

RESEARCH PAPER

Lipoxin A₄ attenuates zymosan-induced arthritis by modulating endothelin-1 and its effects

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BACKGROUND AND PURPOSE

Lipoxin A₄ (LXA₄) is a lipid mediator involved in the resolution of inflammation. Increased levels of LXA₄ in synovial fluid and enhanced expression of the formyl peptide receptor 2/lipoxin A₄ receptor (FPR2/ALX) in the synovial tissues of rheumatoid arthritis patients have been reported. Endothelins (ETs) play a pivotal pro-inflammatory role in acute articular inflammatory responses. Here, we evaluated the anti-inflammatory role of LXA₄, during the acute phase of zymosan-induced arthritis, focusing on the modulation of ET-1 expression and its effects.

EXPERIMENTAL APPROACH

The anti-inflammatory effects of LXA₄, BML-111 (agonist of FPR2/ALX receptors) and acetylsalicylic acid (ASA) pre- and post-treatments were investigated in a murine model of zymosan-induced arthritis. Articular inflammation was assessed by examining knee joint oedema; neutrophil accumulation in synovial cavities; and levels of prepro-ET-1 mRNA, leukotriene (LT)B₄, tumour necrosis factor (TNF)- α and the chemokine KC/CXCL1, after stimulation. The direct effect of LXA₄ on ET-1-induced neutrophil activation and chemotaxis was evaluated by shape change and Boyden chamber assays respectively.

KEY RESULTS

LXA₄, BML-111 and ASA administered as pre- or post-treatment inhibited oedema and neutrophil influx induced by zymosan stimulation. Zymosan-induced preproET-1 mRNA, KC/CXCL1, LTB₄ and TNF- α levels were also decreased after LXA₄ pretreatment. *In vitro*, ET-1-induced neutrophil chemotaxis was inhibited by LXA₄ pretreatment. LXA₄ treatment also inhibited ET-1-induced oedema formation and neutrophil influx into mouse knee joints.

CONCLUSION AND IMPLICATION

LXA₄ exerted anti-inflammatory effects on articular inflammation through a mechanism that involved the inhibition of ET-1 expression and its effects.

Abbreviations

ASA, acetylsalicylic acid; ATL, aspirin-triggered lipoxin; ET, endothelin; FPR2/ALX receptor, formyl peptide receptor 2/lipoxin A₄ receptor; i.art., intra-articular; i.pl., intraplantar; LT, leukotriene; LXA₄, lipoxin A₄; RA, rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by synovial hyperplasia, progressive destruction of cartilage and bone, neoangiogenesis and infiltration of the joint synovium by activated inflammatory leucocytes, leading to chronic inflammation of the joints (Harris, 1991). A critical factor that contributes to joint damage is the excessive production of inflammatory mediators by resident and/or infiltrating inflammatory cells. Free radicals, extracellular matrix-degrading enzymes, pro-inflammatory cytokines [including interleukin (IL)-6, IL-1 and tumour necrosis factor (TNF)- α], chemokines (such as CXCL1) and lipid mediators [like leukotriene (LT) B_4] are among the main mediators involved in this process (Maini *et al.*, 1999). Our group has recently shown that endogenous endothelins (ETs) are inflammatory mediators that also play a crucial pro-inflammatory role in articular inflammation by regulating oedema formation, leucocyte influx and the production of inflammatory mediators, including TNF- α , chemokines and LTB $_4$ (Conte *et al.*, 2008). Accordingly, ET-1 levels have been shown to be increased in the serum (Haq *et al.*, 1999; Kuryliszyn-Moskal *et al.*, 2006) and synovial fluids (Vaudo *et al.*, 2004) of RA patients when compared to healthy subjects. ET-1 is a 21-amino acid isopeptide that is mainly produced by endothelial cells from a 200-residue prepropeptide (Inoue *et al.*, 1989).

Lipoxin A $_4$ (LXA $_4$) is another mediator involved in inflammation that is endogenously derived from arachidonic acid metabolism, and has marked anti-inflammatory and pro-resolving effects (Yacoubian and Serhan, 2007). The C-15 epimeric form of LXA $_4$, aspirin-triggered LXA $_4$ (15-epi-LXA $_4$ or ATL), has been shown to potently inhibit neutrophil activity (chemotaxis, adhesion and transmigration) (Filep *et al.*, 2005) and leucocyte trafficking in various inflammatory disorders. The anti-inflammatory effects of both LXA $_4$ and ATL have been demonstrated in several experimental models of inflammation, including ischaemia/reperfusion (Souza *et al.*, 2007), paw oedema (Menezes-de-Lima *et al.*, 2006), air pouch (Chiang *et al.*, 2000) and gastric damage (Fiorucci *et al.*, 2002). Furthermore, its pro-resolution actions have been previously described in periodontitis (Serhan *et al.*, 2003) and lung inflammation evoked by carrageenan or *Escherichia coli* septicemia (El Kebir *et al.*, 2009). LXA $_4$ exerts its anti-inflammatory effects through binding to formyl-peptide receptor-like (FPRL1), also called formyl peptide receptor 2/lipoxin A $_4$ receptor (FPR2/ALX; receptor nomenclature follows Alexander *et al.*, 2009), which is a G protein-coupled receptor

that is primarily expressed on neutrophils and monocytes (Chiang *et al.*, 2006). A recent study has demonstrated that synovial fluid and tissues obtained from RA patients had elevated levels of LXA $_4$ and FPR2/ALX receptor expression, respectively (Hashimoto *et al.*, 2007).

In this study, we investigated the effect of LXA $_4$ treatment on zymosan-induced articular inflammation by evaluating oedema formation, leucocyte influx and the production of pro-inflammatory mediators. In addition, because ET-1 plays a pivotal role in articular inflammation by inducing the production of cytokines, chemokines and LTs, we also investigated the ability of LXA $_4$ to impair ET-1-triggered knee joint inflammation and neutrophil activation *in vitro*.

Methods

Animals

All animal care and experimental procedures performed were approved by the institution's Ethical Committee for Animal Care and Use (CEUA, Fiocruz, Rio de Janeiro, Brazil). Male C57BL/6 mice (20–25 g) were obtained from the Oswaldo Cruz Foundation Breeding Unit (Fiocruz). The mice were kept in plastic cages with free access to food and fresh water in a room with a controlled temperature (22–24°C) and light cycle (12 h light/dark) at the experimental animal facility until use.

Induction of joint inflammation

Joint inflammation was induced by intra-articular (i.art.) injection of zymosan (500 μ g per cavity in 25 μ L sterile saline) or ET-1 (10 pmol per cavity) by inserting a 27.5 G needle through the suprapatellar ligament into the left knee joint cavity, as previously described (Penido *et al.*, 2006; Conte *et al.*, 2008). Control animals received an i.art. injection of an equal volume of sterile saline.

Pretreatments

LXA $_4$ was administered i.art. in doses ranging from 1 to 20 ng per cavity, in a final volume of 25 μ L sterile saline, 60 min before i.art. zymosan stimulation. BML-111, an LXA $_4$ receptor agonist, was dissolved in sterile saline and administered i.art. at 200 ng per cavity 60 min before zymosan stimulation in a final volume of 25 μ L. BOC-1, an antagonist of the G protein-coupled LXA $_4$ receptor, was administered i.art. 90 min before articular stimulation at 20 ng per cavity in a final volume of 25 μ L. Acetylsalicylic acid (ASA; 300 mg \cdot kg $^{-1}$) was diluted in 0.5% carboxymethylcellulose, and given orally (p.o.) 30 min before i.art. zymosan stimulation. Indomethacin

(3 mg·kg⁻¹) was diluted in a 5% bicarbonate solution, and given orally 30 min before zymosan stimulation. Respective control groups were injected with the same volume of sterile saline.

