# Chapter 5

# An expeditious synthesis of DHA-alkyne*

## Introduction

Docosahexaenoic acid (DHA, 22:6 n-3, **1**) is a polyunsaturated fatty acid (PUFA) which is found to be particularly enriched in the brain (Figure 1).[1] In mammals, DHA can be synthesized from α-linolenic acid (ALA, 18:3 n-3) via sequential desaturation and elongation steps. However, as this process is quite inefficient, most mammalian DHA is obtained from dietary sources.[2–4] DHA has a beneficial role in neuronal development[5] and neuroprotection,[6] as well as in chronic inflammatory conditions like rheumatoid arthritis.[7,8] Recent advances in mass spectrometry have allowed the accurate detection and quantification of PUFAs and their metabolites.[9] This led to the discovery of anti-inflammatory metabolites of DHA produced via oxidative pathways. Several oxygenated metabolites of DHA are involved in the resolution of inflammation and are called specialized pro-resolving mediators (SPMs).[10–12]

*The data presented in this chapter was gathered in collaboration with Hugo Minnee, Joost von Hegedus, Joanneke C. Kwekkeboom, Hans van den Elst, Remco Peter, Herman S. Overkleeft, René E. M. Toes, Andreea Ioan-Facsinay, Mario van der Stelt.

To date, investigation of PUFA metabolites, their cellular localization and role in inter-cellular signaling remains a difficult process. To aid this process, the labeling of fatty acids using (radioactive) isotopes[13] and fluorophores[14,15] has proven an invaluable tool. Isotopically labeled lipids have been used primarily in the investigation of the metabolism of many lipid species,[16,17] while fluorescently labeled lipids have been used extensively to visualize lipid localization and trafficking.[15,18] However, these tools have several drawbacks. Radioactive isotopes require special equipment and procedures, while non-radioactive isotopes do not allow for investigation of the localization of the lipids. Fluorescent tags can be introduced to study localization of lipids, but they are relatively large and rigid compared to the labeled molecules, affecting their metabolism and distribution.[19,20] Thus, to trace metabolism and localization of PUFAs, new tools are required.

The discovery of the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) has led to the development of a large toolbox of 'click' chemistry reagents.[21] Alkyne moieties are synthetically accessible and minimally intrusive when placed on the terminal alkyl chain of a lipid. Terminal alkyne analogues for lipid families such as fatty acids,[22,23] sterols[24,25] and sphingolipids, have already been developed.[26,27] Alkyne lipids have several benefits over radio-labeled lipids. For example, no special equipment or permissions are needed to handle alkyne lipids. After conjugation to a reporter group, alkyne lipids can be used to identify metabolites by liquid chromatography-mass spectrometry (LC-MS) analysis or thin-layer chromatography (TLC).[28] Importantly, alkyne-tagged lipids have outperformed established radiolabeled lipids tracers in sensitivity and linearity of detection.[22] Moreover, alkyne lipids can also be used to study localization by *in situ* click ligation to a fluorophore, followed by fluorescence microscopy.[25,29]

A wide range of fluorophore azides are available for the detection of alkyne-modified lipids.[29,30] With these tools, an alkyne-tagged lipid can be used in different types of experiments, allowing parallel investigation on metabolism and distribution of the same molecule.[29] However, the availability of chemical tools based on PUFAs is limited due to their synthetic difficulty. PUFAs and modified derivatives can be synthesized through a number of strategies. The synthetic challenge lies in the double bonds, which for most natural PUFAs are in *cis* configuration. The two main strategies for the synthesis of PUFAs are linking together skipped alkynes followed by partial hydrogenation,[31] or sequential Wittig reactions.[32] However, skipped alkynes are prone to isomerization to reactive allenes and their partial hydrogenation often affords inseparable mixtures of under- and overreduced products.[33] Total synthesis by Wittig reactions suffers from the severe instability of the multiple β,γ-unsaturated aldehyde intermediates whose purity is essential for a successful reaction.[34,35]

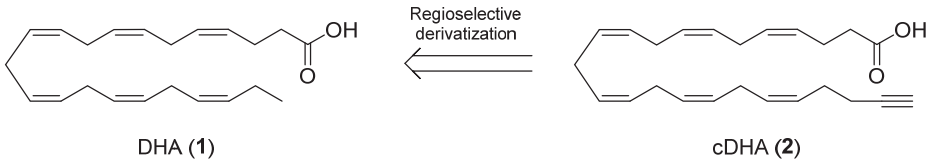



**Figure 1 |** Structures of DHA (**1**) and alkyne-modified derivative, cDHA (**2**)

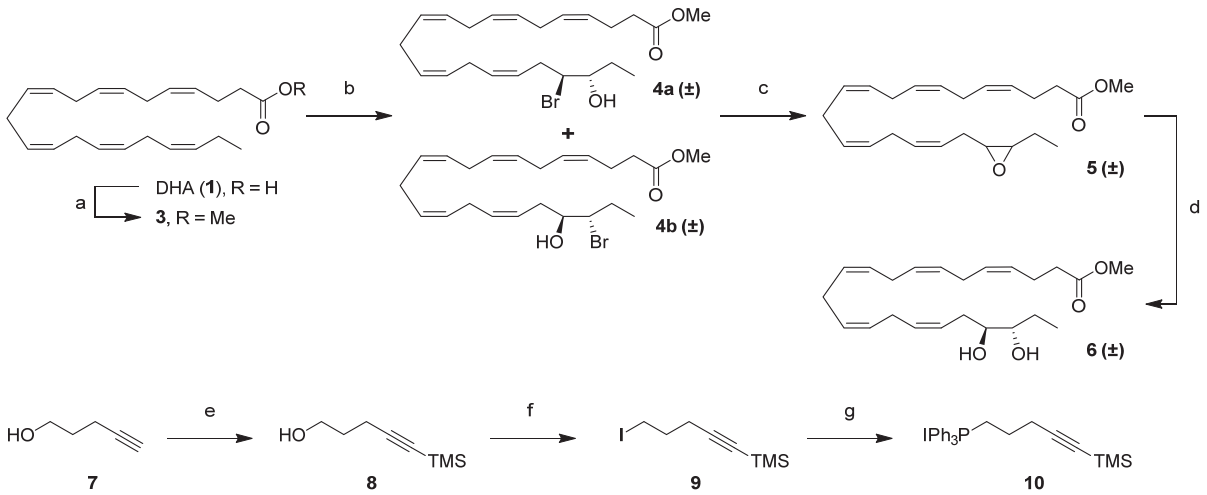
In Chapter 3, the synthesis of a diazirine- and alkyne-modified DHA derivative was achieved by a combination of Wittig reactions and partial hydrogenation of skipped alkynes. However, this was a strenuous process that required HPLC purification of a late-stage intermediate. Here, a concise synthesis of click-DHA (cDHA, **2**) is described which starts from commercially available DHA (Figure 1). Regioselective hydrobromination allowed chemical derivatization of the terminal double bond after which the alkyne was installed using a Wittig reaction. This strategy greatly reduces the number of steps and avoids the use of unstable skipped alkynes, which makes it attractive for the synthesis of other DHA derivatives. As an application cDHA **2** was used to visualize its incorporation into cells using a fluorogenic azidocoumarin and to follow its exchange between primary human cells using flow cytometry.

