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Dietary conjugated linoleic acid expands CD8$^+$ lymphocyte subsets in vivo and enhances their function

by

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A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

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Major Professors: Dean R. Zimmerman and Michael J. Wannemuehler

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CHAPTER 1. GENERAL INTRODUCTION

Introduction

Conjugated linoleic acid (CLA) is a mixture of positional (9,11; 10,12; or 11,13) and

geometric (cis or trans) isomers of octadecadienoic (18:2) acid (Ip et al., 1991). Bartlet et al.

(1961) reported that CLA was an intermediate compound in the microbial biohydrogenation

of linoleic acid to oleic acid. Kepler et al. (1966) described that an intermediate of the

biochemical pathway of biohydrogenation catalyzed by the ruminal bacteria *Butyrivibrio*

fibrisolvens is the CLA isomer cis-9, trans-11. Thus, *B. fibrisolvens* converts dietary linoleic

acid into oleic acid, which is the first step towards the synthesis of stearic acid. Once oleic

acid is synthesized, a different group of bacteria will convert it into stearic acid. Furthermore,

Chin et al. (1991) studied a partially purified isomerase (linoleate isomerase) from *B.*

fibrisolvens and found that most of the added linoleic acid in hydrolyzed safflower oil was

converted into CLA. Therefore, linoleate isomerase is the enzyme expressed by *B.*

fibrisolvens, which converts linoleic acid into the cis-9, trans-11 isomer of CLA.

In 1976, Hansen and Czochanska described that fats from pasture-fed lambs contain

0.1 to 0.7% of cis-9, trans-11-octadecadienoic acid. As a consequence of being produced by a

ruminal bacteria, CLA is found in a greater concentration in ruminant-derived than in non-

ruminant-derived products (Chin et al., 1992), including ruminant processed products such as

cheese (Santha et al., 1992). Nonruminant products will also contain CLA because of the

conversion of free linoleic acid by intestinal gram positive bacteria (Chin et al., 1993; Yang

et al., 1995). Furthermore, Brown and Moore (1960) isolated strains of *B. fibrisolvens* in

human fecal material. Even plant oils contain trace amounts of CLA (Ackman et al., 1981;

Brown et al., 1982). Animal sources are richer in CLA than plant sources and, in general,

foods from ruminants contain more CLA than foods from nonruminants (Werner et al.. 1992;

Chin et al.. 1991) (Table 1).

Until the late 1980s the biological properties of CLA were not known. In 1978. M.

W. Pariza and colleagues at the University of Wisconsin at Madison began to investigate

possible mutagen formation in meat during cooking. a research objective that. initially. did

not seem to be directly related to CLA. Instead. a beef-derived mutagen inhibitor was

Table 1. CLA content in the fat of foods

Food	mg of CLA / g of fat	% of cis9. trans 11 in CLA
Ruminant veal	2.7	75
Lamb	5.6	75
Pork and chicken	0.5 to 0.9	75
Eggs	*	
Turkey	2.5	75
Processed turkey or beef	1.6 to 3.8	75
Milk	30 (Parodi. 1996) 2.4–18 (Griinari et al.. 1995)	
Romana cheese	2.9	75
Colby cheese	6.1	75
Peanut and corn oil	0.2	< 50
Safflower oil	0.7	< 50

Adapted from Chin et al.. 1991.

* Only trace amounts of CLA were detected.

discovered (Pariza et al., 1985). In 1987. Ha, Grimm and Pariza identified that mutagen

inhibitor. They became the first research team that described the anticarcinogenic properties

of CLA. This was the beginning of modern CLA research. Since 1987, subsequent

investigations have allowed scientists to further characterize the biological properties of this

compound. CLA was shown to be anticarcinogenic (Ha et al., 1989; Ha et al., 1990; Pariza,

1991;Shultz et al., 1992a; Shultz et al., 1992b; Ip et al., 1994a; Ip et al., 1994b; Ip et al.,

1994c; Ip et al., 1997; and Liu et al., 1997), antidiabetic (Houseknecht et al., 1998),

antiatherogenic (Nicolosi et al., 1993; Lee et al., 1994) and immunomodulator (Ip et al.,

1991; Cook et al., 1993; Miller et al., 1994; Wong et al., 1997; Chew et al., 1997;

Bassaganya-Riera et al., 1998; Sugano et al., 1998; Bassaganya-Riera et al., 1999; and Hayek

et al., 1999). Although it was initially reported that CLA was an antioxidant (Ha et al., 1990),

based on its structure, it was inferred that there is no chemical evidence that CLA could be an

effective antioxidant (Yurawecz et al., 1995). Finally, Van den Berg et al. (1995) confirmed

the previous inference by reporting that a role for CLA as an antioxidant does not seem

plausible because it did not act as an efficient radical scavenger in any way comparable to

vitamin E or butylated hydroxytoluene (BHT).

Of the various isomers of CLA the cis-9, trans-11 isomer (Figure 1) is preferentially

incorporated into the membrane phospholipids of mice (Ha et al., 1990) and rats (Ip et al.,

1991). These findings have been the origin of the speculation that cis-9, trans-11 is the

biologically active isomer. Li et al. (1998) showed that in rats there was tissue selectivity for

various isomers (e.g., in liver, serum, bone marrow and periosteum c-9, t-11/ t-9, c-11

predominate, whereas in spleen muscle and heart t-10, c-12 predominates). Furthermore they

reported that CLA c-9, t-11/ t-9, c-11 isomers concentrations in bone were the highest of all

Figure 1. Structural differences between the CLA c-9. t-11 isomer and linoleic acid

Linoleic acid
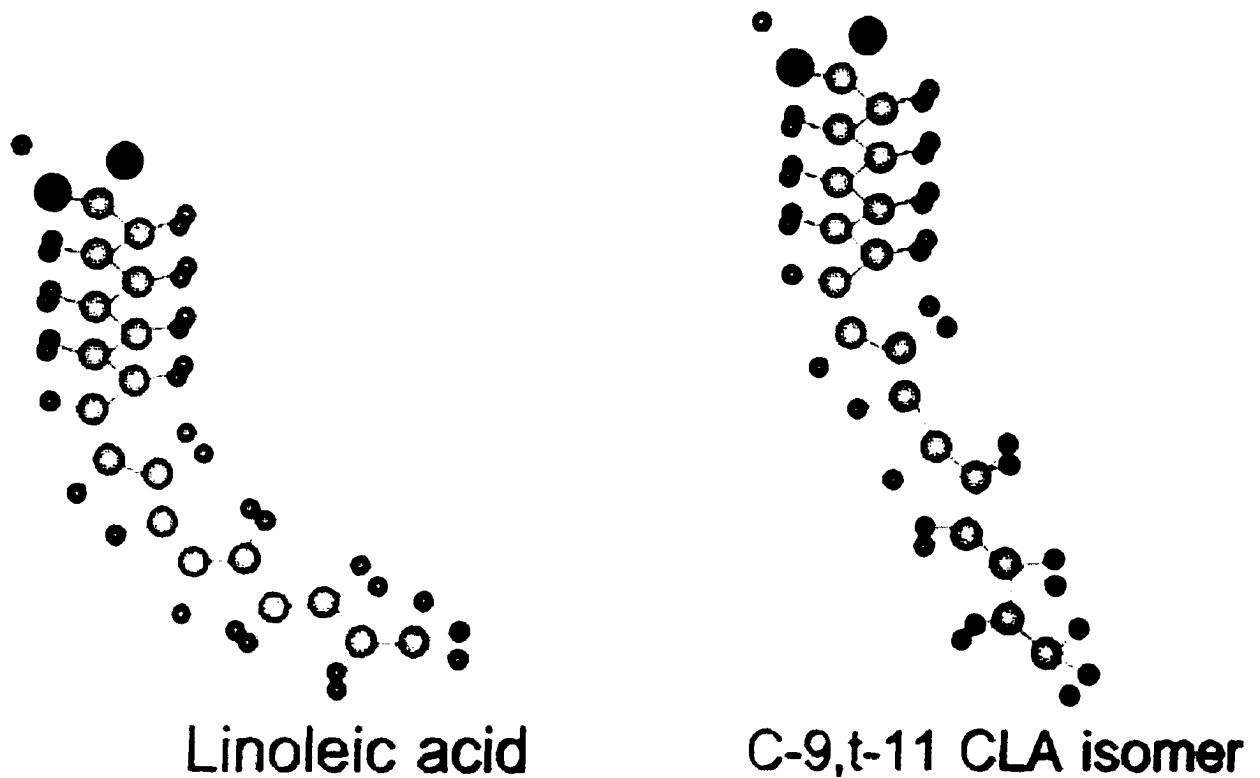
C-9,t-11 CLA isomer

tissues analyzed. The tissue selectivity of c-9. t-11/t-9. c-11 CLA isomers for the bone

marrow and their subsequent accumulation in that tissue indicates that immune cell

progenitors are putatively exposed to high concentrations of CLA.

Kramer et al. (1998) described an accumulation of the 11-cis. 13-trans-18:2 into

cardiac lipids. and in particular into diphosphatidylglycerol (DPG) in the inner mithocondrial

membrane. This could be due to slower metabolism or preferential incorporation. The

incorporation of 11-cis. 13-trans-18:2 into mithocondrial DPG could adversely affect the

activity of key enzymes in mithocondrial energetics, because DPG is imbedded in many of these enzymes (Hoch, 1992; Shigenaga, 1994).

Most of the research conducted on CLA has identified phenomena and not mechanisms. Nevertheless, an exception to that empirical approach is the work conducted at Belury's laboratory at Purdue University where a mechanism of action related to a decrease in arachidonic acid (AA) synthesis and eicosanoid production was postulated (Belury et al., 1997). Later, when investigating the antidiabetic properties of CLA at a molecular level (Houseknecht et al., 1998), they have suggested that CLA could be a peroxisome proliferator-activated receptor gamma (PPAR-γ) ligand. During the last 13 years, the scientific community has described a large list of effects of CLA but the postulated eicosanoid-related mechanism of action does not provide an explanation for all the biological effects caused by this compound. The possible existence of a common mechanistic link, capable of providing a comprehensive explanation for the distinct biological properties of CLA, would require that more CLA research were conducted at the molecular level. In the early years of modern CLA research, Ip et al. (1991) and Chin et al. (1994) suggested that a major emphasis of future research should be on measuring the action of CLA along signal transduction pathways. In the future, scientists conducting CLA research should investigate whether PPAR-γ-CLA binding in a cell environment with low levels of eicosanoids provides a comprehensive explanation for most of the biological properties of CLA.

As mentioned earlier, CLA properties have been partly related to a fatty acid desaturase-related inhibition of the conversion of linoleic acid into AA (Belury et al., 1997; Wong et al., 1997). CLA causes a decrease in hepatic stearoyl-CoA desaturase mRNA expression (Lee et al., 1998) and, therefore, desaturation from linoleic to AA would occur at

a slower rate. AA is a precursor for the synthesis of inflammatory mediators like prostaglandins (PGs) and leukotrienes (LTs) through cyclooxygenase and lipooxygenase activity, respectively. It would be interesting to investigate if that decrease in desaturase mRNA expression derives from PPAR-CLA ligation. Thus, by supplementing diets with CLA, less AA is synthesized in vivo. As a consequence, unless additional dietary AA is provided, eicosanoid synthesis is decreased. Other nutrients have been shown to influence AA metabolism and, therefore, to potentially interact with the effects caused by dietary CLA. For instance, dietary methionine has been shown to stimulate the metabolic conversion of linoleic acid into AA in rats possibly by altering Δ^6-desaturase activity (Choy et al., 1989; Sugiyama et al., 1997). Vitamin B6-deficient diets caused decreases in both the phophatidylcholine (PC)/ phosphatidylethanolamine (PE) ratio and Δ^6-desaturase activity of rat liver microsomes (She et al., 1994).

Although, when comparing n-3 mediated and CLA-mediated effects, the final immunological outcome is completely different, the eicosanoid-driven phenomenon to explain CLA immunmodulatory properties is consistent with the effects described for long chain n-3 polyunsaturated fatty acids (PUFA) on AA metabolism, which also inhibit eicosanoid production (Huang, 1989; and Calder, 1996). N-3 are the PUFA in which the last double bond is located between the third and the fourth carbon from the methyl end of the fatty acid chain, whereas, in n-6 PUFA, the last double bond is located between the sixth and the seventh carbon from the methyl end of the fatty acid chain. Therefore, some isomers of CLA could be considered n-6 (the isomers with the last double bond from the methyl end of the fatty acid chain located in the sixth position; c10, t12/ t10, c12) but not all the other isomers, which could be considered either n-5 (c11, t13/ t11, c13) or n-7 (c9, t11/ t11, c9). As

the definition of CLA includes all the isomers. CLA will not be defined as an n-6 PUFA in this dissertation. The mechanism of n-3-induced inhibition of eicosanoid production has also been used to explain how some PUFA are related to the modulation of inflammation and immune function (Goodwin et al., 1983; Hwang, 1989; Kinsella et al., 1990).

The differences in structure and function of the immune system between vertebrate species suggest that the immunomodulating properties induced by dietary CLA or other dietary immunomodulators could vary from species to species. The recent development of modern immunology has been primarily a story of 'Mice and Men' (Pastoret et al., 1998). Thus, most of the immunological concepts that are currently accepted to describe how the immune system operates have been based on research conducted in humans and inbred mice. However, both gaps in the current knowledge of porcine immunology and evolutionary divergence (Pastoret et al., 1998) between swine and humans or mice suggest that immunomodulation as described in a particular species, will not necessarily apply in the exact same way to other species. Therefore, experimental work on CLA, being a type of research involving both nutrition and immune function in pigs should be built on the basis of strongly supported species-focused scientific knowledge. Particularly, because the effects of dietary nutrients on both immune cell phenotype and immune response are complex (Cunnigham-Rundles, 1994). The enhancement in the understanding of the immune system of species that are being used as models should be an additional objective, and would probably become an obvious consequence of research involving nutrition and immunity.

The broad range of biological effects caused by CLA makes this compound an ideal target for dietary immunomodulation research in swine. I hypothesize that dietary CLA will

modulate the phenotype and function of cells involved in cell-mediated immunity in pigs and that modulation will impact some of the immunological profiles induced by vaccination.

Objectives of the Dissertation

The purpose of the research reported in this dissertation was to measure the effects of dietary polyunsaturated fatty acids on the immune response. More specifically, it was to evaluate the capacity of dietary CLA to modulate cell-mediated immunity in pigs. This objective was accomplished by measuring phenotypic and functional characteristics on porcine T cell subsets. The effects of CLA on pigs of distinct health status and on pigs with different vaccine-induced immunological profiles was also evaluated.

Dissertation Organization

This dissertation is organized in four chapters: a General Introduction including a literature review, a manuscript to be submitted to the Journal of Animal Science, a manuscript to be submitted to the Journal of Immunology, and a General Conclusion.

Literature Review

Nutrition and Immune Function

Background and Precedents of Nutritional Immunomodulation

Growth, differentiation and reproduction are biological processes inherent to all cell types. To successfully accomplish these biological processes cells require seven major molecule types: water, amino acids, nucleotides, sugars, fatty acids, vitamins and minerals (Voet & Voet, 1995). These molecules, after adequate processing, provide structure, function and energy to cells (Stryer, 1995). In higher organisms, the cooperation between the digestive and the circulatory systems make these molecules available to cells (Guyton & Hall, 1996).

Nutrients guarantee that the biological processes of cells, tissues, organs and organisms can be accomplished. Some decades ago, the recognition of the importance of nutrition by the people of developed societies lead to the creation of government policies favorable to support public institutions, which compiled nutritional data and made practical recommendations. The three most important institutions in the world charged to fulfill that purpose are: The Agricultural Research Council (ARC) in the United Kingdom, The National Research Council (NRC) in the United States, and L'Institut National de la Recherche Agronomique (INRA) in France. In 1967, the ARC defined that the requirement for any given nutrient is the amount of that nutrient which must be supplied in the diet to meet the needs of the normal healthy animal given an otherwise completely adequate diet in an environment compatible with good health. Therefore, by that time nutritionists had realized that nutrition and health are two overlapping disciplines the effects of which could be bi-directional in nature. Today, health status is a criterion that is being used by some swine nutritionists to adjust dietary formulations (ISU, 1996). When defining health status from an animal perspective, the immune system becomes a key factor that regulates the balance between health and disease.

Immune function becomes an element of decisive importance for the maintenance of health. Thus, a better understanding of the existing interactions between nutrition and immune function would allow nutritionists to formulate appropriate diets based on the particular health status of the animal population. The NRC, the American homonym of the British ARC, is also including references to immune function into their periodic revisions of the nutrient requirements of domestic animals (Table 2).

Table 2. Importance of immune response based on NRC publications

The nutrient requirements of

Years	Species	Nutrients with immunomodulatory effects
1994	Poultry	Vitamin A, pyridoxine, n-3 / n-6 PUFA
1988 and 1998	Swine	Vitamin A, riboflavin, folacin, panthotenate, pyridoxine
1996	Beef cattle	Vitamin A, selenium, zinc, cobalt
1989	Horses	Vitamin A, pyridoxine, vitamin E
1995	Lab. Animals	Vitamin A, folacin, niacin, panthotenate, zinc
1985	Dogs	Vitamin A, folacin
1993	Fish	Vitamin A, vitamin E, vitamin C*
1989	Dairy cattle	Vitamin A
1981	Goats	Vitamin A

Only were included those nutrients that, based on the respective NRC references, have been reported to elicit direct effects on immune response or on cells of the immune system.

* Indicates that NRC reported that dietary immunomodulation was demostrated to be non-existent.

Remarkably noticeable is that the subcommittee on swine nutrition (NRC, 1998) uses exactly the same immunological generic terms that were already used by the previous subcommittee (NRC, 1988) to discuss the signs of riboflavin, folacin, panthotenic acid and pyridoxine deficiency. Does it mean that during a 10-year period additional research regarding the impact of these vitamins on immune function was not conducted in swine? It could also be inferred that nutritional immunomodulation during the last century's NRC

publications did not occupy a central position in the revision. although there is a clear recognition of the importance of health. even when defining nutrient requirement (ARC, 1967). It is also interesting to notice that Vitamin A effects are consistently observed among distinct animal species and reported in all the reviewed NRC publications.

Nutrient requirements of present-day livestock animals are not the same as when they were first domesticated. A strong genetic selection pressure has changed the body composition and. therefore. the nutrient requirements of most of the livestock animals. Genetic changes induced by humans may also have had an impact on immune cells and on immune response of domestic animal species (e.g.. selection for rapid growth may be inadvertently linked to selection against disease resistance). In the future. nutritionists should formulate diets that both provide the necessary nutrients to allow animals to maximize their genetic potential for growth and reproduction. and to induce the type of immune response most favorable for the environment they are in and the disease status they have acquired. Today. it is clear that small deficiencies of a particular nutrient can induce suppression of the cellular and/or the humoral arm of the immune system. but a more severe deficiency must occur before its effects can be observed in growth performance. An important question that will need to be answered in the coming years is whether the information provided by the NRC on nutrition and immune function is enough to permit nutritionists to formulate immunologically-appropriate diets. Thus. considering that because of the impact of other disciplines such as genetics and immunology on animal characteristics. modern nutrition has become a multidisciplinary science. Should NRC. ARC and INRA include a chapter on nutrition and immune function and another on nutrition and regulation of gene expression in their periodic reviews of the nutrient requirements of the domestic animals?

In humans and in laboratory animals important immunological concepts have been developed at the cellular and molecular level to enhance the understanding of the interaction between nutrition and immunology. However, in pigs the study of immunological concepts at this basic level is a more immature work. For instance, scientists have described the concept of induction of oral tolerance to dietary antigens based on studies conducted in humans and mice (Brandtzaeg, 1996). The subtle understanding of oral tolerance at the cellular and molecular levels is fundamental to better understand mucosal immunology and also to improve the knowledge of the role of $CD8^-$ T cells in the intestine (Sachdev et al., 1993; Li et al., 1995), which might represent between 20 and 40% of IEL (intraepithelial lymphocytes) in swine. Another human- and mice-based concept is how immune cell maturation and differentiation occurs. Based on human studies, we know that the immune system has particular nutrient requirements, which may vary according to the stage of immunologic development (Chandra, 1993). In humans, the immune system is immature at birth, evolves and changes over the span of life and may undergo senescence (Cunningham-Rundles, et al., 1998). McCoy (1998), using human and murine models, suggested that differentiation and maturation of lymphoid cells is highly dependent on the presence and function of appropriate enzymes, macronutrients and micronutrients. Are micro- and macronutrients for immune cell maturation and differentiation required at the same concentrations in swine, humans and mice? To answer that question, scientists should probably elucidate first how the nutrient requirements of the immune system relate to the dietary requirements of each animal species.

Dietary PUFA collaborate in the maintenance of membrane structure, fluidity and function (Calder, 1998). In T cells, membrane dynamics is fundamental for the orchestration

of extracellular interactions (Shaw et al., 1997) and for organization of supramolecular

activation clusters (SMACs) or organized contacts of receptor (T-cell receptor; TCR) and

coreceptor (CD4. CD8. etc.) molecules at the interfaces of physical contact between T cells

and antigen presenting cells (APCs) (Monks et al., 1998). Thus, these three functions

together with a possible PPAR ligation, make PUFAs crucial players in the development of

the immune response and one of the most important groups of dietary immunomodulators. In

spite of that, among all the reviewed NRC publications, only the subcommittee on poultry

nutrition (Table 1) made a direct reference to their immunomodulating properties.

CLA. like other natural dietary immunomodulators may become part of the

nutritional technologies that, during the 21st century, will be applied to enhance the

efficiency of animal agriculture and to improve human health. CLA immunomodulatory

technologies should be understood and applied in the context of a potential interaction with

other nutrients such as vitamin A or other fatty acids. The potential for occurrence of these

interactions is going to be further discussed at the molecular level in a later discussion. The

application of a more mechanistic approach in nutritional research will eventually create a

new science that should be called molecular nutrition.

