

DR. KOICHI FUKUNAGA (Orcid ID : 0000-0001-7460-1990)

Article type : Original Article: Experimental Allergy and Immunology

12-OH-17,18-epoxyeicosatetraenoic acid alleviates

eosinophilic airway inflammation in murine lungs

Short title:

Conversion of EPA to a novel bioactive metabolite

Takao Mochimaru*, Koichi Fukunaga^{*,#}, Jun Miyata*, Masako Matsusaka*, Katsunori Masaki*, Hiroki Kabata*, Soichiro Ueda*, Yusuke Suzuki*, Tomomi Goto^{¶,||}, Daisuke Urabe[¶], Masayuki Inoue[¶], Yosuke Isobe[§], Makoto Arita^{§,†,‡}, and Tomoko Betsuyaku*

* Division of Pulmonary Medicine, Department of Medicine, Keio University School of Medicine, Tokyo, Japan

[§] Laboratory for Metabolomics, RIKEN Center for Integrative Medical Sciences, Kanagawa, Japan

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/all.13297

This article is protected by copyright. All rights reserved.

[¶] Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan

^{||} Pharmaceutical Research Center, Shionogi & Co. Ltd., Osaka, Japan

[†] Graduate School of Medical Life Science, Yokohama City University, Kanagawa, Japan

[‡] Division of Physiological Chemistry and Metabolism, Keio University Faculty of Pharmacy, Tokyo, Japan

Foot note

Corresponding author

Koichi Fukunaga

Correspondence should be addressed to K.F (km-fuku@cpnet.med.keio.ac.jp)

Division of Pulmonary Medicine, Department of Medicine, Keio University, School of Medicine,
Tokyo, Japan

35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

Phone +81-3-3353-1211

Fax +81-3-3353-2502

Abbreviations used in this article: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid;

LC-MS/MS, liquid chromatography–tandem mass spectrometry; OVA, ovalbumin; 17,18-EpETE,

17,18-epoxyeicosatetraenoic acid; 12-OH-17,18-EpETE, 12-hydroxy-17,18-epoxyeicosatetraenoic acid; 18-HEPE, 18-hydroxyeicosapentaenoic acid; 12/15-lipoxygenase, 12/15-LOX.

Grant Support

This work was supported in part by JSPS KAKENHI Grant Numbers 15H05897, 15H05898, 15H04648 (to M.A.), and Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries and Food industry (to M.A.), and a grant from Shionogi & Co., Ltd.

Abstract

Background: Asthma is characterized by airway inflammation and obstruction with eosinophil infiltration into the airway. Arachidonic acid, an omega-6 fatty acid, is metabolized into cysteinyl leukotriene with pro-inflammatory properties for allergic inflammation, whereas the omega-3 fatty acid eicosapentaenoic acid (EPA) and its downstream metabolites are known to have anti-inflammatory effects. In this study, we investigated the mechanism underlying the counter-regulatory roles of EPA in inflamed lungs.

Methods: Male C57BL6 mice were sensitized and challenged by OVA. After EPA treatment, we evaluated the cell count of BALF, mRNA expressions in the lungs by q-PCR, and the amounts of

lipid mediators by Liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based

lipidomics. We investigated the effect of the metabolite of EPA in vivo and vitro study.

Results: EPA treatment reduced accumulation of eosinophils in the airway and decreased mRNA expression of selected inflammatory mediators in the lung. Lipidomics clarified the metabolomic profile in the lungs. Among EPA-derived metabolites, 12-hydroxy-17,18-epoxyeicosatetraenoic acid (12-OH-17,18-EpETE) was identified as one of the major biosynthesized molecules; the production of this molecule was amplified by EPA administration and allergic inflammation. Intravenous administration of 12-OH-17,18-EpETE attenuated airway eosinophilic inflammation through downregulation of C-C chemokine motif 11 (*CCL11*) mRNA expression in the lungs. In vitro, this molecule also inhibited the release of CCL11 from human airway epithelial cells stimulated with interleukin-4.

Conclusion: These results demonstrated that EPA alleviated airway eosinophilic inflammation through its conversion into bioactive metabolites. Additionally, our results suggest that 12-OH-17,18-EpETE is a potential therapeutic target for the management of asthma.

Introduction

Asthma is a common chronic airway inflammatory disease affecting approximately 300 million people worldwide ¹. Although inhaled corticosteroids are an established treatment for asthma and can effectively control asthma symptoms, 5–10% of patients with asthma do not respond to treatment despite optimal medication using high-dose inhaled glucocorticoids plus a bronchodilator and/or oral corticosteroids ². These patients are categorized as having difficult-to-control asthma, a condition that places a great burden on public health. Thus, further studies are needed to develop novel therapeutic approaches or alternative treatments for asthma.

Omega-3 and omega-6 fatty acids are essential fatty acids obtained through the diet. The omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are abundant in fish oil, and epidemiological studies have shown that consumption of a diet rich in omega-3 fatty acids is related to a low prevalence and good control of asthma³⁻⁵. In contrast, excessive intake of omega-6 fatty acids may result in a higher prevalence and poor control of asthma. Arachidonic acid (AA), an omega-6 fatty acid, exhibits potent pro-inflammatory properties partly because it is metabolized into pro-inflammatory lipid mediators (e.g., cysteinyl leukotrienes, prostaglandins, and thromboxanes) via enzymatic reactions with lipoxygenases and cyclooxygenases. Supplementary

intake of omega-3 fatty acids reduces the proportion of omega-6 fatty acids in cellular membranes; therefore, the anti-inflammatory effects of omega-3 fatty acids results from antagonism of the pro-inflammatory effects of omega-6 fatty acids. Alternatively, omega-3 fatty acids have recently been shown to be metabolized into specific mediators (e.g., resolvins, protectins, and maresins) with potent anti-inflammatory properties⁶⁻⁹. For example, intravenous administration of resolvin E1, the first identified anti-inflammatory metabolite of EPA, suppresses airway eosinophilic inflammation in a murine asthma model^{6,9}, and DHA-derived protectin D1 and resolvin D1/D2, promotes the resolution of murine allergic inflammation^{7,8}. Thus, the beneficial effects of omega-3 fatty acids in asthma may at least in part be mediated by these metabolites.

Clarification of the mechanisms underlying the anti-inflammatory effects of omega-3 fatty acids will require analysis of the timing and localization of enzymatic metabolism and resulting biosynthesis of specific lipid mediators. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based lipidomics can simultaneously detect and quantify more than 250 mono- and polyhydroxylated fatty acids¹⁰. Arita et al. have already identified EPA-derived 17,18-epoxy eicosatetraenoic acid (17,18-EpETE) as a new therapeutic target for a murine food allergy model using LC-MS/MS-based lipidomics¹¹. However, EPA metabolism during allergic airway inflammation in the lung has not yet been clarified.

In this study, we investigated the effects of EPA on ovalbumin-induced airway inflammation in mice and identified the metabolic profiles of three fatty acids, i.e., AA, DHA, and EPA, in inflamed murine lungs using LC-MS/MS-based lipidomics. Our findings suggested that 12-OH-17,18-EpETE is an EPA-derived endogenous mediator possessing anti-allergic and anti-inflammatory activities and may therefore serve as a new therapeutic target in patients with allergic airway inflammation and asthma.

Materials and Methods

Mice

Six-week-old male C57BL/6 mice were purchased from Charles River Laboratories (Kanagawa, Japan) and maintained under pathogen-free conditions at Keio University. All protocols for the animal experiments used in this study were approved by the Laboratory Animal Care and Use Committee of Keio University School of Medicine.

