RESEARCH PAPER

Aspirin-triggered lipoxin A4 inhibits atherosclerosis progression in apolipoprotein E^{-/-} mice

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BACKGROUND AND PURPOSE

Atherosclerosis is characterized by a chronic non-resolving inflammation in the arterial wall. Aspirin-triggered lipoxin A4 (ATL) is a potent anti-inflammatory mediator, involved in the resolution of inflammation. However, the therapeutic potential of immune targeting by means of ATL in atherosclerosis has not previously been explored. The aim of the present study was to determine the effects of ATL and its receptor Fpr2 on atherosclerosis development and progression in apolipoprotein E deficient (ApoE^{-/-}) mice.

EXPERIMENTAL APPROACH

ApoE^{-/-} × Fpr2^{+/+} and ApoE^{-/-} × Fpr2^{-/-} mice were generated. Four-week-old mice fed a high-fat diet for 4 weeks and 16-week-old mice fed chow diet received osmotic pumps containing either vehicle or ATL for 4 weeks. Atherosclerotic lesion size and cellular composition were measured in the aortic root and thoracic aorta. Lipid levels and leukocyte counts were measured in blood and mRNA was isolated from abdominal aorta and spleen.

KEY RESULTS

ATL blocked atherosclerosis progression in the aortic root and thoracic aorta of ApoE^{-/-} mice. In addition, ATL reduced macrophage infiltration and apoptotic cells in atherosclerotic lesions. The mRNA levels of several cytokines and chemokines in the spleen and aorta were reduced by ATL, whereas circulating leukocyte levels were unchanged. The ATL-induced athero-protection was absent in ApoE^{-/-} mice lacking the Fpr2 receptor.

CONCLUSION AND IMPLICATIONS

ATL blocked atherosclerosis progression by means of an Fpr2-mediated reduced local and systemic inflammation. These results suggest this anti-inflammatory and pro-resolving agent has therapeutic potential for the treatment of atherosclerosis.

LINKED ARTICLES

This article is part of a themed section on Targeting Inflammation to Reduce Cardiovascular Disease Risk. To view the other articles in this section visit <http://onlinelibrary.wiley.com/doi/10.1111/bph.v174.22/issuetoc> and <http://onlinelibrary.wiley.com/doi/10.1111/bcp.v82.4/issuetoc>

Abbreviations

ApoE, apolipoprotein E; ATL, aspirin-triggered lipoxin A4; HFD, high-fat diet; Ldlr, low-density lipoprotein receptor

Tables of Links

TARGETS

FPR2/ALX

LIGANDS

ATL	CXCL16	IL-23
CCL2	IFN γ	MMP13
CCL5	IL-6	TNF

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

Introduction

Inflammation plays a well-established role in the initiation and progression of atherosclerosis and may represent an important therapeutic target in cardiovascular prevention (Libby *et al.*, 2011). Most of the treatment options evaluated, for example, blocking inflammatory mediators and the induction of immunoregulatory responses, aim to inhibit the pro-inflammatory signalling in atherosclerotic lesions (Bäck and Hansson, 2015). In addition to a continued stimulation of inflammation, chronic inflammation is also favoured by a failure in the resolution of inflammation (Serhan, 2014). As an example, a defective uptake of apoptotic cells (efferocytosis) increases experimental atherosclerosis (Thorpe *et al.*, 2008; Van Vre *et al.*, 2012).

The lipoxygenase metabolism of arachidonic acid yields the pro-inflammatory mediators leukotrienes. Targeting the specific leukotriene receptors reduces atherosclerosis and intimal hyperplasia in different animal models (Bäck *et al.*, 2005; Ketelhuth *et al.*, 2015). In addition, anti-leukotrienes used in the treatment of asthma have been associated with decreased cardiovascular risk in observational studies (Ingelsson *et al.*, 2012), hence reinforcing the potent pro-inflammatory and pro-atherogenic role of this class of lipid mediators. On the other hand, dual lipoxygenation of arachidonic acid yields a group of lipid mediators called lipoxins (Serhan, 1997; Serhan, 2014), which have the opposite effects to leukotrienes. Lipoxins have been shown to induce a resolution of inflammation, by means of, for example, stimulating efferocytosis (Maderna *et al.*, 2010), granulocyte apoptosis (Barnig *et al.*, 2013), leukocyte egress (van Gils *et al.*, 2012), modulate endothelial activation and reduce leukocyte recruitment after ischaemia/reperfusion (Brancaleone *et al.*, 2013; Smith *et al.*, 2015). In addition, the 15-epimer of lipoxin A4, referred to as aspirin-triggered lipoxin A4 (ATL) because of the stimulation of its formation by aspirin-induced cyclooxygenase acetylation (Serhan, 1997), reduces smooth muscle cell responses *in vitro* and intimal hyperplasia after carotid ligation in mice *in vitro* (Petri *et al.*, 2015b). However, the therapeutic potential of ATL in atherosclerosis has remained unexplored.