Oral Tolerance

The gastrointestinal mucosa is one of the largest interfaces in the body with only a

single layer of epithelium separating the exposed external lumen from the internal milieu

(Saito et al., 1998). When either a food component or a soluble protein immunogen is orally

administered it will be denatured by hydrochloric acid, degraded by digestive enzymes and

absorbed into the circulation (Harokopakis et al., 1998). There are exceptions to that

catabolic pathway e.g., intact antigens have been shown to cross the normal gut barrier and

enter the blood stream. even in adults (Brandtzaeg et al.. 1987). The physiologic importance

of oral tolerance may be as a means of preventing immune responses to bacteria that

normally reside as commensals in the intestinal lumen (Abbas et al.. 1997).

Oral tolerance is a complex down-regulatory phenomenon induced via the

gastrointestinal tract (Brandtzaeg. 1998). The oral administration of a protein antigen often

leads to a marked suppression of systemic humoral and cell-mediated immune responses to

immunization with the same antigen (Abbas et al.. 1997). There are three basic

immunological mechanisms that could explain the concept of antigen-driven tolerance:

clonal deletion. clonal anergy. and active suppression by regulatory cells. However. anergy-

driven tolerance (high doses of antigen) and regulatory cell-driven tolerance (low doses of

antigen) were postulated to be the most probable mechanisms of induction of oral tolerance

in humans (Weiner et al.. 1994).

Particularly interesting is the role of CD8$^+$ T cells in oral tolerance. Experiments

using CD8 knock-out mice concluded that CD8$^+$ T cells are crucial for the downregulation of

orally elicited mucosal immunity (Hornquist et al.. 1996). When enterocytes are used as

APCs the result has been stimulation of CD8$^+$ T cells with suppressor function (Brandtzaeg

et al.. 1989). Also. Harper et al. (1996) showed that murine APCs from intestinal origin

(lamina propia and Peyer's patches) induced a suppressive type of T cell response. whereas

when splenic APCs were used. T cell suppression was not observed. Such different responses

may result from distinctive processing and presentation of the antigen by APCs (Harper et

al.. 1996). Furthermore. Li et al. (1995) described that human enterocytes express a ligand

(gp180) that. by interacting with the alpha chain of CD8. may rapidly activate the tyrosine

kinase p56^{lck} (a Src-family kinase which is constitutively associated with the cytoplasmic

domain of the coreceptor molecules CD4 and CD8 alpha chain (Bolen et al., 1997)), and thereby trigger preferentially CD8$^+$ T cells.

At the cellular level, the concept of peripheral tolerance, which might play a role on induction of oral tolerance, involves the elimination of mature antigen-specific T cells after activation to effector function through apoptosis (Von Boehmer et al., 1997). Are antigen-specific CD8$^+$ T cells eliminated by this mechanism? Those CD8$^+$ T cells (CD8$\alpha\alpha$ in pigs) that are immunoregulatory act via secretion of transforming growth factor-β (TGF-β) after antigen-specific triggering (Weiner et al., 1994). Expression of certain cyclins and cyclin-associated proteins such as cyclin-dependent kinases (CDKs) are regulated by specific growth factors (Sherr et al., 1995). As a consequence of the discovery of proteins that bind to and inhibit the catalytic activity of cyclin-CDK complexes, kinase inhibition has been identified as an intrinsic component of cell cycle control (Peter et al., 1994; and Sherr et al., 1995). These cyclin kinase inhibitors (CKIs) induce cell-cycle arrest in response to anti-proliferative signals, including contact inhibition and serum deprivation (Polyak et al., 1994), TGF-β (Reynisdottir et al., 1995), myeloid (Liu et al., 1996) and neuronal differentiation (Lee et al., 1996), and DNA-damage checkpoints (El-Deiry et al., 1993). The identified inhibitors have been grouped into two families based on sequence and functional differences the INK4 and the Kip/Cip family (Russo et al., 1996). The Kip/Cip family of inhibitors, which include p27^{kip1}(Polyak et al., 1994; Toyoshima et al., 1994), p21$^{Cip, WAF-1}$ (El-Deiry et al., 1993; Xiong et al., 1993; Gu et al., 1993; Harper et al., 1993), inhibit cyclin-CDK complexes with preference for the G1 and S phase over the mitotic phase kinase complexes (Russo et al., 1996). The induction of p27^{kip1} by TGF-β is an important example of how cyclins and cyclin-associated proteins are regulated by specific growth factors (Sherr et al.,

1995). Thus, the relationship between TGF-β and regulation of cell cycle could also provide some insights to how the CD8$^+$ T-induced immunoregulatory process occurs. Swine have a large proportion of peripheral T cells that are CD8$\alpha\alpha$. These cells will recirculate between blood and lymphoid tissues such as mesenteric lymph nodes (MLN). Both T and B cells found in porcine MLN preferentially home to the gut (Salmon. 1986). Moreover, in the porcine gut-associated lymphoid tissue (GALT) CD8$\alpha\alpha$ IEL are also abundant. Is the path of some CD8$\alpha\alpha$: from peripheral blood to MLN and from MLN to GALT or most of the IELs and LPLs CD8$\alpha\alpha$ are extrathymic and originated in the GALT? Would these cells elicit similar suppressive effects in pigs?

TCR$\gamma\delta$ IEL are contrasuppressor cells in mice (Kiyono et al.. 1994). They abrogate CD8-driven tolerance. In swine. TCR$\gamma\delta$ can be divided into two subpopulations based on surface expression of the co-receptor molecule CD8$\alpha\alpha$: TCR$\gamma\delta$CD8$\alpha\alpha^+$ cells and TCR$\gamma\delta$ CD8$\alpha\alpha^-$ cells. Adding both populations the result are the TCR$\gamma\delta^+$. and in swine they represent approximately the 35% of peripheral blood mononuclear cells (PBMC). Whereas only a maximum of 20% of small intestine-IEL (SI-IEL) are TCR$\gamma\delta^+$ and in the large intestine (LI-IEL) between 10 and 30% of the lymphocytes are TCR$\gamma\delta^+$ in swine. in mice between 20 and 80% of SI-IEL express TCR$\gamma\delta$ (Goodman et al.. 1988; Bonneville et al., 1988). There are. however. similarities between mice and pigs regarding the percentage of CD8 single positive (CD8 SP) cells in intestinal IEL. In both cases. CD8 SP cells are less prevalent among LI-IEL than SI-IEL (Camerini et al.. 1993). GALT is an important source of peripheral lymphocytes that develop extrathymically. Small cellular clusters called cryptopatches. located within the lamina propia of the small intestine. have been shown to

contain T cell progenitors that are CD4⁻CD8⁻CD3⁻ c-kit positive (c-kit⁺) but lineage marker

negative (Lin⁻) (Saito et al., 1998). These progenitor cells are capable of reconstituting

peripheral and intestinal T cell compartments upon adoptive transfer to immunodeficient

mice (Saito et al., 1998). The phenotype of these progenitor cells found in cryptopatches is

similar to that of a lineage of lymphoid precursors which lack myeloid differentiation

potential found in the bone marrow (Kondo et al., 1997). The presence of greater levels of

TCRγδ cells in the intestine of pigs infected with *Brachyspira hyodysenteriae*, the causative

agent of swine dysentery (SD), may be correlated to a greater severity in both clinical signs

and intestinal lesions than pigs with lower levels of intestinal TCRγδ (Personnal

communication of M. J. Wannemuehler). Therefore, oral tolerance could show scientists that

a dietary-induced manipulation of IEL phenotype together with an inhibition of the intestinal

inflammatory response could be favorably used to inhibit undesirable immune responses of

clinical importance such as food hypersensitivity, autoimmune diseases and infectious

inflammatory diseases in which the immune system is a crucial element in the pathogenesis

of the disease.

In piglets, food hypersensitivity is thought to be responsible for some cases of

postweaning diarrhea and decreased growth performance (Li et al., 1991; Friesen et al.,

1993). If oral tolerance occurs efficiently the undesirable consequences of food

hypersensitivity such as changes in gut morphology and malabsorption will be suppressed.

Sugano et al. (1998) found that CLA decreases the production of IgE and suggested that CLA

mitigates the food-induced allergic reaction. Therefore, CLA could help decrease the

negative consequences of food hypersensitivity potentially by favorably modifying

immunological parameters involved in oral tolerance.

Dietary Fat and Immune Function

There are three overlapping phases on lipids and immunomodulation research and these three phases can also be identified in CLA research. Meade and Mertin (1978) indicated in their review that the interest in the effects of dietary lipids on the immune system dates back many years. That review shows that the first phase of immunomodulatory lipid research was basically supported by empirical studies. In a second phase of lipid research, the elucidation of the roles of eicosanoids derived from AA in modulating inflammation and immunity provided a mechanistic basis to phenomena described in the first phase (Goodwin et al., 1983; Hwang, 1989; and Kinsella et al., 1990; Roper et al., 1994). This eicosanoid-driven research phase has a stronger basis than the empirical research to define phenomena based on the mechanism of action. There is a third phase discussed in more detail under: Approach to the Molecular Basis of Fatty Acid-induced Immunomodulation. The third phase provides the molecular evidence (PPARs) that links dietary fatty acids and regulation of gene expression. Currently, scientists should take into consideration the cumulative results, methods and approaches derived from all three phases to decode the effects that fatty acids elicit on immune response to find ways to induce a fatty acid-derived desired response. In light of the current knowledge, the mechanism of action of fatty acid-induced immunomodulation is multifactorial and at least both eicosanoid- and PPAR-driven.

Prostaglandin E_2 (PGE$_2$) has been shown to inhibit interleukin-2 (IL-2) production (Roitt et al., 1998), which is instrumental for lymphocyte proliferation and enhances the activity of CD8 T cells and NK cells (Kuby, 1997). PGE$_2$ was demonstrated to be supressive to the cellular arm of the immune system (Goldyne and Strobo, 1982). Also, IL-2 deficient mice develop disorders of the hemopoietic and immune systems characterized by anemia,

lymphocytic hyperplasia. and colitis (Contractor et al.. 1998). Wong et al. (1997) and Hayek

et al. (1999) showed that dietary CLA increases IL-2 production in lymphocytes isolated

from spleen of mice. The eicosanoid-based mechanistic explanation for fatty acid-induced

immunomodulation suggests the existence of an immunomodulatory mechanism of AA

metabolism modification that for both n-3 PUFA and CLA is related. in part. to the inhibition

of eicosanoid production. Both types of PUFA seem to decrease PGE_2 production (Fritsche et

al.. 1993: Belury et al.. 1997: Peterson et al.. 1998). However. the final immunomodulatory

outcome when feeding CLA or n-3 PUFA is different. Phenomena derived from feeding

CLA are related to an enhancement of the immune response (Michal et al.. 1992: Miller et

al.. 1994: Wong et al.. 1997: Chew et al.. 1997: Bassaganya-Riera et al.. 1998: Sugano et al..

1998: Bassaganya-Riera et al.. 1999: Hayek et al.. 1999). Whereas. phenomena derived from

feeding n-3 PUFA are related to a suppressive type of immune response (Lee et al.. 1985:

Endres et al.. 1989: Virella et al.. 1989: Meydani et al.. 1991: Kelley et al 1992: Jolly et al..

1997: Kelley et al.. 1998: Peterson et al.. 1998). These different outcomes can be explained

because n-3 PUFA inhibit PGE_2 production but PGE_3 production would be enhanced because

the n-3 PUFA eicosapentaenoic acid (EPA) gives rise to 3 series PGs and 5 series LTs

(Calder. 1998). PGE_3 is more immunosuppressive than PGE_2. When going from PGE_1 to

PGE_3 there is a stronger inhibition in lymphocyte proliferation (Shapiro et al.. 1993). CLA.

therefore. would inhibit PGE_2 production without giving rise to 3 series PGs. Therefore.

based on the described influence of type of PG on immune response. the immunological

impact of dietary CLA supplementation would be greater for swine dietary formulations

containing fish oil (rich in EPA and docosahexanoic (DHA)) than for diets with lower

content in n-3 PUFA.

There is. however. a particular type of research that has not been pursued in the context of fatty acid-induced immunomodulation, which could provide novel and interesting information with regard to how dietary fatty acids modulate immune function. As mentioned earlier when describing the concept of SMACs. T cell membrane dynamics plays a crucial role in the development of the immune response. Shaw and Dustin (1997) indicated that the better understanding of protein interactions in membranes is a new frontier in biology and is a general area in which the T cell activation system is poised to take the lead. Membrane microdomains defined as rafts have been proposed as specialized cell surface platforms used for the selective delivery of membrane proteins (Ledesma et al.. 1999). The understanding of how protein interactions in T cell membranes can be modulated through nutritional technologies capable of altering membrane fluidity in these functionally specialized microdomains. particularly by modifying T cell fatty acid composition. would allow for a subtle nutritional control of T cell activation. Once a model relating membrane fluidity to modulation of immune cell biological responses were established. mixtures of fatty acids and of isomers of fatty acids could be prepared in the proportions that provide the membrane fluidity necessary to achieve the level of protein interaction. both within and between cells. that contributes to optimize particular types of immune responses.

Natural Dietary Immunomodulators as Alternatives to the Use of Antibiotics

In the United States. dietary immunomodulation by using natural products is a strategy that will be more and more used by producers after antibiotic withdrawal from livestock dietary formulations. As long as antimicrobials are allowed for use in production animal diets. bacterial diseases will remain under control because antimicrobials target directly the bacteria. However. in the future, consumer concerns. decreased marketability of

swine products in foreign countries. and development of antibiotic-resistant human pathogens may impose a restriction on use of antimicrobials in livestock diets in the U. S., as it has happened in the European Union. The elimination of antimicrobial utilization at subtherapeutical doses will increase the likelihood that epidemic outbreaks of bacterial diseases occur. In this context. swine dysentery along with other enteric diseases. might threaten the productivity of the swine industry.

CLA and Growth

Cook et al. (1993) showed that dietary CLA was effective in the prevention of growth depression induced by immune stimulation derived from lipopolysaccharide (LPS) injection in chicks. In the same article it was reported that similar findings were observed in rats and mice. Miller et al. (1994) reported that feeding CLA to mice prevented the severe body weight loss caused by LPS injection as observed in mice fed a non-CLA basal diet. However. pigs were not used as a model in this initial work conducted at the University of Wiconsin at Madison. Chin et al. (1994) fed CLA to rat dams during gestation and lactation. Data indicate that pups from dams receiving CLA during gestation and lactation were heavier than pups from control dams.

In weaning pigs. CLA improves feed efficiency but not average daily gain (Bassaganya-Riera et al.. 1999). CLA tended to increase feed efficiency in finisher gilts (Dunshea et al.. 1998). Sparks et al. (1999) showed a quadratic increase in feed efficiency in response to dietary CLA in grower-finisher pigs. However. Eggert et al. (1999) indicate that feed efficiency was not affected by feeding CLA to two genotypes of lean gilts. Thus, the effects of CLA on feed efficiency are not consistent from one experiment to the other. The quality of the source of CLA used. the composition of the basal diet. health status, and the

environmental conditions in which the experiment is held are variables that could potentially influence the final outcome.

CLA and Immune Response

As shown on CLA and growth, CLA does not improve growth unless in the context of changes in immune or inflammatory profiles. Thus, the benefits of CLA on growth might be caused by both an enhancement in the immune response and a decrease in the undesirable consequences of inflammation.

With regard to the effects of CLA on the immune response, both Wong et al. (1997) and Hayek et al. (1999) showed that interleukin-2 (IL-2) production in splenic lymphocytes was enhanced in response to dietary CLA in mice. IL-2 is a cytokine also called T-cell growth factor produced by activated T cells that enhances proliferation and differentiation of most of the lymphocyte subpopulations (Abbas et al., 1997). Thus, increase in IL-2 production in response to dietary CLA would be consistent with enhancement in percentage of $CD8^+$ T cells in pigs described by Bassaganya-Riera et al. (1999).

However, Chew et al (1997), and Michal et al. (1992) described in porcine PBMC that CLA decreased IL-2 production in vitro. The experiments indicating enhancement of IL-2 production in response to CLA were conducted in vivo, whereas the experiments indicating a decrease in IL-2 production were conducted in vitro. A possible explanation for that inconsistency in CLA effects is that CLA added directly into cell cultures could become toxic for cells. Chew et al (1997) and Michal et al. (1992) did not investigate if the percentage of necrotic and/or apoptotic cells was increased after CLA addition directly into cultures was increased. In both in vitro studies 1.78, 3.57 and 7.141×10^{-5} M CLA were added into cell cultures. Although 1×10^{-5} M CLA is considered physiological in human blood (Iverson et

al.. 1985). fetal calf serum (FCS). which was also used as an ingredient of the culture media is known to contain CLA (Park. 1996). It would increase the final concentration of CLA in the culture media.

Spleen lymphocyte proliferation in mice fed 0.3% and 0.9% CLA (Wong et al.. 1997) and 0.5% CLA (Miller et al.. 1994) was enhanced in phytohemagglutinin (PHA)-induced cultures. Neither Miller et al. (1994) nor Wong et al. (1997) showed the number of counts per minute (cpm) in the non-stimulated wells obtained in their lymphocyte blastogenesis test. and therefore. it is not known if CLA increased the background proliferation and the mitogen-induced proliferation or the mitogen-induced proliferation alone. T cell mitogens. such as PHA. concanavalin A (ConA). and pokeweed mitogen (PKM) induce lymphocyte aggregation by binding to different carbohydrate residues in membrane glycoproteins. A greater mitogen-induced proliferation due to CLA addition suggests that CLA makes lymphocytes more capable of producing IL-2. which is consistent with the in vivo results of Wong et al. (1997) and Hayek et al. (1999). Both mechanisms. inhibition of eicosanoid production and PPAR-binding could explain the increase of IL-2 production induced by CLA. The mechanism of inhibition of eicosanoid production by CLA has been discussed earlier. PPAR-CLA binding could influence IL-2 production because PPAR-gamma ligands modulate the activities of transcription factors including AP-1. STAT. and NF-κB (Ricote et al.. 1998) which are involved in the transcriptional regulation of most cytokine genes. including the IL-2 gene. The initiation of cytokine gene transcription requires several signaling pathways which activate different DNA-binding proteins simultaneously. The regulatory or enhancer region of the IL-2 gene. includes a 5' region of 300 base pairs in

which are located binding sites for several transcription factors. including AP-1, NF-κB. Oct-1 and nuclear factor of activated T cells (NFAT) (Abbas et al.. 1997).

Swine IL-2 shares a high degree of sequence conservation with other mammalian IL-2 molecules (Bazan. 1992). IL-2 was shown to play an important role in fighting against viral infections (African swine fever virus. paramyxovirus. and poxvirus) because it induced killer activity to porcine PBMCs (Scholl et al.. 1989: Steinmassl and Wolf. 1990).

The assessment of the impact of a dietary immunomodulator on mitogen-induced responses provides information with regard to its effects on lymphocyte proliferation. However. the practical consequences of these findings sometimes are not clear. The assessment of the impact of CLA on antigen-induced responses would provide more subtle and valuable information with regard to potential applications of this nutritional enhancer of cellular immunity.

CD8⁺ T Cells

Immunologists have used lymphocyte surface markers as a means to identify distinct lymphocyte subpopulations. These surface molecules are crucial for T cell functions such as cell-cell interactions that are important for the development of a T cell response. In humans and mice membrane changes that occur after T cell activation are being elucidated at a molecular level. Kinetic studies about the binding affinity of the T cell receptor (TCR) for the peptide presented on the major histocompatibility complex (MHC). which in swine is called the swine leukocyte antigen complex (SLA). by the APC. gave very low values if compared with those of the antibodies (Matsui et al.. 1991; Weber et al.. 1992). This explains. in part. that to overcome this low affinity recognition. accessory molecules are needed to achieve an

effective T cell response. Thus CD4 and CD8, along with other adhesion molecules, play an auxiliary role in antigen recognition by T cells (Weber et al., 1992).

Some of the surface molecules expressed by T cells (i.e., CD2, CD4, CD8) have in common that their extracellular domain is similar in size to the TCR (approximately 50 KDa) (Dustin et al., 1998). Furthermore, Dustin et al. (1998) and Monks et al. (1998) also suggested that cosegregation of $(\alpha\beta)_2$TCR/CD3, CD4 or CD8, CD2 (LFA-3 receptor), and CD28 occurs on T cells in response to activation signals. When these surface proteins interact respectively with their counterparts found on the APC membrane: $(\alpha\beta)_2$TCR/CD3 and CD8 or CD4 with MHC I or II respectively, LFA-3 (CD48 in mice and CD58 in humans) with CD2, and B7.1/B7.2 with CD28, a distance of 15 nm separates both cells (Dustin et al., 1998). These molecules, when co-segregated, constitute the central-SMAC (c-SMAC) (Monks et al., 1998). Larger surface molecules, like CD45, and LFA-1 (CD54), which bind to ICAM-1, are excluded from the contact area between the two cells. They span a gap of 30–40 nm (Staunton et al., 1990) and constitute the peripheral-SMAC (p-SMAC) (Monks et al., 1998). Dustin et al. (1998) suggested that T cell activation induces the binding of CD2 cytoplasmic domain and a CD2-associated protein (CD2AP), which promotes receptor patterning and cytoskeletal polarization.

The better understanding of $CD8^+$ T cells is of crucial importance for this dissertation because I have shown for the first time that dietary CLA increases the percentage and total number of CD8 in pigs. $CD8^+$ T cells must be understood as functionally distinct when compared, for instance, with CD4 T cells or B cells, but both CD4 and CD8 cells will bear on their surfaces the membrane complexity described above. The CD8 glycoprotein, although very important to phenotypically and functionally classify T cells, becomes, along with the

other molecular machinery of T cell activation, a single element which plays an important

role on the development of T cell responses.

Most peripheral lymphocytes bearing the TCRαβ develop from bone marrow stem

cells but their progenitors migrate to the thymus where they mature (van Ewijk, 1991).

Progression of thymocytes through distinct developmental stages is marked by induced or

repressed expression of numerous regulatory components and cell surface markers such as

CD4 and CD8 (Ellemeier et al., 1999). In the thymus, lymphocytes undergo positive

selection and negative selection. Positive selection favors survival of lymphocytes capable of

recognizing a foreign antigen in the context of self-MHC molecules (Naylor et al., 1997).

