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EFECTO DEL ÁCIDO LINOLEICO CONJUGADO EN EL DESARROLLO DEL SISTEMA INMUNITARIO EN RATAS

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RESULTADOS

ARTÍCULO 1

Respuesta inmunitaria humoral en ratas suplementadas con CLA durante la gestación y lactancia

Humoral immune response in CLA-supplemented rats during gestation and suckling

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- En presentación oral: *Potentiation of systemic humoral immune response in suckling rats by Conjugated Linoleic Acid (CLA)*, en "First International Immunonutrition Workshop", Valencia, 3-5 Octubre, 2007.

RESUMEN

Objetivo: Evaluar el efecto del suplemento dietético de CLA (mezcla 80:20 de los isómeros *cis*9,*trans*11 y *trans*10,*cis*12), durante la gestación y lactancia, sobre la respuesta inmunitaria sistémica de ratas Wistar al final del período de lactancia (21 días de edad).

Material y métodos: Se dispuso de 4 grupos experimentales, de los cuales tres de ellos fueron suplementados con CLA. Dos grupos recibieron CLA durante un período total de 5 semanas. En estos grupos CLA llegó a las crías vía materna durante el período de gestación a través de la placenta (2 semanas) y en la lactancia (3 semanas) a través de la leche materna (grupo 21/5) o por sonda oral (21/5'). El tercer grupo recibió CLA únicamente durante la lactancia por sonda oral (21/3). El grupo 21/Ref no recibió el suplemento dietético. A lo largo del estudio se determinaron variables morfométricas tales como peso, índice de masa corporal, entre otras. Al final del estudio (21 días de edad) se cuantificó el contenido de CLA en leche de las madres y plasma de las crías. A partir de la leche se obtuvo suero lácteo en el cual se cuantificaron IgA, IgG e IgM, al igual que en suero procedente de las crías. Por otra parte, se aislaron esplenocitos para determinar su capacidad de producción de IgG e IgM y citocinas *in vitro*, además de la respuesta linfoproliferativa inducida por mitógeno.

Resultados: Al final del estudio, tanto el peso de las madres como el de las crías no se modificaron tras recibir el suplemento de CLA. La leche de las madres que recibieron CLA presentó mayor contenido de CLA y mayor concentración de IgA e IgG que la de madres alimentadas con dieta estándar. El plasma de las crías de los grupos 21/5, 21/5' y 21/3 presentó 6, 12 y 9 veces mayor contenido del isómero *cis*9,*trans*11 de CLA que el plasma de las crías del grupo 21/Ref. Las ratas suplementadas durante la gestación y lactancia a través de leche materna (21/5) mostraron mayor concentración sérica de IgG que las del resto de grupos (22,14 $\pm$ 2,14 *vs* ~ 5 mg/mL, $p<0,05$). Además, los animales de los grupos suplementados durante la gestación y lactancia (21/5 y 21/5') presentaron aproximadamente el doble de producción de IgM esplénica que las de los grupos 21/3 y 21/Ref. Por el contrario, la suplementación con CLA no modificó de forma significativa la respuesta linfoproliferativa esplénica ni la secreción de citocinas *in vitro*.

Conclusión: La suplementación dietética durante la gestación y lactancia con una mezcla 80:20 de los isómeros de CLA *cis*9,*trans*11:*trans*10,*cis*12 incrementa la producción de los isotipos mayoritarios de inmunoglobulinas *in vitro* e *in vivo* en ratas Wistar.

Immunoglobulin production by CLA-supplemented rats during gestation and suckling

Running title: Ig production in CLA-supplemented suckling rats

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ABSTRACT

The conjugated linoleic acid (CLA) has been reported to exert beneficial physiological effects on body composition and immune system. However, little information is available on the influence of CLA on immune function during early life periods. This study evaluates the effect of feeding during gestation and suckling an 80:20 mixture of *cis*9,*trans*11 and *trans*10,*cis*12 CLA isomers, on systemic immune response of weaned Wistar rats. Pups received dietary CLA from dams through the placental barrier and during suckling by breast milk (group A) or by oral gavage (group B). Pups from group C only received CLA during suckling by oral gavage. Group D constituted the reference group. Milk from dams fed CLA diet had high content of CLA and higher IgA and IgG concentration than rats fed standard diet. Plasma of pups from groups A, B and C showed 6, 12 and 9 times higher content of the *c*9,*t*11 CLA isomer than that of group D pups. Rats from group A exhibited higher serum IgG concentration than rats from the rest of the groups (22.14 ± 2.14 *vs*. ~ 5 mg/ml, $P<0.05$), whereas rats from groups A and B showed approximately 2-fold higher splenocyte IgM production than rats from groups C and D. However, CLA supplementation did not influence significantly the splenocyte proliferative response or the cytokine secretion. Supplementation during gestation and suckling with an 80:20 *cis*9,*trans*11:*trans*10,*cis*12 CLA mix, enhances the production of the main *in vivo* and *in vitro* Ig isotypes in Wistar rats.

INTRODUCTION

The systemic and mucosal immune systems are continuously in development, and their function is highly influenced by maternal, environmental, dietary and behavioural factors.[1-3] In rats and humans, before birth, the immunoglobulin (Ig) G crosses the placenta and reaches the foetus, thereafter the milk constitutes the communication route between the maternal and infant immune system. It represents an active mechanism directing and educating the immune system, metabolism, and gut microflora within the infant, while conferring multiple means of protection from pathogens.[4-5] Breast milk has Ig and many other bioactive molecules, such as growth factors, cytokines, nucleotides, cellular components and lipids, which promote maturation of the developing immune system.[5-7]

It has been suggested that PUFAs, specifically docosahexanoic and arachidonic acids, which constitute a relatively low fraction of the total fatty acids in human breast milk, participate in neonate immune development.[8] In parallel, conjugated linoleic acid (CLA), a PUFA found in breast milk, has been also suggested to contribute to immune development.[9-12] The predominant CLA isomer in dairy products is *cis*9,*trans*11 CLA (*c*9,*t*11 CLA), also called rumenic acid, which ranges in human milk from 83% to 100% of total CLA.[9,11] The *trans*10,*cis*12 CLA isomer (*t*10,*c*12 CLA), is also found in dairy products, but in lower proportion. In human milk, this isomer is in lower amounts than rumenic acid.[11,13] Even very low doses of *trans*10,*cis*12 CLA isomer, seem to have large biological effects.[14]

CLA has been reported to exert beneficial physiological effects on the development of cancer,[15] atherosclerosis,[16] diabetes,[17] and on body composition.[18] The immunomodulatory properties of CLA in both rodents and humans show controversial results ranging from stimulation to inhibition.[19-22] These discrepancies are mainly due to the different mixtures of CLA isomers used in the studies, since each isomer has specific biological effects. The *t*10,*c*12 CLA isomer is responsible for body fat reduction,[23-25] although both CLA isomers have shown immunomodulatory effects.[19-22]

Although many studies have been carried out with CLA isomer mixtures, most of them have used 50:50 mixtures of *c*9,*t*11 and *t*10,*c*12 isomers and have been carried out in animals at least 3-wk-old. Based on the predominance of *c*9,*t*11 CLA isomer in breast milk and that CLA intake during developmental phases might have effects later in life,[26-27] we hypothesized that CLA would be transferred from dams to pups and exert immunoenhancing effects. Thus, the aim of this immunonutrition study was to investigate the effects of supplementing Wistar rats from gestation to the end of suckling with an 80:20 isomer mixture of *c*9,*t*11 and *t*10,*c*12 CLA, on systemic immune response in Wistar rats. We quantified CLA

transfer to pups, serum and milk Ig concentration, *in vitro* splenocyte Ig production and spleen cell proliferation and cytokine secretion ability, as biomarkers of immune development.

MATERIAL 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 12h:12h light:dark cycle, and had access to food and water *ad libitum*. The rats were monitored daily and allowed to deliver at term. The day of birth was registered as day 1 of life. Litters were unified to ten pups per lactating dam; pups had free access to the nipples and rat diet. Animals were daily identified and weighed, and handling was done in the same time range to avoid the influence of biological rhythms. Body weight and body length (nose-anus length) were used to determine the following morphometrical parameters: body mass index (BMI), calculated as body weight/length^2 (g/cm^2) and Lee index, calculated as $^3\sqrt{}$weight/length (g/cm)**,** as markers of obesity in rats and other mammals.[28]

At the weaning day (day 21), rats were anaesthetized with ketamine/xylazine (ketamine 90 mg/kg plus xylacine 10 mg/kg of rat weight) to obtain spleen and blood for serum and plasma samples, which were immediately frozen at -80ºC until processing. 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 approved by the Catalonian Government (CEEA 303/05, UB/DMA 3242).

Pregnant rats were randomly assigned to 1 of the following 4 dietary groups, according to total period of CLA supplementation and administration route used in the pups (**Fig. 1**):

- Group A: Pups from dams fed 1% CLA diet during the last two weeks of gestation and throughout the suckling period. During suckling, pups received CLA through the dam's milk. Total period of supplementation: 5 wk.

- Group B: Pups from dams fed during gestation 1% CLA diet and during suckling standard diet (AIN-93G). Pups were CLA-supplemented daily during suckling by oral gavage. Total period of supplementation: 5 wk.

- Group C: Pups from dams fed standard diet during gestation and suckling. Pups received CLA by daily oral gavage throughout the suckling period. Total period of supplementation: 3 wk.

- Group D: Pups from dams fed standard diet throughout the study. These animals constitute the reference diet group. Total period of supplementation: 0 wk.

Diets

The standard diet corresponded to the American Institute of Nutrition (AIN)-93G formulation,[29] containing 7% soybean oil. A 1% CLA diet was obtained from modified standard flour AIN-513 (Harlan) containing 10 g CLA/kg (**Table 1**). Thus, the supplemented diet contained 6% soybean oil plus 1% CLA oil. The CLA isomer mixture used was approximately 80% *c*9,*t*11 and 20% *t*10,*c*12 from the total CLA isomers in oil. The CLA mixture had 0,69% free fatty acids as oleic acid, a peroxide value of 0.2 mEq/kg, 5.6% saturated fatty acids and less than 5% of minor CLA isomers. CLA oil was kindly supplied by Loders Croklaan, Lipid Nutrition, Wormerveer, The Netherlands.

The 1% CLA diet in suckling animals corresponded to a daily administration of 1.5 mg CLA oil provided/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, USA) 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.

Collection and processing of dam milk

Milk was collected from dams on day 21 post-partum. Pups were separated from dams 1 h before milk extraction to allow it to accumulate in the mammary glands. Then females were anaesthetized intramusculary (i.m) with ketamine (90 mg/kg rat) and then treated i.m with 2 IU of oxytocin (Novartis, BCN, Spain) 10 min prior to milking. By gentle hand stripping of teats, milk droplets were collected into a test tube using silastic tubing connected to a gentle suction. Total milk was used for CLA quantification, whereas Ig determination was performed in milk whey: supernatants after 600 *g,* 30 min 4 ºC centrifugation and fat layer discarding.

Quantification of CLA isomers in pup plasma and in dam milk

Content of *c*9,*t*11 and *t*10,*c*12 CLA isomers in the plasma of pups and fatty acid composition in the milk of dams was quantified by fast gas chromatography using a capillary column (40 m x 0.18 mm x 0.20 μm), coated with RTX-2330 non-bonded stationary phase (poly 90% biscyanopropyl-10% cyanopropylphenyl) siloxane from Thames Restek UK (Saunderton,UK) as previously described.[30] The identities of sample methyl ester peaks were determined by comparison of their relative retention times with those of well-known FAMEs standards. Quantification was based on the amount of the internal standard recovered. Fatty acid composition of dams' milk was also evaluated. The results were expressed in relative amounts (% total fatty acids). CLA isomers content was also evaluated in 1% enriched diet after manufacturing process.

Isolation and culture of spleen cells

Spleen cell suspensions were obtained by passing the tissue through a steel mesh (Cellector;™ Bellco, Vertieb, Austria) in sterile conditions. Cells were then centrifuged and resuspended in phosphate buffer solution (PBS). Erythrocytes were lysed by adding distilled water to the cell suspension, and tonicity was restored by adding PBS 10x. Afterwards, cells were centrifuged, washed, and finally resuspended in RPMI-10% fetal bovine serum containing 0.05 mmol/L 2-mercaptoethanol (Merck, Darmstadt, Germany), 100 IU/ml streptomycin-penicillin (Sigma, Sigma Chemical Co, St. Louis, MO, USA), and 2 mmol/L L-glutamine (Sigma). Cell viability was determined by double staining with acridine orange and ethidium bromide (Sigma). Cells were plated and cultured in different conditions according to the assay.

Lymphocyte proliferation

Spleen cells were cultured at $1x10^5$ cells/100 μl in a 96-well plate and stimulated with phorbol myristate acetate (PMA) plus ionomycin both at 250 ng/ml (Sigma). Lymphocyte proliferation was determined by a modified ELISA technique using Cell Proliferation Biotrak™ (Amersham Biosciences, Munich, Germany) after 72 h incubation. This assay is based on the measurement of 5-bromo-2´-deoxyuridine incorporation into proliferating cells during DNA synthesis. Absorbance (Ab) values correlate directly to the amount of DNA synthesized and therefore, to the number of proliferating cells in culture. The proliferation rate (%) was expressed considering 100% for the reference diet group, as follows:

Proliferation rate (%) = (A/B) x 100, where,

$A= [(Ab_{\text{stimulated cells}} - Ab_{\text{non-stimulated cells}})/Ab_{\text{non-stimulated cells}}]_{\text{CLA diet}}$

194 $B= [(Ab_{stimulated\ cells} - Ab_{non\text{-}stimulated\ cells})/Ab_{non\text{-}stimulated\ cells}]_{standard\ diet}$

195 A parallel plate was cultured with the same samples and conditions to determine cell viability.

196

197 ***In vitro* cytokine production**

198 Splenocytes were cultured at $3x10^6$ cells/ml in a 24-well flat-bottom plate (TPP, Trasadingen,
199 Switzerland) and stimulated with PMA plus ionomycin, (250 ng/ml) for 24 h. The
200 concentration of interleukin-2 (IL-2), interferon-γ (IFN-γ), IL-4 and IL-10 in the supernatants
201 were quantified using rat ELISA sets from Biosource (Nivelles, Belgium) and BD
202 Pharmingen (Erembodegem, Belgium), following the manufacturers' instructions.

203

204 **Splenocytes *in vitro* Ig production and Ig concentration in serum and milk whey**

205 Concentration of IgG and IgM secreted during 7 days by non-stimulated spleen cells and
206 serum and milk IgA, IgM and IgG concentration were quantified by ELISA. Briefly, 96-well
207 polystyrene plates (Nunc Maxisorp, Wiesbaden, Germany) were coated with anti-rat IgA,
208 anti-rat IgM, or anti-rat IgG mAbs (BD Pharmingen) at 2, 2.5, and 10 μg/ml in PBS,
209 respectively (overnight in a humidified chamber). The remaining binding sites were then
210 blocked with PBS-1% bovine serum albumin (BSA) during 1 h at room temperature (RT).
211 Plates were washed (3x with PBS-0.05% Tween-20 and once with PBS), and the
212 supernatants, milk whey, serum and standard Ig dilutions (BD Pharmingen) in PBS-Tween
213 1% BSA were then added and incubated (3 h, RT). Plates were washed again and incubated (2
214 h, RT) with biotinylated anti-rat IgA or IgM (BD Pharmingen) at 0.625 and 1 μg/ml,
215 respectively, Subsequently, extravidin-peroxidase conjugate (4 μg/ml in PBS-Tween 1%
216 BSA) was added to the plates for 30 min at RT. A purified peroxidase anti-rat Ig antibody
217 (Sigma) was used for IgG detection. Ig were detected by addition of the substrate solution
218 (o-phenylenediamine dihydrochloride plus H_2O_2 in 0.2 mol/l phosphate-0.1 mol/l citrate
219 buffer, pH 5). The enzyme reaction was stopped with H_2SO_4 3 mol/l and absorbance was
220 measured at 492 nm.

221

222 **Statistical Analysis**

223 SPSS 14.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Conventional
224 one-way ANOVA was performed considering the experimental group based on CLA
225 supplementation as independent variable. When CLA supplementation had a significant effect
226 on the dependent variable, Bonferroni's and Scheffe's tests were applied. Content of Ig in rat

milk whey was analyzed by the Student's T test. Significant differences were accepted at $P<0.05$.

RESULTS

Body weight

Body weight from dams and pups was monitored daily throughout the study. Pregnant dams starting body weight at 7 days' gestation was 227.4±4.2 g (mean±SEM). The 1% CLA diet did not modify the dams' body weight increase during the gestation period: dams fed 1% CLA or standard diet gained similar weight up to day 21 of gestation (308.7±8.4 g and 309.2±10.4 g, respectively). Moreover, from day 7 to 21 of gestation, dams from both dietary groups had similar chow intake (1% CLA diet, 5.9±0.3 and standard diet, 5.5±0.5 g/100 g rat/day). To assess the effect of CLA supplementation during gestation, pups from dams in the groups A and B were taken together and compared on the day of birth to pups from dams fed standard diet (C and D groups). The weight but not BMI and Lee index of neonates from CLA-supplemented dams (5.7±0.1 g, 0.2±0.1 g/cm^2, and 0.3±0.1 g/cm, respectively) was lower than that from neonates whose dams received standard diet during gestation (6.7±0.1 g, 0.2±0.1 g/cm^2 and 0.4±0.1 g/cm, respectively) ($P<0.05$).

Despite the slight differences among groups during the first week of life, CLA-supplemented animals exhibited a similar body weight pattern when compared to pups from group D throughout the suckling period (**Fig. 2**), and at the end of the suckling period (day 21): weight, BMI and Lee index were similar among the groups (49.1±0.6 g, 0.4±0.1 g/cm^2, and 0.3±0.1 g/cm, respectively). As we have described in other previous experimental nutrition designs in this early life period,[3] there were no deaths in any of the groups in this study. Animals' behaviour and organs weight and appearance were recorded as markers of CLA side effects and there was no evidence of abnormality.

CLA content and fatty acid composition of dam milk

At the end of suckling (day 21), milk from dams fed CLA diet during gestation and suckling showed higher concentration of *c*9,*t*11 and *t*10,*c*12 CLA isomers than rats fed standard diet ($P<0.001$) (**Table 2**). The proportion of these two isomers in milk, 86:14, varied from that supplemented to dams, 80:20. Moreover, the *n*-6:*n*-3 proportion present in milk of dams fed

CLA was 8.04, whereas in milk from dams fed standard diet was 8.21. CLA induced changes in the fatty acid profile of milk from supplemented rats (Table 2).

