Omega-3 Fatty Acids and Oxylipins as Modulators of Phagocytosis in Human Macrophages

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Deep in the human unconscious is a pervasive need for a logical universe that makes sense. But the real universe is always one step beyond logic.

Frank Herbert

Contents

1 Introduction and Scope

Dietary intake of n–3 and n–6 polyunsaturated fatty acids (PUFA) is vital for humans. There is growing evidence that a high intake of n–3 PUFA is associated with beneficial health outcomes such as a decreased risk for cardiovascular diseases. However, the typical Western diet includes high amounts of n–6 PUFA (e.g., from plant-based oils such as soy and sunflower oil), while n–3 PUFA intake, especially long-chain n–3 PUFA, is low [1, 2]. Additionally, the conversion of alpha-linolenic acid to long-chain n–3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is discussed to be low [3, 4], but remains controversial [5].

Blood and tissue levels of EPA and DHA are low in humans in Europe and the US consuming an average Western diet [6, 7]. This is reflected by a high n–6:n–3 ratio and high %n–6 in highly unsaturated fatty acids (HUFA) values [7, 8], which are both biomarkers associated with an increased risk for cardiovascular events [9]. Hence, by changing the n–3 PUFA status, e.g., by consuming n–3 PUFA supplements, health and immune functions can be affected [10–12].

PUFA are important components of the cell membranes and mediate their effects via various mechanisms: they affect membrane fluidity, the composition of lipid rafts, membrane-associated proteins, gene expression and are precursors for the formation of oxylipins [13]. Several oxylipins are lipid mediators involved in the regulation of physiological and inflammatory processes.

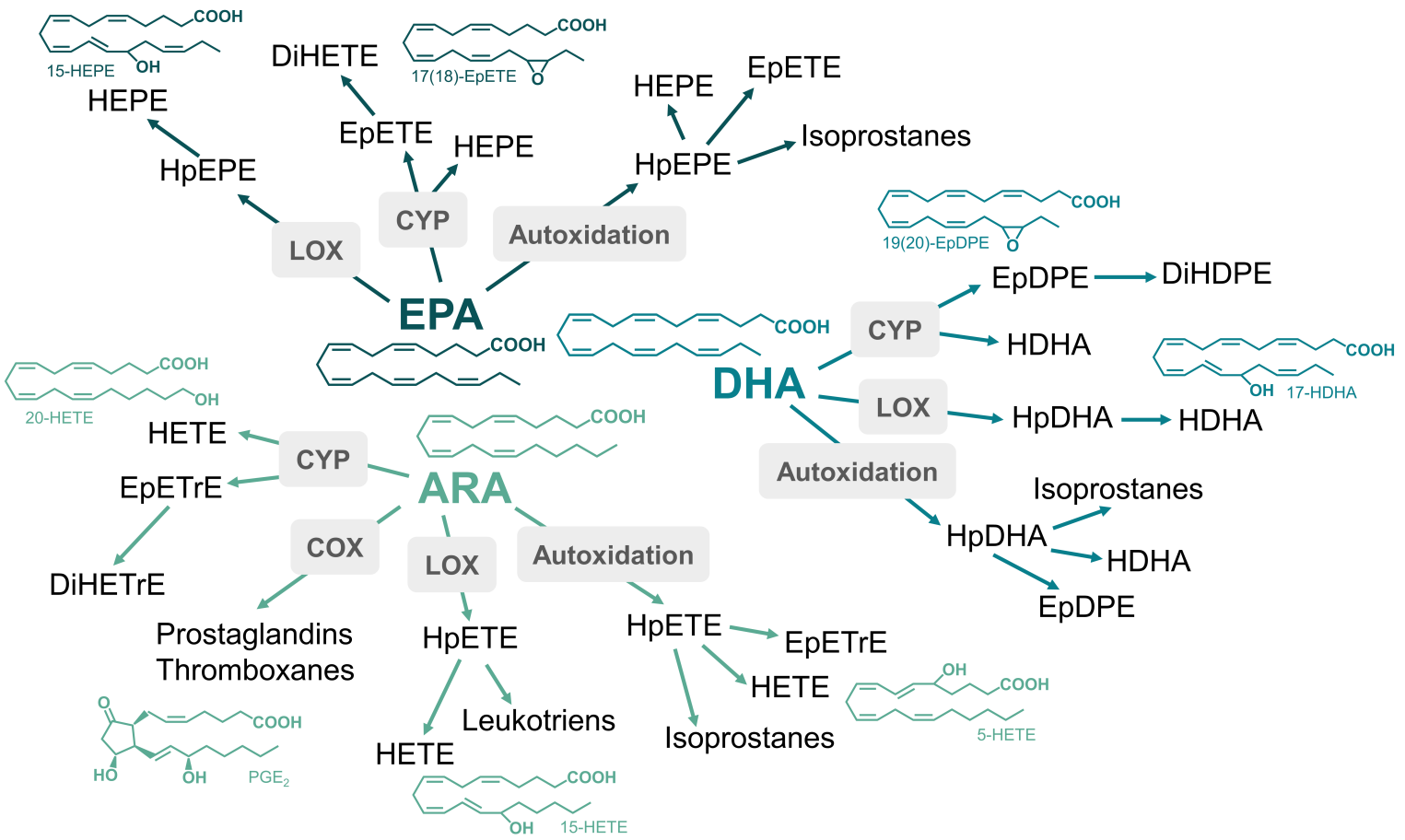

Figure 1.1: Simplified overview over selected branches of the ARA cascade. Only a few exemplary products from the PUFA ARA, EPA, and DHA are shown.

Oxylipins are formed via enzymatic and non-enzymatic oxidation of PUFA giving rise to a variety of different structures (Figure 1.1): (i) The conversion by cyclooxygenases (COX) leads to highly potent prostaglandins (PG) and thromboxanes (Tx) [14], and hydro(pero)xy-FA as side products [15, 16]. The isoform COX-1 is constitutively expressed in almost all tissues maintaining homeostatic functions [17]. COX-2 expression is highly restricted during basal conditions, but dramatically upregulated during inflammation [18]. This makes it an important target for anti-inflammatory drugs such as nonsteroidal anti-inflammatory drugs (NSAID) or glucocorticoids.

(ii) Lipoxygenases (LOX) catalyze the formation of regio- and stereoselective hydroperoxy-PUFA which are further reduced to hydroxy-FA in the cells [19]. There are six human LOX isoforms named by the position of the hydroperoxy group which is inserted into arachidonic acid (ARA). 15-LOX-1 has a dual reaction specificity resulting in n–6 and n–9 products (i.e., 15-/12-HETE) in a distinct ratio specific for each PUFA [20], whereas 15-LOX-2 only leads to the formation of the n–6 product. Of note, both isoforms oxidize not only free, but also esterified PUFA such as phospholipids [20, 21].

(iii) Cytochrome P450 monooxygenases (CYP) form hydroxy-FA carrying the hydroxy-functionality at the ω and ω–n position or epoxy-FA [22]. The latter can be easily hydrolyzed by the soluble epoxide hydrolase to the corresponding *vic*-dihydroxy-FA.

(iv) Non-enzymatic conversion by autoxidation leads to hydroperoxy-FA, hydroxy-FA, PG-like isoprostanes and epoxy-FA among other oxylipins [23]. Autoxidation results in the formation of a racemic mixture of the products. This is a

major difference to the enzymatic conversion of PUFA, which always gives rise to stereoselective products.

Oxylipins exert their biological functions via G protein-coupled receptors (GPCR) [24], nuclear receptors such as peroxisome proliferator-activated receptors (PPAR) [25, 26] or by interfering with other signal transduction pathways [27–29]. Oxylipins are involved in both, pro-inflammatory and anti-inflammatory, pro-resolving immune processes: n–6 PUFA derived oxylipins, especially prostaglandins and leukotriens derived from ARA, are well-investigated pro-inflammatory mediators [22], involved in pain, fever and asthma [30]. In contrast, several n–3 PUFA derived oxylipins are regarded having anti-inflammatory properties, e.g., reducing inflammation and helping to return to homeostasis [22]. Interestingly, increasing the intake of n–3 PUFA not only shifts the PUFA pattern of cells towards n–3, but also changes the oxylipin profile: n–6 PUFA are less converted, while n–3 PUFA formation is increased [31].

There is growing evidence that a high n–3 PUFA status, e.g., achieved by intake of n–3 PUFA supplements, impacts immune and inflammatory functions [10–12]. However, the underlying mechanisms by which n–3 PUFA exert their effects on immune functions, remain to be elucidated. A shift in the oxylipin pattern is considered to be at least part of the mode of action of n–3 PUFA.

The gold standard for investigating the effects and modes of action of n–3 PUFA are human nutrition intervention studies or clinical trials. However, carrying out these studies is challenging, particularly the monitoring of the diet and intake of supplements of participants requires a lot of resources. Considering the analysis of analytes such as oxylipins, blood sampling has to be standardized to ensure

reliable results [32]. These challenges may contribute to the fact, that results of these studies on the effects of n–3 PUFA on immune cells are inconsistent [33], highlighting the need for further research.

For a mechanistic investigation of n–3 PUFA and their oxylipins without the need for human *in vivo* studies, a supplementation strategy in human immune cells would be a valuable tool therefore. For this purpose, an *ex vivo* n–3 PUFA supplementation strategy was developed in primary human macrophages which is described in chapter 2. The developed *ex vivo* n–3 PUFA supplementation strategy allows to change the FA pattern of monocytes from subjects with a low n–3 PUFA status – typical for people in Europe and the US – to macrophages with a FA pattern comparable to subjects with a high n–3 PUFA status. Key parameters for all steps of the supplementation strategy were identified in order to provide a reproducible and reliable strategy, allowing to alter the PUFA and oxylipin profile comparable to human intervention studies. Hence, the developed supplementation strategy allows mechanistic investigation of n–3 PUFA and oxylipins in primary human immune cells under controlled conditions without the need for human intervention studies.

Oxylipins modulating inflammation and immune functions are formed and released by immune cells such as macrophages. Macrophages are important cells of the first line host defense, recognizing foreign molecules and releasing cytokines, chemokines and oxylipins [34]. Additionally, they are capable of phagocytosis, engulfing pathogens and cell debris. Phagocytosis is a fundamental process for the elimination of microbial invaders and apoptotic cells involving many receptors and phases [35]. Briefly, for detection of pathogens/particles, receptors on

the surface of phagocytes such as pattern-recognition receptors (PRR) recognize specific pathogen-associated molecular patterns (PAMP), priming the cell for phagocytosis [36]. The internalization process is activated by various signaling pathways leading to reorganization of the actin cytoskeleton in order to completely cover the pathogen with membrane and form a new vesicle inside the cell, so called phagosome. This phagosome undergoes maturation steps involving the fusion with several endosomes, enabling the degradation of the ingested pathogen by enzymes under acidic conditions [35]. There is growing evidence that various signaling pathways and receptors are involved in phagocytosis, but several are not fully understood to date [35]. Overall, phagocytosis prevents the progression of inflammation and enables tissue recovery by direct neutralization of pathogens and by prevention of secondary necrosis engulfing cell debris [37].

Macrophages can be divided in different phenotypes, reflecting a spectrum of activated phenotypes rather than clearly defined subpopulations [38–40]. Moreover, macrophages are highly plastic cells adapting their functions in response to microenvironmental stimuli [41], leading to inflammatory (M1-like) and anti-inflammatory (M2-like) phenotypes. For cell culture experiments polarization of macrophages usually involves different polarizing agents mimicking the exposure of macrophages to distinct microenvironmental conditions [40]: For M1-like macrophages typically interferon (IFN)γ is used, while for polarization towards M2-like macrophages interleukin (IL)-4 or IL-13 are used [42]. Of note, these polarization conditions reflect the conditions *in vivo* only to a limited extent, but enable a comparatively simple and cost-efficient investigation of the extremes of macrophage phenotypes of a continuous spectrum of different phenotypes.

Depending on the cytokines used for polarization, macrophage phenotypes differ in their gene expression, cell surface receptors, proteins and activity [40]. Differences in these phenotypes involve abundance and activity of the enzymes of the ARA cascade resulting in different oxylipin profiles of these cells [43, 44]. However, the involvement of the ARA cascade enzymes and oxylipins regulating phagocytosis in different macrophage phenotypes is poorly understood.

For this purpose, a phagocytosis assay was established and optimized in primary human macrophages, followed by the investigation of the role of the COX branch of the ARA cascade in phagocytosis, which is described in chapter 3. The COX product PGE_2 was found to inhibit phagocytosis via EP4 receptor signaling, which could explain, why LPS exerts differential effects on phagocytosis in 'inflammatory' M1- and 'anti-inflammatory' M2-like macrophages. A deeper insight into the regulation of phagocytosis was gained highlighting the importance of the link between the COX branch of the ARA cascade and the regulation of phagocytosis.

Phagocytic activity was shown to be highly associated with PUFA composition of the cell membranes [45–47]. Additionally, several oxylipins such as multihydroxy oxylipins derived from EPA and DHA are discussed to modulate phagocytosis [48, 49]. Consequently, shifting the PUFA and oxylipin profile of macrophages via n–3 PUFA supplementation is likely to modulate phagocytosis. In order to investigate the impact of n–3 PUFA and derived oxylipins on phagocytosis, the *ex vivo* supplementation strategy was combined with the phagocytosis assay as described in chapter 4. Changing the n–3 PUFA status of the cells from low to high, accompanied by changes in the oxylipin pattern, resulted in an elevated phagocytosis of the macrophages. This effect could be partially explained by the reduced formation

of prostaglandins such as PGE_2 in the n–3 PUFA supplemented cells. n–3 PUFA derived oxylipins, which were increased by the n–3 PUFA supplementation, have only limited impact on phagocytosis. This demonstrates that subjects with a low n–3 PUFA status could benefit from a n–3 PUFA supplementation altering PUFA and oxylipin profile and thus, elevating phagocytosis of the macrophages.

Overall, this thesis provides detailed strategies for the supplementation of human macrophages with n–3 PUFA and for the investigation phagocytosis. This allows a deeper insight into the mode of action of n–3 PUFA and derived oxylipins in human macrophages contributing to a better understanding of the modulation of immune functions by diet.

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2 An Optimized Ex Vivo n–3 PUFA Supplementation Strategy for Primary Human Macrophages Shows That DHA Suppresses Prostaglandin E2 Formation*

Evidence suggests beneficial effects of long-chain n–3 polyunsaturated fatty acids (PUFA) in inflammatory diseases. However, the underlying mechanisms are still subject of research. For this purpose, we developed an ex vivo n–3 PUFA supplementation strategy. M2-like macrophages were supplemented for 2–3 days with 20–40 μM docosahexaenoic acid (DHA) during differentiation. Quality parameters include <3% oxylipins for PUFA-preparation, total fatty acids (FA) <10 mM, and low oxylipins in plasma, n–3 PUFA <0.25 mM for the selection of donors of plasma as well as %n–6 in highly unsaturated fatty acids (HUFA) ≥70% for donors of cells. Following supplementation, PUFA pattern of cells was shifted toward one described for blood and tissue from subjects with higher n–3 and lower n–6 PUFA. This was accompanied by a decrease of arachidonic acid-derived oxylipins in a dose- and time-dependent manner in favor of n–3 PUFA ones. Stimulation with LPS resulted in decreased levels of pro-inflammatory prostaglandins in the DHA-supplemented cells, but no changes in cytokines. in vitro supplementation studies with n–3 PUFA need rigorous controls to exclude the background formation of oxylipins. By accounting for these possible confounders the described approach allows the mechanistic investigation of n–3 PUFA in primary human immune cells, offering an alternative for intervention studies.

* modified from R. Kirchhoff, N. Kampschulte, C. Rothweiler, N. Rohwer, K-H. Weylandt, and N. H. Schebb. "An Optimized Ex Vivo n–3 PUFA Supplementation Strategy for Primary Human Macrophages Shows That DHA Suppresses Prostaglandin E2 Formation". In: *Molecular Nutrition & Food Research*, **2025** *69*(1), e202400716. DOI: 10.1002/mnfr.202400716.

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2.1 Introduction

Dietary intake of n–3 and n–6 polyunsaturated fatty acids (PUFA) is essential for humans. In Western countries, n–6 PUFA are consumed in large amounts (e.g., by intake of plant oils from soy, sunflower, and corn) whereas the n–3 PUFA intake is low [1, 2]. In addition, the conversion of alpha-linolenic acid to long-chain n–3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is discussed to be low [3, 4], particularly because the n–6 PUFA linoleic acid competes for the same enzymes for its conversion to arachidonic acid (ARA) [5]. However, the conversion rate of PUFA to long-chain PUFA in humans remains controversial [6]. Blood and tissue levels of EPA and DHA are low in humans on a Western diet [7, 8], indicated by a high n–6:n–3 PUFA ratio and high %n–6 in highly unsaturated fatty acids (HUFA) values, respectively [8, 9]. These values are biomarkers for increased health risks, such as cardiometabolic events [10]. A large number of studies show that a high n–3 PUFA status, for example, by consuming n–3 PUFA supplements, influences immune and inflammatory functions [11–13]. However, the underlying mechanisms of action by which n–3 PUFA exert their anti-inflammatory effects are not fully understood.

Being key components of cell membranes, fatty acid (FA) composition impacts the fluidity of membranes which in turn influences the function of membrane-associated proteins [14]. Moreover, PUFA undergo enzymatic and non-enzymatic oxidation giving rise to multiple oxylipins: conversion by cyclooxygenases (COX) leads to highly potent prostaglandins (PG) and thromboxanes (Tx) [15]. Lipoxygenases (LOX) form regio- and stereoselective hydroperoxy-PUFA which are further reduced to hydroxy-FA in the cells [16]. Cytochrome P450 monooxygenases

(CYP) form hydroxy-FA carrying the hydroxy-functionality at the ω and ω–n position or epoxy-FA. The latter can be easily hydrolyzed by the soluble epoxide hydrolase to *vic*-dihydroxy-FA. Non-enzymatic conversion by autoxidation leads to hydroperoxy-FA, hydroxy-FA, and PG-like isoprostanoids [17].

Several oxylipins are potent bioactive lipid mediators involved in the regulation of physiological processes such as fever and inflammation [18]. ARA-derived PG formed by COX activity and leukotrienes formed by 5-LOX activity are well-investigated pro-inflammatory mediators [18]. n–3 PUFA reduce the conversion of n–6 PUFA and gives rise to a distinct set of n–3-PUFA-derived oxylipins. Several of those are discussed to have an anti-inflammatory effect, including 15-hydroxy-eicosapentaenoic acid (HEPE) [19] or 7(*S*),17(*S*)-dihydroxy-docosahexaenoic acid (DiHDHA) (resolvin [Rv]D5) [20]. Consequently, a shift in the oxylipin pattern is considered to be at least part of the anti-inflammatory mode of action of n–3 PUFA.

For elucidating the effects and the mode of action of n–3 PUFA, human intervention studies or clinical trials are ultimately necessary [21–23]. However, controlling nutrition and supplementation is challenging, and thus, the results regarding anti-inflammatory effects on immune cells are inconsistent [24]. Moreover, blood sampling and analysis of analytes such as oxylipins in clinical settings can lead to large variations [25]. In order to enable mechanistic investigation of immune cells without the need for human *in vivo* supplementation studies, we developed an *ex vivo* supplementation strategy for the detailed investigation of the physiological effects of n–3 PUFA and derived oxylipins in human macrophages. For all steps of the strategy, key parameters were identified and defined ensuring a reli-

able supplementation strategy. Applying the model to investigate the effect of the inflammatory stimulus LPS, changes in mRNA, protein, and oxylipin levels of the supplemented cells were examined.

2.2 Experimental Section

2.2.1 Chemicals and Biological Materials

Human AB plasma was provided by the blood donation center at University Hospital Düsseldorf (Düsseldorf, Germany) and bought from BioIVT (West Sussex, UK) and Biowest (local distributor VWR, Darmstadt, Germany). Lymphocyte separation medium 1077 was obtained from PromoCell (Heidelberg, Germany). Recombinant human colony-stimulating factors CSF-1 (macrophage colony-stimulating factor [M-CSF]), CSF-2 (granulocyte-macrophage colony-stimulating factor [GM-CSF]), interferon (IFN)γ, and interleukin 4 (IL-4) produced in Escherichia coli were purchased from PeproTech Germany (Hamburg, Germany). Ethylenediaminetetraacetic acid (EDTA) disodium dihydrate and dimethyl sulfoxide (DMSO) were obtained from Carl Roth (Karlsruhe, Germany). DHA was bought from Nu-check Prep, Inc. (DHA >99%) (Elysian, Minnesota, USA). EPA was obtained from Chemo-dex Ltd. (EPA $\geq$97%) (local distributor Biomol, Hamburg, Germany) and Nu-check Prep, Inc. (EPA >99%). BCA reagent A was bought from Fisher Scientific (Schwerte, Germany). The ultrapure water with a conductivity of >18 MΩ*cm was generated by the Barnstead Genpure Pro system from Thermo Fisher Scientific (Langenselbold, Germany). RPMI 1640 cell culture medium, L-glutamine and penicillin/streptomycin (P/S, 5.000 units/mL penicillin, 5 mg/mL streptomycin),

LPS from E. coli (0111:B4), dextran 500 from Leuconostoc spp., and copper sulfate pentahydrate were purchased at Merck (Schnelldorf, Germany).

2.2.2 Cell Cultivation

Primary human macrophages were prepared as described [26]. In brief, peripheral blood monocytic cells (PBMC) were isolated from buffy coats obtained from blood donations at the University Hospital Düsseldorf. Blood samples were drawn with the informed consent of healthy human subjects. The study was approved by the Ethical Committee of the University of Wuppertal. PBMC were isolated from the supernatant after dextran (5%) sedimentation for 30−45 min. The erythrocytes in the remaining sediment were washed with PBS and subsequently frozen at -80 °C. Dextran supernatant was subjected to centrifugation (800 × g without deceleration, 10 min, 20 °C) on lymphocyte separation medium to isolate plasma and a leukocyte ring. Leukocytes were washed twice with PBS and seeded in 60.1 mm^2 dishes in RPMI medium (100 IU/mL penicillin and 100 µg/mL streptomycin [P/S], 2 mM L-glutamine) in a humidified incubator at 37 °C and 5% CO_2 for 1 h. Non-adherent cells were collected by washing them off the dishes. Collected cells were washed two times with PBS and stored as dry pellets frozen at -80 °C until use. RPMI medium (P/S, 2 mM L-glutamine, and 5% human AB plasma) was added to the adherent cells. Monocytes were differentiated into M2-like macrophages using an established protocol by 10 ng/mL M-CSF for 7 days and additional 10 ng/mL IL-4 for the final 48 h [26, 27].

2.2.3 Supplementation Experiments

Following predilution in DMSO (50 mM), the n–3 PUFA preparation (DHA) was gently mixed with human plasma in order to stabilize the solution of the PUFA: for medium supplemented medium, 20 μM n–3 PUFA preparation was added and for high supplemented medium 45 μM. Premixed plasma was added (5%, *v/v*) to RPMI medium (P/S, 2 mM L-glutamine). Medium containing the same plasma, but without additional n–3 PUFA preparation, served as control medium with low n–3 PUFA content (low). Macrophages were incubated with medium or high supplemented medium for either 2, 3, or 7 days. Every 2–3 days the cultivation medium was renewed. Before renewal of the medium, samples of supernatants were collected and frozen at -80 °C for analysis. For LPS stimulation, macrophages were treated with 1 μg/mL LPS (in PBS) 6 h before harvest. Macrophages were harvested by cold shock method [26], and cell pellets were frozen at -80 °C until analysis.

2.2.4 Quantification of Oxylipin, Fatty Acyl, and Protein Levels by LC-MS/MS

Oxylipin determination was carried out in cell pellets, medium, and human plasma as described [28–31]. Briefly, cell pellets were resuspended in PBS, sonicated and protein content was determined via bicinchoninic acid assay [32]. After the addition of internal standards (IS), proteins were precipitated using MeOH (for non-esterified oxylipins) or iso-propanol (for total oxylipins) followed by centrifugation (20 000 × g, 10 min, 4 °C). For quantification of total oxylipin levels, supernatants were saponified using 0.6 M KOH in MeOH/H_2O (75/25, *v/v*) for 30 min at 60 °C.

Oxylipins were purified using solid phase extraction (SPE) and analyzed by means of LC-MS/MS.

The precipitated protein pellet following MeOH precipitation was analyzed for the abundance of key proteins [27, 31]. Briefly, after resuspension in 5% (*w/v*) sodium deoxycholate and precipitation in ice-cold acetone, the pellet was redissolved in 6 M urea, disulfide bridges were reduced with dithiothreitol, and free sulfhydryl groups were alkylated with iodoacetamide. Following tryptic digestion for 15 h and the addition of heavy labeled peptides, samples were subjected to SPE extraction and analyzed by LC-MS/MS.

The samples for oxylipin and peptide analysis were measured with separate methods on 1290 Infinity II LC systems (Agilent, Waldbronn, Germany), coupled to a QTRAP mass spectrometer (Sciex, Darmstadt, Germany) as described [28–31]. Chiral separation of oxylipins was carried on a 50 × 3.0 mm Chiralpak IA-U column, that is, amylose tris(3,5-dimethylphenylcarbamate) material immobilized on 1.6 μm silica (Daicel, Osaka, Japan) at 35 °C with a linear gradient of acidified water and acetonitrile [33]. Fatty acyl concentrations from the same samples as for the total oxylipins were determined by LC-MS/MS as described [34]. Oxylipin, fatty acyl, and peptide concentrations were quantified using external calibrations with IS and were calculated based on the total protein content in the cells. Fatty acyl levels in human plasma were analyzed by GC-FID following HCl-catalyzed transmethylation to fatty acid methyl esters (FAME) [35].

2.2.5 RNA Extraction and Quantitative PCR Analysis

Total RNA from M2-like macrophages was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and QIAshredder spin columns (Qiagen) according to the manufacturer's instructions. First-strand cDNA was synthesized with oligo (dT) primers and the iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories, München, Germany). Gene expression was measured using the SsoFAt EvaGreen Supermix on a CFX96 Real-Time PCR Detection System (both Bio-Rad Laboratories). Relative fold-changes of target gene expression were calculated by the comparative ΔCT method normalizing CT values to the geometric mean of the CT values of the housekeeping genes RPL13A, SDHA, and YWHAZ. Primer sequences are listed in Table A.1. qPCR measurements from the cell suspensions with and without sonication were comparable (Figure A.1) allowing the parallel analysis of gene transcription, protein abundance, and fatty acyl and protein levels.

2.2.6 Statistical Analysis

GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used for the statistical analysis.

2.3 Results and Discussion

Dietary intake of n–3 PUFA is required for human health, and sufficient intake of long-chain n–3 PUFA is associated with beneficial health effects [36, 37]. In order to investigate the effects of these long-chain n–3 PUFA and their oxylipins, a reliable *ex vivo* supplementation strategy was developed allowing to change the

cellular FA pattern of monocytes from subjects following a typical Western diet to macrophages with an FA pattern comparable to subjects having a high n–3 PUFA status. This tool allows mechanistic investigation of the effects of long-chain n–3 PUFA in primary human immune cells under strictly controlled conditions.

2.3.1 Characterization of Key Parameters for the Ex Vivo Supplementation Strategy

In order to enable a reproducible n–3 PUFA supplementation of primary human macrophages, several key parameters such as selection of buffy coats, plasma, n–3 PUFA preparation, and composition of cell cultivation media have to be controlled (Table 2.1).

a. Selection of cells from suitable subjects/buffy coats

FA status of the subjects (blood donors) was characterized based on the components of buffy coats. During the isolation of PBMC from buffy coats, fractions of erythrocytes, plasma, and non-adherent cells were collected and investigated for their FA composition based on %n–6 in HUFA and n–6:n–3 ratio. Both markers of macrophages (e.g., subject 1: %n–6 in HUFA: 73.2% $\pm$ 2.4%; n–6:n–3 ratio: 3.2 $\pm$ 0.8) were reflected best by the erythrocytes (subject 1: %n–6 in HUFA: 72.8% $\pm$ 0.2%; n–6:n–3 ratio: 3.5 $\pm$ 0.1) (Figure A.2).

The PUFA pattern of erythrocytes, described as omega-3 index or %n–6 in HUFA, are established biomarkers for the individual n–3 PUFA status of subjects [10, 38, 39]. They correlate with the PUFA composition of tissues [40, 41] as well as other blood cells [42]. Due to the Western diet, typically containing high amounts of n–6

Table 2.1: Key parameters for the selection of materials for the *ex vivo* supplementation of primary macrophages obtained from buffy coats.

	Parameters	Explanation
Selection of suitable subjects/buffy coats	%n-6 in HUFA ≥70%	Subjects should reflect poor n–3 PUFA status of average subjects in Europe and the US, which can be improved by supplementation.
Selection of human plasma for cell culture medium	Prepared under controlled conditions, resulting in low oxylipin concentrations, e.g., 5-HETE <100 nM, 12-HETE <200 nM, 15-HETE <200 nM, 5-F2t-IsoP <1 nM, 9-HODE <500 nM	The supplementation conditions should reflect the oxylipin pattern of plasma from healthy subjects.
	Total FA concentration <10 mM	The total FA concentration should be in a range of physiological conditions.
	n–3 PUFA concentration <0.25 mM, %n–6 in HUFA >75%	Plasma should reflect the n–3 PUFA status of average subjects in Europe and the US, which can be increased by supplementation.
Selection of n–3 PUFA preparation	Max. 0.5% (*w*/*w*) oxylipins	In order to investigate the effects of the n–3 PUFA and not of the artificially added oxylipins.
Storage of supplemented cell culture medium	Keep medium at 0–4 °C and store supplemented medium max. up to 2 days in the fridge. Replace medium of cells every 2–3 days.	Keeps autoxidative formation of oxylipins low in the medium.

and low amounts n–3 PUFA [43], the average omega-3 index is low (4%–6%) and %n–6 in HUFA is high (60%–85%) in Europe and the US [10, 39, 44]. In order to reflect this, we only included subjects with >70% n–6 in HUFA ensuring that they were representative of persons having a low n–3 PUFA status. Supporting the poor n–3 PUFA status of the German population, erythrocytes obtained from all buffy coats analyzed fulfilled the criterion (%n–6 in HUFA 72% $\pm$ 1% to 82% $\pm$ 1%) (Table A.2).

b. Selection of human plasma for cell cultivation media

Human plasma was selected based on the oxylipin and FA levels. A non-fasted plasma pool obtained from a local blood donation center and two commercially available plasma pools were compared for their oxylipin concentrations. Overall, oxylipin concentrations were up to 10-fold higher for the first and up to 600-fold higher in the second commercial plasma pools reaching the micromolar range for several oxylipins such as 5-HEPE or 14-hydroxy-docosahexaenoic acid (HDHA) (Table A.3). This can only be explained by inappropriate sampling and storage conditions during plasma preparation which leads to the artificial formation of oxylipins by both, non-enzymatic and enzymatic reactions [25, 29, 33]. In order to reflect plasma conditions of healthy subjects [29], a plasma low in oxylipin levels has to be used for the cell culture medium. We defined concentrations of the five following oxylipins as a criterion if the plasma can be used for further experiments: 5-hydroxy-eicosatetraenoic acid (HETE) (formed by autoxidation [45], 5-LOX-activity [46, 47] <100 nM, 12-HETE (formed by platelet 12-LOX during blood coagulation, 12-LOX and 15-LOX activity [48, 49], autoxidation [45]) <200 nM, 15-HETE <200 nM (formed by 15-LOX activity [50], side-product of

COX-2 activity [51], autoxidation [45]), 5-F2t-IsoP <1 nM (formed by autoxidation [52]), and 9-HODE <500 nM (formed by LOX activity [53], autoxidation [45], oxylipin with the highest concentration in plasma).

Plasma samples (Figure A.3) from several healthy donors generated under controlled conditions at a local blood donation center were analyzed. FA concentration and pattern differed markedly (Figure A.3, Table A.4): Due to the different fasting states of the subjects, total FA concentrations were only about half as high in plasma B (7.7 $\pm$ 0.1 mM) and plasma E (7.1 $\pm$ 0.4 mM) compared to plasma C (15 $\pm$ 1 mM). n–3 PUFA concentrations were almost three times higher in plasma C and D (C, 0.48 $\pm$ 0.05 mM; D, 0.45 $\pm$ 0.02 mM) compared to plasma B (0.17 $\pm$ 0.02 mM) and %n–6 in HUFA ranged from 67% $\pm$ 1% (plasma D) to 83% $\pm$ 1% (plasma B). The FA pattern, for example, n–6:n–3 ratio, n–3 index, and %n–6 in HUFA, was also different among the tested materials (Table A.4) consistent with the strong variations reported for plasma even within a subject [42, 54].

For the selection of a plasma, we set the following criteria: (i) Total FA concentration <10 mM, (ii) n–3 PUFA concentrations <0.25 mM, and (iii) %n–6 in HUFA >75%. Applying these criteria ensures that, (i) the addition of n–3 PUFA to plasma leads to FA levels that are in the range of physiological total FA levels—considering plasma triglyceride values of a maximum 3.3 mM [55], as most of FA are bound to triglycerides in plasma—and (ii) FA composition in plasma is comparable to plasma from subjects having a low n–3 PUFA status, typical for Europeans and US-Americans [10]. Plasma B and E fulfilled all criteria, and plasma B was used for the following experiments.

c. Selection of n–3 PUFA preparation

A comparison of commercially available n–3 PUFA preparations regarding their oxidation status revealed that oxylipin content differed more than 10-fold (Tables A.5, A.6). Different extraction and purification methods, but particularly formulation, storage as well as packaging conditions influence autoxidation of the PUFA and thus, could explain different amounts of oxylipins. Considering that concentrations up to 45 μM of the n–3 PUFA preparation are used in the cell culture medium, this could lead to relevant oxylipin concentrations in the medium: for example, using a commercial n–3 PUFA preparation with 3% (*w/w*) oxylipins, 18-HEPE concentrations in the supplemented medium were more than 50-fold higher (59 nM) than in the non-supplemented medium. This makes it impossible to differentiate whether biological effects originate from the n–3 PUFA or the artificially added oxylipins. Thus, only using a n–3 PUFA preparation with <0.5% oxylipins can ensure that mainly effects of the n–3 PUFA are investigated and not of the artificially formed and non-intentionally added oxylipins, as several of those are potent bioactive lipid mediators.

d. Composition and storage of n–3 PUFA-supplemented cell culture media

The addition of DHA led to approximately five- (medium, 20 μM DHA added) or ten-fold (high, 45 μM DHA added) increased DHA concentrations compared to the non-supplemented medium (low). Compared to ARA (22 ± 1 μM), the resulting DHA concentration was equal (medium, 21 ± 1 μM) or twice as high (high, 41 ± 2 μM) (Figure 2.1A (i)). %n–6 in HUFA was decreased from 81.0% ± 0.6% (low) to 53.9% ± 1.8% (medium) or 39.4% ± 0.4% (high) (Figure 2.1A (ii)). In line with this, n–6:n–3 ratio ≥C18 was decreased from 17.4 ± 0.8 (low) to 5.5 ± 0.3

(medium) or 3.1 ± 0.1 (high) and to a similar extent for n–6:n–3 ratio >C18 in both supplemented media (Figure 2.1A (ii)).

