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Modulation of Voltage-Dependent and Inward Rectifier Potassium Channels by 15-Epi-Lipoxin-A4 in Activated Murine Macrophages: Implications in Innate Immunity

Cristina Moreno,*1 Patricia Prieto,*1 Álvaro Macías,*1 Maria Pimentel-Santillana,*1 Alicia de la Cruz,* Paqui G. Través,* Lisardo Boscá,*+ and Carmen Valenzuela*

Potassium channels modulate macrophage physiology. Blockade of voltage-dependent potassium channels (Kv) by specific antagonists decreases macrophage cytokine production and inhibits proliferation. In the presence of aspirin, acetylated cyclooxygenase-2 loses the activity required to synthesize PGs but maintains the oxygenase activity to produce 15R-HETE from arachidonate. This intermediate product is transformed via 5-LOX into epimeric lipoxins, termed 15-epi-lipoxins (15-epi-lipoxin A4 [e-LXA4]). Kv have been proposed as anti-inflammatory targets. Therefore, we studied the effects of e-LXA4 on signaling and on Kv, and inward rectifier potassium channels (Kir), in mice bone marrow–derived macrophages (BMDM). Electrophysiological recordings were performed in these cells by the whole-cell patch-clamp technique. Treatment of BMDM with e-LXA4 significantly reverted LPS effects on Kv and Kir currents. Under these conditions, e-LXA4 decreased the calcium influx versus that observed in LPS-stimulated BMDM. These effects were partially mediated via the lipoxin receptor (ALX), because they were significantly reverted by a selective ALX receptor antagonist. We provide evidence for a new mechanism by which e-LXA4 contributes to inflammation resolution, consisting of the reversion of LPS effects on Kv, and Kir currents in macrophages. The Journal of Immunology, 2013, 191: 6136–6146.

Macrophages play an important role in the inflammatory response, acting as APCs and modifying the cytokine milieu and intensity of T cell signaling. Therefore, macrophages may tune the immune response toward inflammation or tolerance (1–4). The proliferation, activation, and resolution or tolerance of immune cells is mainly modulated by membrane transduction of extracellular signals. Some of these interactions involve changes in transmembrane ion fluxes that, in turn, modulate the network of intracellular signaling (i.e., Ca2+ fluxes). The electrophysiological properties of macrophages depend on their state of functional activation (5). Indeed, changes in membrane potential that occur as a consequence of the modulation of ion channels are among the earliest events after macrophage activation (6, 7).

Macrophages display different activation programs depending on the stimuli received (1, 2, 8). The classic and innate activation can be induced in vitro after challenge of macrophages with IFN-γ + LPS and is associated with high microbial activity, proinflammatory cytokine release, and reactive oxygen and nitrogen species production. The alternative activation can be mimicked in vitro after culture with IL-4, IL-13, glucocorticoids, immune complexes, or IL-10 and is associated with phagocytosis, tissue repair, tumor progression, and humoral immunity (9, 10). IL-13 and, to a lesser extent IL-4, regulate the expression of the lipoxin receptor (ALX) in human enterocytes. Moreover, lipoxin A4 (LXA4) and 15-epi-LXA4 (e-LXA4) are potent inhibitors of agonist-induced IL-8 secretion by enterocytes (11). Importantly, inflammation is involved in several cardiovascular pathologies, such as atherosclerosis, atrial fibrillation, myocardial infarction, and heart failure (12–14).

Consistent data indicate that voltage-dependent potassium channels (Kv) play a pivotal role in the regulation of macrophage immunomodulatory responses: Kv are tightly regulated during proliferation and activation in macrophages, and their functional activity is important for cellular responses (5, 15, 16). Proliferation and innate activation trigger the induction of the Kv outward current, and, in parallel, a decrease of the inward rectifier...
potassium channels (K\textsubscript{v}) 2.1 current, whereas alternative activation downregulates K\textsubscript{v} current (5, 15, 16). Experimental evidence indicates that in macrophages, the major K\textsubscript{v} is a heterotetramer K\textsubscript{1.3}/K\textsubscript{1.5} channel (15, 16). Innate activation changes the stoichiometry of these channels, increasing the K\textsubscript{1.3}/K\textsubscript{1.5} ratio, whereas alternative activation decreases it (15, 16). However, less is known regarding the role of these channels in the resolution phase. Lipoxins are endogenous eicosanoids released during the resolution phase of inflammation (17–21), and they might modulate K\textsubscript{v} activity. The potent anti-inflammatory and proresolving actions of these lipids have been demonstrated in multiple animal models of human diseases (22, 23). Both native LXA\textsubscript{4} and e-LXA\textsubscript{4} (20) bind to and activate the ALX receptor and decrease polymorphonuclear cells infiltration in rodent models of acute peritonitis (24). The endogenous protective role of ALX mediating the biological actions of lipoxins has been demonstrated in mice overexpressing the human ALX in myeloid cells (25). A recent study in which vascular inflammation in wild-type and ALX−/− mice was compared demonstrates that ALX activation controls platelet/neutrophil aggregates to afford LXA\textsubscript{4} synthesis, inhibiting vascular inflammation on reperfusion, at the time that aspirin can jumpstart this circuit by triggering e-LXA\textsubscript{4} synthesis (26). In the present work, we have studied the effects of e-LXA\textsubscript{4} on K\textsubscript{v} and K\textsubscript{ir} activity. The potent anti-inflammatory and proresolving actions of these lipids have been demonstrated in multiple animal models of human diseases (22, 23). Both native LXA\textsubscript{4} and e-LXA\textsubscript{4} (20) bind to and activate the ALX receptor and decrease polymorphonuclear cells infiltration in rodent models of acute peritonitis (24). The endogenous protective role of ALX mediating the biological actions of lipoxins has been demonstrated in mice overexpressing the human ALX in myeloid cells (25). A recent study in which vascular inflammation in wild-type and ALX−/− mice was compared demonstrates that ALX activation controls platelet/neutrophil aggregates to afford LXA\textsubscript{4} synthesis, inhibiting vascular inflammation on reperfusion, at the time that aspirin can jumpstart this circuit by triggering e-LXA\textsubscript{4} synthesis (26). In the present work, we have studied the effects of e-LXA\textsubscript{4} on K\textsubscript{v} and K\textsubscript{ir} in bone marrow–derived macrophages (BMDM) under different activation conditions. Our data show that e-LXA\textsubscript{4} partially inhibits NF-\kappaB activity, protects against LPS-dependent apoptosis, and exerts rapid and specific effects on K\textsubscript{v} and K\textsubscript{ir} currents, compatible with attenuation of the inflammatory response.

