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Gamma-Linolenic and Pinolenic Acids Exert Anti-Inflammatory Effects in Cultured Human Endothelial Cells Through Their Elongation Products

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Scope: Omega-3 fatty acids (FAs) from oily fish reduce cardiovascular disease. This may be partly due to modulation of endothelial cell (EC) inflammation. Fish stocks are declining and there is a need for sustainable alternative FAs. Gamma-linolenic acid (GLA) and pinolenic acid (PLA) are plant-derived FAs, which can fulfil this role.
Methods and results: EA.hy926 cells are exposed GLA and PLA prior to stimulation with tumor necrosis factor (TNF)-α. GLA and PLA are incorporated into ECs, resulting in increases in long-chain derivatives produced by elongase 5, dihomo-gamma-linolenic acid (DGLA), and eicosatrienoic acid (ETA). Both GLA and PLA (50 μM) decrease production of soluble intercellular adhesion molecule-1 (sICAM-1), monocyte chemoattractant protein 1 (MCP-1), and regulated on activation, normal T cell expressed and secreted (RANTES). However, decreases in these mediators are not seen after pre-treatment with GLA or PLA in elongase 5 silenced EA.hy926 cells. DGLA and ETA (10 μM) decrease EC production of sICAM-1, MCP-1, RANTES, and IL-6. All FAs reduce adhesion of THP-1 monocytes to EA.hy926 cells. Both PLA (50 μM) and ETA (10 μM) decrease NFκBp65 phosphorylation.
Conclusion: These effects suggest potential for GLA, PLA and their long-chain derivatives, DGLA and ETA, as sustainable anti-inflammatory alternatives to fish-derived FAs.

1. Introduction

Atherosclerosis is considered to be an inflammatory disease, and endothelial cell (EC) dysfunction plays a pivotal role in its pathogenesis.[1–3] The dysfunctional endothelium, which can arise from unresolved inflammation, promotes an influx of leukocytes, especially monocytes, into the intimal layer of the artery wall.[4] These monocytes differentiate into macrophages which become lipid laden so promoting atherosclerotic plaque formation.[5,6] Very long chain omega-3 (n-3) polyunsaturated fatty acids (PUFAs), particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have anti-inflammatory and anti-atherogenic actions and their consumption is associated with lowering of risk for cardiovascular disease (CVD).[7–11] In order to understand their actions and the mechanisms involved, studies have examined the effects of EPA and DHA on endothelial and leukocyte functions.[12,13] EPA and DHA are found predominantly

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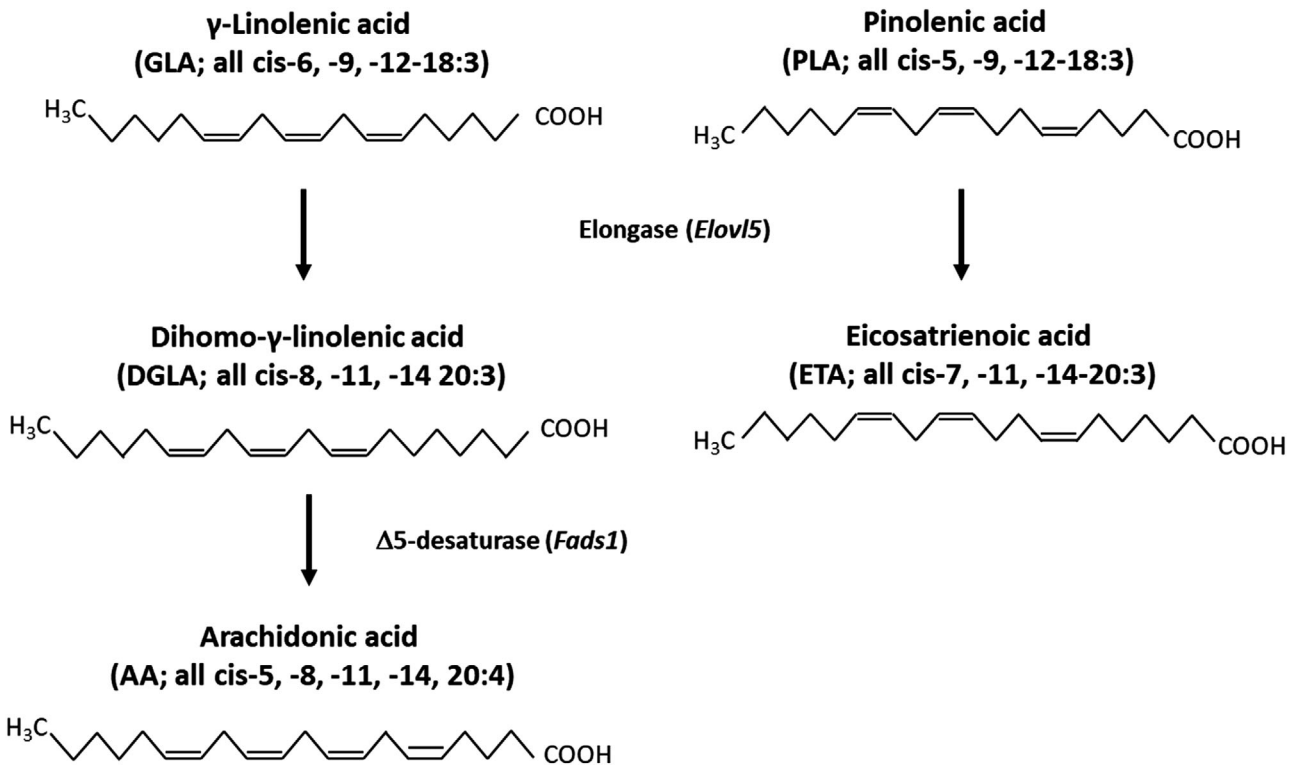

Figure 1. The pathway of metabolic conversion of gamma-linolenic acid and the likely pathway of conversion of pinolenic acid to longer-chain polyunsaturated fatty acids. Genes encoding the various enzymes are shown in brackets.

in fatty fish and many authorities recommend regular consumption of fatty fish to achieve health benefits.[14] However, current fish stocks are declining and so more sustainable sources of health benefiting bioactive fatty acids need to be explored.[15] Gamma-linolenic acid (GLA) and pinolenic acid (PLA) are plant-derived fatty acids, which may provide alternative sustainable sources of bioactive PUFAs that target inflammation.

GLA is found in some leafy green vegetables, vegetable oils, and nuts, including evening primrose (*Oenothera beinnis*) oil and borage (*Borage officinalis*) oil.[16] PLA, a poly-methylene-interrupted PUFA, is found exclusively in pine nut oil (present at about 15% of FAs).[17] There are older studies exploring the anti-inflammatory effects of GLA,[18,19] but its effect on endothelial inflammation is not known. There has been little exploration of the functionality and potential benefits of PLA.

Studies examining the effects of GLA identified that its actions seem to be due to its elongated derivative dihomo-gamma-linolenic acid (DGLA). This conversion, catalyzed by elongase 5, is shown in **Figure 1**. Elongase 5 is encoded by the *ELOVL5* gene. DGLA can be further desaturated to arachidonic acid (Figure 1). When cells, laboratory animals, or humans are provided with GLA, cells, including those involved in inflammation, such as neutrophils and mononuclear cells, become enriched in DGLA.[20] DGLA is a precursor of eicosanoids produced via the cyclooxygenase (COX) and lipoxygenase (LOX) pathways. These include the 1-series prostaglandins, such as prostaglandin E1 (PGE_1), which has been found to possess anti-inflammatory properties, including inhibition of smooth muscle cell proliferation associated with atherosclerotic plaque development.[21] The pathway of conversion of PLA to derivatives analogous to DGLA has not been fully described; however Szu-jung Chen et al. suggested that PLA can be converted to eicosatrienoic acid (ETA) in microglial BV-2 cells and described incubation with PLA to lead to reduced production of several pro-inflammatory mediators, including interleukin (IL)-6, prostaglandin E_2 (PGE_2), and tumor necrosis factor alpha (TNF-α) in response to stimulation with lipopolysaccharide (LPS).[22] Conversion of PLA to ETA is likely to be catalyzed by elongase 5 (Figure 1).

Both EPA and DHA have been shown to have anti-inflammatory effects in ECs, including lowering of surface expression of adhesion molecules, reduced monocyte adhesion, and decreased inflammatory marker expression, at the levels of both protein and messenger RNA.[23–27] These effects are likely to be linked to decreased atherosclerosis. Previously we showed EPA and DHA to have multiple anti-inflammatory effects in the EA.hy926 EC line.[28] In the current study, effects of GLA and PLA, and their elongation products, DGLA and ETA, on the inflammatory responses of EA.hy926 cells were examined.

