Transworld Research Network
37/661 (2), Fort P.O.
Trivandrum-695 023
Kerala, India

Recent Advances in Pharmaceutical Sciences II, 2012: 203-232 ISBN: 978-81-7895-569-8
Editors: Diego Muñoz-Torrero, Diego Haro and Joan Vallès

12. Immunomodulation by conjugated linoleic acid (CLA) in early life

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Abstract. Conjugated linoleic acid (CLA) has been reported to exert beneficial physiological effects on body composition and the immune system. However, little information is available on the influence of CLA on immune function during early life periods. The present study evaluates the effect of feeding an 80:20 mixture of *cis*-9,*trans*-11- and *trans*-10,*cis*-12-CLA isomers during gestation, suckling and early infancy on the systemic and mucosal immune responses of Wistar rats at three different time points: at the end of the suckling period (21-day-old rats), in early infancy (28-day-old rats), and later in life (adulthood). *Cis*-9,*trans*-11- and *trans*-10,*cis*-12-CLA isomers were detected in the milk of CLA-fed dams and in the plasma of all CLA-supplemented pups, and the highest content was achieved in those rats supplemented over the longest period. Dietary supplementation with that CLA mix enhances the systemic production of the main *in vivo* and *ex vivo* immunoglobulin (Ig) isotypes in 21- and 28-day-old rats. Moreover, CLA supplementation during suckling and early infancy also enhances the humoral immune defenses at intestinal level, by means of mucosal IgA increase, whereas down-regulates the systemic lymphoproliferative response. Finally, we described

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herein how feeding a diet enriched with the same isomer mix of *cis*9,*trans*11- and *trans*10,*cis*12-CLA from gestation to adulthood improves the capacity of adult rats to achieve a specific systemic and mucosal immune responses. All these data support the immunomodulatory effects of dietary supplementation of CLA, particularly of the *cis*9,*trans*11-CLA isomer, during early stages of life on immune system development, as well as the long-term effects on the specific immune response in adult age.

Introduction

Birth is for the newborn a transition from a sterile environment to a world full of bacteria and viruses, where protection is crucial. During the first stages of life, antibodies from the mother are transferred to the foetus and the child decrease the number of infectious episodes caused by microorganisms to which exist maternal immunological memory [1,2]. The immune system is in constant evolution, and their function is profoundly influenced by maternal, environmental, dietary, and behavioural 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 [3].

Over the last years, the effect of nutrition on the development of the immune system has acquired great interest and has led to adoption of the term "immunonutrition". Because breast milk is the only natural food for newborns, and dietary contact has a pivotal role in the development of their immune system, significant progress has been made in the characterisation of milk components affecting growth, development and functions of the gastrointestinal tract and the immune system [4]. In this sense, breast milk is rich in, among others, immunoglobulins (Ig) that can bind and neutralise pathogens in the intestinal tract; bactericidal factors such as lactoferrin, lactoperoxidase and lysozyme and growth factors, nucleotides and cytokines that improve immune defence and gut-barrier function [5]. Besides these compounds, the dietary lipids present in breast milk have been studied with special attention. In this sense, it has been suggested that polyunsaturated fatty acids (PUFA), specifically docosahexaenoic acid and arachidonic acid, which constitute a relatively low fraction of the total fatty acids in human breast milk, participate in neonate immune development [6].

Human milk contains measurable quantities of conjugated linoleic acid (CLA), a class of positional and geometric conjugated dienoic isomers of linoleic acid. The predominant CLA isomer in milk and dairy products is *cis*-9,*trans*-11 (*c*9,*t*11)-CLA, also called rumenic acid, which ranges in human milk from 83 to 100% of total CLA [7,8]. The *trans*-10,*cis*-12 (*t*10,*c*12)-CLA isomer is also found in dairy products, although in lower proportion than rumenic acid [8,9], but even very low doses of the *t*10,*c*12-

CLA isomer seem to have large biological effects [10]. The concentration of CLA in milk is influenced by the intake of food of ruminant origin [8].

CLA has been reported to exert beneficial physiological effects on body composition and inhibition of carcinogenesis, atherosclerosis, and diabetes [11-14]. Moreover, CLA isomer mixtures have been shown to modulate immune function *in vitro* and *in vivo* [15-19]. 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. However, little work has been done on the immunomodulatory effects of CLA during the early postnatal periods (lactation or infancy), and even less during the prenatal period (gestation).

The present study evaluates the influence of dietary supplementation during gestation, suckling, and early infancy with an 80:20 isomer mix of *c*9,*t*11- and *t*10,*c*12-CLA on the systemic and mucosal immunity in Wistar rats at the end of the suckling period (21-day-old rats), in early infancy (28-day-old rats), and later in life (adulthood).

1. Dietary supplementation with CLA during early life on the development of the immune system in rats

The aim of this part of the study was to evaluate the effect of dietary supplementation during early life with an 80:20 isomer mixture of *c*9,*t*11- and *t*10,*c*12-CLA on the development of the immune system in Wistar rats. CLA supplementation was performed during three life periods: gestation, suckling, and early infancy. For that purpose, the immunomodulatory effects of dietary CLA during gestation and suckling were evaluated in 21-day-old rats (at the end of the suckling period) [20]. And, on the other hand, the influence of CLA supplementation limited to suckling period and to early infancy was evaluated in 28-day-old rats (early infant rats, 1 wk after weaning) [21]. Moreover, as it has been suggested that CLA intake during early stages of development may have effects later in life [22,23], we have also studied whether CLA supplementation limited to suckling produces effects which can last until early infancy [21].

At both ages, their immune system is still in maturation (i.e., antibody production), however, in 28-day-old rats some immune functions are similar to those of adult animals (i.e., lymphoproliferative response). To investigate the immunomodulatory ability of CLA supplementation on the immune system of 21- and 28-day-old rats, both systemic and mucosal immune responses were evaluated [20,21,24].

1.1. Experimental design

Animals were distributed in eight experimental groups according to the total period of CLA supplementation, administration route used, and age at the time immune status was assessed (21- or 28-day-old) (**Fig. 1**).

Day 21 assessment: Pregnant Wistar rats at 7 days' gestation were randomly assigned to one of the following four dietary groups, and pups from these groups were sacrificed at the end of the suckling period (21- day-old):

- 21/5* group: Pups from dams fed a 1% CLA diet during the last 2 weeks of gestation and throughout the suckling period. During suckling, the pups received CLA through the dams' milk. The total period of supplementation was 5 wk.
- 21/5 group: Pups from dams fed a 1% CLA diet during gestation and a standard diet during suckling. During suckling, pups were CLA-supplemented daily by oral (p.o.) administration. The total period of supplementation was 5 wk.
- 21/3 group: Pups from dams fed the standard diet during gestation and suckling. Pups received CLA daily by p.o. administration throughout the suckling period. The total period of supplementation was 3 wk.
- 21/0 group (Ref): Pups from dams fed the standard diet throughout the study. These animals constitute the reference diet group. The total period of supplementation was 0 wk.

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

- 28/4 group. Pups received CLA daily by p.o. administration during suckling; after weaning, animals were fed 1% CLA diet from day 21 to 28 (early infancy). The total period of supplementation was 4 wk.
- 28/3 group. Pups received CLA daily by p.o. administration during suckling; after weaning, animals were fed standard diet up to day 28. The total period of supplementation was 3 wk.
- 28/1 group. Pups received 1% CLA diet exclusively for one week after weaning (days 21–28). The total period of supplementation was 1 wk.
- 28/0 group (Ref). Pups fed standard diet during suckling and early infancy. These animals constitute the reference diet group. The total period of supplementation was 0 wk.

