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

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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 attenuation of inflammation. However, effects of lipoxins on macrophage are less known, particularly the action of LXA_4 on the regulation of apoptosis of these cells. Our data show that pretreatment of human or murine macrophages with LXA_4 at the concentrations prevailing in the course of resolution of inflammation (nanomolar range) significantly inhibits 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 and 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 antiapoptotic 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, which was required for the observation of the antiapoptotic effects of LXA_4. These data suggest that the LXA_4, released after the recruitment of neutrophils to sites of inflammation, exerts a protective effect on macrophage viability that might contribute to a better resolution of inflammation.
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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] During this process, neutrophils are quickly recruited to sites of infection at which they have a very short life and die rapidly through apoptosis.[3] Macrophages arrive later to phagocytic 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 that are released during the resolution phase of inflammation, in the nanomolar range.[4] Lipoxins are mainly generated by transcellular metabolism from arachidonic acid depending on the cellular context.[5,6] In mammals, lipoxygenase enzymes (LOXs) generate two main native products: lipoxin A_4 (LXA_4) and B_4 (LXB_4), with LXA_4 being the most studied, which exerts potent anti-inflammatory actions modulating leukocyte trafficking and promoting phagocytic clearance of apoptotic cells.[7,8] The effects of LXs as lipid mediators with potent anti-inflammatory actions are well documented, but their role in apoptosis remains controversial.[9] For instance, an apoptotic effect on fibroblasts after treatment with LXA_4 at micromolar concentrations has been described,[10] whereas it has been also reported that this compound inhibits peroxynitrite formation in leukocytes,[11] reduces colonocyte apoptosis[12] and promotes survival of retinal pigment epithelial cells.[13] Therefore, the precise role of LXs as modulators of apoptosis remains elusive and seems to be dependent on the cell type and on the LXA_4 concentration. Given the lack of studies regarding 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,[3,14,15] and LPS/IFNγ challenge 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 antiapoptotic environment, and a blockade of early apoptotic signaling, which involves the suppression of the release of mitochondria-dependent apoptotic mediators and caspases activation. Moreover, our data show that LX treatment of macrophages promotes a rapid activation of the PI3K/Akt signaling that is relevant for protection against apoptosis, in agreement with a previous study describing upregulation of antiapoptotic genes by this pathway, such as *Mcl-1*.[16] In addition to this, treatment of macrophages with LXA_4 induced ERK/Nrf-2 activation, with Nrf-2 being a transcription factor that regulates the expression of *ca.* 100 cytoprotective genes that share in common a sequence termed antioxidant response element (ARE),[17,18] and contributes to the

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antioxidant defense pathway in many cells.[19,20] Activation of this pathway in macrophages by LXA_4 decreased staurosporine-induced apoptosis, without affecting the activity of JNK and p38 MAPKs.

Results

LXA_4 prevents stimuli-dependent apoptosis in macrophages. Preliminary studies in our laboratory suggested that LXA_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 LX to interfere with the apoptosis induced by this drug in macrophages. Incubation for 4 h of RAW 264.7 cells with staurosporine (200 ng/ml) increased the percentage of annexin V-positive cells by 38% (Figure 1a). Preincubation for 2 h 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, through its receptor, as it was interfered after 1 h of incubation with 1 μM BocPLP, a formyl peptide LXreceptor antagonist,[23,24] and was specific for LXA_4, the 15-epi-LXA_4 derivative being less effective (Figure 1a). Figure 1b shows a representative flow cytometry distribution of this annexin V analysis. The protective effect of LX against apoptosis was confirmed by DNA size distribution analysis, observing a significant reduction of the hypodiploid population (Figure 1c). When apoptosis was induced with