CLA content in pup plasma

The concentration of CLA in plasma of pups was determined at weaning day, following dietary supplementation either through gestation and suckling from the dam or by oral gavage. Pups from group D showed a low plasma content of *c*9,*t*11 CLA and no *t*10,*c*12 CLA. The groups A, B and C had approximately 9, 12 and 6 times higher levels of *c*9,*t*11 CLA than the group D, respectively ($P<0.05$) (**Table 3**). Moreover, the groups A and B (both receiving CLA during 5 wk) had higher concentrations of both CLA isomers than the group C (3 wk fed CLA, $P<0.05$). Although the supplemented proportion to dams and pups of *c*9,*t*11 and *t*10,*c*12 CLA was 80**:**20, the CLA content in pups plasma did not show this proportion in any group. CLA isomers proportion was 86:14, 93:7 and 94:6 for groups A, B and C, respectively. Hence, group B presented a higher content of *c*9,*t*11 CLA, but a lower content of *t*10,*c*12 CLA than the group A ($P<0.05$).

Ig concentration in milk whey

Concentration of IgG, IgA and IgM was quantified in dams' milk whey at the end of the suckling period (21 days postpartum) (**Fig. 3**). The predominant Ig present in rat milk was IgG (~280 μg/ml), followed by IgA (~30 μg/ml) and finally IgM (~5 μg/ml). Dams fed CLA diet during gestation and suckling increased the concentration of the main Ig isotypes in rat milk, IgG and IgA, about 6- and 2-fold, respectively ($P<0.05$).

Serum Ig concentration

Serum IgG, IgM and IgA concentration was quantified in 21-day-old animals (**Fig. 4**). Animals from group D showed ~5 mg/ml IgG (**Fig. 4A**), ~95 μg/ml IgM (**Fig. 4B**), and ~2.7 μg/ml IgA (**Fig. 4C**). CLA supplementation for 5 weeks, 2 during gestation and 3 during suckling through the dams' milk (group A), increased the total Ig serum concentration almost 4-fold, mainly by enhancement of IgG (**Fig. 4A**) ($P<0.05$). At this age, there were no differences in IgM serum concentration among the groups (**Fig. 4B**). However, group A exhibited a lower IgA serum concentration than that of the other groups (**Fig. 4C**) ($P<0.05$).

Spleen lymphocyte proliferation, viability, and cytokine secretion

The CLA diet did not modify the *ex vivo* lymphoproliferative capacity in any group, measured 72 h after PMA/Ionomycin stimulation (**Fig. 5A**). To evaluate the effect of CLA and the relationship between proliferation and cell viability, we assessed splenocyte starting viability, which was ~ 95% for all groups, an optimum level for development of the assay. Cell viability was slightly reduced after PMA/Ionomycin stimulation, but this decrease did not differ among groups, indicating that CLA dietary supplementation had no effect on splenocyte viability in 21-day-old animals. IL-2 production, the main proliferative signal for lymphocytes, was measured in supernatants obtained after 24 h of spleen cell stimulation and was not modified by CLA supplementation (**Table 4**). Moreover, IFN-γ was also secreted in similar amounts in all groups after mitogen stimulation. Th2 cytokines, IL-4 and IL-10, were also quantified in same splenocyte supernatants and although no statistical differences were found among groups, due to the big intra-group variability, rats from groups A and B showed almost 2-fold higher values than those observed in groups C and D (Table 4).

Splenocytes *in vitro* Ig production

Spontaneous IgM and IgG production by splenocytes from weaning rats was quantified and IgM was the main isotype found in supernatants, being 40 times higher than IgG ($P<0.05$) (**Fig. 6**). Regarding CLA supplementation, IgM production from both groups supplemented for 5 wk (group A and B, ~900-1200 ng/ml) was higher than that of the groups supplemented for 3 wk (group C) and 0 wk (group D) (both approximately, ~500 ng/ml). However, this increase was only significant when pups received CLA during gestation and suckling by oral gavage (group B) ($P<0.05$) (**Fig. 6A**). Otherwise, IgG production was very low (~10 ng/ml) and no differences were found among groups (**Fig. 6B**). Thus, continuous CLA supplementation during gestation and suckling enhances splenocyte IgM production, increasing total Ig concentration by almost two-fold that of non-supplemented animals.

DISCUSSION

Evidences suggest that CLA has immunomodulatory properties,[19-22] but there are no conclusive results in this regard either in animals or in humans early life. We are not aware of any previous studies reporting the effects of CLA on immune function in suckling rats. Thus, this is the first study describing the influence of length (3, 5 weeks), life period (gestation,

suckling), and route of supplementation (placenta, breast milk, oral gavage) on the immune effects of CLA.

Dietary supplementation with 1% of an 80:20 mix of *c*9,*t*11 and *t*10,*c*12 CLA isomers during gestation did not modify body weight or food intake of dams, in agreement with other studies of dams fed different CLA isomers mixtures during gestation and lactation.[12,14,31] The body weight on the day of birth was lower in pups from CLA-fed dams, than those from dams fed standard diet. Thus, the effect of CLA supplementation during gestation was already evident on the delivery day, probably due to the body fat reduction effects of the *t*10,*c*12 CLA isomer. [18,32] Nevertheless, the effect of this isomer was not evidenced in older animals. This finding contrasts with results obtained in other species after feeding other CLA isomer mixtures.[23-34] Chin et al[12] reported a moderately enhanced body weight gain in rat pups after CLA administration during gestation and suckling. But our result is consistent with others who supplemented rats with a 50:50 isomer mixture during the same life period.[14,31] It is likely that these discrepancies are mainly due to differences in the isomers used in the related studies. Moreover, in the present study there were no negative side effects, as evidenced by the organs weight and appearance, and the animals' behaviour. This is in keeping with CLA safety assessments carried out in animals [12,14,35-36] even when CLA comprised 5% of the diet,[35] and in humans consuming a maximum of 6 g/day.[37] Moreover, evidence from studies using pure isomers have suggested that the adverse effects attributed to CLA may be due to the *t*10,*c*12 CLA isomer.[38] In this sense, in the present study the rat CLA intake based on a consumption of 10 g of 1% CLA diet/100 g of rat/day, is around CLA doses used in human studies, which ranged from 0.7-6.8 g/day [39], but are slightly higher than the CLA intake average of humans in occidental diets, which oscillate between 0.3-2.6 g/day [40].

The results regarding *c*9,*t*11 and *t*10,*c*12 CLA content in the plasma of all supplemented pups, indicated that CLA was transferred from dams to pups during gestation and/or suckling. Plasma CLA content in the groups supplemented with the 80:20 CLA mix during gestation and suckling (groups A and B, 5 wk) was ~1.5 and 2 times higher, respectively, than that of animals supplemented only during suckling (group C, 3 wk). However, these results cannot demonstrate that CLA transfers through the placenta since plasma of newborns were not analyzed. Nevertheless, Chin et al[12], found a 20-fold increase of CLA in liver of foetus (at day 20 of gestation) from dams fed 0.5% CLA diet during gestation than in foetal livers coming from dams fed control diet. This together with our findings of higher content of *c*9,*t*11 and *t*10,*c*12 CLA in milk of rats fed CLA, than in those fed standard diet and the higher content of this isomer in group B plasma than in group C,

359 confirms that CLA is transferred through the placenta to the foetus, as well as through milk to
360 the pup. The highest plasma CLA values in pups from group B may be due to CLA transfer
361 during suckling from the maternal stores accumulated during gestation, besides oral gavage
362 supplementation. Moreover, differences between groups A and B in CLA isomers proportion
363 in pups' plasma may be due to the influence of the food matrix in group A, as milk is one of
364 the major factors that affect PUFAs bioavailability.[41]

365 Despite the fact that an 80:20 isomer mix was used in all supplemented animals, the
366 *c*9,*t*11 and *t*10,*c*12 CLA proportion was maintained neither in dam's milk (86:14), nor in
367 pups' plasma (A 86:14; B 93:7; C 94:6). The lower proportion of *t*10,*c*12 CLA than that
368 initially supplemented is probably due to faster metabolism of this isomer. It has been
369 reported that *t*10,*c*12 CLA activates the β-oxidation system more strongly than *c*9,*t*11 CLA in
370 rats; therefore, the former could easily become oxidized.[42] In addition, the immune effects of
371 CLA seen in this study occurred in the groups supplemented during gestation and suckling,
372 perhaps because of incorporation of *c*9,*t*11 CLA to cell membranes and tissue since gestation,
373 while no effects were observed when CLA supplementation was restricted to the suckling
374 period. This might be explained by the fact that *c*9,*t*11 CLA has been seen to accumulate to a
375 greater extent than *t*10,*c*12 CLA in tissue phospholipids of liver,[43] skin, and bone of
376 experimental animals.[44] Interestingly, CLA isomer proportion in pups' plasma from group A
377 keeps the proportion found in dams' milk after consuming the CLA diet.

378 The presence of a small quantity of *c*9,*t*11 and not of *t*10,*c*12 CLA in milk and plasma
379 of non-supplemented rats supports the concept that rats produce rumenic acid (*c*9,*t*11 CLA),
380 as has been suggested by other authors, by conversion of free linoleic acid by the intestinal
381 bacterial flora[45] or by endogenous conversion of *trans*-vaccenic acid, present in vegetable
382 oils contained in standard diets.[46]

383 Rat milk not only transfers CLA to pups, but also antibodies, such as IgG, IgA and
384 IgM. Dams fed standard diet had milk IgG > IgA > IgM concentration pattern which agrees
385 with that described by Dahlgren et al.[47] This pattern was maintained in dams fed CLA diet,
386 although IgG and IgA concentration was much higher in these rats. It has been described that
387 IgA and IgG present in rat milk have a local mammary gland production,[47] which could be
388 advantageous for rat pups since they absorb these Ig from the ingested milk through the
389 intestinal mucosa and may extend beyond this compartment.

390 The present study also evaluated the effect of feeding an 80:20 CLA isomer mixture
391 during gestation and suckling in the incipient antibody production of weaning rats. As rats can

392 transmit Ig across the placenta, rat neonatal model seems more appropriate to investigate CLA
393 role on Ig production during pregnancy than the piglet model, due to its six-layered structure
394 of the placenta which do not allow transfer of Ab to mother to fetus[48] . Results in rat model
395 show the enhancing properties of CLA on the main *in vivo* serum Ig isotype, IgG. However,
396 this effect was not observed in all the supplemented groups, a fact that underlines the
397 importance of continuous CLA supplementation during gestation and suckling. This immune
398 enhancing effect was already reported in older animals, as by Sugano et al[49] in 7-wk-old rats
399 receiving 1% CLA (50:50 isomer mix), showing an increase in serum IgA, IgG and IgM
400 concentration and a decrease in IgE. Song et al[50] reported a similar effect in humans
401 following supplementation with a 50:50 isomer mixture for 12 weeks. Nevertheless,
402 Yamasaki et al[51] found no significant effect on serum IgA, IgG, or IgM concentration after
403 feeding 5-wk-old rats for 3 weeks with a 50:50 CLA isomer mixture at doses ranging from
404 0.05% to 0.5%.[51] Discrepancies with the study reported by Yamasaki et al[52] might be due to
405 the low doses of CLA tested in that study. Studies carried out in other species during gestation
406 and lactation periods have also reported serum IgG increases,[52-53] in keeping with our results.
407 With respect to the serum IgA decrease after CLA supplementation seen in the present study,
408 Turpeinen et al[54] also detected an IgA reduction in subjects with birch pollen allergy
409 supplemented for 12 weeks with a CLA mixture containing 63.5% *c*9,*t*11**.** Yamasaki et al[50]
410 found a slightly lower concentration of serum IgA after feeding the 0.5% CLA dose, although
411 the reduction was not significant. Considering that IgA is the main Ig in the gut surface
412 (80-90%) and the fact that systemic IgA-plasma cells continuously migrate to the intestinal
413 wall,[55] the serum IgA decrease should not be interpreted as harmful. In fact, this serum IgA
414 decrease is accompanied with an increase of intestinal IgA in CLA supplemented weaned
415 rats.[56] We also have reported an increase of mucosal IgA after specific challenge in adult rats
416 following this CLA diet along life [57]. Moreover, in the current study, CLA supplementation
417 during gestation and suckling enhanced spleen IgM production. These results agree with other
418 studies carried out in older rats, which reported enhancement of splenocyte Ig production after
419 feeding CLA 50:50 isomer mixtures.[49,51] In addition, some authors have reported this
420 increase in old mice after feeding pure *t*10,*c*12 CLA isomer.[58] Although our results
421 confirmed the immunoenhancing effect of the *c*9,*t*11 isomer, we cannot rule out immune
422 functions for *t*10,*c*12 CLA.

423 In the present study, splenocyte proliferation rate and viability did not differ among
424 the groups, partly due to the limited capacity of neonatal spleen lymphocytes to proliferate; at
425 weaning this functional capacity is far less than that of adult rats.[59] Despite our results, a

426 wide range of PUFAs, including CLA, have been found to reduce mitogen-stimulated
427 proliferation of lymphocytes isolated from several species.[60-61] A lack of effect of dietary
428 CLA on lymphocyte proliferation has also been reported by Kelley et al.[19] after feeding
429 8-wk-old mice with pure isomers for 56 days, and in humans either by ingestion of pure
430 isomers[62] or 50:50 and 80:20 isomer mixtures.[19,63] Since IL-2 plays a central role in the
431 cell-mediated immune response by regulating proliferative abilities, it could be expected that
432 if CLA does not modify splenocyte proliferation, it will not affect IL-2 production. Our
433 results are consistent with most other studies in animals and humans, which have found no
434 significant effects on IL-2 and IFN-γ splenocyte secretion among the dietary groups.[58,62-64]
435 Nevertheless, some other studies have reported enhancement of IL-2 splenocyte production
436 after CLA supplementation.[65-67] Once again, the assay conditions, particularly the isomer
437 mixtures used, might hold the key to the differing impact of CLA in these studies. Although
438 no significant effects were found on IL-4 and IL-10 splenocytes production, groups A and B
439 exhibited a higher values tendency, than groups C and D. This IL-10 increase is in line with
440 recent studies showing higher IL-10 production by dendritic cells incubated with *c*9,*t*11 CLA
441 after stimulating with LPS.[68] This effect may be related with the anti-inflammatory
442 properties attributed to CLA.[26,69] Moreover, by increasing IL-4, CLA might be promoting
443 Th2 responses as modulating antibody production and inhibiting several cellular functions,
444 which is in agreement with our results regarding CLA enhancement of the principal *in vivo*
445 and *in vitro* Ig, whereas CLA did not modify splenocyte proliferation.
446 In summary, this study is the first to investigate immune effects after supplementation
447 with an 80:20 *c*9,*t*11:*t*10,*c*12 CLA isomer mixture during gestation and the entire suckling
448 period. CLA supplementation increased milk IgG and IgA concentration, serum IgG
449 concentration and spleen IgM production. These effects were only observed in the CLA
450 supplemented groups from gestation on, and for the long-lasted time, a fact that underscores
451 the importance of this supplementation during gestation. Moreover, these data contribute to
452 the scientific evidence pointing to the potential impact of lipid nutrition on immune system
453 development during early life, particularly the effect of *c*9,*t*11 CLA isomer. To better
454 delineate the importance of dietary supplementation during the early stages of life, further
455 studies should focus on the effects of CLA mixtures in early suckling, when the immune
456 system is even more immature, as well as in older weaning animals, when the immune system
457 bears a high antigenic load with ingestion of the first solid diet.

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659 **LEGENDS**

660

661 **Fig. 1.** Diagram of the experimental design beginning on day 7 of gestation until day 21 of
662 suckling. [1] CLA arrives to foetus by transplacental transfer. [2] CLA arrives to pups through the
663 milk of dams. [3] Indicates total period of CLA supplementation from gestation until the end of
664 suckling.

665

666 **Fig. 2.** Influence of CLA supplementation on body weight from day 1 to 21. Values represent
667 mean ± SEM; n = 15-20 pups per group. There were no significant differences among the
668 groups based on a general linear model (ANOVA) with Bonferroni's adjustment for multiple
669 comparisons. Period of life and total period of supplementation were as follows: A: CLA
670 supplementation during gestation and suckling through dams (5 wk). B: CLA
671 supplementation during gestation and suckling by oral gavage (5 wk). C: CLA
672 supplementation during suckling by oral gavage (3 wk). D: Non-supplemented animals
673 (0 wk).

674

675 **Fig. 3.** Effects of CLA on IgA (A), IgG (B) and IgM (C) of rat milk collected on day 21 of the
676 suckling period. Data are expressed as mean ± SEM; n = 4-7 dams per group. IgA and IgM
677 concentration in milk whey from rats fed CLA diet was higher than in milk whey from rats
678 fed standard diet. Significant T Student differences: $^{*}P<0.05$ *vs* standard diet.

679

680 **Fig. 4.** Effects of CLA supplementation on serum IgG (A), IgM (B) and IgA (C)
681 concentration. Data are expressed as mean ± SEM; n = 15-20 pups per group. Serum IgG and
682 IgA concentration in the group A was significantly different from that of the remaining
683 groups ($P<0.05$), but there was no significant effect of CLA supplementation on serum IgM
684 concentration. Period of life and total period of supplementation were as follows: A: CLA
685 supplementation during gestation and suckling from dams (5 wk). B: CLA supplementation
686 during gestation and suckling by oral gavage (5 wk). C: CLA supplementation during
687 suckling by oral gavage (3 wk). D: Non-supplemented animals (0 wk). Significant one-way
688 ANOVA differences: $^{*}P<0.05$ *vs.* D; $^{\phi}P<0.05$ *vs.* C; $^{\omega}P<0.05$ *vs.* B group.