Materials and Methods

Chemicals

LPS was from Invivogen (San Diego, CA), and e-LXA\textsubscript{4} was from Cayman Chemical (Ann Arbor, MI). e-LXA\textsubscript{4} (55,6R,15R-trihydroxy-7,9,13-trans-11 cis-eicosatetraenoic acid) was dissolved in ethanol and stored protected from light at −80°C to prevent oxidation. Aliquots of a volume and concentration desired for each experimental condition were prepared. Other reagents were from Sigma–Aldrich (St. Louis, MO), Roche (Basel, Switzerland), Invitrogen (Carlsbad, CA), or Merck (Darmstadt, Germany). Cytoines were from PeproTech (Rocky Hill, NJ). Commercial Abs were from Santa Cruz Biotechnology (Santa Cruz, CA), Cell Signaling Technology (Danvers, MA), Abcam (Cambridge, U.K.), R&D Systems (Minneapolis, MN), Sigma–Aldrich, Alomone (Jerusalem, Israel), or PeproTech. Serum and culture media were from BioWhittaker (Walkersville, MD).

Cell culture and transfection

K\textsubscript{1.5} was cloned into a modified pBK plasmid (gift from Dr. M.M. Tamkun, Colorado State University, Fort Collins, CO). K\textsubscript{2.1} was cloned into pEFPP-C1 (gift from Dr. A. Felipe, Universidad de Barcelona, Barcelona, Spain), and K\textsubscript{2.1} was cloned into pcDNA3.1 (gift from Dr. E. Delpón, Universidad Complutense de Madrid, Madrid, Spain). HEK293 cells were cultured in DMEM supplemented with 10% FBS and antibiotics (Sigma–Aldrich). Transfection of K\textsubscript{1.5} (0.5 μg), K\textsubscript{1.3} (0.2 μg), or K\textsubscript{2.1} (0.5 μg) and CD8 reporter plasmid (1.6 μg) was performed by using Fugene-6 in a 3:1 ratio (μl/μg). Before experimental use, cells were incubated with polystyrene microbeads precoated with anti-CD8 Ab (Dynabeads M450, Dynal), as previously described (27).

Treatment of animals and preparation of BMDM

BALB/c mice were housed and bred in our pathogen-free facility. Animal care and experimental procedures were performed according to the 2010/63/ EU Directive of the European Parliament and approved by the Institutional Committee on Bioethics (authorization 28079-37A to the Instituto de Investigaciones Biomédicas). Total bone marrow was obtained by flushing pelvices, femurs, and tibiae with DMEM. BMDM were prepared as follows: bone marrow mononuclear phagocytic precursor cells were propagated in suspension by culturing in DMEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.2 mM recombinant murine M-CSF (PeproTech) in tissue-culture plates. The precursor cells became adherent within 7 d of culture. For priming of BMDM, the cells were maintained in DMEM medium supplemented with 10% FBS for 14 h prior to use. Experiments were carried out in phenol-red free DMEM medium and 2% of heat-inactivated FBS plus antibiotics.

Flow cytometry

Cells were harvested and washed in cold PBS. After centrifugation at 4°C for 5 min and 1000 × g, cells were resuspended in Annexin V binding buffer (10 mM HEPES [pH 7.4], 140 mM NaCl, and 2.5 mM CaCl\textsubscript{2}). Cells were labeled with Annexin V–FITC (BD Pharmingen, San Jose, CA) and/or propidium iodide (PI; 100 μg/ml) for 15 min at room temperature in the dark. PI is impermeable to living and apoptotic cells but stains necrotic and apoptotic dying cells with impaired membrane integrity in contrast to Annexin V, which stains early apoptotic cells (28).

Determination of intracellular calcium concentration

Ca\textsuperscript{2+} concentration was measured in BMDM cells seeded at 50% confluence over coverslips (9 mm) and loaded with fura 2-AM (final concentration 5 μM) at 37°C for 30 min in serum-free medium containing 2 μM/ml pluronic to favor the dispersion of the probe. After loading, the cells were washed twice with PBS and kept in 1 ml phenol red–free RPMI 1640 medium containing 2 mg/ml fatty acid–free BSA. The fluorescence was recorded at 510 nm in an LS50B Perkin–Elmer spectrofluorometer (Perkin–Elmer, Norwalk, CT) provided with continuous stirring and using a dual excitation source at 340 and 380 nm. The maximal and minimal fluorescence of the assay were determined for each sample after addition of 1% SDS and 5 mM EGTA (pH 9), respectively (29).

Preparation of cell extracts

BMDM cells (six-well dishes) were washed twice with ice-cold PBS and homogenized in 0.2 ml buffer A (10 mM Tris-HCl [pH 7.5], 1 mM MgCl\textsubscript{2}, 1 mM EGTA, 10% glycerol, 0.5% CHAPS, 1 mM 2-ME, 0.1 mM PMSF, and a protease and phosphatase inhibitor mixture [Sigma–Aldrich]). The extracts were vortexed for 30 min at 4°C and centrifuged for 15 min at 13,000 × g. The supernatants were stored at −20°C. Proteins levels were determined using the Bio-Rad detergent-compatible protein reagent (Bio-Rad, Richmond, CA). All steps were carried out at 4°C.

