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Title:

Lowering the linoleic acid to alpha-linoleic acid ratio decreases the production of inflammatory mediators by cultured human endothelial cells

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Introduction

Vascular endothelial cells play important roles in atherosclerosis and other inflammatory processes through controlling adhesion and transendothelial migration of circulating immune cells [1,2]. Lowering the production of inflammatory mediators by endothelial cells may be a potential target to inhibit the atherosclerotic process.

Polyunsaturated fatty acids (PUFAs) act through a variety of mechanisms to affect inflammatory processes [3,4]. However, the effects of different PUFAs on these processes may be divergent and interdependent. Long-chain PUFAs such as arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been well explored in the context of inflammation [5,6]. Other PUFAs are less well studied. The plant-derived n-3 PUFA alpha-linolenic acid (ALA), and the n-6 PUFA linoleic acid (LA) are both precursors for further desaturation and elongation that occurs by shared enzymes in a complex biological pathway. ALA may be converted into LC n-3 PUFAs including EPA, docosapentaenoic acid (DPA) and DHA, while LA can be converted to AA and beyond [7]. The conversion of ALA into LC n-3 PUFAs may be important for the physiological effects of ALA [7]. Although the conversion efficiency may be limited in humans [8], ALA may constitute an alternative and important source of LC n-3 PUFAs given the greater availability of sources rich in ALA such as walnuts, flaxseed, green leafy vegetables and plant oils including canola and soybean oils [9] compared to marine-derived LC n-3 PUFAs. Most Western diets contain an excess of LA over ALA [7]. Lowering the ratio of LA to ALA is likely to favour synthesis of EPA [7].

The first step of the pathway of LA and ALA conversion is catalysed by a Δ6-desaturase, which is considered to be the rate-limiting step in the reaction [10]. No interconversion occurs between the n-3 PUFA and n-6 PUFA families, but competition between ALA and LA may occur and includes both substrate competition and product inhibition [11–16]. In addition, previous studies have suggested that the Δ6-desaturase may have a higher substrate preference for ALA than LA [16–19]. However, the importance of such preference is uncertain because the intake of LA in most populations by far exceeds that of ALA [7].

LC n-3 PUFAs may exert inhibitory effects on inflammation by reducing leucocyte chemotaxis, adhesion molecule expression and leukocyte-endothelial adhesive interactions, and production of pro-inflammatory cytokines and of pro-inflammatory eicosanoids from AA [4,5]. They also promote increased production of inflammation-resolving mediators including resolvins,

protectins and maresins [4,5]. However, LC n-3 PUFAs do also give rise to inflammatory eicosanoids, but these are considered less inflammatory compared to those produced from AA [4,5].

The suggested competitive mechanisms between ALA and LA imply that lower ratios of LA:ALA may be associated with a higher conversion efficiency of ALA and a lower production of inflammatory mediators when compared to higher ratios. The effect of changing the ratio of LA to ALA on inflammatory responses has been little studied. Therefore, the objectives of this study were to investigate the incorporation and metabolism of ALA and LA in cultured endothelial cells and to explore the production of inflammatory mediators by these cells when exposed to different concentrations of ALA, LA and ratios of LA:ALA.

Materials and methods

Cell culture conditions and study design

The human hybrid endothelial cell line EA.hy926 (ATCC® CRL-2922™) was obtained from ATCC. The cells were thawed and subcultured in accordance with the suppliers' instructions. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 1% L-glutamine, penicillin and streptomycin solution and HAT Media Supplement (100 µM hypoxanthine, 0.4 µM aminopterin, 16 µM thymidine, Hybri-Max™, Sigma-Aldrich). Prior to the experiments, cells were cultured in Corning T-175 culture flasks at 37°C in 5% CO_2 until cells were confluent.

ALA and LA were obtained from Sigma-Aldrich. Prior to the experimental procedures master stock concentrations of these fatty acids was verified using gas chromatography analyses. The fatty acids were dissolved in 100% ethanol. Subsequently, the fatty acids were diluted in DMEM to obtain final exposure concentrations of a total of 100 µM in 0.1% ethanol. Fatty acid treatments were as follows; 100 µM ALA, 100 µM LA and different ratios of LA:ALA: 1:4 (20 µM LA : 80 µM ALA), R1:1 (50 µM LA : 50 µM ALA), R4:1 (80 µM LA : 20 µM ALA), R9:1 (90 µM LA : 10 µM ALA) or R19:1 (95 µM LA : 5 µM ALA). In experiments evaluating potential competition between ALA and LA, we used a constant exposure concentration of 50 µM ALA with increasing amounts of LA (10 µM, 25 µM, 50 µM and 75 µM).

The incorporation and metabolism of ALA and LA were investigated by scraping and resuspending confluent endothelial cells at a density of 3.6 $x10^5$ cells/ml for culturing with either culture medium or culture medium supplemented with fatty acids corresponding to the exposure

concentrations described above. Cells were seeded at 9 $x10^5$ cells per T-25 culture flask and incubated for 48 hours at 37°C in 5% CO_2. Following incubation, cells were scraped, centrifuged at 1200 rpm for 5 minutes and resuspended in unsupplemented DMEM for cell counting using a Beckman Coulter cell counter. Subsequently, the cells were centrifuged at 1200 rpm for 5 minutes and the pellet was resuspended in 0.9% NaCl to obtain a concentration of $1.25x10^6$ cells. The experiments were conducted in duplicates and repeated three times (3 independent experiments).

The production of inflammatory mediators was explored by culturing endothelial cells with either culture medium with supplements or culture medium with supplements and fatty acids described above for 48 hours followed by 24 hours stimulation with TNF-α (1 ng/ml). Initially, confluent endothelial cells were scraped from culture flasks and resuspended in supplemented DMEM at a density of $2x10^5$ cells/ml based on cell counts using a Beckman Coulter cell counter. The cells were seeded at $1x10^4$ cells/well in a 96-well bottom flat plate. Following incubation, supernatant was removed and stored below -20°C until analysis. These experiments were conducted in triplicates and repeated three times (3 independent experiments).

Lipid extraction and fatty acid analysis

The fatty acid composition of the endothelial cells was determined using gas chromatography. Total lipid was extracted from the resuspended cell pellets of which 800 µl was transferred to glass tubes with 30 µl of internal standard (1 µg/µl 37 FAMES). Subsequently, 5.0 ml chloroform:methanol (2:1) containing butylated hydroxytoluene (50 mg/l) as anti-oxidant was added followed by 1.0 mL 1 M NaCl. The samples were vortexed and centrifuged at 2000 rpm for 10 minutes. The lower phase was collected from each sample using Pasteur pipettes and transferred to glass tubes which were dried under nitrogen at 40°C. Then, 0.5 ml dry toluene and 1.0 ml methanol containing 2% (v/v) H_2SO_4 were added to each sample followed by heating at 50°C for 2 hours. Samples were allowed to cool and 1.0 ml of neutralizing solution (0.25 M $KHCO_3$ (25.03 g/l) and 0.5 M K_2CO_3 (69.10 g/l)) was added. Subsequently, 1.0 ml dry hexane was added and the samples were centrifuged at 1000 rpm for 2 minutes. The upper phase was collected using Pasteur pipettes and dried under nitrogen. Samples were resuspended in 150 µl dry hexane and transferred to a gas chromatograph autosampler vial. The fatty acid analyses were performed using a Hewlett-Packard 6890 chromatograph. A SGE BPX-70 capillary column (30 m x 0.2 mm x 0.25 µm) and temperature programming were used (115°C for 2 min, then increased by 10°C every minute until reaching 200°C which was held for a further 18 minutes). The injector port temperature was 300°C

and the split ratio 2:1. Helium was used the carrier gas. Chromatograms were analysed using Agilent ChemStation software. Fatty acid concentrations were expressed in μg per 10^6 cells.