Ovalbumin (OVA)-challenge mouse model

The OVA-challenge mouse model was prepared as previously reported¹². Briefly, mice were sensitized by intraperitoneal injection of 15 µg OVA (Sigma, St. Louis, MO, USA) with alum on days 0, 7, and 14 and were then challenged with intranasal administration of OVA (10 µg) in 40 µL

phosphate-buffered saline (PBS) on days 21–24. EPA (1000 mg/kg) was administered intraperitoneally on days 25–27. 12(S)-OH-17,18-EpETE (100 ng/mouse) or 17,18-EpETE (1 µg/mouse) was injected intravenously on days 25–27. Control mice were treated with PBS only, PBS plus EPA (no OVA), or OVA plus PBS (no EPA). Mice were killed by pentobarbital overdose. Bronchoalveolar lavage fluid (BALF) was obtained by flushing the lungs with 0.7 mL PBS via tracheal cannulae. The cell counts in the lavage fluids were determined with a hemocytometer, and differential cell counts were determined in cytopsin preparations stained with Diff-Quick (Symex, Kobe, Japan); more than 200 cells were analyzed for each sample using conventional morphologic criteria. The right lung lobes were homogenized for RNA isolation, and the left lobes were fixed in 4% formaldehyde for histological analysis.

Detection of fatty acids and their metabolites in the lungs

LC-MS/MS-based lipidomics was performed to measure the amounts of lipid mediators as previously reported^{10,11}. Briefly, the lipids were extracted by solid-phase extraction using a Sep-Pak C18 cartridge (Waters) with a deuterium-labeled internal standard (AA-d8, 15-HETE-d8, LTB₄-d4, and PGE₂-d4). We then used a triple quadrupole linear ion trap mass spectrometer (QTRAP5500; AB SCIEX) equipped with a 1.7-µm, 1.0 × 150 mm Acquity UPLC BEH C₁₈ column (Waters). MS/MS

analyses were performed in the negative ion mode, and fatty acid metabolites were identified and quantified by multiple reaction monitoring.

Analysis of lipid profile in the lungs after 17,18-EpETE injection

One hour after the intravenous injection of 17,18-EpETE (1µg or 10µg) or PBS into naïve mice (six-week-old male C57BL/6), lung tissues were collected and analyzed using LC/MS/MS-based lipidomics.

Reagents

EPA was obtained from Mochida Pharmaceutical Co., Ltd. (Japan). Recombinant human interleukin (IL)-4 was purchased from R&D Systems. 17,18-EpETE was purchased from Cayman. 12(S)-OH-17,18-EpETE was synthesized as previously reported^{13 14}.

Cell culture

The human bronchial epithelial cell line BEAS-2B was purchased from American Type Culture Collection (Manassas, VA, USA). BEAS-2B cells were cultured in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin (Invitrogen), and 100 µg/mL streptomycin (Invitrogen) at 37°C in an atmosphere containing 5% CO₂. For stimulation, BEAS-2B cells were seeded (5×10^4 cells/well) in 24-well tissue culture plates

(Thermo Scientific, Japan) and allowed to grow to 80% confluence. In all experiments, the indicated concentration of 12-OH-17,18-EpETE and 10 nM of EPA, 17,18-EpETE and 17,18-DiHETE were added to the culture plates 1 h before stimulation. Four hours after stimulation with IL-4, C-C chemokine motif 11 (*CCL11*) mRNA was analyzed by reverse transcription polymerase chain reaction (RT-PCR), as described below. At 24 h after stimulation with IL-4, CCL11 protein in the supernatants from BEAS-2B cells was quantified using enzyme-linked immunosorbent assays (ELISAs), as described below.

RNA isolation and real-time quantitative PCR

RNA was isolated from lung homogenates with RNeasy mini kits according to the manufacturer's instructions (Qiagen, Tokyo, Japan), and cDNA was generated with SuperScript reverse transcriptase (Invitrogen). The expression levels of *IL-5*, *IL-10*, *IL-13*, *IL-17A*, mucin 5AC, oligomeric mucus/gel-forming (*MUC5AC*), *CCL11*, and *CCL24* were measured by real-time quantitative PCR using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and an ABI7500 real-time PCR system (Applied Biosystems). Data were normalized to the expression levels of *Gapdh*. PCR conditions were as follows: one cycle at 95°C for 20 s followed by 50 cycles at 95°C for 3 s and 60°C for 30 s. Primer sequences are as below; *Gapdh*

(5'-TTGATGGCAACAATCTCCAC-3', 3'-CGTCCCGTAGACAAAATGGT-5'), *IL-5*

(5'-CCCACGGACAGTTTGATTCT-3', 3'-GCAATGAGACGATGAGGCTT-5'), *IL-10*

(5'-TGTCAAATTCATTCATGGCCT-3', 3'-ATCGATTCTCCCCTGTGAA-5'), *IL-13*

(5'-CACACTCCATACCATGCTGC-3', 3'-TGTGTCTCTCCCTCTGACCC-5'), *IL-17A*

(5'-TGAGCTTCCCAGATCACAGA-3', 3'-TCCAGAAGGCCCTCAGACTA-5'), *MUC5AC*

(5'-CCATGCAGAGTCCTCA GAACAA -3', 3'-TTACTGGAAAGGCCCAAGCA-5'), *CCL11*

(5'-TAAAGCAGCAGGAAGTTGGG-3', 3'-TCCACAGCGCTTCTATTCCT-5'), *CCL24*

(5'-TCTTATGGCCCTTCTTGGTG-3', 3'-AATTCCAGAAAACCGAGTGG-5').

ELISA

The amount of eotaxin-1 (CCL11) in cell culture supernatants was measured using a Duoset ELISA kit (R&D Systems) according to the manufacturer's protocols.

Statistical analysis

Data are presented as means $\pm$ standard errors of the means (SEMs). Differences in BALF cells were examined using Student's *t* tests. The results of ELISA and mRNA expression analysis were

evaluated using one-way analysis of variance (ANOVA), followed by Dunnett's test as a post hoc test. The data were analyzed with GraphPad Prism 4.0c software (GraphPad Software, San Diego, CA, USA). Differences with *P* values of less than 0.05 were considered significant.

Results

EPA administration attenuated airway inflammation in a murine model of asthma

To determine whether EPA could regulate allergic airway inflammation, EPA (30 mg/body) or PBS were administered intraperitoneally on protocol days 25–27 after allergen sensitization and challenge with OVA (Fig. 1A). Compared with the PBS-treated group, EPA administration significantly decreased the total number of cells (6.43 ± 0.72 versus $2.18 \pm 0.34 \times 10^5/\text{mL}$) as well as eosinophils (4.35 ± 0.59 versus $1.33 \pm 0.27 \times 10^5/\text{mL}$) and macrophages (2.02 ± 0.17 versus $0.78 \pm 0.09 \times 10^5/\text{mL}$) in the BALF (Fig. 1B-D).

EPA administration also decreased the expression levels of several inflammatory mediators in the lung (Fig. 2). For example, in the lungs of OVA-sensitized mice, the expression levels of *IL-5*, *IL-13*, *IL-17A*, *MUC5AC*, and eotaxin (*CCL11* and *CCL24*) mRNAs were elevated as compared with

those in naïve mice. There were no significant changes in the levels of *IL-13* mRNA. In contrast, EPA treatment significantly reduced the expression of *IL-5*, *IL-17A*, *MUC5AC*, and eotaxin mRNAs.

LC-MS/MS-based lipidomics revealed lipid mediator profile in EPA-treated murine lungs

Next, we performed the lipidomics analysis in lung samples from all groups of mice (Fig. 3A). Differential analysis was performed to determine the ratios of lipid metabolites in the lungs for mice treated with OVA plus EPA versus mice treated with OVA plus PBS (Fig. 3B). The results showed that EPA-derived metabolites were greatly increased in the lungs from mice treated with OVA plus EPA.

