

Dietary α -linolenic acid diminishes experimental atherogenesis and restricts T cell-driven inflammation

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Aims

Epidemiological studies report an inverse association between plant-derived dietary α -linolenic acid (ALA) and cardiovascular events. However, little is known about the mechanism of this protection. We assessed the cellular and molecular mechanisms of dietary ALA (flaxseed) on atherosclerosis in a mouse model.

Methods and results

Eight-week-old male apolipoprotein E knockout (*ApoE*^{-/-}) mice were fed a 0.21% (w/w) cholesterol diet for 16 weeks containing either a high ALA [7.3% (w/w); *n* = 10] or low ALA content [0.03% (w/w); *n* = 10]. Bioavailability, chain elongation, and fatty acid metabolism were measured by gas chromatography of tissue lysates and urine. Plaques were assessed using immunohistochemistry. T cell proliferation was investigated in primary murine CD3-positive lymphocytes. T cell differentiation and activation was assessed by expression analyses of interferon- γ , interleukin-4, and tumour necrosis factor α (TNF α) using quantitative PCR and ELISA. Dietary ALA increased aortic tissue levels of ALA as well as of the *n*-3 long chain fatty acids (LC *n*-3 FA) eicosapentaenoic acid, docosapentaenoic acid, and docosahexaenoic acid. The high ALA diet reduced plaque area by 50% and decreased plaque T cell content as well as expression of vascular cell adhesion molecule-1 and TNF α . Both dietary ALA and direct ALA exposure restricted T cell proliferation, differentiation, and inflammatory activity. Dietary ALA shifted prostaglandin and isoprostane formation towards 3-series compounds, potentially contributing to the atheroprotective effects of ALA.

Conclusion

Dietary ALA diminishes experimental atherogenesis and restricts T cell-driven inflammation, thus providing the proof-of-principle that plant-derived ALA may provide a valuable alternative to marine LC *n*-3 FA.

Keywords

α -Linolenic acid • Atherosclerosis • Inflammation • Polyunsaturated fatty acids

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Introduction

Atherosclerosis is a chronic inflammatory disease of the vasculature, which eventually leads to myocardial infarction or stroke.¹ The majority of acute myocardial infarctions can be attributed to modifiable cardiovascular risk factors such as exercise and diet.²

Epidemiological studies have shown an inverse correlation between consumption of dietary long chain *n*-3 fatty acids (LC *n*-3 FA) and cardiovascular events.³⁻⁵ Increased intake of the marine-derived eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA) exert anti-arrhythmic,⁶ lipid-lowering,⁷ antihypertensive, and potentially anti-thrombotic effects.^{8,9} These effects may result in part from a modulation of immunological functions. Indeed, *in vitro* EPA inhibits cytokine-induced cell surface expression of MHC II, as well as inflammatory cytokine expression itself of antigen-presenting cells.^{10,11} Moreover, dietary fish oil decreases natural killer cell and cytotoxic T cell activity decreasing the cell-mediated immune response.¹⁰ These findings set the rationale for international health organizations to adapt nutritional guidelines in favour of an increased intake of marine-derived LC *n*-3 FA.¹² However, the availability of *n*-3 FA from marine origin remains restricted in many countries due to expensive supply, cultural preference, unfavourable geography, and the depletion of menhaden, a marine forage fish (Clupeidae) at the bottom of the food chain capable to generate large quantities of *n*-3 FA from algae.¹³ Therefore, α -linolenic acid (ALA), a plant-derived *n*-3 FA, may provide a valuable cardioprotective alternative to marine *n*-3 FA.

α -Linolenic acid is an essential intermediate (as opposed to long-chain *n*-3 fatty acid, found in high concentrations in vegetable oils such as flaxseed oil, where it accounts for over 50% of the total fatty acid content.¹⁴ Being a precursor of LC *n*-3 FA, ALA is partially converted into EPA and DPA in the body.¹⁵ Data about the efficiency of the conversion of ALA into its longer chain metabolite EPA remain controversial, ranging from 0.05 to 8%.¹⁶⁻¹⁸ Recent epidemiological studies report an inverse association between dietary ALA and sudden cardiac death,⁶ acute myocardial infarction,¹³ and atherosclerotic plaque calcification.¹⁹ Moreover, *in vitro* studies^{20,21} allow some insight into the beneficial effects of marine-derived *n*-3 FA. However, the mechanisms responsible for the potentially protective phenomena upon supplementation of plant-derived *n*-3 FA remain insufficiently understood.

Given the growing epidemiological evidence suggesting ALA as an effective and accessible cardioprotective nutritional supplement, this study aims to investigate the cellular and molecular mechanisms of dietary ALA in a mouse model of atherosclerosis.

Methods

Detailed information about the Methods section is available in Supplemental material online.

Animals and diet

Eight-week-old male C57/BL6 *ApoE*^{-/-} mice were fed a 0.21% (w/w) cholesterol diet for 16 weeks containing either a high ALA [7.3% (w/w), D06080702, Research Diets, USA; *n* = 10] or a low ALA proportion [0.03% (w/w), D06080701, Research Diets; *n* = 10]. All animal experiments were approved by the local Ethics Committee.

Blood analyses

Mice were fasted over night before blood was drawn. Lipids were analysed using the reagents TR13421, TR22421 (Thermo Electron Clinical Chemistry and Automation Systems, USA) and 994-75409 (Wako Chemicals GmbH, Germany). The lipid distribution in plasma lipoprotein fractions was assessed by chromatography gel filtration.

Tissue processing

For *en face* analyses, thoraco-abdominal aortae were excised and opened longitudinally. Plaques were visualized by fat staining. For histological examination, cryosections were obtained. For biochemical analyses, tissue was shock-frozen in liquid nitrogen.

Plaque quantification

Atherosclerotic plaques were analysed *en face* in thoraco-abdominal aortae as described.²²

Complementary analyses of plaque size and composition were performed in serial longitudinal cryosections of the aortic arch as described.²³

Endothelial function and oxidative stress

Aortic rings were obtained and dose-response curves generated using an isometric force transducer (MultiMyograph, DMT, Denmark) as described.²⁴

Key reactive oxygen species generating and scavenging enzymes were assessed in aortic lysates by quantitative PCR (qPCR) and western blot analyses as described below. Total and small molecule antioxidant capacity of plasma was measured using the total antioxidant capacity kit (Abcam) according to the manufacturer's instructions. The direct detection of aortic reactive oxygen species was performed using electron spin resonance spectroscopy as described previously.²⁵ Cytoplasmic reactive oxygen species in aortae were assessed using dihydroethyidine (Sigma, USA) stainings.

Immunohistochemistry and immunofluorescence

Cryosections were blocked and stained using the following antibodies: rat anti-mouse CD68, CD3, and vascular cell adhesion molecule 1 (VCAM-1; Serotec, UK), and goat anti-mouse tumour necrosis factor α (TNF α ; Santa Cruz, USA).

