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Bioavailability and Allergoprotective Capacity of Milk-Associated Conjugated Linoleic Acid in a Murine Model of Allergic Airway Inflammation

Christian Böcking[a] Hani Harb[a] Markus Johannes Ege[b] Nicole Zehethofer[c] Kathleen Fischer[c] Julia Krauß[a] Otto Holst[c] Rolf M. Nüsing[d] Buko Lindner[c] Erika von Mutius[b] Harald Renz[a] Holger Garn[a] Petra Ina Pfefferle[a]

[a]Institute for Laboratory Medicine, Pathobiochemistry and Molecular Diagnostics, Philipps University of Marburg, Marburg, [b]Dr. von Haunersches Children Hospital, University of Munich, Munich, [c]Divisions of Structural Biochemistry, Innate Immunity and Immunochemistry, Research Center Borstel, Leibniz-Center for Medicine and Biosciences, Borstel, and [d]Institute for Clinical Pharmacology, Johann Wolfgang Goethe University of Frankfurt, Frankfurt, Germany

Key Words
Conjugated linoleic acid · Asthma · Eicosanoids · Farm milk · Phospholipids · Eosinophils

Abstract
Background: Cross-sectional epidemiological studies have demonstrated that farm milk from traditional farm settings possesses allergoprotective properties. Up to now, it has not been clarified which milk ingredient is responsible for protection against allergic diseases. As farm milk is rich in conjugated linoleic acids (CLA), it is hypothesized that this n–3 polyunsaturated fatty acid family contributes to the allergoprotective capacity of farm milk. We aim to prove this hypothesis in a murine model of allergic airway inflammation. ***Methods:*** To prove the bioavailability and allergoprotective capacity of milk-associated CLA in a standardized protocol, milk batches that differed significantly in terms of their CLA content were spray dried and incorporated into a basic diet by substituting the regular sunflower fat fraction. Initially, the milk CLA uptake from the diet was monitored via measurement of the CLA content in plasma and erythrocyte membranes obtained from supplemented mice. To determine whether a milk CLA-enriched diet possesses allergoprotective properties, female Balb/c mice were fed the milk CLA-enriched diet ahead of sensitization and a challenge with ovalbumin (OVA) and the parameters of airway inflammation and eisosanoid pattern were measured. ***Results:*** In animals, supplementation with a diet rich in milk CLA resulted in elevated CLA levels in plasma and erythrocyte membranes, indicating bioavailability of milk fatty acids. Though membrane-associated phospholipid patterns were affected by supplementation with milk CLA, this application neither reduced the hallmarks of allergic airway inflammation in sensitized and OVA-challenged mice nor modified the eiconsanoid pattern in the bronchoalveolar lavage fluid of these animals. ***Conclusion:*** Milk-associated CLA was not capable of preventing murine allergic airway inflammation in an animal model of OVA-induced allergic airway inflammation.

C. Böcking, H. Harb, H. Garn, and P.I. Pfefferle contributed equally to this work.

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E-Mail karger@karger.com
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Correspondence to: PD Dr. Dr. Petra Ina Pfefferle
Institute for Laboratory Medicine, Pathobiochemistry and Molecular Diagnostics
Philipps University of Marburg, Biomedical Research Centre
Hans-Meerweinstrasse 2, DE–35043 Marburg (Germany)
E-Mail pfefferl@med.uni-marburg.de

Introduction

In many regions of the world, cow's milk consumption has been a common part of the human diet for more than 7,000 years since ancient farmers developed dairy culture [1]. In industrialized countries, the traditional pasture husbandry system was progressively forced back during the last century in favor of intensive indoor keeping and feeding of livestock. However, in some alpine farm settings, traditional production of cow's milk is still common and there is growing evidence that early and continuing consumption of such 'farm milk' acts protectively against atopy and allergic diseases [2, 3]. A number of potential explanations for the beneficial effect of traditionally produced cow's milk on allergies have been discussed, but to date no profound proof of concept is available [4, 5]. However, epidemiological evidence has pointed to lipid components as putative factors which might prevent allergies. Two studies independently demonstrated that an intake of dairy products with a high amount of milk fat was associated with a reduced risk of asthma development [6, 7]. Controversially, there are recent studies demonstrating the opposite effect [8, 9].

