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## **A vitamin E long-chain metabolite and the inspired drug candidate $\alpha$ -amplexichromanol relieve asthma features in an experimental model of allergen sensitization**

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1 **Vitamin E long-chain metabolite and the inspired drug candidate  $\alpha$ -amplexichromanol**  
2 **relieve asthma features in an experimental model of allergen sensitization**

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7

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36

### 37 **Declarations of interest**

38 None.

39

### 40 **Abbreviations**

41 5-LOX, 5-lipoxygenase; 12-HHT, (12(S)-hydroxyheptadecatrienoic acid;  $\alpha$ -T-13'-COOH,  $\alpha$ -  
42 13'-carboxychromanol;  $\alpha$ -AC,  $\alpha$ -amplexichromanol; AA, arachidonic acid; AHR, airway  
43 hyperreactivity; COX, cyclooxygenase; HETE, hydroxyeicosatetraenoic acid; DHA,  
44 docosahexaenoic acid; EPA, eicosapentaenoic acid; ELISA, enzyme-linked immunosorbent  
45 assay; H&E, Hematoxylin and eosin; IL, interleukin; LTs, leukotrienes; LM, lipid mediator;  
46 LCMs, long-chain metabolites; OVA, ovalbumin; PUFA, polyunsaturated fatty acids; SPM,  
47 specialized pro-resolving mediators; T, tocopherol, TX; thromboxane.

48 **Abstract**

49 Benefits for vitamin E intake in diseases with inflammatory components have been  
50 described and related in part, to endogenously formed metabolites (long-chain metabolites,  
51 LCM). Here, we have evaluated the role of LCM in relieving asthma features. To this aim,  
52 the endogenous vitamin E metabolite  $\alpha$ -13'-carboxychromanol ( $\alpha$ -T-13'-COOH) that acts as  
53 potent 5-lipoxygenase inhibitor has been administered either intraperitoneally or by oral  
54 gavage to BALB/c mice sensitized by subcutaneous injection of ovalbumin (OVA). We also  
55 have taken advantage of the metabolically stable  $\alpha$ -T-13'-COOH derivative  $\alpha$ -  
56 amplexichromanol ( $\alpha$ -AC). Intraperitoneal treatment with  $\alpha$ -T-13'-COOH reduced OVA-  
57 induced airway hyperreactivity (AHR) as well as peri-bronchial inflammatory cell infiltration.  
58  $\alpha$ -AC was more efficacious than  $\alpha$ -T-13'-COOH, as demonstrated by better control of AHR  
59 and in reducing subepithelial **thickening**. Both compounds exerted their protective function  
60 by reducing pulmonary leukotriene C<sub>4</sub> levels. Beneficial effects of  $\alpha$ -AC were coupled to  
61 inhibition of the sensitization process, as indicated by a reduction of IgE plasma levels, lung  
62 mast cell infiltration and Th2 immune response. Metabololipidomics analysis revealed that  
63  $\alpha$ -AC raises the pulmonary levels of prostanoids, their degradation products, and 12/15-  
64 lipoxygenase metabolites. Following oral administration, the pharmacodynamically different  
65 profile in  $\alpha$ -T-13'-COOH and  $\alpha$ -AC was abrogated as demonstrated by a similar and  
66 improved efficacy in controlling asthma features as well as by metabololipidomics analysis.  
67 In conclusion, this study highlights a role for LCM and of vitamin E derivatives as  
68 pharmacologically active compounds that ameliorate asthmatic features and defines an  
69 important role for endogenous vitamin E metabolites in regulating immune response  
70 underlying the sensitization process.

71 **Keywords:**  $\alpha$ -13'-carboxychromanol,  $\alpha$ -amplexichromanol, vitamin E, bronchial  
72 hyperreactivity, cyclooxygenase, lipoxygenase

73 Chemical compounds studied in this article  $\alpha$ -13'-carboxychromanol (PubChem CID:  
74 53481461); carbachol (PubChem CID: 5831); dimethyl sulfoxide (PubChem CID: 679);  
75 ovalbumin (PubChem CID: 71311993)

## 76 **1. Introduction**

77 Allergic diseases, such as asthma, affect approximately 25% of the world's population [1,2].  
78 Asthma is a chronic airway disease characterized by reversible airway obstruction, airway  
79 hyperreactivity, chronic inflammation, and mucus production. The risk of asthma is  
80 determined in infancy and childhood and the phenotypes are determined by both genetic  
81 and environmental exposure [3]. Epidemiological studies have shown that maternal nutrition  
82 during pregnancy is related to the occurrence of asthma during childhood. A reduced intake  
83 of food containing antioxidant and anti-inflammatory molecules such as vitamin E, may  
84 increase the incidence of inflammatory and allergic diseases [4]. Accordingly, maternal  
85 vitamin E intake [5], as well as the administration of vitamin E in children with moderate  
86 asthma, contribute to the improvement of breathing and prevention of asthma [6]. These  
87 beneficial effects seem to be correlated not only to antioxidant properties of vitamin E, but  
88 also to its immunomodulatory effects [7,8]. Vitamin E consists of eight isoforms,  $\alpha$ -,  $\beta$ -,  $\gamma$ -  
89 and  $\delta$ -tocopherol (T) and the respective tocotrienol (TE) isoforms, which exhibit opposite  
90 and competitive effects on asthma [9]. High levels of  $\gamma$ -T are associated with asthma [10]  
91 and dietary supplementation with  $\gamma$ -T increases lung inflammation in response to allergen  
92 [11]. On the contrary, decreased serum levels of  $\alpha$ -T, the most abundant isoform in serum  
93 and human tissues, have been reported in asthmatic compared to healthy patients [12],  
94 suggesting a protective role of this isoform in asthma disease. Indeed, a diet rich in  $\alpha$ -T  
95 displays protective effects against asthma [10]. Accordingly, diet supplementation with  $\alpha$ -T  
96 decreases lung inflammation in response to dust mite in a mouse model of asthma [9].  
97 It is known that endogenous long-chain metabolites (LCMs) of  $\alpha$ -T are responsible, at least  
98 partially, for the anti-inflammatory effects of vitamin E [13-15]. LCMs potently inhibit 5-  
99 lipoygenase (5-LOX) and in turn suppress the biosynthesis of pro-inflammatory  
100 leukotrienes (LTs) in inflammatory cells [13]. Recently, we have demonstrated that  $\alpha$ -13'

