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Oxidized phosphatidylserine mitigates LPS-triggered macrophage inflammatory status through modulation of JNK and NF-kB signaling cascades

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ABSTRACT

Recent evidence suggests that phosphatidylserine (PS) and its oxidized species drive the clearance of apoptotic cells by macrophages with putative immune response modulation. However, it is not clear whether PS and oxidized PS differentially modulate at molecular level the functional responses of macrophages. Therefore, we proposed in this work to explore this question by evaluating the influence of PS oxidation products on the macrophages inflammatory status. Thus, we determined the effects of oxidized 1-palmitoyl-2-linoleoyl-phosphatidylserine (oxPLPS) and PLPS on RAW 264.7 macrophages production of the pro-inflammatory mediator nitric oxide (NO) and on the levels of the inducible NO synthase (*Nos2*) and *Il1β* mRNA. The ability of PLPS and oxPLPS to modulate the lipopolysaccharide (LPS)-triggered macrophage activation was also analyzed. Finally, the effects of PLPS species over canonical inflammation-associated signaling pathways, such as nuclear factor (NF)-κB and mitogen-activated protein kinases (MAPKs) were also disclosed.

The results obtained showed that both PLPS and oxPLPS species are deprived of intrinsic pro-inflammatory activity. Exquisitely, only oxPS were found to significantly inhibit NO production and *iNos* and *IL1β* genes transcription induced by LPS. At a molecular level, these effects were partially due to attenuation of LPS-induced c-Jun-N-terminal kinase (JNK) phosphorylation and p65 NF-κB nuclear translocation. Overall our data suggest that oxPLPS, but not native PLPS, mitigates pro-inflammatory signaling in macrophages, contributing to containment of inflammation during apoptotic cell engulfment.

1. Introduction

Clearance of apoptotic cells by phagocytes is a crucial homeostatic function in several cellular processes such as embryonic development, tissue remodeling and resolution of inflammation [1,2]. The removal of dying cells prior to the loss of plasma membrane integrity prevents the release of toxic or immunogenic intracellular contents, limiting the establishment of deleterious inflammatory cascades and autoimmune responses [3,4].

The role of macrophages as the main cells involved in apoptotic cell removal is well-established. However, the nature of the "eat-me" signals and receptors that lead to cell recognition and engulfment are still in debate. Several receptors on the surface of macrophages such as CD14 [5], CD91 [6] and scavenge receptors [7] have been implicated in the recognition of apoptotic cells. The constitutive expression of the scavenge receptor CD36 on macrophages and dendritic cells was shown to be crucial for their capacity to recognize and engulf dying cells. Furthermore, ectopic expression of the receptor on non-professional phagocytes such as fibroblasts and melanoma cells confers them phagocytic activity over apoptotic cells [8,9].

Regarding *eat-me* signals, the translocation of phosphatidylserine (PS) to the outer leaf of cytoplasmic membrane is the central phagocytosis-associated event occurring in apoptotic cells [10–13]. In fact, masking PS exposure in dying cells was shown to inhibit their engulfment in vitro and in vivo [14,15]. However, the exact nature of the PS species that drive this strong *eat-me* signal is not totally disclosed.

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Greenberg and co-workers in an elegant study about macrophage recognition of apoptotic cells via CD36 have shown that recognition occurs via membrane-associated short chain derivatives of oxidized PS (oxPS) and, to a lesser extent, oxidized phosphatidylcholine (oxPC), but not by non-oxidized phospholipid species [16]. A more recent study highlighted the preferential of externalization of oxygenated PS (oxPS) as major eat me signal in apoptotic macrophages and identified BAI-1 and GAS-6 as novel receptor candidates expressed by macrophages showing selectivity for oxPS, but not for unoxidized PS [17]. Indeed, it is well known that PS is susceptible to alterations under oxidative stress conditions and that these modifications occur before their externalization. During apoptosis, the asymmetric transbilayer distribution of PS is lost, bringing PS to the surface. The appearance of PS in outer monolayer of apoptotic cells membrane was recently found to occur upon caspase-mediated inactivation of flipases [18], but could also result from its oxidation that leads to a misrecognition by aminophospholipid translocases (APT), preventing that way the translocation to the inner leaflet [19,20]. Oxidized PS has been detected in apoptotic cells in innumerous works [17,21,22]. Some published work also showed that oxidized phospholipids tags can be transferred from apoptotic cells to neighboring viable cells via tunneling nanotubes, composed of PS exposed on the plasma membrane [23].

