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Effects of dietary ω3 and ω6 lipids and vitamin E on proliferative response, lymphoid cell subsets, production of cytokines by spleen cells, and splenic protein levels for cytokines and oncogenes in MRL/MpJ-*lpr/lpr* mice

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*ω3 Fatty acid rich fish oil (FO) and vitamin E may delay the progress of certain autoimmune diseases. The present study examined the mechanisms of action of ω3 lipids and vitamin E in autoimmune-prone MRL/*lpr *mice suffering from extensive lymphoproliferation, lupus-like symptoms, and accelerated aging. To determine whether the effects of ω3 lipids in autoimmune disease is linked to vitamin E levels, weanling female MRL/*lpr *and congenic control MRL/++ mice were fed diets containing 10% corn oil (CO) or 10% FO at two levels of vitamin E (75 IU or 500 IU/kg diet) for 4 months. The appearance of lymph nodes was delayed in the mice fed FO, and higher levels of FO offered further protection against the appearance of lymph nodes. Analysis of the spleen cells revealed that the cells positive for Thy.1 and Fas were significantly higher in the MRL/++ mice. The groups fed high levels of vitamin E generally exhibited higher levels of Fas. The proliferative response of splenocytes of MRL/++ mice to mitogens was significantly higher compared with MRL/*lpr *mice. Interleukin (IL)-10 production by spleen cells was significantly higher in FO-fed MRL/*lpr *mice than in CO-fed mice. In mice fed a high level of vitamin E, the production of IL-12 and tumor necrosis factor-α was significantly lower and IL-2 was significantly higher than in animals fed a low level of vitamin E. Proinflammatory cytokines were higher in the MRL/*lpr *mice and both FO and vitamin E lowered the levels of proinflammatory cytokines and lipid mediators. Western blots revealed that c-myc and c-ras were significantly lower and IL-2 and transforming growth factor (TGF)-β1 levels were significantly higher in the spleens of MRL/++ mice. FO lowered c-myc and high levels of vitamin E in the diets normalized the levels of TGF-β1 in MRL/*lpr *mice. The observations from this study suggest that both FO and vitamin E modulate the levels of specific cytokines, decrease the levels of proinflammatory cytokines, inflammatory lipid mediators, and c-myc, and increase TGF-β1 levels in spleens of MRL/*lpr *mice and thus may delay the progress of autoimmune diseases.* (J. Nutr. Biochem. 10:582–597, 1999)

Keywords: autoimmunity; cytokines; immune response; mice; oncogenes; ω3 lipids

Introduction

Defective regulation of inflammatory responses and disordered immune mechanisms are central to the pathologic processes encountered in certain autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). RA is an autoimmune disease characterized by progressive joint destruction and immobility. Alterations in proliferative response to lectins,[1] abnormal levels of serum anti-DNA antibodies,[2] imbalances in pro- and anti-inflam-

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matory cytokines,[3] and increases in the expression of certain proinflammatory cytokines and oncogenes[4] have been observed in RA and SLE. Various cytokines and chemokines have been implicated as important mediators of inflammation and joint destruction in RA and other inflammatory processes and prevention of cartilage erosion would be of great therapeutic benefit to patients.[5] Because several autoimmune diseases are associated with overproduction of cytokines that activate inflammatory cells or responses, downregulating or blocking these proinflammatory cytokines and mediators or supplementing anti-inflammatory cytokines may have potential in delaying the disease.

There are two substrains of mice—MRL/*lpr* and MRL/++ mice—that offer a unique controlled model for investigating dietary and drug effects on autoimmune disease.[6,7] MRL/*lpr* mice spontaneously develop massive lymphadenopathy with hypergammaglobulinemia, autoantibodies, high levels of acute phase proteins, abnormal lymphoid cell subsets, expression of oncogenes in lymphoid tissues, arthritis, and immune complex glomerulonephritis compared with congenic MRL/++ mice.[8] These characteristics are linked closely with the regulatory imbalance of T cells and B cells that display an exceptional constellation of altered membrane markers.[9,10] Until recently, the function of the *lpr* gene had been obscure, but the identification of *lpr* as the *Fas* gene, the product of which mediates a pathway for apoptosis, offers new insight into the mechanism of autoimmunity.[11]

The treatment of autoimmune diseases with nutrition interventions has gained attention in recent years. Nutritional interventions have been accepted as having major therapeutic potential.[12,13] Significant beneficial effects of dietary supplementation with ω3 fatty acids in autoimmune-prone mice[14] and in patients with RA[15] have been reported. Clinical trials using ω3 lipids containing fish oil (FO) on RA patients ameliorated clinical symptoms. RA patients who were taking FO supplements could eventually reduce the dosage of nonsteroidal anti-inflammatory drugs or even discontinue the use of medication.[16] Incorporation of ω3 fatty acids into tissues may modify inflammatory and immune reactions and have a potential therapeutic value for inflammatory diseases.[17] Nutritional intervention with marine lipids containing long chain ω3 fatty acids [eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA)] have been reported to significantly increase the life span and delay the onset of autoimmune disease in autoimmune-prone mice.[18]

The present study was planned to examine some of the mechanisms of action of ω3 lipids and the role of vitamin E on autoimmune disease in a mouse model for RA. The major goal of this study was to investigate the immunologic mechanisms through which ω3 dietary lipids and vitamin E provide protection against autoimmune disease in MRL/*lpr* mice. We have investigated the effects of feeding ω3 lipids in the presence of low and high levels of vitamin E on the proliferative response of spleen cells, lymphoid cell subsets, pro- and anti-inflammatory cytokine levels, and protein levels of specific cytokines and oncogenes in MRL/*lpr* mice.

Table 1 Composition of the experimental diets

Ingredient*	Percent
Casein	20%
Dextrose	45%
Starch	16%
Corn oil or fish oil†	10%
Cellulose	3.5%
AIN salt mixture‡	3.5%
AIN vitamin mixture§	1.5%
DL-methionine	0.3%
Choline	0.2%

*All diet ingredients were purchased from Dyets, Bethlehem, PA, USA.
†Fish oil diet was supplemented with 1% corn oil (fish oil = 9%, corn oil = 1%)
‡The salt mixture was supplemented with 0.0023 mg/kg diet of sodium fluoride.
§Composition of the vitamin mixture (ICN Biochemicals, g/kg of mixture): retinyl acetate (500,000 IU/g) = 1.8 g; vitamin D concentrate (850,000 IU/g) = 0.125 g; DL-α-tocopheryl acetate (250 IU/g) = 20.0 g; ascorbic acid = 45.0 g; inositol = 5.0 g; choline chloride = 75.0 g; menadione = 2.25 g; p-aminobenzoic acid = 5.0 g; niacin = 4.25 g; riboflavin = 1.0 g; pyridoxine hydrochloride = 1.0 g; thiamin hydrochloride = 1.0 g; calcium pantothenate = 3.0 g; biotin = 0.02 g; folic acid = 0.09 g; and vitamin B_{12} = 0.00135 g.

Methods and materials

Experimental animals and diets

Weanling female MRL/*lpr* and MRL/++ mice (10 per group) purchased from Jackson Laboratories (Bar Harbor, ME USA) were fed nutritionally-adequate semipurified diets containing 10% (w/w) corn oil (CO; ICN, Irvine, CA USA) or 10% odor-free menhaden FO (U.S. Department of Commerce, National Marine Fisheries Service, Charleston, NC USA) with low (75 IU/kg diet; LE) and high levels (500 IU/kg diet; HE) of vitamin E. Both dietary oils had equal levels of antioxidant supplements, 1.3 g/kg oil of D-α-tocopherol oil (ICN), 1.2 g/kg oil of γ-tocopherol (U.S. Department of Commerce, National Marine Fisheries Service), and 1 g/kg oil of tertiary butylhydroxyquinone (Dyets, Bethlehem, PA USA), as recommended by the National Institutes of Health to prevent peroxidation during storage. The composition of the diets is presented in *Table 1* and fatty acid composition of the oils is presented in *Table 2* The FO diets were supplemented with 1% corn oil (9% FO and 1% CO) to prevent essential fatty acid deficiency. Fresh diet was provided daily and precautions were taken to prevent oxidation of lipids. The diets were prepared once a week, stored in air-tight containers, and flushed with nitrogen every time before closing the containers. The mice were maintained in plastic cages and with a 12-hour light/dark cycle. Body weights were recorded every 2 weeks. The animals were sacrificed at 4.5 months of age by cervical dislocation.

Histology of kidneys

Kidneys for light microscopic examination was immediately fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin-eosin (H&E) using standard techniques and graded based on a semiquantitative scale.

Spleen cell preparation

Spleens were aseptically collected free of connective tissue and single-cell suspensions were prepared. Spleens were minced gently in RPMI-1640 medium containing 5% heat-inactivated fetal bo-

Table 2 Fatty acid composition of the oils*

Fatty acids	Corn oil %	Fish oil %
14:0	0.03	8.40
16:0	10.33	13.97
18:0	1.99	2.51
20:0	–	0.14
16:1	0.16	11.17
18:1	24.00	10.00
18:2n-6	52.60	0.66
18:3	0.09	
20:4n-6	–	1.45
20:5n-3	–	13.37
22:4n-6	–	0.11
22:5n-6	–	0.39
22:5n-3	–	2.06
22:6n-3	–	8.60
Others	10.8	27.2

*Source: Galloway, S.B. (1989). U.S. Department of Commerce, NOAA, National Marine and Fisheries Service. The fatty acid composition was analyzed by gas chromatograph (Personal Communication).

vine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids.[19] The cell suspension was centrifuged at 100 × g and then washed three times in the same medium. Cell viability was determined by Trypan blue exclusion test.

Proliferation of spleen cells in response to mitogens

Proliferative response of spleen cells to lectins [concanavalin A (Con A), phytohemagglutinin (PHA), and lipopolysaccharides (LPS)] was determined by culturing the cells in the presence of optimal concentrations of these lectins at 37°C for 48 hours. Triplicates of 100 μL of cell suspension containing 5×10^6 cells/mL in the culture medium were placed in the wells of 96-well microtiter plates. One hundred microliters of standard culture medium containing 5% heat inactivated low endotoxin fetal calf serum (FCS) and optimal concentrations of Con A (25 μg/mL; Sigma Chemical Co., St. Louis, MO USA), or PHA (18.75 μg/mL), or LPS (2.5 μg/mL) were incubated at 37°C in a carbon dioxide (CO^2) incubator. After 38 hours of culturing, the cells were pulsed with 0.5 μCi of [^{3}H]-TdR (specific activity-6.7 Ci/mmol; ICN Radiochemicals, Costa Mesa, CA USA) and incubation continued for 12 more hours. Cells were harvested onto glass fiber filters using a cell harvester (Skantron Inc., Sterling, VA USA) and dried, and 5 mL of Scintiverse cocktail was added, and vials were counted in a Beckman LS 6800 liquid scintillation counter (Irvine, CA USA).