Post-treatments

In this set of experiments, mice were treated with LXA₄, BML-111 and ASA 1 and 6 h after zymosan i.art. injection. LXA₄ and BML-111 were administered i.p. at 5 µg·kg⁻¹ in a final volume of 100 µL of sterile saline. ASA (300 mg·kg⁻¹) was diluted in 0.5% carboxymethylcellulose and was given orally (p.o.) in a final volume of 100 µL. Evaluations of oedema formation, total and differential counts were performed 10 h after zymosan stimulation. The control group was injected i.p. with the same volume of sterile saline.

Measurement of knee joint swelling

Knee joint swelling was evaluated by measurement of the transverse diameters of the left knee joints using a digital caliper (Digmatic Caliper, Mitutoyo Corporation, Kanagawa, Japan). Values of knee joint thickness are expressed as the difference (Δ) between the diameter measured before (basal) and after induction of articular inflammation in millimetres (mm).

Collection of synovial fluid and leucocyte counts

Mice were killed by an excess of CO₂ at 6 h after i.art. injection of zymosan. Knee synovial cavities were washed with 300 µL of phosphate-buffered saline (PBS) containing EDTA (10 mM) by inserting a 21 G needle into the mouse knee joint, and the synovial washes were recovered by aspiration. Total leucocyte counts were performed in a Neubauer chamber under an optical microscope, after dilution in Turk's fluid (2% acetic acid). Differential neutrophil counts were performed using May–Grunwald–Giemsa-stained cytopins (1000× magnification) (Cytospin 3, Shandon Inc., Pittsburgh, PA, USA), and values are expressed as numbers of cells per cavity (×10⁵). After cellular counts, the synovial washes were centrifuged at 416× g for 10 min at 4°C, and the supernatant was stored at -80°C for further analysis.

Paw oedema

Mice were injected intraplantarly (i.pl.) with ET-1 (30 pmol per paw) into the right hind paw, following a previously described protocol (Henriques *et al.*, 1987). The contralateral paw was injected with the same volume of sterile PBS and was used as a control. All inflammatory agents were dissolved in sterile PBS before injection. Paw oedema was mea-

sured at 60 min after stimuli using digital plethysmometer (Ugo Basile, Comerio, Italy). LXA₄ (10 ng per paw) was diluted in sterile PBS and concomitantly i.pl. administered with the inflammatory mediators as described by Menezes-de-Lima *et al.* (2006). The final volume injected into the paw (summing agonists and treatments) was never higher than 50 µL. Results are expressed in microlitres, and the difference (Δ) between the right and left paws was taken as oedema volume.

ELISA

Levels of TNF-α and KC/CXCL1 in the knee joint washes were evaluated by sandwich ELISA using matched antibody pairs (Quantikine, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Enzymic immuno-assay for LTB₄ and LXA₄

LTB₄ and LXA₄ levels were evaluated in cell-free synovial washes recovered from zymosan-stimulated C57BL/6 mice after zymosan (500 µg per cavity) stimulation. LTB₄ was assayed by immunosorbent assay (EIA) according to the manufacturer's protocol (Cayman Chemical, Ann Arbor, MI, USA). In the specific case of LXA₄, samples were extracted with C18 Sep-Pak cartridges (Waters, Milford, MA, USA) pre-activated with methanol and deionized water. The column was washed with deionized water followed by petroleum ether. Materials in the ethyl formate eluate were dried under a stream of N₂, and immediately measured by EIA (Neogen, Lexington, KY, USA) according to the manufacturer's instructions.

Real-time RT-PCR

Quantitative PCR (QPCR) was performed as previously described (Verri *et al.*, 2008). Briefly, mice were killed by excessive CO₂ inhalation, 2 h after i.art. injection of zymosan (500 µg per cavity), and knee joint complexes were harvested. Samples were homogenized in Trizol reagent, and total RNA was extracted using the SV Total RNA Isolation System (Promega Biosciences, San Luis Obispo, CA, USA). QPCR was performed in an ABI Prism 7000 Sequence Detection System using SYBR-green fluorescence (Applied Biosystems, Wilmington, NC, USA). The following primers were used: preproET-1, sense: 5'-TGT GTC TAC TTC TGC CAC CT-3', anti-sense: 5'-CAC CAG CTG CTG ATA GAT AC-3'; β-actin, sense: 5'-AGC TGC GTT TTA CAC CCT TT-3', anti-sense: 5'-AAG CCA TGC CAA TGT TGT CT-3'. The expression of β-actin mRNA was used as control for tissue integrity in all samples.

Chemotaxis assay

Neutrophils isolated from C57BL/6 mouse bone marrow were purified by Percoll discontinuous gradients, washed and resuspended in RPMI 1640 supplemented with 10% FBS and then assayed in a 48-well microchemotaxis Boyden chamber (Neuroprobe Inc., Cabin John, MD, USA). The bottom wells of the chamber were filled with 28 μ L of a chemoattractant stimulus, KC (10 nM), ET-1 (1–1000 nM) or RPMI 1640 (control), whereas the upper wells were filled with neutrophils (10^5 cells; 50 μ L) that had been previously incubated with BOC-1 (1 nM) (or medium) for 30 min before LXA₄ (1–100 nM, 60 min), followed by ET-1 (100 nM) stimulation for 60 min (37°C, 5% CO₂). The bottom and upper cells were separated with a 3 μ m polycarbonate filter (Nuclepore, Pleasanton, CA, USA). The chamber was incubated in humidified air with a 5% CO₂ atmosphere at 37°C for 60 min. Cells that migrated completely through the filter were counted under light microscopy ($\times 1000$). Neutrophil chemotaxis was calculated and expressed as the mean number of migrated cells in five random high-power fields per well (in quadruplicate).

Shape change assay

Human peripheral polymorphonuclear leucocytes (PMNLs) were obtained by venepuncture, from male adult healthy volunteers (25–30 years; with full consent), who had taken no systemic medication for at least 72 h before donating blood. About 20 mL of venous blood was taken into 2 mL of sodium citrate anticoagulant (3.8% w/v solution), and neutrophils were isolated and purified by dextran sedimentation followed by Percoll discontinuous gradients, as previously described (Penido *et al.*, 2001). Aliquots of 5×10^5 neutrophils (~90–95% final purity) were incubated in the presence or absence of ET-1 (0.3–10 nM) or KC (0.1 nM) in a 37°C shaking water bath for 6 min, after which the reaction was stopped by the addition of a paraformaldehyde solution (0.25%) at 4°C and placed on ice until analysis. To evaluate the LXA₄ (1–100 nM)-induced shape change, samples were pretreated for 15 min (37°C) before stimulation. Samples were immediately analysed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Acquisition was set using the FL-2 fluorescence channel, through which human neutrophils can be distinguished from eosinophils by means of their auto-fluorescence characteristics. Forward scatter (FSC-H), side scatter (SSC-H) and FL-2 data were saved. Five hundred neutrophils were acquired for each of the triplicate samples. As measurement of shape change, data are reported as

the change in FSC-H compared with buffer-treated cells.

Statistical analysis

Results are reported as the mean \pm SEM, and were statistically analysed by means of ANOVA followed by Newman–Keuls–Student test or Student's *t*-test. Values of $P \leq 0.05$ were regarded as significant.

