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

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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; α -T-13'-COOH, α -
42 13'-carboxychromanol; α -AC, α -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 α -13'-carboxychromanol (α -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 α -T-13'-COOH derivative α -
56 amplexichromanol (α -AC). Intraperitoneal treatment with α -T-13'-COOH reduced OVA-
57 induced airway hyperreactivity (AHR) as well as peri-bronchial inflammatory cell infiltration.
58 α -AC was more efficacious than α -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 C₄ levels. Beneficial effects of α -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 α -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 α -T-13'-COOH and α -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:** α -13'-carboxychromanol, α -amplexichromanol, vitamin E, bronchial
72 hyperreactivity, cyclooxygenase, lipoxygenase

73 Chemical compounds studied in this article α -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, α -, β -, γ -
89 and δ -tocopherol (T) and the respective tocotrienol (TE) isoforms, which exhibit opposite
90 and competitive effects on asthma [9]. High levels of γ -T are associated with asthma [10]
91 and dietary supplementation with γ -T increases lung inflammation in response to allergen
92 [11]. On the contrary, decreased serum levels of α -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 α -T
95 displays protective effects against asthma [10]. Accordingly, diet supplementation with α -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 α -T are responsible, at least
98 partially, for the anti-inflammatory effects of vitamin E [13-15]. LCMs potently inhibit 5-
99 lipoxygenase (5-LOX) and in turn suppress the biosynthesis of pro-inflammatory
100 leukotrienes (LTs) in inflammatory cells [13]. Recently, we have demonstrated that α -13'

101 carboxychromanol (α -T-13'-COOH), an endogenous metabolite of α -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]. α -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 α -tocotrienol (α -amplexichromanol, α -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 α -T-13'-COOH
111 and α -AC in an experimental mouse model of asthma.

112

113 **2. Material and Methods**

114 **2.1. Materials**

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

117

118 **2.2. Animals**

119 Female BALB/c mice (18 ± 2 g body weight, Charles River, Calco, Italy) were housed in a
120 controlled environment (21 ± 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]. α -T-13'-COOH (OVA + α -T-13'-COOH group) and α -AC (OVA + α -AC group) (10
137 mg kg⁻¹) 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% O₂ and 5% CO₂) 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 μm 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. LTC₄ and IL-13 levels**

163 Lungs were isolated and homogenized in **ice-cold** PBS pH 7.4 (**100 mg ml⁻¹**, 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 LTC₄ (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 LTC₄ and IL-13 are expressed as pg mg⁻¹ 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⁻¹) 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-LTB₄, d4-prostaglandin (PG)E₂, d8-5S-hydroxyeicosatetraenoic acid
178 (HETE), d5-lipoxin A₄, d5-resolvin D₂, (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⁻¹ 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 α -T-13'-COOH and α -AC on asthma features**

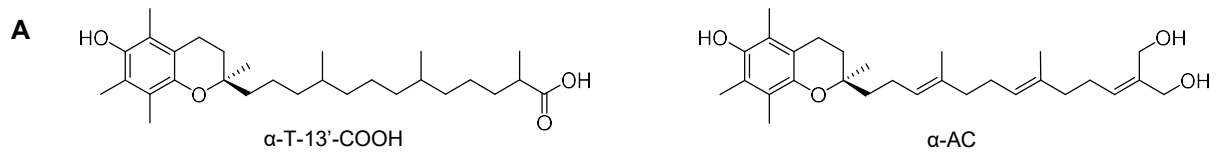
213 OVA sensitization induced airway hyperreactivity (AHR) as evidenced by a significant
214 increase of bronchial reactivity to carbachol compared to the control group (Figure 1C).
215 Allergen-sensitized BALB/c mice receiving (i.p.) the endogenous α -T metabolite α -T-13'-
216 COOH or the semi-synthetic 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