Western blot analysis

Samples of cell extracts containing equal amounts of protein (30 μg/lane) were boiled in 250 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 2% 2-ME and size-separated in 10–15% SDS-PAGE. The gels were blotted onto a polyvinylidene difluoride membrane (GE Healthcare) and processed as recommended by the supplier of the Abs against the murine Ags: NO synthase-2 (NOS-2; sc-7271), cyclooxygenase (COX)-2 (sc-1747), heme oxygenase-1 (HO-1; AB-1284; Millipore), Arg-1 (sc-20150), Arg-2 (sc-20151), p65 (3034; Cell Signaling Technology), Nrf-2 (sc-13032), IκBα (sc-371), p-IκBα (2859;Cell Signaling Technology), p-IκB kinase (IKKβ; 2681; Cell Signaling Technology), iICOS (sc-371i), p-IκBα (2859;Cell Signaling Technology), and β-actin (A-5441). The blots were developed by ECL protocol (GE Healthcare), and different exposition times were performed for each blot with a charged coupling device camera in a luminescent image analyzer (Molecular Imager; Bio-Rad) to ensure the linearity of the band intensities.

Immunofluorescence of K\textsubscript{v} and K\textsubscript{ir} proteins

Cells were seeded into sterile eight-well Chamber Slides (Falcon, Lincoln Park, NJ) and activated for the indicated times. After fixation for 10 min with 2% paraformaldehyde, cells were then permeabilized in ice methyl and incubated with 3% BSA for 30 min. After incubation with Abs against Kv\textsubscript{a} or Kv\textsubscript{w} at 4°C for 18 h, cells were washed with PBS followed by incubation with Alexa 488 anti-rabbit secondary Ab at 4°C for 1 h at room temperature (1:500; Molecular Probes) and Hoechst 33342. Coverslips were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and examined using an Espectral Leica TCS SP5 confocal microscope (Leica Microsystems). Fluorescence intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Measurement of IKK\textsubscript{β} activity

To determine the direct effect of e-LXA\textsubscript{4} in vitro, IKK\textsubscript{β} activity was measured by homogeneous time-resolved fluorescence assay using cloned and expressed IKK\textsubscript{β} and biotinylated IκBα (aa 28–40) as substrate. Fluorescence (excitation at 330 nm and emission at 615 and 665 nm) was recorded after addition of europium cryptate p-Ab recognizing the S32/S36 phosphorylation site.
phosphorylation peptide and streptavidin-XL665. Inhibition of IKKβ by staurosporine was used as control (30).

RNA isolation and RT-PCR analysis
A total of 1 μg total RNA, extracted with TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions, was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit for RT-PCR following the indications of the manufacturer (Roche). Real-time PCR was conducted using specific TaqMan probes on a MyiQ Real-Time PCR System (Bio-Rad). PCR thermocycling parameters were 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min (31). All samples were analyzed for 36B4 expression in parallel as housekeeping control. Each sample was run in duplicate and normalized to 36B4.

Electrophysiological recordings
Whole-cell currents were measured using the patch clamp technique with an Axopatch 200B amplifier (Axon Instruments) and stored on a personal computer with an analog-to-digital converter DigiData 1400A (Axon Instruments). pClamp version 9 software (Axon Instruments) was used for data acquisition and analysis. Currents were recorded at 21–23°C and stimulation frequency of 0.1 Hz and sampled at 4 kHz after anti-alias filtering at 2 kHz. Patch electrodes of 2–4 MΩ were fabricated in a P-87 puller (Sutter Instruments) from borosilicate glass capillary tubes (GD-1; Narishige). Gigohm seal formation was achieved by suction (2–5 GΩ; n = 60). After seal formation, cells were lifted from the bath, and the membrane patch was ruptured with a brief additional suction. The capacitive transients elicited by symmetrical 5-mV steps from −80 mV were recorded at 50 kHz and filtered at 10 kHz for subsequent calculations of capacitative surface area, access resistance, and input impedance. Thereafter, capacitance and series resistance compensation were optimized, and, usually, 80% compensation of the effective access resistance was obtained. Electrodes were filled with an intracellular pipette solution containing (mM): K-aspartate 80, KCl 50, phosphocreatine 3, KH2PO4 10, MgATP 3, HEPES-K 10, and EGTA-K 5 and was adjusted to pH 7.25 with KOH. The bath solution contained (mM): NaCl 130, KCl 4, CaCl2 1.8, MgCl2 1, HEPES 10, and glucose 10 and was adjusted to pH 7.4 with NaOH. Uncompensated series resistances were 4–8 MΩ. As currents evoked were <1 nA, voltage errors from uncompensated series resistance were <2 mV. Inactivation was fit to an exponential process using an equation of the form: \( y = A \exp \left( -\frac{V}{\tau} \right) + C \), where \( \tau \) is the system time constant, \( A \) is the amplitude, and \( C \) is the baseline value.

Statistics
Data are expressed as the mean ± SEM. The significance of differences was established by Student t test or Pearson product-moment correlation coefficient with a two-tailed p value (Prism 4.0; GraphPad) when indicated. The curve-fitting procedure used a nonlinear least-squares (Gauss-Newton) algorithm; results were displayed in linear format. Goodness of fit was judged by the \( F \) criterion and by inspection for systematic nonrandom trends in the difference plot. A p value < 0.05 was considered significant.

