Mucosal IgA increase in rats by continuous CLA feeding during suckling and early infancy

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Abstract The aim of this work was to establish the effect of the *cis*9,*trans*11 conjugated linoleic acid (CLA) isomer on mucosal immunity during early life in rats, a period when mucosal immunoglobulin production is poorly developed, as is also the case in humans. CLA supplementation was performed during three life periods: gestation, suckling, and early infancy. The immune status of supplemented animals was evaluated at two time points: at the end of the suckling period (21-day-old rats) and 1 week after weaning (28-day-old rats). Secretory IgA was quantified in intestinal washes from 28-day-old rats by ELISA technique. IgA, TGFβ, and PPARγ mRNA expression was measured in small intestine and colon by real time PCR, using Taqman® specific probes and primers. IgA mucosal production was enhanced in animals supplemented with CLA during suckling and early infancy: in 28-day-old rats, IgA mRNA expression was increased in small intestine and colon by approximately 6- and 4-fold, respectively, and intestinal IgA protein by ~2-fold. TGFβ gene expression was independent of age and type of tissue considered, and was not modified by dietary CLA. Gene expression of PPARγ, a possible mediator of CLA's effects was also upregulated in animals receiving CLA during early life. In conclusion, dietary supplementation with CLA during suckling and extended to early infancy enhances development of the intestinal immune response in rats.—Pérez-Cano, F. J., C. Ramírez-Santana, M. Molero-Luís, M. Castell, M. Rivero, C. Castellote, and À. Franch. **Mucosal IgA increase in rats by continuous CLA feeding during suckling and early infancy. *J. Lipid Res.* 2009.** 50: **467–476.**

Supplementary key words gestation • mucosal immunity • conjugated linoleic acid

For the newborn, birth is a transition from a sterile environment to a world full of microbes, where protection is crucial. Neonatal life is characterized by a heightened sensitivity to infectious agents, partly due to the lack of pre-existing immunological memory in newborns (1, 2). During the first stages of life, maternal antibodies transferred to the fetus and the child decrease the number of infectious episodes caused by micro-organisms to which the mother has developed immunological memory (1, 3). The mucosal immune system is in constant development because birth (4) and its function is profoundly influenced by maternal, environmental, dietary, and behavioral factors. Although the impact of these factors is greatest during the prenatal and immediate postnatal periods, their influence extends beyond this period. Patterns of development in postnatal life determine many of the immune outcomes in later life (5).

The intestinal immune system is the largest and most complex part of the immune system, and its responses are formed by an interplay of regulatory mechanisms that ensure the maintenance of gut homeostasis (6). Secretory IgA is the principal immunoglobulin on the surface of the mucosa (80–90%) and has the combined task of protecting against foreign substances and microbes, while not subjecting the mucosa to undue inflammation (7, 8). IgA present in milk is transferred to the offspring, conferring protection against mucosal pathogens to which the mother has been exposed (9). In rodents, IgM-secreting cells predominate at weaning and are a key component of the mucosal barrier, whereas IgA secreting cells are less abundant (4). The switch to IgA in the intestine, where it becomes the predominant Ig in this compartment, is directed by postweaning-related challenges before dietary intake. Over the last few years, the effect of nutrition on the development of the immune system has acquired great interest and has led to adoption of the term "immunonutrition." Because breast milk is the only natural food for infants, and dietary contact has a pivotal role in the

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development of the infant's intestinal immune system, the composition of breast milk, in particular of dietary lipids, should be studied with special attention. Human milk contains measurable quantities of conjugated linoleic acid (CLA), a class of positional and geometric conjugated dienoic isomers of linoleic acid, among which, *cis*9,*trans*11 (*c*9,*t*11) and *trans*10,*cis*12 (*t*10,*c*12) CLA predominate (10, 11). The concentration of CLA in milk is influenced by the intake of food of ruminant origin (12). Many biological effects are ascribed to CLA, including altered body composition and inhibition of carcinogenesis, atherosclerosis, and diabetes (13–15). Moreover, CLA isomer mixtures have been shown to modulate immune function in vitro and in vivo (16, 17). Results from these studies show great variability, partly because of differences in the experimental animal species used and the length of the studies, but also because of differences in the isomer mixtures used for supplementation. Recent studies carried out in young animals have shown that immune function is enhanced after feeding *c*9,*t*11 CLA isomer (18, 19). The immune effects of CLA have been also seen in healthy and ill individuals (20, 21). However, little work has been done on the effects of CLA during the early postnatal periods (lactation or infancy), and even less during the prenatal period (gestation).

Diet is known to have several effects on the development of the intestinal immune system, including gene regulation (22). Fatty acids can modulate the expression of a variety of genes coding for cytokines, adhesion molecules, and inflammatory proteins (23, 24). In vitro studies have shown that naturally occurring PUFAs and their metabolites, including CLA, are endogenous peroxisome proliferator-activated receptor (PPAR)-γ ligands (25, 26). PPARs (α, β/δ, and γ) are nuclear receptors that translate nutritional and/or pharmacologic stimuli into changes in gene expression (27). Initially, PPARs were identified as components of adipocyte gene expression (28, 29), but recent reports have described an effect of PPARs on several other biological events, including the pathogenesis of inflammatory bowel disease (30, 31). In addition, it is known that transforming grow factor β (TGFβ)-mediated signaling has a pivotal role in the stimulation of IgA responses at mucosal sites (32).

The aim of this study was to evaluate the effect of dietary supplementation during gestation, suckling, and early infancy with an 80:20 isomer mix of *c*9,*t*11 and *t*10,*c*12 CLA, respectively, on mucosal immunity (small intestine and colon) in Wistar rats, during periods in which their immune system is still in development. We quantified intestinal IgA at both the gene and protein levels as a biomarker of mucosal defense in neonates, and TGFβ and PPARγ mRNA expression as possible mediators of CLA's immunomodulatory effects.