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

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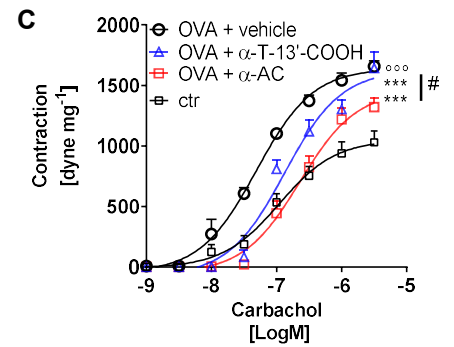
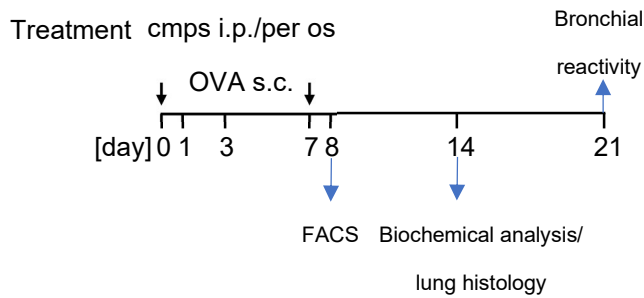
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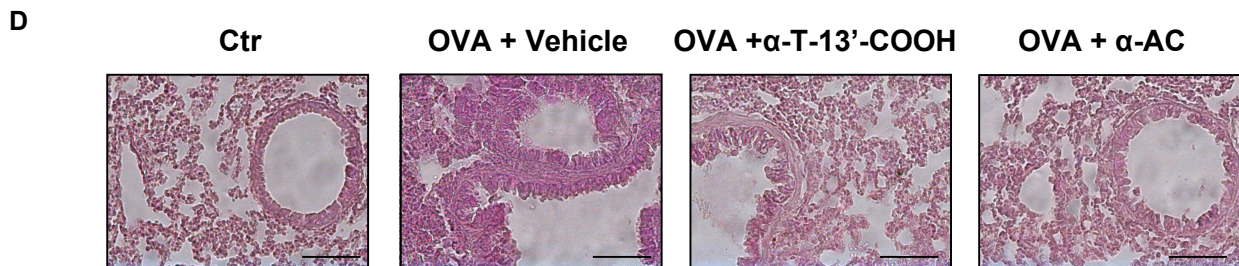
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239 **Figure 1. Effects of α -T-13'-COOH and α -AC on bronchial hyperreactivity and**
240 **pulmonary inflammation in sensitized mice.** A) Chemical structure of α -T-13'-COOH and

241 α -AC. B) Scheme of sensitization and treatment. Female mice were pretreated i.p. with α -
242 T-13'-COOH and α -AC (10 mg kg⁻¹) or vehicle (DMSO 2%, 0.5 mL) 30 min before each

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 μ 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 + α -AC vs OVA + α -T-13'-COOH.