Results
Macrophage profiling upon activation
BMDM were challenged with LPS or IL-4/IL-13, and the expression of genes characteristics of the LPS (NOS-2, COX-2, and IP-10: M1 condition (in the absence of stimuli), and of genes characteristics of each pathway (A) was determined by Western blot. The extent of apoptosis/necrosis under these conditions was determined by the staining with Annexin V and PI, respectively (B). Cells were treated with e-LXA4 (200 nM) for 30 min prior the challenge with LPS (100 ng/ml), and the effect of e-LXA4 on apoptosis/necrosis in LPS-activated BMDM was determined (C). The activation of IKK (D) and the presence of p65 and Nrf-2 in the nuclei of BMDM (E) were determined at 0–120 min. The effect of e-LXA4 (200 nM) on the protein levels of NOS-2, COX-2, HO-1, Arg-1, and Arg-2 were determined at 10–30 h (F). Band intensities were normalized for content in β-actin (D, F) or β-tubulin (E), and the ratios p-IKK/IKK and p-IκBα/IκBα (D), and the p65, Nrf-2 (E), NOS-2, COX-2, and HO-1 levels (F) were represented as percentage over the maximal value in each ratio or lane. e-LXA4 inhibited IKKβ activity in homogeneous time-resolved fluorescence in vitro assays, using staurosporine (Stauro) as control inhibitor (G). Results show a representative experiment out of four (A, D–F) or the mean ± SEM of three experiments. *p < 0.05. **p < 0.01 versus the control condition (in the absence of stimuli), +p < 0.05 versus values obtained in LPS-activated BMDM cells. CTRL, Control.
marketers) and IL-4/IL-13 activation (HO-1 and Arg-1: M2 markers) confirmed the differential phenotype of these populations upon treatment (Fig. 1A). Approximately 20% of LPS-activated M1 macrophages exhibited apoptosis, whereas IL-4/IL-13-activated M2 macrophages did not (Fig. 1B). In agreement with previous data (22) and in part due to the attenuation of the LPS-dependent activation, treatment of BMDM with e-LXA4 protected against apoptosis (Fig. 1C). Treatment of BMDM with e-LXA4 significantly impaired IKK activation and IkBα phosphorylation and subsequent degradation in response to LPS stimulation (Fig. 1D), an effect that was further evidenced by the decreased translocation of p65 to the nucleus and the enhanced accumulation of Nrf-2, a transcription factor activated in response to e-LXA4 (Fig. 1E). In addition to these effects, e-LXA4 exhibited a moderate but significant direct inhibition of IKKβ when analyzed in vitro, using staurosporine as a control inhibitor (Fig. 1G).

**Early effects of e-LXA4 on Kv of BMDM**

Voltage-dependent potassium currents were evoked in BMDM by applying depolarizing pulses from a holding potential of −80 mV to different voltages from −80 to 60 mV in 10-mV steps. Fig. 2 shows original records of Kv currents obtained in control (resting) or activated with LPS or IL-4/IL-13 BMDM (Fig. 2A–C), before and after perfusion (15 min) with e-LXA4. Fig. 2D–F shows the current-voltage relationships (I-V) obtained in the absence and presence of e-LXA4. Resting and IL-4/IL-13–stimulated BMDM elicited small Kv currents of similar magnitude that were not modulated by perfusion with e-LXA4. However, LPS-stimulated BMDM elicited inactivating Kv currents of greater amplitude. Perfusion with e-LXA4 significantly decreased Kv currents at potentials >0 mV (20 ± 3% at +60 mV; n = 12, p < 0.05) (Fig. 2E). Because it has been described that the increase of Kv current induced by LPS is due to an upregulation of Kv1.3 (5), this early inhibition of Kv current produced by e-LXA4 could be due, at least in part, to its effects on Kv1.3. Use-dependent inactivation of certain Kv channels, a unique property of Kv1.3 but not of Kv1.5, is characterized by an inactivation that accumulates after repetitive depolarizing pulses because recovery during the interpulse interval is incomplete (16, 32). Supplemental Fig. 1A–C shows the use-dependency of Kv currents in control BMDM or activated with LPS or IL-4/IL-13 before and after perfusion with e-LXA4, as well as the graphs in which the normalized current was plotted against the number of the pulse during the train. The magnitude of use-dependent inactivation observed in control BMDM was small, 15 ± 5% (n = 10), but increased to 69 ± 3% (n = 10; p < 0.01 versus control) in LPS-activated BMDM. In contrast, stimulation with IL-4/IL-13 led to a marked decrease of the use-dependent inactivation (1.6 ± 3%; n = 8, p < 0.01 versus control). Perfusion with e-LXA4 was unable to modify the degree of use-dependent decrease of the current. As shown in Supplemental Fig. 1D–F, Kv currents are inactivated with a different time course in control versus LPS or IL-4/IL-13–challenged BMDM. Cumulative and use-dependent are useful parameters to evaluate differences in the nature of the tetrameric Kv channel phenotype, because Kv1.3 present cumulative and use-dependent inactivation, which is absent in Kv1.5 (16, 33). Supplemental Fig. 1D–F shows the time course of Kv current inactivation after applying a depolarizing pulse from −80 to +60 mV for 1 s. Although Kv currents are inactivated with a time constant of 1899 ± 1275 ms (n = 5) in control BMDM, this parameter decreased to 456 ± 43 ms in LPS-treated cells (n = 10; p < 0.05 vs. control). However, this process was slower in BMDM activated with IL-4/IL-13 (10,591 ± 5,826 ms; n = 7, p < 0.05 versus control) (Supplemental Fig. 1F). These results further support the notion that Kv currents generated upon depolarization in BMDM are mediated by Kv1 channels with different subunit stoichiometry after activation with LPS or with IL-4/IL-13 (5, 16).

**FIGURE 2.** Early effects of e-LXA4 on Kv currents. Representative traces of Kv currents recorded in control, LPS-, and IL-4/IL-13–activated BMDM (top panel). Cells were untreated (A) or treated with LPS (100 ng/ml) (B) and IL-4/IL-13 (20 ng/ml) (C) for 18 h. Current recordings were obtained in the absence and after perfusion during 15 min with e-LXA4 (500 nM) (A–C). Currents were elicited by applying depolarizing pulses from a holding potential of −80 mV to different depolarizing voltages from −80 to +60 mV in 10-mV steps (250-ms duration). I-V relationships in the absence and presence of e-LXA4 (500 nM) in nonstimulated BMDM (D), LPS (E), and IL-4/IL-13–activated BMDM (F) are shown. *p < 0.05 versus control; n > 10 cells/group. CTRL, Control.
Kir currents were evoked by applying pulses from a holding potential of −80 mV to different voltages from −140 to 40 mV in 10-mV steps (Supplemental Fig. 2A–C). Resting and IL-4/IL-13–activated BMDM showed similar magnitudes of the Kir currents; however, cells activated with LPS elicited Kir currents of smaller amplitude. The mean I-V relationship for Kir currents and the degree of Kir inactivation are shown in Supplemental Fig. 2. In all experimental conditions, e-LXA4 decreased the current at negative potentials to −120 mV (12 ± 4%, n = 10; 18 ± 9%, n = 12; and 23 ± 4%, n = 7) at −140 mV in resting, LPS, and IL-4/IL-13–stimulated BMDM, respectively). The decrease of the current magnitude induced by e-LXA4 was accompanied, in resting and LPS-stimulated BMDM, by a higher degree of inactivation of the current.