689

690 **Fig. 5.** Effects of CLA on proliferation rate in splenocytes stimulated with PMA plus
691 ionomycin. Data are showed as percentage of controls (set at 100%) and expressed as mean ±

692 SEM; n = 5-20 pups per group. CLA dietary supplementation did not modify splenocyte
693 proliferation, regardless of lifespan or supplementation duration. Period of life and total
694 period of supplementation were as follows: A: CLA supplementation during gestation and
695 suckling through dams (5 wk). B: CLA supplementation during gestation and suckling by oral
696 gavage (5 wk). C: CLA supplementation during suckling by oral gavage (3 wk). D: Non-
697 supplemented animals (0 wk).
698
699 **Fig. 6.** Effects of CLA on spleen cell Ig secretion. IgM (A) and IgG (B) concentration in
700 supernatants after 7 days of spleen cell culture. Data are expressed as mean ± SEM; n = 15-20
701 pups per group. IgM splenocyte production from group B was significantly higher than that
702 from groups C and D ($P<0.05$). CLA had no significant effect on IgG splenocyte production.
703 Period of life and total period of supplementation were as follows: A: CLA supplementation
704 during gestation and suckling from dams (5 wk). B: CLA supplementation during gestation
705 and suckling by oral gavage (5 wk). C: CLA supplementation during suckling by oral gavage
706 (3 wk). D: Non-supplemented animals (0 wk). Significant one-way ANOVA differences:
707 $^{*}P<0.05$ *vs.* D; $^{\phi}P<0.05$ *vs.* C group.
708

Table 1. Composition of experimental dam's diets (g/kg of diet)

Ingredient	AIN-93G[1]	1% CLA
Total energy, *kJ*	15767	15763
% as fat	16.7	16.7
% as CLA oil	-----	2.39
% as soybean oil	16.7	14.3
Caseine	200	199.9
L-cysteine	3	2.9
Corn starch	397.5	397.5
Maltodextrin	132	131.9
Sucrose	100	99.9
Cellulose	50	49.9
Mineral mix[2]	35	34.9
Vitamin mix[3]	10	9.9
Choline bitartrate	2.5	2.5
Ter-butylhydroquinone[4]	0.014	0.014
Soybean oil	70	59.9
16:0	7.7	6.7
18:0	2.8	2.4
18:1	16.4	14.2
18:2	37.2	32.2
18:3	5.6	4.8
80:20 *c*9,*t*11:*t*10,*c*12 CLA oil[5]	--	10[6]

[1]The diet was prepared according to AIN guidelines.[(23)]

[2]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.

[3]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 (cholicalciferol, 5000.000 IU/g), 0.075 g vitamin K (phylloquinone), and 974.705 g sucrose.

[4]Antioxidant.

[5]From total oil, 78% corresponded to CLA in triacylglycerol form containing an isomer ratio of 80:20 *c*9,*t*11 and *t*10,*c*12, respectively. The CLA mixture had 0,69% free fatty acids as oleic acid, a peroxide value of 0.2 mEq/kg, 5.6% saturated fatty acids and less than 5% of minor CLA isomers.

[6]CLA isomers content after manufacturing process was ~0.8% and the *c*9,*t*11:*t*10,*c*12 proportion was 80:20.

Table 2. Fatty acid composition of rat milk fed standard or CLA diet (g/100 g total fatty acids)[1-2]

Fatty acid	Standard diet	CLA diet
C8:0	1.67 ± 0.02	1.62 ± 0.04
C10:0	6.31 ± 0.38	6.45 ± 0.10
C11:0	0.06 ± 0.01	0.06 ± 0.00
C12:0	10.14 ± 0.73	9.89 ± 0.05
C14:0	11.97 ± 0.36	11.51 ± 0.12
C14:1	0.03 ± 0.01	0.03 ± 0.00
C15:0	0.16 ± 0.01	0.18 ± 0.00*
C15:1	0.03 ± 0.00	0.02 ± 0.00*
C16:0	26.37 ± 0.32	25.16 ± 0.09
C16:1	1.47 ± 0.01	1.19 ± 0.01*
C17:0	0.13 ± 0.01	0.12 ± 0.01*
C17:1	0.10 ± 0.01	0.09 ± 0.01*
C18:0	3.29 ± 0.16	3.54 ± 0.04
C18:1 *n*-9c	14.29 ± 0.27	13.80 ± 0.14
C18:1 *n*-7	0.75 ± 0.02	0.77 ± 0.01
C18:2t	0.02 ± 0.00	0.01 ± 0.00*
C18:2 *n*-6c	17.71 ± 0.59	16.86 ± 0.49
C18:3 *n*-6c	0.39 ± 0.01	0.29 ± 0.01*
C18:3 *n*-3	2.12 ± 0.01	1.92 ± 0.03*
C20:0	0.07 ± 0.00	0.08 ± 0.01*
_c_9,_t_11 CLA	**0.02 ± 0.00**	**2.93 ± 0.11***
11.13 CLA	0.01 ± 0.00	0.12 ± 0.01*
_t_10,_c_12 CLA	**0.00 ± 0.00**	**0.46 ± 0.02***
C20:1 *n*-9	0.24 ± 0.01	0.22 ± 0.01*
tt CLA	0.02 ± 0.00	0.24 ± 0.01*
C20:2 *n*-6	0.40 ± 0.00	0.23 ± 0.00*
C20:3 *n*-6	0.39 ± 0.01	0.38 ± 0.01*
C20:4 *n*-6	0.95 ± 0.01	0.96 ± 0.02
C22:0	0.00 ± 0.00	0.01 ± 0.00
C22:1	0.03 ± 0.01	0.02 ± 0.00*
C22:2	0.23 ± 0.01	0.21 ± 0.00*

C20:5 *n*-3	0.03 ± 0.00	0.04 ± 0.00*
C22:4 *n*-6	0.16 ± 0.01	0.13 ± 0.01*
C22:5 *n*-6	0.03 ± 0.00	0.04 ± 0.00*
C24:0	0.04 ± 0.00	0.04 ± 0.00
C24:1	0.01 ± 0.00	0.01 ± 0.00
C22:5 *n*-3	0.12 ± 0.00	0.12 ± 0.00
C22:6 *n-3*	0.22 ± 0.00	0.22 ± 0.01

[1]The composition of milk was evaluated on day 21 of suckling.

[2]Values are means ± SEM. n = 4-7.

Significant T Student differences: *$P<0.001$ *vs* standard diet.

Table 3. Relative content of *c*9,*t*11- and *t*10,*c*12-CLA isomers as percentage of total fatty acids in 21-day-old pups plasma[1]

CLA isomer	A		B		C		D	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
*c*9,*t*11	1.34	0.01	1.78	0.05	0.90	0.01	0.15	0.01
*t*10,*c*12	0.21	0.01	0.13	0.01	0.05	0.00	N.D	

[1]Data are expressed as mean ± SEM. n = 10. A: pups supplemented with 1% CLA during gestation and suckling through dams (5 wk). B: pups supplemented with 1% CLA during gestation through dams and during suckling by oral gavage (5 wk). C: pups supplemented with 1% CLA only during suckling by oral gavage (3 wk). D: pups non-supplemented (0 wk). N.D. Non-detectable. Both CLA isomers content in pups' plasma is significantly different among all groups (one-way ANOVA $P<0.001$).

Table 4. Content of cytokines in 24 h splenocytes supernatants stimulated with 250 ng/mL of PMA plus ionomycin[1]

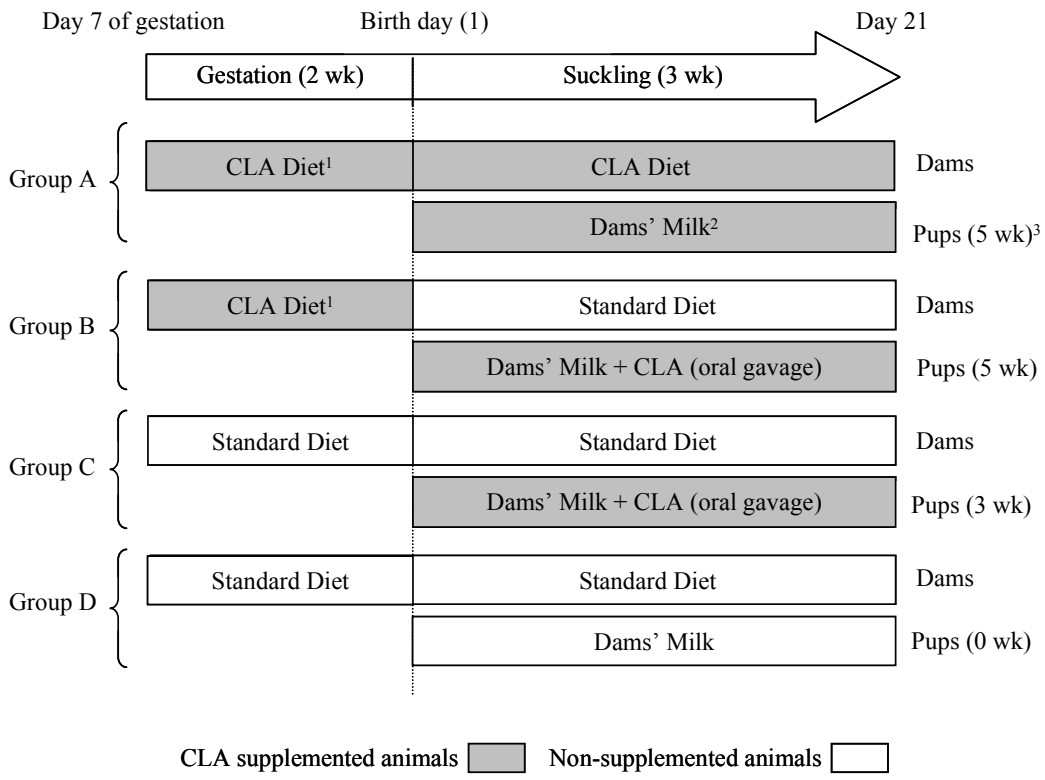
Cytokines	A		B		C		D	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Th1								
IL-2 (ng/ml)	4.10	1.04	4.10	1.01	5.65	0.93	4.10	0.79
IFNγ (ng/ml)	4.61	0.79	5.93	1.02	6.37	1.36	3.70	0.81
Th2								
IL-4 (pg/ml)	29.32	7.97	32.43	5.99[2]	15.86	1.31	12.44	1.82
IL-10 (pg/ml)	181.68	47.24	176.33	43.80	90.59	8.53	108.03	19.62

[1]Data are expressed as mean ± SEM. n = 10. A: pups supplemented with 1% CLA during gestation and suckling through dams (5 wk). B: pups supplemented with 1% CLA during gestation through dams and during suckling by oral gavage (5 wk). C: pups supplemented with 1% CLA only during suckling by oral gavage (3 wk). D: pups non-supplemented (0 wk).

[2] P=0.06 *vs* group D (one-way ANOVA)

862

Figure 1



863

Figure 2

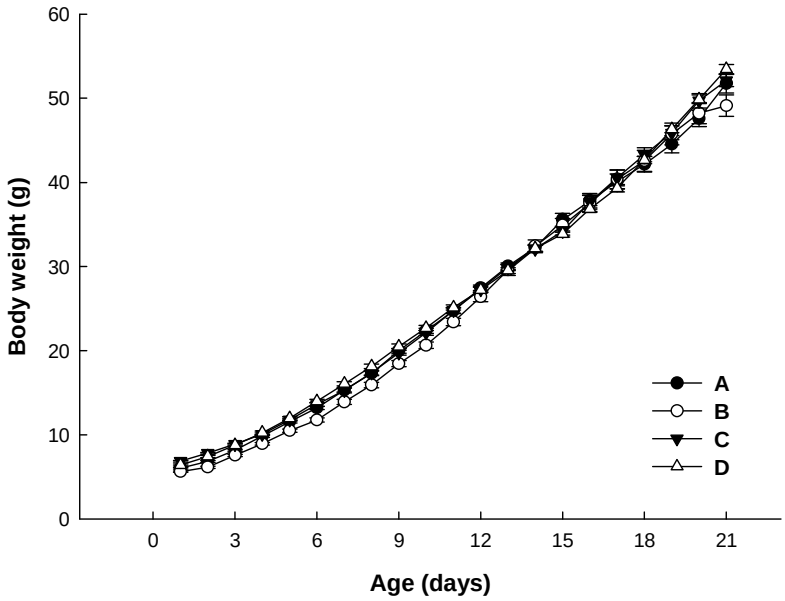
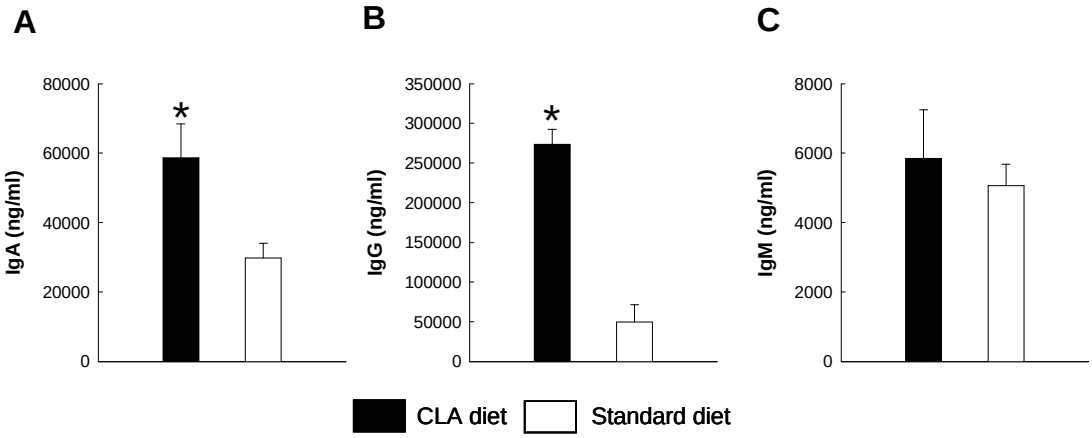
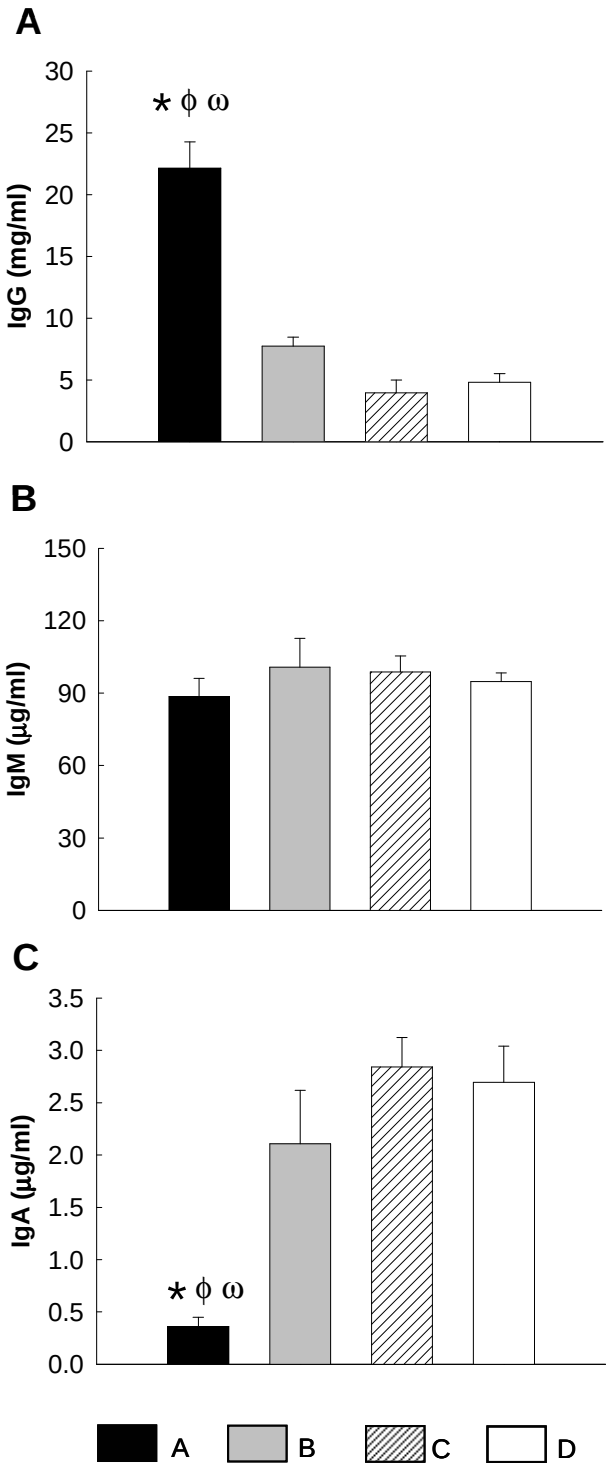


Figure 3


A
B
C
IgA (ng/ml)
IgG (ng/ml)
IgM (ng/ml)
80000
60000
40000
20000
0
350000
300000
250000
200000
150000
100000
50000
8000
6000
4000
2000
*
*
CLA diet
Standard diet

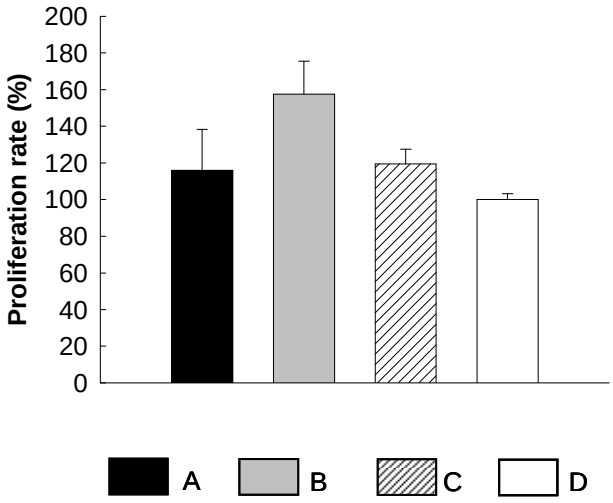
865

Figure 4


A
IgG (mg/ml)
30
25
20
15
10
5
0
* ϕ ω
B
IgM (µg/ml)
150
120
90
60
30
0
C
IgA (µg/ml)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
* ϕ ω
A
B
C
D

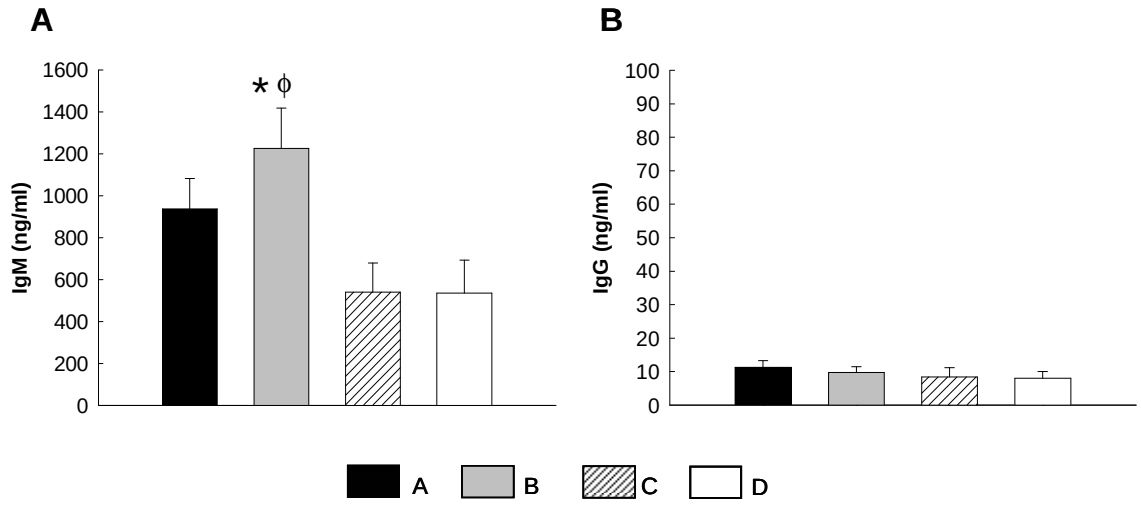
866

Figure 5



867

Figure 6


A
B
IgM (ng/ml)
IgG (ng/ml)
1600
1400
1200
1000
800
600
400
200
0
100
90
80
70
60
50
40
30
20
10
0
* ϕ
A
B
C
D

868

ARTÍCULO 2

Potenciación del sistema inmunitario de ratas jóvenes por la mezcla isomérica de ácido linoleico conjugado 80:20 de *cis*-9,*trans*-11 y *trans*-10,*cis*-12

Infant rat immune enhancement by an 80:20 mixture of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 conjugated linoleic acid

Carolina Ramírez-Santana, Cristina Castellote, Margarida Castell, Montserrat Rivero, Francisco J. Pérez-Cano y Àngels Franch

Journal of Lipid Research (pendiente de aceptación)

Los resultados de este artículo han permitido la presentación de 2 comunicaciones:

- En formato póster: *Effect of CLA feeding on splenocyte proliferation in early infant rats*, en "Fats and Health –Update on Dietary Phytosterols, Trans-fatty Acids and Conjugated Linoleic Acids", Frankfurt, 19-20 Octubre, 2006.
- En presentación oral: *Ácido Linoleico Conjugado (CLA) sobre el sistema inmunitario de ratas lactantes, jóvenes y adultas*, en "II Congrés de la Societat Catalana d'Immunologia", Barcelona, 19-20 Noviembre, 2008.