Culturing spleen cells for in vitro production of cytokines

Spleen cells, at a final concentration of 5×10^6 cells/mL, were cultured in flat bottom microtiter plates (Corning, NY USA) in RPMI-1640 culturing medium containing 5% FCS and the presence of Con A (5 μg/mL, 48 hours), PHA (10 μg/mL, 48 hours), or LPS (10 μg/mL, 24 hours; Sigma Chemical Co.) in a CO_2 incubator. Cell suspensions were centrifuged at 10,000 × g for 1 minute. Cell free supernatants were stored at −70°C for the determination of the concentration of cytokines.

Determination of cytokines in the culture supernatants

The cytokine concentrations in the culture supernatants were determined by enzyme linked immunosorbant assay (ELISA) utilizing mouse cytokine kits purchased from Genzyme Diagnostics (Cambridge, MA USA). The kit protocols were followed. Briefly, Nunc-Maxisorb 96-well ELISA plates were coated with specific anti-mouse capture antibodies in a coating buffer and incubated at 4°C overnight. The following day, capture antibodies were aspirated, the plates were washed with PBS-T [phosphate-buffered saline (PBS) − 0.01 M, pH 7.3) − 0.05% Tween-20]. The plates were incubated with 250 μL of blocking buffer [PBS (0.01 M, pH 7.3) − 3% bovine serum albumin (BSA)] for 2 hours at 37°C. Appropriately diluted samples or standards (final volume 100 μL) were added to test wells in duplicates and incubated overnight at 4°C. The wells were washed and the secondary antibodies were added and incubated for 60 minutes at 37°C. After washes, 100 μL detection reagent (horseradish peroxidase-conjugated Streptavidin) was added and incubated for 15 minutes at 37°C. The wells were washed followed by the addition of 100 μL substrate (3, 3′, 5, 5′-tetramethylbenzidine and hydrogen peroxide solution) to the wells. The reaction was then stopped by the addition of 50 μL of 1 M sulfuric acid. The intensity of the yellow color was read at 450 nm in a microplate reader (EL311s, Bio-Tek Instruments Inc., Winooski, VT USA). The mean absorbance of the standards and samples were calculated. Absorbance was corrected by subtracting background from the mean values of the samples and other standards. A standard curve was constructed to quantitate the concentration of specific cytokines in the samples.

Determination of lipid mediators (prostaglandin E_2, leukotriene B_4 and thromboxane B_2)

Prostaglandin E_2 (PGE_2), thromboxane B_2 (TXB_2), and leukotriene B_4 (LTB_4) levels in cell-free supernatants were determined by ELISA using kits purchased from Neogen Corporation (Lexington, KY USA). These kits were very specific for the lipid mediators to be tested and cross reactivity by were minimal (0.2–1%). Cross reactivity of PGE_2 to PGE_3 was less than 8%. The kits were supplied with 96-well MaxiSorpTM Nunc microplates precoated with anti-PGE_2, TXB_2, or LTB_4 antibodies. Appropriately diluted samples or standards (50 μL) and diluted PGE_2-, LTB_4-, or TXB_2-horseradish peroxidase (50 μL) were added to the test wells. The plates were incubated at room temperature for 1 hour and the wells were washed with enzyme immunoassay (EIA) wash buffer. Then 150 μL of substrate (3,3′,5,5′-tetramethyl benzidine (TMB) and hydrogen peroxide solution) was added. The reaction was stopped by adding 50 μL of 1 M HCl. The intensity of yellow color was read at 450 nm in a microplate reader. Mean absorbance of the standards and samples was calculated. The corrected absorbance was calculated by subtracting background (mean outer diameter of the 0 ng/mL standard) from the mean values of the samples and other standards. A standard curve was constructed to quantitate the concentration of cortisol or estradiol in the samples.

Flow cytometric analysis of lymphoid cell subsets

Red cell-free spleen cells (1×10^6 cells) were stained with anti-mouse FITC-Thy 1.2, FITC-CD4, FITC-CD8, FITC-CD25 (IL-2R α-chain), and FITC-Fas purchased from Pharmingen (San Diego, CA USA). Antibodies were added to 50 μL portions of cell suspension at predetermined optimal staining concentration and incubated in dark for 45 minutes at 4°C. The cells were then washed twice with 2.5 mL of wash buffer (PBS, pH 7.2/3% FBS/0.1% NaN_3) at 500 × g for 6 minutes at 4°C. The final pellet was resuspended in 200 μL fixing buffer (PBS, pH 7.2/3%

BSA/0.1% NaN_3/0.5% paraformaldehyde). Staining profiles of the cells were analyzed by FACScan flowcytometer (Beckton Dickinson, Mountain View, CA USA). The FACScan system was calibrated and optimized for analysis each day by Calbrite beads (Becton Dickinson). Background autofluorescence was determined for each sample and an average of 10,000 cells per sample were counted. Lymphocytes were selected from other cell populations on the basis of their morphologic parameters. The data were acquired and analyzed using the Cell Quest Program (Becton Dickinson).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots

Western blot analysis was carried out as described previously.[20] Proteins were extracted from the spleens by homogenizing tissues in RIPA buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% deoxycholate, 1% Nonidet P-40, 0.2 mM PMSF, 5 ng/mL leupeptin, and 50 ng/mL aprotinin) and electrophoresed through SDS-PAGE. Proteins were electroblotted onto nitrocellulose membranes. Nonspecific binding was blocked by incubating blots for 1 hour at 23°C in TTBS buffer (0.1% Tween-20, 0.1 M Tris-HCl, pH 7.7, and 0.9% NaCl) containing 10% non-fat milk. The blots were then incubated with primary antibody (1–2 μg/mL, Santa Cruz Biotechnologies, Santa Cruz, CA USA) for 2 hours at 23°C. After washing with TTBS (0.1% Tween-20, 0.1 M Tris-HCl, pH 7.7, and 0.9% NaCl), the blots were further incubated at 23°C for 1 hour with affinity-purified secondary antibody (1:5000; Santa Cruz) then washed with TTBS buffer. The blots developed with ECL^+ chemiluminescence system were exposed to X-ray films for 1 to 5 minutes. The intensity of bands was semiquantitated using the Molecular Analyst Program (Bio-Rad Laboratories, Hercules, CA USA).

Statistical analysis

The values are presented as mean ± SEM. Statistical analyses of the data were carried out using Statview 4.0/Super ANOVA package software (Abacus Concepts, Berkeley, CA USA). Data were analyzed by three-way analysis of variance (ANOVA; strain, oil, and vitamin E as main factors) and two-way ANOVA (oil and vitamin E) as main factors. Where a significant F ratio was found ($P < 0.05$) Fisher's protected least significant difference test was used to describe differences in the means among groups ($P < 0.05$).

Results

The effects of feeding CO and FO based diets (at 10% level) containing low or high levels of vitamin E for 4.5 months to MRL/*lpr* and MRL/++ mice on the body weights, production of cytokines, and lipid mediators by lectin-stimulated spleen cells, lymphoid cell subsets, and Western blot analyses of spleens for oncogenes (c-myc, c-ras), interleukin (IL)-2, and transforming growth factor (TGF)-β are presented in this section.

Body weights, incidence of lymph nodes, and survival

Because the lymphoproliferative (*lpr*) gene is overexpressed in the MRL/*lpr* mice, these mice suffer from lymphadenopathy and accelerated aging. The MRL/*lpr* mice developed lymphadenopathy and swollen lymph nodes. The body weight gain of the mice fed CO and FO with low and high levels of vitamin E (for 4.5 months) are presented in *Figure 1*. Generally the body weight gain at 4.5 months was higher in groups fed diets containing high levels of vitamin E, with the exception of the CO-HE MRL/++ group. The body weight gain in the CO-LE MRL/*lpr* group was significantly lower than that of the other MRL/*lpr* groups. In the MRL/++ groups, the weight gain in mice fed the FO-HE diet was significantly higher compared with the CO-LE MRL/++ group.

When the MRL/*lpr* mice were 9 weeks old, all the mice were surviving but the mice fed the CO-LE and FO-LE diets had started to develop lymph nodes (*Table 3*). Fifty percent of the mice in the CO-LE diet developed at 9 weeks whereas those in the HE diets did not exhibit any visible signs of lymph nodes. By 11 weeks of age, only 90% of mice were surviving in the LE groups, whereas 100% of the mice were surviving in the HE groups. At 13 weeks, most of the mice in the LE groups had developed lymph nodes, and there were visible signs of lymph nodes in the FO-HE diet group suggesting ω3 fatty acids containing high levels of vitamin E may offer protection against development of lymph nodes in the MRL/*lpr* mouse model.

Pathology of kidneys

Compared with the MRL/++ mice, the kidneys of MRL/*lpr* mice exhibited mild pathology of the kidneys (*Table 4*). The glomeruli of the MRL/*lpr* mice had mild proliferation of mesangial segments and thickening of glomerular basement membrane, the vessels had mild lymphoid infiltrates, and there was 0 to 20% atrophy of the interstitium.