**Chronic effects of e-LXA4 on Kv and Kir of BMDM**

Because e-LXA4 produces changes in gene expression in addition to early signaling, the long-term effects of e-LXA4 were analyzed on Kv and Kir currents. The effects of 18-h treatment with e-LXA4 in resting or LPS-stimulated BMDM are shown in Fig. 3A and 3B. The effects of e-LXA4 on IL-4/IL-13–stimulated BMDM were not

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**FIGURE 3.** Effect of long-term treatment with e-LXA4 on Kv currents in control and LPS-activated BMDM. Representative traces of Kv currents recorded in control (resting) and LPS-activated BMDM. BMDM were incubated with e-LXA4 (500 nM) (A), with LPS (100 ng/ml), or with e-LXA4 + LPS for 18 h (B). Currents were elicited by applying depolarizing pulses from a holding potential of −80 mV to different depolarizing voltages from −80 to +60 mV in 10-mV steps (250-ms duration). I-V relationships obtained in control BMDM and after incubation for 18 h with e-LXA4 (C). I-V relationships obtained in LPS stimulated BMDM and after incubation with e-LXA4 + LPS during 18 h (D). To measure Kv inactivation, the pulse protocol shown in the middle panel of the figure was applied and representative traces of Kv currents recorded in control or e-LXA4 (500 nM) (E) and in LPS-activated or incubated with e-LXA4 (500 nM) + LPS (100 ng/ml) BMDM (F). Plots show the normalized current measured at the maximum peak current during the application of a train of depolarizing pulses. Current records obtained after applying a depolarizing pulse of 1 s in control cells or incubated with e-LXA4 (G) and in LPS-activated or incubated with e-LXA4 BMDM (H). Note that incubation with e-LXA4 reverts the LPS-induced effects on use-dependent and kinetics of inactivation. *p < 0.05 versus control; n > 10 cells/group. CTRL, Control.
studied due to the slight changes produced by IL-4/IL-13 on K<sub>v</sub> and K<sub>ir</sub> currents. Treatment of BMDM with e-LXA<sub>4</sub> (18 h) did not change the K<sub>v</sub> outward current versus that elicited in resting BMDM at any membrane potential tested (Fig. 3C). However, the combined stimulation of BMDM with e-LXA<sub>4</sub> + LPS significantly decreased K<sub>v</sub> current at membrane potentials > −10 mV, reverting the magnitude current values toward those observed in resting cells (546 ± 152 versus 178 ± 48 pA measured at the end of +60 mV pulses; n = 7–9, p < 0.05; Fig. 3D).

As shown in Supplemental Fig. 1, one of the characteristics of the K<sub>v</sub> currents recorded in LPS-activated BMDM is the use-dependent inactivation observed upon application of a train of stimuli. Fig. 3E and 3F show the long-term effects induced by e-LXA<sub>4</sub> (18 h) on the use-dependent inactivation of the K<sub>v</sub> currents in resting and LPS-stimulated BMDM. e-LXA<sub>4</sub> did not modify the degree of use-dependent inactivation in resting BMDM (Fig. 3E), as shown in the graphs representing the normalized peak K<sub>v</sub> current during the application of train pulses. Interestingly, K<sub>v</sub> generated by BMDM activated by e-LXA<sub>4</sub> + LPS did not exhibit the use-dependent inactivation characteristic of K<sub>v</sub>1,3 (Fig. 3F).

Similarly, incubation with e-LXA<sub>4</sub> did not modify the inactivation kinetics of resting BMDM, whereas it slowed this process in LPS-stimulated cells (1123 ± 335 versus 456 ± 43 ms in cells incubated with e-LXA<sub>4</sub> + LPS and LPS, respectively, n = 6–11, p < 0.05; Fig. 3G, 3H). These results suggest that e-LXA<sub>4</sub> prevents the LPS-induced changes in the stoichiometry of K<sub>v</sub>, leading to the formation of heterotetramers with a lower K<sub>v</sub>1,3:K<sub>v</sub>1,5 ratio. Thus, the resulting Kv phenotype closely resembles that of resting BMDM.

K<sub>v</sub> currents were recorded in BMDM by using the same pulse protocol described in Supplemental Fig. 2 and are shown in the top panel of Fig. 4. Similarly to that observed in K<sub>v</sub> currents, e-LXA<sub>4</sub> did not modify K<sub>v</sub> current magnitude versus resting BMDM (Fig. 4A, 4C). However, in LPS-activated BMDM, e-LXA<sub>4</sub> reverted LPS effects, increasing the K<sub>v</sub> current magnitude at membrane potentials < −90 mV (Fig. 4B, 4D). Interestingly, in all the experiments in which cells were incubated with e-LXA<sub>4</sub> + LPS, e-LXA<sub>4</sub> returned the current biophysical characteristics toward the control situation (Fig. 3.4). These results could be explained either by e-LXA<sub>4</sub>-induced changes in the expression of both K<sub>v</sub> and K<sub>ir</sub> or by posttranslational channel protein modifications. To address this question, the mRNA and protein levels were measured. Our data show an upregulation of the K<sub>v</sub>1,3 mRNA by LPS that was impaired when cells were pretreated with e-LXA<sub>4</sub> (Fig. 5A). e-LXA<sub>4</sub> promoted a modest upregulation of K<sub>v</sub>1,5 mRNA that was suppressed by LPS or IL-4/IL-13. K<sub>v</sub>2.1 mRNA levels were downregulated by LPS, and to a lesser extent by IL-4/IL-13. Interestingly, when the protein levels were determined at 18 h (the time of evaluation of the biochemical and electrophysiological parameters), a significant increase of K<sub>v</sub>1,3 and K<sub>v</sub>2.1 protein levels was observed after LPS challenge, an effect that was blunted by e-LXA<sub>4</sub> (18 h) (Fig. 5B).