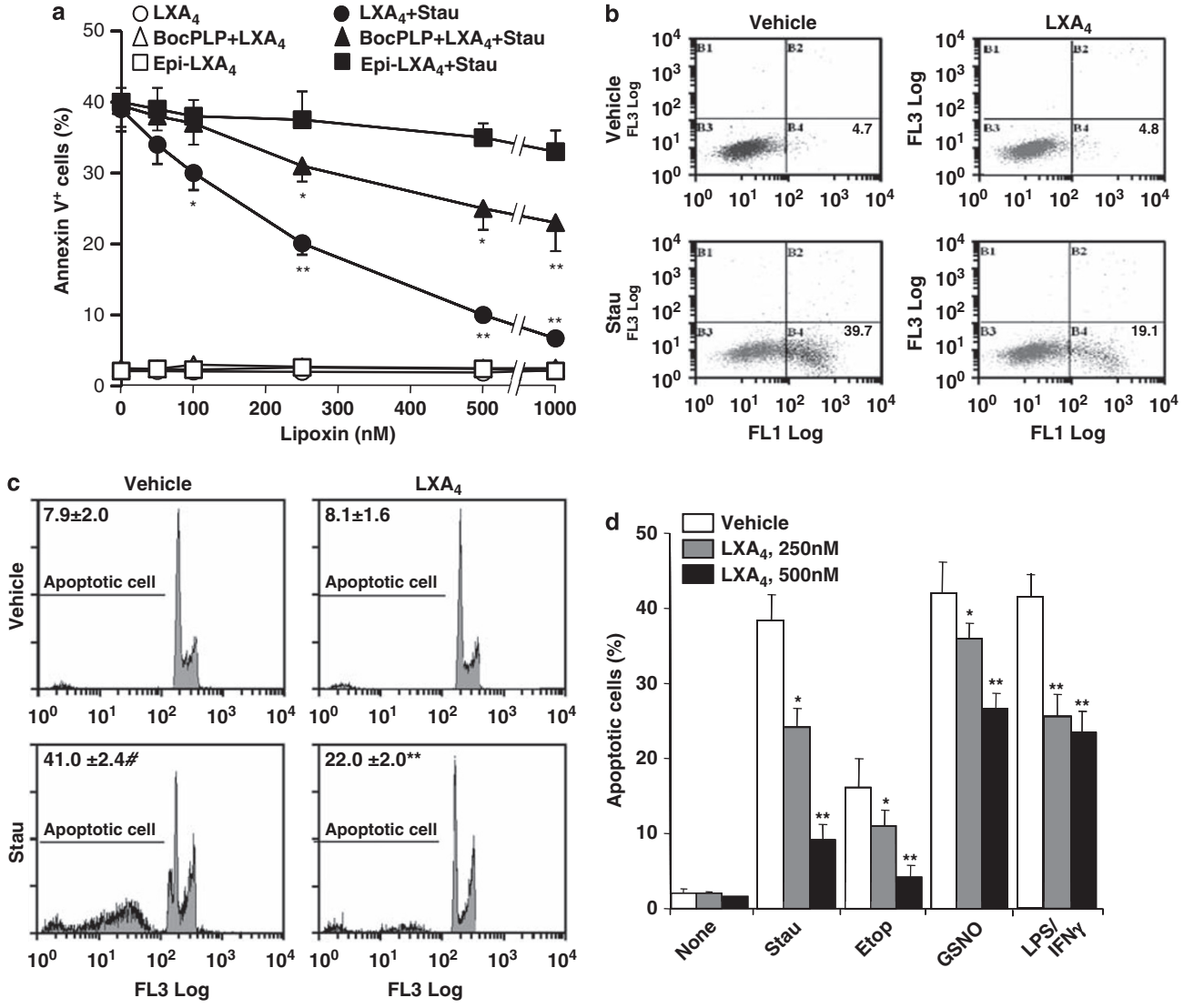

Figure 1 Lipoxin A_4 attenuates apoptosis in RAW 264.7 cells. The percentage of annexin V-positive cells was determined by flow cytometry after preincubation (2 h) of macrophages with the indicated concentrations of epi-LXA_4 or LXA_4 in the absence or presence of 1 μM BocPLP (1 h before LXA_4 treatment), a lipoxin receptor antagonist, followed by apoptosis induction using 200 ng/ml of staurosporine or vehicle for 4 h (**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 18 h, and with LPS (500 ng/ml)/IFNγ (20 ng/ml) for 48 h. Apoptosis was determined by flow cytometry using PI labeling (**d**). Results show the mean ± S.D. values of four different experiments. $^*P<0.05$, $^{**}P<0.01$ *versus* the corresponding condition in the absence of lipoxin

etoposide (1 μM), GSNO (0.5 mM) or after LPS (500 ng/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 (Figure 1d).

LX inhibition of caspase activation and pro-apoptotic signaling in macrophages treated with staurosporine. To study the antiapoptotic effect of LX, the activation of caspases 3, 8 and 9 was analyzed in cells treated with staurosporine. As shown in Figure 2a, the well-known activation of caspases induced after staurosporine treatment (200 ng/ml; 4 h and 18 h, 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; Figure 2b, left panel). Figure 2b (right panel) shows the percentage of cells with activated caspases 3, 8 or 9 after staurosporine treatment. Lipoxin A_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 because this is one of the key events in the commitment of the apoptotic response of macrophages. As shown in Figure 3, the release of cytochrome *c* (panel A) and apoptosis-inducing factor (AIF; panel B) induced after staurosporine challenge was inhibited in LX-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 LX treatment, as reflected by the decrease in DCFH oxidation in the presence of LXA_4 (Figure 3c). Moreover, on 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 LX (Figure 3d).

To investigate the mechanisms mediating the antiapoptotic effects of LXA_4 in macrophages, the levels of apoptosis-related proteins were determined by western blotting. As shown in Figure 4a, treatment with LXA_4 increased the levels of the antiapoptotic proteins, Mcl-1, and to a lesser extent those of x-IAP and Bcl-2, without affecting the Bax levels on staurosporine challenge. Indeed, the ratio between Mcl-1 (and also x-IAP and Bcl-2) and Bax (Figure 4b) well-reflected the protection against apoptosis observed in LX-treated cells.