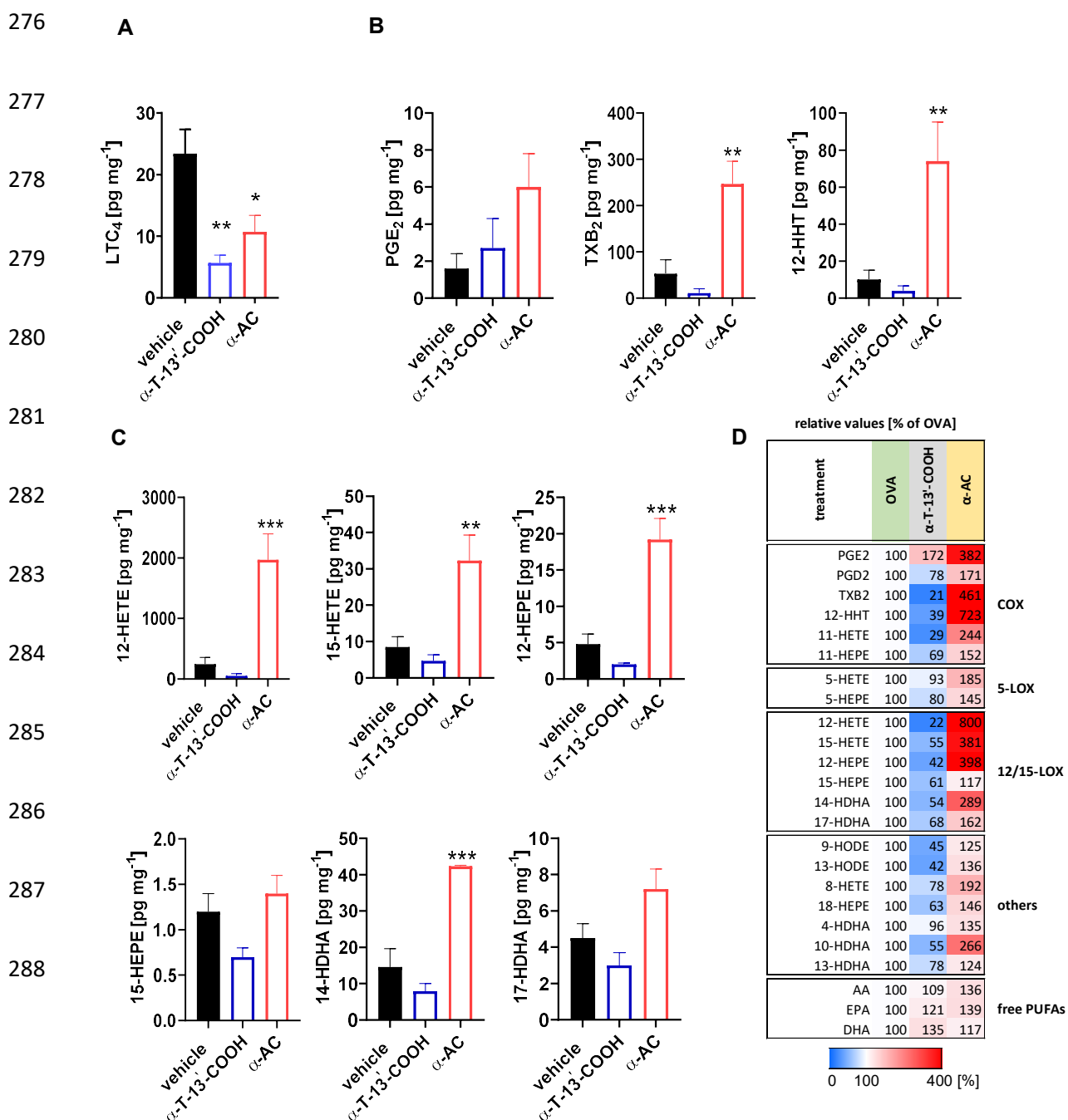
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251 **3.2. α -T-13'-COOH and α -AC interfere on lipid mediator release**

252 To assess if the beneficial effects of α -T-13'-COOH and α -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 α -T-13'-COOH or α -AC during the sensitization
255 phase caused a significant reduction of pulmonary levels of 5-LOX-derived LMs, such as
256 LTC₄ by 76% and 54%, respectively (Fig. 2A). Different effects were observed for
257 cyclooxygenase (COX)-derived LMs, i.e., PGE₂, the stable thromboxane (TX)A₂ metabolite
258 TXB₂, and the PGH₂ degradation product 12(S)-hydroxyheptadecatrienoic acid (12-HHT)
259 (Fig. 2B). While pulmonary PGE₂ levels were (not-significantly) increased both by α -T-13'-
260 COOH and α -AC injection, other COX-derived products, i.e., TXB₂ and 12-HHT, were
261 upregulated by α -AC, but decreased by α -T-13'-COOH (Fig. 2B), which might reflect the
262 superior COX-1-inhibitory activity of the endogenous α -T-13'-COOH as compared to α -AC
263 [13,16]. In addition, α -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 α -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 α -T-13'-COOH and α -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 α -T-13'-COOH and α -AC on lipid mediator levels in sensitized**
290 **mice.** Female mice were pretreated i.p. with α -T-13'-COOH and α -AC (10 mg kg⁻¹) 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) LTC₄ 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.

