Dietary Conjugated Linoleic Acid Modulates Phenotype and Effector Functions of Porcine CD8$^+$ Lymphocytes[1]

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ABSTRACT In vivo vaccination and challenge studies have demonstrated that CD8$^+$ lymphocytes are essential for the development of cell-mediated protection against intracellular pathogens and neoplasic cells. Depletion of peripheral blood CD8$^+$ cells interferes with clearance of viruses and intracellular fungi, induction of delayed type hypersensitivity responses and antitumoral activity. In contrast to humans or mice, porcine peripheral CD8$^+$ lymphocytes are characterized by a heterogeneous expression pattern (i.e., CD8$\alpha\beta$ and CD8$\alpha\alpha$) that facilitates the study of distinctive traits among minor CD8$^+$ cell subsets. A factorial (2×2) arrangement within a split-plot design, with 16 blocks of two littermate pigs as the experimental units for immunization treatment (i.e., unvaccinated or vaccinated with a proteinase-digested *Brachyspira hyodysenteriae* bacterin) and pig within block as the experimental unit for dietary treatment (soybean oil or conjugated linoleic acid) were used to investigate the phenotypic and functional regulation of CD8$^+$ cells by dietary conjugated linoleic acid (CLA). Dietary CLA supplementation induced in vivo expansion of porcine CD8$^+$ cells involving T-cell receptor (TCR)$\gamma\delta$CD8$\alpha\alpha$ T lymphocytes, CD3$^-$CD16$^+$CD8$\alpha\alpha$ (a porcine natural killer cell subset), TCR$\alpha\beta$CD8$\alpha\beta$ T lymphocytes and enhanced specific CD8$^+$-mediated effector functions (e.g., granzyme activity). Expansion of peripheral blood TCR$\alpha\beta$CD8$\alpha\beta$ cells was positively correlated ($r = 0.89$, $P < 0.01$) with increased percentages of CD8$\alpha\beta^+$ thymocytes. Functionally, CLA enhanced the cytotoxic potential of peripheral blood lymphocytes and proliferation of TCR$\gamma\delta$CD8$\alpha\alpha$ cells. Collectively, these results indicate that dietary CLA enhances cellular immunity by modulating phenotype and effector functions of CD8$^+$ cells involved in both adaptive and innate immunity. J. Nutr. 131: 2370–2377, 2001.

KEY WORDS: • *conjugated linoleic acid* • *pigs* • *cellular immunity* • *CD8$^+$ lymphocytes.*

During the last century, frontiers of nutritional sciences were modified to accommodate research beyond the limits defined by prevention of nutrient deficiencies and evaluation of nutrient requirements. Today, a broader scope is given to nutritional research that includes the identification of minor dietary adjustments that enhance homeostasis and health. Nutritional therapy may be important in immunoregulation, inflammation, neoplasia and vascular diseases. In particular, fatty acids influence health through a variety of mechanisms, including immunomodulation (1). Modulation of the immune system and of cellular homeostasis by dietary fatty acids may occur through regulation of arachidonic acid [20:4(n-6)] metabolism and eicosanoid production (2,3), modification of membrane fluidity (4,5) and/or transcriptional regulation of gene expression by peroxisome proliferator-activated receptors (PPAR),[4] which may be activated directly through fatty acids or indirectly through lipid-derived mediators (6–8).

Dietary fatty acids influence several aspects of adaptive immunity. CD28 expression on the membrane of lymphocytes recovered from mice fed a diet enriched in docosahexaenoic acid [22:6 (n-3)] was elevated compared with those recovered from dietary control mice (9). In aged mice, energy-restricted fish oil–supplemented diets have been shown to attenuate the decrease of CD4$^+$ and CD8$^+$ lymphocytes (10). In pigs, although the effects of dietary fat on immune function have been investigated (11,12), these studies usually did not focus on the nutritional modulation of a particular T-cell subset (i.e., CD8$^+$ cells). The characterization of the immunomodulatory properties of fatty acids on the numerically most important lymphocyte subset in pigs could be instrumental in the enhancement of porcine health by nutritional means.

In most mammalian species, CD8 molecules are expressed either as a disulfide-linked $\alpha\alpha$-homodimer or as an $\alpha\beta$-heterodimer on the surface of lymphocytes (13). In humans, most mature peripheral lymphocytes preferentially express the CD8$\alpha\beta$ molecule, whereas the expression of CD8$\alpha\alpha$ within the lymphocytic pool is restricted to a subset of natural killer (NK) cells and on intraepithelial lymphocytes (14). The pre-

[1] Supported by a grant of the National Pork Board (99208) awarded to J.B.-R.
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[4] Abbreviations used: BLT, benzyloxycarbonyl-L-lysine thiobenzyl ester; CLA, conjugated linoleic acid; Con A, Concanavalin A; FACS, fluorescence-activated cell sorting; Ig, immunoglobulin; MHC, major histocompatibility complex; NK, natural killer; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; PPAR-γ, peroxisome proliferator-activated receptor-γ; PUFA, polyunsaturated fatty acids; SP, single-positive; TCR, T-cell receptor.

0022-3166/01 $3.00
Manuscript received 4 April 2001. Initial review completed 30 May 2001. Revision accepted 2 July 2001.

dominance of CD8$\alpha\beta^+$ T cells in humans often masks the functional role of other CD8$^+$ subsets (15). Conversely, porcine CD8$^+$ cells are functionally and phenotypically heterogeneous. Subpopulations of porcine peripheral blood mononuclear cells (PBMC) expressing CD8$\alpha\alpha$ are not overshadowed by a predominance of CD8$\alpha\beta^+$ cells (16,17). Phenotypically, porcine CD8$\alpha\alpha^+$ lymphocytes can be subdivided into four subpopulations as follows: *1*) T-cell receptor (TCR)$\gamma\delta^+$CD4$^-$; *2*) TCR$\alpha\beta^+$CD4$^+$; *3*) CD3$^-$CD16$^+$ (a subset of NK cells); and putatively, according to indirect inferences, *4*) TCR$\alpha\beta^+$CD4$^-$ (18). A fifth subset of porcine CD8$^+$ cells is represented by CD8$\alpha\beta^+$ lymphocytes that express TCR$\alpha\beta$ on the membrane and are CD4$^-$.

