# Toll-like receptor signaling induces a temporal switch towards a resolving lipid profile in monocyte-derived macrophages

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# Toll-like receptor signaling induces a temporal switch towards a resolving lipid profile in monocyte-derived macrophages

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ABSTRACT

Inflammation is a tightly regulated process. During the past decade it has become clear that the resolution of inflammation is an active process and its dysregulation can contribute to chronic inflammation. Several cells and soluble mediators, including lipid mediators, regulate the course of inflammation and its resolution. It is, however, unclear which signals and cells are involved in initiating the resolution process. Macrophages are tissue resident cells and key players in regulating tissue inflammation through secretion of soluble mediators, including lipids. We hypothesize that persistent inflammatory stimuli can initiate resolution pathways in macrophages.

In this study, we detected 21 lipids in LPS-stimulated human monocyte-derived macrophages by liquid chromatography coupled to tandem mass spectrometry. Cyclooxygenase-derived Prostaglandins were observed in the first six hours of stimulation. Interestingly, a switch towards 15-lipoxygenase products, such as the pro-resolving lipid precursors 15-HEPE and 17-HDHA was observed after 24 h. The RNA and protein expression of cyclooxygenase and 15-lipoxygenase were in line with this trend. Treatment with 17-HDHA increased IL-10 production of monocyte-derived macrophages and decreased $LTB_4$ production by neutrophils, indicating the anti-inflammatory property of this lipid.

These data reveal that monocyte-derived macrophages contribute to the resolution of inflammation in time by the production of pro-resolving lipids after an initial inflammatory stimulus.

## 1. Introduction

Inflammation protects the host from infection and injury. However, the process itself can be damaging if not effectively resolved. Resolution of inflammation typically follows acute inflammation allowing the tissue to return to homeostasis. In recent years, it has become evident that resolution is an active and tightly regulated process. An effective resolution program may be able to prevent the progression to persistent chronic inflammation. A body of evidence supports the notion that the molecular mechanisms involved in initiating inflammation can also contribute to resolution [26–29] and that proinflammatory triggers such as lipopolysaccharide (LPS), appear to be indispensable for the resolution process [28]. However, little is known about the cellular mechanisms and mediators involved in this process.

In models studying the different phases of inflammation, two distinct classes of lipid mediators (LM) were identified [12–14]. During the acute phase of inflammation, proinflammatory lipids such as prostaglandins and leukotrienes are predominant. During the resolution phase, however, a more recently described class of pro-resolving lipid mediators (SPM) was discovered: the so-called specialized pro-resolving mediators (SPM). This class includes Lipoxins (e.g. $LXA_4$), Resolvins (e.g. RvD and RvE), Protectins and Maresins [15]. The pro-resolving functions of these SPM include inhibition of neutrophil migration, wound healing, pain relief and tissue regeneration [7]. The synthesis of the different LM requires enzymatic reactions involving cyclooxygenase (COX) and lipoxygenases (LOX). The selective engagement of these enzymatic pathways provides the potential to tailor the lipid mediator response to meet the need of the different phases during the inflammatory response [32].

In self-resolving models in mice, a lipid class-switch was observed from Prostaglandins and Leukotrienes in the beginning of inflammation towards SPM and their precursors at later stages. The switch towards the production of SPM was associated with a decrease in neutrophil infiltration and an accumulation of macrophages [8–10,31]. The

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cellular and molecular mechanisms responsible for the initiation of this lipid class-switch and the subsequent resolution process remain to be determined.

Macrophages play a pivotal role in acute inflammation and are known to be tissue resident cells that regulate local immune responses including resolution [15]. Macrophages have been reported to be able to synthesize both inflammatory LM, as well as SPM via transcellular metabolism [11,16,17,23]. Another report, using a macrophage cell line, suggested that myeloid cells can produce $LXA_4$ through sequential triggering by LPS and extracellular ATP [10]. These findings provide evidence that combinatorial triggering as well as transcellular metabolism can lead to the synthesis of SPM. The most commonly used inflammatory stimulus for macrophages is LPS. Low doses of LPS are associated with non-resolving inflammation [28,29], whereas high doses are implicated to cause acute inflammation followed by resolution [26,27]. Whether this self-limiting inflammatory process involves the production of LM is however unknown.

The goal of our study was to assess whether resolution of inflammation could be orchestrated by (long-term) exposure of a (tissue resident) immune cell to an inflammatory stimulus.

## 2. Materials and methods

### 2.1. Cell isolation and culture conditions of macrophages

Human PBMCs were isolated by ficoll density gradient from healthy donor buffy coats (Sanquin, Amsterdam, The Netherlands). Blood monocytes were isolated by positive selection from PBMCs using MACS CD14 Microbeads (Miltenyi Biotec, Cologne, Germany) and purity was checked by FACS (LSRIII, BD, San Jose, USA), by staining the cells with CD14-PE (clone MφP9). Monocytes were differentiated for seven days in RPMI 1640 (Gibco, Waltham, USA) containing 8% fetal calf serum (FCS), 100 U/ml Penicillin-Streptomycin, GlutaMAX supplement (Thermo Fisher Scientific Inc., Rockford, IL, USA), 5 ng/ml GM-CSF (Miltenyi Biotec, Cologne, Germany) or 150 ng/ml M-CSF (R&D Systems). Medium was replenished on day three and five. Phenotype was checked before experiments by visual inspection by assessing the typical morphology of M-CSF MDMs ("elongated and spindle-like") vs GM-CSF MDM ("egg sunny-side up-like"). Phenotype was also confirmed by preforming IL-12 OptEIA (BD, San Jose, USA), IL-10 PeliPair reagent set (Sanquin, Amsterdam, The Netherlands), and TNFα OptEIA (BD, San Jose, USA) ELISA on supernatant of cells stimulated for 24 h with 10 ng/ml LPS (Merck, Darmstadt,Germany). GM-CSF monocyte-derived macrophages (MDM) secreted high IL-12 and TNFα and low IL-10, while M-CSF MDM secreted low IL-12 and TNFα and high IL-10 levels. Cells were harvested using Accutase (Merck, Darmstadt,Germany) and seeded in 24 well plates containing 250 µl of 10ˆ6 cell/ml. All experiments were performed in phenol red-free RPMI 1640 medium(Gibco), supplemented with 0,1% BSA fatty acid free (Merck, Darmstadt,Germany), Penicillin and streptomycin and, glutamate. When indicated, cells were stimulated with 10 ng/ml LPS (Merck, Darmstadt,Germany) or 10 min stimulation with 4 µM calcium ionophore A23178 (Merck, Darmstadt, Germany). Cells were pretreated with 100 ng/ml 17HDHA (Cayman Chemicals, Ann Arbor, USA) or vehicle, 0,1% HPLC grade ethanol (Fischer scientific, Loughborough, UK).