101 carboxychromanol ( $\alpha$ -T-13'-COOH), an endogenous metabolite of  $\alpha$ -T detected at low  
102 nanomolar concentrations in human plasma, accumulates within immune cells at sites of  
103 inflammation. Accordingly, it significantly reduces *in vivo* the inflammatory reaction in  
104 zymosan-induced peritonitis by inhibiting LT production and increasing the systemic  
105 concentration of distinct specialized pro-resolving mediators (SPM) [13].  $\alpha$ -T-13'-COOH also  
106 effectively modulates allergen-induced hyperreactivity [13]. Starting from endogenous LCMs  
107 as lead structures, many semisynthetic compounds have been synthesized [16,17], and  
108  $12\alpha',13'$ -dihydroxylated  $\alpha$ -tocotrienol ( $\alpha$ -amplexichromanol,  $\alpha$ -AC) has been identified as a  
109 highly potent allosteric 5-LOX inhibitor with prominent *in vitro* and *in vivo* anti-inflammatory  
110 activity [16]. Based on this evidence we have here investigated the effects of  $\alpha$ -T-13'-COOH  
111 and  $\alpha$ -AC in an experimental mouse model of asthma.

112

## 113 **2. Material and Methods**

### 114 **2.1. Materials**

115  $\alpha$ -T-13'-COOH and  $\alpha$ -AC (Fig. 1A) have been semisynthesized respectively from natural  $\delta$ -  
116 garcinoic acid and  $\delta$ -amplexichromanol through previously reported strategies [18,19].

117

### 118 **2.2. Animals**

119 Female BALB/c mice ( $18 \pm 2$  g body weight, Charles River, Calco, Italy) were housed in a  
120 controlled environment ( $21 \pm 2$  °C) and provided with standard rodent chow and water. All  
121 animals were allowed to acclimate for four days before experiments and were subjected to  
122 12 h light – 12 h dark schedule. Each mouse was assigned an identification number and  
123 randomized to different groups so that all experiments were carried out in a blinded manner.  
124 Mice were divided into groups not predetermined by a statistical method but by previous  
125 experimental data in this murine sensitization model [20-23]. Experiments were conducted

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

131

### 132 **2.3 Sensitization and drug treatment**

133 Mice were treated with 0.4 ml s.c. of a suspension containing 100 µg of ovalbumin **from**  
134 **chicken egg white** (OVA, **Sigma-Aldrich, Milan, Italy**) adsorbed to 3.3 mg of aluminum  
135 hydroxide gel (**Merck KGaA, Darmstadt, Germany**) or saline (control group) on days 0 and  
136 7 [18-21].  $\alpha$ -T-13'-COOH (OVA +  $\alpha$ -T-13'-COOH group) and  $\alpha$ -AC (OVA +  $\alpha$ -AC group) (10  
137 mg kg<sup>-1</sup>) or vehicle (dimethyl sulfoxide 4%, 0.5 ml; OVA group, **Sigma-Aldrich, Milan, Italy**)  
138 were administered i.p. 30 min or per os (Fig. 1B) 1 h before each OVA administration,  
139 respectively [13,16]. Animals were sacrificed at days 8, 14, or 21 by an overdose of  
140 enflurane, and lungs, bronchi, mediastinal lymph nodes, and blood were collected.

141

### 142 **2.4 Bronchial reactivity**

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



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

154

## 155 **2.5. Lung histology**

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

161

## 162 **2.6. LTC<sub>4</sub> and IL-13 levels**

163 Lungs were isolated and homogenized in **ice-cold** PBS pH 7.4 (**100 mg ml<sup>-1</sup>**, Sigma Aldrich,  
164 Milan, Italy) **using a FastPrep tissue homogenizer (2 cycles of 20 sec, 6m/s; MP**  
165 **Biomedicals, DBA, Segrate, Italy)**. The homogenate was centrifuged (4 °C, 6000  $\times$  g, 10  
166 min). Commercially available enzyme-linked immunosorbent assay (ELISA) were used to  
167 measure the levels of LTC<sub>4</sub> (Cayman Chemical, BertinPharma, Montigny Le Bretonneux,  
168 France) and interleukin (IL)-13 (Invitrogen, Vienna, Austria) according to the manufacturer's  
169 instructions. The levels of LTC<sub>4</sub> and IL-13 are expressed as pg mg<sup>-1</sup> of tissue.

