

Prominent release of lipoxygenase generated mediators in a murine house dust mite-induced asthma model



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ARTICLE INFO

Keywords:

BALF
HDM
PUFA
Lipid mediators
Airway hyperresponsiveness

ABSTRACT

The profile of activation of lipid mediator (LM) pathways in asthmatic airway inflammation remains unclear. This experimental study quantified metabolite levels of ω 3-, ω 6- and ω 9-derived polyunsaturated fatty acids in bronchoalveolar lavage fluid (BALF) after 4-weeks of repeated house dust mite (HDM) exposure in a murine (C57BL/6) asthma model. The challenge induced airway hyperresponsiveness, pulmonary eosinophil infiltration, but with low and unchanged mast cell numbers. Of the 112 screened LMs, 26 were increased between 2 to > 25-fold in BALF with HDM treatment ($p < 0.05$, false discovery rate = 5%). While cysteinyl-leukotrienes were the most abundant metabolites at baseline, their levels did not increase after HDM treatment, whereas elevation of PGD₂, LTB₄ and multiple 12/15-lipoxygenase products, such as 5,15-DiHETE, 15-HEDE and 15-HEPE were observed. We conclude that this model has identified a global lipoxygenase activation signature, not linked to mast cells, but with aspects that mimic chronic allergic airway inflammation in asthma.

1. Introduction

Asthma is a heterogeneous and complex respiratory disease that comprises a variety of different clinical and molecular phenotypes with wheezing, chest tightness, breathlessness, cough and airway hyperresponsiveness (AHR) as the main clinical symptoms [1]. Lipid mediators (LMs), with pro- and anti-inflammatory properties are important signalling molecules in asthma [2] that are formed following activation of many inflammatory cells, including mast cells [3,4]. An initial hypothesis-generating approach is to delineate their release profile in a murine (C57BL/6) asthma model where airway inflammation is triggered by house dust mite (HDM).

LMs originate from a limited set of enzymes (Fig. 1). Formation of individual LMs can depend on, but are not limited to, specific cell-to-cell interactions where each cell carry different enzymes necessary for their formation. Prostaglandins are generated by the primary action of cyclooxygenase (COX) 1 and 2 and can initiate bronchoconstriction and

relaxation when released in the respiratory tract and also exert vascular effects in the local and systemic circulation. In addition, cysteinyl-leukotrienes (CysLTs) from the lipoxygenase (LOX) pathway are important in asthma, however their role in mice is less clear. They are released by primarily mast cells, but also eosinophils and macrophages. Earlier studies have shown allergen induced release of CysLTs in BALB/c mice whereas it is constantly high in C57BL/6 [5]. Recent research on eicosanoid biology has identified new LMs generated from docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) by LOX enzymes. Some products exhibits properties suggesting a role to promote resolution of inflammation and the name specialized pro-resolving lipid mediators (SPMs) has therefore been introduced [6,7]. Lower levels of arachidonic acid derived lipoxins have been linked to the activity of soluble epoxide hydrolase (sEH) in severe asthmatic patients [8]. However, the contribution of SPMs, or many other LMs, in different asthma phenotypes is not yet completely understood. Here we assess the relative activation of different LM pathways in a murine house dust

Abbreviations: AHR, airway hyperresponsiveness; HDM, house dust mite; SPM, specialized pro-resolving lipid mediators; BALF, bronchoalveolar lavage fluid; LM, lipid mediator; FDR, false discovery rate

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<https://doi.org/10.1016/j.prostaglandins.2018.05.005>

Received 8 February 2018; Received in revised form 6 April 2018; Accepted 9 May 2018
Available online 12 May 2018

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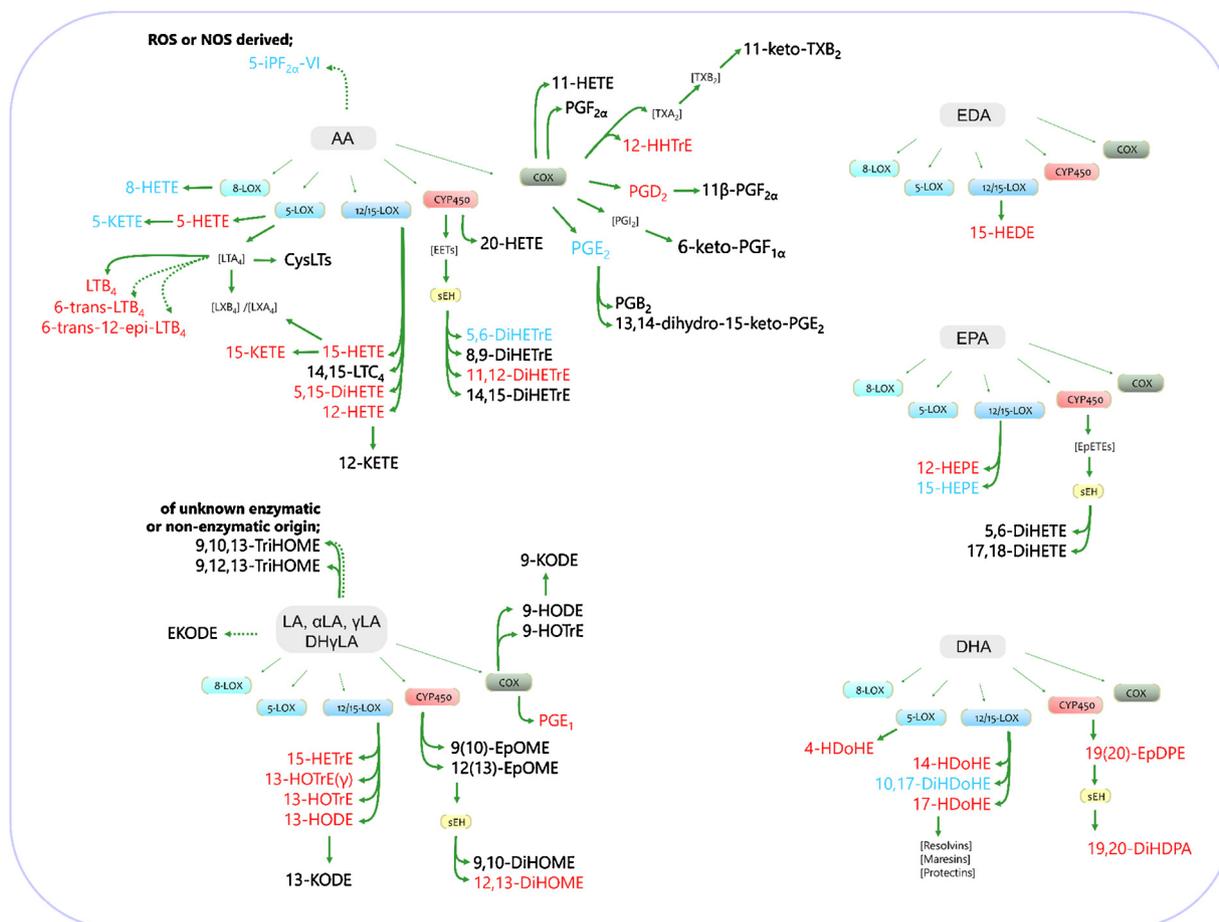


Fig. 1. In total 59 lipid mediators originating from polyunsaturated fatty acids (ω 3, ω 6 and ω 9) metabolism could be detected in murine BALF. Significantly altered LMs are coloured red ($p < 0.05$, FDR = 5%) or blue ($p < 0.05$). Non-altered LMs are coloured in black. Main substrates for LM production are arachidonic acid (AA), linoleic acid (LA), α - and γ -linolenic acids (α LA, γ LA), dihomo- γ -linolenic acid (DH γ LA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and 11,14-eicosadienoic acid (EDA). Non-enzymatic production (dotted line) of LMs occurs to a low degree under baseline conditions, but can be strongly elevated upon release of reactive oxygen, or nitrogen, species (ROS, RNS). 12,13-epoxy-9-keto-10(trans)-octadecenoic acid (EKODE), soluble epoxide hydrolase (sEH), lipoxygenase (LOX), cyclooxygenase (COX) and cytochrome P450 (CYP450). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

mite (HDM) model of asthma.