The %n–6 in HUFA of non-supplemented medium is comparable to plasma %n–6 in HUFA from subjects having a diet low in n–3 and high in n–6 PUFA such as the Western diet in the US and Europe [10]. Thus, incubation of cells with a non-supplemented medium resembles the nutritional status of subjects low in n–3 and high in n–6 PUFA.

Medium supplemented medium with 54% ± 2% n–6 in HUFA is similar to human plasma after supplementation with DHA in human nutrition studies [21, 56] and in populations whose diets are rich in fish [10]. The FA composition in high supplementation medium with DHA concentrations twice as high as ARA resulting in 39% ± 0.4% n–6 in HUFA is slightly higher than what can be reasonably achieved by diet in plasma. However, %n–6 in HUFA values low as 30% have been reported in erythrocytes and human tissues, for example, for subjects from Greenland [10, 57].

As PUFA are prone to autoxidation, we investigated the stability of the cultivation medium after preparation and after incubation under cell culture conditions or in the fridge based on the formation of oxylipins. At baseline, all DHA-derived oxylipins were significantly higher concentrated in the supplemented media compared to the non-supplemented (e.g., 4-HDHA: low, 1.62 ± 0.03 nM; medium, 4.4 ± 0.2 nM; high, 7.8 ± 0.3 nM), whereas oxylipins derived from other PUFA such as ARA or EPA were comparable in the different media (e.g., 5-HEPE: low, 0.31 ± 0.03 nM; medium, 0.40 ± 0.03 nM; high, 0.44 ± 0.04 nM) (Figure 2.1B,

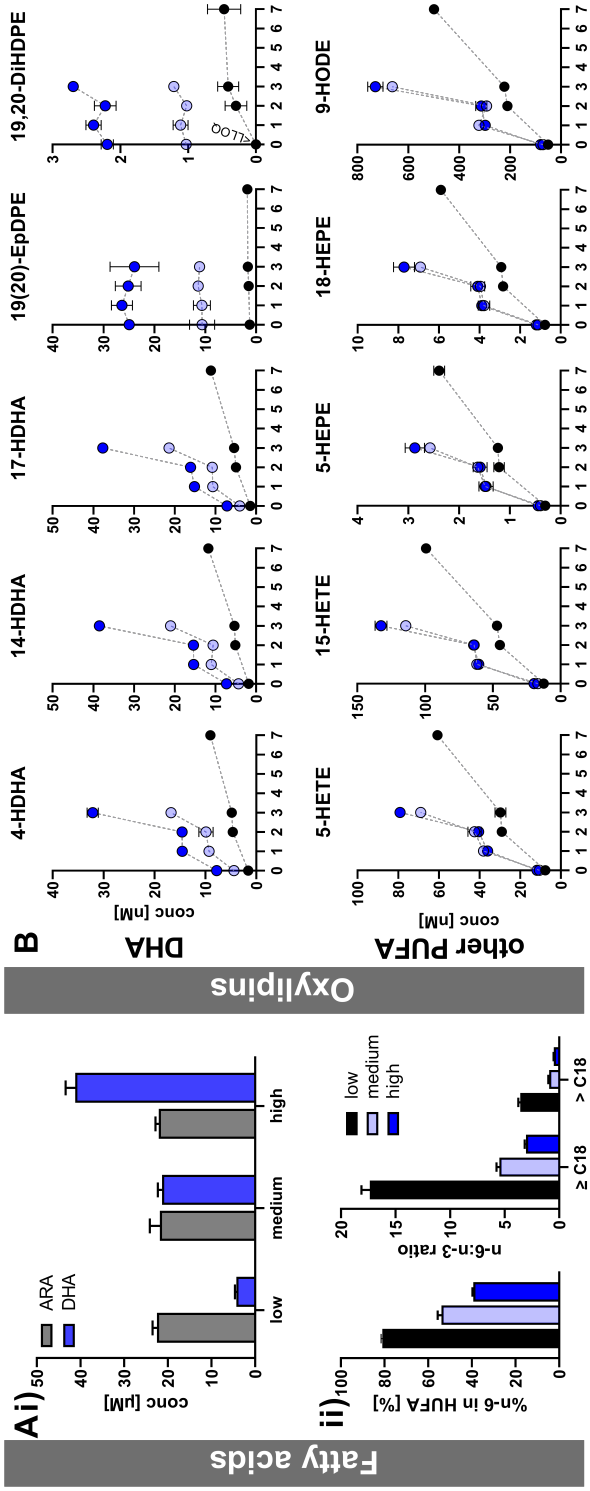

Figure 2.1: Characterization of n–3 PUFA-supplemented cell culture media. Media were prepared by using plasma B and addition of DHA preparation (>99%) to the cell culture medium leading to concentrations of 4 µM (low, non-supplemented control), 21 µM (medium), or 41 µM (high) DHA in the cell culture media. (A) (i) Total FA concentrations were analyzed in the different media by means of LC-MS/MS. (ii) %n–6 in HUFA was calculated from concentrations of C20:3 n–6, C20:4 n–6, C22:4 n–6, C22:5 n–6, C20:3 n–9, C20:5 n–3, C22:5 n–3, C22:6 n–3. n–6:n–3 ratios were determined by FA concentrations of C20:4 n–6, C20:5 n–3 and C22:6 n–3. n–6:n:3 ratio ≥C18 includes additionally concentrations of C18:2 n–6 and C18:3 n–3. (B) Total oxylipin concentrations were analyzed at baseline and after incubation for 1, 2, 3, and 7 days at 37 °C by means of LC-MS/MS. Results are shown as mean ± SD, $n = 3$. Results of the statistical analysis are shown in Table A.7.

Table A.7). This indicates that the majority of DHA-derived oxylipins in supplemented media originated from the PUFA preparation, as plasma contributed only a small part of total DHA oxylipins (Figure A.4) and the oxylipin pattern of the PUFA preparation matched those found in the media (Table A.6, Figure A.4).

When the medium was stored at 37 °C for several days, monohydroxy-FA concentrations increased over time, whereas total FA concentrations, epoxy-FA, and *vic*-dihydroxy-FA were not affected (Figure 2.1B, Table A.7). The slope of oxylipin formation was dose-dependently higher with increasing amounts of supplemented n–3 PUFA in the medium (Table A.7), as the rate of autoxidation is mainly dependent on (i) the number of double bonds being prone to abstraction of a *bis*-allylic hydrogen atom and (ii) the number of hydroperoxides, which are contained or formed in the PUFA preparation (Table A.6), leading to radicals and starting the chain reaction [58].

After 3 days of storage of non-supplemented medium the oxylipin concentrations increased linearly by approximately 3–4-fold. An increase by 6–9-fold was found in the DHA-supplemented media (Figure 2.1B). In order to limit the massive formation of monohydroxy-FA, the supplemented medium was replaced after 48 h of cell cultivation. Of note, supplemented medium stored in the fridge at 4 °C underwent autoxidation resulting in oxylipin levels comparable to storage at 37 °C (e.g., for 14-HDHA in high supplemented medium stored for 1 day: 4 °C, 13.8 $\pm$ 0.4 nM; 37 °C, 15.4 $\pm$ 0.3 nM) (Figure A.5, Figure 2.1B). For this reason, also supplemented media should be prepared freshly and stored at 0–4 °C as short as possible but at most up to 2 days.

Overall, several key parameters such as the selection of buffy coats, plasma, and n–3 PUFA preparation have to be controlled for an *ex vivo* n–3 PUFA supplementation of primary macrophages (Table 2.1). Only then it can be ensured that the effects of the n–3 PUFA are investigated and not of the artificially formed and non-intentionally added oxylipins.

2.3.2 Effects of n–3 PUFA Supplementation on Macrophages FA Pattern

Following supplementation of macrophages for 2, 3, or 7 days with the DHA-supplemented medium, the FA profile and the oxylipin pattern were strikingly changed in the macrophages compared to non-supplemented cells (low) (Figure 2.2): non-supplemented cells showed 84% $\pm$ 2% n–6 in HUFA which is comparable to values found in tissues from subjects having a low n–3 PUFA status [10]. In supplemented macrophages, %n–6 in HUFA was dose- and time-dependently lower (after 2-day supplementation: medium, 51% $\pm$ 2%; high, 35% $\pm$ 2%) (Figure 2.2A). The change in %n–6 in HUFA was strongest during the first 2 days of supplementation, then the decrease flattened with a duration of supplementation indicating that the cells are approaching steady state. After 7 days of supplementation %n–6 in HUFA was decreased to a minimum of 23% $\pm$ 1% or 14% $\pm$ 1% in the cells incubated with medium or high supplemented medium, respectively.

Human nutrition intervention studies reduced %n–6 in HUFA to about 50%–60% in erythrocytes after 3–5 months by daily doses between 1 and 2 g [21–23]. These %n–6 in HUFA values achieved by oral supplementation are comparable with those

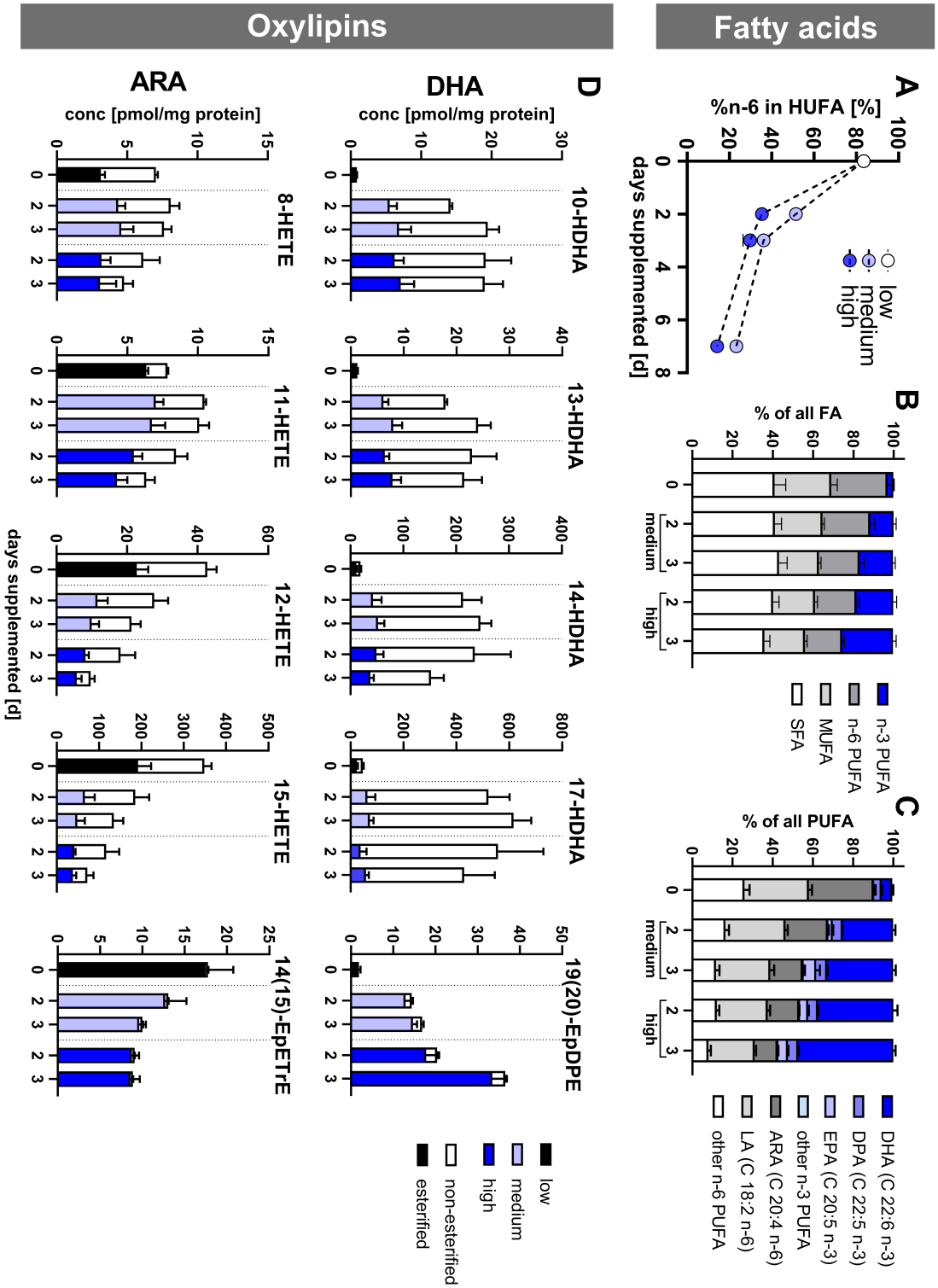
Fatty acids
Oxylipins
A
%n-6 in HUFA [%]
days supplemented [d]
low
medium
high
B
% of all FA
SFA
MUFA
n-6 PUFA
n-3 PUFA
C
% of all PUFA
DHA (C 22:6 n-3)
DPA (C 22:5 n-3)
EPA (C 20:5 n-3)
other n-3 PUFA
ARA (C 20:4 n-6)
LA (C 18:2 n-6)
other n-6 PUFA
D
DHA
ARA
conc [pmol/mg protein]
10-HDHA
13-HDHA
14-HDHA
17-HDHA
19(20)-EpDPE
8-HETE
11-HETE
12-HETE
15-HETE
14(15)-EpETrE
days supplemented [d]
low
medium
high
non-esterified
esterified

Figure 2.2: (previous page) Characterization of FA and oxylipin pattern in macrophages after supplementation with n–3 PUFA. After isolation of monocytes and differentiation into M2-like macrophages, cells were supplemented using media containing 4 μM (low, non-supplemented control), 21 μM (medium), or 41 μM (high) DHA for 2, 3, or 7 days. Cell pellets were analyzed for total FA and non-esterified and total oxylipins by means of LC-MS/MS. (A) %n–6 in HUFA was calculated from total FA concentrations of C20:3 n–6, C20:4 n–6, C22:4 n–6, C22:5 n–6, C20:3 n–9, C20:5 n–3, C22:5 n–3, C22:6 n–3. (B) Relative distribution of n–3 PUFA, n–6 PUFA, MUFA, and SFA. Increase in n–3 PUFA was significantly different as determined by 2-way rmANOVA ($F(12, 24) = 25.87$, $p = 0.012$) (Table A.8). (C) Relative distribution of PUFA including C22:6 n–3, C22:5 n–3, C20:5 n–3, C18:3 n–3 and C22:2 n–6, C22:4 n–6, C22:5 n–6, C20:3 n–6, C20:4 n–6, C18:2 n–6, C18:3 n–6. (D) Concentrations of selected, most abundant oxylipins derived from DHA and ARA in the cells. Results are shown as mean ± SEM, $n = 3-6$.

of the macrophages supplemented with DHA in the cultivation medium (medium) for 2 days (51% ± 2%).

Taking a closer look at the relative FA pattern of the supplemented cells, the relative amount of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and PUFA was unchanged independently from the n–3 PUFA status (Figure 2.2B, Table A.8). Thus, supplementation leads to the replacement of n–6 PUFA by n–3 PUFA (increase of n–3 PUFA from 2.9% ± 0.7% to approximately 12%–25% in a dose- and time-dependent manner). This is consistent with supplementation studies in humans and animals [21–23, 40] and mainly driven by an increase in DHA (from 1.7% ± 0.3% to 8.9%–21%) and a decrease in ARA (from 13%–16% to 9.8%– 13% of all FA) (Figure 2.2C). Although only DHA was supplemented, EPA was also higher in the supplemented cells (0.8%–2.2% of all FA) compared to the control cells (0.09% ± 0.01% of all FA). An increase in EPA in response to DHA supplementation is discussed to be due to a formation of EPA by retroconversion from DHA [21, 59, 60] or due to a slower EPA metabolism in the presence of high DHA levels [56]. Recent studies show that DHA inhibits its own synthesis at the

level of EPA [61]: the higher EPA level could also result from a change in synthesis rates of EPA/DHA from α-linolenic acid. Even though the molecular mechanism is still a matter of debate, an increase of EPA in blood and tissue following DHA-only supplementation is commonly described in human and animal models [21, 56, 60, 62, 63]. The decrease of ARA is similar to previous reports for erythrocytes in human intervention studies, where ARA was decreased from approximately 13%–16% to 9.8%–13% of all FA following n–3 PUFA supplementation [21–23].

In summary, we showed how the PUFA pattern of monocytes from subjects having a low n–3 PUFA status can be modified to macrophages resembling a PUFA pattern with a high n–3 PUFA status under controlled conditions. Moreover, higher n–3 PUFA levels can be achieved when supplementing at a higher dose, allowing the detailed investigation of the effects of an increased n–3 PUFA status in human immune cells.

2.3.3 Effects of n–3 PUFA Supplementation on the Oxylipin Pattern of Macrophages

The oxylipin pattern was changed in the supplemented macrophages compared to control cells (low) in a similar manner as their PUFA precursors (Figure 2.2C, D): Although DHA was increased up to 10-fold, DHA-derived oxylipins increased 10–20-fold, whereas some ARA-derived oxy-lipins decreased about 2-fold in the supplemented cells. Our findings, (i) an increase in n–3 PUFA-derived oxylipins while ARA-derived ones decreased, (ii) that the relative increase in n–3 PUFA concentrations was higher than the decrease in ARA concentrations, and (iii) that

the relative change in oxylipins was higher than that of the corresponding PUFA by supplementation, are consistent with nutrition intervention studies [40, 64–66].

Interestingly, concentrations of non-esterified monohydroxy-DHA were increased to a greater extent by supplementation (approx. 5–20 times more) than esterified oxylipins (Figure 2.2D). In contrast, the ratio of unbound to esterified oxylipins was hardly changed for ARA-derived oxylipins in the supplemented cells. This could indicate that the formation or uptake of DHA-derived oxylipins from the medium was more rapid than their incorporation into lipids such as phospholipids of the cell membrane.

Highest total oxylipin concentrations were found for 17-HDHA in the supplemented cells (430 $\pm$ 120–620 $\pm$ 81 pmol mg^{-1} protein), whereas 15-HETE was the most abundant oxylipin in the non-supplemented cells (low, 349 $\pm$ 73 pmol mg^{-1} protein) (Figure 2.2D). 14- and 17-HDHA concentrations were increased strongest by supplementation (difference between 2-day supplementation with medium supplemented medium and low: 14-HDHA, 194 $\pm$ 51 pmol mg^{-1} protein; 17-HDHA 475 $\pm$ 102 pmol mg^{-1} protein), whereas others such as 10-HDHA were less affected (13.3 $\pm$ 31.2 pmol mg^{-1} protein). 12- and 15-HETE were dose- and time-dependently lower in the supplemented cells, whereas other monohydroxy-ARAs such as 8-HETE were not altered by supplementation (Figure 2.2D).

The observed changes in oxylipin concentrations are likely due to the modified PUFA profile and resulting enzymatic conversion. To investigate this, we compared oxylipins in the medium following a 2-day supplementation period with a medium that was stored under identical conditions, but without cells reflecting autoxidation (Figure 2.3A, Figure A.6A, Table A.9): monohydroxy-PUFA were lower in the

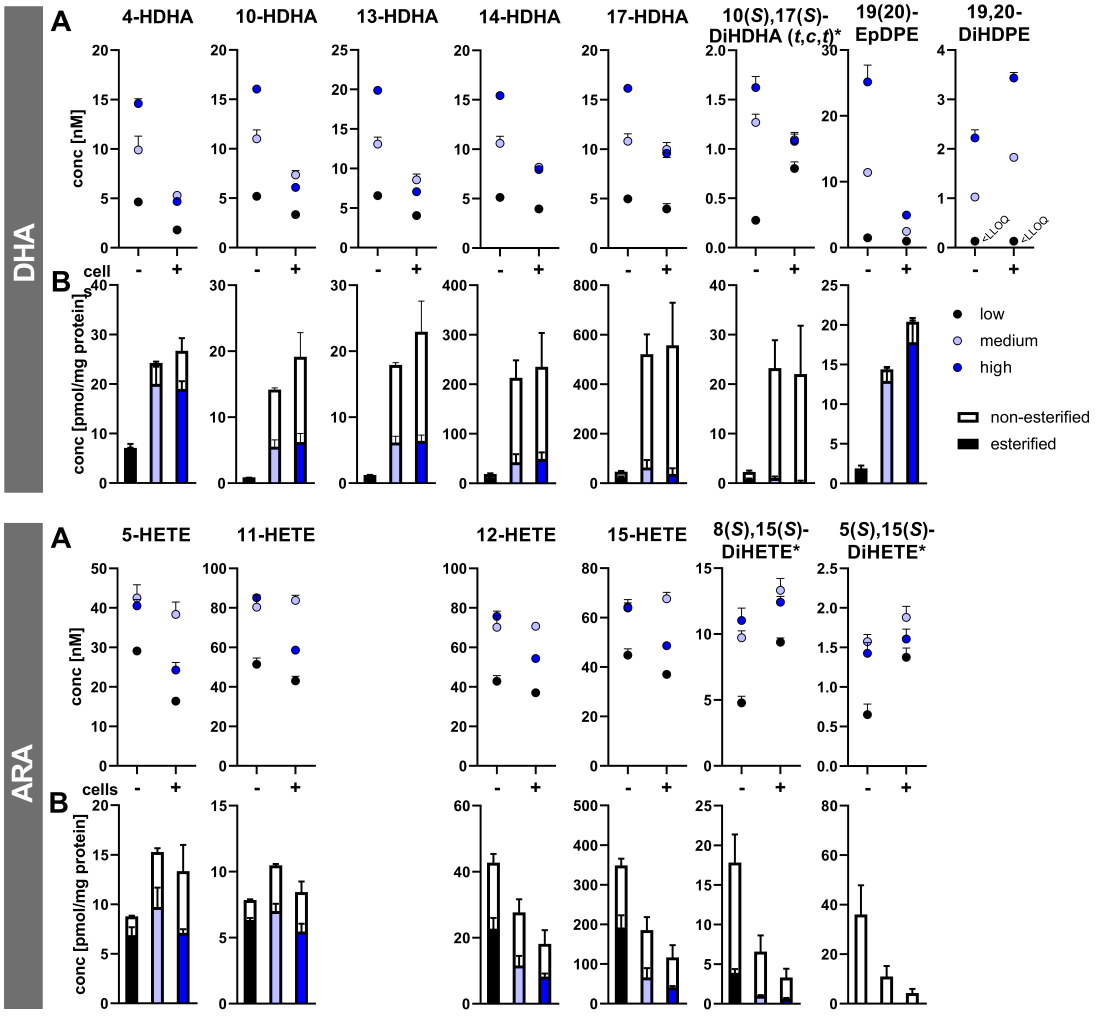

Figure 2.3: Oxylipin pattern of (A) medium (without [-] and with cells [+]) and (B) the macrophages after two days of incubation with different levels of DHA. Monocytes were isolated from buffy coats and differentiated into M2-like macrophages. Incubations were carried out using media containing 4 µM (low, non-supplemented control), 21 µM (medium), or 41 µM (high) DHA for 2 days with and without cells. Cell culture media was analyzed for total oxylipins, and cell pellets for total and non-esterified oxylipins by means of LC-MS/MS. Results are shown as mean ± SD (medium) or mean ± SEM (cells), $n = 3$. Results of the statistical analysis are shown in Table A.9. (*for (A), oxylipin and enantiomer).

cells' supernatant than in the medium without cells under all conditions (Figure 2.3A). The highest difference was observed for the high supplementation medium (~6–10 nM), whereas the difference was smaller in the non-supplemented medium (~1–3 nM). However, within the supplementation groups (low, medium, and high), concentration differences were comparable for all monohydroxy-FA from DHA (e.g., low: 7-HDHA, 1.0 nM, 17-HDHA, 1.0 nM; high: 7-HDHA, 5.9 nM, 17-HDHA, 6.6 nM). As the original concentrations of the various hydroxy-FA are of the same order of magnitude (Figure 2.3A, Table A.6), this suggests that hydroxy-FA are unselectively taken up into the cells in a dose-dependent manner.

In the cells, DHA-derived oxylipins were increased differently by supplementation, for example, 17-HDHA was increased 25-fold stronger than 4-HDHA (18 $\pm$ 6 pmol mg^{-1} protein vs. 490 $\pm$ 200 pmol mg^{-1} protein) (Figure 2.3B top). This could be explained by enzymatic conversion of DHA by 15-LOX-1 activity, which is highly abundant in M2-like macrophages [31]. 15-LOX-1 has a dual reaction specificity which gives rise to both 17- and 14-HDHA [67, 68]. This explains the high concentrations of the second most abundant oxylipin in the supplemented cells, 14-HDHA. Similar findings were observed for 15-LOX-1 products of EPA, 12- and 15-HEPE (Figure A.6). 12- and 15-HETE were both dose-dependently lower in supplemented cells (Figure 2.3B bottom) suggesting a lower conversion of n–6 PUFA by competition with supplemented DHA. This is supported by the finding that 15-LOX-1 preferentially converts DHA and EPA compared to ARA [67]. This also explains the relative higher increase in 14- and 17-HDHA compared to the elevation of DHA following supplementation.

The dual reaction specificity of 15-LOX-1 results in n–6 and n–9 products (i.e., 17-/14-HDHA and 15-/12-HETE) in a distinct ratio, which is specific for the different PUFA [67]. The ratios in the supplemented macrophages (n–6/n–9 ratio: DHA, 2.6 $\pm$ 0.3; EPA, 6.4 $\pm$ 0.8) were similar but slightly higher than the ratios reported for the isolated enzyme (n–6/n–9 ratio: DHA, 1.5; EPA 5.4) [67]. In line with this, chiral analysis of 15-HETE and 17-HDHA revealed that the (*S*)-product is predominantly present in the cells whereas the medium contains the racemate (Figure A.7) supporting that the majority of the 15-LOX products in the cells was formed by enzymatic conversion from PUFA.

Considering other monohydroxy-PUFA such as 4-HDHA, which can be formed by 5-LOX activity, concentration differences were comparable to oxylipins which can be only formed by autoxidation such as 10- or 13-HDHA (Figure 2.3B top). Similar results were observed for EPA-derived 5-HEPE (Figure A.6) and ARA-derived 5-HETE (Figure 2.3B bottom). In contrast to the medium, cells contained mainly the (*S*)-enantiomers of 5- and 8-HETE (Figure A.7). This could indicate a selective uptake of the (*S*)-enantiomers from the medium or a formation of 5(*S*)-HETE by 5-LOX activity in the M2-like macrophages. However, only poor 5-LOX abundance and activity was reported for M2-like macrophages [31].

The concentration of 10(*S*),17(*S*)-DiHDHA (*t*,*c*,*t*) (protectin [P][Dx) was approximately ten-fold higher in the supplemented cells than in the non-supplemented cells (Figure 2.3B top), whereas concentrations in the media were comparable (Figure 2.3A top). For double hydroxylated products from ARA such as 8(*S*),15(*S*)-dihydroxy-eicosatetraenoic acid (DiHETE) and 5(*S*),15(*S*)-DiHETE, concentrations were higher in the medium with cells compared to medium without cells (for

low and medium) (Table A.9), but decreased in the cells following supplementation. This indicates that a formation of double hydroxylated oxylipins in the cells is likely caused by 15-LOX activity converting monohydroxylated oxylipins formed by autoxidation, as the levels of the dihydroxy-FA follow the changes of the monohydroxylated precursor. This was supported by chiral analysis (Figure A.7): 10-HDHA was present as a racemate in the medium and in the high supplemented macrophages indicating a formation by autoxidation and/or uptake from the medium. For 10,17-DiHDHA multiple isomers were detected including the 10(*S*),17(*S*)-DiHDHA (*t*,*c*,*t*) (PDx) and 10(*R*),17(*S*)-DiHDHA (*t*,*c*,*t*), indicating their formation by the conversion of racemic 10-HDHA by 15-LOX activity. 10(*R*),17(*S*)-DiHDHA (*t*,*t*,*c*) (neuroprotectin [NP]D1) coelutes with other isomers in reversed-phase chromatography (Figure A.7), as previously described [69]. Thus, it is difficult or even impossible to support a relevant formation of NPD1 in the cells. For 5,15-DiHETE and 7,17-DiHDHA only one isomer, corresponding to the (*S*),(*S*)-product, was detected by chiral chromatography in the cells [33]. Thus, the formation of double hydroxylated DHA and ARA oxylipins in M2-like macrophages is dependent on 15-LOX-1 activity as well as the amount of added monohydroxylated precursors/PUFA. Other dihydroxy- or trihydroxy-DHA belonging to the class of so-called “specialized pro-resolving mediators” (7-*epi*-maresin [MaR]1, MaR1, MaR2, RvD1, RvD2, RvD3) were not detected in the cells. Retention times and/or the ratio of different transitions did not match those of the corresponding authentic reference standard (Figure A.8). This is consistent with previous reports [70].

Concentrations of 19(20)-epoxy-docosapentaenoic acid (EpDPE) were higher in the medium and high supplemented medium without cells (high: 25.2 $\pm$ 2.6 nM) compared to medium with cells (high: 4.9 $\pm$ 0.5 nM) (Figure 2.3A top, Table A.9). Consistently, the concentrations were strongly and dose-dependently increased in the supplemented macrophages (Figure 2.3B top) suggesting an uptake from the medium and incorporation into the cells. Here, for example, epoxy-PUFA are bound in phospholipids, as oxylipins were mainly found in the esterified form (Figure 2.2D). In contrast, 19,20-dihydroxy-docosapentaenoic acid (DiHDPE) concentrations were higher in the cells' supernatant than in the medium without cells (Figure 2.3A top, Table A.9). This could be explained by the conversion of 19(20)-EpDPE by soluble epoxide hydrolase activity within the cells and subsequent release into the medium. 19,20-DiHDPE was not detected in the cells. Following supplementation, increased concentrations of DHA-derived epoxy-FA and *vic*-dihydroxy-FA were described in human plasma [64] whereby epoxy-PUFA were mainly esterified and dihydroxy-PUFA were non-esterified [65].

Overall, the results indicate that the observed shift in the oxylipin pattern following supplementation is either caused by oxylipin formation within the cells (e.g., 15-LOX products) or uptake of oxylipins from the media in a dose-dependent manner (e.g., 19(20)-EpDPE). Absolute levels of 15-LOX-1 oxylipins such as 17-HDHA or 15-HETE were changed the most by supplementation compared to other oxylipins. This indicates that 15-LOX-1 activity is mainly responsible for the formation of these oxylipins in supplemented M2-like macrophages. Here, the increased DHA- and EPA-derived oxylipins were at the expense of ARA-derived ones. This shift in the oxylipin pattern is in line with that reported in supplementation stud-

ies in mice and humans supporting that the *ex vivo* n–3 PUFA supplementation strategy allows the investigation of the effects of the n–3 PUFA in immune cells.

The change in the PUFA profile leads to a shift of oxylipins toward n–3 PUFA products which may contribute to the beneficial effects of n–3 PUFA on human health. Many oxylipins derived from n–3 PUFA such as 17-HDHA and 10,17-HDHA, which are massively increased by supplementation, are regarded as anti-inflammatory lipid mediators [71–74]. In contrast, oxylipins from ARA, which decrease following supplementation, are predominantly described having pro-inflammatory effects such as fever, pain, or bronchoconstriction [18]. However, further research is needed to investigate the role of oxylipins in the physiological effects of n–3 PUFA supplementation. The developed *ex vivo* supplementation strategy is a promising tool for these experiments.

2.3.4 Effects of n–3 PUFA Supplementation on LPS Stimulation of M2-Like Macrophages

The developed *ex vivo* supplementation strategy was used for investigating changes in the response of the macrophages to the inflammatory stimulus LPS. A high dose of LPS (1 μg/mL) resulted in a highly elevated induction (100-700-fold) of the mRNA levels of pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) or IL-6 (Figure A.9). It should be noted that baseline expression of pro-inflammatory genes is generally low in M2-like macrophages. n–3 PUFA supplementation did not significantly change the expression of cytokines and chemokines, with or without LPS stimulation (Figure 2.4A, B). This is in contrast to experiments in PMA-differentiated human monocyte cell line (THP-1) cells, which

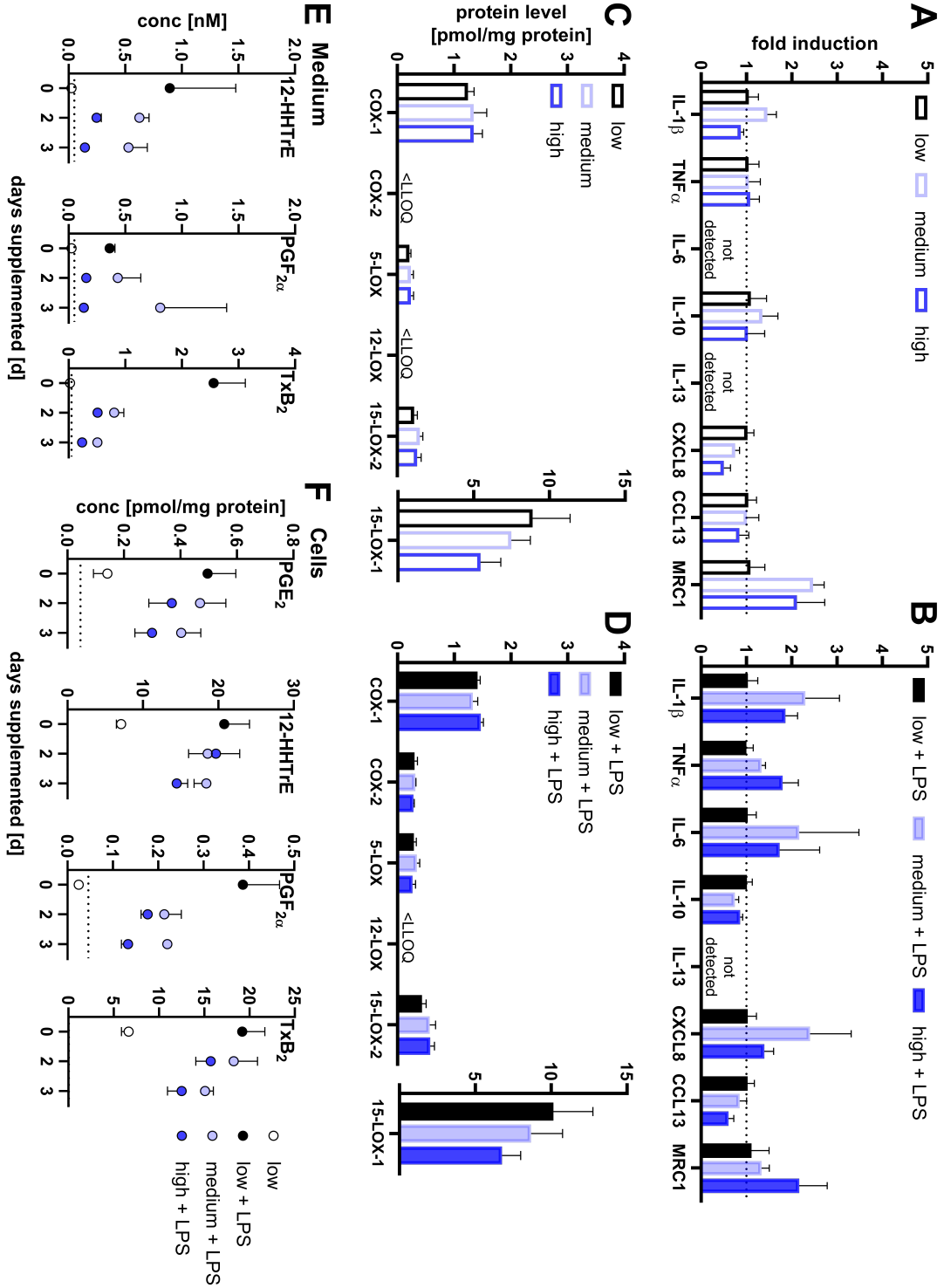
A
fold induction
low
medium
high
not detected
IL-1β
TNFα
IL-6
IL-10
IL-13
CXCL8
CCL13
MRC1
B
low + LPS
medium + LPS
high + LPS
C
protein level [pmol/mg protein]
<LLOQ
COX-1
COX-2
5-LOX
12-LOX
15-LOX-2
15-LOX-1
D
E Medium
conc [nM]
12-HHTrE
PGF2α
TxB2
days supplemented [d]
F Cells
conc [pmol/mg protein]
PGE2

Figure 2.4: (previous page) LPS elicits changes in supplemented versus non-supplemented M2-like macrophages. After isolation of monocytes and differentiation into M2-like macrophages, cells were supplemented using media containing 4 μM (low, non-supplemented control), 21 μM (medium), or 41 μM (high) DHA. For LPS stimulation 1 μg/mL LPS was added during the final 6 h. Following 3 days of supplementation, changes in (A, B) the transcription of selected genes analyzed by qPCR and (C, D) abundance of human enzymes COX-1 and -2, 5-, 12-, 15-LOX-1, and 15-LOX-2 determined by quantitative proteomics. Non-esterified oxylipins in (E) medium and (F) cell pellets determined by LC-MS/MS. Results are shown as mean ± SEM or SD, $n = 3$. Dotted line indicates the lower limit of quantification (LLOQ).

reported downregulation of inflammatory genes following n–3 PUFA supplementation [75–77]. However, PMA-differentiated THP-1 cells cannot be directly compared to the used primary M2-like macrophages having a highly different phenotype and thus, different expression of pro-inflammatory genes. In human supplementation studies, the results regarding inflammatory cytokines are inconsistent [24], highlighting the need for further studies in order to investigate the mechanism of how n–3 PUFA impact inflammatory processes.