### 2.2. Cell isolation and culture conditions of neutrophils

Human polymorphonuclear neutrophils (PMN) were isolated from fresh EDTA stored blood using DextranT500 sedimentation (Pharmacosmos, Holbaek, Denmark) followed by collection of the upper layer and ficoll density gradient centrifugation. The remaining erythrocytes were lysed by adding ice cold water for 20 s. Purity was checked by FACS (LSRIII, BD, San Jose, USA) by staining the cells with 2 CD3-AF700 (clone UCHT1)/ CD15-APC (clone HI98)/CD16-PE (clone 3G8)/CD19-FITC (clone HIB19) and was typically 97%. Isolated neutrophils were resuspended (10ˆ6 cell/ml) in Dulbecco's phosphate buffered saline with MgCl2 and CaCl2 (Merck, Darmstadt, Germany) and were pretreated for 10 min with 5µg/ml 17HDHA (Cayman Chemicals, Ann Arbor, USA) or vehicle, 0,1% HPLC grade ethanol (Fischer scientific, Loughborough, UK), followed by stimulation for 10 min with 4 µM calcium ionophore A23178 (Merck, Darmstadt, Germany).

### 2.3. Lipid isolation and LC-MS/MS

Cells and supernatant were quenched using three volumes of MeOH (Honeywell, 349,661 L) and internal standards containing known concentrations of four internal standards: 500 pg/ml $PGE_2$-d4, 5 ng/ml DHA-d5, 500 pg/ml $LTB_4$-d4 and 500 pg/ml 15S-HETE-d8 were added for subsequent quantification. All samples were stored at −80 °C under argon until analysis. Quenched samples were centrifuged and the supernatant was transferred into an auto sampler vial containing an equal volume of $H_2O$ (Honeywell) before Liquid-chromatography combined with mass spectrometry (LC-MS/MS) analysis was carried out as previously published [1]. Lipid measurements were performed using a QTrap 6500 mass spectrometer in negative ESI mode (Sciex, Nieuwerkerk aan den Ijssel, The Netherlands), coupled to a LC system employing LC-30AD pumps, a SIL-30AC auto sampler, and a CTO-20AC column oven (Shimadzu's-Hertogenbosch, The Netherlands). A Kinetex C18 50 × 2.1 mm, 1.7 µm column combined with a C8 pre column (Phenomenex, Utrecht, The Netherlands), kept at 50 °C. A gradient of water and Methanol with 0.01% acetic acid was used. The injection volume was 40 µL and the flow rate 400 µL/min. MRM transitions used to identify LM were based on previous work by the group of M. Giera [1]. Peaks were integrated with manual supervision and area corrected to corresponding IS (RRT) with MultiQuant™ 2.1 (Sciex, MA, USA). Only peaks with a signal to noise (S/N) > 10 were quantified. Calibration curves were constructed using authentic synthetic standards. 8-HETE, 18-HEPE, 17-HDHA, 15-HEPE, 14,15-diHETE, 19,20-diHDPA, PGF2α, TXB2, PGE2, PGD2, 15-HETE, 17-OHHETE, AA, DHA, LTB4, 5-HETE, 11-HETE, EPA, AdA, LA, 12-HETE, LXA4, AT-LXA4, LXB4, RvD1, RvD2, MaR1, LTD4, LTE4, RvE1, RvE2, PD1, PDx, 15S-HETE-d8, LTB4-d4, PGE2-d4 and DHA-d5 were purchased from Cayman Chemicals (Ann Arbor, MI, USA). RvE1, RvE2, 18S-RvE3 and 18R-RvE3 were kind gifts from Dr. Makoto Arita (Tokyo, Japan).

### 2.4. RNA isolation and real time PCR

Total RNA was extracted using RNA elution columns RNeasy mini kit (Qiagen, San Diego, USA) followed by treatment with DNAse I, amp grade (Invitrogen, Waltham, USA) and recombinant RNasin (Promega, Madison, USA). RNA purity was checked by Nanodrop2000 (Thermo Fisher Scientific Inc., Rockford, IL, USA) and a 260/230 ratio ~2,0 was considered satisfactory. Synthesis of cDNA was performed directly after RNA extraction with SuperscriptIII and Ligo(dt)12-18primer (Invitrogen, Waltham, USA). Real-time PCR for 15-LOX type 2 (gene; ALOX15B) and COX-2 (gen; PTGS2) were performed using SensiFAST probe (Bioline, London, UK), while cPLA2 (gen; PLA2G4A) was performed using SYBR Green PCR Master Mix (Applied Biosystems, Madison, USA). The following forward (5′–3′) and reverse primers (5′–3′) were used: ALOX15: 5′-TGGAAGGACGGGTTAATT CTGA-3′ 5′-GCGAAACCTCAAAGTCAACTCT-3′ ALOX15B: 5′-GACCCTGCT ATACCAGAGCC-3′ 5′-ACCAGTCCCACTTGTCATCAG-3′ PTGS2: 5′-TAAGT GCGATTGTACCCGGAC-3′ 5′-TTTGTAGCCATAGTCAGCATTGT-3′ PLA2 G4A: 5′-ATGGATGAAACTCTAGGGACAGC-3′ 5′-CTGGGCATGAGCAAACT TCAA-3′. The expression of each gene was calculated using the cycle threshold (Ct) method and normalized to the expression of the endogenous references genes Beta-2-Microglobulin (B2M): forward primer 5′-GATCGA GACATGTAAGCAGC-3′, reverse primer 5′-TCAAACATGGAGACAGCAC-3′ and 60s Ribosomal Protein L5 (RPL5): forward primer 5′-TGGAGGTGACT GGTGATG-3′ and reverse primer 5′-GCTTCCGATGTACTTCTGC-3′. Amplification specificity was confirmed by melting curve analysis and standard curves were included during every run to determine efficacy. Fold

induction in expression of each gene was calculated by comparing the mean of three biological triplicates of LPS-treated to untreated samples at each time point.