Lipid profiles

Tissue lipid profiles were analysed using gas-liquid chromatography of fatty acid methyl esters²⁶ after fractionation of lipid classes by solid-phase extraction.

Urine analyses

Twenty-four-hour urines were collected using metabolic cages. Prostaglandins and isoprostanes (iPs) were extracted and quantified utilizing liquid chromatography/mass spectrometry/mass spectrometry analyses as described.²⁷⁻²⁹ Metabolite levels were corrected for urinary creatinine.

Cell culture

Splenocytes were harvested from *ApoE*^{-/-} mice and CD3- and CD11c-positive cells were magnetically separated. Purity was assessed by flow cytometry. CD3- and CD11c-positive lymphocytes were cultured in supplemented RPMI-1640.

Flow cytometry

Flow cytometry analyses were performed in a FACSCanto II using FACSDiva software (both BD, Heidelberg, Germany). Results were expressed in percentage positive cells or mean fluorescence intensity.

Functional *in vitro* assays

For proliferation assays CD3-positive lymphocytes were activated by anti-CD3 and anti-CD28 antibody presentation (BD, USA) before treatment with ALA (Cayman Chemical, USA) or vehicle (ethanol 0.1%, Sigma). Cells were incubated for 78 h including incorporation of ^3H -thymidine during the final 18 h. Thymidine incorporation was measured using liquid scintillation. α -Linolenic acid-mediated cytotoxicity was assessed by colorimetric quantification of lactate dehydrogenase release. Expression analyses in CD11c-positive cells (FACS, qPCR, ELISA) were performed in either stimulated or unstimulated cells.

A capture ELISA was used to quantify cytokine release in the supernatant. For analysis of IL-4, IL-6, p40, IFN γ , and TNF α , BD OptEIA™ ELISA Sets (BD, USA) were used.

RNA analyses

RNA isolation and reverse transcription were performed according to standard protocols. Quantitative PCR was carried out in a Stratagene Mx 3000 P™ machine (Stratagene, USA) using the Stratagene MxPro sequence detection system and software. Expression was calculated using the $\Delta\Delta C_T$ method. Relative gene expression was normalized to murine 12S rRNA expression as an internal control.

Western blot analyses

Western blot analyses of aortic lysates were performed according to standard protocols.

Statistics

Statistical analysis was performed using an unpaired two-tailed Student's *t*-test or one-way ANOVA with Dunnett's correction where appropriate. Significance was accepted at $P < 0.05$. Values are expressed as mean \pm SEM. Sample size was calculated with average values and standard deviations from previous experience (7.5 ± 2.1 and $21.2 \pm 12.6\%$). With an estimated effect size of 1.5, an α -error probability of 0.05, and a power of 0.80, the expected sample size was seven per group. Calculations were performed in GraphPad PRISM 5.01 (GraphPad Software Inc., USA) and G*Power 3.1 (Institute of Experimental Physiology, University of Dusseldorf, Germany).³⁰

Results

Dietary α -linolenic acid diminishes plaque formation

To determine the effects of a diet enriched with ALA on atherosclerotic plaques, male *ApoE*^{-/-} mice were exposed to a 0.21 % (w/w) cholesterol diet for 16 weeks containing either a high concentration of ALA [7.3 % (w/w); $n = 10$] or a low concentration of ALA [0.3 % (w/w); $n = 10$]. Dietary ALA was provided as flaxseed oil, which was compensated for with cocoa butter in the control diet (Table 1). Total fat, protein, and carbohydrate contents were kept constant. To mimic a 'western-type' diet, trans-fat content was 1.7 % (w/w) in both diets. Mice exposed to a high ALA diet revealed a 50% decrease ($P = 0.014$) in *en face* thoraco-abdominal aortic plaque formation (Figure 1A) and in plaques on serial longitudinal sections of the aortic arch

Table 1 Formulas of the high and the low ALA diet, respectively

	Low ALA		High ALA	
	% (w/w)	kcal%	% (w/w)	kcal%
Protein	20	17	20	17
Carbohydrate	50	43	50	43
Fat	21	40	21	40
kcal/g	4.68		4.68	
Ingredient	g	kcal	g	kcal
Casein	195	780	195	780
DL-Methionine	3	12	3	12
Corn starch	50	200	50	200
Maltodextrin 10	100	400	100	400
Sucrose	341	1364	341	1364
Cellulose, BVW200	50	0	50	0
Milk fat, anhydrous	0	0	0	0
Corn oil	10	90	10	90
Cocoa butter	137	1233	0	0
Flaxseed oil	0	0	137	1233
Primex shortening	63	567	63	567
Mineral mix S10001	35	0	35	0
Calcium carbonate	4	0	4	0
Vitamin mix V10001	10	40	10	40
Choline bitartrate	2	0	2	0
Cholesterol USP	2.1	0	2.1	0
Ethoxyquin	0.04	0	0.04	0
FD&C blue dye #1	0	0	0.05	0
FD&C yellow dye #5	0.05	0	0	0
Total	1002.14	4686	1002.14	4686
	% (w/w)	kcal%	% (w/w)	kcal%
C18:3 <i>n</i> -3, ALA	0.03	0.06	7.3	13.90
<i>n</i> -3 FA	0.08	0.15	7.15	13.62
<i>n</i> -6 FA	16.38	3.12	3.61	6.88
<i>n</i> -6/ <i>n</i> -3	20.73		0.51	
Trans-fat	1.7	6.16	1.7	6.16

The source of α -linolenic acid (C18:3 *n*-3, ALA) is flaxseed oil. The fat content in the low ALA diet is compensated for by cocoa butter. The calories/weight and fat including total cholesterol and trans-fat content of the two diets are identical.

($P = 0.016$) (Figure 1B). Surprisingly, no difference in endothelial function was observed between the two groups (see Supplementary material online, Figure S1A), which was paralleled by unchanged levels of aortic reactive oxygen species (see Supplementary material online, Figures S1B and S2) and total antioxidant capacity in plasma (see Supplementary material online, Figure S1C). Interestingly, we observed a slight increase in small molecule antioxidant capacity ($P = 0.0044$) in plasma from mice of the intervention group (see Supplementary material online, Figure S1C). Expression levels of endothelial nitric oxide synthase, p47/*phox*, NOX1 (p22/*phox*), NOX2 (gp91/*phox*) as well as of superoxide dismutase 1–3 in aortic lysates were unchanged (see Supplementary material online, Figure S3).

