In vitro effects of conjugated linoleic acid (CLA) on inflammatory functions of bovine monocytes

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ABSTRACT

The conjugated linoleic acid (CLA) isomers, a group of naturally occurring isomers of the essential fatty acid (FA) linoleic acid, have received special attention in animal and human nutrition. Although they have long been used as dietary integrators in dairy cows, the effects of CLA isomers on bovine immune cells remain mostly undisclosed. The present study aimed to cover this gap and investigate the in vitro effects of CLA on inflammatory functions, including chemotaxis, phagocytosis, killing capability, and extracellular respiratory burst of purified bovine monocytes (CD14⁺). The apoptosis rate of monocytes was addressed as well. Once assessed, the effects of different concentrations (10, 50, 100, and 500 μ M) of the 2 main CLA isomers, namely *cis*-9,*trans*-11 and *trans*-10,*cis*-12, the experiments were carried out using a concentration of 50 μ M of the CLA isomers, both individually and in a mixture (50:50). The immunomodulatory activities of linoleic acid, an essential FA, and stearic acid, a saturated FA, were also investigated. Only the 50:50 CLA mixture was able to reduce monocyte apoptosis and to increase the extracellular respiratory burst during experimental proinflammatory conditions, as assessed by measuring production of reactive oxygen species. Linoleic acid and CLA had no effects on chemotaxis, phagocytosis, or killing capability. Remarkably, treatment of monocytes with stearic acid significantly reduced their chemotactic capability. The present results demonstrated that CLA isomers do have immunomodulatory effects on some functions of bovine monocytes, and that the mixture of the 2 CLA isomers is more effective than the CLA isomers individually.

Key words: conjugated linoleic acid, monocyte, innate immunity, dairy cow

INTRODUCTION

Conjugated linoleic acid is a group of naturally occurring positional and geometrical isomers of the essential n-6 fatty acid (FA) linoleic acid, featuring conjugated double bonds in either *cis* or *trans* configuration (Bhattacharya et al., 2006). Conjugated linoleic acid is synthesized as an intermediate product during the biohydrogenation of linoleic acid by *Butyrivibrio fibrosolvens* rumen bacteria (Churruarín et al., 2009) or through the endogenous conversion of *trans*-vaccenic acid by desaturase-9 in the mammary gland, as previously demonstrated (Griinari et al., 2000).

Among the 28 CLA isomers thus far reported, the *cis*-9,*trans*-11 (c9,t11) and the *trans*-10,*cis*-12 (t10,c12) are the most abundant and the ones that show relevant biological activities (Pariza et al., 2001; Viladomiu et al., 2016). Conjugated linoleic acid is formed in ruminants, in particular the c9,t11 isomer that is the most predominant isomer in milk fat (80 to 90%). The concentration of CLA in blood has been recently measured as 0.54 mg/L, primarily c9,t11-18:2 (Lahlou et al., 2014). The c9,t11 isomer concentration in the milk fat of multiparous late-lactation Holstein-Friesian cows is 0.71 g/100 g of total FA, and 0.02 g/100 g of blood fat, and it increases significantly ($P < 0.01$) up to 2.5-fold after feeding with fresh pasture (Kay et al., 2005). In other studies, the mean concentration of the c9,t11 isomer in bovine milk varied from 5.04 to 11.28 mg/g of fat in animals fed with different diets, showing that CLA milk content is markedly influenced by the composition of the animal's diet (Bauman et al., 1999; Fritsche et al., 1999). The 2 isomers differ in their biological effects, and, furthermore, different activities were demonstrated when they were combined in equal amounts (Pariza et al., 2001). Dietary supplementation of dairy cows with CLA is a frequent and relevant nutrition strategy, as in vivo studies have demonstrated reduction in milk fat and glucose production, allowing more efficient whole-body energy utilization and enhanced milk yield (Selberg et al., 2004; Dänicke et al., 2012; Galamb et al., 2017). Besides the effects on metabolism, supplementing CLA as well as other PUFA in dairy ruminants has