Assessment of inflammatory mediators

The concentrations of the inflammatory mediators including interleukin 6 (IL-6), interleukin 8 (IL-8), vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1), regulated on activation, normal T cell expressed and secreted (RANTES) and intercellular adhesion molecule-1 (ICAM-1) were determined using a purchased premixed Human Luminex Multi-Analyte kit (R&D Systems Europe, Ltd). Reagents and standards were prepared in accordance with the instructions from the manufacturer. Plates were analysed on a calibrated Luminex® 200 reader using Bio-Plex software (version 6.1, Bio-Rad Laboratories Inc. United States). Lower limits of detection are: IL-6 (1.7 pg/mL), IL-8 (1.8 pg/mL), VEGF (2.1 pg/mL), MCP-1 (9.9 pg/mL), RANTES (1.8 pg/mL) and ICAM-1 (87.9 pg/mL).

Evaluation of cell viability

Cell viability was evaluated as mitochondrial activity using a quantitative colorimetric assay. Confluent cells were scraped and resuspended at a density of $2x10^5$ cells/ml. The cells were then seeded at $1x10^4$ cells/well in a 96-well bottom flat plate. The cells were incubated for 48 hours with the various fatty acid exposure concentrations as described previously, followed by 24hour incubation with TNF-α (1 ng/ml). These experiments were conducted in triplicates and repeated three times (n = 9). Following, the medium was replaced by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (5mg/mL) (Sigma-Aldrich) in DMEM (100 μl/well) and incubated for 4hours. After incubation, 75 μl supernatant was replaced by 75 μl DMSO added to each well. The plates were incubated for a further 10 minutes. Absorbance was measured at 540 nm on a plate reader. The absorbance in DMEM controls was set at 100%.

Statistical analyses

We used multiple linear regression to calculate the average mean difference across experiments in the PUFA content and the production of inflammatory mediators with 95% confidence intervals (CIs). In analyses of either ALA or LA alone, we used DMEM as reference. In analyses of the different ratios of LA:ALA the highest ratio (R19) was used as reference, whereas in analyses evaluating effects of adding increasing LA amounts to a constant ALA concentration, we used ALA

alone as reference. In analyses of cell viability we used DMEM control as reference. Model assumptions of normality and variance homogeneity of the observations were assessed visually by inspection of residual plots. Further, the assumption of variance homogeneity was formally tested using robust tests for the equality of variances. In sensitivity analyses and when assumptions were not met, we used robust variance estimation based on bootstrap analyses with 5000 replicates to calculate CI intervals and P-values. Data were analyzed using Stata statistical software (version 14; StataCorp MP), and P-values below 0.05 was considered statistically significant. Graphs were prepared using GraphPad Prism 7.0 (GraphPad Software, La Jolla California USA).

Results

Incorporation and metabolism of PUFAs

In cells incubated with either 100 μM of ALA or LA alone, the absolute content of these PUFAs was statistically significantly higher than in cells incubated with DMEM. Also, the absolute content of ALA increased in a dose-dependent manner with lower ratios of LA:ALA although only statistically significantly when comparing the lowest ratios (4:1, 1:1 and 4:1) with the highest (19:1) (Figure 1A and Supplemental Table 1). The content of LA decreased with lower LA:ALA ratios although only statistically significantly when comparing the lowest ratio with the highest. In cells incubated with 100 μM of ALA, the content the longer-chain metabolites ETA, EPA and DPA increased, whereas the content of DHA decreased when comparing with cells cultured in DMEM. Across ratios of LA:ALA, the content of DPA increased significantly with lower ratios. Also, the content of EPA increased slightly although not statistically significantly. No appreciable differences were observed across ratios for ETA (Figure 1B and Supplemental Table 1). Stearidonic acid could not be identified in any of the samples. In cells exposed to 100 μM of LA, the contents of GLA and AA were lower, whereas the content of DGLA was higher compared cells cultured in DMEM. In cells exposed to the lowest ratio of LA:ALA, there were lower concentrations of DGLA and AA compared cells cultured with the highest ratio of LA:ALA. No appreciable differences in the content of GLA were seen across ratios of LA:ALA (Figure 1C and Supplemental Table 1).

When cells incubated with 50 μM ALA and varying concentrations of LA, we found that cells incubated with both ALA and LA had significantly lower incorporation of ALA compared to cells incubated with 50 μM ALA alone. However, the incorporation did not seem to decrease with increasing exposure to LA (Figure 2 and Supplemental Table 2). The content of the longer-chain

metabolites EPA and DPA was lower in cells incubated with both ALA and LA compared to 50 μM ALA alone although this was only statistically significant for EPA.

Inflammatory mediators

The concentrations of VEGF, RANTES, ICAM-1, MCP-1 and IL-6 were statistically significantly lower in the medium of cells incubated with 100 μM ALA when comparing with TNF-α stimulated control cells cultured in DMEM (Figure 3 and Supplemental Table 3). No statistically significant difference was found for IL-8 in cells incubated with 100 μM ALA. In cells incubated with 100 μM LA, there were statistically significantly lower concentrations of RANTES and MCP-1 in the medium compared with cells incubated with DMEM control. Also, a slight, but not statistically significant, lower concentration of ICAM-I was found in the medium of cells incubated with 100 μM LA. When evaluating different ratios of LA:ALA, the concentrations of VEGF, RANTES, MCP-1 and IL-6 decreased with lower ratios when comparing with the highest ratio. However, the observed lower concentrations of VEGF, MCP-1 and IL-6 were only statistically significant for the two lowest ratios (1:4 and 1:1) when comparing with the highest. Also, the concentration of ICAM-1 was only statistically significantly lower for the lowest ratio compared with the highest. No appreciable nor consistent pattern was observed across ratios for IL-8 concentration.
In cells incubated with either 50 μM ALA or LA, there were statistically significantly lower concentrations of VEGF, RANTES, ICAM-1, MCP-1 and IL-6, whereas no appreciable differences were seen for IL-8, when comparing with cells incubated in DMEM. In cells incubated with 50 μM ALA and varying concentrations of LA, the concentrations of VEGF, RANTES, ICAM-1 and IL-6 decreased slightly with increasing LA concentrations, but these differences were only statistically significant when comparing cells incubated with 50 μM ALA and the two highest LA concentrations (50 μM and 75 μM) (Figure 4 and Supplemental Table 4).