### 2.5. Western blotting and ELISA

Cells were pelleted and lysed using NP-40 lysis buffer: 50 mM Tris-HCl (pH 7.5), 120 mM NaCl, and 1% (*v*/v) nonidet P-40 (NP-40), supplemented with phosphatase inhibitors (25 mM sodium fluoride, 1 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate, 2 μM microcystin LR (Alexis)), and protease inhibitors 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM benzamidine and cell lyzates were kept on ice for 15 min. Next, the samples were centrifuged (10,000 rpm, 10 min, 4 °C) and protein concentrations in the supernatants were determined using the Pierce bicinchoninic acid (BCA) method according to the manufacturer's instructions (Thermo Fisher Scientific Inc., Rockford, IL, USA). Subsequently, equal quantities of the cell lysates (30 μg total protein) were loaded and separated on 10% polyacrylamide gels via electrophoresis (SDS-PAGE) and proteins were electrophoretically blotted onto a nitrocellulose membrane (Odyssey, LI-COR Biosciences, Bad Homburg, Germany). Membranes were incubated in Odyssey blocking reagent (LI-COR Biosciences, Bad Homburg, Germany), followed by treatment with the respective primary antibodies directed against COX-2 (mouse monoclonal, Santa Cruz Biotechnology, sc-19999 Heidelberg, Germany), 5-LO (mouse monoclonal, Santa Cruz Biotechnology, sc-515821, Heidelberg), 15-LO-2 (rabbit polyclonal, Oxford Biomedicals, LX-25, Rochester Hills, MI, USA OLD mouse monoclonal, Santa Cruz Biotechnology, sc-, Heidelberg, Germany NEW), or β-actin (goat, polyclonal, Santa Cruz Biotechnology, sc-1616, Heidelberg, Germany). After several washing steps and incubation with the corresponding IRDye680- or IRDye800-conjugated secondary antibodies (LI-COR Biosciences, Bad Homburg, Germany), immunoreactive bands were visualized on the Odyssey Infrared Imaging System (LI-COR Biosciences, Bad Homburg, Germany).

ELISAs were performed according to the manufacturer's protocols: IL-10 PeliPair reagent set (Sanquin, Amsterdam, The Netherlands), TNFα OptEIA (BD, San Jose, USA) and IL-12/IL-23 p40 (R&D Systems).

### 2.6. Statistical analysis

Heat maps were generated using Multiple Experiment Viewer Java tool (MeV version 4.7 Artistic License version 2.0, Harvard, USA) and hierarchical clustering was performed by Spearman's rank correlation. Data are expressed as the mean ± SD. Different treatment conditions were compared using Wilcoxon matched-pairs signed rank test and Two-way ANOVA in GraphPad PRISM software version 7.00 (San Diego, USA) and a *p*-value of 0.05 or less was considered significant.

## 3. Results

### 3.1. M-CSF and GM-CSF cultured monocyte-derived macrophages have distinct temporal LM profiles upon LPS stimulation

To evaluate the temporal effect of LPS on the LM profile of both M-CSF and GM-CSF MDM, we measured 37 different lipids of which we could confidently quantify 21 in the total fraction containing both supernatant and cells. By calculating the relative level of each lipid in treated relative to untreated samples within a donor, the biosynthesis of each lipid over time could be assessed using a Spearman's rank correlation clustering (Fig. 1).

Many of the mono-hydroxylated LM: 5-Hydroxy-Eicosatetraenoic acid (5-HETE), 17-hydroxy-Docosahexaenoic Acid (17-HDHA), 8-hydroxy-Eicosatetraenoic acid (8-HETE) and 15-hydroxy–Eicosapentaenoic acid (15-HEPE) were detected in GM-CSF but not in M-CSF MDM. In contrast, Di-hydroxylated products: 14,15diHETE and 19,20-DiHDPA were produced concurrently and increased during culture in both types of MDM. Polyunsaturated fatty acids (PUFA): Arachidonic acid (AA), Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA), Adrenic acid (AdA) and Linoleic acid (LA) were also detected in both cell types but their levels decreased during culture. However, the relative abundance of di-hydroxylated products and PUFA did not change upon LPS stimulation (Fig. 1A, B).

Solely based on the lipids that increased upon LPS stimulation, we identified two distinct clusters in GM-CSF and only one cluster in M-CSF MDM, indicating that different macrophage subsets respond differentially to LPS stimulation in relation to the lipids that are produced. $PGD_2$, $PGE_2$, $PGF_2$ and $TxB2_2$, which are all COX derived products from AA, cluster together in both types of MDM. The levels of these COX derived products are low in the untreated MDM but increase upon the first 6 h of LPS stimulation (Fig. 1A, B). Interestingly, in GM-CSF MDM we observed a second cluster of 15-LOX derived ω-3 LM, 15-HEPE and 17-HDHA. Both lipids were detected only from 24 h LPS stimulation onwards (Fig. 1A).

### 3.2. LPS induces a temporal switch from COX derived ω-6 LM towards 15-LOX derived ω-3 mono-hydroxylated FA in GM-CSF cultured monocyte-derived macrophages

To further evaluate the temporal LM profile upon persistent LPS stimulation in GM-CSF MDM we depicted lipid concentrations in the individual donors over time. Despite donor variation in concentrations of different lipids, the trend within donors is clearly in concordance with the observation that $PGD_2$, $PGE_2$, $PGF_2$ and $TxB2_2$ increase in the first 6 h (Fig. 2A) and 15-HEPE and 17-HDHA in the subsequent 24 h (Fig. 2B). Furthermore we observe an increase of 17-HDHA and 15-HEPE (Fig. 2B) but not 15-HETE, a 15-LOX product of AA (Fig. 2A). The increase of different LM and mono-hydroxylated FA upon LPS stimulation is not reflected in the levels of their precursors AA, (Fig. 2A) DHA or EPA (Fig. 2B) which do not change upon LPS stimulation.

Next we evaluated if the enzymes; cPLA2, 5-LOX, COX-2 and 15-LOX, responsible for the synthesis of LM, show the same trend as the lipid levels over time. In parallel to the lipid fraction, RNA was isolated from total cell pellet of the same donors and was used for RT-qPCR. Although cPLA2-expression appeared to be affected by LPS-treatment, donor-to-donor variation was observed: in two of the GM-CSF and in one of the M-CSF cultured macrophage donors an increase in cPLA2 RNA expression was observed in the first 6 h of LPS stimulation (Fig. 3A). 5-LOX was not affected by LPS treatment in either type of MDM (Fig. 3B). In concordance with the lipid data all donors showed an increase in COX-2 RNA expression upon LPS stimulation (300–900 fold) in the first 6 h (Fig. 3C). Although we did not detect 15-LOX type 1 RNA levels (data not shown), 15-LOX type 2 increased (2–6 fold) after 24 h of LPS stimulation in both types of MDM in all donors (Fig. 3D).

On protein level, 5-LOX was not affected by LPS treatment but was constitutively expressed over time (Fig. 4A). Due to technical difficulties, we were unfortunately unable to study expression of cPLA2. The expression of COX-2 was highest at 6 h of LPS treatment (Fig. 4A) and COX-2 expression in GM-CSF MDM was relatively higher compared to M-CSF MDM based on the COX-2/β-actin ratio (Fig. 4B). Comparable to the RNA expression, we were not able to detect 15-LOX type 1 (data not shown), while 15-LOX type 2 protein levels increased after 24 h. This increase by LPS treatment was strongest in the GM-CSF MDM (Fig. 4A, C). Based on both the enzyme and lipid data, these findings indicate a switch from COX-2 to 15-LOX type 2 synthesized lipids during LPS stimulation in GM-CSF MFM. Although we did see a similar expression pattern of the enzymes in M-CSF MDM this was relatively lower on protein level and did not result in the production of 15-LOX derived ω-3 mono-hydroxylated FA.