Besides their role in tagging apoptotic cells for engulfment, it is plausible that oxPS may also contribute to modulate intracellular signaling pathways in phagocytes in order to limit their activation and consequent modulate exacerbation of inflammatory state. However, the role of oxidized phospholipids (oxPLs) in inflammation is unclear and it is reported that oxPLs can present either anti- or pro-inflammatory properties, as comprehensively reviewed by Bochkov and co-authors [24,25]. Nonetheless, this extensive amount of published data tended to focus on the effects of oxPC in the inflammatory response, rather than oxPS. Regarding the role of oxPS, several works attributed them pro-inflammatory properties [26,27], and recent findings reported how low levels of anti-oxPS IgM in humans correlate with the risk of cardiovascular diseases and atherosclerosis [28]. Nevertheless, other studies suggest that some phospholipids exert anti-inflammatory actions and that this ability is improved upon their oxidation [29–31]. Accordingly, it was reported that PS is able to suppress the formation and release of pro-inflammatory mediators in activated macrophages while inducing the production and release of anti-inflammatory cytokines such as transforming growth factor β (TGF-β) and interleukin 10 (IL-10) [32–35]. Moreover, PS liposomes were shown to enhance the anti-inflammatory effect of curcumin in cultured RAW 264.7 macrophages [36] and to reduce the LPS-induced release of NO in microglial cultures from neonatal rat brain [37,38] and in apoptotic PC12 cells [39]. Similarly, it was reported that PS inhibited the production of the inflammatory mediators IL-6, IL-8, vascular endothelial growth factor and prostaglandin E2 in IL-1β-stimulated fibroblast-like synoviocytes from rheumatoid arthritis patients via modulation of NF-κB and JNK/p38 MAPK signaling pathways [40]. Despite these evidences for PS involvement in the modulation of inflammatory responses, knowledge regarding the influence of PS oxidation products is scarce.

Therefore, we sought in present study to evaluate the influence of PS oxidation products in the modulation of pro-inflammatory events in macrophages. We compared the effects of 1-palmitoyl-2-linoleoyl-sn-glycero-3-phospho-L-serine (PLPS) and oxidized PLPS (oxPLPS) on the activation status of lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. LPS is an endotoxin present in the outer membrane of Gram-negative bacteria, being recognized by TLR4 receptor on immune cells in which it promotes strong inflammatory responses. Our results indicate that while both PLPS and oxPLPS are deprived of intrinsic pro-inflammatory activity, oxPLPS may actively contribute to restrain inflammatory signaling cascades in macrophages during apoptotic cell recognition and engulfment.

2. Material and methods

2.1. Material

PLPS was obtained from Avanti polar lipids, Inc. and used without further purification. $FeCl_2$, EDTA and H_2O_2 (30%, *w*/w) were acquired from Merck. Triethylamine (Acros organics), chloroform (HPLC grade), methanol (HPLC grade) and ethanol absolute (Panreac) were used without further purification. TLC silica gel 60 plates with concentrating zone (2.5x20cm) were purchased from Merck. Dulbecco's Modified Eagle Medium (DMEM), penicillin, streptomycin, resazurin and LPS from *Escherichia coli* (serotype O26:B6) were obtained from Sigma–Aldrich Química (St Louis, MO, USA). Fetal calf serum and trypsin were purchased from Invitrogen (Paisley, UK). The protease and phosphatase inhibitor cocktails, cOmplete Mini Protease Inhibitor and PhosSTOP respectively, were obtained from Roche (Mannheim, Germany). Antibodies against phospho-p44/p42 MAPK (ERK1/ERK2), phospho-p38 MAPK, phospho-SAPK/JNK, and anti-NF-kB p65 were from Cell Signaling Technologies (Danvers, MA, USA). Antibodies anti-actin and anti-lamin were from Millipore (Bedford, MA, USA) and Calbiochem (Darmstadt, Germany), respectively. The alkaline phosphatase-linked secondary antibodies and the enhanced chemi-fluorescence (ECF) reagent were obtained from GE Healthcare (Chalfont St. Giles, UK), and the polyvinylidene difluoride (PVDF) membranes were from Millipore Corporation (Bedford, MA). TRIzol® reagent was purchased from Invitrogen (Barcelona, Spain). The iScript Select cDNA Synthesis Kit and SYBR green were obtained from BioRad (Hercules, CA, USA). Primers were from MWG Biotech (Ebersberg, Germany). Unless otherwise stated, all other reagents were from Sigma Chemical Co. (St. Louis, MO, USA) or from Merck (Darmstadt, Germany).

