

Anti-Inflammatory Effects of Alpha Linolenic Acid on Human Corneal Epithelial Cells

Nir Erdinest,¹ Or Shmueli,¹ Yoni Grossman,¹ Haim Ovadia,² and Abraham Solomon¹

PURPOSE. Systemic polyunsaturated fatty acids (PUFAs) were shown to improve the symptoms of dry eye syndrome due to their anti-inflammatory effects. This study evaluated the in vitro anti-inflammatory effects of PUFAs on human corneal epithelial (HCE) cells.

METHODS. HCE cells were incubated for 2 hours with different concentrations of PUFAs: alpha-linolenic acid (ALA), gamma-linolenic acid (GLA), and linoleic acid (LA). Oleic acid (OA) and dexamethasone (DM) served as negative and positive controls, respectively. Cells were stimulated with either polyinosinic: polycytidylic acid (poly I:C) or lipopolysaccharide (LPS) complex. The protein contents and mRNA expression levels of IL-6, IL-8, IL-1 β , and TNF- α were evaluated with multiplex fluorescent bead immunoassay and real-time PCR, respectively. The expression of inhibitory factor- κ B α (I- κ B α) was evaluated with real-time PCR.

RESULTS. The protein and mRNA levels of IL-6, IL-8, IL-1 β , and TNF- α were significantly increased after stimulation with LPS or poly I:C. Following treatment with ALA, a significant decrease was demonstrated in the protein content of TNF- α to 23.81% ($P < 0.001$), IL-6 to 46.71% ($P < 0.001$), IL-1 β to 20.86% ($P < 0.05$), and IL-8 to 52.21% ($P < 0.001$). Similar results were demonstrated at the mRNA level. The anti-inflammatory effects of ALA were similar to those of DM for all of the pro-inflammatory cytokines. The ALA inhibition of the pro-inflammatory cytokines was associated with a significant reduction of I- κ B α .

CONCLUSIONS. ALA may serve as a potent anti-inflammatory agent in ocular surface inflammation. The anti-inflammatory effects of ALA are comparable to those of corticosteroids, and are mediated through NF- κ B signal transduction. (*Invest Ophthalmol Vis Sci.* 2012;53:4396-4406) DOI:10.1167/iov.12-9724

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Many ocular surface disorders are associated with chronic inflammation. Among these are the dry eye syndrome,¹ allergic conjunctivitis, and contact lens intolerance.² These disorders affect a large proportion of the general population. Dry eye syndrome is one of the most frequent ocular disorders, affecting almost 15% of the entire population at all ages. Secondary inflammatory processes, which are associated with these disorders, may cause significant damage to the ocular surface.

Studies indicate that topical anti-inflammatory therapies can inhibit the various inflammatory mediators and reduce the signs and symptoms of dry eye syndrome. Topical corticosteroids show efficacy in treating dry eye associated inflammation, and have a rapid onset of action.³ However, corticosteroids have significant side effects with long term use, such as increased IOP and formation of posterior subcapsular cataracts.^{4,5} Therefore, corticosteroids appear to be most appropriate for short-term pulse therapy.

Cyclosporine A has been introduced in recent years to treat the inflammation associated with dry eyes. However, topical Cyclosporine A may require up to 6 months to achieve a maximal clinical therapeutic effect, and is therefore used for extended courses of treatment. The efficacy of topical Cyclosporine A is controversial, and it is indicated for mild to moderate inflammatory conditions.^{6,7}

Recent studies have shown that dietary supplements of polyunsaturated fatty acids (PUFAs) may improve dry eye symptoms due to their anti-inflammatory effects.⁸⁻¹⁰ PUFAs include α -linolenic acid (ALA; 18:3, n-3) and linoleic acid (LA; 18:2, n-6). ALA and LA elongation and desaturation products are the precursors of eicosanoid molecules, which are key players in inflammatory processes.^{11,12} ALA-derived eicosanoids have anti-inflammatory properties, while LA-derived eicosanoids are considered to have pro-inflammatory characteristics.¹²⁻¹⁴

Studies have confirmed that there is a relationship between PUFAs oral supplementation and improvement in dry eye syndrome and contact lens intolerance.^{9,15} A recent study found that topical ALA decreased the clinical signs of dry eye syndrome in a mouse model.¹⁶ As of today, the direct, local anti-inflammatory effects of n-3 PUFAs in the ocular surface cells have not been studied in vitro.

The authors have, therefore, decided to study the anti-inflammatory effects of ALA on human corneal epithelial (HCE) cells in vitro, and to compare the anti-inflammatory effects with those of corticosteroids in order to evaluate its efficacy as a possible anti-inflammatory agent for the treatment of ocular surface inflammatory disorders.

METHODS

The Hadassah Medical Center Institutional Review Board (IRB) approval was obtained for this study (IRB protocol number and version: EFA-EFE-IV-01), and all of the study procedures were carried out in accordance with the IRB guidelines. This study followed the tenets of the Declaration of Helsinki.

Culture of Human Corneal Epithelial Cells

HCE cells were cultured from human corneoscleral rim explants, taken from several different human donors, provided by the Department of Ophthalmology at the Hadassah Medical Center, using a previously described method.¹⁷

Corneal epithelial cells were cultured in supplemented hormonal epithelial medium.¹⁸ HCE cells were incubated at 37°C under 95% humidity and 5% CO₂. The culture medium was replaced every other day. Cultures were kept for 10 to 14 days until a density of 90% confluence was observed. At this time, cells were passaged and seeded onto 6-well plates at a density of 2.0×10^5 cells/well. Cells were observed by phase-contrast microscopy to ensure uniformity of morphology. The purity of HCE cultures was confirmed by staining for cytokeratin-19 with the indirect immunoperoxidase procedure with monoclonal antibody to human cytokeratin-19 (Abcam, Cambridge, UK). Second generation cells were used in all experiments.

Fatty Acids

LA, ALA, Gamma linolenic acid (GLA; 18:3 n-6) and Oleic acid (OA; omega 9; 18:1 n-9) were obtained as greater than 99% pure sodium salts (Nuchek Prep Inc., Elysian, MN). All fatty acid salts were dissolved in distilled water in a nitrogen chamber, filtered through 0.2- μ m pore size, divided to aliquots, and sealed under nitrogen in opaque Eppendorf tubes (Brinkmann Instruments, Westbury, NY). All fatty acids were stored at -80°C for no longer than 90 days before treatments.

Experimental Designs

HCE cells were seeded into 6-wells plates for 24 to 48 hours before the experiment, at a density of 1.2×10^5 cells/well in 2.0 mL of medium. Culture medium was exchanged every other day, and cultures were maintained until subconfluence. Fatty acids were conjugated with BSA (Fraction V; Mercury, Rosh-Ha'ayin, Israel) at a maximal 5:1 ratio according to a previous study.¹⁹ HCE cells were pre-incubated for two hours with each of the various fatty acids (ALA, LA, GLA, or OA, respectively) before the inflammatory stimulus, based on a previous protocol.²⁰ PUFAs were incubated at doses of 200, 20, and 2.0 μ M for IL-6 and IL-8, while for TNF- α and IL-1 β the doses were 125, 12.5, and 1.25 μ M. In order to avoid oxidative effects, the fatty acids were defrosted once and were not reused again.

