**Lipoxin $A_4$ impairment of apoptotic signaling in macrophages: implication of the PI3K/Akt and the ERK/Nrf-2 defense pathways.**

*Short Title:* *Antiapoptotic effects of lipoxin $A_4$ in macrophages*

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**Abstract**

Lipoxin $A_4$ ($LXA_4$) is an endogenous lipid mediator that requires transcellular metabolic traffic for its synthesis. The targets of $LXA_4$ on neutrophils are well described, contributing to attenuate inflammation. However, the effects of lipoxins on macrophage are less known, in particular the action of $LXA_4$ on the regulation of apoptosis of these cells. Our data show that pre-treatment of human or murine macrophages with $LXA_4$ at the concentrations prevailing in the course of resolution of inflammation (nanomolar range) inhibit significantly the apoptosis induced by staurosporine, etoposide and S-nitrosoglutathione or by more pathophysiological stimuli, such as LPS/IFNγ challenge. The release of mitochondrial mediators of apoptosis as well as the activation of caspases was abrogated in the presence of $LXA_4$. In addition to this, the synthesis of reactive oxygen species induced by staurosporine was attenuated and anti-apoptotic proteins of the Bcl-2 family accumulated in the presence of lipoxin. Analysis of the targets of $LXA_4$ identified an early activation of the PI3K/Akt and ERK/Nrf-2 pathways that was required for the observation of the antiapoptotic effects of $LXA_4$. These pathways activated in response to $LXA_4$ contribute to the upregulation of the antiapoptotic proteins of the Bcl-2 family and to the transcription of cytoprotective genes under the control of the ERK/Nrf-2 signaling. These data suggest that the $LXA_4$ released after recruitment of neutrophils to sites of inflammation exerts a protective effect on macrophage viability that might contribute to a better resolution of inflammation.

## INTRODUCTION

Regulation of apoptosis of immune cells is a critical stage during inflammation, because when the injury is over, accomplishment of a correct resolution, mainly through apoptotic death, prevents the development of chronic inflammatory diseases [1,2,3]. During this process, neutrophils are quickly recruited to sites of infection where they have a very short life and die rapidly via apoptosis [4,5]. Macrophages arrive later to phagocyte apoptotic-cells and pathogens in the affected area. Thus, for an effective resolution of the inflammatory process, it is important that macrophages have a sufficient life span to clear the inflamed area (2-3 days).

Lipoxins (LXs) are endogenous eicosanoids which are released during the resolution phase of inflammation, in the nanomolar range [6,7]. LXs are mainly generated by transcellular metabolism from arachidonic acid depending on the cellular context [8,9]. In mammals, lipoxygenase enzymes (LOX) generate two main native products, lipoxin $A_4$ ($LXA_4$) and $B_4$ ($LXB_4$) being the most studied $LXA_4$, which exerts potent anti-inflammatory actions modulating leukocyte trafficking and promoting phagocytic clearance of apoptotic cells [10,11,12]. The effects of lipoxins as lipid mediators with potent anti-inflammatory actions are well documented, but their role in apoptosis remains controversial [13]. For instance, it has been described an apoptotic effect in fibroblasts after treatment with $LXA_4$ at micromolar concentrations [14], whereas it has been also reported that this compound inhibits peroxynitrite formation in leukocytes [15], reduces colonocyte apoptosis [16] and promotes survival of retinal pigment epithelial cells [17]. Therefore, the precise role of lipoxins as modulators of apoptosis remains elusive and appears to be dependent on the cell type and on the $LXA_4$ concentration. Given the lack of studies concerning the role of $LXA_4$ in macrophage apoptosis and considering the importance of this immune cell in inflammation, we have investigated the effect of native $LXA_4$ on macrophage apoptosis using staurosporine, etoposide and GSNO as strong apoptosis-inducers in many cell types [18,19,20], as well as LPS/IFNγ as a more pathophysiological condition. Our results show that concentrations of $LXA_4$ in the nanomolar range prevent apoptosis in murine and human macrophages through a mechanism consistent with changes in the levels of apoptosis-related proteins, favoring an anti-apoptotic environment, and a blockade of early apoptotic signaling, that involves the suppression of the release of mitochondrial-dependent apoptotic mediators and caspases activation. Moreover, our data show that lipoxin treatment of macrophages promotes a rapid activation of the PI3K/Akt signaling that is relevant for protection against apoptosis, in agreement with previous work describing up-regulation of antiapoptotic genes by this pathway, such as Mcl-1 [21,22]. In addition to this, treatment of macrophages with $LXA_4$ induced ERK/Nrf-2 activation, being Nrf-2 a transcription factor that regulates the expression of *ca.* 100 cytoprotective genes that share in common a sequence termed antioxidant response element (ARE) [23,24], and contributes to the anti-oxidant defense pathway in many cells [25,26,27]. Activation of this pathway in macrophages

by $LXA_4$ decreased staurosporine-induced apoptosis, without affecting the activity of JNK and p38 MAPKs.

## MATERIALS AND METHODS

*Materials*. Staurosporine, MG132, LY294002 and PD098059 were from Calbiochem (San Diego, CA); $LXA_4$ and 15-epi-$LXA_4$ were from Cayman (Ann Arbor, MI) or Calbiochem; etoposide, LPS, PMA and GSNO were from Sigma (St. Louis, MO); IFNγ was form PeProtech (UK); Jo2 was from BD (CA). N-t-Boc-Phe-Leu-Phe-Leu-Phe (BocPLP), a formyl peptide lipoxin-receptor competitive antagonist was from Bachem (Bubendorf, CH); antibodies were from Santa Cruz Biotech (Santa Cruz, CA), BD Transduction Laboratories (San Jose, CA), or Cell Signaling Technology (Danvers, MA). Fluorescent probes were from Molecular Probes (Eugene, OR). Other reagents were from Roche (Mannheim, Germany) or Sigma. Tissue culture dishes were from Falcon (Lincoln Park, NJ), and culture media were from Invitrogen (Carlsbad, CA).

*Animals*. Seven-week old male wild-type C57BL/6 mice and *nrf*-2-knockout littermates [28] were a generous gift from Dr A. Cuadrado (IIBM, Madrid). Mice were housed at RT under 12h light/dark cycle and food and water was provided *ad libitum*. Animals were cared for according to a protocol approved by the Ethical Committee of our institution (following 86/609/EEC, 2003/65/EC directives).

*Isolation of human monocytes.* PBMCs were isolated from blood of healthy donors by centrifugation on Ficoll-Hypaque Plus (Amershan Biosciences, UK) following the manufacturers' protocol. Cells were maintained for 2 h to a density of $10^6$ cells/ml in DMEM supplemented with antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin). After this period, the supernatant was removed and adherent cells were cultured in the same medium supplemented with 10% heat-inactivated FBS. Purity of all cultures was verified by $CD14^+$ staining; on average 87% of the cells presented this surface marker. Cells were maintained overnight with this medium and differentiated with h-MCSF (20 ng/ml, PeProtech) for 7 days followed by treatment with the indicated stimuli.

*Cell culture and transient transfections*. The macrophage cell line RAW 264.7 was maintained in RPMI 1640 medium containing 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated FBS. Before experiments, the medium was challenge to 1% FBS. Elicited peritoneal macrophages were prepared as previously described [29] and used as indicated for the cell line. For transient transfections, cells were grown at 80% confluence and transfected using the Cell Line Nucleofector Kit V, following the manufacturer´s instructions (Amaxa). After nucleofection, cells were stimulated for 6h and luciferase activity was measured using a Dual Luciferase Reporter Assay System (Promega). The human monocytic THP-1 cells were maintained in culture with RPMI 1640 supplemented with 10% FBS and 20 mM HEPES. PMA (50 nM, 48h) was used to induce THP-1 monocytes to differentiate into macrophages. Then, the medium was changed to RPMI 1640 supplemented with 1% FBS and cells were stimulated for the indicated periods of time.