CD8$^+$ cells, particularly CD8$\alpha\beta$TCR$\alpha\beta$ lymphocytes, are central to the induction of cell-mediated responses against viruses, intracellular bacteria, parasites and neoplasia. Depletion of CD8$^+$ cells adversely affects the development of immune responses to a variety of intracellular pathogens such as simian/human immunodeficiency virus (19), *Histoplasma capsulatum* (20), *Cryptococcus neoformans* (21) and *Mycobacterium tuberculosis* (22). In addition, CD8 depletion abrogates antitumor immunity (23). In pigs, an enhancement of numbers of CD8$^+$ cells correlated with enhanced growth performance and feed efficiency (24). Thus, the maintenance of numbers of peripheral blood CD8$^+$ cells enhances health, possibly by increasing the efficacy of cell-mediated immune responses.

Fatty acids containing two double bonds separated by a single bond, chemically defined as conjugated dienes, are nutraceuticals with broad biological activities including health benefits. Conjugated linoleic acid (CLA) is a mixture of positional (9,11; 10,12; or 11,13) and geometric (*cis* or *trans*) isomers of linoleic acid [18:2(n-6)] with anticarcinogenic (25), antidiabetic (26), antiatherogenic (27) and immunomodulatory (28–30) properties. In preliminary studies, we observed that percentages of peripheral blood CD8$^+$ cells increased in pigs fed a diet containing CLA (predominantly *cis*-9, *trans*-11/*trans*-9, *cis*-11; 35.1% and *trans*-10, *cis*-12/*cis*-10, *trans*-12; 25.1% isomers) (29). However, a more complete functional and phenotypic characterization of cell subsets within the CD8$^+$ population was required to define specifically the functional properties of dietary CLA as an immunomodulatory compound.

MATERIALS AND METHODS

Experimental design. From d 0 to 35 of the experiment, a total of 32 pigs ($n = 8$) were on trial. Eight pigs were killed by litter, body weight and gender on d 35 for phenotypic evaluation of thymocytes. From d 35 to 72, 24 pigs were on trial distributed into four ($n = 6$) dietary and/or vaccination treatments as follows: *1*) no supplemented CLA, unvaccinated; *2*) supplemented CLA, unvaccinated; *3*) no supplemented CLA, vaccinated; and *4*) supplemented CLA, vaccinated. Peripheral blood was collected on d 0, 14, 21, 28, 35, 42, 49, 56, 63 and 72.

Dietary and vaccination treatments. Cross-bred pigs ($n = 32$; 14 d old) serologically negative for *Brachyspira hyodysenteriae* were randomly distributed from outcome groups based on litter, body weight and gender to 16 blocks of two contiguous individual pens. The design was chosen to decrease genetic variation associated with the utilization of cross-bred pigs. Either a 1.33 g CLA/100 g of diet or an isocaloric and isonitrogenous soybean oil–supplemented control diet (**Table 1**) was randomly allotted to pens within blocks of two littermate pigs as previously described (29). Pigs were given free access to feed for 72 d in four phases (I, 1–2; II, 3–4; III, 5–8; and IV, 9–11 wk). Between treatments, diets were formulated to be isocaloric and isonitrogenous because modifications in the levels of energy (10) or protein (31) have been shown to influence immunity. Thus, in control diets, 2.21 g CLA/100 g was replaced by 2.21 g soybean oil/100 g to maintain both the CLA-supplemented and the control diets isocaloric within phases. Pigs were fed either a CLA-supplemented or a control diet from d 0 to 72. The source of CLA (alkali-isomerized sunflower oil; ConLinco, Detroit Lakes, MN) contained 61.32% conjugated dienes with *cis*-9, *trans*-11/*trans*-9, *cis*-11 (34.5%), *trans*-10, *cis*-12/*cis*-10, *trans*-12 (24.5%), and *cis*-11, *trans*-13 (19.2%), representing 78% of the isomers. Diets were formulated as previously described (29) to maintain or exceed current recom-

TABLE 1

Composition of control diets (as-fed basis)

	Control diets[1,2]			
	Phase I	Phase II	Phase III	Phase IV
	g/100 g			
Ingredient				
Corn	32.69	51.15	63.11	73.00
Soybean meal (48%)	12.00	21.16	31.16	21.76
Dried whey	22.00	16.00	—	—
Spray-dried plasma[3]	7.50	4.00	—	—
Dried blood cells[4]	—	2.00	—	—
Dry skim milk	21.00	—	—	—
CLA-60[5]	—	—	—	—
Soybean oil[6]	2.21	2.21	2.21	2.21
DL-Methionine	0.18	0.22	0.09	0.05
L-Lysine	—	0.18	0.19	0.20
Sodium chloride	0.25	0.25	0.25	0.25
Dicalcium phosphate	1.09	1.72	1.95	1.39
Calcium carbonate	0.78	0.81	0.74	0.81
Vitamin premix[7]	0.20	0.20	0.20	0.25
Trace mineral[8]	0.05	0.05	0.05	0.06
Selenium premix[9]	0.05	0.05	0.05	0.02
Calculated composition				
Crude protein	24.36	21.38	19.92	16.14
Lysine	1.91	1.67	1.42	1.13
Ether extract	4.49	5.20	5.50	5.78
Methionine + cystine	0.97	0.86	0.72	0.57
Calcium	1.05	0.90	0.81	0.69
Phosphorus	0.88	0.79	0.74	0.60
Ca:P ratio	1.20	1.13	1.10	1.15
Metabolizable energy, *MJ/kg*	15.31	15.36	15.64	15.75

[1] In CLA-supplemented diets, 2.21% of CLA-60 was replaced by 2.21% of soybean oil to maintain the diets isocaloric within phases. Analyzed ether extracts (g/100 g) of diets within each phase were: 4.50 ± 0.11, 5.10 ± 0.15, 5.43 ± 0.14 and 5.68 ± 0.09 for CLA-supplemented diets and 4.48 ± 0.11, 5.21 ± 0.15, 5.48 ± 0.14 and 5.79 ± 0.09 for soybean oil–supplemented diets.

