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Functional differences between primary monocyte-derived and THP-1 macrophages and their response to LCPUFAs

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ABSTRACT

Background: In immune cell models, macrophages are one of the most frequently used cell types. THP-1 cells are often used as model to study macrophage function, however they may act differently from primary human monocyte derived macrophages (MDMs).
Methods: In this study, we investigated the intrinsic baseline differences between the human macrophage cell line THP-1 and human primary MDMs. Additionally, we studied the difference in response to treatment with long-chain polyunsaturated fatty acids (LCPUFAs): well-described immunomodulators.
Results: Although the amount of cells that phagocytose were similar between the cell types, primary MDMs consumed significantly more *E. coli* bioparticles compared to THP-1 macrophages. In M1 macrophages, IL-12 secretion was almost fifty times higher by primary MDMs compared to THP-1 macrophages, thereby increasing the IL-12/IL-10 ratio. Despite this, the IL-12 secretion by THP-1 M1 macrophages was higher that the secretion of IL-10, thereby showing that it is still a suitable M1 type. Cytokine profiles differed between primary MDMs and THP-1 M1 and M2 macrophages. In response to LCPUFAs, primary M1 MDMs and THP-1 M1 macrophages were alike. Interestingly, primary M2 MDMs secreted less IL-10 and CCL22 when treated with LCPUFAs, whereas THP-1 M2 macrophages secreted more IL-10 when treated with LCPUFAs and showed no difference in CCL22 secretion.
Conclusions: In conclusion, in an M1 setting, both THP-1 and primary MDMs are suitable models. However, when interested in M2 models, the model choice highly depends on the research question.

1. Introduction

Macrophages are mononuclear phagocytes that differentiate from monocytes when entering tissues. Furthermore, they are classified as antigen-presenting cells (APCs). As a part of the innate immune system, macrophages respond to pathogens invading tissues, but are also very important in wound repair. Macrophages can be classified as the classically activated, pro-inflammatory M1, which are important in defense against pathogens, or the alternatively activated anti-inflammatory M2, which are important in processes such as wound healing and homeostasis. In general, M1 macrophages are defined as macrophages that have a high IL-12/IL-10 ratio, and M2 macrophages are defined as macrophages that have a high IL-10/IL-12 ratio. In addition, they secrete a large range of other pro-inflammatory cytokines and chemokines (CCL), Over the years, it has become clear that M2 macrophages can be subdivided into multiple phenotypes, e.g. M2a, M2b and M2c [1], and that their polarization is more like a spectrum rather than a hard distinction [2]. Macrophages differentiate towards M1 when stimulated with e.g. lipopolysaccharide (LPS), tumor necrosis factor α (TNFα) and/or interferon γ (IFNγ), and secrete pro-inflammatory cytokines including IL-12, TNFα, IL-1β, IL-6 and IL-23. The M2a macrophage phenotype can be obtained using IL-4 and/or IL-13, resulting in a secretion of IL-10, TGFβ, IL-1RA, CCL17, CCL22 and CCL24. As regulatory macrophage M2c, obtained by incubation with IL-10, also produces IL-10 and TGFβ, it is very hard to distinguish between M2a and M2c macrophages. Finally, M2b macrophages can be obtained using a

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combination of LPS, IL-1RA and immunocomplexes, and produce IL-10, TNFα, IL-1β and IL-6. As there is much overlap in differentiation triggers and a large need for quick polarization from one phenotype to the other in a dynamic environment such as immune organs and mucosal tissues, macrophages have a high plasticity [3].

Macrophages are involved in almost every inflammatory process in the human body. They are not only able to clear a site from pathogens, their secreted cytokines can also actively attract other immune cells such as, in the case of M1, Th1 and natural killer (NK) cells and, in the case of M2, Th2 cells [4]. Therefore, they have been studied extensively in many processes and diseases, including wound healing [5,6], cancer [7,8], myocardial infarctions [9–11], atherosclerosis [12,13], COPD [14] and neuroinflammation [15,16]. In order to properly study macrophages *in vitro*, multiple cell models can be used. The mostly commonly used human cell types are primary monocyte-derived macrophages (MDMs), derived from healthy human donor blood monocytes, or the THP-1 immortalized cell line. The advantage of using primary MDMs is that it will more closely represent the *in vivo* situation. However, some large disadvantages include the large variance in responses due to differences in biological response between donors, and not being able to sub-culture them for a long period of time. Therefore, a cell line such as THP-1 can be advantageous, as they do not show much variation between passages and can be cultured over a longer period of time, which allows to obtain a clearer picture of variation in effects of triggers to which the cells are exposed, rather than this being obscured by phenotypic variation. However, their functional response might not represent the *in vivo* situation as well as primary MDMs do. Previous studies have investigated differences between THP-1 derived macrophages and primary MDMs in gene expression [17,18], phagocytosis and cytokine production [19,20]. These studies already indicated differences between M0, M1 and M2 THP-1 derived macrophages and primary MDMs. Therefore, it would be interesting to further investigate their functional response in immunomodulatory properties which we addressed in this paper by exposing the cells to LCPUFA.

In order to more closely investigate whether the immunomodulatory properties of primary MDMs and THP-1 macrophages are similar, they can be treated with compounds known to modulate the immune response. This has been well-described for long-chain polyunsaturated fatty acids (LCPUFAs), although mostly in dendritic cells (DCs), T-cells and mast cells (MCs)[21]. Omega-3 (n-3) PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have both been described to have an anti-inflammatory effect [22,23]. These LCPUFAs can both be obtained by consuming fatty fish or taking fish or algae oil capsules, or they can, at a low rate, be elongated and desaturation from ALA (α-linolenic acid). Furthermore, omega-6 (n-6) LCPUFA arachidonic acid (AA) has also been shown to modulate immune cells, most of the time in a pro-inflammatory manner [24,25]. AA can be obtained by consuming seeds and (pea)nuts or animal products, or it can be elongated and desaturated from LA (linoleic acid). As both n-3 and n-6 LCPUFAs are immunoregulatory, we will use AA, EPA and DHA in this study to investigate the differences in response to immunomodulators in primary M1 and M2 MDMs and THP-1 M1 and M2 macrophages.