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been identified as a potential strategy to mitigate the effects of the proinflammatory status associated with the oxidative stress related to metabolic and endocrine changes around calving in dairy cows (Sordillo, 2016). Although CLA isomers have been extensively used for improving milk quality and alleviating the magnitude of negative energy balance, the information about their influence on cows' immunity is limited. In vitro studies carried out on a model of mammary gland epithelial cells (BME-UV1) have demonstrated that CLA can modulate inflammation and respiratory burst (Basiricò et al., 2015, 2017; Dipasquale et al., 2018). The effects of CLA on the activity of immune cells have been investigated in bovine peripheral blood mononuclear cells (PBMC) and resulted in contradictory results. Supplementing dams with a commercial CLA preparation (Lutrell Pure, BASF SE, Ludwigshafen, Germany) in the preceding lactation period exerted effects on the ex vivo stimulation ability of bovine PBMC (Dänicke et al., 2012). In an in vitro study assessing the effect of c9,t11 and t10,c12 CLA isomers, the inhibition of bovine isolated PBMC mitogen-activated proliferation was detected, together with a marginal effect of c9,t11 isomer on cells' cytokine expression pattern (Renner et al., 2013). The apparent inconsistency of these results may be related to the fact that most of the studies were not carried out with comparable experimental designs and on an isolated population, but on PBMC, which included both lymphocytes and monocytes. Monocytes are myeloid cells derived from bone marrow. They play a pivotal role in immune defense against infections and injuries and are involved in almost all phases of the immune reaction (Hussen and Schuberth, 2017). Given their pivotal role, the present study aimed to investigate the in vitro effect of c9,t11 and t10,c12-CLA isomers, both individually and using a mixture (50:50) of the 2 isomers, on a sorted bovine monocyte population. To examine whether and how these CLA isomers could play a role in modulating monocytes' inflammatory activities, their effects on chemotaxis, respiratory burst, phagocytosis, killing capability, and apoptosis were evaluated. The effects of other unsaturated FA (linoleic acid) and saturated FA (stearic acid), were assessed as well.

MATERIALS AND METHODS

Materials

Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), EDTA 2 mM, red blood cell lysis buffer, and sterile-filtered Dulbecco's PBS without calcium and magnesium (Sigma-Aldrich, St. Louis, MO)

were used for bovine PBMC isolation. For monocyte purification, we used CD14 MicroBeads, LS (large size) columns, and 30-mm pre-separation filters (Miltenyi-Biotec, Bergisch Gladbach, Germany) and BSA (Sigma-Aldrich). After isolation, cells were resuspended in complete medium, comprising RPMI 1640 medium with 25 mM HEPES and L-glutamine supplemented with 1% nonessential amino acid solution 100× and 1% penicillin streptomycin solution 100× (Euroclone, Milano, Italy), and 10% fetal bovine serum (Sigma-Aldrich). Sterile 96-well Microtest plates (Becton Dickinson and Company, Franklin Lakes, NJ), 384-well black plates, 24-well Transwell migration plates (Costar, Corning Inc., Corning, NY), and cryogenic vials (Sigma-Aldrich) were used for cell culture. The FA 9(E),11(Z)-octadecadienoic acid and 10(E),12(Z)-octadecadienoic acid (c9,t11 and t10,c12 CLA, respectively; Matreya LLC, State College, PA) and linoleic and stearic acid (Sigma-Aldrich) were used for cell treatment.

Zymosan A from *Saccharomyces cerevisiae* and cytochrome C from equine heart, phorbol myristate acetate (PMA; Sigma-Aldrich), fluorescein-labeled *Escherichia coli* K-12 strain bioparticles (Invitrogen, Carlsbad, CA), and *E. coli* ATCC 25922 (strain Seattle 1946; LCG Standards Ltd., Teddington, UK) were used for chemotaxis, reactive oxygen species (ROS) production, phagocytosis, and killing capability assays, respectively.