# Results

## Synthesis of cDHA (**2**) from DHA

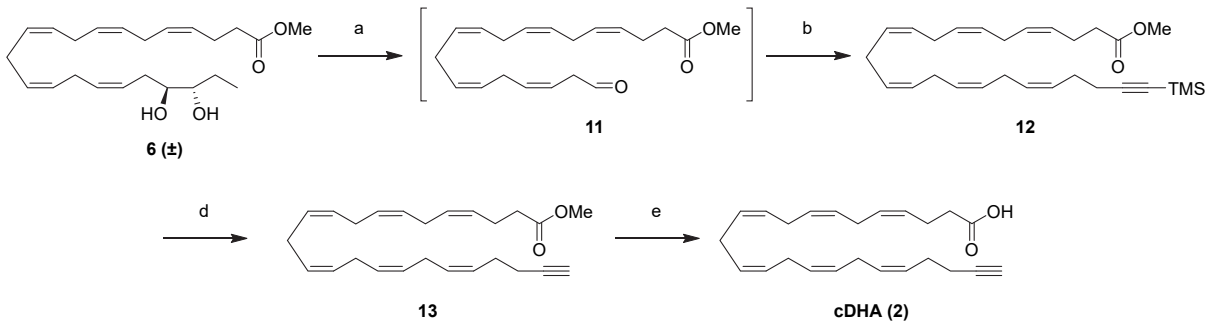
For the introduction of an alkyne moiety to the tail of the molecule, the use of commercially available DHA as a starting point was investigated. The terminal double bond of DHA has a different reactivity compared to the other alkenes in aqueous medium, which makes it available for chemical derivatization. This difference is hypothesized to be due to a coiled conformation of DHA in solution.[36] The design of click lipid **2** was chosen to keep al double bonds intact and to have the minimal space required between the last double bond and alkyne to afford a stable click lipid.[37]

Starting from commercially available DHA (**1**), the carboxylate was protected by methylation to afford ester **3**, which was used in a hydrobromination reaction (Scheme 1).[36] Following a reported procedure, 1.3 eq. *N*-bromosuccinimide (NBS) was added in portions over 8 h to a cooled (0 °C) solution of **3** in dimethoxyethane (DME)/$H_2O$ (4:1), which afforded a mixture of brominated and hydrobrominated products. The hydrobrominated products accounted for a low yield of 16%, of which 70% consisted of the desired regioisomers **4a/b** as judged by $^1$H-NMR. To obtain a higher yield and improve the reproducibility, a syringe pump was used to add a solution of recrystallized NBS in DME/$H_2O$ (4:1) over 24 h. After complete addition the reaction was stirred for another 2 h and quenched by adding $Na_2S_2O_3$ This resulted in a mixture of monohydrobromins **4a/b** with a 27% yield, as well as recovery of 34% starting material **3**, which could be reused. The selectivity for the desired hydrobrominated alkene remained 70%, but formation of brominated DHA and other unwanted products was greatly reduced.




**Scheme 1 | Synthesis of dihydroxide 6 and phosphonium salt 10.** Reagents and conditions: (a) DHA, oxalyl chloride, DCM, 0 °C to rt, 5 h, then NaOMe, MeOH, 1 h, 98%; (b) NBS, DME/$H_2O$ (3:1), 0 °C, 26 h, 27% (41% based on recovered starting material); (c) NaOMe, MeOH, rt, 45 min; (d) $HClO_4$, THF/$H_2O$ (4:3), rt, 7 h, 69% over 2 steps; (e) *n*-BuLi, TMSCl, THF, -78 °C to rt, 16 h, 82%; (f) $I_2$, $PPh_3$, imidazole, THF, rt, 1 h, 93%; (g) $PPh_3$, acetonitrile, reflux, 3 d, 88%.

The mixture of bromohydrins **4a/b** was treated with anhydrous sodium methoxide in methanol to afford epoxide **5** without hydrolysis of the methyl ester. Epoxide **5** was hydrolyzed with perchloric acid to obtain **6** in 69% yield over two steps. To introduce the terminal alkyne, butynol (**7**) was protected as the trimethylsilyl alkyne to form alcohol **8** in 82% yield. Compound **8** was converted into iodide **9** using an Appel reaction in 93% yield. Subsequently, phosphonium salt **10** was obtained in 88% yield by reacting **9** with $PPh_3$ in refluxing ACN for 72 h. Phosphonium salt **10** was used in a Wittig reaction to install the terminal double bond of the DHA scaffold. To this end, **6** was oxidatively cleaved by sodium periodate at 0 °C to afford unstable the β,γ-unsaturated aldehyde (**11**) (Scheme 2).[35,38] After a workup, it was immediately added to a cooled (-110 °C) solution of phosphonium salt **10** pretreated with potassium *tert*-butoxide. The reaction temperature was raised to -90 °C over 1 h and subsequently to 0 °C in 2 h after which the mixture was quenched at -78 °C by addition of a suspension of silica in $Et_2O$. This resulted in the formation of **12** in 19% yield from the dihydroxylated **6**. At this stage, shorter side products resulting from regioisomers in the hydrobromination reaction could be removed by silica gel purification.




**Scheme 2 | Synthesis of cDHA (2).** Reagents and conditions: (a) $NaIO_4$, MeOH/$H_2O$ (10:3), 0 °C, 4 h; (b) **10**, KO*t*Bu, THF, -78 °C, 1 h, then **11**, -110 °C to 0 °C, 3 h, 19% over two steps; (c) TBAF, THF, rt, 30 min, 95%; (d) LiOH, $H_2O$/THF (1:1), rt, 16 h, 82%.

Next, the silyl group of **12** was deprotected with TBAF providing terminal alkyne **13** in 95% yield. Final product **2** was obtained by hydrolysis of the methyl ester in aqueous LiOH in a total of 4.2% yield over 6 steps from DHA.

## Investigation of metabolism of cDHA (**2**) on HPTLC

To investigate the incorporation of clickable lipids into cells, cDHA (**2**) was compared to a saturated palmitic acid-alkyne (cPA, **14**),[39] which was previously used for this application.[22] The lipids (20 µM, 40 nmol) were incubated with mouse neuroblastoma Neuro-2a cells for 2 h. Cellular lipids were extracted and conjugated to fluorogenic hydroxycoumarin azide (**15**) under CuAAC conditions.[27] As a reference, 1 nmol of the free fatty acids were also reacted with **15**. All lipids were then separated on high-performance thin layer chromatography (HPTLC) and visualized by capturing the fluorescent signal (Figure 2B). This showed that cDHA (**2**) could be incorporated in a similar manner to cPA (**14**) into higher-running hydrophobic species, as well as lower-running, hydrophilic species. Interestingly, the labeling pattern indicated that cDHA (**2**), as well as cPA (**14**) were also incorporated in non-overlapping, lipid-specific metabolites.