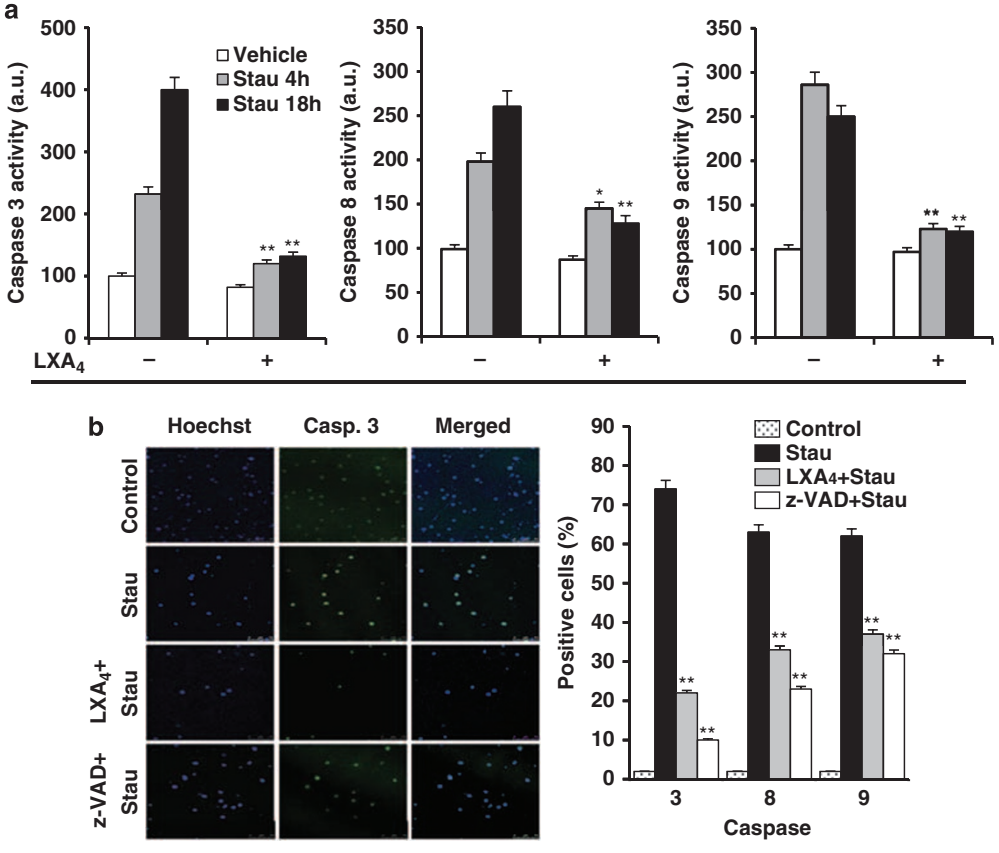

Figure 2 Lipoxin treatment impairs caspase activation in RAW 264.7 cells. Cells were treated for 4 or 18 h with 200 ng/ml staurosporine alone or in combination with 250 nM LXA_4 (2-h pretreatment) and the activity of caspases 3, 8 and 9 was determined in cell extracts (**a**) or using CaspGlow 'in-cell' 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's instructions as positive control. Results show the mean ± S.D. values of three different experiments. *$P<0.05$, **$P<0.01$ *versus* the corresponding condition in the absence of lipoxin