2. Results

2.1. Results of MTT Assay: Effects of GLA and PLA on EA.hy926 Cell Viability

Cell mitochondrial activity was measured using the MTT assay and viability was calculated as a % of control (cells in DMEM plus supplements). Exposure to TNF-α at 1 ng mL^{-1} for 24 h had no effect on cell viability. **Figure 2**A,B shows viability of cells cultured

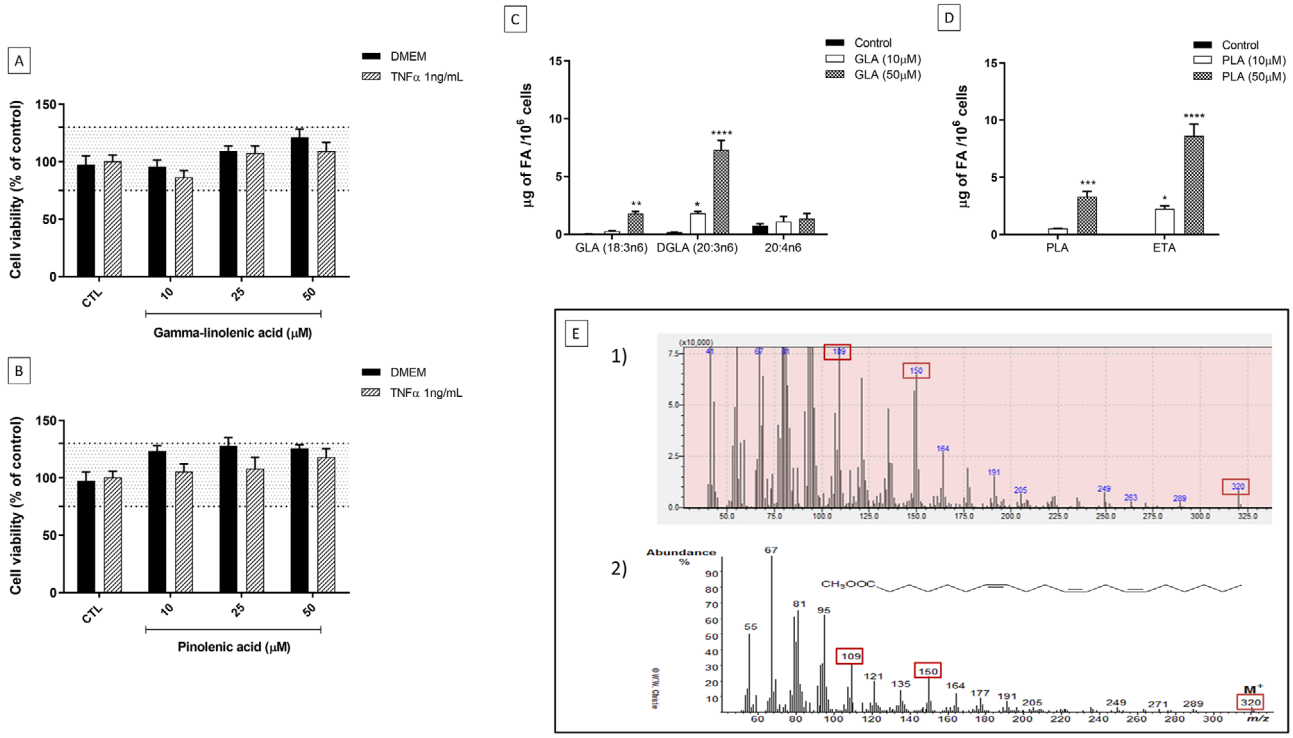

Figure 2. A,B) Viability of EA.hy926 cells after incubation for 48 h with DMEM containing 0.1% of ethanol (control; CTL) or fatty acids (GLA [A] and PLA [B]) at 10, 25, 50, or 100 μM followed by incubation with or without TNF-α (1 ng mL^{-1}) for 24 h ($n = 3$, two-way ANOVA with Tukey's post hoc test: no significant differences). C,D) Incorporation of fatty acids (GLA and PLA) and appearance of elongation products after incubation of EA.hy926 cells for 48 h with DMEM containing 0.1% of ethanol (control) or fatty acids (GLA [C] and PLA [D]) at 10 or 50 μM ($n = 3$, one-way ANOVA with Dunnett's post-hoc test: Fatty acid vs control, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$). E) MS total ion chromatogram depicting 1) the ion fragments of the FA produced in EA.hy926 cells after 48 h treatment with PLA at 50 μM and 2) the total ion chromatogram of eicosatrienoic acid (ETA) taken from the Lipidhome database.[29]

with GLA or PLA at 10, 25, and 50 μM. Treatment with FAs for 48 h at any concentration followed by 24 h 1 ng mL^{-1} TNF-α exposure had no effect on EA.hy926 cell viability. Based on these results, GLA and PLA at all concentrations were found to be suitable for further experiments.

2.2. Identification and Incorporation of FAs in EA.hy926 Cells

FA incorporation into EA.hy926 cells was determined by gas chromatography. Both GLA and PLA were incorporated into EA.hy926 cells in a concentration-dependent manner, with the appearance of longer chain derivatives (Figure 2C,D). PLA treatment led to the increase of an FA that was not identifiable through standard gas chromatography. Gas chromatography–mass spectrometry was used to identify this FA. Figure 2E-1,2 depicts the MS total ion chromatogram of the unknown FA present in EA.hy926 cells after exposure to PLA. The unknown fatty acid was identified to be ETA when compared to standards on the Lipidhome database[29] (Figure 2E-2).

GLA treatment at 10 μM did not enrich cells with GLA, but significantly increased DGLA content ($p < 0.05$) (Figure 2C). GLA treatment at 50 μM led to a significant increase in both GLA ($p < 0.01$) and DGLA ($p < 0.001$) (Figure 2C). Pre-treatment with GLA did not significantly change levels of arachidonic acid in EA.hy926 cells (Figure 2C). Incubation of EA.hy926 cells with PLA at 10 μM led to a significant increase in ETA ($p < 0.05$), while treatment with PLA at 50 μM led to an increase in both PLA ($p < 0.001$) and ETA ($p < 0.0001$) (Figure 2D). Levels of DGLA and ETA exceeded those of GLA and PLA.

2.3. Effects of GLA and PLA on Inflammatory Mediators Produced by EA.hy926 Cells

TNF-α stimulation (24 h at 1 ng mL^{-1}) led to significantly increased production of all inflammatory mediators examined (data not shown). FA exposure had differential effects depending on the individual FA and on FA concentration (**Figure 3**A–E).

At a concentration of 10 μM, GLA did not significantly alter the production of any of the five mediators studied. However, GLA treatment at 25 and 50 μM led to significant reduction in secretion of sICAM-1 ($p < 0.01$, $p < 0.01$, respectively). Exposure of GLA at 50 μM also led to significant decreases in the production of both MCP-1 ($p < 0.001$) and RANTES ($p < 0.01$) by EA.hy926 cells.

PLA treatment at 10 μM decreased the production of sICAM-1 ($p < 0.05$); this effect was also seen after treatment with 25 and 50 μM PLA ($p < 0.01$, $p < 0.001$, respectively). Treatment of EA.hy926 cells with PLA at 50 μM also reduced the production of both MCP-1 ($p < 0.001$) and RANTES ($p < 0.01$).