RESUMEN

Objetivo: Evaluar la influencia del suplemento dietético de CLA (mezcla isomérica 80:20 de *cis*9,*trans*11 y *trans*10,*cis*12), durante la lactancia y primera infancia, sobre la respuesta inmunitaria sistémica de ratas Wistar de 28 días de edad.

Material y métodos: Se dispuso de 4 grupos experimentales, de los cuales tres de ellos recibieron CLA. La suplementación se llevó a cabo de forma continuada durante la lactancia (3 semanas) y primera infancia (1 semana) (28/4), únicamente durante la lactancia (28/3) o durante la semana de primera infancia (28/1). Se dispuso de un grupo de referencia (28/Ref) que consumió pienso estándar a lo largo del estudio. Se evaluó el peso e índice de masa corporal y el día de sacrificio se extrajo el bazo y sangre. Se determinó el contenido de los isómeros de CLA en plasma y la concentración de IgG, IgM e IgA en suero. También se evaluó *in vitro* la capacidad linfoproliferativa y la producción esplénica de inmunoglobulinas y de las citocinas IL-2, IFNγ, IL-4, IL-6 e IL-10.

Resultados: Los animales de los grupos 28/4 y 28/3 presentaron mayor peso corporal que el resto de grupos el día de sacrificio, sin embargo, el índice de masa corporal y el índice de Lee no se modificaron. Aunque los isómeros *cis*9,*trans*11 y *trans*10,*cis*12 de CLA fueron detectados en el plasma de todos los animales que recibieron CLA, su contenido fue superior en el grupo que recibió suplemento durante la lactancia y primera infancia (28/4) (p<0,001). En este grupo, los animales mostraron un incremento de la concentración sérica de Ig totales y una disminución de su capacidad linfoproliferativa esplénica (p<0,05). Además, la producción de citocinas esplénicas no se modificó por efecto de CLA, a excepción de un incremento de IL-6 en el grupo 28/4, en comparación con el resto de grupos (p<0,05). Por otra parte, el suplemento con CLA únicamente durante la lactancia (28/3) incrementó la producción esplénica *in vitro* de IgG e IgM, respecto a los animales alimentados con dieta estándar (p<0,05).

Conclusión: El suplemento dietético con una mezcla 80:20 de los isómeros *cis*9,*trans*11:*trans*10,*cis*12 de CLA durante la lactancia y primera infancia potencia la respuesta inmunitaria sistémica humoral en ratas de 28 días de edad.

Infant rat immune enhancement by an 80:20 mixture of *cis*9,*trans*11 and *trans*10,*cis*12 conjugated linoleic acid

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Running title: Immune response in CLA fed infant rats

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Abbreviations used: CLA, conjugated linoleic acid; PPAR, peroxisome proliferator-activated receptor; IL, interleukin; Ig, immunoglobulin; IFN, interferon.

ABSTRACT

Conjugated linoleic acid (CLA) is a *trans* fatty acid, to which has been attributed several health effects. This study evaluated the influence of dietary supplementation with 1% of an 80:20 mixture of *cis*9,*trans*11 (*c*9,*t*11)- and *trans*10,*cis*12 (*t*10,*c*12)-CLA isomers, during suckling and early infancy on systemic immune response in Wistar rats. Supplementation was performed during suckling and early infancy, only during suckling, or only in early infancy. The content of CLA isomers in plasma and serum IgG, IgM and IgA concentration were determined. Splenocytes were isolated to evaluate proliferation, IL-2, IFNγ, IL-4, IL-6, IL-10 and Ig production. The CLA isomers *c*9,*t*11 and *t*10,*c*12 were detected in plasma of all CLA-supplemented animals, but the greatest content was quantified in rats supplemented during suckling and early infancy ($p<0.001$). These rats also exhibited increased total serum Ig concentration and inhibited splenocyte proliferation ($p<0.05$), without modifying IL-2, IFNγ, IL-4 or IL-10 production, but showed increased IL-6 splenocyte production compared to rats from the rest of groups ($p<0.05$). CLA supplementation only during suckling increased *in vitro* splenocyte Ig production, compared to non-supplemented animals ($p<0.05$). In summary, CLA supplementation with an 80:20 *cis*9,*trans*11:*trans*10,*cis*12-CLA mix during suckling and early infancy enhanced systemic humoral immune response in infant rats.

Keywords: Conjugated linoleic acid; suckling; early infancy; immunoglobulin; proliferation

INTRODUCTION

Historically, considerable attention has been focused on neonatal nutrition, specially, has been highlighted the importance of PUFAs, other than arachidonic acid (AA) and DHA (1). Neonatal development, particularly neonatal immunity is influenced after birth through consumption of breast milk (1). Breast milk includes many bioactive components such as antibodies, growth factors, cytokines, nucleotides, cells and lipids such as conjugated linoleic acid (CLA), all these components mainly influence on maturation of the developing immune system (2-5). In addition, it has been demonstrated in animal models that milk-derived immune benefits can cross the neonatal intestine and influence beyond this compartment (3), and can provide protection through breast milk further than weaning (6).

Conjugated linoleic acid was first identified as a principal component of fried ground beef with anti-carcinogenic properties (7). Since then, to CLA has been attributed many effects as reducing the severity of atherosclerosis (8) and reducing body fat while enhancing lean body mass (9). The variety of biological activities of CLA is due to its variable composition, since is a mixture of more than 20 geometric and positional isomers. However, the primary research is focused on the two biologically active isomers, *cis*9,*trans*11 (*c*9,*t*11 and *trans*10,*cis*12 (*t*10,*c*12) (10). These two isomers have shown additive, independent, or even antagonistic effects. Both CLA isomers have anti-cancer effects and probably immune-modulator properties, whereas the *t*10,*c*12 CLA isomer is known to be responsible for body fat reduction (11). More than 80% CLA naturally occurring isomers in dairy products is the *c*9,*t*11, together with other minor isomers as *t*10,*c*12 (12).

Most of the studies that establish CLA immune effects have been conducted in adult animals using 50:50 isomeric mixtures of *c*9,*t*11 and *t*10,*c*12 CLA. These mixtures have reported to ameliorate viral infectivity and enteric inflammation in animal models (13-14). In addition, some studies in healthy humans have shown that CLA supplementation has no effect

on many immune functions (2,15), whereas others performed in immune imbalance, have suggested an anti-inflammatory effect for *c*9,*t*11 CLA (16).

Little work on CLA immune functions has been carried out in young animals, when immune system is relatively immature. During the first two weeks of life, rat spleen contains few lymphocytes and its immune functionality is very low. In the second half of the suckling period, main cell subsets increased in number and proportion, but at weaning, B and T lymphocytes are not fully developed yet (17). Similar development pattern is found in the mucosal compartment (18-19).

A better understanding of the effect of diet on immune events during early life is necessary in order to ensure healthy immune system development. Therefore, the aim of the present study was to evaluate the effect of dietary supplementation during suckling and early infancy with an 80:20 CLA isomer mixture of *c*9,*t*11- and *t*10,*c*12-CLA. For this purpose, infant rat systemic immunity, which is still in development, was evaluated.

MATERIAL 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 12h:12h light:dark cycle, and had access to food and water *ad libitum*. The rats were allowed to deliver at term. The delivery day was identified as day 1 of life. Litters were randomized and unified to ten pups per lactating dam; pups had free access to the nipples and rat diet. All daily handling was done in the same time range to avoid the influences of biological rhythms. Body weight and body length (nose-anus length) were used to determine the following morphometrical parameters: Body mass index (BMI), calculated as body weight/length^2 (g/cm^2) and Lee index, calculated as $\sqrt[3]{}$weight/length (g/cm).

28-day-old rats were anaesthetized with ketamine/xylazine to obtain spleens and blood for plasma and serum samples, which were immediately frozen at -80 ºC until processing. 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 at the University of Barcelona and Catalonian Government (CEEA 303/05 UB/DMA 3242).

Dietary supplementation: The standard diet corresponded to the American Institute of Nutrition (AIN)-93G formulation (20), containing 7% soybean oil. A 1% CLA diet was obtained from modified standard flour AIN-513 (Harlan) containing 10 g CLA/kg (Table 1). Thus, the supplemented diet contained 6% soybean oil plus 1% CLA oil. The CLA isomer mixture was approximately 80% *c*9,*t*11 and 20% *t*10,*c*12 from the total CLA isomers in oil. The CLA mixture had 0,69% free fatty acids as oleic acid, a peroxide value of 0.2 mEq/kg, 5.6% saturated fatty acids and less than 5% of minor CLA isomers. CLA oil was kindly supplied by Loders Croklaan, Lipid Nutrition, Wormerveer, The Netherlands.

120 The 1% CLA diet in suckling animals corresponded to a daily administration of 1.5
121 mg CLA/g rat from day 1 to 21. Low-capacity syringes (Hamilton Bonaduz AG, Bonaduz,
122 Switzerland) adapted to oral 25- or 23-gauge gavage tubes, 27 mm in length (ASICO,
123 Westment, IL, USA) were used for oral administration before and after day 5, respectively.
124 To allow gastric emptying, litters were separated from dams 1 h before oral supplementation.
125
126 **Experimental design:** The delivery day, animals were distributed in 4 experimental groups (2
127 dams with 10 pups each, n = 20/group), according to the total period of CLA supplementation
128 (Figure 1):
129
130 **- 4 wk group:** Pups received CLA daily by oral gavage during suckling, and after weaning,
131 animals were fed 1% CLA pelleted diet from day 21 to 28. Total period of supplementation, 4
132 wk.
133 **- 3 wk group:** Pups received CLA daily by oral gavage during suckling and after weaning,
134 animals were fed standard diet until day 28. Total period of supplementation, 3 wk.
135 **- 1 wk group:** Pups received 1% CLA-pelleted diet exclusively during one week after
136 weaning (day 21-28). Total period of supplementation, 1 wk.
137 **- Ref group:** These animals constitute the reference diet group. Total period of
138 supplementation, 0 wk.
139
140 **Isolation of spleen lymphocytes:** Spleen cell suspensions were obtained in sterile conditions
141 as previously described (19). Briefly, the tissue was passed through a steel mesh (Cellector^{TM};
142 Bellco, Vertieb, Austria). Erythrocytes were lysed from cell suspension. After washing, cells
143 were resuspended at 10^6 cells/mL in RPMI-10% fetal bovine serum containing 0.05 mmol/L
144 2-mercaptoethanol (Merck, Darmstadt, Germany), 100 IU/mL streptomycin-penicillin

(Sigma) and 2 mmol/L L-glutamine (Sigma). Cell viability was determined by double staining with acridine orange and ethidium bromide (Sigma). Cells were plated and cultured in different conditions according to assay.

***In vitro* cytokine production:** Splenocytes were cultured at 3 x 10^6 cells/mL in a 24-well flat bottom plate (TPP, Trasadingen, Switzerland) and stimulated for 24 h at 37 ºC and 5% CO_2 with phorbol myristate acetate (PMA) plus ionomycin (Io), both at 250 ng/mL (Sigma). IL-2, IFN-γ, IL-4, IL-6, and IL-10 concentration from supernatants was quantified following manufacturer's instructions of rat ELISA sets from Biosource (Nivelles, Belgium) and BD Pharmigen (Erembodegem, Belgium).

Spleen lymphocyte proliferation: Lymphocytes were cultured at 1 x 10^5 cells/100 µl in a 96-well plate and stimulated with PMA plus Io (250 ng/mL) at 37 ºC and 5% CO_2. Lymphocyte proliferation was determined by a modified ELISA technique using Cell Proliferation Biotrak™ (Amersham Biosciences, Munich, Germany) after 72 h of incubation as previously described (17). The proliferation rate (%) was expressed considering 100% for the reference diet group (Ref), results based on absorbance (Ab) quantification were expressed as follows:

Proliferation rate (%) = (A/B) x 100, where,

$A = ((Ab_{\text{stimulated cells}} - Ab_{\text{non-stimulated cells}})/Ab_{\text{non-stimulated cells}})_{\text{CLA diet}}$

$B = ((Ab_{\text{stimulated cells}} - Ab_{\text{non-stimulated cells}})/Ab_{\text{non-stimulated cells}})_{\text{standard diet}}$

A parallel plate was cultured with the same samples and conditions to determine the cell viability.

Splenocytes i*n vitro* Ig production and serum Ig concentration: IgG and IgM produced by non-stimulated splenocytes for 7 days and serum IgG, IgM and IgA concentration were

quantified using a sandwich ELISA technique as previously described (17). Briefly, 96-well polystyrene plates (Nunc Maxisorp, Wiesbaden, Germany) were coated with adequate dilution of capture antibodies (anti-rat IgG, IgM and IgA mAbs). After samples and standards incubation, Ig isotypes were detected by adding biotin-conjugated anti-rat IgG, IgM or IgA mAbs followed by extravidin-peroxidase (Sigma). IgG, IgM and IgA standards and antibodies were provided by BD Pharmingen.

CLA quantification in pup plasma: *C*9,*t*11- and *t*10,*c*12-CLA isomers were quantified in pups' plasma by fast-gas chromatography using an RTX-2330 capillary column (40 m x 0.18 mm x 0.20 μm) as previously described (21).

Statistical Analysis: SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Conventional one-way ANOVA was performed considering the experimental group as an independent variable. When CLA supplementation had a significant effect on the dependent variable, Bonferroni's and Scheffé's tests were applied. Significant differences were accepted at $p<0.05$.

RESULTS

Animal growth: Pups body weight was daily monitored throughout the study. Animals supplemented with CLA during suckling, 4 wk and 3 wk groups, exhibited higher body weight at the end of the study compared to that of animals only supplemented during early infancy (1 wk group, $p<0.05$) and those non-supplemented (Ref group) (Table 2). In spite of body weight differences one week after weaning (day 28), no differences in BMI or Lee index were found among groups.

Relative CLA concentration in pup plasma: CLA bioavailability was determined in 28-day-old animals by quantifying *c*9,*t*11- and *t*10,*c*12-CLA isomers in plasma (Table 3). Plasma from Ref group pups had a low content of *c*9,*t*11-CLA and no *t*10,*c*12-CLA. These values were lower than those found in the rest of the groups ($p<0.05$). CLA-supplemented animals, continuously during suckling and early infancy, showed the highest content of both CLA isomers among groups ($p<0.05$). However, pups from 3 wk group (3 wk during suckling and no supplementation during the last week) presented lower content in plasma of both CLA isomers than pups from 1 wk group (1 wk, from day 21 to 28) ($p<0.05$). Moreover, *c*9,*t*11- and *t*10,*c*12-CLA relative proportion is higher in all CLA supplemented groups (4 wk, 91:9; 3 wk, 90:10; 1 wk, 94:6) than that present in the original mixture (80:20).

Serum immunoglobulin concentration: Serum IgG, IgM and IgA concentrations were quantified in 28-day-old animals (Figure 2). Pups from Ref group had in sera ~2 mg/mL of IgG (Figure 2A), ~80 μg/mL of IgM (Figure 2B), and ~8 μg/mL of IgA (Figure 2C). 4 wk group showed higher IgG, IgM and IgA sera concentration compared to that in 1 wk and Ref groups ($p<0.05$). Moreover, pups from 3 wk group showed more than 3 times IgG concentration than those from 1 wk and Ref groups ($p<0.05$), indicating the importance of

CLA supplementation during suckling on serum Ig production. In short, CLA supplementation during suckling (4 wk and 3 wk groups) increased 4-fold total serum Ig compare to 1 wk and Ref groups, mainly by increasing the predominant *in vivo* Ig isotype concentration, IgG.

Spleen lymphocyte proliferation, viability and cytokine secretion: CLA supplementation during suckling (4 wk and 3 wk groups) reduced *ex vivo* lymphoproliferative ability compared to that in 1 wk and Ref groups, but it was only significant for 4 wk group ($p<0.05$) (Fig. 3A). The decrease in the proliferation rate cannot be attributed to a lower viability caused by CLA, since 4 wk group showed the lowest cell death among groups ($p<0.05$) (Fig. 3B), indicating that CLA supplementation also confers more resistance to the mitogen toxic effects. On the other hand, beyond 24 h of spleen cell stimulation, IL-2, IFN-γ, IL-4 and IL-10 concentration in the culture medium was not modified by CLA supplementation (Table 4). However, IL-6 concentration was increased in the 4 wk group compared to 3 wk and 1 wk groups ($p<0.05$). IL-6 was not detected in Ref group (Table 4).

***In vitro* spleen immunoglobulin production:** After 7 days splenocyte culture, IgM and IgG spontaneous production was quantified. Under these conditions, IgM was the predominant isotype found in supernatants, being ~100 times higher than IgG production ($p<0.05$) (Fig. 4). CLA-supplemented groups during suckling (4 wk and 3 wk group) displayed higher IgM and IgG production than Ref group, but it was only significant in the 3 wk group ($p<0.05$). Spleen Ig production from 1 wk and Ref groups revealed similar values, which point out the importance of CLA supplementation during the suckling period.

DISCUSSION

Mammals neonatal immune responses are limited, less competent and functionally deficient and is during early life when start to gradually acquire the immune status of adult animals (22-23). It is shown the impact of nutritional fatty acids on the development of the early immune system by regulating metabolic processes and gene expression of important proteins such as enzymes and cytokines (24). Some animal studies have evaluated the effect of CLA isomer mixtures at doses ranging from 0.1 to 2% of diet (w/w) on immune function (25), however, most of them have been carried out only in adult animals. Thus, the aim of the present study was to evaluate the immune-modulator effects of an 80:20 mixture of *c*9,*t*11- and *t*10,*c*12-CLA on young weaning Wistar rats, which received CLA during suckling or early infancy.

