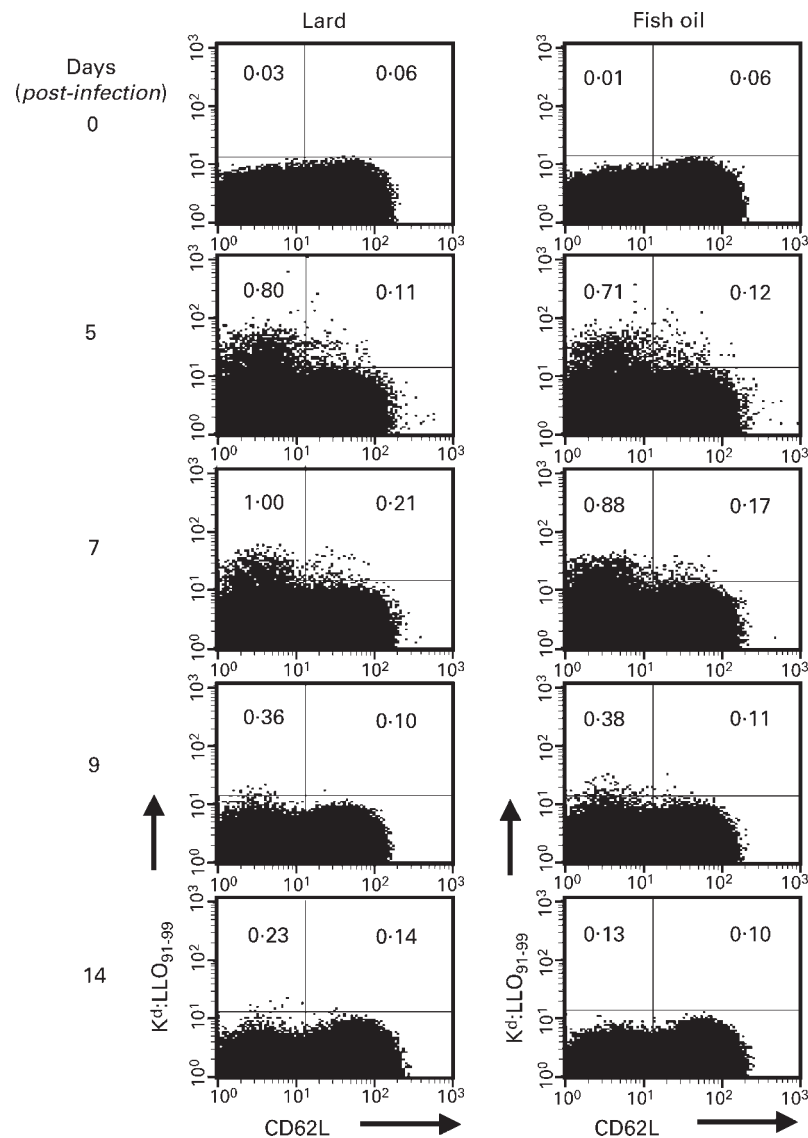
**Fig. 2.** Representative dot plots of the immunodominant antigen-specific CD8$^+$T-cell population in the spleen during the clonal expansion and contraction phases of a primary infection with *Listeria monocytogenes*. CD8$^+$T-lymphocytes isolated from the spleen of mice at indicated times post-*Listeria* challenge were positively selected using magnetic cell sorting; labelled with isotype control antibodies or anti-CD8$\alpha$, anti-CD62L, and MHC tetramer reagent loaded with a listeriolysin O peptide (LLO$_{91-99}$); then analysed by three-colour flow cytometry. For details of procedures, see p. 839. The values shown in the upper quandrants reflect the frequency of tetramer-positive cells expressed as a percentage of total CD8$^+$ cells.