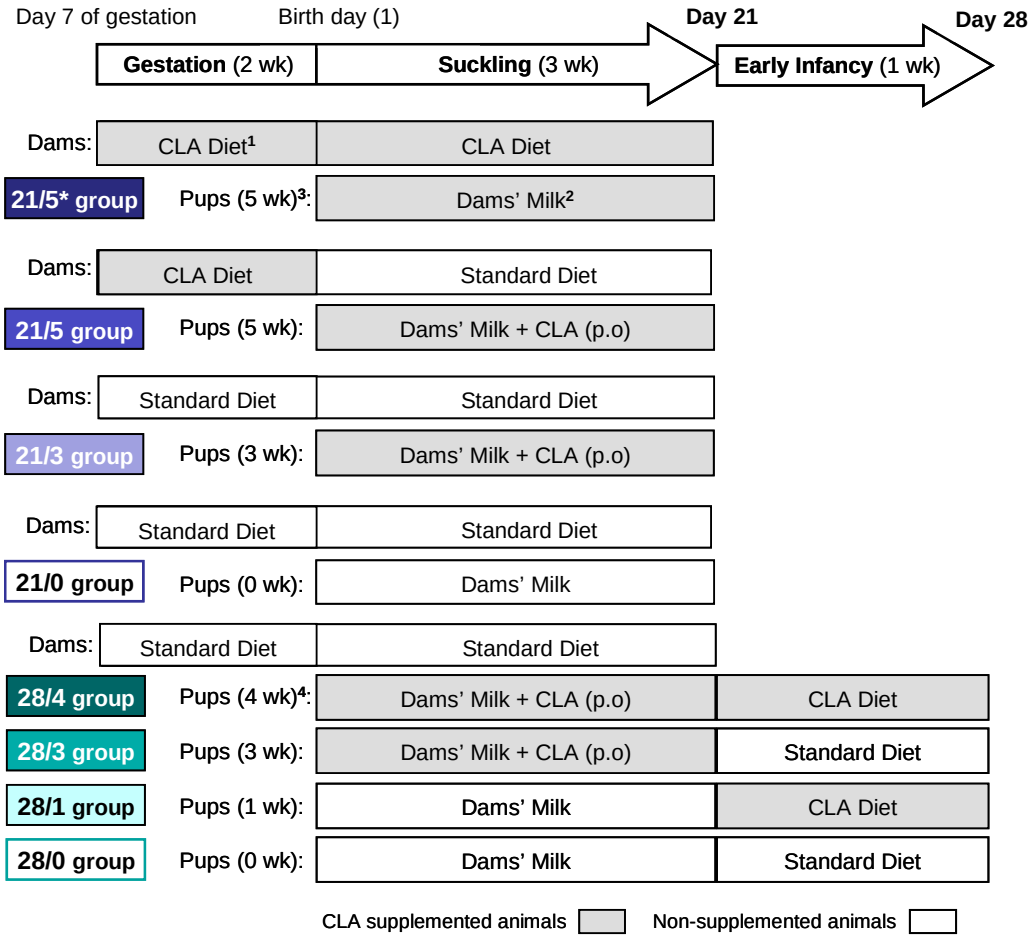

Figure 1. Diagram of the experimental design beginning on day 7 of gestation until day 21 of suckling or day 28 of life. [1] CLA arrives at the foetus by transplacental transfer. [2] CLA arrives at pups through the dams' milk. [3] Total period of CLA supplementation from gestation until the end of suckling. [4] Total period of CLA supplementation from the day of birth until 1 wk after weaning.

1.2. Dietary CLA supplementation

The standard diet used in this study corresponded to the American Institute of Nutrition (AIN)-93G formulation, containing 7% soybean oil. The 1% CLA diet was obtained from modified standard flour AIN-513 (Harlan) containing 10 g CLA/kg [20]. 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. This proportion has been chosen due to its resemblance to that one present in breast milk [7]. 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 of rat body weight from day 1 to 21.

1.3. Relative CLA concentration in dams' milk

In order to confirm the CLA transfer from dams to pups through milk, we collected milk from dams on day 21 post-partum [20] and evaluated the content of *c*9,*t*11- and *t*10,*c*12-CLA isomers by fast gas chromatography, as previously described [25] (**Table 1**). The results showed that, at the end of suckling (day 21), milk from dams fed the CLA diet during gestation and suckling showed higher concentrations of the *c*9,*t*11- and *t*10,*c*12-CLA isomers than rats fed the standard diet. Moreover, the presence of a small quantity of *c*9,*t*11- and not of *t*10,*c*12-CLA in the milk of non-supplemented rats supports the concept that rats produce rumenic acid (*c*9,*t*11-CLA), as has been suggested by other authors, by conversion of free linoleic acid by the intestinal bacterial flora [26] or by the endogenous conversion of trans-vaccenic acid, present in vegetable oils contained in standard diets [27].

On the other hand, the proportion of these two isomers in milk, 86:14, varied from that supplemented to dams, 80:20 (**Table 1**). The lower proportion of *t*10,*c*12-CLA than that initially supplemented is probably due to the faster metabolism of this isomer. It has been reported that *t*10,*c*12-CLA activates the β-oxidation system more strongly than *c*9,*t*11-CLA in rats; therefore, the former could easily become oxidised [28].

Table 1. Relative content of *c*9,*t*11- and *t*10,*c*12-CLA isomers in dams' milk and in the plasma of 21- and 28-day-old pups (% total fatty acids).

	***c*9,*t*11-CLA**[1]	***t*10,*c*12-CLA**[1]
Dams milk: **standard diet**	0.02 ± 0.00	0.00 ± 0.00
Dams milk: **CLA diet**	2.93 ± 0.11**	0.46 ± 0.02**
Plasma: **21/5* group**	1.34 ± 0.01*$^{\sigma\varpi}$	0.21 ± 0.01*$^{\sigma\varpi}$
Plasma: **21/5 group**	1.78 ± 0.05*$^{\sigma}$	0.13 ± 0.01*$^{\sigma}$
Plasma: **21/3 group**	0.90 ± 0.01*	0.05 ± 0.00*
Plasma: **21/0 group**	0.15 ± 0.01	N.D.
Plasma: **28/4 group**	1.31 ± 0.03*$^{\phi\Psi}$	0.13 ± 0.01*$^{\phi\Psi}$
Plasma: **28/3 group**	0.44 ± 0.03*$^{\phi}$	0.05 ± 0.00*$^{\phi}$
Plasma: **28/1 group**	1.23 ± 0.03*	0.08 ± 0.00*
Plasma: **28/0 group**	0.15 ± 0.01	N.D.

[1] Values are expressed as mean ± SEM (n= 4-7 dams/group; n= 10 pups/group). N.D., non-detectable. Significant differences: **P<0.001 *vs.* standard diet group; *P<0.05 *vs.* age-matched reference group (21/0 or 28/0); $^{\sigma}$P<0.05 *vs.* 21/3; $^{\varpi}$P<0.05 *vs.* 21/5; $^{\phi}$P<0.05 *vs.* 28/1; $^{\Psi}$P<0.05 *vs.* 28/3. Modified of Ramírez-Santana *et al.* [20,21].

1.4. Relative CLA concentration in rat plasma

Once confirmed that the CLA from the dams' diet was able to be transferred through dams' milk to pups, the efficiency in the incorporation of such PUFA from milk or directly by p.o. was also evaluated in the pups [20,21]. Therefore, the content of *c*9,*t*11- and *t*10,*c*12-CLA isomers in the plasma of 21- and 28-day-old pups was quantified by fast gas chromatography (**Table 1**). Regarding the concentration of CLA in the plasma of pups determined on the day of weaning, pups from group 21/0 (Ref) showed a low plasma content of *c*9,*t*11-CLA and no *t*10,*c*12-CLA. Groups 21/5*, 21/5 and 21/3 had approximately nine, twelve and six times higher levels of *c*9,*t*11-CLA than group 21/0, respectively. Moreover, the plasma CLA content in the groups supplemented with the 80:20 CLA mix during gestation and suckling (21/5* and 21/5 groups; 5 wk) was ~ 1.5 and 2 times higher, respectively, than that of animals supplemented only during suckling (21/3 group; 3 wk) (**Table 1**).

These results demonstrate that CLA was absorbed by all the supplemented animals since *c*9,*t*11- and *t*10,*c*12-CLA isomers were detected in the plasma of all 21-day-old rats fed CLA. They also indicate that CLA, besides being incorporated after CLA p.o. supplementation, was also able to be transferred from dams to pups during suckling. However, these results cannot demonstrate that CLA transfers through the placenta, since the plasma of newborns was not analysed. Nevertheless, Chin *et al.* [29] found a 20-fold increase of CLA in the liver of foetuses (at day 20 of gestation) from dams fed a 0.5% CLA diet during gestation than in foetal livers coming from dams fed a control diet. This, together with our findings of a higher content of *c*9,*t*11- and *t*10,*c*12-CLA in the milk of rats fed CLA than in those fed the standard diet and the higher content of this isomer in 21/5 group plasma than in 21/3 group, confirms that CLA is transferred through the placenta to the foetus, as well as through the milk to the pup. The highest plasma CLA values in pups from 21/5 group may be due to CLA transfer during suckling from the maternal stores accumulated during gestation, besides supplementation by p.o. administration.

Similarly to that occurred in dams' milk after CLA intake, the supplemented proportion of *c*9,*t*11- and *t*10,*c*12-CLA (80:20) changed in all the groups (**Table 1**). The proportion of CLA isomers was 86:14, 93:7 and 94:6 for groups 21/5*, 21/5 and 21/3, respectively. Hence, group 21/5 presented a higher content of *c*9,*t*11-CLA, but a lower content of *t*10,*c*12-CLA than group 21/5*. Moreover, differences between 21/5* and 21/5 groups in the proportion of CLA isomers in pups' plasma may be due to the influence of the food matrix in 21/5* group, as milk is one of the major factors that affect PUFA bioavailability [30].