The mRNA and protein level changes of K<sub>v</sub>1.3 and K<sub>v</sub>1.5 were in agreement with the electrophysiological results shown above. Moreover, the mRNA levels of K<sub>v</sub>2.1 observed after incubation with LPS were in agreement with the reduced K<sub>v</sub>2.1 recorded under these experimental conditions. However, there are controversial results for the mRNA and protein levels of K<sub>v</sub>2.1 after incubation with e-LXA<sub>4</sub>, e-LXA<sub>4</sub> + LPS, or IL-4/IL-13. Moreover, the increase in the protein levels of K<sub>v</sub>2.1 does not correlate with the electrophysiological results observed, which could be attributed to posttranslational modifications as evidenced by the appearance of two adjacent protein bands in LPS-activated cells. Representative confocal microscopy images of the distribution of the fluorescence of K<sub>v</sub>2.1 show areas of aggregation, perhaps in specific intracellular environments (Fig. 5C).

To assess whether e-LXA<sub>4</sub> produces its effects via a direct interaction with the ion channels or through changes in the LPS signaling pathway, two series of experiments were performed. First, we analyzed the effects of different e-LXA<sub>4</sub> concentrations (1–1000 nM range) on cloned K<sub>v</sub>1,5, K<sub>v</sub>1,3, and K<sub>v</sub>2.1 expressed in HEK293 cells (Supplemental Fig. 3A). As it can be observed, e-LXA<sub>4</sub> did not produce significant effects on any ion channel, suggesting that the effects observed in the current study are not due to a direct interaction between e-LXA<sub>4</sub> and the channels. In the second approach, the effects of low concentrations of e-LXA<sub>4</sub> were analyzed in K<sub>v</sub> and K<sub>ir</sub> in LPS-stimulated BMDM cells (Supplemental Fig. 3B, 3C). e-LXA<sub>4</sub> reverted in a concentration-dependent manner the effects of LPS on K<sub>v</sub> and K<sub>ir</sub> currents with IC<sub>50</sub> of ~250 and ~120 nM, respectively.

Another important issue to be assessed is the relative potency of e-LXA<sub>4</sub> versus LXA<sub>4</sub>. To analyze this issue, we studied the long-term effects of LXA<sub>4</sub> 500 nM in K<sub>v</sub> and K<sub>ir</sub> currents recorded in LPS-stimulated BMDM. As it can be observed, the potency of LXA<sub>4</sub> resulted to be lower than that exhibited by e-LXA<sub>4</sub> (Supplemental Fig. 3D–F).

To gain insight on the mechanism of action of e-LXA<sub>4</sub> in BMDM cells the effects on the endoplasmic reticulum release of Ca<sup>2+</sup> and on the influx via calcium release–activated calcium channels (CRAC) were analyzed. Treatment of BMDM with thapsigargin induced a transient increase in the intracellular Ca<sup>2+</sup> concentration
due to Ca\(^{2+}\) efflux from intracellular stores. Subsequent addition of Ca\(^{2+}\) (1 mM) to the extracellular medium produced a further increase in intracellular Ca\(^{2+}\) concentration indicative of store-operated Ca\(^{2+}\) entry. Supplemental Fig. 4A shows the effects produced by e-LXA\(_4\) in resting and LPS-stimulated BMDM on Ca\(^{2+}\) release and Ca\(^{2+}\) influx. Under all experimental conditions, Ca\(^{2+}\) release from intracellular stores was similar. When analyzing the Ca\(^{2+}\) influx mainly via CRAC, e-LXA\(_4\) did not produce significant changes in the fluorescence under resting conditions. The increase in fluorescence was greater in LPS-stimulated BMDM (\(p, 0.01\)), and, interestingly, e-LXA\(_4\) significantly decreased fluorescence values in LPS-stimulated BMDM (\(p < 0.01\) versus the control condition, \(p < 0.01\) versus the same condition in the absence of e-LXA\(_4\)).

**Blockade of Kv1.3 with MgTx prevents the e-LXA\(_4\) effects**

To know whether the e-LXA\(_4\) effects on the Kv and Kir are critical for its immunomodulatory activity on LPS-stimulated BMDM cells, a series of experiments in the presence of MgTx (10 nM), a selective Kv1.3 blocker, was performed. We observed that, in the presence of MgTx, the effects of e-LXA\(_4\) on NOS-2, IP-10, and Arg-1 were suppressed (Fig. 6A, 6B), suggesting that Kv1.3 activity is required for the action of this eicosanoid. Moreover, the impairing effects of e-LXA\(_4\) on LPS-triggered IKK signaling were also antagonized by MgTx (Fig. 6C).

**e-LXA\(_4\) produces its effects partially via ALX receptor**

The biological effects of e-LXA\(_4\) have been shown to be mediated, at least in part, via ALX receptor (34, 35). Using a selective ALX inhibitor (BocPLP; 1 \(\mu\)M) (36, 37), 85% inhibition of the e-LXA\(_4\)-dependent ERK phosphorylation was observed [data not shown and (22)]. Fig. 7 shows the effects of BocPLP on Kv and Kir currents and the I-V relationships in different combinations of LPS and e-LXA\(_4\) treatments of BMDM. The actions of e-LXA\(_4\) on Kv and Kir appear to be partially mediated via ALX receptor. The biophysical characteristics of Kv currents were very similar to those recorded in cells activated with LPS and to a lesser extent with LPS + e-LXA\(_4\). However, as shown in the Kv I-V relationship (Fig. 7A), the mean magnitude of the current resulted to be smaller than that recorded in cells activated with LPS and somewhat greater than that obtained after stimulating with e-LXA\(_4\) + LPS (Fig. 7A). Kv currents still exhibit cumulative inactivation as occurred in LPS-stimulated cells in the absence of e-LXA\(_4\) (Fig. 7B). Under this line, the Kv inactivation recorded in BMDM stimulated with e-LXA\(_4\) + LPS in the presence of BocPLP was intermediate between that observed in LPS- and in e-LXA\(_4\) + LPS–stimulated cells (Fig. 7C), suggesting that the effects of e-LXA\(_4\) on Kv currents are partially ALX receptor dependent. Regarding the effects of BocPLP on the Kir current in BMDM treated with e-LXA\(_4\) + LPS, the inward current was similar to that recorded in LPS-activated BMDM,
suggesting that Kv effects are more ALX receptor dependent than those on Kir currents.