Cell viability

Cells incubated with LA alone (50 μM or 100 μM) had a moderately lower viability than cells incubated with DMEM although only statistically significantly so for cells incubated with the lowest LA concentration (50 μM) (Figure 5). Cells incubated with ALA (50 μM or 100 μM) alone had an approximately 20% lower viability than cells incubated with DMEM alone although only statistically significantly so for cells incubated with 50 μM ALA. Cells incubated with a LA:ALA ratio of 1:1 had an statistically significantly lower viability, whereas no discernible differences in

viability were at LA:ALA ratios of 1:4, 4:1, 9:1 and 19:1, respectively (Figure 5A). However, cells incubated either 10 μM, 25 μM, 50 μM or 75 μM LA in addition to 50 μM ALA had a statistically significant lower viability compared to cells incubated with DMEM of approximately 25% (Figure 5B).

Discussion

In this in vitro study, we found that TNF-α stimulated endothelial cells preincubated with low LA:ALA ratios had a lower production of VEGF, RANTES, MCP-1 and IL-6 compared to cells preincubated with a high LA:ALA ratio. The observed anti-inflammatory effects were likely to be a result of increased ALA exposure because endothelial cells preincubated with ALA alone had a lower production of the same cytokines compared to DMEM controls, whereas much less of an effect was seen in cells incubated with LA alone. However, LA seemed to lower the production of RANTES and MCP-1. Interestingly, the production of VEGF, RANTES, MCP-1 and IL-6 also seemed to decrease in endothelial cells incubated with increasing LA concentrations while ALA exposure was kept constant. This suggests that LA may have some anti-inflammatory effects, which is in contrast to concerns raised about potential pro-inflammatory properties of LA possibly due to its role as a precursor of AA, which may be converted to a variety of potent pro-inflammatory prostaglandins and leukotrienes [20]. However, a recent review concluded that the current evidence does not support that dietary intake nor plasma levels of LA appear to result in increased tissue AA or in increased in vivo or ex vivo concentrations of inflammatory markers in humans [6]. In this study, we found that both ALA and LA were incorporated into the cells in a dose-dependent pattern, but no appreciable differences were seen in the content of AA in the cells across ratios of LA:ALA. However, the content of AA was markedly lower in cells incubated with LA, ALA and ratios of LA:ALA compared to DMEM control. In endothelial cells incubated with ALA alone, we found higher contents of ETA, EPA and DPA compared to DMEM control suggesting that ALA is metabolised into these longer-chain PUFAs, whereas no discernible differences were seen across ratios of LA:ALA, which could indicate competition from LA on ALA metabolism. This was supported by our findings that the content of ETA increased while EPA decreased slightly when higher concentrations of LA were applied to the cells exposed to constant ALA concentrations although these differences were not statistically significant. We do not have a clear explanation for the observed finding that the content of DPA, but not EPA or DHA, seemed to increase when lowering the LA:ALA ratio. In humans, the conversion of PUFAs occurs predominantly in the liver

[7] and a study has suggested that the conversion of ALA is influenced by the absolute intake of ALA and LA and not by their ratio [21].

The dietary intake of LA has increased markedly throughout the last decades and the balance between n-6 and n-3 PUFAs has been fiercely debated in relation to cardiovascular disease (CVD) risk because of the suggested divergent effects of these PUFAs families on inflammation [22]. Thus, a balance between n-6 and n-3 PUFAs has been suggested. Substantial variations in the dietary LA:ALA ratio exist among different countries [7], but several western countries have LA:ALA ratios of 15-20 [7,23]. Lowering the n-6:n-3 PUFA ratio in the diet has been suggested to be a better predictor of CVD than the absolute intakes of n-3 and n-6 PUFAs [23] although this hypothesis has never been proven or disproven conclusively [24]. Inflammation plays a crucial role in the pathogenesis of atherosclerotic cardiovascular disease [1] and several studies have suggested that n-3 and n-6 PUFAs may influence inflammatory processes through a variety of mechanisms [3,5]. In contrast, limited knowledge exists on the underlying biological effects on inflammatory processes when cells are exposed to a combination of PUFAs. However, a recent study by Yang et al. examined various LA:ALA ratios (1:1, 5:1,10:1 and 20:1) on markers of inflammation, oxidative stress and cell function in endothelial cells and found no appreciable differences in IL-6 and TNF-α concentrations across ratios of LA:ALA in unstimulated cells [25]. In contrast, a previous animal study found that rats fed a high-fat diet based on plant oils with resulting in a low n-6:n-3 PUFA ratio (1:1 and 5:1) had lower serum levels of IL-6, TNF-α and C-reactive protein (CRP) compared with rats fed with a high n-6:n-3 PUFA ratio (20:1) diet [26]. Another study found that rats fed ad libitum diet with an n-3:n-6 PUFA ratio of 1:1 based on plant oils had lower levels of IL-6, TNF-α and CRP compared to control rats fed a diet high in saturated fatty acids (controls), whereas only TNF-α was found to be statistically significantly lower in rats fed with a n-3:n-6 PUFA ratio of 1:4 compared to control rats [27]. The authors did not formally compare the two PUFA diets, but the levels of IL-6, TNF-α and CRP seemed to be lower in rats fed an diet with n-3:n-6 PUFA ratio of 1:1 compared with those fed an n-3:n-6 PUFA ratio of 1:4 [27]. A study in pigs fed with different plant oils to obtain different dietary n-6:n-3 PUFA ratios (1:1, 10:1 and 20:1) found that the lowest ratio resulted in lower levels of IL-6 and IL-β compared to those fed diets with higher ratios (2.5:1, 5:1 and 10:1) [28]. Hintze et al. [29] suggested that the total amount of PUFAs derived from plants may inhibit the LPS-induced acute inflammatory response, whereas the n-6:n-3 PUFA ratio may only be important at higher concentrations of total PUFAs. Importantly, these animal feeding studies were based on supplemental foods, which may provide many nutrients that could influence

inflammatory mediators. Also, the interpretational complexity of a ratio measure makes direct comparisons difficult. Using ratios of fatty acids may be an appealing approach under controlled conditions especially when effects of the numerator and denominator are assumed to be in opposite directions and interdependent. However, this metric should not stand alone and the independent effects the fatty acids under study should also be evaluated. Furthermore, adding combinations of PUFAs by increasing the concentrations of one while keeping another constant may provide some additional insight into the interplay between PUFAs.

Endothelial cells play a pivotal role in the initiation and progression of atherosclerosis through leukocyte recruitment, expression of adhesion molecules and mediating migration of leukocytes into the intima [30]. In this study, we selected a number of inflammatory mediators believed to be important for the inflammatory response that underlies the atherosclerotic process. ICAM-1 is an adhesion molecule belonging to the immunoglobulin superfamily, which is expressed on endothelial cells and involved in leukocyte-endothelium interactions [31]. MCP-1, RANTES and IL-8 are chemoattractants involved in the chemotaxis of leukocytes and migration into the sub-endothelial layer [30]. IL-6 is a cytokine with pleiotropic effects on regulation of the immune response, acute phase response and inflammation [32]. VEGF is involved in angiogenesis, proliferation of endothelial cells, and regulating vascular permeability and migration of monocytes [33]. We used an inflammatory cell model to investigate the interplay between LA and ALA in vitro. However, extrapolation of these study findings into an in vivo situation should be done with caution and further studies are warranted to explore the effects of combinations of fatty acids on inflammation.