Regarding the concentration of CLA isomers quantified in plasma from 28-day-old animals (**Table 1**), plasma from reference rats (28/0 group) had no *t*10,*c*12-CLA and a low content of *c*9,*t*11-CLA, which were lower than those found in the rest of the groups. The animals who received CLA supplementation continuously during suckling and early infancy (28/4 group; 4 wk) showed the highest content of both CLA isomers among the groups. However, rats from the 28/3 group (3 wk) presented lower content of both CLA isomers in plasma than rats from the 28/1 group (1 wk). And, again, as showed for dams' milk and 21-day-old pups' plasma, the relative proportion between *c*9,*t*11- and *t*10,*c*12-CLA was higher in all CLA-supplemented groups (28/4, 91:9; 28/3, 90:10; 28/1, 94:6) than that of the original mixture (80:20).

These results match with the above data concerning 21-day-old animals and with other PUFA studies using similar experimental designs [31]. The highest content of CLA in plasma was achieved in the longest and continuous CLA supplementation (28/4 group), whereas the group that received CLA for 3 wk and then nothing for 1 wk (28/3 group) exhibited lower CLA plasma content than the 28/1 group which only received CLA for the last week. Previous interventional studies have shown that the proportion of fatty acids in the dietary supplement is highly correlated to that one found in plasma and membrane [32]. Therefore, we can suggest that in our study, just finishing the supplementation period, CLA plasma levels are an indicative measure of the CLA incorporated in the cell membranes, in our case for 28/4 and 28/1 groups. But this is not the case for the 28/3 group due to the time lapse between the last CLA intake and the day of quantification of CLA plasma content. The CLA membrane incorporation suggested here agrees with the study of Subbaiah *et al.* [33], which demonstrates that CLA is incorporated into cell membranes to varying extent, depending upon the experimental conditions.

On the other hand, the relative proportion of *c*9,*t*11/*t*10,*c*12 in the plasma of CLA-supplemented animals (~90:10) is higher than in the original isomer mixture (80:20) administered to the animals. These changes in proportions could be attributed to the isomer-differentially β-oxidation induction above commented *(see section 1.3)*. However, although it has been described that both isomers have similar absorption rates in adult rat intestine [34], it is possible that young rodents preferentially absorb the *c*9,*t*11-CLA isomer. Additionally, although no *t*10,*c*12-CLA was found in the plasma of reference animals (28/0), small quantities of *c*9,*t*11-CLA isomer were detected, which would indicate an endogenous production of *c*9,*t*11-CLA, as previously described [26], and, therefore, its influence on the *c*9,*t*11/*t*10,*c*12 ratio observed in plasma.

1.5. Immunoglobulin concentration in dams' milk

One of our objectives was to evaluate the modulation on the Ig response in pups due to CLA, either coming through the milk or directly by p.o. administration. However, it has been described that IgA and IgG have a local mammary gland production [35], and are absorbed by pups from the ingested milk through the intestinal mucosa and extended beyond this compartment. Therefore, to better dissect the direct effect of CLA on Ig production by pups, we first evaluated the CLA influence on IgA, IgG and IgM concentrations in dam's milk whey at the end of the suckling period [20].

As can be shown in **Figure 2**, 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 had a milk concentration pattern of IgG > IgA > IgM, which agrees with that described by Dahlgren *et al.* [35]. This pattern was maintained in dams fed the CLA diet, although IgG and IgA concentrations, the main Ig isotypes in rat milk, were much higher in these rats. Specifically, dams fed the CLA diet during gestation and suckling increased the concentration of IgG and IgA, about 6- and 2-fold, respectively. Thus, rat milk from CLA fed dams not only transfers CLA to pups, but also high concentrations of antibodies.

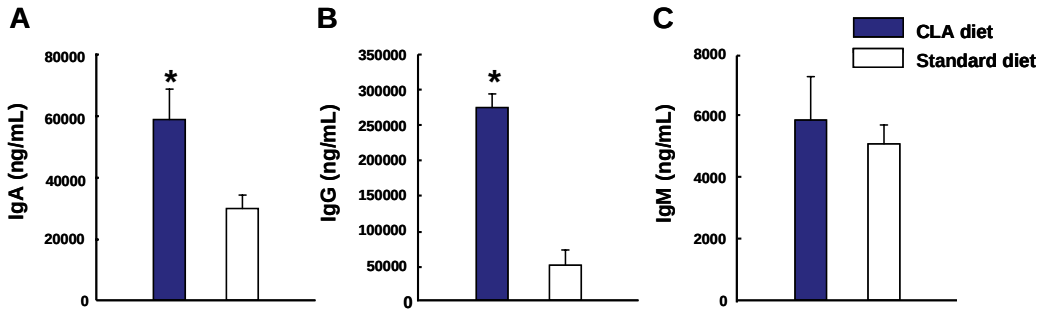

Figure 2. CLA effects on IgA (**A**), IgG (**B**) and IgM (**C**) of rat milk collected on day 21 of the suckling period. IgA, IgG and IgM concentrations were quantified by ELISA in milk whey supernatant fractions after centrifugation and fat layer discarding. Values are expressed as mean ± SEM (n= 4-7). Significant differences: *P<0.05 *vs.* standard diet. Modified of Ramírez-Santana *et al.* [20].

1.6. Effect of CLA on the development of the systemic immunity in rats

As had been mentioned before, this study evaluated the effect of CLA on the systemic immune response in Wistar rats. For that purpose, we quantified serum Ig concentrations, spleen cell proliferation and cytokine secretion ability and *ex vivo* splenocyte Ig production as biomarkers of systemic immune development [20,21].

1.6.1. Serum immunoglobulin concentration

This study evaluated the effect of feeding an 80:20 CLA isomer mixture during gestation and/or suckling and/or early infancy in the incipient antibody production of suckling and early infant rats. For that, serum IgG, IgM and IgA concentrations were quantified in 21- and 28-day-old animals from all the groups included in the exhaustive experimental design (**Fig. 3**).

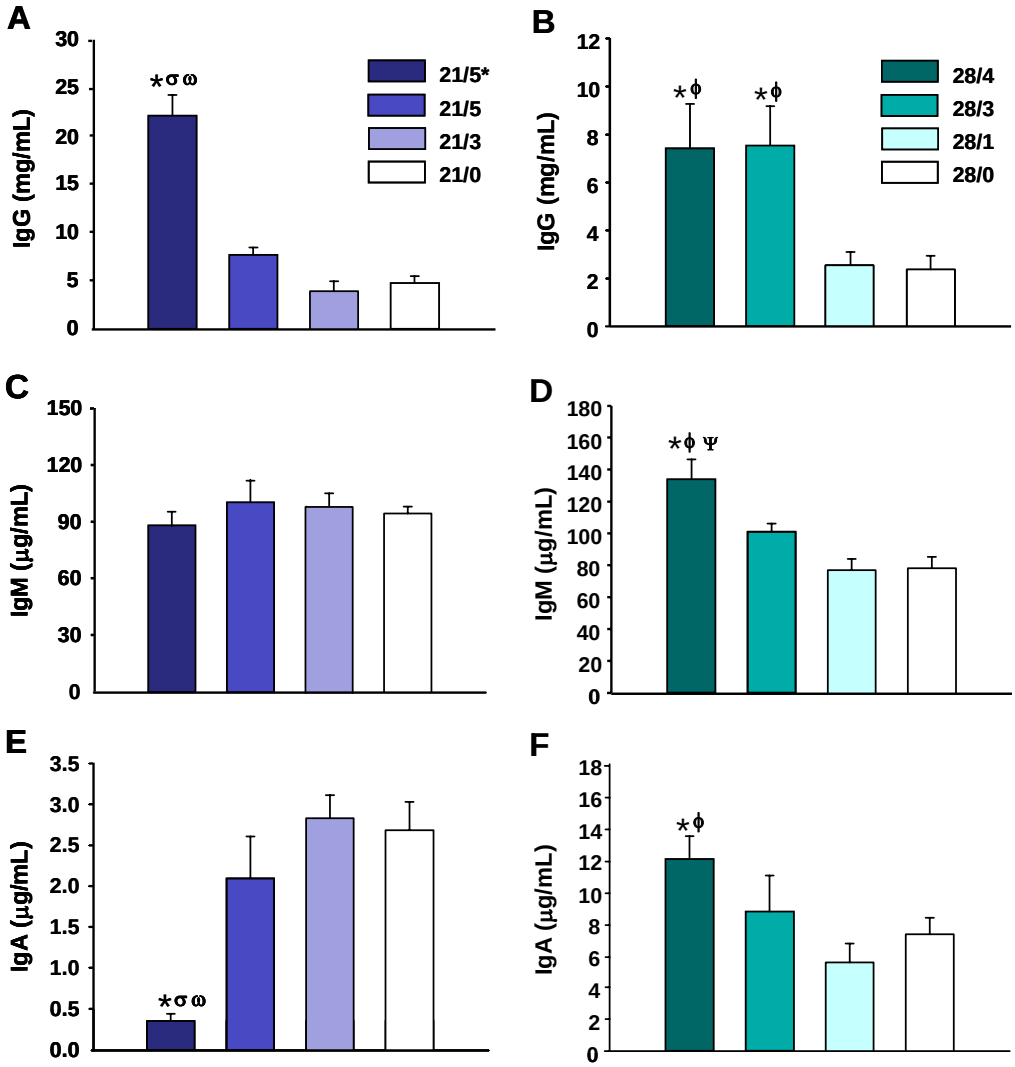

Figure 3. CLA effects on serum IgG, IgM and IgA from 21- and 28-day-old animals. Serum IgG (**A**, **B**), IgM (**C**, **D**) and IgA (**E**, **F**) concentrations were quantified by ELISA at the end of the suckling period (day 21; **A**, **C**, **E**) and 1 week after weaning (day 28; **B**, **D**, **F**). Values are expressed as mean ± SEM (n= 15-20). Significant differences: *P<0.05 *vs.* age-matched reference group (21/0 or 28/0); $^{\sigma}$P<0.05 *vs.* 21/3; $^{\varpi}$P<0.05 *vs.* 21/5; $^{\phi}$P<0.05 *vs.* 28/1; $^{\Psi}$P<0.05 *vs.* 28/3. Modified of Ramírez-Santana *et al.* [20,21].