Lipoxin A4 and ATL share their signalling pathway by means of the FPR2/ALX receptor (Bäck *et al.*, 2014) with other both pro-inflammatory (e.g. amyloid and antibacterial) peptides (Ye *et al.*, 2009) and pro-resolving mediators, such as

annexin A1 (Hayhoe *et al.*, 2006) and resolvin D1 (Krishnamoorthy *et al.*, 2012). Three major cell types within human atherosclerotic lesions express FPR2/ALX, namely, macrophages, vascular smooth muscle cells and endothelial cells (Petri *et al.*, 2015a). In atherosclerosis-prone low-density lipoprotein receptor deficient (Ldlr^{-/-}) mice, pro-inflammatory ligands for the murine homologue of FPR2/ALX (termed Fpr2) appear to dominate to promote leukocyte recruitment and activation in atherosclerotic lesions (Petri *et al.*, 2015a). Increased atherosclerotic lesion size has also been reported in hyperlipidaemic Fpr2^{-/-} mice at early time-points, associated with defective pro-resolving signalling of the FPR2/ALX peptide agonist annexin A1 (Drechsler *et al.*, 2015). Although FPR2/ALX signalling apparently is activated in atherosclerosis, the effects of ATL as a pro-resolving mediator and a possible therapeutic molecule for the treatment of atherosclerosis have not previously been evaluated.

The aim of the present study was to unravel the potential therapeutic value of ATL in atherosclerosis, and to explore the mechanisms involved, in terms of receptor activation and key local and systemic pro-atherogenic processes during initiation and progression of the disease.

Methods

Generation of ApoE and Fpr2 double knockout mice

All experiments were performed according to the animal regulations and guidelines of Karolinska Institutet (ethical permit #N138/12). Fpr2 deficient mice were generated at the William Harvey Institute (London, UK) as previously described through insertion of the gene cassette and a GFP reporter in reverse orientation into intron 1 of Fpr2, which prevented transcriptional read-through of the Fpr2 as well as Fpr3 genes (Dufton *et al.*, 2010). Therefore, these mice have also been termed Fpr2/Fpr3 knockout mice (Brancaleone *et al.*, 2013), but will be referred to as Fpr2^{-/-} in the present report. The mice were backcrossed for at least seven generations in to a C57BL/6 background before being crossed to apolipoprotein E (ApoE)^{-/-} mice from Taconic (Ry, Denmark) for another two generations to achieve ApoE^{-/-} × Fpr2^{-/-} mice. Female mice were kept in a 12 h

light/dark cycle with either chow diet (D12102, Research Diets Inc, New Brunswick, NJ) or high-fat diet (HFD, 21% fat, D12108C, Research Diets Inc, New Brunswick, NJ) and water *ad libitum*. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath & Lilley, 2015).

ATL treatment protocol

In an initial set of experiments, four groups of mice were studied. ApoE^{-/-} × Fpr2^{+/+} and ApoE^{-/-} × Fpr2^{-/-} mice were implanted with osmotic pumps (Alzet Mod #2004) under general anaesthesia (1.5% isoflurane; 1 L·min⁻¹) at 16 weeks of age. Mice were randomized to receive osmotic pumps loaded with either vehicle (15% of ethanol) or ATL (10 µg·kg⁻¹; Millipore) as previously described (Petri *et al.*, 2015b). The skin was sutured with 5-0 Vicryl and 0.1% of buprenorphine injected s.c. at the moment of the pump implantation and every morning for the next 2 days. The mice were kept on chow diet during the whole experiment. After the mice had been put to death by CO₂ inhalation, a blood sample was withdrawn by cardiac puncture, and the heart, spleen and aorta were harvested.

In a second set of experiments, three groups of ApoE^{-/-} × Fpr2^{+/+} mice were studied. The first group was put to death at 16 weeks of age, representing the baseline before the treatment protocols began. The remaining 16-week-old mice were implanted with osmotic pumps and randomized to the same treatment protocols as indicated above, to obtain a replication of the findings and to determine if ATL treatment either reduced progression or induced regression of atherosclerotic lesions.