Inhaled allergens first encounter the airway epithelium which is defined as an important regulator of the inflammatory responses in the lung [9]. One of the most prevalent allergens associated with asthma in large parts of the world is HDM [10]. This complex allergen can affect the epithelial cells and resting immune cells via several mechanisms such as IgE-dependent activation of immunological cells, pattern recognition receptors and via proteolytic activity of HDM constituents. Together these components therefore act as both allergens and adjuvants of allergy and asthma through activation of both the adaptive and innate parts of the immune system [11]. To define the release signature at the site of respiratory inflammation we profiled the LM expression in bronchoalveolar lavage fluid (BALF) after four weeks of continuous HDM exposure in mice. We utilized a comprehensive mass spectrometry profiling approach which enabled us to detect LMs from multiple enzymatic pathways, also including cytochrome P450 (CYP450) generated LMs. We hypothesized that the BALF lipid profile would reveal which LMs that are activated by the induction of this asthma like airway inflammation.

2. Methods

2.1. Chemicals

Lipid mediator standards were bought from Cayman Chemical (Ann Arbor, USA) and Larodan (Solna, Sweden). Methanol, isopropanol, acetonitrile, acetic and formic acid were obtained from Fisher Scientific (Waltham, MA, USA). Disodium phosphate and citric acid monohydrate were obtained from Merck (Darmstadt, Germany). Milli-Q ultrapure deionized water was used (Millipore Corp., Billerica, MA, USA). Mobile phase solvents were of LC/MS grade (Fisher Scientific, GmbH, Germany). Solid phase extraction cartridges, Evolute Express ABN 60 mg/3 mL, were purchased from Biotage (Uppsala, Sweden).

2.2. Allergic sensitization of mice

Animal handling and experiments were approved by the CIBIR Bioethics Committee (ref. 03/12, Logroño, Spain) and the Regional Committee of Animal Experimentation Ethics (N152/15; Stockholm, Sweden). The study was performed in *Igf1^{R/R}* mice in a C57BL/6 enriched background as described in [12] and were not inbred with any CRE containing mice, exhibiting no alteration of phenotype. The mice were housed with a 12-hour light/dark cycle and given food from Special Diets Services (SDS) (Essex, UK, Ref No. 801010-RM1A(P)) and

water *ad libitum*. The allergic sensitization and induction of inflammation was induced on 8–10 weeks old female mice by intranasal challenge of 20 μ L HDM (2 mg HDM protein/mL; Greer, USA) five times per week for four weeks given during light anaesthesia (3.5% Isoflurane, Abbot Scandinavia, Solna, Sweden) as previously described [13]. Control mice were challenged with PBS in a similar fashion.

2.3. *In vivo* measurement of pulmonary mechanics

Mice were anesthetized by subcutaneous injection with a combination of hypnorm (2.5 μ L/g; VetaPharma Ltd, Leeds, U.K) and midazolam (12.5 μ g/g; Hameln Pharmaceuticals GmbH Hameln, Germany) 24 h after the last HDM challenge. The mice were placed on a heating pad (37 °C) and tracheotomized with a blunted 18-gauge needle connected to a flexiVent™ animal ventilator (Scireq, Montreal, Canada). Ventilation was performed at 2.5 Hz and tidal volume was set to a 12 mL·kg⁻¹ body weight with a positive end-expiratory pressure (PEEP) of 3 cm H₂O. To equalize pleural pressure and to eliminate the possibility of spontaneous chest wall movements interfering with lung mechanics measurements, a bilateral thoracotomy was performed as previously described [14]. 10 μ L of aerosolized acetyl- β -metacholine (0, 15.6, 62.5, 250 and 500 mg/mL; Sigma-Aldrich, Sweden) were delivered to the lung via an Aeronex nebulizer (Scireq, Montreal, QC, Canada). Lung resistance (R_L) and compliance (C_L) were measured assuming the single-compartment linear model [14,15].

2.4. Collection and preparation of BALF and lung specimens

Following measurement of pulmonary mechanics, the lungs of each mouse were flushed two times with 800 μ L of cold PBS and the recovered volume was collected as BALF. Immediately following the cytospin separation of the cells, the supernatant was collected and frozen at -20 °C for later analysis. The lungs were fixed in 4% formaldehyde (Histolab, Stockholm, Sweden) and snap-frozen and stored at -80 °C for further analyses.

2.5. Eosinophil and mast cell counts in the lung

Lung sections were stained with hematoxylin and eosin (H&E; Panreac, Barcelona, Spain) and toluidine blue staining. Eosinophilic infiltration index was calculated by counting the number of eosinophils in relation (%) to the total number of inflammatory cells infiltrating the tissue using 6 animals per condition and evaluating 4 different areas under the pulmonary artery and photo-documented under a light microscope (Nikon Instruments, Inc, Tokyo, Japan). Quantification of total mast cell number was performed in areas surrounding the bronchi, assessed on one single lung section per animal in a total of 6 animals per condition and expressed as the number of mast cells per mm² using the Fiji open-source image processing software package v1.48r (<https://fiji.sc>).

2.6. RNA isolation, reverse transcription and qRT-PCR

Lung lobes were homogenized in Trizol Reagent (Invitrogen, Carlsbad, CA) using a T8 Ultra Turrax (IKA Works Inc) homogenizer. Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and treated with 2.72 kU/ μ L RNase-free DNase (Qiagen, Hilden, Germany) and purified through RNeasy columns (Qiagen) following manufacturer instructions. The quantity and quality of total RNA was assessed on a NanoDrop Spectrophotometer and an Agilent 2100 Bioanalyzer, respectively. Isolated RNA was reverse transcribed to cDNA using SuperScript II First-Strand Synthesis System (Invitrogen, Carlsbad, CA). cDNA samples were amplified by qRT-PCR in triplicate reactions on a 7300 Real Time PCR instrument (Applied Biosystems, Foster City, CA), for each primer pair assayed (Table S1), using the SYBR Premix Ex Taq (Takara Bio Inc., Kusatsu, Japan). Results were

normalized using 18S rRNA gene as endogenous control.