170

## 171 **2.7. Profiling of lipid mediators**

172 Lung tissue was homogenized in ice-cold PBS pH 7.4 (100 mg ml<sup>-1</sup>) using an Omni  
173 tissue homogenizer (Omni, Kennesaw, GA; 1-2 min), which was equipped with a flat

174 **bottom generator probe (5 × 75 mm, fine)**. Non-esterified fatty acids and lipid mediators  
175 (LM) were extracted from plasma or lung homogenates using reversed phase cartridges  
176 (Sep-Pak® Vac 6cc 500 mg/6 ml C18; Waters) as previously described [16,17]. Internal  
177 standards added: d4-LTB<sub>4</sub>, d4-prostaglandin (PG)E<sub>2</sub>, d8-5S-hydroxyeicosatetraenoic acid  
178 (HETE), d5-lipoxin A<sub>4</sub>, d5-resolvin D<sub>2</sub>, (200 nM, each, Cayman Chemicals), and d8-  
179 arachidonic acid (AA, 10 μM, Cayman Chemicals). Lipid mediators were separated on an  
180 Acquity UPLC BEH C18 column (2.1 × 100 mm, Waters) using an Acquity UPLC system  
181 (Waters) and detected using a QTRAP 5500 mass spectrometer (SCIEX), equipped with an  
182 electrospray ionization source [25]. Diagnostic ion fragments were determined by scheduled  
183 multiple reaction monitoring in the negative ion mode for peak identification. Absolute  
184 quantification was achieved by automatic peak integration (Analyst 1.6 software, Sciex,  
185 IntelliQuan default settings) and normalization using 6 internal and 45 external standards  
186 [25].

187

## 188 **2.8. Plasma IgE levels**

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

193

## 194 **2.9. Flow cytometry analysis**

195 As previously reported [23], lungs were isolated, digested with 1 U ml<sup>-1</sup> collagenase (Sigma  
196 Aldrich, Milan, Italy), and passed through 70 μm cell strainers. Red blood cells were lysed  
197 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  $\pm$  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 $\alpha$ -T-13'-COOH and $\alpha$ -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  $\alpha$ -T metabolite  $\alpha$ -T-13'-  
216 COOH or the semi-synthetic compound  $\alpha$ -AC (Fig. 1B) showed a reduced AHR (Fig. 1C),  
217 according to what **has been** previously demonstrated [13,16]. However,  $\alpha$ -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  $\alpha$ -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



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

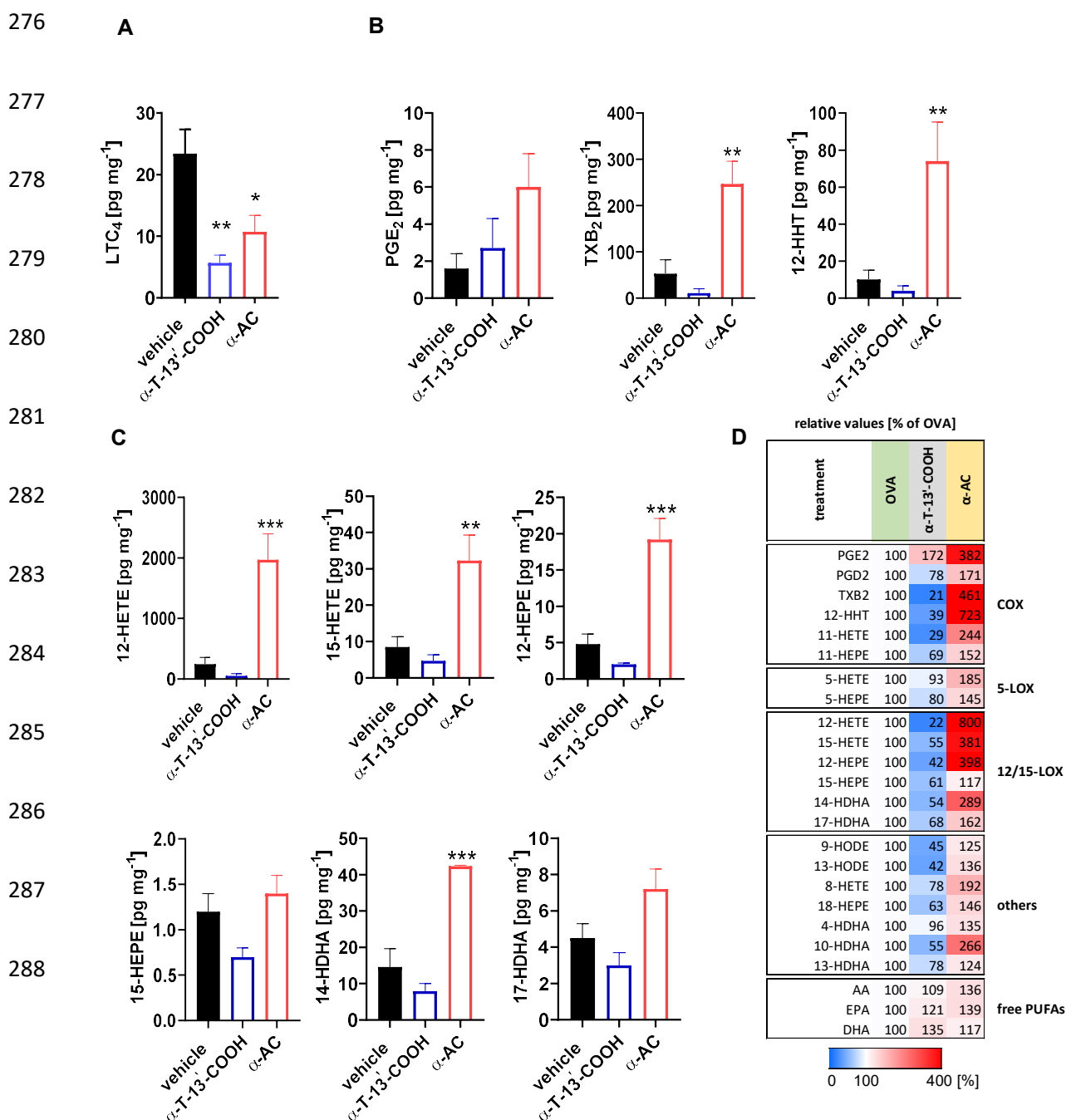
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250