In conclusion, this study suggests that a low LA:ALA ratio exerts anti-inflammatory effects by lowering the production VEGF, RANTES, ICAM-1, MCP-1 and IL-6 in TNF-α stimulated endothelial cells compared to a high ratio, which was likely to be mediated by a higher ALA exposure rather than a lower LA exposure. This study also suggests that LA is not pro-inflammatory in this cell model of endothelial inflammation and may even possess some anti-inflammatory properties.

Declaration of interest: None

Author contributions:

The author responsibilities were as follows: CSB, EJB and PCC conceived the study; CSB and EJB contributed equally to the data acquisition supervised by EAM; CSB conducted the statistical analyses, prepared the tables and figures and wrote the first draft of the manuscript; SLC supervised the planning and conduct of the statistical analyses; CSB, EJB, SLC, EAM and PCC contributed to the interpretation of the data and writing of the manuscript. All authors read and approved the final manuscript.

Fig. 1. Incorporation of LA and ALA (A) and appearance of n-3 (B) and n-6 elongation (C) productions in EA.hy926 cells incubated for 48 hours with LA, ALA and different ratios of LA/ALA. Concentrations are given in means with corresponding standard errors of mean. The experiments were conducted in duplicates and repeated three times (3 independent experiments). LA (100 μM) and ALA (100 μM) alone were compared with DMEM, whereas ratios (R) of LA/ALA including ratio 1:4 (20:80 μM), 1:1 (50:50 μM), 4:1 (80:20 μM) and 9:1 (90:10 μM) were compared with the highest ratio (19:1, 95:5 μM). Statistically significant differences ($p < 0.05$) is marked by (*).

Fig. 2. Incorporation of ALA (A) and appearance of elongation products (B) in EA.hy926 cells incubated with 50 μM ALA and LA concentrations of 10 μM, 25 μM, 50 μM and 75 μM, respectively. Concentrations are given in means with corresponding standard errors of mean. The experiments were conducted in duplicates and repeated three times (3 independent experiments). ALA 50 μM was used as reference. Statistically significant differences ($p < 0.05$) is marked by (*).

Fig. 3. Concentrations of inflammatory mediators including VEGF (A), RANTES (B), ICAM-1 (C), MCP-1 (D), IL-6 (E) and IL-8 (F) in supernatant of EA.hy926 cells incubated for 48 hours with LA, ALA or different ratios of LA/ALA followed by 24 hours TNF-α stimulation. Concentrations are given as means with corresponding standard errors of mean. The experiments were conducted in triplicates and repeated three times (3 independent experiments). LA (100 μM) and ALA (100 μM)

alone were compared with DMEM, whereas ratios of LA:ALA including ratio 1:4, 1:1, 4:1 and 9:1 were compared with the highest ratio (19:1).

Fig. 4. Concentrations of inflammatory mediators including VEGF (A), RANTES (B), ICAM-1 (C), MCP-1 (D), IL-6 (E) and IL-8 (F) in supernatant of EA.hy926 cells incubated for 48 hours with 50 μM ALA and varying LA concentrations (10 μM, 25 μM, 50 μM and 75 μM) followed by 24 hours TNF-α stimulation. Concentrations are given in means with corresponding standard errors of mean. The experiments were conducted in triplicates and repeated three times (3 independent experiments)

Fig. 5. Cells viability of EA.hy926 cells incubated for 48 hours with (A) 100 μM LA, 100 μM ALA or various ratios of LA:ALA or (B) 50 μM LA, 50 μM ALA or various LA concentrations in addition to 50 μM ALA followed by 24 hours TNF-α stimulation. Cell viability are given in percentages with corresponding standard errors of mean. The experiments were conducted in triplicates and repeated three times (3 independent experiments). Standard errors were calculate based on bootstrap analyses with 5000 replications. Cells incubated with DMEM was used as reference.

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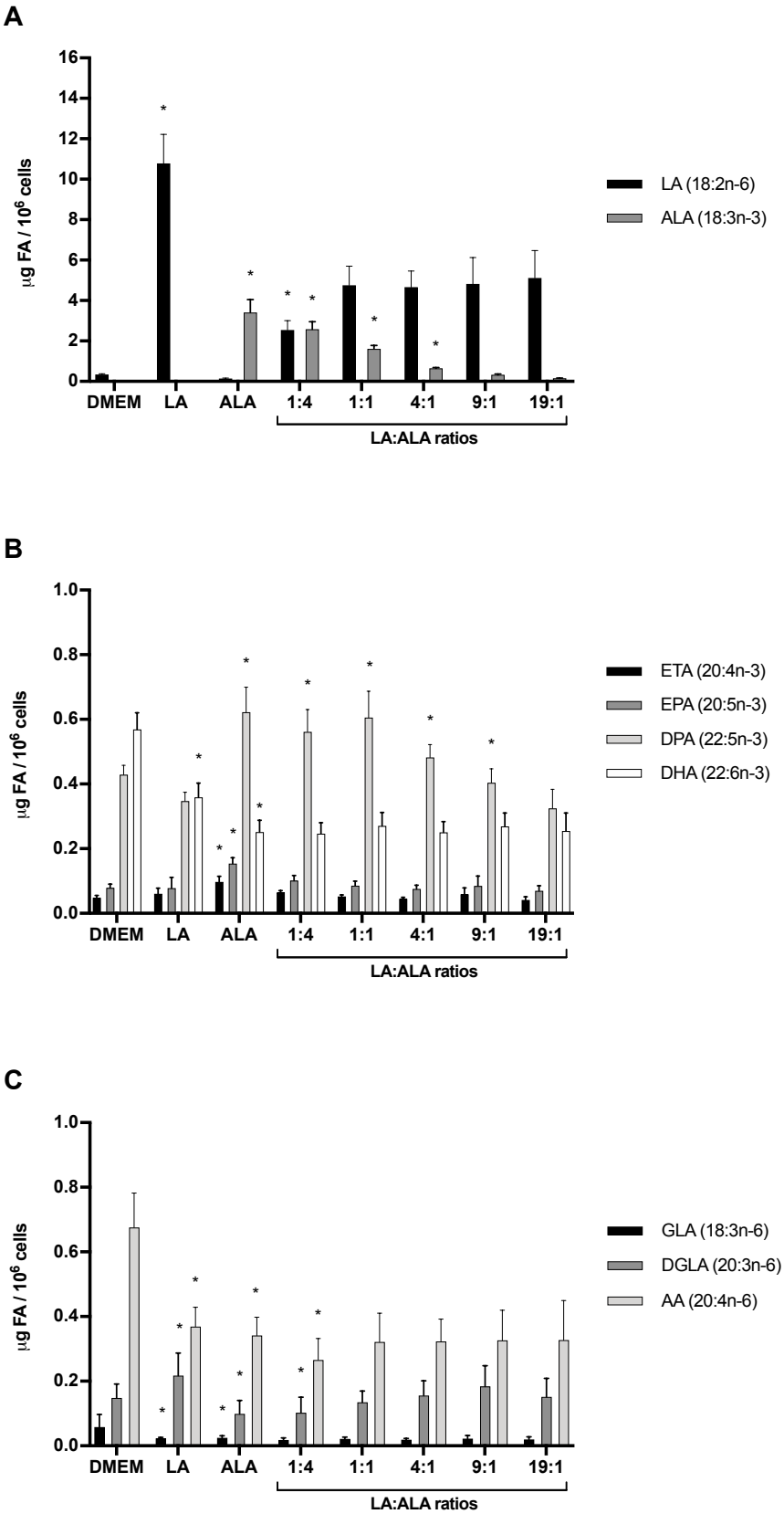
Figure 1