After incubation of the HCE cells with the fatty acids, the cells were not washed out, and were treated with inflammation inducers: either with Lipopolysaccharide (LPS) at a dose of 1000 ng/mL with 500 ng/mL (CD14) and 500 ng/mL LBP (LPS binding protein) as previously described,^{21,22} or with polyribosinoinic: polyribocytidylic acids (poly I:C) at a dose of 25 μ g/ml.²³

For maximal induction of IL-6 and IL-8, the stimulus exposure time lasted 4 hours for protein content measurement and 3 hours for mRNA expression level measurement, after the cells were pre-incubated for 2 hours with PUFAs (ALA, LA, GLA) at concentration 1 to 300 μ M (the total incubation time was 6 and 5 hours for protein and mRNA, respectively). A PUFA concentration of 200 μ M was used for induction of IL-6 and IL-8. The range of 2 to 200 μ M was used for the dose response curves, as this range did not affect the HCE viability. For TNF- α and IL-1 β , the stimulus lasted 15 hours for protein content measurement, and 12 hours for mRNA expression level measurements, after cells were pre-incubated for 2 hours with PUFAs at concentrations 1 to 300 μ M (the total incubation time was 17 and 14 hours for protein and mRNA, respectively). A PUFA concentration 125 μ M was used for induction of TNF- α and IL-1 β . The range of 1.25 to 125 μ M was used for the dose response curves, as this range did not affect the HCE viability.

In addition, DM served as a positive control at a concentration 10^{-5} M,²⁴ while OA at a concentration 200 μ M served as a negative control due to its lack of effect on eicosanoid biosynthesis.^{25,26} At the end of each treatment, the cells were examined with FITC-Annexin V/PI (MBL, Nagoya, Japan). Culture supernatants were collected, aliquoted,

and stored at -80°C for further measurements of the protein contents of the inflammatory mediators. Total RNA was extracted from the cells and stored at -80°C until thawed for cDNA PCR.

Before the experiment, the medium was replaced with 2 mL of serum-free medium (SFM) in each well. All the study experiments were performed in HCE cells that were cultured from human corneoscleral rim explants, taken from four different human donors.

HCE Cells Viability

The apoptosis assay was performed as described previously.²⁷ In brief, at the end of each treatment HCE cells viability was assessed by flow cytometry using an apoptosis detection kit (Annexin V; MBL). One microgram per milliliter of Annexin V-FITC (MBL) and 1 μ g/mL of propidium iodide (PI) was added to the cell suspension, and the mixture was incubated in the dark for 5 minutes at room temperature. Without washing, the cells were placed in 500 μ L of binding buffer, (Annexin V; MBL) kept on ice, and within 5 minutes were evaluated using a Coulter FC-500 flow cytometer (Beckman-Coulter, Miami, FL). Flow cytometry data were analyzed using CXP Analysis 2.0 software (Beckman-Coulter).

A Multiplex Fluorescent Bead Immunoassay (FBI)

The cytokines protein concentration levels were measured using a multiplex fluorescent bead immunoassay (Cytometric Bead Array [CBA] Human Inflammatory Cytokines Kit; BD Biosciences, San Jose, CA). This kit allows the simultaneous measurement of IL-1 β , IL-6, IL-8, IL-10, TNF- α , and IL-12 (p70) protein levels in a single sample. The test was performed and analyzed according to the manufacturer's instructions and was performed as described previously.²⁸ In brief, 50 μ L of six premixed capture beads populations (coated with capturing antibodies specific for different cytokines or chemokines) were mixed with 50 μ L of the provided standards. Eight point standard curves ranging from 20 to 5000 pg/mL were obtained by serial dilutions of the reconstituted lyophilized standards. Fifty microliters of capture beads populations were also mixed with culture supernatant samples, allowing for the different cytokines in the samples to be captured by their analogous beads. Afterwards, the cytokines capture beads were mixed with 50 μ L of phycoerythrin-conjugated detection antibodies to form sandwich complexes. These sandwich complexes were successively incubated in the dark for 3 hours at room temperature. After incubation, the mixture was washed, centrifuged (at 200g for 5 minutes), and the pellet was resuspended in 300 μ L of wash buffer. The flow cytometer (BD LSRII; BD Biosciences) was calibrated with setup beads and 1800 events were acquired for each sample. Results were generated in graphical format and analyzed with array software (FCAP v1.0.1; BD Biosciences).

Western Blot Analysis

Western blot was performed as described previously.²⁹ Briefly, samples were subjected to SDS-PAGE, using a 4% to 20% gradient primary antibody rabbit monoclonal anti-human IL-8 (Abcam) diluted to 0.5 μ g/mL in 1% Difco skim milk (Becton-Dickinson, Sparks, MD). After being washed five times with 0.1% Tween 20 in Tris-buffered saline (TTBS), each membrane was transferred to a 1:100 diluted solution of horseradish peroxidase rabbit antibody (DAKO, Carpinteria, CA) in TTBS and incubated for 90 minutes. Specifically bound antibodies were detected using an immunodetection EZ-ECL kit (Biological Industries, Beit Haemek, Israel), and then exposed to x-ray film (Fuji Super RX Medical, Tokyo, Japan) for 1 minute.

RNA Isolation

Total RNA was extracted from the HCE cell samples with an RNeasy Lysis Kit (Qiagen, Crawley, UK) following the manufacturer's instructions. Quantification of total RNA was performed in a spectrophotometer (ND-1000; NanoDrop). RNAs were stored at -80°C until further utilization.