### 251 **3.2. $\alpha$ -T-13'-COOH and $\alpha$ -AC interfere on lipid mediator release**

252 To assess if the beneficial effects of  $\alpha$ -T-13'-COOH and  $\alpha$ -AC are related to an interference  
253 with LM-driven sensitizing mechanisms, LM levels were measured in the lungs and plasma  
254 of OVA-treated mice. Administration of  $\alpha$ -T-13'-COOH or  $\alpha$ -AC during the sensitization  
255 phase caused a significant reduction of pulmonary levels of 5-LOX-derived LMs, such as  
256 LTC<sub>4</sub> by 76% and 54%, respectively (Fig. 2A). Different effects were observed for  
257 cyclooxygenase (COX)-derived LMs, i.e., PGE<sub>2</sub>, the stable thromboxane (TX)A<sub>2</sub> metabolite  
258 TXB<sub>2</sub>, and the PGH<sub>2</sub> degradation product 12(S)-hydroxyheptadecatrienoic acid (12-HHT)  
259 (Fig. 2B). While pulmonary PGE<sub>2</sub> levels were (not-significantly) increased both by  $\alpha$ -T-13'-  
260 COOH and  $\alpha$ -AC injection, other COX-derived products, i.e., TXB<sub>2</sub> and 12-HHT, were  
261 upregulated by  $\alpha$ -AC, but decreased by  $\alpha$ -T-13'-COOH (Fig. 2B), which might reflect the  
262 superior COX-1-inhibitory activity of the endogenous  $\alpha$ -T-13'-COOH as compared to  $\alpha$ -AC  
263 [13,16]. In addition,  $\alpha$ -AC elevated also the 12/15-LOX-derived product levels that originate  
264 from AA (i.e., 12-HETE and 15-HETE), eicosapentaenoic acid (EPA) (i.e., 12-HEPE and 15-  
265 HEPE) and docosahexaenoic acid (DHA) (i.e., 17-HDHA and 14-HDHA) in respect to  
266 vehicle-treated animals (Fig 2C). This finding is of particular interest because these  
267 hydroxylated fatty acids represent precursors for the biosynthesis of SPMs that orchestrate

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



289 **Figure 2. Effects of  $\alpha$ -T-13'-COOH and  $\alpha$ -AC on lipid mediator levels in sensitized**  
290 **mice.** Female mice were pretreated i.p. with  $\alpha$ -T-13'-COOH and  $\alpha$ -AC (10 mg kg<sup>-1</sup>) or vehicle  
291 (DMSO 2%, 0.5 ml) 30 min before each OVA challenge. Animals were sacrificed at day 14  
292 to evaluate in the lung homogenate A) LTC<sub>4</sub> levels analyzed by ELISA and B) COX products  
293 and C) 12/15-LOX-derived products formed from AA (12-HETE and 15-HETE), EPA (12-  
294 HEPE and 15-HEPE) and DHA (17-HDHA, 14-HDHA) analyzed by UPLC-MS/MS. D) The  
295 heatmap shows percentage changes in lipid mediator levels versus vehicle-treated OVA-  
296 sensitized mice. Values represent mean + S.E.M.; n = 6 mice for each group. Data were  
297 analyzed by one-way ANOVA plus Bonferroni; statistical significance is reported as follows:  
298 \*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001 vs vehicle.

299

### 300 **3.3. Effect of $\alpha$ -T-13'-COOH and $\alpha$ -AC on OVA sensitization**

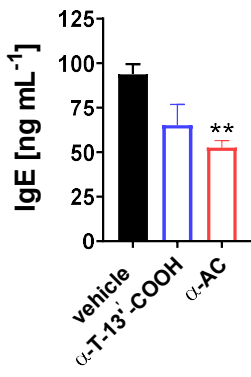
301 Allergen sensitization is sustained by a time-dependent increase in plasma IgE levels, which  
302 correlates with temporal mast cell recruitment and activation in the lung [22]. IgE levels were  
303 decreased in sensitized animals receiving  $\alpha$ -AC (i.p.) in comparison to vehicle-treated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

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

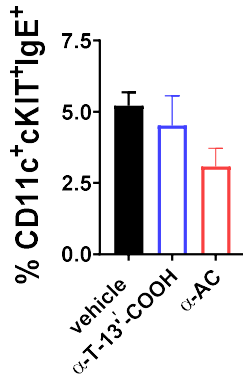
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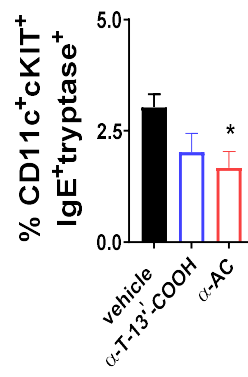
**A**