Cow's milk lipids represent important nutritional sources for n–3 poly-unsaturated fatty acids (n3-PUFA), a family of fatty acids (FA) which has been discussed as a protective factor in chronic airway inflammatory conditions [10]. In this context, it was recently shown that, in comparison to milk from indoor-fed animals, cow's milk from pastured animals kept on traditionally run alpine farms is rich in ruminant milk lipids of the n3-PUFA family. Among them, conjugated linoleic acids (CLA) were found to be most dominantly elevated in milk from pastured cows [11–13]. CLA were shown to possess strong immunomodulatory properties [14] and this led us to hypothesize that CLA might contribute to the allergoprotective effects of farm milk. *c9,t11*-CLA, the most abundant CLA in dairy products, has been previously shown to antagonize the production of inflammatory mediators by inhibiting the sequential steps of prostaglandin (PG) and thromboxane (TX) synthesis [15]. These mediators play a major role in the establishment of inflammatory conditions involved in the pathology of asthma. A first clinical study in mild asthmatics on a diet supplemented with CLA strengthened the assumption that CLA might contribute to protection against airway inflammation in the context of allergic asthma [16]. Another placebo-controlled study conducted in allergic patients revealed that oral CLA supplementation improved symptoms and immunological parameters during pollen season [17]. In line with these findings an experimental approach in a murine model of asthma where mice were fed a synthetically produced CLA derivate underlined that dietary CLA uptake might lead to allergoprotection [18].

However, the concept that cow's milk, which is rich in CLA, is capable of having preventive effects on allergic airway inflammation and thereby may contribute to the effects of farm milk has not yet been proven using an experimental approach. Here, we aim to determine whether CLA-rich farm-collected milk (directly collected on farms) is able to prevent asthma in a well-established murine in vivo model of acute airway inflammation. In accordance with Kanwar et al. [19], we incorporated spray dried milk prepared from freshly collected and subsequently pasteurized milk batches into the mouse diet to standardize milk supplementation. To determine whether alteration of the asthmatic phenotype is a function of the CLA content, we supplied diets with high and low milk-associated CLA contents.

Methods

Milk Collection and Preparation of Milk Fat Diets with Different CLA Contents

Prior to the proof-of-concept experiments, milk batches that differed significantly in terms of their CLA content were prepared. Based on recent data [9, 10], fresh milk was sampled on traditionally run farms in Bavaria and on conventionally run farms in Hessen to obtain high-CLA and low-CLA milk batches. As batches differed significantly in terms of their CLA content (online suppl. fig S1; for all online suppl. material, see www.karger.com/doi/10.1159/000358523), they were immediately centrifuged and pasteurized at 72–73°C for 15 s after collection to exclude side effects of microbial contamination in later experiments. Measurement of the CLA content of the freshly collected milk batches revealed significant differences. In absolute numbers, the high-CLA milk contained 208 μg CLA/ml of milk, while the low-CLA milk contained 130 μg CLA/ml of milk. The integration of spray-dried milk had no influence on this ratio (fig. 1). To ensure a standardized uptake of milk FA by mice, pasteurized milk batches were spray dried and subsequently integrated into a basic diet formulation substituting the original sunflower fat fraction with milk powder (online suppl. fig. S2). The high-CLA diet contained 1.70% *cis*-9, *trans*-11 CLA and 0.10% *trans*-10, *cis*-12 CLA and the low-CLA diet contained 1.08% *cis*-9, *trans*-11 CLA and 0.04% *trans*-10, *cis*-12 CLA, whereas the control diet (C1000) did not contain any CLA isomers. In the diets, the absolute CLA amounts reflected the ratio found in milk batches (53 μg/g diet vs. 38 μg/g diet). The estimated average daily uptake of CLA per gram of body weight was 12.54 μg for mice fed the high-CLA diet and 9.12 μg for mice fed the low-CLA diet, respectively. Control groups received a basic C1000 diet with sunflower oil as the only source of fatty acids.

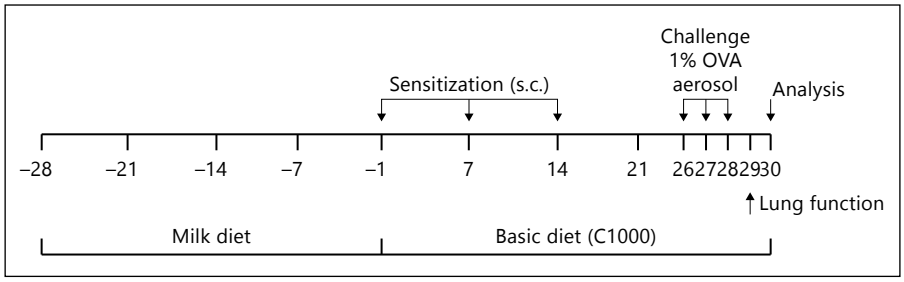

Fig. 1. Protocol of the acute murine model of experimental airway inflammation.