[2] Phase I, 1–2; II, 3–4; III, 5–8; and IV, 9–11 wk.

[3,4] Purchased from American Protein, Ames, IA.

[5] Expressed as CLA-60 (ConLinco, Detroit Lakes, MN) (i.e., 2.21 g/100 g of the diet in CLA-supplemented diets): 2.21 g CLA-60-/100 g diet represented 1.33 g CLA/100 g in the diet. CLA-60 contained: 16:0 (4.7 g/100 g), 16:1 (0.2 g/100 g), 18:0 (5.7 g/100 g), 18:1 (n-9) (21.08 g/100 g), 18:2 (n-6) (6.8 g/100 g), conjugated dienes with *cis*-9, *trans*-11/*trans*-9, *cis*-11 (34.5 g/100 g), *trans*-10, *cis*-12/*cis*-10, *trans*-12 (24.5 g/100 g), and *cis*-11, *trans*-13 (19.2 g/100 g), and 18:3 (n-3) (0.2 g/100 g).

[6] Estimated fatty acid profile of soybean oil (i.e., 2.21 g/100 g of the diet in control diets) expressed as a percentage of total fatty acids: 14:0 (0.1 g/100 g), 16:0 (10.3 g/100 g), 16:1 (0.2%), 18:0 (3.8 g/100 g), 18:1 (n-9) (22.8 g/100 g), 18:2 (n-6) (51 g/100 g) and 18:3 (n-3) (6.8 g/100 g).

[7] Supplied per kilogram of diet: retinyl acetate, 1516 μg; cholecalciferol, 26 μg; *dl*-α-tocopheryl acetate, 22 mg; riboflavin, 6.6 mg; pantothenic acid, 17.6 mg; niacin, 33 mg; and vitamin B-12, 22 μg.

[8] Supplied per kilogram of diet: Zn, 165 mg (ZnO); Fe, 193 mg ($FeSO_4 \cdot H_2O$); Mn, 66 mg (MnO); Cu, 19.29 mg ($CuSO_4 \cdot 5H_2O$); and I, 0.2 mg (ethylene diamine dihydroiodide).

[9] Supplied per kilogram of diet: Se, 0.1 mg (Na_2SeO_3).

mended nutritional requirements of the NRC (32) for pigs. On d 21 and 28, (eight blocks of two pigs), and on d 42 (six blocks of two pigs), the immunization treatments (i.e., squalene control or proteinase-digested *B. hyodysenteriae* bacterin) were randomly assigned to blocks of two pigs each. Pigs were inoculated intramuscularly with 0.002 L of a proteinase-digested *B. hyodysenteriae* bacterin strain B204 in squalene as previously described (33). Briefly, the vaccine was formulated as a pepsin-digested of *B. hyodysenteriae* mixed 1:1 (v/v) with adjuvant. The pepsin-digest was prepared by incubating 0.001 g of pepsin (pH 1.9–2.2) per gram of lyophilized *B. hyodysenteriae* protein for 25 h at 37°C. Unvaccinated groups were inoculated with 0.002 L of a squalene adjuvant preparation alone. The squalene adjuvant preparation contained 880 g/L PBS, 10% squalene/pluronic acid (80:20, v/v) and 2% Tween 80. The vaccination protocol utilized has been shown to be efficacious in enhancing numbers of peripheral blood $CD8\alpha\alpha^+$ cells and in reducing colonic inflammation due to *B. hyodysenteriae* (33–35). The institutional Committee on Animal Care approved animal procedures utilized in this experiment.

Harvesting of peripheral blood mononuclear cells (PBMC). Mononuclear cells were isolated by using a previously described procedure (33). The remaining peripheral blood was used to determine the total white blood cell counts, using a Coulter Z1 Single Particle Counter (Beckman Coulter, Miami, FL). Differential counts were conducted by examining a blood smear stained with Hema 3 Stain Set (Fisher Scientific, Pittsburgh, PA) and by flow cytometry.

Isolation of thymocytes. To isolate thymocytes, a modification of a previously described method (36) was utilized. A sample from the right lobe of the thymus (i.e., 3 cm^2) was removed from the necropsied pigs (4 pigs fed a control diet and 4 pigs fed a CLA-supplemented diet) and standardized using a punch biopsy tool (Miltex Instrument, Bethpage, NY). The standardized thymic tissue was disrupted between two glass surfaces in cold Hanks' balanced salt solution (Sigma, St. Louis, MO), thymocyte suspensions were washed twice in PBS containing 1 g/L sodium azide and thymocytes were enumerated using a Coulter Z1 Single Particle Counter (Beckman Coulter) for use in immunophenotyping (2×10^9 viable thymocytes/L). Thymocytes were standardized against the total number of $CD45^+$ cells (i.e., lymphoid cells).

