RESEARCH REPORT

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Title: Ability of dietary conjugated linoleic acid (CLA-60) to improve the efficacy of a swine dysentery vaccine by enhancing the cell-mediated immune response. **NPB #99-208**

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ABSTRACT

To evaluate the effects of conjugated linoleic acid (CLA) on cell-mediated immunity, early-weaned pigs (n = 32) were distributed into two isocaloric and isonitrogenous (0, and 1.33% CLA) diets. A factorial (2 × 2) arrangement within a split-plot design, with 2 littermate pigs as the experimental unit for vaccination, and pig within litter as the experimental unit for dietary treatment, were used in data analysis. Vaccination with a proteinase-digested *Brachyspira hyodysenteriae* bacterin preparation was performed on d 21, 28, and 42 to evaluate the efficacy of CLA in expanding a $CD8^+$ cell subset enhanced by vaccination. The increase of peripheral CD8□□$^+$ cells induced by vaccination has been shown to be correlated with protection against spirochetal-induced colitis. Thus, the targeted enhancement of particular subsets of peripheral lymphocyte pools, induced either by dietary means or through vaccination, might aid in the induction of effective immune protection against specific groups of pathogens. Activation of $CD8^+$ lymphocytes bearing the TCR□□CD8□□ phenotype (i.e., cytotoxic T cells) is central for the development of protective responses against viruses (i.e., cell-mediated immunity), whereas the TCR□□CD8□□ phenotype would be involved in the development of immune responses against bacterial antigens, mucosal protection, and tolerance.

Polyunsaturated fatty acids, like CLA, modulate fatty acid metabolism and inhibit eicosanoid production, possibly, through a PPAR-□-dependent pathway of gene regulation. While other antiinflammatory agents decrease eicosanoid production but do not affect CD8α expression, CLA does. We hypothesized that the CLA-induced expansion of $CD8^+$ cells involves one or more distinct cell phenotypes and enhances specific $CD8^+$-mediated functions. Here, we demonstrated that dietary CLA promotes an increase of $CD8^+$ lymphocytes. Particularly, we showed that dietary CLA alone expanded TCRαβCD8αβ T lymphocytes (CTL), a critical T cell subset involved in protection against intracellular pathogens such as viruses. CLA alone or in combination with vaccination expanded TCRγδCD8αα T lymphocytes, and $CD3^-CD16^+$CD8αα (NK) cells within the peripheral pool. The expansion of TCRγδCD8αα T lymphocytes could enhance a type of protective immunity that, upon infection with *B. hyodysenteriae*, would ameliorate the signs associated with the onset of disease. The CLA-induced expansion

of peripheral blood TCR$\alpha\beta$CD8$\alpha\beta$ cells was consistent with increased percentages of CD8$\alpha\beta^+$ and CD4$^-$CD8$^-$ thymocytes. Functionally, CLA enhanced the CTL cytotoxic effector potential, and both CLA and vaccination enhanced TCR$\gamma\delta$CD8$\alpha\alpha$ proliferation on d 63. The latter function was not correlated with increased proliferation of CD4$^+$ cells. Collectively, our investigations identified a nutrient that enhances cellular immunity by modulating phenotype and function of CD8$^+$ cells involved in both adaptive and innate immunity. Identification of nutrients, such as CLA, that influence CD8$^+$ lymphocyte kinetics will aid to expand the knowledge regarding the role of distinct CD8$^+$ subsets in porcine health and disease.