Animals

Six- to 8-week-old female Balb/c mice were obtained from Harlan Winkelmann (Borchen, Germany). The mice were kept under specific pathogen-free housing conditions and were provided water and an ovalbumin (OVA)-free diet ad libitum. All experiments were performed in accordance with German and international guidelines and were approved by local authorities.

Sensitization, Challenge, and Feeding of Mice

In accordance with Conrad et al. [20], the mice were sensitized to OVA on days 0, 7, and 14 via subcutaneous injection (s.c.) of 10 μg OVA (grade VI; Sigma, Steinheim, Germany) in 200 μl PBS. Sham-treated groups received 200 μl PBS. On days 26, 27, and 28, the animals were exposed to aerosolized OVA (1% weight/volume in PBS) for 20 min. Lung function was analyzed 24 h after the last aerosol challenge, and another 24 h later the mice were terminally anesthetized with ketamine plus Rompun® for further analyses (fig. 1).

Assessment of Airway Reactivity

Twenty-four hours after the last aerosol challenge, lung function analysis was performed using noninvasive head-out body plethysmography. Airway hyperresponsiveness, measured as described previously [21], is represented in fig. 3 by a lower metacholine concentration that induced a 50% reduction of the baseline EF_{MCh50}.

A principal comparison of noninvasive head-out body plethysmography with invasive Flexivent lung function analysis in our laboratory revealed concordance (data not shown).

BAL and Differential Cell Count

Using a tracheal cannula, BAL was performed using 1 ml PBS containing a protease inhibitor cocktail (Roche). An automated CasyTT cell counter (Schaerfe Systems, Reutlingen, Germany) was used to determine total leukocyte cell counts. For differential cell count analysis, cytospin preparations were prepared, fixed, and stained with Diff-Quick (Merz & Dade AG, Dudingen, Switzerland) and standard morphological criteria were used to identify cell types.

Restimulation of Lymph Node Cells

Single cell suspensions of mediastinal lymph node cells were generated by filtering lymph nodes through a 100-μm cell strainer, washing and resuspending the cells in RPMI medium with 10% FCS. After counting, the lymph node cells were seeded at 2×10^6/ml in 96 wells and incubated with or without OVA (25 μg/ml) for 72 h at 37°C. Cell-free supernatants were collected and stored at –20°C until cytokine measurement.

Measurement of Cytokines

Cell-free supernatants were analyzed for interleukin (IL)-4, IL-5, IL-10, and IFN-γ using a cytometric bead array (CBA; BD Science, San Diego, Calif., USA). The assay was performed as recommended by the manufacturer.

Measurement of Serum Antibodies

Blood samples were taken from the axillary vessels, and serum levels of anti-OVA IgE, anti-OVA IgG_1, and anti-OVA IgG_{2a} were measured by ELISA as previously described [22].

Quantitative Morphological Analysis of Mucin Production

Directly after BAL, the lungs were fixed with 6% paraformaldehyde. Lung tissues were embedded into paraffin, and 3-μm sections were stained with periodic acid-Schiff. Goblet cell metaplasia and the volume of epithelial mucin per surface area of airway basal membrane were determined using the computer-assisted stereology tool box (CAST Grid 2.0; Olympus) as described previously by Conrad et al. [23].

Assessment of FAs in Milk, Serum, and Erythrocyte Membranes

Blood samples were taken and a panel of different FAs in serum and erythrocyte membranes was assessed as described by Böcking et al. [24].

Phospholipid Analysis in Erythrocyte Membranes

The lipid extracts (150 μl) in hexane were evaporated and the residue was redissolved in 200 μl chloroform/methanol = 83/13. Twenty-five microliters of the reconstituted lipid extracts were mixed with 25 μl of a mixture of ^2H internal standards (one per lipid class of interest at 1.43 ng/μl, each in chloroform). Samples were separated by head groups using normal-phase HPLC. The solvents, gradient profiles, and flow rates are summarized in online supplementary table S1. Online detection was performed using a high-resolution hybrid Apex-Qe FT-MS system (Bruker Daltonics, Bremen, Germany) equipped with a 7-tesla actively shielded superconducting magnet and an Apollo Dual ESI/MALDI ion source. Mass spectra were summed according to the respective Rf values of the lipid groups of interest and analyzed using LipID [25], allowing verification of the head groups and the overall FA composition including the degree of saturation. As based on the exact mass only the total number of carbon atoms and double bonds in both FA chains can be determined, MS/MS was applied to identify the FA in phosphatidylcholine (PC) and phosphatidylethanolamine (PE).