MATERIALS AND METHODS

Animals

Pregnant Wistar rats at 7 days' gestation were obtained from Harlan (Barcelona, Spain). The animals were housed in individual cages under controlled temperature and humidity conditions in a 12 h/12 h light/dark cycle, and had access to food and water ad libitum. The rats were monitored daily and allowed to deliver naturally. The day of birth was identified as day 1 of life. Pups from different litters were randomized and unified to 10 pups per lactating dam, with free access to the nipples and rat diet. Animals were identified and weighed daily, and handling was done in the same time range to avoid the influence of biological rhythms. Studies were performed in accordance with the institutional guidelines for the care and use of laboratory animals established by the Ethical Committee for Animal Experimentation of the University of Barcelona and the Catalonian Government (CEEA 303/05, UB/DMA 3242).

Experimental design

Animals were distributed in eight experimental groups (20 animals each) according to the period of dietary CLA supplementation and age at the time immune status was assessed (21 or 28 days old). Total period of CLA supplementation (TPS) is shown in the experimental design.

Day 21 assessment. Pregnant rats were randomly assigned to one of the following four dietary groups, and pups from these groups were sacrificed at the end of the suckling period (21 days old):

21/G+Sd group. Pups from dams fed pellet diet supplemented with 1% CLA during the last 2 weeks of gestation (G) and throughout the suckling period. During suckling, pups received CLA through the milk of dam (Sd). TPS: 5 wk.

21/G+Sog group. Pups from dams fed 1% CLA diet during gestation (G) and standard diet (AIN-93G, Harlan) during suckling. During suckling, pups were CLA-supplemented daily by oral gavage (Sog). TPS: 5 wk.

21/Sog group. Pups from dams fed standard diet during gestation and suckling. Pups received CLA by oral gavage throughout the suckling period (Sog). TPS: 3 wk.

21/Ref group. Pups and dams fed standard diet throughout the study. TPS: 0 wk.

Day 28 assessment. On the day of birth, pups from dams fed standard diet during gestation were randomly assigned to one of the following four dietary groups. All dams were fed standard diet throughout the period of study. Pups from these groups were sacrificed 1 week after weaning (28 days old).

28/Sog+EI group. Pups received CLA by daily oral gavage during suckling (Sog); after weaning, animals were fed 1% CLA diet from day 21 to 28 (early infancy, EI). TPS: 4 wk.

28/Sog group. Pups received CLA by daily oral gavage during suckling (Sog); after weaning, animals were fed standard diet up to day 28. TPS: 3 wk.

28/EI group. Pups received 1% CLA diet exclusively for 1 week after weaning (day 21–28, EI). TPS: 1 wk.

28/Ref group. Pups fed standard diet during suckling and early infancy. TPS: 0 wk.

Dietary CLA supplementation

The standard diet used was AIN-93G (Harlan). Supplemented diet was prepared by adding 1% of a CLA isomer mixture con-

taining approximately 80% *c*9,*t*11 and 20% *t*10,*c*12, among the total of CLA isomers in the oil (Loders Croklaan, Lipid Nutrition, Wormerveer, The Netherlands). The CLA mixture had 0.69% free fatty acids as oleic acid, a peroxide value of 0.2 mEq/kg, and 5.6% saturated fatty acids. The 1% CLA (w/w) chow was produced in the Medicine Development Service of the Faculty of Pharmacy at the University of Barcelona. One percent of the total soybean oil was replaced with the same amount of CLA and added to AIN-513 standard flour. The mixture was pelletized (1 cm-diameter pellets) and dried in a 40°C oven for 24 h. The chow was vacuum-packed to prevent oxidation and contamination by fungi, and kept at 4°C until use. For humidity control, periodic tests were performed in an electronic humidity analyzer (Sartorius MA-45) for 15 min at 105°C, which showed 5% weight loss because of drying. Chow composition is shown in **Table 1**.

Assuming that rats ingest a daily amount of 15 g chow/100 g body weight, 1% CLA diet supplementation to suckling rats by oral gavage is equivalent to a daily volume of 1.5 mg CLA/g rat from day 1 to 21. Low-capacity syringes (Hamilton Bonaduz AG, Bonaduz, Switzerland) adapted to oral 25- or 23-gauge gavage tubes, 27 mm in length (ASICO, Westment, IL) were used for oral administration before and after day 5, respectively. To allow gastric emptying, litters were separated from dams 1 h before oral supplementation.

Extraction of small intestine and colon

Pups aged 21 and 28 days were euthanized by humanitarian methods, and the small intestine and colon were removed from all animals. A maximum of 30 mg of tissue was obtained, corresponding to the distal ileum and proximal colon. Specimens were flash-frozen in liquid N_2 and stored immediately at −80°C until processing. The distal portion of small intestine from 28-day-old animals was weighed, longitudinally opened, cut in 5-mm pieces, and incubated with PBS in a shaker for 20 min at 37°C. The suspension obtained was centrifuged and the supernatant (intestinal wash) was stored at −20°C until IgA quantification by ELISA technique.

TABLE 1. Composition of the experimental diets (g/kg of diet)

Ingredient	AIN-93G	1% CLA
Casein	200	199.98
L-cysteine	3	2.99
Corn starch	397.486	397.545
Maltodextrin	132	131.98
Sucrose	100	99.99
Cellulose	50	49.99
Mineral mix[a]	35	34.99
Vitamin mix[b]	10	9.99
Choline bitartrate	2.5	2.499
Ter-butylhydroquinone[c]	0.014	0.014
Soybean oil	70	59.99
80:20 c9,t11:t10,c12 CLA oil[d]	–	10

[a] Supplied per kg of diet: 357 g calcium carbonate, 196 g potassium phosphate monobasic, 70.78 g potassium citrate, 74 g sodium chloride, 46.6 g potassium sulfate, 24.3 g magnesium oxide, 6.06 g ferric citrate, 1.65 g zinc carbonate, 0.63 g manganous carbonate, 0.31 g cupric carbonate, 0.01 g potassium iodate, 0.01025 g sodium selenate, 0.00795 g ammonium paramolybdate, 1.45 g sodium meta-silicate, 0.275 g chromium potassium sulfate, 0.0174 g lithium chloride, 0.0815 g boric acid, 0.0635 sodium fluoride, 0.0318 g nickel carbonate, hydroxide, tetrahydrate, 0.0066 g ammonium vanadate, and 220.716 g sucrose.