### 3.3. 17-HDHA has anti-inflammatory properties

While COX-2 produced prostaglandins are well known mediators

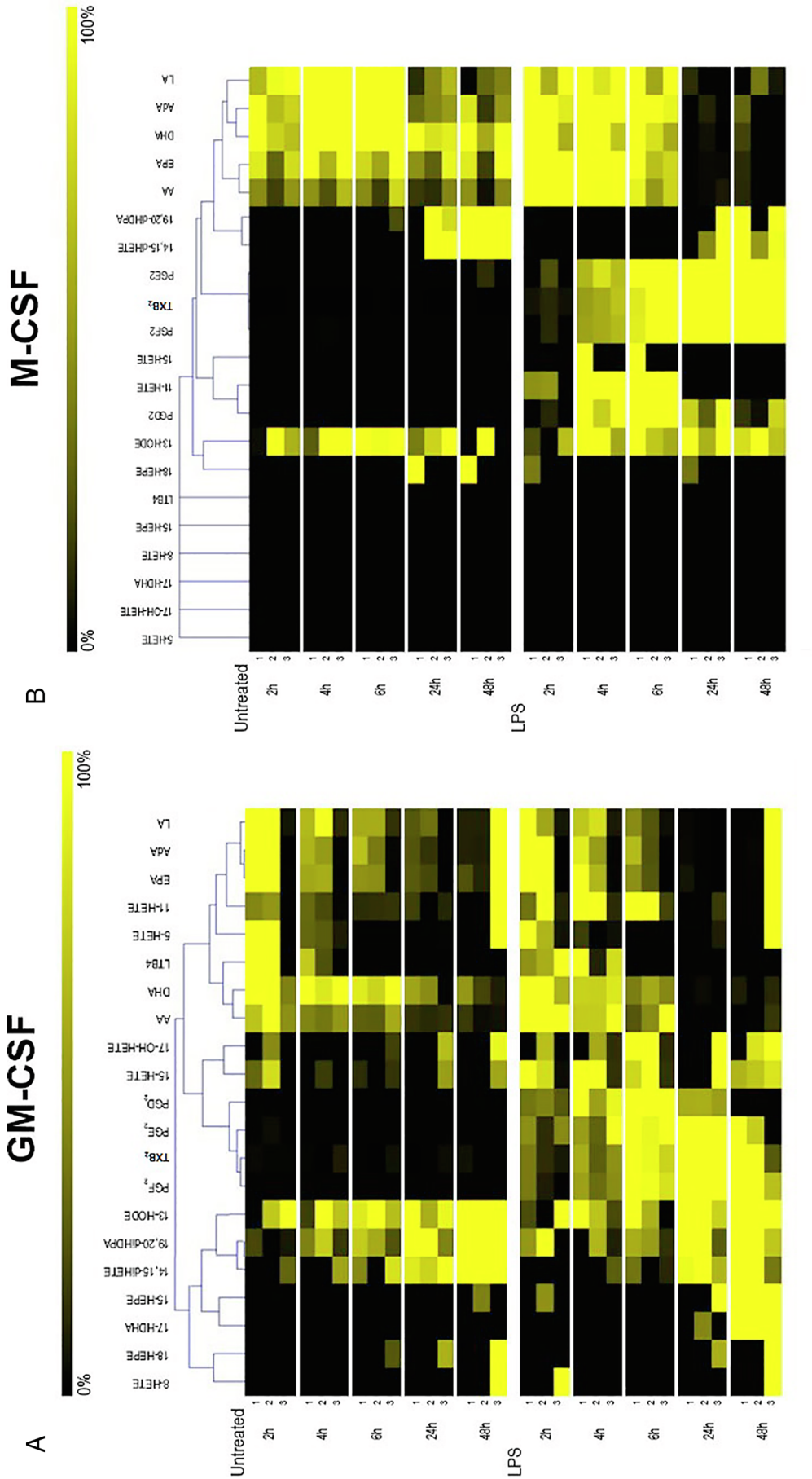
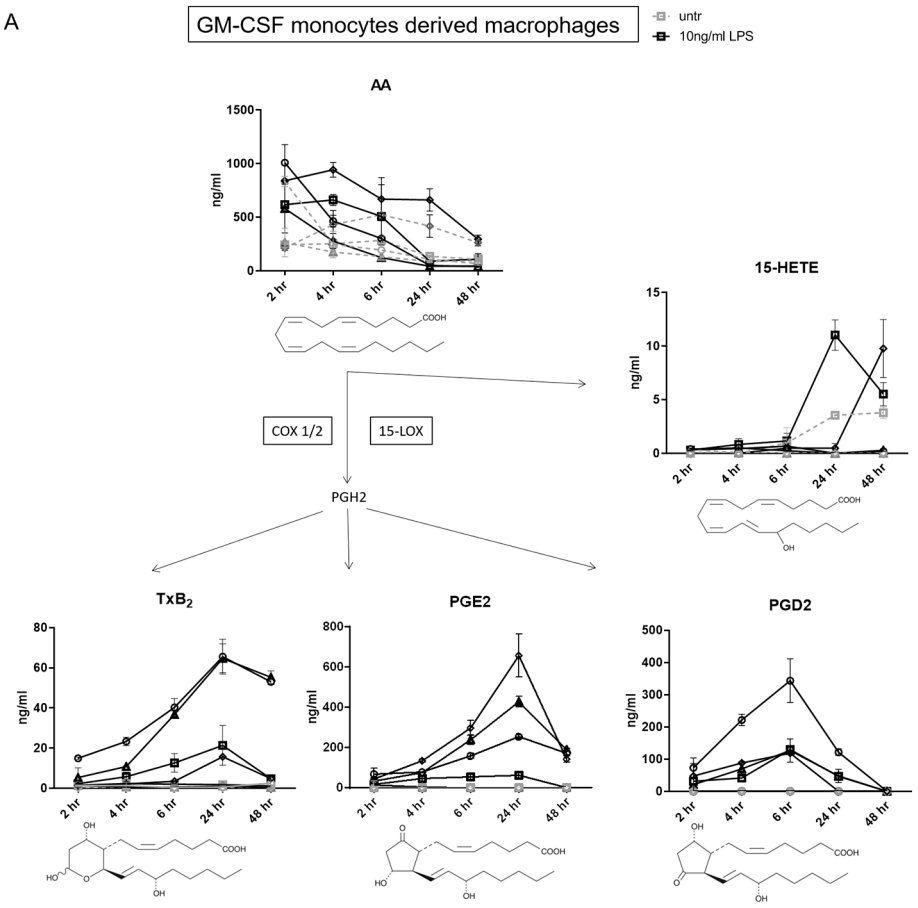



**Fig. 1.** Human monocyte-derived macrophages have distinct temporal LM profiles upon LPS stimulation. Heat map showing the hierarchical clustering of lipids at different time periods of LPS stimulation (y-axis) in monocyte-derived macrophages. Shown are the results for GM-CSF cultured monocyte-derived macrophages(**A**) and M-CSF cultured monocyte-derived macrophages in three individual donors (**B**). The lipid profile was measured by LC-MS/MS and lipids were included if the intensity of the signal in the MS/MS was at least ten times higher than background. Relative expression (%) within donors was calculated by comparing individual lipid levels to the highest concentration measured per type of lipid followed by visualization with Multiple Experiment Viewer. Donors were the same as the donors depicted in Figs. 2 and 3. Clustering was performed by Spearman's rank correlation.