Because the anti-inflammatory actions of e-LXA4 have been also observed at very low concentrations, the effects of 100 nM e-LXA4 were analyzed on K, and Kv currents recorded in BMDM activated with LPS (Supplemental Fig. 4B–E). The effects of 100 nM e-LXA4 on the protein levels of NOS-2, COX-2, HO-1, Arg-1, and Arg-2 were determined at 10–30 h (A). Band intensities were normalized for content in p85 (B). The activation of IKK (C) was determined at 0–30 min. Band intensities were normalized for the content in p85. Results show a representative experiment out of four or the mean ± SEM of three experiments. **p < 0.01, ***p < 0.001 versus the control condition (in the absence of stimuli), *p < 0.01, **p < 0.001 versus values obtained in control plus MgTx, *p < 0.05, **p < 0.01 versus the same experimental condition without MgTx. Veh., Vehicle.

FIGURE 6. MgTx prevents the effects of e-LXA4 on the macrophage profiling pathways. BMDM were maintained in culture and activated for the indicated period with LPS (100 ng/ml). Cells were treated with e-LXA4 (200 nM) for 30 min prior to challenge with LPS (100 ng/ml) and also in the presence of MgTx (10 nM). The effects of e-LXA4 (200 nM) on the protein levels of NOS-2, COX-2, HO-1, Arg-1, and Arg-2 were determined at 10–30 h (A). Band intensities were normalized for content in p85 (B). The activation of IKK (C) was determined at 0–30 min. Band intensities were normalized for the content in p85. Results show a representative experiment out of four or the mean ± SEM of three experiments. **p < 0.01, ***p < 0.001 versus the control condition (in the absence of stimuli), *p < 0.01, **p < 0.001 versus values obtained in control plus MgTx, *p < 0.05, **p < 0.01 versus the same experimental condition without MgTx. Veh., Vehicle.

**Discussion**

This study provides experimental evidence for a new mechanism of action of e-LXA4 by which this eicosanoid contributes to inflammation resolution: on the one hand by directly attenuating NF-κB activity and on the other hand affecting the electrophysiological properties of the macrophage membrane. We show that: 1) activation of BMDM with IL-4/IL-13 produces similar effects on potassium currents than those reported after glucocorticoid treatment; 2) following perfusion, e-LXA4 decreases Kv currents in LPS-elicited BMDM and also Kir currents in resting, LPS-, and IL-4/IL-13–stimulated BMDM at potentials negative to −120 mV, suggesting the involvement of receptor-independent effects; 3) after prolonged incubation of BMDM with e-LXA4, the LPS-induced effects on Kv, and Kir were partially reverted, at the time that the enhanced levels of Kv1.3 and Kir2.1 were downregulated; 4) long-term
exposure to e-LXA₄ reverted the LPS-induced Ca²⁺ influx; and 5) the effects of e-LXA₄ on Kᵥ and Kᵢᵣ in LPS-activated BMDM were partially mediated via ALX receptor. Importantly, the functions of Kᵥ₁.₃ are required for the attenuation of NF-κB activity, because the e-LXA₄ effects on IKK activity and NOS-2 expression were partially reverted in the presence of MgTx.

Potassium channels play a critical role in maintaining the electrochemical gradient required for sustained Ca²⁺ entry in the time frame necessary for activation and effector functions (38). We previously reported that LPS-activated RAW 264.7 macrophages exhibited a 1.6-fold elevation in functional Kᵥ₁.₃ versus the nonstimulated counterparts (16). Also, we described that Kᵥ₁.₃/Kᵥ₁.₅ heteromeric channel expression and their biophysical and pharmacological properties changed differentially upon activation and immunosuppression: LPS induced Kᵥ₁.₃, whereas treatment with dexamethasone resulted in the opposite effect (16). This enhanced Kᵥ₁.₃ expression is likely to promote the Ca²⁺ signaling necessary for coactivation/effector functions in macrophages. Indeed, blockade of Kᵥ₁.₃ suppresses Ag-driven proliferation and cytokine production in T cells, and selective Kᵥ₁.₃ blockers ameliorate experimental allergic encephalomyelitis in rats induced by the adoptive transfer of myelin-specific activated effector memory T cells (39–42). Moreover, several groups have reported the expression of Kᵥ₁.₃ in macrophages and microglia (5, 16, 43–48), and our data add new insights on the fine-tuning of these channels by lipoxins. Indeed, Kᵥ₁.₃ channels, together with Kᵢᵣ₂.₁, Kᵥ₁.₅, Kᵥᵢᵣ₃.₁, and BKᵥᵣ channels, modulate macrophage and microglia function, and, at the same time, pharmacological channel blockers may have effects on the immune response (5, 44, 49, 50).

Early effects of e-LXA₄ on Kᵥ and Kᵢᵣ

The effects of e-LXA₄ were analyzed in nonactivated (resting) and innate or alternatively activated BMDM. In resting cells, the outward Kᵥ current shows small amplitude, a fast activation, and a slow inactivation. Under these conditions, e-LXA₄ did not produce significant effects on the biophysical properties of BMDM. Moreover, most of the Kᵥ current is due to the activity of Kᵥ₁.₅ + Kᵥ₁.₃ tetramers in which Kᵥ₁.₅ predominates (5, 16). These results suggest that e-LXA₄ does not produce significant direct effects on Kᵥ₁.₅ channels, and experiments performed on Kᵥ₁.₅ confirmed these results (see Supplemental Material).