The macrophage marker mannose receptor C-type 1 (MRC1) tended to increase by n–3 PUFA supplementation (Figure 2.4A, B), whereas protein levels of IL1R2, toll-like receptor 2, and toll-like receptor 4 were not changed, indicating only minor or no changes of proteins involved in the polarization of macrophages by n–3 PUFA or LPS. The levels of ARA cascade enzymes such as COX-1, 5- and 15-LOX-2 were low (<2 pmol mg^{-1} protein), whereas 15-LOX-1 was highly abundant (8.9 ± 2.5 pmol mg^{-1} protein) in the M2-like macrophages (Figure 2.4C). This is in line with previously described levels [27, 31]. LPS did not affect the levels of these enzymes (Figure 2.4D). Hence, oxylipins formed by these pathways or autoxidation were less affected by LPS stimulation, and the oxylipin pattern was comparable to that of unstimulated macrophages (Figure A.10).

LPS stimulus resulted in massive elevated COX-2 levels in the macrophages (Figure 2.4C, D). In line with this, concentrations of COX products such as PG were increased in the medium and cells (Figure 2.4E, F, Figure A.10). Concentrations of pro-inflammatory PG such as PGE_2 and TxB_2 were reduced in the macrophages and medium after DHA supplementation, for example, PGE_2 by about 40% in the cells and TxB_2 by about 90% in the medium (Figure 2.4E, F). Similarly, supplementation studies reported decreased levels of PG following n–3 PUFA supplementation [78–81]. Our findings support a reduced conversion of ARA by COX-2 following n–3 PUFA supplementation, which results in lower concentrations of pro-inflammatory PG which is one of the major mechanisms of how the anti-inflammatory effect of n–3 PUFA is mediated.

2.4 Concluding Remarks

Overall, our experiments showed how primary human macrophages can be supplemented with DHA to alter the FA and oxylipin profile, mimicking n–3 PUFA supplementation studies in humans. As n–3 PUFA supplementation in cell culture experiments provides many challenges, key parameters were identified and controlled ensuring a reliable strategy. Autoxidation of PUFA cannot be completely avoided, but the extent can be reduced and should be carefully monitored during the process. This is particularly important because several oxidized PUFA are biologically active lipid mediators, and thus, low concentrations might interfere with investigated inflammatory processes. Here, we present an *ex vivo* supplementation strategy for primary human macrophages addressing these challenges. The observed changes in the PUFA profile and the shift in the oxylipin pattern toward

n–3 PUFA are in line with intervention studies in humans. Applying the model to investigate the inflammatory stimulus LPS, decreased levels of pro-inflammatory PG were observed in the DHA-supplemented macrophages which may be one of the mechanisms by which n–3 PUFA mediate their anti-inflammatory effects. Thus, the developed *ex vivo* supplementation strategy is a helpful tool for mechanistic investigations of n–3 PUFA and oxylipins in primary human immune cells.

2.5 Conclusion

The developed *ex vivo* n–3 PUFA supplementation strategy allows to change the FA pattern of monocytes from subjects with a typical Western diet to macrophages with an FA pattern comparable to subjects having a high n–3 PUFA status. By controlling key parameters for the selection of buffy coats, plasma and n–3 PUFA preparation as well as preparation of the medium, a reliable supplementation is possible, allowing to alter the FA and oxylipin patterns of macrophages comparable to human intervention studies. Thus, this tool allows mechanistic investigation of long-chain n–3 PUFA and arising oxylipins in primary human immune cells under strictly controlled conditions without the need for human intervention studies.

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3 Phagocytosis is differentially regulated by LPS in M1- and M2-like macrophages via PGE_2 formation and EP4 signaling*

Phagocytosis is a key process in human innate immune response. Human macrophages are important phagocytes engulfing and neutralizing pathogens and cell debris. In addition, they modulate the inflammatory process by releasing cytokines and lipid mediators. However, the link between oxylipins and phagocytosis in different macrophage phenotypes remains poorly understood. In order to better understand the link between phagocytosis and the arachidonic acid (ARA) cascade, we established a phagocytosis assay in primary human 'inflammatory' M1- and 'anti-inflammatory' M2-like macrophages from peripheral blood mononuclear cells (PBMC), representing extremes of macrophage phenotypes. The branches of the ARA cascade were investigated by quantitative targeted proteomics and metabolomics. M1-like macrophages show a higher abundance of cyclooxygenase (COX)-2 and its products particularly after LPS stimulus compared to M2-like macrophages. LPS increased phagocytosis in M2-like, but not in M1-like macrophages. We demonstrate that the COX product prostaglandin E2 (PGE_2) modulates the differential effects of LPS on phagocytosis: Via the EP4 receptor PGE_2 signaling suppresses phagocytosis in primary human macrophages. Thus, blockage of COX, e.g. by non-steroidal anti-inflammatory drugs (NSAID), leads to an increase of phagocytosis also in 'inflammatory' M1-like macrophages. This supports the well-described anti-inflammatory effects of these drugs and underscores the importance of the link between the COX branch of the ARA cascade and the regulation of phagocytosis in human macrophages.

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3.1 Introduction

Macrophages are important cells of the first line innate immunity and involved in host defense. Contact to pathogens such as gram-negative bacteria initiates the inflammatory response leading to the release of cytokines, chemokines [1], and activation of cytosolic phospholipase A2 [2]. Cytosolic phospholipase A2 catalyzes the liberation of polyunsaturated fatty acids (PUFA) from membrane phospholipids [3]. These PUFA can be converted via enzymatic and non-enzymatic pathways resulting in a large number of different oxylipins such as prostanoids, hydroperoxy-, mono- and multihydroxy-PUFA as well as epoxy-PUFA [4]. Several of those are key regulators of the inflammatory process: Arachidonic acid (ARA)-derived prostaglandins (PG) formed by cyclooxygenase (COX) activity and leukotrienes formed by 5-lipoxygenase (LOX) activity are well-investigated pro-inflammatory mediators [4, 5] involved in pain, fever and asthma [6]. However, several multihydroxy-PUFA are discussed to have anti-inflammatory effects involved in the active resolution of inflammation [7] but remain controversial [8].

In addition to the release of signaling molecules, macrophages are capable of directly neutralizing pathogens by phagocytosis. This process is not only important for killing bacteria, but also contributes to the active resolution of inflammation: By ingesting apoptotic cells and cell debris, they prevent secondary necrosis and thus enable healing and restoration of healthy tissue [9]. Hence, macrophages participate in both, the initiation and subsequent resolution of inflammation. Due to their high cellular plasticity, they adapt their functions in response to microenvironmental stimuli [10]. This leads to different phenotypes such as inflammatory (M1) and anti-inflammatory (M2) macrophages [11]. However, it is widely ac-

cepted that macrophages represent a spectrum of activated phenotypes, rather than discrete stable subpopulations [12–14]. Differences in the phenotypes involve abundance of the enzymes of the ARA cascade and result in distinct oxylipin patterns [15, 16]. However, the ARA cascade enzymes and oxylipins contribute to the regulation of phagocytosis in different macrophage phenotypes remains poorly understood.

Here, we investigated how the inflammatory stimulus bacterial lipopolysaccharide (LPS) impacts phagocytosis in primary human 'inflammatory' M1- and 'anti-inflammatory' M2-like macrophages, representing extremes of macrophage phenotypes. Particularly the role of the COX branch of the ARA cascade in the regulation of phagocytosis was evaluated. Prostaglandin E2 (PGE_2) signaling via the prostaglandin E2 receptor 4 (EP4) was found to be a key regulator suppressing the increase of phagocytosis in inflammatory M1-like macrophages.

3.2 Materials and Methods

3.2.1 Chemicals and Biological Material

Human AB plasma was obtained from the blood donation center at University Hospital Düsseldorf (Düsseldorf, Germany). Lymphocyte separation medium 1077 was bought from PromoCell (Heidelberg, Germany). Recombinant human GM-CSF, M-CSF, IFNγ and IL-4 produced in Escherichia coli were purchased from PeproTech Germany (Hamburg, Germany). RPMI 1640 cell culture medium, L-glutamine and penicillin/streptomycin (5.000 units penicillin and 5 mg/mL streptomycin), LPS from E. coli (0111:B4), dextran 500 from Leuconostoc spp., Cy-

tochalasin D from Zyosporium masonii and copper sulfate pentahydrate were purchased at Merck (Taufkirchen, Germany). Latrunculin A, prostaglandin E2 (PGE_2), PF-04418948 and GW627368 were bought from Cayman Chemical (local distributor Biomol, Hamburg, Germany). Accutase, DMSO and formaldehyde were obtained from Carl Roth (Karlsruhe, Germany). Resazurin was purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany). Fluorescence-labeled polystyrene microspheres (latex beads) were bought as 2% or 2.5% solutions from Merck (Taufkirchen, Germany) and Thermo Fisher Scientific (Langenselbold, Germany). BCA reagent A was obtained from Fisher Scientific (Schwerte, Germany). The ultra-pure water with a conductivity of >18 MΩ*cm was generated by the Barnstead Genpure Pro system from Thermo Fisher Scientific (Langenselbold, Germany). Heavy labeled (lys: uniformly labeled (U)-$^{13}C_6$; U-$^{15}N_2$; arg: U-$^{13}C_6$; U-$^{15}N_4$) peptide standards were purchased from JPT Peptides (Berlin, Germany).

3.2.2 Isolation and Differentiation of Primary Human Macrophages

Primary human macrophages were isolated as described [17]. In brief, PBMC were isolated from buffy coats provided by blood donations at the University Hospital Düsseldorf, Germany or at Deutsches Rotes Kreuz West, Hagen, Germany. Blood was drawn with the informed consent of healthy human subjects and the study was approved by the Ethical Committee of the University of Wuppertal. PBMC were isolated by dextran (5%) sedimentation for 30−45 min and subsequent centrifugation (800 × g without deceleration, 10 min, 20 °C) on lymphocyte separation medium. The leucocyte ring was isolated, washed twice with PBS and cells

were seeded in 60.1 mm^2 dishes in RPMI medium (1% P/S, 1% L-glutamine) in a humidified incubator at 37 °C and 5% CO_2 for 1 h. Non-adherend cells were removed by washing, and RPMI medium (1% P/S, 1% L-glutamine) supplemented with 5% human AB plasma was added to the cells. For polarization towards M1-like macrophages, 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) was added to the medium for 7 days and additional 10 ng/mL interferon γ (IFNγ) for the final 48 h. For polarization towards M2-like macrophages, 10 ng/mL macrophage (M)-CSF was added for 7 days and additional 10 ng/mL interleukin 4 (IL-4) for the final 48 h.

3.2.3 Phagocytosis Assay

A schematic overview over the phagocytosis assay workflow is shown in Figure 3.1. After differentiation, cells were harvested using accutase (5/10 mL (M1-/M2-like) incubation for 20 min at 37 °C). Cells were transferred into 96-well plates at 50,000 cells/well using 100 µL cell culture medium. After resting overnight, cells were preincubated with test substances or vehicle control (0.1% DMSO) for 2−4 h at 37 °C. Cytotoxic effects of the tested substances were excluded by resazurin (almar blue) assay [18] and lactate dehydrogenase assay (Figure B.1) and only non-toxic concentrations were used. For phagocytosis, fluorescence-labeled polystyrene microspheres (beads) (Table B.1) were used. Only beads having a fluorescence readout proportional to their concentration were used. After washing, the beads were opsonized in human plasma for 1.5−2 h at 37 °C.

For the phagocytosis assay, supernatants of the 96-well plate were discarded after preincubation with the test substances, and opsonized bead solution, which had

been diluted with medium ($6.25 \cdot 10^{-3}$% beads, 5% human plasma), was added together with the preincubated test substance to the cells for 2 h at 37 °C in a humidified incubator. Phagocytosis was stopped by discarding the supernatants and carefully washing the cells three times with 100 µL PBS. Cells were fixated using 4% formaldehyde for 10 min at 37 °C. After three times washing with 100 µL PBS, fluorescence of beads was measured using a plate reader (Infinite 200 PRO, Tecan). For DAPI staining, 10 µg/mL DAPI was added to the cells for 15 min at room temperature followed by three times washing with PBS and fluorescence measurement with the plate reader (λ_{ex} 358 nm, λ_{em} 461 nm).

3.2.4 Quantification of Oxylipin and Protein Levels by LC-MS/MS

Differentiated macrophages were harvested from cell culture dishes via the cold shock method [17] and dry pellets were stored at -80 °C until use. Oxylipin and protein levels were analyzed from the same cell pellet. Cell pellets were resuspended in MeOH/H_2O (50/50, *v/v*) containing antioxidant solution (0.2 mg/mL BHT, 100 µM indomethacin, 100 µM soluble epoxide hydrolase inhibitor trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (t-AUCB) in MeOH) [19, 20], sonicated and protein content was determined via bicinchoninic acid assay (BCA) [21]. Internal standards (IS) for oxylipin analysis were added to the samples before proteins were precipitated using MeOH for at least 30 min at -80 °C, followed by centrifugation (20 000 × g, 10 min, 4 °C). The supernatant was used for the oxylipin analysis and the precipitated protein pellet for the analysis of the protein level [15].

Oxylipin determination was carried out according previously published methods [19, 20]. In brief, oxylipins were purified by solid phase extraction (SPE) on a non-polar (C8)/strong anion exchange mixed mode material (Bond Elut Certify II, 200 mg, Agilent, Waldbronn, Germany), and analyzed using an 1290 Infinity II LC system (Agilent), coupled to a QTRAP mass spectrometer operated in electrospray ionization (ESI(-))-mode (Sciex, Darmstadt, Germany) operated in scheduled selected reaction monitoring. Quantification was carried out by external calibration using IS [15, 22]. Quantitative protein analysis was carried out by LC-MS/MS as described [15, 16, 23]. In brief, the protein pellet was dissolved in 5% (*w/v*) sodium deoxycholate with protease inhibitor (100/1, *v/v*), proteins were precipitated using acetone and centrifuged (15 000 × g, 10 min, 4 °C). After subsequent incubations with 200 mM dithiothreitol, 200 mM iodoacetamide, and again 200 mM dithiothreitol, 100 μg/mL trypsin (trypsin-to-protein ratio of 1:50) was added to digest the proteins for 15 h. The digestion was stopped using acetic acid (pH 3-4). IS (heavy labeled peptides) were added and samples were purified on Strata-X SPE 33 μm Polymeric Reversed Phase cartridges (100 mg/3 mL, Phenomenex LTD, Aschaffenburg, Germany). Peptides were reconstituted in 50 μL 15% ACN/0.1% acetic acid and analyzed on an 1290 Infinity II LC system (Agilent) coupled to QTRAP instrument (Sciex) in ESI(+)-mode operated in scheduled selection reaction monitoring mode. Quantification was carried out by external calibration using IS [15, 23]. Analyst (Sciex, version 1.7) was used for instrument control and Multiquant software (Sciex, version 3.0.2) was used for data analysis. The concentrations of oxylipins and proteins were calculated based on the protein content determined via bicinchoninic acid assay [21].

3.2.5 Statistical Analysis

Data are presented as mean ± standard error of mean (SEM) or standard deviation (SD) as indicated. Statistical analysis and visualization of data was performed using the Prism software (GraphPad Software, version 8.4., San Diego, CA, USA). For statistical analysis, 1-way or 2-way ANOVA following Dunnett's, Sidak's or Tukey's multiple comparison tests were performed as indicated in the figure captions. Statistical significance was described as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. In order to evaluate the quality of the phagocytosis assay and suitability of the controls used, the z-factor (z) [24] was calculated using the means (μ) and standard deviations (σ) of the inhibitor latrunculin A (μ_{neg}, σ_{neg}) and of the LPS stimulation (μ_{pos}, σ_{pos}):

$$z = 1 - 3 \times \frac{(\sigma_{pos} - \sigma_{neg})}{\mu_{pos} + \mu_{neg}} \tag{3.1}$$

A z-factor of >0.5 is recommended indicating a good assay, whereas a z-factor from $0-0.5$ indicates a marginal assay and a z-factor <0 a non-useful assay to investigate an effect [24].

3.3 Results

For the investigation of phagocytosis in human macrophages, we established and optimized a phagocytosis assay in primary human M1- and M2-like macrophages. Both types of macrophages showed phagocytic activity using fluorescence-labeled polystyrene microspheres (beads) (Figure 3.1A). In order to optimize the phagocytosis assay, several parameters of the assay, such as cell number, duration of phagocytosis, and number of wash steps, were carefully selected (Figure 3.1B, C,

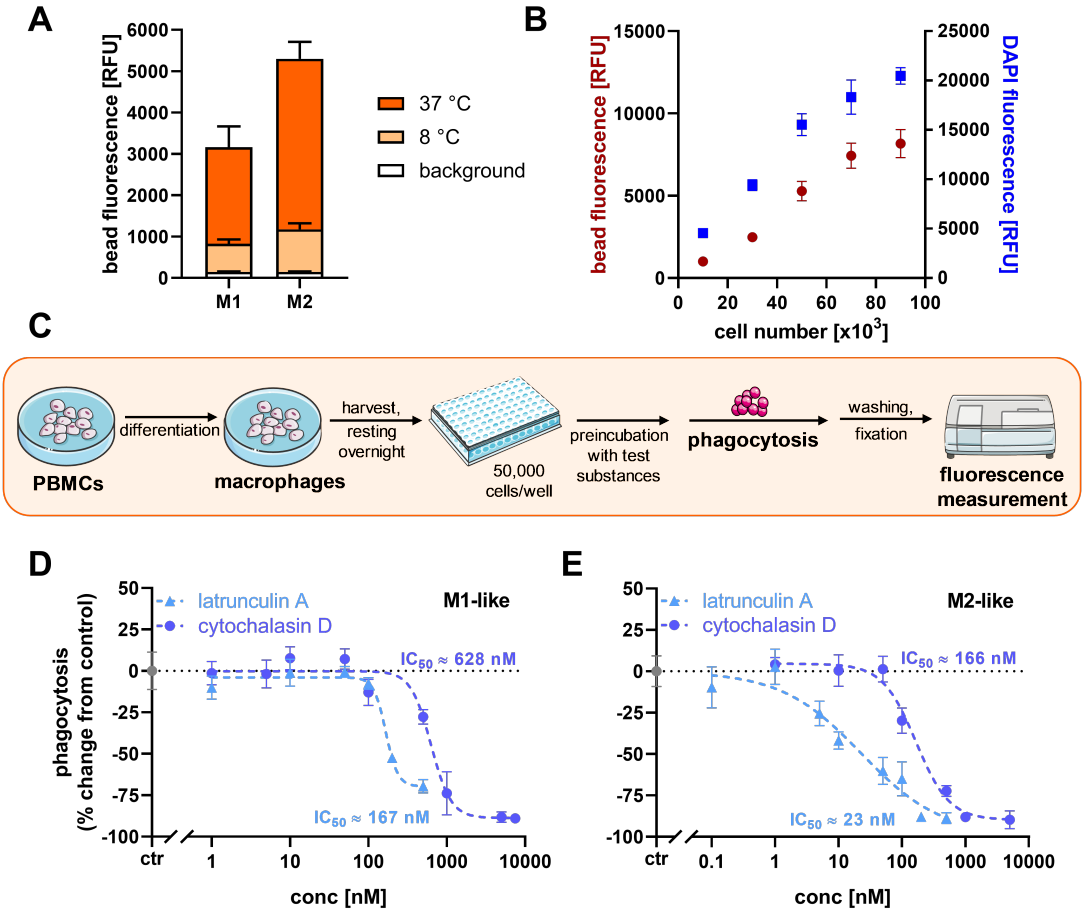

Figure 3.1: Characterization of phagocytosis in M1- and M2-like macrophages. Human monocytes were differentiated into M1-like or M2-like macrophages using 10 ng/mL GM-CSF or M-CSF (M1-/M2-like) for 7 days and additional 10 ng/mL IFNγ or IL-4 (M1-/M2-like) for the last 2 days. (A) Phagocytosis was carried out at 8 °C or 37 °C for 2 h using 50,000 cells per well (post differentiation). Shown is the bead fluorescence at 610 nm after excitation at 575 nm as mean ± SD for $n = 6-9$ replicates from a pool of 3 subjects. (B) Phagocytosis in M2-like macrophages using different cell numbers for 2 h followed by DAPI staining. Shown is the bead and DAPI fluorescence for the different cell numbers as mean ± SD for $n = 8$ replicates from a pool of 3 subjects. (C) Schematic overview over phagocytosis assay. (D-E) Dose dependent inhibition of phagocytosis in M1-like and M2-like macrophages by latrunculin A or cytochalasin D. Substances were preincubated at the indicated concentrations for 2 h. Shown is phagocytosis as % change from vehicle control (grey) as mean ± SD for $n = 5-6$ replicates from a pool of 4 subjects. The inhibitory potencies (IC_{50}) were calculated based on % phagocytosis relative to vehicle control (0.1% DMSO).

Figure B.2). Both, latrunculin A and cytochalasin D inhibited phagocytosis in M1- and M2-like macrophages in a dose-dependent manner, with IC_{50} values in the low nM range (Figure 3.1D, E). Overall, optimization resulted in a z-factor higher than 0.5 indicating a good bioassay (Figure B.3) using the following procedure: After differentiation of primary human macrophages, cells are transferred into 96-well plates (50,000 cells/well), rested overnight and subsequently preincubated with test substances or vehicle control for 2−4 h. For phagocytosis, supernatants of the 96-well plate are discarded, and opsonized bead solution, diluted in medium, is added together with the preincubated test substance to the cells for 2 h. Phagocytosis is stopped by discarding the supernatants and carefully washing the cells with PBS. Cells are fixated using 4% formaldehyde. The fluorescence of the beads is measured using a plate reader. Afterwards, the cells are stained with DAPI, the residual DAPI is washed away and the fluorescence of DAPI is analyzed as an indirect measure of cell number (details are shown in the Appendix B).

The inflammatory stimulus LPS was found to increase phagocytosis in M2-like macrophages in a dose- and time-dependent manner (Figure 3.2C, D). Incubation times between 2 to 24 h and concentrations between 10−100 ng/mL resulted in the highest increase in phagocytosis (1.6-fold). In contrast, no effect of LPS on phagocytosis was observed in M1-like macrophages (Figure 3.2A). In order to elucidate the reasons for these differences, ARA enzyme abundance in the two types of macrophages after LPS stimulation was analyzed and revealed 1.6-fold higher COX-2 abundance in the M1-like type compared to M2-like (Figure 3.3A). In line with this, concentrations of COX products were approximately 2-fold higher (Figure 3.3B). Therefore, we hypothesized that the different effect of LPS on pha-

gocytosis in the two macrophage types may be mediated by COX activity. In order to test this, the impact of COX inhibitors and dexamethasone on phagocytosis was evaluated (Figure 3.2A, B, Table B.2): Indeed, LPS increased phagocytosis in both macrophage types (M1-like: 1.4-fold, M2-like 1.7-fold) in the presence of the three compounds. Moreover, PGE_2, one of the main COX products, inhibited phagocytosis in both macrophage types in a dose-dependent manner (IC_{50}(M1-like) $\approx$ 2.5 nM, IC_{50}(M2-like) $\approx$ 17 nM) (Figure 3.4A). Among the four PGE_2 receptors, EP2 and EP4 are present in the macrophages (Figure B.4) [25–27]. Using an antagonist against EP4, inhibition of phagocytosis by PGE_2 was restored, whereas the antagonist against EP2 showed no effect (Figure 3.4B). This demonstrates that phagocytosis is regulated via EP4 signaling in human M1- and M2-like macrophages.

3.4 Discussion

Phagocytosis is a key process of innate immune cells such as macrophages to eliminate foreign invaders and cell debris during inflammatory processes. Here, we show the optimization of a phagocytosis assay to investigate, how the COX branch of the ARA cascade is involved in the regulation of phagocytosis.

3.4.1 Optimization of the Phagocytosis Assay

For the investigation of phagocytosis, a variety of methods have been described using different bacteria/particles to be engulfed. The readout includes labor- and cost-intensive techniques such as confocal microscopy and flow cytometry [28,

29]. Moreover, simple readouts based on fluorescence measurements using a plate reader and a 96-well plate format are described [30–32]. Here, we established such an assay with fluorescence-labeled polystyrene microspheres (beads) for pri-

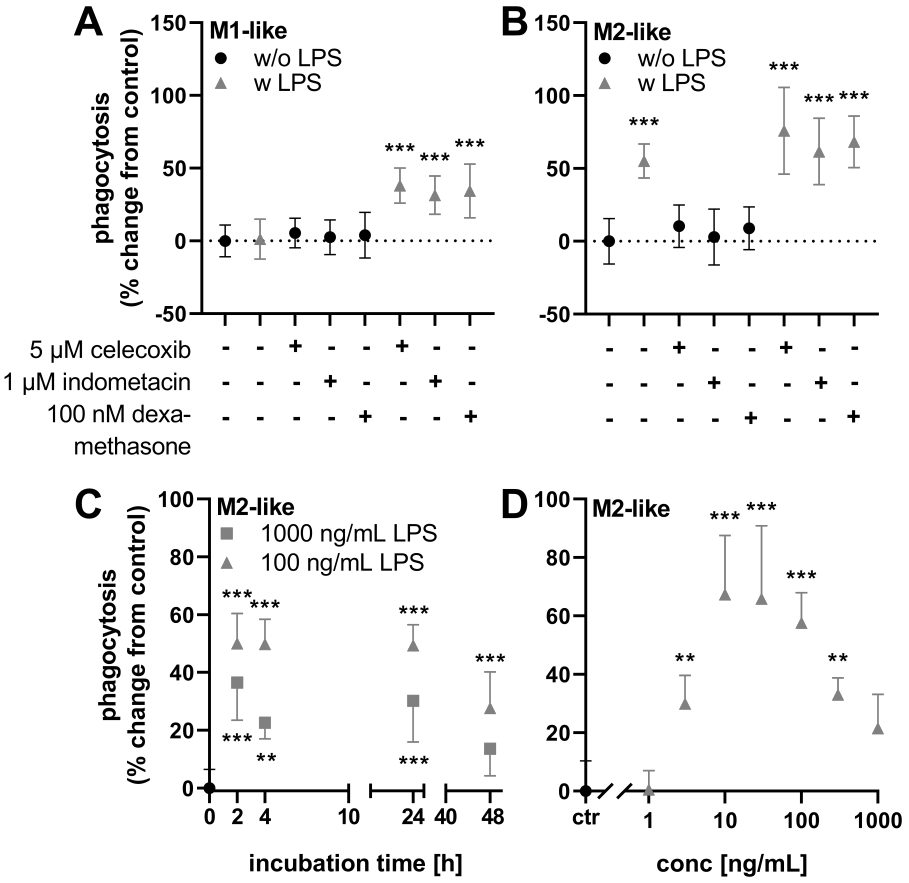

Figure 3.2: Phagocytosis is differentially modulated by LPS in M1- and M2-like macrophages. (A) M1-like or (B) M2-like macrophages were preincubated with celecoxib (5 µM), indometacin (1 µM) or dexamethasone (100 nM) for 30 min before addition of 0.1 µg/mL LPS for 4 h. (C) M2-like macrophages were preincubated with 0.1 or 1 µg/mL LPS for different periods of time or (D) with the indicated concentrations for 4 h. Shown is phagocytosis as % change from vehicle control (0.1% DMSO) as mean ± SD for $n = 5-6$ replicates from a pool of 3−4 subjects. Statistical analysis was performed by 1-way ANOVA followed by Dunnett's multiple comparison test. Differences from vehicle control were considered significant at p values ≤0.01 (**) or ≤0.001 (***).

mary human macrophages. Using the amount of nucleic acids stained with DAPI, the cell number was examined indirectly and optimized for the assay (Figure 3.1B) resulting in a sufficient high cell number leading to an optimal fluorescence read-out, but ensuring sufficient space for the cells. Further parameters such as resting time after transfer of cells to 96-well plates, number of cells per well, the length of phagocytosis, and number of required washing steps to remove >90% of not internalized beads (Figure B.2), were optimized. However, analysis of the fluorescence signals after phagocytosis at 8 °C (Figure 3.1A) showed that beads cannot completely be removed by washing. The fluorescence following an incubation of the cells at 8 °C was comparable for M1- and M2-like macrophages (Figure 3.1A), indicating a similar number of beads adhering unspecifically to the cell surface in both phenotypes. This is consistent to other reports showing that beads are not engulfed, but adhere to the cell surface at 8 °C [30]. Therefore, we calculate fluorescence/phagocytosis data relative to a vehicle control and established controls for the assay.

Comparing the differently polarized macrophage types following an incubation at 37 °C, M2-like macrophages showed 1.5-fold higher fluorescence intensities, indicating a higher phagocytic activity compared to M1-like macrophages (Figure 3.1A). This is in line with previous reports [33, 34] and could be due to the high abundance of scavenging receptors in M2-like macrophages which support phagocytic functions [35, 36].

Cytochalasin D and latrunculin A, both interfering with actin polymerization [37, 38] and thus inhibiting phagocytosis, were able to inhibit phagocytosis in a dose-dependent manner, whereby maximum inhibition was achieved at lower concen-

trations for latrunculin A (200 nM) than for cytochalasin D (1 μM) (Figure 3.1D, E). M2-like macrophages were more sensitive to the inhibitors as indicated by lower IC_{50} values (Figure 3.1D, E). Similar inhibitory concentrations (12−120 nM) were reported for latrunculin A for mouse peritoneal macrophages [39] and higher concentrations (10−40 μM) for cytochalasin D for mouse peritoneal macrophages and for J774A.1 cells, a murine macrophage cell line [40, 41] which may be explained by the different cell type. Cytochalasin D and latrunculin A are suitable chemical probes used as control to inhibit phagocytosis in the assay.

3.4.2 Phagocytosis is differentially modulated by LPS in M1- and M2-like macrophages

Information on substances that reliably enhance phagocytosis is limited in the literature, in contrast to the well-described inhibitors. We investigated LPS, since it has been widely described, that LPS impacts a variety of macrophage functions such as phagocytosis [42–44]. Indeed, in M2-like macrophages, LPS increased phagocytosis in a dose- and time-dependent manner (Figure 3.2C, D). In contrast, LPS showed no effect on phagocytosis in M1-like macrophages (Figure 3.2A). Interestingly, both – stimulation and inhibition of phagocytosis – have been described in the literature following a stimulation with LPS [44–48]: Whereas LPS increased phagocytosis in mouse bone-marrow derived macrophages and human monocytes using similar conditions (1 h or 20−24 h, using concentrations of 10−100 ng/mL) [45, 48], phagocytosis was suppressed in mouse peritoneal macrophages [44, 46]. This leaves the role of LPS in the regulation of phagocytosis controversial. In order to elucidate the differential modulation of phagocytosis by

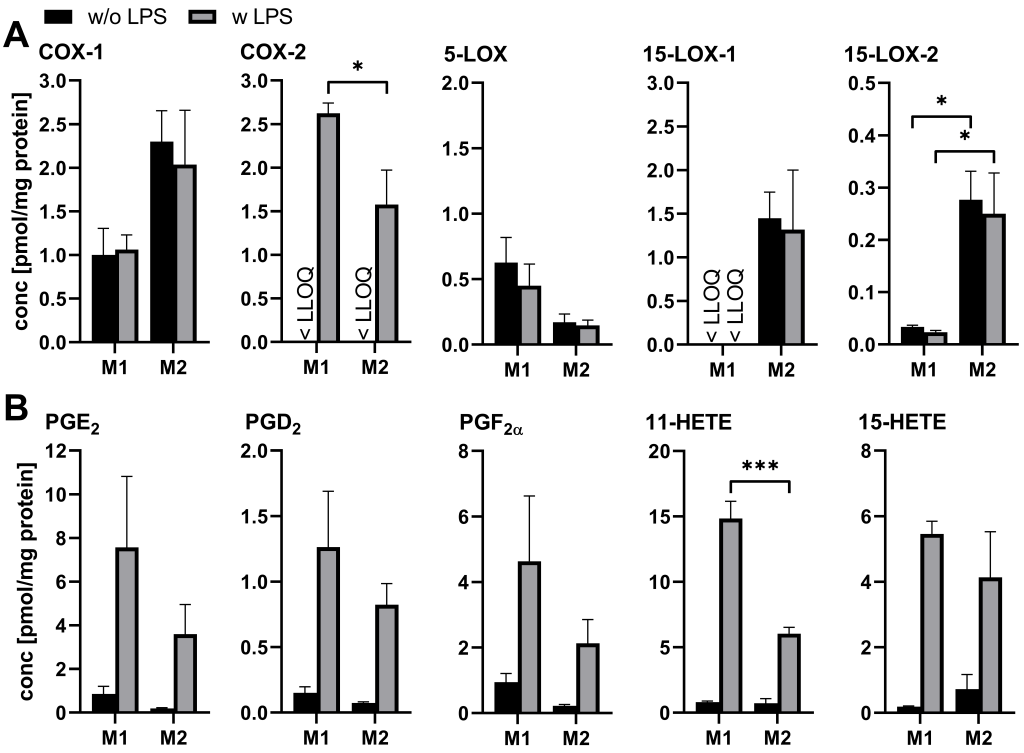

Figure 3.3: ARA enzyme abundance and COX activity of macrophages with and without LPS stimulation. (A) Macrophages were analyzed for ARA enzyme abundance by quantitative proteomics and (B) free oxylipins by quantitative metabolomics with (w) and without (w/o) additional LPS (0.1 μg/mL) treatment for 6 h. Shown are mean ± SEM for $n = 3$ subjects. Statistical analysis was performed by 1-way ANOVA followed by Tukey's multiple comparison test. Differences were considered significant at p values $\leq$0.05 (*) or $\leq$0.001 (***).