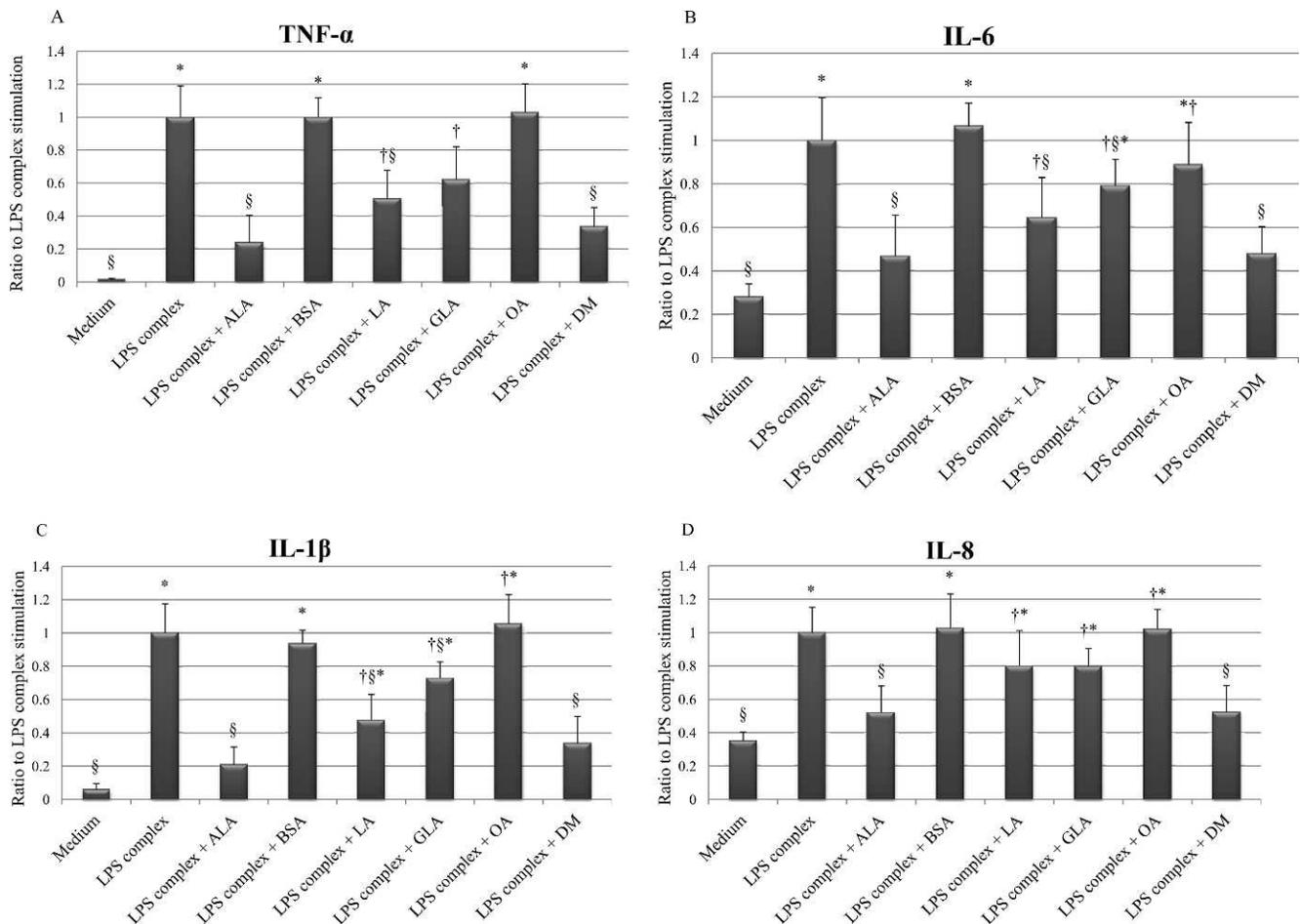


FIGURE 1. The effects of PUFAs on the protein contents of pro-inflammatory mediators in HCE cells stimulated by LPS complex (A–D). For TNF- α and IL-1 β detection, the HCE cells were stimulated in SFM with LPS complex for 15 hours and cells pre-incubated for 2 hours with PUFAs (ALA, LA, GLA) at 125 μ M (A, C). For IL-6 and IL-8 detection the HCE cells were stimulated with LPS complex for 4 hours and cells pre-incubated for 2 hours with PUFAs at 200 μ M (B, D). Data shown are the ratio to stimulus with LPS complex only (mean \pm SD) from four independent experiments on four different cell lines cultured from different donors ($n = 4$). Bars with the same sign were not significantly different ($P > 0.05$), while bars with different signs were significantly different ($P < 0.05$).

cDNA Synthesis

cDNA was synthesized from purified and concentrated 0.5 μ g RNA from each sample using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). A 20 μ L total reaction volume was made with 10 μ L RNA, 2 μ L 10X RT buffer, 0.8 μ L dNTP Mix (100 mM), 2.0 μ L 10X RT random hexamer primers, 1.0 μ L MultiScribe reverse transcriptase (Applied Biosystems), 1 μ L RNase inhibitor and 3.2 μ L nuclease-free water. Synthesis was carried out in a thermocycler (7900 ABI; Applied Biosciences) and reaction conditions were 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes. cDNA samples were stored at –20°C.

Real-Time Polymerase Chain Reaction

Real-time PCR was performed using TaqMan Gene Expression Assays (Applied Biosciences) in the sequence detection system (ABI Prism 7900HT; Applied Biosciences) as described previously.^{30,31} Negative controls were included to evaluate DNA contamination of isolated RNA and reagents.

Real-time PCR assays for the selected cytokines were: TNF- α , IL-1 β , IL-6, IL-8, and an Inhibitor of κ B α (Applied Biosciences). An amount of 1 μ L cDNA was loaded in each total volume of 20 mL of reaction mixture and assays were performed in triplicates.

The fold change in gene expression was normalized to the endogenous gene hypoxanthine phosphoribosyltransferase 1 (Applied Biosciences). Quantitative analysis was performed using the comparative ($\Delta\Delta C_T$) method, in which C_T value is defined as the cycle number that the detected fluorescence exceeds the threshold value, ΔC_T is the difference between C_T of a target gene and the endogenous control, and $\Delta\Delta C_T$ is the difference between ΔC_T of the analyzed sample and the calibrator (control sample).^{30,31} The results were analyzed by DataAssist Software V 2.0 (Applied Biosciences).

Study Statistical Analysis

All tests were carried out on four independent cell cultures, and performed in triplicates for each of the treatments. The levels of the cytokines protein secretion and mRNA expression were manifested as a ratio relative to stimulus alone. The dose response curve was expressed as percentage of inhibition relative to the stimulus.

Statistical analysis and multiple comparisons were performed by one-way ANOVA using the InStat software version 3.0 (GraphPad Software Inc., San Diego, CA).

The median effective concentration (EC 50) of ALA was calculated with the sigmoid Emax model. The pharmacokinetic modeling was performed using WinNonlin 6.2 software (Pharsight Corporation, Mountain View, CA).