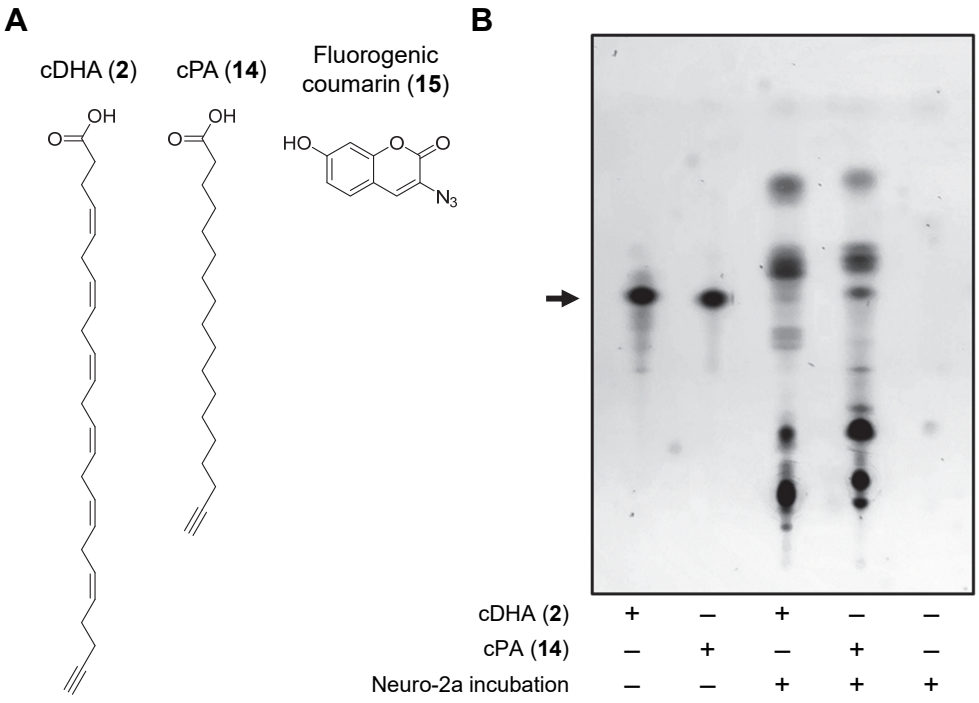



**Figure 2 | cDHA (2) and cPA (14) can be used to follow lipid metabolism. (A)** Structures of clickable lipids **2** and **14** and hydroxycoumarin azide (**15**). **(B)** Fluorescent signal of hydroxycoumarin-labeled lipids. Neuro-2a cells were incubated for 2 h with 20 µM cPA (**14**), cDHA (**2**) or vehicle (EtOH) and the total lipid extracts or 1 nmol of indicated click lipid were subjected to a click reaction with hydroxycoumarin azide, separated on HPTLC and the fluorescent signal was imaged. The arrow indicates the elution height of the coumarin-conjugated free acids.

## Uptake and exchange of cDHA (**2**) by macrophages and fibroblasts

Next, the cellular uptake and exchange of clickable lipids was studied using flow cytometry. First, fibroblasts isolated from synovial membrane of osteoarthritis patients and expanded *in vitro*, as well as GM-CSF-stimulated macrophages differentiated from healthy donor peripheral blood monocytes were separately incubated with clickable lipid **2** (10 nM, 0.5 pmol) or unlabeled DHA for 24 h. The cells were fixed and subjected to click chemistry with AlexaFluor-647-$N_3$ (AF647) and analyzed by flow cytometry.[40] Over 90% of macrophages (Figure 3A) and fibroblasts (Figure 3B) were successfully be labeled using cDHA (**2**).

To study if cDHA (2) can be used to visualize lipid exchange between macrophages and fibroblasts, macrophages were treated with cDHA (**2**) (10 nM, 0.5 pmol) or DHA for 4 h, washed thoroughly and subsequently cocultured with unlabeled fibroblasts for an additional 24 h. Detection of the alkyne handle using AF647 showed that 97% of the fibroblasts were fluorescently labeled by AF647, indicating that these cells have incorporated cDHA (**2**) or cDHA derivatives transferred from the macrophages (Figure 3C).