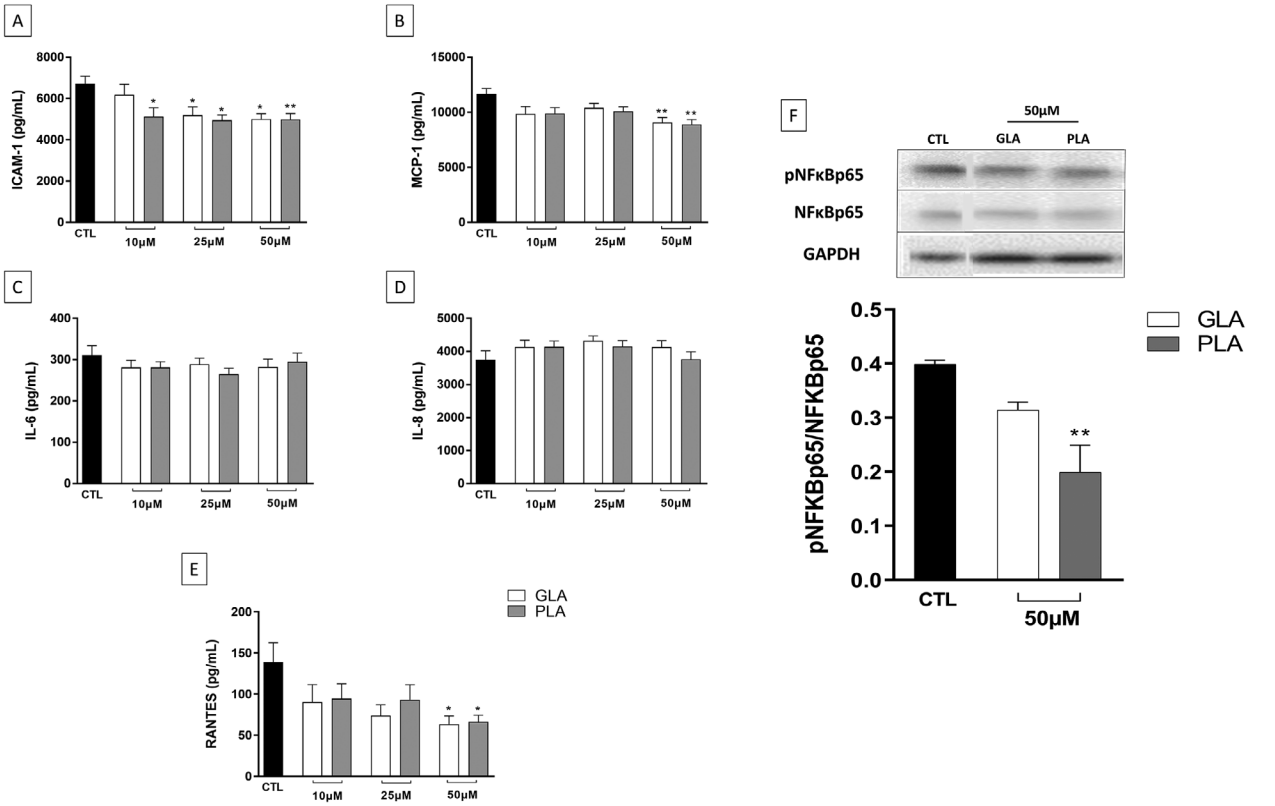

Figure 3. Concentrations (pg mL^{-1}) of A) ICAM-1, B) MCP-1, C) IL-6, D) IL-8, and E) RANTES in the medium of EA.hy926 cells incubated for 48 h with DMEM containing 0.1% of ethanol (CTL) or fatty acids (GLA or PLA) at 10, 25, or 50 μM, followed by incubation with TNF-α (1 ng mL^{-1}) for 24 h (n = 3, two-way ANOVA with Tukey's post hoc test: Fatty acid vs control, $^{*}p < 0.05$ and $^{**}p < 0.01$). F) Protein expression of pNFκBp65 and NFκBp65 in EA.hy926 cells incubated for 48 h with DMEM containing 0.1% of ethanol (control, CTL) or fatty acids (GLA or PLA) at 50 μM, followed by incubation with TNF-α (1 ng mL^{-1}) for 1 h (data are normalized to loading control (GAPDH)) (n = 3, one-way ANOVA with Tukey's post hoc test: Fatty acid vs control, $^{**}p < 0.01$).

2.4. Effects of GLA and PLA on NFκBp65 Protein Expression in EA.hy926 Cells

Pre-treatment with GLA (50 μM) had a tendency to decrease the ratio of phosphorylated nuclear factor kappa-light-chain-enhancer of activated B cells (pNFκB)p65/NFκBp65, although this was not significant (Figure 3F). In contrast, PLA (50 μM) treatment significantly decreased the ratio of pNFκBp65/NFκBp65 ($p < 0.01$) (Figure 3F).

2.5. Effects of GLA and PLA on Expression of ICAM-1 on EA.hy926 Cells

TNF-α was shown to up-regulate ICAM-1 cell-surface expression on EA.hy926 cells.[28] GLA and PLA treatment at either concentration (25 or 50 μM) did not affect either the level of ICAM-1 expression (MFI) (**Figure 4**A) or the % of cells expressing ICAM-1 (Figure 4B) compared to stimulated control cells.

2.6. Effects of GLA and PLA on THP-1 Adhesion to EA.hy926 Cells

Adhesion of calcein-labeled THP-1 cells to EA.hy926 cells was shown to significantly increase with TNF-α stimulation of the ECs (data not shown). Neither GLA nor PLA had any effect on THP-1 adhesion at a concentration of 25 μM; however both GLA and PLA reduced adhesion of THP-1 cells to EA.hy926 cells when used at a concentration of 50 μM (Figure 4C). Treatment with GLA and PLA at 50 μM reduced adhesion of THP-1 cells by ≈28% and ≈23%, respectively ($p < 0.001$, $p < 0.01$) compared to stimulated control cells (Figure 4C). Figure 4D shows images of fluorescence-labelled THP-1 monocytes bound to EA.hy926 cells with and without FA exposure at 50 μM.

2.7. Effects of siRNA Exposure on ELOVL5 Gene Expression in EA.hy926 Cells

Incubation of EA.hy926 cells with ELOVL5 siRNA significantly decreased relative gene expression of ELOVL5 compared to non-siRNA treated cells ($p < 0.001$) (**Figure 5**A). ELOVL5 relative gene expression was decreased by ≈90% after ELOVL5 siRNA exposure across all conditions; control (DMEM alone) (−90%), GLA-treated cells (50 μM) (−87%), and PLA-treated cells (50 μM) (−88%). Control siRNA resulted in unaltered relative gene expression in EA.hy926 cells under all conditions (DMEM, GLA, and PLA), indicative of ELOVL5 siRNA specificity.

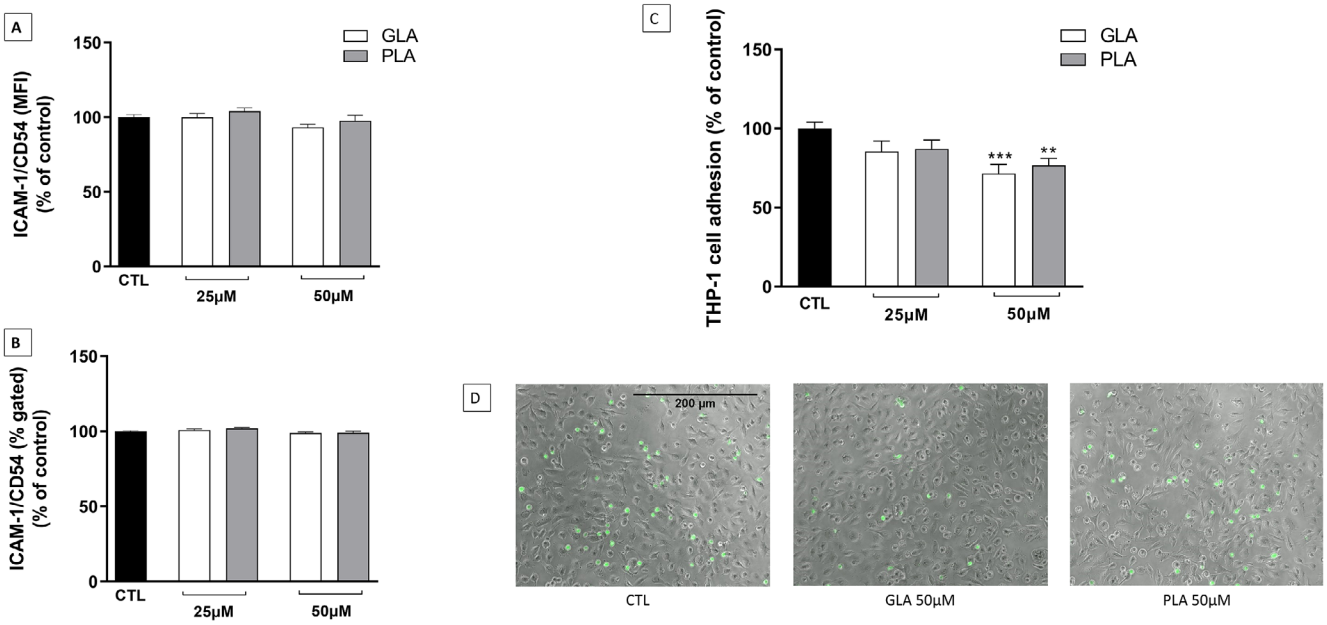

Figure 4. A,B) Cell surface expression of ICAM-1 (CD54) in EA.hy926 cells and C) adhesion of THP-1 cells to EA.hy926 cells incubated for 48 h with DMEM containing 0.1% of ethanol (CTL) or fatty acids (GLA or PLA) at 25 or 50 μM, followed by incubation with TNF-α (1 ng mL^{-1}) for 6 h (data are expressed as % of control [A, C] and % difference from control [B], $n = 3$, one-way ANOVA with Tukey's post hoc test: Fatty acid vs control, $^{**}p < 0.01$ and $^{***}p < 0.001$). Attached THP-1 cells were visualized by fluorescence microscope Nikon Elipse Ti at a magnification of 10× under transmitted light (control, GLA, or PLA [3 D]).

2.8. Effects of Silencing ELOVL5 on Incorporation of FAs in EA.hy926 Cells

FA incorporation into EA.hy926 cells was determined by gas chromatography. FAs were successfully incorporated by EA.hy926 cells, with the appearance of specific metabolic elongation products depending on FA (as seen previously). GLA incubation of non-siRNA treated cells (50 μM) led to a significant increase in DGLA ($p < 0.001$) (Figure 5B); similarly after PLA treatment (50 μM) there was a significant increase in ETA ($p < 0.001$) (Figure 5B). ELOVL5 siRNA treatment of cells significantly inhibited elongation of GLA and PLA to DGLA and ETA ($p < 0.0001$ and $p < 0.001$, respectively) (Figure 5B,C). ELOVL5 silencing in GLA-treated cells led to a build-up of GLA in the cells (Figure 5B); however PLA build up was not seen in PLA-treated silenced cells (Figure 5C).