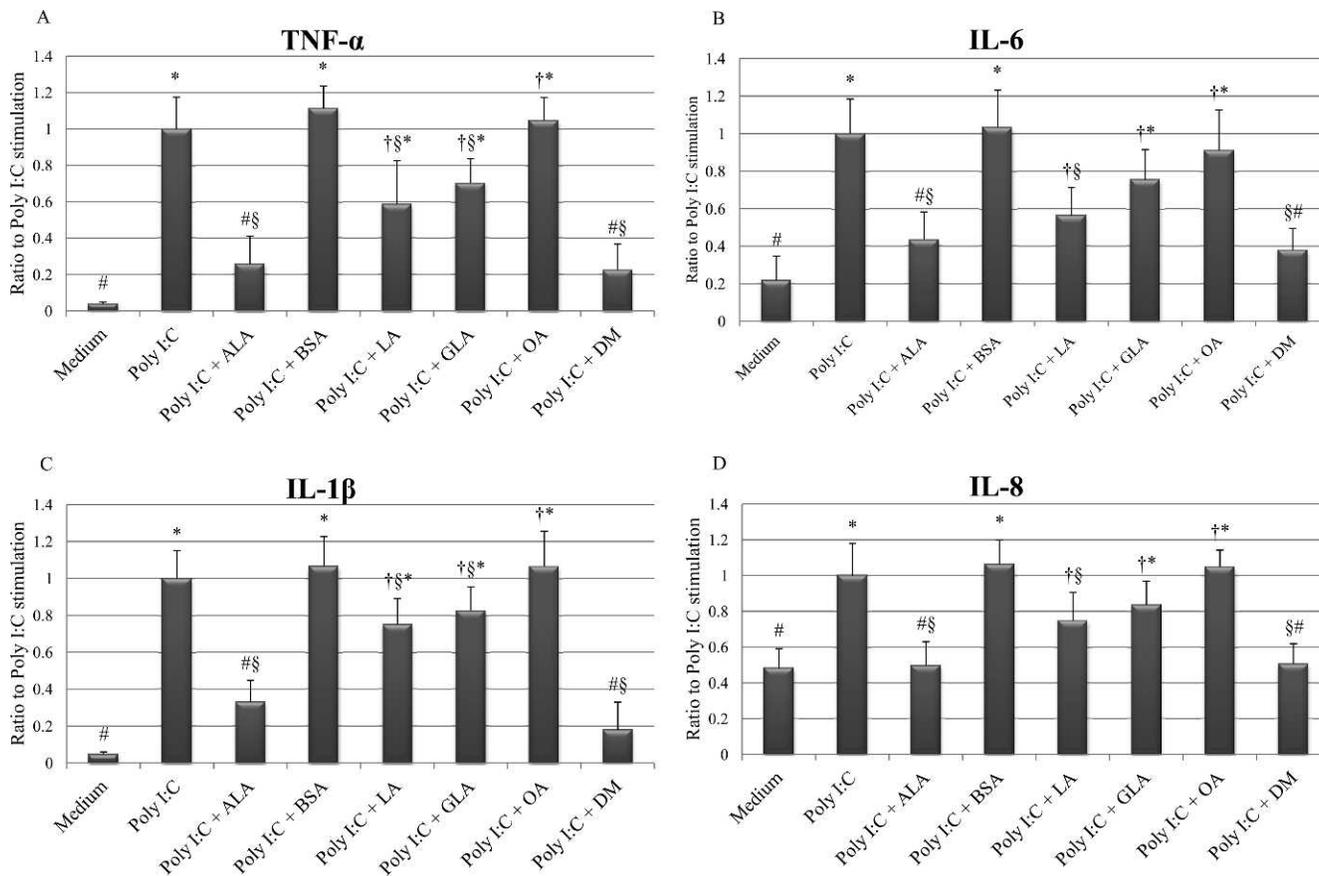


FIGURE 2. PUFAs affects on poly I:C-induced pro-inflammatory cytokines protein secretion in HCE cells (A–D). For TNF- α and IL-1 β detection, the HCE cells were stimulated in SFM with poly I:C (25 μ g/mL) for 15 hours and cells pre-incubated for 2 hours with PUFAs (ALA, LA, GLA) at 125 μ M (A, C). For IL-6 and IL-8 detection, the HCE cells were stimulated with poly I:C for 4 hours and cells pre-incubated for 2 hours with PUFAs at 200 μ M (B, D). Data shown are the ratio to stimulus with poly I:C only (mean \pm SD) from four independent experiments ($n = 4$). Bars with the same sign were not significantly different ($P > 0.05$), while bars with different signs were significantly different ($P < 0.05$).

RESULTS

Cell Viability following Fatty Acid Treatments

HCE cells were cultured with fatty acids in variant concentrations and periods of time as described earlier. Cell viability following fatty acid and the control groups were examined with Annexin V-FITC PI assay (MBL) at the end of each treatment. The viability of the HCE cells at the end of the treatments was 94% to 96% in comparison to the medium only, which served as a negative control (data not shown). There were no significant differences in cell viability among group treatments (PUFAs, OA, DM) and the negative control. In addition, there were no significant differences in cell viability among each of the PUFAs treatment (ALA, GLA, LA) and the negative control.

Stimulation of HCE Cells (Multiplex FBI)

Data from multiplex FBI demonstrate that LPS stimulation combined with CD14 and LBP (LPS complex) increased cytokines production in HCE cells in relation to incubation with LPS alone in cell culture. It was therefore decided to use the LPS complex as an inducer of inflammation in all experiments. The incubation of corneal epithelial cells with the LPS complex elicited up to 10-fold higher levels of IL-6 ($P < 0.001$), 4-fold IL-1 β ($P < 0.001$), 20-fold TNF- α ($P < 0.001$), and 2.5-fold IL-8 ($P < 0.001$) compared with the corresponding levels from cells incubated in medium alone (data not shown).

Corneal epithelial cells incubated with poly I:C expressed up to 35-fold higher levels of IL-6 ($P < 0.001$), 15-fold IL-1 β ($P < 0.001$), 60-fold TNF- α ($P < 0.001$), and 4-fold IL-8 ($P < 0.001$) from cells compared with incubation with medium alone (data not shown).

PUFAs Inhibit LPS and Poly I:C-Induced Cytokines Protein Production (Multiplex FBI)

Following treatment with ALA in HCE cells, which were stimulated with LPS complex, a significant decrease was demonstrated in the protein content of TNF- α to 23.81% ($P < 0.001$), IL-6 to 46.71% ($P < 0.001$), IL-1 β to 20.86% ($P < 0.05$), and IL-8 to 52.21% ($P < 0.001$) compared with the corresponding protein contents of HCE cells incubated with LPS complex only (Figs. 1A–D).

These anti-inflammatory effects of ALA were comparable with the effects of the DM. Incubation of HCE cells with DM (which served as a positive control to ALA) resulted in decreased TNF- α protein content to 33.6% ($P < 0.001$), IL-6 to 48.02% ($P < 0.001$), IL-1 β to 33.7% ($P < 0.05$), and IL-8 to 52.44% ($P < 0.001$), compared with the protein contents of these cytokines after incubation of HCE cells with LPS complex only (Figs. 1A–D).

Interestingly, there were no significant differences between the amount of decrease of the cytokines after treatment with either ALA or DM in HCE cells after stimulation with LPS complex (Figs. 1A–D). This observation suggests a similar

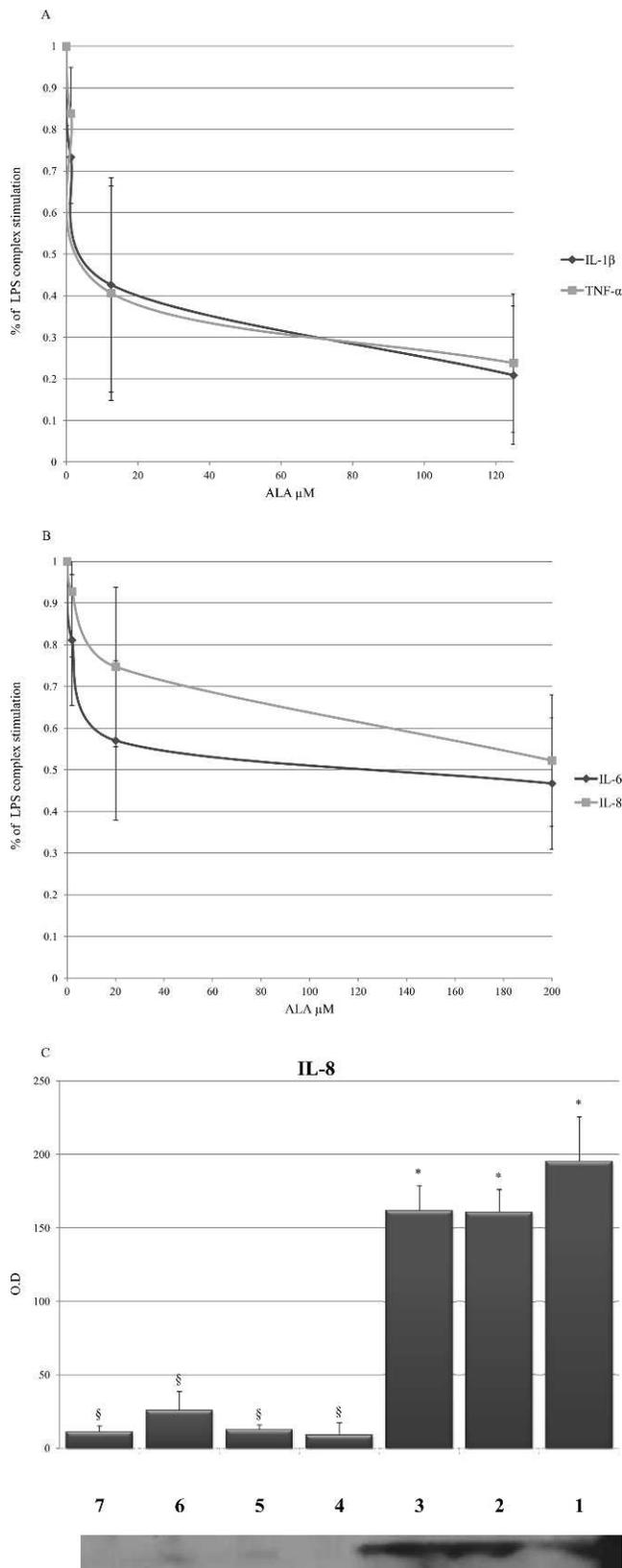


FIGURE 3. A dose-response reduction of TNF- α , IL-1 β (A), IL-6, IL-8 (B), and IL-8 (C) by ALA in LPS complex stimulated in HCE cells. For TNF- α and IL-1 β detection, the HCE cells were incubated in SFM with LPS complex for 15 hours and cells pre-incubated for 2 hours with ALA at 125, 12.5, and 1.25 μ M (A). For IL-6 and IL-8 detection, the HCE cells were incubated in SFM with LPS complex for 4 hours and cells pre-

efficacy of ALA and DM as potent anti-inflammatory agents on cultured HCE cells.