LPS in the two macrophage types, the involvement of ARA cascade enzymes was investigated.

The ARA cascade enzyme pattern showing higher levels of COX-1, 15-LOX-1 and -2 and lower levels of 5-LOX in M2-like compared to M1-like macrophages (Figure 3.3B) and was comparable to previous reports [15, 16]. LPS stimulation led to increased COX-2 abundance in both macrophage types [17], leading to a 1.6-fold

higher COX-2 abundance in the M1-like type (Figure 3.3A). The COX activity was relevantly higher in M1-like macrophages as well as the levels of its products such as PGE_2, PGD_2 and $PGF_{2\alpha}$ were $2-4$-fold higher in M1-like macrophages at basal levels and after stimulation with LPS (e.g., PGE_2 M1 vs M2, basal: 0.85 ± 0.35 vs 0.20 ± 0.02 pmol/mg protein; LPS: 7.57 ± 3.25 vs 3.59 ± 1.36 pmol/mg protein) (Figure 3.3B).

This was in line with levels of 11-HETE (Figure 3.3B), which is a well-known side product of COX-2 activity [49, 50]. 15-HETE showed basal higher concentrations in M2-like macrophages compared to M1-like presumably due to 15-LOX-1 activity; after LPS stimulus levels were increased. This can be explained by COX-2 activity, with 15-HETE being one of the main side products [49, 50]. Both, enzyme abundance and oxylipin levels underline that M2-like macrophages have lower COX-2 abundance and activity after LPS stimulus compared to M1-like macrophages. Therefore, we hypothesized that COX (products) may contribute to the differential effect of LPS on phagocytosis observed in M1-like macrophages (no effect) compared to M2-like macrophages (stimulation).

In order to investigate the impact of COX activity on phagocytosis, COX-inhibitors and dexamethasone were tested at concentrations allowing to effectively block COX activity in primary human macrophages [15]: The tested substances alone had no effect on phagocytosis (Figure 3.2A, B) which fits to the previous finding that basal COX activity is very low in both macrophage types. However, in combination with LPS, phagocytosis was increased in both macrophage types, whereas the increase was higher for M2-like macrophages (M1-like, 35%; M2-like, 69%) (Table B.2). Similar findings, an upregulation of phagocytosis, were already re-

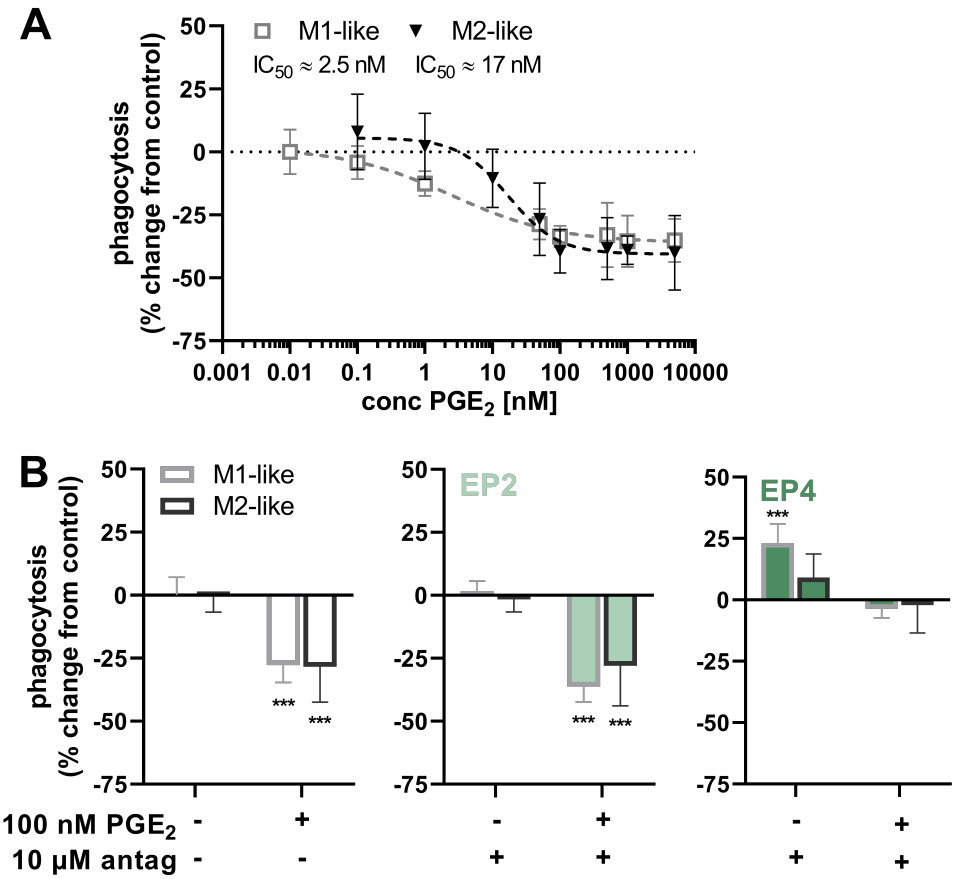

Figure 3.4: Regulation of phagocytosis by COX-product PGE_2. (A) M1-like or M2-like macrophages were incubated with different PGE_2 concentrations for 2 h. (B) Macrophages were treated with 100 nM PGE_2 for 2 h or EP2 antagonist (PF-04418948, 10 µM) or EP4 antagonist (GW627368, 10 µM). Antagonists were tested alone and in combination with 100 nM PGE_2 and added 30 min before PGE_2 incubation. Shown is phagocytosis as % change from vehicle control (0.1% DMSO) as mean ± SD for $n = 5-6$ replicates from a pool of 3−4 subjects. Statistical analysis was performed by 2-way ANOVA followed by Dunnett's multiple comparison test. Differences from vehicle control were considered significant at n values ≤ 0.001 (***).

ported for murine bone marrow derived macrophages and alveolar macrophages treated with celecoxib [51, 52], for rat alveolar macrophages treated with celecoxib or indomethacin [53, 54], as well as for human monocytes with dexametha-

sone [55]. This indicates that (some) COX products counteract the stimulating effect of LPS. Thus, the strong upregulation of COX-2 abundance and activity by LPS in M1-like macrophages can explain why LPS could not increase phagocytosis in M1-like macrophages. However, besides induction of COX-2, LPS induces a variety of other changes in the cells that are discussed affecting phagocytosis such as cytokines [45], number of specific surface receptors [44] and the cytoskeletal network [44]. This is a limitation of this study and further research is needed to elucidate which other LPS mediated effects are involved in the regulation of phagocytosis in M1- and M2-like macrophages.

PGE_2 was the highest abundant prostaglandin in M1-like macrophages after LPS stimulus (Figure 3.3B). Thus, we tested if the LPS effect is mediated by this prostaglandin. PGE_2 inhibited phagocytosis in a dose-dependent manner in both macrophage types with a half-maximal inhibition (IC_{50}) in the low nM range (Figure 3.4A). Observed IC_{50} values were slightly lower for M1-like indicating these cells were more sensitive to PGE_2. Our results are in line with the majority of the studies which reported an inhibitory effect of PGE_2 on phagocytosis *in vitro* [45, 53, 54, 56–60] and *in vivo* [61, 62]. Moreover, the extent of inhibition of phagocytosis by PGE_2 up to 40% is comparable to values found for other monocyte/macrophage systems (approximately $25-85\%$) shown in the literature [45, 54, 58, 60]. Interestingly, PGD_2 has been reported to have opposite effects on phagocytosis in rat alveolar macrophages [53].

PGE_2 affects changes in cell functions through activation of distinct G protein-coupled E prostanoid (EP) receptors (EP1-EP4). The presence of EP receptors in primary human M1- and M2-like macrophages was analyzed by quantitative

proteomics. EP2 (approximately 3−5 fmol/mg protein) and EP4 (approximately 5−7 fmol/mg protein) were present in the cells while EP1 and EP3 could not be detected (Figure B.4). The expression of the two receptors in macrophages is in line with the literature [25–27]. In order to determine, which of the two receptors is involved in the regulation of phagocytosis by PGE_2 in human M1- and M2-like macrophages, we tested specific antagonists against EP2 and EP4 (Figure 3.4B). The EP2 antagonist PF-04418948 did not affect phagocytosis by PGE_2 in both cell types. In contrast, EP4 antagonist GW627368 entirely blocked the inhibitory effect of PGE_2. This indicates, that exogenously added PGE_2 mediates its inhibitory effects on phagocytosis through EP4 activation in human macrophages. GW627368 alone increased phagocytosis in M1-like macrophages (by approximately 23%), suggesting that already basal formed levels of PGE_2 (without LPS stimulus) are high enough to elicit inhibitory effects on phagocytosis. This fits to the comparatively high levels of approximately 1 pmol/mg protein PGE_2 in M1-like macrophages determined by LC-MS/MS (Figure 3.3A). However, the unselective COX inhibitor indomethacin alone did not increase phagocytosis in M1-like macrophages, which could be explained by residual levels of PGE_2 being present prior to inhibitor treatment.

Both receptors, EP2 and EP4, signal predominantly via stimulatory G protein (Gs), activating adenylyl cyclase (AC) and increasing the intracellular levels of cAMP [63]. There is evidence that PGE_2 inhibits phagocytosis via a cAMP dependent pathway [54, 56, 59, 60, 64], which fits to our findings. However, reports are inconsistent, which of the EP receptors are involved in signal transduction: For rat alveolar macrophages and mouse bone marrow derived macrophages, an EP2

dependent signaling was reported [52, 54, 56]. However, other studies reported the involvement of both receptors in rat alveolar macrophages [53] and in THP-1 derived macrophages [59] indicating that the PGE_2 signaling may be highly cell type dependent.

Our results indicate, that PGE_2 signaling on phagocytosis is mainly through EP4 receptor in primary human macrophages. The different PGE_2 levels in M1- and M2-like macrophages as a result of different COX-2 activities are involved in the modulation of phagocytosis by LPS. This shows the importance of the link between the COX branch of the ARA cascade and regulation of phagocytosis in human macrophages and provides a mechanistical explanation of the well-investigated anti-inflammatory effects of an COX-2 inhibition [65]: By suppressing the formation of pro-inflammatory PGE_2, e.g., by using COX inhibitors, phagocytosis can be increased which in turn limits inflammation and enables tissue restoration.

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4 Phagocytosis of primary human macrophages is elevated by ex vivo supplementation with n–3 PUFA*

Epidemiologic studies show that a high n–3 polyunsaturated fatty acid (PUFA) status is beneficial for health and inflammatory diseases. However, results of nutrition studies investigating the impact of n–3 PUFA intake on immune functions, such as phagocytosis, are contradictory. In order to gain more insights into the role of n–3 PUFA on phagocytosis, we investigated the modulation of phagocytosis by n–3 PUFA and derived oxylipins in human macrophages. Using an established ex vivo supplementation strategy, primary human macrophages were supplemented with docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). The PUFA pattern of the cells was shifted from a low n–3 PUFA status towards a high n–3 PUFA status. This was accompanied by a shift in the oxylipin pattern, reduced pro-inflammatory prostaglandin levels, increased phagocytosis in the supplemented macrophages, and reduced inhibitory effect of PGE_2 on phagocytosis. However, when tested alone, n–3 PUFA derived oxylipins did not impact phagocytosis. Under controlled conditions, an increased n–3 PUFA status of macrophages resulted in an elevation of phagocytosis. Less formation of prostaglandins could contribute to this effect, whereas n–3 PUFA derived oxylipins, particularly multihydroxy-PUFA, appear to have a limited impact on phagocytosis following n–3 PUFA supplementation.

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4.1 Introduction

Dietary intake of polyunsaturated fatty acids (PUFA) is vital for humans and a high n–3 PUFA status is associated with beneficial health outcomes such as lowering the risk for cardiovascular diseases [1]. Whereas the typical diet in Europe and the US includes large amounts of n–6 PUFA, the intake of n–3 PUFA – especially long-chain n–3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) – is low [2, 3]. In contrast, a high n-3 PUFA status, e.g. by supplementing n-3 PUFAs from fish or algae oil, results in a lower %n-6 in highly unsaturated fatty acids (HUFA), a biomarker strongly correlated with cardiovascular diseases such as sudden cardiac death [4]. The impact of n-3 PUFAs has been extensively studied during the past decades; however, their multiple mechanisms of action and overall impact on immune functions remain only partially understood.

PUFA are important components of the cell membranes affecting fluidity, membrane-associated proteins, formation of lipid rafts and the pattern of formed oxidized PUFA such as eicosanoids and other oxylipins [5]. Several oxylipins are lipid mediators involved in the regulation of physiological and inflammatory processes: n–6 PUFA derived oxylipins such as prostaglandins (PG) formed by cyclooxygenase (COX) activity and leukotrienes formed by 5-lipoxygenase (LOX) activity from arachidonic acid (ARA) are well-investigated pro-inflammatory mediators [6, 7] involved in e.g., pain, fever and asthma [8]. Increased intake of n–3 PUFA shifts the oxylipin pattern by a reduced conversion of n–6 PUFA, and an increased in the formation of n–3 PUFA derived oxylipins [9]. Of note, genetic variations in genes involved in the PUFA metabolism such as FADS1 and FADS2 are associated with inflammatory diseases and impact PUFA and oxylipin profiles [10,

11]. Consequently, oxylipins are likely to contribute to the beneficial effects of n–3 PUFA on immune functions such as phagocytosis as has been shown recently for alpha linolenic acid [12].

Phagocytosis is a key mechanism of innate host defense directly neutralizing pathogens. Additionally, phagocytosis plays a role in the active resolution of inflammation by removing apoptotic cells and cell debris, thereby enabling restoration of healthy tissue [13]. Several oxylipins, especially multihydroxy oxylipins derived from EPA and DHA, are discussed to have anti-inflammatory effects, such as modulation of phagocytosis [14–20], but their formation and presence in human macrophages remains controversial [21, 22].

In order to elucidate how n–3 PUFA impact phagocytosis and whether n–3 PUFA derived oxylipins contribute to their effects, primary human 'inflammatory' M1- and 'anti-inflammatory' M2-like macrophages, representing extremes of macrophage phenotypes, were supplemented with DHA and EPA using an established *ex vivo* strategy [23]. Changes in FA and oxylipin pattern of macrophages following n–3 PUFA supplementation were quantified by means of LC-MS/MS. Phagocytic activity following n–3 PUFA supplementation as well as the impact of n–3 PUFA derived oxylipins, which were increased by the supplementation, were investigated in both phenotypes of primary macrophages.

4.2 Materials and Methods

4.2.1 Chemicals and Biological Material

Non-fasted human AB plasma was obtained from the blood donation center at University Hospital Düsseldorf (Düsseldorf, Germany). Lymphocyte separation medium 1077 was purchased from PromoCell (Heidelberg, Germany). Recombinant human GM-CSF, M-CSF, IFNγ and IL-4 produced in Escherichia coli were bought from Thermo Fisher Scientific (Langenselbold, Germany). RPMI 1640 cell culture medium, L-glutamine and penicillin/streptomycin (5.000 units penicillin and 5 mg/mL streptomycin), LPS from E. coli (0111:B4), dextran 500 from Leuconostoc spp. and copper sulfate pentahydrate were from Merck (Taufkirchen, Germany). Docosahexaenoic acid (DHA >99%) and eicosapentaenoic acid (EPA >99%) were bought from Nu-check Prep, Inc. (Elysian, Minnesota, United States). Prostaglandin E2 (PGE_2), 5-HEPE, 4-HDHA, 7-HDHA, 12(*S*)-HEPE, 15(*S*)-HEPE, 14(*S*)-HDHA, 17(*S*)-HDHA, 17(18)-EpETE and 19(20)-EpDPE were bought from Cayman Chemical (local distributor Biomol, Hamburg, Germany). Accutase, DMSO and formaldehyde were purchased at Carl Roth (Karlsruhe, Germany). Fluorescence-labeled polystyrene microspheres (latex beads) were obtained as 2% solution from Thermo Fisher Scientific (Langenselbold, Germany). BCA reagent A was bought from Fisher Scientific (Schwerte, Germany). The ultra-pure water with a conductivity of >18 MΩ*cm was generated by the Barnstead Genpure Pro system from Thermo Fisher Scientific (Langenselbold, Germany).

4.2.2 Isolation and differentiation of primary human macrophages

Primary human macrophages were isolated as described [24]. In brief, peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats provided by blood donations at the University Hospital Düsseldorf, Germany or at Deutsches Rotes Kreuz West, Hagen, Germany. The blood donations were drawn with the informed consent of healthy human subjects and the study was approved by the Ethical Committee of the University of Wuppertal. PBMC were isolated by dextran (5%) sedimentation for 30 min followed by centrifugation (800 $\times$ g without deceleration, 10 min, 20 °C) on lymphocyte separation medium. The leucocyte ring was isolated and washed twice with PBS. The cells were seeded in 60.1 mm^2 dishes in RPMI medium (100 IU/mL penicillin and 100 μg/mL streptomycin (P/S), 2 mM L-glutamine) in a humidified incubator at 37 °C and 5% CO_2 for $1-2$ h. Non-adherent cells were removed by gently washing, and RPMI medium (P/S, 2 mM L-glutamine) supplemented with 5% human AB plasma was added to the cells. Using an established protocol [24], cells were polarized towards M1-like macrophages using 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) for 7 days and additional 10 ng/mL interferon γ (IFNγ) for the final 48 h or towards M2-like macrophages using 10 ng/mL macrophage (M)-CSF for 7 days and additional 10 ng/mL interleukin 4 (IL-4) for the final 48 h [25].

4.2.3 Supplementation of human macrophages with n–3 PUFA

Two independently performed supplementation experiments were carried out (experiment 1 and 2), each with four human subjects. Supplementation experiments were carried out as described [23]. In brief, for the selection of a suitable plasma for the cell culture medium, five non-fasting human AB plasmas were obtained at the University Hospital Düsseldorf, Germany and analyzed for fatty acid levels and oxylipin concentrations (Figure C.1, Tables C.1, C.2). Plasmas C and E were pooled (pool 1) resulting in a plasma which fulfilled all following criteria: %n–6 in HUFA >75%, n–3 PUFA <0.25 mM, total FA <10 mM, low oxylipin concentrations [23].

For selection of suitable subjects/buffy coats which reflect the poor n–3 PUFA status of average subjects in Europe and the US, only subjects having a %n–6 in HUFA $\leq$70% in erythrocytes were selected for the supplementation experiments (Table C.3) [23]. For supplementation, following predilution in DMSO (50 mM), EPA and DHA were slowly mixed with human plasma and added (5% (*v/v*)) to RPMI medium (P/S, 2 mM L-glutamine) (10.3 μM DHA, 5.5 μM EPA) (Figure 4.1). Medium containing the same plasma, but without added DHA and EPA, served as control medium with low n–3 PUFA content (low). For n–3 PUFA supplementation, macrophages were incubated with supplemented medium (high) for 2 days. Macrophages were harvested by cold shock method [24], and cell pellets were frozen at -80 °C until analysis.

4.2.4 Phagocytosis assay

Phagocytosis was carried out as described [26]. Briefly, differentiated and supplemented macrophages were harvested using accutase and transferred into 96-well plates at 50,000 cells/well. After resting overnight, cells were preincubated with test substances or vehicle control as indicated. For phagocytosis, supernatants were discarded and fluorescence-labeled polystyrene microspheres (beads) diluted in medium ($6.25 \cdot 10^{-3}$% beads, 5% human plasma) were added together with the preincubated test substance to the cells for 2 h. Phagocytosis was stopped by discarding the supernatants, carefully washing the cells with PBS and fixating the cells with 4% formaldehyde. Fluorescence of beads was measured using a plate reader (Infinite 200 PRO, Tecan) (λ_{ex} 535 nm, λ_{em} 575 nm). For DAPI staining 10 µg/mL DAPI was added to the cells for 15 min at room temperature followed by three times washing with PBS and fluorescence measurement using the plate reader (λ_{ex} 358 nm, λ_{em} 461 nm).

4.2.5 Quantification of fatty acyl and oxylipin levels by means of LC-MS/MS

Oxylipin determination was carried out in cell pellets, medium and human plasma as described[27–30]. Briefly, cell pellets were resuspended in MeOH/H_2O (50/50, *v/v*), and sonicated. After addition of internal standards (IS), proteins were precipitated using MeOH (for non-esterified oxylipins) or iso-propanol (for total oxylipins) followed by centrifugation (20 000 × g, 10 min, 4 °C). For quantification of total oxylipin levels, supernatants were saponified using 0.6 M KOH in MeOH/H_2O

(75/25, *v/v*) for 30 min at 60 °C. Oxylipins were purified using solid phase extraction (SPE) and analyzed by means of LC–MS/MS using an 1290 Infinity II LC system (Agilent), coupled to a QTRAP mass spectrometer operated in electrospray ionization (ESI(-)) mode (Sciex, Darmstadt, Germany) operated in scheduled selected reaction monitoring. Fatty acyl concentrations from the same samples as for the total oxylipins were determined by LC-MS/MS as described [31]. Oxylipin and fatty acyl concentrations were quantified using external calibrations with IS.

Analyst (Sciex, version 1.7) was used for instrument control and Multiquant software (Sciex, version 3.0.2) was used for data analysis. The concentrations of oxylipins and fatty acids in the macrophages were calculated based on the protein content determined via bicinchoninic acid assay [32].

4.2.6 Data analysis

Data are presented as mean ± standard deviation (SD). Statistical analysis and visualization of data was performed using the Prism software (GraphPad Software, version 8.4., San Diego, CA, USA). For statistical analysis, unpaired t-tests, 1-way ANOVA following Dunnett's or Sidak's multiple comparisons tests or 2-way ANOVA following Sidak's multiple comparisons tests were performed as indicated in the figure captions. Statistical significance is depicted as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. %n–6 in HUFA and n–6:n–3 ratios were calculated from total FA concentrations (μM or μmol/g protein) of the PUFAs indicated in the following equations (n–6 PUFA C20:3 n–6 (A), C20:4 n–6 (B), C22:4 n–6 (C), C22:5 n–6 (D), the n-9 PUFA C20:3 n-9 (E), and the n–3 PUFA C20:5 n–3 (F), C22:5 n–3 (G) and C22:6 n–3 (H)) [33]:

$$\%\text{n–6 in HUFA} = 100 \times \frac{(\text{A} + \text{B} + \text{C} + \text{D})}{(\text{A} + \text{B} + \text{C} + \text{D} + \text{E} + \text{F} + \text{G} + \text{H})} \tag{4.1}$$

$$\text{n–6:n–3 ratio} > \text{C18} = \frac{\text{C20:4 n–6}}{(\text{C20:5 n–3} + \text{C22:6 n–3})} \tag{4.2}$$

$$\text{n–6:n–3 ratio} \geq \text{C18} = \frac{(\text{C20:4 n–6} + \text{C18:2 n–6})}{(\text{C20:5 n–3} + \text{C22:6 n–3} + \text{C18:3 n–3})} \tag{4.3}$$

4.3 Results

4.3.1 Ex vivo supplementation of primary human macrophages with n–3 PUFA

The n–3 PUFA supplementation was carried out in two independent experiments (experiment 1 and 2), each with four human subjects. In order to enable a reproducible supplementation, key parameters for selection of buffy coats, plasma, n–3 PUFA and composition of cell culture media [23] were controlled (Figures C.1, C.2, C.3; Tables C.1, C.2, C.3). The cell culture medium was prepared using 5% (*v/v*) of plasma (non-supplemented control, n–3 PUFA low) and plasma with 10.3 µM DHA and 5.5 µM EPA (n–3 PUFA high). This resulted in equal concentrations of ARA and EPA + DHA in the medium, a decrease of the %n–6 in HUFA by approximately 20%, and of the n–6:n–3 ratio by factor 10 for both supplementation experiments (Figure 4.1). For supplementation of the cells, macrophages were incubated with the n–3 PUFA supplemented medium (high) for two days.

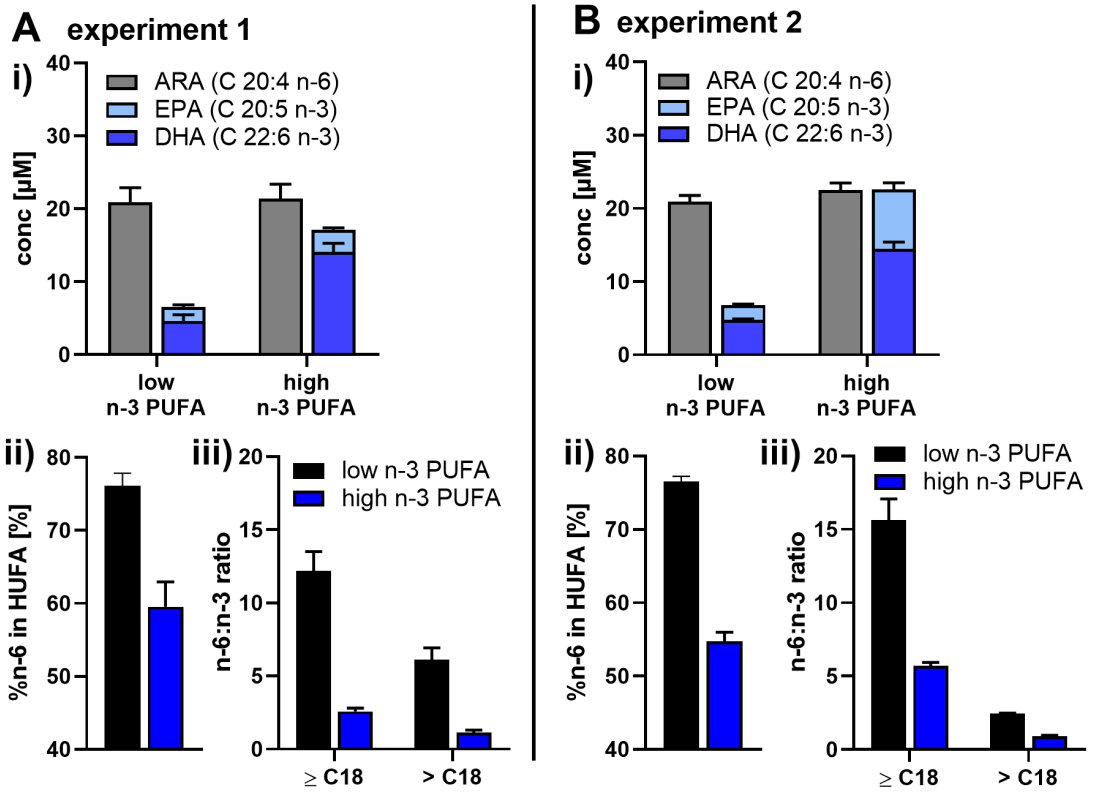

Figure 4.1: The FA pattern of cell culture medium is changed by addition of n–3 PUFA. For two supplementation experiments (experiment 1 and 2) (A, B), medium was prepared by using 5% (*v/v*) plasma pool 1 (pooled plasma of plasmas C and E, Figure C.1, Tables C.1, C.2) (low n-3 PUFA). In the supplemented medium (high n-3 PUFA), 10.3 µM DHA (>99%) and 5.5 µ EPA (>99%) were added. i) Total FA concentrations were determined by means of LC-MS/MS. ii) %n–6 in HUFA and iii) n–6:n–3 ratios were determined based on total FA concentrations. Results are shown as mean ± SD, $n = 3$.

n–3 PUFA supplementation decreased %n–6 in HUFA of macrophages from 85 ± 2% to 61 ± 4% (experiment 1) and 85 ± 1% to 56 ± 1% (experiment 2) (Figure 4.2 (i)). The relative amount of n–3 PUFA, especially DHA and EPA, was increased in the cells by approximately 4−7%, whereas n–6 PUFA such as ARA were decreased by approximately 3%; MUFA and SFA were not or only hardly changed (Figure 4.2 (ii-iii)). Similar to the changes in the FA pattern, oxylipins derived

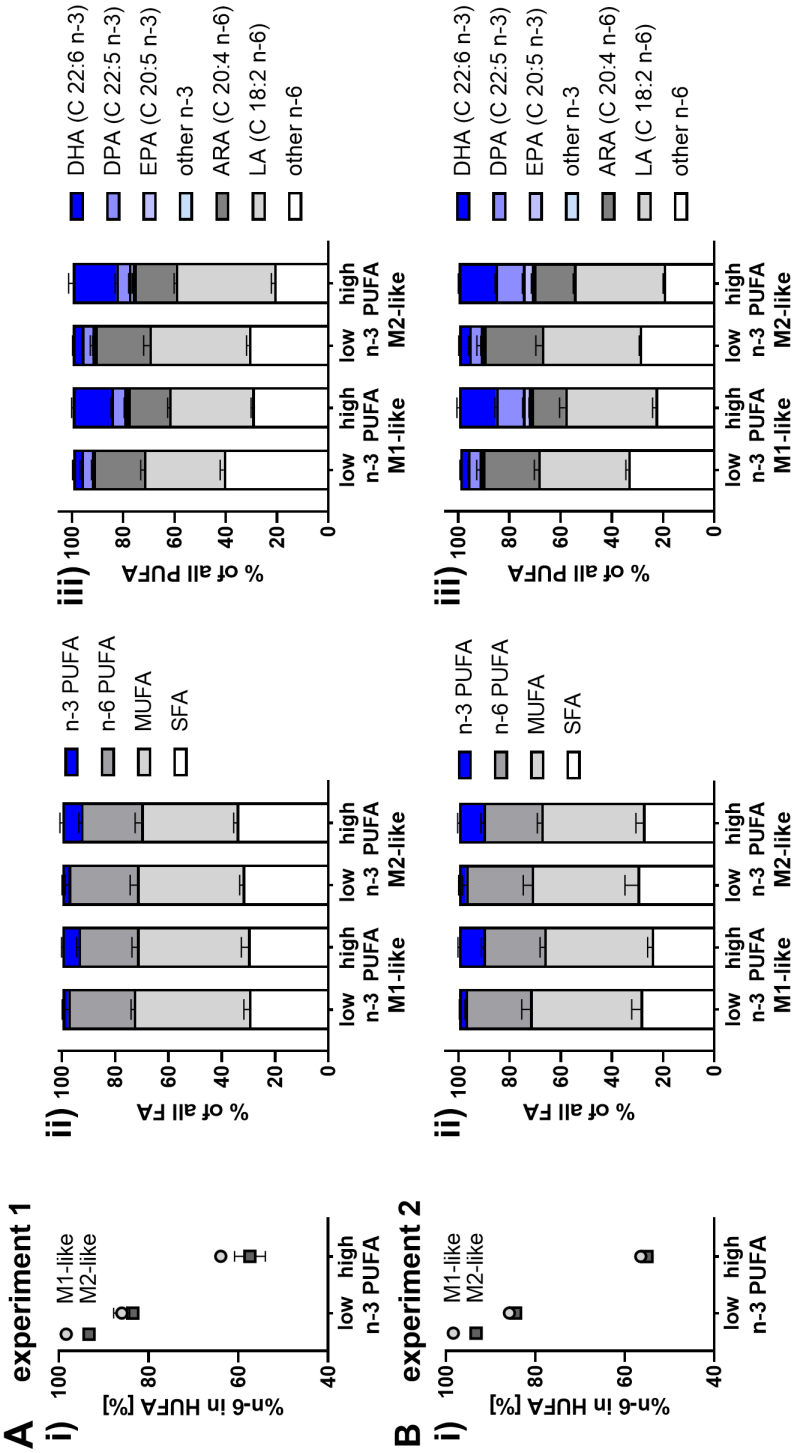
A experiment 1
i)
%n-6 in HUFA [%]
M1-like
M2-like
low high
n-3 PUFA
ii)
% of all FA
n-3 PUFA
n-6 PUFA
MUFA
SFA
iii)
% of all PUFA
DHA (C 22:6 n-3)
DPA (C 22:5 n-3)
EPA (C 20:5 n-3)
other n-3
ARA (C 20:4 n-6)
LA (C 18:2 n-6)
other n-6
low high
n-3 PUFA
M1-like
M2-like
B experiment 2

Figure 4.2: (previous page) FA pattern in macrophages is shifted towards cells with a high n–3 PUFA status following supplementation with n–3 PUFA in both supplementation experiments (A, B). After isolation of monocytes, cells were differentiated into macrophages and supplemented using medium with 5% (*v/v*) plasma pool 1 (low n-3 PUFA) or medium containing plasma pool 1, 10.3 μM DHA (>99%) and 5.5 μM EPA (>99%) (high n-3 PUFA) for 2 days 4.1. Total FA were quantified in the cell pellets by means of LC-MS/MS. i) %n–6 in HUFA of macrophages. ii) Relative distribution of n–3 PUFA, n–6 PUFA, MUFA and SFA. iii) Relative distribution of PUFA. Results are shown as mean ± SD, $n = 3$ pooled cells of 4 subjects for each experiment. Results of the statistical differences between macrophages with (high n-3 PUFA) and without (low n-3 PUFA) n-3 PUFA supplementation, analyzed using an unpaired t-test or two-way ANOVA followed by Sidak's multiple comparisons test, are shown in the Appendix C in Table C.7.

from EPA and DHA increased (e.g., 4-HDHA by approx. 200-300%; 5-HETE by approx. 200−600%) whereas ARA-derived oxylipins were decreased (e.g., 5-HETE by approx. 25%) in a comparable manner in both macrophage types (Figure 4.3). However, 15-LOX products were more affected in the M2-like phenotype: e.g., 17-HDHA was increased by approx. 3−10fold by supplementation in M2-like, but not changed in M1-like; 15-HETE was decreased by 60−70% in M2-like, but only 20−30% in M1-like. Dihydroxy-PUFA such as 5,15-diHETE or 5,15-diHEPE were below the lower limit of quantification (LLOQ). A clear decrease of COX products from ARA such as the PGE_2 breakdown product 12-HHTrE (by approx. 60−70%) and prostaglandins (e.g., PGE_2 by approx. 55−70% in experiment 1) was detected in both macrophage phenotypes following supplementation with n–3 PUFA (Figure 4.3).

4.3.2 Ex-vivo supplementation with n–3 PUFA increases phagocytosis in macrophages

Phagocytic activity of non-supplemented (low) and n–3 PUFA supplemented (high) macrophages was compared using an established phagocytosis assay with fluorescent labeled polystyrene beads [26]. Phagocytosis increased slightly but significantly in both macrophage phenotypes following n–3 PUFA supplementation (Figure 4.4A, Figure C.3). The increase in phagocytosis was more pronounced for the M1-like phenotype (e.g., for experiment 1: M1-like 117 ± 9%, M2-like 108 ± 12%).

