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Long-term stimulation of toll-like receptor-2 and -4 upregulates 5-LO and 15-LO-2 expression thereby inducing a lipid mediator shift in human monocyte-derived macrophages

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ABSTRACT

Macrophage polarization switches during the course of inflammation along with the lipid mediators released. We investigated the lipid mediator formation in human monocyte-derived macrophages during *in vitro* differentiation and pathogen stimulation. For this, peripheral blood monocytes were differentiated into M1 (CSF-2/IFNγ) or M2 (CSF-1/IL-4) macrophages followed by stimulation with the toll-like receptor (TLR) ligands zymosan (TLR-2), Poly(I:C) (TLR-3) or bacterial lipopolysaccharides (TLR-4) mimicking fungal, viral and bacterial infection, respectively. Expression of enzymes involved in lipid mediator formation such as 5- and 15-lipoxygenases (LO), the 5-LO activating protein and cyclooxygenase-2 (COX-2) was monitored on mRNA and protein level and lipid mediator formation was assessed. In addition, cytokine release was measured. *In vitro* differentiation of human peripheral blood monocytes to M1 and M2 macrophages considerably attenuated 5-LO activity. Furthermore, while TLR-2 and -4 stimulation of M1 macrophages primarily triggered pro-inflammatory cytokines and lipid mediators, persistent stimulation (16 h) of human M2 macrophages induced a coordinated upregulation of 5- and 15-LO-2 expression. This was accompanied by a marked increase in IL-10 and monohydroxylated 15-LO products in the conditioned media of the cells. After additional stimulation with Ca^{2+} ionophore combined with supplementation of arachidonic, eicosapentaenoic and docosahexaenoic acid these cells also released small amounts of SPM such as lipoxins and resolvins. From this we conclude that activation of TLR-2 or -4 triggers the biosynthesis of pro-inflammatory 5-LO and COX-2 derived lipid mediators in human monocyte-derived M1 macrophages while persistent stimulation of M2 macrophages induces a shift towards pro-resolving 15-LO derived oxylipins.

1. Introduction

Inflammation is the response of the immune system to injury or infection aiming at eliminating the inciting stimulus in order to restore organ function [1]. During the time-course of an acute inflammatory event, the sequential influx of different leukocyte subsets such as neutrophils, monocytes/macrophages and B and T lymphocytes assures the effective development of an acquired immune response, the clearance of pathogens and cell debris as well as tissue repair and finally resolution [2]. A complicated network of soluble mediators released by immune cells such as cytokines, reactive oxygen species and bioactive lipids coordinates this process [3].

Macrophages belong to both the innate as well as the acquired immune system. During inflammation, these versatile cells clear

Abbreviations: AA, arachidonic acid; β2M, β_2 microglobulin; COX, cyclooxygenase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FLAP, 5-LO activating protein; fMLF, *N*-formylmethionine-leucyl-phenylalanine; LO, lipoxygenase; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; LLOQ, lower limit of quantification; LT, leukotriene; MDM, monocyte-derived macrophage; PAMP, pathogen-associated molecular pattern; PBMC, peripheral blood mononuclear cells; PG, prostaglandin; LX, lipoxin; Rv, resolvin; SPM, specialized pro-resolving mediator

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pathogens by phagocytosis, engulf cellular debris, shape the adaptive immune response by antigen presentation and release a mediator cocktail containing cytokines, lipid mediators and reactive oxygen species [3]. Polarization of macrophages can switch during inflammation depending on the inflammatory milieu [4]. M1 macrophages differentiate in the presence of IFNγ, TNFα and pathogen-derived molecules such as bacterial LPS. These cells are active phagocytes which clear the inciting stimulus, recruit other immune cells and trigger Th_1 responses [5]. In contrast, M2 macrophages differentiate in the presence of the Th_2 cytokines IL-4 or IL-13 and possess anti-inflammatory and tissue repairing properties [5]. These cells display low phagocytic activity, participate in tissue remodeling and wound-healing. Furthermore, a group of pro-resolving macrophages is triggered by anti-inflammatory mediators such as IL-10 or glucocorticoids. These macrophages emerge at the turning point of an inflammatory reaction to shape the adaptive immunity and resolve inflammation by efferocytosis of apoptotic debris thereby dampening the ongoing immune response [6]. Along with the switch in the cell's phenotype during inflammation, profound changes in the cytokine and lipid mediator pattern of the macrophages take place from pro-inflammatory to anti-inflammatory / pro-resolving mediators [7].

Lipid mediators derive from various polyunsaturated fatty acids such as arachidonic (AA), docosahexaenoic (DHA) or eicosapentaenoic (EPA) acid. Leukotrienes (LT) are the first lipid mediators released by invading granulocytes and monocytes/macrophages during inflammation upon encounter with an inciting stimulus. For this, AA is readily released from membrane phospholipids by phospholipase A_2 enzymes and subsequently converted by 5-lipoxygenase (5-LO) with the help of FLAP (5-LO activating protein) to LTB_4 and cysteinyl LTs which are potent inducers of leukocyte influx and vascular permeability [8]. From animal data it is known that after a few hours of persistent inflammation, the production of LTs is followed by an elevation in prostaglandin (PG) release. Here, danger-associated molecular patterns such as bacterial lipopolysaccharides, viral RNA/DNA and fungal cell membrane constituents trigger AA liberation and upregulate the expression of the central PG synthase cyclooxygenase-2 (COX-2) along with its' downstream synthases. PGs such as PGE_2 play a central role in inflammation contributing to the initiation of pain, swelling, fever and also immune cell differentiation and maturation thereby triggering the cardinal signs of inflammation. Furthermore, PGs are also involved in chronic inflammation as well as immune suppression and resolution [9,10]. Biosynthesis of specialized pro-resolving lipid mediators (SPM) such as lipoxins (LX), D- and E-series resolvins (RvD/E), maresins and protectins is thought to take over during the switch from inflammation to resolution [11]. In murine inflammation, these mediators were shown to terminate leukocyte trafficking, to shape the adaptive immunity and to coordinate efferocytosis of dying neutrophils as well as the egress of macrophages into the lymphatics.

Most interestingly, SPM such as LX and Rv are formed by the concerted action of two enzymes already centrally involved in the biosynthesis of pro-inflammatory lipid mediators. LX are formed from AA by the interplay of 5-LO with 15- or 12-LO whereas D- and E-series Rvs are DHA- and EPA-derived 15-LO/5-LO products [12]. Supplementary fig. 1A–D depicts the time-course of inflammation as well as the biosynthesis pathways of important pro-inflammatory and pro-resolving lipid mediators derived from AA, EPA and DHA.

In the recent years, macrophage pro-resolving lipid mediator formation has been extensively studied in exudates from murine inflammation models such as zymosan induced peritonitis. In addition, supplementation of SPM has been proven to ameliorate a number of diseases in animal models [13,14]. Nevertheless, literature on the formation and stability of SPMs in human tissues during health and disease are contradictory at the moment. For the development of resolution supportive therapies, profound knowledge on the molecular details of SPM biosynthesis during the transition from inflammation to resolution is crucial [15]. However, on the cellular level the nature of the participating immune cells, the stimuli triggering the lipid mediator switch in these cells as well as the complete intracellular enzymatic machinery needed for SPM formation are far from understood in men and mice. Some details of transcellular LX and Rv biosynthesis have been elucidated in human leukocytes so far [14,16–20]. In contrast, SPM formation in human one-cell systems such as macrophages which co-express the essential LOs is less understood and the reported lipid mediator yields are considerably lower compared to pro-inflammatory lipid mediators such as PGs and LTs. In addition, human cells can express two 15-LO isoforms (15-LO-1/-2) while mice have only one 12-/15-LO, the orthologue of human 15-LO-1 with a distinct 12-/15-HETE ratio [21].

In this study, we decided to investigate the lipid mediator formation in human monocyte-derived macrophages (MDM) during *in vitro* generation and pathogen stimulation. For this, peripheral blood monocytes were differentiated into M1 (CSF-2/IFNγ) or M2 (CSF-1/IL-4) macrophages followed by stimulation with bacterial LPS, fungal zymosan or Poly(I:C). Representing the far edges of macrophage polarization, stimulated M1 and M2 macrophages might help to better understand the lipid mediator switch during inflammation as well as unravel the enzymes involved.

2. Material and methods

2.1. Drugs, chemical reagents and other materials

Ultrapure bacterial LPS from *E. coli* (serotype O111:B4) and low molecular weight Poly(I:C) were purchased from InvivoGen (Toulouse, France). Zymosan, dextran, $CaCl_2$, BSA, fMLF and calcium ionophore (A23187) were obtained from Sigma-Aldrich (Steinheim, Germany). PBS, RPMI 1640 cell culture medium and penicillin-streptomycin concentrates were purchased from Gibco Life Technologies (Paisley, UK). Recombinant human colony stimulating factors CSF-1 (M-CSF), CSF-2 (GM-CSF) as well as IFNγ and IL-4 were from PeproTech (Hamburg, Germany). EDTA and SDS were purchased from Merck KGaA (Darmstadt, Germany). Peroxide free AA and EPA as well as DHA were bought from Cayman Chemical (Ann Arbor, MI, USA). Human serum was obtained from the DRK-Blutspendedienst Baden-Württemberg/Hessen (Frankfurt a.M., Germany). UPLC grade methanol and D(+)-glucose were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Ultrapure TRIS, NaCl, Triton X-100, Nonidet P40, Tween 20, glycerol and glycine were purchased from AppliChem GmbH (Darmstadt, Germany). Lipid mediator standards RvE1, RvD1, 17(*R*)-RvD1, RvD2, RvD3, RvD5, MaR1, 7(*S*)-MaR1, PDX, LXA_5, 6(*R*)-LXA_4, 15(*R*)-LXA_4, 6(*S*)-LXA_4, and LXB_4 as well as the deuterated internal standards $^2H_5$-RvD1, $^2H_5$-RvD2, $^2H_5$-LXA_4, $^2H_4$-LTB_4, and $^2H_4$-9,10-DiHOME were purchased from Cayman Chemical (Ann-Arbor, MI, USA). Deuterated 18(*R*)-RvE2, 18(*R*)-RvE3 and 18(*S*)-RvE3 where kindly provided by the lab of Makoto Arita (RIKEN Center for Integrative Medical Sciences, Yokohama City, Japan).

2.2. Purification of monocytes from human peripheral blood mononuclear cells (PBMC)

Monocytes were freshly isolated from leukocyte concentrates (DRK-Blutspendedienst, Frankfurt, Germany). Blood samples were drawn with the informed consent of the patients. For monocyte isolation, leukocytes were isolated by dextran sedimentation for 30 min followed by 10 min centrifugation, without deceleration, at 800 *g* on lymphocyte separation medium (LSM BioWhittaker, Lonza, Cologne, Germany). PBMC sedimented on top of the separation medium were collected and washed twice with PBS. The cell pellet was finally resuspended in wash medium (RPMI1640 supplemented with 1% penicillin/streptomycin).

2.2.1. Purification method I (adherence method)

The cell suspension was transferred to Petri dishes for adherence

(eight 10 cm dishes per donor). After 1 h incubation (37 °C, 5% CO_2, humidified atmosphere), adherent monocytes were washed twice with wash medium to remove the non-adherent cells and were subsequently overlaid with full growth medium (RPMI1640 plus 1% penicillin/streptomycin, 5% heat inactivated human serum) supplemented with cytokines.

2.2.2. Purification method II (CD14 positive selection)

Monocytes were isolated from the PBMC fraction by magnetic bead separation. For this, PBMCs were resuspended in running buffer (0.5 M EDTA/PBS, 0.5% BSA) followed by magnetic bead CD14 positive selection (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions.

2.3. Macrophage differentiation and TLR treatments

Freshly purified, adherent monocytes were differentiated into different macrophage phenotypes for seven days. For this, the cells were grown (37 °C, 5% CO_2, humidified atmosphere) in full growth medium supplemented with either 10 ng/mL CSF-2 (M1) or 10 ng/mL CSF-1 (M2) for 7 days. Medium was replenished every other day during differentiation. For the last 48 h cells were additionally treated with 10 ng/mL IFNγ (M1) or 10 ng/mL IL-4 (M2) (Fig. 2). For TLR activation, differentiated macrophages were treated with either zymosan (50 μg/mL; TLR-2/dectin-1), Poly(I:C) (10 μg/mL; TLR-3) or bacterial LPS (0.1 μg/mL; TLR-4) at different time-points.

2.4. Lipid mediator formation

For determination of lipid mediators in macrophage conditioned media, cell culture supernatants were drawn during incubation and subjected to centrifugation (800*g*, 5 min, 4 °C) to get rid of cell debris. Cell supernatants were then stored at −80 °C until further analysis by LC-MS/MS.

To assess lipid mediator formation in ionophore or fMLF stimulated macrophages, cells were harvested using a cold-shock method. For this, cell supernatants were removed and the adherent cells were incubated for 20 min in ice-cold PBS containing 5 mM EDTA at 8 °C. Subsequently, the now loosely adherent cells were gently scraped with a spatula. After an additional washing step with PBS, cells corresponding to one 10 cm dish of differentiated cells were resuspended in 500 μL PGC buffer (PBS supplemented with 1 mg/mL glucose). After supplementation with $CaCl_2$ (1 mM), lipid mediator formation was triggered by the addition of different stimuli for 15 min at 37 °C in a water bath. Cells were either stimulated with 1 μM fMLF or 5 μM Ca^{2+}-ionophore (A23187). Ionophore stimulation was accompanied by supplementation of precursor fatty acids: either 20 μM AA alone or a lipid mediator cocktail containing AA, EPA and DHA (6 μM each). Subsequently, the reaction was stopped by the addition of 500 μL ice-cold methanol. After this, the samples were centrifuged (800*g*, 10 min, 4 °C) and supernatants were stored at −80 °C until analysis by LC-MS/MS.