Sometimes CD8 cells are misselected and express TCRs that do not recognize class I MHC.

Pestano et al. (1999) described that there is a postthymic quality control mechanism that

purges misselected CD8. The failure of mature CD8 cells to simultaneously engage their

TCR and CD8 coreceptor triggers an activation process that begins with inhibition of CD8

gene expression through remethylation and concludes with up-regulation of surface Fas and

Fas ligand and apoptosis. Negative selection eliminates thymocytes whose TCRs recognize

self peptide: self MHC complexes (Fink et al., 1978). CD4-expressing cells express TCRs

specific for MHC class II, whereas CD8⁺ T cells are MHC class I restricted. In humans, CD4

are almost always helper cells that produce cytokines and CD8 have a cytotoxic phenotype

(granzyme and perforin producers). These cytotoxic T lymphocytes (CTLs) express the

CD8αβ molecule on their surface, which I will further characterize later. Most TCRγδ-IEL

and many TCRαβ-IEL, unlike thymus-derived T cells express a unique CD8αα homodimer

instead of a CD8αβ heterodimer and develop extrathymically in the intestinal mucosa

(Kohyama et al., 1999). Although today the concept that mucosal CD8αα can have

extrathymic development. whereas CD8αβ cannot. should be updated. Levelt et al. (1999) found that CD8αβ IEL development was independent of the presence of CD8αβ T cells in the thymus. spleen. or lymph nodes. These findings suggest that CD8αβ IELs were not derived from mature peripheral CD8αβ cells. Mora et al. (1999) showed that NF-κB signaling is more important for the thymus dependent TCRαβCD8αβ population than for other CD8 lineages. Thus NF-κB could be a regulator of the number. function. and normal balance of the peripheral CD8 susbsets. Robey et al. (1996) reported that expression of an activated form of Notch1. a transmembrane receptor that controls cell fate decisions. in developing T cells of the mouse leads to both an increase in CD8 and a decrease in CD4 lineage T cells. Furthermore. notch activity favors the γδ T cell fate over the αβ T cell fate (Washburn et al.. 1997). Levelt et al. (1999) showed that the affinity of the peptide presented on the MHC for the TCR was critical for the development of mucosal CD8αα and CD8αβ T lymphocytes. They showed that high-affinity peptides induce maturation of CD8αα IELs. whereas low-affinity peptides stimulate the development of both CD8αβ and CD8αα IELs.

The requirements for survival of CD8 T cells are important to understand the kinetics of that cell subset. Tanchot et al. (1997) showed that maintenance of murine CD8 T cell memory requires TCR-MHC class I interactions. but memory CD8 T cells have a lower functional activation threshold than naïve CD8 T cells that facilitates secondary responses. Cho et al. (1999) showed that murine memory CD8 T cells differed from naïve cells in requiring IL-15 for proliferation and in having a greater tendency to undergo apoptosis in vitro. On antigen stimulation in vivo, however. they proliferated more rapidly than naïve cells. Another mechanism required for survival of correctly selected CD8 has been explained

earlier and is related to the inhibition of a death signal through continued TCR-CD8

coengagement of MHC molecules.

CD8 Molecule

CD8 is a cell surface 30-35 kDa dimeric glycoprotein that functions as a coreceptor

with the TCR (Janeway, 1992). It binds to MHC class I and transmits signals to the nucleus

through association with the cytoplasmic tyrosine kinase $p56^{lck}$ (Gabert et al., 1987; Dembic

et al., 1987; Barber et al., 1989). Furthermore, lck can also be found non-covalently

associated with the cytoplasmic tail of CD4. On the other hand, a different tyrosine kinase

called Fyn is associated with components of the TCR/CD3 complex. Both Fyn and Lck are

implicated in proximal signal events that lead to TCR activation. CD8 can be expressed in

two distinct molecular forms: either as a disulfide-linked $\alpha\alpha$ homodimer or $\alpha\beta$ heterodimer

(Norment et al., 1988). Mice, in addition, can generate a distinct isoform of the α chain (α')

that lacks the cytoplasmic tail by alternative splicing of the CD8α mRNA (Zamoyska et al.,

1989). In humans the major form expressed in thymocytes and mature T cells is the $\alpha\beta$

heterodimer, but on a subset of NK cells and intestinal IELs only the CD8$\alpha\alpha$ form is present

(Sun et al., 1997). Technologies, such as CLA, capable to increase the numbers of

lymphocytes bearing CD8$\alpha\alpha$, could be used as a means to better understand the role of these

subsets in humans, which numerically represent a very small percentage of the overall T cell

population but functionally they might be very important.

Human and chicken PBMCs have been shown to express both the $\alpha\alpha$ homodimer and

$\alpha\beta$ heterodimer. Since the $\alpha\alpha$ homodimer has been found at a relatively lower density on the

surface of human and chicken PBMC, it has been suggested that $\alpha\beta$ is more efficiently

expressed than αα (Moebius et al.. 1991; Breed et al.. 1996). In pigs this relationship is the

same. but currently. there is no evidence suggesting that this variability is due to a

differential efficiency in the expression of CD8αα or CD8αβ

The CD8β chain. unlike CD8α. is not directly involved in T cell signaling. The

cytoplasmic tyrosine kinase p56[lck] is non-covalently. but tightly. associated with the alpha

chain of CD8 (Sun et al.. 1997) and it is involved in the early signal transduction events that

occur upon T cell recognition of peptide-MHC complexes on APCs (Abbas et al.. 1997). In

mice. the CXCP motif in the cytoplasmic tails of CD8α and CD4 binds to the cyteine 20 and

cysteine 23 located in the unique motif of Lck (Shaw et al.. 1990). The C-terminus tyrosine

(Y505 in Lck) renders the protein inactive upon phosphorylation because this allows the

interaction with the SH2 domain on the same molecule. The protein folds over the non-

regulatory domain. hiding the catalytic domain. CD45 dephosphorylates Y505. which

exposes the catalytic domain and the tyrosine in the catalytic domain can be phosphorylated.

The Lck molecule is then active and can interact with its substrates: e.g.. ITAMs located in

the CD3 molecule and components of the MAP/ERK kinase pathway (Cooper and Howell.

1993). Wheeler et al. (1992) used a chimeric molecule containing the extracellular domains

of CD8β linked to the transmembrane and cytoplasmic portions of CD8α to show that

CD8αβ had enhanced coreceptor function relative to CD8αα. A possible explanation for that

enhanced coreceptor function in CD8αβ is because CD8 beta chain increases the sensitivity

of the TCR by enhancing recruitment of the protein tyrosine-kinase Lck to the CD8 alpha

chain (Levelt et al.. 1999). However. these differences in coreceptor function between

CD8αα and CD8αβ are not because of higher affinity of CD8αβ for MHC class I (Garcia et

al.. 1996). Lck is a protein tyrosine kinase (PTK) of the Src family expressed mainly by T

lymphocytes and thymocytes. Differences in function and cell distribution among members

of Src-PTK are due to specific features found at the N-terminus or unique domain, which has

myristoylation and palmitoylation signals. The rest of the molecular elements: SH3 and SH2

domains. kinase domain and the C-terminus or regulatory domain are highly conserved

(Bijlmakers and Marsh. 1999). The lck molecule contains two tyrosine residues at the

catalytic and the C-terminus domains that regulate the PTK activity through a process of

phosphorylation-dephosphorylation.

The presence of CD8αα or CD8αβ is also used to further define the T cell phenotype

and to relate that phenotype to a function. In humans and mice. as almost all peripheral T

cells bear the CD8αβ heterodimer on their surface. not much additional information is

provided when measuring the type of CD8 molecule expressed. However in swine. as

opposed to what happens in humans or mice where CD8αβ predominates. between 20 and

40% of PBMC bear the CD8αα homodimer on their surface. whereas only between 7 to 13%

of porcine PBMC are CD8αβ (CTLs). Thus. pigs might be a good model to further

functionally characterize the role of CD8αα subsets in humans.

Cellular Immunology in Swine

Distinctive Characteristics of the Porcine Immune System

As I have mentioned earlier. evolutionary diversion between swine and humans or

mice (Pastoret et al.. 1998) makes the porcine immune system different from the two species

whose immune systems have been studied the most. There are some known differences with

no apparent functional relevance for studies of nutritional immunomodulation for instance,

porcine lymph nodes are structurally inverted compared to other domestic species: lymphatic

vessels enter into the lymph node through the hilus and lymph leaves through the cortex

(Roth. 1999). Also. NK cells activity in most species. is associated with large granular

lymphocytes because of bigger size and greater granularity than other lymphocyte subsets,

whereas in pigs. NK activity is mediated by a small agranular lymphocyte subset that bears

the CD2-T cell marker on its surface (Ferguson et al.. 1986: Duncan et al.. 1989). Most

porcine NK cells also bear $CD8\alpha\alpha$ on their surface. The complete phenotype of porcine NK

cells is $Ig^-CD2^+CD3^-CD4^-CD8^{low}$ (Zuckermann et al.. 1998). As it has been shown in other

species. porcine NK cells serve as an early defense against certain intracellular infections. In

humans. 5 to 10% of the peripheral blood lymphocytes are NK cells. They were first

described as null cells displaying cytotoxicity against tumors without previous immunization

to that tumor (Kuby. 1997). In swine. NK cell activty is high in peripheral blood and spleen.

low levels in lymph nodes and no NK activity in the thymus (Pinto and Ferguson. 1988).

However. there are other differences. which might be of crucial importance to better

understand porcine immune responses. Two important differences of functional importance

are the large number of cells bearing TCRγδ in swine (Binns et al.. 1992: Binns. 1994: Binns

et al.. 1994). which is also important in other ungulates (Roth. 1999). and the presence of

mature extrathymic CD4CD8 double positive T cells (Pescovitz et al.. 1994: Zuckerman and

Husmann. 1996: Summerfield et al.. 1996).

γδ T cells are capable of recognizing intact antigen molecules. similar to the way

antibodies recognize antigen (Kaufmann. 1996). In humans and mice. 90% of peripheral T

cells express TCRαβ on their surface and only 10% TCRγδ. whereas the latter represent a

major T cell population of the epithelia in skin and mucosae (Kaufmann. 1996). In swine,

TCRγδ cells represent a major peripheral T cell subpopulation. Porcine TCRγδ cells were initially studied by differential identification (PBMC minus CD8 and CD4 and B cells). When swine immunologists did not have a monoclonal antibody (mAb) anti-TCRγδ there was a population of lymphocytes defined as null cell population. The reason being that these lymphocytes did not express CD4. CD8 or B cell markers (IgM) on their membrane. However. after a mAb specific for a conserved region of TCRγδ (PGBL22A) was developed. it was concluded that the majority of porcine null cell population was primarily made up of TCRγδ cells (Davis et al.. 1998). PGBL22A. the only mAb that detects all CD2$^+$ and CD2$^-$ γδ T cells. might recognize a highly conserved determinant on one of the three described porcine γ chains (Thome et al.. 1993. 1994) or on the δ chain (Davis et al.. 1998).

γδ T cells can express the TCRγδ alone or together with CD8αα. However. Davis et al. (1998) include in their definition of null cell population those TCRγδ cells also expressing CD8αα on their surface. I believe that this is not an accurate definition of the null cell population. The reason is that TCRγδCD8αα cells would be included in the CD8^{low} subpopulation when measuring CD4 versus CD8 therefore. they were not indeed a part of the population of cells not bearing CD4. CD8 or B cell markers on their surface. which is the definition of null cell population. Licence et al. (1995) described that γδ T cells were mainly thymus-dependent. but a small population of thymus-independent γδ T cells was also described and followed for 2 years after thymectomy.

An additional degree of complexity is added to the γδ T cell population when considering surface CD2 expression. The CD2$^+$ subpopulation is comprised of subsets that are negative or positive for CD8αα. Approximately 40 to 50% of the TCRγδCD2$^+$ T cells

coexpress CD8. whereas CD4 positive γδ T cells have not been observed (Davis et al., 1998).

The TCRγδCD2⁺ T cell subset is low in concentration in peripheral blood and high in the

spleen. suggesting that this population differs from the CD2⁻ subset in propensity for homing

and regionalization in specific tissues and lymphoid organs (Saalmuller et al., 1990). The

TCRγδCD2⁻ T cell subset is high in concentration in peripheral blood and low in

concentration in lymphoid organs (Saalmuller et al., 1990). TCRγδCD2⁻ T cells have a high

rate of turnover with minimal residency time in various lymphoid organs and tissues (Binns.

1994). In mice γδ T cells participate in antimicrobial immunity. but they perform primarily

compensatory functions in the absence of αβ T cells (Kaufmann. 1996). Accordingly. murine

γδ T cells differ from αβ T cells in their localization and kinetics rather than in their effector

functions. It would be interesting to investigate whether what occurs in porcine γδ T cell

subsets with regard to CD2 expression is actually a matter of differential localization or when

TCRγδCD2⁻ T cells reach a lymphoid organ initiate surface CD2 expression and decrease

recirculation rate.

The larger number of CD8αα recirculating lymphocytes in swine relative to mice or

humans is an important distinctive characteristic of porcine cellular immunity. This is

because several cellular subsets bear CD8αα on their surface. I am going to discuss the

characteristics of these subsets later. Now I will discuss a peripheral T cell subset which is

typical of swine. but is not found in other species. Other species express CD4 CD8 DP T

cells only in the thymus and they are immature lymphocytes. In pigs it was initially thought

that CD4CD8 DP were premature thymic emigrants. but this hypothesis was discounted

when the absence of CD1 surface expression (CD1 is expressed in thymic immature T cells)

was demonstrated on peripheral CD4CD8 DP T cells (Pescovitz et al.. 1990). Thus.

extrathymic coexpression of CD4 together with CD8 at low density (CD8$\alpha\alpha$) in swine

mature lymphocytes has been shown to identify lymphocytes which functionally can be

defined as T helper cells. and as indicated by the fact that CD4CD8 DP T cells increase with

age. they are involved in recall-type responses (Pescovitz et al.. 1994; Zuckerman and

Husmann. 1996; Summerfield et al.. 1996). Therefore. the concept of divergence in

developmental programming. used by human and mice immunologists to explain that genes

involved in the killing of target cells are activated in CD8$\alpha\beta$ T cells (CTLs). whereas various

cytokine genes are activated in effector CD4 T cells (Von Boehmer et al.. 1989). cannot be

strictly applied to swine immunology. In the future. it will be crucial for the development of

swine immunology to elucidate what advantage is provided to the porcine immune response

through the expression of CD8$\alpha\alpha$ on CD4 T cells. Are these CD4CD8 DP SLA complex

class I and SLA complex class II restricted compared to CD4 which are only SLA class II

complex restricted? At the present time we do not have an answer to that question. However.

it is known that CD4CD8DP T cells express the TCR$\alpha\beta$ and not TCR$\gamma\delta$ on their surface. and

might have developed extrathymically (Licence et al.. 1995).

Finally. another important immunological difference between swine and other animal

species is that the diversity of the variable region of the δ chain (V$_\delta$) of the TCR$\gamma\delta$ is the

greatest in pigs among all animal TCRs studied (Yang et al.. 1995). In humans and mice

because numbers of the variable (V). diversity (D) and joining (J) gene segments for TCR$\gamma\delta$

are low. rearrangements alone are insufficient for creating an equally large diversity as they

do in Ig and TCR$\alpha\beta$. However. a similarly large repertoire is achieved through the

generation of immense junctional diversity (Kaufman. 1996). That slightly decreased

diversity described in humans and mice due to low numbers of TCRγδ V. D and J genes.

could negatively influence the efficacy of an immune response in which TCRγδ cells play a

fundamental role. Maybe that fundamental role would only be compensatory functions in the

absence of αβ T cells. such as in mice. or perhaps in swine γδ T cells perform a more

important function. However if either γδ T cells are functionally more or less important in

swine. the enormous diversity of V_δ could partly overcome the genetic limitations that in

humans and mice have been imposed to the TCRγδ.

Immune Cell Phenotype and Function in Swine

CD8 Cell Population

The CD8 lymphocyte population is numerically and functionally the most important

lymphocyte subset in pigs. Both phenotypically and functionally. CD8 lymphocytes

constitute a non-homogeneous population made up of several subsets. Porcine CD8

lymphocytes are also of particular importance to this dissertation because I have shown for

the first time that the percentage and total number of porcine CD8 cells is increased in

response to dietary CLA. Phenotypic differences are potentially correlated with distinct CD8

lymphocyte subsets. There are basically five distinct lymphocyte subsets bearing the CD8

molecule on their surface in swine. and four of them bear the CD8αα homodimer. whereas

only one subset bears the CD8αβ heterodimer (TCRαβCD8αβ). A subset of CD8^{+} cells in

swine preferentially express SLA complex class II on their surface (Lunney. 1987). It is not

known why some porcine CD8^{+} cells express SLA complex class II on their surface, whereas

CD8⁻ cells in other species do not express MHC class II. Another unique feature of porcine

SLA complex is that it maps across the centromere of chromosome 7 (Smith et al.. 1995).

At the present time. porcine CD8 genes have not been cloned and sequenced.

therefore. to relate a particular CD8 phenotype to a cell subset. some inferences have to be

made. The development of a mAb specific for either an epitope of the β chain or for a new

epitope formed by the combination of the α and β porcine CD8 chains (PG164A) allowed

swine immunologists to infer that CD8^{high} lymphocytes represented the TCR$\alpha\beta$CD8$\alpha\beta$

(CTL) T cell subset (Yang and Parkhouse. 1997; Zuckermann et al.. 1998). Porcine

TCR$\alpha\beta$CD8$\alpha\beta$ cells. such as NK cells. have a cytotoxic function. TCR$\alpha\beta$CD8$\alpha\beta$ play an

important role in the adaptative. and NK in innate. cell-mediated immune responses to

viruses or other intracellular type pathogens. CTL specific activity for hog cholera virus and

African swine fever has been demonstrated in pigs recovered from infection (Martins et al..

1993; Pauly et al.. 1995).

The mechanism of action of TCR$\alpha\beta$CD8$\alpha\beta$ has been postulated in mice and humans.

There are not mechanistic studies in the pig with regard to how cytotoxicity occurs. It is

assumed. although it has not been demonstrated. that porcine CTLs have a mechanism of

action similar to human or murine CTLs. TCR$\alpha\beta$CD8$\alpha\beta$ can be functionally divided into

two subsets: Pre-CTLs and CTLs. Pre-CTLs are T cells that are committed to the CTL

lineage. have undergone thymic maturation. and are already specific for a particular foreign

antigen (Abbas et al.. 1997). Pre-CTLs will differentiate into CTLs after two signals: specific

antigen recognition of antigen on a target cell and co-stimulation which may be provided

either by co-stimulators expressed on professional APCs or by cytokines such as IL-2 and

interferon-γ (INF-γ) produced by helper cells (Kagi et al.. 1996). Differentiation from pre-CTLs to CTLs involves. among other effects such as INF-γ production or surface Fas ligand expression. the activation of genes coding for the membrane pore-forming protein called perforin and the enzymes with serine esterase activity called granzymes (Abbas et al.. 1997).

When a TCRαβ on the CTL surface recognizes a peptide/ MHC class I complex on a target cell. a tight junction formation occurs in a way similar to the formation of SMACs described earlier. Also. similarly to what occurred in SMAC organization. there is a reorientation of the cytoskeleton. but in this case the objective is to focus the release of effector molecules (granzme and perforin) at the site of contact with the target cell (Janeway et al.. 1999). Perforin will make a pore in the membrane of the target cell and granzyme will go through that pore to activate the caspase cascade. which will result in induction of apoptosis (Kagi et al.. 1994). In my research. I have measured granzyme activity as an indicator of cytotoxicity. thus I have assumed that the mechanism of cytotoxicity in mice. humans and swine is similar. There are two additional mechanisms of induction of apoptosis not involving the caspase cascade.

NK cells are cytotoxic lymphoid cells capable of killing a variety of nucleated cells without previous antigenic stimulation (Roth. 1999). Thus. NK cells are a part the innate cell-mediated immunity. whereas CTLs are a part of the adaptative cell-mediated immunity. The involvement of CTLs in the cellular response to African swine fever described above has its NK cell homonym: Evans et al. (1993) showed that swine NK cells are capable of lysing cells infected with transmissible gastroenteritis virus and pseudorabies virus. Enhancement of cytotoxicity as a result of cytokine co-stimulation. as mentioned when discussing the differentiation from pre-CTLs to CTLs, also occurs when NK cells differentiate into

lymphokine-activated killer (LAK) cells (Cho et al., 1996). Swine NK cells respond to an

interferon inducer (poly I:C), IL-2, human interferon-α (IFN-α), and human IL-1α with

enhanced NK activity (Lesnick et al., 1988; Knoblock et al., 1992; Evans et al., 1993). In

contrast to what occurrs in CTL function which is a cytokine- or/and a surface-APCs-co-

stimulator-driven phenomenon, in the case of NK cells, it would be only a cytokine-driven

co-stimulation. The disadvantage of NK cells compared to CTLs is that they are not antigen-

specific cells. The advantage is that NK cells are MHC-nonrestricted, therefore, they can kill

a target cell, even when there is a downregulation of MHC class I expression, which may

occur in certain viral infections.

Earlier, I have described the structural differences (size and granularity) between

porcine NK and other species NK cells along with the fact of being a part of the CD8

population of cells, whereas human NK cells, when discovered, were considered a part of the

null cell population (Kuby, 1997), therefore some human NK cells do not express CD8αα on

their surface. Surface expression of CD8αα alone does not indicate that a particular porcine

cell is a NK cell. The reason being that CD8αα molecules will be expressed on the surface of

some of the TCRγδ cells, also on CD4CD8 DP cells and some CD8 SP T cells. Therefore, to

relate a particular phenotype to a cell subset and to a function, additional cell surface markers

should be considered. G7 and PNK-E are two mAb that enhance porcine NK cell activity by

binding to cytolytic trigger molecules which exist as a molecular complex on porcine NK

cells (Johnson et al., 1991). The PNK-E molecule seems to be distinct from CD2 and CD16

(Johnson et al., 1991). I have shown that almost all porcine G7 and/or PNK-E positive cells

also bear CD8αα molecules on their surface in vivo.