Regarding the results obtained from the 21-day-old animals, those from the group 21/0 (Ref) showed ~5 mg/mL of IgG (**Fig. 3A**), ~95 μg/mL of IgM (**Fig. 3C**) and ~2.7 μg/mL of IgA (**Fig. 3E**). CLA supplementation for 5 wk, 2 wk during gestation and 3 wk during suckling through the dams' milk (21/5* group), increased the total Ig serum concentration almost 4-fold, mainly by enhancement of IgG. At this age, there were no differences in serum IgM concentration among the groups. However, 21/5* group exhibited a lower IgA serum concentration than those of the other groups.

With respect to 28-day-old animals, rats from the 28/0 group (Ref) had ~2 mg/mL of IgG (**Fig. 3B**), ~80 μg/mL of IgM (**Fig. 3D**) and ~8 μg/mL of IgA (**Fig. 3F**). Similarly to that observed in younger animals, those that received CLA diet during and after suckling (28/4 group) showed higher serum IgG, IgM and IgA concentrations than those receiving CLA only for 1 week after suckling (28/1) and rats from the 28/0 group. Moreover, rats from the 28/3 group, i.e., those receiving CLA diet only during suckling, showed 3 times the IgG concentration than those from the 28/1 and 28/0 groups. Considering total serum Ig, CLA supplementation during suckling (28/4 and 28/3 groups) increased the Ig concentration 4-fold compared to the 28/1 and 28/0 groups, mainly by increasing IgG.

All these results show the enhancing properties of CLA on the main *in vivo* serum Ig isotype, IgG. However, this effect was not observed in all the supplemented groups, a fact that underlines the importance of continuous CLA supplementation during gestation and suckling (21 day assessment), or during suckling and early infancy (28 day assessment). This immuno-enhancing effect has already been reported in older animals, as by Sugano *et al.* [15] in 7-week-old rats receiving 1% CLA (50:50 isomer mix), showing an increase in serum IgA, IgG and IgM concentrations and a decrease in IgE. Song *et al.* [36] reported a similar effect in human subjects following supplementation with a 50:50 isomer mixture for 12 wk. Nevertheless, Yamasaki *et al.* [37] found no significant effect on serum IgA, IgG or IgM concentrations after feeding 5-week-old rats for 3 wk with a 50:50 CLA isomer mixture at doses ranging from 0.05 to 0.5%. Discrepancies with the study reported by Yamasaki *et al.* [37] might be due to the low doses of CLA tested in that study. Studies carried out in other species during gestation and lactation periods have also reported serum IgG increases [38,39], in keeping with the present results.

With respect to the serum IgA decrease observed in the present study after CLA supplementation during gestation and suckling, Turpeinen *et al.* [40] also detected an IgA reduction in subjects with birch pollen allergy supplemented for 12 wk with a CLA mixture containing 63,5% of *c*9,*t*11.

Yamasaki *et al.* [37] found a slightly lower concentration of serum IgA after feeding the 0.5% CLA dose, although the reduction was not significant. Considering that IgA is the main Ig in the gut surface (80–90%) and the fact that systemic IgA-plasma cells continuously migrate to the intestinal wall [41], the serum IgA decrease should not be interpreted as harmful. In fact, this serum IgA decrease is accompanied by an increase in intestinal IgA in CLA-supplemented weaned rats [24; *see section 1.7.1.*]. We also have reported an increase in mucosal IgA after a specific challenge in adult rats following this CLA diet through life [42; *see section 2.3.*].

1.6.2. *Ex vivo* spleen immunoglobulin production

The CLA immunoenhancing effects on antibody synthesis, observed upon the supplementation of *c*9,*t*11- and *t*10,*c*12-CLA isomers at 80:20 ratio *in vivo* in suckling and infant rats, were also studied *ex vivo.* With this purpose, IgM and IgG production by unstimulated spleen cells, from animals corresponding of all groups, were quantified after 7 days of culture [20,21].

The spontaneous IgM and IgG production by splenocytes from 21-day-old rats showed that IgM was the main isotype found in supernatant fractions (**Fig. 4A**), being forty times higher than IgG (**Fig. 4B**). Regarding CLA supplementation, IgM production from both groups supplemented for 5 wk (21/5* and 21/5 groups, ~900–1200 ng/mL) was higher than that of the groups supplemented for 3 wk (21/3 group) and 0 wk (21/0 group) (both ~500 ng/mL). However, this increase was only significant when pups received CLA during gestation and suckling by p.o. administration (21/5 group) (**Fig. 4A**). Otherwise, IgG production was very low (~10 ng/mL) and no differences were found among groups (**Fig. 4B**). Thus, continuous CLA supplementation during gestation and suckling enhances splenocyte IgM production, increasing total Ig concentration by almost 2-fold that of non-supplemented animals.

With respect to 28-day-old assessment, IgM was also the predominant isotype found in supernatants (**Fig. 4C**), being ~100 times higher than IgG production (**Fig. 4D**). The groups supplemented with CLA during suckling (28/4 and 28/3 groups) synthesised higher IgM and IgG levels than the reference group (28/0), but it was only significant in the 28/3 group. This fact indicates the lasting of the CLA effect on the Ig synthesis later in life, or at least 1 week after finishing the CLA intake. Spleen Ig production from the 28/1 and 28/0 groups was similar.

Overall, in the present study, CLA supplementation in early life enhanced spleen IgM production. These results agree with other studies carried out in older rats, which reported enhancement of splenocyte Ig

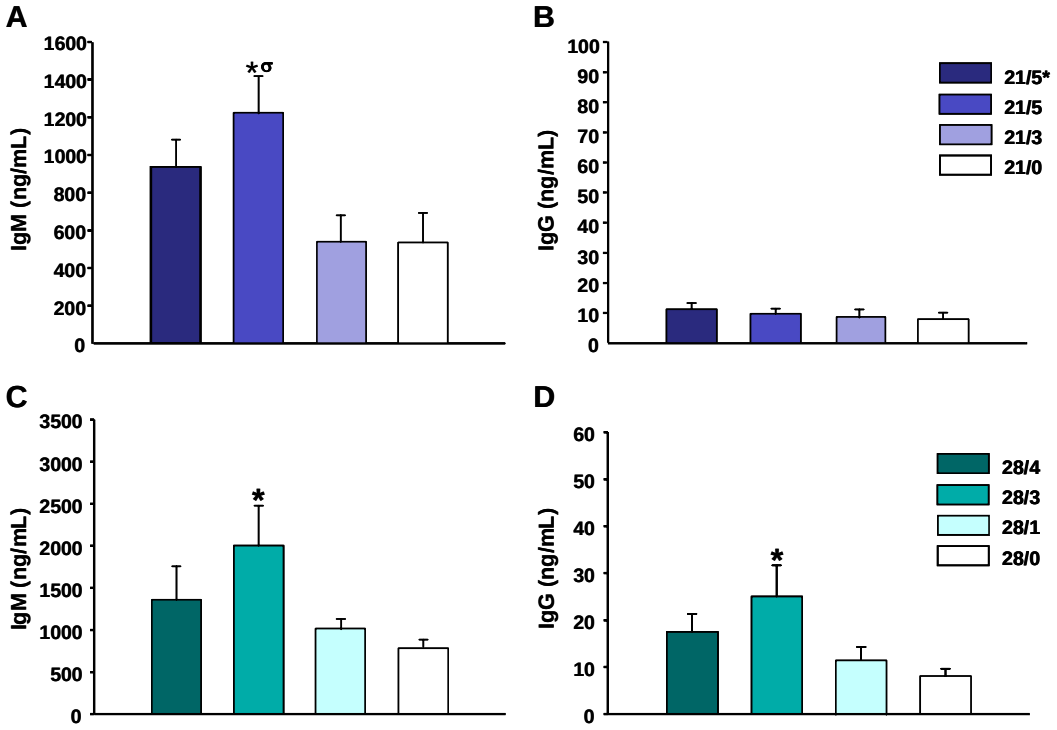

Figure 4. CLA effects on spleen cell Ig production from 21- and 28-day-old animals. IgM (**A**, **C**) and IgG (**B**, **D**) concentrations in supernatants after 7 days of spleen cell culture were quantified by ELISA at the end of the suckling period (day 21; **A**, **C**) and 1 week after weaning (day 28; **B**, **D**). Values are expressed as mean ± SEM (n= 15-20). Significant differences: *P<0.05 *vs.* age-matched reference group (21/0 or 28/0); $^{\sigma}$P<0.05 *vs.* 21/3. Modified of Ramírez-Santana *et al.* [20,21].

production after feeding 50:50 CLA isomer mixtures [15,37]. In addition, some authors have reported this increase in old mice after feeding the pure *t*10,*c*12-CLA isomer [43]. Although the present results confirmed the immunoenhancing effect of the *c*9,*t*11 isomer, we cannot rule out immune functions for *t*10,*c*12-CLA.