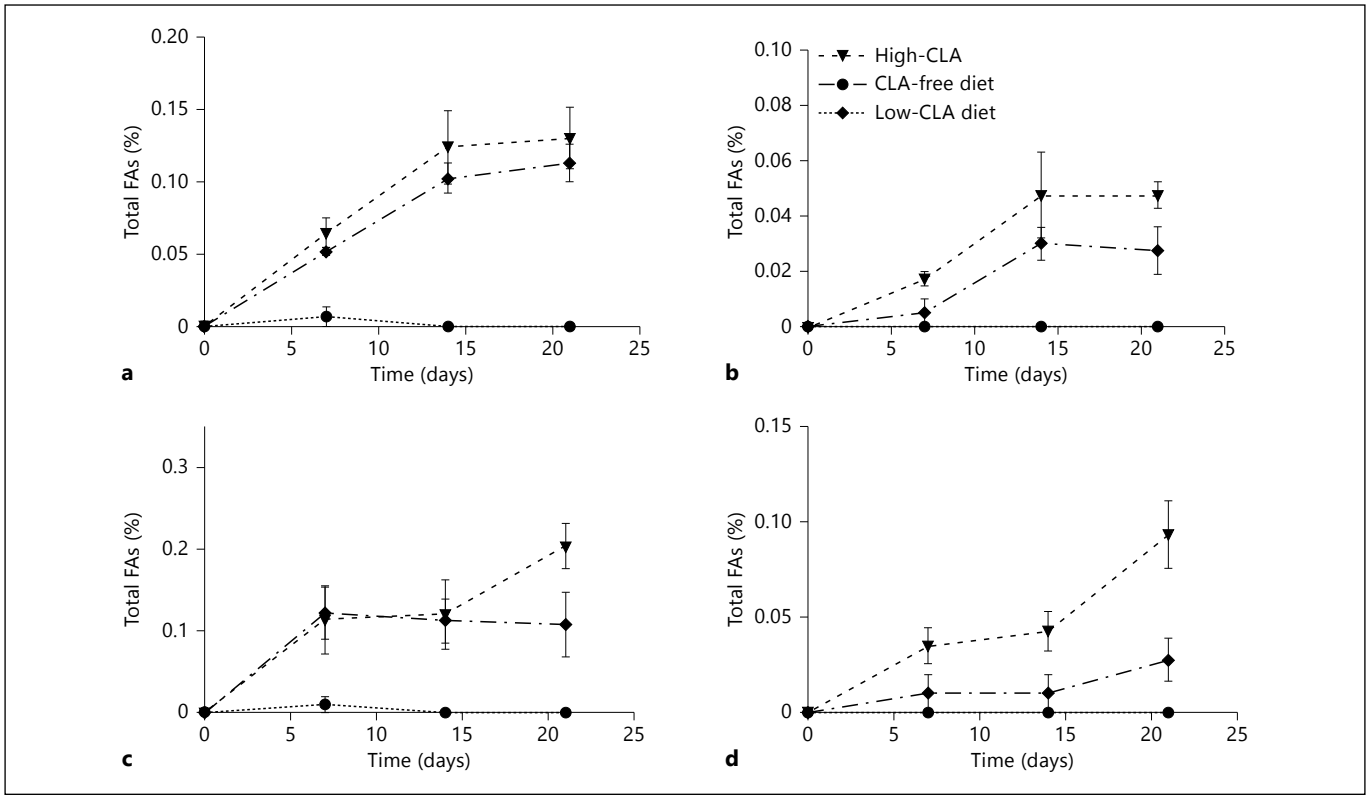

Fig. 2. Kinetics of CLA uptake into plasma and erythrocyte membranes. Female Balb/c mice (n = 4 per group) were fed a basic diet (C1000), a high-CLA, and and low-CLA diet, respectively, ad libitum. The FA composition in plasma and erythrocytes was determined on a weekly basis by gas chromatography. C18:2 c9,t11 (**a**) and C18:2 t10,c12 (**b**) in plasma and C18:2 c9,t11 (**c**) and C18:2 t10,c12 (**d**) in erythrocytes.

Measurement of Eicosanoids in BAL

The amounts of PG D_2 (PGD_2), E_2 (PGE_2), F_{2a} (PDF_{2a}), and 6-keto-PGF_{1a}, as well as TXB_2, leukotriene (LT) B_4 (LTB_4), 5S-HETE, and 15S-HETE in bronchoalveolar lavage (BAL) fluid were determined by LC-MS/MS as described by Schmidt et al. [26] and Maier et al. [27] using an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Darmstadt, Germany).