For normalization to total protein content, the corresponding cell pellets were resolved in hot (95 °C) lysis buffer (2.2% SDS, 11% glycerol, 56 mM TrisHCl) and total protein content of each sample was measured with the Pierce bicinchoninic acid kit according to the manufacturer's instructions (Thermo Fisher Scientific Inc., Rockford, IL, USA).

2.5. Extraction and detection of lipid mediators by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

2.5.1. Method I

Lipid mediators were analyzed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) as described previously [22]. In brief, analytes were extracted from 200 μL sample using solid phase extraction after spiking with a mixture of isotopically labelled internal standards. Calibration standards and quality control samples were prepared using PBS as surrogate matrix. 200 μL PBS were spiked with 20 μL of a working solution and processed like the samples. Chromatographic separation of the analytes was done using an Agilent 1260 LC system equipped with a Lux Amylose-1® column (250 × 4.6 mm, 3 μm particle size and 1000 Å pore size, Phenomenex, Aschaffenburg, Germany). Analytes were eluted from the column using water: FA (100:0.1, *v/v*) (phase A) and ACN:MeOH:FA (95:5:0.1, *v/v/v*) (phase B) in gradient elution mode. The separation was achieved with within 25 min with a flow rate of 700 μL/min. The quantification of all analytes was performed using a hybrid triple quadrupole-ion trap mass spectrometer QTRAP 5500 (Sciex, Darmstadt, Germany) equipped with a Turbo-V-source operating in negative ESI mode. The analysis was done in scheduled multiple reaction monitoring mode. For all analytes, the concentrations of the calibration standards, quality controls and samples were evaluated by Analyst software 1.6.3 and MultiQuant software 3.0.2 (Sciex) using the internal standard method (isotope-dilution mass spectrometry). Calibration curves were calculated by linear regression with 1/x weighting. Lower limit of quantification (LLOQ) was between 0.1 and 0.2 ng/mL in injected sample extract.

2.5.2. Method II

Extraction of lipid mediators was carried out as described before [23,24]. In brief, 10 μL deuterated IS (100 nM, containing 20 IS including $^2H_8$-12-HETE, $^2H_8$-5-HETE, $^2H_5$-RvD1, $^2H_5$-RvD2, $^2H_5$-LXA$_4$, $^2H_4$-LTB$_4$, $^2H_4$-9,10-DiHOME, $^2H_4$-PGE$_2$, $^2H_4$-PGD$_2$, $^2H_4$-TxB$_2$ and $^2H_{11}$-14,15-DiHETrE), 10 μL antioxidant mixture in methanol (0.2 mg/mL butylated hydroxytoluene, 100 μM indomethacin, 100 μM soluble epoxide hydrolase inhibitor trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (t-AUCB)) and 0.9 mL methanol were added to 1 mL sonicated cell suspension in PGC buffer: methanol (50:50, *v/v*). After 30 min at −80 °C samples were centrifuged (10 min, 20,000*g*, 4 °C), supernatants were transferred to glass vials and evaporated to <50% methanol under a gentle N_2 stream. Samples were diluted with disodium hydrogen phosphate buffer (adjusted to pH 6 with acetic acid) and applied to the preconditioned SPE cartridges (Bond Elut Certify II, 200 mg, 3 mL; Agilent, Waldbronn, Germany). Lipid mediators were eluted with 2 mL ethyl acetate:*n*-hexane (75:25, *v/v*) with 1% acetic acid and solvent was evaporated in a vacuum concentrator. Sample extracts were reconstituted in 50 μL methanol containing 40 nM IS2 (1-(1-(ethylsulfonyl)piperidin-4-yl)-3-(4-(trifluoromethoxy)phenyl)urea) and 5 or 10 μL were injected into the LC-MS/MS system for analysis of the mono- and dihydroxylated fatty acid derivatives plus LTB$_4$, PG and the SPM, respectively. LC-MS/MS analysis was carried out as described before [23,24] and SPMs were quantified using one abundant transition. LLOQ was between 0.2 and 2 nM in injected sample extract (for further information see Table 2).

Data are presented as mean lipid mediator formation per total protein [ng/μg]. Data below LLOQ where set 0 for calculation of means.

2.6. Detection of LTC_4 by enzyme-linked immunosorbent assay (ELISA)

LTC_4 was determined from the same samples that were used for the LC-MS/MS measurements *via* ELISA technique. For this, the Cisbio Bioassays' LTC_4 assay (Cisbio Bioassays, Codolet, France) was used. Experiments were carried out according to the manufacturer's instructions. Afterwards, the signals generated by homogeneous time resolved fluorescence (HTRF) were measured using the HTRF certified Tecan spark system (Tecan Group Ltd., Crailsheim, Germany) with automatic mirror (Dichroic 510) settings. The acceptor wavelength was 665 nm with a bandwidth of 8 nm and an optimal gain of 97. For the donor 620 nm as wavelength with a bandwidth of 10 nm with an optimal gain of 93 was used. Lag time of both data acquisitions was 100 μs.

2.7. Protein extraction and Western blot analysis

Cell pellets from cells corresponding to one Petri dish (10 cm in diameter) were resuspended in 100 μL lysis buffer (20 mM TrisHCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% TritonX100, 0.5% NP-40) supplemented with protease inhibitors (cOmplete™ Mini, Roche Diagnostik GmbH, Mannheim, Germany) and kept on ice for 15 min. Afterwards, the samples were centrifuged (10,000 rpm, 10 min, 4 °C). Protein concentrations in the supernatants were determined using the Pierce bicinchoninic acid method according to the manufacturer's instructions (Thermo Fisher Scientific Inc., Rockford, IL, USA) employing a multiplate reader (infinite M200, Tecan Group Ltd., Crailsheim, Germany). Subsequently, equal quantities of the cell lysates were separated on 10% or 16% 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 (sc-1745 or sc-19999, Santa Cruz Biotechnology, Heidelberg, Germany), COX-1 (4841S, Cell Signalling Technology, Danvers, MA, USA), 5-LO (sc-515821, Santa Cruz Biotechnology, Heidelberg, Germany or 6A12 antibody produced in-house), 15-LO-1 (kind gift from Prof. Erik Claesson, Karolinska Institutet, Stockholm, Sweden or ab119774, abcam, Cambridge, UK), 15-LO-2 (LX-25, Oxford Biomedical Research, Rochester Hills, MI, USA or sc-271290, Santa Cruz Biotechnology, Heidelberg, Germany), FLAP (ab53536, abcam, Cambridge, UK) or β-actin (sc-47778 or sc-1616, Santa Cruz Biotechnology, 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).

2.8. RNA isolation and quantitative RT-PCR

Macrophages were harvested and lysed using the TRIzol® reagent (Ambion life technologies, Carlsbad, USA). Subsequently, mRNA was isolated following the manufacturer's protocol. Absence of RNA degradation was confirmed by investigation of the 28S and 18S rRNA quality *via* denaturing agarose gel electrophoresis. DNA contaminations in intact mRNA samples were then digested using DNase (DNase I, RNase-free Kit; Thermo Scientific, Waltham, USA) and mRNA concentrations were determined with a NANODROP2000 spectrophotometer (Thermo Scientific, Waltham, USA). Afterwards, reverse transcription of intact RNA samples was performed using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, USA) following the manufacturer's protocol. Finally, qRT-PCR was performed in the presence of SYBR green fluorescent dye (Applied Biosystems, Foster City, USA) with a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, USA) using primers for human 5-LO, 15-LO-1 and 15-LO-2 (Table 1) which were validated before the experiments to assure specificity. Relative mRNA expression was determined using the $2^{-\Delta\Delta Ct}$ method. For this, data were normalized to the housekeeping gene β_2 microglobulin (β2M). All samples were measured in triplicates per run.

2.9. Cytometric bead array

Cytokines in macrophage cell culture supernatants were quantified employing a bead-based immunoassay (cytometric bead array - CBA, BD Biosciences, Heidelberg, Germany). For this human IL-1β, IL-10, IL-12p70, CCL-1, CCL-5 and TNF Flex Sets were used and the assay was conducted according to the manufacturer's protocol. Data analysis was performed with FCAP Array Software v3.0 (BD Biosciences, Heidelberg, Germany).

Table 1
Primer sequences and nucleotide accession numbers of the genes investigated.

Human gene	Nucleotide accession number	Primer sequence
ALOX5 (5-LO)	NM_000689.4	F: GTTCC GAATGGCTGCAAC R: GGCAATGGGGACAATCTTG
ALOX15 (15-LO-1)	NM_001140.4	F: CTTCAAGCTTATAATTCCCCAC R: GATTCCTTCCACATACCGATAG
ALOX15B (15-LO-2)	NM_001141.2	F: GACAAGTGGGACTGGTTGCT R: TTGATGTGCAGGGTGTATCG
B2M (β_2 microglobulin)	NM_001101.4	F: TGCTGTCTCCATGTTTGATGTATCT R: CTCTGCTCCCCACCTCTAAGT

2.10. Statistical analysis

Data are presented as mean + SEM. GraphPad Prism version 7.04 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Data were subjected to one-way ANOVA coupled with Dunnett's post test for multiple comparisons.

3. Results

3.1. Enzyme expression and lipid mediator formation during differentiation of human peripheral blood monocytes to M1 or M2 macrophages

The lipid mediator formation in human MDM during *in vitro* generation from human peripheral blood monocytes was investigated. For this purpose, human monocytes were isolated from the PBMC fraction of leukocyte concentrates after density gradient centrifugation by seeding of the PBMCs to cell culture dishes thereby allowing the monocyte population to adhere to the dish surface. After this, non-adherent cells were washed away. The adherent monocytes were then differentiated into M1 or M2 macrophages for 7 days by treatment with different cytokines: For M1 differentiation monocytes received CSF-2 (10 ng/mL) for 7 days plus IFNγ (10 ng/mL) for the final 48 h, while M2 cells were differentiated with CSF-1 (10 ng/mL) for 7 days receiving additional IL-4 (10 ng/mL) in the last 48 h (see differentiation scheme, Fig. 1).

The fully differentiated M1 and M2 macrophages were harvested after 7 days and mRNA expression of ALOX5, ALOX15 and ALOX15B was analyzed by qRT-PCR analysis (Fig. 1A). Cells without cytokine supplementation during the 7 day differentiation period (serum only) served as controls and β2M was used as housekeeping gene. ALOX5 mRNA was upregulated in M1 macrophages compared to the serum controls while ALOX15 and ALOX15B levels were higher in M2 cells. To confirm these data, we next analyzed the protein expression of 5-LO, 15-LO-1 and -2 as well as FLAP during the differentiation of the monocytes to M1 or M2 macrophages *via* Western blot analysis (Fig. 1B). For this, a part of the respective PBMC fraction was subjected to CD14 positive selection to receive untouched monocytes while the remaining PBMCs were seeded to petri dishes followed by macrophage differentiation *via* the adherence method. Freshly isolated peripheral blood monocytes expressed high amounts of 5-LO while FLAP and 15-LO-2 expression was low. 15-LO-1 was not expressed on protein level in these cells. Upon seeding and differentiation with CSF-1 or -2 for 5 days the protein expression pattern changed. Here, CSF-2 treated cells retained the high 5-LO expression and additionally upregulated their FLAP levels while 15-LO-2 was almost absent in these cells. 15-LO-1 was not detectable. In contrast, CSF-1 treated cells showed extenuated 5-LO expression compared to the monocytes after 5 days. FLAP and 15-LO-2 levels stayed in a comparable range and 15-LO-1 was also not expressed. Fully differentiated M1 macrophages treated with the combination of CSF-2/IFNγ for seven days showed an expression pattern comparable to the CSF-2 treated cells after 5 days. This tendency was

Table 2
LLOQs as well as ISTDs used for lipid mediator quantification in LC-MS/MS method II.