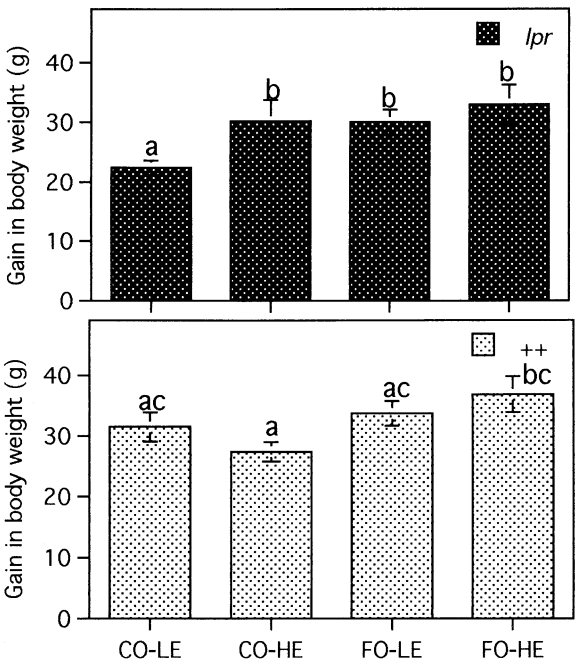

Figure 1 Effects of dietary lipids and vitamin E on body weight gain of MRL/*lpr* and MRL/++ mice. Values are mean ± SEM. $n = 10$ mice/group. Means with different superscripts are significantly different at $P < 0.05$ as revealed by Fisher's protected least significant difference test. CO, corn oil; FO, fish oil; LE, low levels of vitamin E (75 IU/kg diet); HE, high levels of vitamin E (500 IU/kg diet).

Table 3 Effects of dietary lipids and vitamin E levels on incidence of lymph nodes and survival in MRL/*lpr* mice

Age (wks)	Oil	LE				HE			
		0	+	++	Survival*	0	+	++	Survival*
9	CO	5	5	–	10	9	1	–	10
	FO	8	1	1	10	10	–	–	10
11	CO	1	5	4	9	1	5	4	10
	FO	7	–	2	9	9	1	–	10
13	CO	–	2	7	9	–	6	2	8
	FO	7	1	2	10	9	–	–	9

*Survival is reported for 10 mice/group. The scores are arbitrarily assigned to evaluate incidence and size of lymph nodes in mice as follows: 0, no lymph nodes; +, 2–3 lymph nodes; ++, several large lymph nodes.
LE–low levels of vitamin E (75 IU/kg diet). HE–high levels of vitamin E (500 IU/kg diet). CO–corn oil. FO–fish oil.

Lymphoid cell subsets

FO significantly lowered Thy.1 levels compared with CO (*Table 5*). Analysis of data by three-way ANOVA (strain, oil, and vitamin E as main factors) indicated that the percent positive cells for Thy.1 and Fas were significantly higher in the MRL/++ group compared with MRL/*lpr* group ($P < 0.01$). The groups fed the high levels of vitamin E generally had significantly higher levels of Fas with the exception of MRL/*lpr* group fed CO-HE diet. When the data were analyzed by two-way ANOVA (oil and high vitamin E as main factors for each strain), both the type of oil ($P < 0.0001$) and vitamin E ($P < 0.03$) had significant effects on the number of Thy.1 positive cells in the MRL/*lpr* mice. In the MRL/++ mice, the type of oil had a significant effect on the $CD4^+$ ($P < 0.005$) and vitamin E levels had a significant effect on number of Fas positive cells ($P < 0.04$).

Proliferative response of spleen cells to lectins

The effects of low and high levels of vitamin E on proliferative response of MRL/*lpr* and MRL/++ mice splenocytes to Con A, PHA, and LPS is presented in *Figure 2*. The proliferative response of splenocytes of MRL/++ mice to all the three tested mitogens was significantly higher compared with MRL/*lpr* mice (Con A, 4-fold; PHA, 10-fold; and LPS, 4-fold). When the data were analyzed by three-way ANOVA (strain, oil, and vitamin E as main factors), the results revealed that both strain (Con A, $P < 0.0001$; PHA, $P < 0.0001$; LPS, $P < 0.0006$) and vitamin E levels (Con A, $P < 0.007$; PHA, $P < 0.03$; LPS, $P < 0.05$) had significant effects on proliferative response of splenocytes to the three mitogens tested. When data were analyzed by two-way ANOVA (oil and vitamin E) for each strain of mice, proliferative response to Con A was significantly lower in the spleen cells of the MRL/lpr fed the FO-HE diet than in the other groups and the response to PHA was significantly lower in the CO-LE group.

In vitro production of cytokines by spleen cells stimulated with lectins IL-2 and INF-γ

The IL-2 production by lectin-stimulated spleen cells ranged between 45 and 800 pg/mL (*Figure 3*). Analysis of data by three-way ANOVA revealed that production of IL-2 in response to Con A by cultured spleen cells was significantly affected by both strain ($P < 0.0001$) and vitamin E levels ($P < 0.0002$). The production of IL-2 was significantly higher in MRL/++ mice (5-fold in the CO groups and 9- to 10-fold in the FO groups). The IL-2 production in mice on high levels of vitamin E diets was significantly higher (at least 2-fold) than the low levels of vitamin E groups. The interferon (IFN)-γ levels ranged between 1,500 and 20,000 pg/mL in response to Con A and between 130 and 1,700 pg/mL in response to PHA. The production of IFN-γ by spleen cells in response to Con A ($P < 0.0002$) and PHA ($P < 0.0001$) were significantly affected by strain.

When data were analyzed by two-way ANOVA (oil and vitamin E as main factors) for each strain, both the type of oil ($P < 0.0007$) and high level of vitamin E ($P < 0.0001$)

Table 4 Effects of dietary lipids and vitamin E levels on histopathology of kidneys in MRL/*lpr* mice

Indices	MRL/++	MRL/*lpr* CO-LE	MRL/*lpr* CO-HE	MRL/*lpr* FO-LE	MRL/*lpr* FO-HE
Quality grade	0.3 ± 0.1^{a}	1.1 ± 0.3^{ac}	1.4 ± 0.2^{b}	1.5 ± 0.4^{bc}	1.3 ± 0.4^{bc}
Glomeruli	0.4 ± 0.2^{a}	1.4 ± 0.3^{b}	1.9 ± 0.3^{b}	1.7 ± 0.3^{b}	0.4 ± 0.2^{a}
Vessels	0.3 ± 0.2^{a}	0.9 ± 0.3^{ab}	0.7 ± 0.3^{ab}	0.5 ± 0.2^{ab}	1.1 ± 0.4^{b}
Interstitium	0.0 ± 0.0^{a}	0.3 ± 0.2^{a}	0.7 ± 0.3^{a}	0.8 ± 0.3^{a}	0.5 ± 0.5^{a}

Values are mean ± SEM of 6–8 mice/group. Values in the same row without common superscript are significantly different.
The kidneys were preserved in buffered-formalin, sectioned, mounted on slides, stained with H&E dye and evaluated for pathology based on the scale as follows: glomeruli: 0 = normal, 1 = increased matrix, mild proliferation, 2 = mesengial and segmental glomerular basement membrane (GBM) thickening; 3 = diffused proliferation, wire loops, 4-glomerulosclerosis; intestitium: 0 = normal, 1 = 0 to 20%, 2 = 20 to 80%, 3 = 80 to 100% casts and/or atropy; vessels: 0 = normal, 1 = mild, 2 = moderate, 3 = severe lymphoid infiltrates.
CO–corn oil. LE–low levels of vitamin E (75 IU/kg diet). HE–high levels of vitamin E (500 IU/kg diet). FO–fish oil.

Table 5 The effects of dietary lipids and vitamin E levels on lymphoid cell subsets in MRL/*lpr* and MRL/++ mice

Strain	Subsets	CO-LE	CO-HE	FO-LE	FO-HE
MRL/++	Thy.1	28.3 ± 2.5^{a}	26.6 ± 2.1^{ac}	20.7 ± 2.3^{bc}	19.0 ± 2.5^{b}
	$CD4^{+}$	4.1 ± 0.4^{a}	4.1 ± 0.6^{a}	3.6 ± 0.5^{a}	3.3 ± 0.2^{a}
	$CD8^{+}$	5.9 ± 0.9^{a}	5.0 ± 0.6^{a}	5.2 ± 0.9^{a}	5.2 ± 0.9^{a}
	$CD25^{+}$	6.1 ± 1.1^{a}	7.4 ± 0.6^{a}	4.8 ± 1.5^{a}	6.4 ± 1.8^{a}
	Fas	1.8 ± 0.1^{a}	2.7 ± 0.4^{b}	2.0 ± 0.1^{b}	2.3 ± 0.3^{b}
MRL/*lpr*	Thy.1	24.7 ± 1.3^{a}	22.1 ± 0.8^{a}	17.4 ± 1.0^{b}	13.4 ± 2.4^{b}
	$CD4^{+}$	3.9 ± 0.7^{a}	3.3 ± 0.5^{a}	3.1 ± 0.3^{a}	3.2 ± 0.3^{a}
	$CD8^{+}$	5.3 ± 1.5^{a}	4.9 ± 0.7^{a}	3.5 ± 0.5^{a}	3.1 ± 0.7^{a}
	$CD25^{+}$	4.5 ± 1.2^{a}	5.1 ± 0.8^{a}	5.3 ± 0.8^{a}	3.5 ± 1.0^{a}
	Fas	2.0 ± 0.2^{a}	1.6 ± 0.2^{a}	1.5 ± 0.1^{a}	2.0 ± 0.4^{a}

Spleen cells (1×10^{6}) were stained with FITC or phycoerythrin (PE)-labeled monoclonal antibodies and analyzed by flow cytometry. Values are mean ± SEM of 8–10 mice/group. Means with different superscripts are significantly different at $P < 0.05$.
CO–corn oil. LE–low levels of vitamin E (75 IU/kg diet). HE–high levels of vitamin E (500 IU/kg diet). FO–fish oil.