1.6.3. Spleen lymphocyte functionality: Proliferation and cytokine secretion

Besides the capacity of *in vivo* Ig production, the influence of dietary CLA supplementation on the functionality of *ex vivo* isolated spleen lymphocytes was also evaluated by their ability to proliferate and to produce cytokines.

At the end of suckling (21-day-old assessment), the CLA diet did not modify the lymphoproliferative capacity in any group (21/5*, 21/5, 21/3), measured 72 h after mitogen-stimulation when compared to reference animals (21/0) (**Fig. 5A**). However, in the assessment of older animals (1 wk after weaning), CLA supplementation during suckling (28/4 and 28/3 groups)

reduced *ex vivo* spleen lymphoproliferative ability compared to that in the 28/1 and 28/0 groups, but it was only significant for the 28/4 group (**Fig. 5B**). Cell viability was slightly reduced after mitogen-stimulation, but, this decrease did not differ among groups in 21-day-old evaluation, indicating that CLA dietary supplementation had no effect on splenocyte viability. On the other hand, CLA supplementation effect on 28-day-old animals conferred more resistance to the mitogen toxic effects, as is demonstrated by the lowest mortality of 28/4 group among the groups. Thus, the decrease in the proliferation rate cannot be attributed to a lower viability caused by CLA diet.

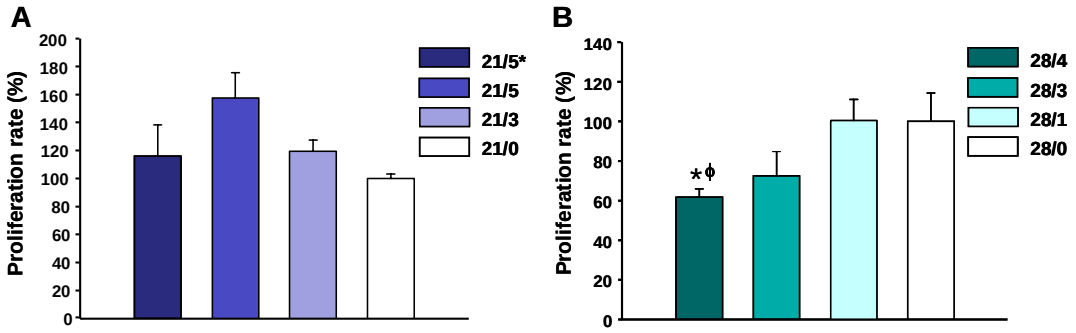

Figure 5. CLA effects on proliferation rate in mitogen-stimulated spleen cells from 21- and 28-day-old animals. Lymphoproliferative response was determined after stimulating with phorbol myristate acetate (PMA) plus ionomycin (Io) for 72 h at the end of the suckling period (day 21; **A**) and 1 week after weaning (day 28; **B**). The proliferation rate (%) are expressed relative to the age-matched reference group (21/0 or 28/0), which was set at 100%. Values are expressed as mean ± SEM (n= 15-20). Significant differences: *P<0.05 *vs.* age-matched reference group (21/0 or 28/0); $^{\phi}$P<0.05 *vs.* 28/1. Modified of Ramírez-Santana *et al.* [20,21].

Moreover, interleukin (IL)-2 production, the main proliferative signal for lymphocytes, was measured in supernatant fractions obtained after 24 h of spleen cell stimulation and was not modified by CLA supplementation either in 21- or 28-day-old assessment (**Table 2**).

The lack of CLA dietary effect on lymphocyte proliferation found in 21-day-old rats is in agreement with the results reported by Kelley *et al.* [16] in 8-week-old mice after feeding pure isomers of CLA for 56 days, and in human subjects either by ingestion of pure isomers [44] or 50:50 and 80:20 isomer mixtures [16,45]. Since IL-2 plays a central role in the cell-mediated immune response by regulating proliferative abilities, it could be expected that if CLA does not modify splenocyte proliferation, it will not affect IL-2 production. The present results are consistent with most other studies in animals and human subjects, which have found no significant effects on IL-2 splenocyte secretion among the dietary groups [43,44,46].

Table 2. Content of cytokines in 24 h splenocyte supernatants after mitogen (PMA/Io) stimulation[1].

Cytokines (day 21)	21/5* group	21/5 group	21/3 group	21/0 group
IL-2 (ng/mL)	4.1 ± 1.1	4.1 ± 1.0	5.6 ± 0.9	4.1 ± 0.8
IFNγ (ng/mL)	4.6 ± 0.8	5.9 ± 1.0	6.4 ± 1.4	3.7 ± 0.8
IL-4 (pg/mL)	29.3 ± 7.9	32.4 ± 6.0 $^{(*)}$	15.9 ± 1.3	12.4 ± 1.8
IL-10 (pg/mL)	181.7 ± 47.2	176.3 ± 43.8	90.6 ± 8.5	108.0 ± 19.6
Cytokines (day 28)	**28/4 group**	**28/3 group**	**28/1 group**	**28/0 group**
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
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*$^{\phi\Psi}$	40.1 ± 12.9	45.7 ± 15.1	N.D.

[1] Values are expressed as mean ± SEM (n= 10). N.D., non-detectable. Significant differences: $^{(*)}$ P=0.06 *vs.* reference group (21/0); *P<0.05 *vs.* reference group (28/0); $^{\phi}$P<0.05 *vs.* 28/1; $^{\Psi}$P<0.05 *vs.* 28/3. Modified of Ramírez-Santana *et al.* [20,21].

Despite the above results, a wide range of PUFA, including CLA, have been found to reduce the mitogen-stimulated proliferation of lymphocytes isolated from several species [47,48]. Thus, the lack of effect observed in 21-day-old animals, may be probably due to the immaturity of this function (which is acquired later in life) at a very early age [49]. In contrast, the mitogen-induced splenocyte proliferation on 28-day-old animals was down-modulated in the longer-lasting CLA supplementation, similarly to that described in adults [42,50,51]. Thus, the age of 28 days is optimal to evaluate CLA effects, since at this age CLA still increases antibody production and is also able to down-modulate lymphoproliferation. Calder and Newsholme [52] reported that some PUFA inhibited lymphocyte proliferation without decreasing IL-2 concentration, a fact that is consistent with the present results showing that CLA supplementation did not modify IL-2 production. In this sense, many studies have described conformation changes in IL-2 receptors by PUFA, specifically modifying lipid rafts [53,54]. Thus, CLA, even with trans double bonds, could potentially alter membrane structure, including lipid rafts, by preventing IL-2Rα migration to soluble membranes, where IL-2 signalling occurs and T-cell activation and proliferation are consequently induced [53,55,56]. In addition, the anti-proliferative lymphocyte effect exhibited by dietary CLA in the present study could be mediated by the nuclear peroxisome proliferator-activated receptor (PPAR)γ,

because intestinal PPARγ gene expression has been found up-regulated after CLA supplementation *(see section 1.7.2.)* [24]. The PPARγ-dependent CLA effect was first described by Bassaganya-Riera and Hontecillas [57] in a pig--inflammatory bowel disease model, where dietary CLA resulted in intestinal disease amelioration and PPARγ gene expression up-regulation.

Besides IL-2 quantification, in the present study other cytokines were also analyzed in splenocyte supernatants after mitogen-stimulation (**Table 2**). Interferon (IFN)-γ, a T helper 1 (Th1) cytokine, was also secreted in similar amounts in all experimental groups. T helper 2 (Th2) cytokines, IL-4 and IL-10, were also quantified in the same splenocyte supernatant fractions and although no statistical differences were found among groups, due to the large intra-group variability, rats from 21/5* and 21/5 groups showed almost 2-fold higher values than those observed in 21/3 and 21/0 groups (**Table 2**). This IL-10 increase is in line with studies showing higher IL-10 production by dendritic cells incubated with *c*9,*t*11-CLA after stimulating with lipopolysaccharide [58]. This effect may be related to the anti-inflammatory properties attributed to CLA [22,59]. Moreover, by increasing IL-4, CLA might be promoting T helper 2 (Th2) responses, such as modulating antibody production and inhibiting several cellular functions, which is in agreement with the present results regarding CLA enhancement of the principal *in vivo* and *in vitro* Ig.

