A vitamin E long-chain metabolite and the inspired drug candidate α-amplexichromanol relieve asthma features in an experimental model of allergen sensitization

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Declarations of interest

None.

Abbreviations

5-LOX, 5-lipoxygenase; 12-HHT, (12(S)-hydroxyheptadecatrienoic acid; α-T-13′-COOH, α-13′-carboxychromanol; α-AC, α-amplexichromanol; AA, arachidonic acid; AHR, airway hyperreactivity; COX, cyclooxygenase; HETE, hydroxyeicosatetraenoic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ELISA, enzyme-linked immunosorbent assay; H&E, Hematoxylin and eosin; IL, interleukin; LTs, leukotrienes; LM, lipid mediator; LCMs, long-chain metabolites; OVA, ovalbumin; PUFA, polyunsaturated fatty acids; SPM, specialized pro-resolving mediators;T, tocopherol, TX; thromboxane.

Abstract

Benefits for vitamin E intake in diseases with inflammatory components have been described and related in part, to endogenously formed metabolites (long-chain metabolites, LCM). Here, we have evaluated the role of LCM in relieving asthma features. To this aim, the endogenous vitamin E metabolite α-13′-carboxychromanol (α-T-13′-COOH) that acts as potent 5-lipoxygenase inhibitor has been administered either intraperitoneally or by oral gavage to BALB/c mice sensitized by subcutaneous injection of ovalbumin (OVA). We also have taken advantage of the metabolically stable α-T-13′-COOH derivative α-amplexichromanol (α-AC). Intraperitoneal treatment with α-T-13'-COOH reduced OVA-induced airway hyperreactivity (AHR) as well as peri-bronchial inflammatory cell infiltration. α-AC was more efficacious than α-T-13'-COOH, as demonstrated by better control of AHR and in reducing subepithelial **thickening**. Both compounds exerted their protective function by reducing pulmonary leukotriene C_4 levels. Beneficial effects of α-AC were coupled to inhibition of the sensitization process, as indicated by a reduction of IgE plasma levels, lung mast cell infiltration and Th2 immune response. Metabololipidomics analysis revealed that α-AC raises the pulmonary levels of prostanoids, their degradation products, and 12/15-lipoxygenase metabolites. Following oral administration, the pharmacodynamically different profile in α-T-13′-COOH and α-AC was abrogated as demonstrated by a similar and improved efficacy in controlling asthma features as well as by metabololipidomics analysis. In conclusion, this study highlights a role for LCM and of vitamin E derivatives as pharmacologically active compounds that ameliorate asthmatic features and defines an important role for endogenous vitamin E metabolites in regulating immune response underlying the sensitization process.

Keywords: α-13′-carboxychromanol, α-amplexichromanol, vitamin E, bronchial hyperreactivity, cyclooxygenase, lipoxygenase

Chemical compounds studied in this article α-13′-carboxychromanol (PubChem CID: 53481461); carbachol (PubChem CID: 5831); dimethyl sulfoxide (PubChem CID: 679); ovalbumin (PubChem CID: 71311993)

1. Introduction

Allergic diseases, such as asthma, affect approximately 25% of the world's population [1,2]. Asthma is a chronic airway disease characterized by reversible airway obstruction, airway hyperreactivity, chronic inflammation, and mucus production. The risk of asthma is determined in infancy and childhood and the phenotypes are determined by both genetic and environmental exposure [3]. Epidemiological studies have shown that maternal nutrition during pregnancy is related to the occurrence of asthma during childhood. A reduced intake of food containing antioxidant and anti-inflammatory molecules such as vitamin E, may increase the incidence of inflammatory and allergic diseases [4]. Accordingly, maternal vitamin E intake [5], as well as the administration of vitamin E in children with moderate asthma, contribute to the improvement of breathing and prevention of asthma [6]. These beneficial effects seem to be correlated not only to antioxidant properties of vitamin E, but also to its immunomodulatory effects [7,8]. Vitamin E consists of eight isoforms, α-, β-, γ- and δ-tocopherol (T) and the respective tocotrienol (TE) isoforms, which exhibit opposite and competitive effects on asthma [9]. High levels of γ-T are associated with asthma [10] and dietary supplementation with γ-T increases lung inflammation in response to allergen [11]. On the contrary, decreased serum levels of α-T, the most abundant isoform in serum and human tissues, have been reported in asthmatic compared to healthy patients [12], suggesting a protective role of this isoform in asthma disease. Indeed, a diet rich in α-T displays protective effects against asthma [10]. Accordingly, diet supplementation with α-T decreases lung inflammation in response to dust mite in a mouse model of asthma [9].

It is known that endogenous long-chain metabolites (LCMs) of α-T are responsible, at least partially, for the anti-inflammatory effects of vitamin E [13-15]. LCMs potently inhibit 5-lipoxygenase (5-LOX) and in turn suppress the biosynthesis of pro-inflammatory leukotrienes (LTs) in inflammatory cells [13]. Recently, we have demonstrated that α-13′-

carboxychromanol (α-T-13′-COOH), an endogenous metabolite of α-T detected at low nanomolar concentrations in human plasma, accumulates within immune cells at sites of inflammation. Accordingly, it significantly reduces *in vivo* the inflammatory reaction in zymosan-induced peritonitis by inhibiting LT production and increasing the systemic concentration of distinct specialized pro-resolving mediators (SPM) [13]. α-T-13'-COOH also effectively modulates allergen-induced hyperreactivity [13]. Starting from endogenous LCMs as lead structures, many semisynthetic compounds have been synthesized [16,17], and 12α′,13′-dihydroxylated α-tocotrienol (α-amplexichromanol, α-AC) has been identified as a highly potent allosteric 5-LOX inhibitor with prominent *in vitro* and *in vivo* anti-inflammatory activity [16]. Based on this evidence we have here investigated the effects of α-T-13'-COOH and α-AC in an experimental mouse model of asthma.

2. Material and Methods

2.1. Materials

α-T-13'-COOH and α-AC (Fig. 1A) have been semisynthesized respectively from natural δ-garcinoic acid and δ-amplexichromanol through previously reported strategies [18,19].

2.2. Animals

Female BALB/c mice (18 ± 2 g body weight, Charles River, Calco, Italy) were housed in a controlled environment (21 ± 2 °C) and provided with standard rodent chow and water. All animals were allowed to acclimate for four days before experiments and were subjected to 12 h light – 12 h dark schedule. Each mouse was assigned an identification number and randomized to different groups so that all experiments were carried out in a blinded manner. Mice were divided into groups not predetermined by a statistical method but by previous experimental data in this murine sensitization model [20-23]. Experiments were conducted

during the light phase. The experimental procedures were approved by the Italian Ministry according to International and National law and policies (EU Directive 2010/63/ EU and Italian DL 26/2014 for animal experiments, ARRIVE guidelines, and the Basel declaration including the 3R concept). Animal studies are reported in compliance with the ARRIVE guidelines [24].