*Preparation of total protein cell extracts***.** Cells were homogenized in a medium containing 10 mM Tris-HCl, pH 7.5; 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 0.5% CHAPS, 1 mM β-mercaptoethanol and 0.1 mM PMSF and a protease and phosphatase inhibitor cocktail (Sigma).

The extracts were vortexed for 30min at 4ºC and after centrifuging for 20min at 13000g, the supernatants were stored at 20ºC. Protein levels were determined with Bradford reagent (Bio-Rad, Hercules, CA).

*Preparation of cytosolic and nuclear protein cell extracts*. Stimulated cells were harvested into ice-cold PBS, resuspended in 200 µl of cytosolic buffer (10 mM HEPES, pH 8; 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40) and left to swell on ice for 15min. Cells were vortexed and centrifuged at 12000g for 30s. The pellet was resuspended in 50 µl of ice-cold nuclear buffer (20 mM HEPES, pH 8; 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 20% glycerol) and vortexed at 4ºC for 30min. Following centrifugation (12000g at 4°C for 20min), the supernatant was transferred to a fresh tube. All buffers contained protease and phosphatase inhibitor Cocktail (Sigma).

*Cytochrome c release assay***.** Cell pellets were resuspended in buffer A (0.32 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5, and protease inhibitor cocktail) and homogenized by repeated passage through a 27-G needle. Then cells were centrifuged for 15min at 4ºC and 10000g. The supernatants were saved as cytosolic fractions and stored at -80ºC. Pellets were resuspended again in buffer A and were saved as 'particulate' fractions containing the mitochondria and stored at -80ºC until cytochrome c quantification.

*Western blot analysis***.** Equal amounts of protein (20-50 µg) from each fraction obtained were loaded into a 10-12% SDS-PAGE. Proteins were size fractionated, transferred to a Hybond-P membrane (Amersham) and, after blocking with 5% nonfat dry milk, incubated with the corresponding Abs. The blots were developed by ECL protocol (Amersham) and different exposition times were performed for each blot with a Charged Coupling Device camera in a luminescent image analyzer (Molecular Imager, BioRad) to ensure the linearity of the band intensities. Values of densitometry were determined using Quantity One software (Bio-Rad).

*In vitro and in vivo caspase activity assays***.** For the *in vitro* determination, total cell extracts were prepared and supernatants were used to measure caspase activities by cleavage of specific substrates in accordance with the supplier´s instruction (BD, Pharmingen). Caspase activity was quantified fluorimetrically by following the cleavage of Ac-DEVD-AMC for caspase 3, Ac-IETD-AFC for caspase 8 and Ac-Leu-Glu-His-Asp-AFC for caspase 9. Cell lysates were incubated for 60min at RT with corresponding substrates and the activity was measured with excitation-emission wavelengths of 340-460 nm for caspase 3 and 400-480 for caspase 8 and 9. CaspGLOW fluorescein Active Caspases Staining kit was also used to detect activated caspases in living cells by fluorescence microscopy, according to manufacturer instructions (Biovision Res, CA). Briefly, cells were treated with the indicated stimuli and incubated in the presence of a fluorescent marker conjugated to FITC that irreversibly binds to activated caspases in apoptotic cells. This label allows for direct detection of apoptotic cells by fluorescence microscopy.

*Flow cytometry***.** Analysis was carried out using a FC 500 Becton Dickinson FACScan flow cytometer (Mountain View, CA) with a CXP Software (Beckman Coulter).

*Cell death detection***.** Cells were harvested and washed in cold PBS. After centrifugation at 4ºC for 5min and 1000g, cells were resuspended in annexin V binding buffer (10 mM Hepes, pH 7.4; 140 mM NaCl, 2.5 mM $CaCl_2$). Cells were labeled with annexin V-FITC solution (BD Pharmingen) and propidium iodide (PI) (100 μg/ml) for 15min at RT in the dark. The percentage of cells with hyplodiploid DNA was used as another marker of apoptosis. For DNA detection, cells were suspended in 0.5 ml of 100 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100.

*Immunofluorescence microscopy***.** RAW 264.7 cells were seeded into sterile 8-wells Chamber Slides (Falcon, Lincoln Park, NJ), 16-24h before treatment. MitoTraker Red CMXRos (50 nM, Molecular Probes) was added to the culture medium for 30min before washing cells with PBS followed by fixation with 2% paraformaldehyde for 10min. Cells were then permeabilized in iced methanol and incubated with 3% BSA for 30min. After incubating with a rabbit Ab against Apoptosis-Inducing Factor (AIF) (Santa Cruz Biotechnology) at 4ºC for 1h, cells were washed with PBS followed by incubating with Alexa 488 anti-rabbit secondary antibody at 4ºC for 1h at RT (1:500; Molecular Probes). Coverslips were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and examined using an Espectral Leica TCS SP5 confocal microscope. Values of intensity fluorescence and colocalization quantification were performed with Image J software (NIH, Bethesda, MD).

*ROS and reactive nitrogen intermediates (RNI) determination***.** ROS generation in RAW 264.7 cells was analyzed by flow cytometry monitoring the oxidation of 2´,7´-dichlorodihydrofluorescein (DCFH). Cells were loaded with 0.4 μM DCFH for 30min, pelleted and resuspended in PBS followed by flow cytometry. RNI synthesis was determined incubating cells with 200 μM DAF-2 diacetate.

*Microarray analysis of apoptosis-related genes.* Total RNA was isolated from cells with TRIzol Reagent (Invitrogen, California, USA) and further cleaned up using the Qiagen RNeasy MiniKit (Hilden, Germany) with one step of DNAse I digestion. 3 μg of RNA were used for cDNA synthesis with $RT_2$ first Standard kit (SuperArray Bioscience, Frederick, MD). The mouse apoptosis PCR array was performed according to the manufacturer's protocol, using the Profiler PCR Array System and the SYBR Green/Fluorescein qPCR master mix (SuperArray Bioscience) on a MyiQ Real-Time PCR System (Bio-Rad). Gene expression was compared with the web-based software package for the PCR Array System (http://www.superarray.com/pcr/arrayanalysis.php). This software automatically performs all ΔΔCt based fold-change calculations from the specific uploaded raw threshold cycle data. Results were analyzed as described Mayoral *et al* [30] and RT-PCR of selected genes was used to validate the microarray data.

*Statistical analysis***.** The values in graphs correspond to the means±SD. The statistical significance was estimated with a Student *t* test for unpaired observation. Data were analyzed by the SPSS for Windows statistical package, version 9.0.1.

