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Long-term influence of lipid nutrition on the induction of $CD8^+$ responses to viral or bacterial antigens

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Abstract

Porcine $CD8^+$ lymphocytes are critical for the development of cellular immune responses to bacterial (i.e. $CD8\alpha\alpha^+$) and viral (i.e. $CD8\alpha\beta^+$ lymphocytes) pathogens. Vaccination and challenge modulate the kinetics of appearance of $CD8^+$ cells in peripheral blood. In addition to antigen-mediated modulation, nutritional modulation can also influence cell-mediated immunity. We had previously observed that diets supplemented with a mixture of conjugated linoleic acid (CLA) isomers expanded porcine $CD8^+$ peripheral blood mononuclear cells (PBMC). The present study aimed to investigate the influence of prior consumption of a nutraceutical, (i.e. dietary CLA) on phenotypes and effector functions of porcine PBMC following immunization with a bacterin or a modified-live viral vaccine. It was demonstrated that the effects of dietary CLA on immune cell phenotype (i.e. numbers of $CD8\alpha\beta^+$ cells) persisted after the compound was withdrawn from the diet (i.e. 67 days), whereas effector functions (i.e. antigen-stimulated proliferation and cytotoxycity) disappeared earlier (i.e. 25 days). Specifically, numbers of $CD8\alpha\beta^+$ PBMC in pigs that had been fed diets suplemented with CLA were greater than in pigs fed control (i.e. isoenergetic and unsupplemented) diets, regardless of the vaccination treatment. Furthermore, prior dietary CLA supplementation interacted with viral immunization (i.e. modified-live pseudorabies virus (PRV) vaccine) by enhancing both pseudorabies-specific proliferative responses of $CD8\alpha\beta^+$ PBMC and granzyme activities of PBMC.

Keywords: Pigs; Pseudorabies virus; Conjugated linoleic acid

1. Introduction

A wide variety of bacterial and viral infections and/or the associated clinical signs of disease are currently prevented through vaccination. The type of immunogen inoculated (i.e. viral versus bacterial) contributes to defining the phenotype of cellular subsets mediating effector/memory responses. In pigs, most peripheral blood mononuclear cell (PBMC) (from 20 to 65% depending on the age and genetic origin) express the CD8 coreceptor molecule on their surface. Furthermore, immune responses to bacterial [1] and viral [2,3] antigens are mediated by $CD8^+$ cells. Phenotypically, porcine $CD8^+$ PBMC are subdivided into five subpopulations based on the type of T cell receptor (TCR) and coreceptor (i.e. CD4 or CD8) molecules expressed: (1) $TCR\gamma\delta^+CD4^-CD8\alpha\alpha^+$ (i.e. immunoregulatory $\gamma\delta$ T cell phenotype) [4,5]; (2) $TCR^-CD4^-CD8\alpha\alpha^+$ (i.e. subset of natural killer cells) [6]; (3) $TCR\alpha\beta^+CD4^-CD8\alpha\beta^+$ (i.e. cytotoxic T cells) [2]; (4) $TCR\alpha\beta^+CD4^+CD8\alpha\alpha^+$ double positive (DP) (i.e. memory/effector T cells) [7], and according to indirect inferences $TCR\alpha\beta^+CD4^-CD8\alpha\alpha^+$ (i.e. immunoregulatory $\alpha\beta$ T cells) [8]. The first two cell subsets are MHC-unrestricted ($CD6^-$) [2,9].

Vaccination with a proteinase-digested *Brachyspira hyodysenteriae* bacterin, the extracellular bacterial pathogen that causes swine dysentery, primes $CD8^+$ cells for recall response to antigen [10]. We have recently shown that the subsets of porcine lymphocytes responding to a proteinase-digested *B. hyodysenteriae* vaccine express the CD8 coreceptor molecule as an alpha/alpha homodimer (i.e. $CD4^+CD8\alpha\alpha^+$ DP and $TCR\gamma\delta^+CD4^-CD8\alpha\alpha^+$) but not as an alpha/beta heterodimer [11]. Conversely, vaccination with a modified life pseudorabies virus (PRV) vaccine stimulates proliferation of $CD8\alpha\beta^+$, $CD4^+CD8\alpha\alpha^+$ DP, $TCR\gamma\delta^+CD4^-CD8\alpha\alpha^+$, and $CD4^+$ single positive (SP) PBMC [2]. Thus, the major phenotypic difference between a cellular response to a bacterial antigen preparation (i.e. *B. hyodysenteriae*) and to a viral antigen (i.e. PRV) in pigs lays on the responsiveness of $CD8\alpha\beta^+$ cells to recall stimulation with the antigen(s).

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Vaccines that induce cell-mediated protection are sometimes evaluated on the basis of their capacity to favor certain phenotypes and/or to modulate particular effector functions of PBMC. However, there are additional factors (e.g. nutrition, physiological status, stress, etc.) that contribute to define the functional and phenotypic characteristics of host cell-mediated responses to vaccination. Previously, we observed that pigs fed diets supplemented with a mixture of isomers of linoleic acid (i.e. predominantly a 50:50 mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers) with conjugated double bonds (CLA), had greater percentages of $CD8^+$ PBMC than did pigs fed a nutritionally identical diet lacking these isomers in which soybean oil replaced the CLA source [12]. More recently, we have further functionally and phenotypically characterized the lymphocyte subsets that are most influenced by dietary CLA supplementation (i.e. TCR$\gamma\delta$CD8$\alpha\alpha$, natural killer cells and TCR$\alpha\beta$CD8$\alpha\beta$) [13]. When used at 1.33% in the diet, a minimum period of CLA accumulation (i.e. 42 day) was required to effectively modulate T cell responses [12,13]. Although no major immunological benefits were obtained during the first 42 days of dietary CLA-supplementation, inclusion of this nutraceutical in dietary formulations could still be economically feasible if its immunomodulatory properties were long-lived. Thus, additional work was required to investigate the temporal sustainability of the $CD8^+$ enhancement following withdrawal of CLA from the diet. In addition, because immunization modulates numbers and effector functions of lymphocyte subsets also affected by dietary CLA supplementation, the possible interaction between dietary CLA and immunization on subsequent antigen-specific responses was also characterized.