4.3.3 Impact of oxylipins on phagocytosis

In order to investigate if oxylipins contribute to the increase in phagocytosis following n–3 PUFA supplementation, those oxylipins (Table C.4) that were found to be elevated following n-3 PUFA supplementation were tested for their effect on phagocytosis (300 nM each). However, the oxylipins had no or slightly inhibitory effects on phagocytosis in non-supplemented macrophages (Figure 4.4B, Figure C.4). After phagocytosis, concentrations of the oxylipins in the supernatants were quantified demonstrating that target concentrations were achieved throughout the assay (Table C.5). PGE_2 which is known to inhibit phagocytosis in human macrophages [26] was found to be decreased in the supernatants of n–3 PUFA supplemented macrophages, both at baseline and after an inflammatory stimulus (0.1 μg/mL LPS) (Figure 4.5A, B). Interestingly, when a relatively high concentration of PGE_2 is added exogenously to the cells, phagocytosis is 30−70% less inhibited in the macrophages following n–3 PUFA supplementation (Figure 4.5C,

D). In order to test, if the n–3 PUFA derived oxylipins abrogate the inhibitory effect of PGE_2 on phagocytosis, non-supplemented macrophages were incubated with PGE_2 and different oxylipins (Table C.4); concentrations of oxylipins were checked in the supernatants after phagocytosis (Table C.6). However, the tested oxylipins did not abrogate the inhibitory effect of PGE_2 on phagocytosis (Figure 4.5E, Figure C.5).

4.4 Discussion

A sufficient intake of long-chain n–3 PUFA such as EPA and DHA is required for human health, and associated with beneficial health effects [34, 35]. Here, we investigated the impact of these PUFA and their derived oxylipins on phagocytosis in human macrophages.

In order to enable a reliable investigation of the physiological effects of n–3 PUFA without carrying out intervention studies, an *ex vivo* supplementation strategy was used [23], and key parameters for all steps of the supplementation were controlled: Briefly, (i) only subjects reflecting the poor n–3 PUFA status of the population of Europe and the US were included (Table C.3), (ii) a suitable plasma low in oxylipin concentrations, low total FA and n–3 PUFA concentrations – reflecting the low n–3 PUFA status of the average population in Europe and the US – was selected (Figure C.1, Tables C.1, C.2), (iii) only pure n–3 PUFA were used (max. 0.5% (w/w) oxylipins), and (iv) cell culture media were always freshly prepared and replaced every 2-3 days in order to keep autoxidative formation of oxylipins low (Figure C.3).

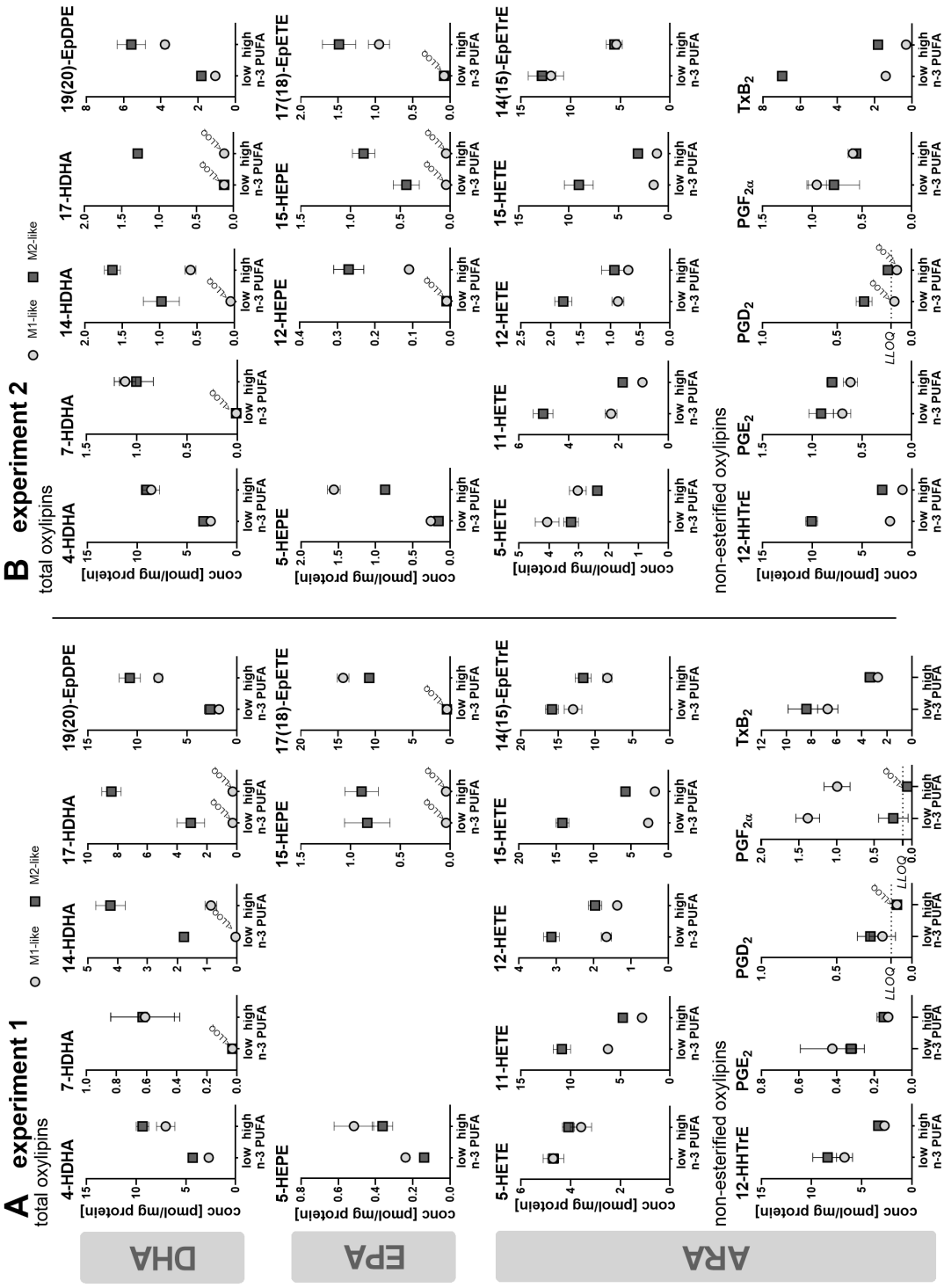
A experiment 1
B experiment 2
total oxylipins
non-esterified oxylipins
M1-like
M2-like
DHA
EPA
ARA
conc [pmol/mg protein]
low high n-3 PUFA
LLOQ
4-HDHA
7-HDHA
14-HDHA
17-HDHA
19(20)-EpDPE
5-HEPE
12-HEPE
15-HEPE
17(18)-EpETE
5-HETE
11-HETE
12-HETE
15-HETE
14(15)-EpETrE
12-HHTrE
PGE2
PGD2
PGF2α
TxB2

Figure 4.3: (previous page) DHA and EPA derived oxylipins are increased, ARA derived oxylipins decreased in macrophages after supplementation with n–3 PUFA. In two independent experiments (A, B), differentiated macrophages were supplemented using medium with 5% (*v/v*) plasma pool 1 (low n-3 PUFA) or medium containing plasma pool 1, 10.3 μM DHA (>99%) and 5.5 μM EPA (>99%) (high n-3 PUFA) for 2 days. Total oxylipins (monohydroxy-PUFA, epoxy-PUFA) and non-esterified oxylipins (12-HHTrE, prostaglandins, TxB_2) were quantified in the cell pellets by means of LC-MS/MS. Concentrations of selected, most abundant oxylipins derived from DHA, EPA and ARA are shown as mean ± SD, $n = 3$ pooled cells of 4 subjects for each experiment. Results of the statistical differences between macrophages with (high n-3 PUFA) and without (low n-3 PUFA) n-3 PUFA supplementation, analyzed using unpaired t-test, are shown in the Appendix C in Table C.8. LLOQ, lower limit of quantification.

Incubation of the macrophages with the n–3 PUFA enriched medium (high) (Figure 4.1) for 2 days remarkedly changed the FA profile (Figure 4.2) as well as the oxylipin pattern (Figure 4.3) of the macrophages: %n–6 in HUFA of non-supplemented macrophages (85 ± 2%) is comparable to values found in tissues from subjects having a low n–3 PUFA status [4], and was decreased to values between 55−64% by supplementation. Similar values for the %n–6 in HUFA were achieved in human nutrition in erythrocytes using oral supplementation and daily doses of 1−2 g [36–38]. Whereas MUFA and SFA levels remained unchanged, n–6 PUFA were replaced by n–3 PUFA such as EPA and DHA (Figure 4.2) which is in accordance with results of supplementation studies in humans and animals [36–39].

Similarly, the increase in n–3 PUFA derived oxylipins and decrease of ARA derived ones (Figure 4.3) is comparable to previous supplementation experiments with primary human M2-like macrophages [23], and consistent with nutrition intervention studies [39–42]. For M1-like macrophages smaller changes in concentrations of 15-LOX products following supplementation were observed compared to

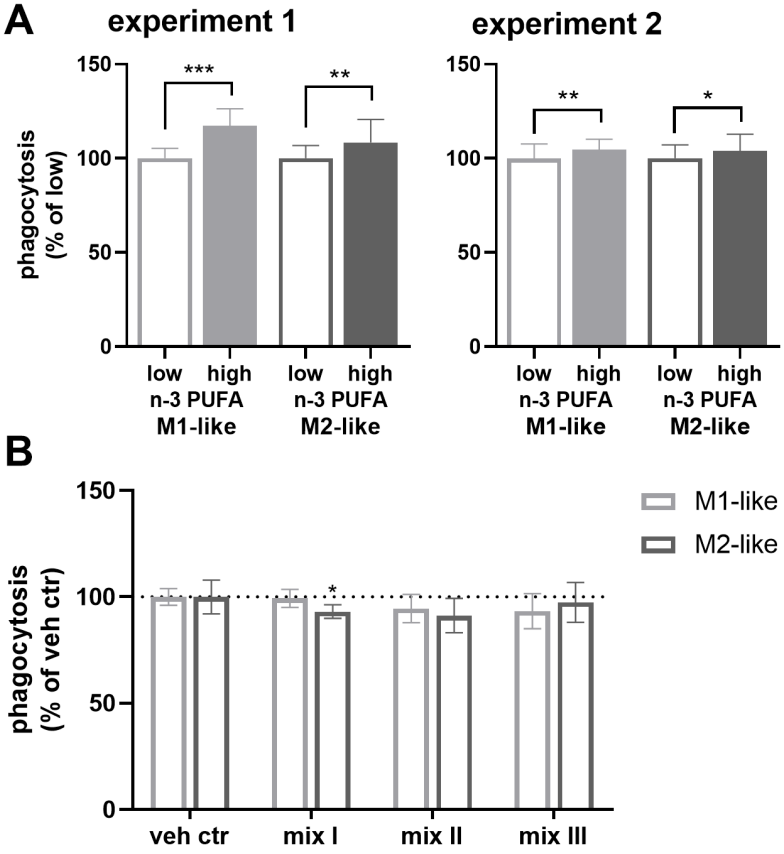

Figure 4.4: n–3 PUFA supplementation, but not the n–3 PUFA derived oxylipins increase phagocytosis in macrophages. A) After isolation of monocytes, cells were differentiated into macrophages and supplemented using medium with 5% (*v/v*) plasma pool 1 (low n-3 PUFA) medium containing plasma pool 1, 10.3 μM DHA (>99%) and 5.5 μM EPA (>99%) (high n-3 PUFA) for 2 days. Shown is phagocytosis as % change from the non-supplemented cells (low). Summarized results are shown as mean ± SD for $n = 26$ (experiment 1) or $n = 58-60$ replicates (experiment 2) from a pool of 4 subjects for each supplementation experiment C.3. B) Non-supplemented macrophages were incubated with 300 nM oxylipins (mix I: 5-HEPE, 4-HDHA, 7-HDHA; mix II: 12(*S*)-, 15(*S*)-HEPE, 14(*S*)-, 17(*S*)-HDHA; mix III: 17(18)-EpETE, 19(20)-EpDPE, Table C.4) or 0.1% DMSO (vehicle control) for 1 h. Summarized results are shown as % change from vehicle control as mean ± SD for $n = 9-10$ replicates from a pool of 4 subjects (Figure C.4). Statistical analysis was performed by 1-way ANOVA followed by A) Sidak's or B) Dunnett's multiple comparisons test. Differences from vehicle control were considered significant at p values ≤ 0.5 (*), ≤ 0.01 (**) or ≤ 0.001 (***).

the M2-like phenotype. This can be explained by the fact that this enzyme is not abundant in the M1-like phenotype, but strongly expressed in the M2-like phenotype [25, 30]. Di- and multihydroxy-PUFA were not detected in the macrophages. This differs from previous studies [23, 43], and could be explained by a sample preparation using 50% (*v/v*) MeOH for dissolving the cell pellet which inhibits enzyme activity and prevents artificial formation of 15-LOX products during sample preparation.

Overall, the FA pattern of monocytes shifted from one comparable to subjects with a typical Western diet to one comparable to subjects with a high n–3 PUFA status. The results of both supplementation experiments are highly comparable which demonstrates the reproducibility of the *ex vivo* supplementation strategy.

In both independent supplementation experiments phagocytosis was increased in the n–3 PUFA supplemented macrophages (Figure 4.4A, Figure C.3). This is consistent with cell culture experiments showing that membrane FA composition is associated with altered phagocytic activity [10, 20, 44]. However, nutrition studies in humans led to conflicting results: Whereas some studies reported an increase of phagocytosis by n–3 PUFA supplementation [17, 45, 46], most studies found no effect [47–51]. Of note, results are difficult to compare as doses, duration and PUFA used for supplementation as well as the method of measuring phagocytosis are highly different between the studies. The finding that n–3 PUFA have no effect in nutrition studies could be related to the fact that studies included all kinds of subjects, with low and high n–3 PUFA status. However, consistent with their role as essential food ingredient, there is growing evidence that only subjects with a low n–3 PUFA status benefit from a supplementation with n–3 PUFA [52–54].

Our results support this finding as only subjects are included with a low n–3 PUFA status, and PUFA profile and immune functions of macrophages are altered by supplementation.

The mechanisms of action by which n–3 PUFA influence phagocytosis are not fully understood: In addition to changes in membrane fluidity [55] or altered gene expression [56], it is hypothesized that the shift of the lipid mediator pattern following n–3 PUFA supplementation contributes to the increase in phagocytosis [22]. In order to investigate the impact of lipid mediators, n–3 PUFA derived oxylipins which were increased following n–3 PUFA supplementation (Figure 4.3) were tested for their impact on phagocytosis (Table C.4): 5-HEPE, 4- and 7-HDHA formed by 5-LOX activity and/or autoxidation [57, 58] (mix I), 12-, 15-HEPE and 14-, 17-HDHA by 15-LOX activity [59–61] (and autoxidation) (mix II) and the epoxy-PUFA 17(18)-EpETE and 19(20)-EpDPE (mix III), which are formed by cytochrome P450 monooxygenases [62], but the increase here is caused by impurities in the PUFA used for supplementation [23]. Of note, the latter are partly converted into the corresponding *vic* dihydroxy-PUFA (Table C.5), presumably by conversion by soluble epoxide hydrolase activity. Using a relatively high concentration (300 nM) of each of the oxylipins did not impact phagocytosis (Figure 4.4, Figure C.4) suggesting that these n–3 PUFA derived oxylipins (alone) are not the reason for the increased phagocytosis following n–3 PUFA supplementation.

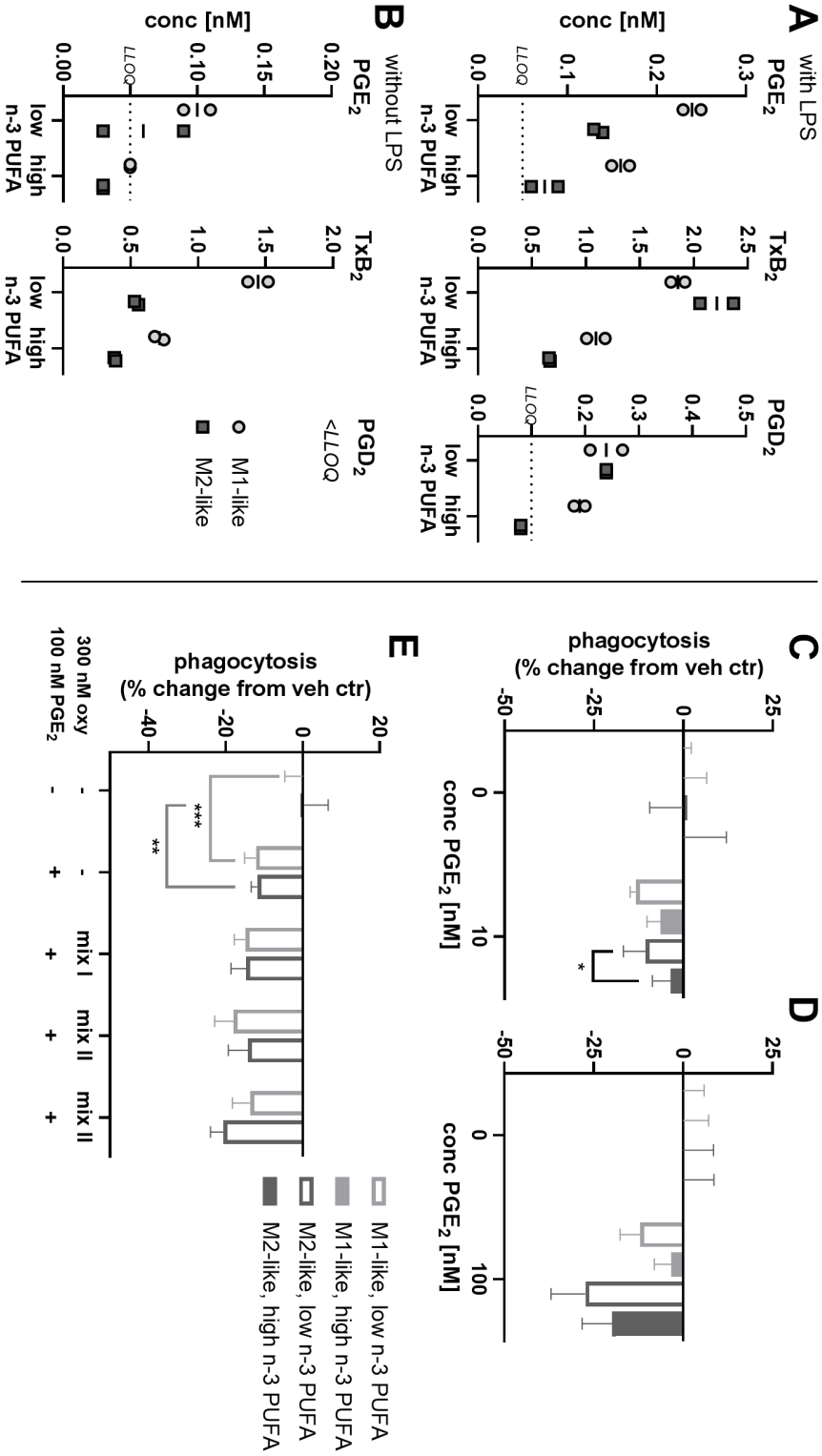
A
with LPS
conc [nM]
PGE2
TxB2
PGD2
LLOQ
low high
n-3 PUFA
B
without LPS
conc [nM]
PGE2
TxB2
PGD2
<LLOQ
M1-like
M2-like
C
phagocytosis
(% change from veh ctr)
conc PGE2 [nM]
D
phagocytosis
(% change from veh ctr)
conc PGE2 [nM]
E
phagocytosis
(% change from veh ctr)
300 nM oxy
100 nM PGE2
mix I
mix II
M1-like, low n-3 PUFA
M1-like, high n-3 PUFA
M2-like, low n-3 PUFA
M2-like, high n-3 PUFA

Figure 4.5: (previous page) n–3 PUFA supplementation reduces inhibitory effect of PGE_2 on phagocytosis. Macrophages were supplemented using medium with 5% (v/v) plasma pool 1 (low n-3 PUFA) or medium containing plasma pool 1, 10.3 μM DHA (>99%) and 5.5 μM EPA (>99%) (high n-3 PUFA) for 2 days. Cells were pooled and transferred into 96-well plates (50,000 cells per well) and incubated A) with 0.1 μg/mL LPS or B) without LPS for 4 h. Non-esterified oxylipin concentrations were quantified in the supernatants by means of LC-MS/MS. Concentrations of COX products are shown for one representative experiment. (C-E) For phagocytosis, cells were preincubated with C) 10 nM or D) 100 nM PGE_2 for 1 h. Results of one representative experiment are shown as mean ± SD for $n = 4-5$ replicates from a pool of 4 subjects. E) Non-supplemented cells were preincubated with 100 nM PGE_2 and 300 nM oxylipins (mix I: 5-HEPE, 4-HDHA, 7-HDHA; mix II: 12(*S*)-, 15(*S*)-HEPE, 14(*S*)-, 17(*S*)-HDHA; mix III: 17(18)-EpETE, 19(20)-EpDPE, Table C.4) or 0.1% DMSO (vehicle control) for 1 h. Shown is phagocytosis as % change from vehicle control as mean ± SD for $n = 3-10$ replicates from a pool of 4 subjects. Statistical analysis was performed by 1-way ANOVA followed by Sidak's multiple comparison test. Differences from vehicle control were considered significant at p values ≤0.5 (*), ≤0.01 (**) or ≤0.001 (***). LLOQ, lower limit of quantification.

n–3 PUFA supplementation resulted in decreased levels of COX products such as prostaglandins in the macrophages (Figure 4.3) and supernatants (Figure 4.5A, B). A reduced formation of prostaglandins, especially PGE_2, could contribute to the increase of phagocytosis by supplementation, as PGE_2 inhibits phagocytosis [26]. Moreover, when high amounts of PGE_2 are added to the cells, phagocytosis is less inhibited in the n–3 PUFA supplemented cells (Figure 4.5C, D). This could indicate that PGE_2 acts differently on phagocytosis when combined with n–3 PUFA derived oxylipins. However, when testing n–3 PUFA derived oxylipins which were increased by n–3 PUFA supplementation (Table C.4), the oxylipins had no impact on the inhibitory effect of PGE_2 on phagocytosis (Figure 4.5E, Figure C.5, Table C.6). Recent studies have shown that 7(*S*),17(*S*)-DiHDHA (RvD5) affects the EP4 receptor through allosteric modulation, leading to a switch in G-protein coupling from Gs to Gi [63]. This changes downstream signaling and thus, reverses the inhibition of phagocytosis by PGE2 in murine macrophages [63]. This fits to our

results that PGE_2 inhibits phagocytosis less in the n–3 PUFA supplemented cells. However, 7(*S*),17(*S*)-DiHDHA, which is discussed to modulate the EP4 receptor, was not detected in the macrophages. Similarly, other multihydroxy-PUFA such as RvE2 and PD1, which were shown to increase phagocytosis in human macrophages [14, 15], could not be detected in the cells. This is consistent with several reports indicating that these compounds do not occur in macrophages [23, 43, 64]. Moreover, formation and signaling of these multihydroxy-PUFA in human macrophages remains controversial [21], indicating that these play only a minor role in increasing the phagocytosis following a n–3 PUFA supplementation. However, an increased phagocytosis is beneficial allowing an efficient defense against pathogens and the rapid clearance of apoptotic cells, both key processes contributing to tissue repair and homeostasis [13, 65].

Our findings support, that subjects with a low n–3 PUFA status – which is typical for people in Europe and the US when having a Western diet – benefit from a n–3 PUFA supplementation altering the PUFA profile and phagocytosis in the macrophages. Additionally, levels of pro-inflammatory prostaglandins are reduced which contributes to the beneficial effect of n–3 PUFA. However, further research is needed elucidating the modes of action by which n–3 PUFA and derived oxylipins impact phagocytosis and immune functions in humans.

4.5 Conclusion

Increasing the n–3 PUFA status of primary human macrophages elevates phagocytosis, indicating an improved potential for the clearance of pathogens and cell debris. This beneficial effect of an n–3 PUFA supplementation could partly be explained by the reduced formation of pro-inflammatory prostaglandins such as PGE_2. The n–3 PUFA derived oxylipins, increased by n–3 PUFA supplementation, alone had no effect. Further studies are needed to understand the mechanisms by which n–3 PUFA elevate phagocytosis, involving the investigation of membrane fluidity, lipid rafts and membrane-associated proteins.

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5 Concluding Remarks and Future Perspectives

Polyunsaturated fatty acids (PUFA) are important components of the cell membranes involved in many physiological processes. They affect membrane fluidity, membrane-associated proteins, the formation of lipid rafts and are precursors for the formation of eicosanoids and other oxylipins [1]. Several oxylipins are lipid mediators playing a key role in the regulation of inflammatory and immune processes [2]. Consequently, oxylipins are likely to contribute to the effects of n–3 PUFA on physiological and immune functions.

This thesis aimed to develop a strategy for the supplementation of human immune cells to investigate the effects of n–3 PUFA and derived oxylipins on immune functions such as phagocytosis. The *ex vivo* supplementation strategy as well as the phagocytosis assay were carefully optimized enabling reliable and reproducible experiments. This allows the mechanistic investigation of n–3 PUFA and derived oxylipins on phagocytosis and contributes to a better understanding of the effects of n–3 PUFA on immune functions and health.

Chapter 2 describes the development of the *ex vivo* supplementation strategy with n–3 PUFA in primary human macrophages. Cell culture experiments allow the investigation of immune cells under strictly controlled conditions, but are challenging, e.g., in terms of autoxidation. For this purpose, key parameters for all steps of the process such as selection of plasma or PUFA for supplementation,

were carefully selected and monitored. This is of importance because PUFA are prone to autoxidation leading to the formation of oxylipins and several of those are biological active lipid mediators, even in concentrations in the low nM range. Autoxidation cannot be completely avoided, but controlled and kept to a minimum. Following the key parameters defined for each step of the supplementation process keeps autoxidative formation of oxylipins low and thus, enables the investigation of the effects of n–3 PUFA and not of the artificially formed oxylipins. PUFA pattern of macrophages is shifted by the *ex vivo* supplementation strategy comparable to human intervention studies being a helpful tool for the mechanistic investigation of the effects of n–3 PUFA on immune cells.

For investigation of the modulation of immune processes by n–3 PUFA and oxylipins, a phagocytosis assay was established in primary human macrophages in chapter 3. Using fluorescence-labeled polystyrene spheres (latex beads) and a 96-well plate format enables a simple and cost-efficient readout. Using the optimized phagocytosis assay for primary human macrophages, LPS increased phagocytosis in M2-like, but not in M1-like macrophages. The different prostaglandin E2 (PGE_2) levels as a result of different cyclooxygenase (COX)-2 activities in the macrophage phenotypes after LPS stimulus contribute to the differential effect of LPS on phagocytosis. LPS induces a variety of changes in the cells besides induction of COX-2 such as cytokines [3], number of specific surface receptors [4] and the cytoskeletal network [4]. However, the mode of action by which LPS elevates phagocytosis in human macrophages remain to be elucidated. Besides the COX branch of the ARA cascade, the role of other branches, such as lipoxygenases (LOX) and cytochrome P450 monooxygenases (CYP) in regulating phagocytosis warrants fur-

ther investigation. For an 'inflammatory' subset of macrophages the presence of the CYP enzymes 1B1, 2E1 and 2B6/7 was described, whereas 'anti-inflammatory' macrophages were reported to have 1B1 and to some extent 2B6/7 [5]. However, cytokines used for polarization into the different macrophage phenotypes impact the expression, abundance and activity of these enzymes. Interestingly, products of 15-LOX enzyme activity, especially multihydroxy-PUFA, are considered anti-inflammatory and have been shown to modulate phagocytosis [6, 7]. However, occurrence, formation and signaling pathways of these oxylipins in human immune cells remain controversial [8], but those oxylipins could be a starting point for the development of chemical probes serving as positive controls for phagocytosis. This is of importance because substances which are suitable positive controls for phagocytosis are scarce in contrast to the well-known and characterized inhibitors of phagocytosis interfering with actin polymerization such as cytochalasin D and latrunculin A [9, 10]. Moreover, for the investigation of phagocytosis many different techniques were developed including flow cytometry and the use of confocal fluorescence microscopes [11, 12] making results of different studies difficult to compare. Thus, finding more positive controls for the elevation of phagocytosis would be highly beneficial for optimization and comparability of different phagocytosis assays.

In order to investigate the impact of n–3 PUFA on phagocytosis, macrophages were supplemented with n–3 PUFA using the *ex vivo* supplementation strategy and phagocytosis was examined using the optimized phagocytosis assay (chapter 4). Supplementation conditions were chosen enabling a shift in the PUFA pattern of the cells comparable to human nutrition studies. Accompanied by the changes

in PUFA and oxylipin pattern, phagocytosis was elevated in the n–3 PUFA supplemented macrophages. PGE_2 formation was suppressed by n–3 PUFA supplementation which could contribute to the elevation of phagocytosis. Further research is needed elucidating the role of membrane fluidity and membrane-associated proteins in the regulation of phagocytosis by n–3 PUFA and derived oxylipins.

Additionally, the inhibitory effect of PGE_2 on phagocytosis was reduced in the n–3 PUFA supplemented macrophages suggesting a combined effect of PGE_2 and n–3 PUFA derived oxylipins. In line with this, it was shown that several DHA derived oxylipins, e.g., 7(*S*),17(*S*)-DiHDHA (Resolvin D5, RvD5), affect the EP4 receptor through allosteric modulation, thereby reversing the inhibition of phagocytosis by PGE_2 in murine macrophages [13]. However, the oxylipins which were described to allosterically modulate the EP4 receptor were not detected in the macrophages after n–3 PUFA supplementation. Of note, this differs from previous studies finding the formation of some dihydroxy-PUFA such as RvD5 in the macrophages [14, 15], and could be explained by the sample preparation using 50% (*v*/*v*) MeOH for dissolving the cell pellet. This inhibits enzyme activity during sample preparation and thus, prevents artificial formation of 15-LOX products.

DHA and EPA derived oxylipins which were increased by n–3 PUFA supplementation did not affect phagocytosis in human macrophages indicating they have only limited impact on phagocytosis following a n–3 PUFA supplementation. In order to investigate if phagocytosis can be affected through allosteric modulation of EP4 similarly in human macrophages, RvD5 in combination with PGE_2 was tested using the phagocytosis assay. First results indicate the potential of RvD5 reversing the inhibitory effect of PGE_2 on phagocytosis at least in M2-like macrophages,

indicating an allosteric modulation of EP4 by RvD5 which is in line with the findings in mouse macrophages. However, further research is needed elucidating the allosteric modulation of the EP4 receptor in human immune cells by n–3 PUFA derived oxylipins.

Using the developed *ex vivo* supplementation strategy and the phagocytosis assay, this thesis enabled new insights into the modulation of phagocytosis by n–3 PUFA and their oxylipins in primary human macrophages. Considering the changes in the PUFA and oxylipin pattern of macrophages using the *ex vivo* n–3 PUFA supplementation strategy, results are in agreement with supplementation studies in humans and animals [16–20]: Levels of n–6 PUFA are decreased in favor of n–3 PUFA, while MUFA and SFA remain unchanged. This is accompanied by decreased levels of n–6 PUFA derived oxylipins, especially pro-inflammatory prostaglandins such as PGE_2, while n–3 PUFA derived oxylipins are increased. For M2-like macrophages especially products of the 15-LOX activity are elevated which could be explained by the fact that this macrophage phenotype has a high abundance and activity of 15-LOX [21, 22]. Moreover, to which extent PUFA and oxylipin pattern are shifted towards n–3 PUFA in the macrophages, can be easily adjusted using different doses and durations for the n–3 PUFA supplementation in the *ex vivo* supplementation strategy.

Regarding the effects of n–3 PUFA and oxylipins on immune functions such as modulation of cytokines or phagocytosis, the results of cell culture and *in vivo* studies are inconclusive: While cell culture studies reported a downregulation of pro-inflammatory genes in immune cells following n–3 PUFA supplementation [23–25], results of cytokine levels in monocytes from n–3 PUFA supplemented

subjects are inconsistent (lower or no changes) [26]. Similarly, for phagocytosis cell culture experiments showed a link between membrane PUFA composition and phagocytic activity [27–29], which was not supported by the majority of human nutrition studies finding no effect of n–3 PUFA supplementation on phagocytosis [30–34]. Of note, supplementation conditions as well as the method for analyzing phagocytosis in the immune cells differ between the studies, making comparison of the results challenging.

The developed *ex vivo* supplementation strategy offers a new approach investigating the effect and mode of action of n–3 PUFA on immune functions combining primary human cells with the ability to use highly controlled conditions for supplementation of the macrophages. Shifting the n–3 PUFA status of cells from low to high by *ex vivo* n–3 PUFA supplementation, results in elevated phagocytosis in the macrophages. This indicates that particularly subjects with a low n–3 PUFA status could benefit from the supplementation, allowing an efficient clearance of pathogens and cell debris. This finding is supported by studies showing that only subjects with a low n–3 PUFA status benefit from a supplementation with n–3 PUFA [35–37]. Moreover, this could explain why phagocytosis is not elevated in most human nutrition studies, because they include subjects with both, low and high n–3 PUFA status.

The shift in the PUFA and oxylipin pattern following n–3 PUFA supplementation has been shown to be one mechanism of action by which phagocytosis is modulated: By increasing the levels of n–3 PUFA, n–6 PUFA levels are reduced and thus, formation of n–6 PUFA derived oxylipins is suppressed. This in turn leads to lower concentrations of pro-inflammatory PGE_2, which is an inhibitor of phagocytosis via EP4 signaling in human macrophages. Hence, reducing the levels of inhibitory PGE_2, is a mechanistic explanation for the elevated phagocytosis caused by n–3 PUFA supplementation.

Overall, this thesis investigated the effects of n–3 PUFA and oxylipins on phagocytosis in human macrophages contributing to a better understanding of the impact of diet on immune functions and health.

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6 Summary

Several oxylipins are lipid mediators formed by oxidation of polyunsaturated fatty acids (PUFA). Both, oxylipins and PUFA are involved in various physiological functions such as regulation of membrane functions, blood coagulation or immunity. There is growing evidence that an increased intake of n–3 PUFA lowers the risk for cardiovascular events and impacts inflammatory diseases. Moreover, increasing the intake of n–3 PUFA lowers the formation of oxylipins derived from n–6 PUFA whereas n–3 PUFA derived oxylipins are increased. Of those oxylipins, especially the multihydroxy-oxylipins derived from n–3 PUFA, are discussed to have anti-inflammatory properties. Consequently, oxylipins are regarded one important mechanism by which PUFA affect immune functions.

Human intervention studies or clinical trials are the gold standard for the investigation of effects and modes of action of n–3 PUFA. However, results regarding the effects of n–3 PUFA on immune functions are inconclusive, presumably because subjects with both, low and high n–3 PUFA status were included. However, there is growing evidence that only subjects with a low n–3 PUFA benefit from the supplementation with n–3 PUFA [1–3]. Additionally, carrying out these studies is challenging and elaborate. For investigation of the effects of n–3 PUFA and derived oxylipins on immune functions without the need for human intervention studies, an *ex vivo* supplementation strategy was developed in chapter 2.