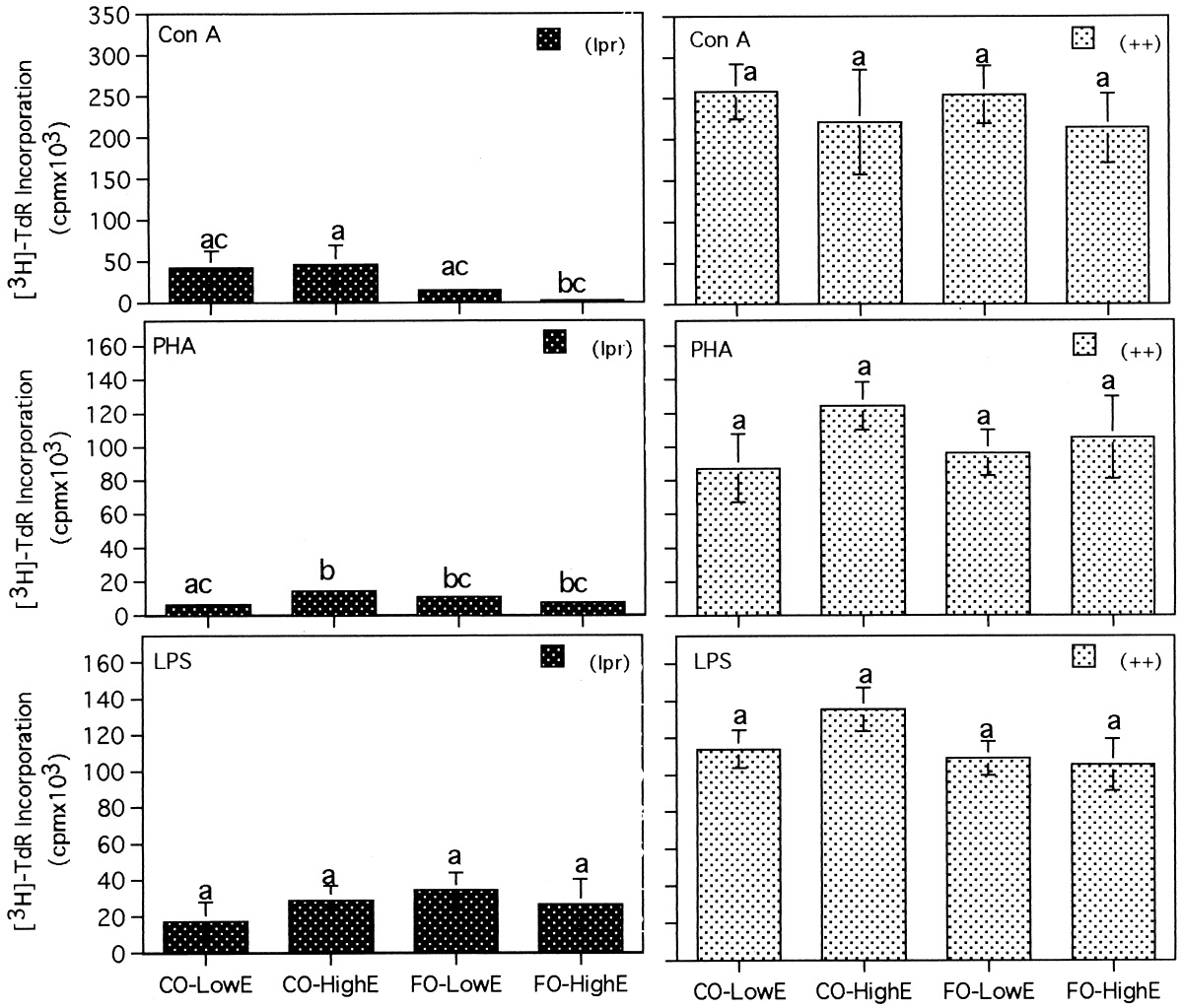

Figure 2 Effects of dietary lipids and vitamin E on proliferative response of spleen cells to lectins. Values are mean ± SEM. $n = 8$ to 10 mice/group. Means with different superscripts for each strain of mice are significantly different at $P < 0.05$ as revealed by Fisher's protected least significant difference test. Con A, concanavalin A; PHA, phytohemagglutinin; LPS, lipopolysaccharides; CO, corn oil; FO, fish oil; LowE, low levels of vitamin E (75 IU/kg diet); HighE, high levels of vitamin E (500 IU/kg diet).

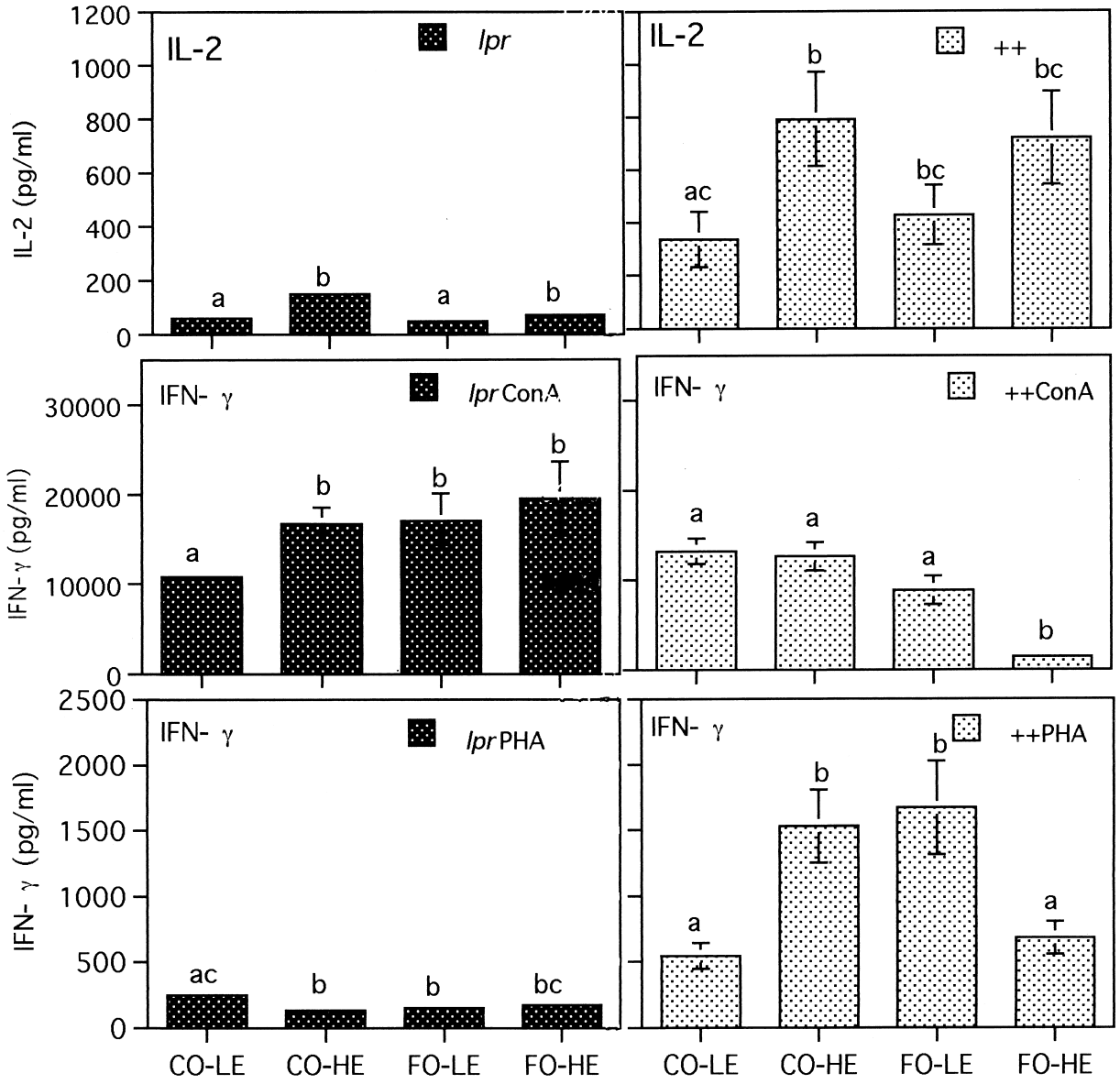

Figure 3 Effects of dietary lipids and vitamin E on production of interleukin (IL)-2 and interferon (IFN)-γ by spleen cells in MRL/*lpr* and MRL/++ mice. Values are mean ± SEM. $n = 10$ mice/group. Means with different superscripts are significantly different for each strain of mice at $P < 0.05$. Con A, concanavalin A; PHA, phytohemagglutinin; LPS, lipopolysaccharides; CO, corn oil; FO, fish oil; LE, low levels of vitamin E (75 IU/kg diet); HE, high levels of vitamin E (500 IU/kg diet).

had a significant effect on IL-2 production by spleen cells in MRL/*lpr* mice whereas only the level of vitamin E had significant effect in the MRL/++ mice. The IFN-γ production by spleen cells stimulated with PHA was not significantly affected by oil or vitamin E level, but IFN-γ production by spleen cells stimulated with Con A was significantly affected by both oil and vitamin E levels in the MRL/*lpr* mice, whereas only the type of oil had significantly influence in the MRL/++ mice.

IL-4, IL-10, and IL-12

The levels of IL-4 in the supernatant ranged between 25 and 300 pg/mL (*Figure 4*). The IL-4 production by spleen cells was 1.5-fold higher in the MRL/++ groups fed the CO-based diets and 8-fold (FO-LE) and 6-fold (FO-HE) higher in the FO groups compared with MRL/*lpr* mice. Analysis of data by three-way ANOVA indicated that IL-4 production by splenocytes in response to Con A was significantly affected by both vitamin E levels (lower in HE groups, $P < 0.05$) and strain (lower in MRL/*lpr* mice, $P < 0.0001$). In the MRL/*lpr* mice, the IL-4 levels were higher in the CO diet fed mice compared with the FO diet groups. High levels of vitamin E were also found to decrease the IL-4 production by spleen cells significantly ($P < 0.05$). The production of IL-4 by spleen cells in response to PHA was significantly affected by dietary oils ($P < 0.01$) and strain ($P < 0.0001$).