Lipid mediator	5-HETE	LTB4	6-trans-LTB$_4$	8-HETE	9-HETE	11-HETE	12-HETE	15-HETE	8,15-DiHETE	5,15-DiHETE	5(S),6(R) LxA$_4$	5(S),6(S) LxA$_4$	LxB$_4$
LLOQ [ng/mL]	0.16	0.084	0.17	0.40	0.80	0.16	0.16	0.40	0.27	0.084	0.018	0.018	0.071
Internal standard	^{2}H$_8$-5-HETE	^{2}H$_4$-LTB$_4$	^{2}H$_4$-LTB$_4$	^{2}H$_8$-12-HETE	^{2}H$_8$-5-HETE	^{2}H$_8$-12-HETE	^{2}H$_8$-12-HETE	^{2}H$_8$-12-HETE	^{2}H$_{11}$-14, 15-DiHETrE	^{2}H$_{11}$-14, 15-DiHETrE	^{2}H$_5$-LXA$_4$	^{2}H$_5$-LXA$_4$	^{2}H$_5$-LXA$_4$
Lipid mediator	5-HEPE	LTB$_5$	8-HEPE	9-HEPE	11-HEPE	12-HEPE	15-HEPE	18-HEPE	LxA$_5$	RvE1	18(*R*)-RvE2	18(*S*)-RvE3	18(*R*)-RvE3
LLOQ [ng/mL]	0.16	0.033	0.20	0.16	0.16	0.20	0.40	0.40	0.026	0.0088	0.033	0.017	0.0084
Internal standard	^{2}H$_8$-12-HETE	^{2}H$_4$-LTB$_4$	^{2}H$_8$-12-HETE	^{2}H$_8$-12-HETE	^{2}H$_8$-12-HETE	^{2}H$_8$-12-HETE	^{2}H$_8$-12-HETE	^{2}H$_8$-12-HETE	^{2}H$_5$-LXA$_4$	^{2}H$_5$-RvD2	^{2}H$_4$-9,10-DiHOME	^{2}H$_4$-9,10-DiHOME	^{2}H$_4$-9,10-DiHOME
Lipid mediator	4-HDHA	7-HDHA	8-HDHA	10-HDHA	11-HDHA	13-HDHA	14-HDHA	16-HDHA	17-HDHA	RvD1	RvD2	RvD5	NPDx
LLOQ [ng/mL]	0.086	0.34	0.17	0.17	0.086	0.17	0.34	0.086	0.69	0.0068	0.014	0.014	0.0065
Internal standard	^{2}H$_8$-5-HETE	^{2}H$_8$-5-HETE	^{2}H$_8$-5-HETE	^{2}H$_8$-12-HETE	^{2}H$_8$-5-HETE	^{2}H$_8$-12-HETE	^{2}H$_8$-12-HETE	^{2}H$_8$-12-HETE	^{2}H$_8$-12-HETE	^{2}H$_5$-RvD1	^{2}H$_5$-RvD2	^{2}H$_4$-LTB$_4$	^{2}H$_4$-9,10-DiHOME
Lipid mediator	PGB$_2$	PGD$_2$	PGE$_2$	PGF$_{2\alpha}$	PGJ$_2$	TXB$_2$	11-dehydro-TXB$_2$	12-HHTrE	TXB$_3$	PGE$_1$	PGF$_{1\alpha}$	15-keto-PGF$_{1\alpha}$	TXB$_1$	Dihomo-PGF$_{2\alpha}$
LLOQ [ng/mL]	0.0134	0.0353	0.8813	0.0249	0.0535	0.0463	0.0184	0.0140	0.0009	0.0115	0.0089	0.0089	0.0186	0.0038
Internal standard	^{2}H$_4$-PGE$_2$	^{2}H$_4$-PGD$_2$	^{2}H$_4$-PGE$_2$	^{2}H$_4$-PGE$_2$	^{2}H$_4$-PGE$_2$	^{2}H$_4$-TXB$_2$	^{2}H$_4$-TXB$_2$	^{2}H$_{11}$-14,15-DiHETrE	^{2}H$_4$-TXB$_2$	^{2}H$_4$-PGE$_2$	^{2}H4-PGE$_2$	^{2}H$_4$-PGE$_2$	^{2}H$_4$-TXB$_2$	^{2}H$_4$PGE$_2$

also true for 5-LO and FLAP expression in M2 macrophages. In contrast, addition of IL-4 triggered the concerted upregulation of 15-LO-1 and -2 in differentiated M2 macrophages.

Next, we investigated the lipid mediator profile during *in vitro* differentiation of human peripheral blood monocytes to M1 or M2 macrophages. For this, freshly isolated monocytes, the corresponding CSF-1 or -2 treated cells after 5 days as well as fully differentiated M1 and M2 macrophages were harvested, resuspended in PGC buffer followed by stimulation with 5 μM Ca^{2+} ionophore A23187 and 20 μM AA for 15 min. Subsequently, the lipid mediator content in the supernatants was quantified using LC-MS/MS technique (method I). Most interestingly, freshly isolated CD14-positive monocytes displayed the by far highest capacity for the formation of the 5-LO derived lipid mediators LTB_4, LTC_4 and 5-HETE while all adherent cell populations formed only low amounts of 5-LO products (Fig. 1C). Among the adherent cell populations, distinct lipid mediator patterns were identifiable. Fitting to the high 5-LO/FLAP expression in M1 macrophages and CSF-2 treated cells after 5 days, LTB_4 and 5-HETE levels were relatively high. In contrast, M2 macrophages and cells treated with CSF-1 for 5 days showed only low levels of 5-HETE. Interestingly, LTC_4 release was highest in CSF-1 treated cells.

12-HETE formation was most abundant in freshly isolated monocytes, most probable due to unavoidable platelet contaminations. In the macrophage preparations 12-HETE formation was only detectable in CSF-1 treated cells after 5 days as well as M2 macrophages. Of note, a basal low level of 15-HETE formation could be detected in all samples. This was potently upregulated in the M2 macrophages corresponding to the high 15-LO expression levels in these cells. In addition to lipoxygenase products, prostaglandin formation was assessed as well (Suppl. Fig. 2). Here, TXA_2 was the most abundant prostanoid in all incubations, while PGE_2, PGD_2 and $PGF_{2\alpha}$ levels were very low. Interestingly, PGE_2 release was highest in monocytes followed by CSF-1 treated cells and M2 macrophages. In contrast, CSF-2 treated cells displayed the highest PGD_2 levels.

3.2. Enzyme expression and lipid mediator formation in M1 and M2 macrophages during short-term (30 min/6 h) stimulation with TLR ligands

Having documented the lipoxygenase expression patterns as well as their activities during differentiation from monocytes to M1 and M2 macrophages *in vitro*, we were interested in the fate of the lipoxygenase enzymes and their lipid mediator biosynthesis capacity during pathogen encounter. Therefore, the cells were stimulated with classical pathogen-associated molecular patterns (PAMP) that shape the immune response by activation of various toll-like receptors (TLR). The bacterial cell wall component lipopolysaccharide (LPS, TLR-4 ligand), the fungal cell wall preparation zymosan (TLR-2 ligand) as well as Poly(I:C) (TLR-3 ligand) mimicking viral double-stranded RNA were chosen to represent TLR stimuli present during bacterial, fungal and viral infection, respectively. The cells were then treated with the TLR ligands up to 16 h and lipoxygenase expression and activities were monitored under various stimulation conditions. In addition, 5-LO activating protein (FLAP) expression which is essential for 5-LO derived lipid mediator formation was studied. Furthermore, the expression and activity of cyclooxygenase-2 (COX-2) the central enzyme in the formation of prostaglandins was simultaneously investigated.

Leukotrienes, potent chemotactic factors, are among the first lipid mediators released from pro-inflammatory myeloid cells upon the encounter of pathogens. Here, TLR-2 and -4 activation was shown to prime cells for LT biosynthesis by substantially elevating the intracellular free AA levels within minutes after the stimulation [25–27]. To test this TLR-dependent early leukocyte activation in our M1 and M2 macrophages, the cells were harvested, suspended in PGC buffer and then primed with either Poly(I:C) (10 μg/mL), zymosan (50 μg/mL) or LPS (0.1 μg/mL) for 30 min at 37 °C. Unprimed M1 and M2 macrophages served as controls. After this, 1 μM N-formylmethionine-leucyl-

Table 3
Lipid mediator formation in co-incubations of M1 and M2 macrophages.[a]

	Cell type	LLOQ [ng/mL]	M1	M2	M1 (LPS)	M2 (LPS)	M1 + M2	M1 (LPS) + M2 (LPS)
	Mediator [ng/μg protein]							
5-LO/FLAP	5-HETE	0.16	0.69 ± 0.82	0.067 ± 0.022	0.42 ± 0.16	0.13 ± 0.10	0.40 ± 0.48	0.24 ± 0.11
	LTB_4	0.084	0.21 ± 0.23	0.011 ± 0.0046	0.12 ± 0.036	0.022 ± 0.017	0.10 ± 0.13	0.055 ± 0.023
	6-trans-LTB_4	0.17	0.09 ± 0.12	0.0047 ± 0.0022	0.044 ± 0.022	0.0081 ± 0.0064	0.045 ± 0.058	0.021 ± 0.012
Mono-/di-hydroxylated	9-HETE	0.80	0.016 ± 0.017	0.012 ± 0.0039	0.0067 ± 0.0022	0.010 ± 0.0074	0.023 ± 0.026	0.0084 ± 0.0027
	11-HETE	0.16	0.046 ± 0.044	0.058 ± 0.019	0.136 ± 0.063	0.065 ± 0.046	0.089 ± 0.097	0.11 ± 0.044
	12-HETE	0.16	0.070 ± 0.11	0.076 ± 0.052	0.013 ± 0.0074	0.13 ± 0.13	0.18 ± 0.27	0.05367905 ± 0.030
	15-HETE	0.40	0.13 ± 0.14	0.84 ± 0.31	0.16 ± 0.077	1.35 ± 0.91	1.15 ± 1.32	0.64 ± 0.28
	8,15-DiHETE	0.27	0.0018 ± 0.0018	0.0058 ± 0.0026	0.00088 ± 0.00032	0.0082 ± 0.0057	0.0079 ± 0.0094	0.0036 ± 0.0019
	5,15-DiHETE	0.084	0.0066 ± 0.0069	0.015 ± 0.0054	0.018 ± 0.0075	0.053 ± 0.033	0.038 ± 0.044	0.040 ± 0.015
SPM	5(S),6(R) LxA_4	0.018	< LLOQ	0.000071 ± 0.00012	< LLOQ	0.00053 ± 0.00040	0.000049 ± 0.000085	0.00040 ± 0.00027
	5(S),6(S) LxA_4	0.018	< LLOQ	< LLOQ	< LLOQ	0.00042 ± 0.00029	< LLOQ	0.00020 ± 0.00024
	LxB_4	0.071	< LLOQ	< LLOQ	< LLOQ	0.00020 ± 0.00034	< LLOQ	< LLOQ
5-LO/FLAP	5-HEPE	0.16	1.35 ± 1.60	0.15 ± 0.058	0.87 ± 0.34	0.26 ± 0.21	0.87 ± 1.08	0.51 ± 0.26
	LTB_5	0.033	0.13 ± 0.12	0.0092 ± 0.0033	0.096 ± 0.027	0.017 ± 0.014	0.077 ± 0.092	0.049 ± 0.022
Mono-hydroxylated	8-HEPE	0.20	0.086 ± 0.087	0.064 ± 0.019	0.037 ± 0.012	0.063 ± 0.042	0.13 ± 0.15	0.051 ± 0.016
	9-HEPE	0.16	0.091 ± 0.09	0.073 ± 0.022	0.038 ± 0.013	0.067 ± 0.047	0.14 ± 0.16	0.054 ± 0.016
	11-HEPE	0.16	0.12 ± 0.12	0.11 ± 0.036	0.097 ± 0.042	0.10 ± 0.072	0.19 ± 0.21	0.098 ± 0.036
	12-HEPE	0.20	0.14 ± 0.18	0.22 ± 0.12	0.040 ± 0.016	0.33 ± 0.30	0.41 ± 0.54	0.15 ± 0.066
	15-HEPE	0.40	0.067 ± 0.07	1.18 ± 0.47	0.033 ± 0.015	1.64 ± 1.18	1.54 ± 1.77	0.77 ± 0.42
	18-HEPE	0.40	0.38 ± 0.38	0.25 ± 0.085	0.15 ± 0.056	0.21 ± 0.15	0.53 ± 0.59	0.20 ± 0.069
SPM	LxA_5	0.026	< LLOQ	< LLOQ	< LLOQ	0.00019 ± 0.00024	< LLOQ	0.00035 ± 0.00027
	RvE1	0.0088	0.000022 ± 0.000038	< LLOQ	0.00043 ± 0.00023	< LLOQ	< LLOQ	0.00012 ± 0.00014
	18(*R*)-RvE2	0.033	0.038 ± 0.033	0.029 ± 0.0094	0.024 ± 0.0075	0.028 ± 0.020	0.059 ± 0.065	0.027 ± 0.0090
	18(*S*)-RvE3	0.017	< LLOQ	0.0022 ± 0.00097	< LLOQ	0.0031 ± 0.0016	0.0013 ± 0.0011	0.0015 ± 0.0013
	18(*R*)-RvE3	0.0084	< LLOQ	0.00064 ± 0.00064	< LLOQ	0.0012 ± 0.00073	0.00016 ± 0.00021	0.00065 ± 0.00070
Mono-hydroxylated	4-HDHA	0.086	0.042 ± 0.044	0.024 ± 0.0097	0.018 ± 0.0060	0.020 ± 0.015	0.058 ± 0.066	0.021 ± 0.0075
	7-HDHA	0.34	0.035 ± 0.039	0.016 ± 0.0044	0.020 ± 0.0090	0.016 ± 0.012	0.039 ± 0.045	0.017 ± 0.0068
	8-HDHA	0.17	0.045 ± 0.045	0.031 ± 0.012	0.020 ± 0.0067	0.026 ± 0.020	0.067 ± 0.076	0.024 ± 0.0091
	10-HDHA	0.17	0.015 ± 0.016	0.01029 ± 0.0038	0.0068 ± 0.0023	0.0091 ± 0.0066	0.022 ± 0.025	0.0085 ± 0.0029
	11-HDHA	0.086	0.018 ± 0.018	0.014 ± 0.0053	0.0076 ± 0.0026	0.014 ± 0.011	0.028 ± 0.033	0.011 ± 0.0034
	13-HDHA	0.17	0.029 ± 0.031	0.020 ± 0.0083	0.029 ± 0.014	0.018 ± 0.014	0.042 ± 0.050	0.021 ± 0.0077
	14-HDHA	0.34	0.021 ± 0.027	0.044 ± 0.019	0.0066 ± 0.0024	0.067 ± 0.050	0.076 ± 0.095	0.034 ± 0.018
	16-HDHA	0.086	0.021 ± 0.024	0.0085 ± 0.0045	0.0076 ± 0.0033	0.0073 ± 0.0060	0.025 ± 0.031	0.0078 ± 0.0025
	17-HDHA	0.69	0.046 ± 0.052	0.33 ± 0.10	0.022 ± 0.0097	0.59 ± 0.37	0.43 ± 0.49	0.25 ± 0.10
SPM	RvD1	0.0068	< LLOQ	0.00015 ± 0.000050	< LLOQ	0.00014 ± 0.000066	0.00020 ± 0.00030	0.00012 ± 0.000067
	RvD2	0.014	< LLOQ	0.00013 ± 0.00015	< LLOQ	0.00029 ± 0.00029	0.000031 ± 0.000053	0.000066 ± 0.00011
	RvD5	0.014	0.0027 ± 0.0030	0.014 ± 0.0056	0.0021 ± 0.0011	0.038 ± 0.025	0.017 ± 0.018	0.015 ± 0.0090
	MaR1	0.0361	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	7-*epi* MaR1	0.0090	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	NPDx	0.0065	< LLOQ	0.00054 ± 0.00028	< LLOQ	0.00076 ± 0.00054	0.00074 ± 0.00089	0.00035 ± 0.00024
	PGB_2	0.0134	0.0017 ± 0.0017	0.0007 ± 0.0003	0.0010 ± 0.0005	0.0007 ± 0.0005	0.0019 ± 0.0022	0.0008 ± 0.0003
	PGD_2	0.0353	0.0117 ± 0.0122	0.0140 ± 0.0064	0.0186 ± 0.0134	0.0146 ± 0.0094	0.0214 ± 0.0247	0.0182 ± 0.0119
	PGE_2	0.8813	0.0119 ± 0.0143	0.0177 ± 0.0118	0.3132 ± 0.2310	0.0346 ± 0.0371	0.0346 ± 0.0458	0.1860 ± 0.1302
	6-keto-$PGF_{1\alpha}$	0.0669	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ	< LLOQ
	$PGF_{2\alpha}$	0.0249	0.0516 ± 0.0451	0.0505 ± 0.0216	0.1088 ± 0.0706	0.0723 ± 0.0684	0.0712 ± 0.0723	0.0942 ± 0.0618
	PGJ_2	0.0535	< LLOQ	< LLOQ	0.0005 ± 0.0005	0.0008 ± 0.0007	< LLOQ	0.0009 ± 0.0006
	TXB_2	0.0463	0.2455 ± 0.1632	0.5433 ± 0.1213	0.4790 ± 0.1560	0.5176 ± 0.2739	0.6122 ± 0.5294	0.4974 ± 0.1752
	11-dehydro-TXB_2	0.0184	< LLOQ	< LLOQ	0.0005 ± 0.0006	0.0002 ± 0.0003	< LLOQ	< LLOQ
	12-HHTrE	0.0140	0.3191 ± 0.2429	0.6250 ± 0.1871	0.5524 ± 0.2322	0.5784 ± 0.3493	0.7700 ± 0.7461	0.5779 ± 0.2633