Next, we analyzed the EPA metabolome, focusing especially on omega-3 hydroxylation and epoxygenation pathway, since these metabolic pathways are considered to represent the endogenous mechanisms acting as anti-inflammatory cascades of EPA¹⁵. Among those, 12-OH-17,18-EpETE was one of the most abundant metabolites found in the lungs of mice treated with OVA plus EPA (Fig. 3C). Since 12-OH-17,18-EpETE has been previously reported to have an anti-inflammatory effect in mouse acute peritonitis model¹³, we next determined whether

12-OH-17,18-EpETE could affect eosinophilic airway inflammation. 12-OH-17,18-EpETE was intraperitoneally administered on days 25–27 using the OVA model. Similar to EPA, 12-OH-17,18-EpETE administration decreased the number of total cells (4.67 ± 0.55 versus $3.07 \pm 0.42 \times 10^5/\text{mL}$) and the number of eosinophils (3.18 ± 0.41 versus $1.92 \pm 0.30 \times 10^5/\text{mL}$) in the BALF compared with the PBS group (Fig. 4B, C). In contrast, the metabolic precursor of 12-OH-17,18-EpETE, namely 17-18-EpETE, did not alleviate eosinophilic airway inflammation (Fig. 4B, C).

Moreover, in contrast to the results with EPA, the expression levels of *IL-5*, *IL-10*, *IL-17A*, *MUC5AC*, and *CCL24* mRNAs were not decreased by 12-OH-17,18-EpETE in the lungs of OVA-sensitized mice. Notably, in 12-OH-17,18-EpETE-treated lungs, only eotaxin-1 (*CCL11*) mRNA expression was suppressed (Fig. 5).

12-OH-17,18-EpETE inhibited the mRNA expression of CCL11 and the release of CCL11 protein induced by IL-4 in BEAS-2B cells

CCL11 is a major chemotactic factor involved in recruitment of eosinophils, and epithelial cells are the primary cellular source of CCL11. To investigate the effects of 12-OH-17,18-EpETE on *CCL11* mRNA expression and CCL11 protein release from epithelial cells, we added various concentrations of 12-OH-17,18-EpETE to BEAS-2B cells in the presence of IL-4.

12-OH-17,18-EpETE inhibited the IL-4-induced expression of *CCL11* mRNA in a concentration-dependent manner (Fig. 6A). Moreover, 12-OH-17,18-EpETE also significantly inhibited CCL11 protein release from epithelial cells stimulated with IL-4 for 24 h (Fig. 6B). On the other hand, EPA, 17,18-EpETE and other EPA metabolites did not suppress the expression of *CCL11* mRNA under the same conditions (data not shown).

Discussion

Omega-3 fatty acids such as EPA and DHA are known to be effective for alleviating inflammatory diseases, including allergic airway inflammation and asthma. Morin et al. showed that monoacylglyceride form of EPA reduced lung inflammation and airway hyper-responsiveness in OVA-sensitized guinea pigs¹⁶. In this study, we examined the effects of administration of EPA on eosinophilic inflammation in an OVA-sensitized murine model and further evaluated the specific metabolites responsible for the effects of EPA using LC-MS/MS-based lipidomics. Our findings demonstrated that EPA treatment prevented eosinophilic airway inflammation, and EPA-derived metabolite 12-OH-17,18-EpETE potently blocked the recruitment of eosinophils into the airway. It has been already reported that 100 ng/mouse of RvE1, another anti-inflammatory metabolite derived from EPA, suppressed the production of the proinflammatory cytokines such as IL-23, -6 and

IL-17 and Th2 cytokines such as IL-4, -5 and -13 in a murine model of asthma^{6,9}. It is unlikely that

12-OH-17,18-EpETE is the only important EPA-derived metabolite to counteract eosinophil recruitment. However, to our knowledge, this is the first report to identify 12-OH-17,18-EpETE as a biologically active metabolite of EPA in a murine asthma model. Thus, our data provided important insights into the beneficial effects of omega-3 fatty acid supplementation in eosinophilic inflammation in allergic asthma.

Because the metabolic pathways of omega-3 and omega-6 fatty acids share the same enzymes, i.e., lipoxygenases and cyclooxygenases, the anti-inflammatory effects of omega-3 fatty acids are thought to be derived from competition for metabolic enzymes with omega-6 fatty acids, which generate pro-inflammatory mediators. However, in our study, EPA treatment did not decrease the ratio of omega-6 metabolites in the lungs. Thus, these results suggested that an alternative EPA-derived anti-inflammatory mediator may be generated in inflamed lungs after administration of EPA in mice.

Among these EPA metabolites, we focused on omega-3 hydroxylation and epoxygenation pathway, since these metabolic pathways represent endogenous mechanism acting as anti-inflammatory cascades of EPA¹⁵. 18-HEPE prevents pressure overload-induced maladaptive cardiac remodeling¹⁷, and its metabolites, including E-series resolvins, have an anti-inflammatory

effect on many disease models ¹⁸⁻²². Additionally, 17,18-EpETE, a metabolite produced via the

epoxide pathway, has relaxing effects on arterial and smooth muscle *in vitro* ^{23 24} and suppresses food

allergies in the gut ¹¹. 12-OH-17,18-EpETE is also the downstream metabolite of the epoxide

pathway, biosynthesized, presumably, with 12-LOX in the lung ^{13 25}, and exhibits potent

anti-inflammatory effects on zymozan-induced peritonitis ¹³. Notably, in our experimental condition,

12-OH-17,18-EpETE was one of the most abundant EPA-derived metabolites in the lung.

Administration of 100 ng 12-OH-17,18-EpETE per mouse decreased eosinophilic recruitment into the

airway, whereas 17,18-EpETE had no effect, even when administered at 1 µg/mouse. Thus,

12-OH-17,18-EpETE exhibited potent anti-inflammatory activity, whereas its metabolic precursor

17,18-EpETE was essentially devoid of activity in this model. As one reason of ineffectiveness of

17,18-EpETE in our model, 17,18-EpETE is rapidly metabolized to 17,18-DiHETE, not to

12-OH-17,18-EpETE (Supplemental Fig.1). Based on this result, we suppose that 17,18-EpETE is

rapidly inactivated by epoxide hydrolases to its less active diol 17,18-DiHTET, and not readily

converted to its active metabolite 12-OH-17,18-EpETE in murine lung tissue.

CCL11 is a major eosinophil chemoattractant ²⁶, and airway epithelial cells are one of the

major sources of this chemokine ²⁷. In this study, we found that 12-OH-17,18-EpETE blocked the

expression of *CCL11* in the lung, whereas EPA decreased the expression of multiple inflammatory

mediators, including *IL-5*, *IL-17A*, *MUC5AC*, and *CCL24*. These data indicated that

12-OH-17,18-EpETE inhibited the recruitment of eosinophils to the airway through suppression of CCL11 production from the airway epithelial cells in the context of IL-4 stimulation. Notably, in patients with asthma, *CCL11* mRNA expression is elevated in bronchial biopsies²⁸, and CCL11 levels are increased in the sputum after allergen challenge²⁹. Thus, our results suggested that 12-OH-17,18-EpETE may be a potential therapeutic target for the management of CCL11-driven eosinophilic inflammation.