2. Material and methods

2.1. Monocyte isolation

Primary monocytes were isolated as described previously [26]. Briefly, purchased buffy coats from healthy donors (Sanquin, Nijmegen, The Netherlands) were diluted 1:1 with phosphate-buffered saline (PBS) + 2% fetal bovine serum (FBS) (HyCloneTM Fetal Bovine Serum, Fisher Scientific, Loughborough, UK) and loaded onto Greiner Bio-One™ LeucoSEPTM Polypropylene Tubes. The peripheral blood mononuclear cells (PBMCs) were washed and loaded onto MACS columns, using the CD14 microbead kit according to the manufacturer's protocol (Miltenyi Biotec, Leiden, The Netherlands). All cells were frozen in FBS with 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen until further use.

2.2. Macrophage differentiation

Primary monocytes were plated into a 24-well plate at a concentration of 0.5×10^6 cells/mL in RPMI-1640 with HEPES and Glutamax (Lonza, Basel, Switzerland) supplemented with 10% FBS, 1% penicillin and streptomycin, 1% sodium pyruvate (all from Thermo Fisher, Landsmeer, The Netherlands) and 1% non-essential amino acids (Sigma Aldrich, Zwijndrecht, The Netherlands). Medium was refreshed at days 3 and 5 and the cells could be used from day 7 onwards.

THP-1 acute monocytic leukemia cells (ATCC, Manassas, VA, USA) were sub-cultured twice a week at a concentration of 0.25×10^6/mL in a T75 flask in RPMI-1640 with HEPES and Glutamax (Lonza, Basel, Switzerland) supplemented with 10% FBS and 1% penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO, USA).

For the experimental setting, cells were plated into a 24-well plate at a concentration of 0.5×10^6 cells/mL in 1 mL To initiate differentiation towards THP-1 macrophages (M0), 100 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Zwijndrecht, The Netherlands) was added. Following 48 h of differentiation, the cells were washed twice with medium and rested for 5 days. For both primary MDMs and THP-1 M1 macrophages, the medium was supplemented with 50 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF;R&D Systems, Minneapolis, USA) and for M2 macrophages, the medium was supplemented with 50 ng/mL macrophage colony-stimulating factor (M-CSF;R&D Systems, Minneapolis, USA). Cells were either differentiated towards M1 using 1 μg/mL LPS (Sigma Aldrich, Zwijndrecht, The Netherlands) and 20 ng/mL IFNγ (Sigma Aldrich, Zwijndrecht, The Netherlands) or towards M2 using 20 ng/mL IL-4 (Sigma Aldrich, Zwijndrecht, The Netherlands) for 24 h.

2.3. PUFA experiments

Before differentiation towards M1 or M2, macrophages were incubated with 50 μM of either AA, EPA or DHA and 25 μM vitamin E in FBS and 75 μM vitamin C (all from Sigma Aldrich, Zwijndrecht, The Netherlands) for 48 h. Vitamins were added to prevent lipid oxidation. To control for the effect of vitamins C and E, a vitamin control was included in the assay. Then, without changing the medium, the macrophages were differentiated towards M1 or M2 (see Section 2.2) and the cells and supernatants were harvested 24 h later.

2.4. Phagocytosis assay

After collection of the supernatant, 0.5 mL medium + 4 μg/mL AlexaFluor-conjugated *Escherichia coli* (K-12 strain) BioParticles® (Molecular Probes, Life Technologies, Eugene, OR, USA) was added to each well. The cells were incubated for 1 h at 37 °C and washed twice with PBS. Macrophages were harvested using 0.25% Trypsin/EDTA and measured using a CytoFlex (Beckman Coulter, Brea, USA) on the FITC-A channel. MFI was presented in arbitrary units (AU).

2.5. Enzyme-linked immunosorbent assay (ELISA)

In the supernatant of the macrophages, IL-12/IL-23(p40), IL-10 and CCL22 were measured by ELISA according to the manufacturer's protocol (BioLegend, Koblenz, Germany). All samples were measured using a Tecan Infinite 200PRO (Tecan, Männedorf, Switzerland). As CCL22 is an M2 marker, only the supernatants of M2 macrophages were measured.

2.6. LEGENDplex

To obtain a broader overview of differences in cytokine secretion

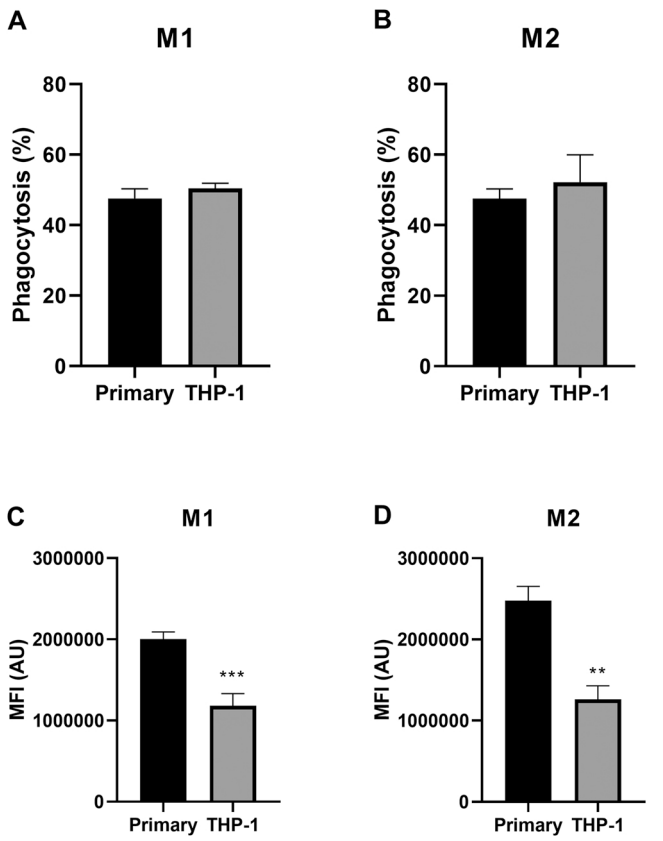

Fig. 1. Phagocytosis of fluorescently labeled E. coli by M1 and M2 primary MDMs and THP-1 macrophages. (A) the percentage of M1 macrophages that consumed E. coli particles. (B) the percentage of M2 macrophages that consumed E. coli particles. (C) the amount of E. coli particles consumed by M1 macrophages. (D) the amount of E. coli particles consumed by M2 macrophages. * * $p < 0.01$, * ** $p < 0.001$.