To investigate the prolonged influence of previous nutritional regimens on subsequent immune responses, pigs that had been previously fed two distinct diets (i.e. 0 or 1.33% CLA) were immunized with a proteinase-digested *B. hyodysenteriae* vaccine, a modified-live PRV vaccine, a combination of both vaccines, or adjuvant alone. PBMC recovered from immunized pigs that had previously been fed CLA had greater proliferative responses to PRV antigens than those recovered from immunized pigs that had been fed a control diet. Furthermore, numbers of CD8$\alpha\beta^+$ PBMC in pigs that had been fed diets suplemented with CLA were greater than in pigs fed control (i.e. isoenergetic, isoproteic, and supplemented with soybean oil replacing CLA) diets, regardless of the vaccination treatment. Thus, previous dietary regimens influenced subsequent antigen-specific immune responses and favored a CD8$\alpha\beta^+$ T cell phenotype in peripheral blood.

2. Materials and methods

2.1. Dietary and vaccination treatments

Sixteen 14-day-old PIC pigs were randomly distributed from outcome groups based on litter, initial body weight, and gender into blocks of two contiguous individual pens. The experimental design was chosen to decrease genetic variation associated with the utilization of cross-bred pigs. Either a 1.33% CLA 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 [12,13]. Pigs were fed either a CLA-supplemented or a control diet from day −72 to 0 of the experiment. Soybean oil was chosen to

Table 1
Composition of the control diets fed from day −72 to day 0 (as-fed basis)

Item	Control diets[a,b,c]			
	Phase I	Phase II	Phase III	Phase IV
Ingredient (g/100 g)				
Corn	32.19	50.21	62.00	71.66
Soybean meal (48%)	13.50	22.00	29.00	23.10
Dried whey	21.00	16.10	–	–
Spray-dried plasma	7.50	4.00	–	–
Dried blood cells	–	2.00	1.27	–
Dry skim milk	21.00	–	–	–
CLA-60[d]	–	–	–	–
Soybean oil[e]	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[f]	0.20	0.20	0.20	0.25
Trace mineral[g]	0.05	0.05	0.05	0.06
Selenium premix[h]	0.05	0.05	0.05	0.02

[a] Control diets (i.e. soybean oil-supplemented) listed above or CLA-supplemented diets (i.e. replacing soybean oil by CLA) were fed to pigs from day −72 (i.e. 15 days of age) to 0 of the experiment.

[b] In CLA-supplemented diets, 2.21% of CLA-60 replaced 2.21% of soybean oil to maintain the diets isocaloric within phases. Analyzed ether extracts (g/100 g) of diets within each phase were: four. 83 ± 0.21, 5.19 ± 0.18, 5.53 ± 0.11, and 5.68 ± 0.13 for CLA-supplemented diets and 4.80 ± 0.10, 5.25 ± 0.17, 5.52 ± 0.10, and 5.67 ± 0.14 for soybean oil-supplemented diets.

[c] Phase I, −72 to −58; II, −57 to −42; III, −41 to −11; and IV, −10 to 0 day.

[d] Expressed as CLA-60 (ConLinco Ltd., 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).

[e] 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: 16:0 (12.33 g/100 g), 16:1 (0.5%), 18:0 (4.63 g/100 g), 18:1 (n-9) (30.35 g/100 g), 18:2 (n-6) (51. 83 g/100 g), and 18:3 (n-3) (0.26 g/100 g).

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

[g] 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).

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

replace CLA in the control diet because it contains more than 50% linoleic acid, a fatty acid with the same carbon length that CLA and with the same number of unsaturated carbons (i.e. two double bonds). The fundamental difference between CLA isomers and linoleic acid consists on the conjugated double bond system (i.e. two double bonds separated by a single bond) found in CLA and absent in linoleic acid (i.e. *cis*-9, *cis*-12 octadecadienoic acid) which may have important structural and functional implications. Soybean and their respective oils are rich in linoleic acid whose concentration in typical United States porcine dietary formulations greatly exceeds the nutritional requirement for maintenance, reproduction or growth (i.e. 0.1% per kilogram of diet) [14]. Thus, by replacing linoleic acid by CLA a nutritional deficiency of linoleic was not induced, there was only a lower amount of excess linoleic acid in CLA-supplemented diets than in control diets. All the diets utilized in this experiment either matched or exceeded the nutritional requirements of pigs [14] defined by the National Research Council (1998), including those for linoleic acid. On day 1, the same diet supplemented with soybean oil replacing CLA was administered to all the pigs for 75 additional days (Table 2); from day 1 to 75 of the experiment. Prior and after day 0, phase feeding was utilized to match the changing nutritional needs of the growing pigs with the nutritional composition of the diets. On days 0, 20, and 50, pigs were intramuscularly inoculated with 2 ml of squalene adjuvant preparation alone ($n = 4$), 2 ml of proteinase-digested *B. hyodysenteriae* B204 in squalene ($n = 4$), 2 ml of PR-Vac Plus® (Pfizer Inc., Groton, CT) ($n = 4$), or 2 ml a combination of both vaccine preparations ($n = 4$).

Table 2
Composition of the diets fed from day 0 to 75 (as-fed basis)[a,b]

Item	Phase V	Phase VI
Ingredient (g/100 g)		
Corn	80.23	81.24
Soybean meal (48%)	15.02	14.00
Soybean oil	2.21	2.21
L-Lysine	0.19	0.20
Sodium chloride	0.25	0.25
Dicalcium phosphate	0.93	0.93
Calcium carbonate	0.90	0.90
Vitamin premix[c]	0.20	0.20
Trace mineral[d]	0.05	0.05
Selenium premix[e]	0.02	0.02

[a] Diets listed above were fed to pigs from day 0 to 75 of the experiment.
[b] Phase V, 0–20 and VI, 21–75 day.
[c] Supplied per kilogram of diet: retinyl acetate, 1516 μg; cholecalciferol, 26 μg; dl-alpha tocoperyl acetate, 22 mg; riboflavin, 6.6 mg; pantothenic acid, 17.6 mg; niacin, 33 mg; and vitamin B-12, 22 μg.
[d] 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).
[e] Supplied per kilogram of diet: Se, 0.1 mg (Na_2SeO_3).