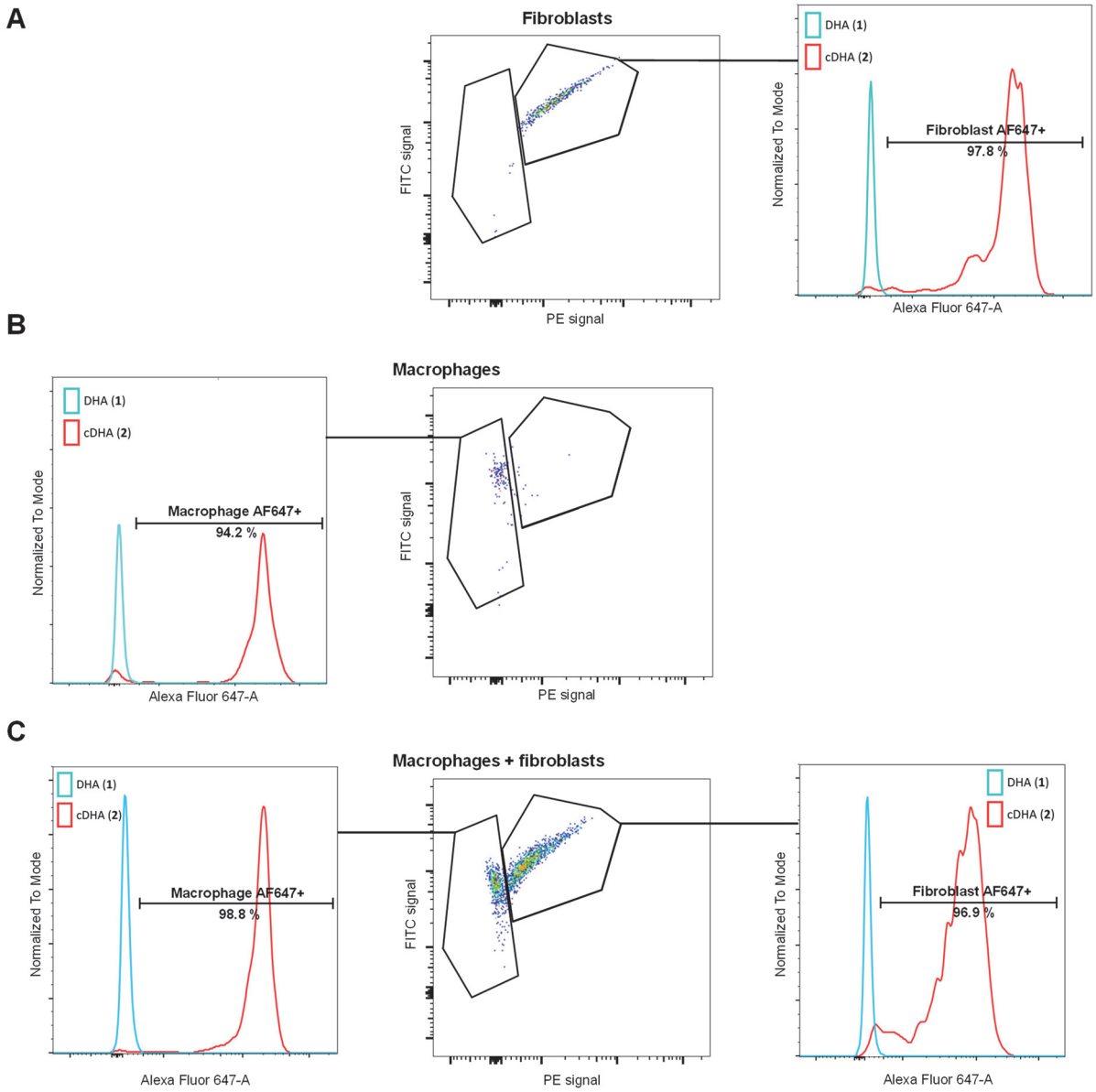



**Figure 3 | cDHA (2) exchange is detectable between macrophages and fibroblast.** Cells were incubated with indicated lipid, harvested and treated with AlexaFluor-647-$N_3$ under CuAAC conditions and analyzed using flow cytometry. **(A)** Treatment of fibroblasts with clickable lipid **2** results in labeling of 97.8% of cells compared to control. **(B)** Treatment of macrophages with clickable lipid **2** results in labeling of 94.2% of cells compared to control. **(C)** Coculturing fibroblasts with macrophages that had been pretreated with cDHA (**2**) results in detectable exchange of the clickable lipid between the different cell types. Gating strategy included the exclusion of debris and doublets based on FSC/SSC and separation of macrophages and fibroblasts in the coculture was based on the autofluorescence observed in both the PE and FITC channel (Figure S1).

# Conclusion

Alkyne-modified lipids such as clickable fatty acids, sphingolipids and cholesterol are versatile tools to study localization, metabolism and exchange of these diverse biomolecules. In recent years, innovative methods to use these tools have been developed, many of which from the lab of Dr. Thiele.[22,27–29] Importantly, these methods to study alkyne lipids require no specialized equipment and use commercially available reagents. However, alkyne-modified PUFAs are scarcely available, due to their synthetic difficulty. This difficulty arises from many inseparable side products, as well as the instability of many intermediates.[33] In this work, a concise synthetic route for an alkyne-modified DHA derivative is reported starting from commercially available DHA. Regioselective hydrobromination allowed chemical derivatization of the terminal double bond after which the alkyne was installed using a Wittig reaction. cDHA was obtained in 6 steps from DHA without making use of unstable skipped alkynes. It is envisioned that this new synthetic route enables the synthesis of other DHA derivatives through Wittig reactions with synthetically accessible phosphonium salts.

As an application, cDHA (**2**) was used to visualize of its incorporation into cells using a fluorogenic azidocoumarin, as well as its exchange between primary human cells using flow cytometry. As little as 0.5 picomoles of cDHA (**2**) was demonstrated to efficiently label cells and to allow for visualization of lipid exchange between human macrophages and fibroblasts using flow cytometry. Quantification and identification of the exchanged lipids under pro-inflammatory or pro-resolution conditions could provide information on the role of PUFA metabolites as lipid signaling molecules.[41]

# Experimental procedures

## General

Solvents were dried over activated 4 Å molecular sieves for at least 24 hours. Reactions were monitored by TLC using silica gel 60 F254 coated aluminum sheets from Merck. DHA was obtained from Cayman chemicals (90310). 3-Azido-7-hydroxycoumarin azide (**15**) and cPA (**14**) were prepared as previously reported in literature.[39,42] The hydroxycoumarin azide was unstable, therefore small aliquots (44.5 mM in DMSO) were stored under nitrogen at -20 °C and discarded after a single use. NBS was recrystallized from $H_2O$ at 90 °C and dried under reduced pressure. Phosphate buffer A was prepared by dissolving $KH_2PO_4$ (0.5 M) in Milli-Q and adjusting the pH value to 7.0 with aq. NaOH. All other reagents were purchased from Alfa Aesar, Sigma Aldrich/Merck or Acros and used without further purification.

## Cell culture

### *Neuro-2a culture*

Neuro-2a cells were cultured at 37 °C under 7% $CO_2$ in DMEM (Sigma Aldrich, D1145) containing stable glutamine, 10% (v/v) New Born Calf Serum (Thermo Fisher) and penicillin and streptomycin (200 µg/mL each, Duchefa). Cells were passaged twice a week at 80-90% confluence by resuspension in fresh medium. Cell lines were purchased from ATCC and were tested regularly for mycoplasma contamination. Cultures were discarded after 2-3 months of use.

### *Primary cell culture*

This study was approved by the local medical ethical committee of the LUMC (METC), and written informed consent was given by all donors. Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient from healthy donor buffy coats (Sanquin). Blood monocytes were isolated by positive selection from PBMCs using MACS CD14 Microbeads (Miltenyi Biotec) and purity was checked by FACS (LSRIII, BD Biosciences), by staining the cells with CD14-PE (clone MφP9). Monocytes were differentiated for seven days in RPMI 1640 medium (Gibco) containing 8% FCS, 100 U/mL penicillin and streptomycin, 2 mM Glutamax (Thermo Fisher) and 5 ng/mL GM-CSF (Miltenyi Biotec). Medium was replenished on day three and five. Phenotype was checked before experiments by visual inspection by assessing the typical morphology of GM-CSF cultured macrophages ('egg sunny-side up-like'). Phenotype was also confirmed by preforming IL-12 OptEIA (BD Biosciences), IL-10 PeliPair reagent set (Sanquin), and TNFα OptEIA (BD Biosciences) ELISA on supernatant of cells stimulated for 24 hours with 10 ng/mL LPS (Merck). GM-CSF monocyte-derived macrophages (MDM) secreted high IL-12 and TNFα and low IL-10. Cells were harvested using Accutase (Merck) and $2.5x10^5$ cells were seeded in 24-well plates in 250 µL medium. Lipids were added in phenol red-free RPMI 1640 medium (Gibco), supplemented with 0.1% (w/v) fatty acid free BSA (A7030, Sigma). For isolation of fibroblasts, left-over synovial tissue was collected anonymously from patients undergoing total knee replacement surgery in the context of standard clinical care at the Departments of Orthopedic Surgery in the LUMC or Alrijne Hospital in Leiderdorp. Preparation of synovial tissue cells was performed less than 6 hours after the surgical procedure. The tissue was kept in PBS at room temperature. All tissue material was obtained with permission from the ethical committee and with patient approval. Whole synovial tissue was cut into small pieces and digested for 1.5 - 3 h at 37 °C under constant roller movement with 6.47 mg (245 U/mg) collagenase type 2 (Worthington Biochemical Corporation) in 5 mL serum-free IMDM medium (Lonza). To remove remaining undigested tissue and collect synovial tissue cells, the cell suspension was filtered and mashed using a 70 µm cell strainer (Falcon, Corning Incorporated). The cell suspension was washed 3 times with 10 mL warm serum-free IMDM medium (Lonza) through centrifugation at 195 g for 10 minutes. Cells were cultured at a density of $2.5x10^4$ cells/$cm^2$ at 37 °C with 5% $CO_2$ in appropriate culture dishes in DMEM-F12 complete medium. Synovium cells were harvested with 2 mL 0.25% trypsin for a T75 flask (Thermo Fisher) for 4-5 min at 37 °C for further passaging to obtain fibroblast-like cells.