2.7. LC-MS/MS analysis of lipid mediators in bronchoalveolar lavage fluid

Five days after BALF sample collection the samples were thawed for LC-MS/MS analysis. A pooled quality control (QC) sample was created by pooling 10 μ L of each study sample creating a representative murine BALF QC pool. The pooled QC sample was split into several aliquots, which were subsequently used throughout the analysis to monitor LM extraction and LC-MS/MS method performance. An internal standard mixture of 42 deuterated LMs, concentration 5–100 ng/mL, was added to each sample followed by 800 μ L BALF extraction buffer, pH = 5.6; 58/42 (v/v) of 0.2 M Na₂HPO₄/0.1 M citric acid hydrate solution. Samples were vortexed and 1.6 mL sample loaded onto a pre-conditioned polymeric solid phase extraction cartridge (SPE) and washed with 10% methanol. LMs were eluted using 2 mL of 100% methanol. SPE was performed and controlled by Extrahera™ and dried with nitrogen using TurboVapLV™ (Biotage, Uppsala, Sweden). Samples were reconstituted in two steps by first adding 60 μ L methanol and vortex. Then 10 μ L of water was added followed by vortex. The final extract was then filtered through a 0.1 μ m nylon filter (Amicon Ultrafree-MC) using an Eppendorf benchtop centrifuge. The samples were immediately put in the autosampler until LC-MS/MS analysis. Lipid profiling was performed by injecting 7.5 μ L of the reconstituted BALF extract onto a BEH C₁₈, 2.1 x 150 mm, 1.7 μ m (Waters, Milford, USA), for chromatographic separation of lipids and quantitative mass spectral data acquired using multiple reaction monitoring mode using Xevo TQ-S™ instrument (Waters, Milford, USA) operated in negative scan mode and adapted from previously [16]. Cysteinyl leukotrienes were quantified using a separate chromatographic method using 0.2% formic acid in water (mobile phase A) and 0.2% formic acid in acetonitrile (mobile phase B) while acquiring data in positive scan mode. An eleven-point external calibration curve spiked with the internal standards were established for both methods. To calculate LM concentrations in BALF the calibration curve used 1/X weighting and with the following calibration curve point criteria; signal-to-noise > 5, < 25% residuals at limit of quantification (LOQ). Concentration calculations were performed in TargetLynx (Waters, Milford, USA). Total cysteinyl leukotriene concentration, CysLT(tot.), was calculated by summarizing the concentrations of LTC₄, LTD₄ and LTE₄ in each animal. Association of lipid levels was performed against total inflammatory cells and eosinophils in BALF previously obtained [13].

2.8. Preparation of HDM extract

A volume of 1 mL freshly prepared HDM extract (2 mg HDM protein/mL) was filtered through a spin-filter by centrifugation at 10,000 rcf for 4 min to remove protein residues. A volume of 60 μ L of the filtered HDM extract was spiked with 10 μ L internal standard solution and injected using the LC-MS/MS platform to determine the presence and concentration of individual LMs.

2.9. Statistical analysis

Changes in LMs levels were evaluated by multiple *t*-testing using a false discovery rate (FDR) of 5%. Statistical analysis of lung mechanics, histology, mRNA and LMs was performed using Graphpad Prism v.6 (GraphPad, La Jolla, CA) and SPSS Statistics Software v21.. Spearman rank correlation (for LMs) and Mann-Whitney U test (for lung mechanics, histology, mRNA) were used for non-parametric tests. Results are shown as mean values + standard error of the mean (SEM). For all analysis, a *p*-value < 0.05 was considered statistically significant.

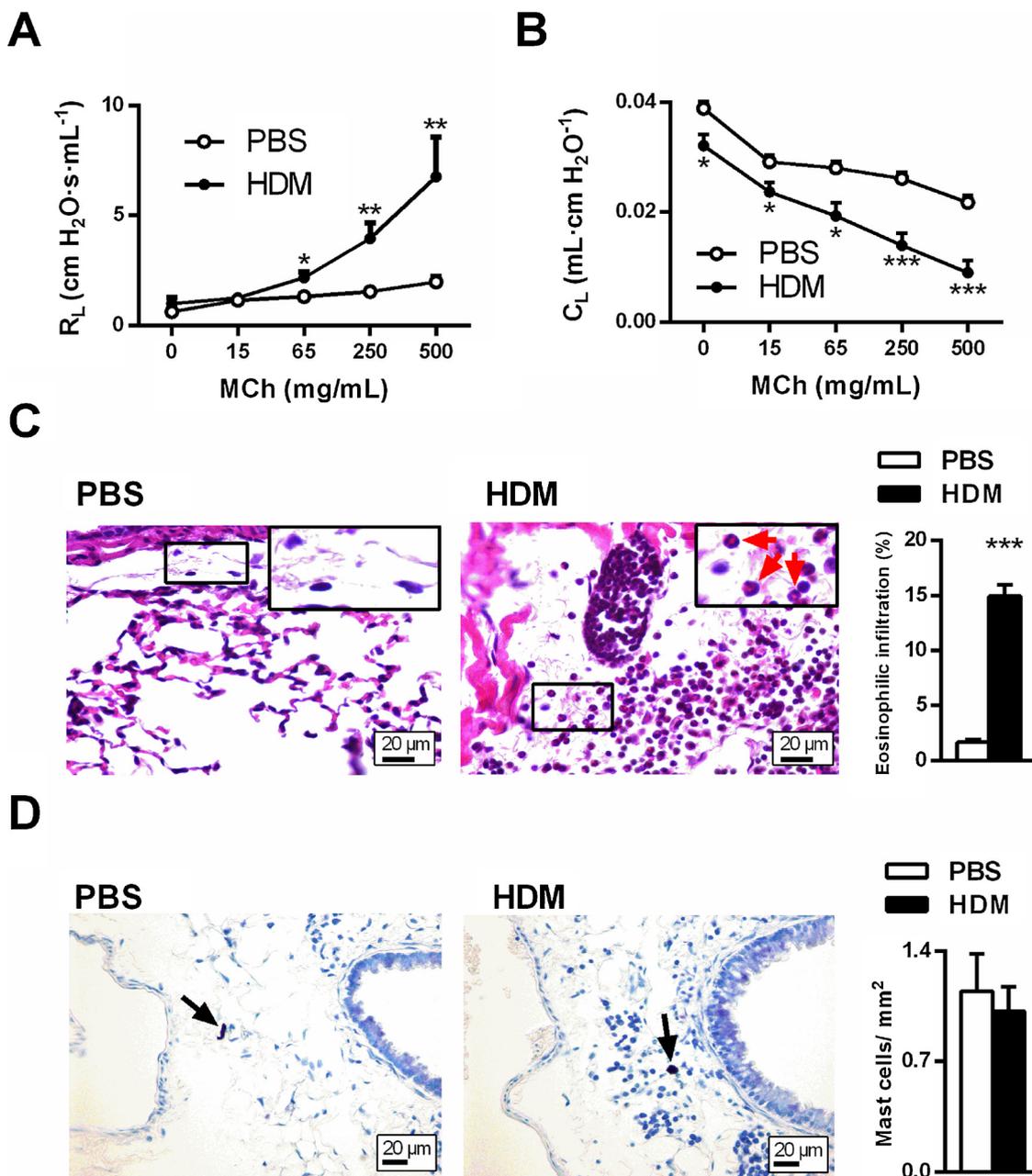


Fig. 2. HDM exposure increases AHR and allergic airway inflammation. Total lung resistance (R_L) (A), and compliance (C_L) (B) (PBS, $n = 11$ HDM, $n = 9$). Representative lung sections and quantification of eosinophilic infiltration grade (C), and number of mast cells (D) ($n = 6$ per condition in PBS and HDM exposed lungs). Data are expressed as mean + SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Magnification was 60x in panel C and 40x in panel D. Airway hyperresponsiveness (AHR), phosphate buffer saline (PBS), house dust mite (HDM).

3. Results

Airway responsiveness and cellular inflammation

AHR was determined 24 h after the last exposure to HDM by measuring lung resistance (R_L) and compliance (C_L). After challenging mouse airways with increasing concentrations of aerosolized methacholine, there was a 3.4 ± 0.9 -fold increase in lung resistance (Fig. 2A) in the HDM exposed group while compliance (Fig. 2B) was reduced 2.4 ± 0.3 -fold indicating a marked AHR induced by HDM. Stained lung sections revealed that the percentage of eosinophils was significantly higher ($14.9 \pm 1.0\%$ vs. $1.6 \pm 0.2\%$) in HDM treated mice whereas no differences were observed for mast cell counts (Fig. 2C and 2D). In BALF, eosinophil counts reached 30.1% in the HDM group versus 7.8% in the control group as reported elsewhere [13].