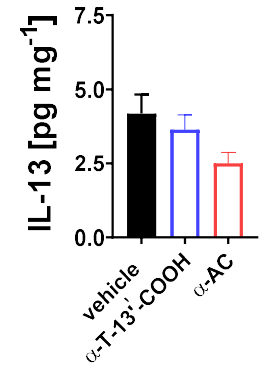
**B**



**D**



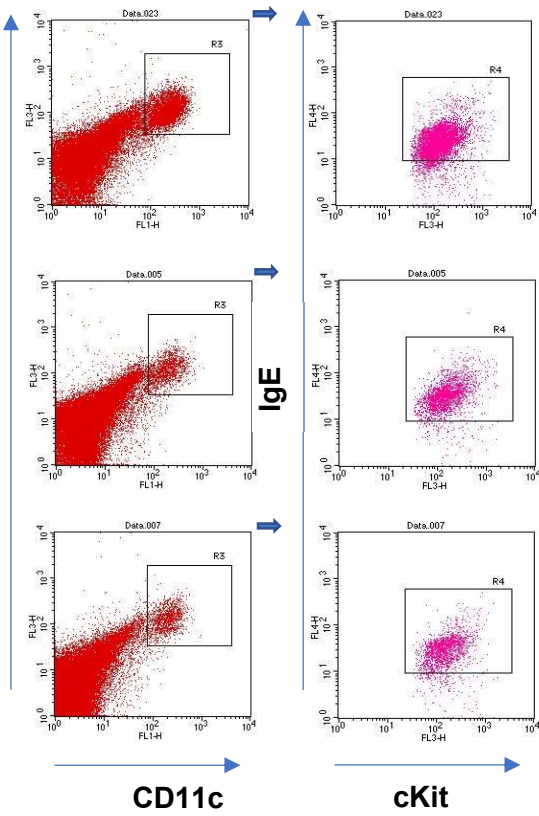
**F**



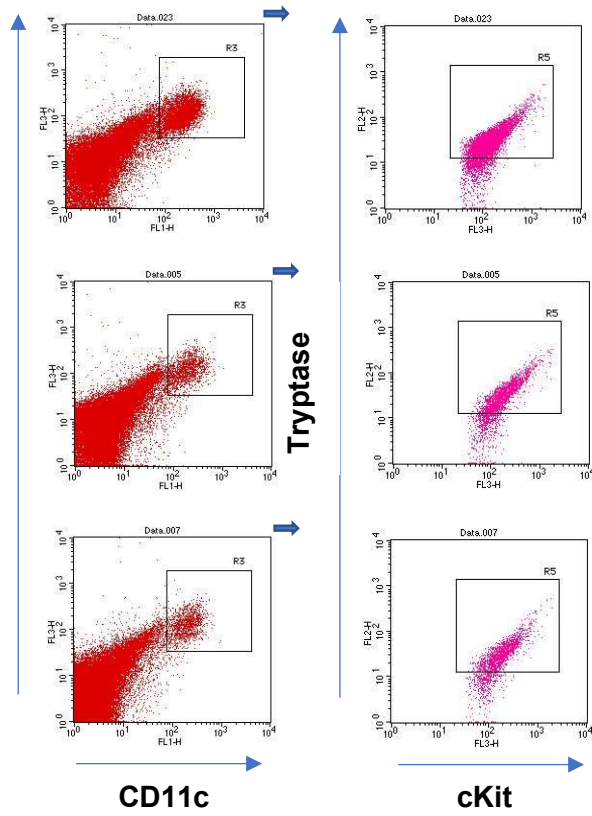
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**C**



**E**



**Vehicle**

**α-T-13'-COOH**

**α-AC**

328

329

330

331



332 **Figure 3. Effects of  $\alpha$ -T-13'-COOH and  $\alpha$ -AC on IgE levels and mast cell infiltration and**  
333 **activation in sensitized mice.** Female mice were pretreated i.p. with  $\alpha$ -T-13'-COOH and  
334  $\alpha$ -AC (10 mg kg<sup>-1</sup>) or vehicle (DMSO 2%, 0.5 ml) 30 min before each OVA challenge.  
335 Animals were sacrificed at day 8 or 14 days to evaluate A) plasma IgE levels, pulmonary  
336 percentage (day 8) of B) CD11c<sup>+</sup>IgE<sup>+</sup>c-KIT<sup>+</sup> and D) CD11c<sup>+</sup>IgE<sup>+</sup>c-KIT<sup>+</sup> tryptase cells  
337 and F) pulmonary levels of IL-13. C and E are representative dot plots for B and D.  
338 Values represent mean + S.E.M.; n = 6 mice for each group. Data were analyzed by one-  
339 way ANOVA plus Bonferroni; statistical significance is reported as follows: \*P < 0.05, \*\*P <  
340 0.01 vs vehicle.

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### 343 **3.4. Effect of $\alpha$ -T-13'-COOH and $\alpha$ -AC on the Th2 response**