2.3 Sensitization and drug treatment

Mice were treated with 0.4 ml s.c. of a suspension containing 100 µg of ovalbumin **from chicken egg white** (OVA, **Sigma-Aldrich, Milan, Italy**) absorbed to 3.3 mg of aluminum hydroxide gel (**Merck KGaA, Darmstadt, Germany**) or saline (control group) on days 0 and 7 [18-21]. α-T-13'-COOH (OVA + α-T-13'-COOH group) and α-AC (OVA + α-AC group) (10 mg kg^{-1}) or vehicle (dimethyl sulfoxide 4%, 0.5 ml; OVA group, **Sigma-Aldrich, Milan, Italy**) were administered i.p. 30 min or per os (Fig. 1B) 1 h before each OVA administration, respectively [13,16]. Animals were sacrificed at days 8, 14, or 21 by an overdose of enflurane, and lungs, bronchi, mediastinal lymph nodes, and blood were collected.

2.4 Bronchial reactivity

OVA-sensitized mice were sacrificed on day 21 by enflurane overdose, exsanguinated, and lungs were removed. Main bronchi were rapidly dissected and cleaned from fat and connective tissue. Rings of 1-2 mm length were cut and mounted in 2.5 ml isolated organ baths containing Krebs solution, at 37 °C, oxygenated (95% O_2 and 5% CO_2) and connected to an isometric force transducer (type 7006, Ugo Basile, Comerio, Italy) associated to a Powerlab 800 (AD Instruments). Rings were initially stretched until a resting tension of 0.5 g was reached and allowed to equilibrate for at least 30 min during which tension was adjusted, when necessary, to 0.5 g and the bathing solution was periodically changed. In

each experiment bronchial rings were previously challenged with carbachol **(Sigma-Aldrich, Milan, Italy)** and then a cumulative concentration-response curve (10^{-9} – 3 × 10^{-6} M) was performed. Results are expressed as dyne per mg tissue [20-23].

2.5. Lung histology

Left lung lobes harvested from mice were paraffin-embedded and 7 µm sections were cut. Histological changes in the lungs were evaluated by hematoxylin and eosin (H&E, **Kaltek, Padua, Italy**) staining [22,23]. Images were acquired by blinded operators using a Leica DFC320 video-camera (Leica, Milan, Italy; 40× magnification) connected to a Leica DM RB microscope using the Leica Application Suite software V.4.1.0.

2.6. LTC_4 and IL-13 levels

Lungs were isolated and homogenized in **ice-cold** PBS pH 7.4 **(100 mg ml^{-1},** Sigma Aldrich, Milan, Italy) **using a FastPrep tissue homogenizer (2 cycles of 20 sec, 6m/s; MP Biomedicals, DBA, Segrate, Italy)**. The homogenate was centrifuged (4 °C, 6000 × g, 10 min). Commercially available enzyme-linked immunosorbent assay (ELISA) were used to measure the levels of LTC_4 (Cayman Chemical, BertinPharma, Montigny Le Bretonneux, France) and interleukin (IL)-13 (Invitrogen, Vienna, Austia) according to the manufacturer's instructions. The levels of LTC_4 and IL-13 are expressed as pg mg^{-1} of tissue.

2.7. Profiling of lipid mediators

Lung tissue was homogenized in ice-cold PBS pH 7.4 (100 mg ml^{-1}) using an Omni tissue homogenizer (Omni, Kennesaw, GA; 1-2 min), which was equipped with a flat

bottom generator probe (5 × 75 mm, fine). Non-esterified fatty acids and lipid mediators (LM) were extracted from plasma or lung homogenates using reversed phase cartridges (Sep-Pak® Vac 6cc 500 mg/6 ml C18; Waters) as previously described [16,17]. Internal standards added: d4-LTB_4, d4-prostaglandin (PG)E_2, d8-5*S*-hydroxyeicosatetraenoic acid (HETE), d5-lipoxin A_4, d5-resolvin D_2, (200 nM, each, Cayman Chemicals), and d8-arachidonic acid (AA, 10 μM, Cayman Chemicals). Lipid mediators were separated on an Acquity UPLC BEH C18 column (2.1 × 100 mm, Waters) using an Acquity UPLC system (Waters) and detected using a QTRAP 5500 mass spectrometer (SCIEX), equipped with an electrospray ionization source [25]. Diagnostic ion fragments were determined by scheduled multiple reaction monitoring in the negative ion mode for peak identification. Absolute quantification was achieved by automatic peak integration (Analyst 1.6 software, Sciex, IntelliQuan default settings) and normalization using 6 internal and 45 external standards [25].

2.8. Plasma IgE levels

Blood was collected by intracardiac puncture using trisodium citrate **(Sigma-Aldrich, Milan, Italy)** as an anticoagulant. Then, plasma was obtained by centrifugation at 800 × g at 4 °C for 10 minutes and immediately frozen at -80 °C. Total IgE levels were measured by ELISA kit (Bethyl Laboratories, USA; BD OptiEIA, Montgomery, TEXAS).

2.9. Flow cytometry analysis

As previously reported [23], lungs were isolated, digested with 1 U ml^{-1} collagenase (Sigma Aldrich, Milan, Italy), and passed through 70 μm cell strainers. Red blood cells were lysed by means of RBC lysis Buffer (ThermoScientific srl, USA). Cell suspensions were used for

198 flow cytometry analysis of different cell subtypes. Cells (10^6) were incubated with the
199 following antibodies: CD11c-FITC, cKit-PeCy5.5, IgE-APC, CD3PeCy5.5, CD4-FITC, IL-4-
200 APC (eBioscience, San Diego, CA, USA). Appropriate isotype controls were used.

201

202 ***2.10. Statistical analysis***

203 The results are expressed as mean ± S.E.M of n observations, where n represents the
204 number of animals. Statistical evaluation was performed by one-way or two-ways ANOVA
205 using GraphPad InStat (Graphpad Software Inc., San Diego, CA) followed by a Bonferroni
206 post-hoc test for multiple comparisons. Post hoc tests were run only if F achieved $P < 0.05$
207 and if there was no significant variance in the homogeneity. A P value < 0.05 was used to
208 define statistically significant differences between mean values. Outliers were identified by
209 Grubbs' test.