[b] Supplied per kg of diet: 3 g nicotinic acid, 1.6 g calcium pantothenate, 0.7 g pyridoxine HCl, 0.6 g thiamin HCl, 0.6 riboflavin, 0.2 g folic acid, 0.02 g D-biotin, 2.5 g vitamin B12 (0.1% in mannitol), 15 g DL-α tocopherol acetate (500 IU/g), 0.8 g vitamin A palmitate (5000,000 IU/g), 0.2 g vitamin D3 (cholicalceferol, 5000,000 IU/g), 0.075 g vitamin K (phylloquinone), and 974.705 g sucrose.

[c] Antioxidant.

[d] In the total oil, 78% corresponded to conjugated linoleic acid (CLA) in triacylglycerol form, containing an isomer ratio of 80:20 *c*9, *t*11 and *t*10,*c*12, respectively.

ELISA IgA quantification

Ninety-six-well polystyrene plates (Nunc Maxisorp, Wiesbaden, Germany) were coated with anti-rat IgA monoclonal antibody (mAb) (2 μg/ml, BD Pharmingen, San Diego, CA) in PBS overnight in a humidified chamber. The remaining binding sites were blocked with PBS-1% BSA for 1 h at room temperature (RT). Plates were then washed (3× with PBS-0.05% Tween and once with PBS), and appropriate samples and standard IgA (BD Pharmigen) dilutions in PBS-Tween-1% BSA were added and incubated (3 h, RT). Plates were washed again and incubated (2 h, RT) with biotinylated anti-rat IgA mAb (BD Pharmigen) at 0.0625 μg/ml. Subsequently, extravidin-peroxidase conjugate (SIGMA, 4 μg/ml in PBS-Tween-1% BSA) was added and plates were incubated for 30 min at RT. IgA was detected by addition of the substrate solution (o-phenylenediamine dihydrochloride plus H_2O_2 in 0.2 mol/L phosphate-0.1 mol/L citrate buffer, pH 5). The enzyme reaction was stopped with H_2SO_4 3M and absorbance was measured at 492 nm. Data were interpolated into the IgA standard curve, and IgA concentrations were expressed as ng/gut weight (g).

Assessment of mRNA gene expression

Tissue homogenization and RNA isolation were performed as previously described, with some modifications (33). Briefly, tissue samples were homogenized (Polytron R, Kinematica, Switzerland) with 1 ml of TRI Reagent™ (Sigma) and then centrifuged (12,000 *g*, 10 min, 4°C) to remove insoluble material. RNA was isolated and redissolved in 100 μl of H20-DEPC (Diethyl pyrocarbonate, Sigma). A Nanodrop spectrophotometer and Nanodrop IVD-1000 v.3.1.2 software (Nanodrop Technologies, Wilmington, DE) were used to quantify the amount of RNA obtained. The observation of two sharp bands in a denaturing electrophoresis gel, corresponding to 18S and 28S rRNA, allowed us to evaluate RNA integrity.

Two hundred ng of total RNA was converted to cDNA in a thermal cycler PTC-100 using random hexamers [Applied Biosystems (AB) Weiterstadt, Germany] (MJ Research, Waltham, MA). A final volume of 1 μL was used to confirm the reaction of each sample by conventional PCR using rat β-actin primers and conditions previously established in our laboratory (34).

PCR Taqman® primers and probes were specific for rat IgA, and the TGFβ and PPARγ genes. glyceraldehyde dehydrogenase and β-actin genes were employed as endogenous controls (Assays on Demand,™ Gene Expression Products, AB). The PCR was performed in the ABI Prism 7000 detection system (AB) The comparative Ct method was used for relative quantification of gene expression. The amount of target mRNA, normalized with an endogenous control (glyceraldehyde dehydrogenase and β-actin) and relative to a calibrator (tissue samples from 21/Ref and 28/Ref for 21- and 28-day-old animals, respectively), is given by $2^{-\Delta\Delta Ct}$, where Ct is the cycle number at which the fluorescence signal of the PCR product crosses an arbitrary threshold set within the exponential phase of the PCR, and $\Delta\Delta Ct = [(Ct_{\text{target (unknown sample)}} - Ct_{\text{endogenous control (unknown sample)}})] - [(Ct_{\text{target (calibrator sample)}} - Ct_{\text{endogenous control (calibrator sample)}})]$. Results are expressed as the mean ± SEM of the percentage of these values for each experimental group compared with its reference age group, which represents 100% gene expression.

Evaluation of histological specimens

For histological evaluation of colon and small-intestine tissue, a 2 cm portion of each was removed and fixed in 10% formalde-

hyde. Five paraffin sections from each rat of the highest supplemented groups (21/G+Sd and 28/Sog+EI with TPS 5 wk and 4 wk, respectively) and age-matched control groups (21/Ref and 28/Ref, both with TPS 0 wk) were stained with hematoxylin-eosin using standard techniques (35). Grading was determined upon microscopic analysis of cross-sections of the colon and small intestine based on signs of disruption of normal bowel architecture. Evaluation was performed by two experienced and blinded examiners.

Statistical analysis

SPSS 10.0 (SPSS Inc., Chicago, IL) was used for the statistical analysis by conventional one-way ANOVA considering the experimental group as an independent variable. When CLA supplementation had a significant effect on the dependent variable, the Least Significant Differences test was applied. Significant differences were accepted at $P < 0.05$.

RESULTS

Animal growth

Body weight from dams and pups was monitored daily throughout the study. Supplementation with 1% CLA during gestation, suckling, and/or early infancy did not modify the body weight increase of dams or pups. All groups showed a similar growth pattern compared with the age-matched reference group: 21/Ref, ~45–52 g and 28/Ref, ~72–88 g. There were no deaths in any of the study groups.