3.1. Gene expression of cytokines and lipid mediator-pathway enzymes

To investigate if alterations of LM levels could be attributed to mRNA expression differences, the expression of 14 key LM enzymes were assessed in lung homogenates from HDM and PBS treated mice. There was a general tendency for a numerical decrease in enzyme expression (7 out of 9 transcripts) in the HDM treated mice (Fig. 3), but it was only *Alox12* which demonstrated a small but statistically significant decrease in HDM compared to PBS treated mice ($p = 0.047$). As a positive control for HDM treatment, we documented the expected significant increase in specific mRNA transcripts of the cytokines TNF α , IL-10 and IL-13 in the HDM group.

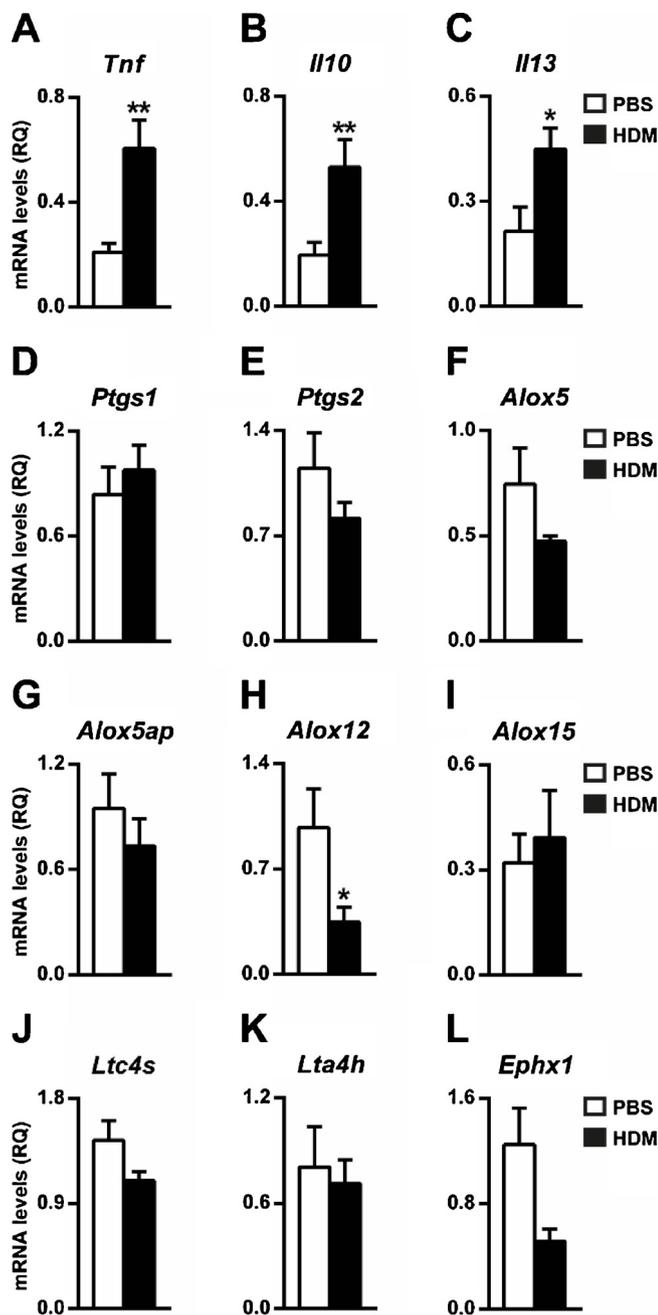


Fig. 3. Lung tissue mRNA expression demonstrate an increase in Th1 and Th2 cytokines, but not LM producing enzymes with HDM exposure. Panel A–C, increased expression of *Tnf* (Th1), *Il10* and *Il13* (Th2). Panel D–L, key LM pathway enzymes. Data are expressed as mean + SEM ($n = 5$). * $p < 0.05$, ** $p < 0.01$. Phosphate buffer saline (PBS), house dust mite (HDM) and relative quantification (RQ).

3.2. Detection and quantification of lipid mediators in the bronchoalveolar lavage fluid

Out of a panel of 112 LMs derived from the polyunsaturated fatty acids AA, linoleic acids (LA), α - and γ -linolenic acids (α LA, γ LA), di-homo- γ -linolenic acid (DH γ LA), 11,14-eicosadienoic acid (EDA), mead acid, DHA and EPA, 57 could successfully be quantified in BALF from control animals, with an additional 2 following HDM treatment (Fig. 1). For 49 of the detected LMs, the pooled QC sample coefficients of variation (CV) were $< 30\%$ and in total, 24 LMs had $< 10\%$ CV, demonstrating the repeatability of the procedure of BALF sample extraction and the LC–MS/MS analysis.

After HDM exposure, 26 of the 59 reported LMs increased > 2 -fold, at $p < 0.05$ (FDR = 5%) (Table 1, Fig. 4). Furthermore, 8 of those 26 LMs demonstrated a > 10 -fold increase. When grouping enzymatic origin of significantly altered LMs, a relatively larger proportion was generated from 12/15-LOX activity followed by 5-LOX, as compared to other LM producing enzyme (12/15-LOX $>$ 5-LOX $>$ sEH $>$ COX $>$ 8-LOX = CYP450).

3.3. Arachidonic acid-derived lipid mediator products

A substantial number of the AA-derived LMs that were elevated belong to the LOX pathways. The level of the primary 5-LOX product 5-HETE was increased 3-fold (Fig. 5A), and the corresponding dehydrogenated product, 5-KETE (Table 1), was also elevated (FDR > 0.05). Leukotriene B₄ (LTB₄) demonstrated a strong elevation (20-fold) (Fig. 5B). In addition, levels of 6-*trans*-LTB₄ and 6-*trans*-12-*epi*-LTB₄, formed by non-enzymatic hydrolysis of leukotriene A₄, were also increased (Table 1). Leukotriene C₄, D₄ and E₄, summed as CysLT(tot.), were altogether the most abundant metabolites in BALF, but their concentration did not increase by the HDM exposure (Fig. 5C).

Prostaglandin D₂ (PGD₂) and E₂ (PGE₂) formed by the COX pathways were elevated 3- and 6-fold, respectively, after HDM treatment (Fig. 5D, E). By contrast, no significant alterations of the levels of PGF_{2 α} (Table 1) and PGI₂ derived 6-keto-PGF_{1 α} (Fig. 5F) were observed.

The primary 15-LOX metabolite 15-HETE evidenced an almost 7-fold increase (Fig. 6A) and its dehydrogenated product 15-KETE was elevated more than 2-fold (Table 1). The combined 15-LOX and 5-LOX product 5,15-diHETE increased 23-fold (Fig. 6B). The products of mouse specific 8-LOX and 12-LOX enzyme activity, 8-HETE and 12-HETE, were elevated 9.3- and 4.7-fold, respectively (Table 1), however these products are likely to be produced also by free radical peroxidation.

None of the CYP450-derived epoxyeicosatrienoic acids (EETs) were observed. Instead, their secondary downstream metabolites, produced by sEH, demonstrated a trend towards elevated levels, with 11,12-DiHETE significantly elevated after HDM treatment (Table 1). Among the isoprostanes (non-enzymatically formed oxidation products of arachidonic acid) 5-IPF_{2 α} -VI was strongly elevated following HDM treatment (Table 1).