GLA and LA achieved only minor anti-inflammatory effects compared with ALA and DM (Fig. 1). As expected, OA did not achieve a similar anti-inflammatory effect and, therefore, served as negative control (Fig. 1). This finding was expected based on previous studies that used OA as a control fatty acid in dietary PUFA intervention trials due to its lack of effect on eicosanoid biosynthesis.^{25,26}

In order to evaluate another type of inflammatory stimulus, the authors have repeated this set of experiments with stimulation with poly I:C, a stimulus that mimics viral antigens. Similarly, a significant increase of cytokine production was evident following stimulation with poly I:C, as demonstrated for the LPS complex. ALA treatment significantly decreased TNF- α protein secretion to 25.87% ($P < 0.05$), IL-6 to 43.53% ($P < 0.001$), IL-1 β to 33.11% ($P < 0.05$), and IL-8 to 49.66% ($P < 0.001$), compared with poly I:C stimulus alone (Figs. 2A-D). OA, which served as a negative control, did not cause a significant decrease in cytokines protein contents. These anti-inflammatory effects of ALA were comparable with that of DM, which decreased TNF- α protein secretion to 22.41% ($P < 0.05$), IL-6 to 38.03% ($P < 0.001$), IL-1 β to 18.0% ($P < 0.05$), and IL-8 to 50.53% ($P < 0.001$), compared with incubation of HCE cells with poly I:C only. There was no significant difference between the decrease in cytokines after treatments with either DM or ALA in HCE cells after stimulated with poly I:C (Fig. 2), suggesting again a similar anti-inflammatory effects of ALA and DM.

Similar to what was noted for the LPS complex stimulation, GLA and LA induced only minor anti-inflammatory effects compared with ALA and DM after stimulation with poly I:C.

A Dose-Response Reduction of Cytokines by ALA in LPS- and Poly I:C-Stimulated HCE Cells (Examined by Multiplex FBI)

HCE cells were further incubated with ALA at concentrations of 125, 12.5, and 1.25 μ M, respectively, for TNF- α and IL-1 β protein analysis, and at 200, 20, and 2.0 μ M, respectively, for IL-6 and IL-8 analysis. Significant dose response curves were demonstrated for ALA treatment doses for all of the inflammatory mediators (Figs. 3, 4). The dose response curves were analyzed for LPS complex (Figs. 3A, 3B) as well as for poly I:C-stimulated HCE cells (Figs. 4A, 4B).

The EC 50 of ALA values were 4.9 μ M \pm 0.7 for IL-1 β , 4.32 μ M \pm 0.6 for TNF- α , 3.91 μ M \pm 0.5 for IL-6, and 19.94 μ M \pm 3.73 for IL-8 after LPS complex stimulation (the dose-response correlation coefficients were > 0.935 for all the EC 50 values, $P < 0.05$). For poly I:C stimulation, the EC 50 values were 3.51 μ M \pm 0.2 for IL-1 β , 13.08 μ M \pm 0.4 for TNF- α , 20.46 μ M \pm 9.22 for IL-6, and 55.85 μ M \pm 5.9 for IL-8 (the dose-response correlation coefficients were > 0.9882 for all the EC 50 values, $P < 0.05$).

The relative protein contents of IL-8, one of the major inflammatory mediators in the ocular surface inflammatory response,¹ was further evaluated by Western blot analysis to validate the multiplex FBI results, and to assess a dose-response

incubated for 2 hours with ALA at 200, 20, and 2.0 μ M (B). Western blot analysis ($n = 3$) of IL-8 protein expression (C). Lane 1: IL-8 Recombinant Protein (10 kDa) 0.1 μ g; lane 2: LPS Complex + BSA; lane 3: LPS Complex; lane 4: LPS Complex + ALA 200 μ M; lane 5: LPS complex + ALA 20 μ M; lane 6: LPS complex + ALA 2.0 μ M; lane 7: medium only untreated HCE cells supernatant. Bars with the same sign were not significantly different ($P > 0.05$), while bars with different signs were significantly different ($P < 0.05$).

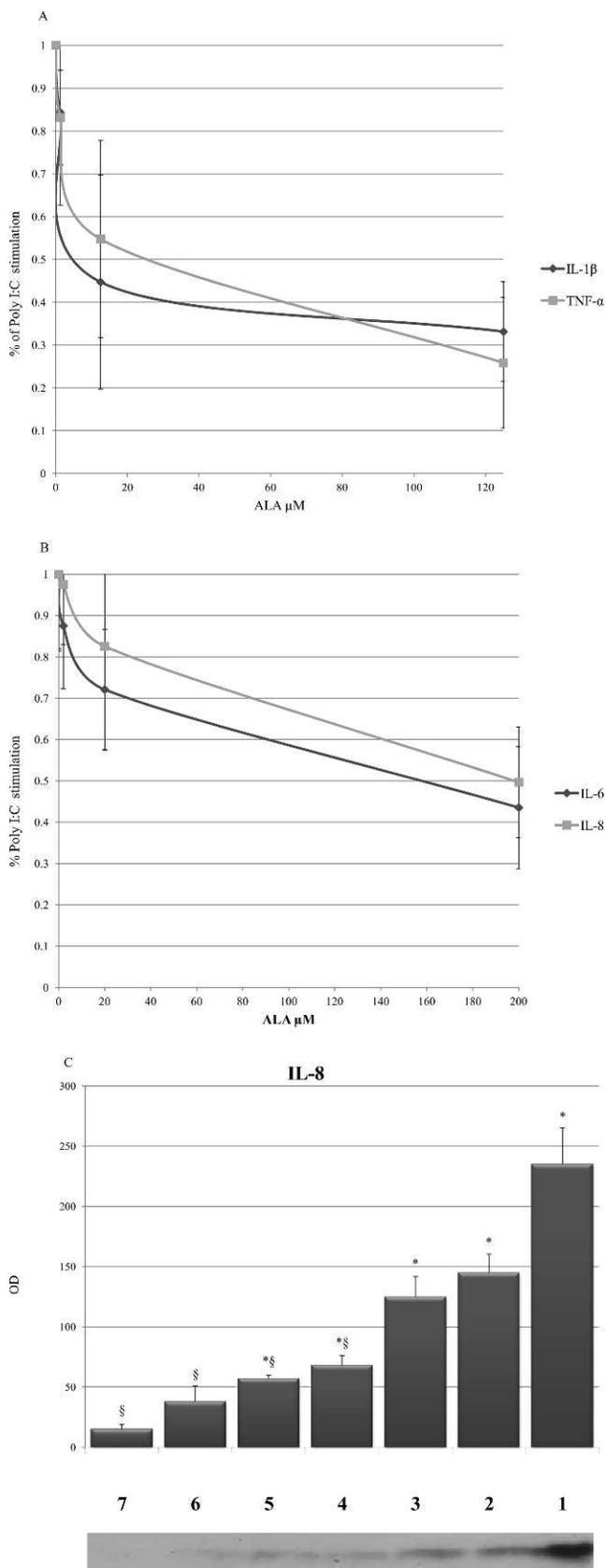


FIGURE 4. A dose-response reduction of TNF- α , IL-1 β (A) IL-6, IL-8 (B), and IL-8 (C) by ALA in poly I:C (25 μ g/mL) stimulated in HCE cells. For TNF- α and IL-1 β detection, the HCE cells were incubated in SFM with poly I:C for 15 hours and cells pre-incubated for 2 hours with ALA at

relationships (Figs. 3C, 4C). Similar results were demonstrated by Western blot analysis, as measured by multiplex FBI.