LA (100 µM) **ALA** (100 µM) **LA:ALA 1:4** [20:80 µM] **LA:ALA 1:1** [50:50 µM] **LA:ALA 4:1** [80:20 µM] **LA:ALA 9:1** [90:10 µM] **LA:ALA 19:1** [95:5 µM]

Figure 2

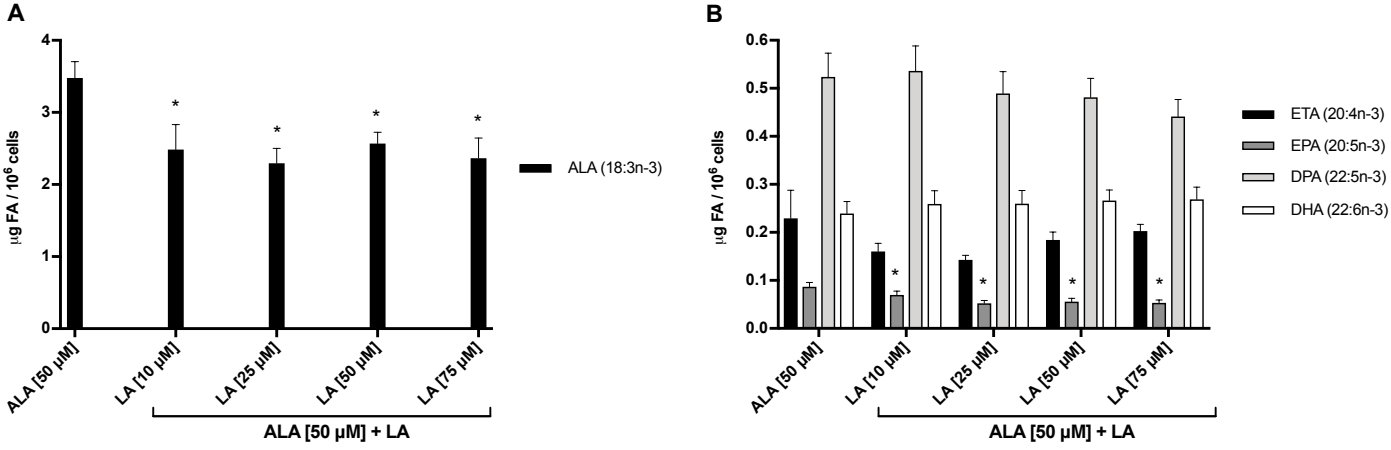
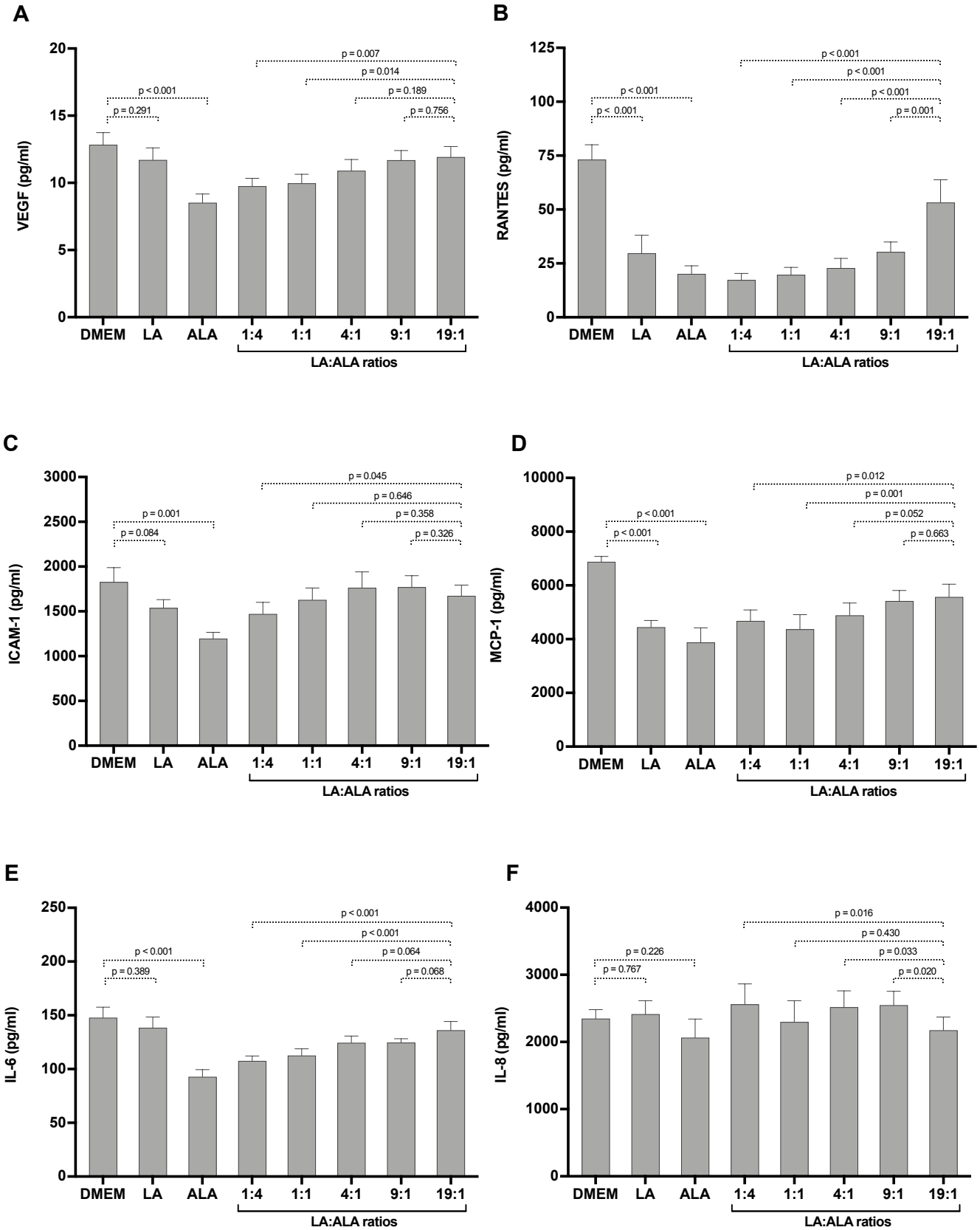