Gene expression of IgA, TGFβ, and PPARγ in small intestine and colon

Small intestine and colon RNA were obtained from five animals per group and converted to cDNA to assess gene expression by real-time PCR. Target genes (IgA, TGFβ, and PPARγ) were quantified relative to β-actin, an endogenous housekeeping gene. **Fig. 1** shows the small intestine and colon gene expression results from reference animal samples (21/Ref and 28/Ref groups). PPARγ expression levels were similar in the two age groups but were higher in small intestine tissue than in colon (Fig. 1C, $P < 0.05$). TGFβ mRNA expression was independent of age and tissue (Fig. 1A). IgA expression was very low during suckling, although the levels increased after weaning ($P < 0.05$); no differences were found for tissue distribution (Fig. 1C).

CLA dietary supplementation and expression of constitutive genes

Because two different endogenous housekeeping genes were used, the first step was to demonstrate their constitutive expression in all animals, regardless of the dietary CLA supplementation they received. No differences were detected in the GADH/β-actin ratio among the groups, in either 21- or 28-day-old animals (~1 in all groups), independently of the administration period (suckling and/or early infancy) or the total period of CLA supplementation (5, 4, 3, 1, or 0 weeks).

Effect of CLA on PPARγ gene expression in small intestine and colon

PPARγ gene expression in small intestine and colon was assessed at the end of the suckling period (day 21) and 1 week after weaning (day 28) (**Fig. 2**). At both ages, there were no differences in PPARγ gene expression in small intestine between CLA and nonsupplemented groups (Figs. 2A, C). However, PPARγ was up-regulated in colon tissue, particularly in 21-day-old animals fed CLA (21/G+Sd, 21/G+Sog, and 21/Sog), when compared with reference animals. This 2-fold up-regulation was only significant in the 21/G+Sd group ($P < 0.05$). Similar to the results found in 21-day-old animals, colon PPARγ gene expression was also up-regulated in 28-day-old animals fed CLA (28/Sog+EI, 28/Sog, 28/EI), when compared with reference animals. Nevertheless, only the 28/Sog and 28/EI groups showed statistical differences ($P < 0.05$), since 28/Sog+EI group showed a great variability (Fig. 2D).

Effect of CLA on TGFβ gene expression in small intestine and colon

TGFβ gene expression was similar among the groups during early life; no statistical differences were found concerning age, tissue, or duration of CLA supplementation. Thus, CLA dietary supplementation during suckling

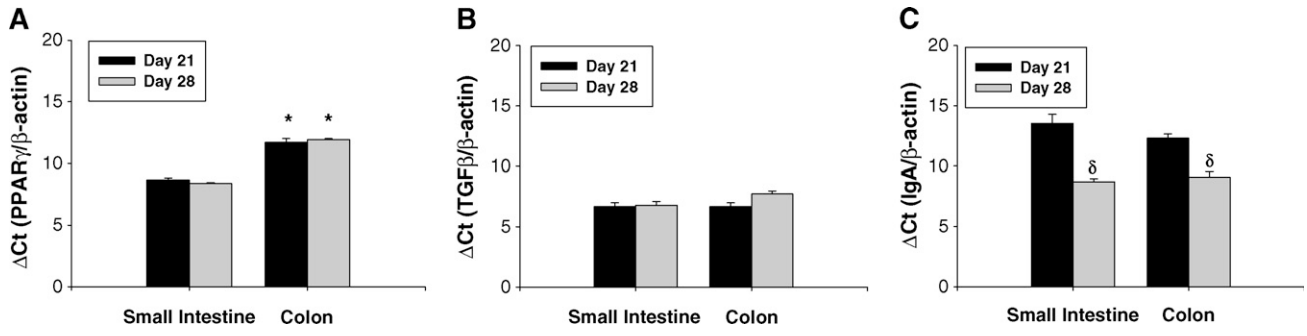

Fig. 1. PPARγ, TGFβ, and IgA gene expression in small intestine and colon. Expression levels of PPARγ (A), TGFβ (B), and IgA (C) genes in small intestine and colon of reference animals are expressed relative to β-actin, an endogenous housekeeping gene. Levels are shown as ΔCT, thus lower bars mean higher gene expression than taller bars. Results correspond to the mean ± SEM of five animals per group. Statistical differences: * $P < 0.05$ colon vs. small intestine, and $^{\delta}P < 0.05$ day 28 vs. day 21.