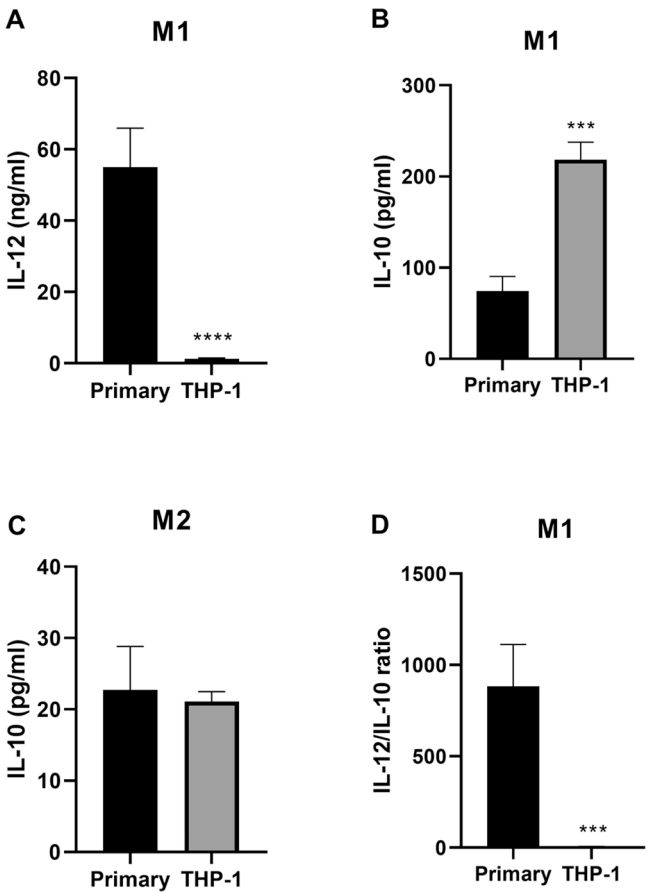

Fig. 2. IL-12p40 and IL-10 secretion of primary MDMs and THP-1 macrophages. (A) IL-12p40 secretion of M1 macrophages. (B) IL-10 secretion of M1 macrophages. (C) IL-10 secretion of M2 macrophages. (D) The IL-12/IL-10 ratio of M1 macrophages. * ** $p < 0.001$, * ** * $p < 0.0001$.

between primary MDMs and THP-1 macrophages, a LEGENDplex panel for Human Macrophage/Microglia, was used according to the manufacturer's protocol (BioLegend, Koblenz, Germany). Cytokines in the Human Macrophage/Microglia panel included IL-12p70, TNFα IL-6, IL-4, IL-10, IL-1β arginase, TARC, IL-1RA, IL-12p40, IL-23, IFNγ, and IP-10. All samples were measured using a CytoFLEX flow cytometer (Beckman Coulter, Woerden, The Netherlands), and data were analyzed using the BioLegend LEGENDplex cloud-based software.

2.7. Statistics

All experiments were repeated six times independently with different primary donor cells or THP-1 passages (n = independent biological replicates). Statistical analyses were carried out using Graphpad Prism 8. All parameters are presented as means ± SEM. A One-way ANOVA with a Dunnett post-hoc to correct for multiple comparisons was used to assess the parameters for significance ($p < 0.05$; $p < 0.1$ is considered a trend), except for the comparisons between primary and THP-1 macrophages, where an unpaired t-test was used. All groups were only compared to the non-treated control (NT). Outliers were identified using the ROUT method with Q= 1%. Graphs were plotted using Graphpad Prism 8.

3. Results

3.1. Phagocytosis activity of primary MDMs and THP-1 macrophages

To evaluate phagocytosis, both the percentage (%) of macrophages that consumed the fluorescent *E. coli* particles as well as the amount of *E. coli* particles that was consumed per macrophage (mean fluorescent intensity, MFI) were taken into account. There was no difference between the percentage of cells that consumed *E. coli* particles by primary MDMs and THP-1 macrophages, in both M1 and M2 (Fig. 1A and B). However, both M1 and M2 primary MDMs consumed significantly more *E. coli* particles compared to the THP-1 macrophages ($p = 0.0009$ and $p = 0.0016$, respectively) (Fig. 1C and D). Furthermore, both primary MDMs and THP-1 M2 macrophages consumed significantly more *E. coli* particles compared to M1 macrophages ($p = 0.04$ and $p = 0.03$, respectively), but the percentage of macrophages that consumed *E. coli* particles did not differ (supplemental Fig. 1).

3.2. Differences in IL-12 and IL-10 secretion by M1 macrophages

A typical marker for M1 macrophages is IL-12, whilst a marker for M2 macrophages is IL-10. Primary M1 MDMs secreted around 50 times more IL-12p40 compared to THP-1 M1 macrophages ($p = 0.0006$) (Fig. 2A). Neither primary MDMs nor THP-1 M2 macrophages produced IL-12p40. Whilst there was no difference between M2 IL-10 secretion, THP-1 M1 macrophages secreted significantly more IL-10 compared to primary M1 MDMs ($p = 0.0003$) (Fig. 2B and C). Noteworthy, both M1 THP-1 macrophages and primary MDMs secreted significantly more IL-10 compared to M2 macrophages ($p = 0.01$ and $p < 0.0001$, respectively). However, the IL-10/IL-12 ratio would still be higher in M2 macrophages, as no IL-12 is secreted, and similar between primary MDMs and THP-1 macrophages as the IL-10 secretion is also similar. Finally, the IL-12/IL-10 ratio in primary M1 MDMs was significantly