A
µg FA / 10^6 cells
4
3
2
1
0
*
*
*
*
ALA (18:3n-3)
ALA [50 µM]
LA [10 µM]
LA [25 µM]
LA [50 µM]
LA [75 µM]
ALA [50 µM] + LA
B
µg FA / 10^6 cells
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ETA (20:4n-3)
EPA (20:5n-3)
DPA (22:5n-3)
DHA (22:6n-3)
ALA [50 µM]
LA [10 µM]
LA [25 µM]
LA [50 µM]
LA [75 µM]
ALA [50 µM] + LA

Figure 3



LA (100 µM) **ALA** (100 µM) **LA:ALA 1:4** [20:80 µM] **LA:ALA 1:1** [50:50 µM] **LA:ALA 4:1** [80:20 µM] **LA:ALA 9:1** [90:10 µM] **LA:ALA 19:1** [95:5 µM]

Figure 4

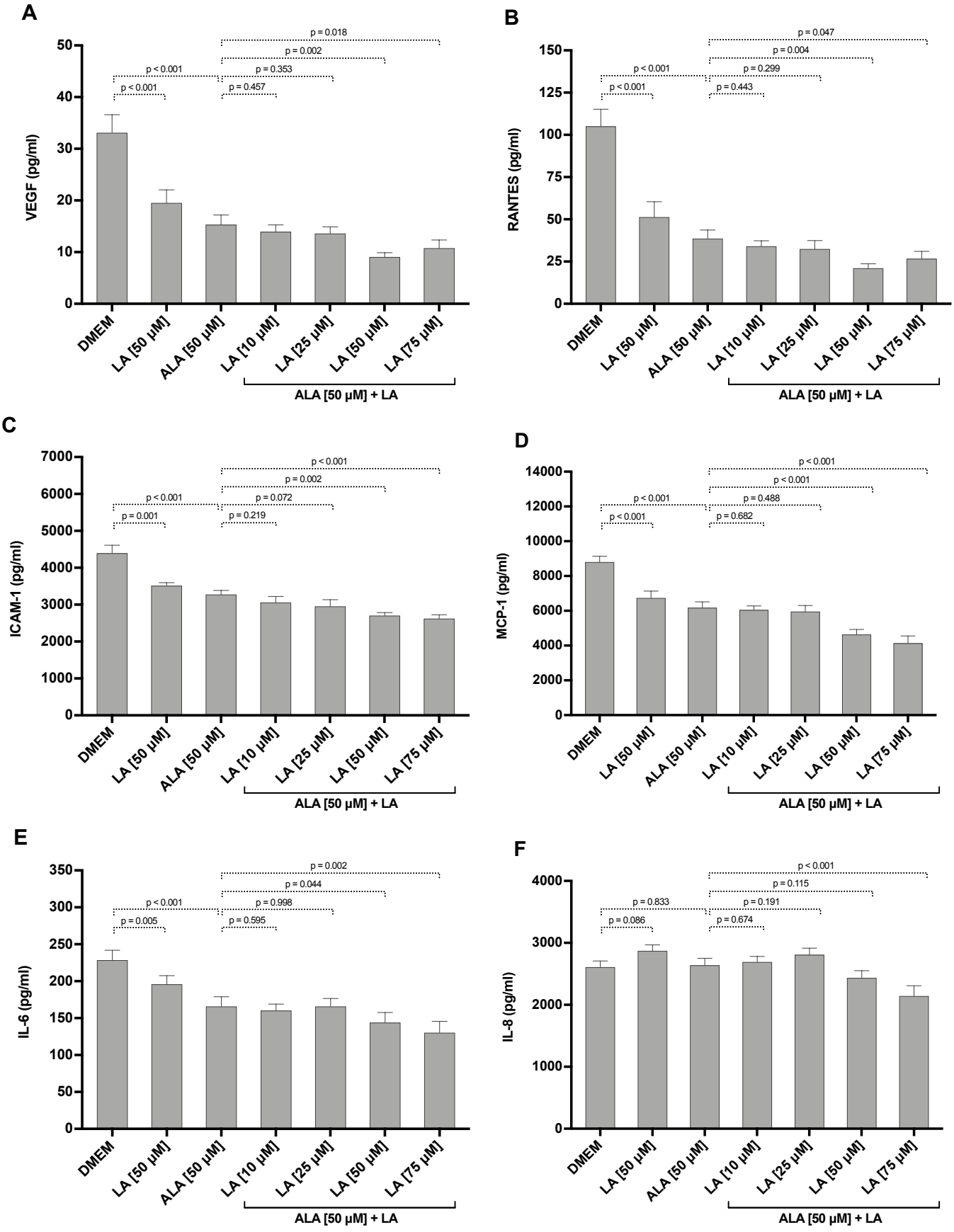
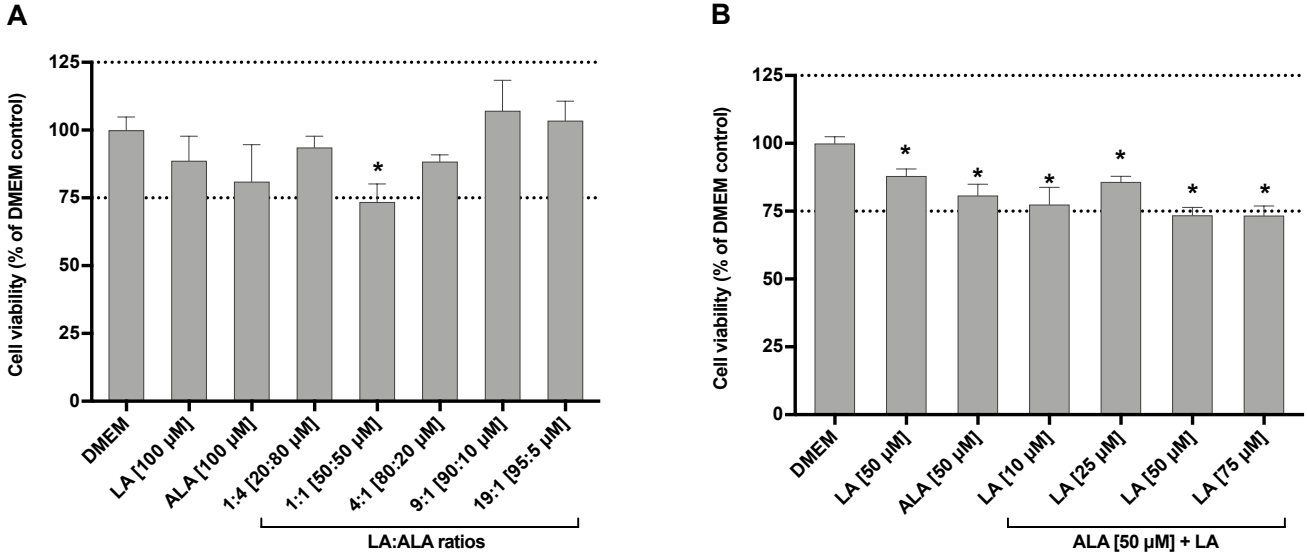