Recently. the G7 molecule has been cloned and identified as porcine FcγRIIIAα chain

(Halloran et al.. 1994). This molecule would most likely be involved in antibody-dependent

cellular cytotoxicity (ADCC). ADCC is an immunological mechanism that serves NK cells

to overcome the problem. which is intrinsic to their non-specific nature. of how to be directed

against specific target cells. NK cells express on their membrane receptors capable of binding

the Fc region of IgG (FcγR). NK cells along with macrophages. monocytes. neutrophils. and

eosinophils use ADCC as a means to enhance their specificity (Kuby. 1997). However.

among all these cellular types. only NK cells will express both CD8αα and FcγRIIIAα

(CD16). The IgG bound to the FcγR will have antigen-specificity in the Fab region. which if

it is specific for a surface antigen presented on a target cell a cytotoxic-type of response will

occur. The molecular mechanism of NK cell cytotoxicity can be the same described above

for CTL cytotoxicity (granzyme and perforin).

There is not a definitive direct proof of the existence of porcine TCRαβCD8αα T

cells. The reason being that. in swine. a mAb for the TCRαβ has not been developed. Based

on the type of CD8 dimer expressed on the cell surface. TCRγδ⁻CD3⁺ populations. which

were inferred to be TCRαβ⁻. can be further divided into two cell subsets cells expressing

CD8^{high} (TCRαβCD8αβ) and cells expressing CD8^{low}. The CD8^{low} population will be a

CD8αα dimer-expressing cell subset. including TCRαβCD4CD8ααDP. NK (CD3⁻

G7⁺CD8αα). and potentially TCRαβCD8αα cells. Dot plots showing CD4CD8ααDP T

cells and NK cells as separate populations are available from several publications

(Zuckerman et al.. 1996; Zuckermann et al.. 1998; Johnson et al.. 1991). However, because

of the lack of available mAb anti-porcine TCRαβ. at the present time. there is no direct flow

cytometric evidence showing the porcine TCRαβCD8αα T cells subset as a separate TCRγδ⁻ CD3⁺ T cell subset. Research by Yang and Parkhouse (1996) represent the only published proof. yet indirect. of TCRαβCD8αα T cells existence in pigs. After developing a mAb anti-porcine CD3 epsilon chain (Yang et al.. 1996), they conducted a three-color flow cytometric analysis assaying for CD4. CD8 and CD3 or TCRγδ. The percentage of porcine TCRαβCD8αα T cells in PBMCs was calculated by substracting the percentage of TCRγδCD8αα in PBMCs from the percentage of CD3CD8αα in PBMCs. From these calculations. it was reported that only 3.7 ± 1.4 % of PBMC were TCRαβCD8αα T cells.

Two important porcine CD8 subsets (TCRαβCD4CD8αα DP. and TCRγδCD8αα) along with TCRγδ cells have been discussed together with other distinctive characteristics of the porcine immune system. therefore. they will not be discussed again here. Because activated effector CD4 cells are involved in cytokine production. this subset will be discussed along with some porcine cytokines important for CLA-induced immunomodulation.

B Cell Population

In swine. surface IgM expression is used as a B cell marker. Porcine B cells. such as B cells of other species. differentiate into plasma cells. the effector cells of the humoral immune system. capable of secreting antibodies. The first antibody will be IgM and after that. through isotype switching IgG. IgA and IgE will be produced. Isotype switching is regulated through interleukins produced by Th cells (CD4). Th cells located in lymph nodes and spleen favor switch to IgG. whereas Th located in mucosae (e.g. GALT and BALT) induce B cells to switch to IgA/ IgE (Roth. 1999). Sugano et al. (1998) described that. in mice. dietary CLA induces an increase in IgA. IgG and IgM. and a decrease in IgE in

lymphocytes from both spleen and MLN. In pigs it has not been reported that CLA influences humoral immunity.

Cytokines

CLA influences cytokine production through the inhibition of eicosanoid production. Also. if CLA is a PPAR ligand. it might be involved in the transcriptional inhibition of pro-inflammatory cytokine production. There are two distinct cytokine-driven events that could be regulated by CLA: cell proliferation and differentiation. and inflammatory responses. Firstly. the effects of CLA on cell proliferation and differentiation involve an increase in IL-2 production. which I have discussed earlier. Secondly. the putative decrease in inflammation caused by dietary CLA could be explained through either a decrease in pro-inflammatory cytokine production (PPAR-γ ligands decrease pro-inflammatory cytokine production). or by blocking the effects of pro-inflammatory cytokines (CLA blocks the effects of TNF-α on protein degradation (Park. 1996)) or both.

Inflammatory cytokine (IL-1. IL-6. IL-8. TNF-α. TNF-β) expression is induced principally by cell wall products of gram-negative bacteria and activators of the transcription factor NF-κB (Pastoret et al.. 1998). Inflammatory cytokines are produced by macrophages and/or T cells. and induce inflammatory responses.

The revolutionary concept of Th1-Th2 paradigm that Mosmann et al. (1986) proposed was based on mice studies. They proposed that CD4 T cells were divided into two subsets based on the type of cytokines that they produced and. therefore. the subsequent immune response derived from that distinct cytokine production. Mosmann et al. (1986) indicated that a Th1-type response will produce IFN-γ, whereas a Th2-type response will induce IL-4, IL-5 and IL-6 production. There is no definitive evidence of the existence of the paradigm in non-

murine models. therefore. if the concept is to be applied in swine. T helper responses should be defined as Th1-like and Th2-like responses.

Swine Dysentery

Swine dysentery (SD) is an inflammatory. mucohemorrhagic diarrheal disease that affects pigs during the growing-finishing period caused by *Brachyspira hyodysenteriae* (Harris et al.. 1999). It has been estimated to cause about 180 million dollars of financial loss annually (Joens. 1990). These losses can be attributed to three main reasons: mortality. decreased growth. and the cost of antibiotic therapy. Antimicrobials have been used at sub-therapeutical levels in the diet to prevent SD along with other bacterial diseases. However. restrictions to the use of antimicrobials were imposed in the European Union and probably. in the future. similar restrictions will be adopted by the United States. Therefore. antimicrobial-free prevention of infectious inflammatory diseases such as SD should be approached differently. A possible therapeutical approach is to induce a type of immune response in the animal that is prone to decrease inflammation which would be favorable to the prevention or to the resolution of the disease. That antimicrobial-free prevention strategy could be achieved by using effective vaccination protocols and/or natural dietary immunomodulators capable of inducing the desired type of immune response.

Based on the similar effects elicited by dietary CLA (Bassaganya-Riera. et al.. 1998; Bassaganya-Riera et al.. 1999) and a SD vaccine (Waters et al.. 1999; Waters et al.. 2000) in increasing the percentage of porcine peripheral CD8$^+$ cells. an experiment with the purpose to measure the effects of CLA in the efficacy of a SD vaccine was included in this dissertation. Waters et al.. (2000) showed that the increase in percentages of CD8$\alpha\alpha^+$ cells. possibly driven by cytokines produced by CD4$^+$ cells. corresponded with the onset of

resolution of SD infection in pigs. Therefore. immunomodulatory strategies capable of increasing $CD8\alpha\alpha^-$ cells might contribute to prevent SD.

Although the mechanism by which CLA or the SD vaccine induce the increase in porcine peripheral blood lymphocytes phenotype is not known. a possibility is that both. in different ways. are modulating cytokine expression. The proposed role of CLA as a PPAR ligand. along with interesting discoveries suggesting novel therapies for human inflammatory bowel disease (IBD). could open the door to new applications for dietary CLA.

Because many enteric inflammatory diseases. such as SD. display similarities in their pathogenesis. SD is a good model to study the impact of other enteric inflammatory diseases in gastrointestinal health and function. For instance. clinically. SD resembles IBD. a chronic debilitating disease that affects millions of people worldwide. Recent research on IBD conducted in cytokine-knockout mice models (Elson et al.. 1995) indicates that this inflammatory disease is the result of loosing the balance that maintains the intestinal mucosa in a quiescent state of inflammation. Intestinal epithelium is the interface between a highly antigenic luminal environment and the mucosal immune system (Su et al.. 1999). Interestingly. enterocytes are not simple passive players in the regulation of the interactions between the intestinal lumen and the mucosa. They have been shown to express various cytokine receptors (Reinecker et al.. 1995). and cytokines and complement factors (Andoh et al.. 1993: Williams. 1998) that could potentially influence the maintenance of the quiescent state of inflammation.

Su et al. (1999) proposed a novel therapy for IBD-induced colitis that consists of the utilization of PPAR-γ ligands to inhibit the epithelial inflammatory response. They showed that PPAR-γ not only regulates growth and differentiation but also regulates immune

responsiveness by interfering with NF-κB activation and subsequently with the ability of colonocytes to express immunomodulatory cytokines. They mentioned a series of PPAR-γ ligands that could be used as therapeutic agents. Amongst these PPAR-γ ligands they used a new class of oral antidiabetic agents called thiazolidinediones (TZDs) to test their hypothesis. The result was that TZDs markedly reduced colonic inflammation in a mouse model of IBD. An additional advantage of using PPAR-γ ligands in prevention and therapy of intestinal inflammatory disease would be derived from inhibiting the activation of NF-κB induced by bacterial lipoproteins (BLPs) which play an important role in the initiation of inflammation (Aliprantis et al.. 1999).

As I am going to further elaborate later. currently. there is enough information in the literature to infer that CLA is a PPAR-γ ligand. Therefore. based on the work conducted by Su et al. (1999) it is proposed that dietary CLA could be utilized as a novel therapeutical and/or preventive approach for enteric inflammatory diseases such us SD and IBD.

Approach to the Molecular Basis of Dietary Fatty Acid-induced Immunomodulation

As mentioned earlier. the third phase of fatty acid research is the phase with the strongest molecular basis. The origin of that molecular basis is attributed to a group of nuclear receptors. which were initially lacking known ligands (Kastner et al.. 1995). called orphan receptors. When nature spends energy in creating something and evolution does not eliminate that process. it is because it must have a function. It is likely that orphan receptors not only interact with hormones. but they might represent the missing link between nutrients and genes.

Nuclear receptors are ligand-inducible transcriptional regulators involved in embryonic development, cell differentiation, homeostasis, and are implicated in cancer, diabetes or hormone resistance syndromes (Gronemeyer and Laudet, 1997). Dietary components are often competent to interact with nuclear receptors and to activate their gene regulatory networks (Gustafsson et al., 1998). PUFAs (e.g., linoleic, linolenic and AA), monounsaturated fatty acids (e.g., oleic and elaidic), saturated fatty acids (e.g., palmitic and stearic), PGs and LTs interact with the orphan receptor peroxisome proliferator-activated receptor (PPAR) (Gottlicher et al., 1992; Gustafsson et al., 1998). PPARs belong to the superfamily of hormone receptors (e.g., steriod hormones, retinoic acid, vitamin D, and thyroid hormones represent other compounds eliciting their actions on other members of that superfamily) (Wolf, 1998). All members of this family have in common: a DNA-binding domain, a gene-activating domain and a ligand-binding domain. When the ligand binds to the receptor, the receptor can interact with DNA and thereby activate or deactivate specific genes (Wolf, 1998). Many genes harbor a peroxisome-proliferator-receptor element (PPRE) sequence. They are two sites consisting of the nucleotide sequence AGGTCA separated by a single base pair which bind a PPAR/RXR dimer (RXR is the retinoid X receptor) (Wolf, 1998; Desvergne et al., 1995; Semba, 1998). This information provided at the molecular level indicates that a potential for interaction between vitamin A and CLA exists.

Houseknecht et al. (1998) showed that dietary CLA is associated with elevated levels of a number of genes that are responsive, in part, to PPAR-γ. Activation of PPAR-γ by PPAR-γ ligands has been linked to decreased circulating glucose levels and improved insulin action in animals and humans (Saltiel et al., 1996). Furthermore, PPAR-γ1 protein expression in WBC undergoes a dynamic change with endotoxin challenge (Leininger et al., 1999),

which indicates that PPAR-γ1 may play an important role in the development of an immune response to lipopolysaccharide (LPS).

Also. CLA has structural and physiological characteristics similar to peroxisome proliferators (Su et al.. 1999). Peroxisome proliferators are compounds that cause an increase in the number and/or the size of liver peroxisomes. Peroxisomes are cytoplasmic organelles surrounded by a lipid membrane which contain a group of enzymes active in lipid catabolism (Desvergne et al.. 1995). PPARs are ligand-activated transcription factors that affect gene expression. Three [PPAR-α. PPAR-β (also called PPAR-δ. FAAR or NUCI). and PPAR-γ] subtypes of PPARs have been described (Grindflek et al.. 1998). Because orphan receptors sometimes cannot be described by their function the existence of several genes with the same name is an important problem. Recently. with the purpose to solve that problem. a unified nomenclature system for the nuclear receptor superfamily was developed. Based on that system NR1C1. NR1C2 and NR1C3 represent PPAR-α. PPAR-β and PPAR-γ. respectively. CLA is able to activate PPARs while not being a peroxisome proliferator (Moya-Camarena et al.. 1999). PPAR-γ is expressed at high levels on colonic cells and regulates growth and differentiation (Su et al.. 1999). inhibits production of monocyte-derived inflammatory cytokines (Jiang et al.. 1998). and modulates the activities of transcription factors including AP-1. STAT. and NF-κB (Ricote et al.. 1998). These transcription activators are involved in the transcriptional regulation of inflammatory cytokine expression (e.g.. IL-1. IL-6. and TNF-α) which can induce a decrease in growth and feed intake. Therefore. compounds capable of decreasing the expression of inflammatory cytokines could provide health benefits in diseases where inflammatory cytokine production

contributes to the pathogenicity of the disease.

PPAR-γ elicits other important biological effects. Rosen et al. (1999) described that PPAR-γ is required for differentiation of adipose tissue in vitro and in vivo. PPAR-γ is abundantly expressed in both white and brown adipose tissue and is induced before activation of most adipose-specific genes (Kliewer et al., 1994). Mutations of PPAR-γ that destroy receptor function have been found in sporadic colon cancer (Mansen et al., 1996; Brockman et al., 1998; Elstner et al., 1998; Mueller et al., 1998; Sarraf et al., 1998, 1999; Tonotoz et al., 1998). PPAR-γ is also required for placental and cardiac development. Its deficiency interferes with terminal differentiation of the trophoblast and placental vascularization, leading to severe myocardial thining and death (Barak et al., 1999).

The porcine PPAR-γ gene encodes a 1.8 kb mRNA transcript and shares 99, 96 and 97% amino acid sequence identity to the human, mouse and cow PPAR-γ molecule (Houseknecht et al., 1998), respectively. In swine, two isoforms derived from alternate promoter usage and differential splicing of PPAR-γ, γ1 (spleen) and γ2 (adipose tissue), have been characterized (Grindflek et al., 1998). Spurlock et al. (1999) reported that PPAR-γ2 mRNA was increased 4-fold in pigs by soybean oil compared to a non-added fat diet. PPAR-γ1 isoform is expressed in lymphocytes. Thus, dietary CLA, as well as other dietary fatty acids, could modulate the transcription of cytokine mRNA, which inhibits inflammatory responses and enhances lymphocyte proliferation. Both linoleic acid and CLA should be able to bind PPARs (Gottlicher et al., 1992; Houseknecht et al. 1998; Gustafsson et al., 1998). However, the effects derived from linoleic acid binding are distinct from those derived from CLA-binding. When CLA is the PPAR ligand there is no peroxisome proliferation, whereas

it occurs when other fatty acids bind to PPAR (Houseknecht et al. 1998). The environment in which ligation and activation occurs should be an important element in defining the final outcome. PPAR binding in a context of high eicosanoid levels (high dietary linoleic acid) might have a different outcome than binding in a CLA-derived low eicosanoid environment.

Although not included as an objective for this dissertation. the study of CLA at the molecular level should be included as a recommendation for any future research on CLA and immune function. A possible application of that type of research would be to identify the anti-inflammatory properties of CLA. After proving that CLA is an anti-inflammatory compound on the whole animal. claims regarding the use of CLA as a natural product that enhances gastrointestinal health and function could be made.

Possible Interaction Between Vitamin A and CLA

To look at the interaction between CLA and vitamin A is not the purpose of this dissertation. However. there is enough molecular basis to suggest that interactions between the compounds may occur. Both vitamin A (Table 3) and CLA elicit important immunomodulatory effects. which in the case of vitamin A have already been reported in several NRC publications. However most of these NRC reviews focussed exclusively in general aspects of the maintenance of epithelial integrity and not on immune cell phenotype and function or immune response.

In 1915. McCollum and Davis described that fat soluble A (vitamin A was a component of fat-soluble A). had growth promoting activity. Drummond (1920) suggested the designation as vitamin A. Green and Mellanby (1928) described vitamin A as the 'anti-infective' vitamin. Since that phenomena was described. science has provided additional information regarding sources of vitamin A, absorption. metabolism. toxicity, existence of

Table 3. Immune response functions that could be regulated by retinoids

Element	References
Natural killer cell function	Zhao et al., 1994
T cell function	Rahman et al., 1997
IL-2 production	Colizzi et al., 1985
Monocyte/ macrophage function	Szabo et al., 1994
TGF-β production	Fisher et al., 1992; Szabo et al., 1994
Phospholipase A_2 production	Hope et al., 1990
Cytotoxic T lymphocyte function	Malkovsky et al., 1983; Sijtsma et al., 1990

Adapted from Semba, 1998.

intracellular binding proteins. and its pleiotropic effects of vitamin A derivatives (on vision, reproduction. embryonic development. growth, keratinization. enzyme activity and immune function). which are transduced by two families of nuclear receptors. With the exception of vision. where retinal and retinol are the derivatives used. retinoic acid has been shown to be the active vitamin A derivative (Ghyselinck et al., 1999).

Two families of nuclear receptors. all-trans retinoic acid receptors (RARs) and 9-cis retinoic acid receptors (RXRs) have been identified. which consist of three isotypes (α, β and γ) that control gene expression by retinoid signals (Chambon. 1996). RARs and RXRs form RAR/RXR heterodimers and RXR/RXR homodimers and bind to retinoic acid response elements (RAREs) and retinoid X receptor response elements (RXRE) of target genes, respectively. As indicated earlier. a consensus sequence 5'-AGGTCA-3' is the core

recognition element for the entire family of nearly 150 non-steroid nuclear receptors to which

RXR. PPAR and RAR belong (Mangelsdorf and Evans. 1995). Interestingly, all RXR

heterodimers preferentially bind response elements composed of two AGGTCA sites

arranged in a direct repeat configuration with characteristic inter-half-site spacings of 1–5 bp

called DR1–DR5 (Mangelsdorf and Evans. 1995). RXR/RXR may inhibit the formation of

heterodimers (Pfahl et al.. 1996). RXRs may also form heterodimers with the thyroid

hormone receptor (TR). vitamin D_3 receptor (VDR). nerve growth factor induced-B (NGFI-

B) receptor. PPARs and other orphan receptors (Semba. 1998).

Table 4. PPAR and retinoid receptors

Item	PPAR	Retinoid receptors
Nuclear receptor	PPAR	RAR / RXR
Receptor subtypes	alpha. beta and gamma	alpha. beta and gamma
Dimers formed	RXR / PPAR	RAR /RXR. RXR / RXR
Region in the DNA	PPRE	RARE and RXRE
Ligand-binding region	Fatty acids. PGs. LTs	all-trans/ 9-cis retinoic

Adapted from Semba. 1998 and Wolf. 1998.

The core sequence of the DNA-binding domain (DBD) is highly conserved in the

nuclear receptor superfamily. This sequence is comprised of two zinc-nucleated modules and

two α-helices that fold into a single globular domain (Rastinejad et al.. 2000). Gronemeyer

and Moras (1995) raised a fundamental question critical for the molecular and functional

understanding of nuclear receptors: Considering that DBD and its core binding sequence are

highly conserved among different nuclear receptors. how are distinct signalling pathways created in this transcription factor superfamily?

PPAR-RXR heterodimers serve as the core of signaling units (Barak et al., 1999). Mukherjee et al. (1997) showed that RXR-specific ligands were capable of promoting typical PPAR-γ activities. such as macrophage differentiation. in cultured cells alone and in synergy with PPAR-γ ligands. Knock-out mice studies showed that RXR deficiency phenotypes arise primarily from a failure to execute PPAR-γ functions (Barak et al.. 1999). Elstner et al. (1998) showed that ligands for peroxisome proliferator-activated receptor gamma and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells in vitro and in mice. RARs and RXR may interact with multiple transcriptional mediators and/or corepressors. adding an enormous level of complexity to regulation of retinoic acid responses (Semba. 1998). This complexity would become greater when retinoic acid responses were investigated with the addition of other variables such as PPARs (dietary fatty acids) or other orphan receptors.

Thus. the molecular interaction between CLA and vitamin A could provide a stronger dietary immunomodulation than when CLA is fed alone. Vitamin A deficiency impairs CD8 cytotoxic T lymphocyte activity in chickens infected with Newcastle virus (Sijtsma et al.. 1990). CLA increases the percentage of CD8 T cells in pigs (Bassaganya-Riera et al.. 1998; Bassaganya-Riera et al.. 1999). The fact that both CLA and vitamin A have an impact on CD8 T cell subsets indicates that this molecular relationship might have biological synergy in the whole animal.