Materials

PBS, Tween-20, *o*-phenylenediamine dihydrochloride, EDTA sodium salt, Hank's balanced salt solution, BSA, ET-1 and ASA were purchased from Sigma Chemical Co. (St Louis, MO, USA). TNF- α and KC/CXCL1 matched antibody pairs were obtained from R&D Systems. LXA₄ [5(*S*),6(*R*),15(*S*)-trihydroxyeicosa-7-*trans*-9-*trans*-11-*cis*-13-transtetraenoic acid] and BML-111 [5(*S*),6(*R*),7-trihydroxyheptanoic acid methyl ester] were purchased from Cayman Chemical Company. BOC-1 (*N*-*tert*-butoxy-carbonyl-methionyl-leucyl-phenylalanine) was purchased from MP Biomedicals (Solon, OH, USA).

Results

Pretreatment with LXA₄ and the lipoxin receptor agonist BML-111 reduced zymosan-induced articular inflammatory response

Previous reports from our group have demonstrated that i.art. injection of zymosan induced an articular inflammatory response within 6 h, characterized by a significant increase in oedema formation and massive neutrophil influx (approximately 90% of total leucocytes) (Penido *et al.*, 2006; Conte *et al.*, 2008). We examined the anti-inflammatory effects of LXA₄ in this murine model of articular inflammation, and observed that the i.art. pretreatment of mice with all doses of LXA₄ (1, 10 and 20 ng per cavity) clearly reduced zymosan-induced oedema formation at 6 h, achieving a 74% inhibition at 20 ng per cavity (Figure 1A). Interestingly, LXA₄ inhibited zymosan-induced total leucocyte influx (Figure 1B) only at the highest dose, mainly due to the inhibition of neutrophil influx (60% inhibition) as measured by synovial washes at 6 h (Figure 1C).

In order to address the involvement of the FPR2/ALX receptor in the therapeutic effects of exogenous lipoxins in this model, mice received the FPR2/ALX receptor antagonist BOC-1 (20 ng per cavity; i.art.), 30 min prior to LXA₄ treatment (20 ng per cavity; i.art.). As expected, BOC-1 reversed the therapeutic effects of LXA₄ (20 ng per cavity) on zymosan-induced oedema formation (Figure 1D), total

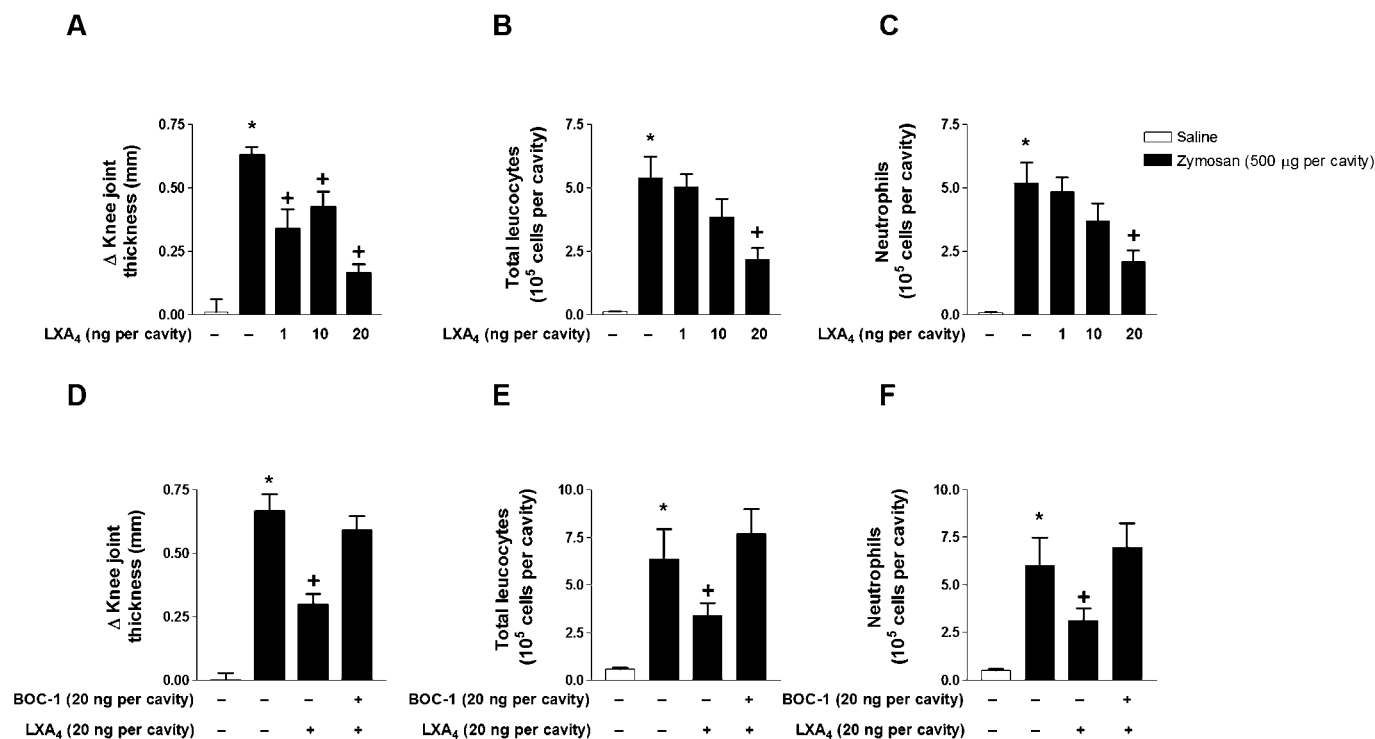


Figure 1

LXA₄ inhibits zymosan-induced oedema formation and neutrophil migration through FPR2/ALX receptors. Pretreatment with exogenous LXA₄ (1–20 ng per cavity; i.art.) 60 min before zymosan (500 µg per cavity; i.art.) stimulation reduced zymosan-induced oedema formation (A), total leucocyte (B) and neutrophil influx (C) in the knee joint cavity of C57BL/6 mice. In another set of experiments, treatment with BOC-1 (20 ng per cavity; i.art., 90 min prior to stimulation) and exogenous LXA₄ (20 ng per cavity; i.art., 60 min prior to stimulation) before i.art. injection of zymosan (500 µg per cavity) prevented the anti-inflammatory actions of LXA₄ on zymosan-induced oedema formation (D), total leucocyte (E) and neutrophil influx (F). Respective control groups were injected with the same volume of sterile saline. Knee joint diameter was evaluated with a digital caliper, and knee synovial cells were recovered 6 h after zymosan stimulation. Results are expressed as the mean ± SEM from at least six animals per group. * $P \leq 0.05$, significant differences between stimulated (zymosan) and non-stimulated groups; + $P \leq 0.05$, significant differences between treated (BOC-1) and untreated groups.

leucocyte (Figure 1E) and neutrophil (Figure 1F) accumulation in synovial washes at 6 h. To confirm the participation of the FPR2/ALX receptor in the anti-inflammatory effects mediated by LXA₄ in zymosan-induced articular inflammation, C57BL/6 mice were pretreated with a LXA₄ receptor agonist, BML-111 (200 ng per cavity; i.art.). This pretreatment significantly inhibited zymosan-induced oedema formation (Figure 2A), total leucocyte (Figure 2B) and neutrophil influx (Figure 2C) into the knee joint cavity.