## RESULTS

***$LXA_4$ prevents stimuli-dependent apoptosis in macrophages.*** Preliminary studies in our laboratory suggested that lipoxin $A_4$ enhanced the viability of activated macrophages. Using staurosporine as a potent and broad inducer of apoptosis in mammalian cells, we evaluated the ability of lipoxin to interfere the apoptosis induced by this drug in macrophages. Incubation for 4h of RAW 264.7 cells with staurosporine (200 ng/ml) increased the percentage of annexin V positive cells by 38% (Fig. 1A). Preincubation for 2h of the cells with $LXA_4$ in the range of concentrations found under inflammatory conditions resulted in a dose-dependent inhibition of the pro-apoptotic effect of staurosporine ($EC_{50}$ 250 nM). This protective action of $LXA_4$ was mediated, at least in part, via its receptor since it was interfered after 1h of incubation with 1 μM BocPLP, a formyl peptide lipoxin-receptor antagonist [31,32], and was specific for $LXA_4$, being the 15-epi-$LXA_4$ derivative less effective (Fig. 1A). Fig. 1B shows a representative flow cytometry distribution of this annexin V analysis. The protective effect of lipoxin against apoptosis was confirmed by DNA size distribution analysis, observing a significant reduction of the hypodipliod population (Fig. 1C). When apoptosis was induced with etoposide (1 μM), GSNO (0.5 mM) or after LPS (500ng/ml)/IFNγ (20 ng/ml) challenge, treatment of cells with $LXA_4$ significantly decreased the apoptotic response in all these cases, suggesting the interference with a common pro-apoptotic signaling step (Fig. 1D).

***Lipoxin inhibition of caspase activation and pro-apoptotic signaling in macrophages treated with staurosporine.*** To study the antiapoptotic effect of lipoxin, the activation of caspases 3, 8 and 9 was analyzed in cells treated with staurosporine. As Fig. 2A shows, the well-known activation of caspases induced after staurosporine treatment (200 ng/ml; 4h and 18h, respectively) was significantly impaired in the presence of $LXA_4$. In addition to these determinations, the "*in vivo*" activation of caspases was measured using cell-permeant fluorescent probes (representative example for caspase 3; Fig. 2B, *left panel*). Fig. 2B (*right panel*) shows the percentage of cells with activated caspases 3, 8 or 9 after staurosporine treatment. $LXA_4$ significantly reduced the percentage of cells with activated caspases, an effect that was in the range of that observed in cells treated with the broad caspase inhibitor z-VAD (20 μM).

In view of these data, the release of pro-apoptotic mediators from the mitochondria was investigated since this is one of the key events in the commitment of the apoptotic response of macrophages. As Fig. 3 shows, the release of cytochrome c (panel A) and AIF (panel B) induced after staurosporine challenge was inhibited in lipoxin-pretreated-cells and both proteins were retained in the mitochondrial/particulate compartment. In addition to this, the oxidative stress induced by staurosporine in macrophages was also attenuated after lipoxin treatment, as reflected by the decrease in DCFH oxidation in the presence of $LXA_4$ (Fig. 3C). Moreover, using DAF-2 as RNI scavenger, a dose-dependent decrease in the oxidation of this probe promoted by staurosporine was observed in cells pretreated with lipoxin (Fig. 3D).

To investigate the mechanisms mediating the anti-apoptotic effects of $LXA_4$ in macrophages, the levels of apoptosis-related proteins were determined by Western blot. As Fig. 4A shows, treatment with $LXA_4$ increased the levels of the anti-apoptotic proteins Mcl-1 and to a lesser extent those of x-IAP and Bcl-2, without affecting Bax changes upon staurosporine challenge. Indeed, the ratio between Mcl-1 (and also x-IAP and Bcl-2) and Bax (Fig. 4B), well reflected the protection against apoptosis observed in lipoxin-treated cells. To gain further insight into the differential apoptotic response of staurosporine-treated and lipoxin-pretreated cells, we used a specific mouse apoptosis microarray to compare the gene expression profile after these treatments. Fig. 4C summarizes the changes in the expression of genes whose transcription is up/down 2-folds over the threshold value, among the apoptosis-related genes analyzed. The data show that staurosporine did not alter significantly the expression of the majority of the genes analyzed ($p<0.01$). Moreover, using RT-PCR to confirm the array data, it was observed that, over the genes modified by staurosporine, $LXA_4$ was capable to reduce significantly the expression of some genes, such as IL-10 (Fig. 4D), that exerts a pro-apoptotic activity in macrophages [33]. Indeed, lipoxin did not alter significantly the expression of most of the genes involved in staurosporine-induced apoptosis, suggesting the involvement of post-transcriptional mechanisms in its mechanism of action.

***Lipoxin-dependent survival involves AKT and ERK activation.*** The rise in Mcl-1 levels after $LXA_4$ treatment suggests the activation of the PI3K/Akt pathway [21]. As Fig. 5A shows, $LXA_4$ promoted a rapid Akt phosphorylation in a PI3K-dependent way, as deduced by the inhibitory effect observed by LY294002 incubation. In addition to this and since MAPKs are implicated in the regulation of basic cellular processes, such as apoptosis, survival, proliferation, and differentiation, and our preceding data suggest that $LXA_4$ is influencing signaling steps upstream mitochondrial pro-apoptotic targeting, the effects of lipoxin on the activity of these kinases were investigated. As Fig. 5B,C illustrate, staurosporine induced the phosphorylation of p38 and JNK from 30 to 120min; however, this treatment did not activate ERK over its low basal level. $LXA_4$ pre-treatment did not affect p38 or JNK phosphorylation, but promoted a rapid activation of ERK after staurosporine challenge. Inhibition of this ERK activation with PD098059 (1 μM), or PI3K activity with LY294002 (10 μM) abolished the protective effect exerted by $LXA_4$ against apoptosis (Fig. 5D). In addition to this, the translocation to the nucleus and activation of the transcription factor Nrf-2 was also observed in $LXA_4$–pretreated cells following kinetics similar to ERK activation (Fig. 5E). Interestingly, unlike staurosporine, $LXA_4$ was able to activate an ARE-luciferase reporter gene upon transfection, whereas the ERK inhibitor PD098059 impaired this response, suggesting that the cytoprotective pathway dependent on Nrf-2 activity [23,24] is activated in response to $LXA_4$. MG132 was used as a positive control of ARE activation (Fig. 5F). Finally, using peritoneal macrophages from Nrf-2 deficient mice (Fig. 5G), it was observed a

significant decrease in the anti-apoptotic activity mediated by $LXA_4$. Taken together, these data suggest a link between Akt and ERK/Nrf-2 activation and the protective effects against apoptosis exerted by $LXA_4$ in macrophages.

***Protection from apoptosis by lipoxin $A_4$ of murine and human macrophages.*** To validate the results obtained in RAW 264.7 cells in other sources of macrophages, we carried out some key experiments in murine peritoneal macrophages. As Fig. 6A shows, the treatment with lipoxin promoted a rapid Akt phosphorylation in these cells that was inhibited in the presence of LY294002. Following the same line, challenge with staurosporine produced a rise in the phospho-ERK levels in cells treated with lipoxin, a receptor-mediated effect, since it disappeared when these macrophages were pre-treated with BocPLP prior to $LXA_4$ challenge (Fig. 6B). As in RAW 264.7 cells, apoptosis was significantly prevented in $LXA_4$ pretreated cells after stimulation with staurosporine, LPS/IFNγ or the anti-mouse FAS Jo2. In agreement with our previous data, inhibition of the ERK or PI3K pathways attenuated the antiapoptotic effect exerted by lipoxin (Fig. 6C). To extent our results, we corroborated that similar effects in terms of apoptosis protection against staurosporine were obtained in cultured human monocyte/macrophages and in the monocytic cell line THP-1. These effects were also mediated by the Akt and ERK pathways as demonstrated using the corresponding inhibitors (Fig. 6D). $LXA_4$ also promoted the translocation to the nucleus of Nrf-2 in these cells as shows in Fig. 6E being capable to maintain the antiapoptotic effects previously reported in murine macrophages.