2.2. Oxidation of phosphatidylserine by Fenton reaction

Ammonium hydrogen carbonate buffer (5 mM, pH 7.4) was added to 1 mg of phospholipid and the solution was vortex-mixed and sonicated for the formation of vesicles. Oxidative treatments using Fe (II) and H_2O_2, were carried out by adding 40 μM $FeCl_2$ and 10 mM of H_2O_2 to total a volume of 500 μL of solution. The mixture was left to react at 37 °C in the dark for several hours with agitation [41–43].

2.3. Cell culture

RAW 264.7, a mouse leukaemic monocyte macrophage cell line from American Type Culture Collection (ATCC number: TIB-71), was cultured in DMEM supplemented with 10% non-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO2. Along the experiments, cells were monitored by microscope observation in order to detect any morphological change.

2.4. Determination of cell viability by resazurin assay

Assessment of metabolically active cells was performed using 7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt (resazurin) colorimetric assay as previously reported [44]. Briefly, RAW 264.7 cells (60×10^5 cells/well) were plated and allowed to stabilize for 12 h in 200 μL medium in 96 well plates. Following this period, cells were either maintained in culture medium (control) or incubated with 5 different concentrations of the lipids to be tested. After 20 h, a resazurin stock solution (500 μM in phosphate buffered saline) was added to a final concentration of 50 μM and cells further incubated at 37 °C for 4 h, in a humidified atmosphere of 95% air and 5% CO_2. The bioreduction of the dye was quantified by absorbance measurement at 570 nm, with a reference wavelength of 620 nm in an automated plate reader (Multiskan 60, Thermo Scientific). Results are presented as a supplementary

information (Fig. S1).

2.5. Measurement of nitrite production

The production of nitric oxide (NO) was measured by the accumulation of nitrite in the culture supernatants, using a colorimetric reaction with the Griess reagent [45]. Briefly, 150 µL of culture supernatants were diluted with equal volumes of Griess reagent [0.1% (*w/v*) N-(1-naphthyl)-ethylenediamine dihydrochloride and 1% (w/v) sulphanilamide containing 5% (w/v) H_3PO_4] and maintained during 30 min, in the dark. Culture medium was used as blank and nitrite concentration was determined from a regression analysis using serial dilutions of sodium nitrite as standard. The absorbance at 55 nm was measured in an automated plate reader (Multiskan 60, Thermo Scientific).

2.6. RNA extraction

RAW cells were plated at 2×10^6 cells/well in 6-well microplates in a final volume of 2 mL and treated during 24 h with PLPS (30 µg/mL) or oxPLPS (30 µg/mL). When testing the effect of lipids over LPS stimulation, the cells were exposed to the lipid during 1 h prior to the addition of 1 µg/mL of LPS. Total RNA was isolated from cells with the TRIzol® reagent according to the manufacturer's instructions. Briefly, cells were washed with ice-cold PBS harvested and homogenized in 1 mL of Trizol by pipetting vigorously. After addition of 200 µL of chloroform the samples were vortexed, incubated for 2 min at room temperature and centrifuged at 12,000 ×*g*, for 15 min, at 4 °C. The aqueous phase containing RNA was transferred to a new tube and RNA precipitated with 500 µL of isopropanol for at least 10 min at room temperature. Following a 10 min centrifugation at 12,000*g*, the pellet was washed with 1 mL 75% ethanol and resuspended in 100 µL 60 °C heated RNA Storage Solution (Ambion, Foster City, CA, USA). The RNA concentration was determined by OD260 measurement using a Nanodrop spectrophotometer (Wilmington, DE, USA) and quality was inspected for absence of degradation or genomic DNA contamination, using the Experion RNA StdSens Chips in the Experion™ automated microfluidic electrophoresis system (Bio-Rad Hercules, CA, USA). RNA was stored at −80 °C until use.