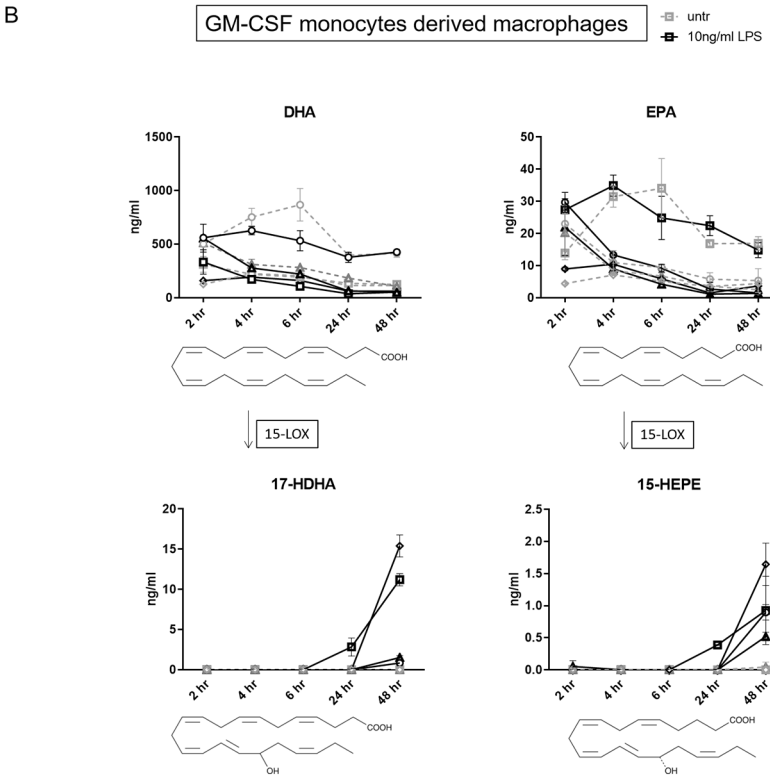




**Fig. 2.** LPS induces a temporal switch from COX derived ω-6 LM's towards 15-LOX derived ω-3 mono-hydroxylated FA's in GM-CSF cultured monocytes. Concentrations of PUFA's and their downstream products at different time periods of LPS stimulation in GM-CSF cultured monocyte-derived macrophages measured by LC-MS/MS in ng/ml (ng/10^6 cells). (**A**) Depicted are the concentrations of AA (omega 6) COX products TxB2, $PGE_2$, $PGD_2$ and 15-LOX derived 15-HETE, (**B**) concentrations of DHA and EPA (omega 3) and their downstream 15-LOX products 17-HDHA (DHA) and 15-HEPE (EPA) at different time periods of LPS stimulation in GM-CSF cultured monocyte-derived macrophages. Samples contained both supernatant and cells of four donors (D1 = O, D2 = □, D3 = Δ, D4 = ◊). Donor 1–3 were the same donors as the ones depicted in Figs. 1 and 3. Data are depicted of four donors as mean ± SD of triplicates that were generated by stimulating GM-CSF cultured macrophages in parallel wells.