2.2. Lymphocyte proliferation assay

Peripheral blood was drawn from all of the pigs on day −72, 0, 25, 43, 67 and 75 of the experiment. PBMC were isolated from whole blood by using a previously described procedure [10]. Briefly, 10 ml of peripheral blood (1:4 dilution in phosphate buffered saline (PBS)) were overlaid on to lymphocyte separation medium with a density of 1.077 (Mediatech, VA) and centrifuged for 40 min at 400 g. The mononuclear cell layer was aspirated using sterile Pasteur pipettes, transferred into a 50 ml tube containing sterile PBS, washed three times with PBS and the final resuspension was in complete RPMI. Complete medium was prepared by supplementing RPMI 1640 (Mediatech) with 25 mM HEPES buffer (Sigma, St. Louis, MO), 100 units per milliliter penicillin (Sigma), 0.1 mg ml^{-1} streptomycin (Sigma), 5×10^{-5} M 2-mercaptoethanol (Sigma), 1 mM essential amino acids (Mediathech Inc., Herndon, VA), 1 mM non-essential amino acids (Sigma), 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Sigma) and 10% FBS (HyClone, Logan, UT). Media pH was measured with a pH meter (Orion Research Inc., Beverly, MA) and adjusted to 7.4 with addition of a solution of 7.5% sodium bicarbonate (Fisher Scientific).

Wells of 96-well flat-bottomed microtiter plates (Becton Dickinson, Lincoln Park, NJ) were seeded with 2×10^5 mononuclear cells in a total volume of 200 μl per well. Wells contained either *B. hyodysenteriae* antigen (5 μg ml^{-1}), PRV antigen (1:100 dilution of the PRV-infected cell lysate kindly provided by Dr. Zuckermann, University of Illinois), both PRV and *B. hyodysenteriae* antigen, or medium alone (non-stimulated). Plates were incubated for 5 days at 37 °C in 5% CO_2 humidified atmosphere. After 5 days [11,15], 0.5 μCi of methyl-[^{3}H] thymidine (specific radioactivity 6.7 Ci $mmol^{-1}$; Amersham Life Science, Arlington Heights, IL) in 10 μl of medium was added to each well and plates were incubated for an additional 20 h. Well contents were harvested onto glass fiber filters using a PHD cell harvester (Cambridge Technology, Cambridge, MA) and incorporated radioactivity measured by liquid scintillation counting (Packard, Meriden, CT). For each individual animal and type of antigen, samples were run in triplicate. Stimulation indices were calculated by dividing counts per minute of antigen-stimulated wells by counts per minute from non-stimulated wells.

Separate PBMC cultures were stained with PKH2 as previously described [11], stimulated as stated above and incubated at 37 °C in 5% CO_2 humidified atmosphere for 5 days. Following culture (i.e. replicates of six wells per pig and antigen), PBMC were prepared for immunophenotyping. Relative proliferation indices for CD8αβ$^+$ and CD4CD8 DP PBMC were calculated by dividing percentages proliferating PBMC (i.e. PKH2^{dim}) bearing the markers of interest in the viable cell gate of antigen-stimulated wells by percentages of their counterparts in the viable cell gate from non-stimulated wells.

2.3. Lymphocyte apoptosis assay

Morphologic changes in the plasma membrane appear in early stages of programmed cell death (apoptosis) [16]. Specifically, the membrane phospholipid phosphatidylserine is translocated from the inner to the outer leaflet of the plasma membrane. This feature of early apoptotic cells has been used to assess apoptosis [17,18]. PBMC cultured with the antigens or medium alone (in triplicate), as stated above, were washed twice in cold PBS and resuspended in binding buffer (BD PharMingen, San Diego, CA). PBMC were stained with Annexin V-PE (5 μl) (BD PharMingen) and 7-amino-actinomycin (7-AAD 5 μl) or biotinylated anti-pig-CD8α (50 μl). PBMC were incubated for 15 min at room temperature (20–25 °C) in the dark, washed and analyzed by flow cytometry.

2.4. Flow cytometry

Mononuclear cells were labeled with primary antibodies in 50 μl of fluorescence-activated cell sorter (FACS) buffer: PE-labeled IgG_{2b} mouse anti-swine-CD4, biotinylated IgG_{2a} mouse anti-swine-CD8α (76-2-11) [19], IgG_{2a} mouse anti-swine-CD8β (PG164A), IgG_1 mouse anti-swine-CD3 (8E6), IgG_1 mouse anti-swine-TCRγδ (PGBL22A) (VMRD Inc., Pullman, WA), and appropriate isotype control antibodies. After a 15 min incubation, cells were washed with FACS buffer and resuspended in 50 μl volume of secondary antibody dilution (PE-conjugated goat anti-mouse IgG_1 (Southern Biotechnology Associates Inc., Birmingham, AL), streptavidin-conjugated CyChrome (Pharmingen, San Diego, CA), PE-conjugated goat anti-mouse IgG_{2a} (Southern Biotechnology Associates Inc.). Cells were incubated for 15 min, washed twice and analyzed by flow cytometry. A total of 10,000 events within the cell viable gate (except for apoptosis assays) were saved, and data analysis was performed using the CellQuest software (Becton Dickinson). Viable cells were defined by forward and 90° side light scatter gating that excludes dead cells. Two-color flow cytometric analysis was performed using a Coulter XL (Beckman Coulter Corp.). The quadrants were set on the fluoresecense obtained with the isotype-matched irrelevant immunoglobulin controls. Electronic compensation was utilized to eliminate spectral overlaps between individual fluorochromes in two and three-color flow cytometric analysis.