Introduction

It has been estimated by the Congressional Office of Technology Assessment that infectious diseases cost the livestock industry approximately $17 billion annually. However, if the use of antibiotics at growth promoting and disease prevention levels were restricted by law, the resulting financial loss in U.S. agriculture would be great because of decreased feed efficiency during the onset of disease (i.e., In the European Union the ban of Mechadox as a growth promoting compound has caused an increase in the prevalence and severity of swine dysentery, a gastrointestinal disease that causes important loses in feed efficiency), additional time to market, loss of carcass quality, and increased mortality. Consumer concerns, animal welfare considerations, decreased marketability of animal products in foreign countries, and the development of antibiotic-resistant human pathogens may soon force restrictions on the use of antimicrobials in livestock diets in the United States which might cause the emergence of diseases that, such as swine dysentery, were previously prevented by using antibiotics at growth promoting levels. Restrictions on the use of antibiotics in animal diets have already been imposed in the European Union. The development of efficacious alternative strategies to the use of antibiotics to control livestock animal infectious diseases and promote growth should focus on human safety, animal welfare, and consumer appeal. Our long-term goal was to develop alternative approaches to the use of antibiotics that enhance animal health and performance by supplementing antibiotic-free diets with nutrients and nutraceuticals that are orally active, and natural. Nutrients with nutraceutical properties, such as conjugated linoleic acid (CLA), could be used in animal agriculture to enhance animal health, to enhance vaccine efficacy, and to promote growth without increasing the risk of selecting antibiotic-resistant pathogens. The use of antibiotics at subtherapeutic levels does not eliminate bacterial agents, but provides an advantage to the animal in a host-agent relationship by targeting microorganisms. As a consequence, bacteria might develop mechanisms of resistance in order to remain within their ecological niche. In contrast, nutraceuticals such as CLA would affect the host rather than the infectious agent, therefore, avoiding the selective pressure of compounds that target the microorganism (e.g., antibiotics and antimicrobial peptides).

Objectives

1. To characterize phenotypic and functional changes in the CD8 T cell subsets (e.g., CD8 □□-homodimers and CD8 □□-heterodimers) in response to dietary CLA supplementation.
2. To examine the impact of CLA on distinct lymphoid compartments, T cell subsets will be determined in the gut (LPL), thymus, spleen, and mediastinal lymph nodes.
3. To measure by flow cytometry the size of antigen-specific CD8$^+$ T cells in subsets responding to both CLA and a vaccine challenge.

Procedures

Experimental design

From d 0 until d 35 there were a total of 32 pigs (n = 8) on trial. Eight animals were euthanized on d 35 for phenotypic evaluation of thymocytes, gut lymphocytes, spleenocytes, and lymphocytes isolated from mesenteric lymph nodes. From d 35 until d 72 there were 24 pigs on trial distributed into four (n = 6) dietary and/or vaccination treatments: no CLA, non-vaccinated; CLA, non-vaccinated; no CLA, vaccinated; and CLA, vaccinated. Peripheral blood was collected on d 0, 14, 21, 42, 49, 56, 63, and 72.

Dietary and vaccination treatments

Thirty-two 14-d-old cross-bred pigs serologically negative for *B. hyodysenteriae* were randomly distributed from outcome groups based on litter, body weight and sex to 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 % CLA or a control isocaloric and isonitrogenous diet was randomly allotted to pens within blocks of two littermate pigs as previously described **(1)**. Pigs were fed either a CLA or a control diet from d 0 until d 72. On d 21, 28 and 42, pigs in the vaccinated group were intramuscularly inoculated with 2 ml of a PD *B. hyodysenteriae* B204 in squalene. Non-vaccinated groups were inoculated with 2 ml of a squalene adjuvant preparation alone. The vaccine was prepared using a modification of a previously described method **(2)**.

Harvesting of PBMC

PBMC were isolated by using a previously described procedure **(2)**. The remaining peripheral blood was used to determine the total WBC. Differential counts were conducted by performing a blood smear, which was stained with Hema 3 Stain Set (Fisher Scientific, Pittsburgh, PA).

Isolation of mucosal lymphocytes and thymocytes

Large intestine and small intestine IEL were isolated using a modification of the methods described previously **(3, 4)**. Briefly, on d 35, four dietary control pigs (two vaccinated and two non-vaccinated) and four pigs fed CLA (two vaccinated and two non-vaccinated) were euthanized and necropsied. IEL were harvested from 45 to 80% interface, washed twice in PBS, and enumerated for phenotypic examination.