PUFA were without effect (Kelley *et al.* 1992). DTH responses involve a complex array of inflammatory mediators (e.g. eicosanoids, chemokines and cytokines) and immune cells (e.g. monocytes, dendritic cells and T-cells) (Kobayashi *et al.* 2001). Thus, the DTH test can only provide indirect evidence about *in vivo*, antigen-driven T-lymphocyte function. Furthermore, DTH is a recall response, primarily of CD4$^+$T-cells, that is intrinsically different from the initial response to antigen by naïve T-cells (Dubey *et al.* 1996; Merica *et al.* 2000). Apart from DTH tests, we are aware of four studies in which modulation of antigen-driven lymphocyte proliferation by *n*-3 PUFA has been investigated. First, Byleveld *et al.* (2000) reported that, compared to cells derived from mice consuming beef tallow, lymphocytes from mice consuming fish oil-containing diets rich in *n*-3 PUFA showed significantly increased proliferation in response to an *in vitro* recall

infection with influenza virus. A year later, Harbige & Fisher (2001) investigated *n*-3 PUFA modulation of antigen-specific immune responses in mice. In their study, mice were fed experimental diets for 2 weeks, then immunized with antigen (ovalbumin) in complete Freund's adjuvant. A week later, proliferation of a crude splenocyte preparation was measured in response to re-exposure to the antigen *in vitro*. Unfortunately, the design of the two previous studies did not allow the investigators to distinguish CD4$^+$ from CD8$^+$T-cell responses, and like the DTH test, these data were for a recall response. Recall responses are not the same as a primary response of naïve T-cells to antigen (Farber & Ahmadzadeh, 2002). In contrast, two studies have examined the impact of *n*-3 PUFA on naïve, antigen-specific T-cell responses. Using a transgenic mouse model in which most CD4$^+$T-cells possess identical T-cell receptors (i.e. specific for ovalbumin$_{323-339}$), we
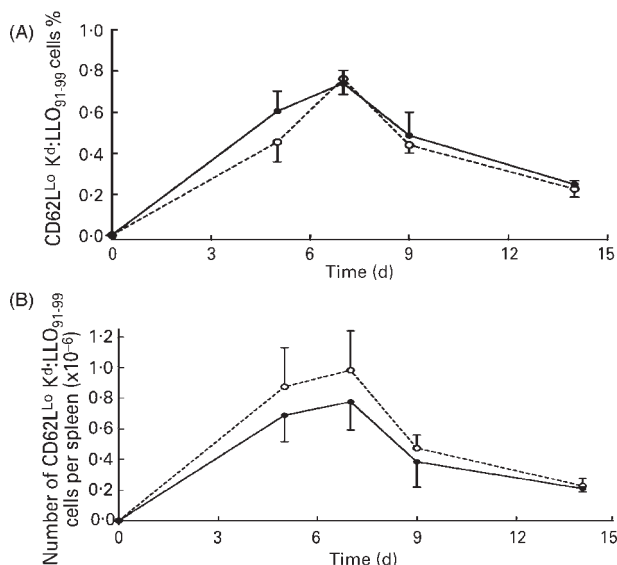
**Fig. 3.** Dietary *n*-3 PUFA do not alter the frequency (A) or absolute number (B) of $K^d$-restricted $CD8^+$T-lymphocytes specific for the immunodominant epitope of *Listeria monocytogenes*, $LLO_{91-99}$. Values are means from five independent experiments with their standard errors depicted by vertical bars, with data from a single such experiment represented in Fig. 2. $CD8^+$T-cells were gated to determine the percentage of $CD62L^{Lo}$ $K^d$:$LLO_{91-99}$-positive cells, and total cell yield data were used to derive the total number of antigen-specific cells per spleen. Statistical analysis was performed by one-way ANOVA. ●, Lard; ○, fish oil.

reported that feeding mice a diet rich in *n*-3 PUFA resulted in a 50% reduction in *ex vivo* lymphocyte proliferation in response to cognate antigen (Pompos & Fritsche, 2002). However, culture conditions (i.e. the presence of homologous serum) significantly influenced the *in vitro* results and conclusions, which is similar to reports from other researchers (Yaqoob *et al.* 1994; Jeffery *et al.* 1996). Using the same transgenic mouse model in conjunction with adoptive transfer, we investigated the impact of dietary *n*-3 PUFA on *in vivo*, antigen-driven proliferation of $CD4^+$T-cells. In contrast to our *in vitro* data, we found that *in vivo* proliferation of $CD4^+$T-cells was unaffected by diet *n*-3 PUFA enrichment (Anderson & Fritsche, 2004). The present study complements these results, and suggests that consumption of diet rich in *n*-3 PUFA does not affect *in vivo*, antigen-driven T-cell responses for $CD8^+$T-cell subpopulations either. However, an important caveat is that we only measured $CD8^+$T-cell response in the spleen. The immune response to infection is known to differ in different organs within the same host (Engwerda & Kaye, 2000). The liver and gastrointestinal tract are other major sites of *Listeria* infection and $CD8^+$T-cell responses (Pope *et al.* 2001). Assessing the impact of *n*-3 PUFA on T-cell responses in those organs would provide a more complete understanding of how these fatty acids might affect systemic T-cell responses.