## HPTLC

HPTLC experiments were performed according to published procedure[22] with adjustments. Neuro-2a cells were plated on a 6-well plate and grown to confluency. They were washed with PBS (1 mL) and subsequently DMEM (2 mL) supplemented with 0.1% (w/v) fatty acid free BSA (A7030, Sigma) was added containing indicated click lipid (from 10 mM ethanolic stock, 20 µM final) or vehicle. After 2 h incubation, the medium was aspirated and the cells were washed with PBS with 0.1% (w/v) fatty acid free BSA, then PBS, after which they were harvested by pipetting in PBS (0.3 mL). Cells were transferred to 2 mL tubes and methanol (600 µL) and $CHCl_3$ (150 µL) were added, after which the tubes are vortexed (10 s) and centrifuged (14,000 *g*, 2 min). The supernatant was transferred to new 2 mL tubes and $CHCl_3$ (300 µL) and 0.1% (v/v) aq. acetic acid (600 µL) were added, followed by vortexing (10 s) and centrifugation (14,000 *g*, 5 min). The lower organic layer was transferred to 1.5 mL eppendorfs and concentrated in a speed-vac. The lipid pellet was redissolved in 7 µL $CHCl_3$ and 30 µL click mix was added (click mix: 5 µL of 44.5 mM 3-azido-7-hydroxycoumarin in DMSO, 500 µL of 10 mM $[acetonitrile]_4CuBF_4$ in acetonitrile, 2 mL ethanol) after which the tube was left in a heating block at 42 °C for 3 h until all solvent condensed under the cap. The tubes were briefly centrifuged and vortexed and 3 µL was applied to a HPTLC silica gel 60 plate (Merck). The plate was developed with a solvent system consisting of $CHCl_3$/MeOH/$H_2O$/AcOH = 65:25:4:1 until 50% of the length of the plate, which was then dried under a stream of nitrogen in the dark and developed again in hexane/EtOAc = 1:1 for 90% of the length of the plate. The plate was dried under a stream of nitrogen in the dark and then dipped in a solution of 4% (v/v) DIPEA in hexane. After allowing most solvent to evaporate in the dark it was then imaged by placing it over a UV-lamp and capturing the fluorescent signal through a Perspex plate. Images were processed using FIJI.[43]

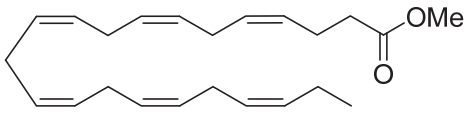
## Flow cytometry

Upon differentiation/expansion, fibroblasts and macrophages were washed with PBS, enzymatically harvested (fibroblasts using trypsin, macrophages using accutase) and resuspended in PBS. They were spun down and resuspended in DMEM/F12 (Sigma) supplemented with 0.5% (w/v) fatty acid free BSA (A7030, Sigma), 2 mM Glutamax (Thermo Fisher), and penicillin and streptomycin (200 µg/mL each). The cells were counted and $2.5x10^4$ cells were plated on a 96-well plate in 50 µL medium. After 24 h, DHA or cDHA was added (5 µL of a 11X concentrated solution in medium, 10 nM final) and the cells were incubated for 4 h. For the monocultures (Figure 3A/B), cells were then washed twice with DMEM/F12 and cultured for 24 h before FACS analysis. For the coculture (Figure 3C), the macrophages were washed twice with DMEM/F12 and then $2.5x10^4$ fibroblasts were added and then incubated for 24 h. Flow cytometry analysis of macrophages and fibroblasts was performed following previously reported procedure with minor modifications.[40] In brief, the cells were washed with PBS (100 µL) and accutase (30 µL) was added and incubated at 37 °C for 10 min. The cell suspension was then placed in V-bottom plates and the wells washed with PBS (100 µL). The plate containing the harvested cells was centrifuged (2,000 *g*, 3 min) and the supernatant was removed. The cells were fixed in 100 µL 4% (w/v) paraformaldehyde (PFA) in PBS (10 min, rt). The cells were spun down (2,000 *g*, 3 min) and washed with PBS (100 µL) and nonspecific binding sites were blocked by incubation with 1% (w/v) BSA in PBS (100 µL) for 30 min. Cells were then washed with PBS and resuspended in 50 µL click mix (2.5 µL 100 mM $CuSO_4$, 1.5 µL 1 M NaAsc, 0.5 µL 100 mM THPTA and 1 µL 2 mM AF647-$N_3$ (Invitrogen), 44.5 µL 100 mM HEPES). After incubation for 1 h at rt, the cells were pelleted (2,000 *g*, 3 min) and washed with PBS and 1% (w/v) BSA in PBS. The cells were resuspended in 1% (w/v) BSA in PBS with 400 nM DAPI (Molecular Probes, D1306) and analyzed with a LSRIII (BD Biosciences, with lasers: 405 nm, 488 nm and 633 nm). Gating strategy included the exclusion of dead cells and doublets based on FSC/SSC (Figure S1). Separation of macrophages and fibroblasts in the co-culture was based on the autofluorescence observed in both the PE and FITC channel. Analysis was preformed using BD FACSDiva™ software.

## Chemistry

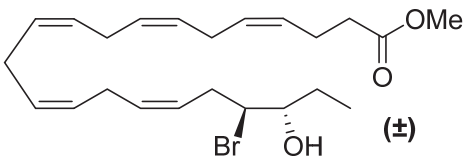
A NE-1000G Future Chemistry syringe pump was used in combination with BD Discardit II plastic syringes. For a 10 mm syringe, the diameter setting was 15.6 mm. The reactions were performed under an inert atmosphere of nitrogen gas unless stated otherwise. $^{1}$H NMR and $^{13}$C NMR spectra were recorded on a Bruker AV-400 (400 MHz) or AV-500 (500 MHz) spectrometer. Chemical shift values are reported in ppm relative to the tetramethylsilane signal for $^{1}$H NMR (δ = 0 ppm) and relative to the solvent signal of $CDCl_3$ for $^{13}$C NMR (δ = 77.16 ppm). Data are reported as follows: Chemical shifts (δ), multiplicity (s = singlet, d = doublet, dd = doublet of doublets, ddt = doublet of doublet of triplets, td = triplet of doublets, t = triplet, q = quartet, p = pentet, bs = broad singlet, m = multiplet), coupling constants *J* (Hz), and integration. Flash column chromatography was performed using SiliCycle silica gel type SiliaFlash P60 (230-400 mesh). TLC analysis was performed on Merck silica gel 60/Kieselguhr F254, 0.25 mm. Compounds were visualized using $KMnO_4$ stain ($K_2CO_3$ (40 g), $KMnO_4$ (6 g), and water (600 mL)). High resolution mass spectra (HRMS) were recorded by direct injection on a q-TOF mass spectrometer (Synapt G2-SI) equipped with an electrospray ion source in positive mode with Leu-enkephalin (m/z = 556.2771) as an internal lock mass. The instrument was calibrated prior to measurement using the MS/MS spectrum of [Glu$^{1}$]-fibrinopeptide B.