In conclusion, our findings showed that EPA-treated mice exhibited reduced eosinophilic airway inflammation and that EPA was metabolized into several bioactive metabolites in inflamed lungs. Among those, 12-OH-17,18-EpETE was abundantly produced, and effectively reduced CCL11 production and eosinophil recruitment to the airway through inhibition of CCL11 production from bronchial epithelial cells. The actions of 12-OH-17,18-EpETE were not shared by EPA and 17,18-EpETE suggesting a structure activity relationships that points to a unique receptor interaction for this essential fatty acid metabolite. The molecular characterization of this putative receptor will be a focus on further investigation. Taken together, our results indicate that the beneficial effects of EPA supplementation and endogenous production of bioactive metabolite, i.e., 12-OH-17,18-EpETE, could be physiologically and clinically relevant in eosinophilic inflammation in allergic asthma. Thus,

development of stable analogs of 12-OH-17,18-EpETE could lead to novel, promising therapeutic approaches for chronic eosinophilic inflammation, such as asthma.

Acknowledgments

We thank Mie Honda, Miyuki Yamamoto, and Shizuko Kagawa for their skillful technical assistance.

References

1. Fanta, C. H. Asthma. *N. Engl. J. Med.* 2009;360: 1002–1014.
2. Hekking, P. P., R. R. Wener, M. Amelink, A. H. Zwinderman, M. L. Bouvy, and E. H. Bel. The prevalence of severe refractory asthma. *J. Allergy Clin. Immunol.* 2015;135: 896–902.
3. Horrobin, D. F. Low prevalences of coronary heart disease (CHD), psoriasis, asthma and rheumatoid arthritis in Eskimos: are they caused by high dietary intake of eicosapentaenoic acid (EPA), a genetic variation of essential fatty acid (EFA) metabolism or a combination of both? *Med. Hypotheses.* 1987;22: 421–428.

- Accepted Article
4. Li, J., P. Xun, D. Zamora, A. Sood, K. Liu, M. Daviglus, C. Iribarren, et al. Intakes of long-chain omega-3 (n-3) PUFAs and fish in relation to incidence of asthma among American young adults: the CARDIA study. *Am. J. Clin. Nutr.* 2013;97: 173–178.
 5. Barros, R., A. Moreira, J. Fonseca, L. Delgado, M. G. Castel-Branco, T. Haahtela, et al. Dietary intake of alpha-linolenic acid and low ratio of n-6:n-3 PUFA are associated with decreased exhaled NO and improved asthma control. *Br. J. Nutr.* 2011;106: 441–450.
 6. Haworth, O., M. Cernadas, R. Yang, C. N. Serhan, and B. D. Levy. Resolvin E1 regulates interleukin 23, interferon-gamma and lipoxin A4 to promote the resolution of allergic airway inflammation. *Nat Immunol.* 2008;9: 873–879.
 7. Levy, B. D., P. Kohli, K. Gotlinger, O. Haworth, S. Hong, S. Kazani, et al. Protectin D1 is generated in asthma and dampens airway inflammation and hyperresponsiveness. *J. Immunol.* 2007;178: 496–502.
 8. Rogerio, A. P., O. Haworth, R. Croze, S. F. Oh, M. Uddin, T. Carlo, et al. Resolvin D1 and aspirin-triggered resolvin D1 promote resolution of allergic airways responses. *J. Immunol.* 2012;189: 1983–1991.

9. Aoki, H., T. Hisada, T. Ishizuka, M. Utsugi, A. Ono, Y. Koga, et al. Protective effect of resolvin E1 on the development of asthmatic airway inflammation. *Biochem. Biophys. Res. Commun.* 2010;400: 128–133.
10. Arita, M. Mediator lipidomics in acute inflammation and resolution. *J. Biochem.* 2012;152: 313–319.
11. Kunisawa, J., M. Arita, T. Hayasaka, T. Harada, R. Iwamoto, R. Nagasawa, et al. Dietary omega3 fatty acid exerts anti-allergic effect through the conversion to 17,18-epoxyeicosatetraenoic acid in the gut. *Sci. Rep.* 2015;5: 9750.
12. Kabata, H., K. Moro, K. Fukunaga, Y. Suzuki, J. Miyata, K. Masaki, T. Betsuyaku, S. Koyasu, and K. Asano et al. Thymic stromal lymphopoietin induces corticosteroid resistance in natural helper cells during airway inflammation. *Nat. Commun.* 2013;4: 2675.
13. Kubota, T., Arita M, Isobe, Y., Iwamoto, R., Goto, T., Yoshioka, T. et al. Eicosapentaenoic acid is converted via omega-3 epoxidation to the anti-inflammatory metabolite 12-hydroxy-17,18-epoxyeicosatetraenoic acid. *FASEB.* 2014;28(2):586–93.
14. Goto, T., Urabe, D., Masuda, K., Isobe, Y., Arita, M., and Inoue, M. Total synthesis of four stereoisomers of (4Z,7Z,10Z,12E,16Z,18E)-14,20-Dihydroxy-4,7,10,12,16,18-docosahexaenoic acid and their anti-inflammatory activities. *J. Org. Chem.* 2015;80(15):7713–26.

- Accepted Article
15. Isobe, Y., and Arita, M. Identification of novel omega-3 fatty acid-derived bioactive metabolites based on a targeted lipidomics approach. *J. Clin. Biochem. Nutr.* 2014;55(2):79–84.
 16. Morin, C., S. Fortin, A. M. Cantin, and E. Rousseau. MAG-EPA resolves lung inflammation in an allergic model of asthma. *Clin. Exp. Allergy.* 2013;43: 1071–1082.
 17. Endo, J., M. Sano, Y. Isobe, K. Fukuda, J. X. Kang, H. Arai, et al. 18-HEPE, an n-3 fatty acid metabolite released by macrophages, prevents pressure overload-induced maladaptive cardiac remodeling. *J. Exp. Med.* 2014;211: 1673–1687.
 18. Arita, M., F. Bianchini, J. Aliberti, A. Sher, N. Chiang, S. Hong, et al. Stereochemical assignment, antiinflammatory properties, and receptor for the omega-3 lipid mediator resolvin E1. *J. Exp. Med.* 2005;201: 713–722.
 19. Tjonahen, E., S. F. Oh, J. Siegelman, S. Elangovan, K. B. Percarpio, S. Hong, et al. Resolvin E2: identification and anti-inflammatory actions: pivotal role of human 5-lipoxygenase in resolvin E series biosynthesis. *Chem. Biol.* 2006;13: 1193–1202.
 20. Isobe, Y., M. Arita, S. Matsueda, R. Iwamoto, T. Fujihara, H. Nakanishi, et al. Identification and structure determination of novel anti-inflammatory mediator resolvin E3, 17,18-dihydroxyeicosapentaenoic acid. *J. Biol. Chem.* 2012;287: 10525–10534.

21. Seki, H., K. Fukunaga, M. Arita, H. Arai, H. Nakanishi, R. Taguchi, et al. The anti-inflammatory and proresolving mediator resolvin E1 protects mice from bacterial pneumonia and acute lung injury. *J. Immunol.* 2010;184: 836–843.
22. Takamiya, R., K. Fukunaga, M. Arita, J. Miyata, H. Seki, N. Minematsu, et al. Resolvin E1 maintains macrophage function under cigarette smoke-induced oxidative stress. *FEBS Open Bio.* 2012;2: 328–333.
23. Morin, C., M. Sirois, V. Echave, E. Rizcallah, and E. Rousseau. Relaxing effects of 17(18)-EpETE on arterial and airway smooth muscles in human lung. *Am. J. Physiol. Lung Cell Mol. Physiol.* 2009;296: L130–139.
24. Khaddaj-Mallat, R., E. and Rousseau. MAG-EPA and 17,18-EpETE target cytoplasmic signalling pathways to reduce short-term airway hyperresponsiveness. *Pflugers Arch.* 2015;467: 1591–1605.
25. Yoshimoto, T., and Takahashi, Y. Arachidonate 12-lipoxygenases. *Prostaglandins Other Lipid Mediat.* 2002;68-69:245–62.