Purification of Monocytes from Blood

Peripheral blood from 33 healthy pluriparous late-lactation Holstein-Friesian cows was collected during routine slaughtering procedures at a local slaughterhouse, in sterile flasks containing 1.8 mg K₂EDTA per mL of blood as anticoagulant. Monocytes (CD14⁺ cells) were isolated through Ficoll 1.077 g/mL density gradient centrifugation, as previously described (Dilda et al., 2012), with a few modifications. Briefly, blood was first centrifuged at $1,260 \times g$ for 30 min at 4°C, and the buffy coat (PBMC ring) was collected. The PBMC were then diluted 1:5 in sterile cold PBS without Ca²⁺ and Mg²⁺, containing 2 mM EDTA, layered on Ficoll, and centrifuged without breaks at $1,700 \times g$ for 30 min at 4°C. The PBMC were recovered at the interface, washed with PBS without Ca²⁺ and Mg²⁺, and treated with red blood cell lysis buffer for red blood cell elimination. Two subsequent centrifugations ($500 \times g$ for 7 min with cold PBS without Ca²⁺ and Mg²⁺, with 2 mM EDTA) were carried out to remove platelets. The CD14⁺ monocyte purification was carried out using the magnetic-activated cell sorting technique. Isolated PBMC were incubated with anti-human CD14 microbeads for 15 min at 4°C, and CD14⁺ cells were isolated

from a LS (large size) column, using a MD (MidiMACS) separator, according to the manufacturer's instructions. The homogeneity of the sorted cells (>98%) was determined using an automatic cell counter (Sysmex Corp., Kobe, Japan). The working concentration of monocytes was then adjusted with complete medium.

Unsaturated and Saturated FA Preparation

Stock solutions of unsaturated and saturated FA were prepared. The 2 CLA isomers (c9,t11 and t10,c12 CLA), linoleic acid, and stearic acid were reconstituted in ethanol at concentrations of 357 mM for CLA isomers and linoleic acid and 70.3 mM for stearic acid. Stock solutions were stored at -20°C , and fresh dilutions with the complete medium were prepared when needed. The amounts of CLA isomers used for preliminary studies ranged from 0 to 500 μM . The authors only used one mixture containing both CLA isomers in a 50:50 proportion at a final concentration of 50 μM . The rest of the fatty acids (linoleic acid and stearic) were prepared individually in aqueous solutions (medium) at final concentration of 50 μM . During the first part of the study, the working concentration of CLA isomers was determined by testing the different concentration of individual c9,t11 and t10,c12 CLA isomers on monocyte apoptosis. Once the CLA working solution was determined, it was tested on monocyte immune-related functions, including chemotaxis, phagocytosis, respiratory burst, killing capability, and apoptosis.

Apoptosis Assay

Apoptosis assay was performed in triplicate on 50×10^3 sorted monocytes seeded in 384-well black plates. The experiment was carried out on cells purified from 8 animals. First, to determine the CLA working concentration, the cells were incubated overnight at 39°C in a humidified atmosphere of 5% CO_2 , with increasing concentrations of c9,t11 and t10,c12 CLA isomers (10, 50, 100, and 500 μM) or with 0.1% ethanol as control (vehicle), this being the concentration of ethanol found in 500 μM of CLA isomers. At this concentration of ethanol, no effects on viability on bovine PBMC were observed, as assessed by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetra-zolium bromide (MTT)-based assay (data not shown). Once the CLA working concentration was determined (50 μM), the effects of other unsaturated and saturated FA on apoptosis of monocytes were measured by incubating the cells with 50 μM of each CLA isomer, the 50:50 mixture of the 2 isomers, linoleic acid, stearic acid, and ethanol (vehicle) as the control, with the same concentration of ethanol found in the 50 μM FA solutions (0.014%). The apop-

tosis rate was measured after overnight incubation, using the Apo-ONE Homogeneous Caspase-3/7 kit (Promega, Madison, WI). The caspase-3/7 reagent was added to each well, and the fluorescence intensity was measured using a fluorescence plate reader Fluoroscan Ascent (Thermo Fisher Scientific Oy Ratastie, Vantaa, Finland) at 485/538 nm (absorbance/emission) every 30 min up to 4 h, as previously described for bovine monocytes (Cecilianí et al., 2007).