A
VEGF (pg/ml)
p = 0.018
p = 0.002
p = 0.353
p = 0.457
p < 0.001
p < 0.001
DMEM
LA [50 µM]
ALA [50 µM]
LA [10 µM]
LA [25 µM]
LA [50 µM]
LA [75 µM]
ALA [50 µM] + LA
B
RANTES (pg/ml)
p = 0.047
p = 0.004
p = 0.299
p = 0.443
p < 0.001
p < 0.001
C
ICAM-1 (pg/ml)
p < 0.001
p = 0.002
p = 0.072
p = 0.219
p < 0.001
p = 0.001
D
MCP-1 (pg/ml)
p < 0.001
p < 0.001
p = 0.488
p = 0.682
p < 0.001
p < 0.001
E
IL-6 (pg/ml)
p = 0.002
p = 0.044
p = 0.998
p = 0.595
p < 0.001
p = 0.005
F
IL-8 (pg/ml)
p < 0.001
p = 0.115
p = 0.191
p = 0.674
p = 0.833
p = 0.086

Figure 5


A
Cell viability (% of DMEM control)
125
100
75
50
25
0
DMEM
LA [100 µM]
ALA [100 µM]
1:4 [20:80 µM]
1:1 [50:50 µM]
4:1 [80:20 µM]
9:1 [90:10 µM]
19:1 [95:5 µM]
LA:ALA ratios
B
Cell viability (% of DMEM control)
125
100
75
50
25
0
DMEM
LA [50 µM]
ALA [50 µM]
LA [10 µM]
LA [25 µM]
LA [50 µM]
LA [75 µM]
ALA [50 µM] + LA

Supplemental material

Supplemental Table 1. Mean differences in the content of PUFAs and their metabolites in EA.hy926 cells incubated with LA, ALA or various LA:ALA ratios

	n-3 PUFAs					n-6 PUFAs			
	18:3n3	20:4:n3	20:5n3	22:5n3	22:6n3	18:2n6	18:3n6	20:3n6	20:4n6
DMEM			Reference					Reference	
LA [100 µM]	0.00 (-1.00; 0.99)	0.01 (-0.03; 0.06)	0.00 (-0.07; 0.06)	-0.08 (-0.23; 0.06)	-0.21 (-0.33; -0.09)	10.44 (8.21; 12.67)	-0.03 (-0.64; -0.01)	0.07 (0.02; 0.12)	-0.31 (-0.41; -0.20)
ALA [100 µM]	3.39 (2.39; 4.39)	0.05 (0.00; 0.10)	0.07 (0.01; 0.14)	0.19 (0.05; 0.34)	-0.32 (-0.44; -0.19)	-0.21 (-2.44; 2.03)	-0.03 (-0.06; 0.00)	-0.05 (-0.10; 0.00)	-0.33 (-0.43; -0.23)
LA:ALA 19:1			Reference					Reference	
LA:ALA 9:1	0.18 (-0.11; 0.47)[a]	0.02 (-0.01; 0.05)	0.01 (-0.03; 0.06)	0.08 (0.02; 0.14)[a]	0.01 (-0.03; 0.06)	-0.30 (-1.61; 1.01)	0.00 (0.00; 0.01)	0.03 (-0.01; 0.08)	0.00 (-0.05; 0.05)
LA:ALA 4:1	0.50 (0.21; 0.78)[a]	0.00 (-0.02; 0.03)	0.01 (-0.04; 0.05)	0.16 (0.09; 0.22)[a]	0.00 (-0.05; 0.04)	-0.46 (-1.77; 0.85)	0.00 (-0.01; 0.00)	0.00 (-0.04; 0.05)	0.00 (-0.05; 0.04)
LA:ALA 1:1	1.46 (1.12; 1.80)[a]	0.01 (-0.02; 0.04)	0.02 (-0.02; 0.06)	0.28 (0.19; 0.37)[a]	0.02 (-0.03; 0.06)	-0.36 (-1.67; 0.95)	0.00 (0.00; 0.01)	-0.02 (-0.06; 0.03)	-0.01 (-0.05; 0.04)
LA:ALA 1:4	2.43 (1.74; 3.13)[a]	0.02 (0.00; 0.05)	0.03 (-0.01; 0.07)	0.24 (0.13; 0.34)[a]	-0.01 (-0.05; 0.04)	-2.58 (-3.89; -1.27)	0.00 (-0.01; 0.00)	-0.05 (-0.10; 0.00)	-0.06 (-0.11; -0.02)

Cells were incubated for 48 hours with either culture medium (DMEM) or culture medium supplemented with ALA, LA or various LA:ALA ratios for 48 hours. Values are presented as absolute differences in PUFA content in µg/10^6 cells with 95% corresponding confidence intervals (CI) (experiments, n = 6). LA (100 µM) and ALA (100 µM) alone were compared with DMEM, whereas ratios of LA:ALA including ratio 1:4 (20:80 µM), 1:1 (50:50 µM), 4:1 (80:20 µM) and 9:1 (90:10 µM) were compared with the highest ratio (19:1, 95:5 µM). Values superscripted (a) was based on standard errors calculated from bootstrap analyses with 5000 replications.

Supplemental Table 2. Mean differences in the content PUFAs and their metabolites in EA.hy926 cells incubated with ALA and varying LA concentrations

	n-3 PUFAs					n-6 PUFAs			
	18:3n3	20:4:n3	20:5n3	22:5n3	22:6n3	18:2n6	18:3n6	20:3n6	20:4n6
DMEM			Reference					Reference	
LA [50 µM]	-0.04 (-0.43; 0.35)	-0.06 (-0.21; 0.09)	-0.02 (-0.04; -0.01)	-0.03 (-0.12; 0.06)	-0.18 (-0.23; -0.12)	5.92 (4.76; 7.08)	-0.11 (-0.32:0.11)	-0.01 (-0.03; 0.02)	-0.24 (-0.29; -0.19)
ALA [50 µM]	3.43 (3.04; 3.82)	0.01 (-0.14; 0.16)	0.05 (0.03; 0.06)	0.18 (0.09; 0.26)	-0.22 (-0.27; -0.16)	-0.16 (-1.31; 1.00)	-0.05 (-0.27;0.16)	-0.06 (-0.09; -0.04)	-0.24 (-0.30; -0.19)
ALA [50 µM]			Reference					Reference	
ALA + LA[10 µM]	-0.99 (-1.60; -0.38)	-0.07 (-0.15; 0.01)	-0.02 (-0.03; 0.00)	0.01 (-0.07; 0.10)	0.02 (-0.03; 0.07)	1.46 (0.75; 2.17)	-0.06 (-0.14; 0.03)	0.02 (-0.02; 0.07)[a]	0.01 (-0.04; 0.07)
ALA + LA[25 µM]	-1.18 (-1.79; -0.57)	-0.09 (-0.16; -0.01)	-0.03 (-0.05; -0.02)	-0.03 (-0.12; 0.05)	0.02 (-0.03 (0.07)	2.81 (2.10; 2.51)	-0.06 (-0.14; 0.02)	0.02 (-0.01; 0.04)[a]	-0.05 (-0.10; 0.01)
ALA + LA[50 µM]	-0.91 (-1.52; -0.30)	-0.05 (-0.12; 0.03)	-0.03 (-0.05; -0.02)	-0.04 (-0.13; 0.05)	0.03 (-0.03; 0.08)	4.55 (3.84; 5.25)	-0.06 (-0.14; 0.03)	0.03 (-0.01; 0.08)[a]	-0.07 (-0.13; -0.02)
ALA + LA[75 µM]	-1.11 (-1.72; -0.50)	-0.03 (-0.10; 0.05)	-0.03 (-0.05; -0.02)	-0.08 (-0.17; 0.00)	0.03 (-0.2; 0.08)	4.92 (4.21; 5.62)	-0.05 (-0.13; 0.03)	0.01 (-0.02; 0.04)[a]	-0.07 (-0.12; -0.02)