2.5. PBMC granzyme activity assay

PBMC were cultured as stated above. After five days, mouse anti-porcine CD3 mAb (8E6) and anti-mouse IgG_1 was added into each well (5 μg per well) to broadly activate T cells relative to NK cells. Addition of anti-CD3 PBMC causes an increase in cytolytic activity [20] and anti-IgG_1 promotes receptor crosslinking. After six days, the cultured cell suspension (run in triplicate) was harvested, centrifuged ($400 \times g$) for 5 min, and supernatant was separated from the cell pellet. Cultured lymphocytes (i.e. cell pellet) at 2×10^6 cells per milliliter were lysed in PBS-0.05% 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.

PBMC cytotoxicity was evaluated by measuring BLT-esterase activity using a modification of a previously described procedure [13,21]. A total of 40 μl of supernatant was added to 40 μl of the reaction mixture (0.2 M Tris–HCl, pH 4.5, 2×10^{-4} M benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) (Calbiochem-Behring, La Jolla, CA), 2.2×10^{-4} M 2-nitrobenzoic acid (5,5′-dithiobis, Sigma) and incubated for 20 min (room temperature). Absorbance (at 405 nm) of the BLT-esterase-induced color change was measured using an ELISA reader (BioTek Instruments, Winooski, VT). BLT-activity indices were calculated by dividing the absorbance of the BLT-esterase-induced color change of the antigen-stimulated wells by the absorbance of color change from non-stimulated wells.

2.6. Statistics

Data were analyzed as a 2×4 factorial arrangement of treatments (two previous dietary treatments and four present immunization treatments) within a split-plot design being the immunization treatment (i.e. squalene adjuvant preparation alone, proteinase-digested *B. hyodysenteriae* bacterin, modified life PRV vaccine, or a combination of both vaccine preparations) the whole plot and the previous dietary treatment (i.e. conjugated linoleic acid (CLA) or soybean oil-supplemented diets) the sub-plot. The whole plot error (i.e. error A) was block within vaccine and the sub-plot error (i.e. error B) was the residual degrees of freedom after accounting for the previous dietary treatment variance and the variance for the interaction between former diet and present immunization. Analysis of variance was performed using the general linear model (GLM) procedure of SAS using the test statement to define the components of the split-plot design [22] as previously described [13]. A $P < 0.05$ was considered to be significant. The statistical model utilized was $Y_{ijk} = \mu + \text{present vaccine}_i + \text{error } A_{ik} + \text{former diet}_j + (\text{present vaccine} \times \text{former diet})_{ij} + \text{error } B_{ijk}$, μ being the general mean, present vaccine_i being the main effect of the ith level of the present immunization effect, former diet_j being the main effect of the jth level of the effect of former diets, $(\text{present vaccine} \times \text{former diet})_{ij}$ being the interaction effect between immunization and diet, and errors A and B representing the random errors for the whole plot and the sub-plot, respectively. When an interaction or main effects of former dietary treatment were found to be significant, no further testing was performed. However, when significant differences in present vaccination were found, three contrasts were utilized to separate vaccination means (i.e. unimmunized versus the average of the other three, combination of *B. hyodysenteriae* and PRV versus the average of the other two vaccines, and *B. hyodysenteriae* versus PRV

vaccine). In tables and figures, statistically significant differences between treatments attributed to the main effects of vaccine, diet, and the interaction between vaccine and diet are reported by utilizing *, †, and, ¥, respectively. Values for measurements represented in tables are expressed as the mean ± individual S.E.M.

3. Results

3.1. Proliferation of antigen-stimulated PBMC

To examine the influence that consumption of a nutraceutical (i.e. dietary CLA supplementation) had on the magnitude of recall responses to bacterial and/or viral antigens, lymphoproliferative responses to PRV, *B. hyodysenteriae*, or a combination of both antigen preparations were evaluated in pigs that had been fed one of two isocaloric and isonitrogenous dietary regimens (i.e. CLA-supplemented or soybean oil-supplemented control diets) prior to immunization. Twenty-five days after withdrawing CLA from the diet, PBMC recovered from PRV-immunized pigs fed a CLA-supplemented diet had greater lymphoproliferative responses to the PRV antigen than PBMC recovered from PRV-immunized pigs that had been fed a control diet (Fig. 1). Conversely, PBMC recovered from *B. hyodysenteriae*-immunized pigs that had been fed CLA-supplemented diets elicited antigen-specific recall responses of a lower magnitude than *B. hyodysenteriae*-immunized pigs that had been fed a control diet (Fig. 1). While antigen-specific responses were demonstrable in each immunized group, the effects of dietary CLA supplementation on the stimulation indices were not maintained 43, 67, or 75 days after withdrawing CLA from the diet (data not shown).

3.2. Phenotype of peripheral blood lymphocytes and rate of apoptosis of $CD8^+$ cells

To determine the long-term effects of dietary CLA supplementation on the phenotype of peripheral blood lymphocytes, PBMC were recovered from pigs that had been fed the CLA-supplemented diet or a control diet and were evaluated by flow cytometry. Before the dietary treatment was administered (i.e. day −72 of the experiment), no differences in numbers of $CD8\alpha\beta^+$ PBMC were detected in pigs that would be fed either a CLA-supplemented or a control diet (i.e. 1.1 and 1.2 $CD8\alpha\beta^+$ per milliliter of blood ($\times 10^{-6}$)). On day 0 of the experiment, pigs fed a CLA-supplemented diet had greater numbers of $CD8\alpha\beta^+$ PBMC than pigs fed a control diet (Fig. 2). Furthermore, numbers of $CD8\alpha\beta^+$ PBMC were greater in pigs that had been fed CLA-supplemented diets than in pigs that had received an isocaloric and isonitrogenous control diet for 67 days following the withdrawal of CLA from the diet (Fig. 2). The increased numbers of $CD8^+$ PBMC in the pigs that had previously been fed CLA-supplemented diets did not correlate with decreased apoptosis of mature $CD8^+$ (Table 3) PBMC.