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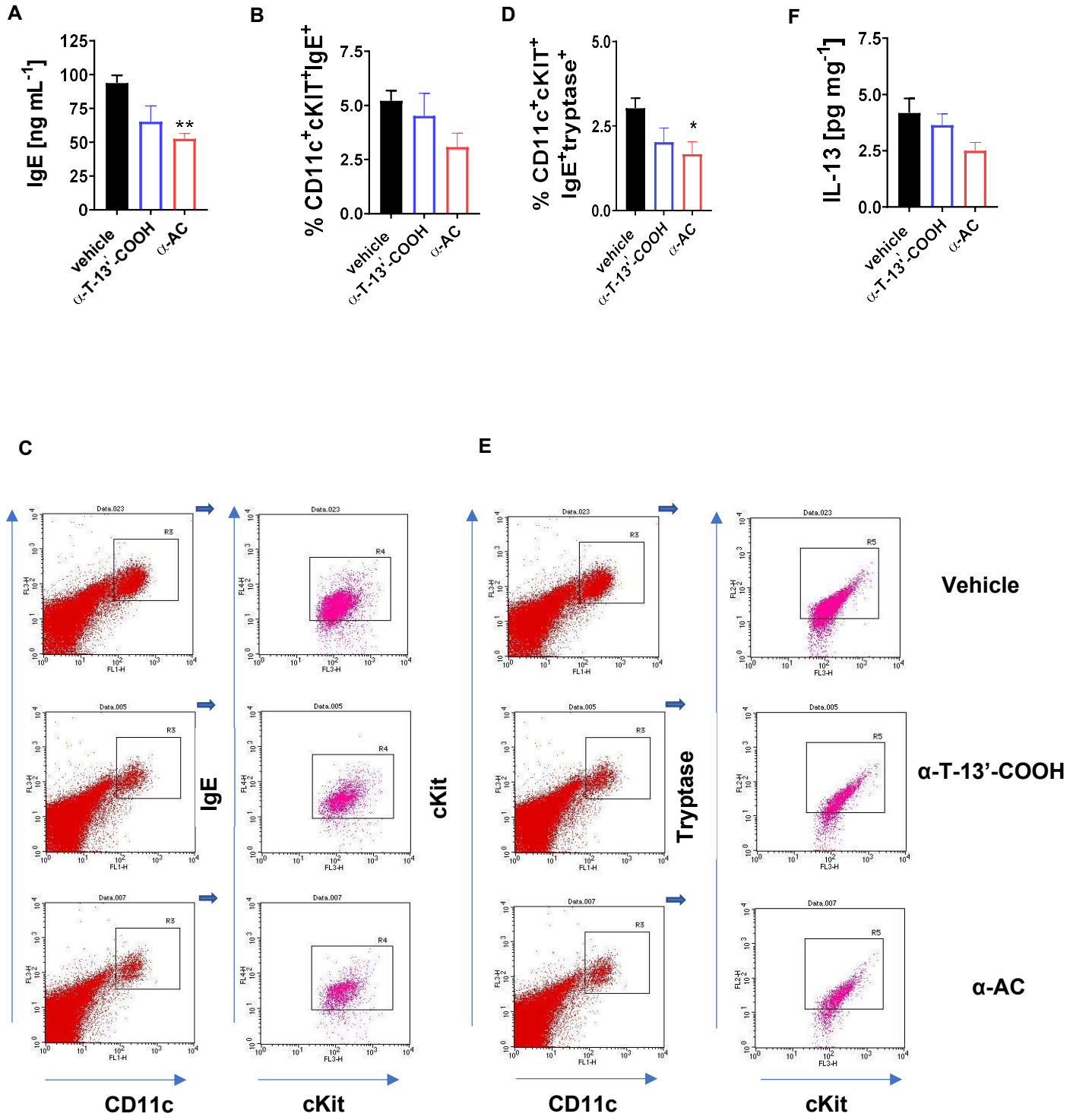
300 **3.3. Effect of α -T-13'-COOH and α -AC on OVA sensitization**

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 α -AC (i.p.) in comparison to vehicle-treated mice,
304 whereas α -T-13'-COOH only showed a partial and not significant effect (Fig. 3A).