Using the strategy developed, primary human macrophages can be supplemented with n–3 PUFA altering FA and oxylipin profile comparable to nutrition studies. Identification and monitoring of key parameters addressing challenges such as autoxidation of PUFA during the supplementation process, ensures reliability and reproducibility of the supplementation strategy. Thus, the developed supplementation strategy is a helpful tool for mechanistic investigation of n–3 PUFA and oxylipins in primary human immune cells.

For a deeper insight into the regulation of immune functions by n–3 PUFA and oxylipins, phagocytosis was investigated in primary human macrophages in chapter 3. For this purpose, a phagocytosis assay was established and several parameters such as cell number, duration of phagocytosis, used fluorescence-labeled beads and removing of non-ingested beads were optimized. Our results indicate that the cyclooxygenase (COX) branch of the arachidonic acid (ARA) cascade is involved in the differential modulation of phagocytosis in M1- and M2-like macrophages by LPS: Following LPS stimulation, M1-like macrophages have a higher COX-2 abundance and activity compared to M2-like macrophages. This was accompanied by higher concentrations of the COX product prostaglandin E2 (PGE_2) in M1-like macrophages. PGE_2 inhibits phagocytosis via EP4 signaling which is a mechanistic explanation, why phagocytosis is not elevated in M1-like macrophages following LPS stimulus. This shows the importance of the link between the COX branch of the ARA cascade in the regulation of phagocytosis. Additionally, by inhibiting COX-2 activity, e.g., by COX inhibitors such as several nonsteroidal anti-inflammatory drugs (NSAID), formation of pro-inflammatory PGE_2 is suppressed, resulting in an elevated phagocytosis in both macrophage phenotypes following LPS stimulus.

This in turn limits inflammation and enables tissue restoration, highlighting the potential of the well-investigated anti-inflammatory COX inhibitors.

In order to better understand the role of n–3 PUFA and derived oxylipins in the regulation of phagocytosis, the *ex vivo* supplementation strategy and the optimized phagocytosis assay were combined in chapter 4. Phagocytosis was elevated following n–3 PUFA supplementation of macrophages comparable to human nutrition studies which indicates an improved potential for the clearance of pathogens and cell debris of the supplemented macrophages. PGE_2 formation was suppressed by n–3 PUFA supplementation which could contribute to the elevation of phagocytosis. However, n–3 PUFA derived oxylipins which were elevated by supplementation, appear to have only limited impact on phagocytosis.

Overall, this thesis provides a reliable approach for the supplementation of primary human macrophages with n–3 PUFA for the mechanistic investigation of n–3 PUFA and derived oxylipins. For a deeper insight into the mode of action of n–3 PUFA and derived oxylipins on immune functions, the modulation of phagocytosis was examined using an optimized phagocytosis assay in human macrophages. By changing the PUFA pattern of macrophages e.g., by n–3 PUFA supplementation, formation of pro-inflammatory n–6 PUFA derived oxylipins such as PGE_2 is suppressed, while levels of n–3 PUFA derived oxylipins are increased. This in one mechanism of action by which n–3 PUFA elevate phagocytosis and thus, impact immune functions. This highlights the potential of n–3 PUFA and oxylipins in the regulation of immune processes and helps to get a better understanding of the modulation of immune functions by diet.

A Appendix for Chapter 2

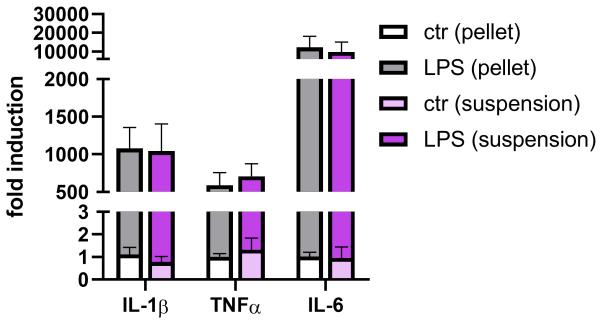

Figure A.1: Relative mRNA expression of macrophages using different sample preparation methods. Primary human monocytic cells were differentiated into macrophages using 10 ng/mL M-CSF for 7 days and additional 10 ng/mL IL-4 for the final 48 h. For LPS stimulation 1 µg/mL LPS was added 6 h before harvest. Cells were harvested by cold shock method and either stored as dry pellet (pellet) or resuspended in PBS, homogenized by sonication and stored as cell suspension in PBS (suspension) at -80 °C followed by mRNA analysis. Results are shown as mean ± SEM, n = 3 subjects.

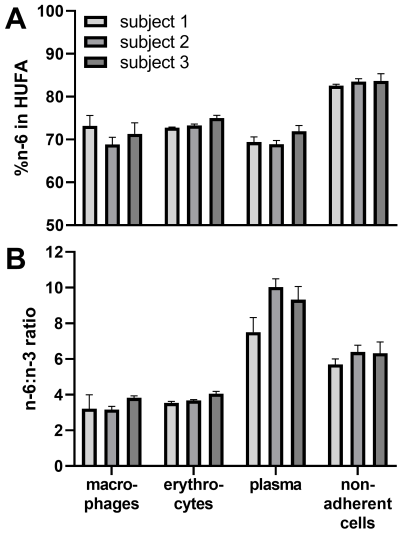

Figure A.2: (previous page) Characterization of FA status of three exemplarily human subjects based on A) %n–6 in HUFA and B) n–6:n–3 ratio of buffy coat fractions. Fractions from buffy coats of three subjects were collected: erythrocytes after dextran sedimentation, plasma after density gradient centrifugation and remaining non-adherent cells after monocytic cells getting adherent for $1-2$ h. Monocytic cells were differentiated with 10 ng/mL M-CSF for 7 days and additional 10 ng/mL IL-4 for final 48 h into M2-like macrophages. Cell pellets and plasma were analyzed for total fatty acid concentrations by LC-MS/MS. A) %n–6 in HUFA was calculated from total fatty acid concentrations of C20:3 n–6, C20:4 n–6, C22:4 n–6, C22:5 n–6, C20:3 n-9, C20:5 n–3, C22:5 n–3, C22:6 n–3 by LC-MS and B) n–6:n–3 ratio was calculated from C18:3 n–3, C18:2 n–6, C20:4 n–6, C20:5 n–3 and C22:6 n–3. Results are shown as $\pm$ SEM, $n = 3$.

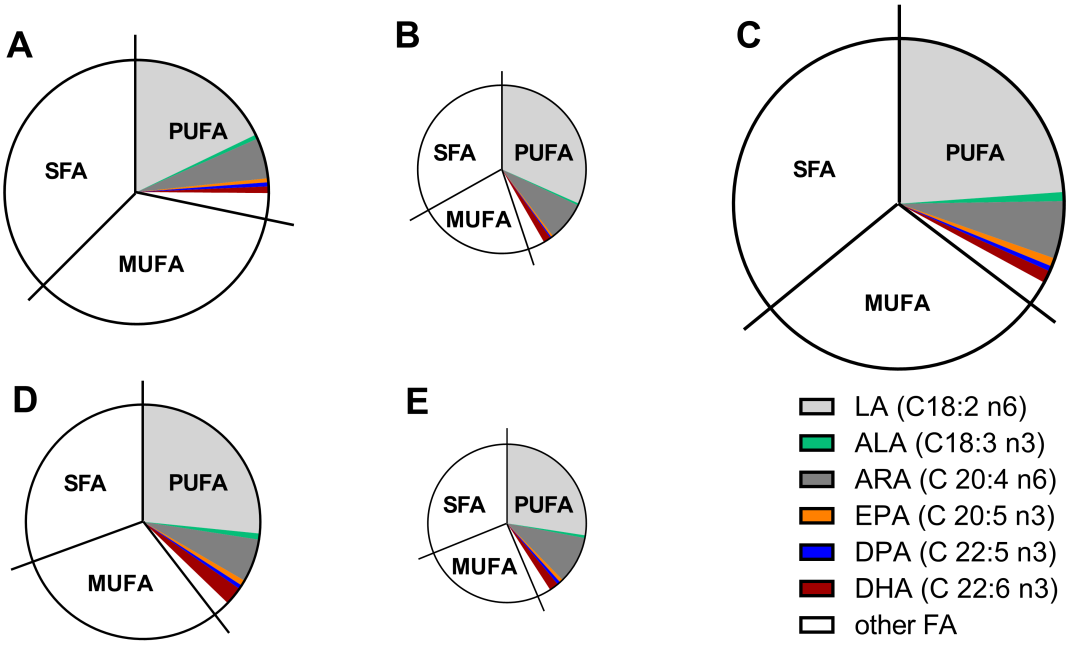

Figure A.3: Relative fatty acid profiles of human plasmas. Non-fasting plasma of five different subjects from a local blood donation center was analyzed for total FA concentrations by means of GC-FID. Sizes of the circles indicate total fatty acid concentrations relatively on plasma's C total fatty acid concentration (plasma A, 11.9 $\pm$ 1.3 mM, 80%; plasma B, 7.7 $\pm$ 0.1 mM, 52%; plasma C, 14.9 $\pm$ 1.1 mM, 100%; plasma D, 10.6 $\pm$ 0.3 mM, 71%; plasma E, 7.1 $\pm$ 0.4 mM, 48%).

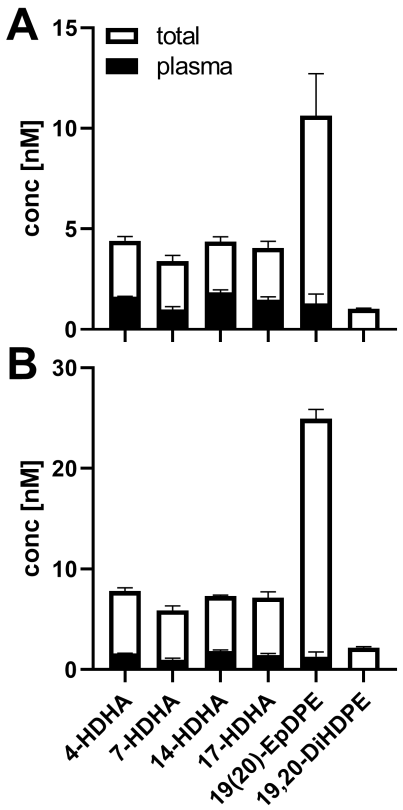

Figure A.4: Human plasma DHA derived oxylipins in A) low and B) high supplemented medium. A) Low and B) high supplemented medium and plasma used were analyzed for total oxylipin concentrations. Plasma oxylipin concentrations were subtracted proportionally from those of the medium. Results are shown as mean ± SD, $n = 3$.

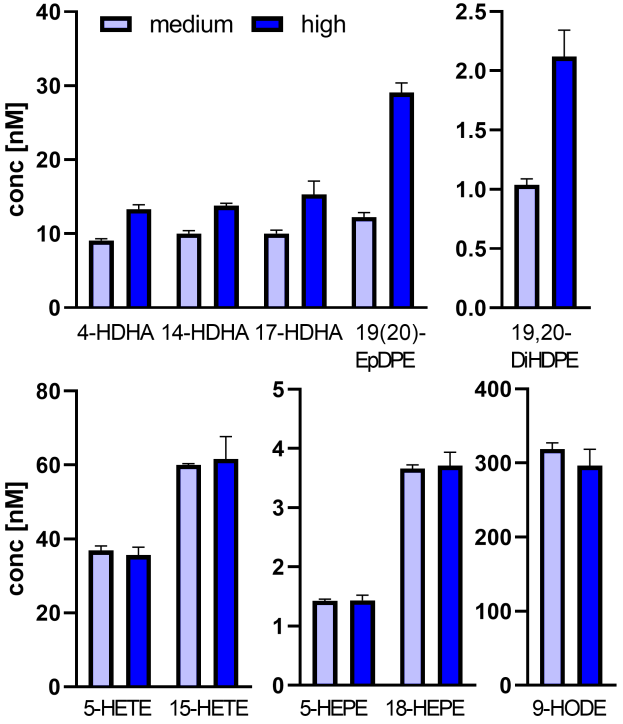

Figure A.5: Oxylipin levels of supplemented media after storage at 4 °C for one day. Medium and high supplemented medium were stored in the fridge (4 °C) for one day and analyzed for total oxylipin concentrations by means LC-MS/MS. Shown are representative oxylipins derived from ARA, DHA and ALA. Results are shown as mean ± SD, $n = 3$.

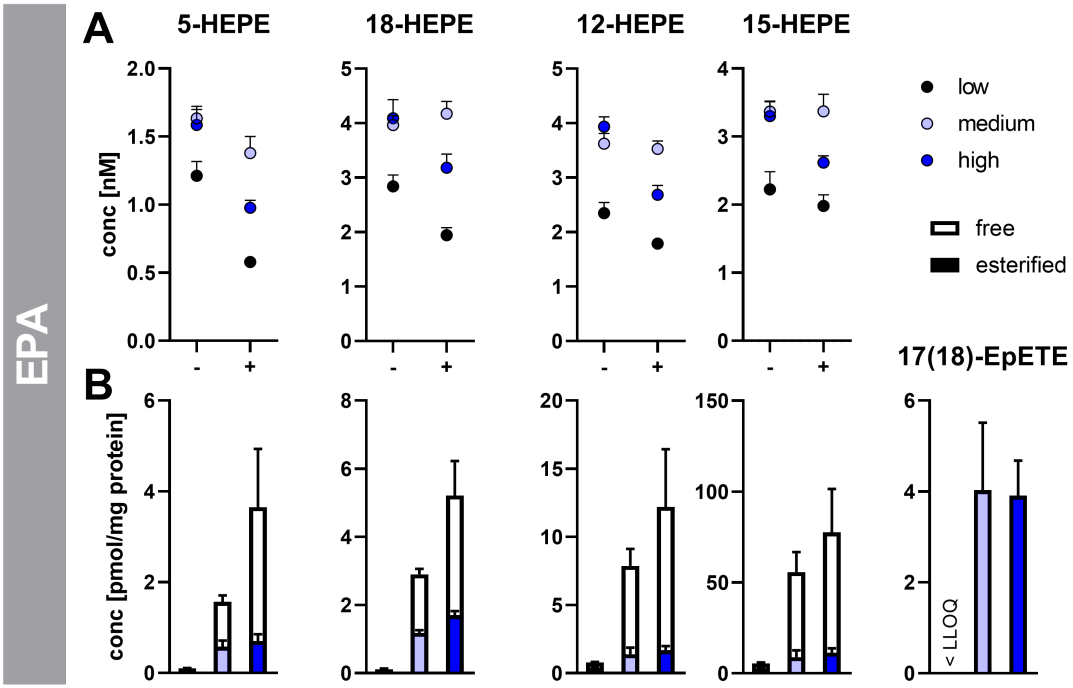

Figure A.6: Oxylipin levels of supplemented media after storage at 4 °C for one day. Medium and high supplemented medium were stored in the fridge (4 °C) for one day and analyzed for total oxylipin concentrations by means LC-MS/MS. Shown are representative oxylipins derived from ARA, DHA and ALA. Results are shown as mean $\pm$ SD, $n = 3$.

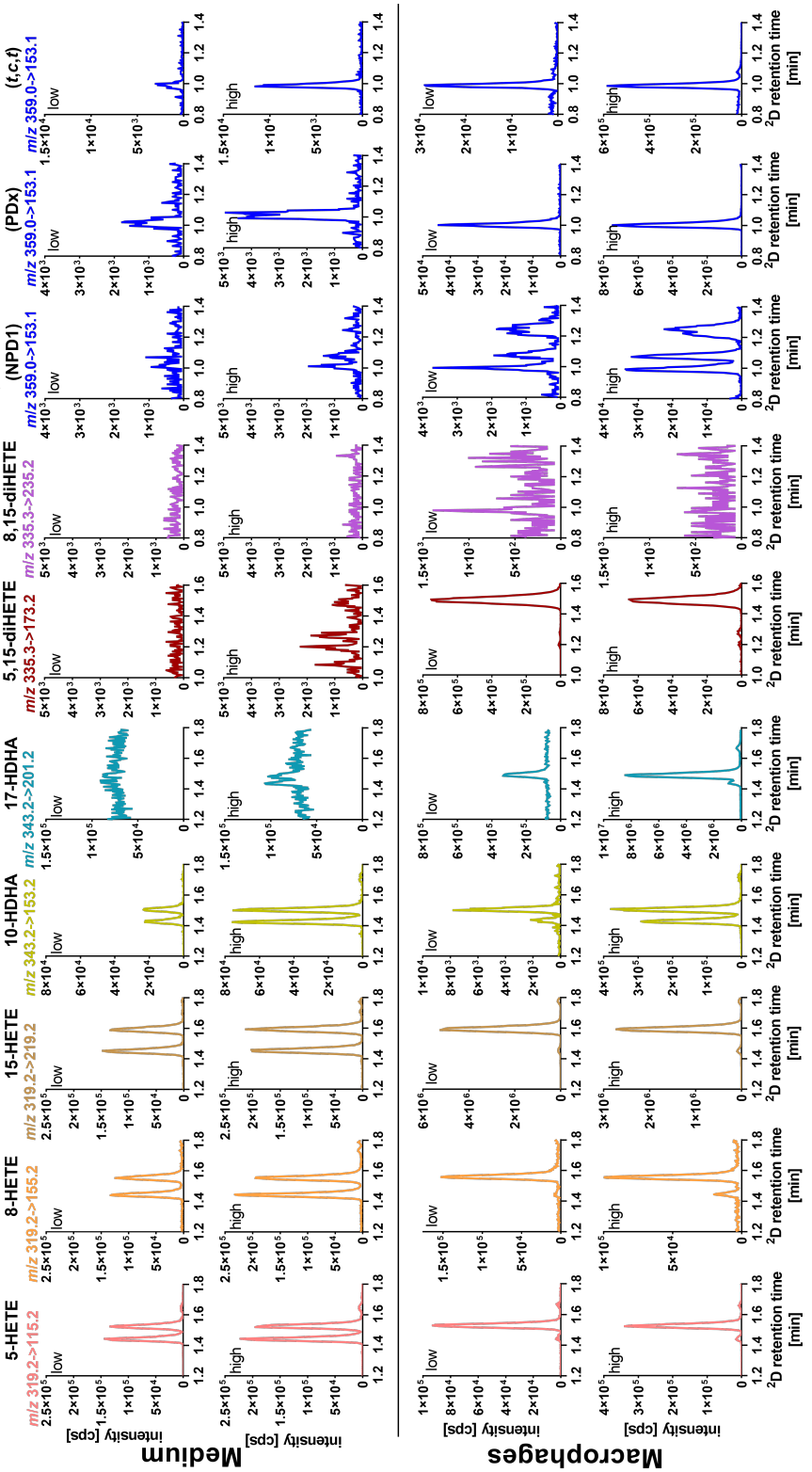

Figure A.7: Chiral LC analysis of oxylipins in medium and macrophages. Media were prepared by adding no (low, non-supplemented control) or 45 µM (high) DHA preparation (>99%) to cell culture medium. Primary human macrophages were supplemented using the different media for 2 days. Shown are the chromatograms of the chiral LC analysis of the indicated oxylipins in the cells and media stored at 37 °C for two days.

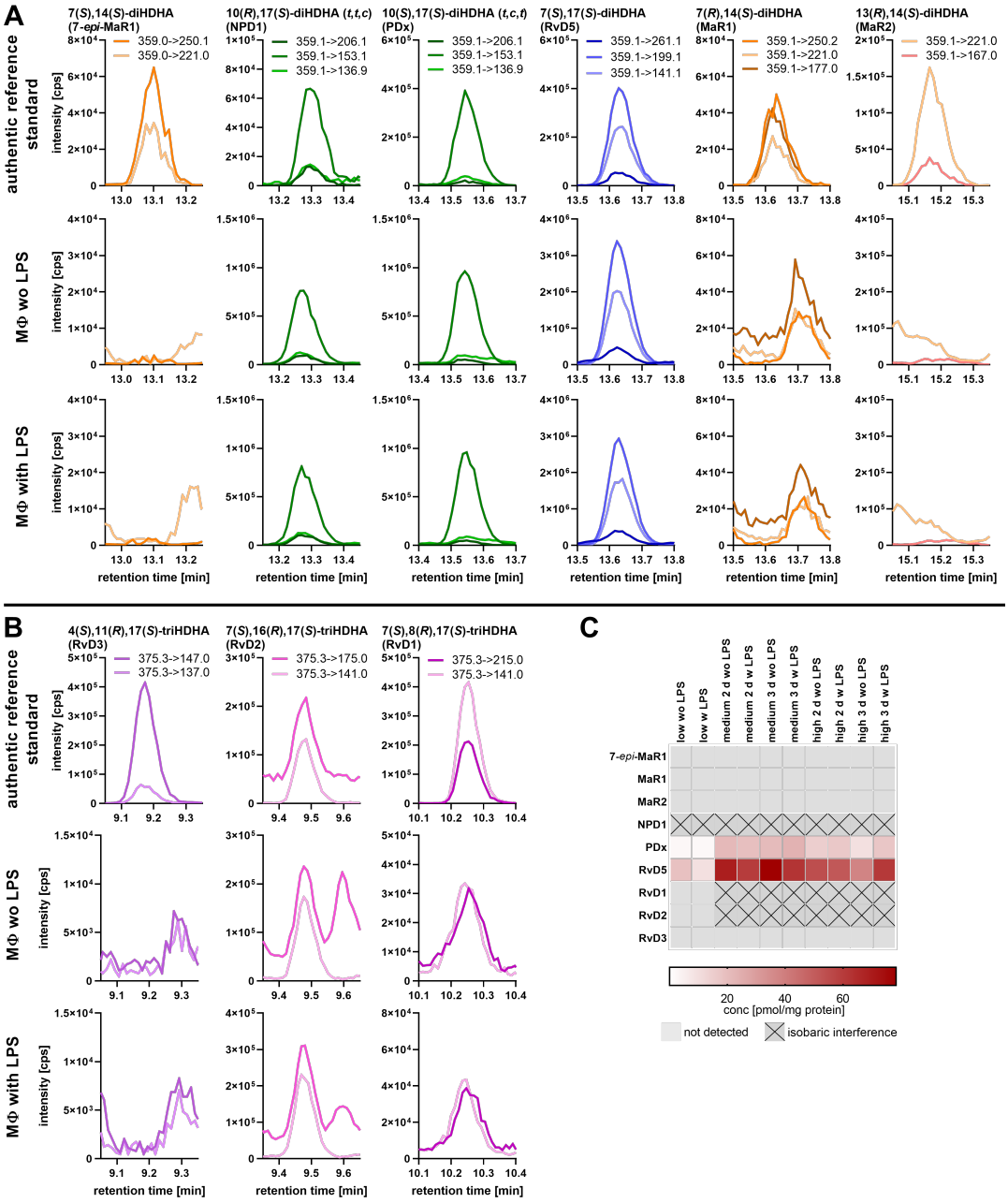
A
authentic reference standard
MΦ wo LPS
MΦ with LPS
intensity [cps]
retention time [min]
7(S),14(S)-diHDHA (7-epi-MaR1)
359.0->250.1
359.0->221.0
10(R),17(S)-diHDHA (t,t,c) (NPD1)
359.1->206.1
359.1->153.1
359.1->136.9
10(S),17(S)-diHDHA (t,c,t) (PDx)
359.1->206.1
359.1->153.1
359.1->136.9
7(S),17(S)-diHDHA (RvD5)
359.1->261.1
359.1->199.1
359.1->141.1
7(R),14(S)-diHDHA (MaR1)
359.1->250.2
359.1->221.0
359.1->177.0
13(R),14(S)-diHDHA (MaR2)
359.1->221.0
359.1->167.0
B
4(S),11(R),17(S)-triHDHA (RvD3)
375.3->147.0
375.3->137.0
7(S),16(R),17(S)-triHDHA (RvD2)
375.3->175.0
375.3->141.0
7(S),8(R),17(S)-triHDHA (RvD1)
375.3->215.0
375.3->141.0
C
low wo LPS
low w LPS
medium 2 d wo LPS
medium 2 d w LPS
medium 3 d wo LPS
medium 3 d w LPS
high 2 d wo LPS
high 2 d w LPS
high 3 d wo LPS
high 3 d w LPS
7-epi-MaR1
MaR1
MaR2
NPD1
PDx
RvD5
RvD1
RvD2
RvD3
20
40
60
conc [pmol/mg protein]
not detected
isobaric interference

Figure A.8: (previous page) Analysis of di- and tri-OH-DHA oxylipins in DHA-supplemented macrophages. Primary human macrophages were supplemented using media containing 45 μM (high) DHA for 2 days without (wo) or with LPS stimulation (1 μg/mL) for 6 h. Shown are the MRM chromatograms (2−3 transitions) of A) di- and B) tri-OH-DHA oxylipins in the authentic reference standard and the macrophages (MΦ) without (wo) and with (w) LPS stimulation of one exemplarily subject and C) overview about analyzed di- and tri-OH-DHA in the macrophages.

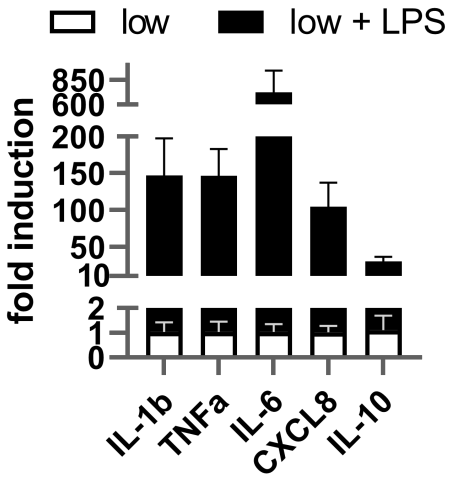

Figure A.9: Relative mRNA induction of pro-inflammatory cytokines after LPS stimulus. Primary human monocytic cells were differentiated into macrophages using 10 ng/mL M-CSF for 7 days and additional 10 ng/mL IL-4 for the final 48 h. For LPS stimulation 1 μg/mL LPS was added into the medium 6 h before harvest. After harvest, cells were analyzed for mRNA levels by qPCR. Results are shown as mean ± SEM, $n = 3$ subjects.

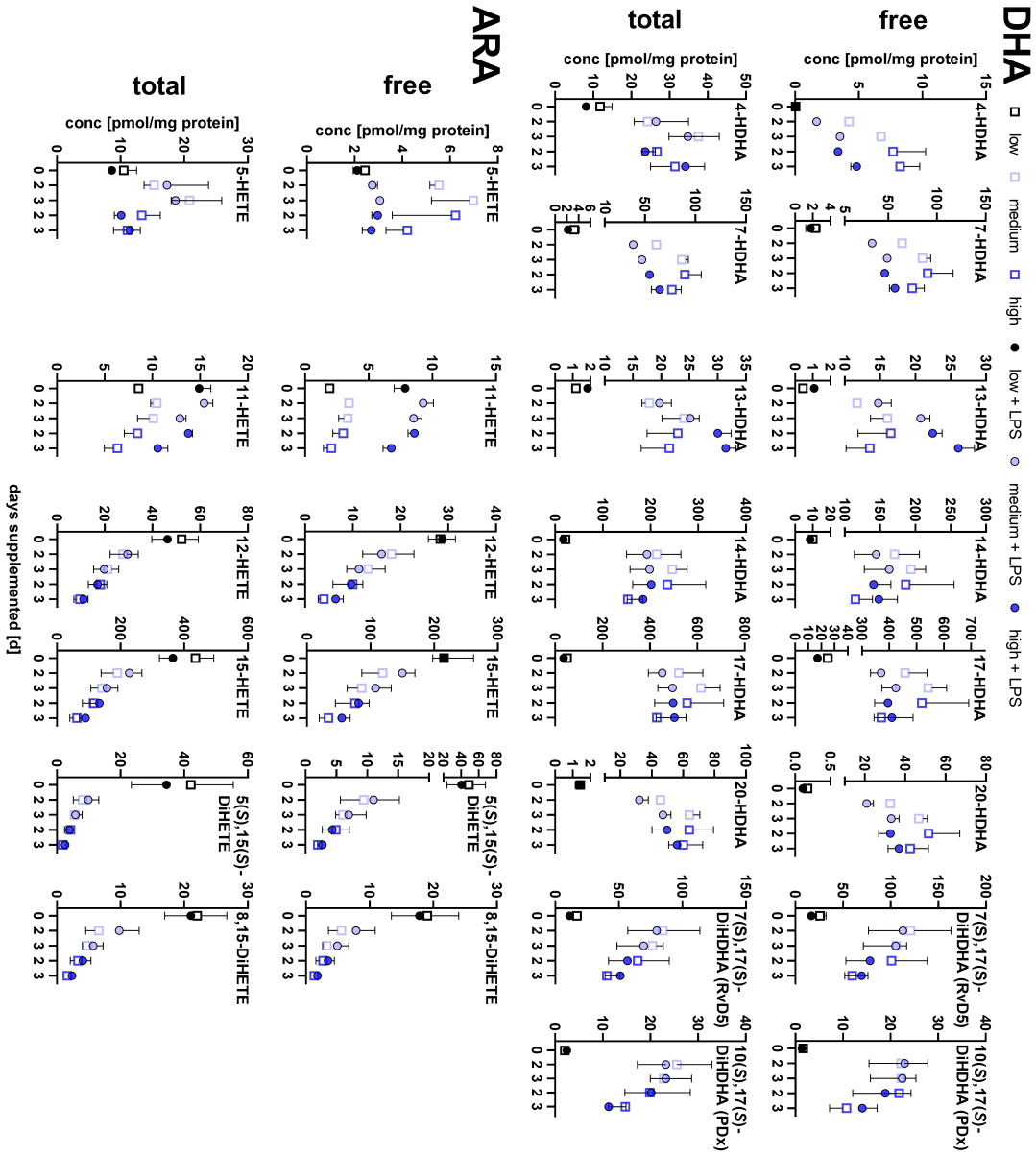

Figure A.10: Free and total oxylipins in DHA-supplemented macrophages with and without LPS stimulus. Primary human monocytic cells were differentiated into macrophages using 10 ng/mL M-CSF for 7 days and additional 10 ng/mL IL-4 for the final 48 h. For LPS stimulation 1 µg/mL LPS was added into the medium 6 h before harvest. After harvest, cells were analyzed for total and free oxylipins by means of LC-MS/MS. Results are shown as mean ± SEM, $n = 3$ subjects.

Table A.1: Primer sequences used for quantitative real-time PCR.

target	primer sequences
CCL18	forward: 5'- CAA ACT GGG CAA GGA GAT CTG-3' reverse: 5'-GGC CCA GGT GTT TCA TAT AAT TCT-3'
CXCL8	forward: 5'-GAT CCA CAA GTC CTT GTT CCA-3' reverse: 5'-GCT TCC ACA TGT CCT CAC AA-3'
IL1B	forward: 5'-GGG CCT CAA GGA AAA GAA TC-3' reverse: 5'-TTC TGC TTG AGA GGT GCT GA-3'
IL6	forward: 5'-TAC CCC CAG GA AAG ATT CC-3' reverse: 5'-TTT TCT GCC AGT GCC TCT TT-3'
IL10	forward: 5'-GCC TAA CAT GCT TCG AGA TC-3' reverse: 5'-TGA TGT CTG GGT CTT GGT TC-3'
MRC1	forward: 5'-AGC CAA CAC CAG CTC CTC AAG-3' reverse: 5'-AAC GCT CGC GCA TTG TCC-3'
RPL13A	forward: 5'-CCT GGA GGA GAA GAG GAA AGA GA-3' reverse: 5'-TTG AGG ACC TCT GTG TAT TTG TCA A-3'
SDHA	forward: 5'-ACG TCA CGA AGG AGC CGA TCC-3' reverse: 5'-ATG TAC CGA GCC ACA GGC GG-3'
TNFA	forward: 5'-TCC TTC AGA CAC CCT CAA CC-3' reverse: 5'-AGG CCC CAG TTT GAA TTC TT-3'
YWHAZ	forward: 5'-ACT TTT GGT ACA TTG TGG CTT CAA-3' reverse: 5'-CCG CCA GGA CAA ACC AGT AT-3'

Table A.2: Fatty acid status of the human subjects. Erythrocytes were analyzed for total fatty acid concentrations by means of LC-MS/MS and %n–6 in HUFA was calculated from fatty acid concentrations of C20:3 n–6, C20:4 n–6, C22:4 n–6, C22:5 n–6, C20:3 n-9, C20:5 n–3, C22:5 n–3, C22:6 n–3. All 12 donors were healthy, voluntary blood donors from a local blood donation center. Results are shown as mean $\pm$ SD, $n = 3$.

subject	**%n–6 in HUFA** [%]
1	72.8 ± 0.2
2	73.3 ± 0.3
3	75.0 ± 0.2
4	75.1 ± 0.3
5	74.7 ± 0.4
6	72.2 ± 1.0
7	76.6 ± 0.2
8	75.1 ± 1.3
9	75.1 ± 1.0
10	82.9 ± 0.6
11	77.7 ± 0.3
12	74.6 ± 0.6

Table A.3: (next page) Oxylipin levels in different human plasmas. Human plasma from a local blood donation center (pool of 4 subjects) as well as two commercially available human plasmas were analyzed for total oxylipin concentrations by means of LC-MS/MS. Representative oxylipins derived from different precursor PUFA covering different classes are shown as mean $\pm$ SD, $n = 3$.

	precursor PUFA	**Human plasma from local blood donation center** conc [nM]	**Commercial human plasma 1** [1] conc [nM]	**Commercial human plasma 2** conc [nM]
5-F2t-IsoP (5-iPF$_{2\alpha}$-VI)	ARA	0.8 ± 0.2	3.5 ± 0.1	92 ± 19
9-HODE	LA	330 ± 10	2800 ± 150	68000 ± 5700
13-HOTrE	ALA	9.3 ± 0.6	72 ± 44	6000 ± 8600
5-HETE	ARA	72 ± 5.1	430 ± 23	16000 ± 1600
12-HETE	ARA	90 ± 10	880 ± 74	24000 ± 3400
15-HETE	ARA	95 ± 0.7	370 ± 33	23000 ± 2200
5-HEPE	EPA	11 ± 0.1	34 ± 2.0	2000 ± 240
12-HEPE	EPA	22 ± 1.2	120 ± 7.7	3900 ± 420
15-HEPE	EPA	18 ± 0.8	110 ± 4.6	3400 ± 440
4-HDHA	DHA	33 ± 1.0	150 ± 1.1	5000 ± 530
7-HDHA	DHA	23 ± 0.8	130 ± 1.3	3200 ± 270
14-HDHA	DHA	30 ± 1.9	140 ± 1.0	4200 ± 740
17-HDHA	DHA	25 ± 2.3	150 ± 2.0	4600 ± 620
12(13)-EpOME	LA	82 ± 14	230 ± 78	520 ± 110
14(15)-EpETrE	ARA	28 ± 2.3	71 ± 24	140 ± 48
12,13-DiHOME	LA	10 ± 1.0	30 ± 1.2	75 ± 5.2
14,15-DiHETrE	ARA	0.72 ± 0.01	1.0 ± 0.1	10 ± 0.8

Table A.4: Fatty acid pattern of human plasmas. Non-fasting plasma of five different subjects from a local blood donation center was analyzed by means of GC-FID. Results are shown as mean ± SD, $n = 3$. %n–6 in HUFA was calculated from fatty acid concentrations of C20:3 n–6, C20:4 n–6, C22:4 n–6, C22:5 n–6, C20:3 n-9, C20:5 n–3, C22:5 n–3, C22:6 n–3.