However, in 28-day-old animals, the concentration of IL-6, which was also included in the study, was increased in the 28/4 group compared to the 28/3 and 28/1 groups, while IL-6 was not detected in the 28/0 group (**Table 2**). This result is of interest because IL-6 is clearly defined as a prominent regulator of T-cell proliferation and differentiation of Ig-secreting B cells [60]. Therefore, it might be suggested that the effects on proliferation -in early infant rats- and antibody production -in both suckling and early infant rats- induced by CLA could be due to an up-regulation of IL-6 production. Further studies should confirm this hypothesis and ascertain the mechanism involved.

1.7. Effect of CLA on the development of the mucosal immunity in rats

As it had been mentioned before, this study also evaluated the effect of CLA-supplementation during gestation, suckling, and/or early infancy on mucosal immunity (small intestine and colon) in Wistar rats. In these periods, their mucosal immune response is still in development. In fact, 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 [61]. For that reason, in this study we quantified intestinal IgA at both the gene and protein levels as a biomarker of mucosal immune development, and TGFβ and PPARγ gene expression as possible mediators of CLA's immunomodulatory effects [24]. Finally, a broad array of genes was studied in that compartment to elucidate other mediators in CLA action.

1.7.1. Effect of CLA on IgA gene expression and IgA protein secretion in intestinal mucosa

IgA gene expression in small intestine and colon was assessed in 21- and 28-day-old animals from all experimental groups. Dietary CLA did not modify IgA gene expression in small intestine or colon at the end of the suckling period. However, IgA gene expression in animals continuously CLA-supplemented during suckling and early infancy (28/4 group; 4 wk) was up-regulated almost 5-fold (**Fig. 6A, 6B**). This increase was seen in both tissues analyzed as compared with the reference group (28/0). Supplementation limited to the suckling or early infancy periods failed to produce this immunoenhancing effect (**Fig. 6A, 6B**).

In addition to detection of changes in gene expression, IgA protein concentration was quantified in intestinal washes of 28-day-old animals (**Fig. 7**). IgA content was statistically higher in animals CLA-supplemented for 4 weeks (28/4 group) than in animals in the 28/3, 28/1 or 28/0. These results demonstrate that continuous CLA dietary supplementation for a longer period, during suckling and early infancy, increases expression of the IgA

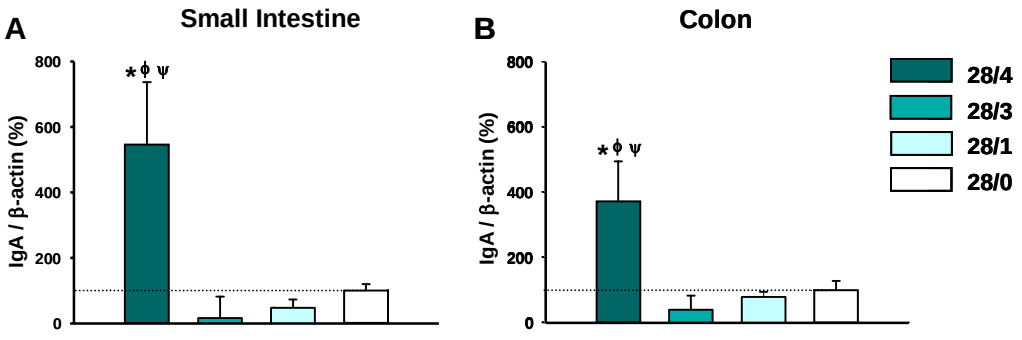

Figure 6. Effect of CLA on IgA gene expression in 28-day-old animals. Gene expression was evaluated in small intestine (**A**) and colon (**B**) by Real Time PCR. IgA gene expression was normalized using β-actin and are showed as percentage relative to values in age-matched reference animals (21/0 or 28/0). Values are expressed as mean ± SEM (n= 5). Significant differences: *P<0.05 *vs.* reference group (28/0); $^{\phi}$P<0.05 *vs.* 28/1; $^{\Psi}$P<0.05 *vs.* 28/3. Modified of Pérez-Cano *et al.* [24].

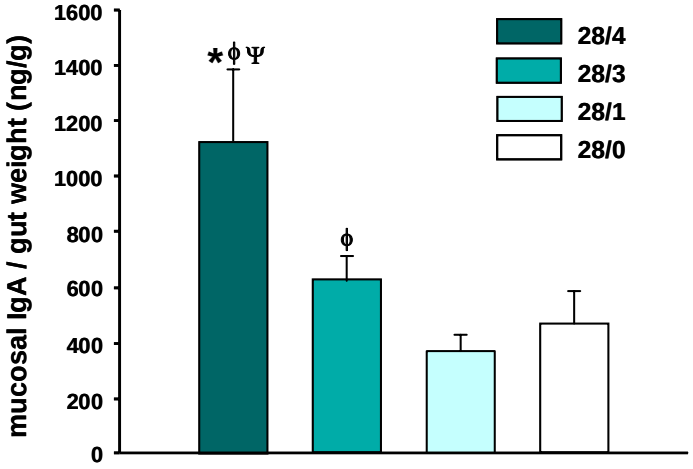

Figure 7. Effect of CLA on IgA in intestinal washes from 28-day-old rats. Secretory IgA was quantified in intestinal washes of small intestine by ELISA. Results are expressed as IgA protein (ng) referred to intestinal weight (g) used for the wash. Values are expressed as mean ± SEM (n= 9-10). Significant differences: *P<0.05 *vs.* 28/0; $^{\phi}$P<0.05 *vs.* 28/1; $^{\Psi}$P<0.05 *vs.* 28/3. Modified of Pérez-Cano *et al.* [24].

gene and protein, thereby enhancing development of the rat's mucosal defense system. This is the first *in vivo* report, to our knowledge, showing an increase of mucosal IgA after feeding CLA during early life [24]. In line with our data, Sugano *et al.* [15] reported an increase in IgA secretion from cultured mesenteric lymph nodes of 7-week-old rats, fed a 1% CLA 50:50 isomer mix.

The specific mechanism by which CLA enhances IgA levels at mucosal sites remains unknown. But since CLA has been shown to suppress IL-4 production *in vitro* [62], attenuate Th2 responses in challenged animals [63], and regulate the number and effectors functions of several lymphocytes [64], further studies should be addressed to elucidate whether it exist a direct enhancer mechanism of CLA on IgA-producing cells.

1.7.2. Effect of CLA on TGF-β and PPARγ gene expression in small intestine and colon

Because IgA gene expression and intestinal production were increased after feeding CLA, we also studied molecules which could have a role in this process. In this sense, transforming growth factor (TGF)β gene expression was a candidate due to its involvement in the isotype switching process from IgM to IgA [65,66]. However, intestinal TGFβ gene expression was not modified in any of the CLA-supplemented groups during gestation, suckling and/or early infancy. Nonetheless, an influence of CLA on TGFβ cannot be

ruled out. If CLA is modulating the effects of TGFβ on IgA production, it is probably due to posttranscriptional and/or translational regulation, which are important in this cytokine, because it has been suggested that TGFβ mRNA levels do not completely correlate with the quantity of protein produced [67]. 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 [68].