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

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332 **Figure 3. Effects of α -T-13'-COOH and α -AC on IgE levels and mast cell infiltration and**
333 **activation in sensitized mice.** Female mice were pretreated i.p. with α -T-13'-COOH and
334 α -AC (10 mg kg⁻¹) 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⁺IgE⁺c-KIT⁺ and D) CD11c⁺IgE⁺c-KIT⁺ 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.

341

342

343 **3.4. Effect of α -T-13'-COOH and α -AC on the Th2 response**

344 To evaluate the effect of α -T-13'-COOH and α -AC on the allergen-induced immune
345 response, CD4⁺IL4⁺ 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 α -T-13'-COOH or α -AC significantly reduced the percentage of CD4⁺IL4⁺ cells
348 in the lymph nodes in comparison to vehicle-sensitized mice. The reduction of CD4⁺IL4⁺
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 α -AC (Fig. 4C and D).

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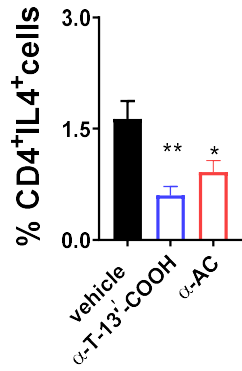
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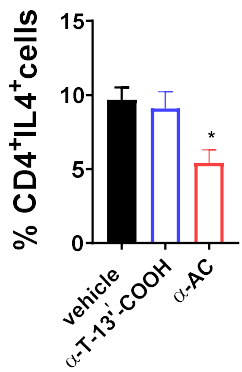
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C

Lungs



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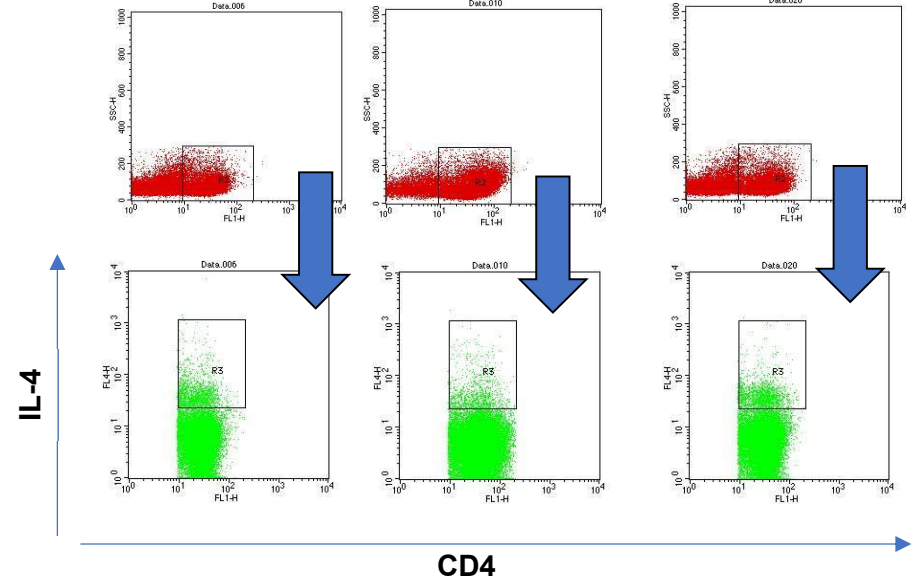
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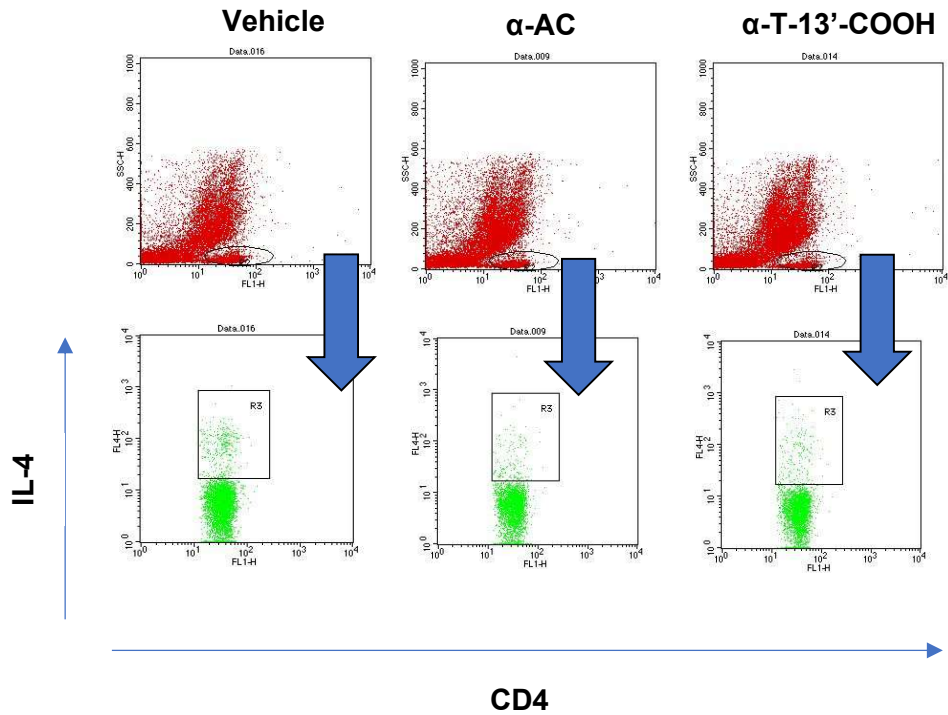
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B