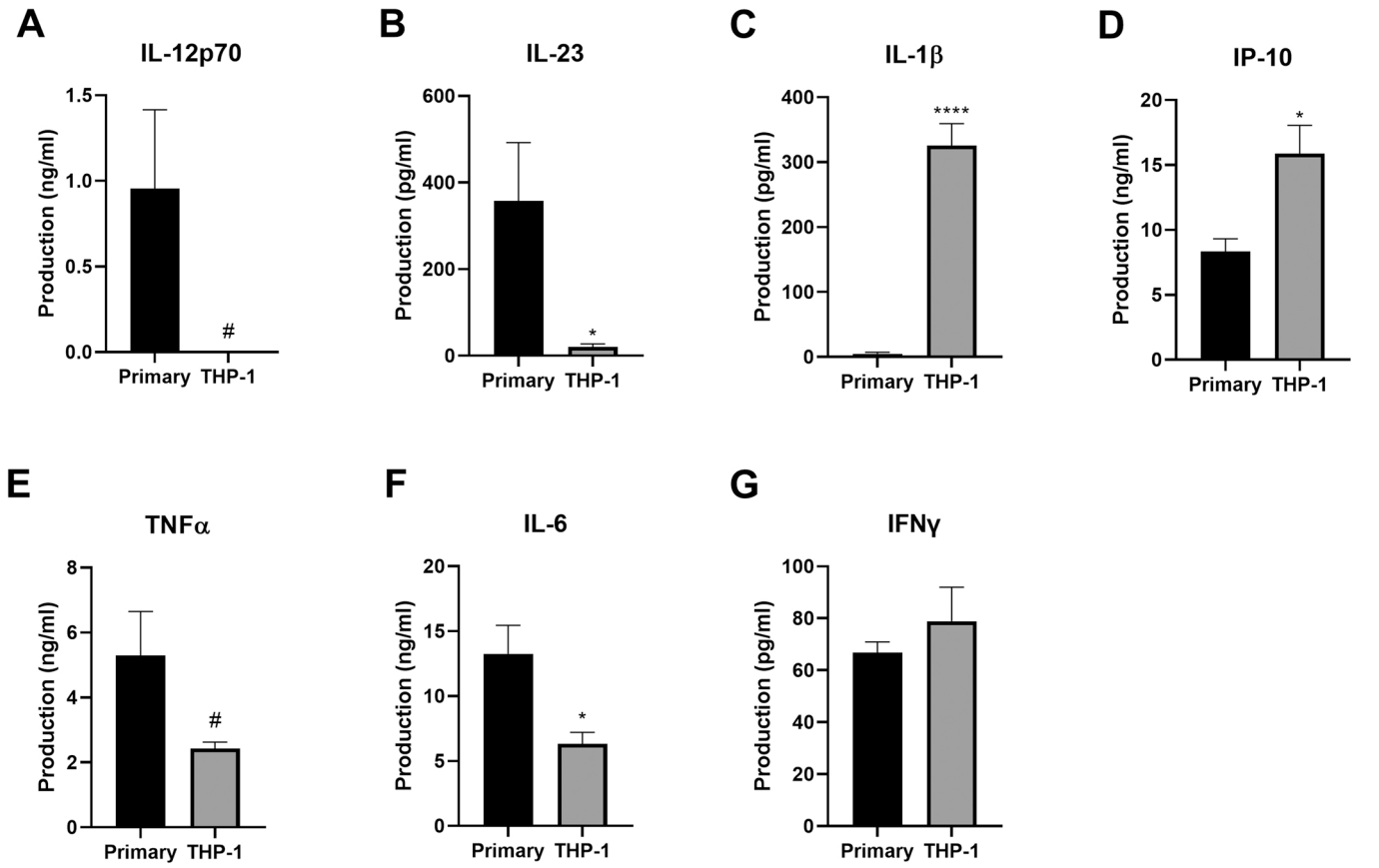

Fig. 3. Secretion of cytokines by M1 macrophages. (A) IL-12p70 secretion. (B) IL-23 secretion. (C) IL-1β secretion. (D) IP-10 secretion. (E) TNFα secretion. (F) IL-6 secretion. (G) IFNγ secretion. # $p < 0.1$, * $p < 0.05$, * ** * $p < 0.0001$.

higher compared to M1 THP-1 macrophages, reflected by the high IL-12 secretion by primary M1 MDMs ($p = 0.002$) (Fig. 2D). Primary M1 MDMs secreted almost 900 times more IL-12 than IL-10, whist THP-1 M1 macrophages secreted 6 times more IL-12 than IL-10.

3.3. Differences in M1 and M2 cytokine secretion

In order to obtain more insight in differences between the general cytokine and chemokine secretion pattern of primary MDMs and THP-1 macrophages, several more M1 (IL-12p70, IL-23, TNFα, IL-1β, IL-6, IFNγ and IP-10) and M2 (IL-4, CCL17, CCL22, IL-1RA) cytokines were measured (Figs. 3 and 4 and Supplemental Table 1 and 2). As expected, following the pattern found in IL-12p40 secretion, IL-12p70 and IL-23 were secreted in higher amounts by primary M1 MDMs compared to THP-1 M1 macrophages (Fig. 3A and B and Supplemental Table 1) ($p = 0.08$ and $p = 0.03$, respectively), although not significant for IL-12p70. By contrast, IL-1β and IP-10 were secreted significantly more by THP-1 M1 macrophages (Fig. 3C and D and Supplemental Table 1) ($p < 0.0001$ and $p = 0.01$, respectively). Furthermore, the secretion of IL-6 was significantly higher in primary M1 MDMs, and TNFα showed the same trend (Fig. 3E and F and Supplemental Table 1) ($p = 0.02$ and $p = 0.06$, respectively). Finally, the secretion of IFNγ was similar in primary M1 MDMs and THP-1 M1 macrophages (Fig. 3G and Supplemental Table 1).

Regarding M2 macrophages, the secretion of IL-4 and IL-1RA were similar between primary M2 MDMs and THP-1 M2 macrophages (Fig. 4A and D and Supplemental Table 2). CCL17 and CCL22 secretion was higher in primary MDMs compared to THP-1 macrophages (Fig. 4B and C and Supplemental Table 2) ($p = 0.03$ and $p < 0.0001$, respectively). Noteworthy, IL-1RA secretion was significantly higher in THP-1 M1 macrophages compared to THP-1 M2 macrophages (Supplemental Table 1 and 2).

To obtain more insight in the propensity of both MDM and THP-1 macrophages towards M1 or M2 differentiation, all secreted cytokines were compared between the M1 and M2 phenotypes of each cell type (Table 1). Except for IL-1β and IFNγ, which were in both phenotypes near the lower detection limit, all M1 cytokines are secreted significantly more by the primary M1 MDMs compared to the primary M2 MDMs, although IL-12p70 and IL-1RA only showed a trend. All M1 cytokines are significantly more secreted by THP-1 M1 macrophages compared to THP-1 M2 macrophages, except for IL-12p70, which was near the lower detection limit in both phenotypes. Interestingly, M2 cytokine IL-10 was secreted significantly more by both primary M1 MDMs and THP-1 M1 macrophages than M2 samples. M2 cytokine CCL17 was secreted more by primary M2 MDMs, but no difference was observed between CCL17 secretion by THP1 M1 and M2 macrophages. M2 cytokine IL-4 was in both primary MDMs and THP-1 macrophages detected in higher amounts in M2 samples, however variation was too large to detect statistical significance. CCL22 secretion was only measured in the M2 macrophages, and can therefore not be included in the analysis.