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

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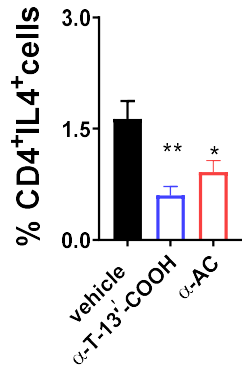
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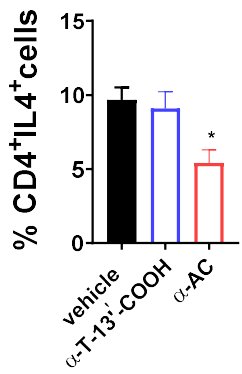
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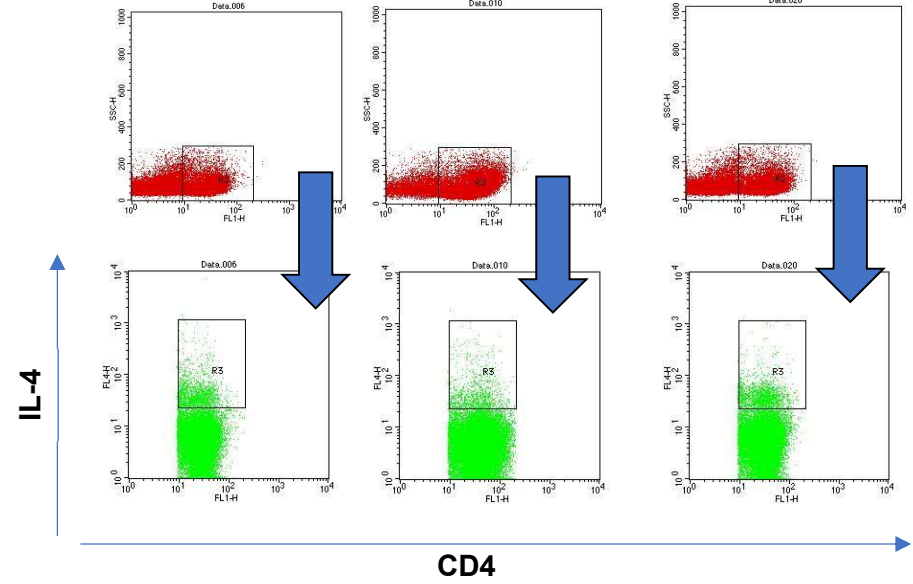
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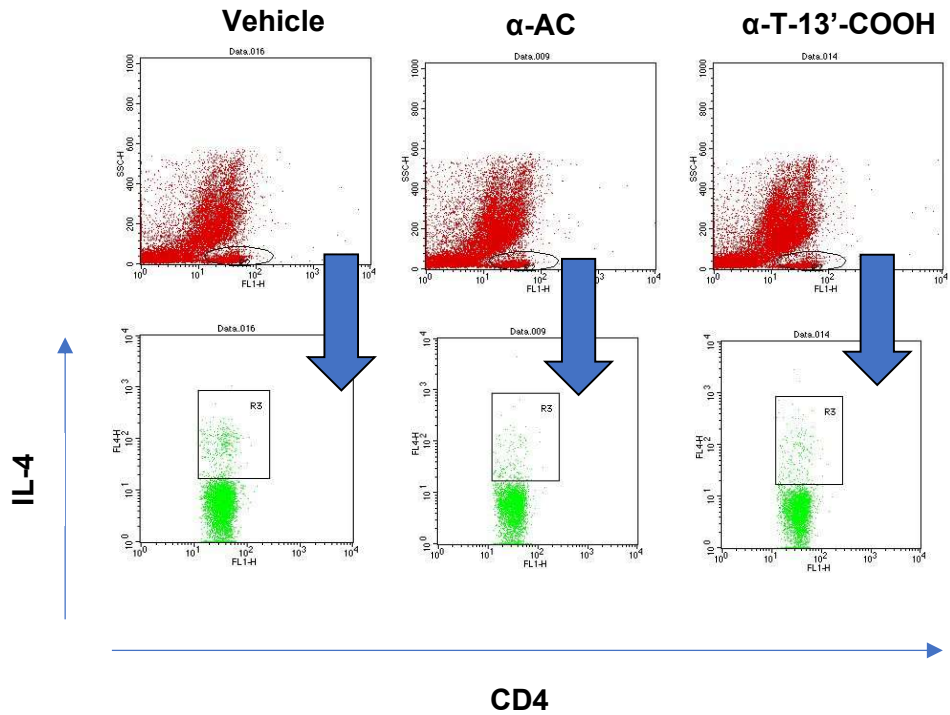
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B



D



375 **Figure 4. Immunomodulatory effects of  $\alpha$ -T-13'-COOH and  $\alpha$ -AC in sensitized mice.**

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

382

383

384 **3.5. Effect of oral administration of  $\alpha$ -T-13'-COOH and  $\alpha$ -AC in sensitized mice**

385 The effects of  $\alpha$ -T-13'-COOH and  $\alpha$ -AC on allergic airway inflammation were also evaluated  
386 following oral administration. Both compounds abrogated AHR in sensitized mice (Fig. 5A)  
387 as demonstrated by the overlapping concentration-response curves (carbachol/contraction)  
388 obtained in control and sensitized mice upon treatment. This effect was associated with  
389 increased pulmonary levels of COX- (Fig. 5B,**D**) and 12/15-LOX-derived metabolites (Fig.  
390 5C,**D**). The accumulation of LMs seems to be driven by an increase in the pulmonary levels  
391 of free PUFAs (Fig. 5D). Systemic PUFA and LM levels followed the same trend (Fig. S2).  
392 The protective effects of  $\alpha$ -T-13'-COOH and  $\alpha$ -AC were associated with an impaired Th2-  
393 dependent immune activation, as indicated by the drop of IgE in plasma (Fig. 6A) and the  
394 inhibition of CD4<sup>+</sup>IL4<sup>+</sup> cell infiltration in the lung (Fig. 6B,**C**) relative to vehicle-treated  
395 sensitized mice.