Statistical Analyses

Data analyses of animal experiments were performed using GraphPad Prism® (GraphPad Software Inc., La Jolla, Calif., USA). All numerical data are expressed as means ± SEM and were analyzed for significance using one-way ANOVA and Tukey tests. $p < 0.05$ was considered statistically significant.

Results

Milk Fat CLA Are Bioavailable after Feeding

Prior to the proof-of-concept experiments, we investigated the bioavailability of milk-derived CLA incorporated into the diet. For this purpose, 6-week-old female Balb/c mice were fed a high-CLA, low-CLA, or control diet (C1000) for 21 days and the kinetics of the CLA uptake in plasma and erythrocyte membranes were monitored in each group. Blood was taken from the mice of each group (n = 4) on days 0, 7, 14, and 21. Determination of CLA levels in plasma and erythrocyte membranes showed an increasing presence of the CLA isomers *cis9, trans11*-CLA and *trans10, cis12*-CLA over time in milk diet-fed mice (high-CLA and low-CLA diets) compared to controls. However, this increase only reached statistical significance for C18:2 t10,c12 in the erythrocyte membranes of mice fed the high-CLA-diet (fig. 2).

A Diet Rich in Milk CLA Does Not Affect Allergic Sensitization and Airway Inflammation in Mice

To analyze whether milk CLA-enriched diets may prevent the development of allergic airway inflammation, we employed a well-established acute model of murine experimental asthma based on s.c. sensitization with OVA [20]. Six-week-old female Balb/c mice were fed high-CLA and