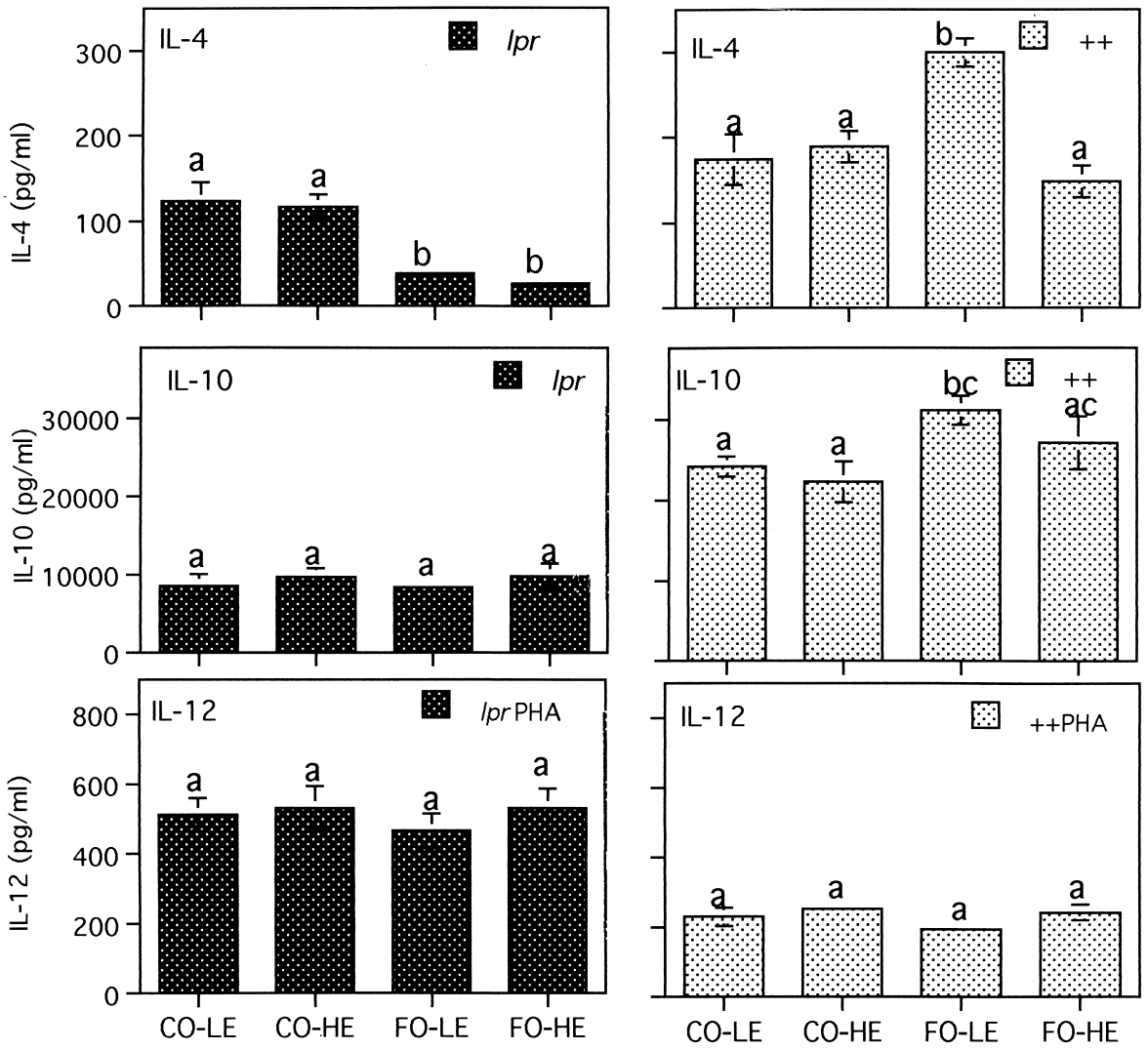

Figure 4 Effects of dietary lipids and vitamin E on production of interleukin (IL)-4, IL-10, and IL-12 by spleen cells in MRL/*lpr* and MRL/++ mice. Values are mean ± SEM. $n = 8$ to 10 mice/group. Means with different superscripts for each strain of mice are significantly different at $P < 0.05$. PHA, phytohemagglutinin; CO, corn oil; FO, fish oil; LE, low levels of vitamin E (75 IU/kg diet); HE, high levels of vitamin E (500 IU/kg diet).

When the data were analyzed by two-way ANOVA for the two strains separately, the type of oil had a significant effect ($P < 0.001$) on IL-4 production by spleen cells stimulated with Con A in MRL/*lpr* mice, whereas both oil and vitamin E levels had significant effects in the MRL/++ mice.

IL-10 production by spleen cells ranged between 8,000 to 32,000 pg/mL (*Figure 4*). The production of IL-10 by spleen cells in response to Con A was significantly higher in the FO groups than in the CO groups ($P < 0.002$) and lower in the MRL/*lpr* strain than in the MRL/++ strain ($P < 0.0001$), as revealed by three-way ANOVA. The IL-10 levels in the supernatants were 3-fold higher in the MRL/++ groups compared with the MRL/*lpr* groups. When the data were analyzed separately for the two strains by two-way ANOVA, oil had a significant effect on the production of IL-10 by spleen cells stimulated with Con A.

IL-12 production by spleen cells ranged between 190 and 700 pg/mL (*Figure 4*). IL-12 production in response to PHA was significantly affected by dietary oils ($P < 0.04$) and strain ($P < 0.0001$; 1.5-fold higher in MRL/*lpr* mice). IL-12 production in response to LPS was significantly higher in MRL/*lpr* mice compared with MRL/++ mice ($P < 0.0001$). Analysis of the data by two-way ANOVA for oil and vitamin E levels separately, showed the level of vitamin E in the diet had significant effect (MRL/*lpr*: $P < 0.04$); MRL/++ $P < 0.013$) on the production of IL-12 by spleen cells stimulated with Con A.

IL-1β, IL-6, and TNF-α

The IL-1β levels in the supernatants ranged between 25 and 45 pg/mL (*Figure 5*). Analysis of data by three-way ANOVA suggested that the production of IL-1β by splenocytes in response to LPS was higher in MRL/*lpr* mice than

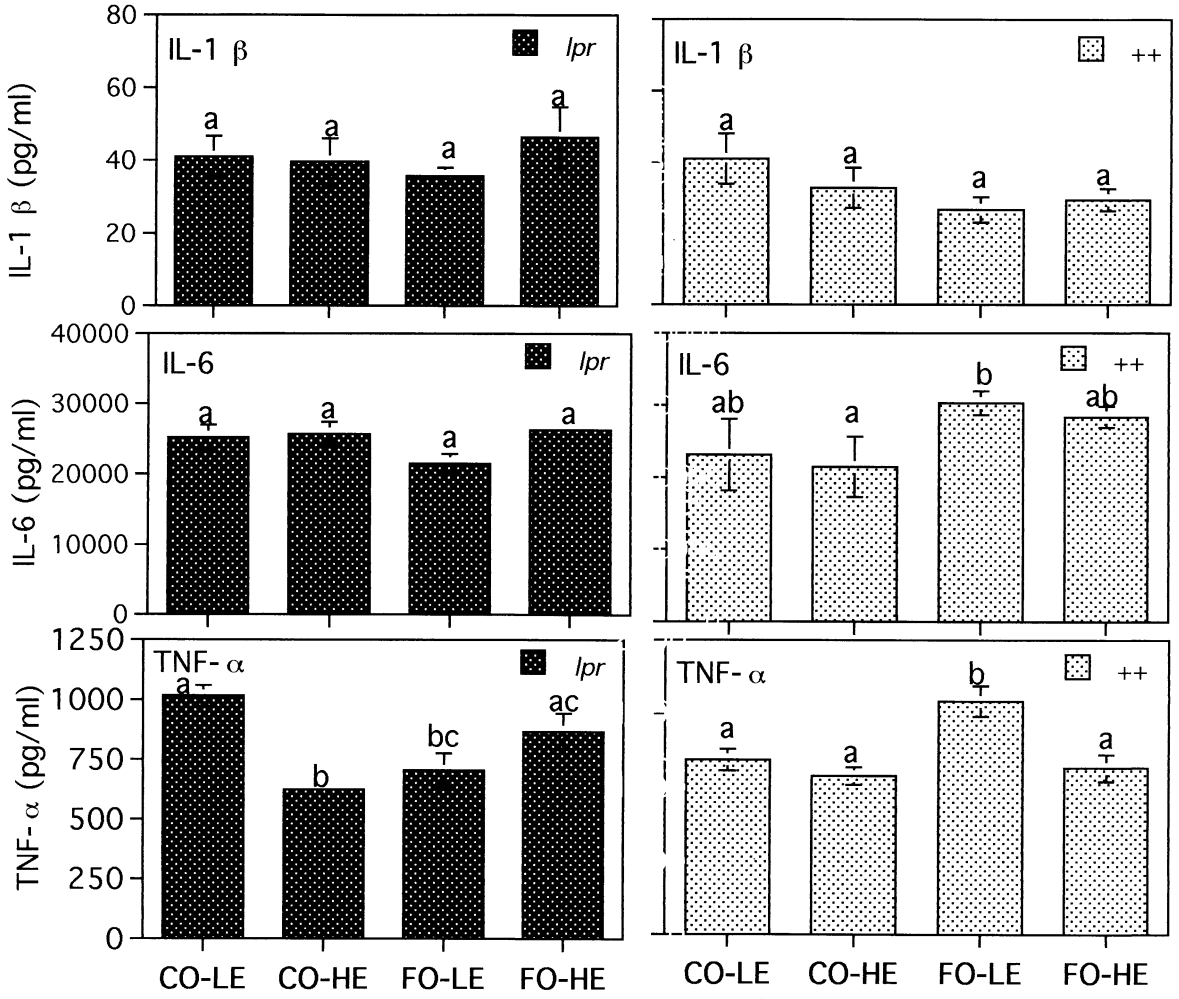

Figure 5 Effects of dietary lipids and vitamin E on the production of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α by spleen cells stimulated with lipopolysaccharides in MRL/*lpr* and MRL/++ mice. Values are mean ± SEM. n = 8 to 10 mice/group. Means with different superscripts with in each panel are significantly different at $P < 0.05$. CO, corn oil; FO, fish oil; LE, low levels of vitamin E (75 IU/kg diet); HE, high levels of vitamin E (500 IU/kg diet).

in the MRL/++ mice ($P < 0.04$). IL-1β levels were lower in the MRL/++ groups fed FO-based diets, but this effect was not statistically significant. When the data were analyzed by two-way ANOVA for each strain, there was no significant effect of oil or vitamin E on the production of IL-1 by spleen cells in response to LPS in both the strains.

The IL-6 levels in supernatants ranged between 20,000 and 30,000 pg/mL. Strain, dietary oils, and vitamin E levels did not affect IL-6 levels in the supernatants. Type of oil had a significant effect on the production of IL-6 ($P < 0.03$) in spleen cells stimulated with LPS in the MRL/++ mice, but not in the MRL/*lpr* mice as revealed by two-way ANOVA.

The TNF-α levels in the supernatants ranged between 600 and 1,600 pg/mL. The production of TNF-α by splenocytes cultured in the presence of LPS was affected by both vitamin E levels ($P < 0.0015$) and strain ($P < 0.0001$). When the data were analyzed by two-way ANOVA for the two strains separately, TNF-α levels were significantly affected by vitamin E levels in both the strains (MRL/*lpr*: $P < 0.008$; MRL/++: $P < 0.0009$), but the type of oil had significant effect ($P < 0.006$) only in the MRL/++ mice.