In summary, primary MDMs secreted higher amounts of five out of eight measured M1 cytokines and two out of five measured M2 cytokines/chemokines compared to THP-1 macrophages. THP-1 macrophages secreted higher amounts of two out of eight measured M1 cytokines and none of the M2 cytokines. Secretion of one of the M1 cytokines and three of the M2 cytokines were similar. Both primary MDMs and THP-1 macrophages are committed to the M1 phenotype when exposed to LPS and IFNγ, but primary MDMs seem to be slightly more committed to the M2 phenotype, as based on the IL-10/IL-12 ratio, CCL17 and CCL22 secretion.

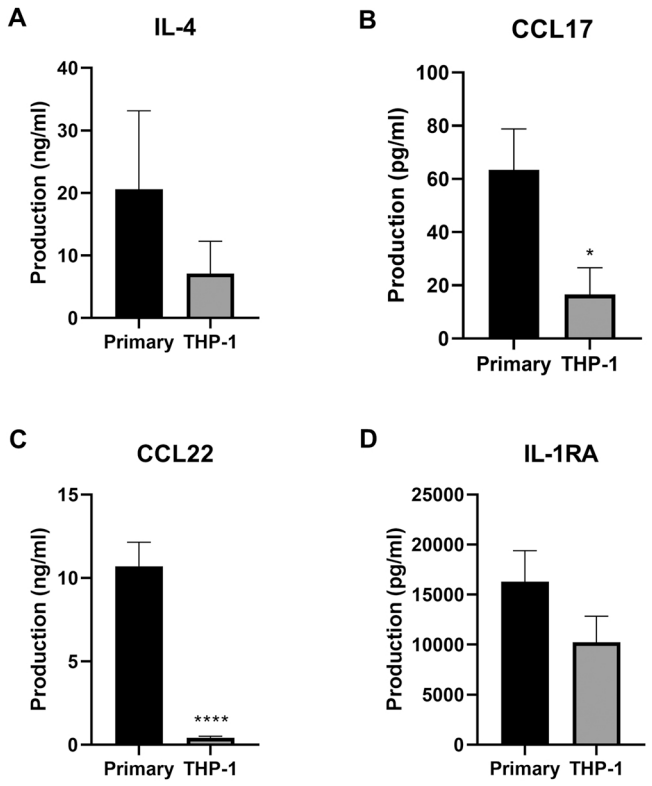

Fig. 4. Fig. 3: Secretion of cytokines by M2 macrophages. (A) IL-4 secretion. (B) CCL17 secretion. (C) CCL22 secretion. (D) IL-1RA secretion. * $p < 0.05$, * ** * $p < 0.0001$.

3.4. M2 primary MDMs respond differently to LCPUFAs compared to THP-1 macrophages

When treated with LCPUFAs prior to differentiation, phagocytosis remained unchanged in both primary M1 and M2 MDMs and THP-1 M1 and M2 macrophages (supplemental Fig. 2). This was observed for both the percentage of macrophages consuming *E. coli* particles (%), as well as the number of *E. coli* particles that were consumed per macrophage (MFI).

Between primary M1 MDMs and THP-1 M1 macrophages, a slightly different trend in cytokine response could be observed. In both primary MDMs and THP-1 macrophages, AA, EPA and DHA decreased the IL-12p40 secretion significantly compared to the untreated control (Fig. 5A) ($p = 0.002$, $p = 0.006$ and $p = 0.04$, respectively). EPA also significantly lowered the IL-12p40 secretion in THP-1 macrophages ($p = 0.05$), whereas AA and DHA showed a trend in lowering the IL-12p40 secretion ($p = 0.08$ and $p = 0.07$, respectively). IL-23, which is strongly correlated to IL-12 secretion, was lowered, although not significantly, by EPA in both primary M1 MDMs and THP-1 M1 macrophages (Fig. 5B) ($p = 0.1$ and $p = 0.09$, respectively). Additionally, AA and DHA lowered the secretion of IL-23 non-significantly by THP-1 macrophages, but not primary MDMs ($p = 0.1$ and $p = 0.08$, respectively). Furthermore, AA, EPA and DHA all significantly lowered the IL-1β secretion in THP-1 macrophages ($p = 0.006$, $p = 0.001$ and $p = 0.003$, respectively), but not in primary MDMs, where the secretion of IL-1β was already very low (Fig. 5C). Finally, the secretion of TNFα was significantly lowered by AA in THP-1 M1 macrophages ($p = 0.0007$) and a lowering trend was observed after treatment with EPA ($p = 0.09$), whereas in primary MDMs, it was similar to the untreated control (Fig. 5D). Interestingly, IL-6 secretion was significantly higher when THP-1 macrophages were treated with vitamin alone ($p = 0.05$) (Supplemental Fig. 3). No differences were found in secretion of IL-6, IFNγ, IP-10 and IL12p70 after treatment with LCPUFAs by primary M1 MDMs or THP-1 M1 macrophages (Supplemental Fig. 3).

Between primary M2 MDMs and THP-1 M2 macrophages, the response to LCPUFA treatment differed largely. Both EPA and DHA decreased IL-10 secretion in primary MDMs ($p = 0.002$ and $p = 0.01$, respectively), whereas in THP-1 macrophages, DHA increased the secretion of IL-10 (Fig. 6A) ($p = 0.04$). Furthermore, although not significantly, AA increased the secretion of IL-10 in THP-1 macrophages ($p = 0.07$), but not in primary MDMs. These large differences could also be found for CCL22 secretion, which was significantly lowered by AA and DHA in primary MDMs ($p = 0.02$ and $p = 0.001$, respectively), but not in THP-1 macrophages (Fig. 6B). IL-1RA secretion was significantly lowered by DHA in primary MDMs ($p = 0.03$), but not in THP-1 M2 macrophages (Fig. 6C). CCL17 and IL-4 secretion were not affected by LCPUFA treatment in both primary M2 MDMs and THP-1 M2 macrophages (Supplemental Fig. 4).

In summary, THP-1 M1 macrophages seem to be more sensitive to modulation by LCPUFAs. Surprisingly, not only are primary M2 MDMs more sensitive to modulation by LCPUFAs, they lower the secretion of IL-10, CCL22 and IL-1RA, whereas in THP-1 M2 macrophages, they increase the secretion of IL-10.