2.9. Effects of Silencing ELOVL5 and FA Treatment on Inflammatory Mediators Produced by EA.hy926 Cells

GLA and PLA both significantly decreased the secretion of sICAM-1 compared to stimulated control cells ($p < 0.001$) (Figure 5D). This reduction was not seen in ELOVL5 siRNA silenced cells: incubation of these cells with GLA or PLA led to no changes in levels of sICAM-1. GLA treatment also led to the significant decrease of RANTES ($p < 0.05$) by EA.hy926 cells compared to stimulated control cells (Figure 5H). This effect of GLA treatment was not seen in ELOVL5 siRNA treated cells. MCP-1 production was decreased after treatment with both FAs (Figure 5E), with a greater reduction observed after GLA treatment compared to PLA ($p < 0.001$ and $p < 0.05$, respectively). ELOVL5 silencing prevented the effects of PLA on MCP-1. GLA incubation in ELOVL5 silenced cells still led to the same significant decrease in MCP-1 as seen in unsilenced cells ($p < 0.001$). Neither IL-6 nor IL-8 production by EA.hy926 cells was affected by GLA or PLA with or without ELOVL5 siRNA treatment.

2.10. Results of MTT Assay: Effects of DGLA and ETA on EA.hy926 Cell Viability

Cell mitochondrial activity was measured using the MTT assay and viability was calculated as a % of control (cells in DMEM plus supplements). **Figure 6**A,B shows viability of cells cultured with DGLA and ETA at 5, 10, 25, and 50 μM. There was a significant reduction in viability of cells cultured with DGLA and ETA at 25 μM ($p < 0.0001$) and 50 μM ($p < 0.0001$), compared to stimulated control cells (<60%, <70%, <25% viability, respectively). Based on these results, a maximum concentration of 10 μM for each of these FAs was chosen for further experiments.

2.11. DGLA and ETA Incorporation into EA.hy926 Cells

Both DGLA and ETA were incorporated into EA.hy926 cells in a concentration-dependent manner at 5 and 10 μM (Figure 6C,D). DGLA treatment at 5 and 10 μM led to a significant increase in DGLA ($p < 0.05$, $p < 0.001$, respectively) in EA.hy926 cells (Figure 6C). Similarly, ETA treatment at 5 and 10 μM led to a significant increase in ETA ($p < 0.05$, $p < 0.001$, respectively) (Figure 6D).

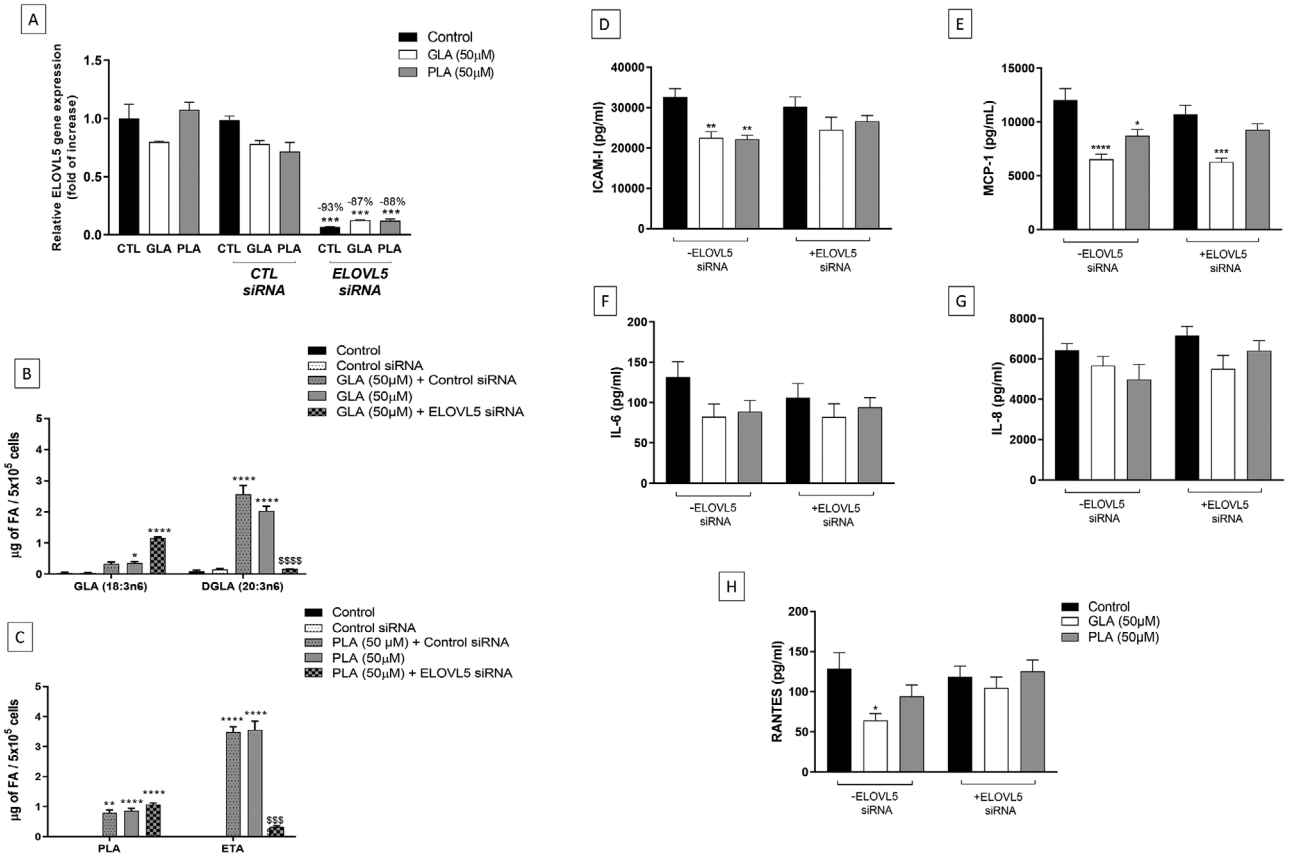

Figure 5. A) Expression of ELOVL5 gene in EA.hy926 cells treated with siRNA and incubated for 48 h with DMEM containing 0.1% of ethanol (CTL) or fatty acids (GLA or PLA) at 50 μM, followed by incubation with TNF-α (1 ng mL^{-1}) for 24 h ($n = 3$, one-way ANOVA with Tukey's post hoc test: ELOVL5 siRNA vs control, ***$p < 0.001$). B,C) Incorporation of fatty acids (GLA and PLA) and appearance of elongation products (DGLA and ETA) after incubation of EA.hy926 cells for 48 h with DMEM containing 0.1% of ethanol (control) or fatty acids (GLA [B] and PLA [C]) at 50 μM with or without ELOVL5 siRNA ($n = 3$, two-way ANOVA with Dunnett's post hoc test: Fatty acid vs control, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$; Fatty acid vs Fatty acid (GLA or PLA) + ELOVL5 siRNA, $$$$$p < 0.001$). Concentrations (pg mL^{-1}) of D) ICAM-1, E) MCP-1, F) IL-6, G) IL-8, and H) RANTES in the medium of EA.hy926 cells incubated for 48 h with DMEM containing 0.1% of ethanol (CTL) or fatty acids (DGLA or ETA) at 50 μM, followed by incubation with TNF-α (1 ng mL^{-1}) for 24 h with or without ELOVL5 siRNA ($n = 3$, two-way ANOVA with Tukey's post hoc test: Fatty acid vs control, *$p < 0.05$ and **$p < 0.01$).

2.12. Effects of DGLA and ETA on Inflammatory Mediators Produced by EA.hy926 Cells

Both FAs led to significant decreases in sICAM-1, MCP-1, IL-6, and RANTES at both 5 and 10 μM (**Figure 7**A–E). DGLA treatment at 5 and 10 μM lead to significant reduction in secretion of sICAM-1 ($p < 0.0001$, $p < 0.001$, respectively). Production of both MCP-1 and RANTES by EA.hy926 cells was significantly decreased after exposure to DGLA at both concentrations used ($p < 0.0001$). DGLA treatment also led to a significant decrease in IL-6 secretion at 5 and 10 μM ($p < 0.01$) compared to stimulated control cells.

Similarly, ETA exposure led to significant decreases in sICAM-1 at 5 μM ($p < 0.01$) and 10 μM ($p < 0.001$). MCP-1 and IL-6 production were also decreased by ETA exposure at 5 μM ($p < 0.01$, $p < 0.0001$, respectively) and 10 μM ($p < 0.0001$, $p < 0.01$). RANTES secretion was significantly reduced by ETA at both concentrations ($p < 0.0001$).

Neither DGLA nor ETA had any effect on production of IL-8 by EA.hy926 cells.

2.13. Effects of DGLA and ETA on NFκBp65 Protein Expression in EA.hy926 Cells

Pre-treatment with either DGLA or ETA (10 μM) had a tendency to decrease the ratio of pNFκBp65/NFκBp65, with ETA having greater potency reaching near significance ($p = 0.06$) (Figure 7F).

2.14. Effects of DGLA and ETA on Expression of ICAM-1 on EA.hy926 Cells

Neither DGLA nor ETA treatment at either concentration (5 or 10 μM) had any effect on the level of ICAM-1 expression (MFI) (**Figure 8**A) or the % of cells expressing ICAM-1 (Figure 8B) compared to stimulated control cells.