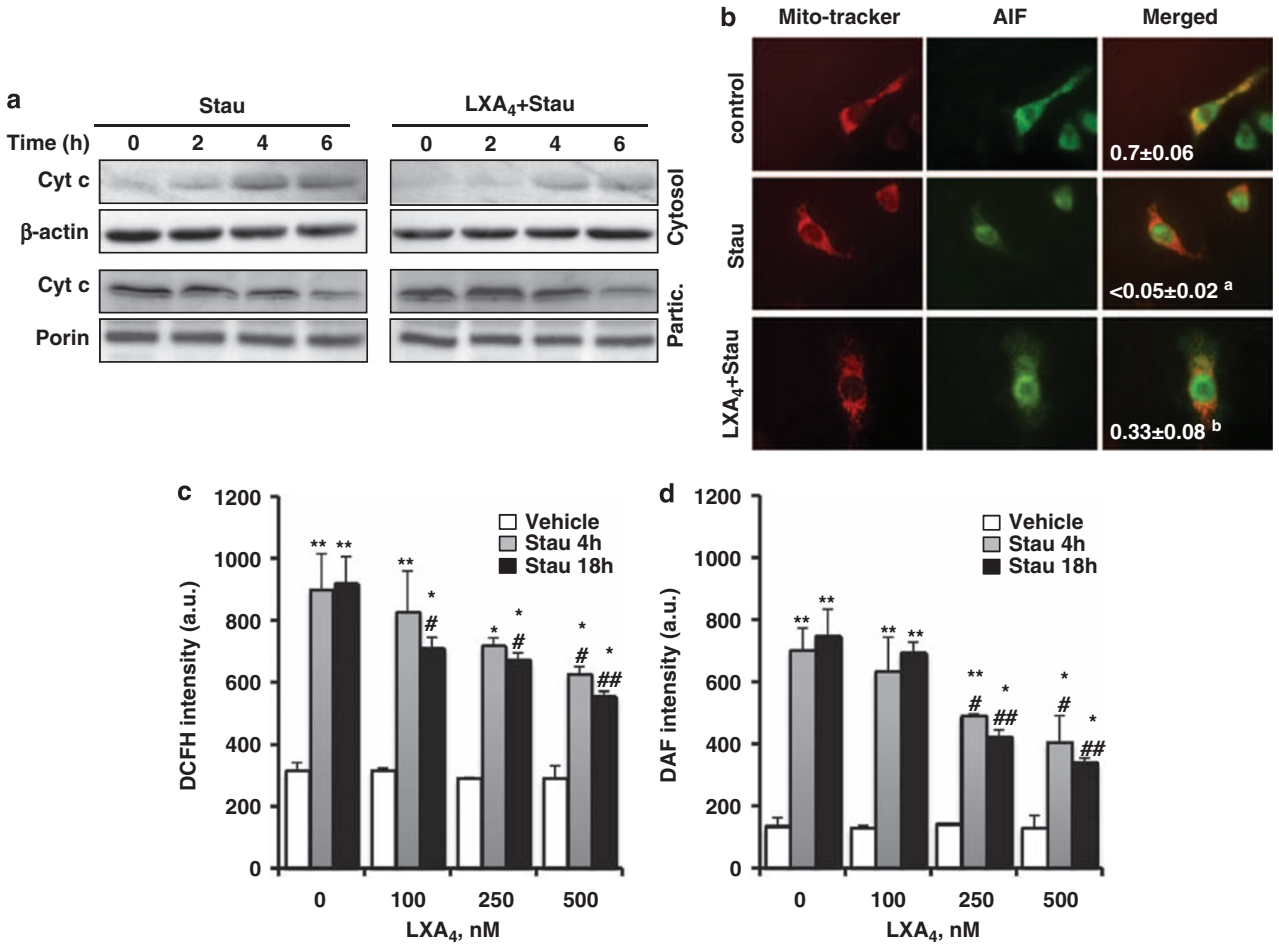

Figure 3 Release of mitochondrial apoptotic mediators and oxidative stress were reduced by lipoxin treatment in RAW 264.7. Cells were pretreated for 2 h with LXA_4 (250 nM) and challenged with 200 ng/ml of staurosporine for the indicated periods of time. Particulate (containing mitochondria) and cytosolic 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 loading controls (**a**). The subcellular distribution of AIF was determined 4 h 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 30 min at 37°C and its oxidation was monitorized by flow cytometry (**c**). Using the same treatment, cells were loaded with DAF-2 for 30 min at 37°C and its oxidation was determined to measure RNI production (**d**). Data are presented as mean ± S.D. values of three different experiments or a representative blot (**a**) or cell image (**b**). $^{a}P<0.01$ *versus* control, $^{b}P<0.01$ *versus* the staurosporine condition (**b**). $^{*}P<0.05$, $^{**}P<0.01$ *versus* vehicle-treated cells; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *versus* the corresponding condition in the absence of lipoxin

To gain further insight into the differential apoptotic response of staurosporine-treated and LX-pretreated cells, we used a specific mouse apoptosis microarray to compare the gene expression profile after these treatments. Figure 4c summarizes the changes in the expression of genes, transcription of which is up/down by two-folds over the threshold value, among the apoptosis-related genes analyzed. Data show that staurosporine did not significantly alter 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 able to significantly reduce the expression of some genes, such as *IL-10* (Figure 4d), that promote a pro-apoptotic activity in macrophages.[25] Indeed, LX did not significantly alter the expression of most of the genes involved in staurosporine-induced apoptosis, suggesting the involvement of post-transcriptional mechanisms in its mechanism of action.

LX-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.[16] As shown in Figure 5a, LXA_4 promoted a rapid Akt phosphorylation in a PI3K-dependent manner, as deduced by the inhibitory effect observed by LY294002 incubation. In addition to this and as MAPKs have been implicated in the regulation of basic cellular processes, such as apoptosis, survival, proliferation and differentiation, and our preceding data that suggest that LXA_4 influences signaling steps upstream mitochondrial pro-apoptotic targeting, the effects of LX on the activity of these kinases were investigated. As illustrated in Figure 5b and c, staurosporine induced the phosphorylation of p38 and JNK from 30 to 120 min; however, this treatment did not activate ERK over its low basal level. Pretreatment with LX A_4 did not affect p38 or JNK phosphorylation, but promoted a rapid activation of ERK after staurosporine challenge. Inhibition of