3.4. Linoleic, linolenic and di-homo- γ -linolenic acid-derived lipid mediators

From the precursors α LA, γ LA and DH γ LA, the LMs 13-HOTrE, 13-HOTrE(γ) and 15-HETrE are produced by the 15-LOX enzyme. They demonstrated an approximate elevation of 6.5–12.7 times after HDM exposure (Table 1). 13-hydroxyoctadecadienoic acid (13-HODE), produced by enzymatic or non-enzymatic oxidation of LA, was 3-fold elevated, whereas its NAD⁺ dependent dehydrogenase derivative 13-KODE was elevated to a lesser extent (Table 1).

3.5. DHA, EPA and EDA derived lipid mediators

Two major ω 3-fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which were abundantly found in the PBS treated mice, appeared in about 5 times higher in concentration after HDM treatment. Notably, the ratio DHA:EPA was similar (24:1) after HDM treatment (Table S-2). A significant finding was the results of 12/15-lipoxygenase activity of DHA, which produced 12-fold higher levels of 17-HDoHE (Fig. 6C). In addition, 10,17-DiHDoHE demonstrated a > 20 -fold elevation, but was not significant per the FDR criterion of 5% (Fig. 6D). From EPA the 12/15-LOX products, 12-HEPE and 15-HEPE, were both elevated > 13 -fold, and from 11,14-eicosadienoic acid (EDA), 15-HEDE was 25-fold elevated after HDM treatment.

Table 1

After four weeks of HDM exposure a total of 26 lipid mediators were significantly altered, $p < 0.05$ (FDR = 5%) in murine BALF. Of those, 19 LMs originated from LOX enzyme activity, 4 LMs from COX enzyme activity, 2 LMs from CYP450 enzyme activity, 2 LMs from sEH enzyme activity and 1 non-enzymatically produced isoprostane.

Name	PBS (ng/ mL)	HDM (ng/ mL)	Fold change HDM/PBS (PBS/ HDM)	p -value (FDR = 0.05)*	Total inflammatory cells in BALF, correlation (ρ)	Eosinophils in BALF, correlation (ρ)	Enzyme	Fatty acid substrate (series)
5-KETE	n.d	0.0297	> 25	0.029	0.47	0.37	5-LOX	AA (ω 6)
10,17-DiHDoHE	n.d	0.0014	> 25	0.037	0.78**	0.79**	5-LOX,12/15- LOX	DHA (ω 3)
5,15-DiHETE	0.0002	0.0054	25.0	0.010*	0.80**	0.68**	5-LOX, 12/15- LOX	AA (ω 6)
15-HEDE	0.002	0.051	24.8	< 0.001*	0.75**	0.87**	12/15-LOX	EDA (ω 6)
LTB ₄	0.0008	0.0154	20.3	0.004*	-0.3	-0.51	5-LOX, LTA ₄ H	AA (ω 6)
19,20-EpDPE	0.002	0.043	18.6	0.045	0.73**	0.80**	CYP450	DHA (ω 3)
15-HEPE	0.003	0.056	17.9	0.033	0.77**	0.87**	12/15-LOX	EPA (ω 3)
6-trans-LTB ₄	0.002	0.033	15.0	< 0.001*	0.57**	0.57**	5-LOX	AA (ω 6)
13-HOTrE(γ)	0.001	0.018	13.2	0.004*	0.75**	0.78**	12/15-LOX	γ LA (ω 6)
12-HEPE	0.007	0.089	13.2	0.003*	0.76**	0.74**	12/15-LOX	EPA (ω 3)
17-HDoHE	0.12	1.40	12.1	0.013*	0.79**	0.89**	12/15-LOX	DHA (ω 3)
14-HDoHE	0.24	2.46	10.3	0.009*	0.68**	0.80**	12/15-LOX	DHA (ω 3)
13-HOTrE	0.019	0.182	9.7	< 0.001*	0.55	0.58**	12/15-LOX	α LA (ω 3)
8-HETE	0.011	0.097	9.3	0.005*	0.71**	0.80**	8-LOX	AA (ω 6)
6-trans-12-epi- LTB ₄	0.003	0.026	8.8	< 0.001*	0.4	0.56**	5-LOX	AA (ω 6)
19,20-DiHDPA	0.021	0.173	8.4	0.042	0.70**	0.73**	CYP450, sEH	DHA (ω 3)
15-HETE	0.063	0.433	6.9	0.001*	0.75**	0.85**	12/15-LOX	AA (ω 6)
15-HETrE	0.007	0.049	6.7	< 0.001*	0.70**	0.83**	12/15-LOX	DH γ LA (ω 6)
4-HDoHE	0.0012	0.0083	6.7	< 0.001*	0.49	0.42	5-LOX	DHA (ω 3)
PGD ₂	0.016	0.106	6.5	0.025*	0.64**	0.39	COX	AA (ω 6)
12-HHTrE	0.023	0.12	5.3	0.013*	0.54	0.49	COX	AA (ω 6)
5-iPF _{2α} -VI	0.0015	0.0075	5.0	< 0.001*	0.70**	0.80**	NE	AA (ω 6)
12-HETE	0.77	3.61	4.7	< 0.001*	0.79**	0.82**	12/15-LOX	AA (ω 6)
5,6-DiHETrE	0.0005	0.0019	4.0	0.05	-0.02	-0.14	CYP450, sEH	AA (ω 6)
11-HETE	0.019	0.069	3.6	0.014*	0.67**	0.58**	COX	AA (ω 6)
13-HODE	0.41	1.43	3.5	< 0.001*	0.65**	0.69**	12/15-LOX	LA (ω 6)
5-HETE	0.012	0.036	3.0	< 0.001*	0.55	0.37	5-LOX	AA (ω 6)
PGE ₂	0.13	0.36	2.8	0.039	0.64**	0.51	COX	AA (ω 6)
12,13-DiHOME	0.10	0.28	2.7	0.002*	0.02	-0.16	CYP450, sEH	LA (ω 6)
11,12-DiHETrE	0.0066	0.0164	2.5	< 0.001*	-0.03	-0.12	CYP450, sEH	AA (ω 6)
PGE ₁	0.006	0.015	2.4	0.014*	0.26	0.02	COX	DH γ LA (ω 6)
15-KETE	0.0065	0.0148	2.3	0.014*	0.64**	0.72**	12/15-LOX	AA (ω 6)
13-KODE	0.049	0.093	1.9	< 0.001*	0.06	0.24	12/15-LOX	LA (ω 6)
CysLT(tot.)	6.2	3.9	0.6 (1.6)	0.141	-0.53	-0.59*	5-LOX, LTC ₄ S	AA (ω 6)

The table comprise a list of murine BALF LMs that have met the following selection criteria; > 1.9 median fold change (or more than a 20% decrease) and statistically significant ($p < 0.05$), with the exception of included CysLT(tot.) $p > 0.05$. Primary ω 3-substrates EPA and DHA also passed the selection criteria, but are listed in Table S2. At a false discovery rate (FDR) of 5% significantly altered lipid mediators are highlighted with a (*). (**) are LMs having a significant association by Spearman rank correlation (ρ) to total inflammatory cell, or eosinophil, count in BALF. A combined total leukotriene LM was abbreviated CysLT(tot.). Lipid mediators formed non-enzymatically or having unknown enzymatic origin are annotated with NE. Arachidonic acid (AA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), linoleic acid (LA), α -linolenic acid (α LA), γ -linolenic acid (γ LA), dihomo- γ -linolenic acid (DH γ LA), 11,14-eicosadienoic acid (EDA), soluble epoxide hydrolase (sEH), leukotriene A₄ hydrolase (LTA₄H) and leukotriene C₄ synthase (LTC₄S). Phosphate buffer saline (PBS) and house dust mite (HDM).