Proliferation assay. A total of 2×10^7 PBMC from each pig (recovered using 1.077 lymphocyte separation medium, Mediatech, VA) were prepared to perform proliferation assays (Sigma) as previously described (34,37). Briefly, cells were labeled with PKH2-GL (Sigma), washed and, after assessing viability using propidium iodide fluorescence (Sigma), adjusted to 2×10^9 viable PBMC/L of complete media. Cells (10^{-4} L) were added to 96-well flat-bottomed microtiter plates (Corning, Corning, NY) containing 10^{-4} L of medium (nonstimulated), medium plus 0.005 g/L of a *B. hyodysenteriae* B204 whole-cell lysate, or medium plus 0.005 g/L of concanavalin A. Samples from each pig were cultured in replicates of six for each ex vivo treatment. Cells were incubated at 37°C in 5% CO_2 humidified atmosphere for 6 d. As cells divide, PKH2-GL membrane staining diminishes, resulting in a decreased mean fluorescence intensity (37). After 6 d, cultured cells from the six wells of the same ex vivo treatment and pig were pooled and prepared for immunophenotyping.

Flow cytometry. Mononuclear cells or thymocytes (i.e., 2×10^9 viable cells/L) were labeled with primary antibodies in 50 μL of fluorescence-activated cell sorting (FACS) buffer: phycoerythrin (PE)-labeled anti-swine-CD4 (clone 74–12-4), biotinylated immunoglobulin IgG2a mouse anti-swine-CD8α (clone 76–2-11) (originally provided by Dr. Joan K. Lunney; USDA, Beltsville, MD) (38), IgG2a mouse anti-swine-CD8β (PG164A), IgG1 mouse anti-swine-CD45 (K252.1E4, pan-leukocyte marker) (VMRD, Pullman, WA), IgG1 mouse anti-swine-CD3ϵ (8E6), IgG2a mouse anti-swine-major histocompatibility complex (MHC) Class II (TH81A5 and TH14B, VMRD), IgG1 mouse anti-swine-TCRδ chain (PGBL22A, VMRD Inc), IgG2b mouse anti-swine-SWC3a (74–22-15, VMRD), IgG1 mouse anti-swine-CD16 (G7) (NK cell marker kindly provided by Dr. Yoon B. Kim, Chicago, IL) (39,40) or appropriate isotype control antibodies. The percentages of $TCR\alpha\beta^+$ cells were assessed by subtracting $TCR\gamma\delta^+$ cells from overall $CD3^+$ cells as previously described (18). After a 15-min incubation, cells were washed with FACS buffer and resuspended in a 5×10^{-5} L volume containing the secondary antibody dilution [PE-conjugated goat anti-mouse IgG1 (Southern Biotechnology, Birmingham, AL), streptavidin-conjugated CyChrome (Pharmingen, San Diego, CA), PE-conjugated goat anti-mouse IgG2a (Southern Biotechnology)]. Cells were incubated for 15 min, washed twice and analyzed by flow cytometry. Three-color flow cytometric data acquisition of the PKH2-stained cultured cells was performed using a FACScan (Becton Dickinson, San Jose, CA). A total of 10,000 events were saved, and data analysis on the viable cell gate (previously determined) was performed by using CellQuest software (Becton Dickinson). Two-color flow cytometric analysis was performed in a Coulter XL (Beckman Coulter). Electronic compensation was utilized to eliminate spectral overlaps between individual fluorochromes in two- and three-color flow cytometric analysis.

Assay for benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT)-esterase activity. PBMC were cultured as stated above. After 5 d, anti-CD3 mAb (8E6) was added into each well (5 μg/well) to broadly activate T cells relative to NK cells. Addition of anti-CD3 to cytotoxic T lymphocytes ($TCR\alpha\beta CD8\alpha\beta$ cells) causes an increase in cytolytic activity in mice (41) and pigs (42). At 6 d, the cultured cell suspension was harvested, centrifuged ($400 \times g$) for 5 min and the supernatant separated from the cell pellet. Cultured lymphocytes (i.e., cell pellet) at 2×10^9 cells/L were lysed in PBS-0.5% NP-40 (Sigma) for 30 min on ice with vortexing at 5-min intervals. Both supernatant and cell lysates were frozen at −70°C for later analysis of BLT-esterase activity.

BLT-esterase activity was measured using a modification of a previously described procedure (43). Granzyme activity assessed using BLT-esterase activity was shown to be highly correlated with cytotoxicity (44). Briefly, a total of 4×10^{-5} L of supernatant was added to 40 μL of the reaction mixture [0.2 mol/L Tris-HCl, pH 4.5, 0.22 mmol/L BLT (Calbiochem-Behring, La Jolla, CA), 0.22 mmol/L 2-nitro benzoic acid (5, 5′dithiobis, Sigma)] and incubated for 20 min (room temperature). Absorbance of the BLT-esterase–induced color change was measured in an ELISA reader (BioTek Instruments, Winooski, VT) at a wavelength of 405 nm.

Statistical analysis. Data were analyzed as a 2×2 factorial arrangement of treatments (2 vaccines and 2 diets) within a split-plot design with 16 blocks of two littermate pigs as the experimental unit for immunization treatment (i.e., unvaccinated or vaccinated with a proteinase-digested *B. hyodysenteriae* bacterin) and pig within block as the experimental unit for dietary treatment (soybean oil or conjugated linoleic acid). The whole plot error (i.e., error A) was block within vaccine (i.e., 10 df when $n = 24$) and the subplot error (i.e., error B) was the residual degrees of freedom after accounting for the dietary treatment variance and the variance for the interaction between diet and vaccine (i.e., 10 df when $n = 24$). ANOVA was performed using the general linear model procedure of SAS using the TEST statement (45). A $P < 0.05$ was considered to be significant. The statistical model utilized was $Y_{ijk} = \mu + \text{Vaccine}_i + \text{error } A_{ik} + \text{Diet}_j + (\text{Vaccine} \times \text{Diet})_{ij} + \text{error } B_{ijk}$, where μ is the general mean, Vaccine_i is the main effect of the ith level of the immunization effect, Diet_j is the main effect of the jth level of the dietary effect, $(\text{Vaccine} \times \text{Diet})_{ij}$ is the interaction effect between immunization and diet, and errors A and B represent the random errors for the whole plot and the subplot, respectively.