ASA pretreatment reduced articular zymosan-induced inflammation

In another set of experiments, we addressed the effects of ASA, a non-steroidal anti-inflammatory drug that produces endogenous aspirin-triggered lipoxins (ATLs), during the zymosan-induced knee articular inflammatory response. As demonstrated in Figure 3, ASA (300 mg·kg⁻¹; p.o.) treatment clearly reduced zymosan-induced oedema formation, total leucocyte and neutrophil (Figure 3A–C) influx into

the knee joint cavity of C57BL/6 mice within 6 h compared to the zymosan-stimulated group (50, 68 and 68% inhibition respectively). Interestingly, BOC-1 (20 ng per cavity; i.art.) significantly reversed the anti-inflammatory effects of ASA on zymosan-induced total leucocyte (Figure 3B) and neutrophil (Figure 3C) influx, whereas it did not alter zymosan-induced articular oedema formation (Figure 3A). In addition, pretreatment with BOC-1 (20 ng per cavity; i.art.) alone did not alter zymosan induced-oedema formation and cellular influx. Moreover, as demonstrated in Figure 3D, the i.art. injection of zymosan failed to induce endogenous production of lipoxin within the first 6 h, in contrast with the significant increase in lipoxin production observed at later time-points. The increased lipoxin production at a later time-point (72 h) was accompanied by a significant decrease in zymosan-induced neutrophil influx (at 24 h, 10.75 ± 2.26 ; at 72 h, $1.42 \pm 0.67 \times 10^5$ neutrophils per cavity; $n = 6$, $P \leq 0.05$) and of knee joint swelling (at 24 h, 0.92 ± 0.22 ; at 72 h, 0.30 ± 0.07 Δ mm; $n = 6$, ANOVA, $P \leq 0.05$).

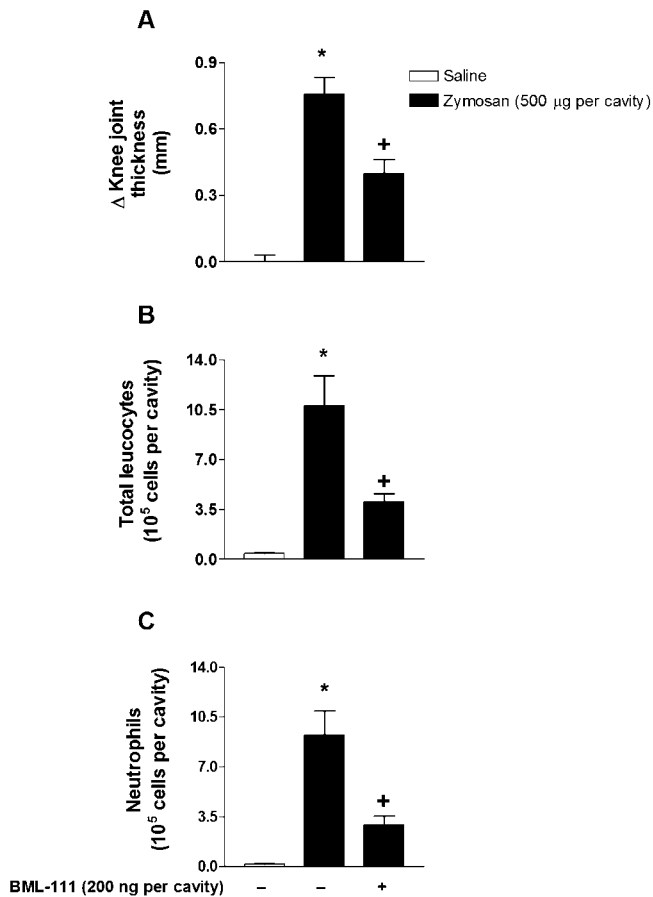


Figure 2

BML-111 inhibits 6 h zymosan-induced oedema formation and neutrophil migration. Pretreatment with BML-111 (200 ng per cavity; i.art.) 60 min before i.art. injection of zymosan (500 μg per cavity) inhibited zymosan-induced oedema formation (A), total leucocyte (B) and neutrophil influx (C) into the knee joint cavity of C57BL/6 mice. The control group was injected with the same volume of sterile saline. Knee joint diameter was evaluated with a digital caliper, and knee synovial cells were recovered 6 h after zymosan stimulation. Results are expressed as the mean ± SEM from at least six animals per group. * $P \leq 0.05$, significant differences between stimulated (zymosan) and non-stimulated groups; + $P \leq 0.05$, significant differences between treated (BML-111) and untreated groups.

Confirming the participation of ATLs acting through FPR2/ALX receptors on zymosan-induced arthritis, treatment of mice with indomethacin (3 mg·kg⁻¹), a non-steroidal anti-inflammatory drug that does not trigger ATL formation, led to a significant reduction of zymosan-induced oedema formation (Figure 3E), whereas no alteration was observed on the total leucocyte influx (Figure 3F). Furthermore, no significant change was observed when BOC-1 (200 ng per cavity) was co-administered to mice that received zymosan or indomethacin (3 mg·kg⁻¹, i.p.).

LXA₄ pretreatment reduced the levels of pro-inflammatory mediators in articular zymosan-injected knee joints

To investigate if LXA₄ exerts its anti-inflammatory effects by modulating the production of inflammatory mediators, we analysed its effects on the expression levels of the ET-1 peptide precursor, preproET-1, mRNA, and the production of KC/CXCL1, LTB₄ and TNF-α. As demonstrated in Figure 4, preproET-1 mRNA expression in articular tissue, as well as TNF-α, KC/CXCL1 and LTB₄ production in synovial washes, was increased after i.art. zymosan injection. *In situ* pretreatment with LXA₄ markedly reduced zymosan-induced preproET-1 mRNA expression, as well as TNF-α, KC/CXCL1 and LTB₄ levels (Figure 4A–D).

LXA₄ impaired human neutrophil activation and chemotaxis in vitro

Our previous results showed that ET-1 triggered neutrophil accumulation *in vivo* (Conte *et al.*, 2008), and data from another group demonstrated ET_{A/B} receptor expression by human neutrophils (Mencarelli *et al.*, 2009). Together, these data led us to assess the effects of LXA₄ on ET-1-induced neutrophil activation and migration *in vitro*. As shown in Figure 5, ET-1 (100 nM) induced a significant increase in neutrophil shape change that was blocked by pretreatment with LXA₄, as demonstrated in the representative dot plots of side scatter (SSC-H) versus forward scatter (FSC-H). As a control for the assays, possible cytotoxic effects were evaluated. ET-1 (1 and 100 nM) or LXA₄ (10 and 100 nM) pretreatment did not induce neutrophil cell death (data not shown). As shown in Figure 6A, ET-1 (1–1000 nM) increased the number of migrating neutrophils compared to basal migration in control wells. In addition, it is noteworthy that ET-1 (100 and 1000 nM) induced higher levels of neutrophil transmigration than KC (10 μM), a rodent chemokine used as positive control. LXA₄ (1–100 nM) *in vitro* pretreatment, 60 min prior to ET-1 (100 nM) stimulation, blocked neutrophil migration in all doses used (Figure 6B). Confirming the *in vitro* participation of FPR2/ALX receptor in LXA₄ anti-inflammatory effects on ET-1-induced neutrophil migration, BOC-1 (30 min before LXA₄ pretreatment) inhibited neutrophil migration induced by ET-1 (Figure 6C).