As an infection resolves, the vast majority (>90%) of pathogen-specific $CD8^+$T-cells die off, while the remaining ones become long-lived memory cells (Busch *et al.* 1998). The mechanisms involved in memory cell generation are mostly unknown. Recent studies, however, show that $CD8^+$T-cell contraction is also programmed (Badovinac *et al.* 2002). In the present study we included two sampling

periods (days 9 and 14 post-infection) that allowed us to assess the impact of *n*-3 PUFA on the contraction of effector $CD8^+$T-cells. We found no indication that *n*-3 PUFA affected the early phase of $CD8^+$T-cell contraction following the resolution of *L. monocytogenes* infection. The present findings contrast with reports that *n*-3 PUFA enrichment is associated with a small, but statistically significant increase in activation-induced cell death of $CD4^+$T-cells following *in vitro* polarization to a Th1 phenotype (Switzer *et al.* 2003, 2004). At present it is unclear whether the disagreement between these findings reflect a fundamental difference between the impact of *n*-3 PUFA on $CD4^+$ *v.* $CD8^+$T-cells or are a consequence of the experimental approaches used (i.e. *in vivo v. in vitro*). It is possible that *n*-3 PUFA may affect long-term survival of memory T-cells and that such effects would not have been apparent in the present study. This may help explain why we observed a small, but statistically significant, reduction in host protection from re-infection 35 d following a primary challenge with *L. monocytogenes* (Irons *et al.* 2003).

CD62L (L-selectin) is a surface marker that causes homing of lymphocytes to lymph nodes, and it is down-regulated upon activation. Others have reported that *n*-3 PUFA can diminish *in vitro* CD62L expression on rat lymphocytes (Sanderson & Calder, 1998). At the time of this investigation, it was not known whether *n*-3 PUFA affect CD62L expression on $CD8^+$T-cells. We found that, as expected, CD62L expression on the immunodominant $CD8^+$T-cell population was down-regulated in response to the *L. monocytogenes* challenge, yet was unaffected by *n*-3 PUFA intake.

In order to maximize the ability to discern an effect of *n*-3 PUFA on T-cell proliferation, the diets used in the present study represented two extremes for *n*-3 PUFA intake. The control diet contained lard as the sole source of fat and was devoid of long-chain *n*-3 PUFA. In contrast, the test diet provided nearly 8% of energy (en%) from long-chain *n*-3 PUFA (approximately 5 en% from eicosapentaenoic acid and approximately 3 en% from docosahexaenoic acid). These levels meet or exceed those of other animal-based reports on which the paradigm of immunosuppression by *n*-3 PUFA has been based. Furthermore, 8 en% represents intake levels that greatly exceed what is possible in man even for those consuming *n*-3 PUFA supplements; and exceeds by 30-fold the levels currently recommended for optimal cardiovascular health (Wijendran & Hayes, 2004). While we did not present any fatty acid data in the present study, we do not believe that the lack of an effect on *in vivo* T-cell response can be attributed to our treatments not altering immune cell *n*-3 PUFA levels. We have previously demonstrated that immune cells (i.e. T-cells and macrophages) from mice fed diets with fatty acid compositions identical, or nearly so, to those used in the present study are significantly enriched with *n*-3 PUFA and have diminished arachidonic acid content (Huang *et al.* 1992).

In summary, this is the first investigation to use MHC tetramers to directly measure the impact of *n*-3 PUFA on an *in vivo*, antigen-driven $CD8^+$T-cell response. Use of MHC tetramers in conjunction with flow cytometric analysis proved to be both a sensitive and novel approach to address this question. In contrast to a large body of *in vitro* evidence, we did not observe a significant effect of dietary *n*-3 PUFA on $CD8^+$T-cells during an *in vivo* immune response, even at a very high level of intake. The present data suggest that caution

should be exercised when interpreting results from studies of fatty acid modulation of T-cell function that rely entirely on *in vitro* and *ex vivo* approaches.

## Acknowledgements

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