26. Garcia-Zepeda, E. A., M. E. Rothenberg, R. T. Ownbey, J. Celestin, P. Leder, and A. D. Luster. Human eotaxin is a specific chemoattractant for eosinophil cells and provides a new mechanism to explain tissue eosinophilia. *Nat. Med.* 2: 449–456.
27. Rothenberg, M. E. Eotaxin. An essential mediator of eosinophil trafficking into mucosal tissues. *Am. J. Respir. Cell Mol. Biol.* 1996;21: 291–295.
28. Ying, S., Q. Meng, K. Zeibecoglou, D. S. Robinson, A. Macfarlane, M. Humbert, et al. Eosinophil chemotactic chemokines (eotaxin, eotaxin-2, RANTES, monocyte chemoattractant protein-3 (MCP-3), and MCP-4), and C-C chemokine receptor 3 expression in bronchial biopsies from atopic and nonatopic (Intrinsic) asthmatics. *J. Immunol.* 1999;163: 6321–6329.
29. Zeibecoglou, K., A. J. Macfarlane, S. Ying, Q. Meng, I. Pavord, N. C. Barnes, et al. Increases in eotaxin-positive cells in induced sputum from atopic asthmatic subjects after inhalational allergen challenge. *Allergy.* 1999;54: 730–735.

Figure Legends

Figure 1. EPA administration attenuated airway inflammation in a murine asthma model.

(A) Protocol for the OVA-induced allergic mouse model. EPA (30 mg) or PBS was administered intraperitoneally on days 25–27. (B, C) The number of total BALF cells (B), the number of eosinophils (C) and of neutrophils, lymphocytes and macrophages (D) on protocol day 28. Values are expressed as means and SEMs; $n = 12$ per group. $*p < 0.05$, $***p < 0.001$ compared with the PBS group (Student's t-tests).

Figure 2. EPA decreased the expression levels of inflammatory mediators in the lung during the resolution of allergic inflammation.

The expression levels of mRNAs were analyzed by RT-PCR in OVA-naïve, control nonallergic control mice (white) and OVA-induced allergic mice given PBS (black) or EPA (gray). The results are expressed as the ratio of intensity to the GAPDH signal and calculated as the fold increase compared with that in naïve mice. Values are expressed as means and SEMs; $n = 12$ per group. $**p < 0.01$, $***p < 0.001$ compared with the PBS group (one-way ANOVA).

Figure 3. Analysis of fatty acid metabolites in lungs by LC-MS/MS.

(A) Lung tissue was obtained on protocol day 28 during the resolution phase from OVA-naïve, nonallergic mice given PBS or EPA and OVA-exposed allergic mice given PBS or EPA. (B) The heatmap shows the relative amounts of lipid metabolites in the lungs on protocol day 28. The color corresponds to the log-fold change ($\log_{10}$) of the OVA-challenged and EPA-administered group to the OVA-challenged and PBS-administered group. Dark blue color indicates -1, white color indicates 0, and dark red color indicates 1. Four mice were evaluated for each group. (C) Levels of EPA-derived metabolites in the lung were evaluated by LC-MS/MS. Lung tissues were sampled on protocol day 28 from OVA-naïve, nonallergic mice given PBS (white) or EPA (gray) and OVA-sensitized and -challenged mice given PBS (black) or EPA (hatched). Values are expressed as means and SEMs; $n = 4$ per group.

Figure 4. 12-OH-17,18-EpETE promoted the resolution of airway inflammation. (A)

17,18-EpETE (1 μg), 12-OH-17,18-EpETE (100 ng), or PBS was administered intravenously on days 25–27 during resolution of airway eosinophilic inflammation after cessation of OVA exposure. (B–D) The number of total BALF cells (B), the number of eosinophils (C), and the number of neutrophils, lymphocytes and macrophages (D) in BALF were determined on protocol day 28. BALF was

collected from mice given 12-OH-17,18-EpETE (black) or 17,18-EpETE (gray). Values are expressed as means and SEMs; 12-OH-17,18-EpETE group, n = 14–15, 17,18-EpETE group, n = 4 per group.

* $p < 0.05$, ** $p < 0.01$ compared with the PBS group (Student's t-test).

Figure 5. 12-OH-17,18-EpETE decreased *CCL11* mRNA expression in the lung. Lung tissues were obtained on protocol day 28 during the resolution phase from OVA-naïve, nonallergic mice given PBS (white) and OVA-exposed allergic mice give PBS (black) or 12-OH-17,18-EpETE (gray).

mRNA expression levels were analyzed by RT-PCR. The results are expressed as the ratio of the intensity to GAPDH and calculated as the fold increase compared with naïve mice. Values are expressed as means and SEMs; n = 4 per group. * $p < 0.05$, ** $p < 0.01$ compared with the PBS group (one-way ANOVA).

Figure 6. 12-OH-17,18-EpETE decreased *CCL11* mRNA expression and *CCL11* protein release from BEAS-2B cells stimulated with IL-4.

(A) *CCL11* mRNA expression was analyzed by RT-PCR. The results are expressed as the ratio of the intensity to GAPDH and calculated as the fold increase compared with the control. (B) Cell

supernatants were analyzed for CCL11 protein by ELISA. Values are expressed as means and SEMs;

n = 4 per group. * $p < 0.05$, ** $p < 0.01$ compared with IL-4 alone (one-way ANOVA).