2.15. Effects of DGLA and ETA on THP-1 Adhesion to EA.hy926 Cells

Figure 8D shows images of fluorescence-labelled THP-1 monocytes bound to EA.hy926 cells with and without FA exposure at

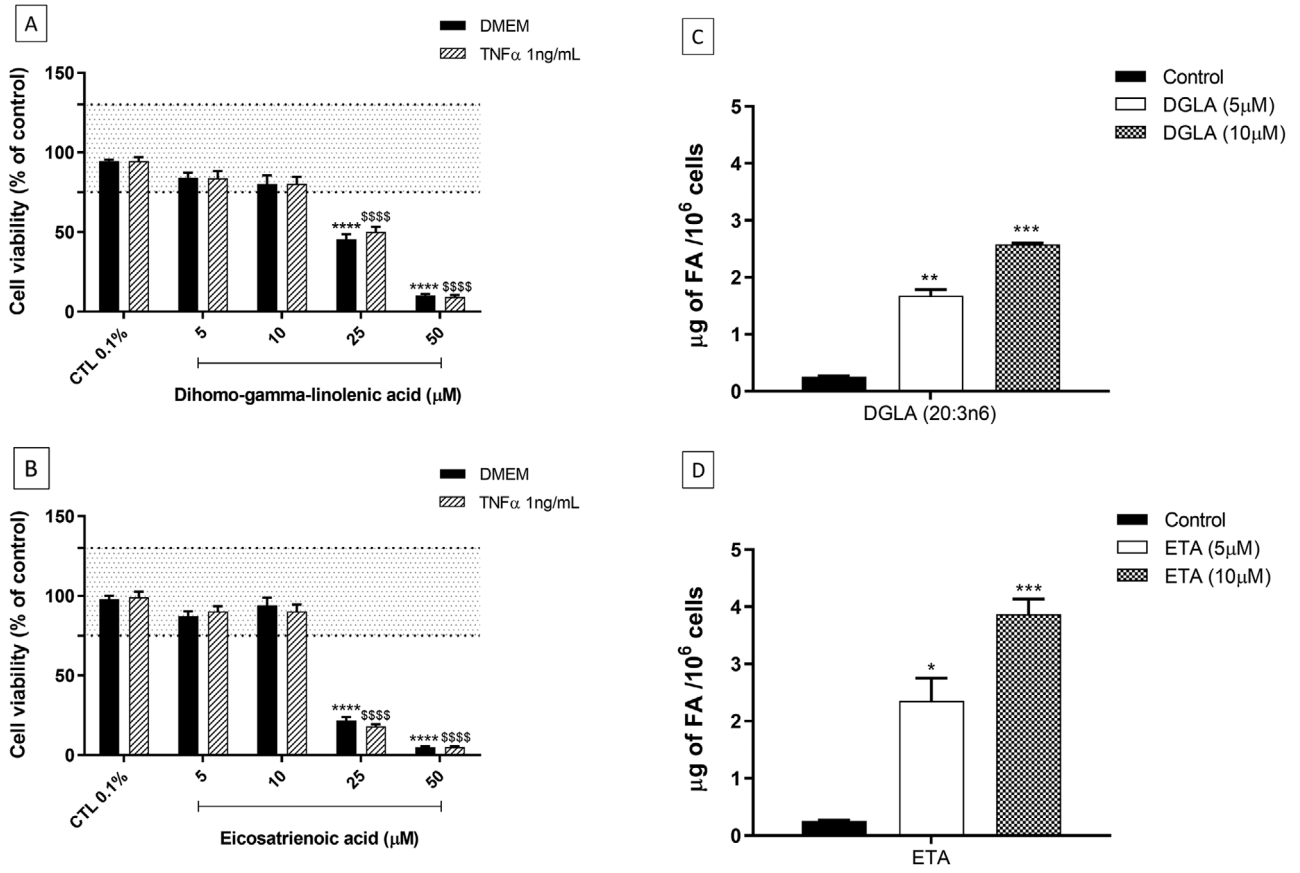

Figure 6. A,B) Viability of EA.hy926 cells after incubation for 48 h with DMEM containing 0.1% of ethanol (control; CTL) or fatty acids (DGLA [A] and ETA [B]) at 5, 10, 25, or 50 μM followed by incubation with or without TNF-α (1 ng mL^{-1}) for 24 h ($n = 3$, two-way ANOVA with Tukey's post hoc test: Fatty acid vs control, unstimulated [DMEM], ***$p < 0.01$; ****$p < 0.001$; Fatty acid vs control, stimulated [TNF-α 1 ng mL^{-1}], \$$p < 0.05$; \$\$\$$p < 0.0001$). C,D) Incorporation and appearance of fatty acids (DGLA and ETA) after incubation of EA.hy926 cells for 48 h with DMEM containing 0.1% of ethanol (control) or fatty acids (DGLA [C] and ETA [D]) at 5 or 10 μM, ($n = 3$, one-way ANOVA with Dunnett's post hoc test: Fatty acid vs control, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

10 μM. DGLA and ETA significantly reduced adhesion of THP-1 cells to EA.hy29 cells at both concentrations (5 and 10 μM) (Figure 8C). Treatment with DGLA at 5 and 10 μM reduced adhesion of THP-1 cells by ≈25% and ≈40%, respectively ($p < 0.05$, $p < 0.001$) compared to stimulated control cells (Figure 8C). Treatment with ETA at both 5 and 10 μM reduced adhesion of THP-1 cells by ≈50% ($p < 0.0001$) compared to stimulated control cells (Figure 8C).

3. Discussion

The functionality of plant-derived FAs such as GLA and PLA has been underexplored; this study examines the potential for these FAs to serve as sustainable alternative sources to LC n-3 PUFAs for human health. GLA and PLA may play a role in controlling inflammatory processes, including EC responses. In the current study, both GLA and PLA decreased inflammatory responses of TNF-α stimulated ECs, with PLA being more potent than GLA. The observed effects of GLA and PLA appear to be the result of metabolism to their longer chain derivatives, DGLA from GLA and ETA from PLA, as demonstrated by silencing of elongase 5 which reduced or abolished most of the anti-inflammatory effects of GLA and PLA. In accordance with this, DGLA and ETA were both shown to exert potent anti-inflammatory effects in this EC model.

The study demonstrated various anti-inflammatory effects of GLA and PLA in EA.hy926 cells exposed to TNF-α. TNF-α has been shown to be crucially involved in the pathogenesis and progression of atherosclerosis and alters both EC function and EC-leukocyte interactions through increases in pro-inflammatory gene expression and cell adhesion molecules.[30,31] TNF-α is used frequently as a stimulus in various inflammatory EC models[24,26,28,32,33] and was therefore deemed an appropriate stimulus within this model.

The MTT assay was used as a measure of cell viability in this study. Neither GLA nor PLA at any concentration had an effect on MTT readings; however DGLA and ETA were both shown to reduce MTT assay output at higher concentrations (25 and 50 μM). This suggests DGLA and ETA may influence mitochondrial activity. Recent findings by Gallagher et al. suggest DGLA improves macrophage mitochondrial bioenergetic profile by decreasing proton leak, increasing non-mitochondrial respiration, sparing respiratory capacity, and coupling efficiency.[34] As far as