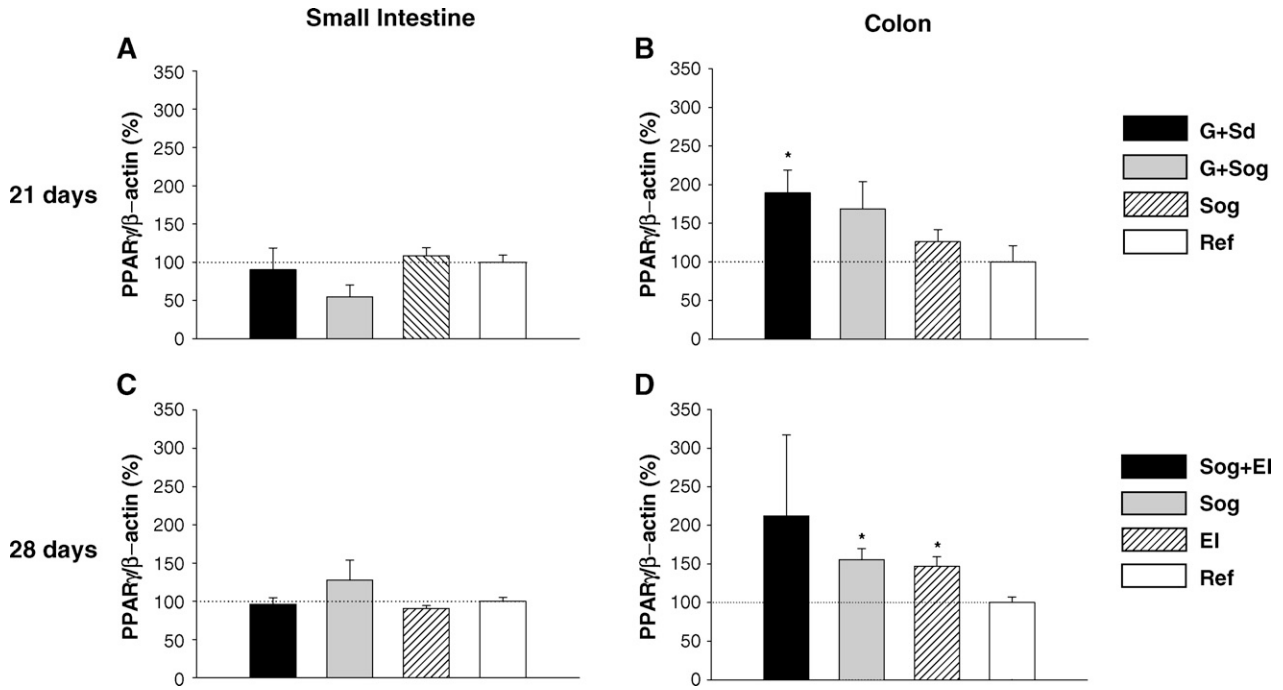

Fig. 2. Effect of conjugated linoleic acid (CLA) on PPARγ gene expression in 21- and 28-day-old animals. The 21-day experimental design establishes four groups according to the period of life and total period of supplementation (TPS) as follows: 21/G+Sd: CLA supplementation during gestation and suckling from dams (TPS 5 wk). 21/G+Sog: CLA supplementation during gestation and suckling by oral gavage (TPS 5 wk). 21/Sog: CLA supplementation during suckling by oral gavage (TPS 3 wk). 21/Ref: Nonsupplemented animals (TPS 0 wk). Gene expression was evaluated in small intestine (A) and colon (B). The 28-day experimental design establishes four groups according to the period of life and TPS as follows: 28/Sog+EI: CLA supplementation during suckling by oral gavage and during early infancy through chow (TPS 4 wk). 28/Sog: CLA supplementation during suckling by oral gavage (TPS 3 wk). 28/EI: CLA feeding through chow during early infancy (TPS 1 wk). 28/Ref: Nonsupplemented animals (TPS 0 wk). Gene expression was evaluated in small intestine (C) and colon (D). PPARγ gene levels were normalized using the gene β-actin and are expressed as percentage relative to values from age-matched reference animals (21/Ref or 28/Ref). Results correspond to the mean ± SEM of five animals per group. Statistical differences: * $P < 0.05$ vs. age-matched reference group.

and/or early infancy failed to modulate mRNA levels of TGFβ (**Fig. 3**).

Effect of CLA on IgA gene expression in small intestine and colon

Dietary CLA did not modify IgA gene expression in small intestine or colon at the end of the suckling period (**Fig. 4A, B**). However, IgA gene expression in animals continuously CLA-supplemented during suckling and early infancy (1 wk after weaning, 28/Sog+EI) was up-regulated almost 5-fold. This increase was seen in both tissues analyzed as compared with the 28/Ref group ($P < 0.05$) (Fig. 4C, D). Supplementation limited to the suckling or early infancy periods failed to produce this immune-enhancing effect.

Effect of CLA on secretory IgA in the small ontestine

In addition to detection of changes in gene expression, IgA concentration was quantified in intestinal washes of 28-day-old animals (**Fig. 5**). IgA content was statistically higher in animals CLA-supplemented for 4 weeks, first by oral gavage (Sog, 3 wk) and later through the solid diet (EI, 1 wk), than in animals in the 28/Sog, 28/EI or 28/Ref groups ($P < 0.05$). These results demonstrate that CLA dietary supplementation during early life increases expression of the IgA gene and protein, thereby enhancing development of the rat's IgA defense system.

Effect of CLA on the microscopic analysis of colon and small intestine

Representative histological slides show no dietary-related microscopic findings in any tissue examined (**Fig. 6**). There was no evidence of aberrant crypt foci or bowel architecture disruption in the colon or small intestine examinations of the control and CLA-supplemented animals.

DISCUSSION

The mucosal immune system of the rat continues developing during the suckling period and early infancy, and, as occurs in humans, mucosal Ig production is poor. Previous studies have shown that IgM production by lamina propia cells begins during the second week of life in parallel to the phenotypic development of B cells in rat intestine, and later, weaker production of IgA initiates (4). In keeping with this pattern of IgA development, we found that intestinal specimens from 21-day-old animals (end of

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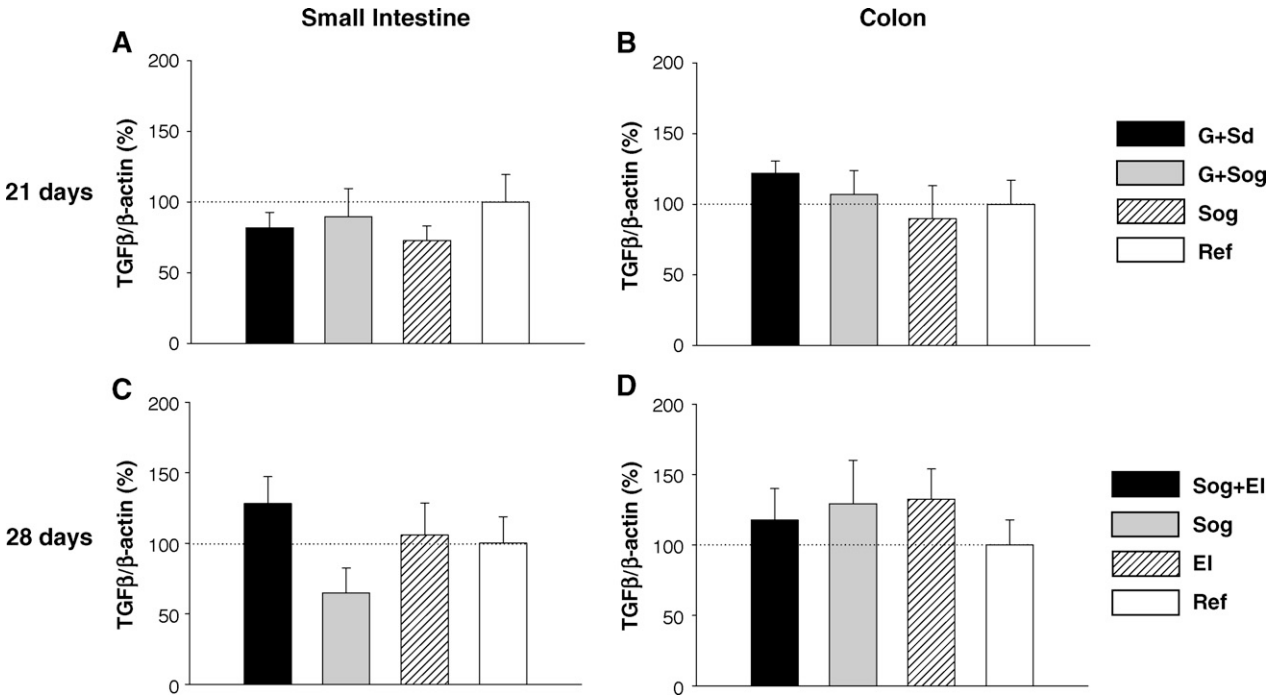