210

211 **3. Results**

212 **3.1. Effect of α-T-13'-COOH and α-AC on asthma features**

213 OVA sensitization induced airway hyperreactivity (AHR) as evidenced by a significant
214 increase of bronchial reactivity to carbachol compared to the control group (Figure 1C).
215 Allergen-sensitized BALB/c mice receiving (i.p.) the endogenous α-T metabolite α-T-13'-
216 COOH or the semi-synthetic compound α-AC (Fig. 1B) showed a reduced AHR (Fig. 1C),
217 according to what **has been** previously demonstrated [13,16]. However, α-T-13'-COOH
218 induced only a shift of the carbachol concentration-response **curve** with respect to vehicle-
219 treated animals (OVA+ vehicle group), while α-AC reduced the maximal carbachol-induced
220 contractility, too. The functional data, suggesting a different pharmacodynamic effect, were
221 confirmed by histological analysis. H&E, performed on pulmonary sections, showed a

reduction in peribronchiolar and perivascular infiltration of inflammatory cells in the lung induced by α-T-13'-COOH and α-AC. However, inhibition of subepithelial **thickening** occurred only in sensitized α-AC-treated mice (Fig. 1D).

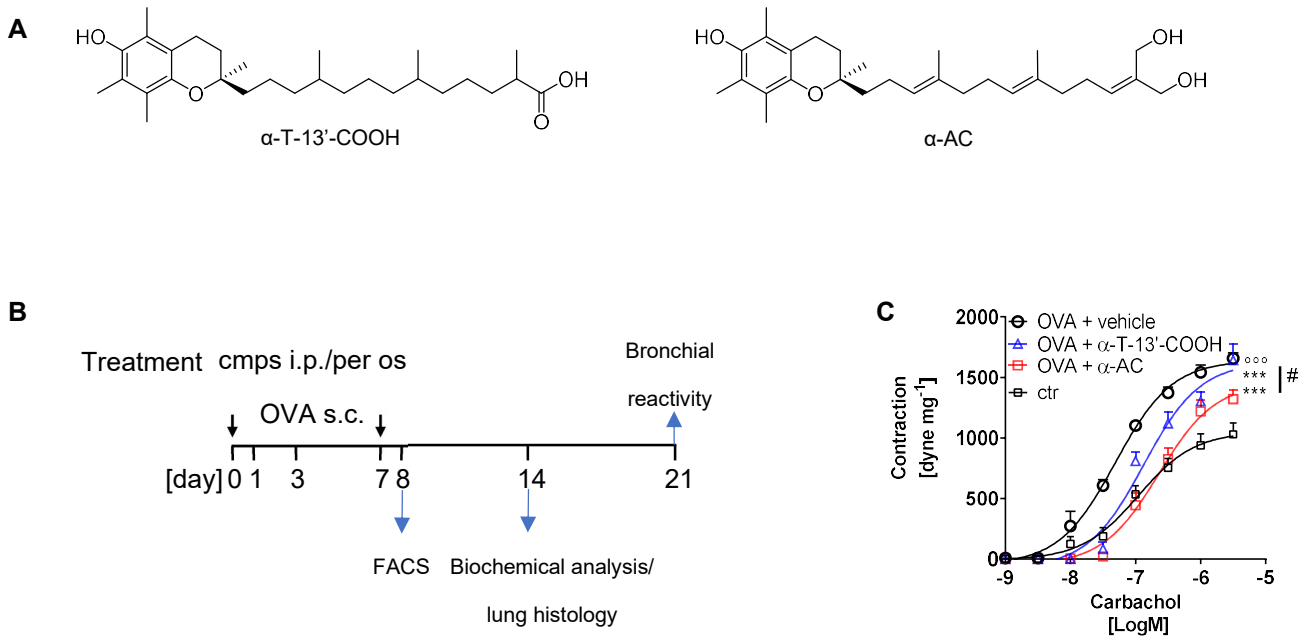


Figure 1. Effects of α-T-13'-COOH and α-AC on bronchial hyperreactivity and pulmonary inflammation in sensitized mice. A) Chemical structure of α-T-13'-COOH and α-AC. B) Scheme of sensitization and treatment. Female mice were pretreated i.p. with α-T-13'-COOH and α-AC (10 mg kg-1) or vehicle (DMSO 2%, 0.5 mL) 30 min before each

OVA challenge. Animals were sacrificed to evaluate C) bronchial reactivity to carbachol and C) lung histology. Values represent means ± S.E.M.; n = 6 mice for each group. D) Representative photomicrographs of hematoxylin and eosin (H&E) on lung sections. Scale bars = 50 μm. Data were analyzed by two-way ANOVA plus Bonferroni (C). Statistical significance is reported as follows: °°° $P < 0.001$ vs control (ctr, without OVA); *** $P < 0.001$ vs OVA + vehicle; # $P < 0.001$ OVA + α-AC vs OVA + α-T-13'-COOH.

3.2. α-T-13'-COOH and α-AC interfere on lipid mediator release

To assess if the beneficial effects of α-T-13'-COOH and α-AC are related to an interference with LM-driven sensitizing mechanisms, LM levels were measured in the lungs and plasma of OVA-treated mice. Administration of α-T-13'-COOH or α-AC during the sensitization phase caused a significant reduction of pulmonary levels of 5-LOX-derived LMs, such as LTC_4 by 76% and 54%, respectively (Fig. 2A). Different effects were observed for cyclooxygenase (COX)-derived LMs, i.e., PGE_2, the stable thromboxane (TX)A_2 metabolite TXB_2, and the PGH_2 degradation product 12(S)-hydroxyheptadecatrienoic acid (12-HHT) (Fig. 2B). While pulmonary PGE_2 levels were (not-significantly) increased both by α-T-13'-COOH and α-AC injection, other COX-derived products, i.e., TXB_2 and 12-HHT, were upregulated by α-AC, but decreased by α-T-13'-COOH (Fig. 2B), which might reflect the superior COX-1-inhibitory activity of the endogenous α-T-13'-COOH as compared to α-AC [13,16]. In addition, α-AC elevated also the 12/15-LOX-derived product levels that originate from AA (i.e., 12-HETE and 15-HETE), eicosapentaenoic acid (EPA) (i.e., 12-HEPE and 15-HEPE) and docosahexaenoic acid (DHA) (i.e., 17-HDHA and 14-HDHA) in respect to vehicle-treated animals (Fig 2C). This finding is of particular interest because these hydroxylated fatty acids represent precursors for the biosynthesis of SPMs that orchestrate

the resolution of inflammation [26]. Conversely, a tendency for reduction, although not significant, in LM levels was observed following α-T-13'-COOH treatment (Fig. 2C). The availability of polyunsaturated fatty acids (PUFAs), which are released from phospholipids and eventually converted into LMs, was slightly elevated by both treatments without gaining statistical significance (Fig. 2D). Thus, we conclude that α-T-13'-COOH and α-AC did not essentially shape the LM profile by targeting fatty acid release. The pronounced effects of the two compounds on the lung LM network were only partially observed in plasma (Fig. S1).