4. Discussion

In this study, we have shown that there are some crucial differences between primary MDMs and THP-1 macrophage models. In addition to the existing literature comparing THP-1 derived macrophages and primary MDMs, this is the first study that compares the functional

Table 1
Comparison between M1 and M2 cytokine secretion. Bold text indicates a significant expected difference between M1 and M2. Red indicates a significant difference, orange indicates a trend.

Cytokine	Primary (pg/mL)			THP-1 (pg/mL)		
M1 cytokines						
	M1	M2	P-value	M1	M2	P-value
IL-12p40	$5.5 \times 10^4 \pm 1.1 \times 10^4$	0 ± 0	**0.004**	$0.1 \times 10^4 \pm 0.02 \times 10^4$	0 ± 0	**0.002**
IL-12p70	$9.6 \times 10^2 \pm 4.6 \times 10^2$	1.2 ± 0.8	**0.09**	0.4 ± 0.2	0 ± 0	0.1
IL-23	$3.6 \times 10^2 \pm 1.4 \times 10^2$	4.7 ± 2.9	**0.04**	21 ± 6.6	1.6 ± 1.0	**0.04**
TNFα	$5.3 \times 10^3 \pm 1.4 \times 10^3$	9.0 ± 5.0	**0.009**	$2.4 \times 10^3 \pm 0.2 \times 10^3$	7.2 ± 4.7	**< 0.0001**
IL-1β	4.7 ± 2.3	9.5 ± 4.8	0.4	$3.3 \times 10^2 \pm 0.3 \times 10^2$	20 ± 3.6	**0.0003**
IL-6	$1.3 \times 10^4 \pm 0.2 \times 10^4$	6.0 ± 0.7	**0.002**	$6.3 \times 10^3 \pm 0.9 \times 10^3$	1.7 ± 0.3	**0.0008**
IFNγ	67 ± 4.1	36 ± 20	0.2	75 ± 12	0.6 ± 0.5	**0.003**
IP-10	$8.3 \times 10^3 \pm 1.0 \times 10^3$	17 ± 5.1	**0.0004**	$1.6 \times 10^4 \pm 0.2 \times 10^4$	$1.7 \times 10^2 \pm 0.4 \times 10^2$	**0.0008**
IL-1RA	$2.8 \times 10^4 \pm 0.7 \times 10^4$	$1.6 \times 10^4 \pm 0.3 \times 10^4$	**0.08**	$2.2 \times 10^4 \pm 0.3 \times 10^4$	$1.0 \times 10^4 \pm 0.3 \times 10^4$	**0.008**
M2 cytokines						
IL-10	74 ± 16	23 ± 6.1	0.007	$2.2 \times 10^2 \pm 19$	21 ± 1.4	0.00002
IL-4	0.9 ± 0.4	$2.1 \times 10^4 \pm 1.3 \times 10^4$	0.2	1.2 ± 0.4	$0.7 \times 10^4 \pm 0.5 \times 10^4$	0.2
CCL17	14 ± 1.3	66 ± 19	**0.06**	9.3 ± 1.2	17 ± 10	0.5

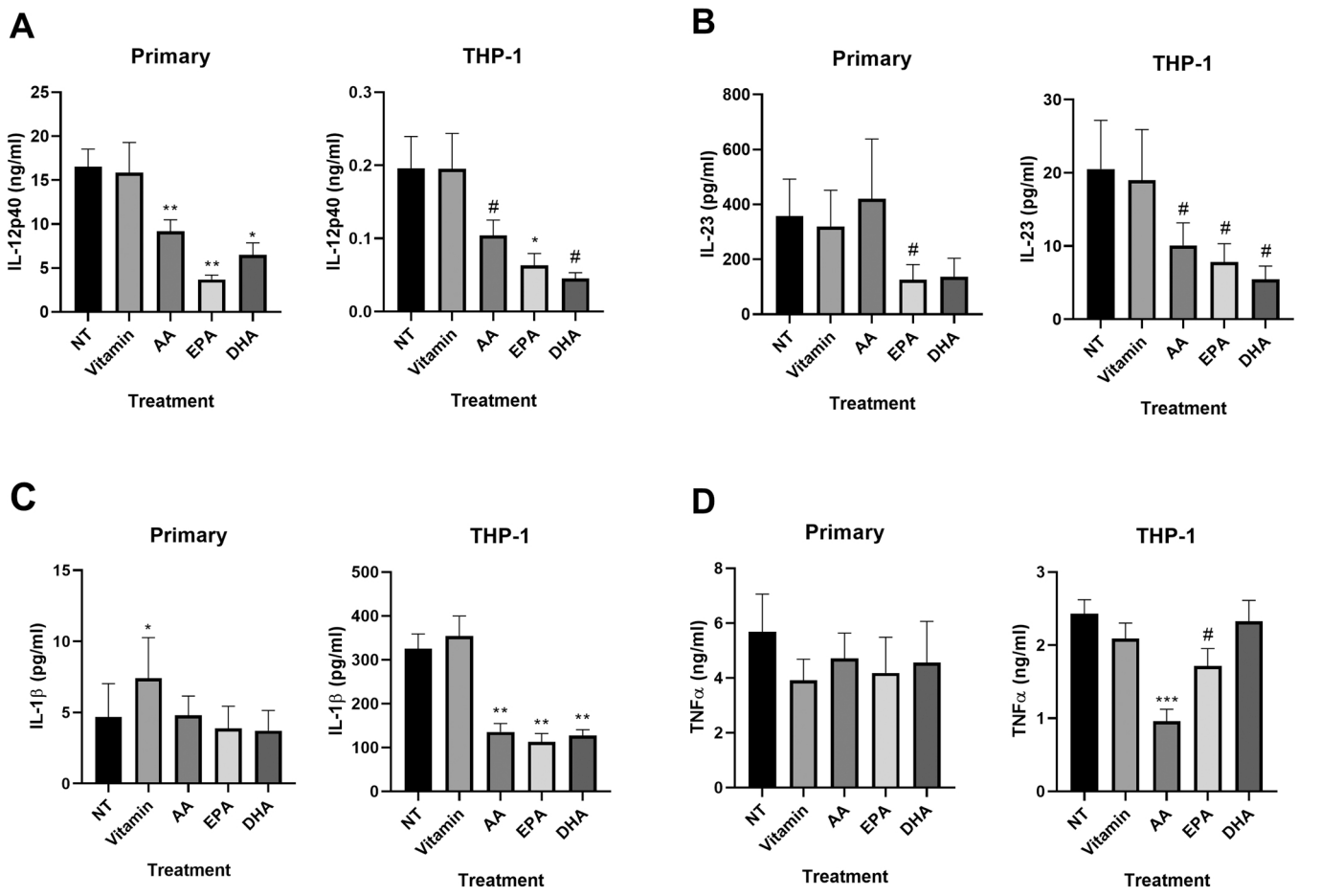

Fig. 5. M1 macrophage cytokine secretion in response to treatment with LCPUFAs. (A) IL-12p40 secretion by primary MDMs and THP-1 macrophages. (B) TNFα secretion by primary MDMs and THP-1 macrophages. (C) IL-1β secretion by primary MDMs and THP-1 macrophages. (D) IL-6 secretion by primary MDMs and THP-1 macrophages. # $p < 0.1$, * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$.

responses of two human macrophage models both with and without LCPUFA treatment.