**DISCUSSION**

The present study provides evidence for a new mechanism of action of $LXA_4$, by which this compound contributes to inflammation resolution, delaying apoptosis in murine and human macrophages. Prolonged macrophage survival is required for phagocytosis of death cells in the inflamed area, avoiding excessive accumulation of cellular debris into the injured tissues. Lipoxins are natural compounds detected in several tissues in the nanomolar range, but exhibiting a certain cell-specificity. In this regard, lipoxins have been involved in the pathogenic mechanism of some microbes. For example, in addition to the anti-apoptotic effects of $LXA_4$ it has been described that this lipoxin prevents $PGE_2$ synthesis and this strategy is used by virulent Mycobacterium tuberculosis strains to avoid repair of the macrophage plasma membrane and to promote necrosis at the time the pathogen evades macrophage function [12,34]. Therefore, although their role as anti-inflammatory compounds is well-documented, the implication in apoptosis has been a matter of debate [2,5,8,35]. Opposite actions of lipoxins on the balance between survival/apoptosis have been reported in neutrophils when signaling through the pleiotropic formylpeptide receptor like-1/$LXA_4$ receptor [31]. This receptor binds a series of molecules including $LXA_4$, acute-phase reactant serum amyloid A (SAA) and the glucocorticoid-inducible protein annexin-1. Whereas annexin 1 and lipoxins accelerate resolution/apoptotic death of neutrophils via caspase 3 activation, SAA rescues the cells by preventing mitochondrial dysfunction [36,37,38]. In contrast to neutrophils, macrophages arrive later to the inflamed area and they contribute to tissue clearance. Thus, as our results indicate, as far as the synthesis of lipoxins occurs they act as anti-apoptotic molecules, preserving macrophage function during resolution.

We previously described that the release of NO by activated macrophages contributes transiently to expand macrophage viability by inhibiting caspase processing and activation, via the formation of S-nitrosothiols in these enzymes [39]. In line with this, the results reported in this work stand directly on the mechanism of action of lipoxin in the resolution of inflammation. The data show that lipoxin pre-treatment of macrophages diminishes staurosporine, etoposide, GSNO and LPS/IFNγ-induced apoptosis. Moreover, in murine peritoneal macrophages and in human monocyte/macrophages that exhibit an apoptotic response via the Fas/FasL pathway, treatment with $LXA_4$ also exhibited a protective effect. The fact that the protective effect of $LXA_4$ against apoptosis was observed in human and murine primary cultures of macrophages and in macrophage cell lines suggests the existence of common mechanisms of response to lipoxins in these cells. The action of lipoxin was independent of the time of pre-treatment from 12h to 30min, prior to staurosporine challenge (data not shown). These data suggest that the mechanisms involved in protection against apoptosis are mainly regulated by short-term responses to lipoxins, rather than by profound changes in the expression of genes that regulate cell viability.

The apoptosis induced by staurosporine involves essentially the mitochondrial pathway [18,40,41]. Activation of apoptosis-related proteins is considered a basic step into the apoptotic

progression being fundamental those of the Bcl-2 family, which includes both anti and pro-apoptotic members, and other related proteins. Using microarrays of apoptosis-related genes, it was observed that $LXA_4$ fails to promote changes in the expression of these genes. However, in the presence of lipoxin the protein levels of Bcl-2, x-IAP and Mcl-1, all anti-apoptotic proteins, were maintained, indicating post-transcriptional regulation. Moreover, our data show that incubation of macrophages with $LXA_4$ promotes a rapid activation of the PI3K/Akt pathway that has a relevant role in the inhibition of apoptosis [42]. Among the antiapoptotic genes regulated through this pathway upregulation of Mcl-1 levels have been identified in various cell types [23,24], a situation also prevailing in macrophages treated with $LXA_4$. Indeed, incubation with lipoxin preserved the mitochondrial integrity as deduced by the minimal release of cytochrome c and AIF to the cytosol. Oxidative stress is another determinant which regulates the balance between viability/apoptosis [25,43,44,45,46]. We have analyzed the effect of lipoxin on the liberation of some important reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI), using fluorescent probes. Our results indicate that lipoxin inhibits ROS and RNI release, reducing the oxidative stress associated with apoptosis. Consistent with this, lipoxin at nanomolar concentrations also diminishes the caspase activation induced after staurosporine treatment. This inhibitory action was detected for all caspases analyzed, being the most potent effect on caspase 3 and to a lesser extent on both caspase 8 and 9. The results from the experiments using the pan-caspase inhibitor z-VAD, confirmed the importance of lipoxin reduction of caspase activation, leading to similar levels of apoptosis in macrophages.

In addition to this, it is known that macrophage apoptosis is controlled by a complex network of signaling pathways, including MAPKs pathways [45,47,48,49,50]. The present study provides evidence that lipoxin might affect these pathways. Incubation of macrophages with staurosporine induces a rapid activation of JNK and p38 MAPKs, but not of ERK, a classic survival pathway [51]. Interestingly, lipoxin pre-treatment was able to promote ERK phosphorylation in the presence of staurosporine without affecting neither JNK nor p38 activities. Furthermore, MAPKs pathway associates with the modulation of Antioxidant Response Elements (ARE)-driven gene expression via Nrf-2 activation, a transcription factor that is considered as the "*guardian of redox homeostasis"*. It has been reported that ERK activation is involved in the translocation of Nrf-2 to the nucleus where this transcription factor binds to the ARE motifs [27,52]. Nrf-2 regulates the transcription of *ca.* one hundred of genes involved in cytoprotection and regulation of oxidative stress [23]. Recently it has been described the $LXA_4$-dependent apoptosis of human neutrophils involves the inhibition of myeloperoxidase-induced ERK, that results in a reduction in the expression of the anti-apoptotic gene Mcl-1 [53]. However, our results provide a new mechanism by which lipoxin impairs macrophage apoptosis, involving Nrf-2 activation through an ERK dependent pathway [26,27,54].

Although multiple studies exist about the regulation of innate immunity by lipoxins, including SOCS2-dependent ubiquitinylation of TRAF-2 and -6 [55,56], our results demonstrate that $LXA_4$ prolongs macrophages survival by: a) activation of the PI3K/Akt pathway, leading to the expression of antiapoptotic proteins of the Bcl-2 family; b) activation of the ERK/Nrf-2 pathway upon challenge with an apoptotic stimulus, such as staurosporine; c) preservation of mitochondrial function and integrity, and enhancement of the anti-oxidant defense systems in cells; d) impairment of caspase activation due to the absence of pro-apoptotic signaling dependent on the mitochondrial pathway or the death-receptor pathway. Therefore, these observations provide an additional anti-apoptotic mechanism for $LXA_4$ in macrophages and might contribute to the establishment of a hitherto unrecognized potential of lipoxins for the treatment of inflammatory diseases.

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**LEGENDS TO FIGURES**

**Figure 1. Lipoxin $A_4$ attenuates apoptosis in RAW 264.7 cells.** The percentage of annexin V-positive cells was determined by flow cytometry after preincubation (2h) of macrophages with the indicated concentrations of epi-$LXA_4$ or $LXA_4$ in the absence or presence of 1 μM BocPLP (1h before $LXA_4$), a lipoxin receptor antagonist, followed by apoptosis induction by 200 ng/ml of staurosporine or vehicle for 4h (A). Representative dot plot diagrams are shown (250 nM of $LXA_4$) (B). This cell population was also analyzed by flow cytometry for nuclear DNA content (C). Cells were incubated with staurosporine (200 ng/ml), etoposide (1 μM) or GSNO (0.5 mM) alone or in combination with $LXA_4$ for 18h, and with LPS (500 ng/ml)/IFNγ (20 ng/ml) for 48h. Apoptosis was determined by flow cytometry using PI labeling (D). Results show the mean±SD of four different experiments. **P*<0.05, ***P*<0.01 *vs.* the corresponding condition in the absence of lipoxin.