To isolate the thymocytes, spleen lymphocytes, and mediastinal lymph node lymphocytes a modification of a previously described method **(5)** was utilized. A 3 cm^2 sample of the tissue was removed from the necropsied pigs (4 dietary control pigs, and 4 pigs fed CLA), the tissue was disrupted between two glass surfaces in cold HBSS, cell suspensions were washed twice in PBS, and lymphocytes were enumerated for use in immunophenotyping.

Proliferation assay

A total of 2×10^7 PBMC were separated to perform PKH2-GL proliferation assays (Sigma) as previously described **(6, 7)**. Briefly, cells were centrifuged ($400 \times g$) for 5 min, supernatants aspirated and cells resuspended in 1 ml of diluent C (Sigma). Cells, in diluent C, were added to 1 ml of the membrane intercalating agent PKH2 (4×10^{-6} M) (Sigma), and incubated 5 min followed by the addition of 2 ml of FBS. Cells were washed three times with RPMI 1640. PKH2 stained PBMC were enumerated and the cell concentration was adjusted to 2×10^6 PBMC/ml of complete media. Cell suspensions (100 μl) were added to 96-well flat bottomed microtiter plates containing 100 μl of medium (non-stimulated), medium plus 5 μg/ml of a WCS *B. hyodysenteriae* B204, or medium plus 5 μg/ml of Con-A. Samples were run in replicates of six for each animal and ex vivo treatment. Cells were incubated at 37º C in 5% CO_2 humidified atmosphere for 6 d. As cells divide, PKH2 membrane staining diminishes resulting in a decreased mean fluorescence intensity **(6)**. After a 6-d period, cultured cells from the six wells of the same ex vivo treatment and pig were pooled and prepared for immunophenotyping.

Flow cytometry

Mononuclear cells were labeled with primary antibodies in 50 μl of FACS buffer: PE-labeled anti-swine-CD4 (74 - 12 - 4), biotinylated IgG2a mouse anti-swine-CD8α (76 - 2 - 11) (kindly provided by Dr. Joan K. Lunney; Beltsville, MD) **(8)**, IgG2a mouse anti-swine-CD8β (PG164A), IgG1 mouse anti-swine-CD3 (8E6), IgG1 mouse anti-swine-TCRγδ (PGBL22A) (VMRD Inc, Pullman, WA), IgG1 mouse anti-swine-CD16 (G7) (NK cell marker kindly provided by Dr. Yoon B. Kim; Chicago, IL) **(9)** 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 IgG1 (Southern Biotechnology Associates Inc., Birmingham, AL), streptavidin-conjugated CyChrome (Pharmingen, San Diego, CA), PE-conjugated goat anti mouse IgG2a (Southern Biotechnology Associates Inc.). 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 in a FACScan (Becton Dickinson, San Jose, CA). A total of 10,000 events were saved, and data analyisis on viable cell gate was performed by using the CellQuest software (Becton Dickinson). Two-color flow cytometric analysis was performed in a Coulter XL (Beckman Coulter Corp.).

Assay for 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 induce cytotoxic activity. Anti-CD3 addition to CTLs causes an increase in cytolytic activity or death via internal action of TNF-□ **(10)**. At 6 d, the cultured cell suspension was harvested, centrifuged (400 × g) for 5 min, and supernatant was separated from pellet. Cultured lymphocytes at 2×10^6 cells/ml 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 –70º C for later analysis of BLT-esterase activity.

BLT-esterase activity was measured using a modification of a previously described procedure **(11)**. A total of 40 μl of supernatant were 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-nitro benzoic acid (5, 5'dithiobis, Sigma) for 20 min (RT). Absorbance of the BLT-esterase-induced color change was measured in an ELISA reader (BioTek Instruments, Winooski, VT) at a wavelength of 405 nm.