Fig. 3. Effect of CLA on TGFβ gene expression in 21- and 28-day-old animals. The 21-day experimental design establishes four groups according to the period of life and total period of supplementation (TPS) as follows: 21/G+Sd: CLA supplementation during gestation and suckling from dams (TPS 5 wk). 21/G+Sog: CLA supplementation during gestation and suckling by oral gavage (TPS 5 wk). 21/Sog: CLA supplementation during suckling by oral gavage (TPS 3 wk). 21/Ref: Nonsupplemented animals (TPS 0 wk). The gene expression was evaluated in small intestine (A) and colon (B). The 28-day experimental design establishes four groups according to the period of life and total period of supplementation (TPS) as follows: 28/Sog+EI: CLA supplementation during suckling by oral gavage and during early infancy through chow (TPS 4 wk). 28/Sog: CLA supplementation during suckling by oral gavage (TPS 3 wk). 28/EI: CLA feeding through chow during early infancy (TPS 1 wk). 28/Ref: Nonsupplemented animals (TPS 0 wk). Gene expression was assessed in small intestine (C) and colon (D). Levels of the TGFβ gene were normalized using β-actin and are expressed as percentage relative to values from age-matched reference animals (21/Ref or 28/Ref). Results correspond to the mean ± SEM of five animals per group.

suckling period) presented a lower IgA concentration than specimens from animals a week older (28-day-old weaned rats). Moreover, IgA expression was similar in small intestine and colon, in both 21- and 28-day-old animals, suggesting a similar distribution of IgA plasma cells along the intestinal tissue.

Growing evidence from experimental studies has indicated that CLA enhances the humoral immune response. In 7-week-old rats fed a 1% CLA (50:50 isomer) mix, Sugano et al. (16) reported increases in serum IgA, IgG, and IgM concentrations and a drop in IgE. Song et al. (36) reported a similar effect in humans after supplementation with a 50:50 CLA isomer mixture for 12 wk. However, Yamasaki et al. (37) found no significant effects on serum IgA, IgG, or IgM concentration after feeding 5-wk-old rats a 50:50 CLA isomer mixture for 3 wk at doses ranging from 0.05% to 0.5% (38). Other studies have additionally suggested that the effects of CLA intake during developmental phases are manifested beyond the supplementation period (30). A study carried out in another species during gestation and lactation has reported serum IgG increase (39), a fact that supports humoral enhancement effects of CLA in early age.

This is the first in vivo report, to our knowledge, showing an increase of mucosal IgA after feeding CLA during early life. This IgA increase was manifested by greater gene expression and higher protein values in 28-day-old animals. Moreover, higher protein values cannot be attributed to bowel disruption, since the architecture of the small intestine and colon of 28- as well as 21-day-old animals was preserved regardless of the diet. The immunomodulator effect on IgA gene expression was seen in the group supplemented with CLA for a longer period, during suckling and early infancy (TPS 4 wk); nonetheless, higher IgA concentration was observed in intestinal washes of Sog+EI and Sog groups. Thus, early, continuous CLA supplementation has important influences on immune response during infancy. In line with our data, Sugano et al. (16) reported an increase in IgA secretion from cultured mesenteric lymph nodes of 7-week-old rats, fed a 1% CLA 50:50 isomer mix.

The specific mechanism by which CLA enhances IgA levels at mucosal sites remains unknown. But since CLA has been shown to suppress IL-4 production in vitro (39), attenuate Th2 responses in challenged animals (40), and regulate the number and effectors functions of several lymphocytes (41), further studies should be addressed to elucidate whether there exists a direct enhancer mechanism of CLA on IgA-producing cells.

Because IgA gene expression and intestinal production was found to increase after feeding CLA, we also studied