(*continued on next page*)

Table 3 (*continued*)

Cell type	LLOQ [ng/mL]	M1	M2	M1 (LPS)	M2 (LPS)	M1 + M2	M1 (LPS) + M2 (LPS)
Mediator [ng/μg protein]							
TXB_3	0.0009	0.1270 ± 0.0925	0.2533 ± 0.0589	0.1165 ± 0.0416	0.2293 ± 0.1248	0.2792 ± 0.2529	0.1528 ± 0.0604
PGE_1	0.0115	< LLOQ	< LLOQ	0.0102 ± 0.0058	0.0003 ± 0.0006	< LLOQ	0.0063 ± 0.0036
$PGF_{1\alpha}$	0.0089	0.0054 ± 0.0038	0.0026 ± 0.0006	0.0072 ± 0.0036	0.0026 ± 0.0020	0.0063 ± 0.0055	0.0057 ± 0.0030
15-keto-$PGF_{1\alpha}$	0.0089	0.0000 ± 0.0000	< LLOQ	0.0003 ± 0.0003	< LLOQ	< LLOQ	0.0002 ± 0.0001
TXB_1	0.0186	0.0034 ± 0.0016	0.0064 ± 0.0010	0.0048 ± 0.0008	0.0032 ± 0.0018	0.0088 ± 0.067	0.0046 ± 0.0014
dihomo-$PGF_{2\alpha}$	0.0038	0.0090 ± 0.0064	0.0052 ± 0.0008	0.0144 ± 0.0070	0.0058 ± 0.0045	0.0113 ± 0.100	0.0123 ± 0.0055

[a] M1 and M2 macrophages were either treated with bacterial LPS (100 ng/mL) for 16 h or left unstimulated. Then, cells were harvested and resuspended in PGC buffer. Subsequently, the lipid mediator formation of the single macrophage phenotypes as well as the mixed macrophage populations was assessed after stimulation of the cells with a cocktail containing 5 μM Ca^{2+} ionophore plus a fatty acid cocktail (DHA/AA/EPA, 6 μM each) for 15 min. Each reaction was stopped by addition of ice-cold methanol and the lipid mediators formed were measured by LC/M-MS method. Lipid mediator concentrations were normalized to total protein content of the preparations (ng/μg protein). Data are presented as mean ± SEM from 4 independent experiments. Mean total protein concentrations in the macrophage preparations were 0.40 ± 0.13 for M1, 0.37 ± 0.071 for M2, 0.57 ± 0.17 for $M1_{LPS}$ and 0.54 ± 0.12 for $M2_{LPS}$ phenotypes.

phenylalanine (fMLF) was used as stimulus to trigger lipoxygenase activity. fMLF is a formylated tripeptide with immune stimulating properties released by proliferating bacteria during inflammation which triggers intracellular AA release as well as lipoxygenase activation. The reaction was terminated after 15 min. Priming of M1 macrophages with the TLR-4 ligand LPS substantially elevated 5-LO activity, as expected while zymosan treatment had no influence (Suppl. Fig. III). Interestingly, TLR-3 stimulation completely abolished LT synthesis in these cells. In contrast, TLR-3 stimulation triggered LT formation in M2 macrophages, while TLR-2 and -4 stimulation had no influence on the already low levels of LTs. SPM were not detected (below the LLOQ) in these incubations.

Following the immediate release of LTs after pathogen encounter, pro-inflammatory macrophages are known to upregulate PG biosynthesis within 4–6 h after TLR stimulation due to a rapid induction of COX-2 expression, the central pro-inflammatory PG synthase [28]. In this context, the fate of the macrophage lipoxygenases after 6 h of persistent TLR stimulation has been less studied so far. Therefore, M1 and M2 macrophages were treated again with zymosan, Poly(I:C) or bacterial LPS for 6 h. Control cells were left untreated. After 6 h, protein expression of 5-LO, FLAP, 15-LO-1, 15-LO-2 and COX-2 were assessed *via* Western Blotting technique. In addition, macrophage conditioned media were collected and lipid mediator release was measured by LC-MS/MS (method I). As expected, COX-2 was almost non-detectable in unstimulated M1 and M2 macrophages, while its' enzyme expression was considerably upregulated after stimulation of TLR-2 (zymosan) or -4 (LPS) (Suppl. Fig. IVB). This upregulation was more pronounced in the M1 phenotype and these data also fitted to the elevated PG levels we found in the conditioned media of these cells (Suppl Fig. IVC). Of note, although Poly(I:C) treatment did not induce COX-2 expression it led to a pronounced upregulation of PGs in both macrophage phenotypes. Prostacyclin was not detected in any of the incubations.

In contrast to the COX/PG lipid mediator axis, no change in the expression of the lipoxygenase enzymes and FLAP was found after 6 h of TLR stimulation compared to the control cells (Suppl. Fig. IVA, B).

3.3. Enzyme expression and lipid mediator formation in M1 and M2 macrophages during persistent stimulation with TLR-2, -3 and -4 ligands for 16 h

Next, enzyme expression and lipid mediator biosynthesis capacity under persistent TLR stimulation was explored in M1 and M2 macrophages. For this, the cells were treated again with zymosan (50 μg/mL), Poly(I:C) (10 μg/mL) or LPS (0.1 μg/mL) for 16 h. Control incubations were left untreated. We then analyzed the mRNA expression of ALOX5 (5-LO), ALOX15 (15-LO-1) and ALOX15B (15-LO-2) by quantitative RT-PCR. TLR ligand untreated cells served as controls. While ALOX5 and ALOX15 mRNA expression was not influenced by persistent TLR-2 and -4 activation in M1 and M2 macrophages, these treatments triggered an elevation of ALOX15B mRNA levels up to 20–50 fold over control after 16 h (Fig. 2A). Poly(I:C) treatment had no influence on any lipoxygenase mRNA. Next, we had a closer look on the protein expression levels of 5-LO, FLAP, 15-LO-1, 15-LO-2 and COX-2 *via* Western blotting (Fig. 2B,C). Expression of 5-LO, FLAP and 15-LO-1 did not change in M1 macrophages treated with the TLR ligands for this prolonged time. Also, the anticipated increase in 15-LO-2 protein expression due to the elevated ALOX15B mRNA levels in the qPCR experiments could not be confirmed in these cells. Apparently, the mRNA was not efficiently translated in this macrophage phenotype. In contrast, M2 macrophages showed an upregulation of 15-LO-2 on protein level after zymosan (TLR-2) and LPS (TLR-4) stimulation in accordance with the ALOX15B mRNA data (Fig. 2B). In addition, M2 cells simultaneously upregulated 5-LO expression on the protein level, although ALOX5 mRNA was not induced in the preceding qPCR experiments. M2 macrophage FLAP and 15-LO-1 levels were not influenced by the TLR ligands (Fig. 2C).

Together with a 15-lipoxygenating enzyme, 5-LO is thought to play

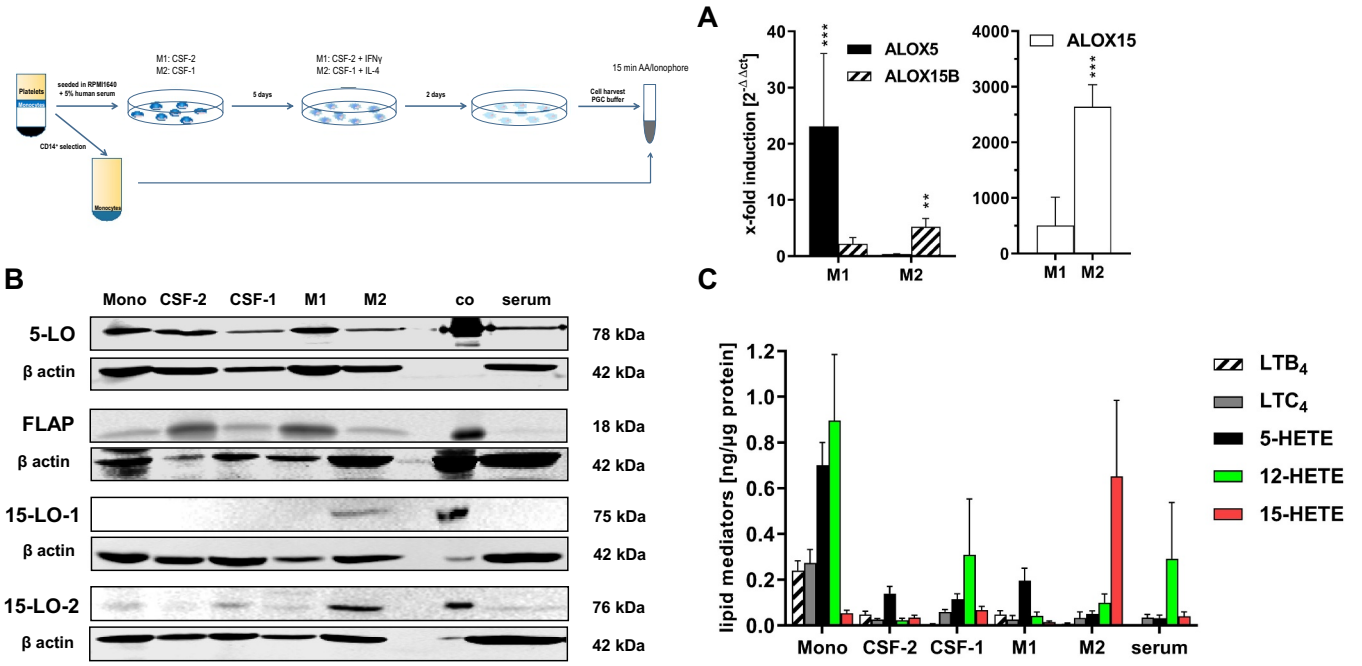

Fig. 1. Lipoxygenase and FLAP expression as well as lipid mediator formation during differentiation of human peripheral blood monocytes to M1 and M2 macrophages. M1 and M2 macrophages were differentiated from human peripheral blood monocytes (isolated by adherence method). For this, the cells were treated with 10 ng/mL CSF-2 (M1) or CSF-1 (M2) for seven days. Additionally, the cells received 10 ng/mL IFNγ (M1) or IL-4 (M2) for the final 48 h. (A) mRNA expression of ALOX5, ALOX15 and ALOX15B genes in M1 and M2 macrophages. Each gene was normalized to the housekeeping gene β2M as well as serum treated controls ($2^{-\Delta\Delta ct}$). Data are presented as mean ± SEM of 4–7 independent experiments. (B) Protein expression of 5-LO, 15-LO-1, 15-LO-2 and FLAP in freshly isolated peripheral blood monocytes, CSF-1 or -2 stimulated differentiating macrophages after 5 days as well as fully differentiated M1 and M2 macrophages after 7 days. Equal loading was assured by measurement of the housekeeping gene β actin. A single representative experiment out of 3–4 is shown. (C) Formation of lipoxygenase derived lipid mediators in human peripheral blood monocytes (isolated by CD14 positive selection), macrophages differentiating in the presence of CSF-1/−2 after 5 days as well as fully differentiated macrophages after 7 days. The cells were resuspended in PGC buffer followed by stimulation with 5 µM Ca^{2+} ionophore (A23187) and 20 µM AA for 15 min. Subsequently, the reaction was stopped by addition of ice-cold methanol. LTC_4 concentrations were assessed by ELISA while the other lipid mediators were analyzed by LC/MS-MS technique (method I). Data represent the mean ± SEM out of 3–8 (LC-MS/MS) and 2–4 (ELISA) independent experiments. Lipid mediator concentrations were normalized to total protein content of the preparations. SPM formation was below the LLOQ in all experiments and is therefore not depicted.