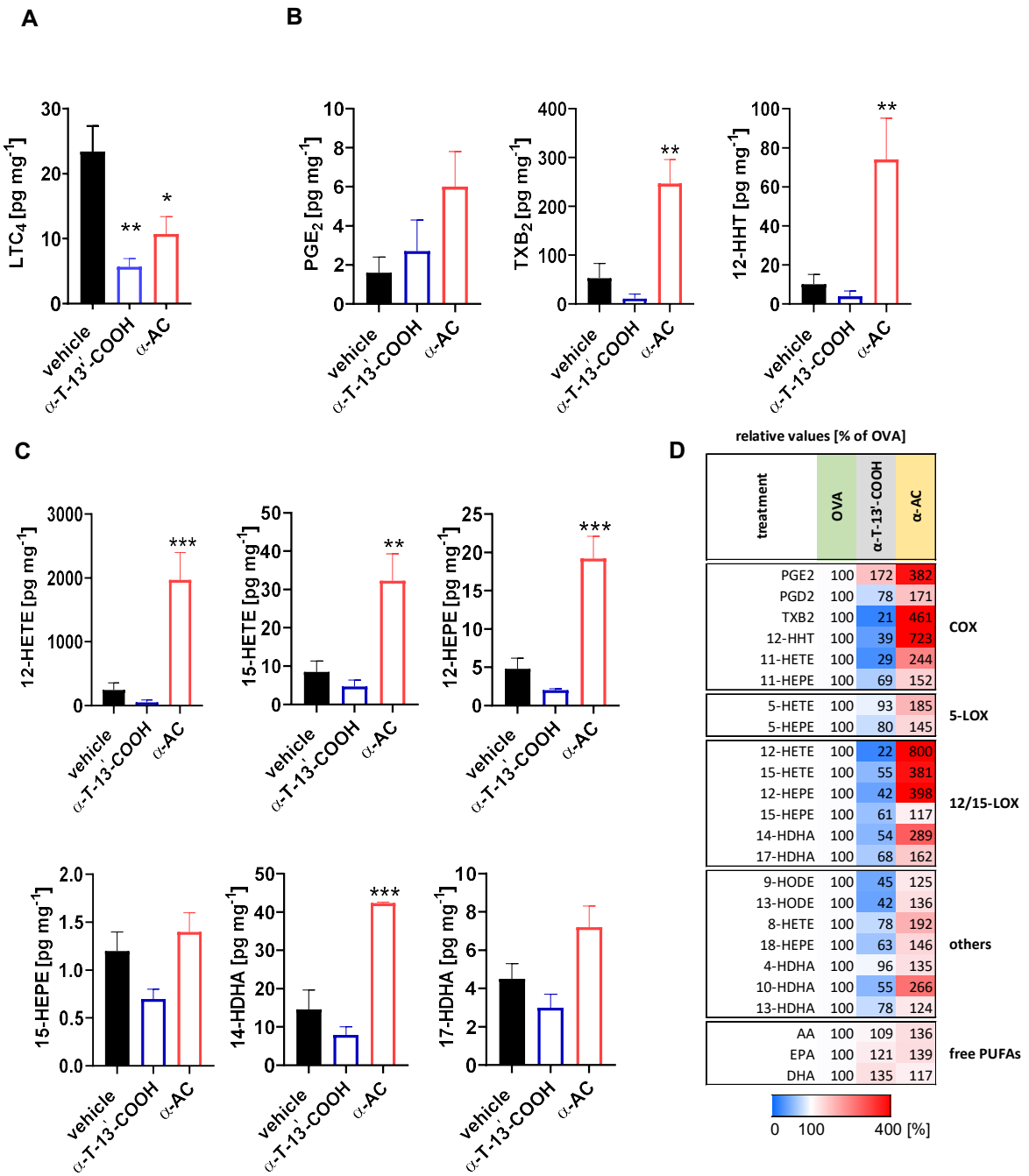
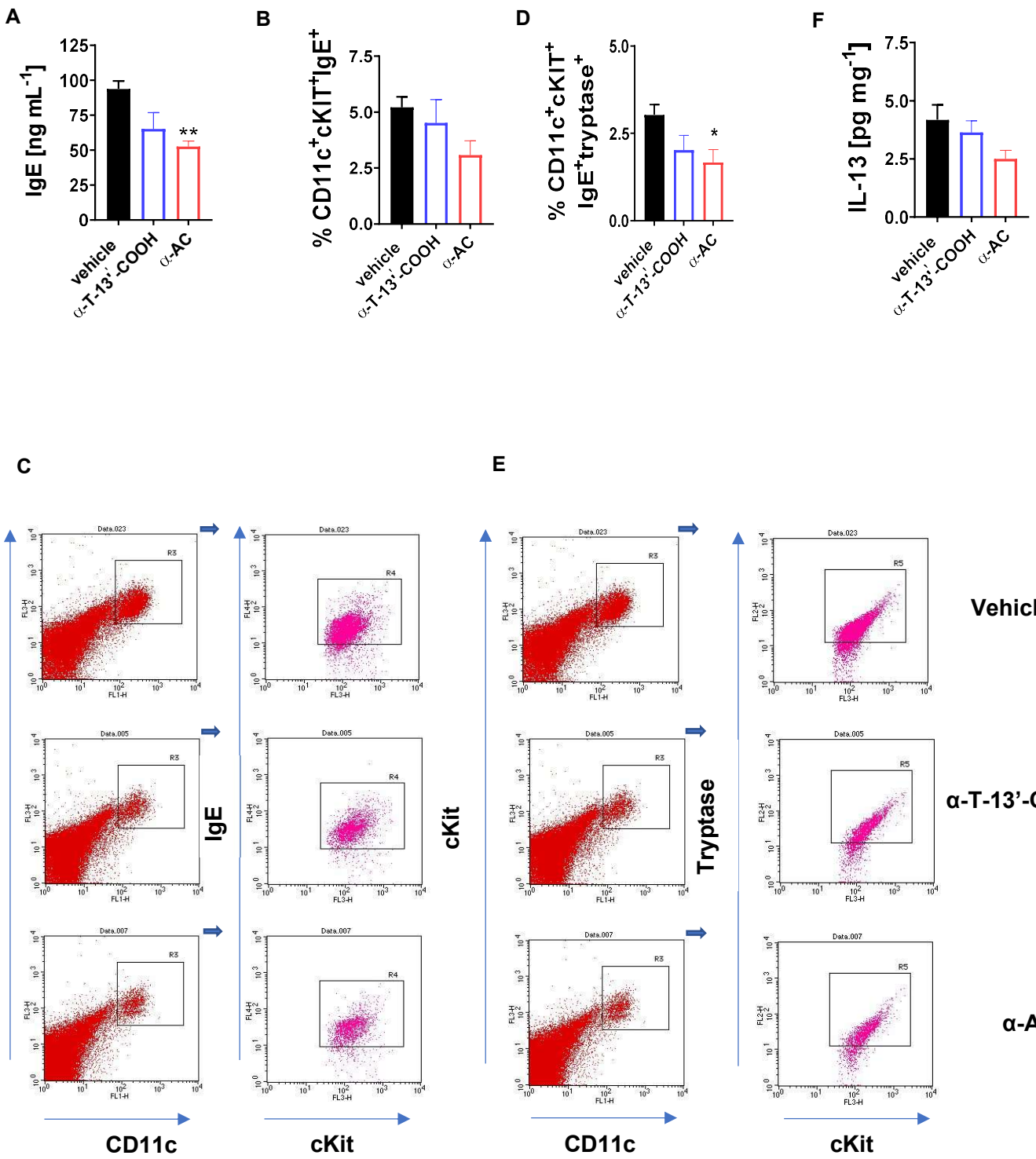