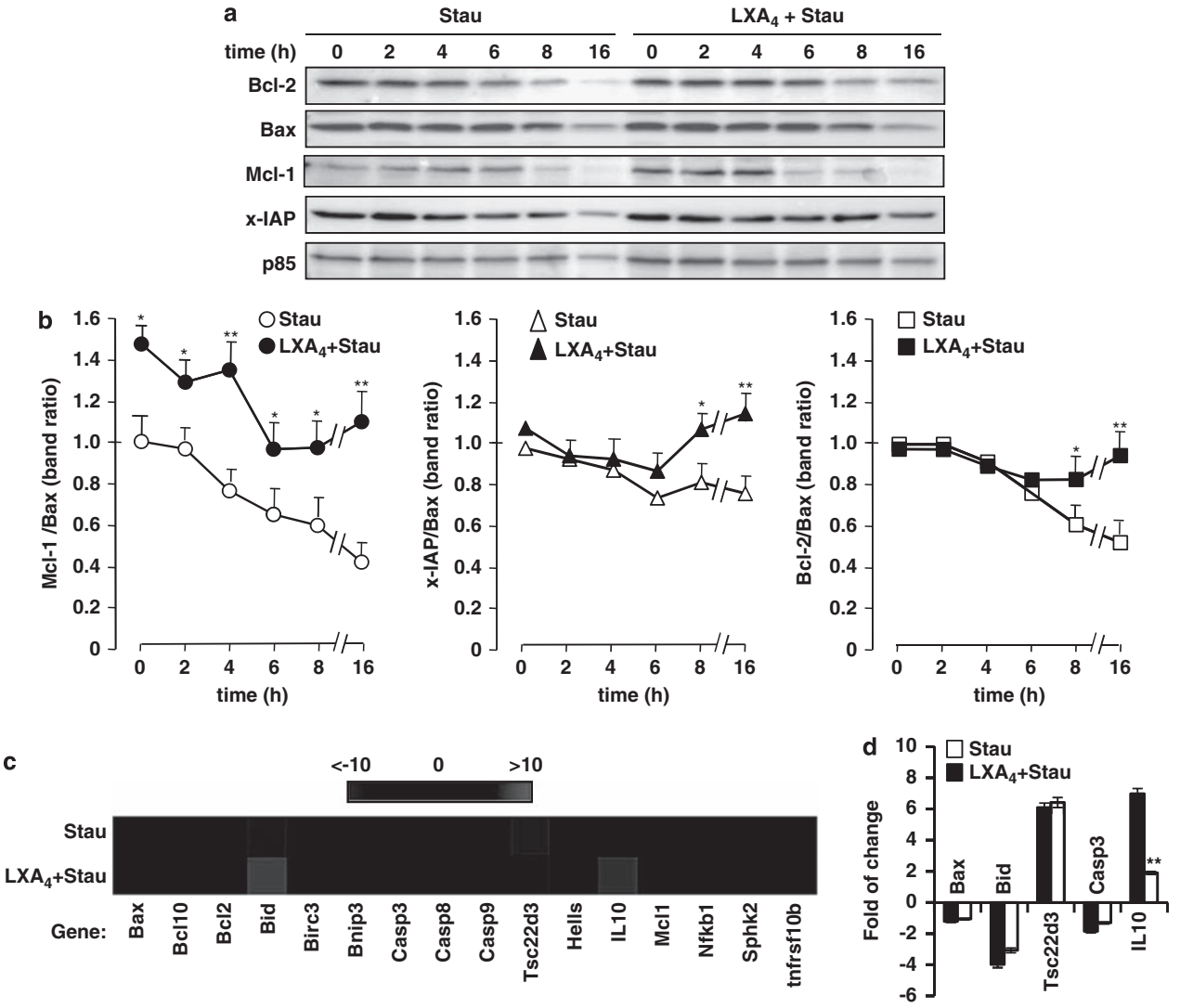

Figure 4 Changes of apoptotic-related genes and proteins in lipoxin-treated cells. RAW 264.7 cells were incubated with staurosporine (200 ng/ml) and LXA_4 (250 nM) and analyzed at the indicated times. The protein levels of Bcl-2, Bax, Mcl-1 and x-IAP were determined by western blot. The levels of the 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 *versus* 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 transcription of which is up/down (red/green) by two-fold in gene expression after staurosporine or lipoxin plus staurosporine treatment *versus* control (4 h) is 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 ± S.D. value of band ratios. $^*P < 0.05$; $^{**}P < 0.01$ *versus* the corresponding condition in the absence of lipoxin

this ERK activation using PD098059 (1 μM) or PI3K activity using LY294002 (10 μM) abolished the protective effect exerted by LXA_4 against apoptosis (Figure 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 that of ERK activation (Figure 5e). Interestingly, unlike staurosporine, LXA_4 was able to activate an ARE–luciferase reporter gene on transfection, whereas ERK inhibitor, PD098059, impaired this response, suggesting that the cytoprotective pathway dependent on Nrf-2 activity[17,18] is activated in response to LXA_4. MG132 was used as a positive control of ARE activation (Figure 5f). Finally, on using peritoneal macrophages from Nrf-2-deficient mice (Figure 5g), a significant decrease was observed in the antiapoptotic 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 LXA_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 shown in Figure 6a, the treatment with LX 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