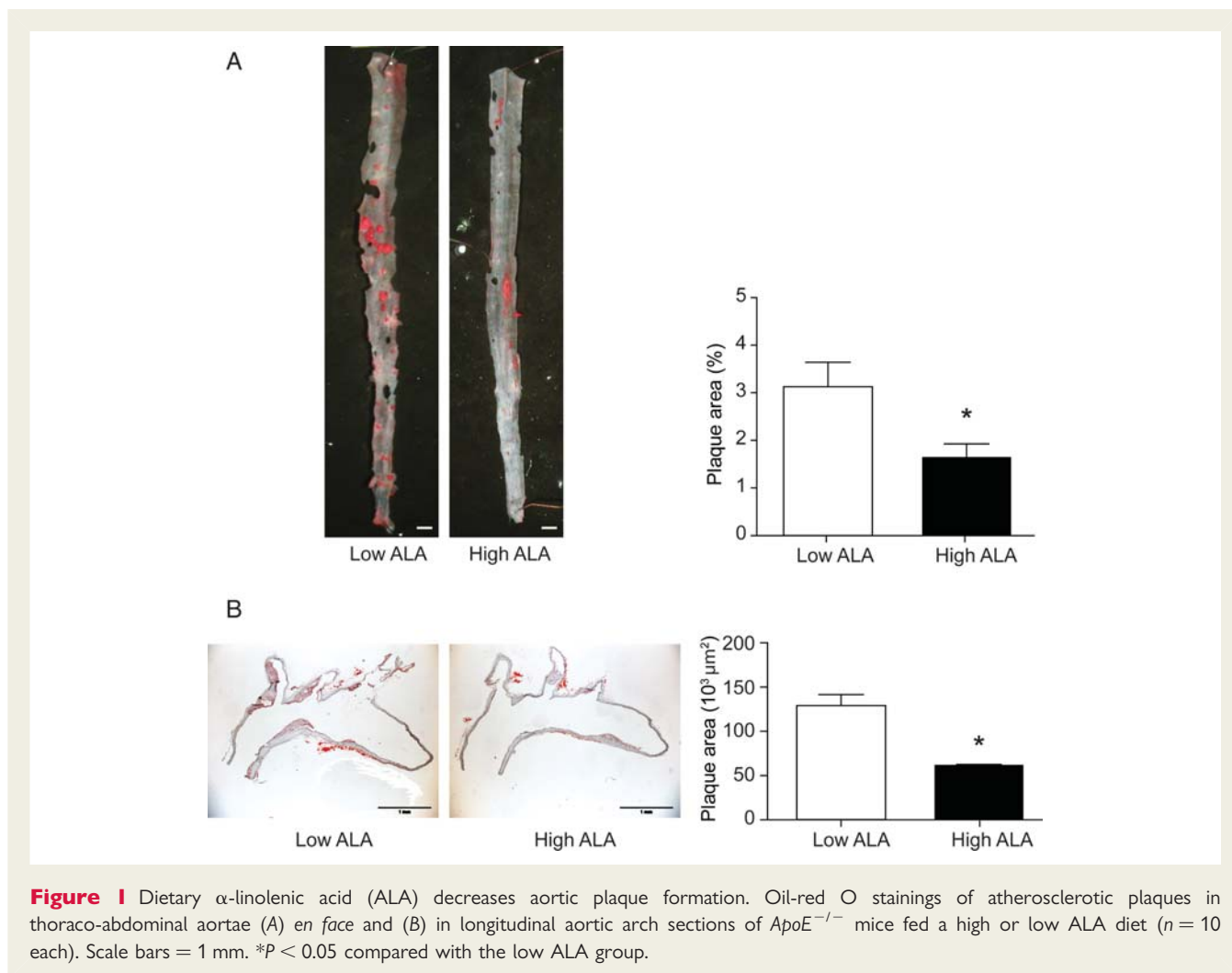


Figure 1 Dietary α -linolenic acid (ALA) decreases aortic plaque formation. Oil-red O stainings of atherosclerotic plaques in thoraco-abdominal aortae (A) *en face* and (B) in longitudinal aortic arch sections of $ApoE^{-/-}$ mice fed a high or low ALA diet ($n = 10$ each). Scale bars = 1 mm. * $P < 0.05$ compared with the low ALA group.

Dietary α -linolenic acid restricts plaque T cell content, tumour necrosis factor α , and vascular cell adhesion molecule 1 expression

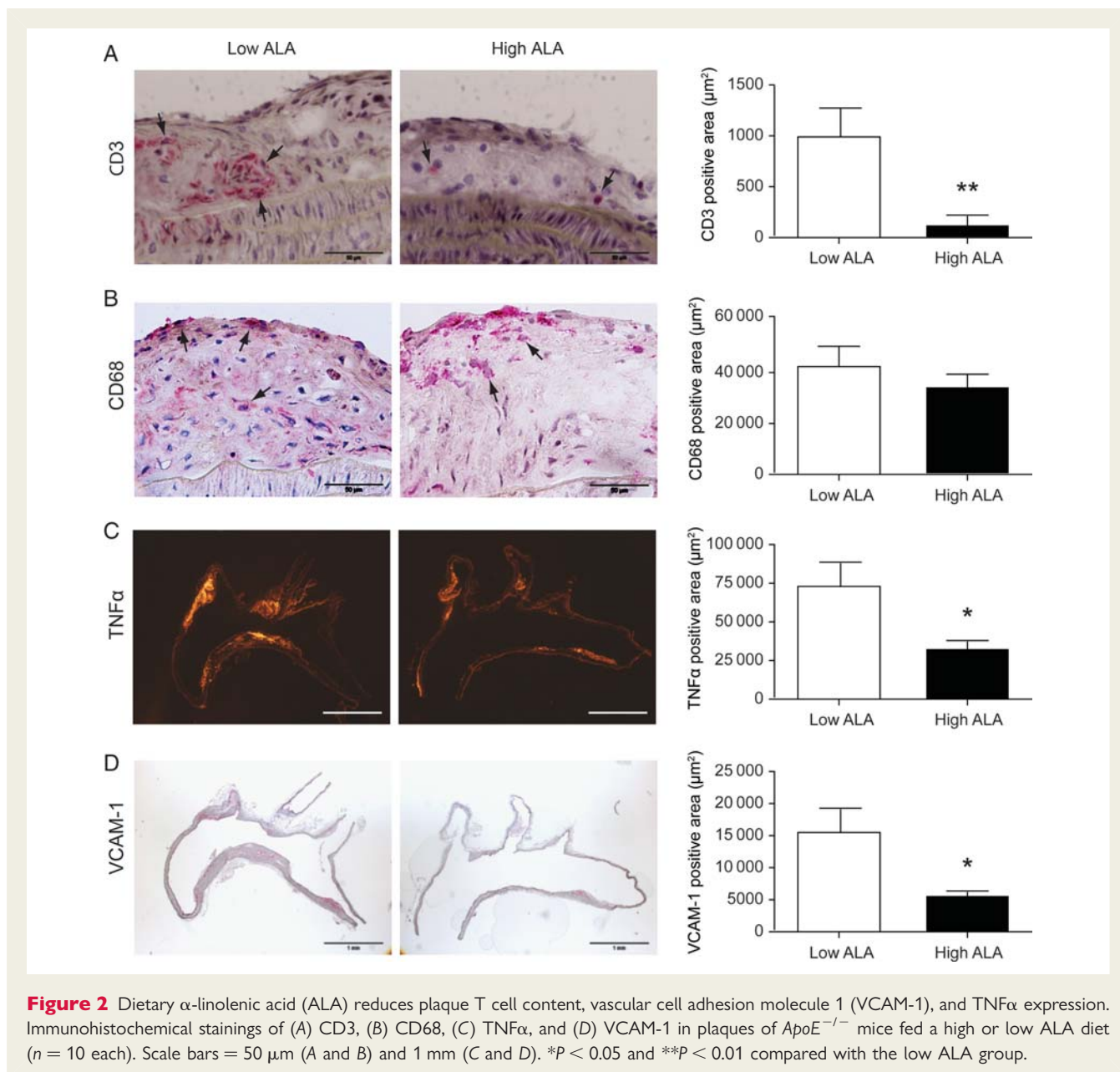
To investigate the effects of dietary ALA on plaque inflammation, we performed immunohistochemical stainings for T cells (CD3) and macrophages (CD68) (Figure 1A and B). We observed an 85% decrease ($P = 0.0065$) in T cell content in the intervention group compared with the control group. The CD68-positive area remained unchanged, suggesting that macrophage content did not differ between the groups. Blood counts did not reveal any differences in circulating monocytes or lymphocytes (see Supplementary material online, Table S1C). Interestingly, dietary ALA reduced TNF α and VCAM-1 expression in plaques ($P = 0.017$ and $P = 0.016$) (Figure 2C and D), consistent with attenuation of endothelial activation, successive leucocyte adhesion, and subsequent intimal infiltration.