CLA supplementation since birth during suckling and early infancy (4 wk group) or only during suckling (3 wk group) resulted in increased body weight in 28-day-old animals in contrast to those of animals only supplemented during early infancy (1 wk group) and with those from reference group (Ref group). Since *c*9,*t*11 CLA isomer represents 80% of the mixture used, our results are in line with the data of rodents growth improvement by the *c*9,*t*11-CLA isomer, suggested by Pariza *et al.* (10) and Yamasaki *et al.* (26). CLA supplementation in spite of increasing body weight did not modify morphometrical parameters as BMI or Lee index among groups. These results were similar to those reported for non-supplemented rats at similar age (27).

After CLA supplementation during suckling and/or early infancy, *c*9,*t*11- and *t*10,*c*12-CLA isomers were detected in plasma of 28-day-old animals, which demonstrates CLA intestinal absorption and agrees with results of other PUFAs in similar experimental dietary designs (28-29). The highest content of CLA in plasma was achieved at longest CLA supplementation (4 wk group), showing its accumulation in plasma. However, the 3 wk

group that received CLA for 3 wk and after 1 wk null, exhibited lower CLA plasma content than 1 wk group, that only received CLA for the last wk. It might be due to the time go by between the last CLA intake and the day of quantification of CLA plasma content. CLA could have been incorporated in tissues or eliminated, is for that reason that CLA content in tissues and faecal samples should be measured in further studies. On the other hand, there is a highest relative proportion of *c*9,*t*11/*t*10,*c*12 in plasma of CLA-supplemented animals (~90:10) in comparison to the original isomer mixture (80:20). In this sense, it has been reported that *t*10,*c*12-CLA activates the β-oxidation system more strongly than *c*9,*t*11-CLA and consequently is more easily oxidized (30-31). Although it is described that both isomers present similar absorption rates in adult rat intestine (30), it is also possible that young rodents absorb preferentially the *c*9,*t*11 isomer and therefore its higher proportion after CLA supplementation. On the other hand, although no *t*10,*c*12-CLA was found in rats plasma, small quantities of *c*9,*t*11-CLA isomer were also detected in Ref animals, supporting endogenous production (32). This basal *c*9,*t*11-CLA amount in young animals may influence the final plasma *c*9,*t*11/*t*10,*c*12 ratio found in CLA-supplemented rats.

Regarding humoral immune responses on 28-day-old animals, the concentration of serum Ig isotypes was higher than that from younger animals (17), indicating an age-dependent evolution. Moreover, supplementation with an 80:20 mix of *c*9,*t*11- and *t*10,*c*12-CLA during suckling (3 wk group) plus early infancy (4 wk group) showed higher Ig serum concentration than non-supplemented animals, by means of an increase in all isotypes analyzed, IgG, IgM and IgA. The IgA increase was also found in the mucosal compartment of young rats after 4 wk of same CLA mix supplementation, where both IgA gene expression and protein was up-regulated in the intestine (33). Other studies conducted in older animals (34-35) and humans (16) also reported enhancement of serum Ig production after dietary CLA.

295 Moreover, CLA supplementation enhanced the spleen ability to secrete Ig in
296 28-day-old rats, in agreement with others in older animals (26,34,36). In contrast with serum
297 Ig concentration, *in vitro* splenocyte IgM and IgG production was significantly higher in those
298 animals supplemented exclusively during suckling (3 wk group), although animals
299 supplemented during suckling and early infancy (4 wk group) showed also an increase
300 tendency.

301 Th2 cytokines, proteins involved in T cell polarization to Ig production as IL-4, IL-6
302 and IL-10 were also measured in splenocytes supernatants. Although IL-4 and IL-10
303 concentration were similar among all groups, IL-6 level was approximately 6-fold higher in
304 the 4 wk group compared to 3 wk and 1 wk CLA-supplemented groups, whereas IL-6 was not
305 detected in Ref group. Though in the present study was not evaluated the mechanisms of IL-6
306 upregulation by CLA, the Ig concentration increase exhibited *in vivo* and *ex vivo* could be
307 mediated by IL-6 activation, since IL-6 is clearly defined, besides a prominent regulator of T
308 cell proliferation and differentiation, as an inductor of Ig secreting B cells (37).

309 Regarding lymphocyte mitogen-induced proliferation, it was down-modulated by CLA
310 supplementation during suckling and early infancy or only during suckling, although the
311 inhibitory effect was exclusively significant in the 4 wk group animals without affecting IL-2
312 production. In this regard, different reports have shown that dietary PUFAs suppress mitogen-
313 stimulated proliferation of lymphocytes isolated from rat lymph nodes, spleen and lymphatic
314 duct, from mouse spleen, pig lymph nodes or human peripheral blood (38-41). Most of these
315 studies found a good correlation between lymphocyte proliferation inhibition and IL-2
316 concentration decrease. However, Calder and Newsholme (38) reported that PUFAs did not
317 decrease the IL-2 concentration, although inhibited lymphocyte proliferation; which is
318 consistent with our results. Moreover, since in the current study CLA supplementation did not
319 modify IL-2 production, proliferation inhibition might be regulated through IL-2 receptors.

Many studies have described the modification of IL-2 receptors by PUFAs, specifically modifying lipid rafts (42-43). CLA, even with *trans* double bonds, probably acts modifying lipid rafts components as many other PUFAs, thus preventing IL-2Rα migration to soluble membranes, where IL-2 signalling occurs and T cell activation and proliferation is induced (42, 44-45).

Finally, the antiproliferative lymphocyte effect exhibited by CLA in the present study could be mediated by the nuclear peroxisome proliferator-activated receptor (PPAR)γ, since we have found in these animals colon PPARγ gene expression up-regulation (33). The involvement of PPARγ in the antiproliferative effect of CLA has been recently reported in other cell type (46). PPAR activation and expression is controlled by a diverse set of natural and synthetic molecules, including nutrients as PUFAs, non-nutrient endogenous ligands and drugs. Their main biological function is the sensing of intracellular nutrient concentrations—i.e., PUFAs and lipoproteins—and regulation of gene expression (47). CLA effect PPARγ-dependent was first described by Bassaganya-Riera & Hontecillas (14) in a pig-inflammatory bowel disease (IBD) model, where dietary CLA resulted in IBD amelioration and PPARγ gene expression upregulation (14).

The results presented herein demonstrate that supplementation with an 80:20 *c*9,*t*11: *t*10,*c*12-CLA mix during suckling and early infancy modulates immune function in young rats: increases serum Ig concentration and splenocyte Ig production and reduces splenocyte proliferation. Although further studies should be carried out to elucidate CLA signalling mechanisms, all these data support the early life immune enhancing properties of *c*9,*t*11-CLA, the main CLA isomer present in breast milk.

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FIGURE LEGENDS

FIGURE 1

Diagram of the experimental design beginning on the day of birth until day 28 of life. [1]Indicates total period of CLA supplementation from the day of birth until 1 wk after weaning.

FIGURE 2

Modulation of IgG (A), IgM (B) and IgA (C) sera concentration by dietary CLA. Values represent mean ± SEM; n = 15-20 pups per group. Serum IgG, IgM and IgA concentration from 4 wk group was significantly higher than that from 1 wk and Ref groups ($p<0.05$). Serum IgM concentration was also significantly higher than that from 3 wk group ($p<0.05$). 3 wk group had also higher serum IgG concentration than that in 1 wk and Ref groups ($p<0.05$) Total period of supplementation was as follows: 4 wk group: CLA supplementation during suckling by oral gavage and during early infancy through chow. 3 wk group: Suckling by oral gavage. 1 wk group: CLA fed pups during early infancy. Ref: Non-supplemented animals. Significant one-way ANOVA differences: $^{*}p<0.05$ *vs* Ref; $^{\phi}p<0.05$ *vs* 1 wk group; $^{\omega}p<0.05$ *vs* 3 wk group.

FIGURE 3

CLA effects on proliferation rate (A) and cell death (B) in mitogen-stimulated spleen lymphocytes. Values represent mean ± SEM; n = 15-20 pups per group. CLA dietary supplementation decreased cell death and proliferative response in 4 wk group compared to those in 1 wk group and Ref groups ($p<0.05$). Total period of supplementation was as follows: 4 wk group: CLA supplementation during suckling by oral gavage and during early infancy through chow. 3 wk group: Suckling by oral

552 gavage. 1 wk group: CLA fed pups during early infancy. Ref: Non-supplemented
553 animals. Significant one-way ANOVA differences: $^{*}p<0.05$ *vs* Ref; $^{\phi}p<0.05$ *vs* 1 wk
554 group; $^{\omega}p<0.05$ *vs* 3 wk group.
555
556 **FIGURE 4**
557 Dietary CLA changes in splenocyte Ig production. IgM (A) and IgG (B) concentrations
558 in supernatants after 7 days of spleen cell culture. Values represent mean ± SEM;
559 n = 15-20 pups per group. IgM and IgG splenocyte production from 3 wk group was
560 significantly higher than that from Ref group (one way ANOVA, $^{*}p<0.05$). Total period
561 of supplementation was as follows: 4 wk group: CLA supplementation during suckling
562 by oral gavage and during early infancy through chow. 3 wk group: Suckling by oral
563 gavage. 1 wk group: CLA fed pups during early infancy. Ref: Non-supplemented
564 animals.

Table 1. Composition of the Experimental Diets (g/kg of diet)

Ingredient	AIN-93G[1]	1% CLA
Total energy, *kcal*	3766	3765
% as fat	16.7	16.7
% as CLA oil	-----	2.39
% as soybean oil	16.7	14.31
Caseine	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[2]	35	34.99
Vitamin mix[3]	10	9.99
Choline bitartrate	2.5	2.499
Ter-butylhydroquinone[4]	0.014	0.014
Soybean oil	70	59.99
16:0	7.7	6.7
18:0	2.8	2.4
18:1	16.4	14.2
18:2	37.2	32.2
18:3	5.6	4.8
80:20 *c*9,*t*11:*t*10,*c*12 CLA oil[5]	--	10[6]

[1]The diet was prepared according to AIN guidelines.[(23)]

[2]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,

599 tetrahydrate, 0.0066 g ammonium vanadate, and 220.716 g
600 sucrose.
601 [3]Supplied per kg of diet: 3 g nicotinic acid, 1.6 g calcium
602 pantothenate, 0.7 g pyridoxine HCl, 0.6 g thiamin HCl, 0.6
603 riboflavin, 0.2 g folic acid, 0.02 g D-biotin, 2.5 g vitamin
604 B12 (0.1% in mannitol), 15 g DL-α tocopherol acetate (500
605 IU/g), 0.8 g vitamin A palmitate (5000.000 IU/g), 0.2 g
606 vitamin D3 (cholicalciferol, 5000.000 IU/g), 0.075 g vitamin
607 K (phylloquinone), and 974.705 g sucrose.
608 [4]Antioxidant.
609 [5]From total oil, 78% corresponded to CLA in triacylglycerol
610 form containing an isomer ratio of 80:20 *c*9,*t*11 and *t*10,*c*12,
611 respectively. The CLA mixture had 0,69% free fatty acids as
612 oleic acid, a peroxide value of 0.2 mEq/kg, 5.6% saturated
613 fatty acids and less than 5% of minor CLA isomers.
614 [6]CLA isomers content after manufacturing process was
615 ~0.8% and the *c*9,*t*11:*t*10,*c*12 proportion was 80:20.
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Table 2. Morphometrical parameters in 28-day-old rats[1]

	4 wk	3 wk	1 wk	Ref
Body weight (g)	88.39 ± 1.65$^{*\phi}$	86.49 ± 3.38$^{\phi}$	70.21 ± 3.24	75.74 ± 3.75
BMI (g/cm^2)	0.41 ± 0.01	0.39 ± 0.01	0.37 ± 0.01	0.37 ± 0.01
Lee index ($\sqrt[3]{}$g/cm)	0.30 ± 0.01	0.29 ± 0.01	0.30 ± 0.01	0.30 ± 0.01

[1]Body weight, body mass index (BMI) and Lee index are expressed as mean ± SEM (n = 20). 4 wk group, pups supplemented with CLA during suckling by oral gavage and from 21- to 28-day-old (early infancy) through solid diet. 3 wk group, pups supplemented with CLA during suckling by oral gavage. 1 wk group, pups supplemented with CLA diet only during early infancy. Ref, pups from reference group non-supplemented. There was a significant effect of CLA supplementation period (one-way ANOVA, $^{*}p<0.05$ *vs* Ref group; $^{\phi}p<0.05$ *vs* 1 wk group).

Table 3. Relative content of *c*9,*t*11- and *t*10,*c*12-CLA isomers as percentage of total fatty acids in plasma of 28-day-old pups[1]

CLA isomer	4 wk	3 wk	1 wk	Ref
*c*9,*t*11	1.31 ± 0.03	0.44 ± 0.03	1.23 ± 0.03	0.15 ± 0.01
*t*10,*c*12	0.13 ± 0.01	0.05 ± 0.00	0.08 ± 0.00	N.D

[1]All values are mean ± SEM, n = 10. 4 wk group, pups supplemented with CLA during suckling by oral gavage and from 21- to 28-day-old (early infancy) through solid diet. 3 wk group, pups supplemented with CLA during suckling by oral gavage. 1 wk group, pups supplemented with CLA diet only during early infancy. Ref, non-supplemented pups from reference group. N.D, Non-detectable. The content of both CLA isomers in pups' plasma is significantly different among all groups (one-way ANOVA, $p<0.001$).

Table 4. Content of cytokines in 24 h splenocytes supernatants stimulated with 250 ng/mL of PMA plus ionomycin[1]

Cytokines	4 wk	3 wk	1 wk	Ref
Th1				
IL-2 (ng/ml)	6.2 ± 0.8	6.1 ± 0.8	7.2 ± 1.1	6.8 ± 1.2
IFNγ (ng/ml)	10.9 ± 1.4	7.1 ± 0.9	7.3 ± 0.8	6.0 ± 1.3
Th2				
IL-4 (pg/ml)	27.8 ± 4.0	18.8 ± 2.8	28.7 ± 7.4	17.3 ± 2.4
IL-10 (pg/ml)	244.4 ± 41.0	224.9 ± 29.2	222.4 ± 34.5	191.6 ± 24.2
IL-6 (pg/ml)	219.0 ± 38.0*	40.1 ± 12.9	45.7 ± 15.1	N.D

[1]Data are expressed as mean ± SEM. n = 10. 4 wk group: pups supplemented with CLA during suckling and early infancy. 3 wk group: pups supplemented with CLA during suckling. 1 wk group: pups supplemented with CLA during early infancy. Ref group: pups non-supplemented.

* $p<0.05$ *vs* 3 wk and 1 wk groups (one-way ANOVA)

Figure 1

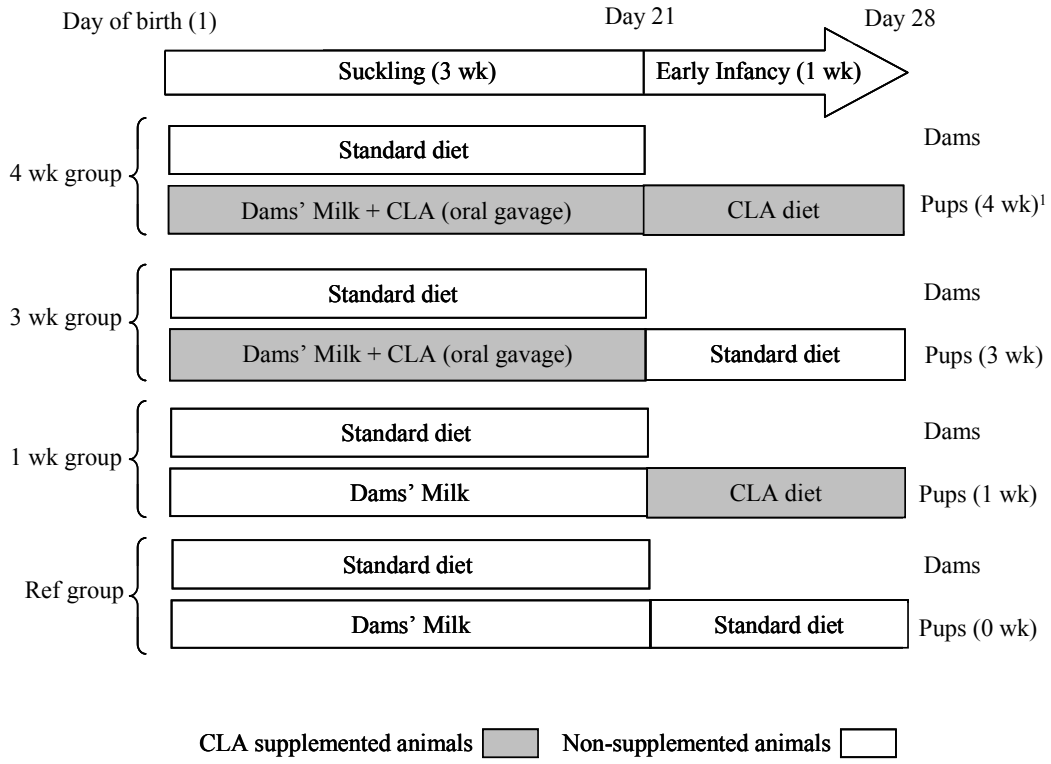
Day of birth (1)
Day 21
Day 28
Suckling (3 wk)
Early Infancy (1 wk)
4 wk group
Standard diet
Dams
Dams' Milk + CLA (oral gavage)
CLA diet
Pups (4 wk)1
3 wk group
Standard diet
Dams
Dams' Milk + CLA (oral gavage)
Standard diet
Pups (3 wk)
1 wk group
Standard diet
Dams
Dams' Milk
CLA diet
Pups (1 wk)
Ref group
Standard diet
Dams
Dams' Milk
Standard diet
Pups (0 wk)
CLA supplemented animals
Non-supplemented animals