On the other hand, two main mechanistic theories have been proposed to explain the immunoenhancing effects of dietary CLA: a PPARγ-dependent and a PPARγ-independent pathway [69]. The present study investigates PPARγ gene expression in the small intestine and colon of 21- and 28-day-old animals fed standard and CLA-supplemented diets (**Fig. 8**). At both ages, there were no differences in PPARγ gene expression in small intestine between CLA and non-supplemented groups. However, PPARγ was up-regulated in colon tissue, particularly in 21-day-old animals fed CLA (21/5*, 21/5, and 21/3 groups), when compared with reference animals (21/0) (**Fig. 8A**). This 2-fold up-regulation was only significant in the 21/5* group. Similar to the results found in 21-day-old animals, colon PPARγ gene expression was also up-regulated in 28-day-old animals fed CLA (28/4, 28/3, and 28/1), when compared with reference animals (28/0) (**Fig. 8B**). Nevertheless, only the 28/3 and 28/1 groups showed statistical differences, since 28/4 group had a great variability. Overall, CLA-supplemented rats showed higher PPARγ expression than non-supplemented animals. Specifically, the effects seem to be related to a

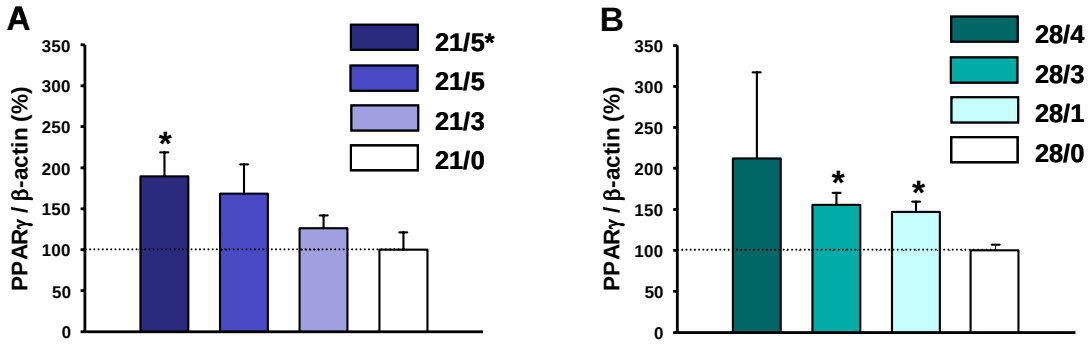

Figure 8. Effect of CLA on colon PPARγ gene expression in 21- and 28-day-old animals. Gene expression was evaluated by Real Time PCR at the end of the suckling period (day 21; **A**) and 1 week after weaning (day 28; **B**). PPARγ gene levels were normalized using the β-actin gene and are expressed as percentage relative to values from age-matched reference animals (21/0 or 28/0). Values are expressed as mean ± SEM (n= 5). Significant differences: *P<0.05 *vs.* age-matched reference group (21/0 or 28/0). Modified of Pérez-Cano *et al.* [24].

dose-dependent manner that was proportional to the duration of supplementation over gestation, suckling, or early infancy. Thus, CLA modulated PPARγ expression in all the dietary conditions examined, even when animals were supplemented for only 1 week.

These results concur with findings from studies showing an increase of PPARγ mRNA expression associated with CLA supplementation in colon of healthy and ill mice and pigs [22,57,63,69]. Moreover, PPARγ up-regulation by CLA is in line with the results of Takamura *et al.* [70], 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 [69].

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 [71,72]. Hence, the effects of CLA found in the present study may be due to the interaction of CLA with PPARγ1. 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.* [73] 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 [74,75], 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 [76], suggesting that CLA may influence the natural mechanisms involved in intestinal homeostasis regulation. Lastly, it has been demonstrated that PPARγ regulates the epithelial differentiation process [77]. 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 [78].

1.7.3. Effect of CLA on rat mesenteric lymph nodes gene expression profiles

The data reported in above *sections 1.7.1.* and *1.7.2.* provide scientific evidence of the impact of lipid nutrition, particularly the influence of the *c*9,*t*11-CLA isomer, on mucosal immunomodulation. On the other hand, it is known that food components play a role in influencing, either directly or

indirectly (through hormonal regulation), the expression of genes involved in immune responses [79]. Thus, further studies were developed to define the mechanism of action CLA at genic level in the intestinal compartment [80].

For that purpose, the gene expression profile in animals supplemented with CLA during gestation and suckling of mesenteric lymph nodes, sites of activation and proliferation of lymphocytes coming from the intestinal tissue (i.e. an inductor site), was determined. The specific GeneChip® Rat Genome 230 2.0 (Affymettrix) and bioinformatic analyses (GeneSpring GX software) were used. It led to the identification of 123 genes differentially expressed in all CLA dietary approaches with respect to the reference group. Generation of a biological association network evidenced several genes, such as connective tissue growth factor (Ctgf), tissue inhibitor of metalloproteinase 1 (Timp1), galanin (Gal), synaptotagmin 1 (Syt1), growth factor receptor bound protein 2 (Grb2), actin gamma 2 (Actg2) and smooth muscle alpha actin (Acta2), as highly interconnected nodes of the resulting network [80]. All these genes modulated by CLA supplementation may have a role on lymphoproliferation and mucosal immune responses in early life.

2. Long-term feeding of CLA from gestation to adulthood: Effect on the immune system in adult rats

As previous studies have suggested that CLA intake during developmental phases may have effects later in life [22,23], this second part of the study was performed from gestation to adulthood. The aim 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 *c*9,*t*11- and *t*10,*c*12-CLA, respectively [42].

2.1. Experimental design

Pregnant Wistar rats at 7 days' gestation were assigned to 1 of the 2 dietary groups and after delivery, litters were kept with their dams until weaning (day 21). Thereafter, pups consumed the same diet as their mothers. The 2 dietary groups were the CLA group and the reference (REF) group (**Fig. 9**). The CLA group was constituted by rats whose dams were fed a 1% CLA diet *(see section 1.2.)* 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), animals were also fed 1% CLA diet. The total period of supplementation was 17 wk. Rats from the reference group were fed a standard diet throughout the 17 wk of study. Nine-week-old

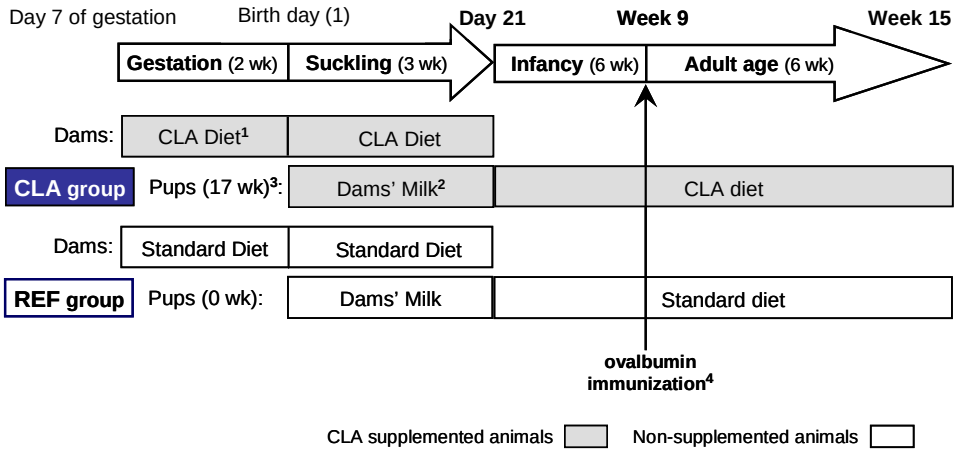

Figure 9. Diagram of the experimental design beginning on day 7 of gestation until week 15 of life. [1] CLA arrives at the foetus by transplacental transfer. [2] CLA arrives at pups through the dams' milk of dams. [3] Total period of CLA supplementation from gestation until 15 weeks of age. [4] Ovalbumin immunization by i.p. injection to 9-week-old rats.

rats from both groups were immunized with ovalbumin (OVA) emulsified with alum adjuvant by intraperitoneal (i.p.) injection. Six weeks after immunization, 15-wk-old rats were sacrificed and immune status was assessed (**Fig. 9**).

2.2. Effect of CLA on the polyclonal and antigen-specific cell immune response

Although the main goal was to examine whether a long-term CLA diet was able to modulate the capacity to generate an antigen (Ag)-specific cell immune response, the *ex vivo* capacity to generate a mitogen-induced immune response was also evaluated in isolated spleen lymphocytes by means of their ability to proliferate and to secrete IL-2 [42]. The **Figure 10A** shows that spleen cells from rats fed CLA throughout the study (17 wk) had a ~10% lower proliferative response than reference rats after mitogen-stimulation. This down-regulatory effect by dietary CLA was not due to cell viability loss, because viability from the CLA after mitogen addition was comparable to that of reference cells. Secretion of IL-2 was also lower in cell cultures of CLA-fed rats than in those of rats fed the standard diet (**Fig. 10B**). These results agree with those found in the 28-day-old assessment *(see section 1.6.3.)* and those of Tricon *et al.* [44], who showed that peripheral blood mononuclear cells from subjects fed either *c*9,*t*11- or *t*10,*c*12-CLA isomers, after mitogen-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 [45,47,81].