an important role in the formation of SPMs such as lipoxins and resolvins. While in mice there is only one 12-/15-LO enzyme present, it is not known which of the two human 15-LO isoforms contributes to the formation of SPM in man. Due to the coordinated upregulation of 5- and 15-LO-2 found in M2 macrophages after persistent stimulation of TLR-2 and -4, we speculated that these cells might readily form SPM. Therefore, we investigated the lipid mediator profile of the macrophage conditioned media after persistent TLR stimulation by LC-MS/MS (method I). While 5-LO-derived LTB_4 was not detectable in any of the conditioned media (Fig. 2D), the monohydroxylated AA derivatives 5-, 12- and 15-HETE were found in all supernatants, the pattern depending on the stimulus used. 5-LO derived 5-HETE was present in M1 controls as well as M1 macrophages treated with LPS while Poly(I:C) and zymosan treated cells did not release this oxylipin. Most interestingly, 5-HETE release from M2 controls was much higher compared to M1 cells despite the low 5-LO/FLAP expression in these cells. Comparably, release of 12-HETE was higher in M2 compared to M1 macrophages. TLR ligand treatment reduced this oxylipin in both macrophage phenotypes. Release of 15-HETE could be found in all macrophage conditioned media investigated. Again, M2 controls displayed higher 15-HETE levels compared to M1 cells. Treatment with zymosan or LPS elevated 15-HETE release in M2 cells, fitting to the enzyme expression pattern found in these cells. SPMs were not detected (below the LLOQ) in any of the macrophage conditioned media. In addition to the lipoxygenase derived lipid mediators, prostaglandin release was measured as well. As expected, TLR-2 and -4 ligands potently stimulated prostaglandin release in M1 macrophages. Especially PGE_2, TXA_2 and $PGF_{2\alpha}$ release was potently elevated in zymosan and LPS treated cells (Fig. 2D). This elevation in PG release could also be found in zymosan or LPS treated M2 macrophages although to a much lower extent.

Despite the elevated release of 15-LO derived oxylipins into the macrophage conditioned media, TLR stimulation alone was not sufficient to trigger SPM release in our M2 preparations. Therefore, 1 µM fMLF was used as additional stimulus to trigger lipoxygenase activity. The cells were harvested after 16 h of LPS stimulation, resuspended in PGC buffer and stimulated with fMLF. TLR ligand untreated M2 cells served as controls. After 15 min the reaction was terminated by addition of ice-cold methanol and the lipid mediator content in the preparations was measured by LC-MS/MS (method I). Upon stimulation with fMLF, M2 control cells released substantial amounts of the 15-LO products 15-HETE and 17-HDHA as well as TXB_2 while 5-LO products and PGE_2 were barely detectable (Fig. 3). This held also true for M2 macrophages treated with LPS overnight. When we added an additional 30 min priming step prior fMLF stimulation to M2 macrophages that were already treated overnight with LPS lipid mediator biosynthesis was potently inhibited. This effect might assure that pro-resolving lipid mediator biosynthesis is not untimely triggered in macrophages. Again, lipoxins, resolvins, protectins and maresins were not detected (below the LLOQ) in the fMLF stimulated cells.

Although we found abundant amounts of SPM precursors such as 15-HETE and 17-HDHA in in our fMLF stimulated preparations no SPMs were detectable. For this reason, we decided to stimulate the macrophages with the even stronger stimulus Ca^{2+} ionophore A23187 combined with exogenous fatty acid supplementation. Here, we chose a fatty acid mix containing equal amounts of AA, DHA and EPA. This combination of potent intracellular Ca^{2+} elevation together with

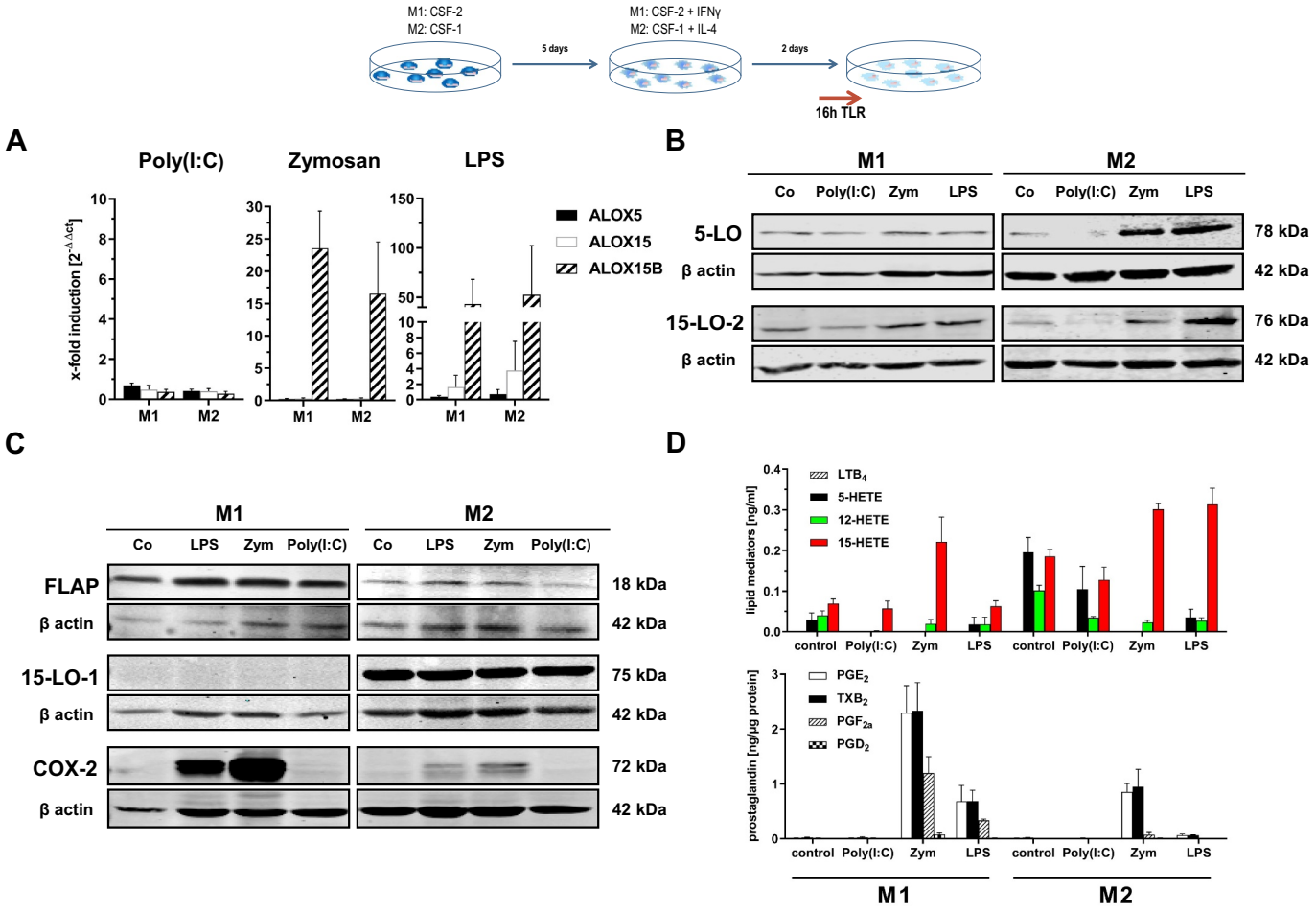

Fig. 2. Lipoxygenase, FLAP and COX-2 expression in as well as lipid mediator release from M1 and M2 macrophages treated with TLR ligands for 16 h. M1 and M2 macrophages were differentiated from human peripheral blood monocytes (isolated by adherence method). For this, the cells were treated with 10 ng/mL CSF-2 (M1) or CSF-1 (M2) for seven days. For full differentiation, the cells received 10 ng/mL IFNγ (M1) or IL-4 (M2) for the final 48 h. After this, the macrophages were treated with either LPS (0.1 µg/mL), zymosan (50 µg/mL) or Poly(I:C) (10 µg/mL) for 16 h. (A) mRNA expression of ALOX5, ALOX15 and ALOX15B genes in the TLR ligand treated M1 and M2 macrophages. Each gene was normalized to the housekeeping gene ß2M and was additionally normalized to TLR untreated M1 or M2 macrophages ($2^{-\Delta\Delta ct}$). Data are presented as mean ± SEM of 4 independent experiments. (B,C) Protein expression of 5-LO, 15-LO-1, 15-LO-2, FLAP and COX-2 in the TLR ligand treated M1 and M2 macrophages assessed by western blotting. Equal loading was assured by measurement of the housekeeping gene β actin. A single representative experiment out of 4–11 is shown. (D) Lipoxygenase and cyclooxygenase derived lipid mediator release into the conditioned media from TLR ligand treated macrophages. Cell supernatants were analyzed using LC-MS/MS technique (method I). Data represent the mean ± SEM from 3 independent experiments. SPM formation was below the LLOQ and is therefore not depicted.

exogenous supplementation of the fatty acid precursors should maximize the lipid mediator formation in our M1 and M2 macrophages. It is conceivable that a heterogeneous mix of macrophage phenotypes consisting of still pro-inflammatory, 5-LO^{high} macrophages as well as freshly differentiated alternatively activated 15-LO^{high} cells might be present at the side of inflammation during the switch to resolution. Therefore, mixed M1 and M2 populations (1:1) were investigated alongside the M1 and M2 populations. Once again, macrophages treated with LPS (0.1 µg/mL) for 16 h as well as LPS untouched cells were harvested and suspended in PGC buffer. Then, the cell suspensions were stimulated with 5 µM Ca^{2+} ionophore plus the fatty acid cocktail (AA, EPA, DHA, 6 µM each) for 15 min. Afterwards, reactions were terminated by addition of ice-cold methanol and free lipid mediators in the preparations were analyzed employing LC-MS/MS technique (Method II).

We found abundant amounts of monohydroxylated fatty acids such as the hydroxyeicotetraenoic (HETE), the hydroxyeicosapentaenoic (HEPE) and the hydroxydocosahexaenoic acids (HDHA) as well as the prostanoids TXB_2, PGE_2 and $PGF_{2\alpha}$ in our incubations (Fig. 4). Compared to DHA, the C-20 fatty acids AA and EPA were overall more readily accepted by the different lipoxygenases. Especially, formation of 5-LO derived DHA products was comparably low. As expected, a switch from 5- to 15-LO products was evident in M2 macrophages compared to M1 and was even more pronounced in cells treated overnight with LPS. TXB_2, PGD_2 and $PGF_{2\alpha}$ levels did not substantially change between the treatments while PGE_2 release was highest in preparations that contained LPS treated M1 macrophages. As expected, PGI_2 was not detected in any of the incubations.

Formation of the dihydroxylated product 5,15-DiHETE as well as RvD5 was about 25 fold lower compared to the monohydroxlated AA products and prostanoids. Here, 5,15-DiHETE and RvD5 were most abundant in M2 macrophages treated overnight with LPS. Formation of lipoxins, E-series resolvins as well as the resolvins D1 and D2 was very low. While the amount of LTB_4 released was comparable to that of RvD5 and 18(*R*)-RvE2 in LPS stimulated M2 macrophages, the formation of the other SPM was considerably lower by a factor of 10 to 100, even more so compared to the monohydroxylated fatty acid derivatives and the prostanoids by a factor of 500 up to 8000. RvE2 was detected in all samples without any variation suggesting LO independent formation and RvE1 was only detected in M1 macrophages treated overnight with LPS. Interestingly, after treatment with LPS for 16 h M2 macrophages overexpressing 5- and 15-LO-2, readily formed LXA_4, LXB_4, RvE3 as

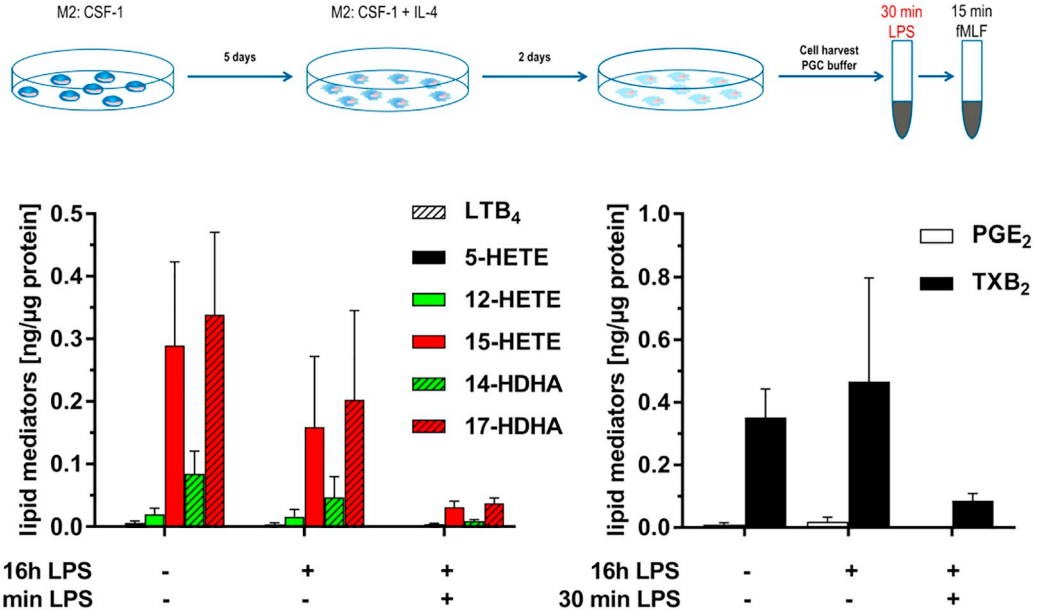

Fig. 3. Lipid mediator formation of M2 macrophages after persistent (16 h) LPS stimulation followed by stimulation with fMLF. M2 macrophages were treated overnight (16 h) with or without LPS (0.1 µg/mL). The next day, the cells were harvested, resuspendend in PGC buffer and primed for 30 min with LPS (0.1 µg/mL) before they were stimulated with 1 µM fMLF for 15 min. M2 macrophages stimulated with fMLF alone were used as controls. Formation of COX and LO derived lipid mediators released into the cell supernatants was assessed by LC-MS/MS (method I) and is presented as mean ± SEM of 4 independent experiments. SPM, PGD_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ were below the LLOQ and are therefore not depicted. Lipid mediator concentrations were normalized to total protein content of the preparations.

well as RvD2 with the highest capacity. RvD1 was produced in comparable concentrations in M2 and $M2_{LPS}$ macrophages as well as co-incubations of M1 and M2 cells. For detailed information on the lipid mediator profile measured in this experiment see Table 3.