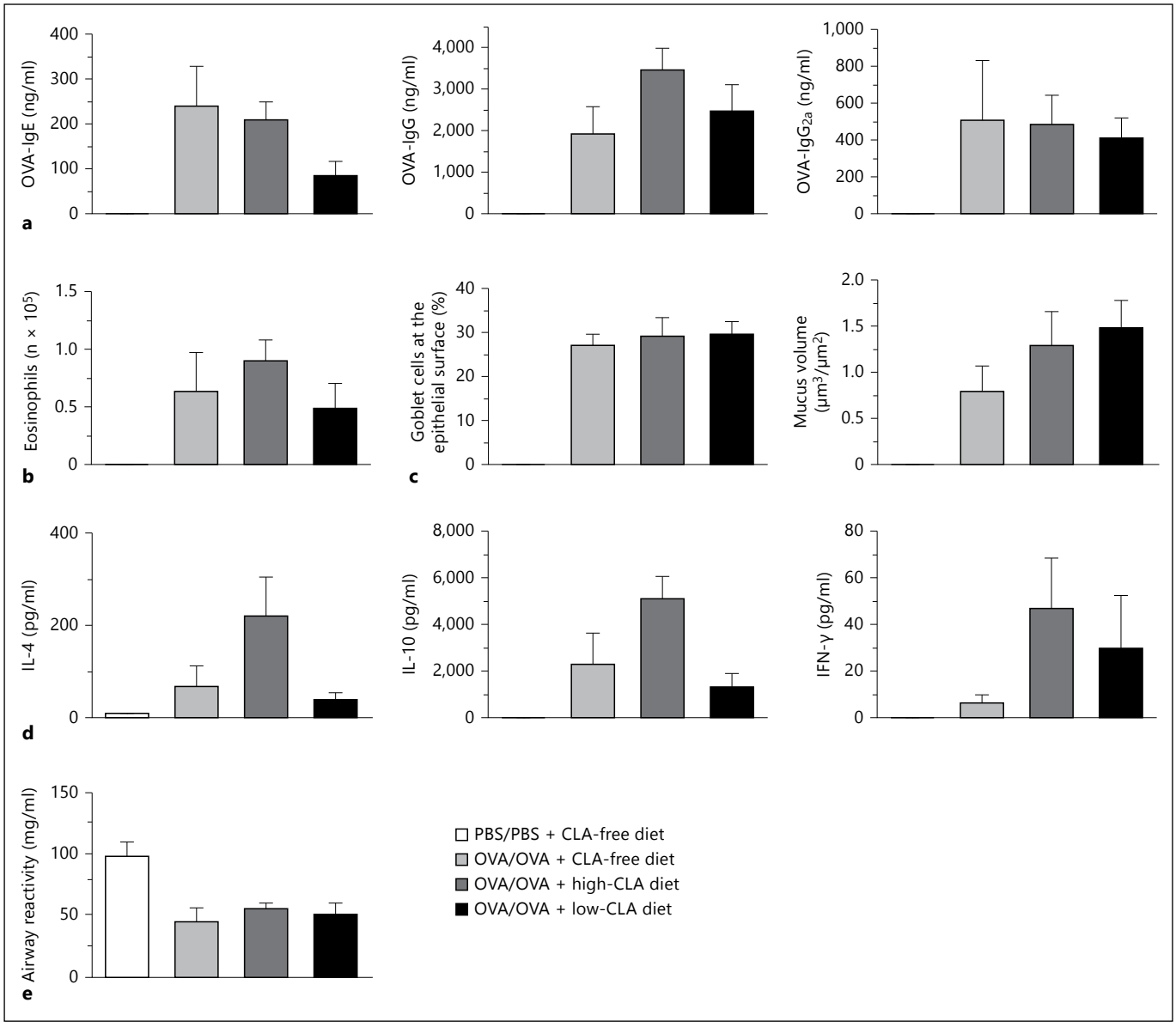

Fig. 3. Effects of a milk CLA-enriched diet on the hallmarks of acute airway inflammation in Balb/c mice subjected to an OVA (s.c.)-induced model of experimental asthma. **a** OVA-specific antibody levels in serum. **b** Differential eosinophil cell counts in BAL fluids. **c** Number of mucus-producing goblet cells and mucus volume (V_{GC}) quantified by the CAST system in periodic acid-Schiff-stained histologic lung sections. **d** In vitro cytokine production of OVA-restimulated lymph node cells (72 h), measured with ELISA. **e** Airway hyperresponsiveness to metacholine (MCh_{50}) measured by head-out body plethysmography (n = 8). Given is the metacholine concentration that induced a 50% reduction of the baseline EF_{MCh50}. Statistical testing was carried out using a one-way ANOVA with Tukey test analysis in relation to the non-CLA group and between CLA groups.

low-CLA milk diets prior to sensitization, while control animals received a milk-free control diet (C1000). To establish a preventive condition, mice were fed the CLA-enriched diet for 4 weeks prior to sensitization and aerosol challenge. To determine whether allergic sensitization was affected by consumption of CLA-containing diets, we assessed the OVA-specific IgE, IgG_1, and IgG_{2a} production in serum. No significant changes were observed in the milk-fed OVA-sensitized/challenged groups compared to the sham-treated OVA-sensitized/challenged group (fig. 3a).

Further, we analyzed differential cell counts in the BAL fluid. Neither BAL total leukocyte numbers (data

not shown) nor eosinophils showed any significant difference between milk diet-fed and control diet-fed mice (fig. 3b). Quantification of mucus-producing goblet cells and mucus volume in the different experimental groups showed an increased number of goblet cells and augmented mucus production in OVA-sensitized/challenged mice. Animals fed the high-CLA diet or the low-CLA diet did not show a reduction of goblet cell numbers and mucus production (fig. 3c).

The assessment of T_H1/T_H2 cytokine profiles in OVA-restimulated mediastinal lymph node cells showed no significant differences in milk diet-fed and OVA-sensitized/challenged animals compared to OVA-sensitized/challenged animals fed the CLA-free control diet (fig. 3d).

Additionally, we did not observe any improvement of lung function in milk diet-fed mice compared to controls (fig. 3e).

Milk CLA Supplementation Did Not Alter the CLA Distribution in Erythrocyte PC and PE

Next, we analyzed the distribution of linoleic acid isomers in erythrocyte PC and PE as these phospholipid subtypes are substrates for phospholipase A2 generating free FAs (FFA) as major players in the eicosanoid-mediated initiation of inflammation [28, 29]. Therefore, erythrocyte membranes were prepared from blood samples taken on day 28. Linoleic acid residues were found in PE 34:2, PE 38:6, and PE 36:2 and in PC 34:2, PC 36:3, and PC 36:2. Though we found differences in CLA content in the erythrocyte membranes after feeding with the milk CLA-enriched diets compared to the control diet, we did not observe any changes in the phospholipids of the erythrocyte membranes (table 1).

Table 1. Distribution of PE and PC species containing linoleic acid

Lipid	High-CLA diet, %	Low-CLA diet, %	Control diet, %
16:0–18:2 PE	18	17	19
18:0–18:2 PE	5	4	8
20:4–18:2 PE	77	76	73
16:0–18:2 PC	62	54	60
18:0–18:2 PC	21	21	26
18:1–18:2 PC	17	25	14

The lipid extracts of each group (n = 8) were pooled, evaporated and redissolved and injected into the HPLC system. Lipid fractions containing linoleic acid residues were isolated and fragmented using IRMPD. Linoleic acid isomers containing fractions are shown as percentages of the total 18:2 isomers containing lipids.