2.7. Real-time RT-PCR

One microgram of total RNA was reverse transcribed using the iScript Select cDNA Synthesis Kit. Briefly, 2 µL of random primers and the necessary volume of RNase-free water to complete 15 µL, were added to each RNA sample. The samples were heated at 65 °C, for 5mim, and snap-chilled on ice for 1 min. After this, 5 µL of a Master Mix containing 1 µL of iScript reverse transcriptase and 4 µL of 5x Reaction Buffer were added to each sample. A protocol for cDNA synthesis was run on all samples (5 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C and then put on hold at 4 °C). After the cDNA synthesis, the samples were diluted with RNase-free water up to a volume of 100 µL. Real-time PCR was performed in a 20 µL volume containing 5 µL cDNA (50 ng), 10 µL 2x Syber Green Supermix, 2 µL of each primer (250 nM) and 1 µL H_2O PCR grade. Samples were denatured at 95 °C during 3 min. Subsequently, 40 cycles were run for 10s at 95 °C for denaturation, 30 s at the appropriate annealing temperature and 30 s at 72 °C for elongation. Real-time RT-PCR reactions were run in duplicate for each sample on a Bio-Rad My Cycler iQ5. Primers were designed using Beacon Designer® Software v7.2, from Premier Biosoft International and thoroughly tested. Primer sequences are given in Table 1.

Amplification reactions were monitored using a SYBR-Green assay. Gene expression changes were analyzed using the built-in iQ5 Optical system software v2. The software enables analyzing the results with the Pfaffl method [7], a variation of ΔΔCT method corrected for gene-specific efficiencies, and to report gene expression changes as relative fold changes compared to control samples. The results were normalized using a reference gene, *Hprt1*, determined with Genex® software (MultiD Analyses AB) as the most stable for the treatment conditions used.

Table 1
Oligonucleotide primer pairs used for real-time RT- PCR.

PRIMER	5′-3' SEQUENCE	REFSEQ ID
	F: FORWARD; R; REVERSE	
HPRT1	F: GTTGAAGATATAATTGACACTG R: GGCATATCCAACAACAAAC	NM_013556
IL1B	F: ACCTGTCCTGTGTAATGAAAG R: GCTTGTGCTCTGCTTGTG	NM_008361
NOS2	F: GCTGTTAGAGACACTTCTGAG R: CACTTTGGTAGGATTTGACTTTG	NM_010927
ARGINASE 1	F: GTGCCCTCTGTCTTTTAG R: GCTCCGATAATCTCTAAG	NM_007482.3

2.8. Western blot

Total cell lysates were prepared using the RIPA buffer [50 mM Tris–HCl (pH 8.0), 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS and 2 mM EDTA] freshly supplemented with 1 mM DTT, protease and phosphatase inhibitor cocktails. Cytoplasmic and nuclear extracts were obtained by a commercial nuclear extract kit (Active Motif, Rixensart, Belgium), according to the manufacturer instructions. Protein concentration of cell lysates was determined by the bicinchoninic acid protein assay. Cell lysates were denaturated at 95 °C, for 10 min, in sample buffer [0.125 mM Tris (pH 6.8), 2% (*w/v*) SDS, 100 mM DTT, 10% glycerol and bromophenol blue].

Proteins were subjected to SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes. Non-specific binding was blocked with 5% (w/v) of skimmed milk in TBS-T (100 mM Tris pH 8.0, 1.5 mM NaCl and 0,1% Tween-20) during 1 h at room temperature and under soft and continuous stirring. Membranes were then incubated overnight at 4 °C with antibodies against phospho-ERK1/2, JNK, p38 MAPKs and against NF-$_k$B p65 subunit diluted (1:1000) in TBS-T. The membranes were then washed and incubated with secondary antibodies associated to the alcaline phosphatase, for 1 h room temperature. The immune complexes were detected using the enhanced chemifluorescence reagent on the Typhoon FLA 9000 (GE Healthcare, Chalfont St. Giles, UK) and analyzed with the software ImageQuant TL® (GE Healthcare, Chalfont St. Giles, UK). To demonstrate equivalent protein loading, membranes were stripped and re-probed with antibodies against actin or lamin or the total forms of MAPKs.

2.9. Statistical analysis

The results were expressed as means and standard deviation (mean ± SD) for each experimental group. To obtain the correlations and similarities between groups a 1-way ANOVA independent-measures test followed by the Dunnett's test was used and differences were considered significant at $p < .05$. Graph pad (version 6.0) was used for all comparisons.