As previously reported, LPS increases Kᵥ and decreases Kᵢᵣ currents (5, 15, 16). The increase in Kᵥ current induced by LPS appeared concomitantly with a use-dependent decay and a C-type inactivation, both characteristics of Kᵥ₁.₃. Moreover, LPS up-regulated both the mRNA and protein levels of Kᵥ₁.₃. Under these conditions, e-LXA₄ decreased the magnitude of the Kᵥ current in LPS-stimulated BMDM but did not modify the use-dependent decrease or the C-type inactivation of the current. Although these effects point out to a direct interaction with Kᵥ₁.₃, the analysis of the response to e-LXA₄ in HEK293 cells expressing these channels rules out this hypothesis, suggesting that the e-LXA₄ actions are the consequence of some effect on a signaling pathway present in macrophages but not in HEK293 cells. Finally, after stimulation of BMDM with IL-4/IL-13, Kᵥ currents exhibited similar magnitudes to those recorded in resting cells. Interestingly, although activation of BMDM with IL-4/IL-13 did not modify the amplitude of the current, it decreased the use-dependent effects, resembling the actions of dexamethasone (16). These results suggest that alternative activation can change the stoichiometry of Kᵥ, leading to the formation of channels with a decreased Kᵥ₁.₃/Kᵥᵢᵣ₁.₃ ratio. These conclusions are supported by the slight increase in the mRNA levels for Kᵥ₁.₅ after IL-4/IL-13 activation. In BMDM stimulated with IL-4/IL-13, e-LXA₄ did not produce significant effects on the magnitude nor on the electrophysiological properties of Kᵢᵣ currents.

In resting and alternatively activated BMDM, Kᵢᵣ current exhibits the typical characteristics of the inward rectifying potassium current. e-LXA₄ decreased the amplitude of the Kᵢᵣ₂.₁ current at negative membrane potentials under the three experimental conditions tested. In resting BMDM, but not in LPS- or IL-4/IL-13-stimulated BMDM, the reversal potential became more positive, which leads to a depolarization of the membrane potential, thus avoiding Ca²⁺ entry into the cell. As it was previously reported, LPS decreased Kᵢᵣ current, consistent with a downregulation in the mRNA for Kᵢᵣ₂.₁ (5).
Long-term effects of e-LXA4 on $K_r$ and $K_v$

Because the early inhibitory effects of e-LXA4 on NF-$k$B and proinflammatory signaling are extended at later periods (>10 h), the electrophysiological effects of long-term incubation with e-LXA4 were studied at these times. Under these conditions, e-LXA4 did not modify the magnitude of the $K_r$ or the $K_v$ currents in resting BMDM. On the contrary, it was able to revert partially the electrophysiological effects produced by LPS, decreasing the amplitude, the use-dependent decrease, and the C-type inactivation of the $K_v$ current. In a parallel way, e-LXA4 increased the magnitude of the $K_r$ current previously decreased by LPS. Using a selective inhibitor of ALX (22), it was shown that the actions of e-LXA4 on $K_r$ currents are, at least in part, dependent on the interaction with ALX receptor. Indeed, the $K_{r,1.5}$ protein levels were not regulated by e-LXA4, whereas $K_{v,1.3}$ was upregulated by LPS and downregulated by e-LXA4 in LPS-stimulated BMDM. Because $K_r$ currents recorded in BMDM stimulated with LPS are the consequence of the activation of heterotrimers of $K_{r,1.5}$ and $K_{v,1.3}$ with different stoichiometry, the contribution of $K_{r,1.5}$ to the total of this current can explain the apparently low dependency of the effects of e-LXA4 on $K_r$. On the contrary, the $K_v$ current measured in the presence of BocPLP in cells treated with e-LXA4 + LPS was similar to that recorded in BMDM activated with LPS, suggesting that the e-LXA4 effects on $K_v$ involves the interaction with the ALX receptor.

The influx of extracellular Ca$^{2+}$ is an essential requirement for the activity of many cellular processes (6, 51, 52). Therefore, $K_{v,1.3}$ and the Ca$^{2+}$-activated $K_v$ channel $K_{v,3.1}$ regulate Ca$^{2+}$ influx through the Ca$^{2+}$ release-activated Ca$^{2+}$ channel, which consists of the Ca$^{2+}$-sensor stromal interaction molecule 1 and the pore-forming protein CRACM1 (Orai1) (53–56). In T cells, this crucial influx of Ca$^{2+}$ is only possible if they can keep their membrane potential negative by a counterbalance of $K_v$ efflux through $K_{v,1.3}$ and/or $K_{v,3.1}$ (38, 57), and both channels are regarded as targets for immunosuppression (38). In the current study, we demonstrated that e-LXA4 inhibits $K_{v,1.3}$ in LPS-stimulated cells. This inhibition should lead to a depolarization of the membrane potential and therefore to an inhibition of the Ca$^{2+}$ influx through CRAC, which is a crucial event in the activation of macrophages (58). Indeed, we demonstrated that e-LXA4 is able to decrease extracellular Ca$^{2+}$ influx (Supplemental Fig. 4A). Therefore, e-LXA4 may produce anti-inflammatory effects through this new mechanism of action.

In conclusion, lipoxins were the first mediators exhibiting both anti-inflammatory and proresolving actions. The beneficial actions of aspirin in the cardiovascular system have been attributed to the capability of aspirin to block PG and prothrombotic thromboxone generation via acetylation of COX. Aspirin acetylation of COX-2 not only inhibits prostanooid formation but also alters the active site of COX-2 and thereby favors the conversion of arachidonic acid into 15$\beta$-HETE in vascular endothelial cells. This compound can be further transformed to epimeric lipoxins by leukocytes and other immune cells contributing to the fine tuning of the inflammatory response. Our data describe a direct inhibition of e-LXA4 on IKK$\beta$ activity, a broad inhibition on NF-$k$B activity, and a cytoprotective mechanism of macrophages during resolution. Also, the function of $K_{v,1.3}$ channels are required for the attenuation of NF-$k$B activity, because the e-LXA4 effects on IKK activity and NOS-2 expression are partially reverted by MgTx. Finally, and in addition to this, Ca$^{2+}$ fluxes are negatively modulated by e-LXA4 through the inhibition of $K_{v,1.3}$ channels in LPS-stimulated cells; all these mechanisms converging in the attenuation of the inflammatory response as far as e-LXA4 accumulates in the medium.
MODULATION OF Kᵥ AND Kᵢᵣ CHANNELS BY e-LXA₄