Inhibition of LPS-Induced Gene Expression of Cytokines Examined by Real-Time PCR

The mRNA expression levels of IL-6, IL-1 β , TNF- α , and IL-8 were significantly increased in HCE cells upon stimulation with LPS complex (Figs. 5A-D) and poly I:C (Figs. 6A-D), compared with unstimulated cells.

ALA treatment in HCE cells after stimulation with LPS complex induced a significant reduction of the mRNA expression levels of TNF- α to 20.94% ($P < 0.001$), IL-6 to 43.15% ($P < 0.01$), IL-1 β to 16.33% ($P < 0.001$), and IL-8 to 44.0% ($P < 0.01$), compared with incubation of the HCE cells with LPS complex alone (Figs. 5A-D). Incubation of corneal epithelial cells with DM after stimulation with LPS complex decreased TNF- α mRNA expression levels by 19.29% ($P < 0.001$), IL-6 by 39.84% ($P < 0.01$), IL-1 β by 15.24% ($P < 0.001$), and IL-8 by 33.3% ($P < 0.05$), compared with incubation of the epithelial cells with LPS complex alone.

ALA treatment in HCE cells after stimulation with poly I:C (Figs. 6A-D) elicited a significant reduction of TNF- α mRNA levels to 38.76% ($P < 0.001$), IL-6 to 44.47% ($P < 0.001$), IL-1 β to 28.85% ($P < 0.001$), and IL-8 to 46.53% ($P < 0.001$), compared with incubation of the cells with poly I:C only.

Effects of ALA on Inhibitory Factor- κ B α (I- κ B α) mRNA Expression Levels

ALA treatment in HCE cells after stimulation with either LPS complex or poly I:C elicited a significant reduction of I κ B α mRNA expression levels to 30.06% ($P < 0.001$) and 34.06% ($P < 0.001$), respectively (Fig. 7A, 7B), while DM treatment decreased I κ B α mRNA expression levels to 23.17% ($P < 0.001$) after stimulation with LPS complex, and to 34.06% ($P < 0.001$) after stimulation with poly I:C.

DISCUSSION

This study demonstrates a highly potent anti-inflammatory effect of the PUFA ALA. ALA dramatically reduced the poly I:C and LPS complex stimulated production of the pro-inflammatory cytokines TNF- α , IL-6, IL-1 β , and the chemokine IL-8 in cultured HCE cells. These effects were evident at both the protein and the gene expression levels for each of these inflammatory mediators. A significant dose-dependent reduction was demonstrated by ALA for all of these anti-inflammatory mediators.

The anti-inflammatory effects of ALA were stronger than those of the two other PUFAs (GLA and LA) that were investigated. In addition, ALA showed inhibitory effects on the protein secretion of the inflammatory mediators, which were equal to those of DM. Furthermore, stimulated HCE cells treated

125, 12.5, and 1.25 μ M (A). For IL-6 and IL-8 detection, the HCE cells were incubated in SFM with poly I:C for 4 hours and cells pre-incubated for 2 hours with ALA at 200, 20, and 2.0 μ M (B). Western blot analysis ($n = 3$) of IL-8 protein expression (C), Lane 1: IL-8 Recombinant Protein (10 kDa) 0.1 μ g; lane 2: poly I:C + BSA; lane 3: poly I:C; lane 4: poly I:C + ALA 2.0 μ M; lane 5: poly I:C + ALA 20 μ M; lane 6: poly I:C + ALA 200 μ M; lane 7: medium only untreated HCE cells supernatant. Bars with the same sign were not significantly different ($P > 0.05$), while bars with different signs were significantly different ($P < 0.05$).

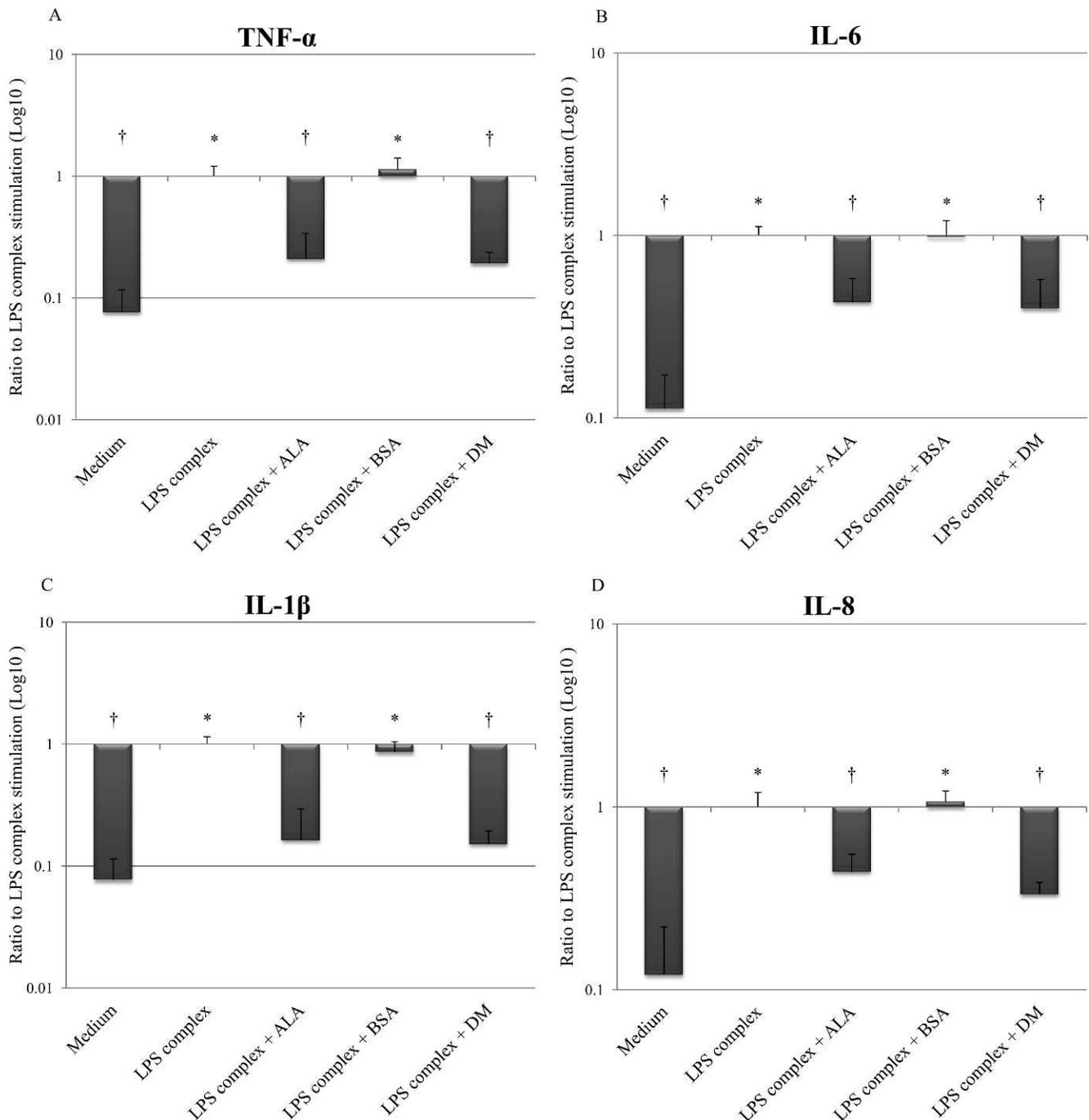


FIGURE 5. ALA's affects on LPS complex-induced cytokines mRNA expression levels in HCE cells, examined by real-time PCR (A–D). mRNA expression levels are presented on a logarithmic scale. Data are expressed as percentage (mean \pm SD) of the expression found after LPS complex stimulation, which was normalized to 1.0, and derived from four independent experiments ($n = 4$). Bars with the same sign were not significantly different ($P > 0.05$), while bars with different signs were significantly different ($P < 0.05$).

with ALA demonstrated a decrease in $\text{I}\kappa\text{B}\alpha$ mRNA expression, suggesting that the anti-inflammatory effects of ALA involve regulatory effects of the nuclear factor kappa B (NF- κ B) pathway.