Effects of CO and FO with low and high levels of vitamin E on PGE_2, TXB_2, and LTB_4 production by spleen cells stimulated with LPS

The PGE_2 levels in supernatants ranged between 30 and 130 ng/mL (*Figure 6*). The production of PGE_2 by spleen cells in response to LPS was significantly effected by the type of oil ($P < 0.0001$) and by strain ($P < 0.0007$), as indicated by three-way ANOVA. The production of PGE_2 was significantly higher in the MRL/*lpr* mice fed the CO than in the FO groups. PGE_2 levels in the CO groups were 4-fold higher compared with the FO groups. When the data were analyzed by two-way ANOVA (oil and vitamin E as main factors), oil had a significant effect on PGE_2 levels (PGE_2 level in MRL/lpr mice: $P < 0.0015$; MRL/++ mice: $P <$

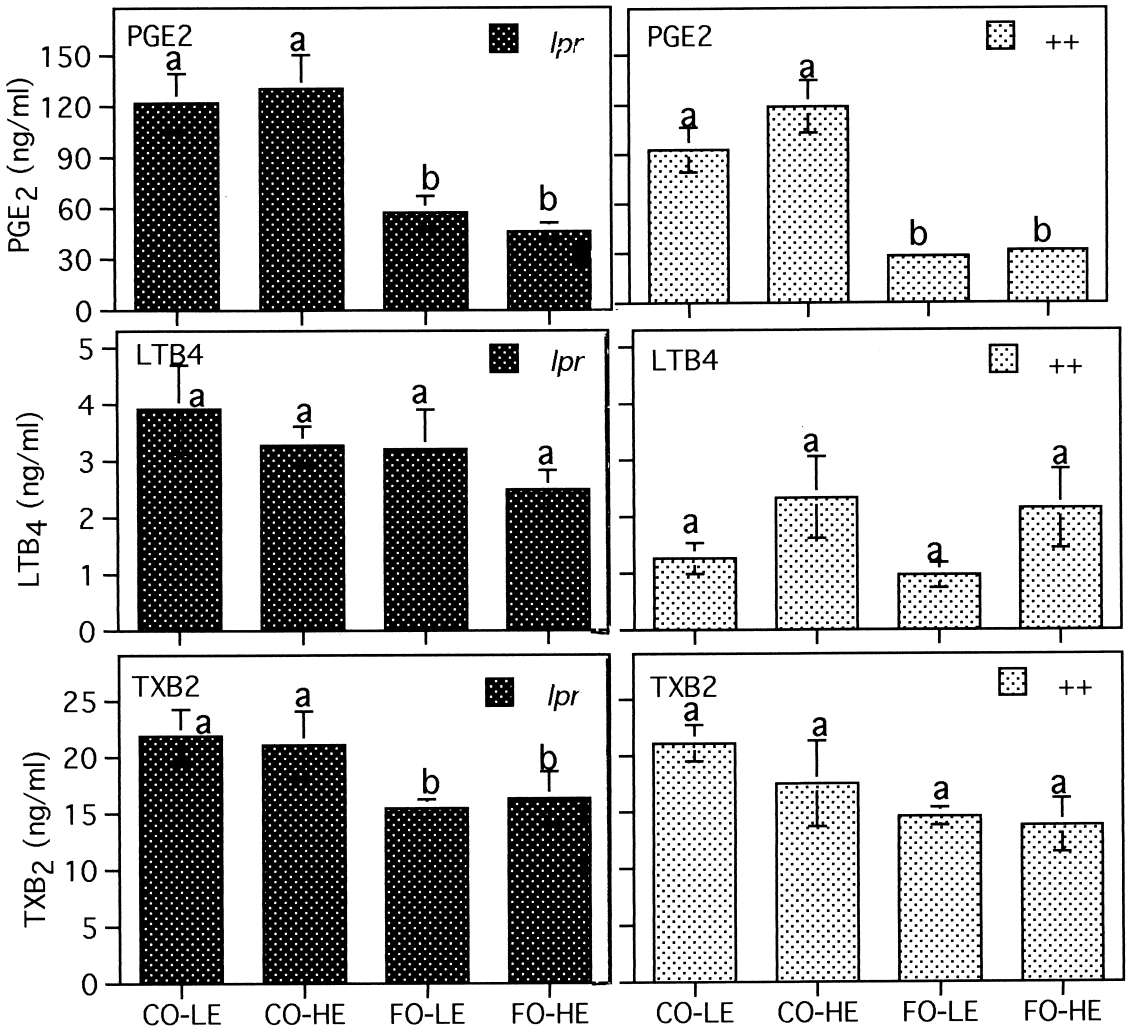

Figure 6 Effects of dietary lipids and vitamin E on production of lipid mediators [prostaglandin$_2$ (PGE_2), leukotriene B_4 (LTB_4), and thromboxane B_2 (TXB_2)] by spleen cells stimulated with lipopolysaccharides in MRL/*lpr* and MRL/++ mice. Values are mean ± SEM. $n = 8$ to 10 mice/group. Means with different superscripts for each strain of mice are significantly different at $P < 0.05$. CO, corn oil; FO, fish oil; LE, low levels of vitamin E (75 IU/kg diet); HE, high levels of vitamin E (500 IU/kg diet).

0.0002; and TXB_2 levels in MRL/++ mice: $P < 0.07$) and level of vitamin E had an effect on MRL/++ mice.

The TXB_2 levels ranged between 13 and 22 ng/mL in supernatants. The production of TXB_2 by spleen cells in response to LPS was significantly effected by the type of oil ($P < 0.004$). The supernatant LTB_4 levels ranged between 1 to 4 ng/mL. The production of LTB_4 by spleen cells in response to LPS was significantly effected by strain ($P < 0.003$). LTB_4 production was significantly higher in the MRL/*lpr* mice compared with the MRL/++ mice. The production of LTB_4 was threefold higher in the MRL/*lpr* groups than in MRL/++ groups. In the MRL/++ groups, the HE groups had twofold higher levels of LTB_4 than LE groups.

Western blot analyses

Densitometric analysis of the Western blots of proteins extracted from spleens revealed significantly lower levels of c-myc and c-ras oncogenes in the spleens of MRL/++ mice compared with the MRL/*lpr* groups (*Figure 7* and *Table 6*). The levels of c-myc were lower in the MRL/*lpr* mice fed FO-based diets. Vitamin E had no effects on the levels of c-myc and c-ras in the spleens of these mice. Splenic IL-2 levels were significantly higher in MRL/++ mice compared with the MRL/*lpr* groups (*Figure 8* and *Table 6*). Although the MRL/*lpr* group fed the FO-HE diet had higher levels of IL-2 compared with the MRL/*lpr* mice in the other groups, this effect was not statistically significant. Splenic TGF-β1 levels were normalized by high levels of vitamin E in the diet irrespective of the type of oil (*Figure 8* and *Table 6*). In the MRL/*lpr* mice fed low levels of vitamin E, the TGF-β1 levels were significantly lower than the MRL/++ mice.

Discussion

The present study was conducted to examine the mechanism of action of ω3 lipids and antioxidants on autoimmune

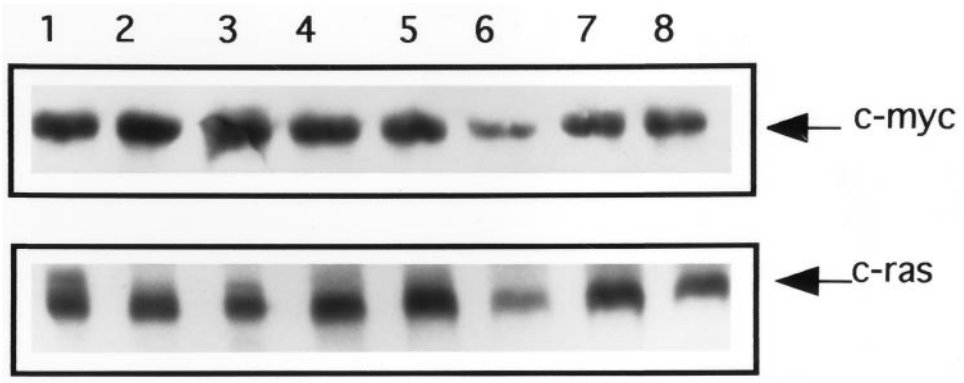

Figure 7 Western blot analysis of c-myc and c-ras protein levels in spleens of MRL/*lpr* mice fed corn oil (CO) and fish oil (FO) based diets containing low (LE) or high (HE) levels of vitamin E. Spleen proteins (40 μg) were electrophoresed by SDS-10%PAGE for c-myc (MW = 67,000) and 16% PAGE for c-ras (MW = 21,000), electroblotted on nitrocellulose membrane, and probed with c-myc or c-ras antibodies. The bands were developed using enhanced chemiluminescence system and the blots were exposed to X-ray film. The bands were semiquantitated using the Molecular Analyst Program. Lanes 1 and 2, CO-LE; 3 and 4, CO-HE; 5 and 6, FO-LE; 7 and 8, FO-HE.

disease. Results from this study indicated that both FO and high levels of vitamin E delayed the visible appearance of lymph nodes in the MRL/*lpr* mice. The MRL/*lpr* mice have a very different cytokine profile, oncogene levels, lymphoid cell subsets, and inflammatory mediators, and several of these factors may contribute to the disease. Strain, oil, and vitamin E level had significant effects on IL-2 and IL-4 levels. The type of oil had a significant effect on certain lymphoid cell subsets, proliferative response, and production of IL-2, IL-4, IL-12, PGE_2, and TXB_2 by spleen cells in response to lectins and vitamin E had a significant effect on IL-2, IL-4, and TNF-α levels, suggesting that it may be possible to delay certain abnormalities existing in cytokine, inflammatory mediators, subsets, oncogene levels, and the like by dietary intervention with ω3 lipid and vitamin E. Once the lymph nodes start to appear in the MRL/*lpr* mice, dietary intervention may not help, and it is important to start the dietary ω3, vitamin E therapy at very early stages in these mice to delay or prevent the appearance of lymph nodes.