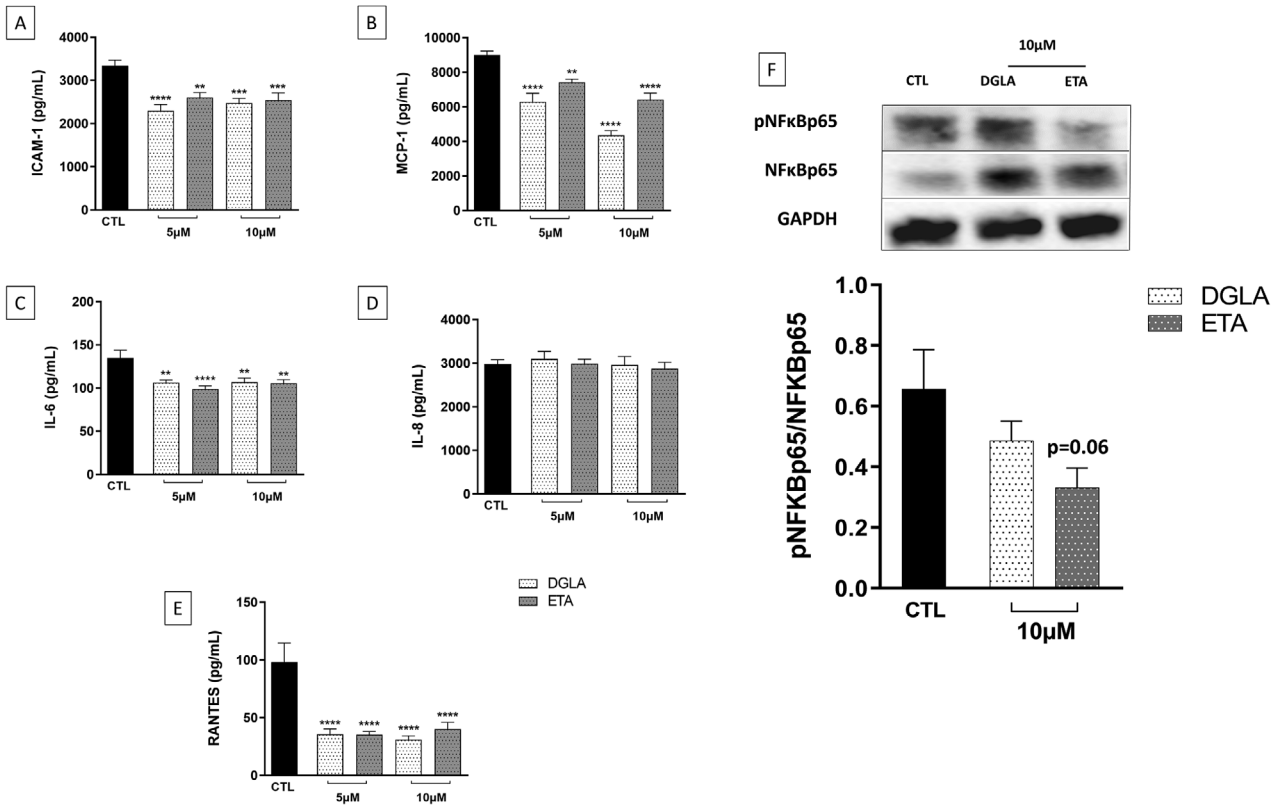

Figure 7. Concentrations (pg mL^{-1}) of A) ICAM-1, B) MCP-1, C) IL-6, D) IL-8, and E) RANTES in the medium of EA.hy926 cells incubated for 48 h with DMEM containing 0.1% of ethanol (CTL) or fatty acids (DGLA or ETA) at 5 or 10 μM, followed by incubation with TNF-α (1 ng mL^{-1}) for 24 h ($n = 3$, two-way ANOVA with Tukey's post hoc test: Fatty acid vs control, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$). F) Protein expression of pNFκBp65 and NFκBp65 in EA.hy926 cells incubated for 48 h with DMEM containing 0.1% of ethanol (control, CTL) or fatty acids (DGLA or ETA) at 10 μM, followed by incubation with TNF-α (1 ng mL^{-1}) for 1 h (data are normalized to loading control [GAPDH], $n = 3$, one-way ANOVA with Tukey's post hoc test: no significant differences).

we are aware no one has examined the effects of ETA on mitochondrial activity.

GLA and PLA at a concentration of 50 μM were both shown to decrease the production of chemokines (MCP-1 and RANTES) and an adhesion molecule (sICAM-1). These chemical signals are all involved in the initiation and development of the atherosclerotic plaque.[1–6] Similar to effects observed here, Kim et al. reported reduced MCP-1 production in rat kidney epithelial cells (NRK-52E) and in mesangial cells after treatment with GLA at 10 and 100 μM.[35] Gallagher et al. also described reduced ICAM-1 gene expression in stimulated THP-1 cells after treatment with GLA (100 μM).[34] However, very few studies have reported effects of GLA on ECs and there are no studies of PLA and ECs. Neither GLA nor PLA had any effect on IL-6 or IL-8. Erdinest et al. also reported lack of effect of GLA on IL-6 and IL-8 production by LPS-stimulated human corneal ECs,[36] whereas Chen et al. described reduced IL-6 production in LPS-stimulated murine microglial BV-2 cells after PLA treatment.[22] Similarly, Chen et al. also reported lowered IL-6 production after treatment of differentiated THP-1 cells with PLA.[37] These different findings might indicate different sensitivities of different cell types to GLA and PLA or they may reflect differences in experimental conditions (e.g., inflammatory stimulant, fatty acid concentration, incubation time). However, taken as a whole, the findings of the current study, along with these earlier studies,[22,34–37] indicate that both GLA and PLA have the potential to decrease the production of a range of key inflammatory chemokines and cytokines.

Neither GLA nor PLA had any effects on the surface expression of ICAM-1 in EA.hy926 cells. Similarly, De Caterina et al. reported little effect of GLA on VCAM-1 surface expression on human saphenous vein ECs.[25] Effects of PLA on cell surface expression of adhesion molecules in ECs have not been previously described.

Elongase 5 catalyzes the conversion of GLA to DGLA and of PLA to ETA; elongase 5 is encoded by ELOVL5. ELOVL5 silencing led to the significant reduction in elongation products after incubation with GLA or PLA. Incubation with GLA led to a small increase of GLA in silenced cells, however this was not significant. PLA did not appear to accumulate in PLA-incubated silenced cells; this may indicate that these cells are resistant to accumulating too much PLA, possibly due to its unusual structure.

ELOVL5 silencing led to the full reversal of the effects observed after treatment with PLA: production of sICAM-1, MCP-1, and RANTES by siRNA treated cells stimulated with TNF-α was similar to that seen for control cells (i.e., ECs stimulated with TNF-α but without prior exposure to a fatty acid). This suggests that the anti-inflammatory effects seen with PLA treatment are due to a metabolic product beyond elongase 5, perhaps ETA.

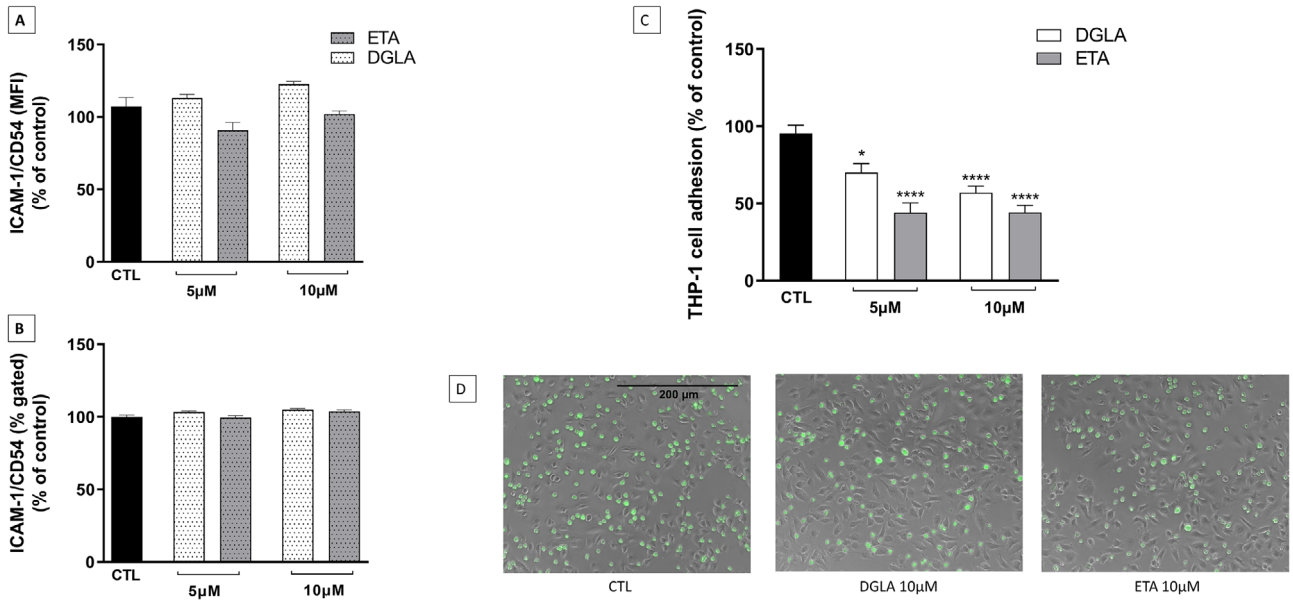

Figure 8. A,B) Cell surface expression of ICAM-1 (CD54) in EA.hy926 cells and C) adhesion of THP-1 cells to EA.hy926 cells incubated for 48 h with DMEM containing 0.1% of ethanol (CTL) or fatty acids (DGLA or ETA) at 25 or 50 μM, followed by incubation with TNF-α (1 ng mL^{-1}) for 6 h, (data are expressed as % of control [A, C] and % difference from control [B], $n = 3$, one-way ANOVA with Tukey's post hoc test: Fatty acid vs control, $^{**}p < 0.01$ and $^{***}p < 0.001$). Attached THP-1 cells were visualized by fluorescence microscope Nikon Elipse Ti at a magnitude of 10× under transmitted light (control, DGLA or ETA [7 D]).