Milk CLA-Enriched Diets Do Not Alter the Local Eicosanoid Release

Several in vivo studies have proposed an influence of CLA on the production of eicosanoids by inhibiting the activity of enzymes such as carboxygenase and lipoxygenase or competing with other FAs as a substrate for eicosanoid synthesis [30–32]. The lipid mediators PGD_2 and LTB_4 are involved in the asthma pathogenesis [33]. In order to investigate whether consumption of CLA and their uptake into cell membranes alter the release of eicosanoid patterns, we determined the concentrations of PGD_2, E_2, F_{2a}, and 6-keto-PGF_{1a} as well as TXB_2, LTB_4, and 5S- and 15S-HETE by LC-MS/MS.

The lipid mediators PDF_{2a}, TXB_2, and 5S- or 15S-HETE were not detectable in the BAL fluid, whereas the asthma-relevant eicosanoid PGD_2 and LTB_4 and also PGE_2 and 6-keto-PDF_{1a} were found in the BAL fluid in each of the sensitized groups (fig. 4). Nonetheless, analyses did not reveal any significant changes in the concentration of these lipid mediators between the control group and the experimental groups.

Discussion

Findings from the ALEX study suggest that farm milk from traditional farm settings might contribute independently of other farm exposures to the allergoprotective farm effect [2]. So far, it has been a matter of speculation which farm milk ingredients might be responsible for this effect. Cow's milk is a heterogenic product that varies broadly in its biochemical and microbial composition depending on the region of origin and the husbandry conditions on the farms [34]. Our data indicate that the CLA content might be a marker for farm milk sampled from cows traditionally kept in the alpine region as this milk batch was characterized by a significantly elevated CLA level in comparison to milk sampled on conventionally run farms located elsewhere. These results are in line with former studies on CLA content in cow's milk samples from the alpine region in comparison to other origins [11, 12].

The study diet employed here enabled us to investigate possible milk CLA effects independently of associated microbes or heat-sensitive ingredients as these components were separated or denatured during pasteurization. In contrast, the milk lipid fraction was not affected by milk processing as no significant loss of CLA was ob-

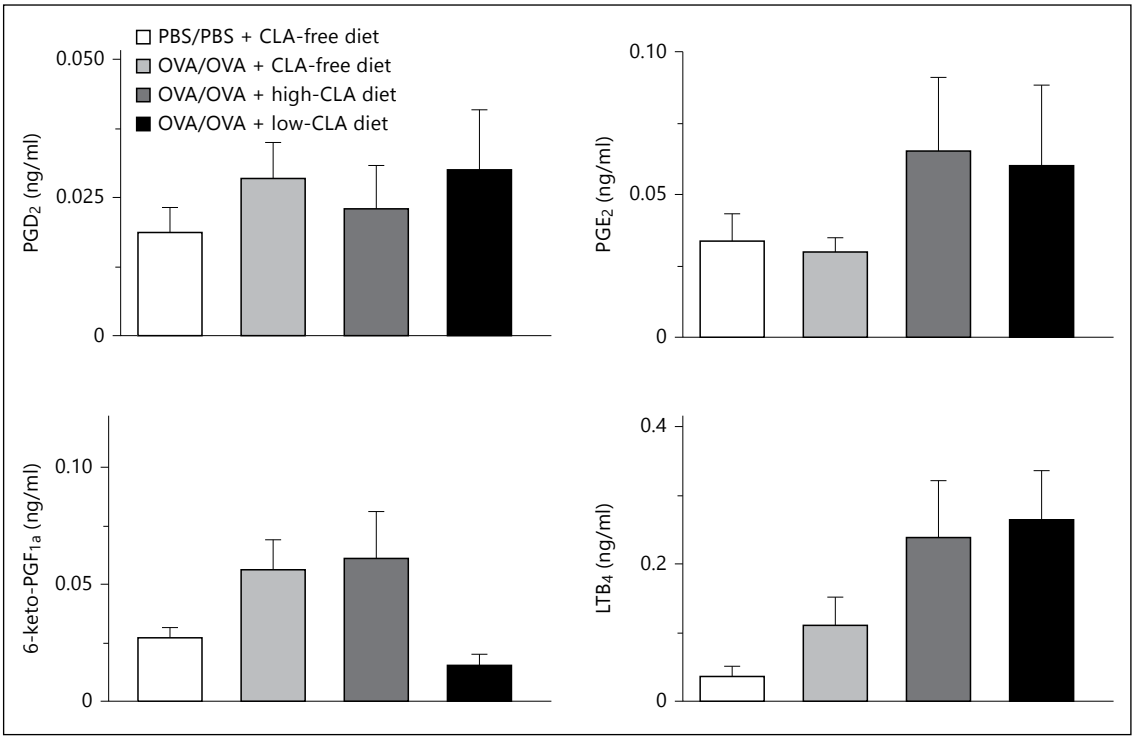

Fig. 4. Milk CLA supplementation does not affect the eicosanoid pattern in BAL. PGD_2, PGE_2, 6-keto-PGF_{1a}, and LTB_4 were measured in BAL fluid using LC-MS/MS, 48 h after the last challenge. PGF_{2a}, TXB_2, and 5S- and 15S-HETE were not detectable in the BAL fluid.