Chemotaxis Assay

Monocyte chemotaxis toward zymosan-activated serum (ZAS) was measured as previously reported (Lecchi et al., 2008; McClelland et al., 2010) with some minor modifications. The experiment was carried out on cells purified from 8 animals. Monocytes were first pretreated overnight, in absence of chemoattractant, with 50 μM of each CLA isomer, the 50:50 mixture of the 2 isomers, linoleic acid, stearic acid, or 0.014% ethanol as the control, in 24-well Transwell migration plates (Corning Inc.), equipped with a 5- μm pore size membrane. A total of 1×10^5 monocytes (100 μL final volume) were added in triplicate in the upper chamber, and FA and migration medium (RPMI 1640 with 1% fetal bovine serum) were added in both chambers to a final volume of 750 μL . Cells were incubated overnight at 39°C in a humidified atmosphere of 5% CO_2 . After pretreating the cells, chemotaxis was measured by adding 3 mg/mL of the chemoattractant ZAS to the lower chamber, in the presence of newly added FA (50 μM) or ethanol (vehicle) as control, and again incubating for 2 h at 39°C in a humidified atmosphere of 5% CO_2 . For the negative control, cells were incubated with the vehicle without ZAS. Finally, the upper chambers were removed, nonmigrated cells on the upper part of the membrane were gently eliminated using a swab moistened with PBS, and migrated cells were stained with Diff-Quick (Sigma-Aldrich) and counted in 10 different fields, using light microscopy.

Determination of Respiratory Burst by Measuring ROS Production Under Normal and Proinflammatory Conditions

The production of extracellular superoxide anion (O_2^-) was determined using the cytochrome C reduction method, as previously described (Lecchi et al., 2016). The experiment was carried out on cells purified from 8 animals. A total of 1×10^5 monocytes (50 μL) were seeded in complete RPMI 1640 medium without phenol red, in duplicates, in 96-well sterile plates. Cells were then incubated overnight at 39°C in a humidified atmosphere of 5% CO_2 , with 50 μM (50 μL) of each

CLA isomer individually, the 50:50 mixture of isomers, linoleic acid, stearic acid, or 0.014% ethanol (vehicle) as control at a final volume of 100 μL . At the end of the incubation time, 10 μL of cytochrome C and 90 μL of Hanks' balanced salt solution (HBSS) or 100 μL of HBSS alone, as negative control, were added to each well to make up a final volume of 200 μL . Finally, to mimic possible proinflammatory conditions, the second set of experiments was performed by adding PMA (2.5 $\mu\text{g}/\text{mL}$ final concentration). The absorbance was measured every 30 min for 4 h at 550 nm with a LabSystems Multiskan plate reader spectrophotometer (LabX, Midland, Canada).

Phagocytosis Assay

Monocytes' phagocytic activity was determined as previously described (Lecchi et al., 2011). The experiment was carried out on cells purified from 9 animals. First, 6×10^8 fluorescein-labeled *E. coli* bioparticles (K-12 strain) were opsonized, by incubating them with 20% autologous serum for 30 min at 37°C. The suspension was then centrifuged at $800 \times g$ for 15 min and suspended in HBSS. Opsonized bacteria were stored at -20°C upon use. A 100- μL suspension containing 3×10^5 monocytes was seeded in duplicates in 96-well sterile plates with 50 μM (50 μL) of each CLA isomer individually, the 50:50 mixture, linoleic acid, stearic acid, or 0.014% ethanol (vehicle) as the control. The medium was then added, to a final volume of 200 μL . Afterward, cells were incubated overnight at 39°C in a humidified atmosphere of 5% CO_2 . Cells were then washed with sterile HBSS, and 45 opsonized fluorescein-labeled *E. coli* bioparticles (100 μL) per cell were added. Monocytes were then incubated again for 2 h at 39°C in a humidified atmosphere of 5% CO_2 . Cells were washed twice with HBSS, and 0.4% trypan blue was added to quench the fluorescence from noninternalized bacteria. Finally, cells were again washed with HBSS, and the fluorescence intensity of fluorescein-labeled *E. coli* bioparticles was measured using a Fluoroscanner Ascent FL (Thermo Fisher Scientific, Waltham, MA) at 485/538 nm (absorbance/emission).