D



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

376 Female mice were pretreated i.p. with α -T-13'-COOH and α -AC (10 mg kg⁻¹) 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.

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

385 The effects of α -T-13'-COOH and α -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 α -T-13'-COOH and α -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⁺IL4⁺ cell infiltration in the lung (Fig. 6B,**C**) relative to vehicle-treated
395 sensitized mice.

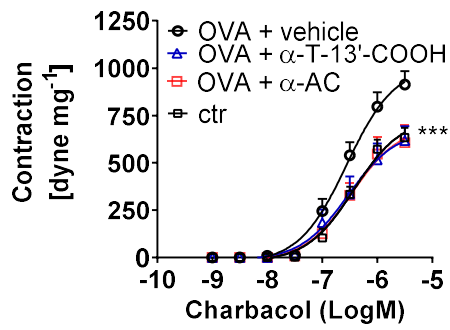
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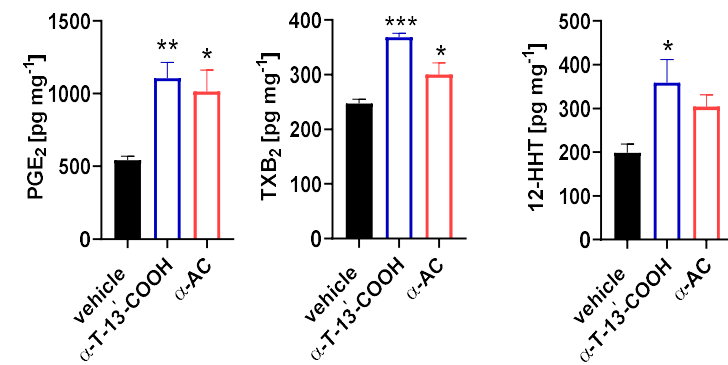
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A