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CHAPTER 2. EFFECTS OF DIETARY CONJUGATED LINOLEIC ACID (CLA) IN NURSERY PIGS OF DIRTY AND CLEAN ENVIRONMENTS ON GROWTH, EMPTY BODY COMPOSITION AND IMMUNE COMPETENCE

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Abstract

Early-weaned pigs (n=64) averaging 5.3 ± .3 kg and distributed into two environments (dirty and clean) were used to evaluate effects of CLA on growth performance. immune competence and empty body composition. A factorial (2 × 4) arrangement within a split-plot design with four littermate pigs as the experimental unit for the environment. pig within litter as the experimental unit for dietary treatment. and d 0 body weight used as a covariate were used in data analysis. Diets were formulated to contain CLA at 0. .67. 1.33 or 2% and to exceed NRC (1988) nutrient needs of pigs. and were self-fed for 7 wk in three phases (I. 1 to 2: II. 3 to 5: and III. 6 to 7 wk). Within phases. diets were isocaloric and isonitrogenous. In phase I as CLA concentration increased. ADFI decreased (P<.05) linearly. In phase II. ADG increased quadratically (603. 623. 622. and 548 g/d: P<.01). ADFI decreased linearly (873. 840. 867. and 717 g/d: P<.02) and gain:feed increased linearly (691. 742. 715. and 763: P<.07). In phase III. no differences in growth performance were attributed to dietary treatments. Pigs in the clean room had a greater cumulative ADG (P<.01) and a greater ADFI

(P<.01) than pigs in the dirty room. The greater PUN observed in clean pigs on d 14 (P<.03) was attributed to greater protein intake associated with a greater ADFI. of pigs in the clean room. On d 42. dietary CLA induced an increase in percentages of $CD8^+$ lymphocytes (21.7. 22.3. 28.0. and 32.7 %: P<.001). AGP levels were used as indirect indicators of cytokine-driven responses. and showed a linear increase on d 14 (P<.02). 28 (P<.01) and 42 (P<.01) as dietary CLA increased. On d 42. three pigs per treatment per room were killed. ground. sampled and protein. fat and water content were measured. As dietary CLA increased, a linear decrease in fat (10.8. 10.5. 9.8. and 8.6 %: P<.07) was observed. These data suggest that dietary CLA supplementation modulates porcine $CD8^+$ immune cell phenotype. improves feed efficiency and decreases body fat.

Introduction

CLA is a mixture of positional (9.11: 10.12: or 11.13) and geometric (cis or trans) isomers of conjugated octadecadienoic (18:2) acid with broad biological activities. CLA is anticarcinogenic (Ha et al.. 1989: Ha et al.. 1990: Pariza. 1991:Shultz et al.. 1992a: Shultz et al.. 1992b: Ip et al.. 1994a: Ip et al.. 1994b: Ip et al.. 1994c: Ip et al.. 1997: and Liu et al.. 1997). antidiabetic (Houseknecht et al.. 1998). antiatherogenic (Nicolosi et al.. 1993: Lee et al.. 1994) and immunomodulatory (Cook et al.. 1993: Miller et al.. 1994: Wong et al.. 1997: Chew et al.. 1997: Bassaganya-Riera et al.. 1998: Sugano et al.. 1998: Bassaganya-Riera et al.. 1999: and Hayek et al.. 1999). CLA is a natural product that enhances immune function while decreasing. at the same time. the negative effects of inflammatory responses. In the European Union. the use of antimicrobials at subtherapeutical levels has been prohibited. In the United States. dietary immunomodulation by using natural products might become a

strategy adopted by producers as a means to maintain their competitivity after antibiotic withdrawal from livestock dietary formulations.

CLA diminishes both the catabolic response and the decreased growth associated with an LPS-induced inflammatory response in chickens and mice (Cook et al.. 1993. and Miller et al.. 1994). Thus. the effects of CLA on growth seem to be linked to changes in immune or inflammatory profiles. We hypothesized that CLA would enhance cellular immunity. and that CLA effects on growth performance and body composition would be influenced by the environment. The objectives of this experiment were to determine how the health status of the pig influences the response to dietary CLA supplementation on growth, immune response and body composition.

Materials and Methods

Dietary and Management Treatments

Sixty-four 14-d-old early-weaned pigs averaging 5.3 ± .3 kg were randomly distributed from outcome groups based on litter and body weight to eight blocks of four contiguous individual pens within environment (dirty or clean). In each environment. 0. .67. 1.33 or 2% CLA-60 isocaloric and isonitrogenous diets were randomly allotted to pens within block. Diets were formulated to exceed NRC (1988) nutrient needs of pigs (Table 1), and were self-fed for 7 wk in three phases (I, 1 to 2: II, 3 to 5: and III, 6 to 7 wk). Two distinct management schemes were used to create the two experimental environments. To create the dirty environment. older pigs were introduced into the experimental room 1 wk before starting the experiment and no biosecurity rules were applied to this room. To create the clean environment. pigs were injected with a third generation cephalosporin (Naxcel; Upjohn,

Kalamazoo. MI) every other day from the farrowing date until d 0 of the experiment, and biosecurity rules were applied to the clean room during the entire experimental period.

Data and Sample Collection

Pigs were weighed and feed intakes were measured weekly to determine ADG. ADFI and gain : feed ratio (G:F). Peripheral blood was obtained through vena cava puncture on d 0, 14, 28 and 42 and collected in 10 ml heparanized evacuated tubes (Monoject, St Louis, MO). White blood cells in peripheral blood were counted with a Coulter Particle Counter (Beckman Coulter Corp., Miami, FL) after erythrocyte lysis with whole blood lysis reagents (Coulter Immunology, Hialeah, FL). Plasma was separated after centrifugation at 800 × g for 20 min, and stored at -20° C until it was analyzed for α1-acyl glycoprotein (AGP) with a radial immunodiffusion assay and plasma urea nitrogen (PUN) using the colorimetric automated technique described by Marsh et al. (1965).

Table 1. Dietary composition (as-fed basis).

Ingredient, %	Control diets		
	Phase. I[a]	Phase. II[a]	Phase. III[a]
Corn	31.57	50.21	62.18
Soybean meal (48%)	12.00	21.16	31.16
Dried whey	22.00	16.00	—
Spray-dried plasma	7.50	4.00	—
Dried blood cells	—	2.00	—
Dry skim milk	21.00	—	—
CLA-60[b]	—	—	—
Soy oil	3.33	3.33	3.33
DL-Methionine	.18	.22	.09
Sodium chloride	.25	.25	.25

Table 1. (continued)

Dicalcium phosphate	1.09	1.72	1.95
Calcium carbonate	.78	.81	.74
Vitamin premix[c]	.20	.20	.20
Trace mineral[d]	.05	.05	.05
Selenium premix	.05	.05	.05
Nutrient composition, %			
Crude protein	24.18	21.20	19.74
Lysine	1.90	1.66	1.40
Methionine+ cystine	.96	.85	.71
Calcium	1.05	.90	.81
Phosphorus	.87	.79	.73
Ca:P ratio	1.21	1.14	1.11
ME kcal/kg	3725	3737	3804

[a]Phase I. wk 1–2: Phase II. wk 3–5: Phase III. wk 6– 7.

[b]Expressed as CLA-60: 1.12. 2.21. and 3.33 representing .67. 1.33 and 2% CLA experimental diets. respectively. CLA-60 contains a 60% CLA in an approximate 50: 50 mixture of c9. t11/t9. c11 and c10. t12/ t10. c12.

[c]Supplied 4,409 IU vitamin A: 1,102 IU vitamin D_3: 22 IU vitamin E: 6.6 mg riboflavin: 17.6 mg pantothenic acid: 33 mg niacin: and 22 µg vitamin B_{12} per kilogram of diet.

[d]Supplied 165 ppm Zn: 193 ppm Fe: 66 ppm Mn: 19.29 ppm Cu: and .2 ppm Iodine per kilogram of diet.

Lymphocyte Blastogenesis Assay

Complete medium was prepared by supplementing RPMI 1640 with 25mM HEPES buffer (Sigma. Saint Louis. MI), 100 units/ml penicillin (Sigma). .1 mg/ml streptomycin (Sigma). 5×10^{-5} M 2-mercaptoethanol (Sigma). 1 mM essential amino acids (Mediathech Inc.). 1 mM non-essential amino acids (Sigma). 2 mM L-glutamine (Sigma). 1 mM sodium pyruvate (Sigma) and 10% FBS. Media pH were measured with a pH meter (Orion Research

Inc.. Beverly. MA) and brought to 7.4 with addition of a solution of 7.5% sodium bicarbonate (Fisher Scientific. Pittsburgh. PA).

Wells of 96-well flat-bottomed microtiter plates (Falcon 3072: Becton Dickinson. Lincoln Park. NJ) were seeded with 100 µl of whole blood and 100 µl of either media alone (non-stimulated wells) or media containing concanavalin A (Con-A) (5 µg/ml) (Sigma). Plates were incubated for 5 d at 37° C in 5% CO_2 humidified atmosphere. After 5 d of culture. 0.5 µCi of methyl-$[^3H]$ 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 incubated for an additional 20 h. Well contents were harvested onto fiber filters with a PHD cell harvester (Cambridge Technology. Cambridge. MA) and incorporated radioactivity measured by liquid scintillation counting (Hewlett-Packard. Meriden. CT). Samples were run in triplicate and stimulation indices (SI) calculated by dividing counts min^{-1} (cpm) of stimulated wells by counts min^{-1} from non-stimulated wells.

Preparation of Blood for Flow Cytometric Analysis of CD4 and CD8α Surface Expression

Lymphocytes were analyzed for expression of CD4 and CD8α cell surface antigens by flow cytometry. A volume of 10 µl of whole blood was added into a round-bottomed microtiter plate (Becton Dickinson. Lincoln Park. NJ) and stained with 15 µl of primary antibody dilution in phosphate buffered saline (PBS) (1:50): IgG2b mouse anti-swine-CD4 PE-labeled (74-12-4). IgG2a mouse anti-swine-CD8α FITC-labeled (76-2-11) (hybridoma supernatants kindly provided by Dr. Joan K. Lunney: Beltsville. MD). and appropriate isotype control antibodies: mouse IgG2a-Quantum Red (Sigma) for anti-CD8α and mouse IgG2b-PE (Sigma) for anti-CD4. After a 30-min incubation. cells were washed twice with PBS and

centrifuged 8 min at 200 × g in a Beckman TJ-6 centrifuge (Beckman Coulter Corp., Miami,

FL). Cells were washed with 100 µl of lysing solution dilution in PBS (1:25) (Immuno-lyse;

Beckman Coulter Corp.) and 10 µl of fixative solution (Fixative liquid reagent: Coulter

Corp.) was added for a 2-min period. Finally, cells were washed twice and brought to a final

volume of 500 µl. A two-color flow cytometric analysis with a Coulter XL (Beckman Coulter

Corp.) was conducted on lymphocytes.

Measurement of Plasma Porcine α1-acyl glycoprotein (AGP) Concentrations

AGP levels were measured with radial immunodiffusion test according to

manufacturer instructions (Cardiotech Services Inc., Louisville. KY). Briefly, a standard

curve (250. 500. 750 and 1000 µg AGP/ ml) was prepared with appropriate dilution of

standard solution A (1000 µg AGP/ ml) or standard solution B (250 µg AGP/ ml) both

provided in the measurement kit. A volume of 5 µl of each sample was added into one of the

10 wells contained in each of the test plates. The cover was firmly secured, and incubated in a

humidified atmosphere at 37° C for a 24-h period. After 24 h. a plastic scale provided with the

kit was used to measure the external diameter of each ring of precipitation. The diameters of

the samples were converted into AGP concentration values based on the standard curve.

Measurement of Empty Body Composition

A total of 24 pigs were killed and necropsied at the end of the experiment to assess

empty body composition. Gastrointestinal contents were removed: the empty body of the pig

was sawed into pieces and ground once through a 12.5 mm plate and once through a 6.5-mm

plate of a large-size grinder. After grinding, the ground tissue was mixed by hand to

maximize homogeneity. Two 200-g samples of ground tissue were obtained. Water content of

the empty body was determined by freeze-drying samples to a constant weight. Tissue

samples were then dried in a 100° C oven. Protein was determined with the Kjeldahl method

(AOAC. 1990). Fat was determined using an ether extraction procedure described by AOAC

(1990).

Experimental Design

The experiment was designed as a split-plot design with pig within block being the

experimental unit for dietary treatment (sub-plot) and eight blocks of four littermate pigs

within room being the experimental units for environmental treatment (whole plot).

Statistical Analysis

Data were analyzed as a 2 × 4 factorial arrangement of treatments within the split-plot

design using the general linear model (GLM) procedure of SAS (1988) (SAS Institute Inc..

Cary. NC). ANOVA and linear and quadratic contrasts were analyzed. Significance was

assessed at P <.05.

Results

Growth Performance

An interaction (P<.01) was described between environment and dietary treatment only

on phase I. In the dirty room. pigs fed CLA had lower ADG and ADFI than control pigs but

in the clean room that decrease was not observed. In phase II. as dietary CLA increased. ADG

increased and then decreased quadratically (603. 623. 622. and 548 g/d: P<.01), ADFI

decreased linearly (873. 840. 867. and 717 g/d: P<.02) and gain:feed increased linearly (691.

742. 715. and 763: P<.07). In phase III. no differences in growth performance were attributed

to dietary treatments. Through the entire experiment. differences in ADG and ADFI were

attributed to the environment. Pigs in the clean room grew faster and ate more than did pigs in

the dirty room. Pigs in the clean environment had a greater phase I (P<.0001), phase II

(P<.0001) and cumulative (P<.01) ADG than pigs in the dirty environment. Also, ADFI in

phases I (P<.001), II (P<.005) and cumulative (P<.01) were greater in pigs exposed to a clean

environment than in pigs exposed to a dirty environment (Table 2).

Table 2. Effect of CLA and environment on growth performance of weanling pigs[a,b].

		Environmental treatments									P value[c]	
		Dirty				Clean						
Item	phase[d] CLA,%	0	.67	1.33	2	0	.67	1.33	2	SEM[e]	L	Q
ADG, g	I	245	158	197	168	275	286	342	224	19	.02	.21
	II	576	593	558	473	630	653	685	623	26	.05	.01
	III	764	735	767	740	789	764	810	820	24	.54	.60
	Cum.	535	509	514	462	575	580	617	565	15	.04	.06
ADFI, g	I	296	197	228	216	341	325	419	275	24	.05	.56
	II	835	795	799	625	910	885	935	809	55	.02	.15
	III	1295	1227	1289	1213	1249	1190	1319	1221	47	.65	.73
	Cum.	810	745	772	616	849	820	892	804	42	.04	.25
G:F	I	828	802	864	778	806	880	816	815	74	.49	.96
	II	690	746	698	757	692	738	733	770	117	.07	.65
	III	590	599	595	610	632	642	614	672	47	.32	.36
	Cum.	660	683	666	750	677	707	692	703	124	.11	.43

[a]Least squares means.

[b]An environment × diet interaction was described on phase I for ADG (P<.01) and ADFI (P<.01).

[c]P-value of a linear (L) and quadratic (Q) effect of dietary treatment.

[d]Phase I, wk 1 to 2; ph II, wk 3 to 5; ph III, wk 6 to 7; and cumulative (cum), wk 0 to 7.

[e]Subplot SEM.

Lymphocyte Phenotype and Function

On d 42. as the level of CLA in the diet increased. a linear increase in percentages of lymphocyte subsets contained in the CD4⁻CD8⁺ lymphocyte population (P<.001) and in TCRαβCD4⁻CD8αα T lymphocytes (P<.06) were observed. Also. TCRαβCD4⁺ (P<.02) T cells percentage decreased linearly as the CLA content was augmented. although numerically the decrease in CD4⁻ lymphocytes was not as large as the increase in CD8⁺ lymphocytes (Table 3).

Table 3. Effect of CLA and environment on porcine lymphocyte subsets phenotype (% of total lymphocytes)[ab].

| | | Environmental treatments[c] | | | | | | | | | P value[d] | |
| | | Dirty | | | | Clean | | | | | | |
Phenotype	CLA,%	0	.67	1.33	2	0	.67	1.33	2	SEM[e]	L	Q
TCRαβCD4⁻		27.0	27.1	27.4	24.1	27.5	24.9	24.6	21.3	1.70	.02	.41
TCRαβCD4⁻CD8αα		7.2	8.9	9.7	11.8	6.9	7.0	10.4	9.1	1.69	.06	.83
CD4⁻CD8⁺ subsets[f]		21.9	23.1	24.3	29.0	21.6	21.4	31.7	36.3	1.80	.0001	.13
CD4⁻CD8⁻[g]		45.4	43.1	37.3	33.4	43.6	47.6	31.9	31.9	2.40	.0001	.37

[a]Least squares means.

[b]An environment × diet interaction (P<.03) was described on CD4⁻CD8⁺ subsets.

[c]Pigs in the clean room had greater percentages (P<.02) of CD4⁻CD8⁺ subsets than dirty room pigs.

[d]P-value of a linear (L) and quadratic (Q) effect of dietary treatment.

[e]Subplot SEM.

[f]The CD4⁻CD8⁻ cell subset includes TCRγδCD8αα. CD3⁻CD16⁻CD8αα (NK cells), TCRαβCD8αβ. and putatively TCRαβCD8αα T cells.

[g]This lymphocyte fraction includes B cells and TCRγδCD8αα⁻ lymphocytes

An environment × diet interaction (P<.03) was described on CD4⁻CD8⁺ subsets which indicated that the CLA-induced increase in percentages of CD4⁻CD8⁺ cells was greater in pigs found in the clean room. Also, pigs in the clean room had greater percentages (P<.02) of CD4⁻CD8⁺ subsets than did pigs in the dirty room. No differences caused by dietary CLA supplementation were found in cells proliferating in response to Con-A addition into the media (data not shown). However, in the non-stimulated (non-stim) wells (media alone) pigs fed CLA had a greater proliferative response, measured as methyl-[^{3}H] thymidine incorporation, than pigs fed a control diet on d 14 (P<.04 L) and 28 (P<.07 Q) (Table 4). On d 42, proliferation of cells from pigs in the dirty environment was greater (P<.01) than in cells obtained from clean environment pigs.

Table 4. Effect of CLA on the porcine lymphocyte proliferation[ab] (cpm in non-stimulated wells[c]).

| | | Environmental treatments[d] | | | | | | | | | P value[e] | |
| | | Dirty | | | | Clean | | | | | | |
Day	CLA, %	0	.67	1.33	2	0	.67	1.33	2	SEM[f]	L	Q
14		742	1054	1471	2041	947	600	1518	916	353.1	.04	.99
28		1025	1792	2135	1091	1185	1249	2052	1356	481.1	.57	.07
42		881	523	801	604	315	632	437	357	146.6	.53	.58

[a]Least squares means.

[b]No environment × diet interaction.

[c]Methyl-[^{3}H] thymidine incorporation in proliferating lymphocytes.

[d]On d 42, lymphocyte proliferation of pigs in the dirty environment was greater (P<.01) than that of pigs located in the clean environment.

[e]P-value of a linear (L) and quadratic (Q) effect of dietary treatment.

[f]Subplot SEM.

On d 14 and 42. a trend towards an increase in white blood cell (WBC) counts. which

included lymphocytes. neutrophils. eosinophils. basophils and monocytes, as dietary CLA

increased was found (P<.09 Q) and (P<.09 L). respectively. regardless of the room (Table 5).

That increase was probably caused by increased numbers of lymphocytes.

Table 5. Effects of CLA on WBC counts[a][b].

Day	CLA, %	Environmental treatments[c]								SEM[e]	P value[d]	
		Dirty				Clean					L	Q
		0	.67	1.33	2	0	.67	1.33	2			
14		38.0	42.1	38.3	32.6	14.8	18.1	13.9	16.3	2.31	.17	.09
28		26.6	28.9	24.0	21.6	23.6	25.1	20.7	31.0	3.37	.92	.67
42		19.6	24.5	22.9	17.4	45.3	36.4	41.3	35.8	2.84	.09	.40

[a]Least squares means.

[b]No environment × diet interaction.

[c]On d 14. dirty room pigs had greater WBC counts than clean room pigs (P<.0001) and. on d 42. clean room pigs had greater WBC counts (P<.0001) than dirty room pigs.

[d]P-value of a linear (L) and quadratic (Q) effect of dietary treatment.

[e]Subplot SEM.

Empty Body Composition

Empty body composition analyses of killed pigs indicated that pigs fed CLA had a

trend towards lower percentages of body fat (P<.07) (Table 6) than control animals did. The

percentage of fat in pigs from the clean room was greater (P<.009) than that of pigs in the

dirty room. An environment × diet interaction for water content (P<.02) suggested that. in the

dirty room. pigs fed CLA had greater water content than pigs fed CLA in the clean room. Pigs

in the dirty room had greater water percentages (P<.005) than pigs in the clean room.

Table 6. Effects of CLA and environment on empty body composition, %[a][b].

Item,	CLA, %	Dirty				Clean				SEM[e]	P value[d]	
		0	.67	1.33	2	0	.67	1.33	2		L	Q
Protein		16.1	15.8	17.0	15.1	16.4	19.2	15.8	16.3	.90	.52	.33
Fat		9.7	8.4	10.0	6.8	11.9	12.5	9.6	10.4	.70	.07	.60
Water		74.5	74.9	71.5	75.6	70.4	70.4	74.5	71.8	.60	.20	.67

[a]Least squares means.

[b]An environment × diet interaction (P<.02) for fat content was described.

[c]Fat content was greater (P<.009) in pigs of the dirty room.

[d]P-value of a linear (L) and quadratic (Q) effect of dietary treatment.

[e]Subplot SEM.

Plasma AGP Concentration

No environment × diet interaction was found for plasma AGP levels. Throughout the

experiment, pigs fed CLA had greater levels of plasma AGP than pigs fed a control diet

(Table 7). On d 28 and 42 pigs in the dirty room had greater plasma AGP (P<.01) and

(P<.0001) respectively, than did pigs in the dirty room.

Plasma Urea Nitrogen Levels

On d 14 and 28. pigs in the clean room had greater PUN levels (P<.03) and

(P<.0006). respectively. than pigs in the dirty room. Overall. dietary CLA did not influence

PUN levels (Table 8). However. on d 14. an environment × diet interaction (P<.06) indicates

that in the clean room CLA numerically decreases PUN levels but. in the dirty room. CLA

does not modify PUN levels.

Table 7. Effects of CLA on plasma AGP concentration ($\mu g/ml$)[a,b].

| | | Environmental treatments[c] | | | | | | | | | P value[d] | |
| | | Dirty | | | | Clean | | | | | | |
Day	CLA, %	0	.67	1.33	2	0	.67	1.33	2	SEM[e]	L	Q
14		970	825	982	1056	726	745	1140	1261	156.6	.02	.28
28		626	652	779	1145	475	520	658	848	58.1	.0001	.004
42		612	584	574	854	448	475	496	616	32.7	.0001	.0001

[a]Least squares means.