Killing Capability Assay

The intracellular bacteria-killing capability of monocytes was evaluated according to Lecchi et al. (2013). The experiment was carried out on cells purified from 7 animals. Briefly, *E. coli* ATCC 25922 (LCG Standards) was opsonized with 20% bovine serum at 37°C for 30 min. The bacteria were washed twice by centrifuging at $1,500 \times g$ for 10 min and suspended in sterile HBSS. A total of 3×10^5 bovine monocytes (100 μL) were

suspended in complete medium-containing cryogenic vials (300 μL final volume; Sigma-Aldrich) and treated overnight with 50 μM (50 μL) of each CLA isomer individually, the 50:50 mixture of isomers, linoleic acid, stearic acid, or 0.014% ethanol as vehicle, at 39°C in a humidified atmosphere of 5% CO_2 . Cells were then washed and incubated with 1×10^7 opsonized live *E. coli* and incubated for 1 h at 39°C in a humidified atmosphere of 5% CO_2 . Monocytes were centrifuged at $110 \times g$ for 5 min to remove unbound bacteria and treated further for 1 h with 100 $\mu\text{g}/\text{mL}$ of gentamicin to kill any remaining extracellular bacteria. Gentamicin was eliminated by washing with HBSS and centrifuging the cells at $110 \times g$ for 5 min. Finally, cells were lysed using 0.5% Triton X-100 (Sigma-Aldrich) for 10 min, and the surviving *E. coli* were counted on MacConkey agar plates. Results are expressed in colony-forming units.

Statistical Analysis

Statistical analyses were performed in GraphPad Prism 8.0.2 (San Diego, CA). For the data normality assessment, the Shapiro-Wilk test was applied. Repeated measures 1-way ANOVA and Tukey's multiple comparison test were used to evaluate ROS production, under normal and proinflammatory conditions, in normally distributed data. Repeated measures Friedman test and Dunn's multiple comparison test were used for apoptosis, chemotaxis, phagocytosis, and killing capability, in non-normally distributed samples. Statistical differences were accepted at $P \leq 0.05$.

RESULTS

Effects of c9,t11 and t10,c12 CLA Isomers on Bovine Monocyte Apoptosis

To determine optimal concentrations of c9,t11 and t10,c12 CLA isomers, a preliminary study on monocytes was carried out by incubating the cells overnight with increasing concentrations of CLA isomers (10, 50, 100, and 500 μM) or with the vehicle (ethanol), and assessing their apoptosis rates (Supplemental Figure S1; <https://doi.org/10.3168/jds.2020-18659>). No effects were observed with any of the CLA isomers at increasing concentrations on the sorted population. The following experiments were then carried out using a concentration of 50 μM .

In the second part of the study, the differential effects of additional unsaturated (linoleic acid and the 50:50 mixture of both CLA isomers) and saturated FA (stearic acid) on monocyte apoptosis were compared. Compared with the vehicle (ethanol), after exposing

Figure 1. In vitro effect of saturated and unsaturated fatty acids on bovine monocytes apoptosis. Caspase-3/7 enzymatic activity of bovine monocytes after overnight incubation with fatty acids or vehicle (0.014% ethanol). CLA isomers: 9c,11t = *cis*-9,*trans*-11; 10t,12c = *trans*-10,*cis*-12. Data are means $\pm$ SEM of 8 independent experiments. Significance was declared at $P < 0.05$ (*).

the cells with the FA overnight, apoptosis (Figure 1) was found to be reduced ($P = 0.013$) only by the 50:50 mixture of CLA isomers.