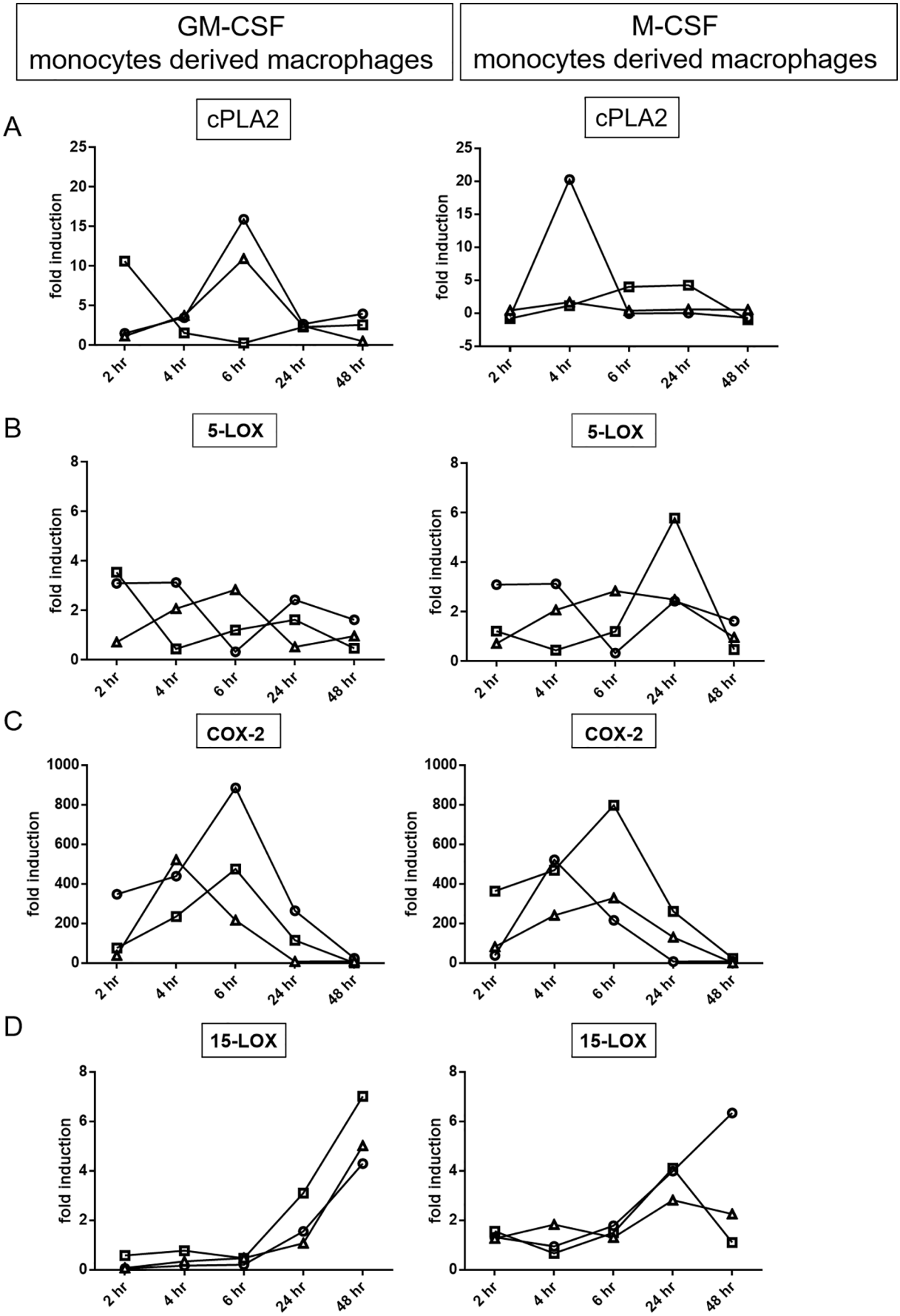



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**Fig. 3. LPS** induced expression of first COX-2 RNA followed by expression of 15-LOX type 2 RNA in human monocyte-derived macrophages. Temporal expression of (**A**) cPLA2, (**B**) 5-LOX, (**C**) COX-2 and (**D**) 15-LOX type 2 in LPS treated monocyte-derived macrophages. RNA levels were determined in cell pellets of both GM-CSF and M-CSF cultured monocytes by RT-qPCR. Fold induction was calculated by comparing RNA levels in LPS-treated samples to untreated samples at corresponding time periods using the mean of duplicate measurement that were normalized to household gene (RPL5). Graph shows three donors (D1 = O, D2 = □, D3 = Δ) These donors are the same donors as depicted in Figs. 1 and 2.

that initiate the acute proinflammatory phase triggered by LPS, whether 15-LOX derived ω-3 mono-hydroxylated FA can have an opposite function is less well studied. To determine if 17-HDHA has anti-inflammatory properties, we measured its effects on MDM and neutrophils. The LPS-induced TNFα and IL-10 levels were measured upon pre-treatment with 17-HDHA in both types of MDM. The level of IL-10 was below our detection limit in GM-CSF MDM and TNFα was very low in M-SCF MDM (data not shown). The levels of TNFα in LPS treated GM-CSF MDM were within the linear range of the ELISA but did not significantly decrease or increase when pretreated with 17-HDHA. In contrast, IL-10 levels were significantly ($p < 0,05$) increased in LPS-treated M-CSF MDM upon pre-treatment with 17-HDHA (Fig. 5A). In

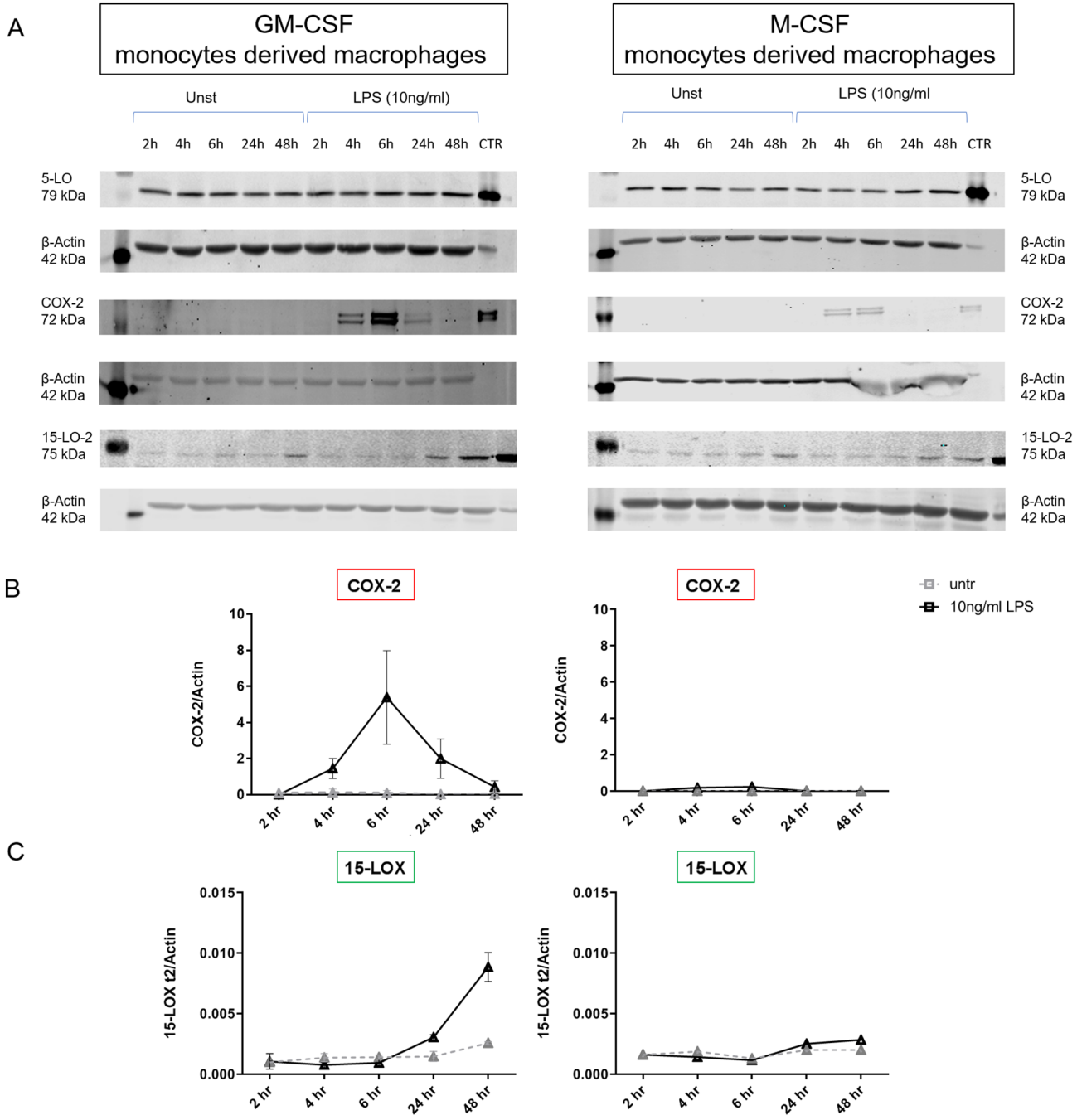


**Fig. 4.** LPS induced expression of COX-2 and 15-LOX type 2 protein levels in a temporal fashion in human monocyte-derived macrophages. Temporal expression of 5-LOX, COX-2 and 15-LOX type 2 in LPS treated monocyte-derived macrophages. (**A**) Data of one representative donor are shown for the protein expression of 5-LOX, COX-2 and 15-LOX type 2. (**B**) Relative levels of COX-2 or (**C**) 15-LOX type 2 to β-actin at different timepoints are presented. Values represent mean ± SD of biological triplicates ($n = 3$).