3.3. Phenotype of antigen-stimulated PBMC

Relative proliferation indices indicate that on day 25 post-withdrawal, $CD8\alpha\beta^+$ PBMC recovered from PRV-immunized pigs that had been fed CLA-supplemented diets were more responsive to stimulation with PRV antigens than $CD8\alpha\beta^+$ PBMC recovered from PRV-immunized pigs that had been fed the control diet (Fig. 3). Conversely, relative proliferation indices of *B. hyodysenteriae*-stimulated $CD4^+CD8\alpha\alpha^+$ DP PBMC recovered on day 25 from *B. hyodysenteriae*-inoculated pigs were greater in pigs that

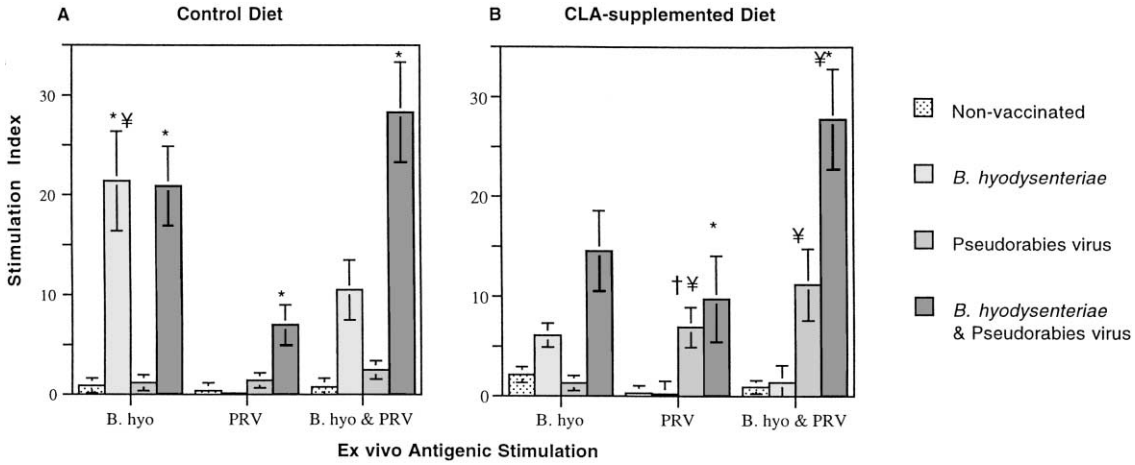

Fig. 1. Ex vivo antigen-specific proliferation of peripheral blood mononuclear cells isolated on day 25 post-withdrawal of dietary CLA from pigs that had been fed either a control diet (A) or a CLA-supplemented diet (B) and cultured for 5 days with *B. hyodysenteriae*, PRV, or the two antigens. Data represents the mean stimulation index of cells. Pigs were immunized on days 0 and 20. Significant differences ($P < 0.05$) caused by vaccine, diet, and/or interaction between vaccination and dietary treatment are indicated by *, †, and ¥, respectively.