[b]No environment × diet interaction.

[c]On d 28 and 42 pigs in the dirty room had greater plasma AGP (P<.01) and (P<.0001) respectively, than did pigs in the clean room.

[d]P-value of a linear (L) and quadratic (Q) effect of dietary treatment.

[e]Subplot SEM.

Table 8. Effects of CLA and environment on plasma urea nitrogen (mg/dl)[a,b].

| | | Environmental treatments[c] | | | | | | | | | P value[d] | |
| | | Dirty | | | | Clean | | | | | | |
Day	CLA, %	0	.67	1.33	2	0	.67	1.33	2	SEM[e]	L	Q
14		10.3	13.5	9.8	11.0	14.8	12.0	11.9	12.2	1.00	.14	.75
28		8.8	9.6	8.5	9.7	13.4	14.3	12.5	10.5	1.33	.56	.30
42		13.5	14.2	13.8	12.5	13.2	12.9	12.8	9.7	1.36	.11	.30

[a]Least squares means.

[b]An environment × diet interaction (P<.06) was described on d 14.

[c]PUN levels on d 14 and 28 were greater (P<.03) and (P<.0006), respectively, in pigs of the clean room.

[d]P-value of a linear (L) and quadratic (Q) effect of dietary treatment.

[e]Subplot SEM.

Discussion

Growth Performance. Previous data show that the effects of CLA on feed efficiency were not consistent from one experiment to the other. CLA tended to improve feed efficiency in finisher gilts (Dunshea et al., 1998). Sparks et al. (1999) showed a quadratic improvement in feed efficiency in response to dietary CLA in grower-finisher pigs. However, Eggert et al. (1999) indicate that feed efficiency was not affected by feeding CLA to two genotypes of lean gilts. The quality of the source of CLA used, the composition of the basal diet, health status, and the environmental conditions in which the experiment is held are variables that will potentially influence the final outcome. We have shown that CLA improved feed efficiency in phase II, regardless of the environment. However, in phase I, CLA decreased ADG and ADFI of pigs in the dirty room. Although CLA induced an enhancement in cellular immunity after 40 d, that future enhancement in the immune response will not have an immediate benefit to pigs exposed to an environment contaminated with pathogens from older pigs.

In phases I and II, pigs in the dirty room had lower ADFI than pigs in the clean room, which relates to the lower PUN levels found on d 14 and 28 in pigs in the dirty room. The reason being that pigs in the dirty room, as a consequence of their decreased feed intake, will also decrease the intake of protein.

Body composition. In our experiment, dietary CLA only slightly modified body composition of the pigs by decreasing the percentage of fat. Two month-old pigs, which are mainly depositing lean mass during growth, will show less pronounced dietary-induced differences in fat content than grower-finisher pigs.

Immune Competence. We showed a trend towards an increase in WBC counts due to CLA supplementation. which is consistent with earlier work conducted by Cook et al. (1998). In the light of the lymphocyte phenotype data showing increased percentages of certain lymphocyte subsets. the increase in WBC counts could be attributed to an increase in the total number of lymphocytes. In swine. CD8⁺ lymphocytes represent the numerically larger peripheral lymphocyte sub-population. We have shown for the first time that. after a period of feeding CLA. percentages of TCRαβCD4⁺CD8αα T cells and CD4⁻CD8⁺ cell subsets were linearly increased. The latter phenotype includes distinct cell subsets with different functions: CD3⁻CD16⁻CD8αα lymphocytes (NK cells). TCRαβCD8αβ T cells (cytotoxic T lymphocytes). TCRγδCD8αα T cells and putatively TCRαβCD8αα T cells. The type of dietary immunoenhancement induced by CLA and. therefore. the diseases that CLA could contribute to prevent depend on the function of the CD8⁺ cell subset most influenced by dietary CLA. In future experiments we are planning to further characterize both phenotypically and functionally the nature of the cell subsets most affected by dietary CLA supplementation.

We have shown that lymphocytes from pigs fed CLA proliferate more in culture without mitogen stimulation than do lymphocytes from control pigs. Wong et al. (1997) and Hayek et al. (1998) described an increase in interleukin-2 (IL-2) production in murine splenic lymphocytes. IL-2. which is a T cell growth factor. could be involved in the enhanced proliferation of lymphocytes from pigs fed CLA. Our AGP data. an indirect indicator of IL-2 production. are also consistent with a role of CLA in regulating cytokine production because, throughout the experiment. pigs fed CLA had greater levels of plasma AGP than did control pigs. However. based on the findings indicating that CLA has antiinflammatory properties

(Cook et al.. 1993) we can conclude that increased plasma AGP concentrations was not synonymous of increased pro-inflammatory cytokine production. Therefore, AGP levels alone are not reliable indicators of either immune responsiveness or health status, and AGP data should be interpreted in the context of other immunological assays. CLA decreases prostaglandin E_2 (PGE$_2$) production (Belury et al.. 1997) by interfering in the metabolism of linoleic acid into arachidonic acid, which is the precursor for the synthesis of 2-series prostaglandins and 4-series leukotrienes. PGE$_2$ was demonstrated to be suppressive to the cellular arm of immunity (Goldyne and Strobo. 1982) and to decrease IL-2 production (Roitt et al.. 1998). Therefore. the CLA-derived decrease in PGE$_2$ might diminish the negative impact that PGE$_2$ elicits on cellular immunity and IL-2 production. CLA might also modulate cytokine production and immune function through peroxisome proliferator-activated receptors (PPAR) ligation. Houseknecht et al. (1998) showed that dietary CLA is associated with elevated levels of a number of genes that are responsive. in part. to PPAR-γ. Also. CLA has structural and physiological characteristics similar to peroxisome proliferators (Su et al.. 1999) and is able to activate PPARs while not being a peroxisome proliferator (Moya-Camarena et al.. 1999). As PPARs are involved in modulating the activities of important transcription factors that regulate cytokine expression (Ricote et al.. 1998). that could also explain why CLA causes peripheral CD8$^+$ lymphocyte expansion and modulates cytokine production.

Implications

Dietary CLA increases porcine CD8$^+$ lymphocyte subsets and enhances overall lymphocyte proliferation in both clean and dirty environments. Thus. CLA is a cellular

immune enhancer that could be used to improve, at the cellular level, vaccination efficacy and to control diseases where CD8⁺ cells are critical.

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CHAPTER 3. DIETARY CONJUGATED LINOLEIC ACID-INDUCED PHENOTYPIC AND FUNCTIONAL MODULATION OF CD8⁺ CELL SUBSETS VERSUS BACTERIAL ANTIGEN-DERIVED CELLULAR REGULATION

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Abstract

The heterogeneity of the porcine CD8⁺ lymphocyte subpopulation. which contains distinct cell subsets. makes pigs good models for functional and phenotypic investigations on CD8⁺ cells. In contrast to that. a more uniform CD8 expression pattern described in humans complicates the study of differences among CD8⁺ subsets. Biological enhancers of the numbers of CD8⁺ cells might improve the knowledge of the role of CD8⁺ cells in cellular immunology. Conjugated linoleic acid (CLA) is a mixture of positional (9, 11; 10, 12; or 11, 13) and geometric (cis or trans) isomers of octadecadienoic acid that decreases prostaglandin E_2 (PGE_2) production by modulating arachidonic acid (AA) metabolism. 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 distinct CD8⁺ cell subsets and enhances specific CD8⁺-related functions. Here. we confirmed our preliminary findings by showing a CLA-dependent increase in CD8⁺ lymphocytes. Furthermore. we report for the first time. an observation that links a nutritional supplement to

increased CD8α expression. CLA causes in vivo expansion of peripheral TCRγδCD8αα T

cells. CD3⁻CD16⁺CD8αα lymphocytes (NK cells) and TCRαβCD8αβ T cells pre-cytotoxic T

lymphocytes (pre-CTLs). Interestingly. the CLA-induced expansion of peripheral

TCRαβCD8αβ cells was consistent with increased percentages of TCRαβCD8αβ and double

negatives thymocytes. whereas thymic CD8αα⁻ subpopulations percentages were unaffected

by dietary CLA. Moreover. these phenotypic changes are functionally related to an

enhancement in effector CTL cytotoxicity. Collectively. these data suggest that dietary CLA

causes in vivo expansion of CD8⁻ cells involved in both adaptative and innate immunity.

Introduction

Immunological concepts are sometimes developed without taking into consideration

the influence that external factors such as diet. environment or genetics elicit on the animal

host that is the source of experimental biological material (e. g.. lymphocytes. cytokines.

nuclear transcription factors). A substantial amount of work has been completed regarding the

impact of dietary fatty acids in eicosanoid production (Calder. 1996. 1998). or more recently.

the fatty acid-induced transcriptional regulation of cytokine gene expression (Jiang et al..

1998). both related to regulation of the immune response. However. the lack of specific

studies addressing the effects of nutrients on the phenotype of distinct T cell subsets has

particularly limited the understanding of the mechanisms of action of nutrients at the cellular

and molecular levels. For instance. to induce a measurable expansion of cells within a

particular phenotype in vivo. antigen-induced lymphocyte stimulation represents the

traditional immunological tool. in which the pathway of antigen presentation plays an

important role. This approach is supported by the clonal selection theory (Burnet. 1959)

which has been utilized to explain the expansion of naïve lymphocytes into clones of antigen-

specific effector lymphocytes and has become one of the main pilars of cellular immunology. We propose a novel approach. which is nutritional in nature. to modulate the phenotype of particular lymphocyte subsets in vivo. The modification of T cell phenotype through a nutritional intervention differs from reponses derived from antigenic challenge on that expanded cell subsets cannot be described as specific for one single epitope. The impact on $CD8^+$ cellular immunity of a mixture of positional (9. 11: 10. 12: or 11. 13) and geometric (cis or trans) isomers of octadecadienoic (18:2) acid known as CLA will be described here in detail. CLA is a nutrient with broad biological activity and is known to be anticarcinogenic (Ha et al.. 1989: Ha et al.. 1990: Pariza. 1991:Shultz et al.. 1992a: Shultz et al.. 1992b: Ip et al.. 1994a: Ip et al.. 1994b: Ip et al.. 1994c: Ip et al.. 1997: and Liu et al.. 1997). antidiabetic (Houseknecht et al.. 1998). antiatherogenic (Nicolosi et al.. 1993: Lee et al.. 1994). and immunomodulator (Cook et al.. 1993: Miller et al.. 1994: Wong et al.. 1997: Chew et al.. 1997: Bassaganya-Riera et al.. 1998: Sugano et al.. 1998: Bassaganya-Riera et al.. 1999: and Hayek et al.. 1999). Currently. there are no studies relating the role of CLA-induced enhancement of a particular lymphocyte subset to tumor immunology. Although CLA has been shown to increase IL-2 production in vivo (Hayek et al.. 1999). the lack of a cellular basis in CLA research indicates that the mechanistic understanding of its functional role as an anticarcinogen is in a very immature stage. In humans and mice. abundant data suggest that $CD8^+$ cells may have an essential role in antitumor immunity.

Both CTLs and natural killer (NK) cells bear the CD8 molecule on their surface. In most mammalian species. CD8 molecules are expressed either as a disulfide-linked αα-homodimer or as an αβ-heterodimer on the surface of lymphocytes (Norment et al.. 1988). In humans. thymocytes and most mature peripheral lymphocytes preferentially express the

CD8$\alpha\beta$ molecule, but on a subset of NK cells and intraepithelial lymphocytes (IEL) only the CD8$\alpha\alpha$ form is present (Sun et al., 1997). The functional significance of the tissue-dependent CD8 expression patterns in lymphocytes is not entirely known.

In humans, most recirculating CD8$^+$ lymphocytes are phenotypically TCR$\alpha\beta$CD8$\alpha\beta$ cells which are, functionally, resting or quiescent cells (pre-CTLs) containing granzymes and other lytic molecules within their lytic granules. Pre-CTLs have the potential to become, upon appropriate TCR/CD3 complex stimulation, degranulating activated effector cells (CTLs). In human immunology, the term CTL or pre-CTL is indeed used synonymously with CD8 lymphocyte. A weakness of the human model of CD8 research derives from the uniformity of that cell subset, which is reflected by the predominance of TCR$\alpha\beta$CD8$\alpha\beta$CD18^{dim} T cells which express CD18, a β_2 integrin used for endothelial adherence, at low densities might mask the functional role of other CD8$^+$ subsets. For instance, because the human TCR$\alpha\beta$CD8$\alpha\beta$CD18^{bright} cell subset is a very reduced peripheral cell subset compared with TCR$\alpha\beta$CD8$\alpha\beta$CD18^{dim}, detection of IL-12 induced activation of the first subset was masked by non-responssiveness of the latter (Gollob et al., 1998). In contrast to what happens in humans, porcine CD8$^+$ lymphocytes represent a cell population that is both functionally and phenotypically heterogeneous, in which CD8$\alpha\alpha$ expression predominates in thymocytes and mature peripheral lymphocytes (Pescovitz et al., 1994; Zuckermann and Husmann, 1996; Summerfield et al., 1996; Zuckermann et al., 1998). Phenotypically, porcine CD8$\alpha\alpha^+$ lymphocytes can be further subdivided into TCR$\gamma\delta^+$CD4$^-$, TCR$\alpha\beta^+$CD4$^-$, or CD3$^-$CD16$^+$ (natural killer) and putatively, according to indirect inferences (Yang and Parkhouse, 1996) TCR$\alpha\beta^+$CD4$^-$. All CD8$\alpha\beta^+$ T cells bear the TCR$\alpha\beta$ on their membrane. The enormous CD8

hetereogeneity described in pigs. which contrasts with the uniform CD8 pattern described in humans or mice. facilitates the detection of small changes in a broad range of both peripheral and tissue-specific $CD8^+$ lymphocyte subsets. Therefore. based on that peculiarity, pigs are good models for studies of several aspects of cellular immunology. particularly those related to the surface expression of $CD8\alpha$ in lymphocytes.

Our studies were designed to determine the effects of CLA on phenotype and function of $CD8^+$ lymphocyte subsets and to better understand how these populations regulate or are regulated by the immune response induced by an ongoing bacterial antigenic challenge. Based on our previous studies. which indicated that peripheral $CD8^+$ cells linearly increased as we increased the amount of CLA in the diet (Bassaganya-Riera et al.. 1999). we proposed that CLA would increase distinct $CD8^+$ lymphocyte subsets and that might influence lymphocyte function. In our preliminary experiments. to study how CLA influenced T cell phenotype. CD4 and $CD8\alpha$ surface expression was assayed on peripheral lymphocytes (Bassaganya-Riera et al.. 1999). In the studies reported here. we have assayed for peripheral blood mononuclear cell (PBMC) surface expression of $CD8\alpha$ or $CD8\beta$ along with the necessary surface markers to further phenotypically characterize all the distinct $CD8^+$ cell subsets (e.g.. δ chain of TCR. CD16. CD4 and CD3). A model of vaccination with an extracellular bacterial antigen (proteinase-digested *Brachyspira hyodysenteriae* B204 strain bacterin) that causes clonal selection and expansion of $CD4^+$ T cells which. in turn. are responsible for a cytokine-driven expansion of $CD8\alpha\alpha$ cells. was adopted to evaluate how the non-specific increase in lymphocyte subsets induced by dietary CLA modifies and /or interacts with a bacterial clonally-derived antigen-specific immune response.

Materials and Methods

Dietary and Vaccination Treatments

Thirty-two 14-d-old cross-bred pigs were randomly distributed from outcome groups based on litter. body weight and sex to blocks of two contiguous individual pens. Either a control or a 1.33% CLA isocaloric and isonitrogenous diet was randomly allotted to pens within block. On d 21. 28 and 42 four blocks of two pigs each were intramuscularly inoculated with 2 ml of a proteinase-digested (PD) *B. hyodysenteriae* strain B204 bacterin in squalene. and four blocks of two pigs each were inoculated with 2 ml of a squalene adjuvant preparation alone. Briefly. the squalene adjuvant preparation contained 88% phosphate buffered saline (PBS). 10% squalene/pluronic acid (80/20: v:v) and 2% Tween 80.

Harvesting of Peripheral Blood Mononuclear Cells (PBMC)

Peripheral blood was obtained through vena cava puncture on d 0. 14. 28. 42. 49. 56. 63 and 72. PBMC were isolated by gradient centrifugation. Peripheral blood was diluted 1:4 (v/v) in PBS. overlayed onto lymphocyte separation medium (Mediatech Inc. Herndon. VA) and centrifuged at 400 × g for 40 min. PBMC were obtained from the separation medium/plasma interface with a Pasteur pipette. washed three times with PBS. and counted with a hemocytometer (Coulter Particle Counter: Beckman Coulter Corp.. Miami. FL) for use in phenotypic and functional assays. The remaining peripheral blood was saved for later total white blood cell (WBC) after erythrocyte lysis with whole blood lysis reagents (Coulter Immunology. Hialeah. FL) and differential counts were conducted with a blood smear and staining with Hema 3 Stain Set (Fisher Scientific. Pittsburgh. PA).

Isolation of Mucosal Lymphocytes and Thymocytes

Large intestine and small intestine intraeptithelial lymphocytes (IELs) and lamina

propia lymphocytes (LPLs) were isolated using a modification of a previously described

procedure (VanCott et al.. 1994). Briefly, on d 35, four control pigs (two vaccinated and two

non-vaccinated) and four pigs fed CLA (two vaccinated and two non-vaccinated) were killed

and necropsied. Sections (15 cm) of large and small intestine were removed, opened

longitudinally. and cut into 1 cm pieces totaling 10 to15 g. Sections were rinsed with Hank's

balanced salt solution without Ca^{+2} and Mg^{+2} (HBSS. Sigma. St Louis. MO), placed into

flasks containing 15 ml of HBSS with 5 mM ethylenediamine tetraacetic acid disodium salt

dihydrate (EDTA. Sigma) and 0.29 µg/ml dithiothreitol (DTT. Sigma), incubated for 15 min

at 37° C with constant stirring. and supernatants harvested for IEL isolation. Remaining tissue

fragments were rinsed with RPMI 1640 (Mediatech Inc.) followed by three incubations in 15

ml RPMI containing 0.25 mg/ml DNase (Boehringer Mannheim: Indianapolis. IN), 300 U/ml

collagenase (Sigma). 5% heat-inactivated (56° C for 30 min) fetal bovine sera (FBS:

HyClone. Logan. UT). 200 mM HEPES (Sigma). at 37° C for 15 min with constant stirring.

Supernatants were collected after each incubation for isolation of LPL. Isolation of IEL and

LPL from supernatants was by discontinuous Percoll (Sigma) gradient centrifugation. Briefly,

supernatants were centrifuged at 250 × g for 10 min and cells resuspended in 45% Percoll.

Cells in 45% Percoll were overlayed onto 80% Percoll and centrifuged at 400 × g for 40 min

at 4° C. IEL and LPL were harvested from 45 to 80% interface. washed twice in PBS, and

counted for use in immunophenotyping. To isolate the thymocytes a 3 cm^2 sample of thymus

was removed from the necropsied pigs, the tissue was disrupted between two glass surfaces in

cold HBSS. the cell suspension was washed twice. and lymphocytes counted on a hemocytometer for use in immunophenotyping.

Lymphocyte Blastogenesis Assay

Complete medium was prepared by supplementing RPMI 1640 with 25mM HEPES buffer (Sigma). 100 units/ml penicillin (Sigma). 0.1 mg/ml streptomycin (Sigma). 5×10^{-5} M 2-mercaptoethanol (Sigma). 1 mM essential amino acids (Mediathech Inc.). 1 mM non-essential amino acids (Sigma). 2 mM L-glutamine (Sigma). 1 mM sodium pyruvate (Sigma) and 10% FBS. Media pH was measured with a pH meter (Orion Research Inc.. Beverly. MA) and brought 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 whole cell sonicate (5 μg/ml. WCS) or (5 μg/ml. PD) or concanavalin A (5 μg/ml. Con-A: Sigma). or medium alone (non-stimulated). Plates were incubated for 5 d at 37° C in 5% CO_2 humidified atmosphere. After 5 d. 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 incubated for an additional 20 h. Well contents were harvested onto fiber filters with a PHD cell harvester (Cambridge Technology. Cambridge. MA) and incorporated radioactivity measured by liquid scintillation counting (Hewlett-Packard. Meriden. CT). Treatments were run in triplicate and stimulation indices (SI) calculated by dividing counts min^{-1} of stimulated wells by counts min^{-1} from non-stimulated wells.

CD8$^+$ Subpopulations Proliferation Assay

A total of 2×10^7 PBMC were separated to perform PKH2-GL proliferation assays (Sigma). 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), which is a green fluorescent dye, and incubated 5 min followed by a 1 min incubation with 2 ml of FBS to stop the reaction. Cells were washed three times with RPMI 1640. Then, stained PBMC were counted in a Coulter Particle Counter. PBMC numbers were adjusted to be at a concentration of 2×10^6 PBMC/ml complete media. Standarized cell suspensions (100 µl) were added to a 96-well flat bottomed microtiter plate containing 100 µl of medium (non-stimulated), medium plus 5 µg/ml of a WCS *B. hydysenteriae* bacterin, medium plus 5 µg/ml of a PD *B. hydysenteriae* bacterin, or medium plus 5 µg/ml of Con-A. Treatments were run in replicates of six for each animal and ex vivo treatment. Cells were incubated at 37° C in 5% CO_2 humidified atmosphere for 6 d. 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 of the distinct CD8$^+$ cell subsets.

Preparation and Analysis of Cells for Flow Cytometric Analysis of Surface Antigen Expression.