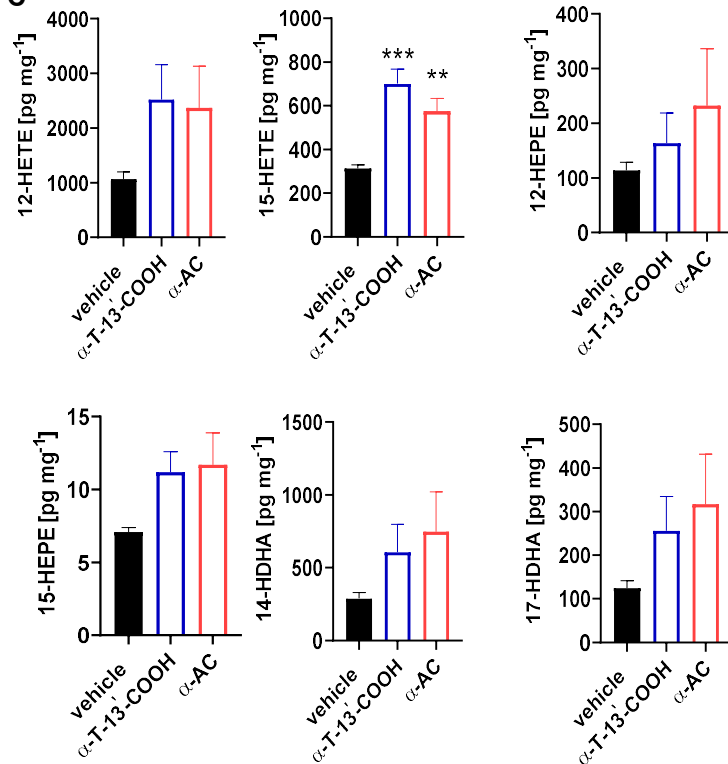
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B



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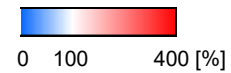
C



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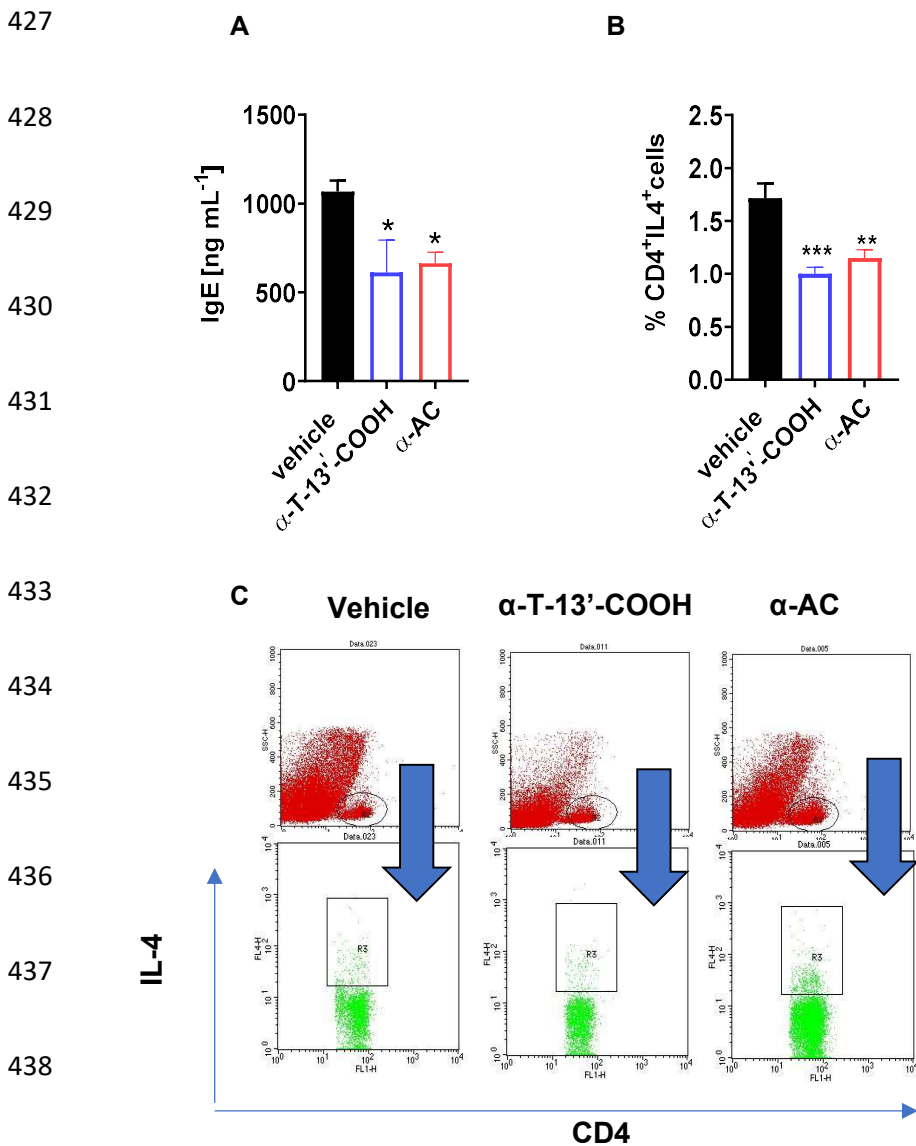
D