LXA₄ inhibited ET-1-induced articular and paw inflammation

According to our previous results (Conte *et al.*, 2008), ET-1 (10 pmol per cavity; i.art.) injection induced a significant articular inflammatory

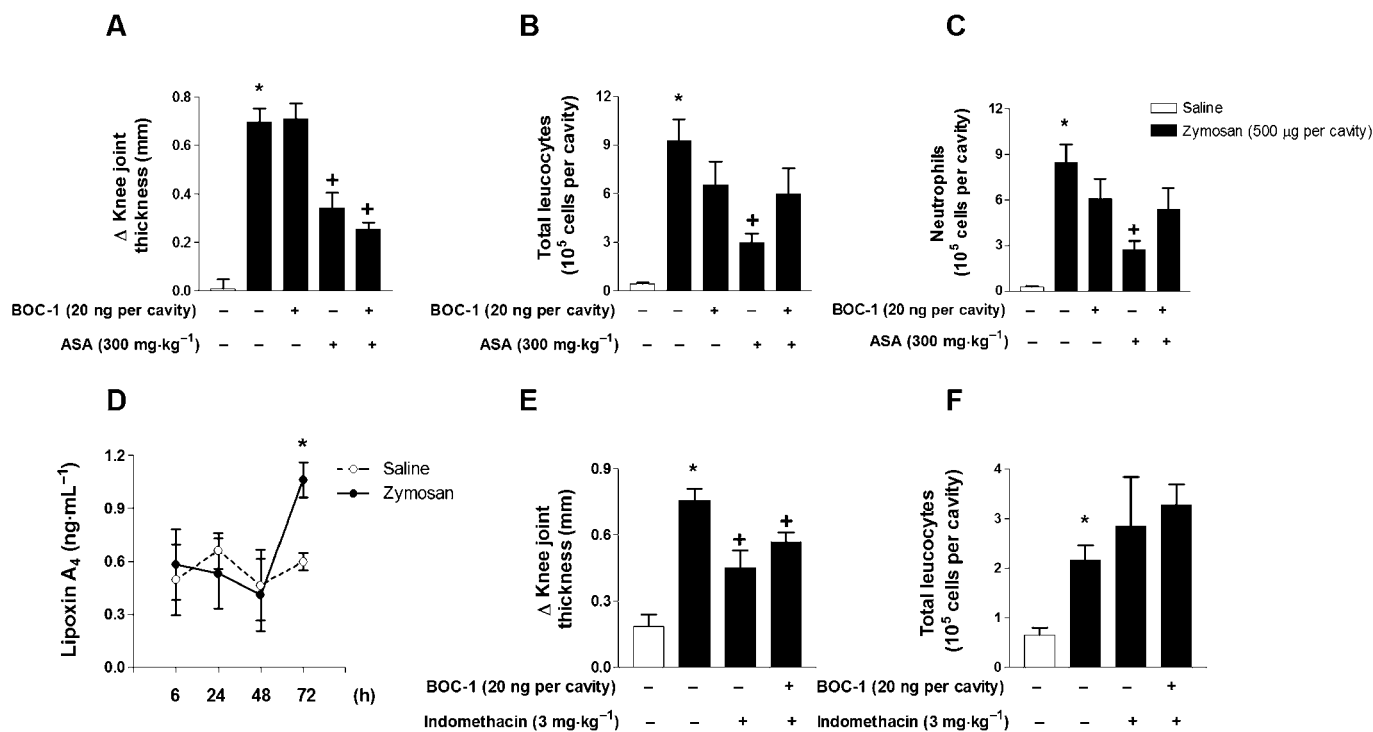


Figure 3

ASA significantly reduces articular neutrophil influx and oedema formation induced by zymosan. Pretreatments with BOC-1 (20 ng per cavity; i.art.) and ASA (300 mg per cavity; p.o.) were performed 60 and 30 min, respectively, prior to zymosan (500 μ g per cavity; i.art.) stimulation. BOC-1 pretreatment blocked the inhibitory actions of ASA on zymosan-induced oedema formation (A), total leucocyte (B) and neutrophil influx (C) into the knee joint cavity of C57BL/6 mice. Zymosan-induced LXA₄ levels in knee synovial washes (D) were determined by EIA 6, 24, 48 and 72 h after stimulation. In another set of experiments, C57BL/6 mice were pretreated with BOC-1 (20 ng per cavity; i.art.) and indomethacin (3 mg·kg⁻¹; p.o.) 60 and 30 min, respectively, prior to zymosan (500 μ g per cavity; i.art.) stimulation. BOC-1 did not reverse the effects of indomethacin pretreatment on zymosan-induced oedema formation (E) and total leucocyte influx (F) within 6 h after stimulation. The control group was injected with the same volume of sterile saline. Knee joint diameter was evaluated with a digital caliper, and knee synovial cells were recovered 6 h after zymosan stimulation. Results are expressed as the mean \pm SEM from at least six animals per group. * $P \leq 0.05$, significant differences between stimulated (zymosan) and non-stimulated groups; + $P \leq 0.05$, significant differences between treated (BOC and ASA) and untreated groups.

response that was characterized by marked articular oedema formation and neutrophil accumulation in synovial washes. LXA₄ (20 ng per cavity) pretreatment significantly reduced ET-1-induced oedema formation (Figure 7A), and corroborating previous published data (Kassuya *et al.*, 2008), we also observed that i.pl. injection of ET-1 (30 pmol per paw) induced marked increase in paw oedema 1 h after stimulation that was significantly inhibited by LXA₄ (20 ng per paw; i.pl.) co-administration (inset). Moreover, LXA₄ (20 ng per cavity) pretreatment significantly decreased ET-1-induced total leucocyte accumulation (Figure 7B), which was mainly due to reduced neutrophil influx (Figure 7C) and TNF- α production (Figure 7D).

LXA₄ post-treatment reduced zymosan-induced articular inflammatory response

Corroborating the results obtained with experiments using pretreated mice, post-treatments with

LXA₄ (5 μ g·kg⁻¹; i.p), BML-111 (5 μ g·kg⁻¹; i.p) and ASA (300 mg·kg⁻¹; p.o.) also reduced oedema formation (Figure 8A), total leucocyte (Figure 8B) and neutrophil influx (Figure 8C) to zymosan-stimulated joints at 10 h.

Discussion

In the present study, we demonstrated that LXA₄ exerted an anti-inflammatory effect on zymosan-induced arthritis through a mechanism that involved activation of FPR2/ALX receptors. We have also shown for the first time that the LXA₄ anti-inflammatory effect on articular inflammation involves the blockade of ET-1 production and action.

Several human arthropathies, including RA, are characterized by significant joint oedema, movement-induced joint hyperalgesia and the infiltration of inflammatory cells (Kraan *et al.*, 2000). In