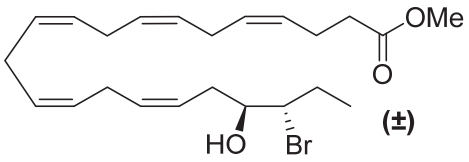
**Methyl (4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*)-docosa-4,7,10,13,16,19-hexaenoate (3).**




DHA (0.888 g, 2.70 mmol) was coevaporated with toluene thrice and dissolved in DCM (44 mL). Oxalyl chloride (0.75 mL, 8.75 mmol) was added dropwise to the reaction mixture at 0 °C. The reaction was allowed to reach room temperature over 5 h. Then, a methanolic solution of NaOMe (1.3 M, 4.5 mL, 2.70 mmol) was added and the reaction was stirred for 5 min. The reaction mixture was diluted with $Et_2O$ (450 mL) and washed with $H_2O$ (2 x 200 mL) and brine (200 mL). The combined aq. layers were extracted with $Et_2O$ (2 x 150 mL) and the combined organic fractions were dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford the title compound as a yellow oil (0.904 g, 2.64 mmol, 98%). $R_f$ = 0.5 (pentane/$Et_2O$ = 96:4). $^{1}$H NMR (400 MHz, $CDCl_3$): δ 5.47 – 5.26 (m, 12H), 3.67 (s, 3H), 2.92 – 2.75 (m, 10H), 2.46 – 2.32 (m, 4H), 2.08 (m, 2H), 0.97 (t, *J* = 7.5 Hz, 3H). $^{13}$C NMR (100 MHz, $CDCl_3$): δ 173.6, 132.1, 129.4, 128.7, 128.4, 128.4, 128.3, 128.2, 128.2, 128.0, 127.1, 51.7, 34.1, 25.8, 25.7, 25.7, 22.9, 20.7, 14.4. Spectra were consistent with previously reported data.[44]

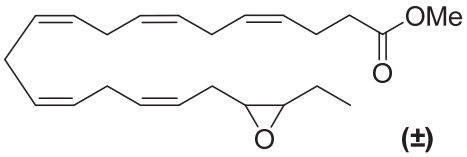
**Methyl (4*Z*,7*Z*,10*Z*,13*Z*,16*Z*)-19-bromo-20-hydroxydocosa-4,7,10,13,16-pentaenoate (4a) & Methyl (4*Z*,7*Z*,10*Z*,13*Z*,16*Z*)-20-bromo-19-hydroxydocosa-4,7,10,13,16-pentaenoate (4b).**



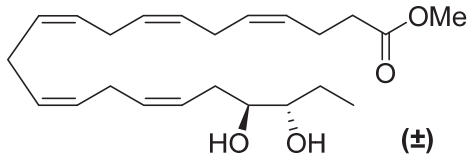

Methyl ester **3** (0.534 g, 1.56 mmol) was dissolved in dimethoxyethane/$H_2O$ (22.9 mL, 4:1 (v/v)) and cooled to 0 °C. A solution of freshly recrystallized NBS (0.399 g, 2.42 mmol) in dimethoxyethane/$H_2O$ (5.5 mL, 4:1 (v/v)) was added over 24 hours with the use of a syringe pump. After an additional 2 h of stirring, aq. $Na_2S_2O_3$ (6.0 mL, 10 wt%) was added dropwise and the reaction mixture was allowed to reach room temperature. The mixture was diluted with $Et_2O$ (250 mL), washed with $H_2O$ (3 x 125 mL) and brine (100 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (pentane/$Et_2O$ = 96:4 to 74:26) to afford the starting material **3** as a colorless oil (0.183 g, 0.53 mmol, 34%) and a mixture of monobromohydrins including **4a** and **4b** as a colorless oil (0.183 g, 0.42 mmol, 27%). $R_f$ = 0.3 (pentane/$Et_2O$ = 4:1). $^{1}$H NMR (400 MHz, $CDCl_3$): δ 5.62 – 5.28 (m, 10H), 4.13 – 3.99 (m, 1H), 3.68 (s, 3H), 3.60 – 3.38 (m, 1H), 2.91 – 2.71 (m, 10H), 2.45 – 2.33 (m, 4H), 1.68 – 1.54 (m, 2H), 0.98 (t, *J* = 7.4 Hz, 3H).$^{13}$C NMR (100 MHz, $CDCl_3$): δ 173.7, 132.2, 131.4, 131.2, 129.4, 128.7, 128.6, 128.6, 128.3, 128.2, 128.1, 128.0, 128.0, 127.8, 127.1, 126.2, 125.9, 124.8, 74.4, 73.4, 72.3, 66.1, 63.1, 51.7, 34.1, 34.0, 33.8, 29.3, 29.1, 26.1, 26.0, 25.8, 25.7, 22.9, 12.7, 10.1. HRMS: Calculated for $[C_{23}H_{35}BrO_3+H]^+$ 439.1842, found 439.1842.

**Methyl (4*Z*,7*Z*,10*Z*,13*Z*,16*Z*)-18-(3-ethyloxiran-2-yl)octadeca-4,7,10,13,16-pentaenoate (5).**



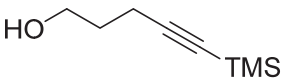

The mixture of **4a** and **4b** (0.116 g, 0.26 mmol) was coevaporated thrice with toluene and dissolved in a methanolic solution of NaOMe (0.2 M, 6.6 mL, 1.3 mmol). After stirring for 45 min at room temperature, the reaction mixture was diluted with $Et_2O$ (100 mL) and 0.2 M HCl (7 mL) was added dropwise. The organic layer was washed with $H_2O$ (2 x 50 mL) and brine (50 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude **5** was used in the next reaction without further purification. $R_f$ = 0.5 (pentane/$Et_2O$ = 4:1). $^1$H NMR (400 MHz, $CDCl_3$): δ 5.52 – 5.34 (m, 10H), 3.68 (s, 3H), 2.98 – 2.85 (m, 10H), 2.43 – 2.35 (m, 4H), 2.30 – 2.20 (m, 2H), 1.61 – 1.53 (m, 2H), 1.04 (t, *J* = 7.5 Hz, 3H). $^{13}$C NMR (100 MHz, $CDCl_3$): δ 173.69, 130.53, 129.43, 128.53, 128.36, 128.32, 128.26, 128.22, 128.04, 127.98, 124.65, 58.48, 56.66, 51.73, 34.15, 26.32, 25.95, 25.79, 25.72, 22.94, 21.22, 10.79. HRMS: Calculated for $[C_{23}H_{34}O_3+H]^+$ 359.2581, found 359.2583.