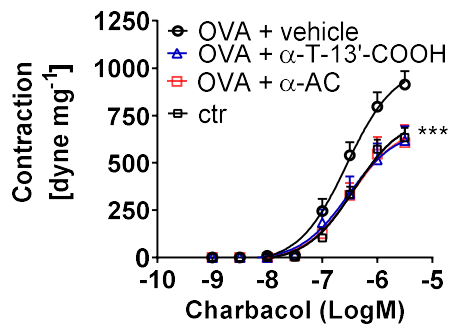
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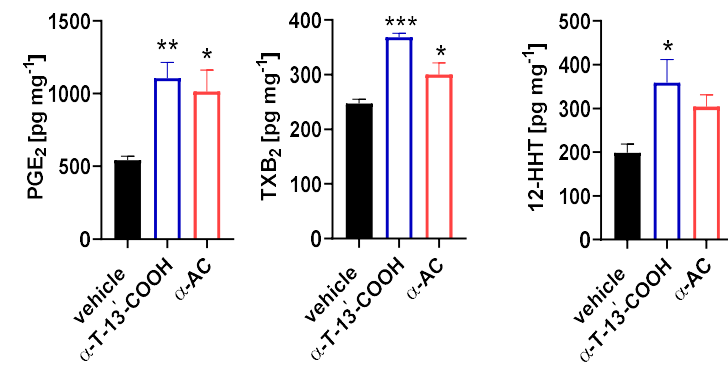
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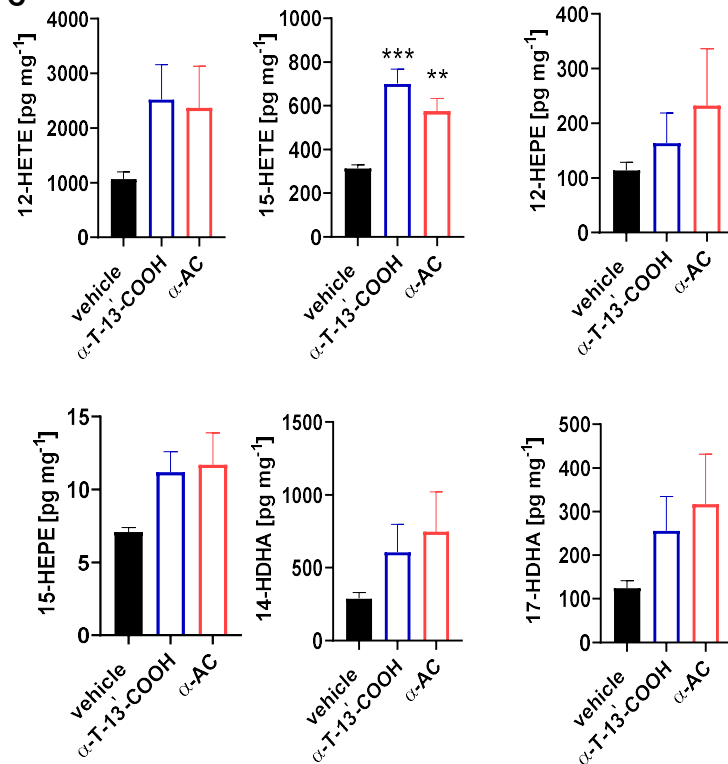
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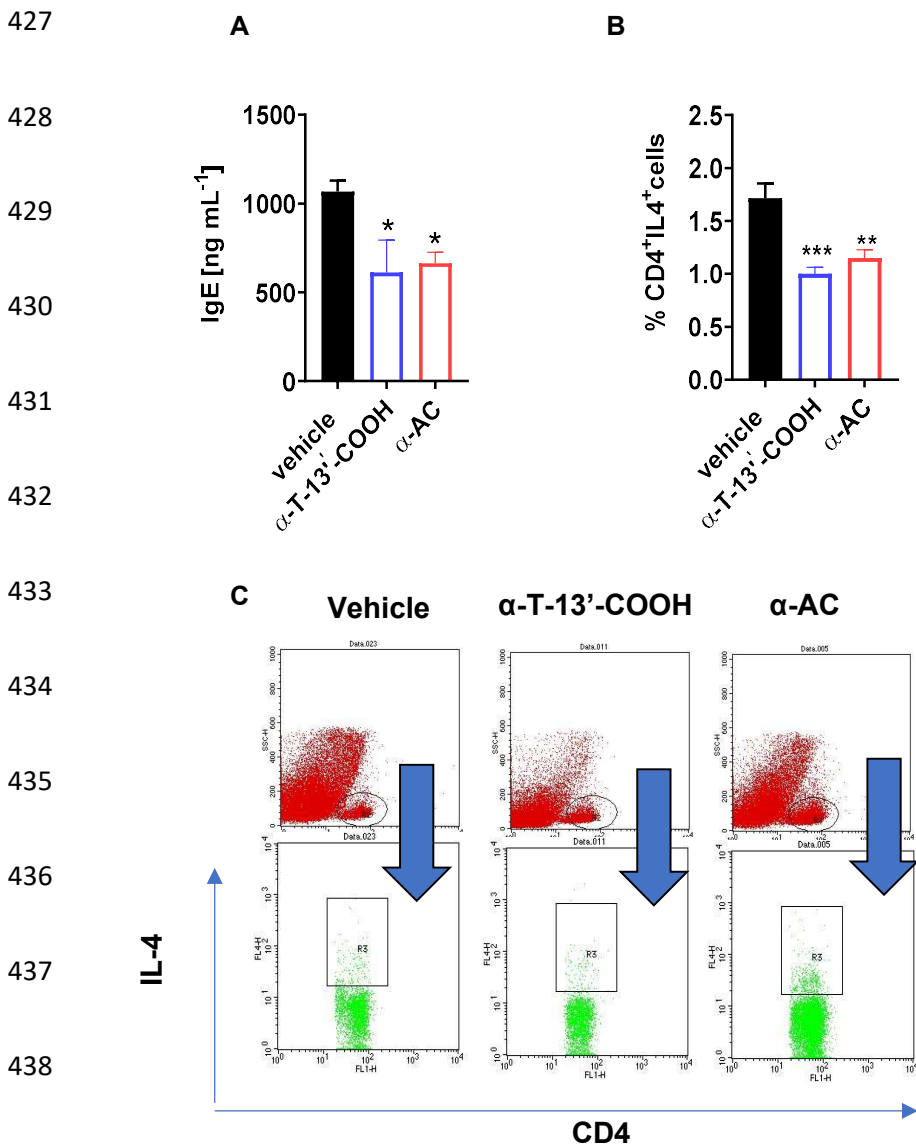
D