Statistics

Data were analyzed as a 2 × 2 factorial arrangement of treatments (2 vaccines, and 2 diets) within a split-plot design. ANOVA was performed using the general linear model (GLM) procedure of SAS **(12)**. In tables I to III, main effects of vaccination, diet and the interaction between diet and vaccination are reported within the table. In tables IV and V, statistically significant differences between treatments attributed to the main effects of vaccine, diet, or the interaction vaccine by diet (V × D) are reported by utilizing *, †, and, ¥, respectively. Values for measurements represented in tables are expressed as the mean ± individual SEM. A $p < 0.05$ was considered to be significant.

Results

Objective 1: To characterize phenotypic and functional changes in the CD8 T cell subsets (e.g., CD8 □□-homodimers and CD8 □□-heterodimers) in response to dietary CLA supplementation. A major phenotypic modification observed in pigs fed CLA was the expansion of TCR□□CD8□□ T cell subset (CTL). As anticipated based on data obtained in preliminary studies, expansion of CD8□□$^+$ T cells occurred after 56 days of dietary CLA supplementation and it is associated with an enhancement of the PBMC cytotoxic potential. Vaccination interacted with dietary CLA to expand TCR□□CD8□□ T cells and NK cells. The expansion of CD8□□$^+$ T cells induced by CLA

suggests a possible role of CLA as a natural stimulator of immune responses against viruses. CD8□□$^+$ T cells, along with NK cells, are involved in protection against intracellular infections.

Dietary CLA induces expansion of peripheral CTL, NK cells and TCRγδCD8αα cells

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 until d 49 no significant differences in numbers of peripheral blood lymphocytes expressing CD8 were attributed to either the dietary or the vaccination treatments (data not shown). Changes in the phenotypic profiles of porcine CD8$^+$ peripheral lymphocytes induced by dietary CLA supplementation were first detected on d 49. CLA induced an earlier expansion (Table I) of TCRγδCD8αα lymphocytes (49 to 63 d) followed by a subsequent expansion of CD8□□$^+$ (Fig. 1), and NK cells (56 to 72 d). Vaccination, in combination with dietary CLA supplementation, induced a greater expansion of NK cells (Fig. 2) than dietary CLA supplementation alone. CLA expanded the TCRαβCD8αβ T cell subset (CTL), whereas vaccination did not (data not shown).

Dietary CLA increases CTL granzyme activity regardless of the vaccination treatment

Because major phenotypic changes were noted within the CD8□□ population, the effects of dietary CLA on CTL cytotoxic potential were utilized as a criterion of effector function. PBMCs isolated from animals fed CLA, during the period in which phenotypic data showed enhanced CTL numbers, had greater BLT-esterase activities (i.e., granzyme activity) than PBMCs from pigs not fed CLA, regardless of the vaccination treatment (Fig. 3).

The effects of both dietary CLA supplementation and vaccination on TCRγδCD8αα cells suggest that **CLA might be as efficacious as the vaccine in preventing the onset of clinical disease upon infection with *B. hyodysenteriae*, the causative agent of swine dysentery.**

Objective 2: To examine the impact of CLA on distinct lymphoid compartments, T cell subsets will be determined in the gut, thymus, spleen, and mediastinal lymph nodes.

No phenotypic differences in lymphocytes isolated from spleen or mediastinal lymph nodes were attributed to either dietary CLA supplementation or vaccination. However, dietary CLA increased percentages of CD8αβ$^+$ cells in the thymus, and interacted with vaccination to increase percentages of intestinal CD4$^+$CD8αα-intraepithelial lymphocytes (IEL).

Dietary CLA supplementation increases CD8αβ$^+$ thymocyte subsets

Percentages of CD8αβ$^+$ and CD4CD8 thymocytes were significantly increased by dietary CLA supplementation (Table II), at day 35. Vaccination interacted with diet to modulate the percentages of TCR□□CD8□□ thymocytes; pigs fed CLA and not vaccinated had greater percentages of TCR□□CD8□□ thymocytes than dietary control pigs. No differences in phenotypic profiles of intestinal lamina propia lymphocytes (LPL) were observed between pigs fed CLA and pigs fed an isocaloric control diet.