Regarding the long-term effects of dietary CLA supplementation on Ag-specific immune response, the lymphoproliferative capacity after OVA addition was evaluated. Reference and CLA groups had a higher (2- to 3--fold) splenocyte proliferation after OVA stimulation than unstimulated cells and after control protein addition (**Fig. 11**). In terms of specific proliferative response, splenocytes recovered from OVA-immunized rats fed CLA had

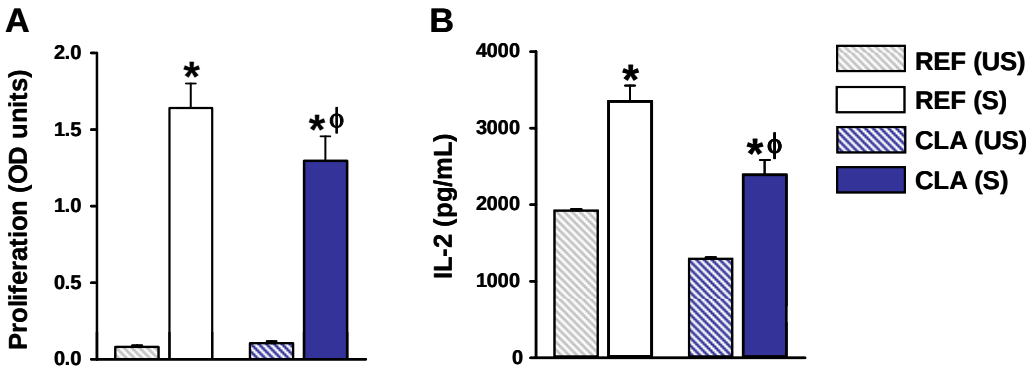

Figure 10. Proliferative response (**A**), and IL-2 production (**B**) of unstimulated (US) and mitogen-stimulated (S) spleen cells from rats fed a CLA or standard diet (CLA or REF groups). Polyclonal proliferative response was determined after stimulating with PMA/Io for 72 h. IL-2 was quantified by ELISA in 24-h supernatant cultures. Data are means ± SEM (n= 20). Significant differences: *P<0.05 *vs.* US cells within the same dietary group; $^{\phi}$P<0.05 *vs.* reference group. Modified of Ramírez-Santana *et al.* [42].

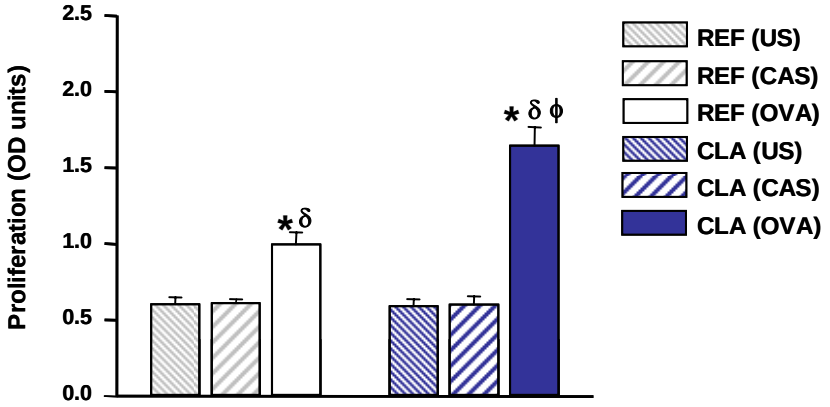

Figure 11. OVA-specific proliferative response of spleen cells from rats fed the CLA or the standard diet (CLA or REF groups). Specific response was determined after OVA stimulation for 96 h. Control protein (casein, CAS) and only medium (unstimulated cells, US) were used as negative reference controls. Data are means ± SEM (n= 20). Significant differences: *P<0.05 *vs.* US cells within a diet group; $^{\delta}$P<0.05 *vs.* CAS-stimulated cells within a diet group. $^{\phi}$P<0.05 *vs.* reference group. Modified of Ramírez-Santana *et al.* [42].

higher (~275%) lymphoproliferative response to OVA than splenocytes recovered from OVA-immunized rats fed the standard diet (~165%). The OVA-specific splenocyte proliferation enhancement by CLA found here agrees with that reporting a higher specific proliferative response of T CD8+ lymphocytes from pigs fed a CLA diet (~50:50 isomers mix) [50,51]. In addition, following hepatitis B vaccination, specific lymphocyte proliferation was higher in humans fed CLA 50:50 than in the control group [45]. Conversely, Kelley *et al.* [82] showed no effect on influenza-specific proliferation in humans after feeding CLA, but in this case, the two 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.

2.3. Effect of CLA on the Ag-specific systemic and mucosal humoral immune responses

To ascertain long-term CLA diet effects on systemic humoral immune response, we have quantified serum OVA-specific antibody concentration, *ex vivo* spleen anti-OVA antibody production, and spleen anti-OVA-antibody-secreting cells (SC) number. Long-term dietary CLA did not modify the humoral immune response against the OVA-specific challenge either in serum or in splenocyte supernatants. However, using enzyme-linked immunosorbent spot technique (ELISPOT) we counted spontaneous anti-OVA IgG-, IgM-, and IgA-SC in spleens and we found that OVA-immunized rats had more spleen anti-OVA IgA-SC in CLA-fed rats (15.6 ± 3.5; mean ± SEM) than in reference group (11.9 ± 1.9). These results show overall that, although OVA-primed spleen B cells produced specific anti-OVA antibodies after later OVA contact, 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 antibodies. Our results agree with others carried out in humans and animals fed CLA [51,82,83], although Albers *et al.* [45] showed a higher concentration of anti-B hepatitis antibodies 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 [16,81,82] but disagrees with a human study that reported increased IgM and IgA plasma concentrations after consuming CLA [36]. Nevertheless, better humoral enhancing effects were observed in developing states *(see section 1.6.)*, although specific adaptive responses were not addressed in such studies [20,21].

Regarding mucosal sites, we found interesting CLA results in this particular immune compartment: the dietary CLA modulated mucosal IgA production. Long-term dietary CLA increased the anti-OVA IgA synthesis in the intestinal mucosa ~75% (**Fig. 12**), although CLA did not modify total gut IgA. These data suggest that the CLA diet had a restricted enhancement effect on OVA-specific IgA intestinal production but not a general effect on humoral immunity.

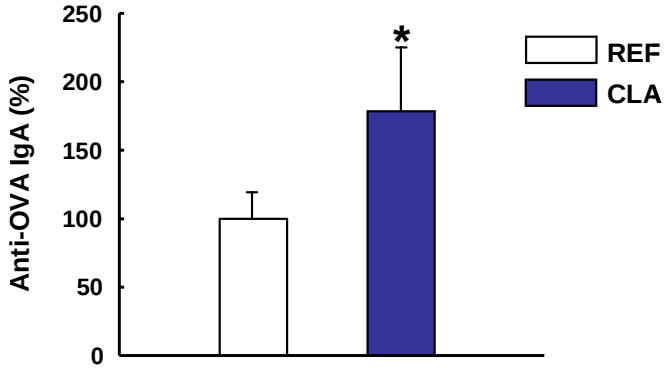

Figure 12. OVA-specific IgA in intestinal washes from rats fed the CLA or the standard diet (CLA or REF groups). Six weeks after immunization, secretory IgA was quantified in intestinal washes of small intestine by ELISA. Results are expressed relative to the reference group, which was set at 100%. Data are means ± SEM (n= 20). Significant differences: *P<0.05 *vs.* reference group. Modified of Ramírez-Santana *et al.* [42].

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 [84]. 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 or serum antibodies, it is plausible to suggest that CLA may be enhancing B cells present in the lamina propria or even promoting the IgA-secreting cell 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.* [64] reported a higher percentage of a particular immune cell subset, but not of others, in swine fed CLA.

3. Conclusions

The data reported in this chapter contribute to the scientific evidence pointing to the potential impact of lipid nutrition on immune system development during early life, particularly the effect of the *c*9,*t*11-CLA isomer, the main CLA isomer present in breast milk. Although further studies should be carried out to elucidate CLA signalling mechanisms, the results presented herein by the dietary supplementation with an 80:20 *c*9,*t*11:*t*10,*c*12 CLA mix demonstrate that:

- CLA is transferred from the dams' diet to breast milk and later is efficiently incorporated by their pups. This supplementation also increases dam's milk IgG and IgA concentrations.
- The capacity of immune cells to synthesise antibodies, i.e. humoral immune response, is enhanced by CLA during gestation, suckling and/or early infancy. It is demonstrated, *in vivo* and *ex vivo*, by the increase of serum IgG concentration and spleen IgM production, respectively, in CLA fed animals.
- CLA supplementation during suckling and early infancy down-regulates the systemic lymphoproliferative response and increases the secretion of some Th2 cytokines during early age.
- CLA increases the intestinal immune defenses of 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 early infant rats fed CLA along life. Moreover, PPARγ gene expression levels were up-regulated in a supplementation period-dependent manner.
- It is clearly shown that the effects of CLA are more pronounced the earlier and more long-lasting CLA dietary supplementation. Specifically, the importance of the continuous supplementation during gestation-suckling or suckling-early infancy is evidenced by the observation of some immunomodulatory effects only produced when CLA is received during these periods.
- CLA diet administered from gestation to adulthood enhances specific systemic cell-mediated immunity as well as the mucosal IgA immune response, whereas it down-regulates the polyclonal activation of the immune system. These data support the long-term effects of dietary CLA on the immune system.

Acknowledgements

The authors are grateful to Dr. Jordi Xaus, who reviewed and improved a first version of the manuscript. The present study was supported by fundings

from the FBG-303225 and the Generalitat de Catalunya (SGCR-2005-00833). C. R-S. had a grant from Agencia Española de Cooperación Internacional (AECI).

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