| treatment    | relative values [% of OVA] |              |      |            |
|--------------|----------------------------|--------------|------|------------|
|              | OVA                        | α-T-13'-COOH | α-AC |            |
| PGE2         | 100                        | 203          | 186  | COX        |
| PGD2         | 100                        | 272          | 237  |            |
| PGF2α        | 100                        | 183          | 161  |            |
| TXB2         | 100                        | 149          | 121  |            |
| 12-HHT       | 100                        | 181          | 153  |            |
| 11-HETE      | 100                        | 193          | 176  |            |
| 11-HEPE      | 100                        | 133          | 130  |            |
| LTB4         | 100                        | 205          | 266  | 5-LOX      |
| LTB4 isomers | 100                        | 214          | 222  |            |
| 5-HETE       | 100                        | 225          | 216  |            |
| 5-HEPE       | 100                        | 156          | 169  |            |
| 5,15-diHETE  | 100                        | 180          | 181  |            |
| 5S,6R-diHETE | 100                        | 243          | 351  |            |
| 12-HETE      | 100                        | 236          | 222  | 12/15-LOX  |
| 15-HETE      | 100                        | 224          | 183  |            |
| 12-HEPE      | 100                        | 143          | 203  |            |
| 15-HEPE      | 100                        | 158          | 165  |            |
| 14-HDHA      | 100                        | 210          | 259  |            |
| 17-HDHA      | 100                        | 233          | 215  |            |
| MaR2         | 100                        | 198          | 320  |            |
| 9-HODE       | 100                        | 192          | 157  | others     |
| 13-HODE      | 100                        | 185          | 154  |            |
| 8-HETE       | 100                        | 273          | 258  |            |
| 18-HEPE      | 100                        | 133          | 130  |            |
| 4-HDHA       | 100                        | 162          | 193  |            |
| 7-HDHA       | 100                        | 206          | 223  |            |
| 10-HDHA      | 100                        | 205          | 254  |            |
| 13-HDHA      | 100                        | 238          | 242  | free PUFAs |
| PDX          | 100                        | 250          | 294  |            |
| AA           | 100                        | 236          | 213  |            |
| EPA          | 100                        | 149          | 166  |            |
| DHA          | 100                        | 225          | 213  |            |

0 100 400 [%]

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

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



440

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

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#### 452 **4. Discussion**

453 Here, we demonstrate that the endogenous  $\alpha$ -T metabolite  $\alpha$ -T-13'-COOH and the  
454 semisynthetic derivative  $\alpha$ -AC relieve typical asthma hallmarks such as AHR and pulmonary  
455 inflammation, by modulating the immune response underlying **OVA sensitization**. The  
456 beneficial effects of LCMs were sustained by their ability to modulate LM levels such as  
457 LTC<sub>4</sub>, COX- and 12/15-LOX-derived metabolites. Studies in both mice and humans have  
458 shown that these LM play a crucial role in all aspects of Th2 immunity, including the  
459 promotion and regulation of type 2 inflammation. Here we demonstrate that  $\alpha$ -T-13'-COOH  
460 and  $\alpha$ -AC affect Th2 immune response during sensitization by modulating pro-inflammatory  
461 and anti-inflammatory mediator levels, with focusing on 5-LOX, COX and 12/15-LOX derived  
462 metabolites.

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

468 PUFA like AA, EPA and DHA, released by phospholipase A<sub>2</sub> isoenzymes, are subsequently  
469 converted into LMs which orchestrate the inflammatory reaction. The acute phase of  
470 inflammation is dominated by excessive formation of AA-derived LMs, i.e. LTs and PGs,  
471 whereas 12/15-LOX products are produced particularly from DHA and EPA in the resolution  
472 phase [26-28].

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

477 Immunohistochemical analysis confirmed a different action of the two compounds showing  
478 that  $\alpha$ -AC inhibited not only pulmonary cell infiltration but also the subepithelial **thickening**.  
479 The analysis of LMs highlighted a marked difference between  $\alpha$ -AC and  $\alpha$ -T-13'-COOH.  
480 Indeed, only  $\alpha$ -AC treatment of sensitized mice increased the pulmonary levels of COX- and  
481 12/15-LOX-derived metabolites. It is plausible that this effect represents an attempt by the  
482 organism to limit ongoing inflammation and protect airways from subsequent damage.  $\alpha$ -AC  
483 promoted a significant increase in pulmonary levels of PGE<sub>2</sub>, TXB<sub>2</sub> and 12-HHT. Several  
484 preclinical and clinical data confirm an active role of PGE<sub>2</sub> as a protective mediator in asthma  
485 [31-34]. Indeed, in asthmatic patients and animal models a negative correlation between the  
486 sputum levels of PGE<sub>2</sub> and eosinophil numbers and AHR has been reported [29-34]. A  
487 positive correlation between TXB<sub>2</sub> and 12-HHT levels with improvement in lung function has  
488 been found as well [35,36]. LMs produced by 12/15-LOX are negatively associated with  
489 pulmonary inflammation. Thus, 15-LOX-derived SPM biosynthesis is downregulated in  
490 eosinophils isolated from patients with severe asthma and eosinophilic chronic rhinosinusitis  
491 [37]. Moreover, systemic administration of SPMs suppressed pulmonary eosinophilic  
492 inflammation in murine models of asthma [38,39], and 12/15-LOX-deficient mice displayed  
493 augmented IL-33-induced lung inflammation [40].

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

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



503 Th2 response. Indeed,  $\alpha$ -AC significantly inhibited CD4<sup>+</sup>IL4<sup>+</sup> cell infiltration in both  
504 mediastinal lymph nodes and lung. Thus,  $\alpha$ -AC not only inhibited immune cell trafficking  
505 between lymph node and lung, but also reduced **the percentage of** CD4<sup>+</sup>IL4<sup>+</sup> cell in the  
506 mediastinal lymph nodes. The ability of  $\alpha$ -AC to counterbalance the pro-inflammatory  
507 response has been confirmed by a significant attenuation of plasma IgE levels and mast cell  
508 activation in the lung.

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

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

534

535

### 536 **Author contributions**

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

543

### 544 **Conflict of interest**

545 The authors declare no conflicts of interest.

546

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550

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556

557 **Data availability statement**

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

559

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