RESULTS

Dietary CLA induces expansion of peripheral $TCR\alpha\beta CD8\alpha\beta$ cells, $TCR\gamma\delta CD8\alpha\alpha$ cells, and a NK cell subset. To determine the effects of dietary CLA-supplementation on peripheral $CD8^+$ cell subsets in vivo, a flow cytometric analysis was performed on isolated PBMC. From d 0 to 49 of the CLA feeding period, no significant differences in numbers of $CD8^+$ peripheral blood lymphocytes were attributed to either the dietary or the vaccination treatments. Changes in the phenotypic profiles of the porcine $CD8^+$ peripheral pool induced by dietary CLA supplementation were first detected on d 49. Chronologically, CLA induced an earlier expansion (**Fig. 1***A* and *B*) of $TCR\gamma\delta CD8\alpha\alpha$ lympho-

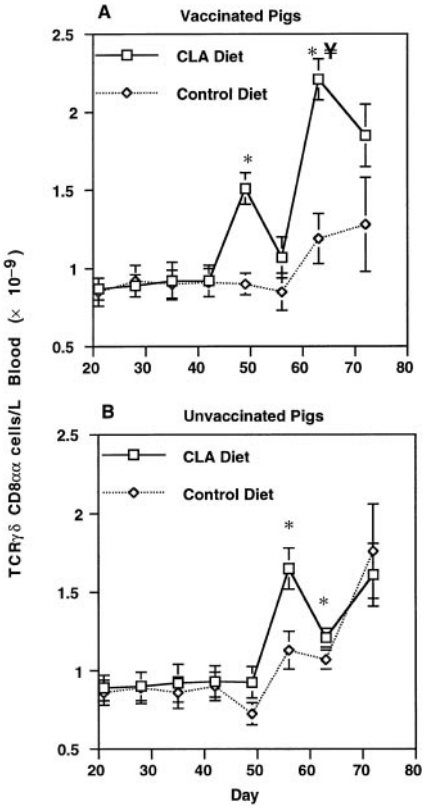

FIGURE 1 Flow cytometric analysis of TCR$\gamma\delta$CD8$\alpha\alpha$ T cells in peripheral blood mononuclear cells recovered from vaccinated (*panel A*) and unvaccinated (*panel B*) pigs fed control or conjugated linoleic acid (CLA)-supplemented diets. Significant differences ($P < 0.05$) between treatments attributed to the main effects of vaccine (†), diet (*) and the interaction between vaccine and diet (V × D) ([¥]) are reported. Values are means ± SEM, from d 0 to 35 ($n = 8$), from d 35 until d 72 ($n = 6$).

cytes (49–63 d), followed by a subsequent expansion (**Fig. 2**) of TCR$\alpha\beta$CD8$\alpha\beta$ lymphocytes (56–72 d) and a subset of NK cells (72 d) (**Fig. 3**). Vaccination, in combination with dietary CLA supplementation, induced a greater expansion of the NK cell subset (Fig. 3) and of the TCR$\gamma\delta$CD8$\alpha\alpha$ subset (Fig. 1A)

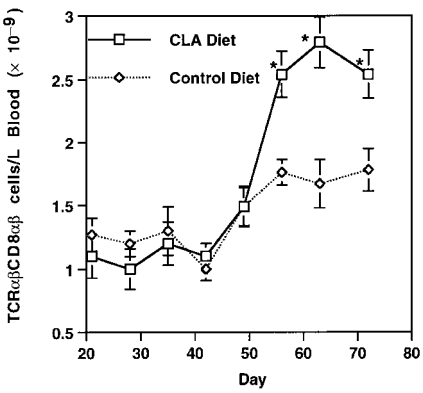

FIGURE 2 Peripheral blood TCR$\alpha\beta$CD8$\alpha\beta$ T lymphocytes of pigs fed control or conjugated linoleic acid (CLA) diets. T lymphocytes were greater in pigs fed CLA (*$P < 0.05$). Data for vaccinated and unvaccinated pigs were pooled within diet. Values are means ± SEM, d 0 to 35 ($n = 16$), d 35 to 72 ($n = 12$).

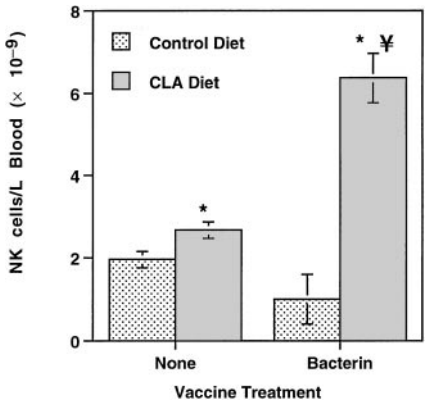

FIGURE 3 The number of a subset of natural killer (NK) cells in the peripheral blood of pigs that were or were not vaccinated and fed control or conjugated linoleic acid (CLA)-supplemented diets. The subset of NK cells was defined as $CD3^{-}CD16^{+}CD8\alpha\alpha^{+}$. Significant differences ($P < 0.05$) between treatments attributed to the main effects of vaccine (†), diet (*) and the interaction between vaccine and diet (V × D) ([¥]) are reported. Values are means ± SEM, $n = 6$.