Vaccination and CLA interacted to increase percentages of small intestinal CD4$^+$CD8αα-IEL

To investigate a possible extrathymic origin of peripherally expanded lymphocyte subpopulations, we isolated large and small intestine IEL. The flow cytometric analysis showed that TCRγδCD8αα-IEL were not significantly increased in the small intestine of pigs receiving dietary CLA supplementation (Table III). Nonetheless, large intestinal TCRγδCD8αα-IEL percentages were increased in non-vaccinated pigs fed CLA, but decreased in vaccinated pigs fed CLA in comparison to the non-vaccinated dietary control pigs. Also, percentages of small intestinal TCR□□CD4$^+$CD8αα-IELs were

increased when both treatments, vaccination and dietary CLA supplementation, were simultaneously administered (Table III).

These results suggest that the peripheral expansion of $CD8^+$ cells probably originated at the thymic level. Also, CLA might be beneficial to gastrointestinal health by modulating intestinal IEL profiles.

Objective 3: To measure by flow cytometry the size of antigen-specific $CD8^+$ T cells in subsets responding to both CLA and a vaccine challenge.

TCR□□CD8□□ cells responded to both dietary CLA supplementation and to vaccination. A PKH proliferation assay allowed for separation of the TCR□□CD8□□cell subsets between proliferating and non-proliferating cells. Thus, the need for evaluating cellular size as a proliferation criterion disappeared. CLA and vaccination induce proliferation of TCR□□CD8□□ cells.

The role of $CD4^+$ T cells on the proliferation of TCR$\gamma\delta$CD8$\alpha\alpha$ T cells

To further characterize the nature of the CLA-induced expansion of TCR$\gamma\delta$CD8$\alpha\alpha$ lymphocytes, proliferative responses of the distinct $CD8^+$ cell subsets were assessed by a proliferation assay coupled with flow cytometric analysis. Analysis of cells recovered from pigs fed CLA demonstrated that CLA had increased the proliferative ability of TCR$\gamma\delta$CD8$\alpha\alpha$ cells. The greater proliferation (63 to 72 d) observed in TCR$\gamma\delta$CD8$\alpha\alpha$ lymphocytes induced by dietary CLA was not correlated with greater percentages of proliferating $CD4^+$ lymphocytes in non-vaccinated pigs (Table IV). However, the enhancement in TCR$\gamma\delta$CD8$\alpha\alpha$ cell proliferation induced by vaccination was correlated with a greater proliferation of $CD4^+$ cells (Table IV).

As anticipated, $CD4^+$ lymphocytes recovered from vaccinated pigs and stimulated in vitro with *B. hyodysenteriae* antigens had greater proliferation rates than antigen-stimulated $CD4^+$ lymphocytes recovered from non-vaccinated pigs (Table V). Following vaccination, dietary CLA augmented the proliferative response of $CD4^+$ T helper cells to specific-antigen (Table V). In contrast to CD8□□ T cells, dietary CLA had no effect on the number of $CD4^+$ PBMC in vivo, however, vaccination increased peripheral blood $CD4^+$ cell numbers (data not shown).

TCR□□CD8□□ cells are involved in the induction of immunologic tolerance. Swine dysentery is a disease that occurs when the causative agent initiates a change in the large intestinal microbial population that turns on a local inflammatory response. TCR□□CD8□□-mediated tolerance might prevent the induction of intestinal inflammation.

Literature Review

1. Bassaganya-Riera, J., R. Hontecillas, K. Bregendahl, M. J. Wannemuehler, and D. R. Zimmerman. 2000. Effects of dietary conjugated linoleic acid in nursery pigs of dirty and clean environments on growth, empty body composition and immune competence. *J. Anim. Sci.:Submitted*.

2. Waters, W. R., B. A. Pesch, R. Hontecillas, R. E. Sacco, F. A. Zuckermann, and M. J. Wannemuehler. 1999. Cellular immune responses of pigs induced by vaccination with either whole cell sonicate or pepsin-digeted *Brachyspira (Serpulina) hyodysenteriae* bacterin. *Vaccine 18:711*.