3.4. Cytokine release from TLR stimulated M1 and M2 macrophages

In addition to the lipid mediator synthesis profile of the TLR stimulated M1 and M2 macrophages, cytokine release into the conditioned media of the cells was assessed after 6 and 16 h of TLR stimulation. For this, IL-1β, TNFα, IL-12p70, CCL2, CCL5 and IL-10 levels were measured *via* cytometric bead array (Fig. 5).

M1 macrophages showed a time-dependent upregulation of IL-1β, IL-12 and CCL5 after stimulation with LPS and zymosan at which stimulation with zymosan was always more potent than LPS. TNFα was also upregulated by TLR-2 and TLR-4 stimulation with its onset earlier compared to the aforementioned cytokines. CCL2 expression was very high in M1 macrophages independent of the stimulus used. As expected, the anti-inflammatory cytokine IL-10 was only expressed in low levels in M1 cells. Here, only persistent (16 h) zymosan stimulation was able to induce low levels of IL-10.

M2 macrophages showed a TLR-2 and -4 triggered upregulation of TNFα and CCL5 comparable to M1 cells while IL-1β and IL-12 were only expressed at low levels in these cells. CCL2 expression was very low in M2 macrophages independent from the stimulus. Most interestingly, stimulation of TLR-2 and -4 potently upregulated IL-10 levels in M2 macrophages in a time-dependent manner. The low IL-12 levels together with the high IL-10 release after 16 h of persistent TLR-2 and -4 stimulation might point to an anti-inflammatory role of these macrophages. Stimulation with Poly(I:C) did not influence the release of the cytokines measured in this study in M1 and M2 macrophages.

4. Discussion and conclusions

4.1. Discussion

In the present report lipoxygenase and cyclooxygenase derived lipid mediator formation in human MDM during *in vitro* generation and pathogen stimulation was investigated. For this, peripheral blood monocytes isolated from the PBMC fraction of human leukocyte concentrates where seeded in petri dishes. After 1 h, the non-adherent cells were washed away leaving only the monocytes which were then differentiated into M1 and M2 macrophages by different cytokine cocktails. Furthermore, the differentiated macrophages were stimulated with various TLR ligands such as bacterial LPS, fungal zymosan and Poly(I:C) that mimicks viral infection. To obtain an optimal response, non-cytotoxic TLR ligand concentrations were chosen which are frequently used in literature.

To study enzyme expression and activity in untouched peripheral blood monocytes the cells were isolated by CD14-positive selection directly from PBMCs instead of employing the monocyte adherence method that was used throughout the rest of the manuscript. This procedure assured the selection of cells that were undisturbed to the largest extent possible. Although monocytes purified *via* CD14-negative selection would be even lesser activated compared to CD14-positive selected cells, we decided against this purification method due to higher platelet and thus 12-LO contamination in these preparations which impedes the interpretation of the formation of 12-lipoxygenation products.

Freshly isolated human monocytes displayed a very potent LT biosynthesis capacity although 5-LO and FLAP expression was not higher compared to the differentiated macrophages. Furthermore, macrophages differentiated without cytokine addition (serum only controls) showed a comparable enzyme expression pattern to monocytes but lipid

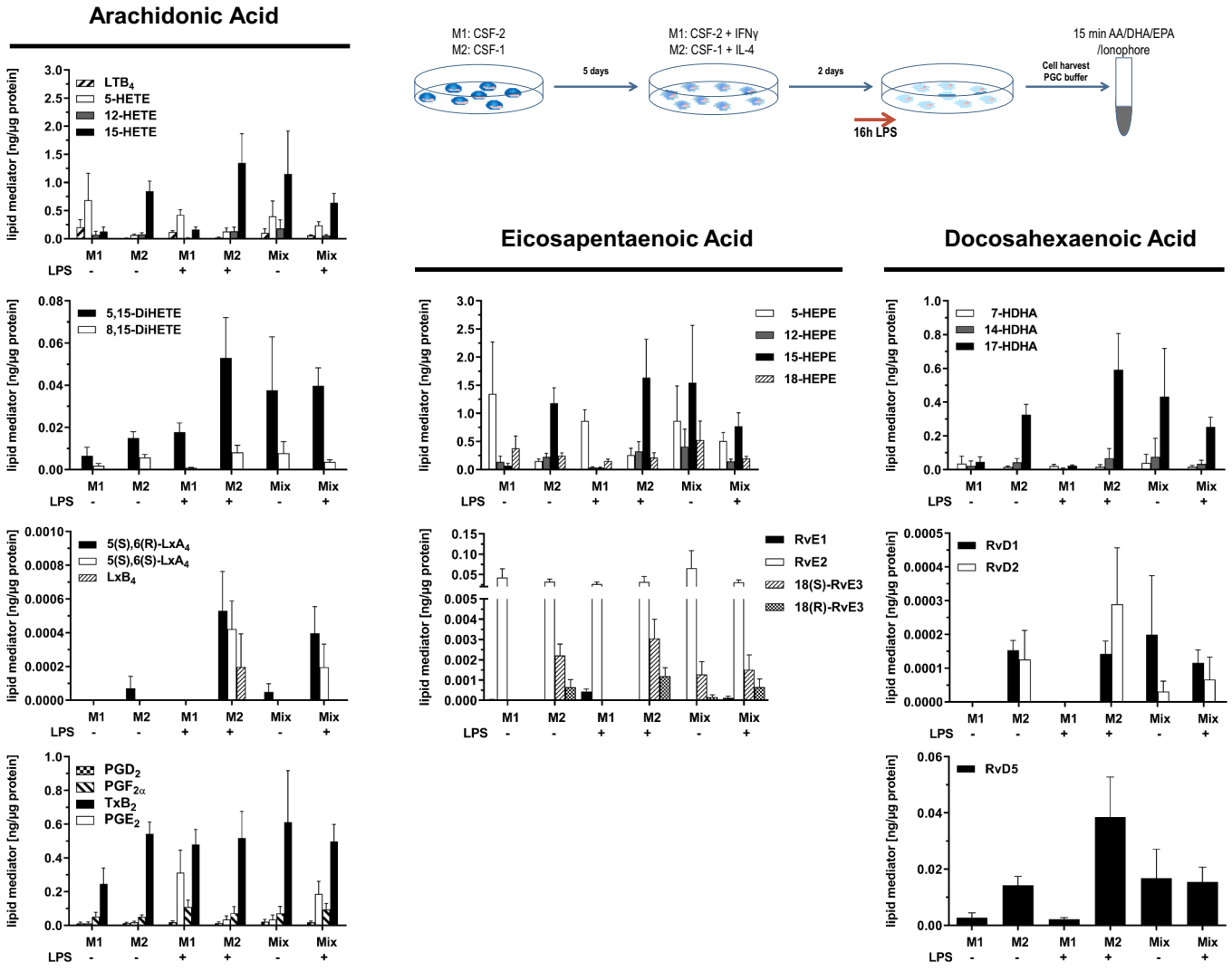

Fig. 4. Lipid mediator formation of M2 macrophages after persistent (16 h) LPS stimulation followed by stimulation with Ca^{2+} ionophore (A23187) plus a fatty acid mix (AA/EPA/DHA). M1 and M2 macrophages were either treated with bacterial LPS (0.1 µg/mL) for 16 h or left unstimulated. Then, cells were harvested and resuspendend in PGC buffer. Subsequently, the lipid mediator formation of the single macrophage phenotypes as well as mixed macrophage M1/M2 populations was assessed after stimulation of the cells with a combination of 5 µM Ca^{2+} ionophore plus a fatty acid cocktail (DHA/AA/EPA, 6 µM each) for 15 min. Each reaction was stopped by addition of ice-cold methanol and the lipid mediators formed were measured by LC/M-MS method (method II). Lipid mediator concentrations were normalized to total protein content of the preparations. Depicted here is a selection of the lipid mediators formed. The complete analysis can be found in Table 2. Data are presented as mean ± SEM from 4 independent experiments. Mean total protein concentrations in the macrophage preparations were 0.40 ± 0.13 mg/mL for M1, 0.37 ± 0.071 mg/mL for M2, 0.57 ± 0.17 mg/mL for $M1_{LPS}$ and 0.54 ± 0.12 mg/mL for $M2_{LPS}$ phenotypes.

mediator synthesis capacity was also strongly attenuated. Apparently, capacity for 5-LO product formation was substantially altered during macrophage differentiation. The cause for this loss of activity is not known so far and requires further investigation in the future. In addition to high 5-LO product formation, the monocyte preparations also generated substantial amounts of 12-HETE. This was certainly due to unavoidable platelet contaminations that are present in freshly isolated cells even after CD14-positive selection. In addition, freshly isolated monocytes released small amounts of 15-HETE most probably due to the low basal expression of 15-LO-2.

Even if not fully differentiated yet, plated monocytes treated with CSF-2 or CSF-1 for 5 days showed marked differences in lipoxygenase and FLAP enzyme expression and activities. While CSF-2 treated cells expressed high levels of 5-LO and FLAP and showed LTB_4 and LTC_4 formation in addition to 5-HETE, CSF-1 treated macrophages expressed lower amounts of both proteins and released less 5-HETE. LTB_4 and LTC_4 were almost absent in these cells. In line with these data, a recent study investigating the lipid mediator formation and expression of enzymes involved in LT and SPM biosynthesis in human MDM differentiated with CSFs supports the differences in 5-LO and FLAP expression found in our experiments [29]. Again, 15-HETE was present in those incubations which can be explained by the low 15-LO-2 expression present in the CSF treated cells.

The distinct enzyme expression patterns of 5-LO and FLAP already detectable after 5 days of CSF-2 or CSF-1 treatment were retained in the fully differentiated cells after seven days past addition of IFNγ (M1) or IL-4 (M2). In contrast, 15-LO-1 was completely absent in monocytes and during the 5 days of CSF incubation, while its' expression changed substantially after the addition of IL-4 to CSF-1 treated cells (M2). Still, in CSF-2/IFNγ (M1) treated macrophages the enzyme was not detectable. This IL-4-triggered upregulation of 15-LO-1 has been well documented in the past for a number of cell types [30]. In contrast to 15-LO-1, 15-LO-2 was already constitutively expressed in monocytes and CSF treated cells even though at a low level. Along with 15-LO-1, 15-LO-2 expression increased substantially in M2 macrophages upon IL-4 stimulation. As a consequence thereof, 15-HETE release was potently