**Methyl (4*Z*,7*Z*,10*Z*,13*Z*,16*Z*)-19,20-dihydroxydocosa-4,7,10,13,16-pentaenoate (6).**



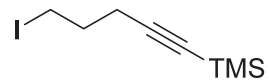

To a cooled (0 °C) solution of crude **5** in THF (4.3 mL) was added dropwise aq. $HClO_4$ (0.4 M, 3.3 mL) and the resulting suspension was stirred for 4 h at rt. Phosphate buffer A (8 mL) was added and the reaction mixture was diluted with $H_2O$ (80 mL). The aq. layer was extracted with EtOAc (3 x 50 mL) and the combined organic layers were washed with brine (50 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (pentane/EtOAc = 4:1) to afford the title compound as a colorless oil (0.068 g, 0.181 mmol, 69% over 2 steps). $R_f$ = 0.4 (pentane/EtOAc = 3:2). $^1$H NMR (400 MHz, $CDCl_3$): δ 5.63 – 5.30 (m, 10H), 3.68 (s, 3H), 3.50 (dd, *J* = 12.0, 8.0 Hz, 1H), 3.39 (dt, *J* = 8.0, 4.0 Hz, 1H), 2.90 – 2.79 (m, 8H), 2.42 – 2.30 (m, 6H), 1.65 – 1.45 (m, 2H), 1.00 (t, *J* = 7.5 Hz, 3H). $^{13}$C NMR (100 MHz, $CDCl_3$): δ 173.81, 131.39, 129.46, 128.53, 128.36, 128.23, 128.01, 125.51, 75.31, 73.53, 51.77, 34.14, 31.93, 29.86, 26.68, 25.93, 25.80, 25.73, 22.94, 10.20. HRMS: Calculated for $[C_{23}H_{36}O_4+Na]^+$ 399.2506, found 399.2508.

**5-(Trimethylsilyl)pent-4-yn-1-ol (8).**



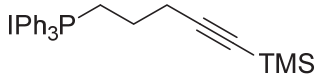

To a cooled (-78 °C) solution of pent-4-yn-1-ol (1.1 mL, 12 mmol) in THF (45 mL) was added dropwise *n*-BuLi (1.6 M solution in hexanes, 16.3 mL, 26.1 mmol) over a period of 20 min affording a light yellow suspension. After stirring for 50 min at rt, the solution was cooled to -78 °C and TMSCl (3.3 mL, 28 mmol) was added dropwise. The reaction mixture was allowed to reach room temperature overnight. 3 M HCl (16.7 mL) was added and after 40 min the solution was diluted with $H_2O$ (60 mL) and extracted with $Et_2O$ (2 x 30 mL). The combined organic fractions were washed with brine (30 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification of the residue by silica gel column chromatography (pentane/EtOAc = 9:1) afforded the title compound as a colorless oil (1.52 g, 9.75 mmol, 82%). Spectra were consistent with previously reported data.[45]

**5-(Trimethylsilyl)pent-4-ynyl-1-iodide (9).**



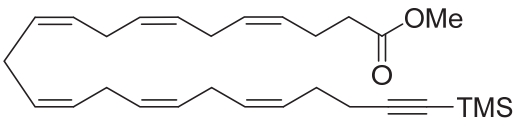

To a cooled (0 °C) solution of **8** (0.179 g, 1.15 mmol) in THF (7.8 mL) was added $PPh_3$ (0.45 g, 1.72 mmol), imidazole (0.12 g, 1.72 mmol) and $I_2$ (0.44 g, 1.72 mmol). The reaction mixture was stirred for 1 h at room temperature and diluted with $Et_2O$ (20 mL) and an aq. $Na_2S_2O_3$ solution (10 wt%, 30 mL). The aq. layer was extracted with $Et_2O$ (20 mL) and the combined organic fractions were washed with brine (20 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was triturated in pentane (4 mL) overnight and the resulting suspension was filtered over a glass frit. The filtrate was concentrated under reduced pressure and the residue was purified by silica gel column chromatography (pentane) to afford the title compound as a yellow oil (0.283 g, 1.06 mmol, 93%). Spectra were consistent with previously reported data.[45]

**5-Trimethylsilylpent-4-ynyltriphenylphosphineiodide (10).**



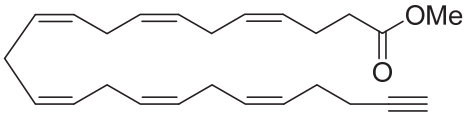

To a solution of **9** (2.45 g, 9.19 mmol) in acetonitrile (46 mL) was added $PPh_3$ (3.62 g, 13.8 mmol) and the suspension heated to reflux for 72 h. After cooling to room temperature, the reaction mixture was concentrated under reduced pressure and the residue was purified by silica gel column chromatography (DCM/MeOH = 95:5) to afford the title compound as a white foam (4.27 g, 8.07 mmol, 88%). $R_f$ = 0.3 (DCM/MeOH = 95:5). $^1$H NMR (400 MHz, $CDCl_3$): δ 7.76 – 7.65 (m, 9H), 7.65 – 7.57 (m, 6H), 3.73 – 3.61 (m, 2H), 2.54 (td, *J* = 6.6, 1.3 Hz, 2H), 1.82 – 1.70 (m, 2H), 0.00 (s, 9H). $^{13}$C NMR (100 MHz, $CDCl_3$): δ 135.03 (d, *J* = 3 Hz), 133.32 (d, *J* = 10 Hz), 130.37 (d, *J* = 13 Hz) 117.48 (d, *J* = 87 Hz), 104.83, 86.46, 21.70 (d, *J* = 5 Hz), 21.42 (d, *J* = 44 Hz), 20.33 (d, *J* = 18 Hz), -0.14. HRMS: Calculated for $[C_{26}H_{30}PSi]^+$ 401.1849, found 401.1855.

**Methyl (4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*)-24-(trimethylsilyl)tetracosa-4,7,10,13,16,19-hexaen-23-ynoate (12).**



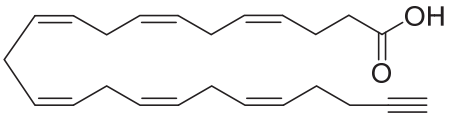

To a cooled (0 °C) solution of **6** (0.085 g, 0.226 mmol) in MeOH (4.4 mL) was added a solution of $NaIO_4$ (0.072 g, 0.339 mmol) in $H_2O$ (1.3 mL). After 3.5 h, the mixture was diluted with $Et_2O$ (50 mL), washed with $H_2O$ (3 x 25 mL) and brine (20 mL), dried over $Na_2SO_4$, filtered and concentrated under reduced pressure. Phosphonium salt **10** (0.300 g, 0.564 mmol) was coevaporated with benzene/$CHCl_3$ (1:1 (v/v)) and twice with benzene, and subsequently suspended in THF (5 mL). The suspension was cooled to -78 °C and KO*t*Bu (1.0 M in THF, 0.56 mL, 0.56 mmol) was added dropwise. The solution was stirred for 1 h and then cooled down to -110 °C. Then, the aldehyde was coevaporated with benzene twice and added to the reaction via cannula in THF (1 mL). The reaction mixture was stirred at -90 °C for 1 h, warmed to 0 °C over 2 h and then cooled to -78 °C. It was diluted with $Et_2O$ (7 mL) and quenched with a suspension of silica (5 mL) in $Et_2O$ (7 mL). The reaction was allowed to reach room temperature and filtered. The silica plug was rinsed with $Et_2O$ (20 mL) and the solvents were concentrated under reduced pressure. The residue was purified by silica gel column chromatography (pentane/$Et_2O$ = pentane to 49:1) to afford the title compound as a clear oil (0.0184 g, 0.042 mmol, 19%). $R_f$ = 0.6 (pentane/$Et_2O$ = 96:4). $^1$H NMR (400 MHz, $CDCl_3$): δ 5.50 – 5.31 (m, 12H), 3.68 (s, 3H), 2.85 (m, 10H), 2.48 – 2.34 (m, 4H), 2.27 (m, 4H), 0.15 (s, 9H). $^{13}$C NMR (100 MHz, $CDCl_3$): δ 173.68, 129.45, 129.25, 128.41, 128.36, 128.31, 128.24, 128.22, 128.02, 107.00, 84.77, 51.73, 34.15, 26.75, 25.84, 25.78, 25.72, 22.93, 20.30, 0.28. HRMS: Calculated for $[C_{28}H_{42}O_2Si+H]^+$ 439.3027, found 439.3024.