3.6. Association of total inflammatory BALF cell numbers with LM concentration

In the HDM group 17 of the significantly elevated LMs were also correlated with total inflammatory cells and eosinophils in BALF, with 16 positively correlated ($\rho = 0.56$ – 0.89 , $p < 0.05$) and CysLT(tot.) being negatively correlated ($\rho = -0.59$, $p = 0.04$). Among the positively correlating LMs the majority were products of lipoxygenase activity, however no such correlation was found for LTB₄ (Table 1).

3.7. Measurement of lipid mediators in the house dust mite extract

We finally evaluated whether the HDM extract itself might contribute to the levels of LMs detected in BALF. Results from the HDM extract characterization revealed only 7 out of the 59 detectable LMs were found in the extract and that each 20 μ L HDM installation contributed a total dose of 280 pg of 13-HODE, followed by descending order of 12,13-DiHOME (126 pg) > 13-KODE (56 pg) > 9,10,13-TriHOME (36 pg) > EPA = DHA (14 pg) > 5-HETE (8 pg). These amounts are comparable with the corresponding levels detected in

BALF of PBS treated animals. However, taking into consideration tissue absorption and metabolism of lipids from the HDM extract following exposure, their contribution to the measured levels in BALF 24 h after the last installation are considered of no importance.

4. Discussion

This is the first study describing the comprehensive pattern of recovered LMs in BALF in a murine HDM asthma model. In the quantification panel of 112 LMs, 59 were detected and 28 of these were increased at least 2-fold with HDM treatment, and some metabolites to a much larger extent. The observed LMs were derived from multiple biosynthetic pathways including enzymatic (e.g., LOX, COX, CYP450 and sEH) as well as non-enzymatic (i.e., reactive oxygen or nitrogen species (ROS, RNS) induced autoxidation routes). The HDM exposure induced elevation of LMs on a pathway-specific basis ranked in descending contribution order as follows; 12/15-LOX > 5-LOX > > COX > 8-LOX = CYP450. As demonstrated by the mRNA expression data from lung tissue homogenates, the increase of LMs was not associated with a concomitant increase of enzyme expression,

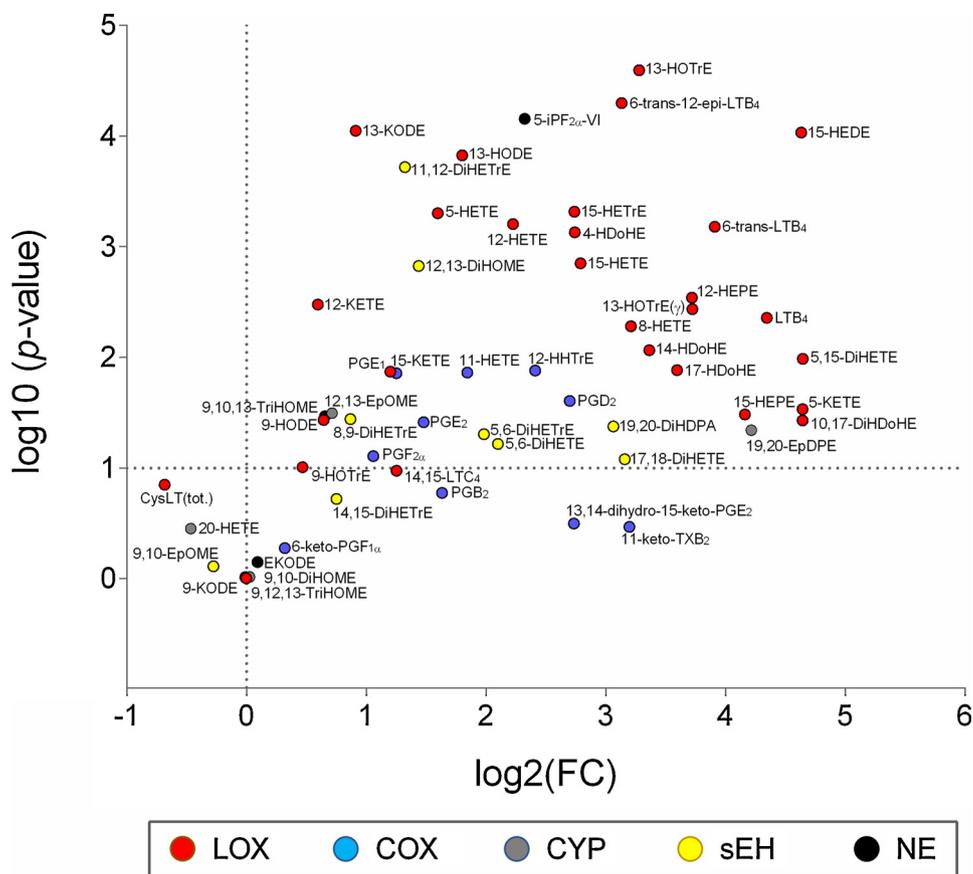


Fig. 4. Volcano plot of 55 LC-MS/MS detected LMs in murine BALF, after 4 weeks of HDM treatment, reveal a prominent elevation LMs from LOX activity. Y-axis represent log₁₀(p-value) and x-axis log₂(FC:fold change). Y-intercept correspond to $p < 0.05$. LMs are coloured according to enzymatic origin. Lipoxygenase (LOX), cyclooxygenase (COX), cytochrome P450 (CYP), soluble epoxide hydrolase (sEH) and non-enzymatic, or unknown, origin (NE).

however, for some lipids the levels were related to total BALF cell numbers. Due to the prominent elevation of 12/15- and 5-LOX derived LMs, lipoxygenase activation is clearly a consequence of the HDM induced allergic inflammation.

Generally, HDM has commonly been used in asthma models using BALB/c mice and rarely in C57BL/6 mice, which is the most common strain used to create transgenic mice. In this study, HDM treatment caused a marked AHR (R_L , C_L) and eosinophilic infiltration to the lung, as well as increased mRNA expression of *Tnf*, *Il10* and *Il13* [13]. Additionally, the HDM-induced inflammation was previously corroborated in animals used in this study (*Igf1^{fl/fl}* mice in a C57BL/6 enriched background) by: i) increased AHR (Rn, G, H), ii) elevated peripheral and BALF eosinophil counts, iii) augmented serum total IgE and IL13 levels in lung homogenates, and iv) increased inflamed lung area, mucus-producing and MUC5AC+ cells, collagen area, as well as smooth muscle and airway thickness [13]. The model is therefore appropriate for this assessment of LM pathways during HDM-induced inflammation.