However, effects seen with GLA were only partially reversed by ELOVL5 silencing. Decreases seen with GLA in both sICAM-1 and RANTES production were reversed in siRNA treated cells, whereas MCP-1 production remained significantly lower in siRNA-treated cells compared to control cells. This may indicate a direct role for GLA in the inhibition of MCP-1 production in ECs. However, just as for PLA, the observations suggest that at least some of the anti-inflammatory effects seen with GLA treatment are due to a metabolic product beyond elongase 5, perhaps DGLA. DGLA was also shown to reduce production of MCP-1 in this model. Others have described a reduction in MCP-1 gene expression in stimulated THP-1 cells after treatment with both GLA (100 μM) and DGLA (50 μM).[34]

Both DGLA and ETA exhibited potent anti-inflammatory effects within this EC model, leading to reduced production of MCP-1 as well as sICAM-1, RANTES, and IL-6. Effects of DGLA and ETA on these outcomes in ECs have not been previously described. Gallagher et al. describe reduced ICAM-1 gene expression in stimulated THP-1 cells after incubation with DGLA (50 μM).[34] In another study Chen et al described reduced IL-6 production in LPS-stimulated BV microglial cells after treatment with ETA (50 μM).[22] Similarly Huang et al. indicated that pre-treatment with ETA led to a reduction in IL-6 production by RAW264.7 macrophages, but the data was not shown.[38]

Both GLA and PLA led to decreased adhesion of THP-1 cells to EA.hy926 cells. No others have explored the effects of either of these FAs on adhesion of monocytes to EC monolayers. However, one study describes reduced migration of THP-1 monocytes after incubation with GLA (50 μM).[34] DGLA and ETA were shown to also reduce adhesion of THP-1 cells to EA.hy926 cells, at lower concentrations and with greater potency than seen with GLA and PLA. Others have described reduced migration of human aortic smooth muscle cells pre-treated with DGLA at 50 μM,[34] but again there are no previous reported studies of effects of ETA on these cell properties.

Together the results from this study clearly suggest that the metabolism of GLA and PLA plays a critical role in the bioactivity of these FAs, with most of their effects likely due to the elongation products DGLA and ETA. GLA and PLA were shown to be incorporated into cells in a concentration-dependent manner with the appearance of elongation products, DGLA from GLA and ETA from PLA. DGLA can be further metabolized via delta-5 desaturase to AA. However, incubation with GLA did not lead to increased AA in EA.hy926 cells. Similar observations have been made in human studies where increased GLA intake has led to increased DGLA in PBMCs and neutrophils with no effect on AA.[39–43] Previous studies have therefore attributed the anti-inflammatory actions of GLA to its conversion to DGLA. DGLA can be metabolized by both cyclooxygenase (COX) 1 and COX 2 to 1 series prostaglandins and by 15-lipoxygenase into 15-(S)-hydroxy-8,11,13-eicosatrienoic acid (15-HETrE).[44,45] These metabolites of DGLA have been shown to suppress inflammation.[21,46,47] DGLA treatment (100 μM) was shown to increase levels of prostaglandin (PG) E_1 produced by THP-1-derived macrophages.[34] Others have described treatment with PGE_1 to reduce several inflammatory processes in ECs, including lowered ICAM-1 and VCAM-1 as well as reduced monocyte adhesion to EC monolayers; these effects were shown to be through suppression of TNF-induced NFκB activation.[48] Therefore, anti-inflammatory actions of GLA and furthermore DGLA in this model are possibly through production of PGE_1.

Very few studies have described effects of ETA on inflammatory processes, and none have examined effects with ECs. It is likely that metabolites of ETA may also suppress inflammation

in a similar way to those produced from DGLA. Huang et al. described reduced pro-inflammatory PGE_2 after treatment of RAW264.7 cells with ETA.[38]

NFκB drives the expression of pro-inflammatory genes.[49,50] EPA and DHA have been shown to inhibit the activity of NFκB, leading to decreases in expression of genes encoding various inflammatory chemokines, cytokines, adhesion molecules, and enzymes.[13,51] These effects are linked to reduced leukocyte-EC adhesive interactions and reduced leukocytic infiltration into tissues.[13,51] These effects might be important in decreasing initiation and development of atherosclerotic plaques.[52] Observations made in the current study suggest that the decreased production of inflammatory mediators by ECs and the decreased THP-1 cell adhesion seen with GLA and PLA (and also with DGLA and ETA) may be due to reduced activation of NFκB. Incubation of EA.hy926 cells with PLA and ETA lead to decreased phosphorylation (activation) of NFκB; GLA and DGLA also appeared to reduce the activation, although not to the same extent. No previous studies have described effects of GLA, DGLA, PLA, nor ETA on NFκB activation in ECs. Also, few studies have examined effects of these FAs on NFκB in other cell types. Cao et al. reported significantly reduced NFκB activity after treatment with GLA at 100 μM in LPS-stimulated primary goat mammary gland epithelial cells.[53] One other study described decreased NFκB activity (nuclear translocation) in LPS-stimulated RAW264.7 macrophages after treatment with both PLA and ETA at 50 and 100 μM.[38]

The need for sustainable, non-fish alternatives to marine-derived FAs requires exploration into plant-derived FAs. GLA and PLA, which are both plant derived, have anti-inflammatory actions within this EC model, reducing production of cytokines and chemokines and THP-1 monocyte adhesion, likely through inhibition of the NFκB pathway. The effects of GLA and PLA did not occur in ELOVL5 silenced cells, suggesting that metabolites of GLA and PLA are mainly responsible for their effects. In agreement with this, greater anti-inflammatory potency was observed with DGLA and ETA than with GLA and PLA. GLA and PLA may provide a sustainable source of bioactive FAs for health benefits, especially in the context of endothelial inflammation and atherosclerosis.

Strengths of the current study include confirmation of the incorporation of the FAs used into the cells; evaluation of the effect of different concentrations of FAs for several of the experiments; integrated examination of the effects of the FAs on aspects of cell signaling, protein expression, secreted proteins, and binding of ECs to monocytes; and the novel examination of the effects of un- or underexplored plant-derived FAs. One limitation of the study is that it examined the effects of only a single period of exposure of the FAs to the ECs (48 h). Furthermore, the study used only a single inflammatory stimulus, TNF-α, and it would be interesting to examine other stimuli such as lipopolysaccharide or IL-6. In order to confirm efficacy of siRNA treatment it would be beneficial to examine elongase 5 protein concentrations alongside ELOVL5 gene expression in ELOVL5 silenced cells; however the EC fatty acid concentration changes reported are consistent with reduced elongase 5 enzyme activity which would result from less protein being present. Finally, the EA.hy926 EC line was used; although this cell line maintains many of the innate properties of primary ECs,[54] it may have some differences in properties or responses. We have previously verified that effects of EPA and DHA seen with EA.hy926 cells[28] are similar to those reported for other types of EC.[23–26,32,55–57]

It is important to also consider the concentrations of the FAs used in the current experiments. The concentrations of GLA, PLA, and ETA used are likely to exceed those present in human plasma; the concentrations of these FAs are not routinely reported in human plasma and are considered to be very low. Studies supplementing humans with GLA report that GLA itself does not accumulate in plasma lipids[39,58] or in white blood cells.[39–43,59] In contrast, DGLA is readily detectable in human plasma and in white blood cells and accumulates when GLA is given.[39–43,58,59] Miles et al.[60] reported DGLA as a weight percentage of fatty acids in plasma triglycerdes, phospholipids, and cholesteryl esters prior to and after 12 weeks of daily supplementation with 2 g GLA. Weight percentages changed from 0.36 to 0.48, 3 to 4.8, and 0.74 to 1.20, respectively. Hodson et al.[61] quote a mol% contribution of DGLA to total plasma fatty acids of 1.1%. Using typical fasting plasma concentrations of triglycerides, phospholipids, cholesteryl esters, and non-esterified fatty acids of 1, 3, 4, and 0.6 mM (equivalent to 3, 6, 4, and 0.6 mM of fatty acids), 1.1 mol% equates to a total DGLA concentration in plasma of 150 μM. This concentration of DGLA might as much as double after supplementation with GLA. Hence, the concentrations of DGLA used in the current study are lower than the total plasma concentration of DGLA that is typically seen. However, most DGLA in plasma is esterified into triglycerides, phospholipids, and cholesteryl esters but in the current experiments free, non-esterified DGLA was used. Hodson et al.[61] state that DGLA typically contributes 0.1 mol% to plasma non-esterified fatty acids; this would equate to a concentration of <1 μM well below the concentrations used here.

It is concluded that the plant-derived FAs GLA and PLA exert anti-inflammatory effects, mainly through conversion to their elongated derivatives DGLA and ETA. The context of the current research is the initiation and progression of atherosclerotic plaques that predispose to coronary heart disease and other CVDs. Effects of GLA and PLA should be further explored in other cell systems, in relevant animal models and in humans.

4. Experimental Section

Endothelial Cell Culture: EA.hy926 cells (ATCC, LGC standards, Middlesex, UK) were cultured in high glucose DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine-penicillin-streptomycin solution, and 1% HAT (100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine); medium and supplements were purchased from Sigma-Aldrich (Gillingham, UK). Cultures were maintained at 37 °C in humidified 95% air and 5% CO_2.

Fatty Acid Treatment of Endothelial Cells: GLA (Sigma-Aldrich), PLA, DGLA, and ETA (Larodan, Sweden) were prepared as 50, 25, 10, or 5 mM stocks in 100% ethanol. Experimental FA concentrations were achieved by diluting stock solutions in complete medium to 5, 10, 25, and 50 μM in a final concentration of 0.1% ethanol.