**Methyl (4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*)-tetracosa-4,7,10,13,16,19-hexaen-23-ynoate (13).**




To a cooled (0 °C) solution of **12** (0.0232 g, 0.052 mmol) in THF (3 mL) was added dropwise TBAF (1 M in THF, 0.079 mL, 0.079 mmol) and the reaction was stirred at rt for 30 min. Then, aq. sat. $NH_4Cl$ (1 mL) and $Et_2O$ (30 mL) were added and the layers were separated. The organic layer was washed with $H_2O$ (3 x 15 mL) and brine (10 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (pentane/$Et_2O$ = 49:1) to afford the title compound as a clear oil (0.0183 g, 0.050 mmol, 95%). $R_f$ = 0.6 (pentane/EtOAc = 19:1). $^1$H NMR (400 MHz, $CDCl_3$): δ 5.50 – 5.32 (m, 12H), 3.68 (s, 3H), 2.86 (dd, *J* = 11.1, 6.6 Hz, 3H), 2.44 – 2.35 (m, 1H), 2.35 – 2.27 (m, 1H), 2.28 – 2.20 (m, 1H), 1.96 (t, *J* = 2.5 Hz, 1H). $^{13}$C NMR (100 MHz, $CDCl_3$): δ 173.63, 129.50, 129.44, 128.36, 128.31, 128.28, 128.22, 128.09, 128.02, 84.09, 68.60, 51.72, 34.14, 26.52, 25.84, 25.78, 25.71, 22.93, 18.88. HRMS: Calculated for $[C_{25}H_{34}O_2+H]^+$ 367.2632, found 367.2632.

**(4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*)-Tetracosa-4,7,10,13,16,19-hexaen-23-ynoic acid (cDHA, 2).**




To a cooled (0 °C) solution of **13** (0.0183 g, 0.050 mmol) in THF (1.6 mL) was added 1 M aq. LiOH (1.6 mL, 1.6 mmol) and the solution was allowed to reach rt overnight. After cooling the reaction mixture to 0 °C, 1 M aq. HCl (1.6 mL) was added and the solution was diluted with $H_2O$ (15 mL). The aq. layer was extracted with EtOAc (3 x 6 mL) and the combined organic fractions were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification of the residue by silica gel column chromatography (DCM/MeOH =199:1) afforded the title compound as a clear oil (0.0145 g, 0.041 mmol, 82%). $R_f$ = 0.6 (DCM/MeOH = 19:1). $^1$H NMR (500 MHz, $CDCl_3$): δ 5.51 – 5.30 (m, 12H), 2.81 (d, *J* = 38.1 Hz, 10H), 2.46 – 2.36 (m, 4H), 2.32 (dt, *J* = 11.3, 7.4 Hz, 2H), 2.28 – 2.21 (m, 2H), 1.96 (t, *J* = 2.6 Hz, 1H). $^{13}$C NMR (125 MHz, $CDCl_3$): δ 177.77, 152.67, 129.88, 129.71, 129.52, 128.44, 128.34, 128.30, 128.15, 128.11, 127.71, 84.21, 68.61, 33.89, 26.53, 25.86, 25.80, 25.75, 22.67, 18.90. HRMS: Calculated for $[C_{24}H_{32}O_2+Na]^+$ 375.2295, found 375.2302.

# Supplementary data

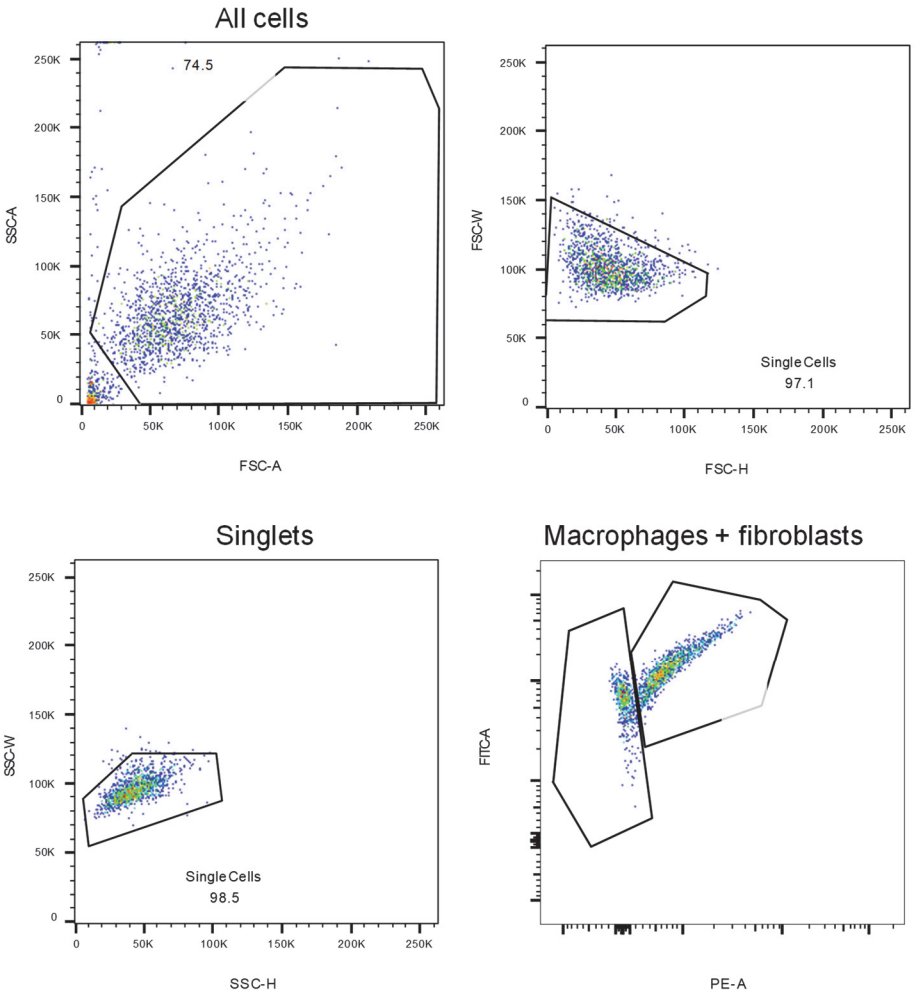



**Figure S1 | Gating strategy and example of coculture of fibroblasts with macrophages pretreated with cDHA.** Gating strategy included the exclusion of debris and doublets based on FSC/SSC. Separation of macrophages and fibroblasts in the coculture was based on the autofluorescence observed in both the PE and FITC channel.

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