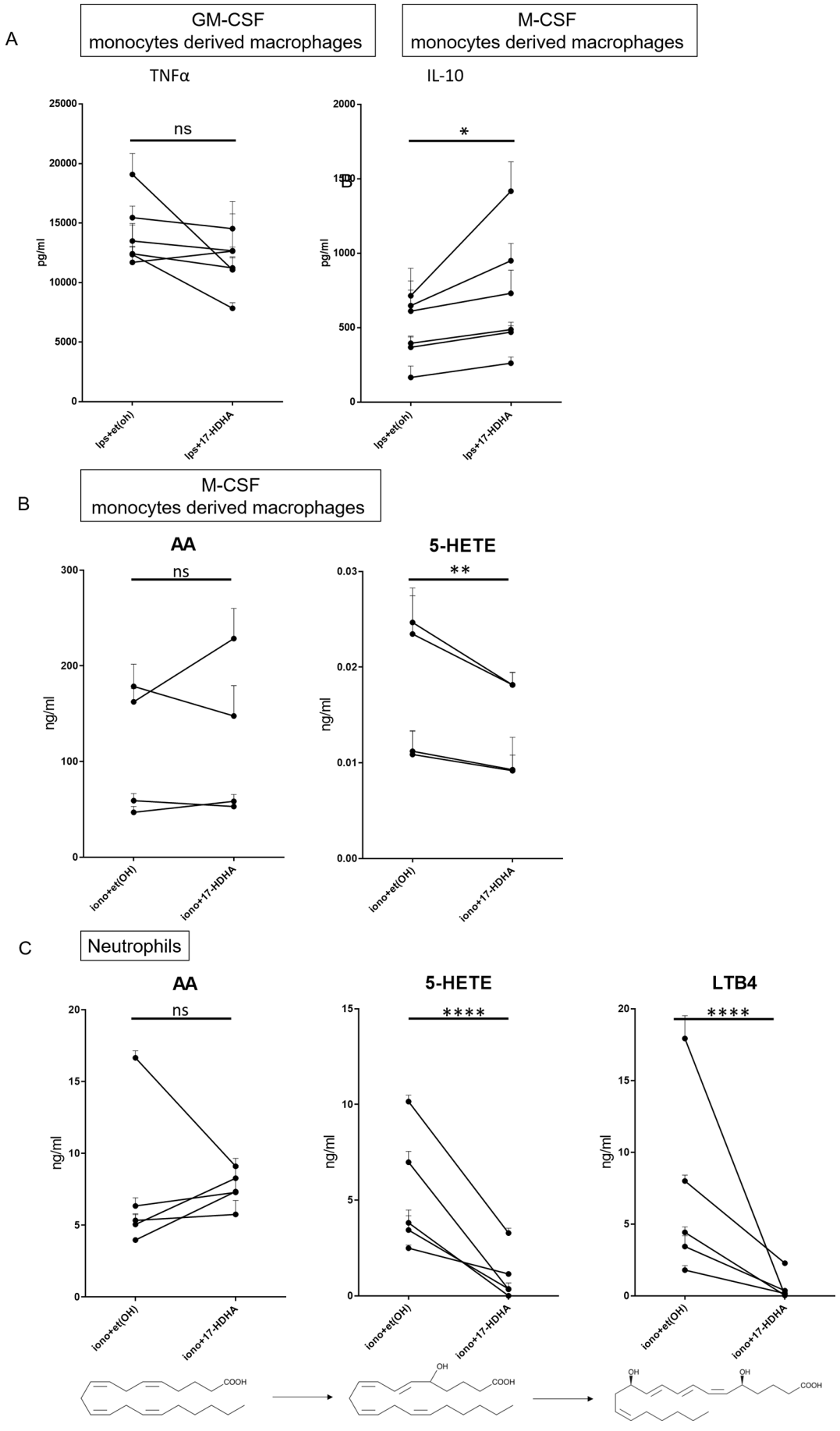


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**Fig. 5.** 17-HDHA has anti-inflammatory properties in M-CSF monocyte-derived macrophages and neutrophils. (**A**) TNFα levels (ELISA) in the supernatant of LPS stimulated GM-CSF monocyte-derived macrophages. IL-10 levels (ELISA) in the supernatant of LPS stimulated M-CSF cultured macrophages. Cells were either pretreated for 24 h with 17-HDHA (100 ng/ml) or vehicle control. Values are mean ± SD of biological triplicate ($n = 6$). Statistically significant differences are indicated by asterisks (Wilcoxon matched-pairs signed rank test, number of pairs = 6, $p < 0.05$) (**B**) AA and 5-HETE (LC-MS/MS) in 10 min ionophore (4 μM) stimulated M-CSF monocyte-derived macrophages ($n = 4$). (**C**) AA, 5-HETE and $LTB_4$ levels (LC-MS/MS) in 10 min ionophore (4 μM) stimulated neutrophils. Cells were either pretreated for 15 min with 17-HDHA (5μg/ml) or vehicle control. Values are mean ± SD of biological triplicate ($n = 5$) Statistically significant differences are indicated by asterisks, (Two-way ANOVA number of pairs = 5, $p < 0.05$) ns $P > 0.05$ * $P \leq 0.05$ ** $P \leq 0.01$ *** $P \leq 0.001$ **** $P \leq 0.0001$.

parallel to the cytokines, we also measured the different LM in LPS treated MDM. $PGD_2$ and $PGE_2$ levels did not change in GM-CSF MDM and showed a slight positive trend in M-CSF MDM after 24 h of 17-HDHA treatment (Suppl table 1.1).

Previous studies have assessed the pro-resolving/anti-inflammatory functions of SPM by evaluating neutrophil infiltration and activity [7,31]. Neutrophils are key players in the acute inflammatory response and perpetuate the response via an autocrine loop that is regulated through the secretion of $LTB_4$ [36]. To address the pro-resolving/anti-inflammatory potential of 17-HDHA, we pretreated human neutrophils with 17-HDHA and measured the $LTB_4$ levels induced upon calcium ionophore stimulation. We observed a strong decrease in the levels of $LTB_4$ and its precursor 5-HETE in human neutrophils (Fig. 5C). M-CSF MDM did not produce $LTB_4$, but when pretreated with 17-HDHA we did observe a trend in the reduction of 5-HETE (Fig. 5B). Interestingly, 15 min incubation of M-CSF MDM and neutrophils with 17-HDHA (5μg/ml) also increased the levels of 15-HETE, a precursor of SPM. (Suppl Table 1.1). To further investigate the activation of the pro-resolving pathways and biosynthesis of classical SPM, we measured LXA4, AT-LXA4, LXB4, RvD1, RvD2, MaR1, RvE1, RvE2,18S-RvE3, 18R-RvE3, PDx and PD1. In GM-CSF MDM and neutrophils pretreated with 5μg/ml 17-HDHA, we did find traces of both PDx and PD1 but those were below our detection limit (Suppl 1.1). These data indicate that 17-HDHA can have anti-inflammatory effects in both MDM and neutrophils.