In a third set of experiments, 4-week-old ApoE^{-/-} × Fpr2^{+/+} and ApoE^{-/-} × Fpr2^{-/-} mice were fed a HFD and put to death after 4 weeks to study the genotype-induced effect on atherosclerosis initiation.

ATL analysis

Osmotic pumps filled with ATL were incubated at 37°C for 4 weeks, and the content was then collected, and two volumes of ice-cold MeOH was added followed by C18 solid phase extraction for liquid chromatography and tandem mass spectrometry (LC-MS-MS; Colas *et al.*, 2014; Stanke-Labesque *et al.*, 2012). The system consisted of Waters Xevo® TQS triple quadrupole equipped with Acquity UPLC System from Waters Corporation and an autosampler cooled to 5°C (Milford, MA, USA). An Acquity UPLC BEH (Ethylene Bridged Hybrid) C18 column (130 Å, 1.7 µm, 2.1 × 150 mm) equipped with a pre-column (Acquity UPLC C18 VanGuard Pre-column, 130 Å, 1.7 µm, 2.1 × 5 mm; Milford, US) was used with gradients A (0.1% acetic acid in water) and B [acetone-trile/isopropanol; 90:10 (v.v⁻¹)] from 80:20 (v.v⁻¹) to 0:100 (v.v⁻¹) in 17 min at a 0.5 mL·min⁻¹ flow rate, which was then equilibrated to initial conditions for 2.5 min. Data acquisition was performed in negative ionization mode, and identification conducted in accordance with published criteria (Colas *et al.*, 2014) with a minimum of six diagnostic ions.

Blood and plasma analysis

Circulating leukocytes were measured by an automated cell counter (ABC vetpack). Cholesterol and triglycerides were measured in plasma by kits from Randox following the

manufacturer's protocol. Protein quantification was performed by ELISA following the manufacturer's protocols: IL-6 (Cat # 431303, BioLegend), MMP13 (cat# LS-F5519, LSBio) and serum amyloid A (SAA; Cat# KMA0021, Invitrogen).

Evaluation of atherosclerosis

The thoracic aorta was opened longitudinally, pinned and stained with Sudan IV solution. A micrograph of the aortic arch was captured using a Leica Microsystems colour video camera, and the image analysis was performed using ImageJ in a blinded fashion. The area of the aortic arch covered by atherosclerotic lesions divided by the aortic arch area was calculated as previously described (Petri *et al.*, 2015a).

In addition, the upper portion of the heart and aortic root was embedded in optimal cutting temperature compound and frozen at -80°C. Serial 10 µm cryosections were collected beginning at the aortic valve cusp for a distance of 800 µm. Oil red-O staining was performed on eight formalin-fixed sections collected every 100 µm from the aortic valve cusps. Lesion area was determined in a blinded fashion by light microscopy using Leica Qwin. The mean value of lipid stained areas (lesion size) of aortic root sections was calculated.

Immunohistochemistry and plaque composition analysis

Antibodies against CD68 (MCA1957, Serotec) and CD4 (553647, BD) were used for the immunodetection of macrophages and T-cells in sections representing the largest lesion area of each aortic root. One section per animal was analysed, and *n* indicates the number of animals. In a subset of animals, four sections from each aortic root were stained for CD68, CD4 and apoptosis detection and yielded similar results as those obtained with the largest lesion size (data not shown). Biotinylated secondary antibodies and Nova Red staining were used to identify positive cells/areas, and sections were counterstained with haematoxylin. Apoptotic cells were detected using an *in situ* apoptosis detection kit (ab206386, Abcam) following the manufacturer's protocol. For collagen quantification, sections were stained with Picrosirius red (HistoLab® HL27150) and analysed under polarized light for the amount of red (thick) and green (thin) fibres, as quantified by Leica Qwin. The slides were mounted, and the sections were analysed as described for oil red-O for quantification of CD68, CD4 and apoptosis detection positive areas.

Real-time PCR

Total RNA was isolated from the abdominal aorta and spleen using QIAzol (Qiagen) and concentrations were measured spectrophotometrically using Nanodrop 1000 (Thermo Fisher Scientific). Reverse-transcription was performed with a high capacity kit (Life Technologies, USA). Real-time PCR was performed on a 7900HT Fast Real-Time PCR system (Perkin-Elmer Applied Biosystems) using TaqMan Assay-on-Demand from Applied Biosystems for IL-6 (Mm00446190_m1), MMP13 (Mm00439491_m1), CCL2 (Mm00441242_m1), CCL5 (Mm01302427_m1), CXCL16 (Mm00469712_m1), IFN γ (Mm01168134_m1), IL-23A (Mm00518984_m1), SAA (Mm00441203_m1) and TNF (Mm00443258_m1). Results are expressed as 2^{-ΔCT} obtained

by comparing the threshold cycle (CT) for the gene of interest with that obtained using hypoxanthine phosphoribosyl-transferase (HPRT; Mm01545399_m1) as a housekeeping gene.