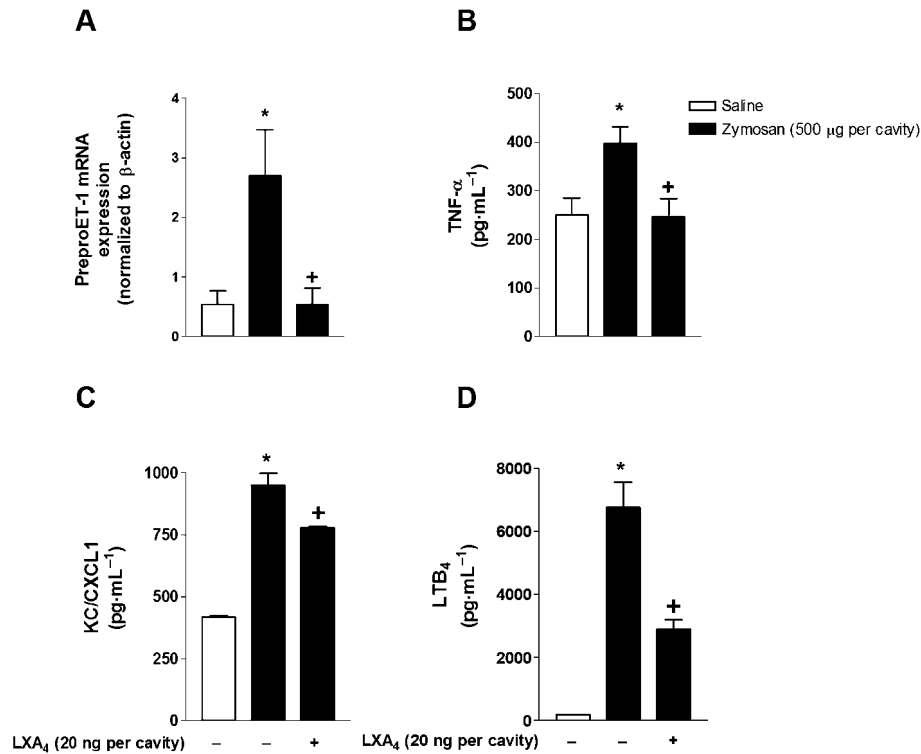


Figure 4

LXA₄ inhibits the zymosan-induced increase of preproET-1 mRNA expression and KC/CXCL1, LTB₄ and TNF-α production. Mice were treated with LXA₄ (20 ng per cavity) 1 h before the i.art. injection of zymosan (500 μg per cavity). After 2 h of zymosan stimulation, samples of the knee joints were collected for real-time PCR analysis (A) (*n* = 5 mice per group, each sample was analysed in triplicate). TNF-α (B), KC/CXCL1 (C) and LTB₄ (D) levels in knee synovial washes were determined 6 h after i.art. injection of zymosan (500 μg per cavity). Results are expressed as the mean ± SEM from at least six animals per group. **P* ≤ 0.05, significant differences between stimulated (zymosan) and non-stimulated groups; +*P* ≤ 0.05, significant differences between treated (LXA₄) and untreated groups.

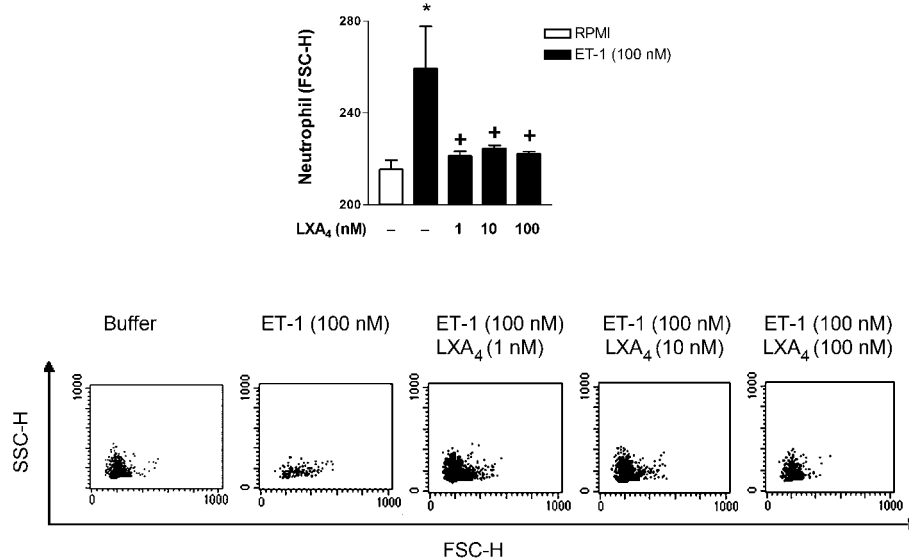


Figure 5

LXA₄ blocks ET-1-induced human neutrophil shape change *in vitro*. Human polymorphonuclear leucocytes purified from peripheral venous blood were pretreated *in vitro* with LXA₄ (1–100 nM) 15 min before ET-1(100 nM)-induced neutrophil shape change. Representative dot plots of FSC-H versus SSC-H of human neutrophils exposed to LXA₄ pretreatment 15 min before the ET-1-induced increase in forward scatter characteristics. Control groups were treated with RPMI under the same conditions. Results are expressed as mean of FSC-H, and data represent the mean ± S.E.M. from at least quadruplicate wells per group of two separate experiments. **P* ≤ 0.05, significant differences between stimulated (ET-1) and non-stimulated groups; +*P* ≤ 0.05, significant differences between treated (LXA₄) and untreated groups.

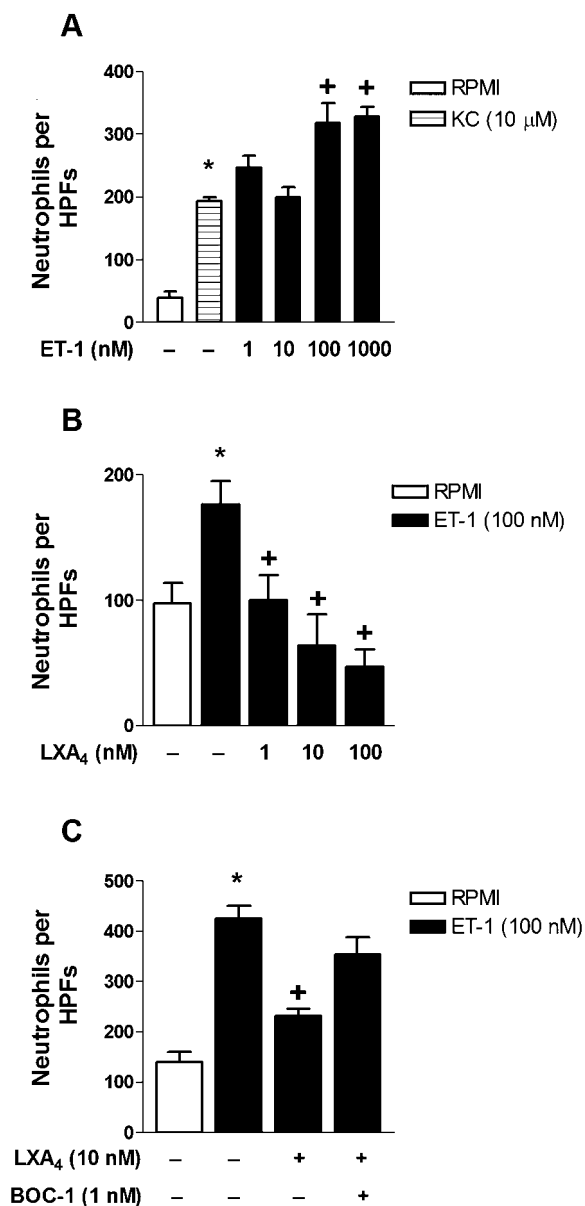


Figure 6

LXA₄ inhibits ET-1-induced murine neutrophil chemotaxis. *In vitro* stimulation with KC (10 μM) or ET-1 (1–1000 nM), for 60 min at 37°C, induced murine bone marrow-derived neutrophil chemotaxis (A). *In vitro* pretreatment with LXA₄ (1–100 nM; 60 min prior to stimulation) inhibited ET-1(100 nM)-induced chemotaxis at all doses used (B). BOC-1 (1 nM; 90 min prior to stimulation) pretreatment reversed LXA₄ inhibition of neutrophil chemotaxis induced by ET-1 (C). Control groups were treated with RPMI under the same conditions. Results are expressed as numbers of neutrophils per high-power fields (HPFs) per well. Data represent the mean ± SEM from at least quadruplicate wells per group of one out of two separate experiments. * $P \leq 0.05$, significant differences between stimulated (ET-1, KC) and non-stimulated groups; + $P \leq 0.05$, significant differences between treated (LXA₄, BOC-1) and untreated groups; ANOVA followed by a Newman–Keuls–Student test.

this regard, the role of neutrophils in the pathogenesis of tissue lesions in arthritis has long been recognized (Jonsson *et al.*, 2005). Previous work from our group has demonstrated that i.art. administration of zymosan into mouse knee joints induced marked acute oedema formation, hypernociception and significant leucocyte influx, mainly due to neutrophil accumulation in knee synovial washes (Penido *et al.*, 2006; Conte *et al.*, 2008; Guerrero *et al.*, 2008), which correspond to features of human RA articular inflammation. Moreover, in accordance with RA clinical data, this murine model was also characterized by a significant increase of important pro-inflammatory mediators, such as ETs, TNF- α and LTB₄, that have pivotal roles in articular oedema formation and leucocyte accumulation (Conte *et al.*, 2008).