Data from animal and human studies have demonstrated that vitamin E may have beneficial effects on symptoms of arthritis.[21] The exact mechanism(s) involved in delaying autoimmune disease by FO is still not clear, although several possibilities have been suggested.[12,13,22] Dietary FO may dramatically downregulate key immunoregulatory cytokines involved in autoimmune disease.[23] In autoimmune diseases, abnormal production of proinflammatory cytokines, or a reduced inhibition of their actions, may lead to an imbalance.[24] The balance of Th_1/Th_2 cytokines and high levels of several oncogenes in lymphoid tissues is thought to play a role in the progression of many autoimmune diseases. Our earlier studies have suggested that high levels of vitamin E in FO diets have additional benefits in lowering the levels of proinflammatory cytokines.[23]

Cytokines and eicosanoids are important biological mediators and production of these are under tight regulation. Overproduction of these mediators may contribute to the pathogenesis of acute inflammatory and autoimmune diseases. Overproduction of IL-1, IL-6, and TNF-α has been implicated in the pathogenesis of several inflammatory diseases such as RA.[25] Generally high levels of IL-6 are detected in synovial fluid and in sera of patients with RA. In the present study, IL-2 production by Con A-activated spleen cells was lower in the MRL/*lpr* mice compared with MRL/++ mice. In vitro studies have established that supplementing di-homo-gamma linoleic acid (precursor to PGE_1) or arachidonic acid (precursor to PGE_2) inhibits IL-2 production. In contrast, EPA (precursor to PGE_3) showed less inhibition of IL-2,[26] indicating the immunosuppressive role of ω3 fatty acids.[23] Significantly higher IL-2 production by spleen cells in response to Con A has been reported in FO-fed B/W mice compared with CO-fed mice.[23] However, several studies have indicated that ω3 fatty acids may decrease or not affect IL-2 levels.[27–29] In our study, high levels of vitamin E enhanced in vitro production of IL-2 by Con A-activated spleen cells in the MRL mice. We observed that lectins (Con A and PHA) had differential effects

Table 6 The effects of dietary lipids and vitamin E levels on densitometric analysis of Western blots

	MRL/++	MRL/*lpr* CO-LE	MRL/*lpr* CO-HE	MRL/*lpr* FO-LE	MRL/*lpr* FO-HE
c-myc	13.2 ± 0.1[a]	31.7 ± 1.7[b]	32.8 ± 3/4[b]	24.1 ± 5.0[b]	23.7 ± 0.2[b]
c-ras	13.1 ± 0.1[a]	26.0 ± 0.4[b]	26.2 ± 3.3[b]	25.8 ± 5.4[b]	26.7 ± 3.7[b]
IL-2	8.3 ± 0.1[a]	5.1 ± 0.2[b]	5.5 ± 0.7[b]	5.2 ± 0.3[b]	6.5 ± 0.5[b]
TGF-β	25.5 ± 0.05[a]	18.9 ± 0.7[b]	24.1 ± 2.6[a]	20.2 ± 1.6[b]	25.4 ± 1.3[a]

Although Western blot analysis was repeated several times for 4 samples/group, the values reported here are for the 2 samples/group seen in the blots presented in *Figures 7 and 8.* Means with different superscripts are significantly different at $P < 0.05$.
The bands detected by specific antibodies shown in *Figures 7 and 8* were semiquantitated by videoimaging using Molecular Analyst IV Program.
CO– corn oil. LE–low levels of vitamin E (75 IU/kg diet). HE– high levels of vitamin E (500 IU/kg diet). FO–fish oil. IL–interleukin. TGF–transforming growth factor.

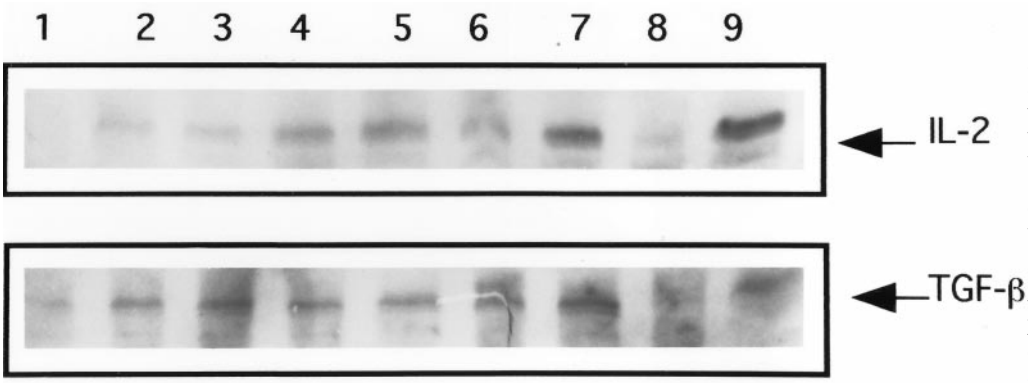

Figure 8 Western blot analysis of interleukin (IL)-2; (MW = 14,000) and tranforming growth factor (TGF)-β (MW = 8,000–10,000) protein levels in spleens of MRL/*lpr* and MRL/++ mice fed corn oil (CO) and fish oil (FO) based diets containing low (LE) or high (HE) levels of vitamin E. Spleen proteins (80 μg) were electrophoresed by SDS-16%PAGE, electroblotted on nitrocellulose membrane, and probed with IL-2 or TGF-β antibodies. The bands were developed using enhanced chemiluminescence system and the blots were exposed to X-ray film. The bands were semiquantitated using the Molecular Analyst Program. MRL/*lpr* mice spleens. Lanes 1 and 2, CO-LE; 3 and 4, CO-HE; 5 and 6, FO-LE; 7 and 8, FO-HE; 9, MRL/++.

on IFN-γ production by spleen cells in MRL/*lpr* mice compared with MRL/++ mice. IFN-γ is an important mediator of the immune system, having profound effects on immune regulation and inflammation. It can also influence the activities of T cells, B cells, and natural killer cells. IFN-γ upregulates the expression of major histocompatibility complex (MHC) class II antigens in a wide variety of cells and accelerates development of autoimmunity in B/W mice.[30] Both increased production of IFN-γ following consumption of ω3 fatty acid[31] and no changes in IFN-γ levels[29] have been reported.

Data from the present study indicated lower IL-4 production by Con A-activated spleen cells in MRL/*lpr* mice compared with MRL/++ mice. IL-4 generally produced by T cells seems to exhibit a coordinated anti-inflammatory action.[32] In synoviocytes, IL-4 is reported to block IL-1–induced PGE_2 and granulocyte-macrophage colony-stimulating factors (GM-CSF), but increase IL-6 production.[33] IL-4 acts in concert to induce activated B lymphocytes to grow, switch isotypes, and ultimately, differentiate into antibody-producing plasma cells. Feeding CO- and FO-based diets did not alter IL-4 production by spleen cells of B/W mice.[23] Our data suggest that in vitro production of IL-4 by spleen cells in response to Con A was lower in the groups of MRL/*lpr* mice fed FO than in the groups fed CO.

IL-10 is a proinflammatory cytokine that is produced by subsets of activated T cells, B cells, and macrophages and mediates a variety of both immunostimulatory and immunosuppressive properties in the mouse and human in vitro.[34] Continuous treatment of lupus-prone B/W mice with anti–IL-10 antibodies is reported to substantially delay the onset of autoimmunity.[35] IL-12 is a potent proinflammatory and immunoregulatory cytokine that plays a key role in innate and adaptive immunity. In the present study, spleen cells from MRL/*lpr* mice produced higher levels of IL-12 compared with MRL/++ mice. Although feeding FO is reported to lower the level of IL-12 in normal strains of mice, we did not observe these effects in the MRL mice.

FO may ameliorate clinical symptoms of RA patients by lowering the levels of proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α. These cytokines are major mediators of inflammation and they may be involved in the pathogenesis of RA. These cytokines can trigger cells to secrete other inflammatory mediators, including other cytokines such as the T-lymphocyte products IFN-γ and IL-2 and lipid mediators such as prostaglandins, leukotrienes, and platelet-activating factor.[36] In addition to TNF-α and IL-1 action, the destructive process appears to be under the control of mediators such as IL-6 and IL-10. It is suggested that IL-1-α, epidermal growth factor, and TGF-β may be important in modulating the contribution of the intracellular and extracellular route of collagen breakdown. The data from our study indicated that both FO and diets containing high levels of vitamin E lowered the TNF-α and IL-6 levels in MRL/*lpr* mice, but not IL-1β levels. LPS-activated spleen cells from MRL/*lpr* mice produced higher levels of IL-1β compared with MRL/++ mice. The expression of MHC class II antigen, production of IL-1 and TNF-α mRNA levels,[12] and arachidonic acid metabolism[37] are reported to be downregulated by ω3 lipids intake. The absence of dramatic changes in IL-1β by ω3 lipids by spleen cells stimulated with LPS in the present study may be a result of peritoneal macrophages being more sensitive to ω3 treatment and IL-1 production compared with spleen cell macrophages. However, TNF-α production by LPS-activated spleen cells was lower in MRL/*lpr* mice.