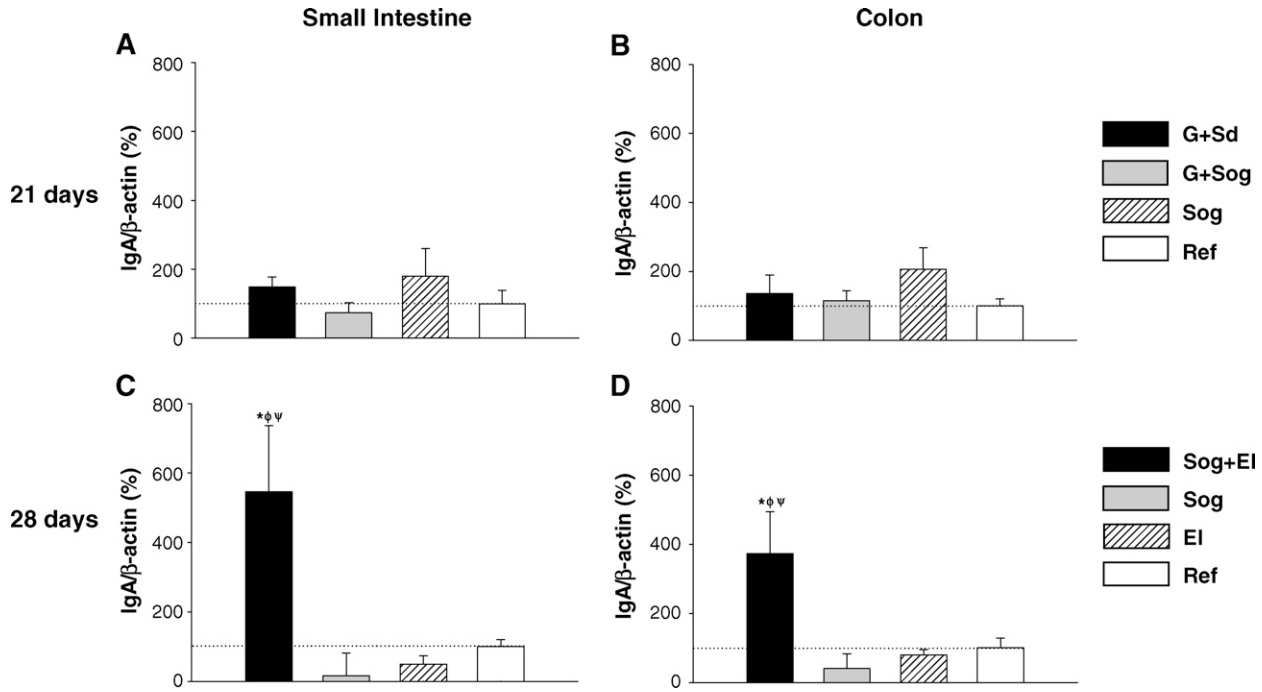

Fig. 4. Effect of CLA on IgA gene expression in 21- and 28-day-old animals. The 21-day experimental design establishes four groups according to period of life and total period of supplementation (TPS) as follows: 21/G+Sd: CLA supplementation during gestation and suckling from dams (TPS 5 wk). 21/G+Sog: CLA supplementation during gestation and suckling by oral gavage (TPS 5 wk). 21/Sog: CLA supplementation during suckling by oral gavage (TPS 3 wk). 21/Ref: Nonsupplemented animals (TPS 0 wk). Gene expression was evaluated in small intestine (A) and colon (B). The 28-day experimental design establishes four groups according to period of life and total period of supplementation (TPS) as follows: 28/Sog+EI: CLA supplementation during suckling by oral gavage and during early infancy through chow (TPS 4 wk). 28/Sog: CLA supplementation during suckling by oral gavage (TPS 3 wk). 28/EI: CLA feeding through chow during early infancy (TPS 1 wk). 28/Ref: Nonsupplemented animals (TPS 0 wk). Gene expression was evaluated in small intestine (C) and colon (D). Levels of the IgA-gene were normalized using β-actin and are expressed as percentage relative to values in age-matched reference animals (21/Ref or 28/Ref). Results correspond to the mean ± SEM of five animals per group. Statistical differences: * $P < 0.05$ vs. an age-matched reference group (21/Ref or 28/EI); $^{\phi}$ $P < 0.05$ vs. 28/EI; $^{\Psi}$ $P < 0.05$ vs. 28/Sog.

TGFβ gene expression because of its involvement in the isotype switching process from IgM to IgA (32, 42). Intestinal TGFβ gene expression showed a similar pattern in 21- and 28-day-old rats, without specific differences between tissue types, showing that the dietary change produced at weaning does not modify expression of this regulatory molecule at the mucosal level. mRNA levels of this gene were not modified in any of the CLA-supplemented groups during gestation, suckling and/or early infancy. Nonetheless, an influence of CLA on TGFβ cannot be ruled out. If CLA is modulating the effects of TGFβ on IgA production, it is probably due to posttranscriptional and/or translational regulation, which are important in this cytokine, because it has been suggested that TGFβ mRNA levels do not completely correlate with the quantity of protein produced (43). On the other hand, the increase of IgA as result of CLA supplementation might be independent of the isotype switching mechanism produced by TGFβ, which has been described, but is not completely defined (44).

As to CLA immunomodulation, two main mechanistic theories have been proposed to explain the immunoenhancing effects of dietary CLA: a PPARγ-dependent and a PPARγ-independent pathway (26). The present study investigates for the first time PPARγ gene expression in the small intestine and colon of 21- and 28-day-old animals fed standard and CLA-supplemented diets. Animals fed standard diet showed similar levels of PPARγ expression at the two ages; however, tissue-specific differences were detected. PPARγ was more highly expressed in small intestine than in colon. This finding is in line with the results reported by Braissant et al. (45), who showed higher PPARγ expression in small intestine than colon of adult Sprague-Dawley rats, but contrasts with other studies showed higher expression of the gene in colon than small intestine (46, 47); still others have focused more on the action of colon PPARγ (30, 48). The discrepancies could be due to differences in expression patterns between species.

CLA-supplemented infant rats showed higher PPARγ expression than nonsupplemented animals. Specifically, the effects were seen in colon from both 21- and 28-day-old animals in a dose-dependent manner that was proportional to the duration of supplementation over gestation, suckling, or early infancy. Thus, CLA modulated PPARγ expression in all the dietary conditions examined, even when animals were supplemented for only 1 week. These results concur with findings from studies showing an increase of PPARγ mRNA expression associated with CLA supplementation in colon of healthy and ill mice and pigs