Among the mediators chronically produced in the joints of RA patients, LXA₄ and ATL have been shown to be increased when compared to osteoarthritis, a phenomenon that occurs in parallel to increased expression of FPR2/ALX receptors in the RA synovium (Hashimoto *et al.*, 2007). Interestingly, LXA₄ and ATLs are described as endogenous 'stop signals' that regulate chronic leucocyte trafficking and neutrophil activity, and promote resolution of the inflammatory process (Takano *et al.*, 1997). Such effects have been demonstrated to be mediated by FPR2/ALX receptors (Takano *et al.*, 1997; Devchand *et al.*, 2003; Chiang *et al.*, 2006) in several inflammatory models (McMahon and Godson, 2004), and, more recently, experimental evidence suggests a protective role for 12/15-lipoxygenase products (including LXA₄) in experimental models of arthritis (Kronke *et al.*, 2009). It is noteworthy that we observed, in an experimental model of zymosan-induced arthritis, an increase in LXA₄ production at later time-points, which was accompanied by a significant amelioration of articular inflammation. This is additional evidence of the anti-inflammatory and pro-resolution actions of LXA₄. Moreover, Zhang *et al.* (2008) have reported the therapeutic effects of BML-111 (FPR2/ALX receptor agonist) in collagen-induced arthritis. In line with these reports, the present study clearly demonstrates that LXA₄, as well as BML-111, markedly reduced articular zymosan-induced neutrophil accumulation through an FPR2/ALX receptor-operated mechanism, because it is reversed by BOC-1 treatment. It is noteworthy that, in mice, BOC-1 antagonizes both FPR2/ALX and FPR1 receptors; however, in this species, LXA₄ has also been shown to bind both of these receptors (Takano *et al.*, 1997; Vaughn *et al.*, 2002; Ye *et al.*, 2009).

The finding that ASA treatment markedly reduced zymosan-induced total leucocyte influx,

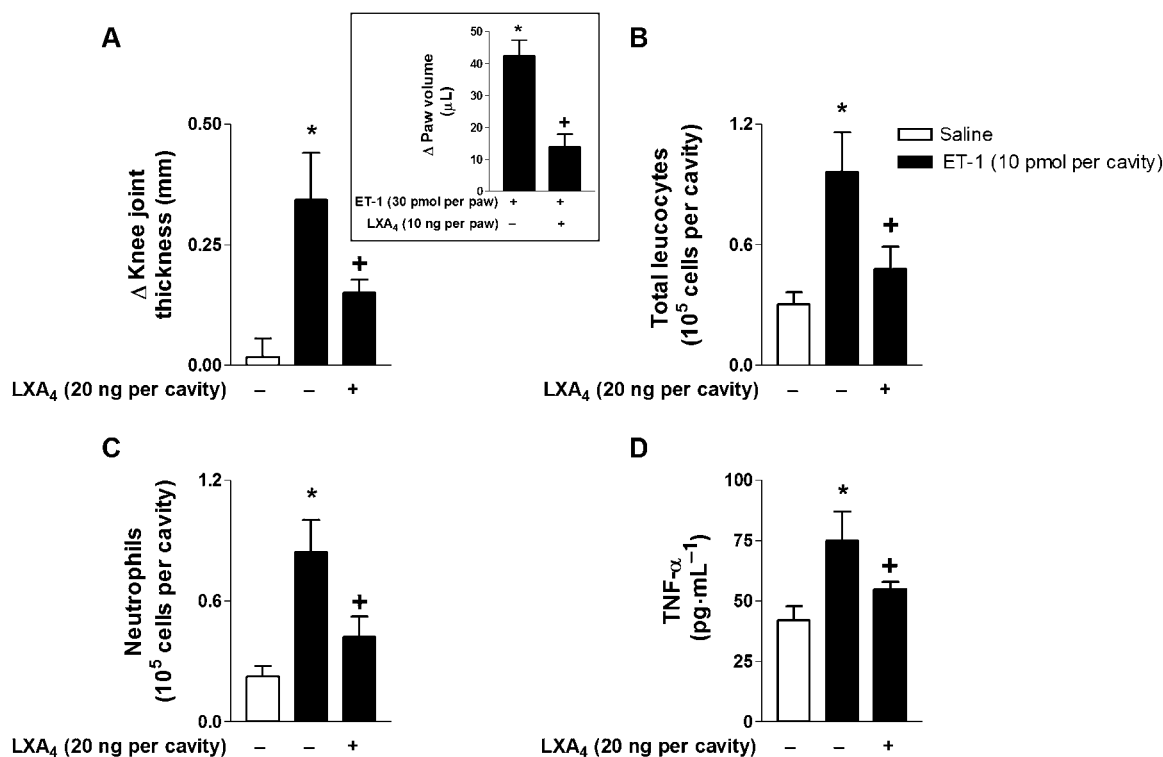


Figure 7

ET-1-induced articular and paw inflammation are impaired by LXA₄ pretreatment. LXA₄ (20 ng per cavity; i.art.) pretreatment 60 min prior to ET-1 (10 pmol per cavity; i.art.) stimulation reduced ET-1-induced oedema formation (A), total leucocyte (B) and neutrophil influx (C) into the knee joint cavity of C57BL/6 mice. Respective control groups were injected with the same volume of sterile saline. Knee joint diameter was evaluated with a digital caliper, and knee synovial cells were recovered 6 h after ET-1 stimulation. The inset shows that paw oedema induced by ET-1 (30 pmol per paw; i.pl.) was inhibited by LXA₄ (20 ng per paw; i.pl.) pretreatment. Values represent the differences of volume (in microlitres) between ET-1- and vehicle-injected paws 60 min after stimulation. Results are expressed as the mean ± SEM from at least five animals per group. **P* ≤ 0.05, significant differences between stimulated (ET-1) and non-stimulated groups; †*P* ≤ 0.05, significant differences between treated (LXA₄) and untreated groups; ANOVA followed by Newman–Keuls–Student test (A–D) or Student’s *t*-test (inset).

mainly due to decreased neutrophil trafficking into the knee synovium, suggests that ASA treatment leads to endogenous ATL production *in vivo* that triggers FPR2/ALX receptor-orchestrated mechanisms, culminating in reduced leucocyte trafficking. This conclusion is supported by the reversal of ASA anti-inflammatory effects on zymosan-induced leucocyte influx induced by BOC-1 (FPR2/ALX receptor antagonist), as well as by the lack of effects of indomethacin on zymosan-induced total leucocyte accumulation in knee synovial washes, in the presence or absence of BOC-1. Together, these results support the proposition that ASA treatment induced ATL production, a phenomenon that is not triggered by other NSAIDs.