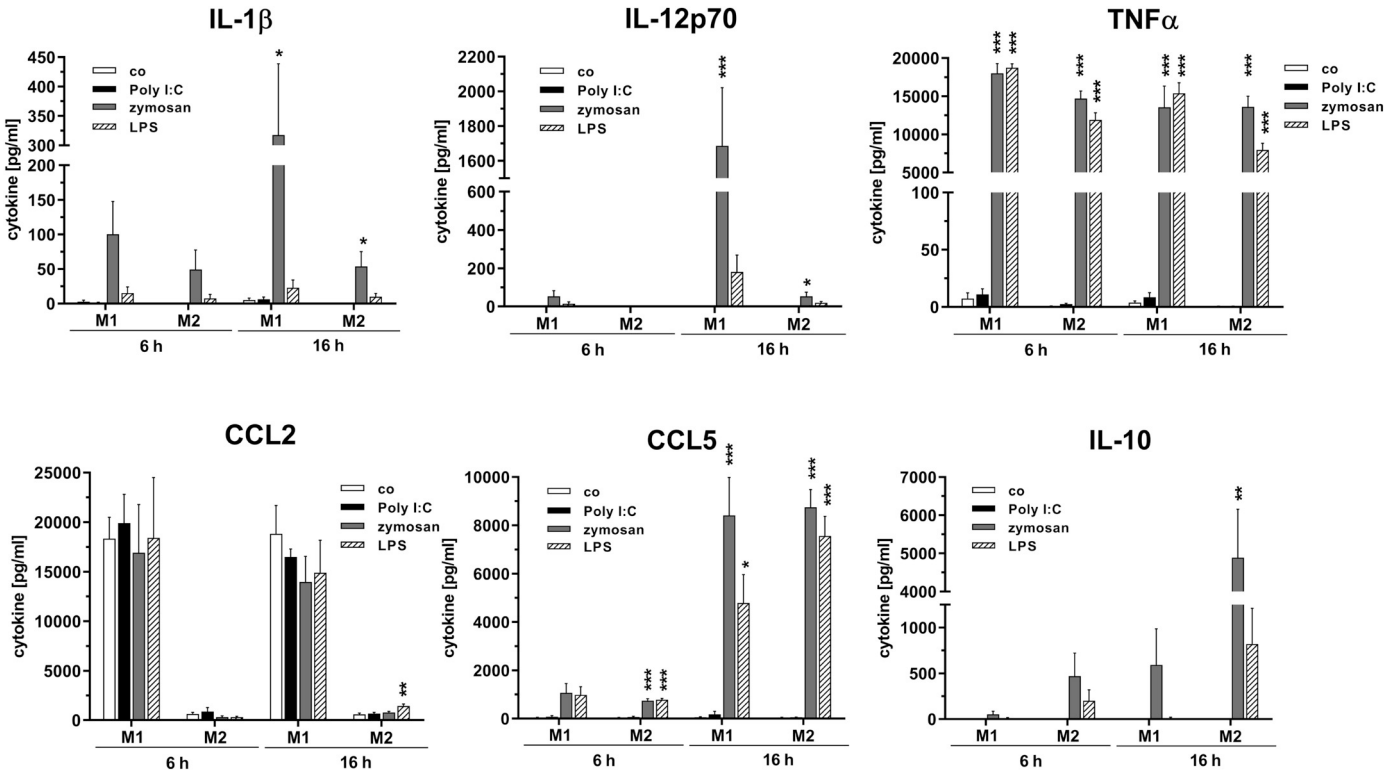

Fig. 5. Cytokine release into the conditioned media of M1 and M2 macrophages treated with TLR ligands for 6 and 16 h. M1 and M2 macrophages were differentiated from human peripheral blood monocytes (isolated by adherence method). For this, the cells were treated with 10 ng/mL CSF-2 (M1) or CSF-1 (M2) for seven days. Additionally, the cells received 10 ng/mL IFNγ (M1) or IL-4 (M2) for the final 48 h. After this, media were supplemented with TLR ligands for 6 or 16 h [bacterial LPS (0.1 μg/mL), yeast-derived zymosan (50 μg/mL) or Poly(I:C) (10 μg/mL)]. Cytokine concentrations in the conditioned media were assessed by cytometric bead array. Data are presented as mean ± SEM of 3 independent experiments. Significant changes *versus* the untreated controls are indicated with an asterisk. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

elevated in M2 macrophages. Taken together, differentiation of human monocyte-derived M1 and M2 macrophages from peripheral blood monocytes resulted in a distinct lipoxygenase expression pattern in our hands: $M1_{(CSF\text{-}2/IFN\gamma)}$ 5-LO^{hi}/$FLAP^{hi}$/15-LO-2^{lo} and $M2_{(CSF\text{-}1/IL\text{-}4)}$ 5-LO^{lo}/$FLAP^{lo}$/15-LO-1^{hi}/15-LO-2^{hi} macrophages (Fig. 6A). The spectrum of lipid mediator released from the macrophages reflected these patterns.

Depending on the inflammatory milieu, monocyte/macrophage differentiation and activation is shaped by endogenous pro- and anti-inflammatory mediators such as cytokines and lipid mediators giving rise to a broad spectrum of macrophage phenotypes. Here, IFNγ activated M1 macrophages and IL-4 triggered M2 cells represent the extremes of this multidimensional continuum of macrophage activation [31]. In addition to endogenous mediators, macrophages are also shaped by pathogen and damage associated molecular patterns (P/DAMP) such as microbial cell wall components, foreign nuclear acids or cellular debris. Therefore, we compared the expression and activities of enzymes involved in lipid mediator biosynthesis in M1 and M2 macrophages during treatment with PAMPs representing viral [Poly(I:C), TLR-3], fungal (yeast derived zymosan, TLR-2/dectin-1) and bacterial (*E. coli* derived LPS, TLR-4) inflammation. M1 macrophages readily responded to TLR-2 and TLR-4 activation with an immediate release of chemotactic LTB_4 while M2 cells did not (Suppl. Fig. III). The immediate release of LTs is of utmost importance to mount a quick immune response upon the encounter of pathogens [32]. Indeed, short-term incubation (20–60 min) with TLR-2 and -4 ligands has been shown to elevate LT levels due to an augmented intracellular AA release triggered by activation of phospholipase A_2 enzymes in pro-inflammatory macrophages [25,33,34].

After 6 h of persistent TLR-2 or TLR-4 stimulation immediate early genes such as COX-2 and TNFα were elevated in M1 macrophages resulting in the formation of pro-inflammatory prostanoids such as PGE_2 and TXB_2. Interestingly, M2 macrophages upregulated COX-2 as well but to a lower extent compared to M1 cells. PG levels in the macrophage conditioned media were time-dependently elevated and showed to be more persistent in M1 macrophages compared to M2 cells. These observations fit well with a number of publications documenting the upregulation of PG biosynthesis during acute inflammation in macrophages treated with TLR-2 and -4 ligands [35,36].

In our hands, PG biosynthesis was more potently triggered by zymosan compared to LPS. This was probably due to the more pronounced and also prolonged upregulation of COX-2 during zymosan treatment. If this is the consequence of the strong cell stimulation due to the additional phagocytic stimulus triggered by zymosan particles has to be speculated. Of note, TLR-3 stimulation by Poly(I:C) potently triggered PG release after 6 h although COX-2 expression was not induced in M1 and M2 macrophages. In line with this, Lundberg et al. have found that TLR-3 stimulation does not activate NFκB in human MDM and dendritic cells although the receptor is expressed. Type I interferon induction and signalling was intact in these cells. This was in stark contrast to murine macrophages in which NFκB inducible genes such as IL-6 and TNF are upregulated upon TLR-3 stimulation [37]. Although, Poly(I:C) treated M1 and M2 macrophages lacked COX-2 expression in our experiments, PG formation was elevated after 6 h. Therefore, it can be reasoned that this elevation is dependent on the activity of constitutively expressed COX-1 instead.

Persistent stimulation (16 h) of M1 and M2 macrophages with the TLR-2 ligand zymosan or the TLR-4 ligand LPS triggered a substantial upregulation of ALOX15B mRNA in both macrophage phenotypes. But, translation into 15-LO-2 protein was only detected in M2 cells. The reason for this is unknown so far, but comparable effects have already been documented for 15-LO-1 protein expression. Here, the regulatory heterogeneous ribonucleoprotein particles (hnRNP) E1 and K are

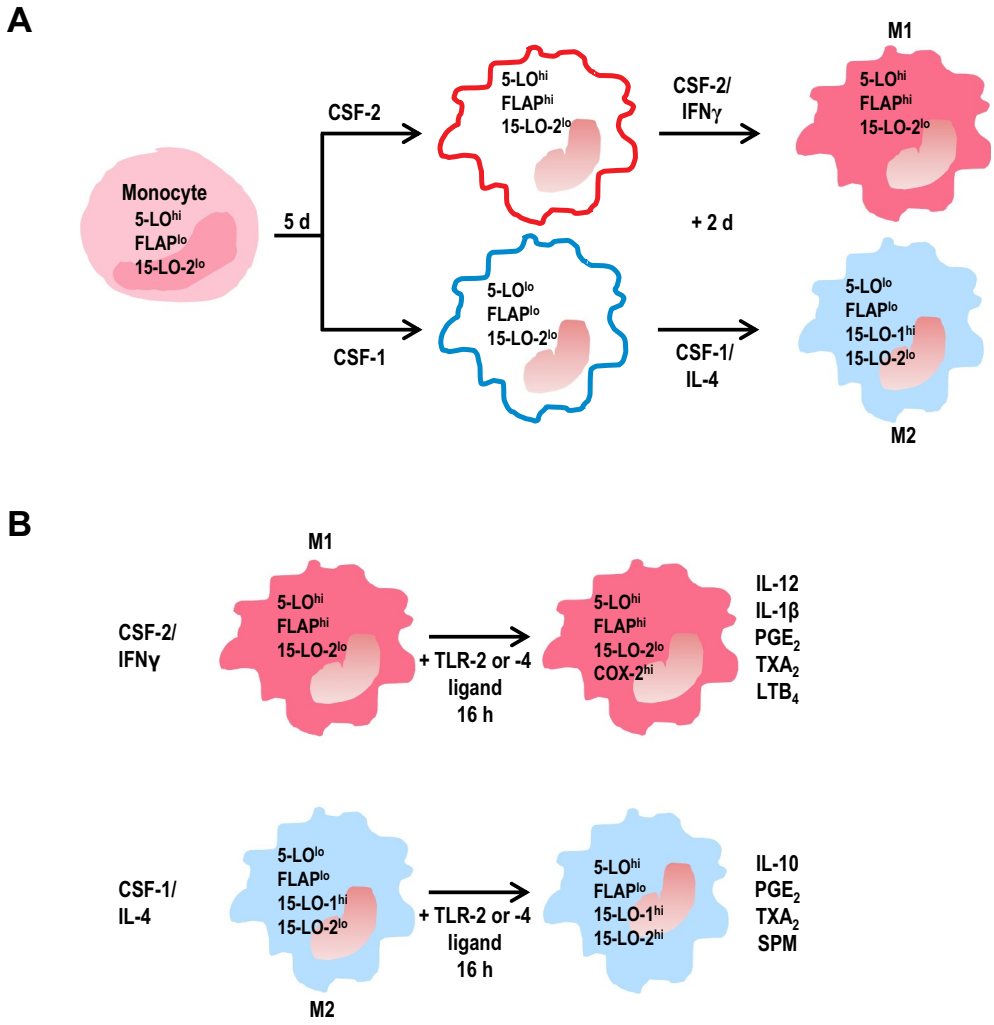

Fig. 6. Summary of enzyme expression and mediator release during differentiation of human peripheral blood monocytes to M1 and M2 macrophages and influence of persistent TLR stimulation. (A) Summary of the expression of the central enzymes involved in lipid mediator biosynthesis during the differentiation from human peripheral blood monocytes to M1 and M2 macrophages. (B) Summary of the influence of persistent TLR-2 and TLR-4 stimulation on enzyme expression, lipid mediator biosynthesis and cytokine release in M1 and M2 macrophages.

reported to bind to the 3'UTR of the ALOX15 mRNA thus preventing translation of the transcript and thereby blocking protein translation [38].

Concerning the expression of 5-LO, the direct opposite happened in our experiments: Here, ALOX5 mRNA was not elevated during TLR stimulation but protein levels increased about 4-fold in M2 macrophages upon long-term stimulation with TLR-2 or -4 ligands. Again, the reason for this is not known so far but it is evident from our data that profound changes in the posttranscriptional regulation take place between M1 and M2 macrophages upon persistent TLR stimulation. We will address these issues in future studies.

As expected the supernatants of M2 cells showed high 15-HETE levels most probably due to the elevation of 15-LO expression. Interestingly, these cells also released higher amounts of 5-HETE although the expression of 5-LO and FLAP was way lower compared to M1 macrophages. LPS stimulated M1 macrophages express high amounts of inducible NO synthases (iNOS) which have been shown to inhibit 5-LO activity in alveolar macrophages [39]. Together with the fact that M2 cells have a NOS deficit, this might explain the low levels of LTs in our M1 and the higher levels of 5-HETE in our M2 preparations. Of note, stimulation with the TLR ligands attenuated 5-HETE release into the macrophage conditioned media.

Detailed knowledge on the molecular details of SPM biosynthesis is needed to tailor resolution supporting therapies. Indeed, macrophage pro-resolving lipid mediator formation has been extensively studied in exudates from murine inflammation and SPM supplementation has been proven beneficial in a number of animal models [12,13,40]. However, on the cellular level the nature of the participating immune cells, the stimuli triggering the lipid mediator switch in these cells as well as the complete intracellular enzymatic machinery needed for SPM formation are far from understood in men and mice. Some details of transcellular LX and Rv biosynthesis have been elucidated in human leukocytes so far [14,16–20]. In contrast, SPM formation in human one-cell systems such as macrophages which co-express the essential LOs is less understood and reported mediator yields are considerably low compared to pro-inflammatory lipid mediators such as PGs and LTs [41]. We could further confirm this in our experiments. To make matters even more complicated, human cells can express two 15-LO isoforms (15-LO-1/-2) while mice have only 12-/15-LO, the orthologue of human 15-LO-1 producing a distinct 12-/15-HETE ratio [21].

Investigation on the formation of pro-resolving mediators in our macrophage incubations turned out to be challenging. We did not find any SPM in the conditioned media of the macrophages as well as in incubations of fMLF stimulated cells. We concluded that either the stimuli used (TLR ligands, fMLF) were too weak to trigger SPM formation in our cells or SPM formation is a transcellular process

dependent on a certain mixture of 5-LO^{high} and 15-LO^{high} macrophage phenotypes.

To investigate this further, the lipid mediator formation in Ca^{2+} ionophore stimulated M1 and M2 macrophages treated with LPS for 16 h was assessed. In addition, co-incubations of different macrophage phenotypes were monitored. To increase lipid mediator yields further, the incubations were supplemented with a mixture containing equal amounts of the SPM precursor fatty acids AA, EPA and DHA. This combination was recently found not to alter enzyme reaction specificities [42]. And indeed, SPMs were not detectable until we combined the strong intracellular Ca^{2+} elevating stimulus (Ca^{2+} ionophore A23187) together with exogenous lipid supplementation to induce a maximum LO activation. This approach gave a good estimate of the maximum enzyme activities possible in the different macrophage phenotypes but it should be kept in mind that the quantities of lipid mediators released *in vivo* will be less since elevation of intracellular Ca^{2+} after physiological stimulation resulting in lipoxygenase activation is way lower.