Figure 2. Effects of α-T-13'-COOH and α-AC on lipid mediator levels in sensitized mice. Female mice were pretreated i.p. with α-T-13'-COOH and α-AC (10 mg kg^{-1}) or vehicle (DMSO 2%, 0.5 ml) 30 min before each OVA challenge. Animals were sacrificed at day 14 to evaluate in the lung homogenate A) LTC_4 levels analyzed by ELISA and B) COX products and C) 12/15-LOX-derived products formed from AA (12-HETE and 15-HETE), EPA (12-HEPE and 15-HEPE) and DHA (17-HDHA, 14-HDHA) analyzed by UPLC-MS/MS. D) The heatmap shows percentage changes in lipid mediator levels versus vehicle-treated OVA-sensitized mice. Values represent mean + S.E.M.; n = 6 mice for each group. Data were analyzed by one-way ANOVA plus Bonferroni; statistical significance is reported as follows: *P < 0.05, **P < 0.01 and ***P < 0.001 vs vehicle.

3.3. Effect of α-T-13'-COOH and α-AC on OVA sensitization

Allergen sensitization is sustained by a time-dependent increase in plasma IgE levels, which correlates with temporal mast cell recruitment and activation in the lung [22]. IgE levels were decreased in sensitized animals receiving α-AC (i.p.) in comparison to vehicle-treated mice, whereas α-T-13'-COOH only showed a partial and not significant effect (Fig. 3A).

Mast cell ($CD11c^+cKit^+IgE^+$ cells) infiltration was analysed in pulmonary tissues by flow cytometry. The percentage of $CD11c^+cKit^+IgE^+$ cells in lung tissues harvested from sensitized mice decreased upon treatment with α-AC (Fig 3.B and C). α-AC also reduced the activation rate of mast cells as demonstrated by a reduced percentage of $CD11c^+cKit^+IgE^+tryptase^+$ cells (Fig. 3D and E). In perfect tune, α-AC reduced pulmonary IL-13 production (Fig. 3F). α-T-13'-COOH did not show a significant effect on any of the evaluated parameters (Fig. 3B-F).


A
IgE [ng mL-1]
125
100
75
50
25
0
**
vehicle
α-T-13'-COOH
α-AC
B
% CD11c+cKIT+IgE+
7.5
5.0
2.5
0.0
vehicle
α-T-13'-COOH
α-AC
D
% CD11c+cKIT+ IgE+tryptase+
5.0
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0.0
*
vehicle
α-T-13'-COOH
α-AC
F
IL-13 [pg mg-1]
7.5
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C
E
cKit
IgE
cKit
Tryptase
CD11c
cKit
CD11c
cKit
Vehicle
α-T-13'-COOH
α-AC

Figure 3. Effects of α-T-13'-COOH and α-AC on IgE levels and mast cell infiltration and activation in sensitized mice. Female mice were pretreated i.p. with α-T-13'-COOH and α-AC (10 mg kg^{-1}) or vehicle (DMSO 2%, 0.5 ml) 30 min before each OVA challenge. Animals were sacrificed at day 8 or 14 days to evaluate A) plasma IgE levels, pulmonary percentage (day 8) of B) $CD11c^{+}IgE^{+}c\text{-}KIT^{+}$ and D) $CD11c^{+}IgE^{+}c\text{-}KIT^{+}$ tryptase cells and **F**) pulmonary levels of IL-13. C and E are representative dot plots for B and D. Values represent mean + S.E.M.; n = 6 mice for each group. Data were analyzed by one-way ANOVA plus Bonferroni; statistical significance is reported as follows: *P < 0.05, **P < 0.01 vs vehicle.

3.4. Effect of α-T-13'-COOH and α-AC on the Th2 response

To evaluate the effect of α-T-13'-COOH and α-AC on the allergen-induced immune response, $CD4^{+}IL4^{+}$ cell trafficking was evaluated by flow cytometry analysis of mediastinal lymph nodes and lungs during the sensitization phase. As shown in Fig. 4A and B, i.p. treatment with α-T-13'-COOH or α-AC significantly reduced the percentage of $CD4^{+}IL4^{+}$ cells in the lymph nodes in comparison to vehicle-sensitized mice. The reduction of $CD4^{+}IL4^{+}$ cells in lymph nodes was connected to a lower proportion of this cell population in the lungs of sensitized mice only when treated with α-AC (Fig. 4C and D).