than dietary CLA supplementation alone. Furthermore, the numbers of TCR$\gamma\delta$CD8$\alpha\alpha$ cells were increased earlier in vaccinated pigs fed CLA than in other treatment groups (Fig. 1A). No differences in numbers of TCR$\alpha\beta$CD8$\alpha\beta$ T cells were caused by vaccination (data not shown). Thus, TCR$\alpha\beta$CD8$\alpha\beta$ T-cell data from both vaccinated and unvaccinated pigs were pooled in each of the dietary treatments. CLA alone caused a stable expansion of cells within the TCR$\alpha\beta$CD8$\alpha\beta$ subset (Fig. 2), whereas the effects of CLA and vaccination on the numbers of TCR$\gamma\delta$CD8$\alpha\alpha$ cells appeared to be cyclic (Fig. 1A and *B*). Vaccination with the bacterin, but not dietary CLA supplementation, increased numbers of peripheral CD4 single-positive (SP) T cells (**Fig. 4**) (40–50 d). Although numbers of white blood cells tended to increase ($P = 0.05$) in pigs fed CLA-supplemented diets, the numbers of cells of myelomonocytic origin (i.e., $SWC3^{+}$ cells) or MHC class II-positive cells in peripheral blood were

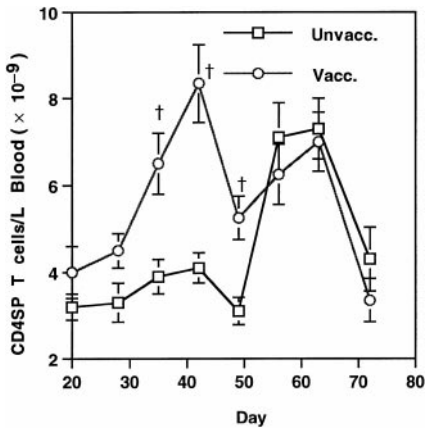

FIGURE 4 Influence of vaccination on numbers of peripheral blood CD4 single-positive (SP) T cells (10^9/L blood) in pigs fed control or conjugated linoleic acid (CLA)-supplemented diets. Data for pigs fed the two diets were pooled within vaccination group. CD4SP T lymphocytes were greater in vaccinated pigs (†$P < 0.05$), regardless of dietary treatment. Values are means ± SEM, d 0 to 35 ($n = 16$), d 35 to d 72 ($n = 12$).

TABLE 2

Influence of dietary and immunization treatments on CD8 expression in thymocytes from pigs fed control or conjugated linoleic acid (CLA)-supplemented diets[1,2]

	Unvaccinated		Vaccinated			*P*-value[3]		
Phenotype	0	1.33	0	1.33	SEM[4]	V	D	V × D
			CLA, g/100 g					
CD8 $\alpha\beta$[5]	8.65	19.15	25.10	32.80	2.47	0.15	0.05	0.06
CD4$^-$CD8$^-$ [6]	44.75	49.80	34.10	40.20	0.38	0.19	0.05	0.83
TCR$\gamma\delta$CD8$\alpha\alpha$	2.79	6.24	8.44	2.93	0.48	0.43	0.53	0.05

[1] Thymus samples were recovered from pigs killed at d 35 and thymocytes were isolated as described in Materials and Methods.
[2] Results are expressed as mean percentage of total cells, $n = 8$.
[3] *P*-value of main effects of vaccine (V), diet (D) and interaction of vaccine by diet (V × D).
[4] Subplot standard error of the mean.
[5] Includes T-cell receptor (TCR)$\alpha\beta$CD8$\alpha\beta$ and TCR$\alpha\beta$CD4CD8$\alpha\beta$ thymocytes.
[6] CD4$^-$CD8$^-$ are progenitor cells that have migrated from the bone marrow to the thymus.

not affected by vaccination or dietary treatment (data not shown).

Dietary CLA supplementation increases CD8αβ thymocyte subsets. The greater numbers of peripheral blood CD8$\alpha\beta^+$ T cells in pigs fed CLA may result from an increase of precursor cells in the thymus. Percentages of CD8$\alpha\beta^+$ and CD4$^-$CD8$^-$ thymocytes were significantly increased by dietary CLA supplementation (**Table 2**) on d 35. Vaccination interacted with diet to lower percentages of TCR$\gamma\delta$CD8$\alpha\alpha$ thymocytes (Table 2); unvaccinated pigs fed CLA-supplemented diets had greater percentages of TCR$\gamma\delta$CD8$\alpha\alpha$ thymocytes than dietary control pigs. On d 35, phenotype of PBMC was not affected by either immunization treatment or dietary CLA supplementation.

Dietary CLA increases PBMC granzyme activity regardless of the vaccination treatment. Because major phenotypic changes were noted within the peripheral CD8$\alpha\beta^+$ population, the effects of dietary CLA on cytotoxic potential of peripheral lymphocytes was assessed as a functional criterion. From d 56 to 72, a sustained increase in TCR$\alpha\beta$CD8$\alpha\beta$ lymphocytes was observed in pigs fed CLA-supplemented diets (Fig. 2). Day 63 was chosen as a representative time point, within the period in which in vivo TCR$\alpha\beta$CD8$\alpha\beta$ lymphocytes were increased, to evaluate PBMC cytotoxicity. PBMC isolated from pigs fed the CLA-supplemented diet had greater BLT-esterase activities than PBMC from those fed the control diet, regardless of the vaccination treatment (**Fig. 5**). The increase in granzyme activity was correlated ($r = 0.73$, $P < 0.04$) with the increase of CD8$\alpha\beta^+$ T cells in peripheral blood (Fig. 2).

The role of CD4SP T cells in the proliferation of TCRγδCD8αα T cells. To further characterize the nature of the CLA-induced expansion of CD8$^+$ lymphocytes, proliferative responses of the distinct CD8$^+$ cell subsets were assessed using a proliferation assay coupled with a flow cytometric analysis. Analysis of cells from pigs fed CLA-supplemented diets demonstrated that dietary CLA supplementation had increased the proliferative ability of TCR$\gamma\delta$CD8$\alpha\alpha$ cells. In unvaccinated pigs, the enhanced proliferation rate (i.e., between 63 and 72 d of dietary CLA supplementation) observed in TCR$\gamma\delta$CD8$\alpha\alpha$ lymphocytes from pigs fed CLA-supplemented diets was not correlated with greater percentages of proliferating CD4SP lymphocytes (**Table 3**). However, the enhancement in TCR$\gamma\delta$CD8$\alpha\alpha$ cell proliferation induced by vaccination was correlated with a greater proliferation rate of CD4SP (CD4$^+$CD8$^-$) cells (Table 3).