3. Results

3.1. PLPS and oxPLPS do not induce an inflammatory status in macrophages

We started to address whether PLPS and oxPLPS trigger per se the production of inflammatory mediators in macrophages. We observed that neither PLPS nor oxPLPS significantly induce NO production in RAW 264.7 cells (Fig. 1). NO is produced under inflammatory

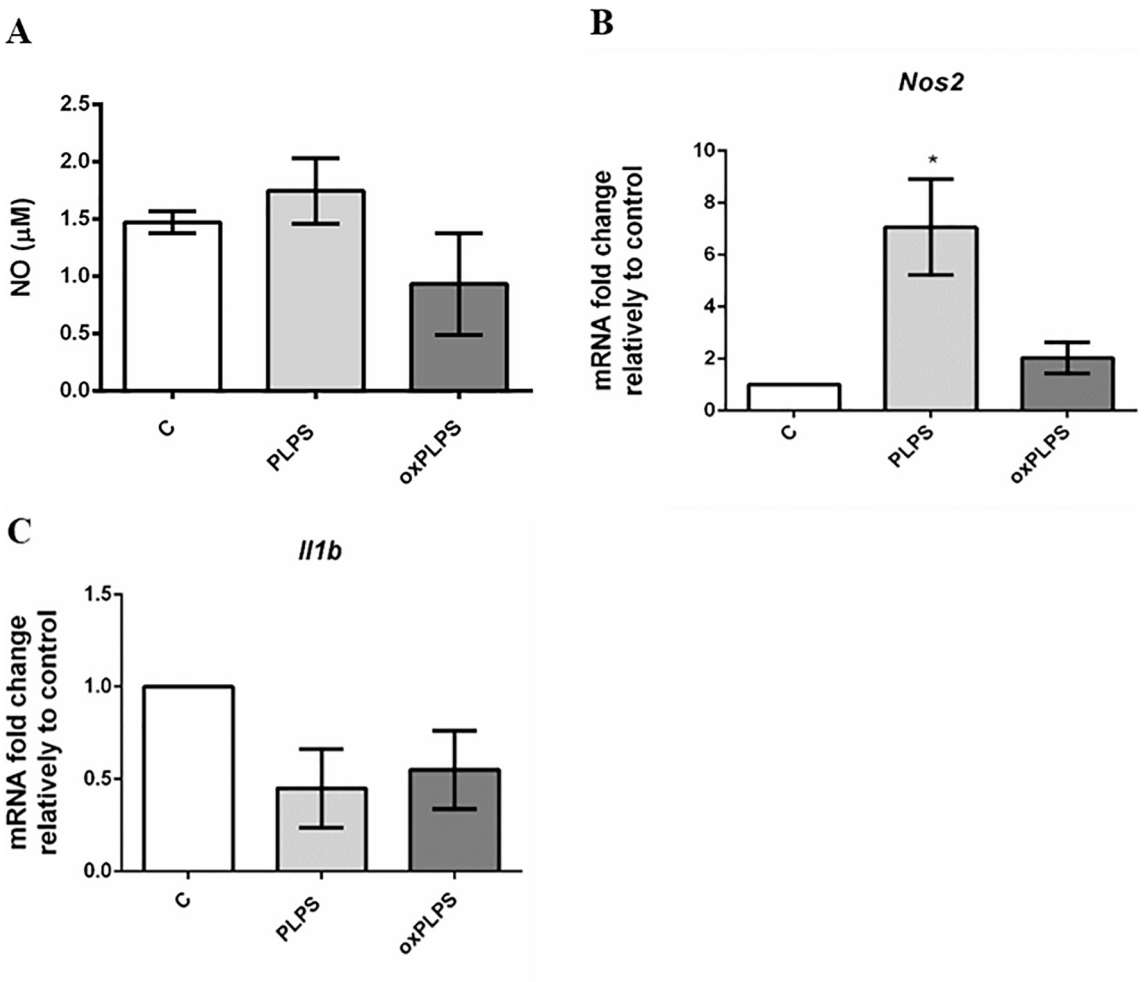

Fig. 1. Effect of PLPS and oxPLPS on NO production and *Nos2* and *Il1b* gene transcription in macrophages. RAW 264.7 macrophages were maintained in culture medium (control) or treated either with non- and oxidized PLPS (30 μg/mL) for 24 h. (A) Nitrite levels (μM)in the culture supernatants were evaluated by the Griess reaction. Each value represents the mean ± SD from at least 3 experiments. (B and C) The mRNA levels of *Nos2* (B) and *Il1b* (C) are presented as normalized fold changes relatively to control. Normalization was performed using *Hprt1* as reference gene. Each value represents the mean ± SD from at least 3 experiments. (* $p < .05$).

conditions mainly by inducible NO synthase (iNOS) a product of the *Nos2* gene, therefore, we analyzed the effects of PLPS species on the transcription of *Nos2*. The results showed that while oxPLPS tends to decrease (although not significantly) *Nos2* mRNA levels, PLPS slightly induced the transcription of this gene ($p < .05$) (Fig. 1B). The increase is, however modest and not sufficient to be reflected in a significant variation in NO production (Fig. 1A). Finally, we also address the effect of phospholipids on the transcription of the canonical pro-inflammatory cytokine IL1-β. As shown in Fig. 1C neither PLPS nor oxPLPS significantly induce the transcription of *Il1b* gene.