Assessment of Cell Viability: Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay which measures cellular mitochondrial activity. EA.hy926 cells were incubated in the presence of FAs at concentrations of 10, 25, or 50 μM for 48 h and further exposed to complete medium with or without TNF-α (1 ng mL^{-1} final concentration) for 24 h. After incubation, supernatant was removed

and replaced with DMEM containing 0.05 mg mL^{-1} MTT (Sigma-Aldrich) (100 µL per well) and samples incubated at 37 °C for 4 h. Supernatants were removed (75 µL) and 75 µL of DMSO added (Sigma-Aldrich). Samples were then incubated for at 37 °C for 30 min before reading the absorbance at 540 nm on a plate reader. The effects of FAs and TNF-α on cell viability were normalized to control (0.1% ethanol, i.e., no FA or TNF-α) cultures (100%).

Determination of Fatty Acid Composition of Endothelial Cells by Gas Chromatography: The FA composition of EA.hy926 cells after culture with various FAs was determined using gas chromatography. Total lipid was extracted from EA.hy926 cells using chloroform/methanol as described elsewhere.[62] Lipid extracts were dried under nitrogen. FAs were cleaved from these isolated lipids and simultaneously methylated by heating with methanol and sulfuric acid at 50 °C for 2 h. The FA methyl esters were extracted into hexane and then separated and analyzed by gas chromatography using conditions described by Fisk et al.[62] FAs were identified by comparison of retention times with those of authentic standards and are expressed as µg per 10^6 cells.

Identification of Unknown Fatty Acid by Gas Chromatography–Mass Spectrometry: The identification of an unknown FA in EA.hy926 cells after culture with PLA was made using gas chromatography/mass spectrometry. Preparation of FA methyl esters from total lipid extracts of EA.hy926 cells was performed as described in the previous section. FA methyl esters were separated by gas chromatography using conditions similar to those described by Fisk et al.[62] The gas chromatograph was fitted with a SGE BPX-70 30 m × 0.2 mm × 0.25 µm capillary column, and helium was used as carrier gas at a total flow rate of 50 mL min^{-1} with 1.58 mL min^{-1} through the column. The injector was kept at 250 °C using the splitless injection mode, and 1 µL injections were made. The temperature was kept constant at 115 °C for 2 min, with a linear increase at 10 °C min^{-1} to 200 °C thereafter; this temperature was held for 18 min after which the column was held at 245 °C for a final 5 min. Total run time was 35 min. The unknown FA methyl ester was subsequently identified by mass spectrometry detection using a Shimadzu QP2010nc system equipped with an AOC5000 autosampler. The quadrupole detector was operated in full scan semi-electron impact (SEI) electrospray ionization mode covering the m/z range of 40–500. Detector voltage was 0.95 kV, and the temperature of the ion source and interface were set to 230 and 250 °C, respectively. FAs were identified according to their mass-to-charge ratio (m/z) and by the comparison of ion fragments to those of authentic standards.[29]

Measurement of Inflammatory Mediator Concentrations: The concentrations of inflammatory mediators (interleukin (IL)-6, IL-8, intercellular adhesion molecule (ICAM)-1, regulated on activation normal T cell expressed and secreted (RANTES) and monocyte chemoattractant protein (MCP)-1) secreted by EA.hy926 cells were determined simultaneously using Human magnetic Luminex Screening Assay kits (R&D Systems, Minneapolis, MN). Assays were conducted in accordance with the instructions from the manufacturer. Plates were analyzed on a calibrated Bio-Plex 200 analyzer using Bio-Plex software (version 6.1, Bio-Rad Laboratories Inc., Berkeley, CA). Lower limits of detection were: IL-6 (1.7 pg mL^{-1}), IL-8 (1.8 pg mL^{-1}), MCP-1 (9.9 pg mL^{-1}), RANTES (1.8 pg mL^{-1}), and ICAM-1 (87.9 pg mL^{-1}).

Assessment of Cell Surface ICAM-1 Expression: Surface expression of ICAM-1 (CD54) on EA.hy926 cells was determined using flow cytometry. Cells were exposed to FAs at 25 and 50 µM for 48 h followed by stimulation with TNF-α (1 ng mL^{-1}) for 6 h. Following this, cells were detached, centrifuged, and stained with phycoerythrin (PE)-conjugated monoclonal anti-human CD54 (BD Biosciences, San Jose, CA) diluted in staining solution (PBS, 2% BSA) for 30 min. Mouse IgG1 (PE) isotype was used as a negative control. After staining, cells were analyzed by flow cytometry using an FACSCalibur flow cytometer (BD Biosciences). A total of 10 000 events were collected. Percentage positive cells and median fluorescence intensity (MFI) were determined.

Protein Extraction and Western Blotting: The expression of NFκBp65, pNFκBp65, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was assessed by Western blotting. Cells were exposed to FAs at 50 µM for 48 h followed by stimulation with TNF-α (1 ng mL^{-1}) for 1 h. Protein was extracted from cells using radioimmunoprecipitation assay buffer. Protein was quantified using the BCA assay (Pierce, ThermoFisher Scientific) following the manufacturer's instructions. Then, samples were diluted and a total of 30 µg of protein loaded onto pre-cast 10% SDS gels (Obtiblot, Abcam, Cambridge, UK) alongside a Prism Ultra Protein Ladder (10–245 kDa). Gels were run for 1.5–2 h and proteins transferred onto a nitrocellulose membrane which was probed with antibodies (Abcam; NFκBp65 (ab32536), phosphorylated NFκBp65 (ab76302), and GAPDH (ab181602). Western blots were quantified using ImageJ software and relative quantification values were presented as the ratio of each protein band relative to the loading control (GAPDH).

Monocyte Adhesion to Endothelial Cells: Adhesion of monocytic THP-1 cells to EA.hy926 cells was determined using the Vybrant Cell Adhesion Assay Kit (ThermoFisher Scientific). EA.hy926 cells were exposed to FAs at 5, 10, 25, or 50 µM for 48 h followed by stimulation with TNF-α (1 ng mL^{-1}) for 6 h. After stimulation, calcein-labeled THP-1 cells were incubated with EA.hy926 cells for 1 h at 37 °C. Non-adherent THP-1 cells were removed by gentle washing, 100 µL PBS added to each well and co-cultures read on the Glomax Discover System (Promega). THP-1 monocyte adhesion was measured as a percentage of control (non-stimulated DMEM treated cells). Images of fluorescence-labeled THP-1 monocytes bound to EA.hy926 cells were taken with a Nikon Elipse Ti using NIS elements software (version 4.30).

Elongase 5 Gene Silencing: Silencing of the elongase 5 gene (ELOVL5) in EA.hy926 cells was achieved by siRNA-mediated inhibition of ELOVL5 using Santa Cruz reagents and protocol (Santa Cruz Biotechnology Inc., Santa Cruz, CA). EA.hy926 cells were incubated in antibiotic-free culture medium for 24 h followed by 6 h incubation with siRNA transfection reagent mixture (1 mL), containing ELOVL5 siRNA or control siRNA and siRNA transfection reagent (all Santa Cruz Biotechnology Inc.). Medium was then removed and replaced with complete culture medium for 24 h. Finally, EA.hy926 cells were incubated in the presence of FAs (GLA or PLA) at a concentration of 50 µM for 48 h; cells were then either removed for FA composition analysis, or further exposed to complete medium with or without TNF-α (1 ng mL^{-1} final concentration) for 24 h, for supernatant inflammatory mediator analysis.

RNA Isolation, cDNA Synthesis, and Real-Time PCR: Changes in relative gene expression were analyzed by RT-PCR. Cells were exposed to siRNA silencing protocol followed by FA treatment for 48 h and finally stimulation with TNF-α (1 ng mL^{-1}) for 24 h. Taqman Gene Expression Primers (ThermoFisher Scientific, Waltham, MA) were used to determine the effect of silencing ELOVL5 (Hs00765730_m1) in EA.hy926 cells. Total RNA was isolated using the ReliaPrep RNA cell Miniprep System (Promega, Southampton, UK). RNA quantity and quality were analyzed by NanoDrop. Analysis of RNA using Agilent Bioanalyzer (RNA Total Eukaryote 2100 Nano) was performed to determine RNA RIN scores. cDNA was synthesized from total RNA using GoScript Reverse Transcriptase (Promega). Housekeeping reference genes were determined using a geNorm Kit (Primerdesign, Camberley, UK). Quantification of relative gene expression was analyzed using B2M, (Hs00187842_m1), GAPDH (Hs02786624_g1), and RPL13A (Hs04194366_g1) as housekeeping genes.

Statistics/Data Analysis: Data were presented as mean ± SEM and were analyzed by two-way analysis of variance (two-way ANOVA) or one-way analysis of variance (one-way ANOVA) followed by post-hoc tests of pairwise differences. Analyses were performed using GraphPad Prism 6.0. Differences were considered significant when $p < 0.05$.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

E.J.B. and P.C.C. designed the experiments; E.J.B., C.A.V., W.v.D., and L.M.F. carried out the experiments under the supervision of E.A.M. and P.C.C.; E.J.B. analyzed the results and drafted the manuscript; P.Y., E.A.M., and P.C.C. had significant input into the manuscript; all authors read and agreed the final version of the manuscript.

Keywords

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