3. VanCott, J. L., T. A. Brim, J. K. Lunney, and L. J. Saif. 1994. Contribution of antibody-secreting cells induced in mucosal lymphoid tissues of pigs inoculated with respiratory or enteric strains of coronavirus to immunity against enteric coronavirus challenge. *J. Immunol. 152:3980*.

4. Waters, W. R., R. E. Sacco, A. D. Dorn, R. Hontecillas, F. A. Zuckermann, and M. J. Wannemuehler. 1999. Systemic and mucosal immune response of pigs to

parenteral immunization with a pepsin-digested *Serpulina hyodysenteriae* bacterin. *Vet. Immunol. Immunopathol. 69:75*.
5. Krenger, W., S. Rossi, and G. A. Hollander. 2000. Apoptosis of thymocytes during acute graft-versus-host disease is independent of glucocorticoids. *Transplantation 69:2190*.
6. Ashley, D. M., S. J. Bol, C. Waugh, and G. Kannourakis. 1993. A novel approach to the measurement of different in vitro leukaemic cell growth parameters: the use of PKH GL fluorescent probes. *Leuk Res 17:873*.
7. Waters, W. R., R. Hontecillas-Magarzo, R. E. Sacco, F. A. Zuckerman, K. R. Harkins, J. Bassaganya-Riera, and M. J. Wannemuehler. 2000. Antigen-specific proliferation of porcine CD8aa T cells to an extracellular bacterial pathogen. *Immunology In press*.
8. Pescovitz, M. D., J. K. Lunney, and D. H. Sachs. 1985. Murine anti-swine T4 and T8 monoclonal antibodies: distribution and effects on proliferative and cytotoxic T cells. *J. Immunol. 134:37*.
9. Halloran, P. J., S. E. Sweeney, C. M. Strohmeier, and Y. B. Kim. 1994. Molecular cloning and identification of the porcine cytolytic trigger molecule G7 as a Fc gamma RIII alpha (CD16) homologue. *J Immunol 153:2631*.
10. Wang, J., S. A. Stohlman, and G. Dennert. 1994. TCR cross-linking induces CTL death via internal action of TNF. *J Immunol 152:3824*.
11. Mobley, J. L., and M. O. Dailey. 1992. Regulation of adhesion molecule expression by CD8 T cells in vivo. I. Differential regulation of gp90^{mel-14} (LECAM-1), Pgp-1, and VLA-4alpha during the differentiation of cytotoxic T lymphocytes induced by allografts. *J. Immunol. 148:2348*.
12. SAS. 1988. *SAS/STAT User's guide (Release 6.0.3).* SAS Inst. Inc., Cary, NC.

Table I. Peripheral TCRγδCD8αα lymphocytes subset expansion ($\times 10^6$ cells/ ml blood).

		Vaccination treatments							
		NON-VACC.		VACC.			P values		
Item	CLA, %	0	1.33	0	1.33	SEM[a]	Vacc	Diet	V × D
50 d		0.72	0.92	0.90	1.51	0.091	0.05	0.04	0.15
56 d		1.13	1.65	0.85	1.07	0.403	0.50	0.05	0.81
63 d		1.07	1.21	1.19	2.21	0.435	0.02	0.02	0.06
72 d		1.79	1.61	1.28	1.85	0.293	0.77	0.68	0.42

[a]Subplot SEM

Table II. Influence of CLA and vaccination on CD8 expression in thymocytes, %

		Vaccination treatments							
		NON-VACC.		VACC.			P value		
Item	CLA, %	0	1.33	0	1.33	SEM[a]	Vacc	Diet	V× D
CD8 αα[b]		58.55	32.15	34.70	36.40	1.626	0.23	0.58	0.32
CD8 αβ[c]		8.65	19.15	25.10	32.80	2.475	0.15	0.05	0.06
$CD4^-$ $CD8^{+d}$		10.82	9.62	20.85	13.05	0.283	0.03	0.04	0.06
$CD4^+$ $CD8^-$		12.20	10.14	18.60	15.30	0.382	0.05	0.11	0.41
$CD4^+$ $CD8^+$ (DP)		32.25	30.45	26.50	31.40	0.778	0.24	0.35	0.17
$CD4^-$ $CD8^-$ (DN)[e]		44.75	49.80	34.10	40.20	0.382	0.05	0.09	0.63
TCRγδCD8αα		2.79	6.24	8.44	2.93	0.488	0.30	0.33	0.08

[a]Subplot SEM.