Data and statistical analysis

Data are expressed as mean $\pm$ SEM. When comparing two groups, either a Student's *t*-test or Mann–Whitney *U*-test was used after a normality test was performed. Either a one or two-way ANOVA was used for multiple comparisons as indicated, followed by a Dunnett's test versus control. $P < 0.05$ was considered significant. All analyses were performed using SigmaPlot version 12.5 (Systat Software Inc). The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

Results

ATL stability

The stability of ATL was assessed, and the results are shown in Figure 1. ATL remained intact following 4 weeks of incubation in osmotic pump at 37°C, with only small amount

(<10%) of potential isomerization as observed by appearance of additional peaks (Figure 1). Its retention time (5.70 min) and MS–MS spectra were identical to that of the 15-epi-LXA4 synthetic standard. Furthermore, it retained its tetraene chromophore with $\lambda_{\max}$ at 300 nm and shoulders at 287 and 315 nm, identical to that of the 15-epi-LXA4 standard (data not shown).

ATL blocks atherosclerosis progression by means of Fpr2

To study the therapeutic potential of ATL on an established disease, 16-week-old ApoE^{−/−} × Fpr2^{−/−} and ApoE^{−/−} × Fpr2^{+/+} mice were randomized to treatment with either vehicle or ATL for 4 weeks followed by atherosclerosis assessment. At the end of the treatment period, ATL-treated ApoE^{−/−} × Fpr2^{+/+} mice exhibited significantly reduced atherosclerosis burden compared with vehicle-treated mice, both at the aortic root level (Figure 2A) and *en face* analysis of the aortic arch (Figure 2B). Furthermore, vehicle-treated ApoE^{−/−} × Fpr2^{−/−} mice exhibited reduced lesion size at both locations compared with vehicle-treated ApoE^{−/−} × Fpr2^{+/+} mice (Figure 2A–B). In contrast, atherosclerotic lesion size was not altered by ATL treatment in ApoE^{−/−} × Fpr2^{−/−} mice. There were no significant differences in lipid levels, blood

Figure 1

LC–MS–MS chromatograph of 15-epi-LXA4 following incubation in osmotic pump. 15-epi-LXA4 in PBS (237.5 ng in 200 μ L) was added to an osmotic pump and incubated in PBS at 37°C. After 4 weeks, samples were placed in two volumes of ice-cold MeOH and taken to solid phase extraction and LC–MS–MS. (A) Multiple reaction-monitoring chromatograms of 15-epi-LXA4 (m/z 351 > 115) from osmotic pump after 4 weeks (top) and 15-epi-LXA4 standard (bottom; Inset: $\times 16$ magnification). (B) Accompanying MS–MS spectra of 15-epi-LXA4 from osmotic pump incubation (top) and standard (bottom; inset: diagnostic ions; M, molecular mass).

Figure 2

Atherosclerotic lesions in the aortic root (A) and *en face* analysis of the aortic arch (B) of 20-week-old ApoE^{-/-} x Fpr2^{+/+} and ApoE^{-/-} x Fpr2^{-/-} treated with either vehicle or ATL for 4 weeks. Below the graphs, representative micrographs of aortic root and *en face* staining of each genotype and treatment are shown. In each group $n =$ at least 6, and lines represent mean $\pm$ SEM. * $P < 0.05$.

cellcounts or body weights between the different groups (Table 1).

To clarify if ATL either regressed or inhibited the progression of atherosclerosis, the treatment protocol was repeated in another series of ApoE^{-/-} x Fpr2^{+/+} mice in which the atherosclerotic lesion size after 4 weeks of either ATL or vehicle treatment was compared with that observed in 16-week-old littermate mice, which corresponded to the start of the treatments. After 4 weeks of treatment with vehicle, 20-week-old mice exhibited significantly increased atherosclerotic lesions in both the aortic root (Figure 3A) and aortic arch (Figure 3B) as compared with the 16-week-old mice at baseline. In contrast, no significant differences compared with baseline were observed after 4 weeks of ATL treatment, indicating that

ATL prevented plaque progression in both the aortic root and aortic arch (Figure 3). ATL did not significantly alter lipids, blood cell counts or body weight of 20-week-old mice (Table 2).