This is the first in vitro study that demonstrates the anti-inflammatory effects of PUFAs at the molecular level on a variety of cytokines that are involved in ocular surface inflammation.

These findings have significant therapeutic implications, as several studies have previously demonstrated enhanced expression of pro-inflammatory mediators including cytokines,

chemokines, protein matrix metalloproteinases, and adhesion molecules in the tears and the ocular surface epithelia of patients with dry eye syndrome, as well as in many animal models.^{32,33} Increased production and activation of IL-1 β , IL-6, IL-8, TNF- α , and TGF- β have been found in dry eye patients, and specifically in Sjögren's syndrome in the conjunctival epithelium compared with healthy controls.¹

The inflammation inducers used in this study were LPS and poly I:C. LPS is a component of the outer membranes of gram negative bacteria, while poly I:C is a molecular stimulant which

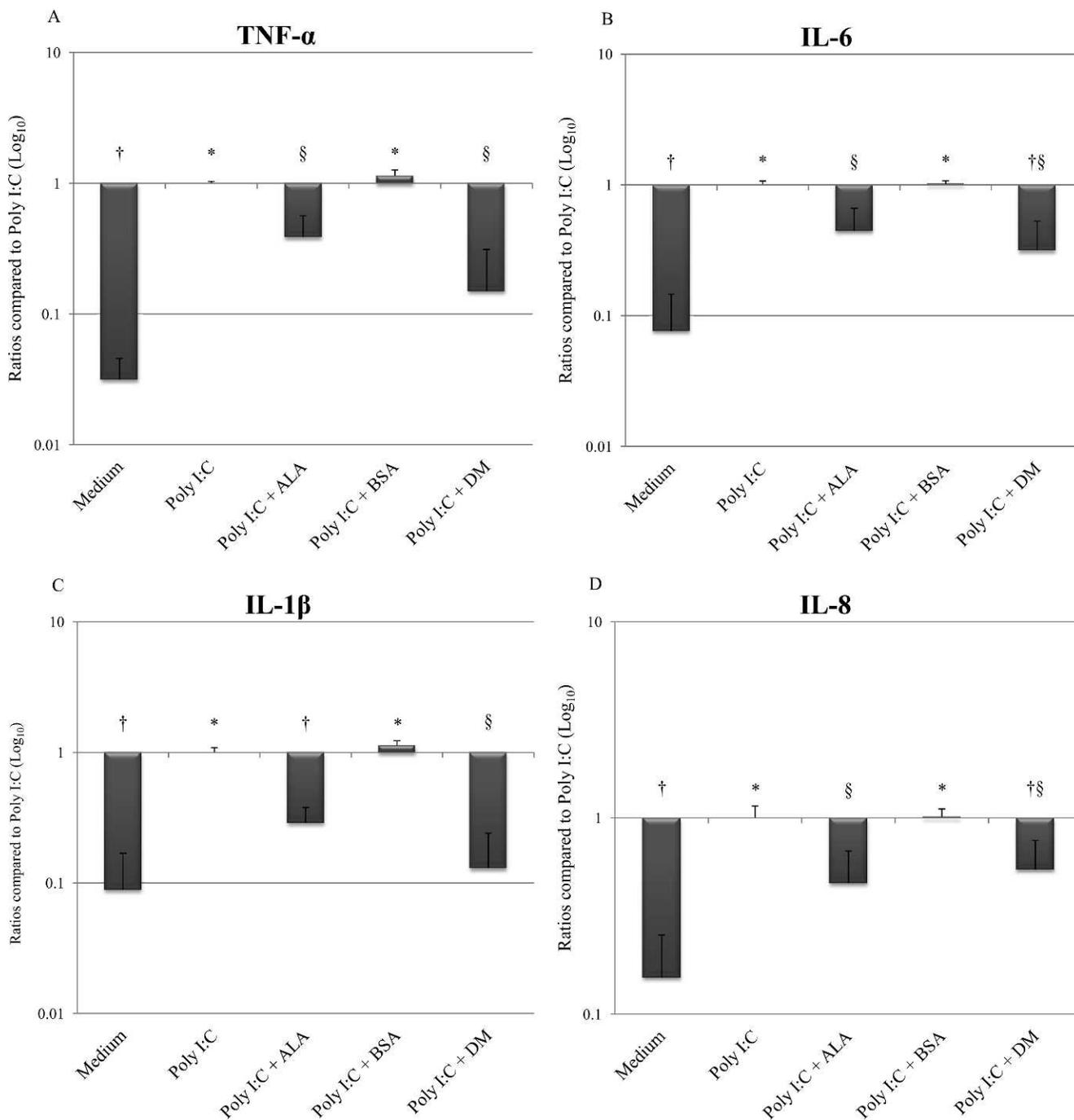


FIGURE 6. ALA's affects on poly I:C-induced cytokines and chemokine mRNA expression levels in HCE cells, examined by real-time PCR (A–D). mRNA expression levels are presented on a logarithmic scale. Data are expressed as percentage (mean ± SD) of the expression found after poly I:C stimulation, which was normalized to 1.0, and derived from four independent experiments (*n* = 4). Bars with the same sign were not significantly different (*P* > 0.05), while bars with different signs were significantly different (*P* < 0.05).

mimics viral infection. Both molecules induce inflammation by stimulating host innate immune system response.^{23,34} LPS binds to intramembranal complex of CD-14 and Toll-like receptors, and induces the release of multiple pro-inflammatory cytokines, including IL-6, IL-8, and TNF- α and an activation of NF- κ B in various cells throughout the body,³⁴ including HCE and fibroblasts.^{35,36} Poly I:C works by triggering of TLR-3, which results in increased production of pro-inflammatory cytokines, including IL-6 and IL-8, and activation of NF-kappa B in HCE cells.²³ Because of their potent effects on the expression and secretion of a large

number of inflammatory mediators, LPS and poly I:C, respectively, were chosen as inflammation inducers in this study.^{23,34–36}

The authors showed that both GLA and LA have a weaker anti-inflammatory effect, compared with ALA, on the inflammatory cytokines of the ocular surface. This finding is in contrast to studies in dry eye patients that had found ocular anti-inflammatory effects of both of these agents when taken systemically,⁹ but agrees with a recent animal model study where topical treatment in dry eye syndrome with either GLA or LA had shown no anti-inflammatory effect.¹⁶