Cells were incubated for 48 hours with either culture medium (DMEM) or culture medium supplemented with ALA and/or LA. Values are presented as absolute differences in PUFA content in µg/10^6 cells with corresponding 95% confidence intervals (CI) (experiments, n = 6). LA (50 µM) and ALA (50 µM) alone were compared with DMEM, whereas cells incubated with ALA (50 µM) and varying LA concentrations of 10 µM, 25 µM, 50 µM and 75 µM were compared with ALA (50 µM) alone. Values superscripted (a) was based on standard errors calculated from bootstrap analyses with 5000 replications.

Supplemental Table 3. Concentrations of inflammatory mediators (pg/ml) in the supernatant of EA.hy926 cells incubated with LA, ALA or different ratios of LA/ALA following TNF-α stimulation

	Mean differences (95 % CI)					
	VEGF	RANTES	ICAM-1	MCP-1	IL-6	IL-8
DMEM	Reference					
LA [100 μM]	-1.12 (-3.27; 1.03)	-43.42 (-60.43; -26.41)	-289.75 (-622.19; 42.70)	-2432.38 (-3383.87; -1480.90)	-9.41 (-31.62; 12.81)	67.69 (-399.90; 535.27)
ALA [100 μM]	-4.30 (-6.45; -2.15)	-52.99 (-70.0; -35.99)	-630.50 (-962.94; -298.06)	-2996.75 (-3948.24; -2045.27)	-55.99 (-77.21; -32.78)	-281.05 (-748.63; 186.54)
LA:ALA 19:1	Reference					
LA:ALA 9:1	-0.24 (-1.78; 1.31)	-22.90 (-35.43; -10.37)	97.50 (-100.85; 295.85)	-149.72 (-840.48; 541.03)	-11.49 (-23.90; 0.91)	372.67 (61.22; 684.12)
LA:ALA 4:1	-1.02 (-2.57; 0.52)	-30.40 (-42.93; -17.87)	91.11 (-107.24; 289.46)	-684.57 (-1375.32; 6.19)	-11.70 (-24.11; 0.71)	340.88 (29.43; 652.33)
LA:ALA 1:1	-1.96 (-3.50; -0.41)	-33.52 (-46.05; -20.99)	-45.30 (-243.66; 153.05)	-1203.17 (-1893.93; -512.42)	-23.58 (-35.99; -11.18)	122.73 (-188.72; 434.18)
LA:ALA 1:4	-2.17 (-3.72; -0.63)	-35.87 (-48.40; -23.34)	-202.86 (-401.22; -4.51)	-895.52 (-1586.27; -204.76)	-28.60 (-41.01; -16.19)	387.37 (75.92; 698.82)

Cells were incubated with either culture medium (DMEM) or culture medium supplemented with ALA, LA or various LA:ALA ratios for 48 hours following 24 hours TNF-α stimulation. Values are presented as absolute differences in pg/ml with corresponding 95% confidence intervals (CI) (experiments, n = 9). LA (100 μM) and ALA (100 μM) alone were compared with DMEM, whereas ratios of LA:ALA including ratio 1:4 (20:80 μM), 1:1 (50:50 μM), 4:1 (80:20 μM) and 9:1 (90:10 μM) were compared with the highest ratio (19:1, 95:5 μM).

Supplemental Table 4. Concentration of inflammatory mediators (pg/ml) in the supernatant of EA.hy926 cells incubated with ALA and varying LA concentrations following TNF-α stimulation

	Mean differences (95 % CI)					
	VEGF	RANTES	ICAM-1	MCP-1	IL-6	IL-8
DMEM	Reference					
LA [50 μM]	-13.59 (-19.78; -7.40)	-53.85 (-73.75; -33.94)	-876.92 (-1325.97; -427.88)	-2056.53 (-2772.80; -1340.27)[a]	-32.72 (-54.35; -11.08)	261.73 (-39.91; 563.38)
ALA [50 μM]	-17.80 (-23.98; -11.61)	-66.60 (-86.51; -46.70)	-1119.12 (-1568.16; -670.07)	-2619.23 (-3307.65; -1930.80)[a]	-62.69 (-84.32; -41.06)	31.10 (-270.55; 332.74)
ALA [50 μM]	Reference					
ALA + LA[10 μM]	-1.38 (-5.11; 2.35)	-4.47 (-16.13; 7.19)	-214.91 (-562.71; 132.90)	-130.16 (-767.55; 507.22)	-5.61 (-26.80; 15.58)	53.47 (-202.00; 308.95)
ALA + LA[25 μM]	-1.73 (-5.46; 2.00)	-6.07 (-17.72; 5.59)	-317.78 (-665.58; 30.03)	-220.46 (-857.85; 416.93)	0.03 (-21.16; 21.22)	167.90 (-87.58; 423.37)
ALA + LA[50 μM]	-6.25 (-9.98; -2.52)	-17.50 (-29.16; -5.84)	-572.00 (-919.80; -224.19)	-1547.38 (-2184.77; -910.00)	-21.86 (-43.05; -0.67)	-203.47 (-458.94; 52.01)
ALA + LA[75 μM]	-4.54 (-8.27; -0.81)	-11.84 (-23.49; -0.18)	-654.92 (-1002.73; -307.11)	-2046.80 (-2684.19; -1409.41)	-35.64 (-56.83; -14.45)	-498.53 (-754.01; -243.15)

Cells were incubated with either culture medium (DMEM) or culture medium supplemented with ALA and/or LA for 48 hours following 24 hours TNF-α stimulation. Values are presented as absolute differences in pg/ml with corresponding 95% confidence intervals (CI) (experiments, n = 9). LA (50 μM) and ALA (50 μM) alone were compared with DMEM, whereas cells incubated with ALA (50 μM) and varying LA concentrations of 10 μM, 25 μM, 50 μM and 75 μM were compared with ALA (50 μM) alone. Values superscripted (a) was based standard errors calculated from bootstrap analyses with 5000 replications.