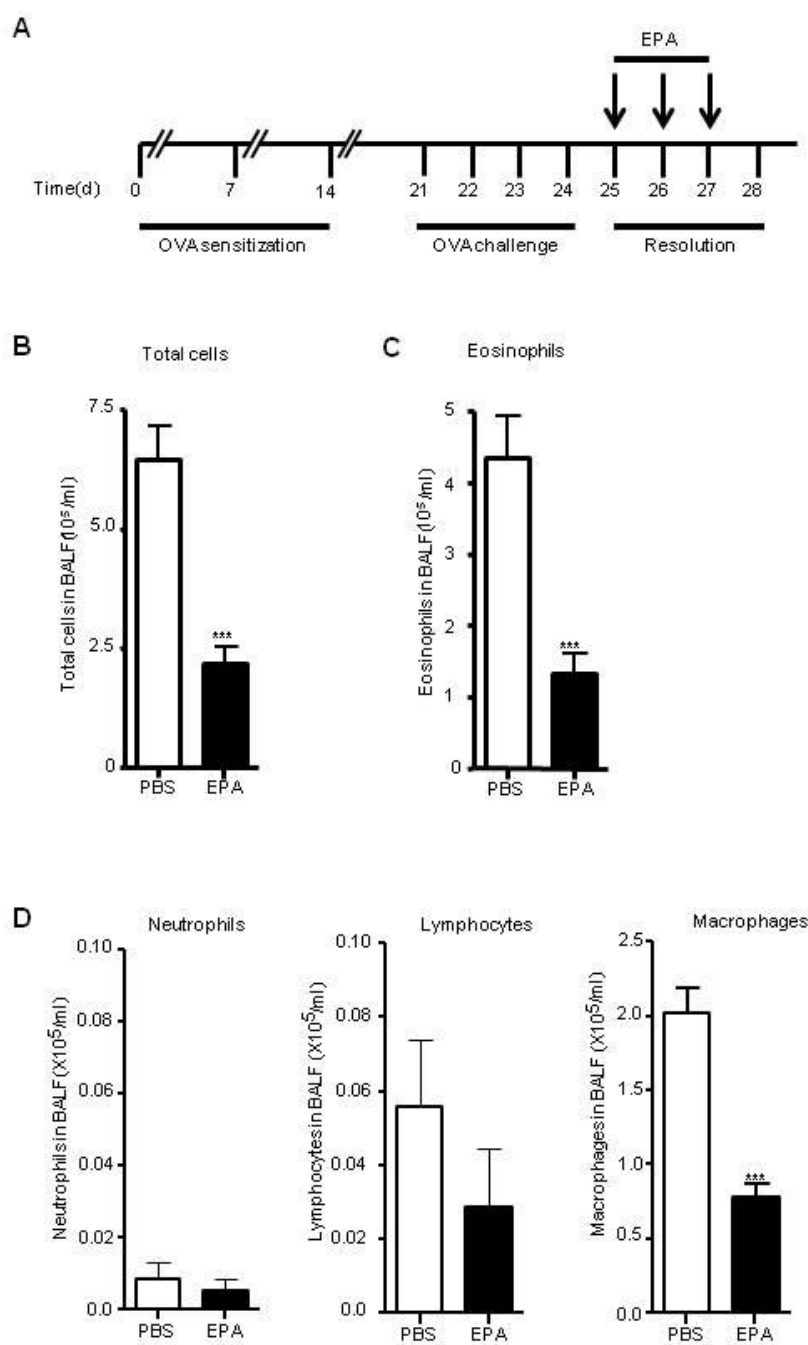
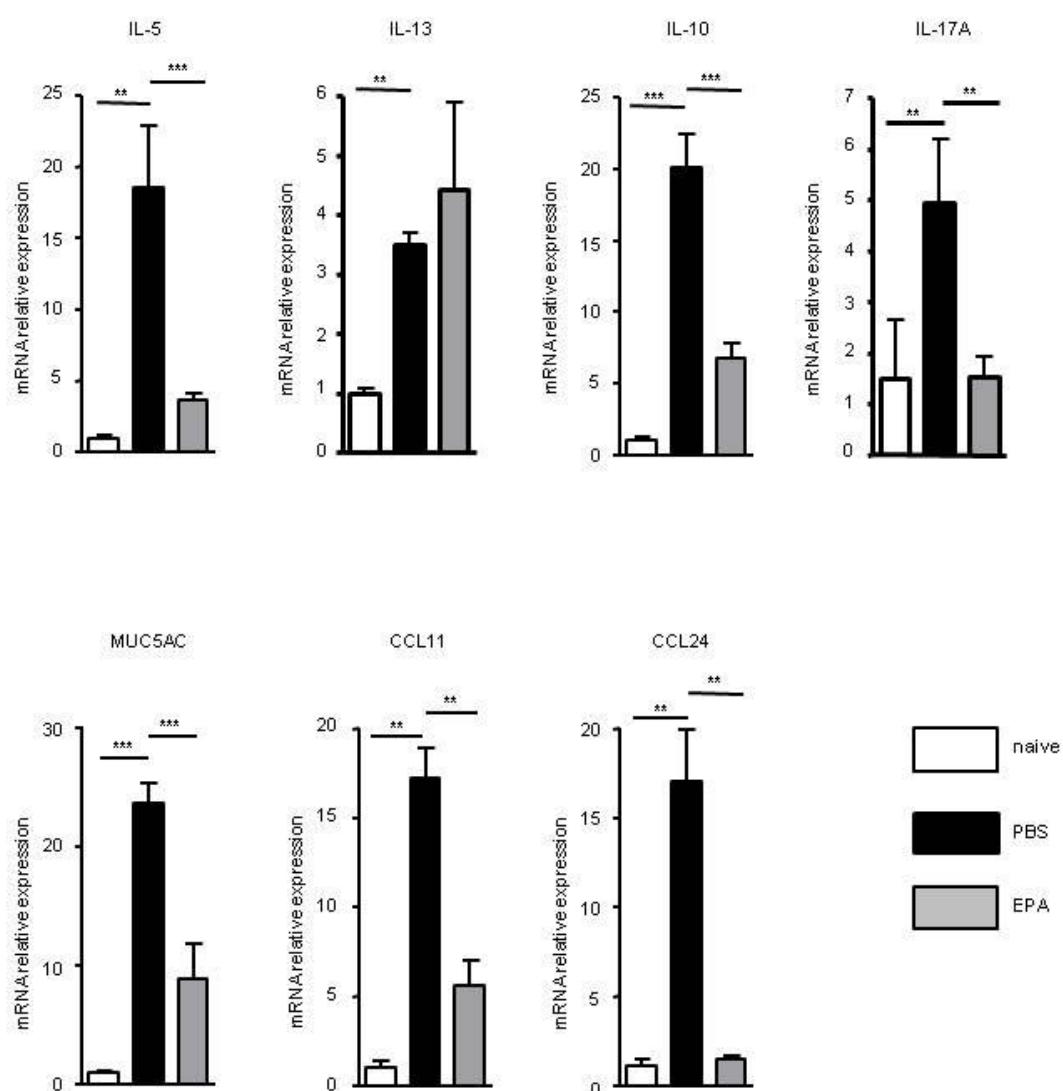
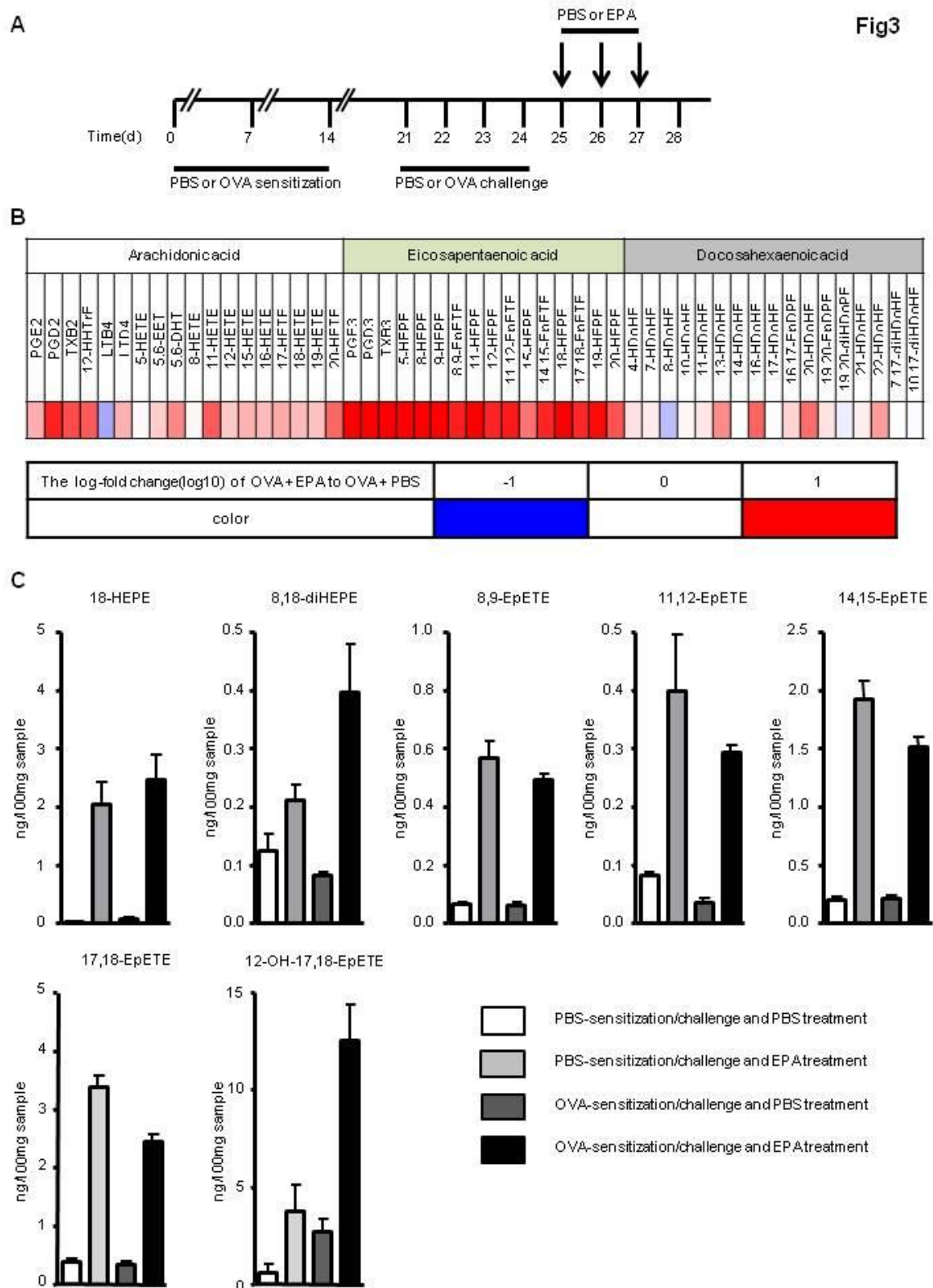