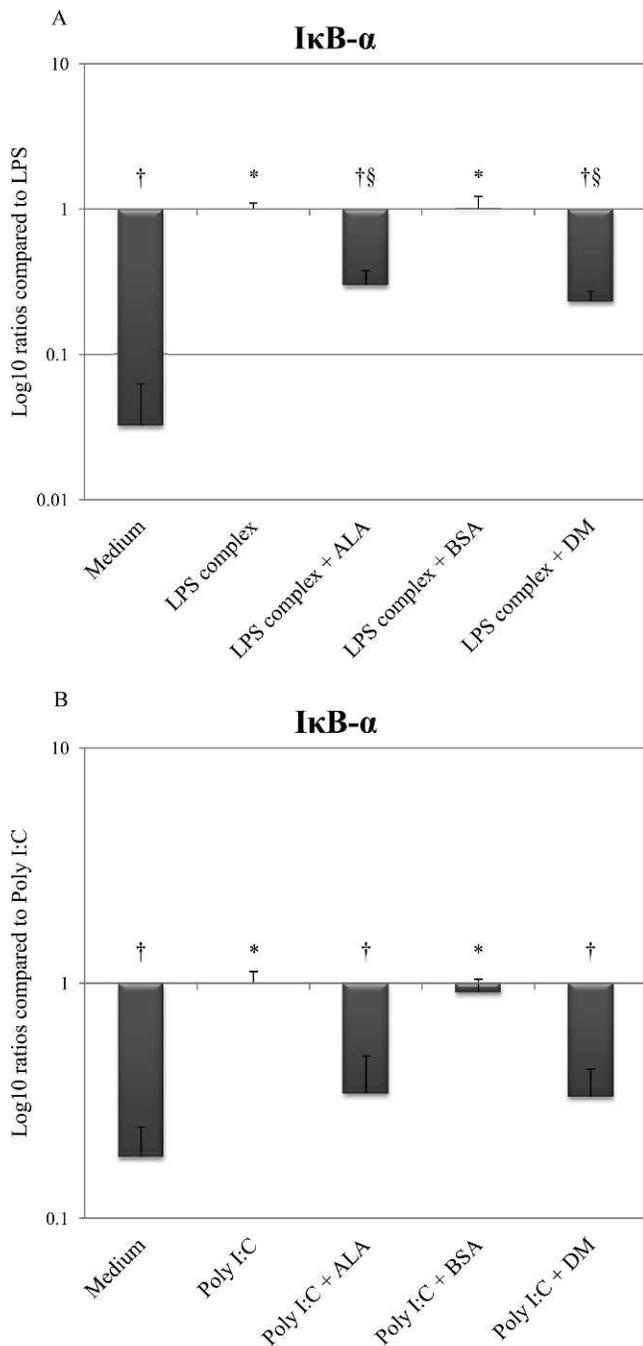


FIGURE 7. ALA's affects on LPS complex (A) and poly I:C-induced (B) IκB α mRNA expression levels in HCE cells. HCE cells were stimulated in SFM with LPS complex or poly I:C (25 μ g/mL) for 12 hours after cells pre-incubated for 2 hours with 200 μ M ALA. mRNA expression levels of IκB α were examined by real-time PCR. mRNA expression levels are presented in a logarithmic scale, data are expressed as percentage (mean \pm SD) of the LPS complex or poly I:C stimulus, which was normalized to 1.0 from three independent experiments ($n = 3$). Bars with the same sign were not significantly different ($P > 0.05$), while bars with different signs were significantly different ($P < 0.05$).

Several clinical trials have demonstrated significant associations between the oral intake of various PUFAs and a systemic anti-inflammatory effect. In these studies, oral intake of the omega-3 fatty acids, including ALA, Docosahexaenoic acid

(DHA), and Eicosapentaenoic acid (EPA) from flaxseed oil or fish oil, respectively, suppressed the systemic production of pro-inflammatory cytokines and eicosanoids, such as TNF- α ,³⁷ IL-1 β ,^{37,38} thromboxane B2, and prostaglandin E2.³⁸

Previous studies have demonstrated beneficial effects of oral treatment with n-3 PUFA on dry eye syndrome. One study found clinical improvement in dry eye symptoms and tear break up time in response to oral n-3 PUFA administration.⁸ Another study found that a high n-3 PUFA intake is associated with decreased risk of developing dry eye syndrome.³⁹ Only minor side effects were described with the systemic intake of n-3 PUFAs, mainly gastrointestinal distress.⁴⁰

Only one animal study, so far, had evaluated the direct topical effects of ALA on the ocular surface.¹⁶ Rashid et al.¹⁶ evaluated whether topical ALA treatment in a murine dry eye model would decrease inflammation and improve Dry eye disease (DED). They showed significant improvement in fluorescein staining, decreased corneal IL-1 α and TNF- α expression, and decreased conjunctival TNF- α expression following topical ALA application. In comparison, either LA, or a combination of LA and ALA, demonstrated lesser anti-inflammatory effects. In addition, topical ALA resulted in a decrease in the leukocyte infiltration, which was demonstrated in the dry eye animal model.¹⁶

Taken together, the clinical data from studies on the systemic effects of orally administered n-3 PUFA, with the recent *in vivo* murine study, demonstrate significant systemic and local anti-inflammatory effects. However, none of these studies have investigated the molecular mechanisms that are responsible for these effects.

The authors' results show that ALA has a significantly stronger inhibition of the inflammatory mediators compared with GLA or LA. The n-3 and n-6 PUFA pathways competitively utilize the same enzymes.⁴¹ Increased consumption of n-3 PUFAs, such as ALA and its products, EPA and DHA, result in increased proportions of those fatty acids in the cell membranes, partly at the expense of the n-6 PUFA arachidonic acid.^{37,42-44} The increased n-3 PUFA proportion in the cell membrane creates increased production of eicosanoids with a more anti-inflammatory profile, such as Leukotriene B5⁴⁴ and PGE3.⁴⁵ In addition, the decreased arachidonic acid proportion in the cell membrane creates decreased production of pro-inflammatory eicosanoids, such as prostaglandin E2,^{37,42} thromboxane B2,⁴² and leukotriene B4 (LTB4)^{43,44} by inflammatory cells.

The gene expression of cytokines is known to be regulated by transcription factors such as nuclear transcription factor κ B (NF- κ B). The authors investigated whether the inhibitory action of ALA on the gene expression of cytokines can be induced through inactivation of NF- κ B.

IκB α is able to regulate rapid but transient induction of NF- κ B activity because of the participation of IκB α in an autoregulatory feedback loop; NF- κ B activation causes the upregulation of transcription of IκB α , which serves to shut off the signal.⁴⁶ As a result, IκB α can oppose the transient effect of inducing agents on NF- κ B activation. The continuing presence of certain inducing agents (i.e., LPS), however, causes NF- κ B to be maintained in the nucleus despite the upregulation of IκB α transcription.⁴⁷ Thus, in continuously stimulated cells, the I- κ B α mRNA level can reflect NF- κ B activation and nuclear translocation.

The authors' study found that continuous stimulation of cells with LPS and poly I:C caused increased I- κ B α mRNA expression in HCE cells. This finding is consistent with previous reports, which demonstrate that continuous induction of NF- κ B activation and nuclear translocation result in increased I- κ B α mRNA expression.⁴⁷

The authors' study shows that ALA inhibited the LPS and poly I:C-induction, and as a result decreased the I κ B α mRNA expression in HCE cells, suggesting decreased NF- κ B activity. This finding is in line with other in vitro studies, which showed that n-3 PUFA inhibited the LPS-induced NF-kappa B activation and subsequent TNF- α expression by decreased I κ B phosphorylation and degradation.⁴⁸ In addition, an in vivo study showed that n-3 PUFA inhibited NF-kappa B activation in an animal inflammation model.⁴⁹

In summary, the authors' study demonstrates several significant direct effects of ALA in reducing inflammation of the ocular surface in vitro. These findings may have clinical relevance to the treatment of dry eye disease, as inflammation plays a central role in its pathogenesis. Topical n-3 PUFA treatment for ocular surface inflammation associated with dry eye syndrome, or with contact lens intolerance, may enable a widely available, inexpensive, and efficient therapy, with less undesired side effects, compared with systemic PUFA administration. Thus, topical ALA may be more beneficial in reducing inflammation associated with dry eye disease, compared with oral PUFA administration. Moreover, topical ALA may be more beneficial than other current anti-inflammatory agents for ocular surface inflammation, which are limited by their undesired long term side effects.

Further clinical investigations are needed in order to assess the efficacy and safety of topical ALA treatment for ocular inflammation and DED in human subjects.

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