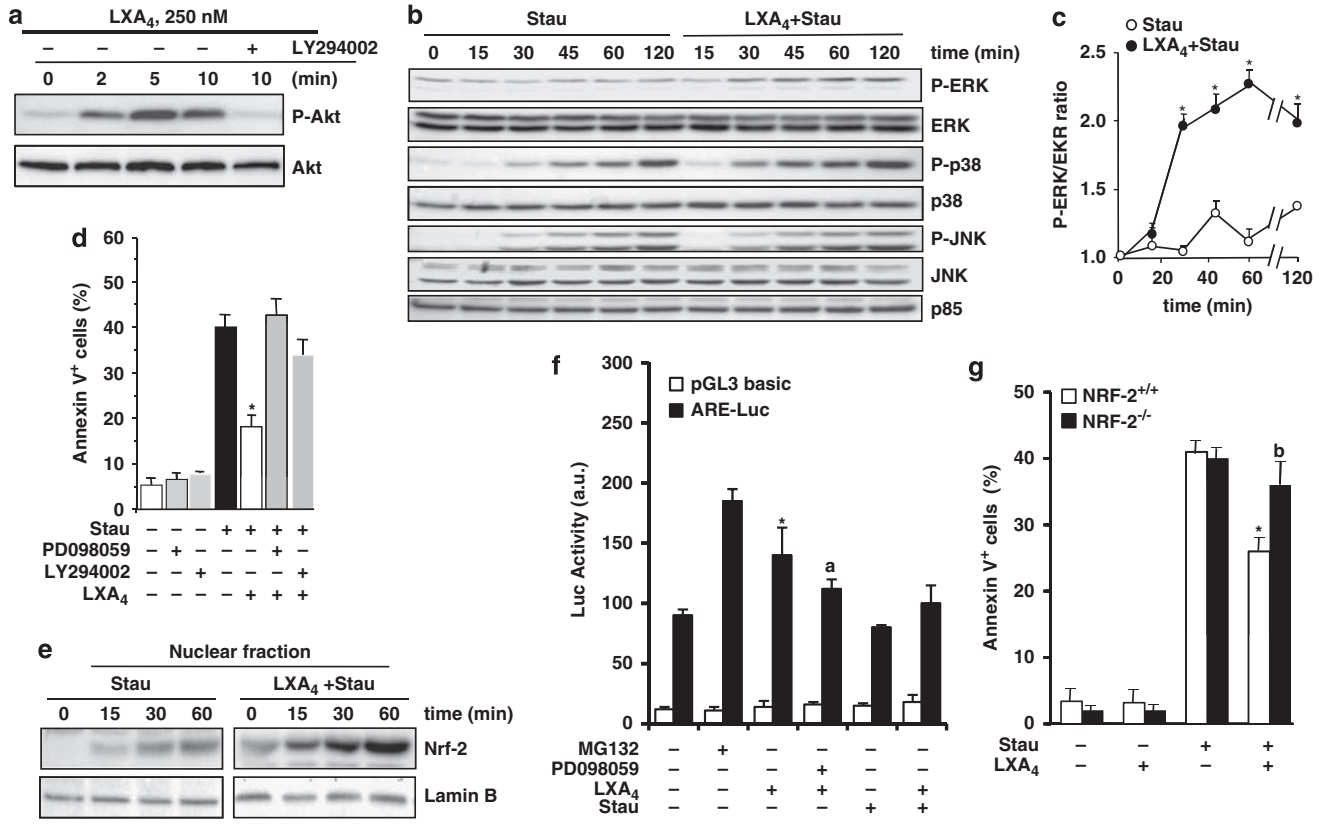

Figure 5 LXA_4 attenuation of macrophage apoptosis involves Akt and ERK/Nrf-2 activation. Cells were treated as described in Figure 1 and the phosphorylation of Akt in S473 was analyzed after LXA_4 challenge. Pretreatment with 20 μM of the PI3K inhibitor, LY294002, inhibited Akt phosphorylation (**a**). The activity of the MAP kinases was followed by western blot analysis using specific antibodies (**b**); the pERK/ERK band ratio was calculated (**c**). Inhibition of PI3K with 10 μM LY294002 (30 min preincubation) or ERK with 1 μM PD098059 (30 min 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 ± S.D. values of four different experiments. $^*P<0.01$ *versus* the corresponding condition in the absence of lipoxin; $^aP<0.05$ *versus* the corresponding condition in the absence of PD098059 (**f**); $^bP<0.01$ *versus* the corresponding value in Nrf-$2^{+/+}$ macrophages (**g**)

in cells treated with LX, a receptor-mediated effect, as it disappeared when these macrophages were pretreated with BocPLP before LXA_4 challenge (Figure 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 pathway attenuated the antiapoptotic effect exerted by LX (Figure 6c). To extend 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 (Figure 6d). Lipoxin A_4 also promoted the translocation of Nrf-2 to the nucleus in these cells as shown in Figure 6e, thereby being capable to maintain the antiapoptotic effects previously reported in murine macrophages.

Discussion

This study provides evidence for a new mechanism of action of LXA_4 by which this compound contributes to inflammation resolution, delaying the 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, LXs have been involved in the pathogenic mechanism of some microbes. For example, in addition to the antiapoptotic effects of LXA_4, it has been described that this LX 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.[8] Therefore, although their role as anti-inflammatory compounds is well documented, their implication in apoptosis has been a matter of debate.[1,3,5,26] Opposite actions of LXs on the balance between survival and apoptosis have been reported in neutrophils when signaling through the pleiotropic formylpeptide receptor like-1/LXA_4 receptor.[23] This receptor binds a series of molecules, including LXA_4, acute-phase reactant serum amyloid A (SAA) and the glucocorticoid-inducible protein annexin-1. Although annexin 1 and LXs accelerate resolution/apoptotic death of neutrophils through caspase 3 activation, SAA rescues the cells by