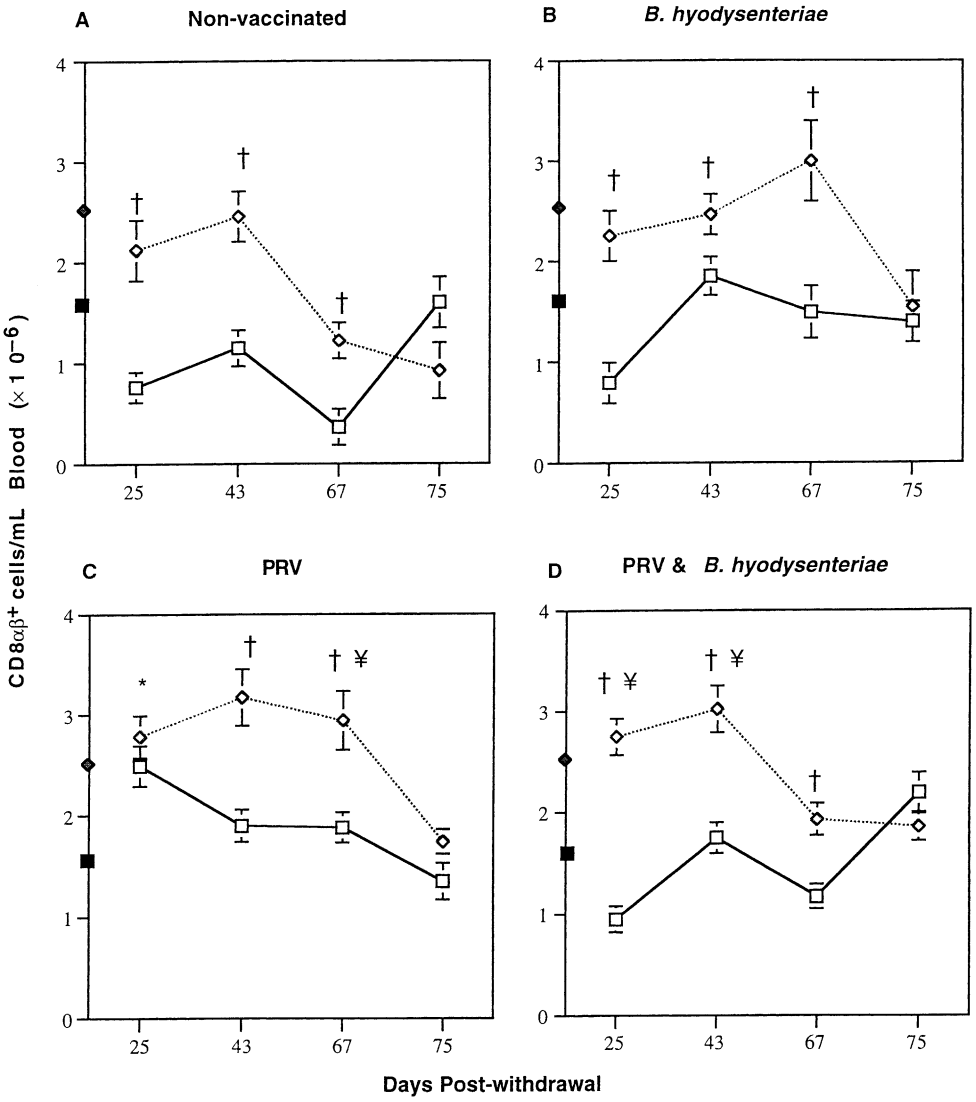

Fig. 2. Flow cytometric analysis of CD8αβ$^+$ peripheral blood mononuclear cells recovered from pigs fed either a control (solid line) or a CLA-supplemented (dashed line) diet. The data in each panel represents the vaccine regimen used: non-vaccinated pigs (A), *B. hyodysenteriae*-immunized pigs (B), PRV-immunized pigs (C), or pigs receiving a combination of both vaccines (D). Two-color flow cytometry was utilized to evaluate PBMC for CD8α and CD8β as described in Section 2. A closed rubi or a closed square are representative of mean numbers of CD8αβ$^+$ cells on day 72 of dietary CLA supplementation (i.e. day 0 post-withdrawal) in pigs fed a CLA-supplemented or a control diet, respectively. Pigs were immunized on days 0, 20, and 50. Significant differences ($P < 0.05$) caused by vaccine, diet, and/or interaction between vaccination and dietary treatment are indicated by *, †, and ¥, respectively.

Table 3
Effects of dietary and vaccination treatments on apoptosis[a] of peripheral blood mononuclear cells (Annexin V$^+$ CD8α$^+$) 43 days after withdrawing conjugated linoleic acid (CLA) from the diet

Antigen/vaccine	Control				CLA				S.E.M.	*P* value		
	NV	*B. hyo*	PRV	*B. hyo* and PRV	NV	*B. hyo*	PRV	*B. hyo* and PRV		Vaccine	Diet	Vaccine × diet
Non-stimulated	1.48	1.74	2.33	0.55*	1.40	2.94	3.29	0.30*	0.44	0.02	0.21	0.37
B. hyodysenteriae	2.66	2.22	3.74	2.64	2.75	2.22	2.15	2.63	0.54	0.64	0.38	0.44
Pseudorabies virus	2.54	2.46	2.42	2.63	2.55	2.63	1.67	3.48	0.40	0.24	0.81	0.39
B. hyodysenteriae and PRV	2.18	2.28	2.11	2.60	2.13	2.24	2.09	2.85	1.88	0.42	0.63	0.84

[a] Data represent the mean percentage of apoptotic CD8α$^+$ PBMC within the viable cell gate following stimulation with medium alone, *B. hyodysenteriae* antigens, PRV antigen, or *B. hyodysenteriae* and PRV antigens. PBMC (2×10^6 ml^{-1}) were cultured for 5 days and assayed for Annexin V and CD8α expression by flow cytometry as described in Section 2.

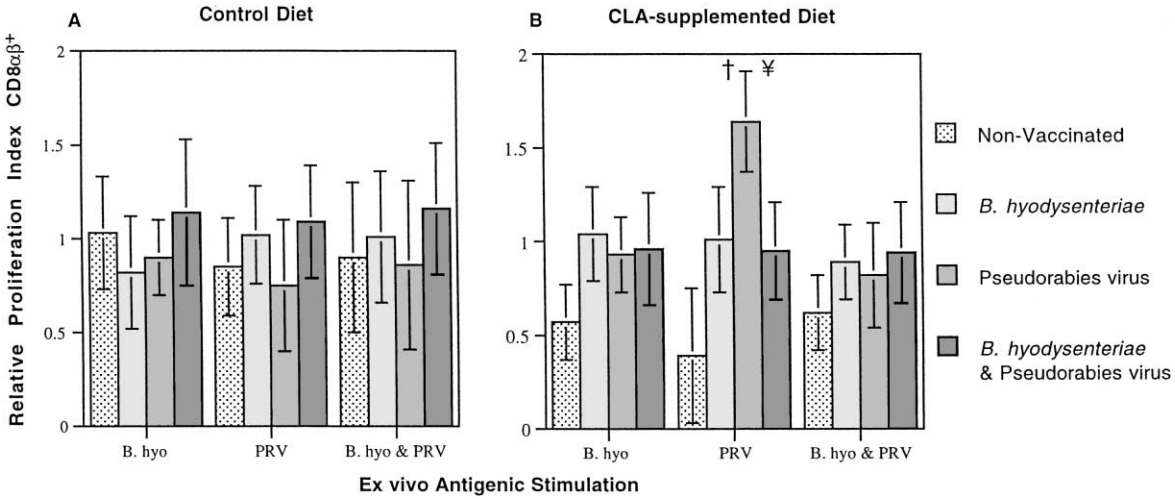

Fig. 3. Relative proliferation indices of CD8αβ+ PBMC isolated on day 25 post-withdrawal of dietary CLA from pigs that had been fed either a control diet (A) or a CLA-supplemented diet (B) and cultured for 5 days with *B. hyodysenteriae*, PRV, or the two antigens. Pigs were immunized on days 0 and 20. Values are expressed as percentages of cells from non-stimulated wells. Significant differences ($P < 0.05$) caused by diet and/or interaction between vaccination and dietary treatment are indicated by † and ¥, respectively.

had been fed the control diet (Fig. 4). Previous dietary treatments did not influence relative proliferation indices of CD8αβ+ and CD4+CD8αα+ DP PBMC on days 43, 67, or 75 or relative proliferation indices of CD4+ SP and TCRγδCD8αα+ cells at any time point following dietary CLA withdrawal.