α -linolenic acid decreases T cell differentiation, activation, and proliferation

Given the marked decrease in CD3-positive cells in the plaques of the intervention group, we investigated the specific effects of ALA

on T cells *in vivo* and *in vitro*. Therefore, CD3-positive lymphocytes from the two mouse groups were isolated from spleens and interferon- γ (IFN γ) as well as interleukin-4 (IL-4) expression was assessed by qPCR, as a surrogate for Th1 and Th2 differentiation, respectively (Figure 3A and B). T cells of animals fed a high ALA diet expressed less IL-4 ($P = 0.011$), suggesting reduced differentiation towards Th2 cells, whereas IFN γ expression remained unchanged. Dietary ALA reduced TNF α expression in T cells by 42% ($P = 0.024$) (Figure 3C). TNF α expression was also reduced in spleens of animals exposed to a high ALA diet ($P = 0.0046$) without changing the amount of CD3-positive cells (Figure 3D and E). TNF α expression colocalized with CD3-positive lymphocytes (Figure 3F).

To substantiate these observations, we performed a dose-response curve of ALA in cultured T cells using primary CD3-positive cells isolated from $ApoE^{-/-}$ mice fed a normal diet. α -linolenic acid doses were chosen to reflect plasma levels achieved upon dietary ALA intake.¹⁵ mRNA levels of IL-4, IFN γ , and TNF α dose-dependently decreased in response to ALA (Figure 4A). These findings were confirmed at the protein level using ELISAs specific for IL-4, IFN γ , and TNF α (Figure 4D–F) in cell culture supernatants.



Since the Th2 cytokine IL-4 is a potent mitogen, we tested the effects of ALA on T cell proliferation. α -linolenic acid administration dose-dependently decreased the proliferation of CD3-positive lymphocytes from *ApoE*^{-/-} mice (Figure 4C). We excluded cytotoxic effects of ALA treatment by measuring LDH release in cell culture supernatants (Figure 4C).

In order to address putative effects of ALA on the cross-talk between T cells and antigen-presenting cells, we determined the expression of co-stimulatory factors in T cells and dendritic cells isolated from *ApoE*^{-/-} mice. We investigated the contribution of the B7 family (B7.1 and 2, L-ICOS, and ICOS) and the TNF/TNF receptor family (CD40 and CD40L, as well as CD137) as well as the secretion of IL-6 and IL-12. In dendritic cells, we observed no regulation upon increasing concentrations of ALA. In T cells, however, increasing doses of ALA led to

decreased expression of ICOS, CD40L, and CD137 as well as IL12R β 2 at the mRNA level. At the protein level, though, no difference was detected (see Supplementary material online, Figures S3 and S4). These data suggest that co-stimulatory pathways do not relevantly contribute to the observed phenotype.

Dietary flaxseed enhances tissue levels of α -linolenic acid and its *n*-3 long chain metabolites

To assess the efficiency of dietary ALA incorporation into target tissues, we quantified ALA in tissue lysates (Table 2). α -Linolenic acid tissue levels within the intervention group were markedly increased ranging from an over 60-fold increase ($P = 0.024 \times 10^{-18}$) in