Effects of Unsaturated and Saturated FA on Bovine Monocyte Chemotaxis

In this part of the study, the capabilities of unsaturated and saturated FA in modulating monocyte chemotactic activity were measured by using transwell migration plates. The chemotactic activity of monocytes was activated using ZAS, after exposing purified cells to 50 μ M of FA or the vehicle (ethanol) overnight. The results are presented in Figure 2. Co-incubation with CLA and linoleic acid did not modulate monocyte chemotaxis. On the contrary, stearic acid induced an evident decrease in the chemotactic ability of monocytes in a statistically significant way ($P = 0.032$) compared with the control (vehicle), the 50:50 mixture of CLA (P

Figure 2. Differential effects of saturated and unsaturated fatty acids on bovine monocytes chemotaxis. Cells were treated with 0.014% ethanol as vehicle control. CLA isomers: 9c,11t = *cis*-9,*trans*-11; 10t,12c = *trans*-10,*cis*-12. Data are means $\pm$ SEM of 8 independent experiments. Significance was declared at $P < 0.05$ (*) and $P < 0.001$ (***).

= 0.049) and t10,c12 CLA ($P = 0.013$) and ($P < 0.001$) with c9,t11 CLA.

Effects of Unsaturated and Saturated FA on Bovine Monocyte ROS Production

Monocytes' production of extracellular superoxide anion under both normal and inflammatory conditions was evaluated through the cytochrome C reduction method. Cells were treated overnight with the FA, and then the ROS production was measured every 30 min for 4 h (Supplemental Figure S2; <https://doi.org/10.3168/jds.2020-18659>). Because the highest ROS levels were observed 60 min after the addition of cytochrome C under both experimental conditions, the effects of the different FA on cells' superoxide anion production were further evaluated and presented only at this time point (Figure 3). Cells under normal conditions did not show any difference in ROS production (Figure 3A)

Figure 3. In vitro effects of saturated and unsaturated fatty acids on bovine monocyte extracellular superoxide anion generation, at 60 min after the addition of cytochrome C, under (A) normal conditions or (B) proinflammatory conditions [phorbol myristate acetate (PMA) stimulation]. Cells treated with 0.014% of ethanol (vehicle) were considered as control. CLA isomers: 9c,11t = *cis*-9,*trans*-11; 10t,12c = *trans*-10,*cis*-12. Data are means $\pm$ SEM of 8 independent experiments. Significance was declared at $P < 0.01$ (**). OD = optical density.

compared with control. On the contrary, an increase with the 50:50 mixture of CLA isomers was detected ($P = 0.002$), after inducing a proinflammatory challenge with PMA (Figure 3B), compared with the control and ($P = 0.003$) with c9,t11 CLA and linoleic acid.

Effects of Unsaturated and Saturated FA on Bovine Monocyte Phagocytosis and Killing Capability of *E. coli*

The last set of experiments was aimed at studying whether co-incubation of isolated bovine monocytes with unsaturated and saturated FA affects their phagocytic and killing capabilities, as determined by fluorescein-labeled *E. coli* bioparticle internalization assay and intracellular *E. coli* killing assay, respectively. The capacity of monocytes to phagocytose (Figure 4A) and kill live *E. coli* (Figure 4B) when treated overnight with saturated and unsaturated FA was not affected compared with the vehicle.