**Figure 2**. **Lipoxin treatment impairs caspase activation in RAW 264.7 cells.** Cells were treated for 4h or 18h with 200 ng/ml staurosporine alone or in combination with 250 nM $LXA_4$ (2h of pretreatment) and the activity of caspases 3, 8 and 9 was determined in cell extracts (A) or using CaspGlow 'in-cell' caspase activation detection (B; *left panel:* representative images of caspase 3 positive cells). The intensity of the cell fluorescence was digitalized and quantified with respect to the total cell number determined by Hoechst labeling (B, right panel). The broad apoptosis inhibitor z-VAD was used at 20 μM following manufacturer instructions as positive control. Results show the means±SD of three different experiments. **P*<0.05, ***P*<0.01 *vs.* the corresponding condition in the absence of lipoxin.

**Figure 3. Release of mitochondrial apoptotic mediators and oxidative stress were reduced by lipoxin treatment in RAW 264.7.** Cells were pretreated for 2h with $LXA_4$ (250 nM) and challenged with 200 ng/ml of staurosporine for the indicated periods of time. Particulate (containing mitochondria) and cytosolic fractions of the cells were prepared and cytochrome c levels were determined by Western blot using specific antibodies and β-actin (cytosol) and porin (mitochondria) as a loading control (A). The subcellular distribution of AIF was determined 4h after staurosporine addition by confocal microscopy, as described in (A). Images were analyzed to determine the co-localization of mitochondrial markers and AIF and the ratio of 'green' (AIF) in 'red' (mito-tracker) of 60-80 cells per condition was quantified (B). To measure ROS production, macrophages were treated with the indicated stimuli, followed by loading with DCFH for 30min at 37ºC and its oxidation was monitorized by flow cytometry (C). Using the same treatment, cells were loaded with DAF-2 for 30min at 37ºC and its oxidation was determined to measure RNI production (D). Data are presented as mean±SD of three different experiments, or a representative blot (A) or cell-image (B). $^{a}P$<0.01 *vs.* control, $^{b}P$<0.01 *vs.* the staurosporine condition (B).

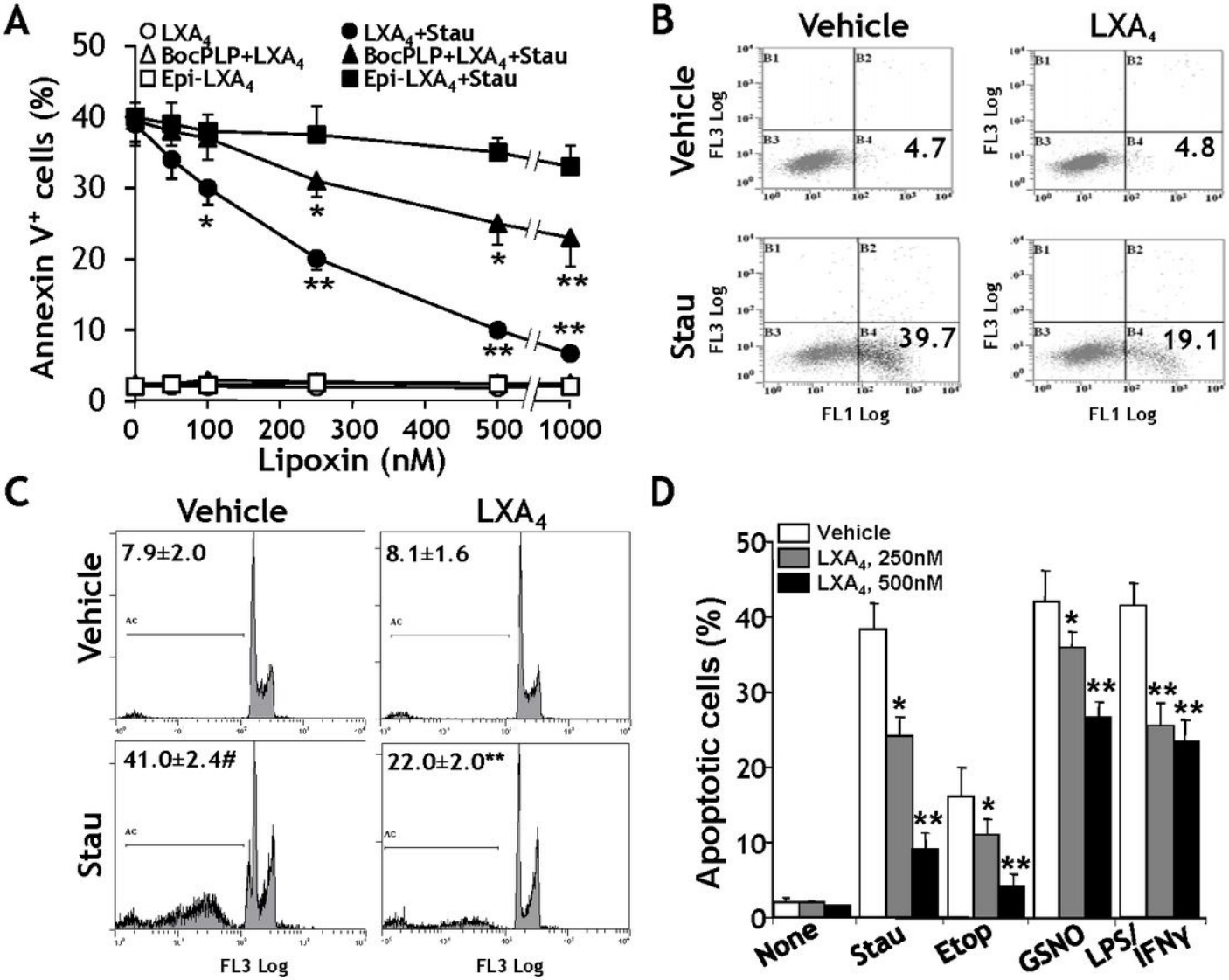
*****$P<0.05$ ******$P<0.01$ *vs.* vehicle-treated cells, $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs.* the corresponding condition in the absence of lipoxin.

**Figure 4**. **Changes of apoptotic-related genes and proteins in lipoxin treated cells**. RAW 264.7 cells were incubated with staurosporine (200 ng/ml) and $LXA_4$ (250 nM) and analyzed at the indicated times. The protein levels of Bcl-2, Bax, Mcl-1 and x-IAP were determined by Western blot. The levels of the p85 subunit of the PI3K were used as control for lane charge (A). After densitometric analysis, the ratios between Mcl-1, x-IAP and Bcl-2 *vs.* Bax were calculated (B). Total RNA was analyzed using the Profiler PCR Array System and the SYBR Green/Fluorescein qPCR master mix (SuperArray Bioscience), and gene expression was compared with the web-based software package for the PCR Array System (http://www.superarray.com/pcr/arrayanalysis.php). Colorimetric diagram with a selection of the apoptosis-related genes analyzed: Genes whose transcription is up/down (red/green) two fold in gene expression after staurosporine or lipoxin plus staurosporine treatment *vs.* control (4h) are shown (C). RT-PCR of specific genes of the array was used to validate the data (D). Results show a representative blot (n=3; *panel* A), and the mean±SD of band ratios. **P*<0.05; ***P*<0.01 *vs.* the corresponding condition in the absence of lipoxin.