Overall in the experimental conditions used in this study, oxPLPS and PLPS did not showed significant pro-inflammatory properties over macrophages.

3.2. OxPLPS, but not PLPS, mitigates LPS-triggered macrophages activation

In order to evaluate the capacity of PLPS and oxPLPS to counteract/modulate inflammatory signals, we tested their effects on LPS-treated cells. LPS is a cell wall component of Gram-negative bacteria, commonly used to induce a pro-inflammatory status in macrophages by acting on its receptor TLR4. First, we assessed the effects of PLPS and oxPLPS on LPS-induced NO production. As shown in Fig. 2A non-oxidized PLPS had no inhibitory effect on the LPS-evoked NO increase. In contrast, oxPLPS inhibited NO production triggered by LPS (Fig. 2A) and this effect was found to be dose-dependent (Fig. 2B). In a second approach we analyzed the effects of PLPS and oxPLPS on LPS-induced transcription of *Nos2* and *Il1b* genes. Data showed that only oxPLPS (30 μg/mL) significantly decrease LPS-induced *Nos2* transcription ($p < .05$), supporting the results obtained for NO quantification (Fig. 2C). As NO production may also be dependent of factors other than iNOS protein levels, we further tested the effect of phospholipids on the transcription of arginase 1 (*Arg1* gene). Arginase 1 is an enzyme that competes with iNOS for the common substrate L-arginine and that can therefore limit NO production. As shown in Fig. 2D, treatment of macrophages with oxPLPS significantly increases the transcription of *Arg1*.

Regarding the analysis of *Il1b* mRNA levels we found that the strong induction triggered by LPS was partially reverted by oxPLPS, while no significant alterations were observed with PLPS treatment ($p > .05$) (Fig. 2E). These results indicate that oxPLPS may present anti-inflammatory properties.

3.3. LPS-triggered JNK phosphorylation is strongly decreased by OxPLPS

The expression of pro-inflammatory molecules is tightly regulated by an intricate network of intracellular signaling pathways and transcription factors. Among these pathways, MAPKs and the transcription factor NF-κB are key elements in innate immunity and inflammation. To