Mononuclear cells either coming directly from PBMC, thymic or mucosal isolation or resulting from a 6-d culture and stained with PKH2, were analyzed for expression of cell surface antigens by flow cytometry. Non-cultured cells (2×10^6/ml) were resuspended in 100 µl of balanced salt solution with 1% FBS and 0.04% sodium azide (FACS buffer). Cultured cells were directly transferred into the plate for surface antigen staining to avoid membrane

damage. Both cultured and non-cultured cells were stained with a volume of 50 μl of the

primary antibody dilution to swine leukocyte surface antigens: IgG2b mouse anti-swine-CD4

PE-labeled (74-12-4). IgG2a mouse anti-swine-CD8α biotinylated (76-2-11) (hybridoma

supernatants kindly provided by Dr. Joan K. Lunney; Beltsville, MD). IgG2a mouse anti-

swine-CD8β (PG164A) (VMRD Inc. Pullman, WA). IgG1 mouse anti-swine-CD3 (8E62)

(VMRD Inc). IgG1 mouse anti-swine-TCRγδ (PGBL22A) (VMRD Inc). IgG1 mouse anti-

swine-PNK-E and IgG1 mouse anti-swine-CD16 (G7) (both NK markers were kindly

provided by Dr. Yoon B. Kim; Chicago, IL) and appropriate isotype control antibodies:

mouse IgG2a-Quantum Red (Sigma) for anti-CD8α and anti-CD8β, mouse IgG2b-PE

(Sigma) for anti-CD4, and a primary mouse IgG1 unlabeled (Southern Biotechnology Inc.)

followed by a secondary goat anti mouse IgG1-PE (Sigma) for anti-CD3, anti-TCRγδ, anti-

CD16, and anti-PNKE. 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) for 8E62, G7, PNK-E and

PGBL22A, streptavidin-conjugated CyChrome (Pharmingen, San Diego, CA) for biotinylated

76-2-11, PE-conjugated goat anti mouse IgG2a (Southern Biotechnology Associates Inc.) for

the PG164A. Cells were incubated for 15 min, washed twice and analyzed by flow cytometry.

In cultured and PKH2 stained cells, the three-color flow cytometric data aquisition was

conducted in a FACScan (Becton Dickinson, San Jose, CA), because we have found that

Coulter XL flow cytometers (Beckman Coulter Corp.) do not provide an accurate flow

cytometric analysis of cultured proliferating cells stained with PKH2 (Harkins et al., 1999).

Analysis of data aquired using FACScan was performed using the CellQuest software

(Becton Dickinson). Two-color flow cytometric analysis with a Coulter XL was conducted on non-cultured and non-stained peripheral or tissue-specific lymphocytes (all lymphocytes except those used in PKH2 proliferation assay).

Granzyme Functional Assays

Wells of 96-well flat-bottomed microtiter plates (Becton Dickinson) were seeded with 2×10^5 mononuclear cells in a total volume of 200 μl per well. Wells contained either *B. hyodysenteriae* antigens (5 μg/ml. WCS) or (5 μg/ml. PD) or Con-A (5 μg/ml). or medium alone (non-stimulated). Plates were incubated for 5 d at 37° C in 5% CO_2 humidified atmosphere. After 5 d. anti-CD3 mAb (8E62) was added into each well at a concentration of 5 μg/well. At 6 d. the cultured cell suspension was harvested. centrifuged (400 × g) for 5 min supernatant was separated from pellet. Anti-CD3 addition causes cytotoxic activity and cell death. regardless of the presence of IL-2 (Wang et al.. 1994). 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 to obtain cell lysates. Both supernatant and cell lysates were frozen at -70° C for later analysis of granzyme activity.

Assay for BLT-esterase Activity

This assay. when compared with the chromium release assay (Towsend and Bodmer. 1989). gave a correlation coefficient (r^2) of 0.878 (Suhrbier et al.. 1991). which indicates its validity as a reliable cytotoxicity assay. Granzyme-derived enzymatic activity was measured using a modification of a previously described procedure (Mobley and Dailey, 1992) by adding 40μl of either the cell lysate or the supernatant 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)

in 96-well Immulo II microtiter plates (Dynatech, Alexandria, VA) for 15 to 30 min at room

temperature. The absorbance of the BLT-esterase-induced color change was measured in the

microplate ELISA reader (BioTek Instruments, Winooski, VT) at a wavelength of 405 nm.

Assay for BAADT-esterase Activity

Granzyme B-specific enzymatic activity was measured using a modification of a

previously described procedure (Caputo et al., 1993) by adding 15 μl of the reaction mixture

(0.2 mM tert-butyloxycarbonyl-Ala-Ala-Asp-thiobenzyl ester (BAADT) (Enzymes Systems

Products, Dublin, CA), 0.2 mM Ellman's reagent in buffer A) into 35 μl of either cell lysate

or supernatant. Buffer A consists of 0.2 M Hepes (Sigma), 0.2 M NaCl (Fisher Scientific),

1 mM EDTA, 0.05% (v/v) Triton X-100 (Sigma). Ellman's reagent was made by dissolving

2-nitrobenzoic acid in dimethylsulfoxide (DMSO) (Sigma) at 11 mM. The absorbance of the

BAADT-aspartase-induced color change was measured in the microplate ELISA reader at a

wavelength of 405 nm.

Experimental Design

From day 0 until the first date of vaccination (d 21) the experimental design was a

randomized complete block (RCB) with pig within block being the experimental unit for

dietary treatment. After d 21, the vaccination treatment was added across blocks and the

design became a split-plot: pig within block being the experimental unit for dietary treatment

(sub-plot) and a block of two littermate pigs being the experimental unit for vaccination

treatment (whole plot).

Statistics

Data were analyzed as a randomized complete block design from d 0 until d 21. From

d 21 to the end of the experiment, data were analyzed as a 2 × 2 factorial arrangement of

treatments within a split-plot design using the general linear model (GLM) procedure of SAS

(1989) (SAS Institute Inc.. Cary. NC).

Results

Dietary CLA Induces Expansion of Peripheral CTLs. NK Cells and TCR$\gamma\delta$CD8$\alpha\alpha$ Cells

To determine the effects of dietary CLA supplementation on peripheral CD8$^+$ cell

subsets. flow cytometric analysis of isolated PBMC was conducted as previously described.

In pigs. four distinct recirculating CD8$^+$ cell subsets have been described: TCR$\gamma\delta$CD8$\alpha\alpha$.

CD3$^-$CD16$^-$CD8$\alpha\alpha$ (NK cells). TCR$\alpha\beta$CD8$\alpha\beta$ (CTLs). and TCR$\alpha\beta$CD4$^-$CD8$\alpha\alpha$.

Table I. Peripheral CD8$^+$ lymphocyte subset expansion ($\times$ 10^6 cells/ ml blood).

Phenotype	Day CLA, %	NON-VACC. 0	1.33	VACC. 0	1.33	SEM[a]	Vacc	Diet	V × D
TCR$\gamma\delta$CD8$\alpha\alpha$	50	0.72	0.92	0.90	1.51	0.091	0.05	0.04	0.15
	63	1.07	1.21	1.19	2.21	0.435	0.02	0.02	0.06
	72	1.79	1.61	1.28	1.85	0.293	0.77	0.68	0.42
TCR$\alpha\beta$CD8$\alpha\beta$	50	1.30	0.99	1.69	2.00	0.177	0.15	0.99	0.22
	63	1.76	2.79	1.58	2.79	0.230	0.80	0.01	0.81
	72	1.92	2.48	1.65	2.61	0.184	0.80	0.02	0.49
CD3$^-$CD8$\alpha\alpha^a$	50	2.25	3.60	2.93	11.50	1.980	0.16	0.12	0.21
	56	2.82	3.88	2.06	5.19	0.661	0.79	0.06	0.33
CD16$^+$CD8$\alpha\alpha^a$	72	1.96	2.67	1.00	6.37	0.587	0.16	0.007	0.02

Note: The table has spanning headers "Vaccination treatments" over NON-VACC./VACC. columns and "P values" over Vacc/Diet/V × D columns.

[a]Subplot SEM

[b]In pigs. both CD3$^-$CD8$\alpha\alpha$ and CD16$^+$CD8$\alpha\alpha$ include only the NK cell subpopulation.

Putatively. there is an additional porcine peripheral $CD8^+$ cell subset ($TCR\alpha\beta CD8\alpha\alpha$) whose existence has been reported (Yang and Parhouse. 1996). although. due to the current lack of a mAb anti-$TCR\alpha\beta$ in pigs. there is no direct evidence pointing to the existence of that sub-population.

We showed that CLA synergizes with vaccination to expand the $TCR\gamma\delta CD8\alpha\alpha$ cells and NK cell subsets. Dietary CLA alone. and not vaccination treatment. expanded the $TCR\alpha\beta CD8\alpha\beta$ T cell subset (pre-CTLs) in vivo (Table I). That increase in numbers of CTLs was sustained until the end of the experiment (Figure 1).

Figure 1. Effects of CLA on $TCR\alpha\beta CD8\alpha\beta$ lymphocytes (CTLs)
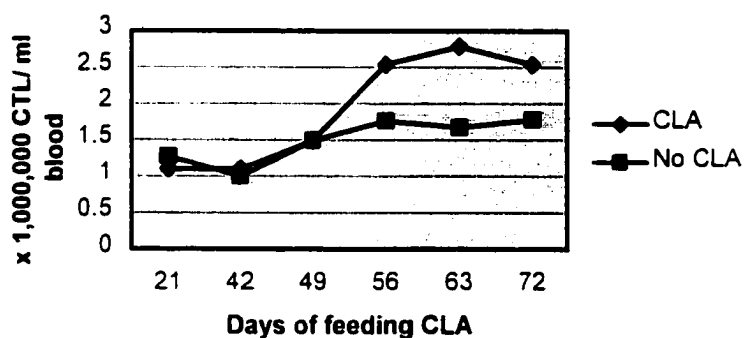
Interestingly. the effects of CLA and vaccination on $TCR\gamma\delta CD8\alpha\alpha$ cells appeared earlier (50 to 63 d) than the effects of CLA on pre-CTLs (63 to 72 d). whereas NK cells are increased due to CLA and vaccination from d 50 to d 72. In a previous study. the increase in $CD8\alpha$ expression appeared at d 42 of dietary CLA supplementation. From d 0 until d 50 no differences on $CD8^+$ lymphocyte subsets were attributed to dietary CLA supplementation.

Dietary CLA Supplementation Increases CD8αβ but not CD8αα Thymocyte Subsets

In an effort to determine the origin (thymic or extrathymic) of the peripheral CD8$^+$

cell subsets expanded as a consequence of dietary CLA supplementation, thymocytes were

isolated and their phenotype was analyzed by flow cytometry. Percentages of TCRαβCD8αβ

thymocytes were increased, but CD8αα$^-$ thymocytes were either decreased or unaffected by

dietary CLA supplementation and by vaccination (Table II). Thus, CTLs are the only CLA-

expanded cell subset with thymic-dependent development.

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

Thymocytes	CLA, %	Vaccination treatments				SEM[a]	P value		
		NON-VACC.		VACC.			Vacc	Diet	V× D
		0	1.33	0	1.33				
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, and putatively TCRαβCD8αα thymocytes.

[c]Only includes TCRαβCD8αβ thymocytes.

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

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

Percentages of TCRγδCD8αα thymocytes were neither altered due to dietary CLA supplementation nor to vaccination. Percentages of CD4⁻CD8⁻ thymocytes were not increased in response to dietary CLA. but there was a trend towards an increase in CD4⁻CD8⁻ double negative (DN) thymocytes.

The CLA-induced NK and TCRγδCD8αα Cell Proliferation is Non-specific

To more fully characterize the nature of the in vivo CLA-induced expansion of CTLs, NK cells and TCRγδCD8αα lymphocytes. the proliferative response of the distinct CD8⁺ cell subsets was assessed ex vivo by a proliferation assay coupled to a flow cytometric analysis. Briefly. PKH2-stained PBMCs isolated from pigs of four different in vivo treatments (No-CLA. non-vaccinated: CLA. non-vaccinated: No-CLA. vaccinated: or CLA. vaccinated) were cultured with or without antigen (5 µg/ml. PD or WCS) or Con-A (5 µg/ml) for 6 d. and then flow cytometrically evaluated for proliferation (Figure 1).

Table III. Influence of CLA and vaccination on TCRγδCD8αα proliferation, %

		NON-VACC.		VACC.			P value		
TCRγδCD8αα[a]	CLA, %	0	1.33	0	1.33	SEM[b]	Vacc	Diet	V× D
Non-stimulated		4.83	7.26	13.65	15.67	0.358	0.001	0.02	0.62
WCS-stimulated		18.60	24.74	55.62	54.59	14.990	0.15	0.88	0.83

[a]PKH2^dim TCRγδCD8αα lymphocytes are proliferating TCRγδCD8αα. The reason being that, as cells divide. PKH2 staining left on the membrane diminishes resulting in a decreased mean fluorescence intensity (Ashley et al.. 1993).
[b]Subplot SEM.

Dietary CLA supplementation and vaccination significantly increased percentages of proliferating TCRγδCD8αα lymphocytes in non-stimulated wells (Table III) but not in antigen- or mitogen-stimulated wells. These results suggest that CLA effects on TCRγδCD8αα lymphocytes are non-specific in nature.

A numerical increase in percentages of proliferating TCRγδCD8αα lymphocytes in wells stimulated with WCS due to vaccination (Table III). although it is only a statistical trend (P<.15). indicates that either the bacterial antigen itself or cytokines produced by antigen-specific cells (bystander effect). are involved in TCRγδCD8αα lymphocyte proliferation in response to vaccination. These results are confirmed by data on thymidine incorporation showing that overall mitogen-induced lymphocyte proliferation was enhanced in PBMCs from pigs fed CLA regardless of the vaccination treatment. This might be explained because CLA decreases PGE$_2$ production by interfering with the in vivo synthesis of arachidonic acid from linoleic acid. PGE$_2$ inhibits IL-2 production and, therefore. T cell proliferation (Roitt et al.. 1998). However. WCS-induced proliferation was mainly enhanced in pigs fed CLA and vaccinated but not in pigs fed CLA without vaccination (Table IV).

Table IV. Influence of CLA and vaccination on PBMC proliferation methyl-[^{3}H] thymidine inc., CPM.

		Vaccination Treatments					P value		
		NON-VACC.		VACC.					
Ex vivo treatment	CLA, %	0	1.33	0	1.33	SEM[a]	Vacc	Diet	V× D
Non-stimulated		2586	1759	2833	3199	629.3	0.31	0.74	0.44
WCS-stimulated		2355	1906	4590	20196	1952.3	0.03	0.06	0.05
Con-A-stimulated		88271	117928	99416	190504	9788.8	0.05	0.02	0.08

[a]Subplot SEM

Vaccination and CLA Interacted to Increase Percentages of Small Intestinal CD4$^+$CD8$\alpha\alpha$-IELs, but not Small or Large Intestinal TCR$\gamma\delta$CD8$\alpha\alpha$-IELs

Gut associated lymphoid tissue (GALT) is an important source of lymphocytes that develop extrathymically. Small cellular clusters called cryptopatches. located within the lamina propia of the small intestine. contain T cell progenitors that are CD4$^-$CD8$^-$CD3$^-$ c-kit positive (c-kit$^-$) but lineage marker negative (Lin$^-$) (Saito et al.. 1998). These progenitor cells are capable of reconstituting peripheral and intestinal T cell compartments upon adoptive transfer to immunodeficient mice (Saito et al.. 1998). Both mice and swine have a greater prevalence of CD8$^+$ cells among SI-IEL and LI-IEL than among LPLs (Camerini et al.. 1993). To investigate a possible extrathymic origin of peripherally expanded lymphocyte subpopulations. we isolated large and small intestine IELs and LPLs. The flow cytometric analyisis shows that TCR$\gamma\delta$CD8$\alpha\alpha$-IELs were not significantly increased either in the small intestine or in the large intestine because of CLA supplementation (Table V). Therefore. CLA-expanded peripheral TCR$\gamma\delta$CD8$\alpha\alpha$ T cells

Table V. Impact of CLA and vaccination in CD8$\alpha\alpha$ expression on GALT IELs, IELs

IELs	CLA, %	NON-VACC. 0	NON-VACC. 1.33	VACC. 0	VACC. 1.33	SEM[a]	P value Vacc	P value Diet	P value V× D
SI-TCR$\gamma\delta$CD8$\alpha\alpha$		8.65	7.74	3.24	14.75	3.097	0.86	0.39	0.34
LI-TCR$\gamma\delta$CD8$\alpha\alpha$		8.65	11.75	22.14	5.83	2.475	0.15	0.05	0.06
SI-CD4$^+$CD8$\alpha\alpha$		1.83	0.81	10.42	14.82	0.191	0.16	0.01	0.01
LI-CD4$^+$CD8$\alpha\alpha$		1.63	1.39	4.93	1.18	1.011	0.29	0.17	0.24

[a]Subplot SEM

cannot be defined as a GALT-originated subset. Nevertheless, percentages of small intestinal

$CD4^+CD8\alpha\alpha$-IELs were increased when both treatments, vaccination and dietary CLA

supplementation, were simultaneously administered (Table V).

Dietary CLA Increases CTL Granzyme Activity Regardless of the Vaccination Treatment

To functionally characterize the cytotolytic activity of PBMC cultured with a bacterial

antigen (presented through the exogenous pathway) that was previously inoculated to half of

the pigs, we used the BLT assay. We found that PBMCs isolated from animals fed CLA had

greater supernatant granzyme activities, after anti-CD3 stimulation, than PBMCs from pigs

not fed CLA, regardless of the vaccination treatment (Table VI). Anti-CD3 addition to pre-

CTLs causes an increase in cytolytic activity or death via internal action of TNF (Wang et al.,

1994). Thus, either anti-CD3 acted on the naïve $TCR\alpha\beta CD8\alpha\beta$ T cells (pre-CTLs) expanded

in vivo by dietary CLA supplementation and induced activation and differentiation to CTL, or

CLA caused a change in expanded pre-CTLs that makes them less prone to undergo anti-

CD3-induced apoptosis. Surviving effector CTLs will release the

Table VI. Influence of CLA on peripheral $TCR\alpha\beta CD8\alpha\beta$ granzyme activity, OD

		Vaccination Treatments						
		NON-VACC.		VACC.			P value	
Granzyme activity	CLA, %	0	1.33	0	1.33	SEM[a]	Vacc Diet	V×D
BLT PD-stim. (supernatant)		0.178	0.213	0.191	0.205	0.002	0.46 0.005	0.11
BAADT ConA-stim. (supernatant)		0.081	0.087	0.084	0.096	0.002	0.21 0.11	0.40
BLT PD-stim. (cell lysate)		0.062	0.074	0.062	0.060	0.001	0.03 0.05	0.02

[a]Subplot SEM

content of lytic granules (e.g.. perforin. granzymes) into the media. NK cells do not express

TCR/CD3 complex on their surface. thus anti-CD3-induced degranulation is not NK-derived.

NK-derived granzyme activity. if any. will only result from a constitutive release of

granzyme. Thus. granzyme activity in the supernatant harvested after anti-CD3 addition is

mainly derived from effector CTLs. These results also indicate that. even when CLA

increases pre-CTL production. it is not decreasing their potential to develop cytolytic activity.

Table VII. Influence of CLA and vaccination on CD4$^+$CD8$^-$ proliferation, %.

| | | Vaccination Treatments | | | | | | | |
| | | NON-VACC. | | VACC. | | | P value | | |
CD4$^+$CD8$^-$	CLA, %	0	1.33	0	1.33	SEM^a	Vacc	Diet	V× D
Non-stimulated		9.12	9.79	13.05	22.90	2.588	0.08	0.17	0.21
WCS-stimulated		22.02	27.27	40.19	78.77	5.268	0.02	0.05	0.08
PD-stimulated		32.36	36.80	42.72	62.14	5.954	0.09	0.18	0.33
Con-A-stimulated		88.71	85.92	94.68	96.15	1.690	0.04	0.73	0.33

^{a}Subplot SEM

Discussion

Here. we report a new method. different from the traditional antigen-induced clonal

selection. to expand in vivo particular peripheral CD8$^+$ lymphocyte subsets. which consists of

feeding a mixture of positional and geometric isomers of conjugated octadecadienoic acid.

We demonstrate that CLA expands pre-CTLs. NK cells and TCRγδCD8αα lymphocytes in

pigs in a non-specific fashion. Interestingly. in vivo expansion of pre-CTLs is independent of

the vaccination. whereas NK cell and TCRγδCD8αα CLA-induced expansion is either

enhanced by vaccination or CLA further enhances the proliferation of cell subsets expanded

by vaccination with a bacterial antigen. That expansion of particular CD8$^+$ lymphocyte

subsets is correlated with increased WBC counts. The CLA-enhanced WBC counts are due to

elevated lymphocyte numbers (data not shown) and not to an increase in polymorphonuclear

cells. This finding is consistent with earlier work conducted by Cook et al. (1998).

Bacterial antigens, such as other extracellular antigens, follow the exogenous pathway

of antigen presentation to the MHC class II (SLA complex class II, in pigs). They are known

to induce clonal selection of CD4$^+$ cells. In the case of the *B. hyodysenteriae* proteinase-

digested bacterin, that T helper enhancement occurs along with enhanced proliferation of

CD8αα$^+$ cell subsets, that it is possibly cytokine-driven. Proliferation assays confirmed that

the bacterial antigen used here induces proliferation of CD4$^+$ cells (Table VII). CLA seems to

potentiate this effect on CD4$^+$ cells, but a CLA-dependent CD4$^+$ expansion was not measured

in vivo. This finding is consistent with our findings regarding *B. hyodysenteriae*-specific

proliferation, indicating that depletion of CD4$^+$ cells inhibits overall proliferation (data not

shown). Even in the face of an ongoing immune response that targets CD4$^+$ cells induced by

spirochetal antigens, CLA alone still increases pre-CTLs, a CD8$^+$ cell subset that is not

affected by the bacterial antigen and would only be affected by non-self antigens presented

through the endogenous pathway. CLA is capable of non-specifically expanding immature

thymocytes with CTL phenotype, pre-CTL numbers and CTL activity. Therefore, based on

these findings, dietary CLA could improve the efficacy of infectious and autologous

vaccination protocols in which particular CD8$^+$ cell subsets are critical for effective immune

protection. In contrast to the specific nature of both CTL expansion caused by an intracellular

antigen or of CD4 expansion caused by an extracellular antigen, CLA-induced expansion of

CD8$^+$ lymphocyte subsets must occur through increased lymphocyte production and not

through clonal selection. Aiming to elucidate which lymphoid tissue containing pre-CTLs or

pre-CTL precursors received the greatest impact from CLA, we measured both peripheral and thymic lymphocyte phenotypes. The basis for a non-clonally-derived expansion of T cell subsets can be partly found in the thymus and partly in the bone marrow, whereas the basis for NK cell expansion is presumably bone marrow related. The increase in TCRαβCD8αβ thymocytes might be related to greater numbers of progenitor cells (DN thymocytes) in pigs fed CLA. DN thymocytes that have migrated from the bone marrow to the thymus, after positive and negative selection, and a process of lineage choice, will be released into the circulation (Janeway, 1999). As CLA has a strong influence on progenitor T cells, in contrast to what would happen upon antigen-specific stimulation that affects mature lymphocytes, CLA should maintain the diversity of the TCR repertoire in peripheral naïve pre-CTLs and TCRγδCD8αα cells. Furthermore, the influence of CLA on progenitor cells is possibly originated in the bone marrow, which is the tissue with the highest concentration of CLA c-9, t-11/ t-9, c-11 isomers in rats (Li et al., 1998). A bone marrow effect of CLA is also supported by the increased percentages of peripheral mononuclear cells. And because the effect of CLA is focused on mononuclear cells, CLA might be targeting a lymphoid precursor population. Kondo et al. (1997) recently described a lymphoid precursor population lacking myeloid differentiation potential, which phenotypically is Lin⁻IL-7R⁺Thy⁻Sca^{lo}CD117^{lo}. If the in vivo expansion of peripheral T cell subsets has originated in the bone marrow and CLA is having the greatest impact on a lymphoid precursor population that could explain the increased numbers of progenitor cells in the thymus.