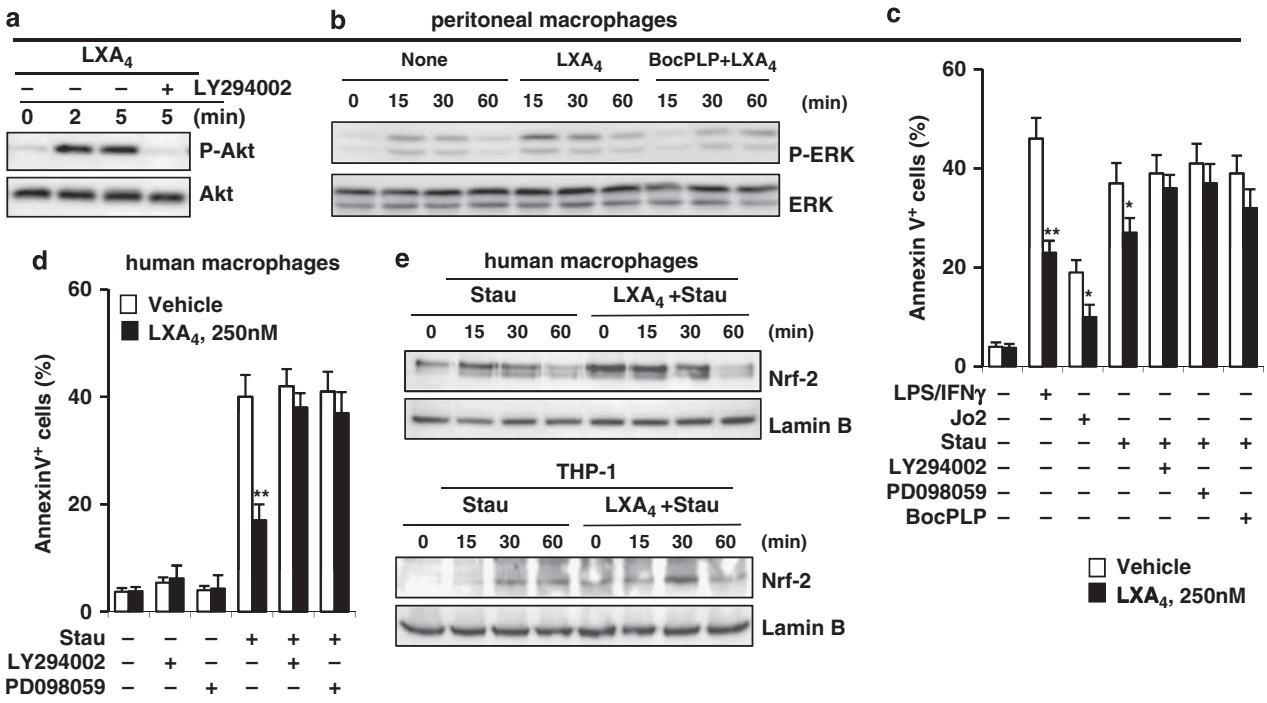

Figure 6 LXA_4 attenuates apoptosis in peritoneal murine macrophages and in human monocyte/macrophages. Primary cultures of murine peritoneal macrophages (**a**–**c**), human peripheral blood monocytes (**d**, **e**-*left*) and the human monocytic cell line THP-1 (**e**-*right*) were incubated as indicated. When cells were treated with PD098059 (1 μM) or LY294002 (10 μM), addition was 30 min before LXA_4 challenge (**a**,**c** and **d**). When Jo2 (1 μg/ml) was used, cells were incubated for 24 h (**c**) BocPLP (1 μM) was added 1 h before lipoxin treatment (**b** and **c**). Nrf-2 was determined by western blot analysis using nuclear extracts of peripheral blood human monocytes or THP-1 cells (**e**). Results show the mean ± S.D. values of four different preparations of cells. $^*P<0.05$; $^{**}P<0.01$ *versus* the corresponding condition in the absence of lipoxin

preventing mitochondrial dysfunction.[27] In contrast to neutrophils, macrophages arrive later to the inflamed area and they contribute to tissue clearance. Thus, our results indicate that as far as the synthesis of LXs in concerned they act as antiapoptotic molecules, preserving the 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 through the formation of *S*-nitrosothiols in these enzymes.[28] In line with this, results reported in this study stand directly on the mechanism of action of LX in the resolution of inflammation. The data show that pretreatment of macrophages with LX diminishes staurosporine-, etoposide-, GSNO- and LPS/IFNγ-induced apoptosis. Moreover, in murine peritoneal macrophages and in human monocyte/macrophages that exhibit an apoptotic response through 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 LXs in these cells. The action of LX was independent of the time of pretreatment, from 12 h to 30 min, before staurosporine challenge (data not shown). These data suggest that the mechanisms involved in protection against apoptosis are mainly regulated by short-term responses to LXs, rather than by profound changes in the expression of genes that regulate cell viability.

The apoptosis induced by staurosporine involves essentially the mitochondrial pathway.[14,29] Activation of apoptosis-related proteins is considered a basic step in the apoptotic progression, being fundamental to those of the Bcl-2 family that 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 LX the protein levels of Bcl-2, x-IAP and Mcl-1, all antiapoptotic 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.[30] Among the antiapoptotic genes regulated through this pathway, upregulation of Mcl-1 levels have been identified in various cell types,[17,18] a situation also prevailing in macrophages treated with LXA_4. Indeed, incubation with LX preserved the mitochondrial integrity as deduced by the minimal release of cytochrome *c* and AIF to the cytosol. Oxidative stress is another determinant that regulates the balance between viability/apoptosis.[31–33] We have analyzed the effect of LX on the liberation of some important reactive oxygen species (ROS) and reactive nitrogen intermediates (RNIs), using fluorescent probes. Our results indicate that LX inhibits ROS and RNI release, reducing the oxidative stress associated with apoptosis. Consistent with this, treatment with LX 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. Results from experiments using the pan-caspase inhibitor z-VAD confirmed the importance of LX 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 MAPK pathways.[32,34,35] This study provides evidence that LX 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.[36] Interestingly, pretreatment with LX was able to promote ERK phosphorylation in the presence of staurosporine without affecting either JNK or p38 activities. Furthermore, MAPK pathway associates with the modulation of antioxidant response element (ARE)-driven gene expression through 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.[20,37] Nrf-2 regulates the transcription of *ca.* 100 genes involved in cytoprotection and regulation of oxidative stress.[17] Recently, it has been described that the LXA_4-dependent apoptosis of human neutrophils involves the inhibition of myeloperoxidase-induced ERK, which results in a reduction in the expression of the antiapoptotic gene *Mcl-1*.[38] However, our results provide a new mechanism by which LX impairs macrophage apoptosis, involving Nrf-2 activation through an ERK-dependent pathway.[19,20]

Although multiple studies exist regarding the regulation of innate immunity by LXs, including SOCS2-dependent ubiquitinylation of TRAF-2 and -6,[39,40] 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 antioxidant defense systems in cells; (d) impairment of caspase activation caused by the absence of pro-apoptotic signaling dependent on the mitochondrial pathway or the death-receptor pathway. Therefore, these observations provide an additional antiapoptotic mechanism for LXA_4 in macrophages and might contribute to the establishment of a hitherto unrecognized potential of LXs for the treatment of inflammatory diseases.