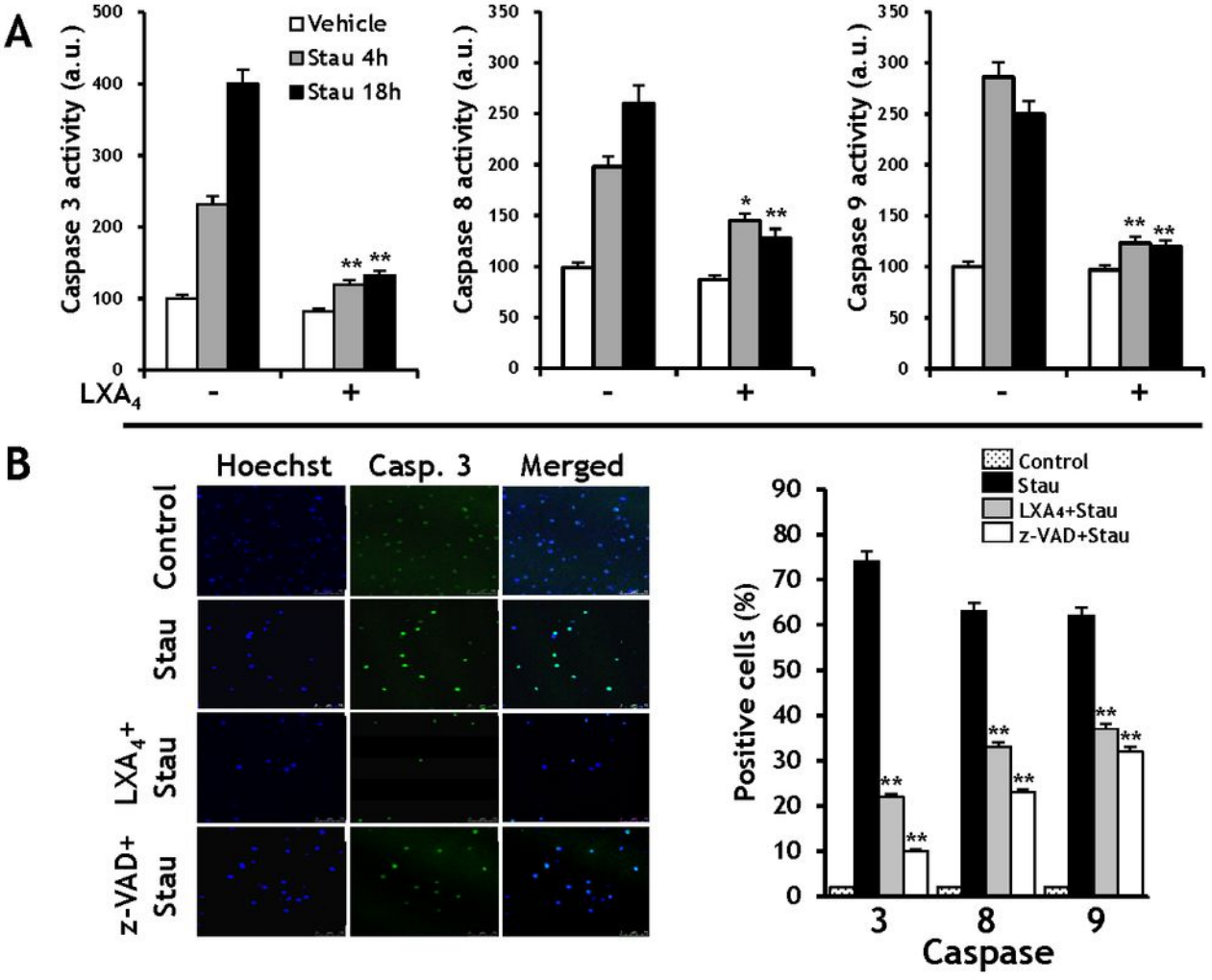
**Figure 5. $LXA_4$ attenuation of macrophage apoptosis involves Akt and ERK/Nrf-2 activation**. Cells were treated as described in Fig. 1, and the phosphorylation of Akt in S473 was analyzed following $LXA_4$ challenge. Pre-treatment with 20 μM of the PI3K inhibitor LY294002 inhibited Akt phosphorylation (A). The activity of the MAP kinases was followed by Western blot using specific antibodies (B); the pERK/ERK band ratio was calculated (C). Inhibition of PI3K with 10 μM LY294002 (30min preincubation) or ERK with 1 μM PD098059 (30min preincubation) abrogated the $LXA_4$-dependent protection against apoptosis (D). Concomitant to ERK activation, Nrf-2 translocated to the nucleus (E) and the activity was increased by MG132 (20 μM; used as positive control) and by $LXA_4$, but inhibited in the presence of PD098059 upon transfection with an ARE-luc reporter vector (F). $LXA_4$-protection from staurosporine induced apoptosis in peritoneal macrophages was attenuated in cells from animals lacking Nrf-2 (G). Data are presented as means±SD of four different experiments. **P*<0.01 *vs.* the corresponding condition in the absence of lipoxin; $^{a}P<0.05$ *vs.* the corresponding condition in the absence of PD098059 (F); $^{b}P<0.01$ *vs.* the corresponding value in Nrf-$2^{+/+}$ macrophages (G).

**Figure 6. $LXA_4$ attenuates apoptosis in peritoneal murine macrophages and in human monocyte/macrophages**. Primary cultures of murine peritoneal macrophages (A-C), human