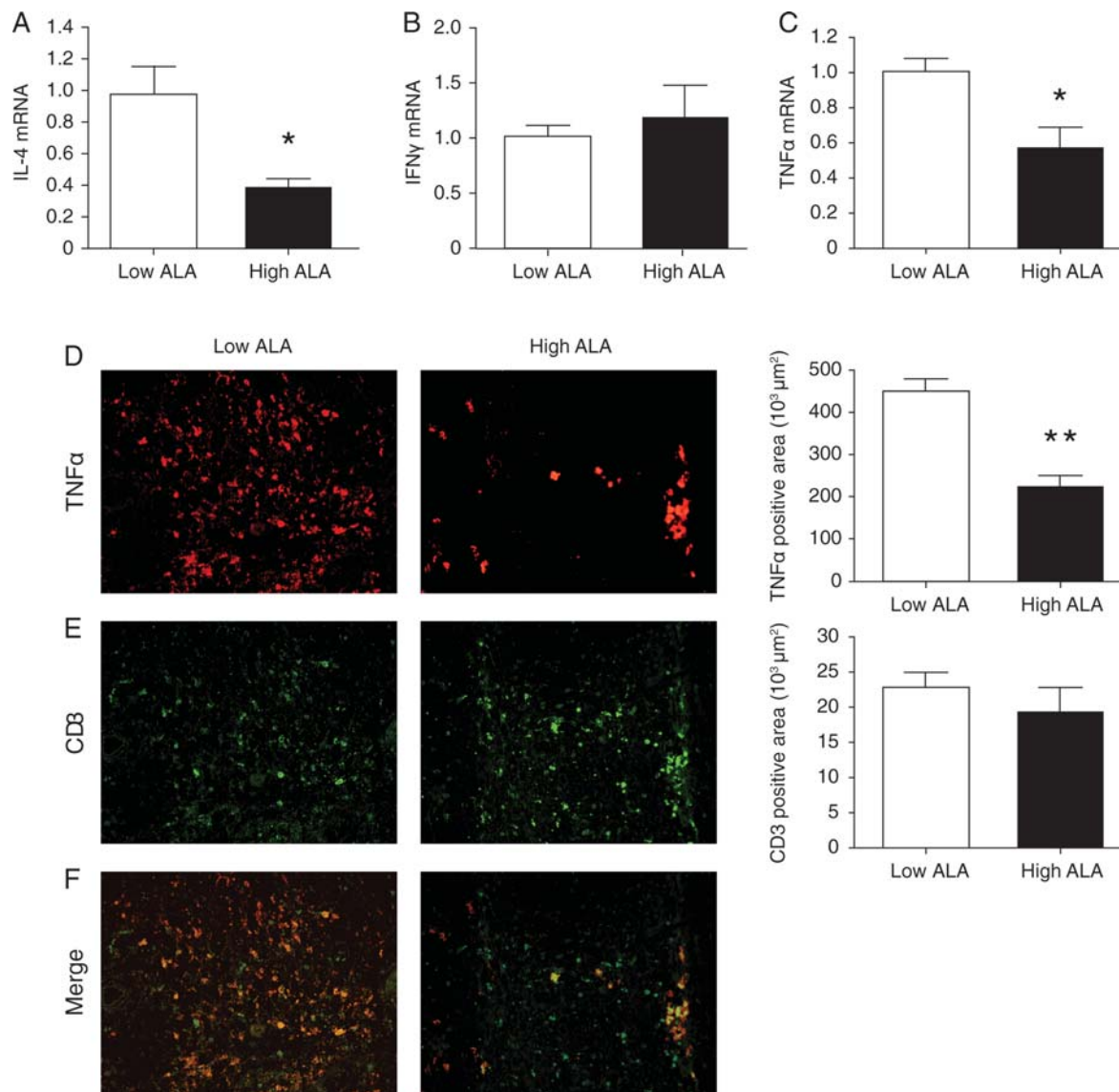


Figure 3 Dietary α -linolenic acid (ALA) decreases differentiation and inflammatory activity of primary murine CD3-positive lymphocytes. (A–C) CD3-positive cells were isolated from spleens of *ApoE*^{−/−} mice fed a high or low ALA diet ($n = 5$ each). T cells were stimulated and cultured for 48 h before harvesting. Gene expression was quantified by quantitative PCR. (D–F) Spleens of *ApoE*^{−/−} mice fed a high or low ALA diet ($n = 5$ each) were harvested and stained for TNF α (red) and CD3 (green). $\times 200$. * $P < 0.05$ and ** $P < 0.01$ compared with the low ALA group.

perirenal white adipose tissue (WAT) to an over 250-fold increase ($P = 0.03 \times 10^{-8}$) in red blood cell (RBC) membranes.

Furthermore, we addressed the conversion of ALA to $n-3$ FAs by measuring EPA, DPA, and docosahexaenoic acid (DHA) (Table 2). In animals receiving an ALA-enriched diet, tissue levels of EPA were increased ranging from a 2.3-fold increase in perirenal ($P = 0.019 \times 10^{-4}$) and subcutaneous WAT ($P = 0.097 \times 10^{-6}$) to a 56-fold increase in RBC membranes (0.012×10^{-9}). Docosapentaenoic acid levels within the intervention group were also elevated between 1.8-fold in aortae and 56-fold in livers ($P = 0.029 \times 10^{-9}$). The same phenomenon was observed with regard to DHA being increased after dietary ALA by 1.8-fold in

RBC membranes ($P = 0.000019$) and 4.6-fold in livers ($P = 0.0016$). These findings indicate that oral flaxseed is sufficient to increase ALA tissue levels and that tissue conversion from ALA to LC $n-3$ FA (EPA, DPA, and DHA) is taking place.

Since $n-3$ and $n-6$ FA metabolisms are interdependent and partially rely on the same mechanisms, we also quantified $n-6$ FA. Arachidonic acid (AA), the $n-6$ equivalent to EPA, was decreased in tissues of animals fed an ALA-enriched diet, ranging from 2.1-fold less in livers ($P = 0.0041$) to 4.8-fold less in aortae. These effects are reflected in the EPA/AA ratio and the total $n-6$ /total $n-3$ FA ratio, which were both reduced in all analysed tissues.