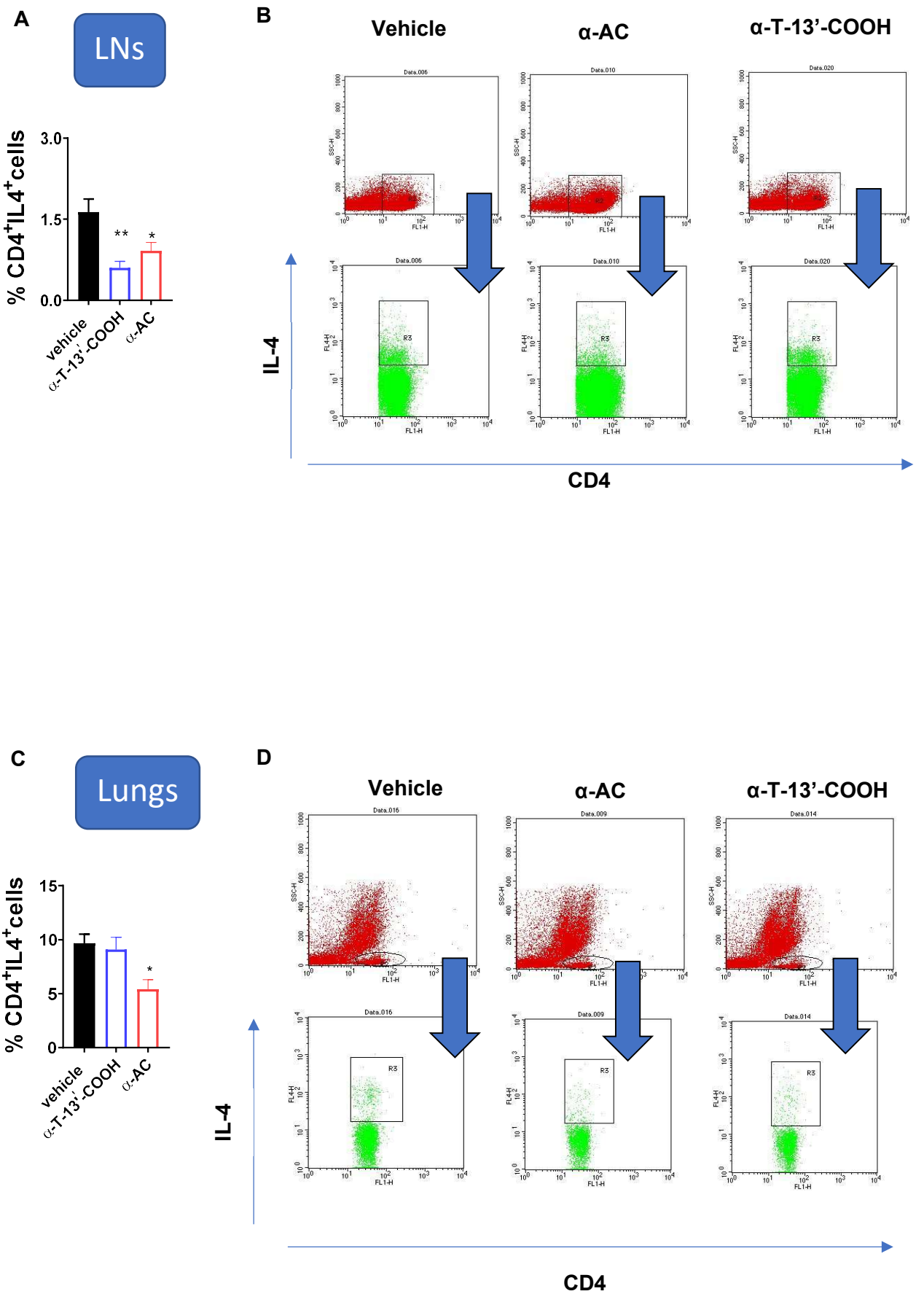
A
LNs
% CD4+IL4+cells
3.0
1.5
0.0
**
*
vehicle
α-T-13'-COOH
α-AC
B
Vehicle
α-AC
α-T-13'-COOH
IL-4
CD4
C
Lungs
% CD4+IL4+cells
15
10
5
0
*
vehicle
α-T-13'-COOH
α-AC
D
Vehicle
α-AC
α-T-13'-COOH
IL-4
CD4

Figure 4. Immunomodulatory effects of α-T-13'-COOH and α-AC in sensitized mice. Female mice were pretreated i.p. with α-T-13'-COOH and α-AC (10 mg kg-1) or vehicle (DMSO 2%, 0.5 ml) 30 min before each OVA challenge. Animals were sacrificed at day 8 to evaluate respectively A) mediastinal lymph node and C) lung percentage of CD4+IL-4+ cells. B and D are representative dot plots for A and C. Values represent mean + S.E.M.; n = 6 mice for each group. Data were analyzed by one-way ANOVA plus Bonferroni; statistical significance is reported as follows: *P < 0.05, **P < 0.01 vs vehicle.

3.5. Effect of oral administration of α-T-13'-COOH and α-AC in sensitized mice

The effects of α-T-13'-COOH and α-AC on allergic airway inflammation were also evaluated following oral administration. Both compounds abrogated AHR in sensitized mice (Fig. 5A) as demonstrated by the overlapping concentration-response curves (carbachol/contraction) obtained in control and sensitized mice upon treatment. This effect was associated with increased pulmonary levels of COX- (Fig. 5B**,D**) and 12/15-LOX-derived metabolites (Fig. 5C,**D**). The accumulation of LMs seems to be driven by an increase in the pulmonary levels of free PUFAs (Fig. 5D). Systemic PUFA and LM levels followed the same trend (Fig. S2). The protective effects of α-T-13'-COOH and α-AC were associated with an impaired Th2-dependent immune activation, as indicated by the drop of IgE in plasma (Fig. 6A) and the inhibition of $CD4^{+}IL4^{+}$ cell infiltration in the lung (Fig. 6B**,C**) relative to vehicle-treated sensitized mice.