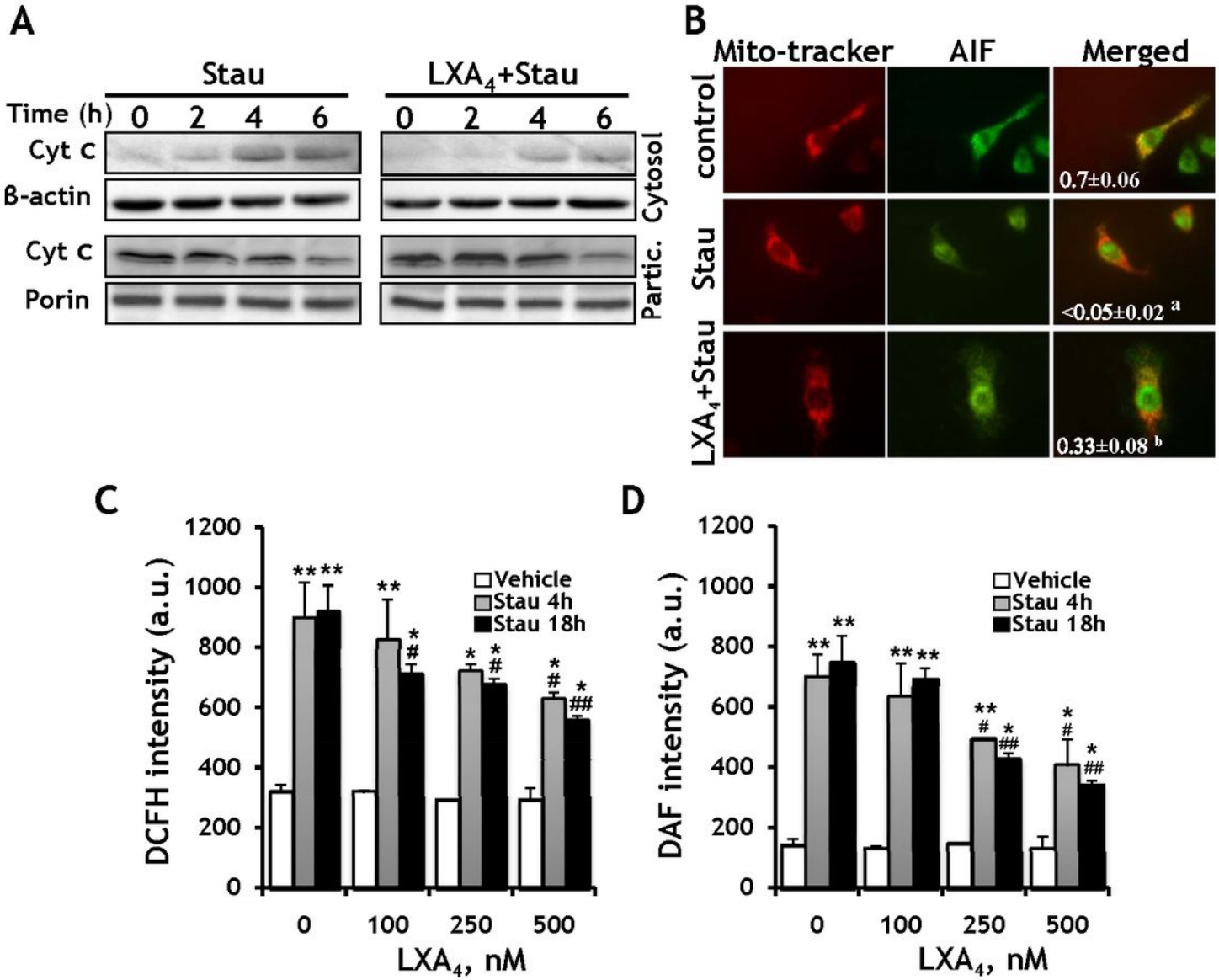
peripheral blood monocytes (D, E-*left*) and the human monocytic cell line THP-1 (E-*right*) were incubated as indicated. When cells were treated with PD098059 (1 μM) or LY294002 (10 μM), addition was 30min prior to $LXA_4$ challenge (A,C,D). When Jo2 (1 μg/ml) was used, cells were incubated for 24h (C); BocPLP (1 μM) was added 1h prior to lipoxin treatment (B,C). Nrf-2 was determined by Western blot using nuclear extracts of peripheral blood human monocytes or THP-1 cells (E). Results show the mean±SD of four different preparations of cells. **P*<0.05; ***P*<0.01 *vs.* the corresponding condition in the absence of lipoxin.