In terms of phagocytosis, both macrophage models are similar. The amount of macrophages that engulfed the *E. coli* particles was similar. Also, in both macrophage models M2 macrophages were more efficient in phagocytosis compared to M1 macrophages, which was also shown in previous research [18,20,27]. Also in line with previous results, primary M1 and M2 MDMs were more efficient in particle uptake compared to the THP-1 macrophages [20]. This could in theory mean that effects on phagocytosis by interventions are more pronounced in primary MDMs. Nonetheless, LCPUFAs did not affect either the amount of cells that phagocytose nor the amount of particles that are taken up per cell, in both macrophage models. In multiple studies that use the murine monocyte/macrophage cell line RAW264.7, DHA and AA enhanced phagocytosis [28,29], which was also seen in microglial cells [30]. Hence, even though the current study shows LCPUFAs do not affect phagocytosis in two different macrophage cell models, in other macrophage models LCPUFAs were effective. This indicates that depending on the environmental triggers and specific characteristics of certain cells, the outcome in biological effects may differ.

M1 and M2 macrophages are often characterized by their IL-12/IL-10 ratio. In both macrophage models, we saw a higher IL-12/IL-10 ratio in M1 macrophages and a higher IL-10/IL-12 ratio in M2 macrophages, indicating that both models can be properly characterized. It is noteworthy that primary M1 MDMs secreted significantly more IL-12p40, IL-12p70, IL-23 and IL-6 compared to THP-1 M1 macrophages, which has been indicated previously for IL-6 both on gene level and cytokine secretion [20]. These differences could be explained by the origin of the cells, as THP-1 cells are derived from a one-year old leukemia patient while the primary monocytes were derived from healthy adult donors. Hence, the latter cells may be more capable of producing a broad panel and larger quantity of mediators. Furthermore, secretion of many cytokines initiates only at the adult level after 4 years of life, and for IL-12, it is comparable at the age of 13 [31]. However, we have also shown that both primary MDMs and THP-1 macrophages secrete the known M1 and M2 cytokines, although primary M1 and M2 MDMs secrete similar amounts of IL-1β and IFNγ, and THP-1 M1 and M2 macrophages secrete similar amounts of IL-4 and CCL17.

The greatest differences between primary MDMs and THP-1 macrophages were observed in the M2 phenotype. CCL17 and CCL22 were both secreted significantly more by primary M2 MDMs compared to THP-1 M2 macrophages, while IL-4 and IL-10 levels were similar. A previous study however reported that CCL22 was expressed more by THP-1 derived macrophages compared to primary MDMs, and that only the THP-1 type M2 macrophages expressed more CCL22 compared to M1 macrophages [20]. However, in the current study CCL22 levels were approximately five times higher in the M2 MDM compared to the THP-1 M2 macrophages. Since MDMs are derived from healthy volunteers, donor variation or procedures followed to obtain blood samples may explain these differences. CCL17 and CCL22 are both CCR4 ligands. CCR4 is mainly expressed by Th2 cells and Treg cells [32], and both CCL17 and CCL22 have been linked to Th2-related diseases such as allergic asthma [33]. This could suggest that primary M2 MDMs may be more efficient in T-cell chemotaxis and activation. Therefore, it is especially interesting to note that AA and DHA were able to lower CCL22 secretion in primary M2 MDMs, as DHA has been previously shown to