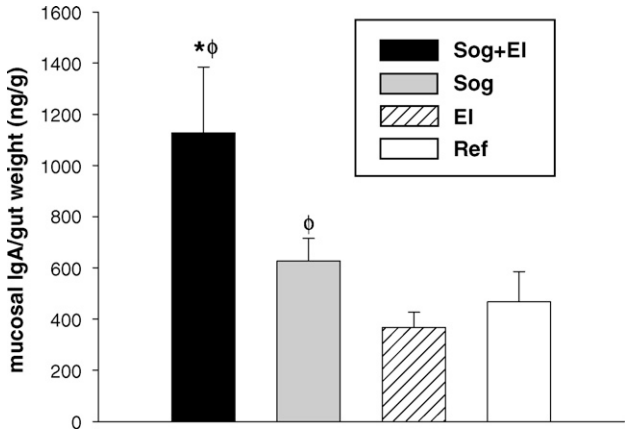

Fig. 5. Effect of CLA on IgA in intestinal washes from 28-day-old Wistar rats. IgA concentration in intestinal washes was quantified in the following groups according to period of life and TPS: 28/Sog+EI: CLA supplementation during suckling by oral gavage and during early infancy through chow (TPS 4 wk). 28/Sog: CLA supplementation during suckling by oral gavage (TPS 3 wk). 28/EI: CLA feeding through chow during early infancy (TPS 1 wk). 28/Ref: Nonsupplemented animals (TPS 0 wk). Results are expressed as IgA protein (ng) referred to intestinal weight (g) used for the wash. Results correspond to the mean ± SEM of 9–10 animals per group. Statistical differences: * $P < 0.05$ 28/Sog+EI vs. 28/Sog, 28/EI and 28/Ref; $^{\phi}$ $P < 0.05$ vs. 28/EI.

(26, 30, 31, 40). Moreover, PPARγ upregulation by CLA is in line with the results of Takamura et al. (49), who showed that specific natural or synthetic ligands of PPARγ can induce a mean 2- to 3-fold expression of this receptor in a positive feedback loop. In vitro studies have also indicated that the PPARγ activating capabilities of CLA are cell type-dependent and isomer specific (26).

PPARγ comprises two isoforms, PPARγ1 and PPARγ2. Both are expressed in adipocytes, but PPARγ1 is expressed in T and B cells, monocytes, dendritic cells, and epithelial cells (50–52). Hence, the effects of CLA found in the present study may be due to the interaction of CLA with PPARγ. There are several possible options through which CLA might act. First, although a direct relationship between PPARγ increase and IgA gene expression has not been described, Ponferrada et al. (53) recently reported that PPARγ agonists can revert stress-induced decrease of IgA production in the colon mucosa, even beyond the IgA-controlled basal concentration. Moreover, it seems that this nuclear receptor acts through modulation of transcriptional factors such as NF-κB, AP1, and STAT1 (48, 54), which are involved in B-cell regulatory processes.

Second, recent research has also indicated close links between intestinal-microbial interactions and regulation of PPARγ expression by epithelial cells of colon tissue (55), where we detected CLA-mediated induction. It seems likely that enhancement of PPARγ expression by microorganisms has a multifactorial mechanism that includes agonistic actions mediated by PUFA generated by the commensal flora (55), and LPS recognition by toll-like receptor 4 (TLR4) in activated epithelial cells (56). In this sense, we can suggest that CLA may influence the natural mechanisms involved in intestinal homeostasis regulation at this age through PPARγ up-regulation.

Lastly, it has been demonstrated that PPARγ regulates the epithelial differentiation process (47). Thus, CLA may be modulating the entry of luminal antigen, the capacity for direct antigenic presentation, or even the transmission of antigen to dendritic cells from the intestinal mucosa. These hypotheses are supported by the fact that dendritic cell immunogenicity is regulated by PPARγ (51).

In summary, CLA dietary supplementation increases the intestinal immune defenses of Wistar rats during the first stages of life. CLA-dependent enhancement of humoral mucosal immune response was demonstrated by the striking

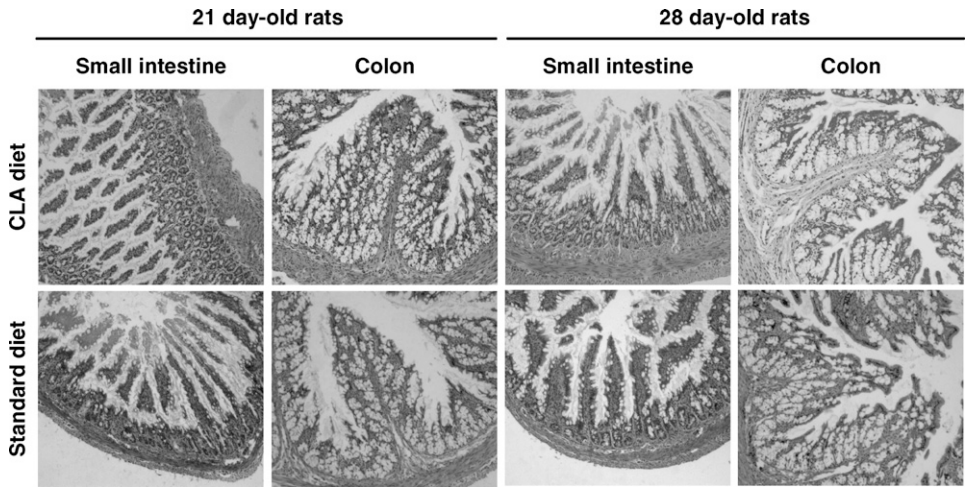

Fig. 6. Effect of CLA on bowel architecture of 21- and 28-day-old Wistar rats. Microscopic analysis was assessed in the following groups: 28/Sog+EI: CLA supplementation during suckling by oral gavage and during early infancy through chow (TPS 4 wk), 21/G+Sd: CLA supplementation during gestation and suckling from dams (TPS 5 wk), 21/Ref and 28/Ref: Nonsupplemented animals (TPS 0 wk). Results are representative hematoxylin-eosin-stained slides from colon and small intestine of CLA and control rats (×100). All specimens show preserved bowel architecture.

increase of intestinal IgA expression in 28-day-old rats fed CLA for 4 weeks during early life. Moreover, this study shows that PPARγ gene expression levels were up-regulated in a supplementation period-dependent manner. Thus, it is clearly shown that the effects of CLA are more pronounced the earlier and more long-lasting CLA dietary supplementation. Although further studies should be developed to define the mechanism of action CLA, the data reported herein provide further scientific evidence of the impact of lipid nutrition, particularly the influence of the *cis*9,*trans*11-CLA isomer, on immunomodulation.

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