The peripheral expansion of pre-CTLs induced by CLA is of particular interest to us for three reasons: firstly, it shows that pre-CTLs are non-specifically expanded by a nutrient for several weeks. The expansion of that cell subset, being non-specific and having its origin

in progenitor cells and not on mature lymphocytes, would maintain the diversity of the T cell

repertoire. secondly. because the expansion of peripheral pre-CTLs is linked to an increase of

bone marrow-originated DN cells and subsequently of $CD8\alpha\beta^+$ thymocytes, which suggest an

effect of CLA on primary lymphoid organs, and, finally, because that modulation in $CD8^+$

cell subset phenotype caused by dietary CLA can be functionally linked to an enhancement in

cytotoxicity. From a functional perspective, we found that PBMCs isolated from animals fed

CLA, after anti-CD3 stimulation, had greater supernatant granzyme activities than did

PBMCs from pigs not fed CLA, regardless of the vaccination treatment. Our ex vivo finding

on cytotoxicity is consistent with in vitro data (Chew et al., 1997) indicating that lymphocyte

cytotoxicity was enhanced by CLA addition into the cell culture. Anti-CD3 addition to pre-

CTLs causes an increase in cytolytic activity or death via internal action of TNF (Wang et al.,

1994). Thus, either anti-CD3 acts on the peripheral naïve $TCR\alpha\beta CD8\alpha\beta$ T cells (pre-CTLs)

expanded in vivo by dietary CLA supplementation and induces activation and differentiation

to CTL and degranulation, or CLA causes a change in expanded pre-CTLs that renders them

less prone to undergo anti-CD3-induced apoptosis. Activated effector $TCR\alpha\beta CD8\alpha\beta$ T cells

and NK cells are granzyme producers and are involved in inducing apoptosis of target cells

presenting foreign epitopes presented to MHC class I (SLA complex class I, in pigs) through

the endogenous pathway of antigen presentation. Thus, they are involved in antiviral immune

responses and in tumor surveillance.

Although the composition of the $CD8^+$ lymphocyte population differs between animal

species, a functional convergence across species appears at the molecular level. Structurally,

the cytoplasmic domain of the $CD8\alpha$ chain is non-covalently associated to the cytoplasmic

tyrosine kinase $p56^{lck}$ (Sun et al., 1997), which suggests an involvement of $CD8\alpha$ in signal

transduction events. On the other hand, the likelihood for a role of the CD8β chain cytoplasmic domain in signal transduction seemed to be very low based on its length of only 19 amino acids and also considering that no protein binding motif has been identified within that amino acid sequence. However, the lack of the 19 amino acid cytoplasmic domain in CD8β chains interferes with the development of mature CD8 T cells (Itano et al., 1994). They also described that different class I-restricted TCRs differ in their dependence on the CD8β cytoplasmic tail. Recently, it has been suggested (Levelt et al., 1999) that the CD8β chain increases the sensitivity of the TCR by enhancing recruitment of p56^{Lck} to the alpha chain. Thus, both CD8α and CD8β chains play important roles in thymocyte development and activation of mature T cells. An important consequence of CD8αβ co-stimulation in the context of appropriate TCR/CD3 stimulus is degranulation of lytic granules, which play an important role in the destruction of target cells by effector CTLs.

NK cells are a part of the innate immunity and do not express the TCR/CD3 complex on their surface. Besides, thymic NK activity has not been described in pigs (Pinto and Ferguson, 1988). Therefore, NK cells are released from the bone marrow into the bloodstream together with immature precursors of T cells and B cells, but do not undergo positive and negative thymic selection. A direct effect of CLA on bone marrow precursors of CD8$^+$ lymphocytes would explain the increased numbers of NK cells and pre-CTLs in blood, and the increased percentages of CD8αβ$^+$ cells in the thymus. With regard to the effects of a bacterial antigen challenge in expanding NK cells and γδ T cells, it is interesting to note that NK cells are known not to be antigen specific, and γδ T cells recognize antigen in a different way than αβ T cells. Therefore, the mechanism by which a bacterial antigen expands these

two cell subsets remains unclear. A possible explanation is that NK cell subset expansion is a cytokine-driven phenomenon. Also, the impact of particular bacterial components, such as bacterial lipoproteins (BLPs), in regulating the innate immune response through NF-κB-activation and cytokine production (Aliprantis et al., 1999) could potentially influence peripheral NK cell numbers. There are no reports either confirming or denying the existence B. hyodysenteriae-derived BLPs. However, Borrelia burgdorferi like B. hyodysenteriae is a spirochete but, in the case of the causative agent of Lyme disease, BLPs have been identified that stimulate a type of immune response involving γδ T cells (Vincent et al., 1998). Also a lipoprotein lipase is expressed in NK cells and plays a role in their cytotoxic activity (Sanctis et al., 1994). We showed that CLA synergizes with vaccination to expand TCRγδCD8αα cells and NK cells. Thus, the effect of a spirochetal antigen on NK cells and γδ T cells expansion could be explained through the influence of BLPs on innate immunity. In that case, a lipase-digested compared with the a proteinase-digested B. hyodysenteriae vaccine preparation will help understand the effects of BLPs on cellular immunity.

Most γδ T cells are thymus-dependent, but a small population of thymus-independent γδ T cells, was followed for 2 years after thymectomy (Licence et al., 1995), which indicates the existence of a thymus-independent γδ T cell subset. Based on those ontogenic findings, the origin of the peripheral CLA-induced expansion of the TCRγδCD8αα cell subset, theoretically, can be explained in two different ways: either the CLA-induced increase in TCRγδCD8αα cells has a thymic origin or an extra-thymic origin. A thymic origin of that peripheral γδ T cell subset is not supported by our data, because TCRγδCD8αα thymocytes were not influenced by dietary CLA supplementation. γδ thymocyte development differs from

that on αβ thymocytes in the major proliferation of double negative thymocytes into

CD4CD8 DP immature thymocytes, that are then subject to positive and negative selection,

and the process of lineage (CD4 versus CD8) choice (Janeway, 1999). We have shown

increased DN thymocyte subsets but not in TCRγδCD8αα thymocytes. It is possible that a

greater αβ thymocyte proliferation might have masked the effects of CLA on γδ thymocytes.

The second way to explain why peripheral TCRγδCD8αα cell subsets were expanded but not

thymic CD8αα⁻ cells is considering an extra-thymic origin of CLA-expanded TCRγδCD8αα

lymphocytes. A GALT origin of that cell subset cannot be supported by the phenotypic

profiles of IELs and LPLs, which indicate that TCRγδCD8αα cells were not increased

because of dietary CLA supplementation. An extrathymic and GALT-dependent origin could

be explained assuming that some of the TCRγδCD8αα lymphocytes originated in the GALT

migrated to the periphery. An extrathymic and GALT-independent origin possibly implies

other MALT such as BALT and CALT in the production of that peripheral cell subset.

In summary, CLA is a complex nutrient that strongly influences phenotype and

function of the distinct CD8⁺ lymphocyte subsets. The knowledge of the impact of CLA on

NK cells, TCRγδCD8αα cells and CTLs will have important applications in both research on

the biology of CD8⁺ cells and, clinically, as a nutritional enhancer of CD8⁺ cells that could be

used either as a complement for infectious or tumoral vaccination protocols, which might be

particularly useful in patients with a suppressed cellular immunity. CLA could have an

influence in most assays of cellular immunity involving utilization of fetal calf serum,

because CLA represents between .5 and .9 % of fatty acids contained in fetal calf serum

(Park, 1996). Of particular interest is that CLA, a compound of known anticarcinogenic

activity, increases two of the cell subsets that perform crucial roles in tumor surveillance. The link between the anticarcinogenic and the immunomodulating properties of CLA was ruled out by Vissonneau et al. (1997) when they showed that CLA was anticarcinogenic in SCID mice. However, they did not use normal mice as controls to see if tumor growth inhibition was greater with normal mice fed CLA than in SCID mice fed CLA. Thus, they showed that there is a mechanism of anticarcinogenicity that is non-immune related, but they did not provide proofs capable of negating that there is indeed an immune-related mechanism. Several experiments relate CTLs and tumor regression. For instance, in mice, migration of CD8$^+$ T cells into ovarian carcinomas and melanomas was associated with tumor regression (Ogawa et al.. 1997), in humans receiving hapten-modified autologous tumor cell vaccines, lymphocytic infiltrates within regressing melanoma metastases are primarily comprised of CD8$^-$ cells (Sensi et al.. 1997). In humans with AIDS, autologous CD8$^+$ T cells activated with IL-2 play an important role in causing regression of Kaposi's sarcoma lesions (Klimas et al.. 1994). In mice. CD8$^-$ T cells are mediators of the anti-leukemic effects of IL-2 (Yang et al.. 1997). The demonstration of CLA involvement in in vivo expansion of cell subsets utilized in tumor surveillance (CTLs and NK cells) should be considered as a possible cellular basis of the mechanism of action of CLA as an anticarcinogen. Chew et al. (1997) indicated that the anticarcinogenic activity of CLA could be mediated through an enhancement of immune function based on in vitro data. Here we provide phenotypic and functional in vivo data to further support the theory that the anticarcinogenic effects of CLA are mediated by a modulation of immune cell phenotype and immune response.

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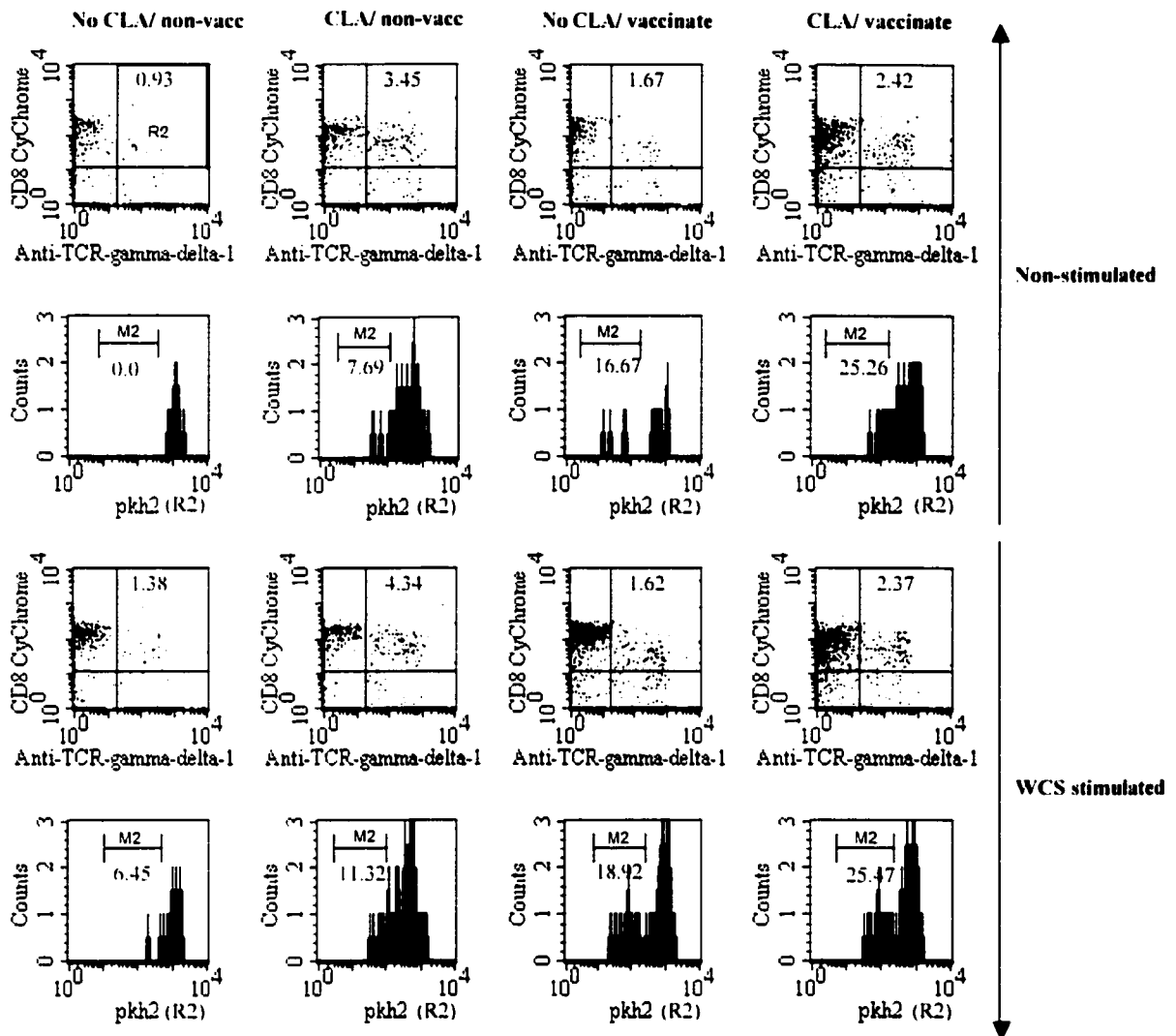
No CLA/ non-vacc   CLA/ non-vacc   No CLA/ vaccinate   CLA/ vaccinate

0.93   R2   3.45   1.67   2.42

CD8 CyChrome   Anti-TCR-gamma-delta-1   Anti-TCR-gamma-delta-1   Anti-TCR-gamma-delta-1   Anti-TCR-gamma-delta-1

Non-stimulated

M2   0.0   M2   7.69   M2   16.67   M2   25.26

Counts   pkh2 (R2)   pkh2 (R2)   pkh2 (R2)   pkh2 (R2)

1.38   4.34   1.62   2.37

CD8 CyChrome   Anti-TCR-gamma-delta-1   Anti-TCR-gamma-delta-1   Anti-TCR-gamma-delta-1   Anti-TCR-gamma-delta-1

WCS stimulated

M2   6.45   M2   11.32   M2   18.92   M2   25.47

Counts   pkh2 (R2)   pkh2 (R2)   pkh2 (R2)   pkh2 (R2)

Figure 1. Effect of CLA and vaccination on TCRγδCD8αα subset proliferation PBMCs from no CLA/non-vaccinated, CLA/non-vaccinated, no CLA/ vaccinated, and CLA/vaccinated pigs were stained with PKH2 and cultured with or without a WCS *B. hyodysenteriae* antigen. At d 6, cells were harvested and assayed for CD8α and TCRγδ surface expression. Non-viable cells were excluded using viability probes (not shown). The TCRγδCD8αα subpopulation within the cell viable gate (R2) was analyzed for PKH2 fluorescence intensity. M2 represents TCRγδCD8ααPKH2^{dim} cells (proliferative fraction).

CHAPTER 4. GENERAL CONCLUSIONS

General Discussion

Nutrition. along with disease status, genetics and management practices are the four

key factors that determine the productivity of livestock operations. For a long time, a greater

productivity was achieved by dietary antimicrobial supplementation at sub-therapeutical

levels. Consumers in Europe and United States demand the suppression of the latter practice.

Alternatives to the use of anti-microbials that are appealing to consumers should be able to

be characterized as safe, natural products with broad biological activity that can be

supplemented into dietary formulations. and that are lacked of antimicrobial connotations in

their label. CLA can be included in that categorization and. therefore. could be considered as

a possible consumer-accepted alternative to the use of antimicrobials.

I have described the effects of CLA on growth performance and body composition. In

my first experiment. I showed that. as the percentage of CLA in the diet increased there was

a decrease in body fat ($P<.07$) and. in phase II. pigs fed CLA had a linear increase in ADG

($P<.05$) and ADFI ($P<.02$). However. the focus of my dissertation. is on the cellular

immunomodulation induced by dietary CLA supplementation. which I have demonstrated for

the first time.

My experimental work has described the impact that CLA has on cell-mediated

immunity in swine. As a result of my investigations, we know which lymphocyte

subpopulations are increased as a result of dietary CLA supplementation (NK cells. CTLs

and TCR$\gamma\delta$CD8$\alpha\alpha$ cells). I have also developed the methodology and assays to evaluate the

interactions between CLA and a swine dysentery vaccine at the cellular level, which could be

easily applied to analyze the interactions with either vaccines against other infectious agents

or tumor vaccines. In the case of the swine dysentery vaccine. I have demonstrated that NK cells and TCRγδCD8αα cell subsets are in vivo expanded by vaccination. Therefore, I have further characterized the immunological response to this vaccine. All that has been done in the context of enhancing the understanding of porcine immunology and of how nutrition modulates immune function.

In summary. I initiated my research with the purpose of developing a nutritional technology capable of enhancing the health status in swine. therefore. the purpose was agricultural in origin. However. in the light of the importance of the findings described here. multiple non-agricultural applications could be derived from the better understanding of the role of CLA in cellular immunity e.g.. dietary supplement for immunosuppressed humans or companion animals: nutritional complement for certain vaccination protocols involving induction of CD8^{+} cells: technology to increase the peripheral lymphocyte pool in HIV^{+} humans: etc.

Recommendations for Future Research

The next step for this research would be to evaluate the cell populations most affected by CLA at a molecular level. A possible option to accomplish that objective would be to measure the impact of CLA on PPARs expressed in CD8^{+} subpopulations. In the light of the results of my research linking CLA and CD8^{+} cells. and considering the importance that PPARs might have in explaining that link. future experiments should be designed to better understand the role of PPARs in cellular immunology. and particularly. how CD8^{+} cells are regulated by PPARs. A possible approach to answer these questions would be to use murine models with altered PPAR expression. e.g. transgenic mice expressing high levels of PPAR-γ and PPAR-γ knockout mice might be good models to study how CD8^{+} cell phenotype and

function are linked to this orphan receptor. Genetically modified murine models could provide valuable hints with regard to the consequences of CLA-PPARs ligation on gene expression and on immune cell phenotype and immune response.

PPAR-γ ligands have been proposed as key elements for a novel therapy for inflammatory bowel disease (IBD) in humans. Research analyzing the relationship between CLA and gastrointestinal health could provide data indicating the potential utilization of CLA as an anti-inflammatory natural product that enhances gastrointestinal health and function. The potential for application of this nutritional anti-inflammatory technology is enormous in both animal agriculture (livestock gastrointestinal inflammatory diseases like swine dysentery) and human medicine (IBD). Individual isomers of CLA should be tested for their binding affinity to distinct PPAR.

From a whole animal perspective, no empirical evidences denying or suggesting an interaction between CLA and vitamin A are available. However the relationship between PPAR and retinoic acid receptors, described at the cellular level (Table 5) suggest that an interaction could occur. The development of a product consisting of a mixture of CLA or specific CLA isomers and vitamin A or specific vitamin A derivatives could potentially become a powerful modulator of cellular immunity.

The CLA-induced expansion of CTLs and NK cells, both involved in anti-tumor and anti-viral activities, could provide the cellular basis for the anticarginogenic properties of CLA. Future studies should analyze the efficacy of the CLA-induced changes in cellular immunity in inhibiting neoplasia formation, enhancing neoplasia regression and in enhancing the efficacy of vaccines developed against viruses.

Finally, other fatty acids might have similar properties than CLA. The identification

of these fatty acids could uncover unknown sources of broad biological like CLA. Based on

the fact that some conjugated linolenic acids (CLnA) (e.g., α-eleostearic acid, punicic acid,

α-parinaric acid), regardless of the configuration of the third double bond, have two double

bonds in the cis-9, trans-11 positions, the CLA isomer that is most abundant in the bone

marrow, might indicate the need to further characterize the biological effects of CLnA. These

conjugated trienes, similarly to what CLA does in cancer prevention, decreased the

development of chemically-induced cancer (Jacobson, 1953), also, similarly to the effects of

CLA in feed intake in laboratory animals, tung oil (a source of CLnA) suppressed food intake

in rats (McPherson et al., 1973). If conjugated trienes are PPAR ligands, additional research

looking at the interaction with vitamin A would also be required.

Although the main focus of this dissertation was CLA, the utilization of a spirochetal

vaccine to measure antigen-specific responses permitted a better understanding of the effects

of this vaccine on cellular immunity. Based on our findings, a future research on swine

dysentery vaccine development should evaluate separately the effects of the vaccine on NK

cells and TCR$\gamma\delta$CD8$\alpha\alpha$ cells, which are potentially induced by bacterial lipoproteins and the

effects on other T cell subsets that would be induced by the peptides found in the proteinase-

digested vaccine. A lipase-digestion of the bacterial preparation could potentially avoid the

effects of vaccination on innate immunity and facilitate the understanding of its effects on

adaptative immunity. Furthermore, the identification of large intestine bacteria that might

contribute to the pathogenesis of the disease, and the development of vaccines with antigens

from these bacteria should also be considered as a means to enhance vaccine efficacy.

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