	SFA conc [mM]	**HUFA** conc [mM]	**MUFA** conc [mM]	**n–6 PUFA** conc [mM]	**n–3 PUFA** conc [mM]	**all FA** conc [mM]	**%n–6 in HUFA** [%]
A	4.70 ± 0.52	3.99 ± 0.44	1.08 ± 0.11	2.91 ± 0.33	0.26 ± 0.03	11.9 ± 1.3	79.2 ± 0.3
B	2.67 ± 0.04	1.67 ± 0.03	0.97 ± 0.02	3.16 ± 0.05	0.17 ± 0.02	7.7 ± 0.1	82.6 ± 1.4
C	5.61 ± 0.42	4.21 ± 0.30	1.49 ± 0.11	4.55 ± 0.32	0.48 ± 0.05	14.9 ± 1.1	73.2 ± 0.8
D	3.42 ± 0.12	3.12 ± 0.08	1.23 ± 0.04	3.59 ± 0.11	0.45 ± 0.02	10.6 ± 0.3	67.1 ± 1.0
E	2.34 ± 0.08	1.76 ± 0.10	1.07 ± 0.05	2.75 ± 0.22	0.24 ± 0.02	7.1 ± 0.4	78.7 ± 0.3

Table A.5: Purity of commercial fatty acid preparations. Different free fatty acid preparations were analyzed for oxylipin concentrations by means of LC-MS/MS. Shown are mean ± SD, $n = 3$.

	Oxylipins [% (*w*/*w*)]
DHA >99%	0.36 ± 0.01
EPA >99%	0.21 ± 0.02
EPA ≥97%	3.30 ± 0.14

Table A.6: Oxylipins in commercial n–3 PUFA preparation used for supplementation without (w/o) and with (w) reduction. DHA >99% (1 M) was analyzed for oxylipin concentrations by means of LC-MS/MS. Reduction of hydroperoxy-FA to hydroxy-FA was carried out using $SnCl_2$ (10 mg/mL). Shown are mean ± SD, $n = 3$.

Oxylipin	**w/o reduction** conc [nM]	**w reduction** conc [nM]
4-HDHA	0.08 ± 0.01	0.34 ± 0.02
7-HDHA	0.07 ± 0.09	0.25 ± 0.02
8-HDHA	0.05 ± 0.04	0.18 ± 0.04
10-HDHA	0.08 ± 0.04	0.19 ± 0.01
11-HDHA	0.09 ± 0.07	0.31 ± 0.02
13-HDHA	0.08 ± 0.01	0.20 ± 0.01
14-HDHA	0.07 ± 0.02	0.16 ± 0.01
16-HDHA	0.07 ± 0.06	0.16 ± 0.01
17-HDHA	0.06 ± 0.03	0.15 ± 0.01
20-HDHA	0.20 ± 0.13	0.45 ± 0.02
10(11)-EpDPE	0.25 ± 0.01	
13(14)-EpDPE	0.24 ± 0.01	
16(17)-EpDPE	0.23 ± 0.01	
19(20)-EpDPE	0.28 ± 0.01	

Table A.7: Increase of oxylipin concentration in n–3 PUFA supplemented medium and statistical analysis. A) Results for linear regression analysis of oxylipin concentrations in different supplemented media (low (l), medium (m), high (h)). B) Differences in the increase of oxylipins over incubation time were determined post-hoc using one-way ANOVA with Tukey's multiple comparisons test. C) Differences in oxylipin concentrations at baseline (t = 0) were determined using one-way ANOVA with Tukey's multiple comparisons test.

A		**slope ± SE**	**95% CI slope**	**y-intercept ± SE**	**R^2**
4-HDHA	l	1.02 ± 0.06	0.89—1.16	1.96 ± 0.23	0.968
	m	3.77 ± 0.40	2.9—4.7	4.45 ± 0.76	0.897
	h	7.31 ± 1.09	4.9—9.7	6.32 ± 2.05	0.818
14-HDHA	l	1.40 ± 0.05	1.3—1.5	1.83 ± 0.22	0.985
	m	4.96 ± 0.64	3.5—6.4	4.33 ± 1.20	0.857
	h	9.37 ± 1.45	6.1—12.6	5.11 ± 2.71	0.807
17-HDHA	l	1.35 ± 0.07	1.2—1.5	1.69 ± 0.26	0.978
	m	5.22 ± 0.62	3.9—6.6	3.90 ± 1.16	0.877
	h	9.25 ± 1.31	6.3—12.2	5.16 ± 2.45	0.834
19(20)-	l	0.06 ± 0.03	-0.01—0.14	1.35 ± 0.13	0.262
EpDPE	m	0.23 ± 0.36	-0.57—1.03	10.6 ± 0.67	0.039
	h	-0.43 ± 0.71	-2.0—1.2	25.8 ± 1.33	0.035
19,20-	l	0.06 ± 0.02	0.02—0.11	0.11 ± 0.08	0.472
DiHDPE	m	0.05 ± 0.02	-0.01—0.10	1.03 ± 0.04	0.286
	h	0.13 ± 0.05	0.03—0.24	2.18 ± 0.09	0.461
5-HETE	l	7.34 ± 0.39	6.5—8.2	9.82 ± 1.52	0.973
	m	18.0 ± 1.50	14.6—21.3	13.1 ± 2.80	0.935
	h	20.6 ± 2.02	16.1—25.1	10.9 ± 3.78	0.912
15-HETE	l	12.1 ± 0.50	11.0—13.2	14.6 ± 1.97	0.983
	m	29.4 ± 2.95	22.8—36.0	20.2 ± 5.52	0.909
	h	34.3 ± 3.82	25.7—42.8	17.9 ± 7.15	0.889
5-HEPE	l	0.287 ± 0.02	0.25—0.33	0.43 ± 0.07	0.963
	m	0.667 ± 0.06	0.54—0.79	0.52 ± 0.11	0.932
	h	0.741 ± 0.08	0.57—0.91	0.48 ± 0.14	0.906
18-HEPE	l	0.710 ± 0.04	0.63—0.79	0.99 ± 0.14	0.975
	m	1.76 ± 0.17	1.4—2.2	1.31 ± 0.33	0.911
	h	1.97 ± 0.21	1.5—2.5	1.27 ± 0.40	0.897
9-HODE	l	62.9 ± 2.46	57.4—68.4	57.3 ± 9.67	0.985
	m	175 ± 23	22.8—227	75.5 ± 43.6	0.849
	h	196 ± 24	143—250	60.7 ± 45.1	0.869

B	**F(2, 33)**	***p* value**		**mean difference**	**adjusted *p* value**	
4-HDHA	21.92	*<0.001*	l vs. m	-2.74	*0.019*	*
			l vs. h	-6.29	*<0.001*	***
			m vs. h	-3.54	*0.002*	**
14-HDHA	9.03	*<0.001*	l vs. m	-3.56	*0.025*	*
			l vs. h	-7.97	*<0.001*	***
			m vs. h	4.41	*0.005*	**
17-HDHA	9.03	*<0.001*	l vs. m	-3.87	*0.007*	**
			l vs. h	-7.90	*<0.001*	***
			m vs. h	-4.03	*0.005*	**
19(20)-EpDPE	0.556	*0.579*	l vs. m	-0.17	*>0.05*	*ns*
			l vs. h	0.49	*>0.05*	*ns*
			m vs. h	0.66	*>0.05*	*ns*
19,20-DiHDPE	2.180	*0.129*	l vs. m	0.01	*>0.05*	*ns*
			l vs. h	-0.07	*>0.05*	*ns*
			m vs. h	-0.09	*>0.05*	*ns*
5-HETE	23.18	*<0.001*	l vs. m	-10.6	*<0.001*	***
			l vs. h	-13.3	*<0.001*	***
			m vs. h	-2.67	*>0.05*	*ns*
15-HETE	17.27	*<0.001*	l vs. m	-17.3	*<0.001*	***
			l vs. h	-22.1	*<0.001*	***
			m vs. h	-4.83	*>0.05*	*ns*
5-HEPE	19.25	*<0.001*	l vs. m	-0.38	*<0.001*	***
			l vs. h	-0.45	*<0.001*	***
			m vs. h	-0.07	*>0.05*	*ns*
18-HEPE	17.95	*<0.001*	l vs. m	-1.05	*<0.001*	***
			l vs. h	-1.27	*<0.001*	***
			m vs. h	-0.22	*>0.05*	*ns*
9-HODE	13.60	*<0.001*	l vs. m	-112	*<0.001*	***
			l vs. h	-133	*<0.001*	***
			m vs. h	-21.7	*>0.05*	*ns*

C	**F(2, 6)**	***p* value**		**mean difference**	**adjusted *p* value**	
4-HDHA	516.0	*<0.001*	l vs. m	-2.79	*<0.001*	***
			l vs. h	-6.18	*<0.001*	***
			m vs. h	-3.39	*<0.001*	***
14-HDHA	684.1	*<0.001*	l vs. m	-2.53	*<0.001*	***
			l vs. h	-5.48	*<0.001*	***
			m vs. h	-2.95	*<0.001*	***
17-HDHA	248.6	*<0.001*	l vs. m	-2.58	*<0.001*	***
			l vs. h	-5.70	*<0.001*	***
			m vs. h	-3.12	*<0.001*	***
19(20)-EpDPE	175.3	*<0.001*	l vs. m	-9.34	*<0.001*	***
			l vs. h	-23.7	*<0.001*	***
			m vs. h	-14.3	*<0.001*	***
19,20-DiHDPE	1187	*<0.001*	l vs. m	-1.03	*<0.001*	***
			l vs. h	-2.19	*<0.001*	***
			m vs. h	-1.16	*<0.001*	***
5-HETE	17.75	*0.003*	l vs. m	-2.91	*0.016*	***
			l vs. h	-4.12	*0.003*	**
			m vs. h	-1.22	*>0.05*	*ns*
15-HETE	110.1	*<0.001*	l vs. m	-4.37	*<0.001*	***
			l vs. h	-7.04	*<0.001*	***
			m vs. h	-2.67	*0.003*	**
5-HEPE	12.36	*0.008*	l vs. m	-0.093	*0.040*	*
			l vs. h	-0.140	*0.007*	**
			m vs. h	-0.047	*>0.05*	*ns*
18-HEPE	13.73	*0.006*	l vs. m	-0.337	*0.018*	*
			l vs. h	-0.427	*0.006*	**
			m vs. h	-0.090	*>0.05*	*ns*
9-HODE	126.4	*<0.001*	l vs. m	-20.6	*<0.001*	***
			l vs. h	-29.6	*<0.001*	***
			m vs. h	-8.98	*0.008*	**

Table A.8: Statistical analysis of changes in relative FA pattern in macrophages after supplementation with n–3 PUFA. Statistical differences were determined using two-way rmANOVA ($F(12, 24) = 25.87$, $p = 0.012$) followed by Dunnett's multiple comparisons test.

		mean difference	95% CI of difference	adjusted *p* value	
SFA	l vs. 2d m	-0.1600	-18.34—18.02	*>0.05*	*ns*
	l vs. 3d m	-2.267	-19.30—14.77	*>0.05*	*ns*
	l vs. 2d h	0.7267	-21.64—23.10	*>0.05*	*ns*
	l vs. 3d h	5.040	-13.96—24.04	*>0.05*	*ns*
MUFA	l vs. 2d m	4.513	-10.37—19.40	*>0.05*	*ns*
	l vs. 3d m	8.420	-4.566—21.41	*>0.05*	*ns*
	l vs. 2d h	7.330	-4.720—19.38	*>0.05*	*ns*
	l vs. 3d h	7.993	-6.566—22.55	*>0.05*	*ns*
n–6	l vs. 2d m	4.207	-1.200—9.613	*>0.05*	*ns*
	l vs. 3d m	7.677	2.169—13.18	*0.026*	*
	l vs. 2d h	7.370	-1.826—16.57	*>0.05*	*ns*
	l vs. 3d h	9.477	-1.172—20.13	*>0.05*	*ns*
n–3	l vs. 2d m	-8.607	-14.44— -2.777	*0.024*	*
	l vs. 3d m	-13.63	-18.37— -8.882	*0.006*	**
	l vs. 2d h	-15.32	-24.40— -6.243	*0.018*	*
	l vs. 3d h	-22.27	-30.21— -14.33	*0.007*	**

Table A.9: Statistical analysis of changes in the oxylipin pattern of medium without (-) and with cells (+). Statistical differences were determined using two-way ANOVA followed by Sidak's multiple comparisons test.

	F(2, 12)	*p* value		mean difference	adjusted *p* value	
4-HDHA	50.51	*<0.001*	l	2.82	*<0.001*	***
			m	4.58	*<0.001*	***
			h	9.91	*<0.001*	***
10-HDHA	132.4	*<0.001*	l	1.84	*<0.001*	***
			m	3.65	*<0.001*	***
			h	9.94	*<0.001*	***
13-HDHA	176.0	*<0.001*	l	2.51	*<0.001*	***
			m	4.52	*<0.001*	***
			h	12.8	*<0.001*	***

	F(2, 12)	***p* value**		**mean difference**	**adjusted *p* value**	
14-HDHA	121.2	*<0.001*	l	1.17	*0.007*	**
			m	2.42	*<0.001*	***
			h	7.49	*<0.001*	***
17-HDHA	57.12	*<0.001*	l	1.01	*>0.05*	*ns*
			m	0.82	*>0.05*	*ns*
			h	6.56	*<0.001*	***
10,17-DiHDHA	73.17	*<0.001*	l	-0.53	*<0.001*	***
			m	0.18	*0.05*	*
			h	0.55	*<0.001*	***
19(20)-EpDPE	119.5	*<0.001*	l	0.51	*>0.05*	*ns*
			m	8.95	*<0.001*	***
			h	20.2	*<0.001*	***
19,20-DiHDPE	26.44	*<0.001*	l	0.00	*>0.05*	*ns*
			m	-0.80	*<0.001*	***
			h	-1.21	*<0.001*	***
5-HETE	56.67	*<0.001*	l	12.73	*<0.001*	***
			m	4.17	*>0.05*	*ns*
			h	16.26	*<0.001*	***
11-HETE	32.95	*<0.001*	l	8.38	*0.023*	*
			m	-3.38	*>0.05*	*ns*
			h	26.5	*<0.001*	***
12-HETE	21.28	*<0.001*	l	5.85	*>0.05*	*ns*
			m	-0.52	*>0.05*	*ns*
			h	21.4	*<0.001*	***
15-HETE	29.41	*<0.001*	l	7.81	*0.003*	**
			m	-3.74	*>0.05*	*ns*
			h	15.6	*<0.001*	***
8(*S*),15(*S*)-DiHETE	9.62	*0.003*	l	-4.60	*<0.001*	***
			m	-3.61	*<0.001*	***
			h	-1.38	*>0.05*	*ns*
5(*S*),15(*S*)-DiHETE	7.84	*0.007*	l	-0.73	*<0.001*	***
			m	-0.31	*0.033*	*
			h	-0.18	*>0.05*	*ns*

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[1] M. Mainka, C. Dalle, M. Pétéra, J. Dalloux-Chioccioli, N. Kampschulte, A. I. Ostermann, M. Rothe, J. Bertrand-Michel, J. W. Newman, C. Gladine, and N. H. Schebb. “Harmonized procedures lead to comparable quantification of total oxylipins across laboratories”. In: *Journal of Lipid Research* 61.11 (2020), pp. 1424–1436. DOI: 10.1194/jlr.ra120000991.

B Appendix for Chapter 3

Table B.1: Properties of the fluorescent labeled polystyrene microspheres (beads) tested in the phagocytosis assay.

	surface modification	**diameter** [μM]	**fluorophore**	λ_{ex} [nm]	λ_{em} [nm]
A	carboxylate	2.0	red	575	610
B	carboxylate	2.0	yellow-green	470	505
C	carboxylate	1.0	yellow-green	470	505
D	amine	1.0	yellow-green	470	505
E	carboxylate	2.0	nile red	535	575

Table B.2: Statistical analysis of the changes in phagocytosis in M1- vs M2-like macrophages after stimulation with COX inhibitors and dexamethasone with and without LPS. Statistical differences were determined using 2-way ANOVA followed by Sidak's multiple comparisons test.

	95% CI of difference	**adjusted *p* value**	
0.1% DMSO	-22.06–22.06	*>0.05*	*ns*
5 μM celecoxib	-30.44–20.50	*>0.05*	*ns*
1 μM indometacin	-25.87–25.07	*>0.05*	*ns*
100 nM dexamethason	-30.47–20.47	*>0.05*	*ns*
5 μM celecoxib + 0.1 μg/mL LPS	-59.91– -15.80	*<0.001*	***
1 μM indometacin + 0.1 μg/mL LPS	-52.25– -8.14	*0.002*	**
100 nM dexamethason + 0.1 μg/mL LPS	-59.40– -8.46	*0.003*	**

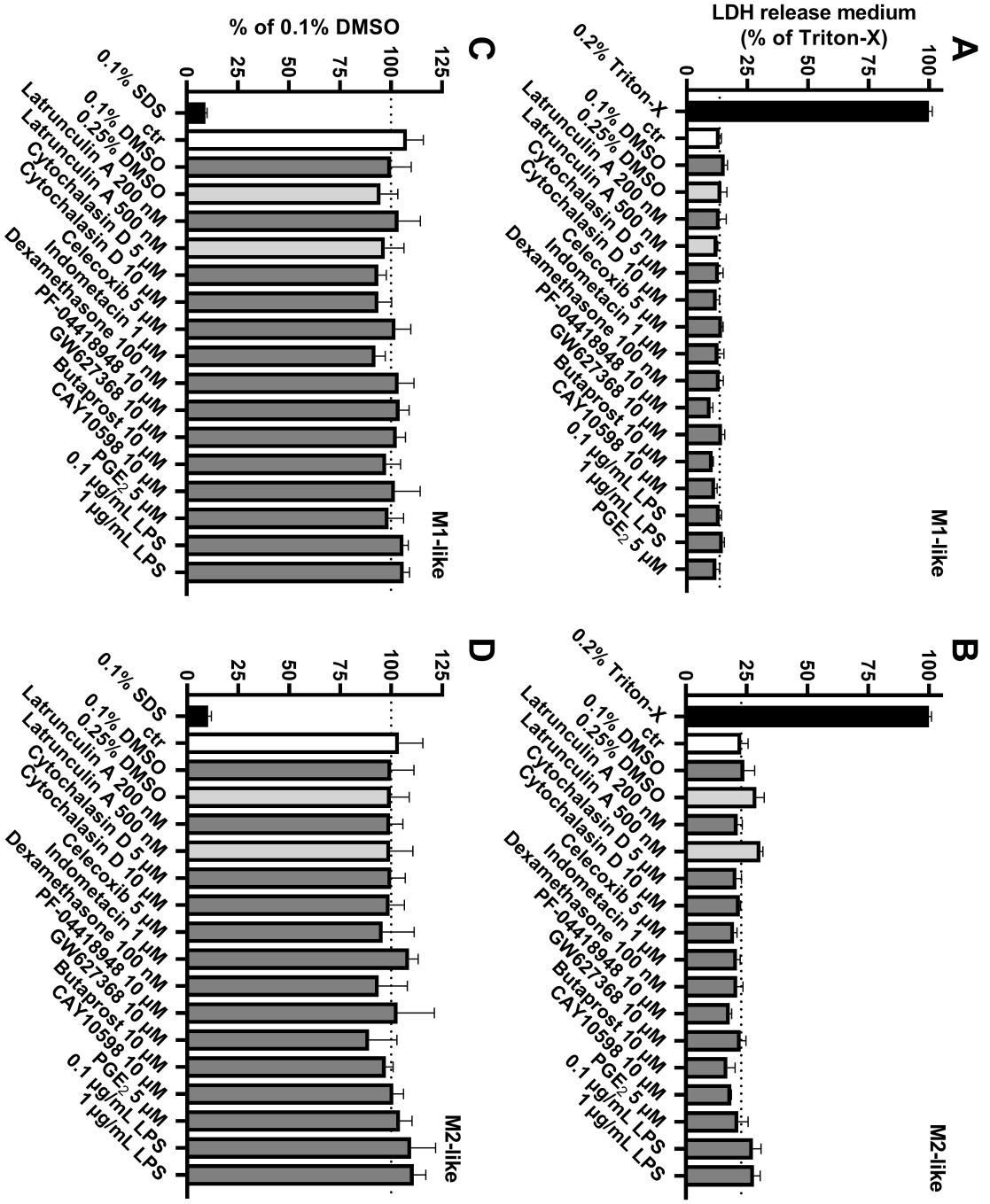

A
LDH release medium
(% of Triton-X)
0
25
50
75
100
0.2% Triton-X
ctr
0.1% DMSO
0.25% DMSO
Latrunculin A 200 nM
Latrunculin A 500 nM
Cytochalasin D 5 µM
Cytochalasin D 10 µM
Celecoxib 5 µM
Indometacin 1 µM
Dexamethasone 100 nM
PF-04418948 10 µM
GW627368 10 µM
Butaprost 10 µM
CAY10598 10 µM
0.1 µg/mL LPS
1 µg/mL LPS
PGE2 5 µM
M1-like
B
M2-like
C
% of 0.1% DMSO
0
25
50
75
100
125
0.1% SDS
ctr
0.1% DMSO
0.25% DMSO
Latrunculin A 200 nM
Latrunculin A 500 nM
Cytochalasin D 5 µM
Cytochalasin D 10 µM
Celecoxib 5 µM
Indometacin 1 µM
Dexamethasone 100 nM
PF-04418948 10 µM
GW627368 10 µM
Butaprost 10 µM
CAY10598 10 µM
PGE2 5 µM
0.1 µg/mL LPS
1 µg/mL LPS
M1-like
D
M2-like

Figure B.1: (previous page) Effect of the tested compounds on cell viability determined by lactate dehydrogenase (LDH) leakage (A, B) or metabolic activity by resazurin assay (C, D) in primary human macrophages. Human monocytes were differentiated into M1-like or M2-like macrophages using 10 ng/mL GM-CSF or M-CSF (M1-/M2-like) for 7 days and additional 10 ng/mL IFNγ or IL-4 (M1-/M2-like) for the last 2 days. The cells were incubated with compounds at the indicated concentrations for 4 h. DMSO served as vehicle control and Triton X (LDH assay) or SDS (resazurin assay) as positive control. Dehydrogenase activity in the medium was measured by the decrease in absorbance at 340 nm for 45 min during the NADH dependent reduction of pyruvate to lactate. LDH leakage was determined by comparing LDH activities in culture medium and lysed cells. Shown are mean ± SD for $n = 3$ replicates from a pool of 3 subjects. Dehydrogenase activity was measured as resorufin formation by fluorometric readout at 590 nm after excitation at 530 nm. Shown are mean ± SD for $n = 3-6$ replicates from a pool of 3 subjects.

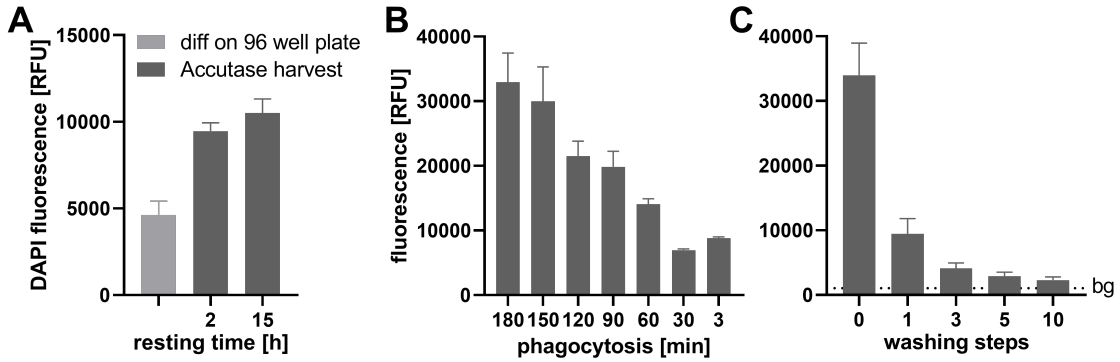


Figure B.2: Characterization of resting time of cells prior phagocytosis assay, duration of phagocytosis assay and number of washing steps. Primary human monocytes were differentiated into M2-like macrophages using 10 ng/mL M-CSF for 7 days and additional 10 ng/mL IL-4 for the last 2 days. A) Cells were either differentiated directly in 96-well plates (light gray) or cultured and differentiated on dishes followed by harvest using accutase (dark gray) and transferred to 96-well plates (50,000 cells/well). The cells were allowed to rest/adhere to the plates for 2 or 15 h. The colonization efficacy (cell number) was determined based on the amount of DNA determined by incubation with DAPI. The cells were washed with PBS and fluorescence (λ_{ex} 358 nm, λ_{em} 461 nm) was measured in order to determine the optimal duration of the phagocytosis. B) Cells were incubated with beads for different periods of time ranging between 3 to 180 min. After washing with PBS, bead fluorescence was analyzed in the cells. C) After phagocytosis assay (for 120 min) cells were washed different times with PBS as indicated and bead fluorescence was measured in the cells in order to determine the necessary number of washing steps to remove adherent, non-ingested beads. All results are shown as mean ± SD, $n = 5-9$ replicates from a pool of 3 human subjects.

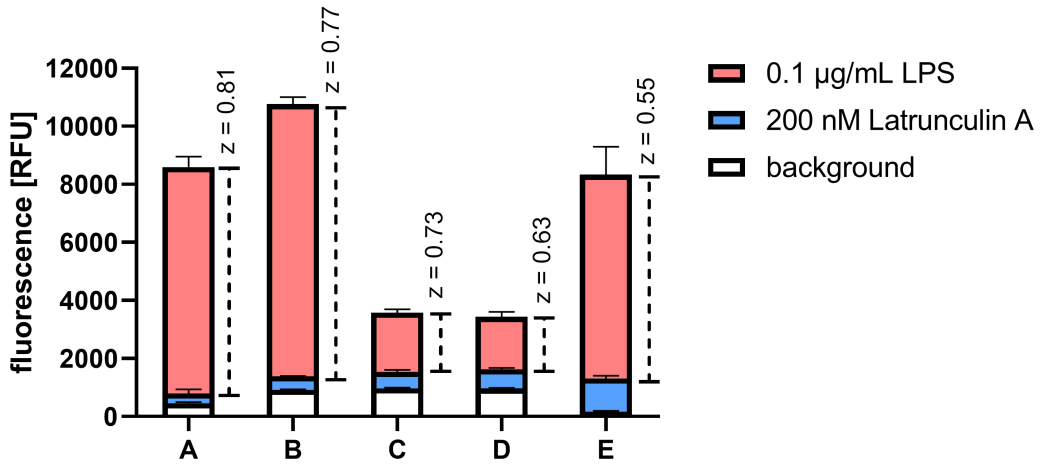
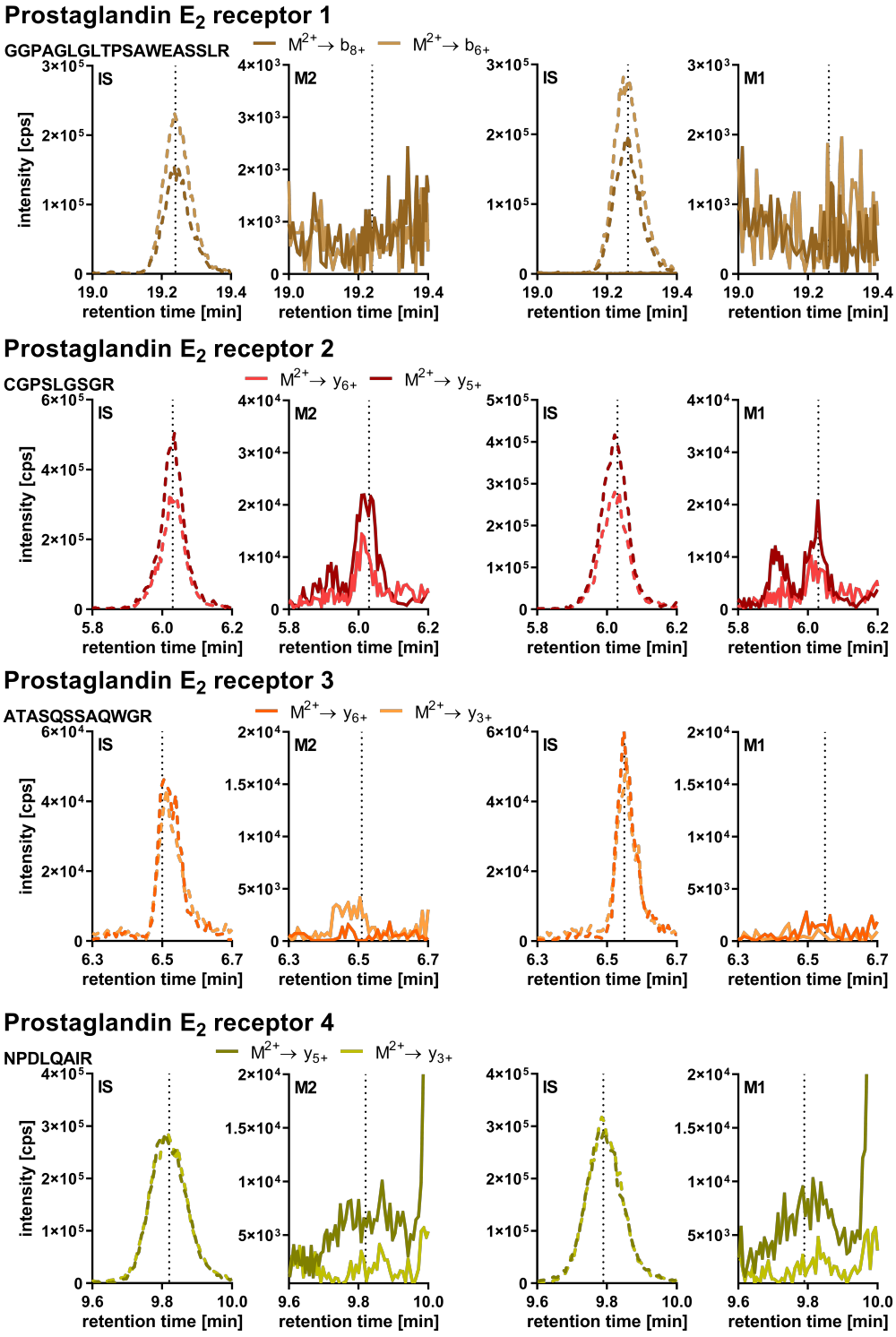


Figure B.3: Impact of type of fluorescent beads on the performance of the phagocytosis assay in M2-like macrophages. Primary human monocytes were differentiated into M2-like macrophages using 10 ng/mL M-CSF for 7 days and additional 10 ng/mL IL-4 for the last 2 days. Cells were preincubated with 0.1 µg/mL LPS (positive control) for 4 h or 200 nM Latrunculin A (negative control) for 2 h before starting phagocytosis. Phagocytosis was carried out using different beads A-E (Table B.2) for 2 h. Results are shown as mean ± SD, $n = 5-9$ replicates from a pool of 3 human subjects. z-factors were calculated based on mean and standard deviation of signals of positive control and negative control.

Figure B.4: (next page) Characterization of the presence of EP receptors in primary macrophages using targeted proteomics. Primary human monocytes were differentiated into M1- or M2-like macrophages using 10 ng/mL GM-CSF or M-CSF for 7 days and additional 10 ng/mL IFNγ or IL-4 for the last 2 days. Cells were investigated for EP receptors based on peptides specific for EP1 (GGPAGLGLTPSAWEASSLR), EP2 (CGPSLGSGR), EP3 (ATASQSSAQWGR) and EP4 (NPDLQAIR) after tryptic digestion. Peptides were determined as described [1, 2]. Shown are the XICs of the peptides and the co-eluting isotope labeled peptide standards (IS).


Prostaglandin E2 receptor 1
GGPAGLGLTPSAWEASSLR
M2+→ b8+
M2+→ b6+
IS
M2
M1
intensity [cps]
retention time [min]
Prostaglandin E2 receptor 2
CGPSLGSGR
M2+→ y6+
M2+→ y5+
Prostaglandin E2 receptor 3
ATASQSSAQWGR
M2+→ y6+
M2+→ y3+
Prostaglandin E2 receptor 4
NPDLQAIR
M2+→ y5+
M2+→ y3+

References

[1] N. M. Hartung, M. Mainka, R. Pfaff, M. Kuhn, S. Biernacki, L. Zinnert, and N. H. Schebb. "Development of a quantitative proteomics approach for cyclooxygenases and lipoxygenases in parallel to quantitative oxylipin analysis allowing the comprehensive investigation of the arachidonic acid cascade". In: *Analytical and Bioanalytical Chemistry* 415 (2023), pp. 913–933. DOI: 10.1007/s00216-022-04489-3.

[2] R. Ohno, M. Mainka, R. Kirchhoff, N. M. Hartung, and N. H. Schebb. "Sterol Derivatives Specifically Increase Anti-Inflammatory Oxylipin Formation in M2-like Macrophages by LXR-Mediated Induction of 15-LOX". In: *Molecules* 29.8 (2024), p. 1745. DOI: 10.3390/molecules29081745.