Eicosanoids such as PGE_2, LTB_4, and TXB_2 are implicated in inflammatory diseases. Hence a decrease in the production of these cytokines and eicosanoids after ω3 fatty acids consumption may slow the pathogenesis of these diseases. Beneficial effects of FO in MRL/*lpr* mice have been reported.[38] We observed that FO decreased PGE_2, TXB_2, and LTB_4 levels compared with CO-based diets. Inflammatory agonists are believed to stimulate eicosanoid synthesis by enhancing the release of arachidonic acid (20:4 ω6) from the intracellular phospholipid pool through activation of phospholipases. Subsequently, the free 20:4 ω6 is metabolized into prostaglandins, thromboxanes, and leukotrienes by the enzymes cyclooxygenase and lipoxygenase. EPA is rapidly incorporated into cell membrane phospholipids, where it replaces 20:4 ω6 as a substrate and is converted into the biologically less active PGE_3, TXA_3, and

LTB_5.[39] ω3 Fatty acids have been shown to be poorly metabolized by cyclooxygenase, thereby reducing the total production of eicosanoids.[40] CO-fed B/W mice had a significantly lower T-cell proliferative response at 6.5 months when compared with FO-fed mice, indicating that an increased 20:4 ω6 fatty acid incorporation in the spleens of the CO-fed group may have contributed to an earlier decline in T-cell function.[23]

The MRL/*lpr* mice develop a defect in the regulatory balance of the immune system and autoimmunity relatively early in life.[9] Mice with the *lpr* gene (a single gene defect) develop generalized massive lymphadenopathy resulting from accumulation of immature $Thy1^+Lyt2\text{-}L3T4^-$ cells[9] due to lack of secretion of adequate regulatory factors such as IL-2, TNF-α, which modulate autoimmunity. In the present study, FO and vitamin E altered the levels of Thy.1 in MRL/*lpr* mice and the level of $CD4^+$ positive cells and vitamin E altered the number of Fas positive cells. FO-fed mice are reported to have higher percentages of $CD4^+Pgp\text{-}1^-$ and $CD8^+Pgp\text{-}1^-$ T cells, including higher $LECAM\text{-}1^+$ cells, compared with CO-fed B/W mice.[23] Increased Pgp-1+ T-cell subsets (memory or Th2 cells) generally increase with age.[41] Several studies have suggested that a high number of Th2 cells are known to contribute to the production of proinflammatory cytokines.[42,43] The lymph nodes of most MRL/*lpr* mice are heavily populated with $Thy.1^+$, dull CD5, CD4, and CD8 cells. In addition, the cells have high expression of cell surface antigens such as CD45 (B220), Ly-6, and CD44.[44] Earlier studies have shown that lymph node cells from MRL/*lpr* mice express large amounts of the myb protooncogene, which is ordinarily only expressed at high levels in the thymus or after mitogenic stimulation of T cells.[45]

Differences in proliferative response in the ω6 and ω3 diets treatments are observed in spleens of normal strains of mice. However, the particular strain of MRL/*lpr* mice age very fast, developing splenomegaly (enlarged spleens). This may be the reason for not observing sufficient differences in proliferative response of spleen cells between ω6 and ω3 lipids fed groups. In addition, the MRL/++ mice are a normal strain and may not require high levels of FO or vitamin E. It is known that normal strains such as C57BL/6 can get sick when fed high levels of FO and high levels of FO may be tolerated as a therapeutic agent only in autoimmune mice. Suppression of mitogenic response in humans after consumption of ω3 polyunsaturated fatty acids has been reported by other investigators.[26]

Autoimmune-prone MRL/*lpr* and B/W mice generally have increased expression of several protooncogenes in their lymphoid tissues.[45,46] High expression of several oncogenes such as c-myc, c-ras, c-fos, and c-myb in the spleens and lymph nodes of MRL/*lpr* mice, which may be responsible for several abnormalities in the MRL/*lpr* mice, have been reported,[22,23,47] but normal strains of mice express very low levels of these oncogenes in steady state. We observed that the MRL/*lpr* mice expressed higher levels of c-ras and c-myc and FO decreased the levels of c-myc in the spleens of these mice, whereas vitamin E had no effect. In the spleens of CO-fed mice, the protein levels for both c-myc and c-ras oncogenes were significantly higher in MRL/*lpr* mice and c-myc levels were lower in the FO-fed groups. TGF-β1 levels were higher in the spleens of HE-fed mice compared with LE-fed groups. The increased oncogenes, c-myc, and c-ras mRNA expression are reported to decrease in the FO-fed mice.[23] The increased oncogenes mRNA levels may be due to in vivo activation of lymphocytes and macrophages, which may increase proinflammatory cytokine levels such as IL-6 and TNF-α. The oncogenes are thought to be involved in several important pathways such as signal-related events, and proliferation of cells.

Activated lymphocytes and macrophages have also been implicated in the production of growth factors including TGF-β, which in turn is known to stimulate fibroblast proliferation and collagen synthesis.[48,49] TGF-β1 is reported to have both immunosuppressive and immunostimulatory effects. It can downregulate $CD8^+$ T-cell function, increase monocyte chemotactic function, and upregulate the expression of integrins and matrix-degrading enzymes.[50]

Although no fatty acid analysis was done for spleen cells in this study (most of the cells were used for setting up immunologic experiments), we carried out fatty acid analyses of spleen cells of autoimmune mice (B/W and MRL/*lpr* mice) in our earlier studies.[6,7,23] These studies suggested that there was extensive incorporation of ω3 fatty acids in the FO-fed groups and ω6 fatty acids into the spleen cell membranes there was incorporation of ω3 lipids in the phospholipids classes and total lipids of spleens and other tissues, in CO fed groups when these diets were fed for 3 to 6 months. The 10% CO fed mice had 18:2 (ω6) 11%; 20:4 (ω6) 9.4%, 20:5 (ω3) 0.76%, and 22:6(ω3) 1.4% in their spleen cells. When 10% FO was fed to the mice, they had 18:2 (ω6) 5%; 20:4 (ω6) 4%, 20:5 (ω3) 3.4%, and 22:6 (ω3) 2.8% in their spleen cells. There also is evidence from other studies that feeding FO does not significantly alter the distribution of phospholipid classes.[51] Our earlier experiments also suggested that feeding ω6, ω3, or saturated fatty acids can significantly alter the fatty acid composition of phospohlipids in splenocytes and membranes such as microsomes, mitochondria, nuclear envelopes, and plasma membranes from other tissues.[6,7] Our earlier experiments on splenic T and B cells of MRL/*lpr* and MRL/++ mice suggested that the fatty acid composition of membranes is genetically determined but could be altered by the manipulation of dietary lipids.[6,7,13] There may be a possibility that immune cells may preserve arachidonic acid pool under certain circumstances, but our earlier work in autoimmune mice has indicated that FO feeding can decrease the levels of arachidonic acid in spleen cells by at least 50% in autoimmune mice.[23]

Most studies using FO report lower levels of monounsaturated fatty acids (MUFA) in FO. Unfortunately, it is difficult to balance the level of MUFAs in the two diets. Studies carried out in $NZB/NZWF_1$ (B/W) mice using EPA and DHA esters have suggested that a combination of these two fatty acids have anti-inflammatory effect in these B/W mice.[23,51] It is difficult to speculate about the contribution by MUFAs in this study; however, most of the observed effects are perhaps due to ω6 and ω3 fatty acids. Some of the recent studies on MUFAs suggests that they have anti-inflammatory effects and may function like ω3 lipids but to a much lesser extent. The effects of MUFAs have

been addressed mainly in connection with cardiovascular diseases where MUFAs may be involved in lipoprotein metabolism. Our earlier studies have suggested that in spite of high levels of MUFAs in CO, they have negative effects on autoimmunity in B/W mice.[13,23] In addition, the effects we see on PGE_2, LTB_4, TXB_2, and the like are mainly due to ω6 and ω3 fatty acids. The effects of MUFAs on the level of cytokines are not very clear at the present time. Some of the recent studies on MUFAs suggest that they have anti-inflammatory effects and may function like ω3 lipids but to a much lesser extent. The effects of MUFAs have been addressed mainly in connection with cardiovascular diseases where MUFAs may be involved in lipoprotein metabolism. The strategy for using FO is because of their effects in lowering IL-1, TNF-α, and inflammatory mediators such as PGE_2, LTB_4, and TXB_2. In future studies it is important to determine the effects of individual ω6 and ω3 fatty acids and balance the levels of saturates and MUFAs to address the role of these in ω6 and ω3 lipid based diets.

Some of the non-lipids and unsaponifiable fractions of FO may be bioactive but it is beyond the scope of this study to speculate the extent to which these fractions may affect our results. There is a possibility that FO from animal sources may contain a certain amount of cholesterol, trace amounts of estrogen, and perhaps other compounds. However, because the major components are EPA and DHA, it is reasonable to speculate that the main effects we observe are because of the ω3 lipids.

In summary, spleen cells from the MRL/*lpr* mice produced lower levels of IL-2, IL-4, IL-10, and TNF-α, and higher levels IL-1β, IFN-γ, IL-12, PGE_2, and LTB_4. FO increased the levels of IL-10 and decreased the production of IL-12 and TXB_2. High levels of vitamin E increased IL-2 and decreased IL-4 and TNF-α production. Both FO and vitamin E are beneficial in specifically modulating the levels of specific cytokines and thereby may affect the immune system onset of autoimmunity. Findings from the present study suggest that there were very significant differences between the two strains in most of the parameters tested. FO may ameliorate autoimmune disease in MRL/*lpr* mice by delaying the appearance of lymph nodes in the MRL/*lpr* model by increasing the production of IL-2 and decreasing IL-4 and lowering PGE_2 and LTB_4 levels. In addition, FO also lowered the levels of the oncogene c-myc, increased levels of splenic IL-2, and normalized TGF-β1 levels. The effects of FO on IFN-γ (Con A stimulated) was different for the two strains of mice. Vitamin E significantly delayed the appearance of lymph nodes and modulated proliferative response, levels of c-myc oncogene, IL-2, IFN-γ IL-12, and TNF-α production by spleen cells in MRL/*lpr* mice. In the MRL/++ mice, the type of oil altered the level of CD4+ positive cells, IFN-γ, IL-4, TNF-α and PGE_2 production by spleen cells and vitamin E altered the levels of Fas positive cells, proliferative response, IL-4, TNF-α, and TXB_2 levels. In the MRL/*lpr* mice, the type of oils had dramatic effects in modulating Thy.1 positive cells, IL-2, IFN-γ, IL-4, and PGE_2 production by spleen cells.

Nutrition intervention should be aimed at reducing the rise in memory T cells and maintaining higher Th_1 (virgin T cells), which are known to secrete higher levels of IL-2, IFN-γ, and TNF-β, that may delay or inhibit the onset of autoimmune disease. ω3 Lipid intake could play a role as a preventive measure against diseases and appears to have promising future to utilize it as an adjunct therapeutic lipid agent to reduce both inflammation and toxicity of drugs in critically ill patients. In the MRL/*lpr* mouse model, the *lpr* gene is overexpressed at very early stages of life. The role of dietary intervention with ω3 lipids and vitamin E in delaying the early expression of the lpr gene remains to be investigated. It is evident from the present study that high levels of vitamin E in the FO diet may be beneficial in delaying appearance of lymph nodes and decreasing abnormal levels of specific proinflammatory cytokines, and oncogenes levels in the MRL/*lpr* mice. Studies in the future should address the role of individual ω6 and ω3 fatty acids on autoimmune disease, the role of FO and vitamin E ligand in apoptosis-related mechanisms, and molecular aspects of the pro- and anti-inflammatory cytokines, role of cytokine receptors, soluble receptors, inhibitors, and oncogenes in the MRL/*lpr* mouse model.

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