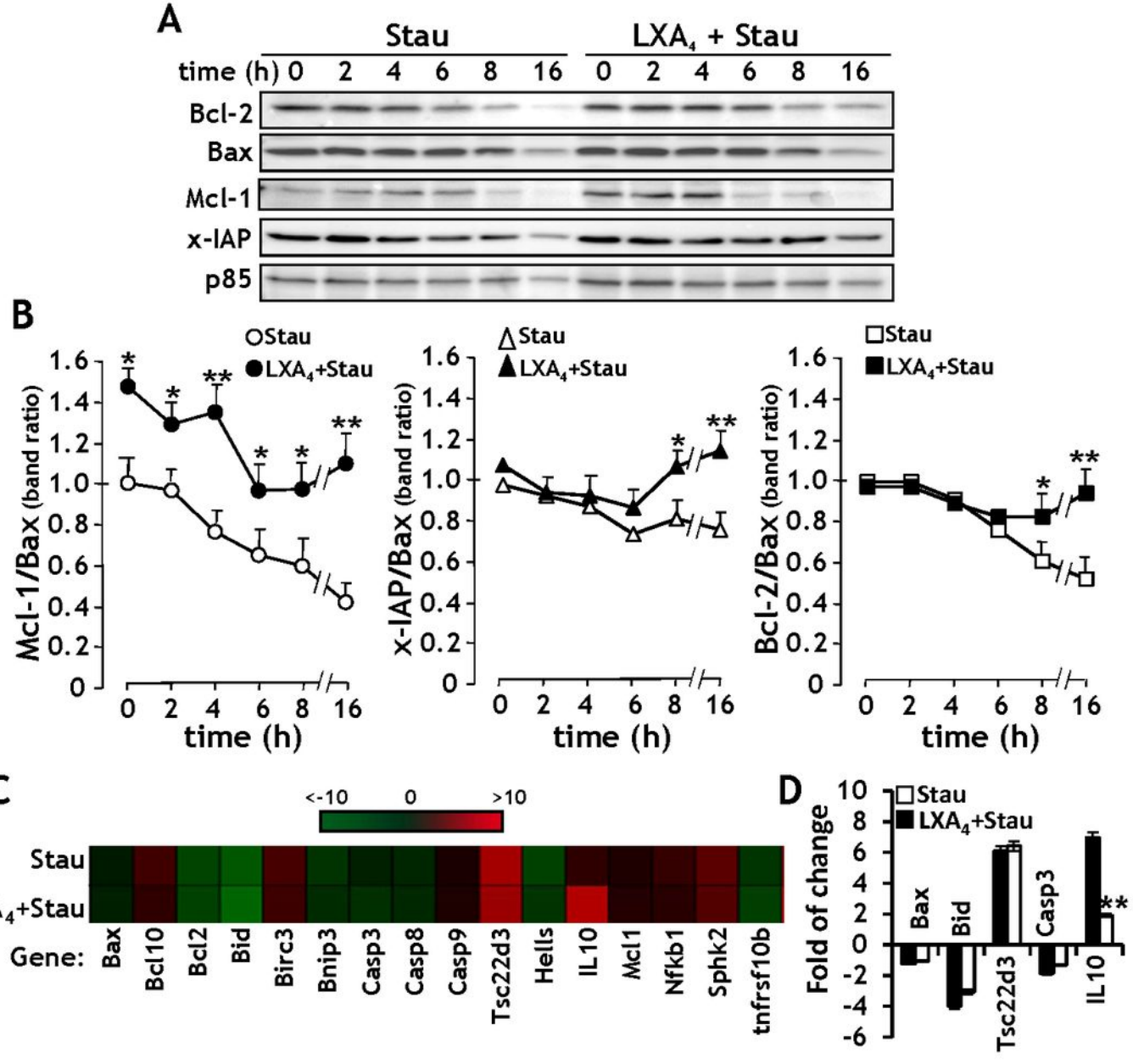

Prieto et al. Fig. 1



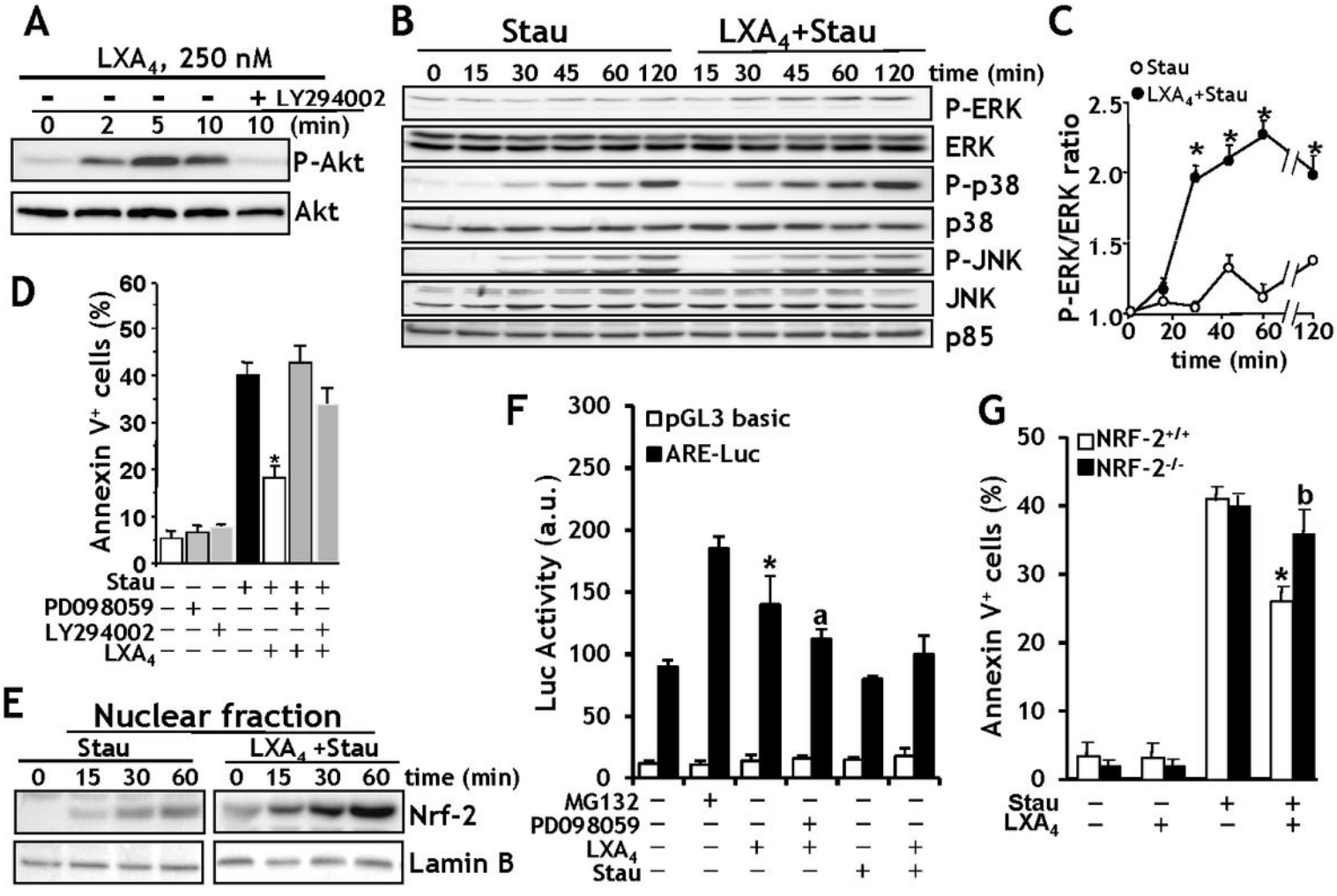

Prieto et al. Fig. 2

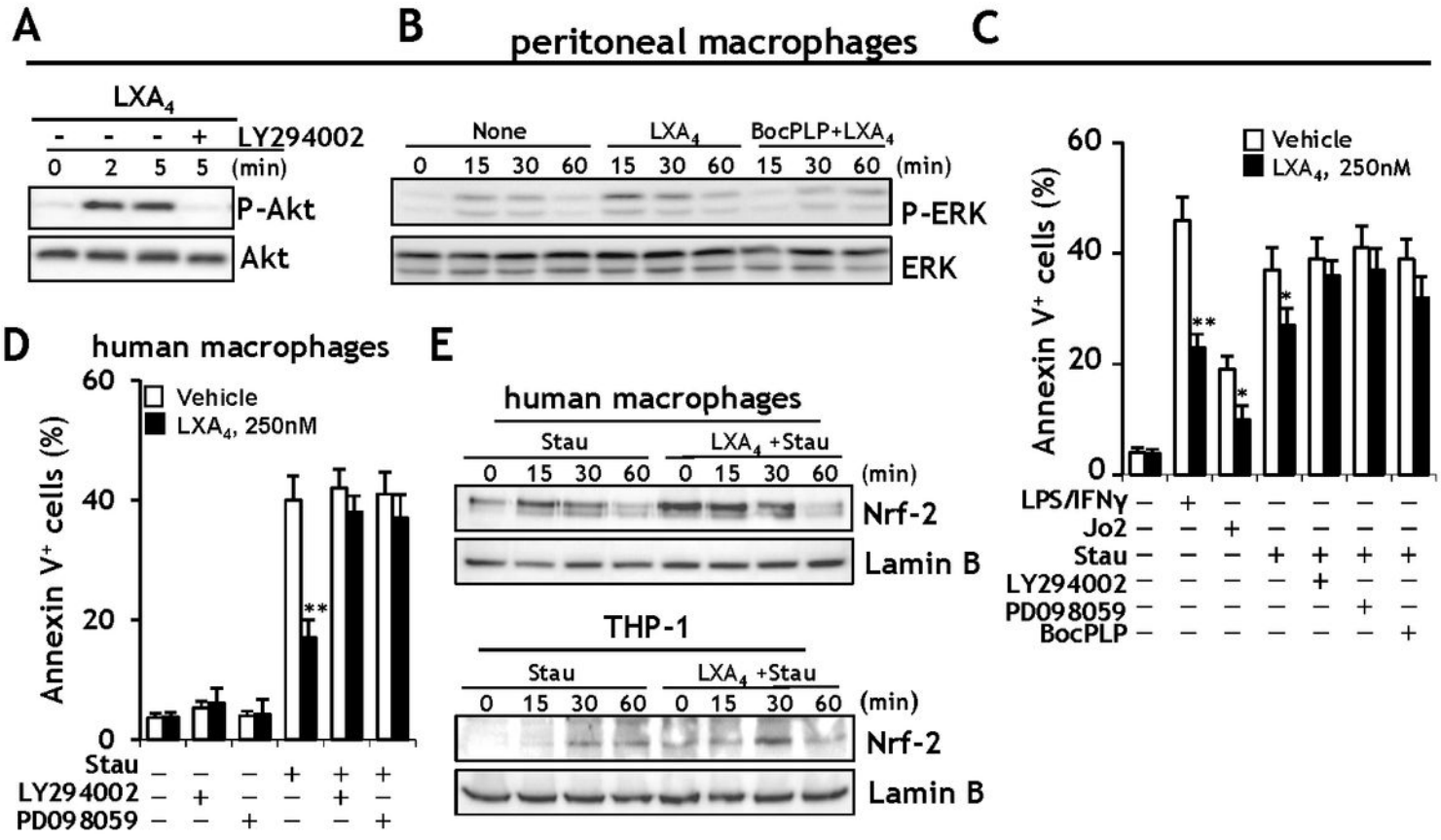



Prieto et al. Fig. 3




Prieto et al. Fig. 4


A
LXA4, 250 nM
- - - - + LY294002
0 2 5 10 10 (min)
P-Akt
Akt
B
Stau
LXA4+Stau
0 15 30 45 60 120 15 30 45 60 120 time (min)
P-ERK
ERK
P-p38
p38
P-JNK
JNK
p85
C
Stau
LXA4+Stau
P-ERK/ERK ratio
time (min)
D
Annexin V+ cells (%)
Stau
PD098059
LY294002
LXA4
E
Nuclear fraction
Stau
LXA4 +Stau
0 15 30 60 0 15 30 60 time (min)
Nrf-2
Lamin B
F
pGL3 basic
ARE-Luc
Luc Activity (a.u.)
MG132
PD098059
LXA4
Stau
G
NRF-2+/+
NRF-2-/-
Annexin V+ cells (%)
Stau
LXA4




Prieto et al. Fig. 6