ATL alters plaque composition

The analysis of the atherosclerotic plaque composition in the aortic root of ApoE^{-/-} x Fpr2^{+/+} mice receiving either ATL or vehicle is shown in Figure 4. Mice receiving ATL exhibited less CD68 positive area compared with vehicle-treated mice (Figure 4A), whereas there were no significant differences in the amount of CD4 positive cells between ATL and vehicle-treatment (Figure 4B). In addition, the number of apoptotic cells was reduced in the ATL-treated

Table 1

Lipid levels and white blood cell counts for ApoE^{-/-} × Fpr2^{+/+} and ApoE^{-/-} × Fpr2^{-/-} treated with either Vehicle or ATL

	ApoE ^{-/-} × Fpr2 ^{+/+}		ApoE ^{-/-} × Fpr2 ^{-/-}		
	Vehicle (n = 11)	ATL (n = 8)	Vehicle (n = 7)	ATL (n = 6)	
Lipids					
Cholesterol (mM)	9.2±0.5	9.4±0.6	9.8 ± 0.5	7.5 ± 0.7	NS
Triglycerides (mM)	1.4±0.1	0.9±0.1	1.2 ± 0.1	1.3 ± 0.1	NS
White blood cell count (10 ⁹ L ⁻¹)	6.2 ± 0.5	5.9 ± 0.5	7.3 ± 0.7	8.6 ± 1.0	NS
Lymphocyte (%)	70.9 ± 1.0	70.8 ± 1.1	71.2 ± 1.8	72.6 ± 2.4	NS
Monocyte (%)	4.3 ± 0.2	3.5 ± 0.2	4.8 ± 0.2	4.7 ± 0.1	NS
Granulocyte (%)	24.5 ± 0.9	25.6 ± 1.0	23.9 ± 1.8	22.6 ± 2.4	NS
Body weight (g)	25.6 ± 0.4	26.7 ± 0.3	25.3 ± 0.5	26.3 ± 0.7	NS

Results are presented as mean ± SEM; NS, non-significant ($P > 0.05$; one way ANOVA).

mice (Figure 4C). The collagen content, as assessed by Picrosirius red staining, revealed a larger proportion of thick collagen fibres (stained in red) in ATL-treated mice (Figure 4D).

Atherosclerosis initiation is not dependent on Fpr2

In order to clarify whether Fpr2 signalling was important for the initiation of atherosclerosis, 4-week-old ApoE^{-/-} × Fpr2^{-/-} and ApoE^{-/-} × Fpr2^{+/+} mice were fed a HFD for 4 weeks. No differences were observed between the two

genotypes in terms of atherosclerotic lesion size (Figure 5), lipid levels or blood count (Table 3).

The reduction of systemic inflammation induced by ATL is dependent on Fpr2

In order to address whether the observed ATL-induced effects were limited to the vasculature, or if they were observed systemically, gene expression profiles were compared between the aorta and the spleen. ATL-treated mice exhibited significantly lower levels of a number of cytokines and chemokines in both the aorta and the spleen, as indicated in Table 4.

Figure 3

Atherosclerotic lesions in the (A) aortic root and (B) *en face* analysis of the aortic arch in ApoE^{-/-} × Fpr2^{+/+} mice at either 16 weeks (baseline, filled diamonds) or 20 weeks (triangles) after 4 weeks of treatment with either vehicle (filled triangles) or ATL (open triangles). Representative micrographs of *en face* staining of each group are also shown. In each group $n = 5$, and lines represent mean ± SEM. * $P < 0.05$

Table 2

Lipid levels and white blood cell counts for either 16 (baseline) or 20-week-old ApoE^{-/-} × Fpr2^{+/-} mice treated for 4 weeks with either Vehicle or ATL

	Baseline (n = 5)	ATL (n = 5)	Vehicle (n = 5)	
Lipids				
Cholesterol (mM)	7.0 ± 0.3	7.3 ± 0.3	5.6 ± 0.4	NS
Triglycerides (mM)	1.2 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	NS
White blood cell count (10⁹ L⁻¹)				
Lymphocyte (%)	74.2 ± 1.2	66.9 ± 1.5	65.7 ± 4.2	NS
Monocyte (%)	4.5 ± 0.2	5.6 ± 0.2	5.3 ± 0.3	NS
Granulocyte (%)	21.2 ± 1.1	27.5 ± 1.5	28.9 ± 3.9	NS
Body weight (g)	24.6 ± 0.3	28.1 ± 0.5*	27.8 ± 0.7*	

Results are presented as mean ± SEM; NS, non-significant ($P > 0.05$; one way ANOVA), * $P < 0.05$ versus baseline.