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	5-LOX
LTB4	100	205	266	
LTB4 isomers	100	214	222	
5-HETE	100	225	216	
5-HEPE	100	156	169	
5,15-diHETE	100	180	181	12/15-LOX
5S,6R-diHETE	100	243	351	
12-HETE	100	236	222	
15-HETE	100	224	183	
12-HEPE	100	143	203	
15-HEPE	100	158	165	others
14-HDHA	100	210	259	
17-HDHA	100	233	215	
MaR2	100	198	320	
9-HODE	100	192	157	
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	
PDX	100	250	294	free PUFAs
AA	100	236	213	
EPA	100	149	166	
DHA	100	225	213	



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 α -AC (10 mg kg⁻¹) 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.



441 **Figure 6. Effects of α -T-13'-COOH and α -AC on IgE plasma levels and Th2 response**
442 **in sensitized mice.** Female mice were pretreated i.p. with α -T-13'-COOH and α -AC (10 mg
443 kg⁻¹) 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 α -T metabolite α -T-13'-COOH and the
454 semisynthetic derivative α -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 LTC₄, 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 α -T-13'-COOH
460 and α -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 A₂ 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 α -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 α -AC also reduced the maximal carbachol-induced contractility.

477 Immunohistochemical analysis confirmed a different action of the two compounds showing
478 that α -AC inhibited not only pulmonary cell infiltration but also the subepithelial **thickening**.
479 The analysis of LMs highlighted a marked difference between α -AC and α -T-13'-COOH.
480 Indeed, only α -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. α -AC
483 promoted a significant increase in pulmonary levels of PGE₂, TXB₂ and 12-HHT. Several
484 preclinical and clinical data confirm an active role of PGE₂ 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 PGE₂ and eosinophil numbers and AHR has been reported [29-34]. A
487 positive correlation between TXB₂ 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 α -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 α -AC as compared to the endogenous
498 metabolite α -T-13'-COOH in inhibiting bronchial hyperreactivity and pulmonary metaplasia
499 in sensitized mice.

500 To confirm our hypothesis on an active role for α -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 α -AC when compared to α -T-13'-COOH in repressing the

503 Th2 response. Indeed, α -AC significantly inhibited CD4⁺IL4⁺ cell infiltration in both
504 mediastinal lymph nodes and lung. Thus, α -AC not only inhibited immune cell trafficking
505 between lymph node and lung, but also reduced **the percentage of** CD4⁺IL4⁺ cell in the
506 mediastinal lymph nodes. The ability of α -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 α -T metabolism [11]. Plasma α -T levels are regulated by α -T transfer protein
515 (α -TTP), a transporter highly expressed in the liver, where it selects α -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 α -TTP in regulating pulmonary levels of α -
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 α -T-13'-COOH and α -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, α -T-13'-COOH and α -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 α -T-13'-COOH as bioactive LCM to relieve asthma features
530 and demonstrated the efficacy of the LCM-inspired semisynthetic derivative α -AC.
531 Favouring the production of precursors for pro-resolving LM likely helps to sustain the effects
532 of α -T-13'-COOH and α -AC and may represent a novel therapeutic strategy targeting the
533 resolution phase of inflammation in asthma.

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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 α -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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