Figure 2

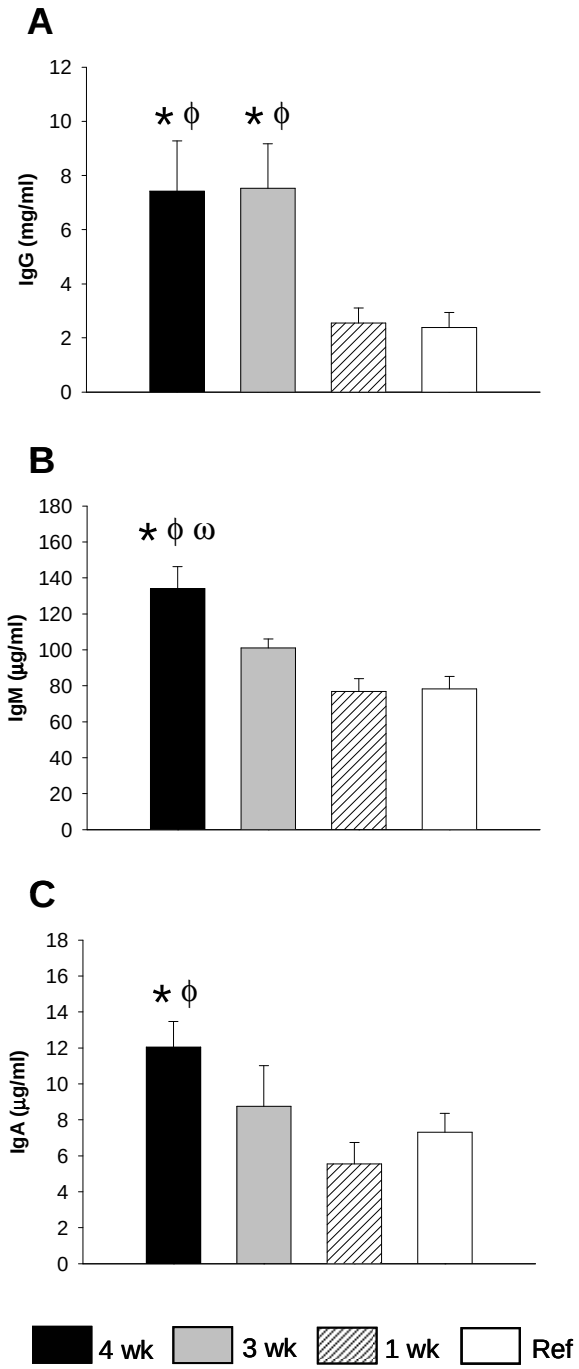

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Figure 3

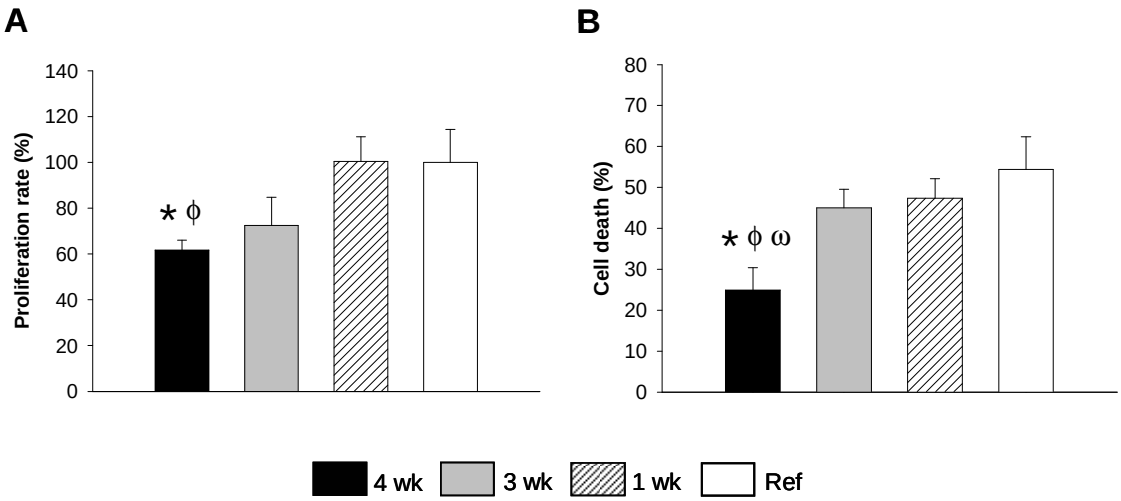

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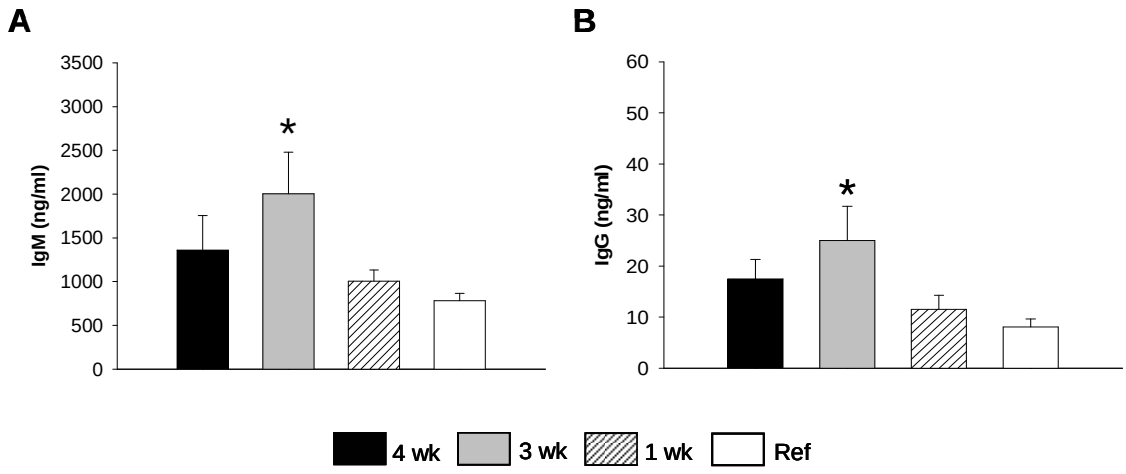
Figure 4



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ARTÍCULO 3

Incremento de IgA mucosal en ratas suplementadas con CLA de forma continuada durante la lactancia y primera infancia

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

Francisco J. Pérez-Cano*, Carolina Ramírez-Santana*, Marta Molero-Luís, Margarida Castell, Montserrat Rivero, Cristina Castellote y Àngels Franch

* Los dos primeros autores contribuyeron equitativamente a este trabajo

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Los resultados de este artículo han permitido la presentación de 2 comunicaciones:

- En presentación oral: *Conjugated Linoleic Acid (CLA) feeding during rat suckling period enhances intestinal IgA production,* en "First International Immunonutrition Workshop", Valencia, 3-5 Octubre, 2007.
- En formato póster: *Small intestine and colon IgA, TGFβ and PPARγ gene expression development pattern during early rat life*, en "6th European Mucosal Immunology Group Meeting", Milán, 8-10 Octubre, 2008.

RESUMEN

Objetivo: Establecer el efecto del isómero de CLA *cis*9,*trans*11 sobre la inmunidad mucosal en ratas durante las primeras etapas de vida, período en el cual la producción de inmunoglobulinas a este nivel está muy poco desarrollada, al igual que sucede en humanos.

Material y métodos: La suplementación dietética con CLA (mezcla 80:20 de los isómeros *cis*9,*trans*11:*trans*10,*cis*12) *se* llevó a cabo durante 3 períodos de vida: gestación, lactancia y primera infancia. La valoración inmunitaria de los animales que recibieron CLA se realizó en dos momentos distintos: al final del período de lactancia (ratas de 21 días de edad) y 1 semana tras el destete (ratas de 28 días de edad). La IgA secretada al lúmen intestinal se determinó en lavado intestinal de ratas de 28 días mediante la técnica de ELISA. La expresión de mRNA de IgA, TGFβ y PPARγ se cuantificó en intestino delgado y colon de animales de 21 y 28 días de edad, a través de PCR a tiempo real, mediante el uso de sondas y cebadores Taqman® específicos.

Resultados: El aporte de CLA durante la lactancia y primera infancia incrementó la producción de IgA intestinal en ratas de 28 días de edad. Así, la expresión de mRNA de IgA incrementó en intestino delgado y colon aproximadamente 6 y 4 veces respectivamente, y la proteína IgA intestinal aproximadamente 2 veces, en relación al grupo de referencia. La expresión del gen TGFβ fue independiente de la edad y del tejido considerado, y no se modificó por la dieta de CLA. En cambio, la expresión génica de PPARγ, posible mediador de los efectos de CLA, incrementó en colon de animales de 21 días, que habían recibido suplemento de CLA durante la gestación y lactancia, y de 28 días de edad que recibieron CLA durante la lactancia o la infancia.

Conclusión: El suplemento dietético con CLA de forma continuada durante la lactancia y primera infancia (4 semanas) potencia el desarrollo de la respuesta inmunitaria intestinal en ratas de 28 días de edad.

MUCOSAL IgA INCREASE IN RATS BY CONTINUOUS CLA FEEDING DURING SUCKLING AND EARLY INFANCY

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Running title: IgA production in CLA-fed suckling rats

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ABSTRACT

The aim of this work was to establish the effect of the *cis*9,*trans*11 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 3 life periods: gestation, suckling and early infancy. The immune status of supplemented animals was evaluated at 2 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.

Key Words: CLA. Early infancy. Gestation. Mucosal immunity. Suckling

INTRODUCTION

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 (Ab) transferred to the fetus and the child decrease the number of infectious episodes caused by microorganisms to which the mother has developed immunological memory (1,3). The mucosal immune system is in constant development since 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 (S-IgA) is the principal immunoglobulin (Ig) 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 (SC) predominate at weaning and are a key component of the mucosal barrier, whereas IgA-SC 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". Since breast milk is the only natural food for infants, and dietary contact has a pivotal role in the 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 beta (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,*c12* 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.

MATERIAL 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 12h:12h 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 ten 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 8 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 1 of the following 4 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 two 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 1 of the following 4 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 containing 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 ºC oven for 24 h. The chow was vacuum-packed to prevent oxidation and contamination by fungi, and kept at 4 ºC until use. For humidity control, periodic tests

were performed in an electronic humidity analyzer (Sartorius MA-45) for 15 min at 105 ºC, 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, USA) 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 ºC 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 ºC. The suspension obtained was centrifuged and the supernatant (intestinal wash) was stored at -20 ºC until IgA quantification by ELISA technique.

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, USA) 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 (3x 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 (12000 *g*, 10 min, 4 ºC) 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, USA) 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, USA). 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. GADH 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 (GADH 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 to 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% formaldehyde. 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, USA) 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 LSD 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 to 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 5 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. Figure 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 2 age groups but were higher in small intestine tissue than in colon (Figure 1C, $p<0.05$). TGFβ mRNA expression was independent of age and tissue (Figure 1A). IgA expression was very low during suckling, although the levels increased after weaning ($p<0.05$); no differences were found for tissue distribution (Figure 1C).

CLA Dietary Supplementation and Expression of Constitutive Genes: Since 2 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) (Figure 2). At both ages, there were no differences in PPARγ gene expression in small intestine between CLA and non-supplemented groups (Figures 2A and 2C). 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 to 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 upregulated in 28-day-old animals fed CLA (28/Sog+EI, 28/Sog, 28/EI), when compared to 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 (Figure 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 and/or early infancy failed to modulate mRNA levels of TGFβ.

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 (Figure 4A and 4B). 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 to the 28/Ref group ($p<0.05$) (Figures 4C and 4D). 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 Intestine: In addition to detection of changes in gene expression, IgA concentration was quantified in intestinal washes of 28-day-old animals (Figure 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, (Figure 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 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 both 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 (4 wk TPS); 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-wk-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.

Since IgA gene expression and intestinal production was found to increase after feeding CLA, we also studied 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 both 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, is probably due to post-transcriptional and/or translational regulation, which are important in this cytokine, since 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 non-supplemented 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 one 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 (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 upregulation.

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 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 is. 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.

Acknowledgments

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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[1]	35	34.99
Vitamin mix[2]	10	9.99
Choline bitartrate	2.5	2.499
Ter-butylhydroquinone[3]	0.014	0.014
Soybean oil	70	59.99
80:20 c9,t11:t10,c12 CLA oil[4]	--	10

[1]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.

[2]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.

[3]Antioxidant.

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

Figure Legends

Figure 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. Statistical differences: $^{*}p<0.05$ colon *vs.* small intestine, and $^{\delta}p<0.05$ day 28 *vs.* day 21.

Figure 2. Effect of CLA on PPARγ gene expression in 21- and 28-day-old animals

The 21-day experimental design establishes 4 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: Non-supplemented animals (TPS 0 wk). Gene expression was evaluated in small intestine (A) and colon (B). The 28-day experimental design establishes 4 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: Non-supplemented 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 5 animals per group. Statistical differences: $*p<0.05$ vs. age-matched reference group.

Figure 3. Effect of CLA on TGFβ gene expression in 21- and 28-day-old animals

The 21-day experimental design establishes 4 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: Non-supplemented animals (TPS 0 wk). The gene expression was evaluated in small intestine (A) and colon (B). The 28-day experimental design establishes 4 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: Non-supplemented 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 5 animals per group.

Figure 4. Effect of CLA on IgA gene expression in 21- and 28-day-old animals

The 21-day experimental design establishes 4 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: Non-supplemented animals (TPS 0 wk). Gene expression was evaluated in small intestine (A) and colon (B). The 28-day experimental design establishes 4 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: Non-supplemented 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 5 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.

Figure 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 total period of supplementation (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: Non-supplemented 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.

Figure 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: Non-supplemented animals (TPS 0 wk). Results are representative hematoxylin-eosin-stained slides from colon and small intestine of CLA and control rats (x100). All specimens show preserved bowel architecture.