Fig1

Fig2





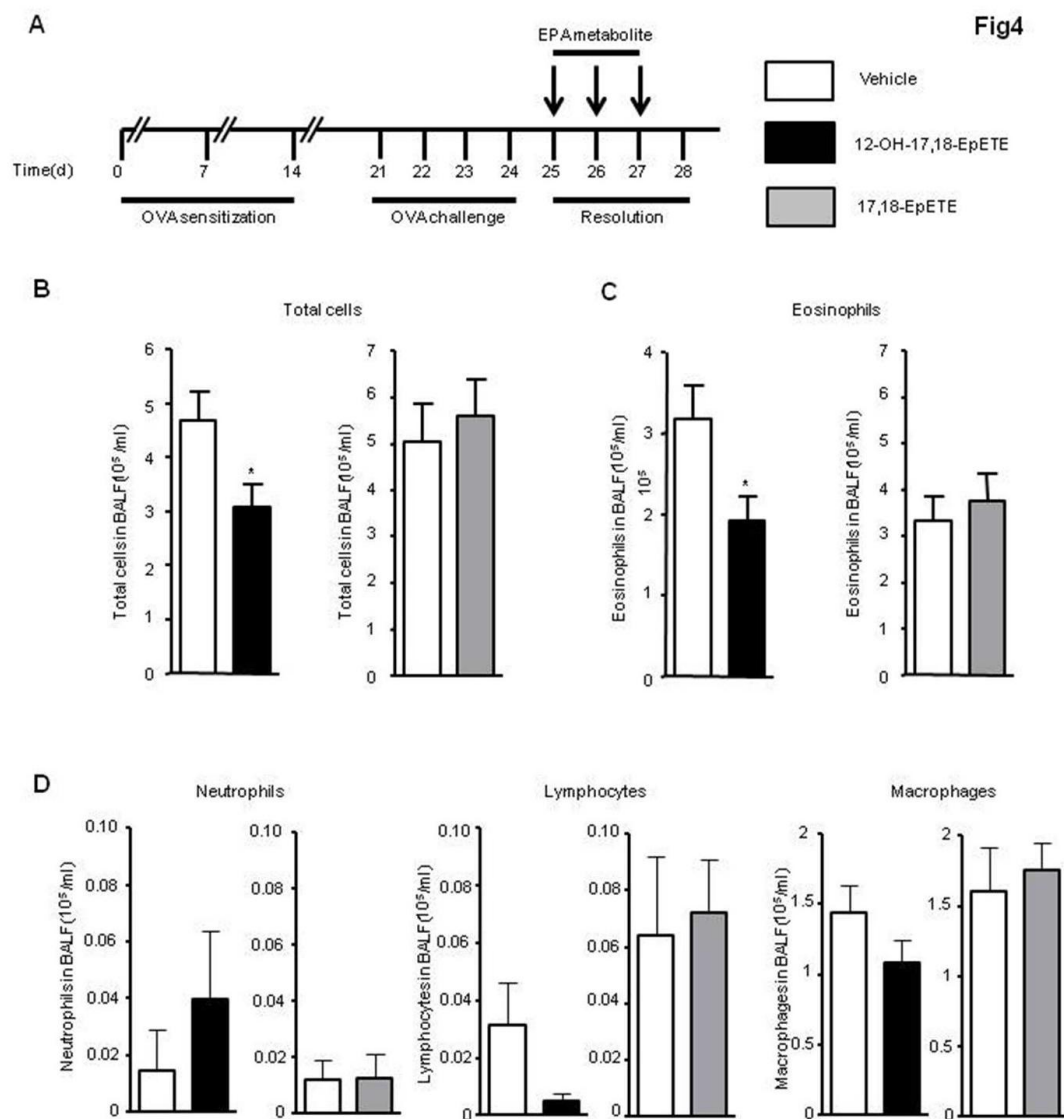


Fig5

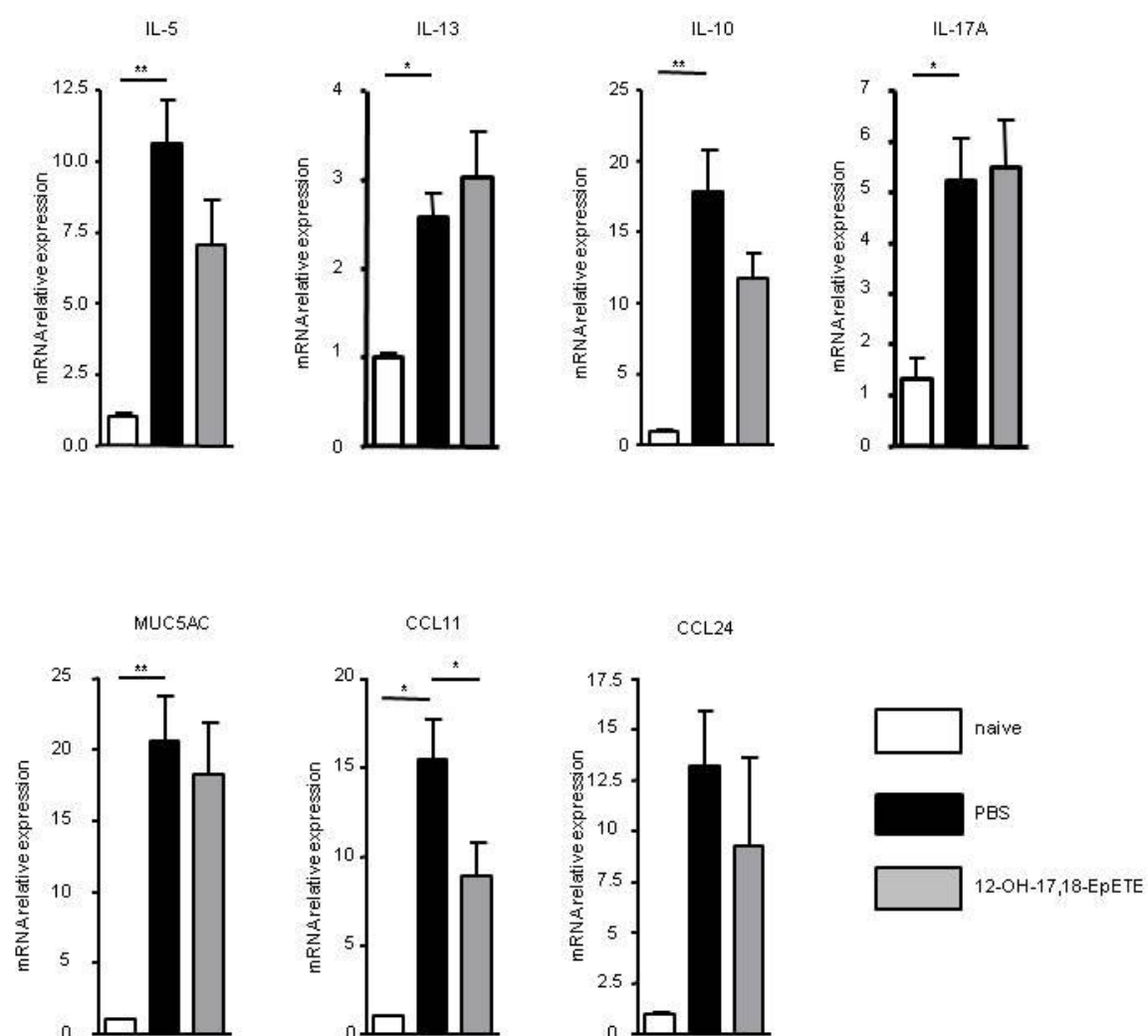
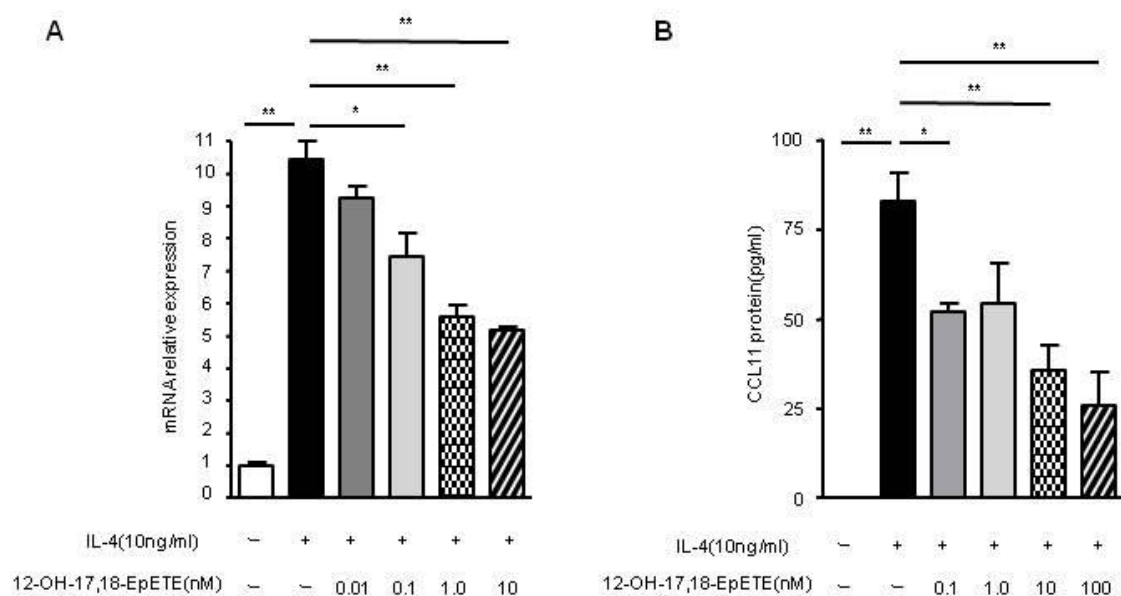
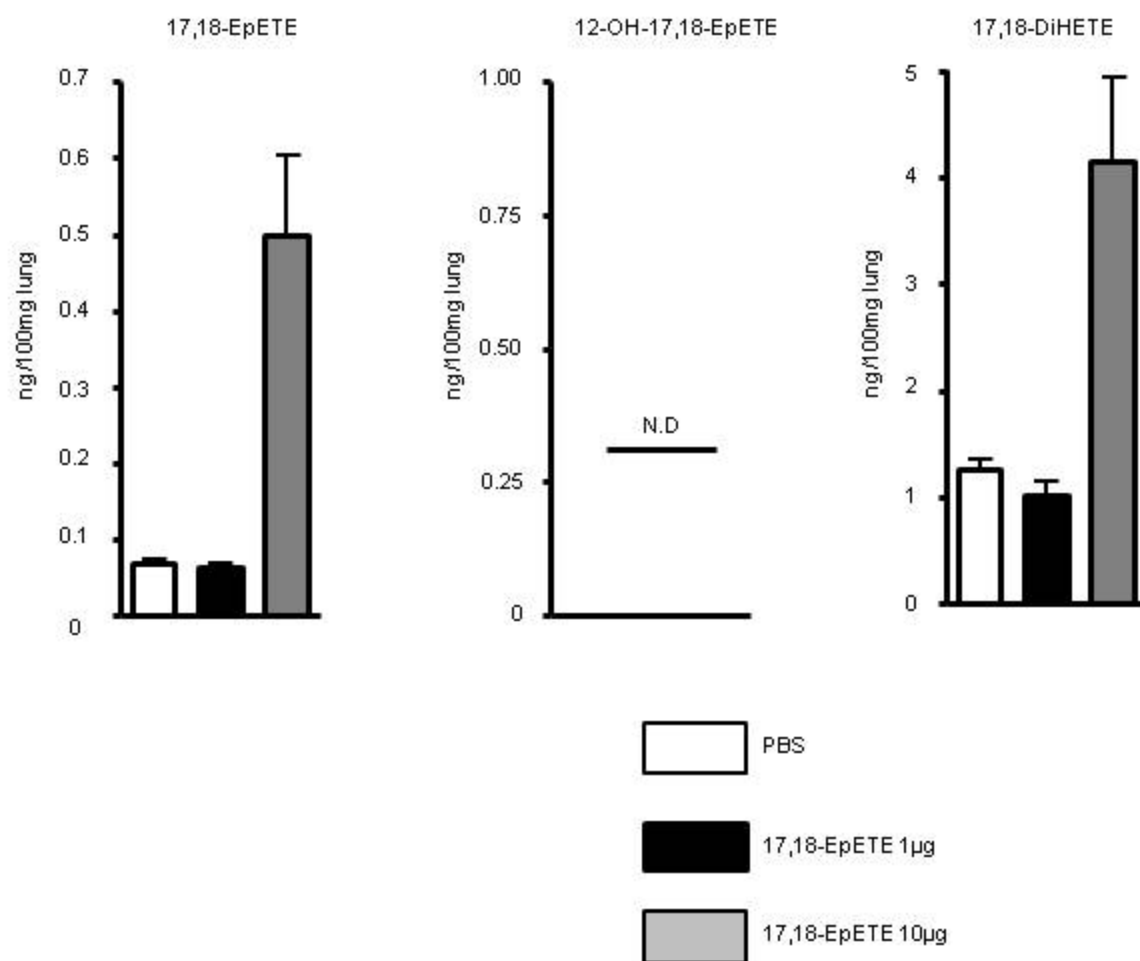


Fig6





Supplementary Figure S1:

We injected 17,18-EpETE (1µg or 10µg) or PBS intravenously and killed the mice 1 h after injection. 12-OH-17,18-EpETE was not detected by LC-MS/MS although the amount of 17,18-DiHETE increased in the 10µg 17,18-EpETE-treated mice. Values are expressed as means and SEMs; n = 4 per group.