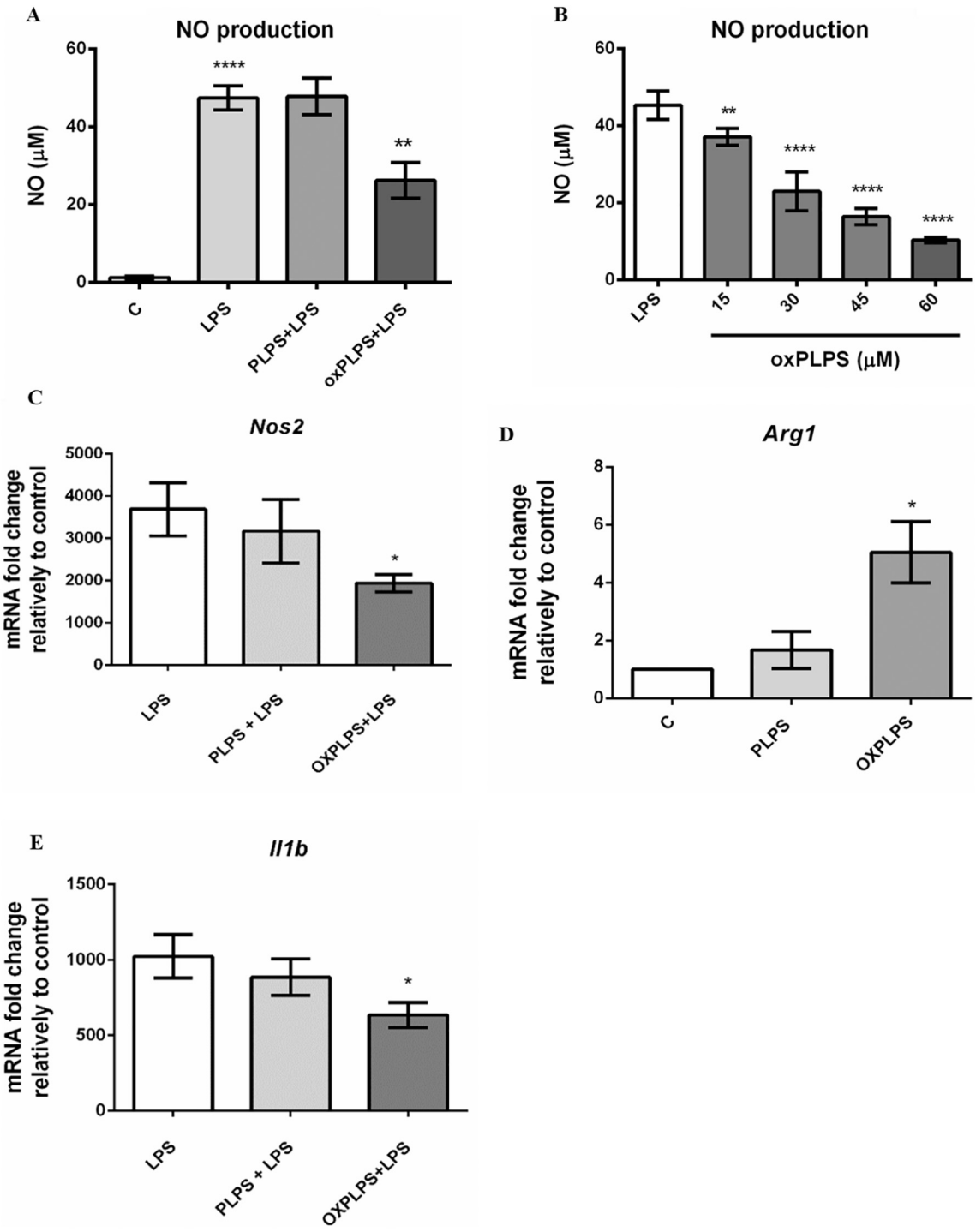

Fig. 2. Influence of PLPS and oxPLPS on LPS-induced macrophage pro-inflammatory status. Raw 264.7 cells were incubated with culture medium alone (Control; C), with LPS (1 μg/mL) and with non-oxidized (PLPS) or oxidized PLPS (oxPLPS; 30 μg/mL except in (B) where concentrations are indicated) for 24 h (A and B). Nitrite levels in the culture supernatants were evaluated by the Griess reaction. The mRNA levels of *Nos2* (C) *Arg1* (D) and *Il1b* (E) are assessed by qPCR and presented as fold changes relatively to control, using *HPRT1* as reference gene. Each value represents the mean ± SD from at least 3 experiments. (* $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$).

elucidate the mechanisms underlying oxPLPS mitigation of LPS-induced macrophages activation, we addressed their effects on MAPKs and NF-κB signaling pathways. Three groups of MAPKs were studied: extracellular signal-regulated kinases (ERK), c-Jun NH_2-terminal kinase (JNK) and p38 MAPK.

PLPS and oxPLPS *per se* do not trigger ERK1/2, JNK1/2 and p38 MAPK activation (evaluated by protein phosphorylation) (Fig. 3). Interestingly, in experiments where cells were co-cultured with LPS and phosphatidylserines, oxPLPS treatment significantly inhibited LPS-induced JNK1/2 phosphorylation (Fig. 3A). Moreover, oxPLPS exposure was also found to down-modulate TNF-α-induced JNK activation (supplementary Fig. S2). Regarding ERK1/2 and p38 MAPKs, no significant modulation was detected for both PLPS and oxPLPS treatments (Fig. 3B and C).