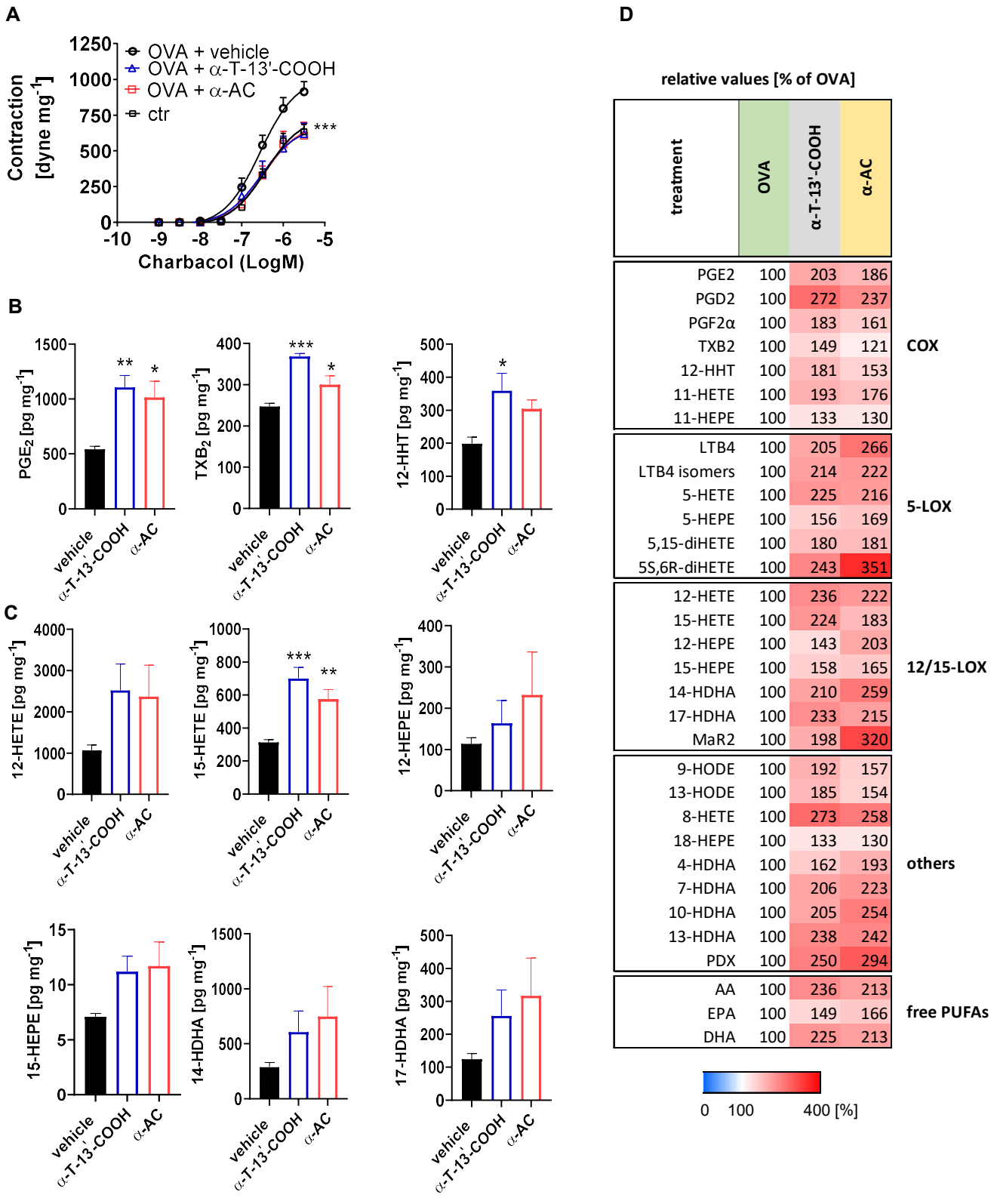

Figure 5. Effects of oral α-T-13'-COOH and α-AC on bronchial hyperreactivity and pulmonary levels of LM in sensitized mice. Female mice were pretreated per os with α-

T-13'-COOH and α-AC (10 mg kg^{-1}) or vehicle (DMSO 2% in CMC 0.5%, 0.5 ml) 60 min before each OVA challenge. Animals were sacrificed at day 21 and 14 to evaluate A) bronchial reactivity to carbachol, pulmonary levels of B) COX products and C) 12/15-LOX-derived metabolites. D) The heatmap shows percentage changes in lipid mediator levels versus vehicle-treated OVA-sensitized mice. Values represent mean + S.E.M.; n = 6 mice for each group. Data were analyzed by two-way ANOVA plus Bonferroni (B) or one-way ANOVA plus Bonferroni (C and D); statistical significance is reported as follows: *P < 0.05, **P < 0.01 and ***P < 0.001 vs OVA + vehicle.

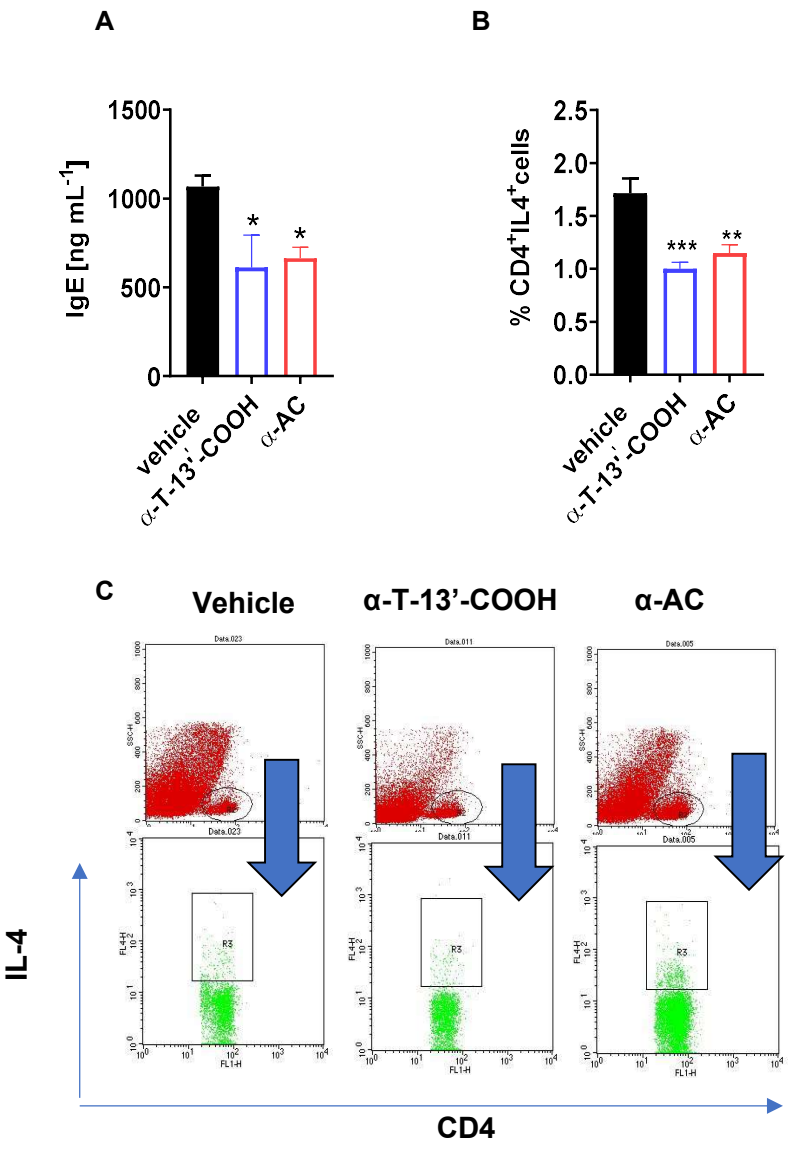

Figure 6. Effects of α-T-13'-COOH and α-AC on IgE plasma levels and Th2 response in sensitized mice. Female mice were pretreated i.p. with α-T-13'-COOH and α-AC (10 mg kg^{-1}) or vehicle (DMSO 2%, 0.5 ml) 30 min before each OVA challenge. Animals were sacrificed to evaluate A) plasma IgE levels and pulmonary percentage of B) CD4+ IL-4+ cells. **C is a representative dot plots for B**. Data are shown as single data points and the mean value. Data were analyzed by one-way ANOVA plus Bonferroni; statistical significance is reported as follows: *P < 0.05, ** P < 0.01, ***P < 0.001 vs vehicle.