One major driver of the observed inflammatory state could be the strong activation of 5-LOX, responsible for production of LTB₄ and 5-HETE (5-KETE). In particular, potent proinflammatory LTB₄ exhibited a 20-fold increase after HDM treatment, and with similar increases for 6-trans- and 6-trans-12-epi-LTB₄. An elevation of LTB₄ levels has been shown in different OVA models of asthma [17,18]. This compound has a short half-life in biological experimental system, generally only minutes, which in this model suggests an ongoing induced synthesis. The carboxy- and hydroxy-metabolites of LTB₄ were not found in BALF, it is therefore possible that the enzymatic degradation of LTB₄ in BALF is low and thereby allowing its accumulation. Basal levels of 5-HETE were low but increased 3-fold, and accordingly, its dehydrogenated product 5-KETE could only be observed after HDM treatment. CysLTs are formed by LTC₄-synthase, but depend on 5-LOX activity generating the precursor LTA₄. Moreover, in our *in-vivo* model using C57BL/6

strain, CysLTs were present at the highest concentration (6 ng/mL) of all LMs in the control group. In contrast to the above-mentioned elevation of 5-LOX products, total CysLTs did not demonstrate a significant change. As LTE₄ is the end-product in the CysLT pathway the significant change observed for LTD₄ is considered less important because its shorter physiological half-life results in rapid conversion into LTE₄. This somewhat surprising finding has been reported in two other studies, one using OVA stimulated C57BL/6 mice [5] and a second study in BALB/c mice with HDM as allergen [19]. In addition, we acknowledge that a basal low number of mast cells in the lung together with the lack of increase in mast cells and CysLTs most likely constitute mice specific characteristics [20], which in humans is an inflammatory component of significant importance to allergic airway inflammation. Hence, an adaptive mechanism may be a possible explanation of the unaltered total CysLT levels observed in our study. It can be speculated that continuous allergen provocation by HDM would completely deplete stores of mast cell granula, and thereby they fail to get stained by the toluidine blue. This is however unlikely, as Li et al. could show elevated mast cells counts after high dose HDM exposure in BALB/c mice and using toluidine staining. Taken together, the lack of increase in total CysLTs clearly differs from human allergen challenge studies [21,22]. This therefore may suggest a different role and regulation for CysLTs in mice, which also may be strain dependent. Furthermore, whereas CysLTs are the most potent known bronchoconstrictors in humans, they do not constrict mice airways [23]. Thus, in the present asthma model the 5-LOX/FLAP pathway activity is markedly increased by HDM exposure, as levels of LTB₄, 6-trans-LTB₄ and 6-trans-12-epi-LTB₄ increased, while the high LTC₄-synthase activity is considered to remain constant.

Levels of HETE products and their relative concentration order in the PBS group (12-HETE > 15-HETE > 11-HETE ≥ 5-HETE ≥ 8-HETE) was similar to previous studies using lung extracts in untreated male C57BL/6 mice [24]. The corresponding mRNA expression of *Alox12*

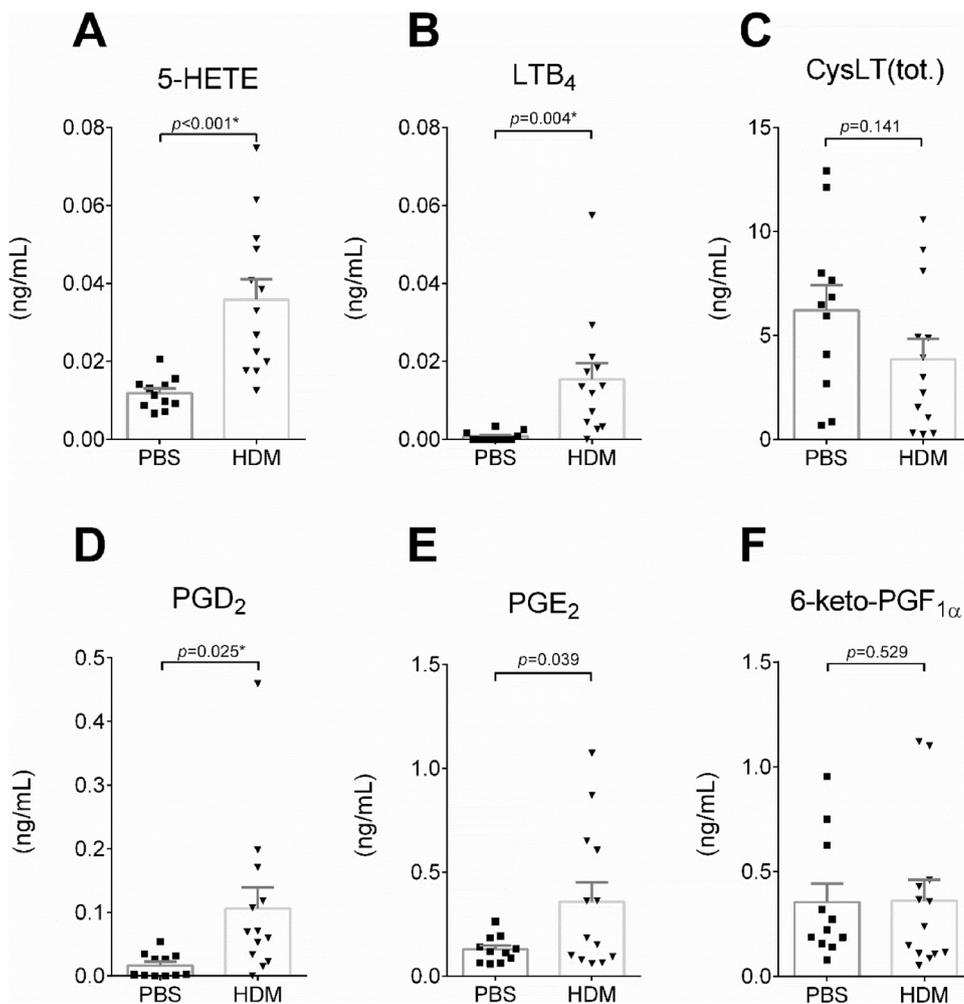


Fig. 5. In murine BALF, a strong increase in 5-lipoxygenase (5-LOX) together with a moderate increase in cyclooxygenase (COX) products were seen after four-week HDM exposure. The increase was significant for 5-HETE and LTB₄ (A, B), but not for the downstream leukotriene C4 synthase products as denoted CysLT(tot.) (C). The main COX-1 derived LM product PGD₂ was 6-fold elevated (D) and the COX-2 product PGE₂ almost 3-fold (FDR \approx 5%) (E). The main PGI₂ metabolite 6-keto-PGF_{1α} was left unchanged with HDM treatment (F). Histogram bars show mean values and standard error of the mean (+SEM). Phosphate buffer saline (PBS) and house dust mite (HDM).

was the only altered transcript, but with lower levels in the HDM group, which excludes its mRNA expression as a likely cause for elevated 12-HETE in BALF. Instead, platelet derived 12-LOX could be a possible source, as well as non-enzymatic oxidation of AA. Moreover, the mice specific 8-LOX product 8-HETE exhibited the largest elevation (9-fold) of all detected HETEs, thereby shifting their relative order (8-HETE \geq 11-HETE \geq 5-HETE) with HDM exposure. In addition to the established role of lipoxygenases in human health and disease, our data support the hypothesis of a functional role for these metabolites in murine models of asthma which has not been assessed [25–27].

In BALF, HDM also elicited an increase in COX products, including PGD₂ and 12-HHTrE. Both of these products are reported to be generated by cultivated mucosal like mast cell by the hematopoietic prostaglandin D-synthase and thromboxane synthase respectively [28]. Since mast cell counts were found not to be increased in lungs of HDM treated mice, this finding implies that the few mast cells present were significantly activated, or additional immune cells and/or structural cells contribute to PGD₂ formation. In addition, macrophages and eosinophils, which carry the necessary enzymes, might also have contributed to their release [29].

Free radical peroxidation during oxidative stress is a common feature during respiratory burst and primary LMs precursor fatty acids thereby easily undergo non-enzymatic free radical peroxidation, producing multiple isoprostanes, HETEs and HODEs [30]. The presence of elevated levels of isoprostane 5-iPF_{2α}-VI, and several of the HODEs and HETEs, supports that HDM also induces a continuous oxidative stress in the lung. These auto-oxidative LM products, together with infiltration of large numbers of leukocytes into the lungs, is thus one important

characteristic of the innate immune response in HDM induced inflammation which has not been highlighted before.