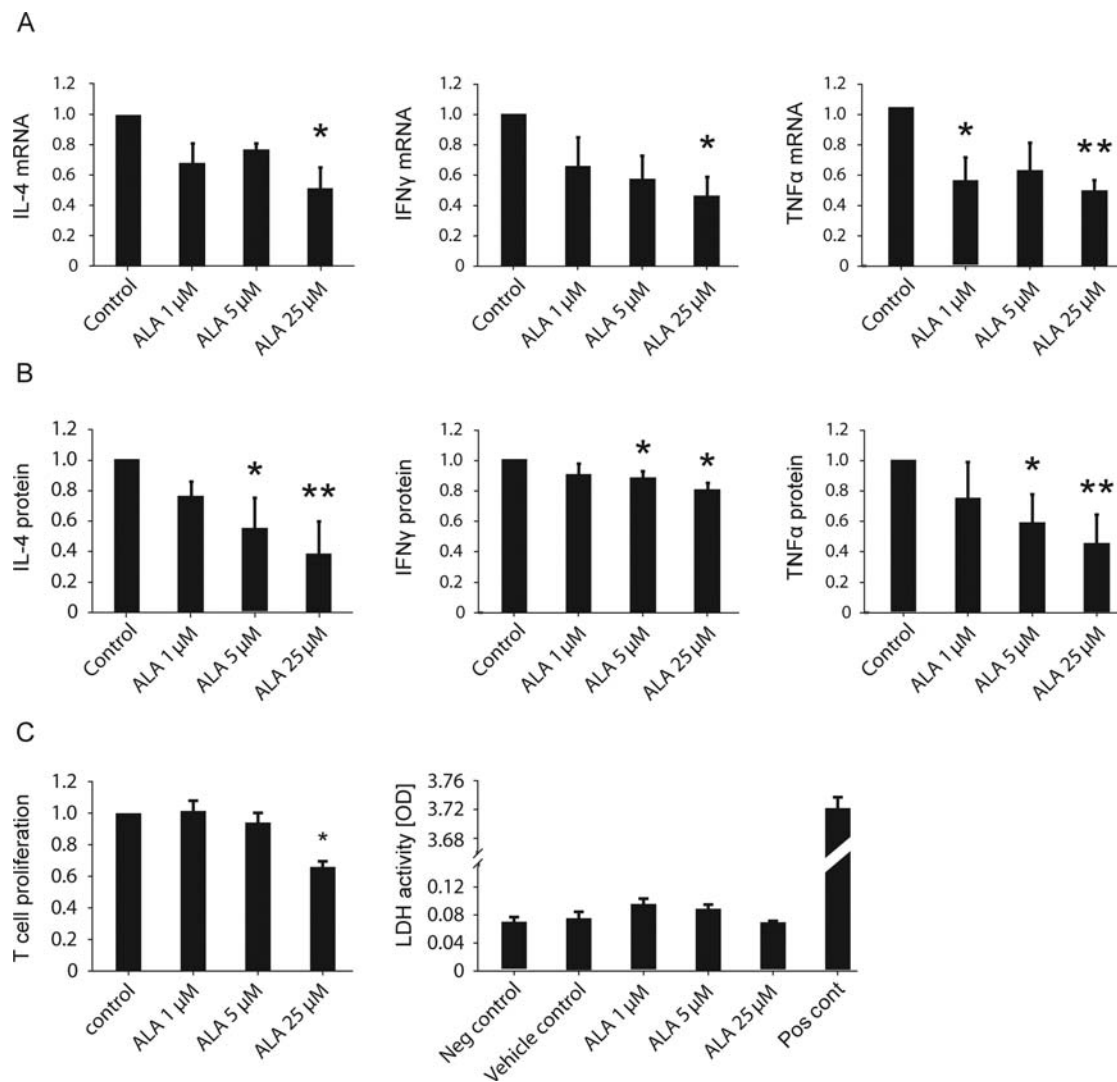


Figure 4 α -Linolenic acid (ALA) decreases differentiation, inflammatory activity, and proliferation of T cells in a dose-dependent manner. CD3-positive cells were isolated from $ApoE^{-/-}$ mice on a normal diet. (A and B) Cells were stimulated for 24 h before ALA was applied in the doses indicated (1, 5, and 25 μ M). After additional 24 h, supernatant and RNA were obtained and analysed by quantitative PCR (A) and ELISA (B), respectively. (C) CD3-positive lymphocytes were stimulated and ALA was applied. Proliferation was assessed by 3H -thymidine incorporation. Cytotoxicity was analysed by LDH quantification in cell culture supernatants. * $P < 0.05$ and ** $P < 0.01$ compared with control conditions.

Dietary α -linolenic acid induces a shift to 3-series prostanoids and isoprostanes

To assess the effects of dietary ALA on FA metabolism and subsequent eicosanoid generation, we quantified urinary metabolites of both $n-6$ - and $n-3$ -derived eicosanoids (Figure 5). Major urinary metabolites of the 2-series prostaglandins E (PGEM), D (PGDM), I (PGIM), and thromboxane B_2 metabolite³¹ were not altered by the high ALA diet. These compounds mirror systemic biosynthesis of the respective AA-derived 2-series prostanoids. In contrast, a significant increase in the corresponding urinary thromboxane A_3 metabolite (Tx B_3 M) was detected. This reflects a shift towards 3-series prostanoid formation. Analyses of iPs, free radical-catalysed prostanoid isomers, revealed a similar

striking shift towards 3-series products. These changes are supported by a marked decrease in the urinary Tx B_2 M/Tx B_3 M and iP-2/iP-3 ratios in animals fed the ALA-rich diet (Figure 5B and D).

Discussion

α -Linolenic acid is a plant-derived intermediate chain $n-3$ fatty acid and precursor of the marine-derived LC $n-3$ FA EPA, DPA, and DHA. Epidemiological studies report an inverse association between dietary ALA and cardiovascular events.^{6,13,19} However, little is known about the mechanisms of a putative atheroprotection.

Table 2 Oral flaxseed intake increases tissue levels of α -linolenic acid and long chain $n-3$ fatty acids

% (w/w) FA	Low ALA	High ALA	Low ALA	High ALA	Low ALA	High ALA
	Red blood cells		Liver		Aorta	
ALA	0.01 \pm 0.002	2.54 \pm 0.1**	0.14 \pm 0.03	15.9 \pm 1.2**	0.2	18.5
C20 5 $n-3$ (EPA)	0.08 \pm 0.005	4.5 \pm 0.13**	0.1 \pm 0.01	2.9 \pm 0.6**	n.d.	0.7
C22 5 $n-3$ (DPA)	0.31 \pm 0.01	3.9 \pm 0.13**	0.01 \pm 0.01	1.5 \pm 0.1*	0.28	0.5
C22 6 $n-3$ (DHA)	2.5 \pm 0.04	4.5 \pm 0.26**	0.7 \pm 0.07	3.2 \pm 0.7**	0.08	0.2
AA	14.1 \pm 0.2	3.0 \pm 0.06	2.7 \pm 0.3	1.3 \pm 0.3	2.4	0.5
AA/EPA	177.7 \pm 11.7	0.7 \pm 0.03**	25.1 \pm 2.3	0.4 \pm 0.03**	n.d.	0.7
$n-6$	28.0 \pm 0.3	19.0 \pm 0.9**	9.6 \pm 0.7	19.5 \pm 1.0**	11.3	18.0
$n-3$	3.0 \pm 0.04	16.0 \pm 1.2**	1.0 \pm 0.08	25.2 \pm 0.8**	0.5	20.2
$n-6/n-3$	9.3 \pm 0.2	1.2 \pm 0.1**	9.4 \pm 0.4	0.8 \pm 0.03**	20.7	0.9
	Periadvent. WAT		Perirenal WAT		Subcut. WAT	
ALA	0.22 \pm 0.01	17.0 \pm 0.4**	0.3 \pm 0.022	18.6 \pm 0.6**	0.2 \pm 0.01	18.7 \pm 0.05**
C20 5 $n-3$ (EPA)	0.02 \pm 0.002	0.3 \pm 0.06**	0.03 \pm 0.002	0.07 \pm 0.004**	0.03 \pm 0.002	0.07 \pm 0.004**
C22 5 $n-3$ (DPA)	0.01 \pm 0.002	0.2 \pm 0.03**	0.01 \pm 0.003	0.09 \pm 0.01*	0.01 \pm 0.01	0.08 \pm 0.01**
C22 6 $n-3$ (DHA)	0.1 \pm 0.01	0.4 \pm 0.13**	0.02 \pm 0.01	0.07 \pm 0.01**	0.02 \pm 0.002	0.05 \pm 0.01**
AA	0.7 \pm 0.04	0.3 \pm 0.07**	0.13 \pm 0.01	0.04 \pm 0.005**	0.13 \pm 0.01	0.04 \pm 0.005**
AA/EPA	35.7 \pm 2.4	0.7 \pm 0.05**	4.2 \pm 0.38	0.4 \pm 0.09**	5.1 \pm 0.25	0.6 \pm 0.09**
$n-6$	8.8 \pm 0.3	18.4 \pm 0.9**	7.4 \pm 0.1	19.0 \pm 0.4**	7.6 \pm 0.1	18.7 \pm 0.4**
$n-3$	0.5 \pm 0.03	18.2 \pm 0.4**	0.3 \pm 0.07	19.0 \pm 0.6**	0.3 \pm 0.03	19.1 \pm 0.5**
$n-6/n-3$	23.6 \pm 1.0	1.0 \pm 0.04**	27.7 \pm 2.9	1.0 \pm 0.03**	29.1 \pm 1.7	0.98 \pm 0.04**

Red blood cells, liver, aorta, and white adipose tissue (WAT) of mice fed a high or low ALA diet were analysed by gas chromatography. All comparisons between the control and intervention groups revealed significant differences. Aortic tissue samples were pooled for analyses.

* $P < 0.01$.