As anticipated, CD4SP lymphocytes recovered from vaccinated pigs and stimulated in vitro with *B. hyodysenteriae* antigens proliferated to a greater extent than antigen-stimulated CD4SP lymphocytes recovered from unvaccinated pigs (**Table 4**). After vaccination, dietary CLA augmented the ex vivo proliferative response of CD4SP T helper cells to specific antigen (Table 4). In contrast to TCR$\alpha\beta$CD8$\alpha\beta$ cells, dietary CLA had no effect on the number of CD4SP PBMC in vivo.

DISCUSSION

Although fatty acids, particularly saturated fatty acids, are often viewed negatively due to the energy value that they add to human diets and the negative effects on health (46), some polyunsaturated fatty acids (PUFA) provide nutritional value beyond the basic requirements of distinct physiologic functions. In the present study, we have identified a mixture of PUFA with functional properties (i.e., expansion of particular subsets of peripheral CD8$^+$ lymphocytes and stimulation of effector functions) beyond the basic nutritional needs. Conjugated linoleic acid modulated the phenotypic profile of pe-

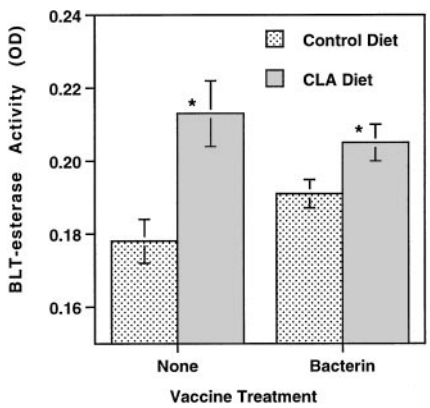

FIGURE 5 Effect of dietary conjugated linoleic acid (CLA) on the cytotoxic potential of peripheral blood mononuclear cells from vaccinated and unvaccinated pigs as measured by benzyloxycarbonyl-L-lysine thiobenzyl ester-esterase activity. *The effect of diet was significant, $P < 0.005$. Values are means ± SEM, $n = 6$. OD = optical density.

TABLE 3

Effects of dietary and immunization treatments on percentages of proliferative TCRγδCD8αα and CD4+ peripheral blood mononuclear cells from pigs fed control or conjugated linoleic acid (CLA)-supplemented diets[1,2]

	Unvaccinated		Vaccinated			*P*-value[3]		
Day	0	1.33	0	1.33	SEM[4]	V	D	V × D
			CLA, g/100 g					
d 63 γδ	8.74	15.35	21.74	53.79	3.90	0.01	0.02	0.26
$CD4^+$	2.47	2.89	5.64	6.66	0.82	0.05	0.30	0.50
d 72 γδ	4.83	7.26	13.65	15.67	2.5	0.04	0.05	0.19
$CD4^+$	9.10	9.69	13.01	22.90	6.2	0.03	0.07	0.06

[1] Peripheral blood mononuclear cells (2×10^9/L) recovered at d 63 from pigs administered each of the immunization (i.e., vaccinated or un-vaccinated) and/or dietary (CLA or soybean oil) treatments and cultured with medium alone.
[2] Results are expressed as mean percentage of proliferating cells, $n = 6$.
[3] *P*-value of main effects of vaccine (V), diet (D) and interaction of vaccine by diet (V × D).
[4] Subplot standard error of the mean.

ripheral lymphocytes as evidenced by a sustained expansion of peripheral blood TCRαβCD8αβ lymphocytes and a transient increase of a subset of a subset of NK cells, and TCRγδCD8αα lymphocytes. On the basis of earlier studies (29), it was anticipated that CLA would influence the function and numbers of porcine $CD8^+$ cells after 42 d of dietary supplementation. However, the complete phenotypic profile of the subpopulation(s) of cells within the $CD8^+$ pool most influenced by dietary CLA supplementation was unknown.

The influence of lipid nutrition on specific aspects of porcine cellular immunity (e.g., immune cell phenotype, proliferation and granzyme activity) was compared with the effects induced by vaccination. Immunization with a *B. hyodysenteriae* vaccine induces an immune response that has been shown to increase the numbers of peripheral blood $CD8\alpha\alpha^+$ lymphocytes (i.e., TCRγδCD8αα and CD4CD8αα), particularly TCRγδCD8αα cells (33) (i.e., $\gamma\delta^+$ T cells coexpressing CD8αα molecules upon activation), a T-cell subset involved in the regulation of mucosal inflammation (47). Both dietary CLA and immunization with a *B. hyodysenteriae* vaccine increased the numbers of peripheral TCRγδCD8αα lymphocytes and a $CD8\alpha\alpha^+$ subset of NK cells. Cyclic changes of peripheral TCRγδCD8αα lymphocytes induced by CLA and vaccination might be attributed to the existence of a feedback mechanism that regulates numbers of recirculating TCRγδCD8αα lymphocytes. Only CLA increased the numbers of peripheral blood TCRαβCD8αβ lymphocytes. Furthermore, in vaccinated pigs fed CLA, the augmentation of numbers of TCRαβCD8αβ lymphocytes induced by dietary CLA supplementation was not overshadowed by the CD4- and CD4CD8-mediated immune response induced by vaccination. In pigs, the reduction in numbers of $CD4^+$ PBMC with age is associated with the expression of the CD8αα molecule on $CD4^+$ cells upon activation to become CD4CD8αα cells (i.e., effector memory cells) (16,17). Vaccination has proved to be effective in controlling infectious diseases by an antigen-dependent mechanism. Here, we demonstrated that nutritional supplementation can significantly influence immunity by an antigen-independent mechanism.