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Figure 1

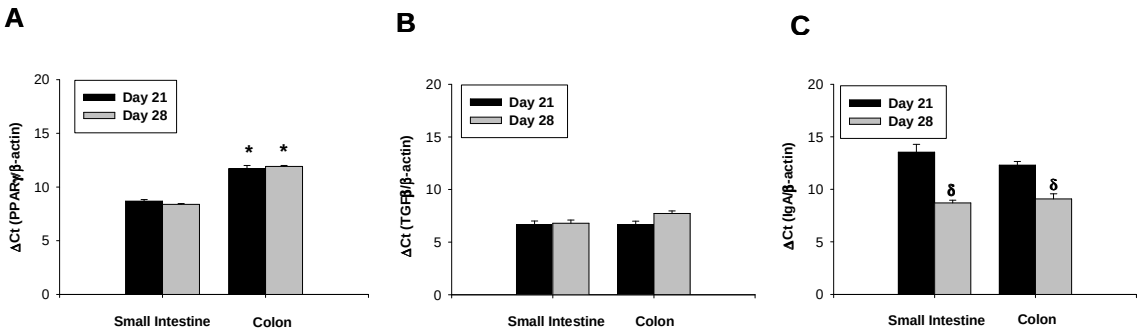

Figure 2

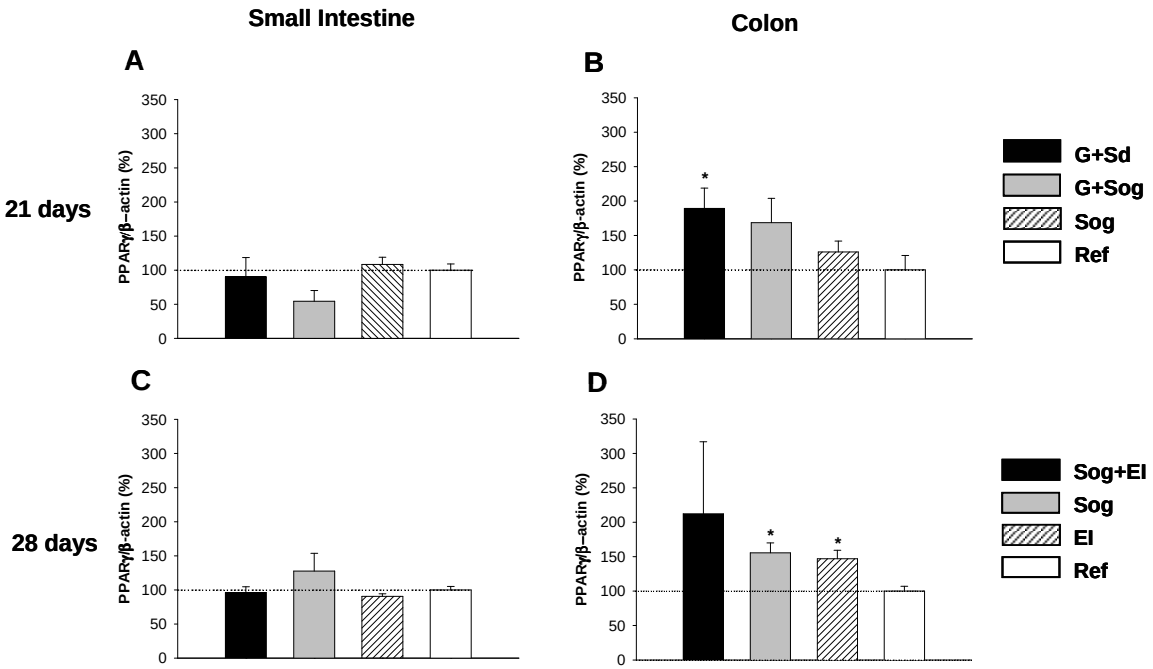

Figure 3

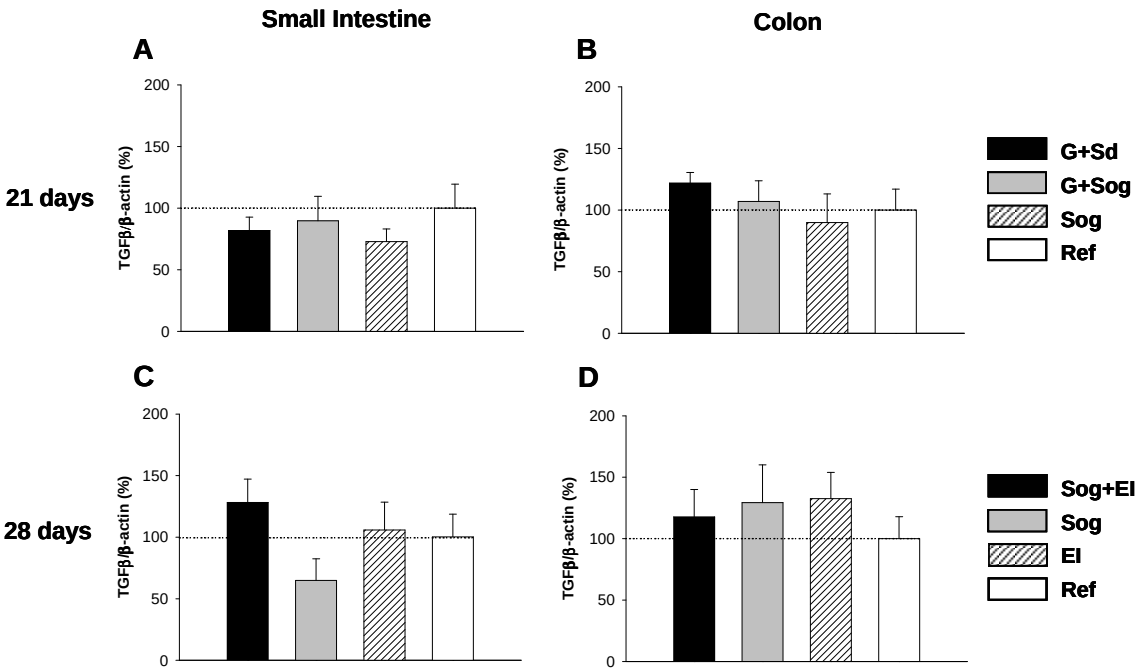

Figure 4

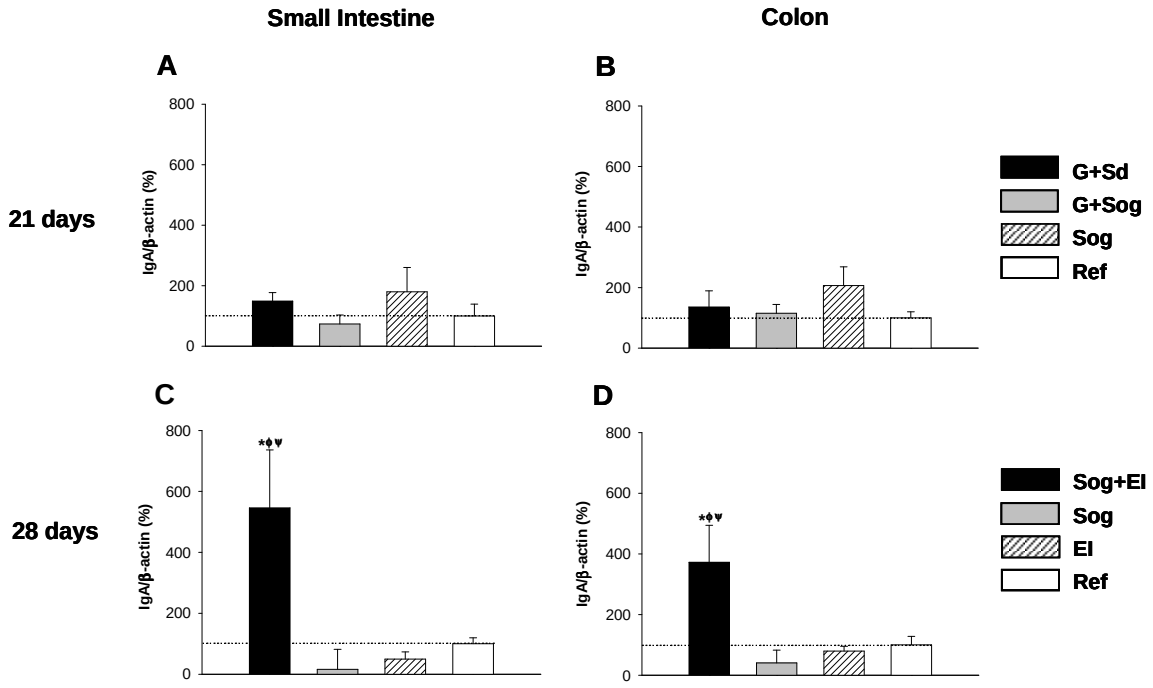

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Figure 5

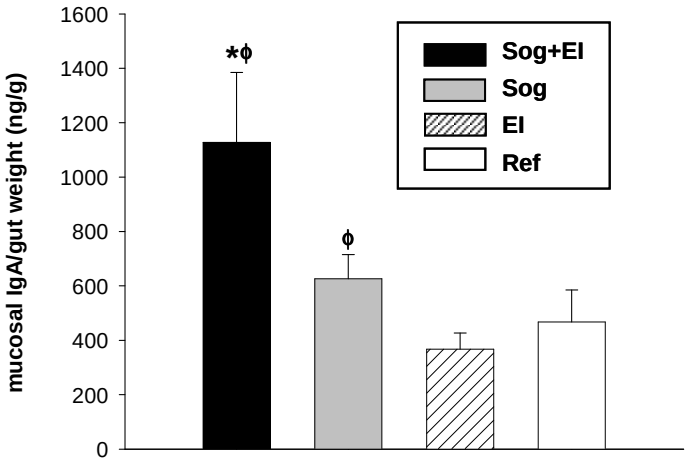
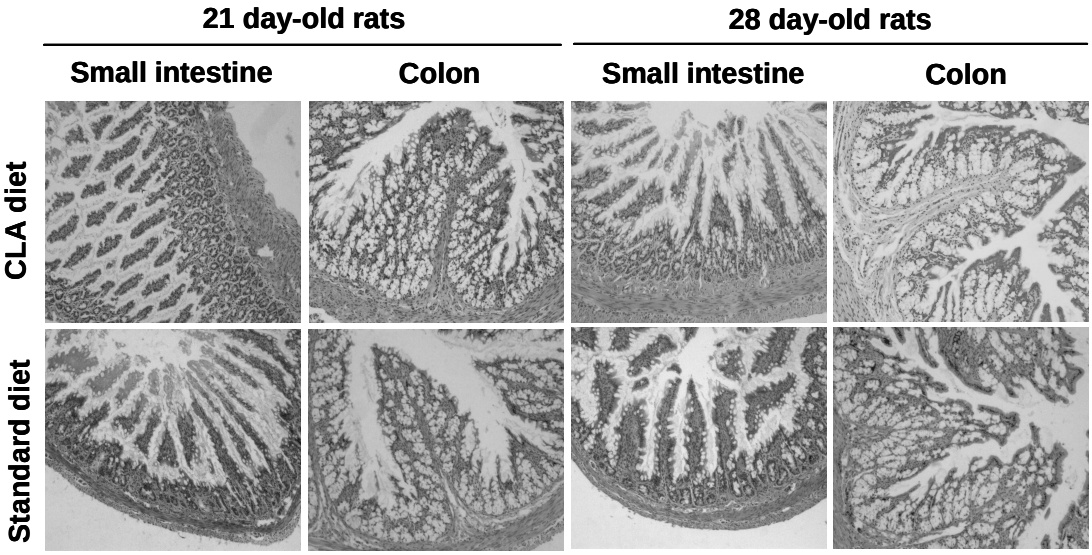


Figure 6



ARTÍCULO 4

La ingesta continuada del isómero *cis*-9,*trans*-11 del ácido linoleico conjugado refuerza la respuesta inmunitaria específica en ratas.

Long-term feeding of the *cis*-9,*trans*-11 isomer of conjugated linoleic acid reinforces the specific immune response in rats

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Los resultados de este artículo han permitido la presentación de 3 comunicaciones:

- En formato póster: *CLA feeding regulates mesenteric lymph node proliferative immune response in adult rats*, en "6th European Mucosal Immunology Group Meeting", Milán, 8-10 Octubre, 2008.
- En formato póster: *Specific mucosal IgA production enhancement in ovalbumin sensitized rats after long term CLA supplementation*, en "6th European Mucosal Immunology Group Meeting", Milán, 8-10 Octubre, 2008.
- En formato póster: *Immune benefits of CLA along rats life*, en "XII Food Studies Meeting: Functional Ingredients", Barcelona, 26-27 Noviembre, 2008.

RESUMEN

Objetivo: Establecer el efecto del suplemento dietético con CLA (mezcla 80:20 de los isómeros *cis*9,*trans*11 y *trans*10,*cis*12 de CLA), desde la gestación y de forma continuada hasta la edad adulta, sobre la capacidad de generar una respuesta inmunitaria específica en ratas Wistar adultas.

Material y métodos: Se dispuso de 2 grupos experimentales, uno que recibió CLA y otro que no recibió el suplemento dietético, constituyendo el grupo de referencia. Ratas Wistar gestantes de 7 días fueron alimentadas con una dieta con 1% de CLA o con una dieta estándar. Las crías una vez destetadas consumieron la misma dieta que las madres hasta las 15 semanas de edad (Ad/17 y Ad/Ref, respectivamente). Las ratas de ambos grupos se inmunizaron con ovoalbúmina (OVA) a las 9 semanas de edad. Se cuantificó la respuesta proliferativa policlonal y específica frente a OVA en esplenocitos y linfocitos de ganglios linfáticos mesentéricos (MLN), además de la producción de IL-2 *in vitro*. Al final del estudio (15 semanas de edad) se determinó también la concentración de anticuerpos anti-OVA en suero, lavado intestinal y sobrenadantes de células en cultivo procedentes de bazo y MLN. Asimismo, se cuantificó el número de células esplénicas secretoras de IgG, IgM e IgA anti-OVA.

Resultados: El suplemento con CLA incrementó la proliferación esplénica específica frente a OVA alrededor de un 50% (p<0,05), y disminuyó la proliferación celular tras estimulación policlonal cerca de un 10-20% (p<0,05). Esta disminución en la proliferación esplénica, se acompañó de una menor secreción de IL-2 (p<0,05). El aporte de CLA durante 17 semanas no incrementó la producción de anticuerpos anti-OVA en suero, bazo o MLN, ni el número de células secretoras de anticuerpos anti-OVA. Sin embargo, cabe destacar, que CLA aumentó la producción de IgA intestinal anti-OVA un 75%, aproximadamente (p<0,05).

Conclusión: El suplemento dietético con un 1% CLA, desde la gestación hasta la edad adulta, potencia la respuesta inmunitaria específica de tipo celular a nivel sistémico y de tipo humoral a nivel intestinal. Estos resultados apoyan los efectos beneficiosos del suplemento prolongado del isómero *cis*9,*trans*11 de CLA de forma continuada a lo largo de la vida sobre el sistema inmunitario.

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Long-Term Feeding of the *cis*-9,*trans*-11 Isomer of Conjugated Linoleic Acid Reinforces the Specific Immune Response in Rats[1,2]

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Abstract

Several effects on the immune system have been ascribed to the *cis*9,*trans*11 conjugated linoleic acid (CLA) isomer. We studied whether feeding a diet enriched with an 80:20 CLA isomer mix of *cis*9,*trans*11 and *trans*10,*cis*12 CLA from gestation to adulthood affects the capacity of adult rats to achieve a specific immune response. Pregnant Wistar rats were fed a 1% CLA diet or a control diet beginning on d 7 of gestation. Weaned pups received the same diet as dams until they were 15 wk old. Rats from both groups were immunized with ovalbumin (OVA) when they were 9 wk old. Dietary CLA enhanced splenocyte OVA-specific proliferation by ~50% ($P < 0.05$) and decreased the mitogen-induced proliferative responses of these cells by ~10–20% ($P < 0.05$). The diminished splenocyte proliferative response was accompanied by a lower interleukin-2 secretion ($P < 0.05$). Long-term CLA supplementation did not increase serum, spleen, or mesenteric lymph node production of OVA-specific antibodies (Ab) or the number of spleen anti-OVA Ab-secreting cells. Interestingly, dietary CLA increased intestinal anti-OVA IgA production by ~75% ($P < 0.05$). In conclusion, a 1% CLA diet administered from gestation to adulthood enhanced specific systemic cell-mediated immunity as well as the mucosal IgA immune response, whereas it downregulated the polyclonal activation of the immune system. These data support the long-term effects of dietary *cis*9,*trans*11 CLA isomer on the immune system. J. Nutr. 139: 76–81, 2009.

Introduction

The influence of dietary fatty acids on the immune function was first studied by Meade and Mertin (1), who focused on the effects of fatty acids on in vitro lymphocyte proliferation. Many studies have subsequently been published and currently it is widely known that dietary fatty acids are able to modify immune responses. The mechanisms involved in these effects influence cell signaling, gene expression, cell membrane structure and function, and the profile of lipid mediator production (2–5).

Conjugated linoleic acid (CLA)[6] is a lipid of great importance. The *cis*9,*trans*11 CLA isomer, also called rumenic acid, is the predominant isoform and is naturally found in beef and dairy products. Several other CLA isomers are industrially produced during vegetable oil processing, with the most abundant among these isomers being the *trans*10,*cis*12 CLA isomer. Many health benefits are ascribed to CLA, including anticancer (6–8), antiatherogenic (9), antidiabetogenic (10,11), and modifying body composition (12) and bone mass (13). However, results from studies in rodents and humans indicate an increase in the concentration of blood sugar and insulin, insulin resistance, VLDL, and reduced blood leptin and HDL (14). In overweight human subjects, the *trans*10,*cis*12 CLA isomer caused a several-fold increase in lipid peroxidation and serum C-reactive protein (15). Additionally, CLA isomer mixtures have been shown to have immunomodulatory properties, ranging from activation to inhibition (16,17). These discrepancies are mainly due to the isomer mixtures used for supplementation. The *trans*10,*cis*12 CLA isomer is responsible for body fat reductions (18), whereas both the latter and the *cis*9,*trans*11 CLA isomer have probable immunomodulatory properties (16). CLA immunomodulation has often been examined by identifying changes in the mitogen-induced immune response (19), but little attention has been drawn to the interaction between CLA and the adaptive immunity after specific antigen (Ag) challenge and even less work has been carried out studying the mucosal compartment.

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[2] Author disclosures: C. Ramírez-Santana, C. Castellote, M. Castell, M. Rivero, M. Rodríguez-Palmero, À. Franch, and F. J. Pérez-Cano, no conflicts of interest.
[6] Abbreviations used: Ab, antibody; Ag, antigen; CAS, casein; CCM, complete culture media; CLA, conjugated linoleic acid; IL-2, interleukin-2; MLN, mesenteric lymph node; OVA, ovalbumin; PMA/Io, phorbol myristate acetate/Ionomycin; SC, secreting cell; TPS, total period of supplementation; US, unstimulated cell.
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The intestinal immune system is the largest and most complex part of the immune system and its responses are elaborated by interaction of regulatory mechanisms that ensure the maintenance of gut homeostasis. Secretory IgA is the main immunoglobulin on the mucosal surfaces (80–90%) and has the shared role of protecting against both ubiquitous foreign substances and microbes while not subjecting the mucosa to undue inflammation (20).

Because previous studies have suggested that CLA intake during developmental phases may have effects later in life (21,22), and that the *cis*9,*trans*11 CLA isomer prevails in breast milk and constitutes ~80% of the total CLA isomers present (23), this study was performed from gestation to adulthood. The aim of this study was to ascertain whether the capacity to produce a specific immune response in ovalbumin (OVA)-sensitized adult rats is influenced by long-term feeding of an enriched diet containing an 80:20 CLA isomer mix of *cis*9,*trans*11 and *trans*10,*cis*12 CLA, respectively.

Materials and Methods

Rats. Pregnant Wistar rats at 7 d of gestation were obtained from Harlan. The rats were housed in individual cages under controlled temperature and humidity conditions in a 12-h-light:12-h-dark cycle and consumed food and water ad libitum. Rats were monitored daily and allowed to deliver at term. Litters were unified to 10 pups per lactating dam; pups had free access to the nipples and rat diet. On the day of weaning, rats were separated by gender and distributed to 3 per cage. Body weight and food intake were monitored weekly. Handling was done in the same time range to avoid the influence of biological rhythms. At the end of the study, we measured body and tail length for each rat, as well as thymus, spleen, and liver weight. Body weight and body length (nose-anus length) were used to determine BMI (kg/m^2). 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 at the University of Barcelona and approved by the Catalonian Government (UB 302–05/DMA 3241).

Experimental groups and diets. Pregnant Wistar rats at 7 d of gestation were assigned to 1 of the 2 dietary groups and after delivery, litters were kept with their dams until weaning (d 21). Thereafter, pups consumed the same diet as their mothers. The 2 dietary groups were the CLA group ($n = 20$) and the control group ($n = 20$). The CLA group were rats whose dams were fed a 1% CLA-enriched diet during gestation (2 wk) and suckling (3 wk); pups received CLA through the placenta and milk, respectively. From weaning until the end of the study (15-wk-old rats), rats were also fed 1% CLA diet [total period of supplementation (TPS), 17 wk]. The control group rats were fed a control diet throughout the 17 wk of study (TPS, 0 wk).

The control diet corresponded to the AIN-93G formulation (24). The 1% CLA diet was obtained from modified standard flour (AIN-513, Harlan) containing 10 g CLA/kg flour (**Table 1**), using a CLA isomer mixture of ~80% *cis*9,*trans*11 and 20% *trans*10,*cis*12 among the total of CLA isomers in the oil (79.5%). CLA oil was kindly supplied by Loders Croklaan, Lipid Nutrition, Wormerveer, The Netherlands. The CLA mixture had 0.69% FFA as oleic acid, a peroxide value of 0.2 mEq/kg, 5.6% SFA, and <5% of CLA minor isomers. The standard flour AIN-513 was modified with 1% less soybean oil and replaced with the same amount of CLA (Table 1). The mixture was pelletized (1-cm-diameter pellets) and dried in a 40°C oven for 24 h. The pelleted diet 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 a 5% weight loss because of drying. This diet was examined and 1% CLA content and absence of PUFA oxidation was confirmed. The 1% CLA (wt:wt) pelleted diet was produced in the Medicine Development Service of the Faculty of Pharmacy at the University of Barcelona.

OVA immunization. Nine-week-old rats received 10 mg/kg of OVA (grade V, Sigma Immunochemicals) emulsified with alum adjuvant (Inject Alum, Pierce) by intraperitoneal injection.

Sample obtaining. Six weeks after immunization, rats were killed and a macroscopic organ observation was performed. Blood was obtained by cardiac puncture and serum was separated and stored at −20°C until use. Spleen and mesenteric lymph nodes (MLN) were excised for lymphocyte isolation. The small intestine was also removed and a distal portion was weighed, longitudinally opened, cut in 5-mm pieces, and incubated with PBS for 20 min at 37°C in a shaker. After centrifugation, the intestinal wash was stored at −20°C until IgA quantification by ELISA.

Lymphocyte isolation and culture

Lymphocytes from spleen and MLN were immediately isolated after organ excision. Spleen cell suspensions were obtained by passing the tissue through a steel mesh in cold sterile conditions as previously described (25). Then, cells were centrifuged at 600 × *g*; 5 min at 4°C and resuspended in PBS (pH 7.2). Erythrocytes were lysed by adding distilled water to the cell suspension and, after restoring tonicity, cells were washed and resuspended in complete culture media (CCM): RPMI-1640 medium, supplemented with 10% fetal bovine serum (Sigma), 0.1 IU/L streptomycin-penicillin (Sigma), 2 mmol/L L-glutamine (Sigma), and 0.05 mmol/L 2-β-mercaptoethanol (Merck).

MLN cell suspensions were obtained in sterile conditions by passing the tissue through a steel mesh. Cell suspensions were kept on ice for 10 min to remove tissue debris by sedimentation and later cells were centrifuged at 600 × *g*; 5 min at 4°C. Cells were then resuspended in CCM. We determined the number and viability of spleen and MLN lymphocytes by double staining with acridine orange and ethidium bromide (Sigma).