Despite elevation of both DHA and EPA, no shift of the DHA-to-EPA ratio was observed, suggesting that there was no selectivity in the demand for those substrates downstream. Two precursors of pro-resolving compounds were found elevated, 14- and 17-HDoHE, which by an additional 5-LOX activity may be converted to SPMs such as maresins and protectins [31]. Although 17-RvD1, RvD1, RvD2, and 7-Maresin-1 were included in the panel of 112 lipid mediators, none of them could be detected at this time point. Therefore, it remains to be established whether other pro-resolving compounds generated from alternative precursors can be found in this mice model at other time points. Accordingly, the current inflammatory environment with a continuous load of HDM into the lungs, maintain a pro-inflammatory status.

Soluble epoxide hydrolase (sEH) activity converts epoxyeicosatrienoic acids (EETs) into less active dihydroxyeicosatrienoic acids (DHETs). The presence of elevated 5,6- and 11,12-DiHETrE, 12,13-DiHOME and 19,20-DiHDPA altogether evidence sEH activity in the model used. LXA₄ has been attributed pro-resolving properties and therefore belong to the SPM family of bioactive lipids [6]. However, despite the 25-fold increase in 5,15-DiHETE, supporting necessary 5- and 15-LOX activity, we could not detect LXA₄.

It is well established that 15-LOX activity is very high in human airway epithelium [32,33], but the functional consequences remain unclear because there is no selective 15-LOX inhibitor that has reached clinical development. There is experimental data suggesting an effect on mucous production [34], but 15-HETE was essentially inactive when inhaled by asthmatics [35].

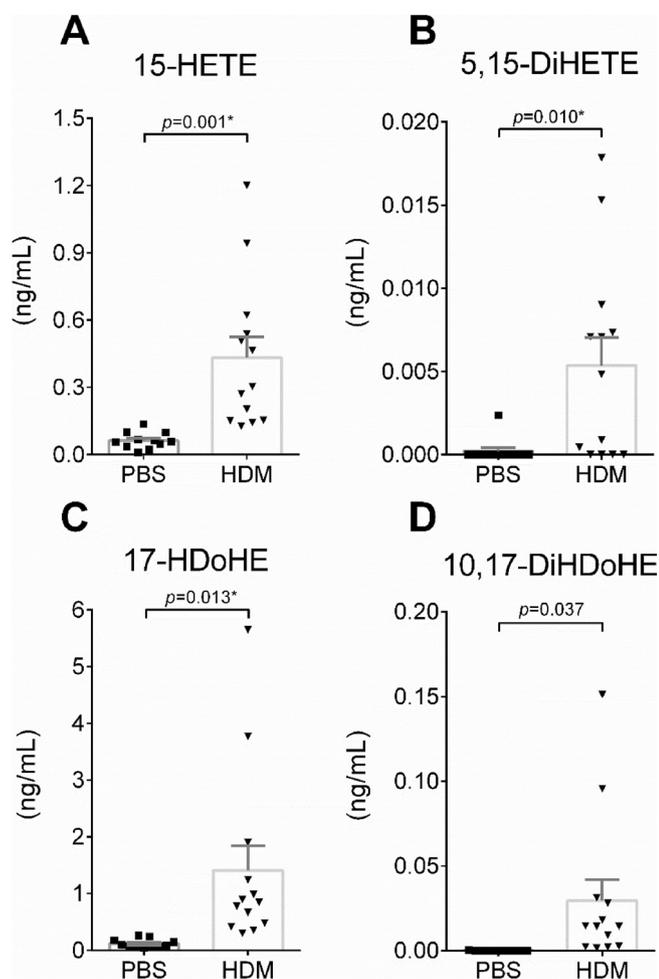


Fig. 6. In murine BALF, increased levels were also seen for arachidonic acid- and DHA-derived LMs by 5- and 12/15-LOX activity with HDM exposure. From arachidonic acid 15-HETE (A) is generated by 12/15-LOX, or COX enzyme activity, by which subsequent 5-LOX activity produces 5,15-DiHETE (B). By the same enzymes, products of DHA revealed elevated concentration in murine BALF of 17-HDoHE (C) and 10,17-DiHDoHE (D). Histogram bars show mean values and standard error of the mean (+SEM). Phosphate buffer saline (PBS) and house dust mite (HDM).

One finding that needs to be highlighted is that the increase of LM levels could not be linked to a concomitant increase in mRNA expression of the enzymes responsible for the biosynthetic production of LMs. Although we did not evaluate specific protein levels in the lung it is possible that levels of active LM enzymes are high at 4 weeks due to an earlier upregulation of mRNA expression, which transiently has faded at the time of lung collection. Nevertheless, it is generally recognized that the limiting factor for biosynthesis of LMs does not depend on the amount of their specific enzymes, but rather is regulated by the substrate availability. Thus the critical step is the cellular activation of cytosolic phospholipase A₂ (cPLA₂), which liberates membrane-bound substrates for LM biosynthesis [36]. Furthermore, with the dense infiltration of leukocytes into the lung tissue, it is likely that the total amount of LM producing enzymes also increased in the HDM treated group, which would contribute to the observed increases in LM levels. This is supported by the association between LMs and eosinophil cell numbers in BALF in the HDM treated animals for 17 out of 26 elevated LMs. Because many LMs are generated by transcellular interactions, it is not expected that the levels of all metabolites correlate with single cell counts.

5. Conclusions

The study has successfully demonstrated that LM products from different key pathways may be quantified in this murine model of asthma. The AHR and eosinophilic cell infiltration was accompanied by a comprehensive LM release indicating strong activation of inflammatory cells and specific enzymatic pathways. While the high levels of CysLTs at baseline remained unchanged with HDM treatment, elevation of PGD₂, excessive production of LTB₄ and multiple 12/15-LOX products, constitute an important signature that would seem to mimic aspects of chronic allergic airway inflammation such as in asthma. However, we acknowledge that a basal low number of mast cells in the lung together with the lack of increase in mast cells and CysLTs most likely constitute mice specific characteristics, which in humans is an inflammatory component of significant importance to allergic airway inflammation. Future interventional studies will define the specific roles of individual metabolic LM pathways by using specific inhibitors that targets key enzymes involved in these mediator cascades.

Author contribution

S.P.-H., J.A.G., I.P.L., M.A. and J.G.P. planned and conceived the animal study. S.P.-H., J.A.G., J.K. and A.F. performed acquisition of data. J.K. and S.P.-H. performed the data analysis, J.K., S.P.-H., C.E.W., M.H., S.-E.D., J.P.G. and M.A. interpreted the results and wrote the manuscript. All authors read, revised and approved the manuscript before submission.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

SP-H thanks the Sistema Riojano de Innovación (Gobierno de La Rioja, Spain) for a PhD grant. SP-H, JAG, IPL, MA and JGP were part of the European Cooperation in Science and Technology COST Action BM1201, Developmental Origins of Chronic Lung Disease. Supportive grants also came from the Fundación Rioja Salud (Gobierno de La Rioja, Spain) to JGP. MA, CEW and S-ED also received grants from the Swedish Heart-Lung Foundation (20150525, 20130636, 20150640, 20140469, 20140533), Swedish Research Council (2016-02798, 2014-3281), the Konsul Th C Berghs research foundation, the ChAMP (Centre for Allergy Research Highlights Asthma Markers of Phenotype) consortium, which is funded by the Swedish Foundation for Strategic Research, the Karolinska Institutet, AstraZeneca & Science for Life Laboratory Joint Research Collaboration, and the Vårdal Foundation. The authors thank Olof Rådmark for critical feedback on lipoxygenase biology.

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