Maturation and lineage choice of T cells (i.e., CD4 vs. CD8) takes place in the thymus. Therefore, if the observed changes in the profile of peripheral blood $CD8^+$ lymphocytes originated in the thymus, phenotypic shifts in this organ were expected. An increase in $CD8\alpha\beta^+$ cells was detected earlier in the thymus (d 35) than in peripheral blood (d 49). The increase in percentages of $CD8\alpha\beta^+$ thymocytes correlated with greater numbers of thymic lymphoid progenitor cells ($CD4^-CD8^-$ or $CD4^+CD8^+$ thymocytes) in pigs fed CLA-supplemented diets. Thus, dietary CLA appears to initially modulate the cellular profiles within primary (i.e., thymus)

TABLE 4

Influence of dietary and immunization treatments on proliferation of CD4+CD8− peripheral blood mononuclear cells from pigs fed control or conjugated linoleic acid (CLA)-supplemented diets[1]

	Unvaccinated		Vaccinated			*P*-value[2]		
Stimuli[3]	0	1.33	0	1.33	SEM[4]	V	D	V × D
			CLA, g/100 g					
Medium	9.12	9.79	13.05	22.90	2.58	0.08	0.17	0.21
Whole-cell sonicate	22.02	27.27	40.19	78.77	5.26	0.02	0.05	0.08
Concanavalin A	88.71	85.92	94.68	96.15	1.69	0.04	0.73	0.33

[1] Peripheral blood mononuclear cells (2×10^6) recovered at d 72 from pigs administered each of the vaccination and/or dietary treatments.
[2] *P*-value of main effects of vaccine (V), diet (D) and interaction of vaccine by diet (V × D).
[3] Peripheral blood mononuclear cells isolated from pigs were cultured for 6 d with either whole *Brachyspira hyodysenteriae* bacterin sonicate (whole cell sonicate), mitogen (concanavalin A) or medium alone (nonstimulated).
[4] Results are expressed as mean percentage of proliferating cells, $n = 6$.

and, subsequently, within the secondary lymphoid pool (i.e., peripheral blood). Thymocytes express PPAR-γ, a nuclear receptor factor activated by PUFA and lipid mediators (48). The DNA-binding domains of PPAR-γ recognize DNA sequences similar to those recognized by retinoid-related orphan receptor-γ, a nuclear receptor factor involved in lymphoid organogenesis and thymopoiesis (49). In vitro studies have shown that CLA is a PPAR-γ ligand (26). Thus, CLA could influence thymocyte differentiation through a PPAR-γ–dependent mechanism. Furthermore, the highest concentration of CLA after dietary supplementation in rats was detected in the bone marrow (50). Future experiments should address whether changes in profiles of thymocytes induced by dietary CLA supplementation are derived from an earlier effect on common lymphoid progenitor cells within the bone marrow or from a direct thymic effect.

Expansion of peripheral blood TCR$\alpha\beta$CD8$\alpha\beta$ lymphocytes caused by dietary CLA supplementation is functionally linked to an enhancement in PBMC cytotoxicity. When examining cytotoxicity, results obtained from assays to determine BLT-esterase activity are highly correlated with those obtained using chromium release assays (45,51). Anti-CD3 stimulated PBMC isolated from pigs fed the CLA-supplemented diet had greater BLT-esterase activities than did PBMC from pigs fed the control diet. Thus, not only were the numbers of CD8$\alpha\beta^+$ T cells increased, but PBMC recovered from pigs fed a CLA-supplemented diet had greater cytotoxic potentials.

Proliferative responses of TCR$\gamma\delta$CD8$\alpha\alpha$ lymphocytes were also influenced by dietary CLA supplementation. TCR$\gamma\delta$CD8$\alpha\alpha$ lymphocytes recovered from pigs fed CLA had greater unstimulated proliferative responses than lymphocytes recovered from pigs fed the isocaloric control diet. Porcine $\gamma\delta^+$ T cells are activated by TCR/CD3-independent mechanisms as shown by the lack of activation of $\gamma\delta^+$ T cells after addition of antibodies against the δ chain of the TCR (52). In line with these findings, bovine $\gamma\delta^+$ T cells have been shown to proliferate in nonstimulated wells (53). Furthermore, in contrast to vaccination-induced proliferation (33,34), TCR$\gamma\delta$CD8$\alpha\alpha$ T-cell proliferation in PBMC recovered from pigs fed CLA was not correlated with enhanced proliferative responses of CD4SP cells. $\gamma\delta$ T cells have been shown to be critical in dampening mucosal inflammation (54). In addition, $\gamma\delta$ T cells are involved in the regulation of the turnover and maturation of enterocytes (55). This suggests a role for CLA as an anti-inflammatory agent that could enhance mucosal integrity during enteric inflammatory diseases (e.g., inflammatory bowel disease). In summary, our results demonstrate for the first time that dietary CLA influences phenotype and effector functions of distinct CD8$^+$ lymphocyte subsets. Elucidating the effect of CLA on lymphocytes in peripheral blood (i.e., a NK cell subset, TCR$\gamma\delta$CD8$\alpha\alpha$ cells and TCR$\alpha\beta$CD8$\alpha\beta$ cells) and thymus (i.e., CD4$^-$CD8$^-$ and CD8$\alpha\beta^+$ thymocytes) will aid in the development of nutritionally based therapeutic applications to augment host resistance to certain infectious, neoplasic or inflammatory diseases.

ACKNOWLEDGMENTS

We thank Y. B. Kim for kindly providing the G7 antibody, and R. E. Sacco, D. C. Beitz and R. C. Ewan for discussions.

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