3.4. Granzyme activity of antigen-stimulated PBMC

Granzyme is a critical effector molecule that mediates lymphocyte cytotoxic responses by inducing apoptosis of target cells [23]. BLT-esterase activities (i.e. granzyme activity) of PBMC cultured with antigens were assayed as an indirect indicator of cytotoxic potential of PBMC. On day 25, even though numbers of CD8αβ+ cells were similar, PRV-stimulated PBMC recovered from PRV-immunized pigs that had been fed CLA-supplemented diets had greater BLT-esterase activity than their counterparts fed control diets (Fig. 5). In addition, BLT-esterase activity of supernatants of PBMC recovered from dually vaccinated pigs fed CLA were greater than those of PBMC recovered from dually vaccinated pigs fed a control diet and stimulated either with PRV alone or along with *B. hyodysenteriae* antigens (Fig. 5). While lymphocytes recovered from pigs immunized

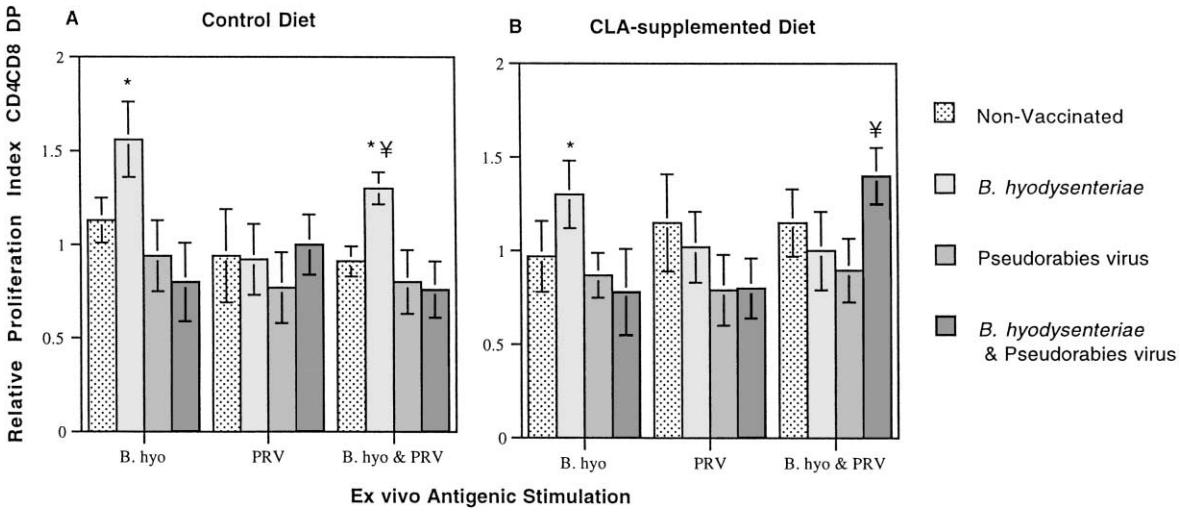

Fig. 4. Relative proliferation indices of CD4+CD8αα+ PBMC isolated on day 25 post-withdrawal of dietary CLA from pigs that had been fed either a control diet (A) or a CLA-supplemented diet (B) and cultured for 5 days with *B. hyodysenteriae*, PRV, or the two antigens. Pigs were immunized on days 0 and 20. Values are expressed as percentages of cells from non-stimulated wells. Significant differences ($P < 0.05$) caused by vaccine and/or interaction between vaccination and dietary treatment are indicated by * and ¥, respectively.

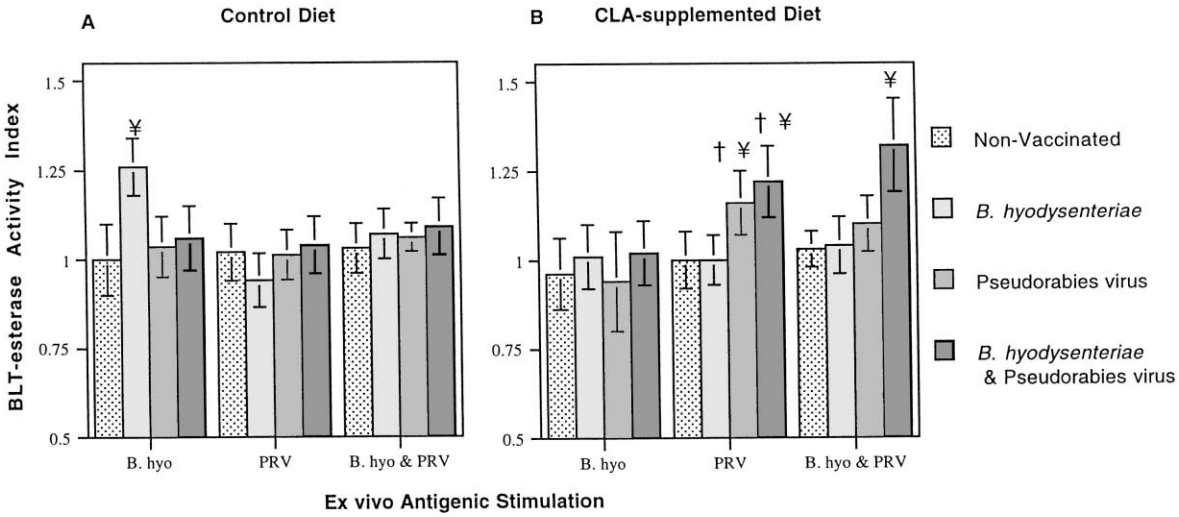

Fig. 5. BLT-esterase activity indices of PBMC isolated on day 25 post-withdrawal of dietary CLA from pigs that had been fed either a control diet (A) or a CLA-supplemented diet (B) and cultured for 5 days with *B. hyodysenteriae*, PRV, or the two antigens. Pigs were immunized on days 0 and 20. Significant differences ($P < 0.05$) caused by diet and/or interaction between vaccination and dietary treatment are indicated by † and ¥, respectively.

with either PRV or the combination of PRV and *B. hyodysenteriae* had greater BLT-esterase activities during the entire experiment, no differences in BLT-esterase activity were attributed to the dietary treatments on days 43, 67, or 75.