After strong stimulation with Ca^{2+} ionophore, LXA_4, RvE1, RvE2, RvE3, RvD1, RvD2, RvD5 and PDx were detectable in incubations of LPS treated (16 h) M2 and M2 control cells as well as in all macrophage co-incubations. M1 cells produced only RvE2 and RvD5 and small amounts of RvE1 when treated with LPS for 16 h. Maresins were below the LLOQ in all incubations. Although SPM formation was considerably upregulated in LPS treated M2 macrophages compared to the other preparations, these cells released huge amounts of prostanoids together with the monohydroxylated LO products and LTB_4 as well. The same was true for the M2 macrophage controls. It is evident that the prostanoids, leukotrienes and monohydroxylated lipids simultaneously produced together with the SPM by far outnumber the pro-resolving mediators in our hands: With 1.35 ng/μg protein 15-HETE was the most abundant AA derivative in LPS treated M2 macrophages followed by TXB_2, 12-HHTrE, 5-HETE and 12-HETE which were less produced by a factor of 2–10 (~100–500 pg/μg protein). These mediators were followed by LTB_4, 5,15-DiHETE, PGD_2, PGE_2 and RvD5, again by a factor of about 10 (~10–50 pg/μg protein). Of note, RvD1, RvD2, NPDx, LXA_4 and LXB_4 were released only in very low amounts of about 0.2 pg/μg protein and were therefore produced about 7000 fold less compared to 15-HETE and about 100–2500 fold less compared to LTB_4 and various prostanoids.

In contrast to the already abundant release of their monohydroxylated precursors into M2 macrophage conditioned media and after fMLF stimulation, SPM were only detectable under Ca^{2+} ionophore stimulation combined with AA/EPA/DHA supplementation in our experiments. Whether the tiny quantity of SPM which are probably released by human macrophages alongside huge amounts of other lipid mediators can have a positive influence on resolution initiation *in vivo* is not known at the moment. At this point, we cannot answer this completely owing to the artificial nature of human *in vitro* macrophage differentiation and stimulation in our experiments. Therefore, studies investigating SPM formation in humans during inflammation and resolution are urgently needed. Nevertheless, our data can help to give a good estimate of the maximum lipid mediator formation capacity of M1 and M2 macrophages representing the far edges of polarization during pathogen encounter as well as in peripheral blood monocytes. What can be clearly seen from our experiments is the marked switch in LO derived lipid mediators from 5-LO product dominated M1 cells that also release huge amounts of prostanoids and pro-inflammatory cytokines towards $M2_{LPS}$ macrophages which primarily release 15-LO-derived oxylipins and IL-10. If the abundant release of 15-LO-derived monohydroxylated fatty acids such as 15-HETE and 17-HDHA plays an important role in transcellular SPM biosynthesis or has so far unknown additional pro-resolving properties has to be investigated in the future.

From the monohydroxylated fatty acid yields it is evident, that EPA-derived 5-LO products were more abundant compared to AA-derived products whereas DHA was only sluggishly converted by 5-LO in our study. This fits well with studies showing a tendency towards substrate preference of EPA over AA for partially purified 5-LO from guinea pig PMNL, as well as the poor conversion of exogenous DHA by human PMNL and RBL-1 preparations [43–45]. In our hands, the macrophage 15-LOs showed a similar preference pattern: EPA and AA products could be found in equal quantities, while DHA-derived monohydroxylated fatty acid derivatives were produced about 50% less. Interestingly, the complete opposite was recently shown in incubations of purified recombinant human 15-LOs, supplemented with a mixture of AA, DHA and EPA. Here, the enzymes preferred DHA as substrate for 17-HDHA formation over the other fatty acids [42]. Apparently, additional factors such as substrate specificities of other enzymes involved in lipid mediator generation such as fatty acid liberating phospholipases make an important contribution to the net conversion ratio found in intact macrophages.

Upon stimulation with Ca^{2+} ionophore, M2 macrophages released substantial amounts of 15-LO products such as 15-HETE and 17-HDHA. This release increased further in the LPS-treated cells. In contrast to this, fatty acid products derived from 12-lipoxygenating activity, such as 12-HETE and 14-HDHA, were very low in these cells. It has been shown that the product spectra achieved by human 15-LO isoforms differ substantially when the isolated enzymes are incubated with DHA. While 15-LO-1 gives rise to equal amounts of 14- and 17-HDHA, 15-LO-2 was reported to form almost exclusively 17-HDHA [42]. Both 15-LO isoforms were present in our unstimulated and LPS stimulated M2 macrophages. So far, it is not known which isoform contributes to the 15-LO products released by these cells. Based on the high 17-HDHA/14-HDHA ratios found in the M2 macrophages, it is tempting to propose that the free 15-LO products released by these cells are primarily derived from 15-LO-2 activity. This could also explain the small quantities of 15-LO products detectable in monocytes, CSF-1 treated macrophages and M1 macrophages, since 15-LO-2 is constitutively expressed in these cells on a low level. Furthermore, the coordinated upregulation of 5- and 15-LO-2 in M2 cells upon persistent TLR stimulation, which is accompanied by a substantial elevation of SPM levels, supports this.

What about 15-LO-1 which is also present in IL-4 treated macrophages? 15-LO-1 is a rather promiscuous enzyme with broad substrate specificity. The enzyme readily converts various polyenoic fatty acids in their free form, as well as phospholipid and cholesterol bound species. Due to its interaction with phospholipids, 15-LO-1 takes part in the break-down and restructuring of biomembranes, thereby influencing cell survival, signalling and phagocytosis [46,47]. Interestingly, activated human M2 macrophages were found to generate ten times more phospholipid-esterified 15-HETE compared to the free lipid [48]. The 15-LO isoform responsible for the formation of phospholipid-bound 15-HETE in these cells was not investigated in this study. Again, it is tempting to speculate that 15-LO-1 mainly produces phospholipid-bound 15-HETE in M2 macrophages, thus influencing membrane fluidity and cell signalling, while 15-LO-2 is important for the formation of free 15-HETE in these macrophages. To give an answer to this question, further experimentation is needed which makes use of 15-LO inhibitors. This is a challenging task, due to a lack in available 15-LO inhibitors that display adequate isoform specificity. Ongoing work in our lab is addressing this issue at the moment. In addition, knock-down experiments for both 15-LO isoforms are ongoing.

Compared to 15-LO-1, the physiological role of human 15-LO-2 has been studied less. The enzyme is known to be upregulated by IL-4 and IL-13 treatment, as well as prolonged stimulation with LPS and hypoxia [49,50]. 15-LO-2 has recently been shown to control cholesterol homeostasis, CCL-17, CXCL-10 secretion and T cell migration in macrophages [51,52]. In addition, the enzyme is highly expressed in carotid plaques, suggesting a role in atherosclerosis [53]. Taking into account the important role of 15-LO-2 in the release of anti-inflammatory / pro-resolving lipid mediators from macrophages, further studies are needed that confirm this anti-inflammatory role in man.

There is another aspect of SPM formation in macrophages that has

led to controversial discussion in recent years: Is SPM formation in macrophages driven by cytosolic 5-LO without the need of translocation and the participation of FLAP? FLAP, a protein which plays a vital role in LT biosynthesis in intact cells, is reported to be much more highly expressed in M1 compared to M2 macrophages [29,54]. We were able to confirm this in our experiments. According to our data, this switch in the expression pattern took place during the stimulation with colony stimulating factors. Here, high FLAP expression was triggered by the presence of CSF-2 and pro-inflammatory stimuli, while low FLAP expression was detected in CSF-1 and also IL-4 treated cells. Employing inhibitors as well as knock-down and overexpression experiments, we could recently show that transcellular biosynthesis of LXA_4 and RvD1 is dependent upon FLAP and 5-LO translocation in intact neutrophils and a monocytic cell line [54]. In line with this, it was recently shown that the FLAP inhibitor Mk-886 blocks the formation of RvD2 and LXA_4 in monocyte-derived M2 macrophages. In contrast, formation of RvD5 as well as PD-1, PDx and maresin-1 (MaR-1) were not influenced in this study [41]. 5-LO is most probably not involved in PD-1, PDx and MaR-1 biosynthesis, therefore the lack of Mk-886 inhibitory activity is not surprising. In contrast, formation of RvD5 should be dependent on 5- and 15-LO, judging from the positions and orientations of the hydroxyl groups. On the basis of these data, FLAP is important for the formation of 5-LO-dependent SPM in intact transcellular as well as one-cell systems such as macrophages. Two alternative conclusions concerning the role of FLAP in macrophage SPM biosynthesis can be drawn from this: 1) Low FLAP protein levels in M2 macrophages are sufficient for SPM formation. In this case, the high levels of FLAP expression in M1 macrophages probably point to a yet unknown additional function of this protein. 2) SPM formation in macrophages is FLAP independent and inhibitors such as Mk-886 interfere with a yet unknown protein that is important in SPM formation in these cells. More investigations are needed in the future to obtain a complete picture of the role of FLAP and other proteins in the formation of SPMs.

In addition to LO-derived lipid mediators released by the different macrophage phenotypes, formation of prostanoids was monitored due to the central role of these lipid mediators in inflammation. Interestingly, prostanoid action is highly context dependent. While prostaglandins such as PGE_2 play an important role in inflammation initiation, they can also contribute to chronic inflammation as well as resolution and immunosuppression. Prostanoids are known to influence cytokine biosynthesis thereby augmenting macrophage and dendritic cell maturation, control adaptive immunity by supporting Th1 and Th17 cell differentiation and foster immunoglobulin class switching [55]. Furthermore, PGE_2 can also be anti-inflammatory and immunosuppressive following early resolution in mice and men thereby preventing the development of autoimmunity [56,57]. Due to this context dependent nature of prostanoid action as well as the expression pattern of prostanoid receptors in the direct vicinity of the macrophages it is almost impossible to correctly predict the inflammatory outcome of the prostanoids released in our assays.

In addition to the profound lipid mediator shift from pro-inflammatory leukotrienes in M1 cells to 15-LO derived products such as SPM in M2 macrophages, long-term treatment with TLR-2 and -4 ligands substantially shifted the cytokine levels of the macrophages. While M1 cells displayed high levels of pro-inflammatory cytokines such as IL-1β, IL-12 and CCL-2, M2 cells released high amounts of IL-10 after persistent stimulation with TLR-2 and -4 ligands. Indeed, long-term stimulation (20 h) of murine bone marrow derived macrophages with LPS (0.1 μg/mL) has been shown to induce efferocytosis of apoptotic shifting the cells to a pro-resolving macrophage phenotype [58]. In addition, it is well known that persistent LPS stimulation mediates the upregulation of IL-10 [59].

4.2. Conclusions

In this study, we investigated the lipid mediator formation in human MDM during *in vitro* differentiation and pathogen stimulation. For this, peripheral blood monocytes were differentiated into M1 (CSF-2/IFNγ) or M2 (CSF-1/IL-4) macrophages followed by stimulation with bacterial LPS, fungal zymosan or Poly(I:C). We found, that M1 macrophages primarily form pro-inflammatory cytokines and lipid mediators while persistent stimulation of human M2 macrophages with the TLR ligands zymosan (TLR-2) or LPS (TLR-4) induced a coordinated upregulation of 5- and 15-LO-2 expression which was accompanied by a substantial increase in the biosynthesis of 15-LO-derived monohydroxylated lipid mediators in the conditioned media of the cells as well as after stimulation with fMLF. Furthermore, stimulation of these macrophages with Ca^{2+} ionophore combined with fatty acid supplementation (AA/EPA/DHA) not only led to the release of huge amounts of monohydroxylated oxylipins but also small amounts of SPM such as lipoxins and resolvins were detectable in these cells. In addition, the cytokines released by these cells shifted from a pro- to an anti-inflammatory pattern suggesting a switch in macrophage phenotype.

Future studies should now focus on the relevance of SPM release during inflammation and resolution in man. In addition, the role of 15-LO-2 in human SPM biosynthesis and resolution as well as the mechanism behind its' TLR-2/-4 induced upregulation warrants further experimentation. In line with this, it has recently been shown that TLR-4 activity is required in the resolution of pulmonary inflammation and fibrosis after acute and chronic lung injury in mice [60]. Therefore, more studies are needed that investigate the *in vivo* relevance of persisting TLR-2/-4 stimulation in presence of Th2 immune responses in inflammation resolution.

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CRediT authorship contribution statement

Roland Ebert:Investigation, Formal analysis, Writing - original draft, Writing - review & editing, Visualization.**Rivelino Cumbana**:Investigation.**Christoph Lehmann**:Investigation.**Laura Kutzner**:Investigation, Formal analysis, Writing - review & editing.**Andy Toewe**:Investigation, Formal analysis.**Nerea Ferreirós**:Formal analysis, Validation, Funding acquisition, Writing - review & editing.**Michael J. Parnham**:Supervision, Writing - review & editing.**Nils H. Schebb**:Validation, Resources, Funding acquisition, Writing - review & editing.**Dieter Steinhilber**:Supervision, Funding acquisition, Resources, Writing - review & editing.**Astrid S. Kahnt**:Supervision, Conceptualization, Project administration, Investigation, Formal analysis, Validation, Funding acquisition, Visualization, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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