C Appendix for Chapter 4

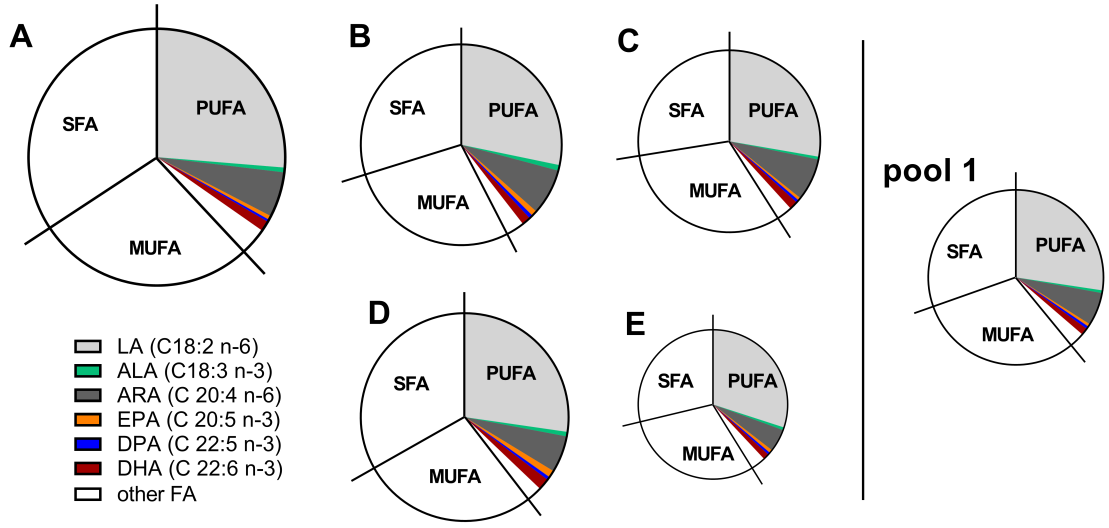
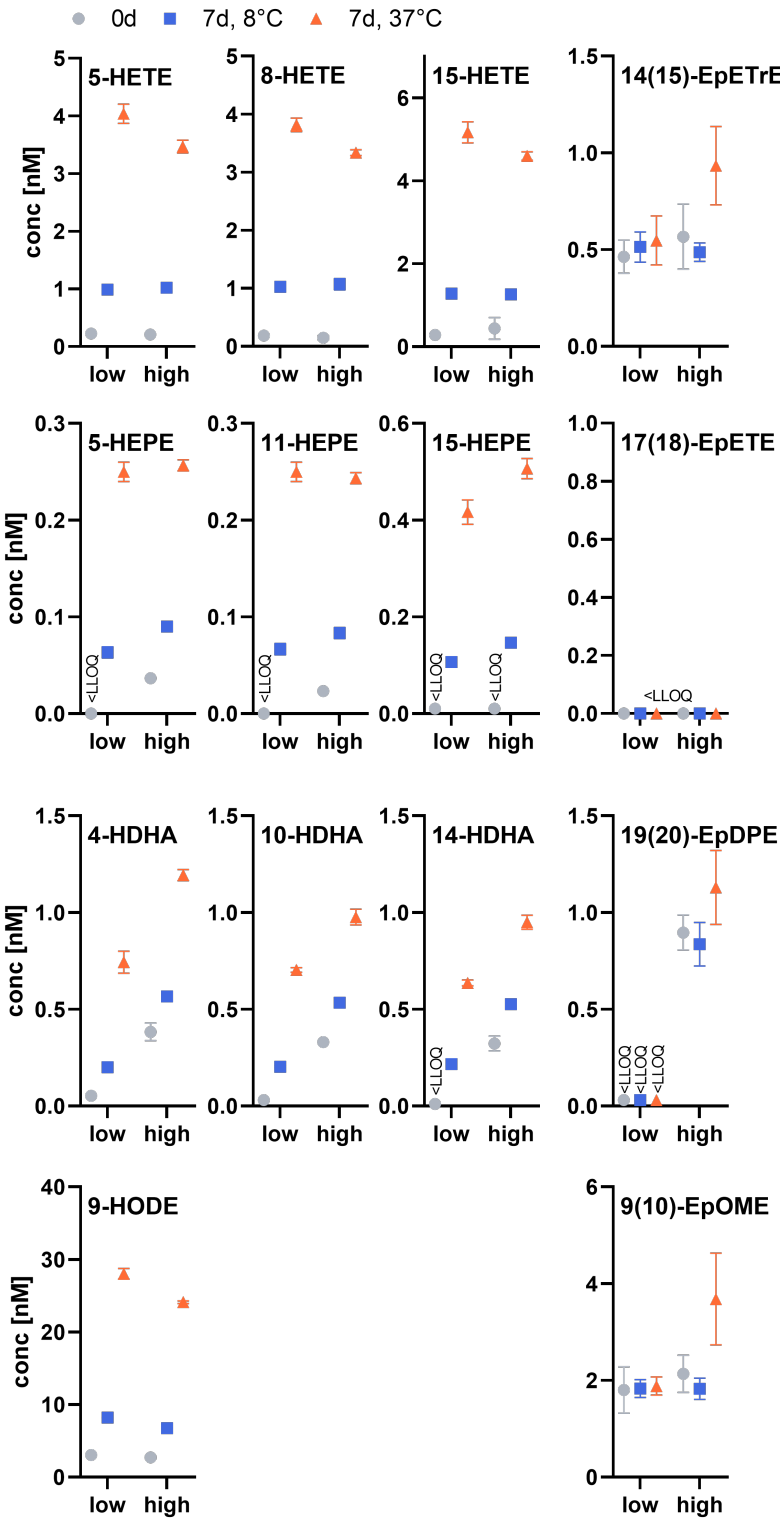


Figure C.1: Relative fatty acid profiles of the tested human citrate plasmas. Non-fasting plasma of five subjects from a local blood donation center was analyzed for total FA concentrations by means of LC-MS/MS. Plasmas C and E were pooled resulting in plasma pool 1. Sizes of the circles indicate relative total fatty acid concentrations (plasma A, 11.0 ± 0.7 mM; plasma B, 8.7 ± 0.7 mM; plasma C, 7.8 ± 0.5 mM; plasma D, 9.0 ± 0.7 mM; plasma E, 6.4 ± 0.4 mM; pool 1, 7.5 ± 0.7 mM). SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Figure C.2: (next page) Oxylipin concentrations of the used cell culture media at baseline and after storage for 7 days. Media were prepared with 5% (*v/v*) plasma pool 1 (non-supplemented medium, low). For the supplemented medium (high), additionally 10.3 µM DHA (>99%) and 5.5 µM EPA (>99%) were added. Concentrations of selected, most abundant oxylipins derived from ARA, EPA, DHA and LA were analyzed at beginning and after 7 days of incubation at 8 °C or 37 °C by means of LC-MS/MS. Results of one representative experiment are shown as mean ± SD, $n = 3$.


0d
7d, 8°C
7d, 37°C
5-HETE
8-HETE
15-HETE
14(15)-EpETrE
5-HEPE
11-HEPE
15-HEPE
17(18)-EpETE
4-HDHA
10-HDHA
14-HDHA
19(20)-EpDPE
9-HODE
9(10)-EpOME
conc [nM]
low
high
<LLOQ

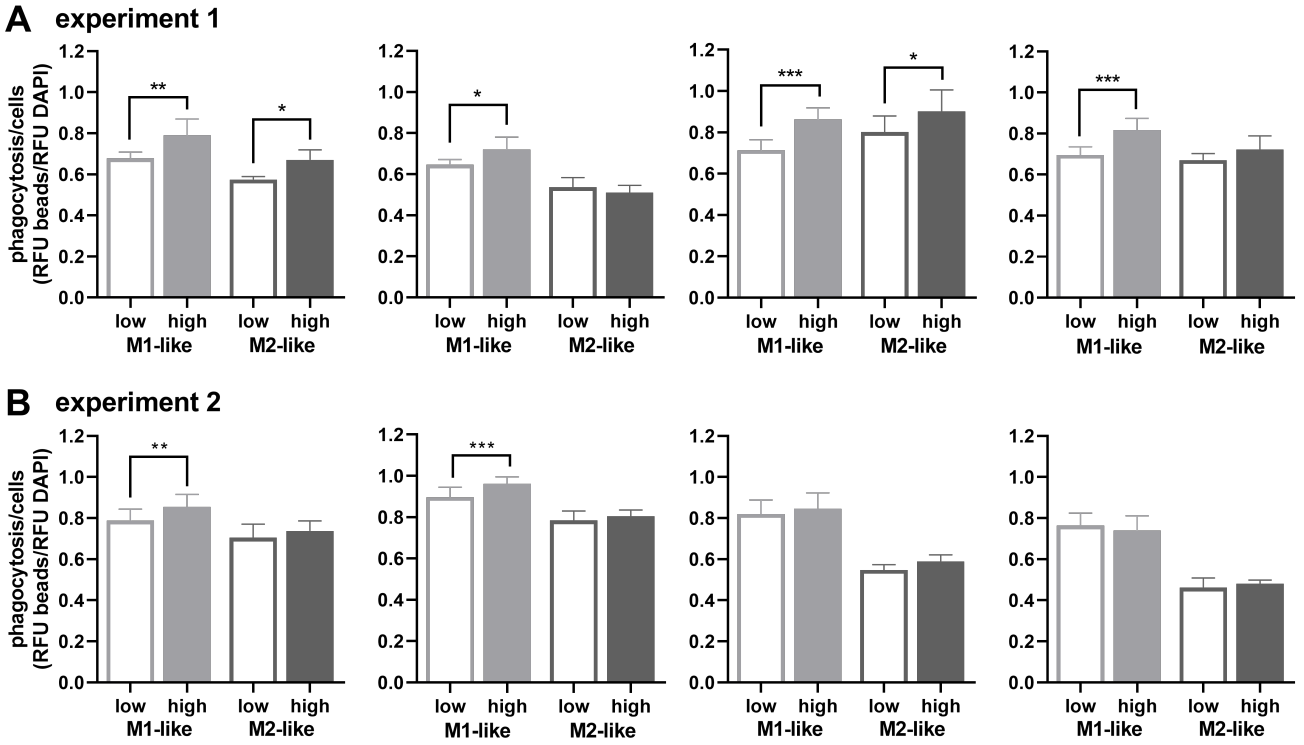


Figure C.3: Impact of n-3 PUFA supplementation on phagocytosis in the two independent supplementation experiments (A, B). After isolation of monocytes, cells were differentiated into macrophages and supplemented using medium with 5% (v/v) plasma pool 1 (low) or plasma pool 1 and 10.3 μM DHA (>99%) and 5.5 μM EPA (>99%) (high) for 2 days. Cells were pooled transferred into 96 well plates (50,000 cells per well) for phagocytosis assay. Shown is phagocytosis as ratio of bead fluorescence to DAPI fluorescence. Results of the four 96 well plates for each supplementation experiment are shown as mean ± SD for $n = 5-8$ (A) or n = 14−15 (B) replicates from a pool of 4 subjects. Statistical analysis was performed by 1-way ANOVA followed by Sidak's multiple comparison test. Differences from vehicle control were considered significant at p values ≤0.5 (*), ≤0.01 (**) or ≤0.001 (***).

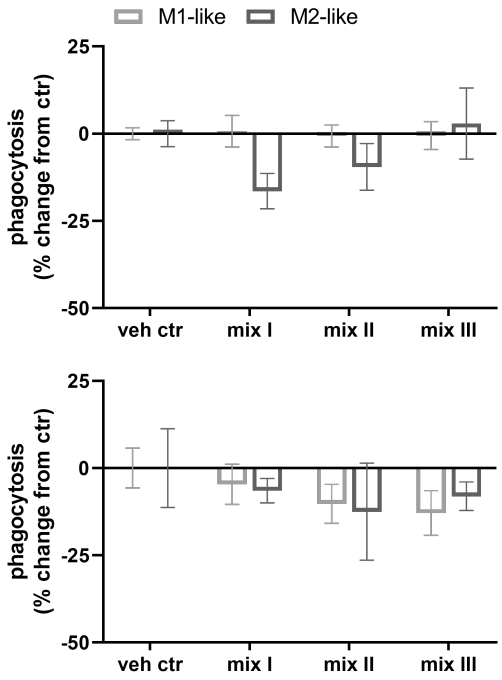


Figure C.4: Impact of mixtures of oxylipins on phagocytosis. Non-supplemented macrophages were transferred into 96-well plates (50,000 cells per well) and incubated with 300 nM oxylipins (mix I: 5-HEPE, 4-HDHA, 7-HDHA; mix II: 12(*S*)-, 15(*S*)-HEPE, 14(*S*)-, 17(*S*)-HDHA; mix III: 17(18)-EpETE, 19(20)-EpDPE, Table C.4) or 0.1% DMSO (veh ctr) for 1 h. Shown are the results of two 96 well plates as % change from vehicle control as mean ± SD for $n = 4–5$ replicates from a pool of 4 subjects.

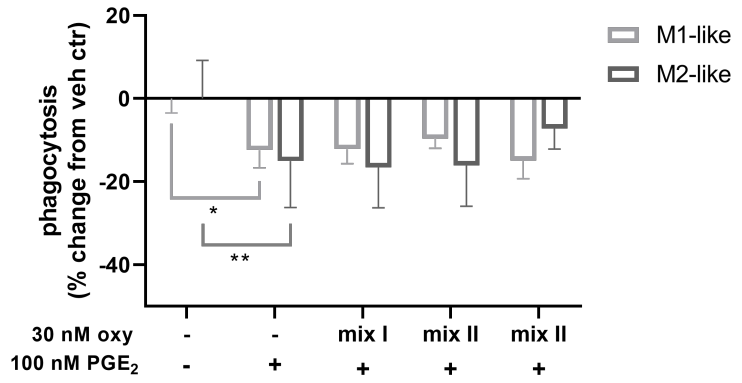


Figure C.5: Impact of n–3 PUFA derived oxylipins on the inhibitory effect of PGE_2 on phagocytosis. Primary human macrophages were preincubated with 100 nM PGE_2 and 30 nM oxylipins (mix I: 5-HEPE, 4-HDHA, 7-HDHA; mix II: 12(*S*)-, 15(*S*)-HEPE, 14(*S*)-, 17(*S*)-HDHA; mix III: 17(18)-EpETE, 19(20)-EpDPE, Table C.4) or 0.1% DMSO (veh ctr) for 1 h, followed by phagocytosis for 2 h. Shown is phagocytosis as % change from vehicle control as mean ± SD for $n = 4–5$ replicates from a pool of 4 subjects. Statistical analysis was performed by 1-way ANOVA followed by Sidak's multiple comparison test. Differences from vehicle control were considered significant at p values ≤ 0.5 (*), ≤ 0.01 (**).

Table C.1: Fatty acid pattern of the tested human citrate plasmas. Non-fasting plasma of five subjects (A-E) from a local blood donation center and plasma pool 1 (pooled plasma of C and E) was analyzed for total FA concentrations by means of LC-MS/MS. Results are shown as mean ± SD, $n = 3$. %n-6 in HUFA was calculated from fatty acid concentrations of C20:3 n-6, C20:4 n-6, C22:4 n-6, C22:5 n-6, C20:3 n-9, C20:5 n–3, C22:5 n–3, C22:6 n–3.

	SFA conc [mM]	**MUFA** conc [mM]	**HUFA** conc [mM]	**n-6 PUFA** conc [mM]	**n–3 PUFA** conc [mM]	**all FA** conc [mM]	**%n-6 in HUFA** [%]
target					<0.25	<10	>75
A	3.8 ± 0.2	3.0 ± 0.3	1.2 ± 0.1	3.8 ± 0.2	0.3 ± 0.02	11.0 ± 0.7	78.2 ± 0.5
B	2.6 ± 0.4	2.4 ± 0.2	1.1 ± 0.1	3.3 ± 0.2	0.34 ± 0.03	8.7 ± 0.7	75.4 ± 1.4
C	2.1 ± 0.1	2.4 ± 0.2	1.0 ± 0.1	2.9 ± 0.2	0.23 ± 0.01	7.8 ± 0.5	78.8 ± 1.4
D	3.0 ± 0.2	2.4 ± 0.2	1.0 ± 0.1	3.1 ± 0.2	0.37 ± 0.03	9.0 ± 0.7	67.3 ± 1.2
E	1.8 ± 0.2	1.9 ± 0.2	0.66 ± 0.03	2.4 ± 0.2	0.21 ± 0.02	6.4 ± 0.6	72.8 ± 1.4
pool 1	2.3 ± 0.3	2.3 ± 0.30	0.8 ± 0.1	2.7 ± 0.2	0.22 ± 0.02	7.5 ± 0.7	76.6 ± 1.3

Table C.2: Oxidation status of the tested human citrate plasmas. Non-fasting plasma of five subjects (A-E) from a local blood donation center and plasma pool 1 (pooled plasma of C and E) was analyzed for total oxylipin concentrations by means of LC-MS/MS. Shown are concentrations of representative oxylipins formed by autoxidation and/or enzymatic activity as mean ± SD, $n = 3$.

	target conc [nM]	**plasma A** conc [nM]	**plasma B** conc [nM]	**plasma C** conc [nM]	**plasma D** conc [nM]	**plasma E** conc [nM]	**pool 1** [%]
5-HETE	<100	17.6 ± 0.6	17.3 ± 0.9	13.6 ± 0.8	15 ± 1	9.0 ± 0.5	12 ± 1
12-HETE	<200	17 ± 1	22 ± 1	14.5 ± 0.6	13.3 ± 0.1	13 ± 1	15 ± 1
15-HETE	<200	21 ± 2	24 ± 2	16.8 ± 0.6	15.2 ± 0.6	9.5 ± 0.9	14.3 ± 0.9
5-F2t-IsoP	<1	0.43 ± 0.04	0.52 ± 0.07	0.21 ± 0.05	0.51 ± 0.12	0.31 ± 0.06	0.35 ± 0.03
9-HODE	<500	137 ± 9	137 ± 7	197 ± 13	214 ± 5	79 ± 7	137 ± 10

Table C.3: Fatty acid status of erythrocytes from the human subjects who donated the monocytes used for n–3 PUFA supplementation experiments. Erythrocytes were analyzed for total fatty acid concentrations by means of LC-MS/MS and %n-6 in HUFA was calculated from fatty acid concentrations of C20:3 n-6, C20:4 n-6, C22:4 n-6, C22:5 n-6, C20:3 n-9, C20:5 n–3, C22:5 n–3, C22:6 n–3. All donors were healthy, voluntary blood donors from local blood donation centers. Results are shown as mean ± SD, $n = 3$.

experiment	**subject**	**%n-6 in HUFA** [%]	**LA** (C18:2 n-6) conc [μM]	**ARA** (C20:4 n-6) conc [μM]	**EPA** (C20:5 n–3) conc [μM]	**DHA** (C22:6 n–3) conc [μM]	**DPA** (C22:5 n–3) conc [μM]
1	1	76.8 ± 0.6	626 ± 93	699 ± 48	36 ± 3	148 ± 16	112 ± 11
	2	74.6 ± 0.6	914 ± 184	745 ± 93	31 ± 5	188 ± 31	116 ± 17
	3	74.7 ± 0.4	538 ± 20	842 ± 56	37 ± 2	207 ± 9	135 ± 5
	4	71.1 ± 0.3	616 ± 40	683 ± 47	35 ± 3	221 ± 12	113 ± 6
2	5	75.4 ± 1.0	764 ± 29	674 ± 23	26 ± 3	222 ± 24	86 ± 6
	6	70.0 ± 0.6	844 ± 41	585 ± 45	50 ± 3	220 ± 9	106 ± 5
	7	76.2 ± 0.3	751 ± 4	709 ± 17	31 ± 1	187 ± 4	105 ± 5
	8	75.9 ± 0.6	1322 ± 117	753 ± 86	44 ± 4	166 ± 15	136 ± 7

Table C.4: Concentrations of oxylipin standards (stock solutions) used for supplementation of the medium analyzed by means of LC-MS/MS.

mix	**analyte**	**concentration**	
		nominal [mM]	measured [mM]
I	4-HDHA	0.29	0.25
	7-HDHA	0.29	0.32
	5-HEPE	0.31	0.18
II	14(*S*)-HDHA	0.29	0.21
	17(*S*)-HDHA	0.29	0.22
	12(*S*)-HEPE	0.31	0.41
	15(*S*)-HEPE	0.31	0.42
III	19(20)-EpDPE	0.29	0.28
	17(18)-EpETE	0.31	0.43

Table C.5: Concentrations of added oxylipins in the media after phagocytosis assay. Macrophages were preincubated with a mix of oxylipins (300 nM) for 1 h. Phagocytosis was started by addition of fluorescent beads and the mix of oxylipins was renewed. After 2 h the medium was collected and analyzed for non-esterified oxylipins by means of LC-MS/MS. Results of one representative experiment are shown as mean ± SD, $n = 3$.

mix	**oxylipin**	**M1-like** conc [nM]	**M2-like** conc [nM]
I	5-HEPE	246 ± 10	173 ± 7
	4-HDHA	162 ± 6	124 ± 9
	7-HDHA	189 ± 5	161 ± 8
II	12(*S*)-HEPE	125 ± 1	105 ± 4
	15(*S*)-HEPE	143 ± 2	115 ± 3
	14(*S*)-HDHA	128 ± 4	110 ± 3
	17(*S*)-HDHA	204 ± 1	158 ± 7
III	17(18)-EpETE	232 ± 8	159 ± 7
	17,18-DiHETE	18 ± 1	26 ± 2
	19(20)-EpDPE	294 ± 13	238 ± 5
	19,10-DiHDPE	14 ± 1	21 ± 1

Table C.6: Concentrations of added oxylipins in the media after phagocytosis assay. Macrophages were preincubated with 100 nM PGE_2 (control) or additionally a mix of oxylipins (300 nM) for 1 h. Phagocytosis was started by addition of fluorescent beads and oxylipins were renewed. After 2 h the medium was collected and analyzed for non-esterified oxylipins by means of LC-MS/MS. Results of one representative experiment are shown as mean ± SD, $n = 3$.

mix	**oxylipin**	**M1-like** conc [nM]	**M2-like** conc [nM]
control	PGE_2	90 ± 2	96 ± 1
I	5-HEPE	12.5 ± 0.4	21 ± 1
	4-HDHA	10 ± 1	14 ± 1
	7-HDHA	12 ± 1	17 ± 2
	PGE_2	86 ± 4	88 ± 2
II	12(*S*)-HEPE	7.9 ± 0.4	10 ± 1
	15(*S*)-HEPE	8.9 ± 0.4	12 ± 1
	14(*S*)-HDHA	8.2 ± 0.4	10 ± 1
	17(*S*)-HDHA	14 ± 2	21 ± 2
	PGE_2	84 ± 2	91 ± 2
III	17(18)-EpETE	17 ± 1	22 ± 2
	17,18-DiHETE	4.1 ± 0.1	2.8 ± 0.2
	19(20)-EpDPE	25 ± 1	29 ± 1
	19,10-DiHDPE	3.0 ± 0.1	1.7 ± 0.2
	PGE_2	87 ± 5	92 ± 9

Table C.7: Statistical analysis of changes in A) %n-6 in HUFA, B) relative FA pattern and C) relative PUFA pattern in macrophages after supplementation with n-3 PUFA. Statistical differences were determined using A) unpaired t-test or B-C) two-way ANOVA followed by Sidak's multiple comparisons test.

A)

experiment	**low vs high**	**t**	**df**	***p* value**	
1	M1-like	20.98	4	*<0.001*	***
	M2-like	12.57	4	*<0.001*	***
2	M1-like	60.98	4	*<0.001*	***
	M2-like	40.49	4	*<0.001*	***

B)

experiment			**mean difference**	**95% CI of difference**	**adjusted *p* value**	
1	low M1 vs high M1	SFA	-0.3	-3.78 to 3.18	*>0.05*	*ns*
		MUFA	1.52	-1.96 to 5.00	*>0.05*	*ns*
		n-6 PUFA	2.62	-0.86 to 6.10	*>0.05*	*ns*
		n-3 PUFA	-3.91	-7.39 to -0.43	0.024	*
1	low M2 vs high M2	SFA	-2.29	-5.66 to 1.07	*>0.05*	*ns*
		MUFA	3.98	0.61 to 7.35	0.017	*
		n-6 PUFA	2.93	-0.43 to 6.31	*>0.05*	*ns*
		n-3 PUFA	-4.65	-8.02 to -1.28	0.005	**
2	low M1 vs high M1	SFA	4.33	-0.04 to 8.70	*>0.05*	*ns*
		MUFA	1.30	-3.07 to 5.67	*>0.05*	*ns*
		n-6 PUFA	1.23	-3.14 to 5.61	*>0.05*	*ns*
		n-3 PUFA	-6.97	-11.3 to -2.60	0.002	**
2	low M2 vs high M2	SFA	2.20	-3.40 to 7.79	*>0.05*	*ns*
		MUFA	1.53	-4.06 to 7.12	*>0.05*	*ns*
		n-6 PUFA	2.94	-2.65 to 8.53	*>0.05*	*ns*
		n-3 PUFA	-6.73	-12.3 to -1.13	0.015	*

C)

experiment			**mean difference**	**95% CI of difference**	**adjusted *p* value**	
1	low M1 vs high M1	other n-6	11.1	9.54 to 12.6	*<0.001*	***
		LA	-1.29	-2.82 to 0.23	*>0.05*	*ns*
		ARA	3.66	2.14 to 5.21	*<0.001*	***
		other n-3	-0.27	-1.79 to 1.26	*>0.05*	*ns*
		EPA	-0.59	-2.12 to 0.94	*>0.05*	*ns*
		DPA	-0.87	-2.40 to 0.66	*>0.05*	*ns*
		DHA	-12.0	-13.5 to -10.4	*<0.001*	***
1	low M2 vs high M2	other n-6	9.87	7.44 to 12.3	*<0.001*	***
		LA	0.43	-2.00 to 2.85	*>0.05*	*ns*
		ARA	4.80	2.38 to 7.22	*<0.001*	***
		other n-3	-0.13	-2.55 to 2.29	*>0.05*	*ns*
		EPA	-0.85	-3.28 to 1.57	*>0.05*	*ns*
		DPA	-0.70	-3.12 to 1.73	*>0.05*	*ns*
		DHA	-13.6	-16.0 to -11.1	*<0.001*	***
2	low M1 vs high M1	other n-6	10.7	8.17 to 13.2	*<0.001*	***
		LA	-0.17	-2.67 to 2.33	*>0.05*	*ns*
		ARA	8.54	5.95 to 11.0	*<0.001*	***
		other n-3	-0.28	-2.79 to 2.22	*>0.05*	*ns*
		EPA	-1.87	-4.38 to 0.63	*>0.05*	*ns*
		DPA	-5.86	-8.36 to -3.36	*<0.001*	***
		DHA	-11.3	-13.8 to -8.82	*<0.001*	***
2	low M2 vs high M2	other n-6	9.29	6.89 to 11.7	*<0.001*	***
		LA	3.26	0.86 to 5.66	0.004	**
		ARA	6.99	4.59 to 9.39	*<0.001*	***
		other n-3	-0.28	-2.69 to 2.12	*>0.05*	*ns*
		EPA	-2.88	-5.28 to -0.48	0.012	*
		DPA	-6.02	-8.42 to -3.62	*<0.001*	***
		DHA	-10.6	-13.0 to -8.17	*<0.001*	***

Table C.8: Statistical analysis of changes in the oxylipin pattern of human macrophages with and without supplementation with n-3 PUFA. Statistical differences were determined using unpaired t-test.

oxylipin	experiment	low vs high	t	df	*p* value	
4-HDHA	1	M1-like	8.12	4	0.0012	**
		M2-like	11.6	4	*<0.001*	***
	2	M1-like	11.2	5	*<0.001*	***
		M2-like	34.8	4	*<0.001*	***
7-HDHA	1	M1-like	4.38	4	0.012	*
		M2-like	4.89	4	0.008	*
	2	M1-like	17.9	4	*<0.001*	***
		M2-like	10.2	4	*<0.001*	***
14-HDHA	1	M1-like	7.25	4	0.002	**
		M2-like	8.22	4	0.001	**
	2	M1-like	12.9	4	*<0.001*	***
		M2-like	4.33	4	0.012	*
17-HDHA	1	M2-like	8.19	4	0.001	**
	2	M2-like	53.0	4	*<0.001*	***
19,20-EpDPE	1	M1-like	25.5	4	*<0.001*	***
		M2-like	11.6	4	*<0.001*	***
	2	M1-like	22.8	4	*<0.001*	***
		M2-like	8.33	4	0.001	**
5-HEPE	1	M1-like	4.46	4	0.011	*
		M2-like	6.70	4	0.003	**
	2	M1-like	23.5	4	*<0.001*	***
		M2-like	42.2	4	*<0.001*	***
12-HEPE	2	M1-like	17.3	4	*<0.001*	***
		M2-like	11.3	4	*<0.001*	***
15-HEPE	1	M2-like	0.35	4	*>0.05*	*ns*
	2	M2-like	4.31	4	0.013	*
17,18-EpETE	1	M1-like	31.1	4	*<0.001*	***
		M2-like	38.8	4	*<0.001*	***
	2	M1-like	10.7	4	*<0.001*	***
		M2-like	10.9	4	*<0.001*	***
5-HETE	1	M1-like	4.04	4	0.016	*
		M2-like	2.18	4	*>0.05*	*ns*
	2	M1-like	3.65	4	0.022	*
		M2-like	5.51	4	0.005	**

oxylipin	experiment	low vs high	t	df	*p* value	
11-HETE	1	M1-like	18.4	4	*<0.001*	***
		M2-like	11.0	4	*<0.001*	***
	2	M1-like	9.05	4	*<0.001*	***
		M2-like	13.7	4	*<0.001*	***
12-HETE	1	M1-like	3.17	4	0.034	*
		M2-like	7.39	4	0.002	**
	2	M1-like	2.87	4	0.045	*
		M2-like	5.75	4	0.005	**
15-HETE	1	M1-like	2.55	4	*>0.05*	*ns*
		M2-like	15.5	4	*<0.001*	***
	2	M1-like	2.86	4	0.046	*
		M2-like	7.05	4	0.002	**
14,15-EpETrE	1	M1-like	6.01	4	0.004	**
		M2-like	5.34	4	0.006	**
	2	M1-like	8.91	4	*<0.001*	***
		M2-like	7.86	4	0.001	**
12-HHTrE	1	M1-like	9.92	4	*<0.001*	***
		M2-like	18.7	4	*<0.001*	***
	2	M1-like	8.59	4	0.001	**
		M2-like	5.76	4	0.005	**
PGE_2	1	M1-like	1.28	4	*>0.05*	*ns*
		M2-like	1.43	4	*>0.05*	*ns*
	2	M1-like	3.02	4	0.040	*
		M2-like	6.50	4	0.003	**
PGD_2	1	M1-like	1.00	4	*>0.05*	*ns*
		M2-like	4.56	4	0.010	*
	2	M1-like	1.94	4	*>0.05*	*ns*
		M2-like	3.55	4	0.024	*
$PGF_{2\alpha}$	1	M1-like	5.73	4	0.005	*
		M2-like	1.49	4	*>0.05*	*ns*
	2	M1-like	2.89	4	0.045	*
		M2-like	1.65	4	*>0.05*	*ns*
TxB_2	1	M1-like	15.3	4	*<0.001*	***
		M2-like	62.5	4	*<0.001*	***
	2	M1-like	8.59	4	0.001	**
		M2-like	5.78	4	0.004	**

List of Abbreviations

AC	adenylyl cyclase
ACN	acetonitrile
ARA	arachidonic acid
BCA	bicinchoninic acid assay
cAMP	cyclic adenosine monophosphate
CE	collision energy
COX	cyclooxygenase
CSF	colony-stimulating factor
CXCL8	C-X-C motif ligand 8
CYP	cytochrome P450 monooxygenase
DAPI	4',6-diamidino-2-phenylindole
DHA	docosahexaenoic acid
DiHEPE	dihydroxy-eicosapentaenoic acid
DiHDHA	dihydroxy-docosahexaenoic acid
DiHDPE	dihydroxy-docosapentaenoic acid
DiHETE	dihydroxy-eicosatetraenoic acid
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
EP	prostaglandin E_2 receptor
EPA	eicosapentaenoic acid

EpDPE	epoxy-docosapentaenoic acid
EpETE	epoxy-eicosatetraenoic acid
ESI	electrospray ionization
FA	fatty acid
FADS	fatty acid desaturase
FID	flame ionization detector
GC	gas chromatography
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
HDHA	hydroxy-docosahexaenoic acid
HEPE	hydroxy-eicosapentaenoic acid
HETE	hydroxy-eicosatetraenoic acid
HHTrE	hydroxy-heptadecatrienoic acid
HODE	hydroxy-octadecadienoic acid
HUFA	highly unsaturated fatty acid
IFNγ	interferon γ
IL	interleukin
IS	internal standard
IsoP	isoprostane
LLOQ	lower limit of quantification
LOX	lipoxygenase
LPS	lipopolysaccharide
MaR	maresin
M-CSF	macrophage colony-stimulating factor

MeOH	methanol
MRC1	mannose receptor C-type 1
MS	mass spectrometry
MUFA	monounsaturated fatty acid
NP	neuroprotectin
NSAID	non-steroidal anti-inflammatory drug
P/S	penicillin/streptomycin
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PG	prostaglandin
PMA	phorbol 12-myristate 13-acetate
PPAR	peroxisome proliferator-activated receptor
PRR	pattern recognition receptor
PUFA	polyunsaturated fatty acid
Rv	resolvin
SD	standard deviation
SEM	standard error of the mean
SFA	saturated fatty acid
SPE	solid phase extraction
SPM	specialized pro-resolving mediator
SRM	selected reaction monitoring
THP-1	human monocyte cell line
TNFα	tumor necrosis factor α

Tx	thromboxane
XIC	extracted ion chromatogram

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Diese Dissertation wurde von März 2021 bis Oktober 2025 am Lehrstuhl für Lebensmittelchemie der Bergischen Universität Wuppertal in der Arbeitsgruppe von Prof. Dr. Nils Helge Schebb angefertigt. Am Ende dieser Jahre, die von Herausforderungen, Lernmomenten und schönen Begegnungen geprägt waren, möchte ich mich bei allen Menschen bedanken, die mich während dieser Zeit begleitet und auf vielfältigste Weise zum Gelingen meiner Dissertation beigetragen haben.

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List of Publications

Publications in peer-reviewed journals

Within the scope of this thesis

Kirchhoff R, Kampschulte N, Rothweiler C, Rohwer N, Weylandt KH, Schebb NH. An optimized *ex vivo* supplementation strategy for primary human macrophages shows that DHA suppresses prostaglandin E2 formation. *Molecular Nutrition and Food Research* **2025** *69*(1), e202400716. DOI: 10.1002/mnfr.202400716.

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Kirchhoff R, Chromik MA, Schebb NH. Phagocytosis of primary human macrophages is elevated by ex vivo supplementation with n–3 PUFA. [Manuscript submitted for publication]

Further publications

Hartung NM, Mainka M, Pfaff R, Kuhn M, Biernacki S, Zinnert L, Schebb NH. "Development of a quantitative proteomics approach for cyclooxygenases and lipoxygenases in parallel to quantitative oxylipin analysis allowing the comprehensive investigation or the arachidonic acid cascade. *Analytical and Bioanalytical Chemistry* **2023**, *415*, 913-933. DOI: 10.1007/s00216-022-04489-3.

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Palmer MA, Kirchhoff R, Hahnefeld L, Aliraj B, Benatzy Y, Weigert A, Schebb NH, Brüne B. Alox8 knockout exacerbates imiquimod-induced psoriasis-like inflammation. [Manuscript submitted for publication]

Conference contributions

Pfaff R, Hartung NM, Mainka M, Zinnert L, Schebb NH. Characterization of human macrophages by LC-MS based analysis of oxylipins and protein abundance from a single cell pellet. Poster presented at *8th European Workshop on Lipid Mediators*; **2022** Jun 29-Jul 1; Stockholm, Sweden.

Pfaff R, Rothweiler C, Schebb NH. Development of an n–3 PUFA supplementation strategy in primary human macrophages. Poster presented at *15th International ISSFAL Congress*; **2023** Jul 2-5; Nantes, France.

Kirchhoff R, Chromik MA, Schebb NH. Modulation of phagocytosis by EPA derived oxylipins in human macrophages. Poster presented at *Annual Meeting of the German Pharmaceutical Society (DPhG)*; **2023** Oct 7-10; Tübingen, Germany.

Kirchhoff R, Rothweiler C, Rohwer N, Weylandt KH, Schebb NH. Ex-vivo supplementation of primary human macrophages with n–3 PUFA. Poster presented at *19th International Winter Eicosanoid Conference*; **2023** Oct 15-17; Baltimore, Maryland, USA.

Kirchhoff R, Rothweiler C, Schebb NH. How to supplement primary human macrophages with n–3 PUFA to mimic nutrition intervention studies. Short-Talk presented at *International School of Pharmacology "Giampaolo Velo"*; **2024** March 25-28; Erice, Italy.

Kirchhoff R, Chromik MA, Schebb NH. Differential regulation of phagocytosis by LPS via Prostaglandin E2 formation and EP4 signaling in primary human macrophages. Poster and Short-Talk presented at *Annual PhD Student & Postdoc Meeting of the German Pharmaceutical Society (DPhG)*; **2025** Feb 26-28; Graz, Austria.