Previous reports demonstrated that LXA₄ and its analogues inhibited plasma leakage in parallel to the impairment of granulocyte migration (Bandeira-Melo *et al.*, 2000; Jin *et al.*, 2007). The effects of lipoxin on plasma leakage have commonly been considered an indirect consequence of the reduction of neutrophil adhesion and transmigration

induced by such lipid mediators (Bandeira-Melo *et al.*, 2000). However, subsequent investigation has revealed that the potent anti-oedematogenic effect of LXA₄ occurred independently of leucocyte accumulation, which suggested that LXA₄ might directly affect endothelial cells (Menezes-de-Lima *et al.*, 2006). Interestingly, our results suggest that LXA₄ inhibits articular plasma leakage independently of neutrophil migration, because LXA₄ significantly inhibited zymosan-induced oedema formation in all doses used, but did not reduce articular neutrophil influx at 1 or 10 ng per cavity. In addition, supporting this notion, we demonstrated that acute paw oedema induced by ET-1 was significantly reduced by LXA₄. Moreover, our data show that the anti-oedematogenic effects of ASA seem to be related to the blockade of cyclooxygenase and arachidonic acid derivatives, rather than by ATL binding to FPR2/ALX receptors. This possibility was supported by the fact that the anti-oedematogenic effects of ASA and indomethacin were not altered by BOC-1 pretreatment. In line with this hypothesis, LXA₄ and

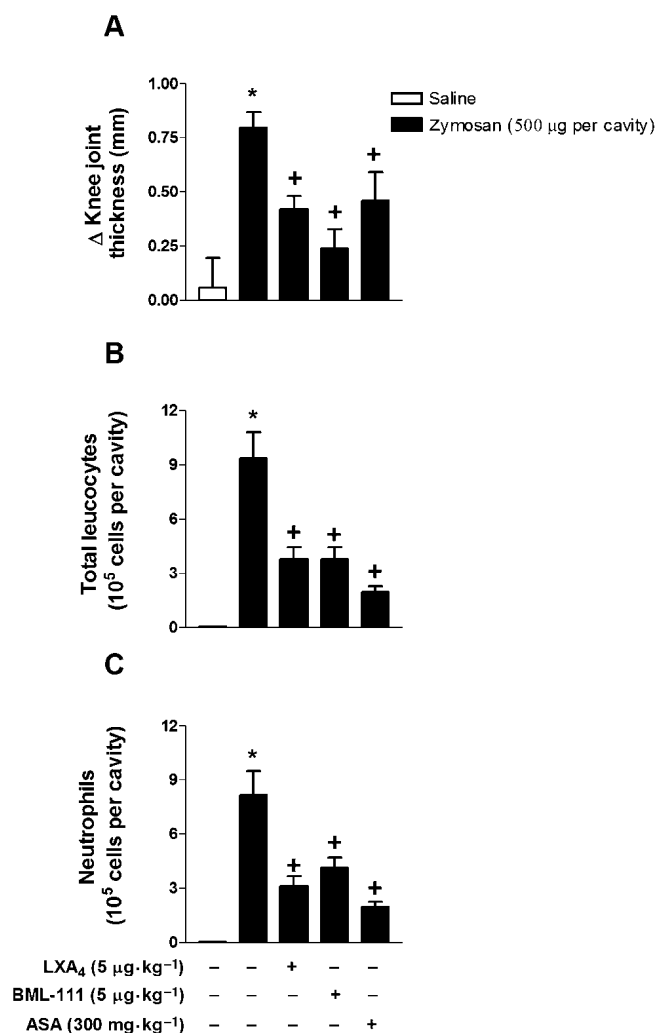


Figure 8

LXA₄, BML-111 and ASA post-treatments inhibited articular inflammation induced by zymosan stimulation. *In vivo* administration of LXA₄ (5 μg·kg⁻¹; i.p.), BML-111 (5 μg·kg⁻¹; i.p.) or ASA (300 mg·kg⁻¹; p.o.) 1 and 6 h following i.art. injection of zymosan (500 μg per cavity; i.art.) reduced oedema formation (A), total leucocyte (B) and neutrophil influx (C) into the knee joint cavity of C57BL/6 mice. The control group received the same volume of sterile saline. Knee joint diameter and knee synovial cell accumulation were analysed 10 h after zymosan stimulation. Results are expressed as the mean ± SEM from at least six animals per group. **P* ≤ 0.05, significant differences between stimulated (zymosan) and non-stimulated groups; +*P* ≤ 0.05, significant differences between treated (LXA₄, BML-111, ASA) and untreated groups; ANOVA followed by Newman–Keuls–Student test.

ATL also directly interacted with vascular CysLT1 receptors, functioning as antagonists (Gronert *et al.*, 2001), in addition to activating FPR2/ALX receptors on leucocytes. This finding could partially explain why BOC-1 did not affect the inhibition by LXA₄ of vascular leakage. In addition, other LXA₄-independent enzymic pathways have been described to be involved in the anti-oedematogenic

effects of ASA. These effects can be triggered by other lipid mediators capable of controlling the resolution of inflammatory response, such as resolvins and protectins, and remain to be investigated (Yacoubian and Serhan, 2007).

Overall, the mechanisms involved in LXA₄ anti-inflammatory effects seem to depend on the blockade of articular pro-inflammatory mediators, including ET-1, TNF-α, CXCL1 and LTB₄. Indeed, we have previously demonstrated that ETs play a crucial pro-inflammatory role in articular inflammation, modulating these mediators (Conte *et al.*, 2008). We additionally demonstrated for the first time that ET-1-induced articular inflammation was prevented by LXA₄ through a mechanism that involves the impairment of TNF-α production. In accordance with this, TNF-α-based therapies are currently clinically employed to treat RA patients. However, some RA patients do not respond successfully to these treatments. New therapeutic targets for the treatment of RA include LTB₄ and its receptors (Mathis *et al.*, 2007), supporting our data that the beneficial effects of LXA₄ also involve the impairment of LTB₄ production in parallel with reduced CXCL1 levels and acute neutrophil accumulation. Moreover, in contrast to current TNF-targeting therapies used in RA (soluble receptors and anti-TNF antibodies), LXA₄ inhibits the production of TNF-α and other mediators. These data therefore suggest that LXA₄ treatment might present additive effects to current anti-TNF-α therapies or at least allow for the use of lower doses of anti-TNF therapies, if used in association with LXA₄, to achieve better clinical outcomes. LXA₄ decreased *in vitro* neutrophil chemotaxis, adherence and transmigration across the vascular endothelium and epithelium (McMahon and Godson, 2004; Filep *et al.*, 2005), CD11b/CD18 expression (Filep *et al.*, 1999) and blocked superoxide generation (Levy *et al.*, 1999) and TNF-α production and actions (Hachicha *et al.*, 1999). Previous *in vitro* data demonstrate that ETs cause direct neutrophil activation (Toffoli *et al.*, 2007), adhesion (Zouki *et al.*, 1999) and migration *in vitro* (Elferink and de Koster, 1994). Interestingly, in the present study, we showed that LXA₄ seemed to directly impair ET-1-induced neutrophil activation and migration through a mechanism that needs further elucidation. Fierro *et al.* (2003) reported that the exposure of PMNLs to LXA₄ and 15-epi-LXA₄ markedly decreased LTB₄-induced transmigration across both human microvessel endothelial and epithelial cells, reinforcing the notion that lipoxins act directly on neutrophils, impairing their activation and transmigration. It is interesting to note that post-treatment with LXA₄ also reduced the established articular inflammation, suggesting that this lipid mediator

might be useful as a putative new anti-inflammatory drug.

Taken together, these results demonstrated a significant anti-inflammatory effect of LXA₄, acting via FPR2/ALX receptors, in a murine model of articular inflammation. Additionally, our data contribute to the understanding of the anti-inflammatory mechanisms mediated by LXA₄ that involve the blockade of ET-1 pro-inflammatory production and effects, and highlight the importance of ETs in the context of articular inflammation. Moreover, our data indicate that LXA₄, ATLs and other FPR2/ALX receptor agonists can represent promising therapeutic tools for the treatment of RA and other articular inflammatory diseases.

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Conflict of interest

There is no conflict of interest to declare.

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