Determination of anti-OVA antibody concentration. Indirect ELISA assays were performed to determine total OVA-specific antibodies (Ab)

TABLE 1 Composition of the experimental diets

Ingredient	AIN-93G[1]	1% CLA
Total energy, *kJ*	15,767	15,763
Fat, *% energy*	16.700	16.700
CLA oil, *% energy*		2.3900
Soybean oil, *% energy*	16.700	14.310
	g/kg	
CAS	200.00	199.98
L-Cysteine	3.0000	2.9900
Corn starch	397.50	397.50
Maltodextrin	132.00	131.90
Sucrose	100.00	99.990
Cellulose	50.000	49.990
Mineral mix	35.000	34.990
Vitamin mix	10.000	9.9900
Choline bitartrate	2.5000	2.5000
Ter-butylhydroquinone	0.014	0.014
Soybean oil	70.000	59.990
16:0	7.7000	6.7000
18:0	2.8000	2.4000
18:1	16.400	14.200
18:2	37.200	32.200
18:3	5.6000	4.8000
80:20 *cis*9,*trans*11:*trans*10,*cis*12 CLA oil[2]		10.000[3]

[1] The diet was prepared according to AIN guidelines (24).
[2] From total oil, 78% corresponded to CLA in triacylglycerol form containing an isomer ratio of 80:20 *cis*9,*trans*11:*trans*10,*cis*12. The CLA mixture had 0.69% FFA as oleic acid, a peroxide value of 0.2 mEq/kg, 5.6% SFA, and <5% of minor CLA isomers.
[3] CLA isomer content after the manufacturing process was ~0.8% and the *cis*9,*trans*11:*trans*10,*cis*12 proportion was 80:20.

in serum, intestinal washes, and spleen and MLN cell supernatants as previously reported (26). Because a standard amount of anti-OVA Ab was not available, we used a pool of OVA-immunized rat serum in each plate to normalize OD results. Results were expressed relative to the control group, which was set at 100%.

Quantification of anti-OVA Ab-secreting cells. We used a previously described enzyme-linked immunosorbent spot technique to quantify anti-OVA IgA-, IgG-, and IgM-secreting cells (SC) from spleen (26).

Specific and polyclonal lymphocyte proliferative response. Specific proliferative response from spleen and MLN cells was determined by culturing 1×10^5 cells in 100 μL of CCM after OVA (10 mg/L) stimulation for 96 h (37°C, 5% CO_2). Addition of control protein [casein (CAS), 10 mg/L] and only medium [unstimulated cells (US)] were used as negative reference controls for each sample.

Polyclonal proliferative response was also quantified by incubating spleen and MLN cells (1×10^5 cells/200 μL CCM) after stimulating with phorbol myristate acetate [(PMA), 250 μg/L] plus ionomycin [(Io), 250 μg/L] or without stimulus in 96-well plates for 72 h.

In both cases, cell proliferation was determined by ELISA using Cell Proliferation Biotra from Amersham Biosciences according to the manufacturer's instructions.

Detection of interleukin-2 in culture supernatants. Interleukin-2 (IL-2) was quantified in 24-h supernatant cultures of spleen and MLN cells (10^6 cells per well) after stimulation with PMA/Io (250 μg/L). Cytokine concentrations were determined using rat ELISA kits from Biosource. ELISA were performed according to the manufacturers' instructions.

Statistical analysis. Data were analyzed by 2-way ANOVA (diet × stimulation) and when the interaction was significant, by the post hoc Scheffé test. Repeated-measures 2-way ANOVA was used to analyze body weight data in male and female rats. Differences were considered significant at $P < 0.05$.

Results

Morphometrical variables. Body weight was monitored throughout the study according to animal gender due to sexual features differentiation after wk 3 of life. Dietary CLA did not modify body weight of males but increased that of females ($P < 0.05$) from 6 wk of age until the end of the study at age 15 wk (**Fig. 1**). Food intake did not differ between the groups (data not shown). At the end of the study, despite differences in females' body weight, BMI of females (5.5 ± 0.1) and males (7.3 ± 0.1) were similar in the dietary groups. Body and tail lengths of males (44 ± 1 cm and 21 ± 1 cm, respectively) and females (40 ± 2 cm and 21 ± 1 cm, respectively) were not affected by dietary CLA. Thymus, spleen, and liver weights in females did not differ between groups (data not shown). However, the spleen weight of males fed CLA was ~17% lower than that of males from the control group (data not shown; $P < 0.05$).

Mitogen-induced cell immune response. Although the main goal was to determine the effect of CLA supplementation on the capacity to generate an Ag-specific immune response, the mitogen-induced immune response was also evaluated.

Spleen lymphoproliferative capacity was evaluated under in vitro conditions (**Fig. 2***A*). Splenocytes from control and CLA rats had a higher proliferation after mitogen stimulation than US ($P < 0.05$). Splenocytes from rats fed CLA throughout the study (TPS, 17 wk) had a ~10% lower proliferative response than control rats after PMA/Io stimulation ($P < 0.05$). This down-regulatory effect by dietary CLA was not due to cell viability loss, because viability from the CLA after PMA/Io addition was comparable to that of control cells.

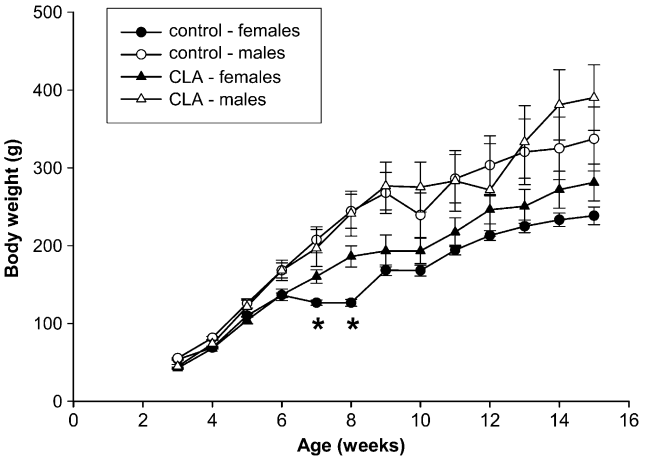

FIGURE 1 Body weight of male and female Wistar rats fed a CLA or control diet from weaning until 15 wk of age. Data are means ± SEM, $n = 20$. *Different from females fed CLA at that time, $P < 0.05$.

IL-2 cytokine secretion was evaluated in 24-h spleen cultures after polyclonal stimulation. The production of this cytokine, responsible for lymphocyte proliferation, was lower in cell cultures of CLA-fed rats than in those of rats fed the control diet ($P < 0.05$; Fig. 2*C*).

The MLN lymphoproliferative response was also evaluated after mitogen stimulation (Fig. 2*D*). MLN cells from rats fed CLA all their lives had a similar proliferative response to rats fed the control diet after mitogen stimulation (Fig. 2*D*). However, IL-2 production and cell viability, measured in 24-h cultures, were not affected by either PMA/Io addition or dietary CLA (Fig. 2*E*,*F*).

Specific anti-OVA cell immune response. To determine the long-term effects of dietary CLA supplementation on specific Ag responses, the lymphoproliferative capacity after OVA addition was evaluated. Control and CLA groups had a higher (2- to 3-fold) splenocyte proliferation after OVA stimulation than unstimulated and control protein addition ($P < 0.05$) (**Fig. 3***A*). In terms of specific proliferative response (mean percentage of increase compared with unstimulated conditions), splenocytes recovered from OVA-immunized rats fed CLA had higher (~275%) lymphoproliferative response to OVA than splenocytes recovered from OVA-immunized rats fed the control diet (~165%) ($P < 0.05$).

The effect of dietary CLA was also established on MLN lymphoproliferative capacity after OVA stimulation (Fig. 3*B*). MLN cells from all OVA-immunized rats proliferated after OVA stimulation compared with unstimulated conditions, but the diet groups did not differ.

Specific anti-OVA humoral immune response. To ascertain long-term CLA diet effects on humoral immune response, we have quantified serum OVA-specific Ab concentration, in vitro anti-OVA Ab (spleen and MLN) production, and spleen anti-OVA-Ab-SC number. Both experimental groups had high anti-OVA Ab concentrations in serum and in splenocyte supernatants (data not shown). Long-term dietary CLA did not modify the humoral response against the OVA-specific challenge in these compartments. Although long-term CLA supplementation tended to increase the in vitro capacity of spleen to produce anti-OVA Ab by 35%, ($P = 0.1$), this was not reflected in the serum concentration of anti-OVA Ab. Moreover, serum total Ig levels were not affected by dietary CLA (data not shown).

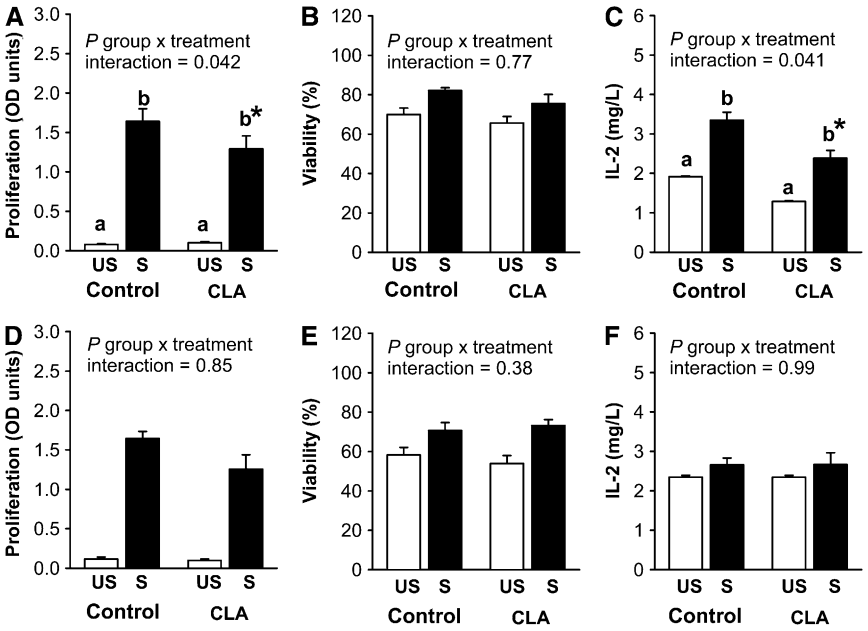

FIGURE 2 Proliferative response (*A*,*D*), viability (*B*,*E*), and IL-2 production (*C*,*F*) of US and PMA/Io-stimulated (S) spleen (*A*–*C*) and MLN (*D*–*F*) cells from rats fed a CLA or control diet. Data are means ± SEM, $n = 20$. Within a diet group, labeled means without a common letter differ, $P < 0.05$. *Different from the corresponding control, $P < 0.05$.

In addition, we counted spontaneous anti-OVA IgG-, IgM-, and IgA-SC in spleens. OVA-immunized rats had more spleen anti-OVA IgG- and IgM-SC than IgA-SC. This pattern was not affected by long-term dietary CLA, although the number of anti-IgA-SC tended to be greater (15.6 ± 3.5) in CLA-fed rats than in control rats (11.9 ± 1.9) ($P = 0.09$).

Anti-OVA Ab levels were also quantified in MLN cell supernatants. These results were similar between both groups (data not shown). However, analysis of intestinal washes showed that dietary CLA modulated mucosal IgA production. Long-term dietary CLA increased the anti-OVA IgA levels in the intestinal mucosa ~75% ($P < 0.05$), although CLA did not modify total gut IgA (data not shown). These data suggest that the 1% CLA diet had a restricted enhancement effect on OVA-specific IgA intestinal production and not a general effect on humoral immunity.

Discussion

In this study, we reported on the effects of long-term feeding a CLA mixture of *cis*9,*trans*11 and *trans*10,*cis*12 isomers (80:20) on the specific and polyclonal immune responses of Wistar rats. We demonstrated that a long-term CLA diet because gestation enhances some aspects of Ag-specific responses, whereas it downregulates polyclonal activation of the immune system.

Because CLA supplementation lasted 17 wk, it was important to assess whether CLA affected rat growth or had toxic effects. The CLA diet did not modify BMI, showing typical values of Wistar rats fed a control diet (27). However, females fed CLA seemed to have improved feed efficiency, because they achieved the plateau adult body weight (~250 g) at least 2 wk earlier than females fed a control diet. In addition, long-term dietary CLA did not cause macroscopic adverse effects compared with control rats. These results agree with those obtained in male rats fed a *cis*9,*trans*11 CLA-enriched diet for 8 wk (28). Conversely, CLA toxic effects were found in subjects fed 3.4 g/d of purified *trans*10,*cis*12 CLA isomer (14). However, as shown here, 20% of this isomer was well tolerated by Wistar rats for 17 wk beginning with the gestation period.

Although the main goal of this study was to examine whether a long-term 1% CLA diet modulates the capacity to generate an Ag-specific immune response, the in vitro capacity to generate a mitogen-induced response was also evaluated. It is the first time, to our knowledge, that this kind of global analysis allows showing that data obtained from the evaluation of an Ag-specific challenge are due to direct effects of CLA on the specific mechanism of immune response and excludes the possibility that the observed findings are due to a global effect on the immune system.

Previous studies concerning other PUFA have demonstrated lymphocyte proliferation reduction (5,29). Accordingly, CLA-fed rats in the present study had lower splenocyte proliferative response and IL-2 production than rats fed the control diet. These results agree with those of Tricon et al. (30), who showed that peripheral blood mononuclear cells from subjects fed either

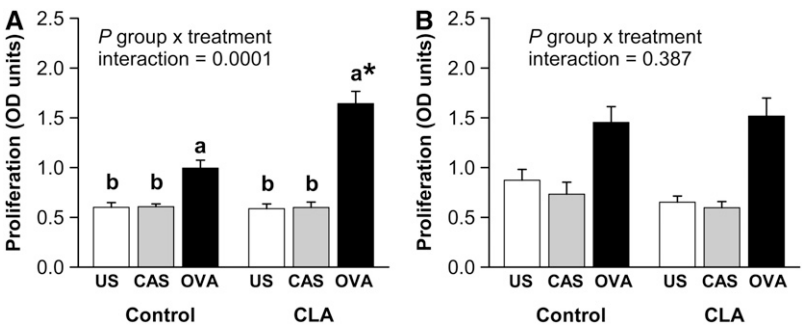

FIGURE 3 OVA-specific immune responses of spleen (*A*) and MLN (*B*) cells from rats fed a CLA or control diet. The proliferative responses were evaluated in US and CAS- and OVA-stimulated cells. Data are means ± SEM, $n = 20$. Labeled means within a diet group without a common letter differ, $P < 0.05$. *Different from corresponding control, $P < 0.05$.

*cis*9,*trans*11 or *trans*10,*cis*12 CLA isomers, after ConA stimulation, decreased CD69 expression, which strongly correlates with lymphocyte proliferation. However, there are other studies using diverse CLA isomer mixtures that described either increased splenocyte proliferation or no effect after stimulus addition (29,31–33).

Besides the polyclonal immunomodulator effects of CLA, the specific anti-OVA immune response also deserves major attention. Some features of the specific systemic and mucosal immune response in OVA-immunized rats have been evaluated. Systemic response was considered by means of specific spleen proliferative response as well as its ability to produce anti-OVA Ab and the serum concentration of anti-OVA Ab. A long-term CLA diet enhanced OVA-specific splenocyte proliferation. This result agrees with that reporting a higher specific proliferative response of T CD8+ lymphocytes from pigs fed a CLA diet (~50:50 isomers mix) (34,35). In addition, following hepatitis B vaccination, specific lymphocyte proliferation was higher in humans fed CLA 50:50 than in the control group (31). Conversely, Kelley et al. (36) showed no effect on influenza-specific proliferation in humans after feeding CLA, but in this case, the 2 main isomers used contributed only 40% of total CLA isomers, whereas in most of the studies affecting proliferative response, the main isomers made up ~80% of all CLA isomers.

OVA-primed spleen B cells produced specific anti-OVA Ab after later OVA contact. Nevertheless, rats fed a CLA diet did not generate a higher systemic (serum and spleen) humoral response against OVA. This might suggest that the presence of 1% CLA in the diet increased neither the number of primed memory B cells nor their ability to produce specific Ab. Our results agree with others carried out in humans and animals fed CLA (35–37), although Albers et al. (31) showed a higher concentration of anti-B hepatitis Ab in subjects consuming CLA 50:50 capsules. On the other hand, CLA feeding did not modify total serum Ig concentrations. This result agrees with many others (33,36,38) but disagrees with a human study that reported increased IgM and IgA plasma concentrations after consuming CLA (39). Nevertheless, better humoral enhancing effects were observed after feeding CLA to young rodents, which reportedly increased concentrations of spleen IgG, IgM, and IgA (40,41), although specific adaptive responses were not addressed in such studies.

Regarding mucosal sites, we found interesting CLA results in this particular immune compartment. CLA supplementation modified neither MLN cell OVA-specific proliferation nor its anti-OVA-Ab production in culture. Nevertheless, a long-term CLA diet increased anti-OVA IgA production at the intestinal level, whereas it did not modify the total IgA concentration in the same samples. The boost of specific intestinal IgA is of great importance, because this Ig is the main isotype present in all mucosa and confers high protection against foreign substances and microbe entry through the intestine, as well as by other mucosal compartments, due to specific secretory IgA homing among mucosal sites (42). Thus, to our knowledge, this is the first time that a CLA supplementation enhancement of Ag-specific mucosal responses has been reported.

Because the CLA diet increased only intestinal-specific IgA, but not spleen, serum, or MLN Ab, it is plausible to suggest that CLA may be enhancing B cells present in the lamina propia or even promoting the IgA-SC migration to the intestine from other immune compartments. This particular type of immunoenhancement induced by CLA, acting on a specific cell subset, is likely, because Bassaganya-Riera et al. (43) reported a higher percentage of a particular immune cell subset, but not of others, in swine fed CLA. Additional experimentation is required to elucidate the mechanism(s) through which CLA enhances specific IgA production in the intestine and whether the *cis*9,*trans*11 CLA isomer is the main isomer responsible for this action.

In conclusion, the present study demonstrates that long-term feeding an 80:20 *cis*9,*trans*11 and *trans*10,*cis*12 CLA mixture influences host immune responses to both polyclonal and specific stimuli. Overall, a long-term 1% CLA diet (from gestation to adulthood) downregulates polyclonal reaction of the immune system, as described for other PUFA, but enhances some aspects of specific cell-mediated immunity as well as the specific IgA intestinal production. These data highlight the evidence of the immunomodulatory effects of CLA, particularly of mixtures rich in the *cis*9,*trans*11 CLA isomer, and suggest that this fatty acid naturally present in dairy products may contribute specifically to mucosal immune defense and/or to counteract situations of immune imbalance.

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