** $P < 0.001$.

We demonstrate that dietary ALA diminishes plaque formation, plaque T cell infiltration, as well as TNF α and VCAM-1 expression using a mouse model of atherosclerosis. Dietary flaxseed increased tissue levels of ALA, EPA, DPA, and DHA. Conversely, it reduced AA in aortas and other tissues and increased urinary excretion of 3-series iPs and TxB₃M. Both dietary ALA and direct ALA treatment restricted T cell proliferation, differentiation, and inflammatory activity.

α -Linolenic acid was delivered in concentrations at the high and low end of an achievable human dietary consumption. Although in humans 3 g/day is sufficient to increase ALA plasma levels from 19.2 to 32.8 μ M, up to 40 g/d have been administered and well tolerated.¹⁵ When designing the diets, we focused on equal energy levels (fat, protein, and carbohydrate) in both diets. α -Linolenic acid was provided as flaxseed and compensated in the control diet using cocoa butter. Cocoa butter consists mainly of the saturated fatty acids (SFA) palmitic and stearic acid as well as the monounsaturated fatty acid oleic acid. *In vivo* and *in vitro* studies report minimal effects of dietary SFA on inflammatory cells.^{15,32,33}

Although the ALA content in the flaxseed-based diet was over 90-fold higher compared with the control diet, linoleic acid (LA), the $n-6$ equivalent of ALA, was also slightly increased in the intervention diet. This diet composition translates into profoundly increased tissue levels of ALA and total $n-3$ fatty acids compared with only mildly increased tissue levels of $n-6$ fatty acids, respectively. As the metabolism of $n-6$ and $n-3$ fatty acids is strongly interdependent, the dietary $n-6/n-3$ ratio confers more biological relevance than their

absolute values. This ratio positively correlates with the incidence of cardiovascular events.³⁴⁻³⁶ Consistent with these findings, all analysed tissues revealed a marked decrease in the $n-6/n-3$ ratio, paralleled by a 50% reduction in plaque area upon dietary ALA. In all tissues assessed, including aortic lysates, a marked increase in ALA was observed. Thus, the diets used in this study provided high and low ALA contents as natural food components, securing a high ALA bioavailability in energetically matched diets.

α -Linolenic acid supplementation decreased atherosclerotic plaque formation in *ApoE*^{-/-} mice, paralleled by a marked reduction in TNF α -, subsequent VCAM-1 expression, and inflammatory CD3-positive T cell infiltration. We observed no changes in macrophage content in *ApoE*^{-/-} mice, in contrast to another study performed in LDL receptor-deficient mice.³⁷ The decreased TNF α expression throughout the plaque observed upon dietary ALA could be attributed to T cells. Our *in vitro* studies showed a profound effect of ALA on T cell activation and proliferation corroborating previous data³² and a decelerated differentiation to the Th1 phenotype, thus conferring atheroprotective properties, such as contributing to reduced plaque destabilization as reported in patients with acute coronary syndromes.³⁸⁻⁴⁰

The observed reduction of T cell activity in response to ALA matches other studies reporting reduced leucocyte TNF α expression upon marine EPA or DHA administration.^{20,32} Without differences in plasma lymphocyte counts, the diminished T cell proliferation after ALA exposure may result from para- or autocrine