Further examples of the anti-inflammatory effects of ATL are shown in Figure 6, namely, the decreased levels of IL-6 and MMP13 mRNA in the spleen and the aorta and their concomitant decreased circulating protein levels in plasma (Figure 6). In contrast, ATL did not significantly reduce the IL-6 and MMP13 mRNA levels in organs derived from ApoE^{-/-} × Fpr2^{-/-} mice (data not shown), indicating that the anti-inflammatory effects of ATL require a functional Fpr2 signalling pathway. Finally, mice treated with SAA, another pro-inflammatory mediator, which is also an Fpr2 agonist, exhibited significantly reduced mRNA levels in the aorta (Figure 6G) and the spleen (Figure 6H) in response to ATL-treatment. Also the circulating protein levels in SAA-treated mice tended to be lower (Figure 6I)

Discussion

The present study raises the notion of a therapeutic potential for ATL in stopping the progression of established atherosclerosis. Furthermore, we show that the effects of ATL were transduced by means of Fpr2, reducing vascular inflammation in terms of for example lowering the number of plaque macrophages and inhibiting pro-inflammatory gene expression. We also show that ATL reduced the systemic inflammation. Taken together, these results suggest that ATL treatment could represent a novel therapeutic strategy to prevent atherosclerosis progression.

Whereas the inhibitory effects of ATL on acute inflammation, such as inflammatory cell recruitment in inflamed dorsal air pouches (Dufton *et al.*, 2010) and microvascular dysfunction after ischaemia-reperfusion (Brancalione *et al.*, 2013; Vital *et al.*, 2016), are well established, its role for resolving chronic inflammation has been less extensively explored. Long-term ATL treatment to mice by means of osmotic pumps has however been established to reveal that this lipid mediator reduces intimal hyperplasia after carotid artery ligation (Petri *et al.*, 2015b). Adopting the latter therapeutic strategy, the present study demonstrates the stability of ATL administered under those conditions and for the first time reveals that 4 weeks of ATL treatment to ApoE-deficient mice with established atherosclerosis significantly reduces

lesion size in the aortic root and arch compared with vehicle-treated mice.

The ATL-induced effects on atherosclerosis were observed in the absence of significant alterations of the plasma lipid profile, suggesting that pro-inflammatory pathways rather than lipid metabolism were targeted by this treatment. In line with this, a reduced number of macrophages was observed in atherosclerotic lesions derived from ATL-treated mice compared with vehicle-treated mice, whereas lesion T-lymphocyte infiltration was not altered by ATL. Macrophages in human atherosclerotic lesions indeed express the ATL receptor FPR2/ALX (Petri *et al.*, 2015a), and lipoxin stimulation of human and murine myeloid cells reduces inflammation *in vivo* and *in vitro* (Devchand *et al.*, 2003; Maderna *et al.*, 2010; Wu *et al.*, 2011; Petri *et al.*, 2015a). Efferocytosis by macrophages is enhanced by lipoxins (Godson *et al.*, 2000) and inhibited in Fpr2^{-/-} mice (Petri *et al.*, 2015a), suggesting that ATL-induced efferocytosis by means of Fpr2 signalling may contribute to its athero-protective effects. In support of the latter, a reduced number of apoptotic cells was observed in atherosclerotic lesions derived from ATL-treated mice in the present study. An increased proportion of thick collagen fibres was also observed in atherosclerotic lesions after ATL-treatment, which is line with previous findings in Fpr2^{-/-} mice (Petri *et al.*, 2015a) and supports the notion that ATL may also induce a more stable lesion phenotype, which is further supported by the decrease in collagenase MMP13 after ATL treatment observed in the present study.

These beneficial effects of ATL were reproduced in a second series of mice, in which the atherosclerotic lesion size at 20 weeks after 4 weeks of osmotic pump ATL treatment was unchanged compared with that observed in 16-week-old mice, corresponding to the start of the treatment period. Based on the latter experiments, we can conclude that ATL treatment blocked the significant progression of atherosclerosis that was observed in vehicle-treated mice. It is also important to stress that the mice had atherosclerotic plaques when treatment was initiated, hence extending previous observations that the Fpr2 agonist annexin A1 prevented atherosclerosis development in younger ApoE knockout mice fed a HFD (Drechsler *et al.*, 2015).