4. Discussion

Here, we demonstrate that the endogenous α-T metabolite α-T-13'-COOH and the semisynthetic derivative α-AC relieve typical asthma hallmarks such as AHR and pulmonary inflammation, by modulating the immune response underlying **OVA sensitization**. The beneficial effects of LCMs were sustained by their ability to modulate LM levels such as LTC_4, COX- and 12/15-LOX-derived metabolites. Studies in both mice and humans have shown that these LM play a crucial role in all aspects of Th2 immunity, including the promotion and regulation of type 2 inflammation. Here we demonstrate that α-T-13'-COOH and α-AC affect Th2 immune response during sensitization by modulating pro-inflammatory and anti-inflammatory mediator levels, with focusing on 5-LOX, COX and 12/15-LOX derived metabolites.

Asthma is sustained by a Th2 immune response involving different phases, starting with the sensing of the stimuli and initiation of the immune response via the release of a wide array of biochemical inflammatory factors and danger signals. This response culminates in the altered tissue physiology and host–effector reactions. Finally, a resolution phase develops that limits the activation of inflammatory cells and helps to promote tissue repair [26,27].

PUFA like AA, EPA and DHA, released by phospholipase A_2 isoenzymes, are subsequently converted into LMs which orchestrate the inflammatory reaction. The acute phase of inflammation is dominated by excessive formation of AA-derived LMs, i.e. LTs and PGs, whereas 12/15-LOX products are produced particularly from DHA and EPA in the resolution phase [26-28].

The i.p. administration of the endogenous α-T-13'-COOH significantly inhibited AHR as demonstrated by the shift of the carbachol concentration-response curve in respect to vehicle-treated sensitized mice as well as pulmonary lung cell infiltration. The semisynthetic analogue α-AC also reduced the maximal carbachol-induced contractility.

Immunohistochemical analysis confirmed a different action of the two compounds showing that α-AC inhibited not only pulmonary cell infiltration but also the subepithelial **thickening**. The analysis of LMs highlighted a marked difference between α-AC and α-T-13'-COOH. Indeed, only α-AC treatment of sensitized mice increased the pulmonary levels of COX- and 12/15-LOX-derived metabolites. It is plausible that this effect represents an attempt by the organism to limit ongoing inflammation and protect airways from subsequent damage. α-AC promoted a significant increase in pulmonary levels of PGE_2, TXB_2 and 12-HHT. Several preclinical and clinical data confirm an active role of PGE_2 as a protective mediator in asthma [31-34]. Indeed, in asthmatic patients and animal models a negative correlation between the sputum levels of PGE_2 and eosinophil numbers and AHR has been reported [29-34]. A positive correlation between TXB_2 and 12-HHT levels with improvement in lung function has been found as well [35,36]. LMs produced by 12/15-LOX are negatively associated with pulmonary inflammation. Thus, 15-LOX-derived SPM biosynthesis is downregulated in eosinophils isolated from patients with severe asthma and eosinophilic chronic rhinosinusitis [37]. Moreover, systemic administration of SPMs suppressed pulmonary eosinophilic inflammation in murine models of asthma [38,39], and 12/15-LOX-deficient mice displayed augmented IL-33-induced lung inflammation [40].

The beneficial effect of α-AC involves not only the inhibition of **pulmonary** pro-inflammatory mediators such as LTs, **but also the increase of anti-inflammatory COX- and 12/15-LOX-derived products.** The differences in the LM profile observed between the two treatments might explain the higher efficacy of the semisynthetic α-AC as compared to the endogenous metabolite α-T-13'-COOH in inhibiting bronchial hyperreactivity and pulmonary metaplasia in sensitized mice.

To confirm our hypothesis on an active role for α-AC in counterbalancing the pro asthmatic inflammatory reaction, we looked at the immune response underlying sensitization. Again, we observed a major efficacy by α-AC when compared to α-T-13'-COOH in repressing the

Th2 response. Indeed, α-AC significantly inhibited $CD4^+IL4^+$ cell infiltration in both mediastinal lymph nodes and lung. Thus, α-AC not only inhibited immune cell trafficking between lymph node and lung, but also reduced **the percentage of** $CD4^+IL4^+$ cell in the mediastinal lymph nodes. The ability of α-AC to counterbalance the pro-inflammatory response has been confirmed by a significant attenuation of plasma IgE levels and mast cell activation in the lung.

Oral vitamin E supplementation has been proposed to have anti-inflammatory and antioxidant properties based on pre-clinical studies [41,42]. However, in the current relevant literature, clinical studies related to vitamin E supplementation produced mixed results and rather point to limited clinical efficacy in inflammatory diseases. The poor efficacy in clinical studies has been ascribed to the different vitamin E states of the patient cohort or individual differences in α-T metabolism [11]. Plasma α-T levels are regulated by α-T transfer protein (α-TTP), a transporter highly expressed in the liver, where it selects α-T among the various vitamin E forms in the diet, transfers the vitamin to plasma lipoproteins and promotes their secretion into the circulation. The pivotal role of α-TTP in regulating pulmonary levels of α-T has been reported also in asthma models [42]. To further confirm the therapeutic advantage of a LCM supplementation in asthma, both compounds were administered to sensitized mice by oral gavage. The oral administration of α-T-13'-COOH and α-AC ameliorated their ability to relieve AHR, suppressing bronchial reactivity to basal values. In addition, the difference in the pharmacodynamic profile of the two compounds was blunted and both displayed a major efficacy. Indeed, α-T-13'-COOH and α-AC increased the pulmonary levels of COX- and 12/15-LOX-derived metabolites, reduced plasma IgE levels and the Th2 response in sensitized mice. This might be related to hepatic conversion into further side-chain truncated metabolites [13], whose biological activities in the context of sensitization are hardly understood. This functional outcome correlates to changes in pulmonary LM production.

In conclusion we have identified α-T-13'-COOH as bioactive LCM to relieve asthma features and demonstrated the efficacy of the LCM-inspired semisynthetic derivative α-AC. Favouring the production of precursors for pro-resolving LM likely helps to sustain the effects of α-T-13'-COOH and α-AC and may represent a novel therapeutic strategy targeting the resolution phase of inflammation in asthma.

Author contributions

A. Rossi, F. Roviezzo and A. Koeberle designed the study, analysed data and wrote the manuscript; I. Cerqua, E. Granato, R. Capasso performed animal studies, cytokine determination and evaluated bronchial reactivity; K. Neukirch performed metabololipidomics analysis; M. Terlizzi and R. Sorrentino performed FACS analysis; E. Caiazzo and C. Cicala performed lung tissue histology, Denis Seraphin and J.J. Helesbeux synthetized α-AC; O. Werz, A. Ialenti and G. Cirino analysed data and revised the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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557 **Data availability statement**

558 The data sets generated for this study are available on request to the corresponding authors.

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