Materials and Methods

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

Animals. The 7-week-old male wild-type C57BL/6 mice and *nrf*-2-knockout littermates were a generous gift from Dr A Cuadrado (IIBM, Madrid). Mice were housed at RT under 12-h light/dark cycle, and food and water were provided *ad libitum*. Animals were cared for according to a protocol approved by the Ethics Committee of our institution (following 86/609/EEC, 2003/65/EC directives).

Isolation of human monocytes. The PBMCs were isolated from blood of healthy donors by centrifugation on Ficoll-Hypaque Plus (GE Healthcare, Barcelona, Spain) following the manufacturers' protocol. Cells were maintained for 2 h at a density of 10^6 cells per 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 were conducted, the medium was challenged with 1% FBS. Elicited peritoneal macrophages were prepared as previously described[21] 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, Cologne, Germany). After nucleofection, cells were stimulated for 6 h and luciferase activity was measured using a Dual Luciferase Reporter Assay System (Promega, Madrid, Spain). The human monocytic THP-1 cells were maintained in culture with RPMI 1640 supplemented with 10% FBS and 20 mM HEPES. For the induction of differentiation of THP-1 monocytes into macrophages PMA (50 nM, 48 h) was used. 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 30 min at 4°C and after centrifuging for 20 min at 13 000 $\times$ *g* the supernatants were stored at 20°C. Protein levels were determined using Bradford reagent (Bio-Rad, Hercules, CA, USA).

Preparation of cytosolic and nuclear protein cell extracts. Stimulated cells were collected 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 and 0.5% NP-40) and left to swell on ice for 15 min. Cells were vortexed and centrifuged at 12 000 $\times$ *g* for 30 s. 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 and 20% glycerol) and vortexed at 4°C for 30 min. After centrifugation (12 000 $\times$ *g* at 4 °C for 20 min), 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 15 min at 4°C and 10 000 $\times$ *g*. The supernatants were saved as cytosolic fractions and stored at -80°C. Pellets were resuspended again in buffer A and were saved as 'particulate' fractions containing mitochondria and stored at -80°C until cytochrome *c* quantification.

Western blot analysis. Equal amounts of protein (20–50 μg) from each fraction obtained were loaded onto a 10–12% SDS-PAGE gel. Proteins were size fractionated, transferred to a Hybond-P membrane (GE Healthcare) and, after blocking with 5% nonfat dry milk, incubated with the corresponding Abs. 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. 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 60 min 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, Mountain View, CA, USA). 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 the 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, USA) using a CXP Software (Beckman Coulter, Brea, CA, USA).

Cell death detection. Cells were collected 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 $CaCl_2$). Cells were labeled with annexin V-FITC solution (BD Pharmingen) and propidium iodide (PI) (100 μg/ml) for 15 min 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. The RAW 264.7 cells were seeded into sterile 8-well Chamber Slides (Falcon) at 16–24 h before treatment. MitoTraker Red CMXRos (50 nM, Molecular Probes) was added to the culture medium for 30 min before washing cells with PBS followed by fixation with 2% paraformaldehyde for 10 min. Cells were then permeabilized in ice-cold methanol and incubated with 3% BSA for 30 min. After incubating with a rabbit Ab against AIF (Santa Cruz Biotechnology) at 4°C for 1 h, cells were washed with PBS followed by incubating with Alexa 488 anti-rabbit secondary antibody at 4°C for 1 h at RT (1:500; Molecular Probes). Coverslips were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and examined using an Espectral Leica TCS SP5 confocal microscope. Values of intensity fluorescence and co-localization quantification were performed using Image J software (NIH, Bethesda, MD, USA).

ROS and RNI determination. Generation of ROS in RAW 264.7 cells was analyzed by flow cytometry monitoring the oxidation of 2′,7′-dichlorodihydro-fluorescein (DCFH). Cells were loaded with 0.4 μM DCFH for 30 min, pelleted and resuspended in PBS followed by flow cytometry. Synthesis of RNI was determined by incubating cells with 200 μM DAF-2 diacetate.

Microarray analysis of apoptosis-related genes. Total RNA was isolated from cells using TRIzol Reagent (Invitrogen) and further cleaned up using the Qiagen RNeasy MiniKit (Hilden, Germany) with one step of DNAse I digestion. A total of 3 μg of RNA were used for cDNA synthesis with RT_2 first Standard kit (SuperArray Bioscience, Frederick, MD, USA). 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 $\Delta\Delta C_t$-based fold-change calculations from the specific uploaded raw threshold cycle data. Results were analyzed as described by Mayoral *et al*[22] and RT-PCR of selected genes was used to validate the microarray data.

Statistical analysis. The values in graphs correspond to mean ± S.D values. The statistical significance was estimated with a Student *t*-test for unpaired observation. Data were analyzed using the SPSS (Madrid, Spain) for Windows statistical package, version 9.0.1.

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