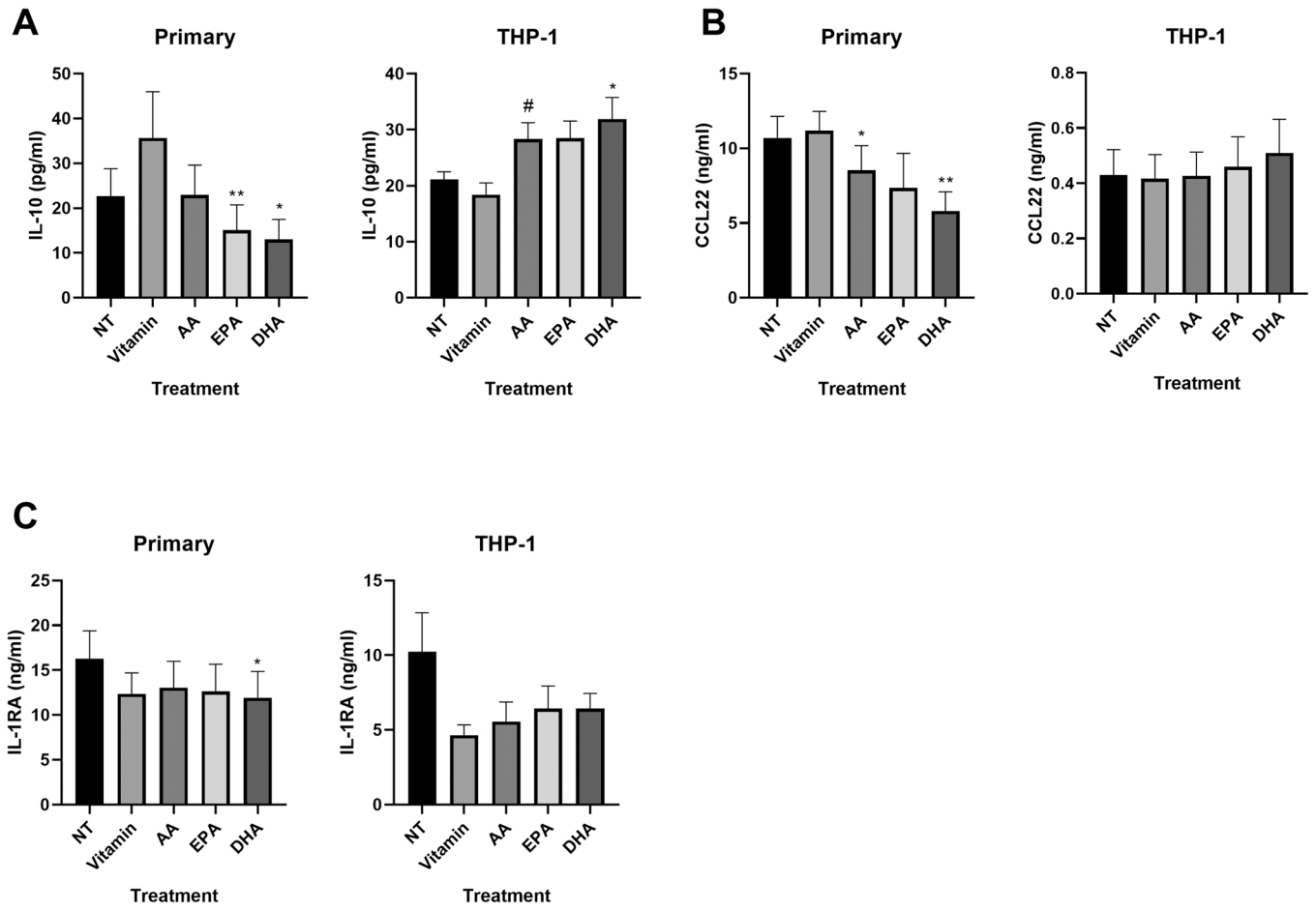

Fig. 6. M2 macrophage cytokine and chemokine secretion in response to treatment with LCPUFAs. (A) IL-10 secretion. (B) CCL22 secretion. (C) IL-1RA secretion. # $p < 0.1$, * $p < 0.05$, * * $p < 0.01$.

reduce allergic sensitization *in vitro* [26] and *in vivo* [34].

In the LCPUFA experiments, vitamins C and E were added to prevent lipid peroxidation of the LCPUFA incorporated in the cell membranes. Antioxidants protect against possible radical induced membrane damage and inflammation when working with LCPUFA. The secretion of IL-12p40 was significantly lowered by LCPUFAs AA, EPA and DHA in primary M1 MDMs, and only EPA showed a significant decrease in THP-1 M1 macrophages. However, the trend was similar. In contrast, IL-23 secretion was lowered, although not significantly, by AA, EPA and DHA in THP-1 M1 macrophages, but in primary M1 MDMs, only EPA had this effect. TNFα was, although not significant, secreted more by primary M1 MDMs compared to THP-1 M1 macrophages, but could only be lowered by AA and, to some extent, by EPA in THP-1 M1 macrophages. IL-1β and IP-10 were secreted significantly more by THP-1 M1 macrophages compared to primary M1 MDMs. Furthermore, IL-1β could be lowered by all LCPUFAs in THP-1 macrophages, but, probably due to the low secretion, no effects were observed in primary MDMs. IL-1β secretion has been mainly associated with tumor-associated macrophages (TAMs) [35]. Since THP-1 cells are tumor cells derived from an acute monocytic leukemia patient, this could possibly explain the differences observed in our study, as the human primary monocytes were derived from healthy donors.

Interestingly, IL-10 secretion was significantly lowered by EPA and DHA treatment in primary M2 MDMs, while DHA and, to a lesser extent AA, elevated IL-10 in THP-1 M2 cells. Lowering of IL-10 by DHA was also observed in previous studies using THP-1 [36] and U937 cells [37]. The THP-1 study used LPS-stimulated M1 macrophages treated with both 25 and 100 μM of DHA, however, we did not observe an effect of any of the LCPUFAs on IL-10 secretion in the THP-1 M1 macrophages. As CCL22 and IL-1RA were also significantly lowered by DHA in primary M2 MDMs but not in THP-1 M2 macrophages, and given the significant differences in cytokine secretion between the two models, primary MDMs seem to be more reliable as an M2 macrophage model compared to THP-1 macrophages.

It is noteworthy that modulation of cytokine secretion in macrophages is mainly observed in M1 macrophages with EPA, whereas DHA has been described as the most prominent inhibitor of cytokine expression and secretion by APCs [26,38,39]. This is in line with a previous study on human asthmatic alveolar macrophages, where the mRNA expression of TNFα and IL-1β and the secretion of TNFα, IL-1β, LTB_4 and PGD_2 was lowered by EPA, but only in a lesser extent by DHA [40]. A study using both *in vivo* mouse models and THP-1 macrophages has elegantly shown that EPA and DHA act on multiple pathways such as the NLRP3 inflammasome, GPR120 (also known as FFA4) and GPR40 activation, thereby decreasing caspase-1 activation and IL-1β secretion [41]. In DCs, the main effects of cytokine modulation by DHA are achieved by acting on PPARγ:RXR heterodimers [42]. In THP-1 macrophages, PPARγ was found to be expressed by both M1 and M2 phenotypes, but the M2 phenotype showed a higher expression. Interestingly, DHA conjugates DHAE and DHA-5-HT only lowered M1 IL-1β, TNFα and MCP1 expression, which could be reversed by adding PPARγ antagonist GW9662 [43]. In the MDMs, it might be possible that the PPARγ is functional in both phenotypes, thereby explaining the different effects of DHA between the THP-1 and MDM M2 models. It would be interesting to use this antagonist in the current model system to investigate if the more prominent effects of DHA in the M2 MDMs compared

to the M1 MDMs are due to activation of PPARγ.

In order to study macrophage responses, many different cell models can be used. In this study, we have shown that there are many differences between the human macrophages that we have used and the in literature described murine macrophage cytokine secretion and modulation by LCPUFAs. Moreover, we also found many differences in cytokine secretion and modulation by LCPUFAs between the human MDMs and THP-1 macrophage models. We highlighted that it is very important to consider the properties of each model and to compare this to the pathological condition that will be reflected by it. Therefore, when testing immunomodulatory compounds, our suggestion would be to validate findings from a murine macrophage cell line or the human THP-1 model in MDMs.

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CRediT authorship contribution statement

All authors participated in the design and interpretation of the reported experiments and results. TH acquired and analyzed all data. All authors participated in drafting and revising the manuscript. TH was responsible for the statistical analysis.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phanu.2022.100322.

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