Figure 4

Plaque composition. Quantification of macrophages, CD68 positive (A); T-lymphocytes, CD4 positive (B); apoptotic cells (*in situ* apoptosis detection) (C); and collagen fibres (Picrosirius red staining) (D), in aortic roots of ApoE^{-/-} × Fpr2^{+/-} treated with either vehicle or ATL. In all the experiments $n =$ at least 6, and bars represent mean $\pm$ SEM. * $P < 0.05$.

Studies in other animal models have shown that lipoxin-induced effects are abolished in mice lacking the murine FPR2/ALX orthologue Fpr2 (Dufton *et al.*, 2010; Brancalione *et al.*, 2013; Petri *et al.*, 2015b; Vital *et al.*, 2016). To address the signalling pathways involved in the observed reduction of atherosclerosis induced by ATL, we generated ApoE and

Fpr2 double knockout mice for the present study. Importantly, ATL-treatment was ineffective in reducing atherosclerosis in ApoE^{-/-} mice lacking Fpr2. This latter finding indicates that Fpr2 is the receptor exclusively mediating the beneficial effects of ATL in terms of atherosclerosis development.

Figure 5

Atherosclerotic lesions in the aortic root (A) and *en face* analysis of the aortic arch (B) of 8-week-old $ApoE^{-/-} \times Fpr2^{+/+}$ (filled circles) and $ApoE^{-/-} \times Fpr2^{-/-}$ (open circles) after 4 weeks of HFD. Next to the graphs, representative micrographs of aortic root and *en face* staining of each genotype are shown. In each group $n =$ at least 6, and lines represent mean $\pm$ SEM.

Another important finding that emerged from the latter analysis was that $Fpr2$ -deficient $ApoE^{-/-}$ mice exhibited reduced atherosclerosis. These results confirm our previous results in $Ldlr$ and $Fpr2$ double knockout mice at similar time points and lesion sizes (Petri *et al.*, 2015a). The similar results obtained with $Fpr2$ deletion in two different murine atherosclerosis models strongly corroborate the notion that pro-inflammatory $Fpr2$ agonists prevail over the anti-inflammatory receptor ligands in atherosclerosis. Indeed, our previous study showed low levels of lipoxins in $Ldlr^{-/-}$ mice fed a HFD compared with the pro-inflammatory $Fpr2$ ligand SAA (Petri *et al.*, 2015a). Taken together, these observations support the hypothesis that murine atherosclerosis

models are associated with low pro-resolving $Fpr2$ agonists, which may contribute to a failure in the resolution of inflammation. Interestingly, ATL-treatment decreased the levels of SAA in the present study, hence potentially further facilitating its pro-resolving $Fpr2$ signalling.

The lack of effects at early stages of atherosclerosis development in the present study are in contrast to a study using a different $Fpr2$ gene targeting strategy, which reported exacerbated atherosclerosis in $ApoE^{-/-}$ mice after 4 weeks of HFD (Drechsler *et al.*, 2015). While it remains to be established whether the different $Fpr2$ genetic targeting techniques or other experimental conditions accounted for these differential results, it should be pointed out that the results of the

Table 3

Lipid levels and white blood cell counts for $ApoE^{-/-} \times Fpr2^{+/+}$ and $ApoE^{-/-} \times Fpr2^{-/-}$ on HFD for 4 weeks

	$ApoE^{-/-} \times Fpr2^{+/+}$ ($n = 8$)	$ApoE^{-/-} \times Fpr2^{-/-}$ ($n = 6$)	
Lipids			
Cholesterol (mM)	18.6 ± 1.6	15.9 ± 0.8	NS
Triglycerides (mM)	1.7 ± 0.1	1.6 ± 0.2	NS
White blood cell count ($10^9 L^{-1}$)			
Lymphocyte (%)	52.9 ± 2.9	55.5 ± 1.6	NS
Monocyte (%)	3.9 ± 0.7	3.4 ± 0.2	NS
Granulocyte (%)	43.1 ± 3.0	41.1 ± 1.6	NS

Results are presented as mean $\pm$ SEM; NS, non-significant ($P > 0.05$; Student's *t*-test).