[b]Includes CD4CD8 DP, TCRγδCD8αα, NK cells and putatively TCRαβCD8αα thymocytes.

[c]Only includes CD8αβ thymocytes.

[d]Includes TCRγδCD8αα (dim), TCRαβCD8αβ (bright), NK cells and putatively TCRαβCD8αα (dim) thymocytes.

[e]$CD3^-CD4^-CD8^-$ are progenitor cells that have migrated from the bone marrow to the thymus.

Table III. Impact of CLA and vaccination in CD8αα expression on GALT IEL

		Vaccination Treatments							
		NON-VACC.		VACC.			P value		
Item	CLA, %	0	1.33	0	1.33	SEM[a]	Vacc	Diet	V× D

SI-TCRγδCD8αα	8.65	7.74	3.24	14.75	3.097	0.86	0.39	0.34
LI-TCRγδCD8αα	8.65	11.75	22.14	5.83	2.475	0.15	0.05	0.06
SI-CD4$^+$CD8αα	1.83	0.81	10.42	14.82	0.191	0.16	0.01	0.01
LI-CD4$^+$CD8αα	1.63	1.39	4.93	1.18	1.011	0.29	0.17	0.24

[a]Subplot SEM

Table IV. Effects of dietary CLA and vaccination on percentages of proliferative TCRγδCD8αα and CD4$^+$ cells[ab]

		Vaccination Treatments			
		NON-VACCINATED		**VACCINATED**	
Day	**DIET**	**No CLA**	**CLA**	**No CLA**	**CLA**
d 63 γδ (n = 6)		8.74 ± 0.5	15.35 ± 0.8*	21.74 ± 1.1†	53.79 ± 2.7*†
CD4 (n = 6)		2.47 ± 1.2	2.89 ± 0.9	5.64 ± 1.5†	6.66 ± 1.3†
d 72 γδ (n = 6)		4.83 ± 0.3	7.26 ± 0.6*	13.65 ± 0.9†	15.67 ± 0.8*†
CD4 (n = 6)		9.10 ± 1.5	9.69 ± 1.9	13.01 ± 1.8†	22.90 ± 3.6*†

[a] PBMC (2×10^6) recovered from pigs administered each of the vaccination and/or dietary treatments and cultured with medium alone.

[b] Results are expressed as mean ± SEM. Within row, *, $p < 0.05$ vs control diet, and †, $p < 0.05$ vs non-vaccinated.

Table V. Influence of CLA and vaccination on $CD4^+CD8^-$ proliferation[a]

		Vaccination Treatments			
		NON-VACCINATED		**VACCINATED**	
Treatment[b]	**DIET**	**No CLA**	**CLA**	**No CLA**	**CLA**
Non-stimulated		9.12 ± 2.1[c]	9.79 ± 2.2	13.05 ± 2.8	22.90 ± 3.2
WCS-stimulated		22.02 ± 3.6	27.27 ± 2.8*	40.19 ± 5.1†	78.77 ± 5.6†*
Con-A-stimulated		88.71 ± 2.4	85.92 ± 3.6	94.68 ± 4.3†	96.15 ± 4.7†

[a] PBMC (2×10^6) recovered from pigs administered each of the vaccination and/or dietary treatments.

[b] PBMC isolated from pigs were cultured for 6 days with either WCS *B. hyodysenteriae* bacterin, mitogen or medium alone.

[c] Results are expressed as mean ± SEM. Six pigs per treatment group. Within row, *, $p < 0.05$ vs control diet, and †, $p < 0.05$ vs non-vaccinated.