served. Since direct administration of cow's milk to mice is conflicting due to incompatibilities and insufficient uptake by this species, Kanwar et al. [19] suggested the incorporation of milk fat fractions into the mouse diet to overcome these problems. Our data indicate that this mode of application does not hamper the bioavailability of milk fat fractions as an increasing uptake of milk CLA over time was observed. Therefore, this mode of milk fat administration to mice seems to be an appropriate approach to prove the physiological impacts of lipids from natural sources in an experimental model.

However, no allergopreventive effects of CLA-rich milk fat were observed in our experiments based on a model that avoided the use of alum as an adjuvant to facilitate the modification of sensitization and effector phase mechanisms [20]. The lack of an allergoprotective effect is surprising, as it has previously been shown that members of the CLA family possess properties that allow them to modulate proinflammatory processes [15, 35]. CLA are capable of interfering with the synthesis of bioactive eicosanoid mediators such as PG, TX, and LT [32]. The metabolism of these lipid mediators is primarily determined by the availability of FAs in membrane phospholipids [36]. In particular, the phospholipid subtypes PC and PE are of main interest since these phospholipids are substrates for phospholipase A2 generating FFA [28, 29]. Enhanced consumption of CLA has been shown to increase the proportions of these PUFA in cell membrane phospholipids, partly at the expense of n–6 PUFA arachidonic acid [37]. Additionally, CLA might reduce eicosanoid synthesis by inhibition of cyclooxygenase-2 and lipoxygenase [38–40]. In particular, PGD_2 and LTB_4 are thought to be involved in the asthma pathogenesis, since the comparison of nonasthmatics and asthmatics has shown increased levels of these eicosanoids in the latter [as reviewed in 33, 41]. In our model, we examined this mechanistic pathway in detail. Though consumption of a CLA-enriched diet increased the CLA content in plasma and erythrocyte membranes, no changes in the composition of the respective phospholipids (PE and PC) was observed. This observation might explain why, despite the increased CLA amount in plasma and erythrocytes, this uptake did not alter the content of the eicosanoids PGD_2, PGE_2, 6-keto-PGF_{1a}, and LTB_4 in the BAL fluid of mice. In contrast to our results, Whigham et al. [42] observed a decrease in inflammatory eicosanoid mediators in com-

parison to the basal release of eicosanoids in CLA-supplemented animals when respiratory organs were challenged with OVA ex vivo. Furthermore, Jaudszus et al. [18] showed that dietary CLA supplementation of mice with a synthetic FA affects sensitization and is capable of reducing the release of the proinflammatory cytokine IL-5. Our experiments, applying CLA in a natural matrix revealed no decrease in T_H2 cytokines in milk diet-fed animals. Moreover, consumption of a CLA-enriched diet did not attenuate any of the hallmarks of allergic airway inflammation. These different findings could be explained by the fact that high-dosage supplementation with a pure synthetic product might be needed to effectively prevent the development of an asthmatic phenotype while exclusive uptake of natural CLA from milk fails to reach the required threshold. Alternatively, it may be speculated that specific CLA, e.g. C9t11-CLA, are responsible for the mediation of protective effects [15]. However, the uptake of this specific CLA did not significantly differ after consumption of the two diets used in this model (fig. 2). Though we demonstrated that farm milk contains significantly higher amounts of CLA compared to dairy milk, this enrichment seems to be insufficient for a protective effect against experimental airway inflammation. Recent data from experimental studies underline this notion by showing that additional supplementation of milk CLA diets with synthetic CLA might reveal concentrations of CLA in the animals that provide protection against airway inflammation [19, 43].

However, milk CLA may have protective effects in synergism with other ingredients of farm milk that do not survive pasteurization. Nevertheless, which milk fraction is responsible for allergoprotection remains uncertain. Therefore, further research is needed to answer the question of whether the health benefits of unpasteurized farm milk can be attributed to a single milk component or to farm milk as a whole.

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