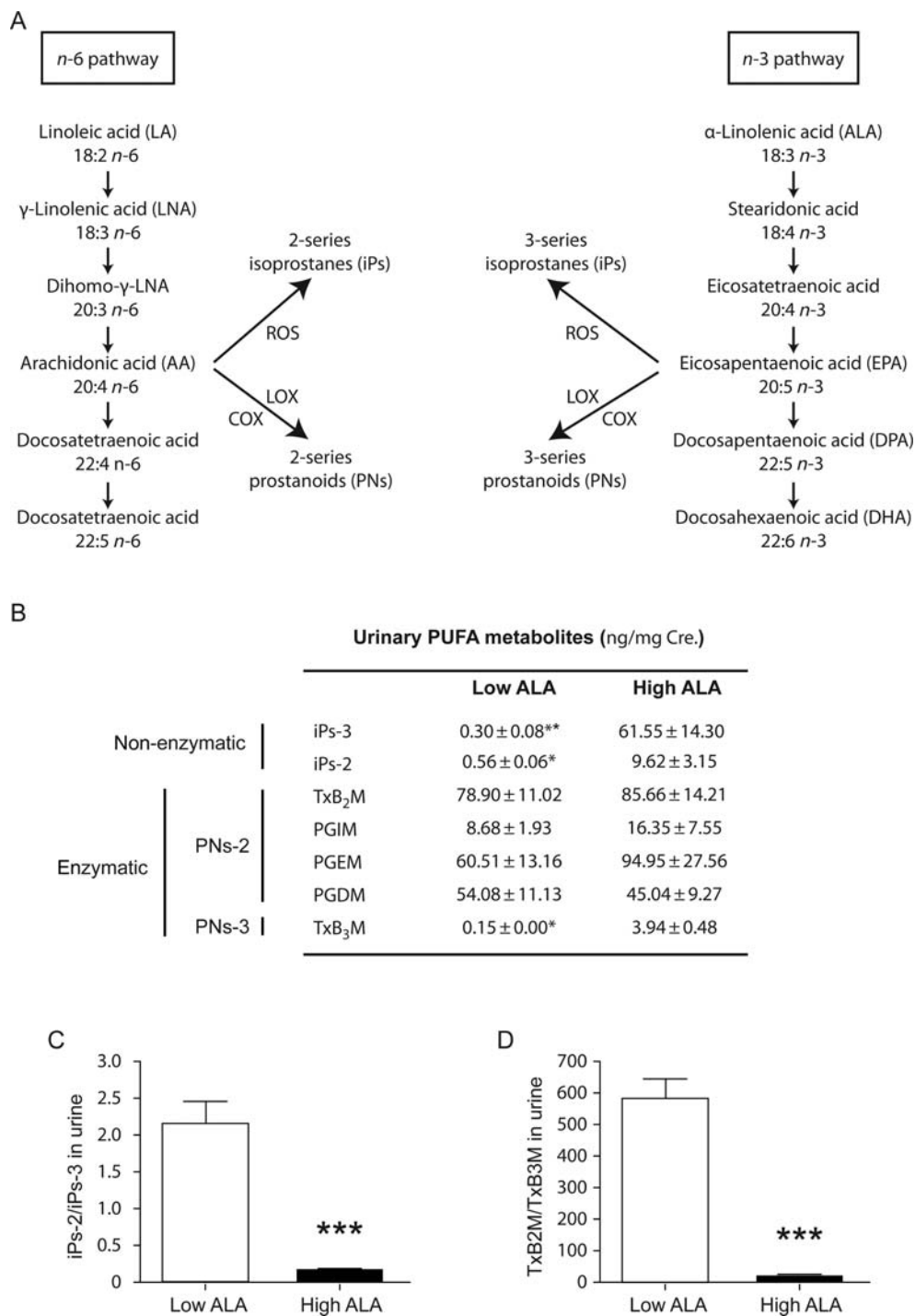


Figure 5 3-Series isoprostanes (iPs-3) and thromboxane B₃ metabolite (TxB₃M) are the chief metabolites of dietary α -linolenic acid (ALA). (A) Eicosapentaenoic acid and arachidonic acid are either subject to radical-catalysed oxidation to isoprostanes or COX-mediated conversion to prostanoids. (B) Urine obtained from *ApoE*^{-/-} mice fed a high or low ALA diet, respectively, were analysed for metabolites of polyunsaturated fatty acids; COX, cyclooxygenase; ROS, reactive oxygen species; LOX, lipoxygenase; iPs, isoprostanes; PNs, prostanoids; TxB₂M, thromboxane B₂ metabolite; TxB₃M, thromboxane B₃ metabolite; PGDM, 11,15-dioxo-9-hydroxy-2,3,4,5-tetranorprostan-1,20-dioic acid (prostaglandin D₂ metabolite); PGEM, 7 α -hydroxy-5,11-diketotetranor-prostane-1,16-dioic acid (prostaglandin E₂ metabolite); PGIM, 2,3-dinor-6-keto prostaglandin F_{1 α} (prostaglandin I₂ metabolite). (C) Ratio of 2-series/3-series isoprostanes. (D) Ratio of thromboxane B₂ metabolite/thromboxane B₃ metabolite **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

effects of reduced IL-4 expression. These findings and the corresponding local events may explain the marked reduction of plaque T cells after ALA supplementation. Notably, expression analyses in dendritic cells and T cells did not reveal a relevant contribution of co-stimulatory pathways to the atheroprotective phenotype. Neither did we observe a difference in endothelial function, in the aortic content of reactive oxygen species, nor the aortic expression of reactive oxygen species generating and scavenging enzymes. Interestingly, plasma small molecule anti-oxidant capacity in the intervention group was slightly increased. However, with small molecules accounting for <30% of the total anti-oxidant capacity, the observed difference was neither sufficient to alter the total anti-oxidant capacity nor to change the absolute quantity of aortic reactive oxygen species. These findings indicate that the reduction in atherosclerotic plaque formation upon dietary ALA is not mediated by differential effects on oxidative stress.

α -Linolenic acid is an essential $n-3$ fatty acid. Whether the effects upon its consumption reflect the activity of ALA itself or of its longer chain $n-3$ fatty acid derivatives remains a matter of debate.¹⁸ The conversion of ALA to EPA and DPA is well known, whereas further conversion to DHA is limited in mammals.¹⁷ Levels of EPA, DPA, and DHA were abundantly increased in our mouse model when ALA was supplemented. This was predominantly the case in circulating RBCs and the liver, but also in white adipose and aortic tissue. In contrast to other studies suggesting that the enzymes (desaturase) for this process have been lost during early evolution,^{41,42} our results imply a substantial degree of chain elongation, allowing a potential *indirect*, eicosanoid-dependent effect. Indeed, we observed diminished AA in aortae paralleled by decreased plaque T cell counts in mice fed a high ALA diet. Arachidonic acid constitutes the substrate for both prostanoid/thromboxane and leucotriene synthesis. Interestingly, previous publications highlight the role of AA-derived leucotriene metabolites on T cell chemotaxis.^{43,44} Our tissue findings corroborate these reports and suggest a link between AA-derived eicosanoids and T cell-driven atherogenesis.

In parallel, our urinary analyses revealed a shift towards 3-series prostanoid formation, most evident in the depressed TxB₂M/TxB₃M ratio. There was a similar shift from $n-6$ - to $n-3$ -derived iPs in mouse urine, reflected by the markedly depressed iP2/iP3 ratio. The compounds measured in this study are the most abundant urinary iPs identified, analogous isomers of PGF_{2 α} and PGF_{3 α} , respectively.²⁸ This striking increase in the iP-3 compound is consistent with the increased susceptibility to peroxidation of $n-3$ compared with $n-6$ FAs. These findings may be of mechanistic relevance as some 3-series analogues such as TxA₃ are less potent ligands at prostanoid receptors than the corresponding AA-derived product.⁴⁵ Given that genetic deletion and pharmacological inhibition of the Tx receptor (TP) decrease atherogenesis in mice and that iPs may act as incidental agonists at the TP, this shift in product formation may contribute to the impact of nutritional ALA on atherogenesis.⁴⁶⁻⁴⁸

A second, *indirect*, EPA- and DHA-mediated mechanism contributing to the observed atheroprotective phenotype may also lie within reduced plasma cholesterol levels. By activating the nuclear receptors LXR α and β , EPA and DHA have been reported to inhibit intestinal cholesterol absorption.⁴⁹ Indeed, we observed

reduced plasma cholesterol levels in the intervention group, whereas plasma triglycerides and lipoprotein subfractions did not differ. However, this effect was much less pronounced than the marked reduction of CD3-positive lymphocytes within the atherosclerotic lesions of the intervention group.

Notably, we observed not only altered expression profiles in T cells after long-term dietary ALA consistent with *indirect* effects, but also after short-term ALA exposure in cell culture experiments. These findings indicate *direct* effects, most likely independent of lipid-derived mediators, i.e. eicosanoids. Taking into account the marked decrease in plaque T cell content, differentiation, inflammatory activity, and proliferation in response to ALA, we propose that the interplay of both *direct* (e.g. TNF α expression) and *indirect* (i.e. eicosanoid-dependent) mechanisms mediate atheroprotection.

Some limitations of this study deserve to be mentioned. The effects observed are based on a mouse model of atherosclerosis and extrapolations to clinical practice have to be made with caution. Mice typically lack vulnerable plaques and differ in the time period needed for plaque formation as well as in their genetic and lipid profile allowing atherogenesis. Moreover, we used a high amount of dietary ALA that may be difficult to widely apply in patients. We consider these limitations to be minor as our goal was to provide a proof-of-principle. We aimed to investigate the effects of dietary ALA on atherosclerosis and investigate cellular and molecular aspects of this concept in a well-characterized mouse model.

In conclusion, we provide evidence that plant-derived dietary ALA diminishes plaque formation and restricts T cell-driven inflammation in atherosclerotic mice. Given the limited availability of $n-3$ FAs from marine origin, ALA may provide a valuable atheroprotective nutritional alternative. Interestingly, two most recent studies provide more insight into this area: an elegant experimental study identified the G protein-coupled receptor 120 (GPR120) in macrophages mediating broad anti-inflammatory effects and increased insulin sensitivity in response to $n-3$ FAs.⁵⁰ The prospective Alpha Omega Trial reported that a low-dose dietary supplementation of ALA did not significantly reduce major cardiovascular events after myocardial infarction, but may be effective in distinct patient subgroups.⁵¹ Thus, further investigation is needed in experimental and clinical studies to determine the optimal dosing of ALA, its key molecular mechanisms, and target patient populations.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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