DISCUSSION

In this study, we reported the effects of c9,t11 and t10,c12 CLA isomers, separately and as a 50:50 mix-

ture, as well of the essential FA linoleic acid, and the saturated FA stearic acid, on several immunoregulatory functions of bovine monocytes (CD14⁺), including apoptosis, chemotaxis, phagocytosis, respiratory burst, and killing capability. Our main finding was that the 50:50 mixture of the c9,t11 and t10,c12 CLA isomers, when used at a concentration of 50 μ M, reduced the apoptosis rate of monocytes. Co-incubating cells with the 50:50 mixture of the 2 isomers also increased the respiratory burst, as determined by an increase of the production of ROS, but only in an experimental proinflammatory environment. On the contrary, CLA does not affect any of the other monocyte immunoregulatory functions herein assessed. Remarkably, we found that stearic acid was capable of a statistically significant reduction of chemotaxis.

Conjugated linoleic acid isomers have been routinely used as a feed supplement for dairy cows due to their beneficial in vivo effects. A decrease in milk fat synthesis and its consequent improvement in energy balance, increase in milk production, improved reproductive performance, and reduction of metabolic-related diseases have been reported (Perfield et al., 2007; de Veth et al., 2009; Basiricò et al., 2017; Csillik et al., 2017). However, their effects on bovine immunity have scarcely been addressed.

Figure 4. (A) Phagocytosis of fluorescein-labeled *Escherichia coli* bioparticles and (B) killing capability of live *E. coli* by bovine monocytes after overnight exposure to fatty acid. The results are expressed as fluorescence intensity (optical density at 485 to 538 nm) and as colony-forming units (cfu), respectively. Cells treated with 0.014% ethanol (vehicle) were considered as control. CLA isomers: 9c,11t = *cis*-9,*trans*-11; 10t,12c = *trans*-10,*cis*-12. Data are means $\pm$ SEM of 9 and 7 independent experiments, respectively.

Monocytes provide a suitable in vitro model to assess the influence of CLA on bovine immunity. Monocytes are circulating blood leucocytes that play a major role in host immune defense against invading pathogens (Chávez-Galán et al., 2015). They fulfill their defensive roles by migrating into inflamed tissues, producing proinflammatory cytokines and ROS, and eventually phagocytosing and killing engulfed pathogens. The adequate display of their functions is critical for an effective immune response.

The rate of apoptosis is regarded as a way to control the activity of blood monocytes, by either increasing or reducing their presence and activity in the inflammatory environment. Therefore, apoptosis is regarded as an integral feature of the immune system (Feig and Peter, 2007). Under this premise, this study tested whether CLA isomers affect apoptosis in sorted monocytes, demonstrating that a 50:50 mixture of the 2 CLA isomers can reduce apoptosis. The effects of CLA on apoptosis have been widely studied in cancer cell models in humans, and the results converge toward a pro-apoptotic and antiproliferative effect (Ochoa et al., 2004; Wang et al., 2008). Studies carried out on bovine cellular models have yielded apparently contradictory results. In bovine mammary cells (MAC-T cell line), co-incubating with 35 μ M concentrations of both CLA

isomers promoted an increase of apoptosis rate (Keating et al., 2008), and in another study the c9,t11 CLA isomer (60 μ M) was able to reduce the caspase-3 activity, thus decreasing apoptosis rate, in bovine aortic endothelial cells (Lai et al., 2005), suggesting the hypothesis that the effects of CLA are cell-specific.

For all the following experiments, the working concentration of CLA was set at 50 μ M. This concentration of CLA was selected after preliminary studies that were carried out by co-incubating the monocytes with different concentrations of CLA isomers, demonstrating that no differences occurred in modulation of apoptosis rate using concentrations ranging from 10 to 500 μ M. The use of a concentration of 50 μ M was set to compare the present results with others from previous studies that used the same CLA concentration (Basiricò et al., 2015, 2017; Dipasquale et al., 2018). Indeed, the concentration of 50 μ M is also close to physiological levels found in human sera (10 to 70 μ M; Basiricò et al., 2015). Moreover, positive effects when using 50 μ M CLA, such as an improved redox status of bovine mammary cells (Basiricò et al., 2015) or atheroprotective properties in human monocytes, have been reported (McClelland et al., 2010).