## 4. Discussion

The data presented in this paper demonstrate that MDM can initiate a self-resolving anti-inflammatory response through biosynthesis of pro-resolving/anti-inflammatory LM induced upon persistent LPS stimulation. We found a LM class switch from COX derived ω-6 LM towards 15-LOX derived ω-3 mono-hydroxylated FA in human GM-CSF MDM. We also show that 17-HDHA, one of the LM that are produced during persistent LPS stimulation, has anti-inflammatory properties in both macrophages and neutrophils. A persistent pro-inflammatory signal could therefore lead to initiation of resolution via the production of 15-LOX derived ω-3 mono-hydroxylated FA.

Despite considerable donor-to-donor variability, overall our data indicate that COX derived LM increase upon the first 6 h of LPS stimulation in GM-CSF MDM. Others have found a similar trend in PGE2 and PGD2 in both murine [10] and human macrophages [17,18] but did not detect ω-3 mono-hydroxylated FA at later timepoints. In their models the dose of the inflammatory signal was however high compared to ours, potentially underlying the observed differences [22,23]. In our experiments the levels of PGE2 and TXB2 are higher than levels of 15-HEPE and 17-HDHA at 24 and 48 h. However, our data also indicate that their levels decrease in time, while the levels of 15-HEPE and 17-HDHA increase after 24 h, suggesting that the relative levels of these lipid classes will be inversed at a later timepoint, although we did not formally prove this.

We did not detect 15-LOX derived ω-3 mono-hydroxylated FA in the M-CSF cultured monocytes. There have been reports of 15-LOX derived LM in M2 like cells in the presence of IL-4 [17,21] and 15-LOX type 1 expression in human macrophages was shown to be dependent on IL-4 [18,21]. We did not culture the MDM in the presence of IL-4 and therefore did not find increased expression of 15-LOX type 1. We did however detect an increase in 15-LOX type 2 RNA levels in both M-CSF and GM-CSF MDM. Both 15-LOX type 1 and 15-LOX type 2 have the potential to catalyze the hydroxylation of PUFA [19,20]. The lack of 15-LOX derived ω-3 mono-hydroxylated FA in M-CSF cultured macrophages could be due to lower protein expression of 15-LOX type 2. Another possibility is that the metabolic status of M-CSF MDM, preferring oxidative metabolism to glycolysis [30], could lead to the usage of FA in beta oxidation to produce acetyl-CoAs that feed into the Krebs cycle to fulfill the energy demands of the cell instead of being used to synthesize ω-3 mono-hydroxylated FA [25].

We performed our experiments in the absence of FCS because we did not wish to introduce high amounts of FA and their autoxidation products in the system. The limited availability of free FA in our culture could explain the LPS independent increase of the di-hydroxylated products 14,15diHETE and 19,20-DiHDPA in both type of MDM. Di-hydroxylated products are generated by the enzyme family cytochrome P450 followed by soluble epoxide hydrolase. Cytochrome P450 is known to increase in macrophages upon serum starvation in vitro [24]. Because during culture PUFA decreased and di-hydroxylated products increased, it is likely that PUFA are converted in their respective P450 products. However, since this effect was independent of LPS, we did not investigate P450 expression to address this further.

Despite the presence of detectable levels of both 5-LOX and 15-LOX in both M-CSF and GM-CSF MDM and the ability of our platform to detect SPM [1,33], we could not detect any of the SPM in our culture system, except traces of PD1 and PDx in cells treated with 17-HDHA for 24 h. We cannot, however, exclude the possibility that 17-HDHA is converted into other lipids and thereby has an indirect effect on the cells. The fact that other studies have shown the presence of SPM in macrophage cultures upon LPS exposure could be due to the presence of serum, which is a rich source of PUFA, especially AA that can be further metabolized into SPM [37].

Compared to others that detect SPM in human macrophages we also excluded secondary signals such as cytokines in our model system. Cytokines and secondary inflammatory signals can alter the expression of the enzymes involved in the synthesis of LM [10,18,39], therefore the synthesis of these lipids by macrophages in vivo will probably be determined by the microenvironment of the tissue in which they reside. Alternatively, it is possible that while an initial inflammatory signal leads to generation of mono-hydroxylated products, a second inflammatory signal is needed for subsequent oxidative steps that lead to generation of the final pro-resolving products [10] While our culture system is merely a simplified model of the in vivo inflammatory milieu, we believe our study provides a proof-of-concept for a qualitative change in macrophages response during long-term stimulation.

The pro-resolving effect of RvDs has been extensively studied in both human and mice [7] however the biological activity of 17-HDHA has been studies far less [2,3,5,6] although they share structural similarities. In murine models 17-HDHA reduces signs of dextran sulfate sodium induced colitis [5], inhibits NLRP3 inflammasome activation in podocytes [4], facilitates the differentiation towards the M2 phenotype and increases the phagocytotic capacity of macrophages [5,6] In humans, increased plasma 17-HDHA levels were associated with lower pain scores in osteoarthritis patients [2] and isolated human B cells differentiate towards antibody-secreting cells [3].

To assess the inflammatory properties of 17-HDHA in vitro we pretreated cells with similar concentrations used in other studies [5,35,38]. Although we did not evaluate the half-life of 17-HDHA, we

could still detect approximately 5.5% of the initial 17-HDHA after 24 h in M-CSF MDM (Suppl 1.1). It was previously shown that 17-HDHA can reduce TNFα levels in a murine cell line [35]. We did not observe a significant change in the LPS-induced TNFα responses in GM-CSF MDM upon pre-incubation with 17-HDHA. This was probably due to the inter-donor variation, as we did observe a trend towards less TNFα and 5-HETE in most donors. Neutrophils are key players in the acute inflammatory response and perpetuate the response via an autocrine loop that is regulated through the secretion of $LTB_4$ [36]. Murine neutrophils produce less 5-HETE and $LTB_4$ after being exposed to 17-HDHA in vivo [5,34], we found similar results in isolated human neutrophils and M-CSF MDM. To our knowledge, this is the first report on the anti-inflammatory effects of 17-HDHA on human primary cells.

One limitation of this study is the low number of samples tested and the donor-to-donor variation in the results. However we believe that our data indicate that LPS stimulated macrophages can initiate a lipid class switch from pro- to anti-inflammatory lipid mediators in a single cell system through temporal regulation of lipid modifying enzymes. These findings support the concept of inflammation and resolution as an active process opening up new opportunities for a better understanding of chronic inflammatory disease.

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## CRediT authorship contribution statement

**Johannes H. von Hegedus:**Investigation, Writing - original draft.**Astrid S. Kahnt:**Investigation.**Roland Ebert:**Investigation. **Marieke Heijink:**Investigation.**Rene E.M. Toes:**Supervision.**Martin Giera:**Investigation.**Andreea Ioan-Facsinay:**Supervision, Investigation, Writing - original draft.

## Declaration of competing interest

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