4. Discussion

Nutritional immunomodulation has not generally been sought as a means to enhance vaccine efficacy. However, the immunomodulatory actions of CLA reported in the present study indicate that nutrition influences host responses to specific antigens. For the first time, it was demonstrated that nutritional immunomodulation (i.e. T cell phenotype) persisted beyond the period of dietary supplementation. Effector functions of lymphocytes and the proliferative response of $CD8\alpha\beta^+$ PBMC to stimulation with specific antigens, were modulated by nutritional means. PRV-stimulated $CD8\alpha\beta^+$ PBMC recovered from PRV-immunized pigs fed CLA-supplemented diets had greater relative proliferation indices than those recovered from PRV-immunized pigs fed a control diet (Fig. 3). The enhanced proliferation of $CD8\alpha\beta^+$ PBMC to PRV antigens in PRV-immunized pigs fed CLA supplemented diets explains, in part, the enhancement in proliferation of total PBMC and the greater granzyme activities of PBMC. The correlation between increased proliferation of $CD8\alpha\beta^+$ PBMC and increased granzyme acitivities of PBMC in PRV-immunized pigs fed CLA-supplemented diets is consistent with studies indicating that CLA increases the cytotoxic activity of lymphocytes [24]. In line with these findings, PRV-specific $CD8\alpha\beta^+$ T cells were shown to be critical in the development of immune responses to PRV [2]. While phenotypic changes were observed on day 0, no functional differences (i.e. proliferation or granzyme activity) were found on day 0 or −72 of the experiment (data not shown). Thus, in these studies, both immunization and CLA supplementation were required for the functional enhancement of $CD8\alpha\beta^+$ PBMC to PRV antigens whereas CLA alone was sufficient to modulate the numbers of $CD8\alpha\beta^+$ lymphocytes in peripheral blood.

$CD4^+CD8\alpha\alpha^+$ DP and $TCR\gamma\delta^+CD8\alpha\alpha^+$ PBMC recovered from *B. hyodysenteriae*-immunized pigs proliferated upon in vitro re-exposure to antigen [10]. Additionally, activation of *B. hyodysenteriae*-specific PBMC correlated with enhanced antigen-specific induction of IFN-γ that was suppressed following in vitro depletion of $CD4^+$ cells [1]. Because *B. hyodysenteriae* antigens do not activate $CD8\alpha\beta^+$ cells [11], the enhanced granzyme activity of *B. hyodysenteriae*-stimulated PBMC recovered from *B. hyodysenteriae*-vaccinated pigs fed unsupplemented diets is unlikely to be mediated by $CD8\alpha\beta^+$ cells. Indirect evidence suggests that activated $CD4^+CD8\alpha\alpha^+$ DP cells mediate cytolytic effector functions [2]. Thus, the $CD4^+CD8\alpha\alpha^+$ DP T cell activation subsequent to *B. hyodysenteriae* stimulation could be involved in the enhancement of granzyme activity in PBMC from *B. hyodysenteriae*-immunized pigs.

In contrast to the functional enhancement, of PBMC and $CD8\alpha\beta^+$ lymphocytes that was observed 25 days after withdrawing CLA from the diet, the modulation of T cell phenotype was longer-lived (i.e. 67 days). Results from initial studies revealed the existence of nutritional modulation of adaptive immunity (i.e. $CD8^+$ cells) while CLA-supplemented diets were fed to pigs after 42 days of dietary supplementation [12]. These results were later confirmed in separate experiments which demonstrated that the $CD8\alpha\beta^+$ subset was the most affected by dietary CLA [13]. Thus, based on collective findings from several trials, numbers of $CD8\alpha\beta^+$ PBMC in pigs fed diets supplemented

with 1.33% CLA are greater than in pigs fed control diets after 42 days of dietary supplementation and continue to be increased after 67 days of withdrawing CLA from the diet. The present study focuses, on the latter observation and demonstrates that the benefits of dietary CLA on cellular immunity (i.e. phenotype and function) are sustained over time and are prolonged beyond the period of dietary CLA supplementation. Because diets were made isocaloric and CLA did not influence feed intake neither in previous experiments [12] nor in this experiment (data not shown), the immunomodulatory effects of CLA in the present study are not related to differences in caloric intake. This is a critical point because it has been shown that the energy level of the diet influences the immune response [25].

We had earlier suggested that CLA might influence thymocyte lineage commitment [12]. It has recently been shown that pigs fed CLA-supplemented diets have greater percentages of immature double negative and $CD8\alpha\beta^+$ thymocytes [13], and greater thymi weights (unpublished observations) than pigs fed control diets. Thus, CLA might modulate T cell phenotype by favoring thymocyte lineage commitment towards $CD8^+$ cells (i.e. preventing apoptosis of $CD8^+$-commited precursor cells) rather than by directly acting on mature T lymphocytes (i.e. preventing apoptosis of mature $CD8^+$ cells), as shown by the normal rate of programmed cell death (Table 1). The mechanistic explanation for this model originates in the capacity of CLA to activate the peroxisome proliferator activated receptor-γ (PPAR-γ) [26,27]. This nuclear transcription factor influences the expression of genes regulated by the retinoid-related orphan receptor-γ (ROR-γ) [28,29]. Activation of ROR-γ has recently been shown to be required for thymopoiesis and thymocyte differentiation [30].

In conclusion, we have demonstrated that CLA has long-term implications on porcine immunity that go beyond the basic nutritional requirements for tissue maintenance and/or growth. Collectively, we have shown that dietary CLA supplementation interacted with vaccination to modulate phenotype and effector functions of PBMC. Specifically, numbers of $CD8\alpha\beta^+$ cells as well as proliferation of and granzyme release by this lymphocyte subset following stimulation by viral antigens were respectively maintained for 67 and 25 days following withdrawal of CLA from the diet. The possible involvement of a mechanism of nutritional regulation of gene expression that acts on immature thymocytes and as a result influences mature $CD8^+$ cells remains to be uncovered.

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