Table 4

Cytokine and chemokine mRNA levels in the aorta derived from mice treated with either vehicle or ATL

	Aorta		<i>P</i>	Spleen		<i>P</i>
	Vehicle (<i>n</i> = 11)	ATL (<i>n</i> = 8)		Vehicle (<i>n</i> = 11)	ATL (<i>n</i> = 8)	
IFN- γ	0.07 $\pm$ 0.01	0.001 $\pm$ 0.00	<0.001	0.03 $\pm$ 0.00	0.01 $\pm$ 0.00	0.02
TNF	0.28 $\pm$ 0.10	0.05 $\pm$ 0.01	0.028	2.12 $\pm$ 0.12	1.19 $\pm$ 0.08	<0.001
CCL2	0.77 $\pm$ 0.17	0.29 $\pm$ 0.04	0.007	0.43 $\pm$ 0.02	0.29 $\pm$ 0.04	0.009
CCL5	0.84 $\pm$ 0.56	0.12 $\pm$ 0.01	NS	6.75 $\pm$ 0.27	4.62 $\pm$ 0.29	<0.001
CXCL16	0.27 $\pm$ 0.09	0.13 $\pm$ 0.04	0.021	2.41 $\pm$ 0.10	1.44 $\pm$ 0.08	<0.001
IL-23a	0.78 $\pm$ 0.76	0.01 $\pm$ 0.01	NS	0.01 $\pm$ 0.00	0.00 $\pm$ 0.00	0.016

Data (mean $\pm$ SEM) are expressed as $2^{-\Delta\text{CT}}$. NS, non-significant ($P > 0.05$; Student's *t*-test).

present study are in line with previous findings that did not reveal any significant effects of Fpr2 deletion at early stages of atherosclerosis in either *Ldlr*^{-/-} mice (Petri *et al.*, 2015a) or after bone marrow transplantation of *Fpr2*^{-/-} bone marrow to *Ldlr*^{-/-} mice (Fredman *et al.*, 2015).

Taken together with the above-mentioned findings, the results of the present study support the postulate that ATL treatment enhances the resolution of inflammation and inhibits the development of atherosclerosis. In support of the latter, a failure in the resolution of inflammation in terms of reduced lipoxin levels has indeed been established in both

experimental atherosclerosis (Petri *et al.*, 2015a) and clinical biomarker studies (Ho *et al.*, 2010). Although not specifically addressed in the present study, it should be noted that aspirin enhances ATL production in atherosclerosis (Brezinski *et al.*, 1992; Ho *et al.*, 2010), and it can hence be anticipated that ATL production may represent an additional beneficial effect of aspirin in cardiovascular prevention, which goes beyond its anti-aggregatory effects on platelets.

As a final approach in the present study, we sought to determine whether the observed Fpr2-mediated reduction

Figure 6

Effects of ATL treatment on IL-6 (A–C) and MMP13 (D–F) and serum amyloid A (SAA; G–I) mRNA levels in the aorta (A, D and G), and spleen (B, E and H) and protein levels in plasma (C, F and I). Data (mean $\pm$ SEM) are expressed as either $2^{-\Delta\text{CT}}$ or pg·mL⁻¹ for *n* = at least 6 in each group. * $P < 0.05$.

in atherosclerosis and lesion macrophage content induced by ATL solely represented a local vascular response or if systemic inflammation was also altered in ATL-treated mice. Based on the lack of significant alterations of the peripheral white blood cell count, we conclude that no apparent myeloid dysfunction was induced by ATL. Nevertheless, ATL reduced the expression levels of a number of pro-inflammatory cytokines, chemokines and enzymes in both the spleen and the aorta, indicating that ATL decreased systemic inflammation. This observation is supported by the decreased circulating proteins levels of IL-6 and MMP13.

In summary, ATL treatment initiated after the establishment of atherosclerosis stopped the progression of the disease over 4 weeks. These effects of ATL were absent in mice lacking Fpr2, the murine orthologue of the human FPR2/ALX receptor, indicating that the beneficial effects of ATL on atherosclerosis are mediated through this signalling pathway. However, in the absence of ATL, a deficiency in Fpr2 protected against atherosclerosis progression, supporting the notion that stimulation of this receptor is involved in the failure in the resolution of inflammation, which can be reversed by the treatment of atherosclerotic mice with the pro-resolving mediator ATL. This is further supported by the reduced macrophage infiltration and inflammatory mRNA levels in ATL-treated mice. In conclusion, stopping atherosclerosis progression by means of the ATL and FPR2/ALX pathway may represent a novel therapeutic option for immune targeting in cardiovascular prevention.

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Author contributions

M.H.P., A.L.F. and M.B. planned and conceived the study. M.H.P., A.L.F. and H.A. performed the experiments. M.H.P., A.L.F., H.A., C.E.W. and M.B. performed the data analysis. M.H.P. and M.B. wrote the manuscript, and A.L.F., H.H., M.P., C.E.W. and G.K.H. revised it critically for important intellectual content.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This [Declaration](#) acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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