To further investigate whether the effects reported were specific for CLA isomers and not related to an

unspecific effect in response to treatment with FA, we compared the differential effects of linoleic acid as n-6 PUFA control and stearic acid as saturated acid, which is also the main FA found in some commercially available CLA supplements, on bovine monocyte apoptosis. We also incorporated a 50:50 mixture of both CLA isomers, because most of the animal studies reporting CLA benefits and commercially available CLA supplements use a mixture of these 2 isomers in roughly equal amounts (Song et al., 2005; Renner et al., 2012). The CLA mixture was the only treatment that caused a significant reduction in monocyte apoptosis compared with the vehicle (ethanol), an effect not observed with the individual CLA isomers.

In the following set of experiments, the ability of CLA isomers to modulate chemotaxis was studied. We did not observe any difference in monocytes' migration toward ZAS when treated with CLA isomers individually or in combination. These results differ from those previously reported in human monocytes, which demonstrated that CLA could modulate monocyte or macrophage chemotaxis by PPAR γ activation and COX-2 inhibition, suggesting atheroprotective properties (McClelland et al., 2010). Intriguingly, a statistically significant reduction of chemotaxis was found when monocytes were co-incubated with stearic acid. These results contrast with previous reports in human monocytes, where stearic acid was found to have proinflammatory activity (Anderson et al., 2012).

In the final set of experiments, further defensive performance of monocytes in the inflammatory focus such as phagocytosis, killing capability, and respiratory burst were measured. Treating isolated monocytes with CLA has no effect on the phagocytic and killing capability of bovine monocytes. These findings are different than the reported effects of CLA on other species, including dogs and pigs, and other cellular targets such as polymorphonuclear cells, where the t10,c12 CLA isomer increased phagocytosis, either indirectly (Kang et al., 2007) or directly (Kang et al., 2009). No differences in the production of extracellular superoxide anion under normal conditions were found. On the contrary, treating cells with PMA, to mimic inflammatory conditions, upregulated ROS production in monocytes treated with the CLA mixture. These results are in agreement with those reported in human macrophages, where both c9,t11 and t10,c12 CLA isomers upregulated ROS synthesis, through a PPAR γ -dependent mechanism (Stachowska et al., 2008). Remarkably, a similar effect of increasing ROS production was recently demonstrated in BME-UV1 mammary gland epithelial cells (Dipasquale et al., 2018). Moreover, the t10,c12 CLA isomer has already been shown to increase oxidative stress in human *in vivo* studies (Risérus et al.,

2002) and in canine polymorphonuclear cells (Kang and Yang, 2008; Kang et al., 2009).

CONCLUSIONS

Conjugated linoleic acid has been shown to present a wide range of beneficial properties for cow health, production, and welfare. However, its effects on immune cell responses and functionality have scarcely been addressed so far. This study demonstrates that CLA exerts an anti-apoptotic activity and can increase ROS production in an inflammatory *in vitro* model, suggesting that CLA may have relevant roles in modulating some *in vitro* monocyte immune functions. The present study provides evidence that the effects of each CLA isomer are different, and a combination of c9,t11 with t10,c12 CLA isomers induces synergic effects on at least 2 important monocyte immune functions: apoptosis and inflammation-induced respiratory burst. In addition to its isomer-dependent activity, effects of CLA are also strictly related to their cellular targets, as the effects observed on mammary gland and endothelial cell lines are different than those on immune-related cells such as monocytes. Several aspects of the potential immunomodulatory effects of n-6 FA are still elusive, particularly the molecular basis of the different mechanisms of action of CLA. Elucidation of these mechanisms would improve our understanding of the actions of CLA in experimental *in vivo* systems and determine its practical biological significance, supporting a more targeted use of CLA in dairy animal nutrition. Given the wide use of stearic acid in dairy animal nutrition, its immunomodulatory effect of reducing chemotaxis deserves to be further explored.

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