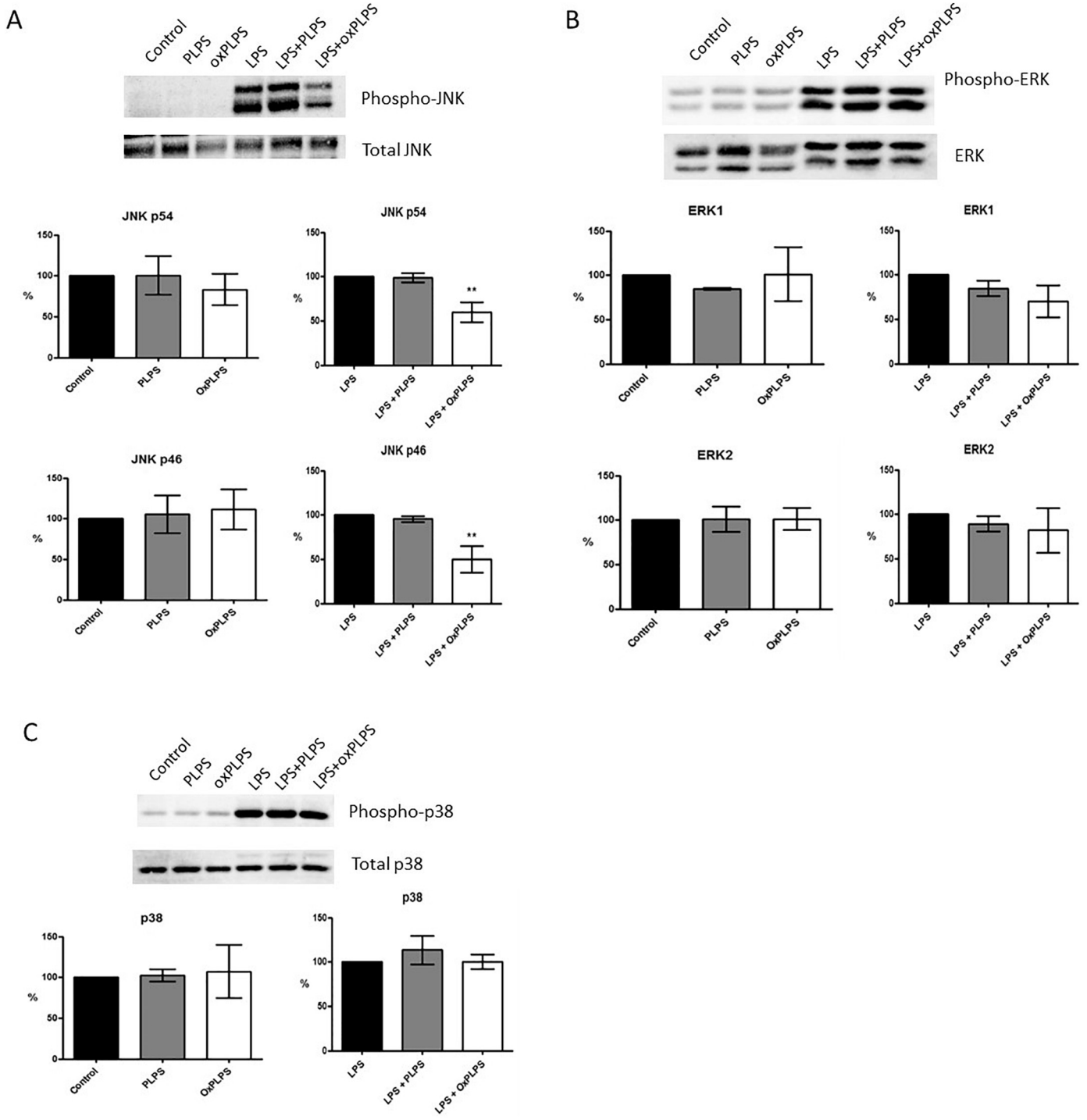

Fig. 3. Influence of PLPS and oxPLPS on MAPKs activation in macrophages and on LPS-triggered MAPKs activation in macrophages. Total cell extracts were analyzed by Western blot using antibodies against (A) phospho-JNK 1/2, (B) phospho-ERK 1/2 and (C) p38MAPK. The results are expressed as percentage of optical densities relative to untreated cells (control) and to LPS-treated cells. Equal protein loading was controlled using antibodies against total (A) JNK, (B) ERK and (C) p38 MAPKs. A representative blot is shown and each value on graphs represents the mean ± SD of 3 to 5 independent experiments.

3.4. oxPLPS inhibits p65 nuclear translocation in LPS-stimulated macrophages

The NF-κB transcription factor regulates the transcription of many genes involved in immune and inflammatory responses, including *Nos2* and *Il1b*. To elucidate whether oxPLPS and PLPS modulate the nuclear translocation of NF-κB p65 in macrophages treated in the absence or in the presence of LPS Western blot analysis was performed to quantify the cytosolic and nuclear p65. The obtained results showed that, oxPLPS and PLPS do not activate NF-κB, as they don't induce the nuclear translocation of cytosolic p65 subunit (Fig. 4A and B). As expected, treatment of macrophages with LPS caused a strong decrease in p65 cytosolic levels and a concomitant increase in the nucleus. The exposure of cells to oxPLPS partially reverted the LPS-induced p65 nuclear translocation, as judging by significantly higher levels in cytosol and decreased levels in the nucleus (Fig. 4A and B). By contrast non-oxidized PLPS had no significant effect neither in cytosolic nor nuclear LPS- induced levels. Taken together these data suggest that oxPLPS may

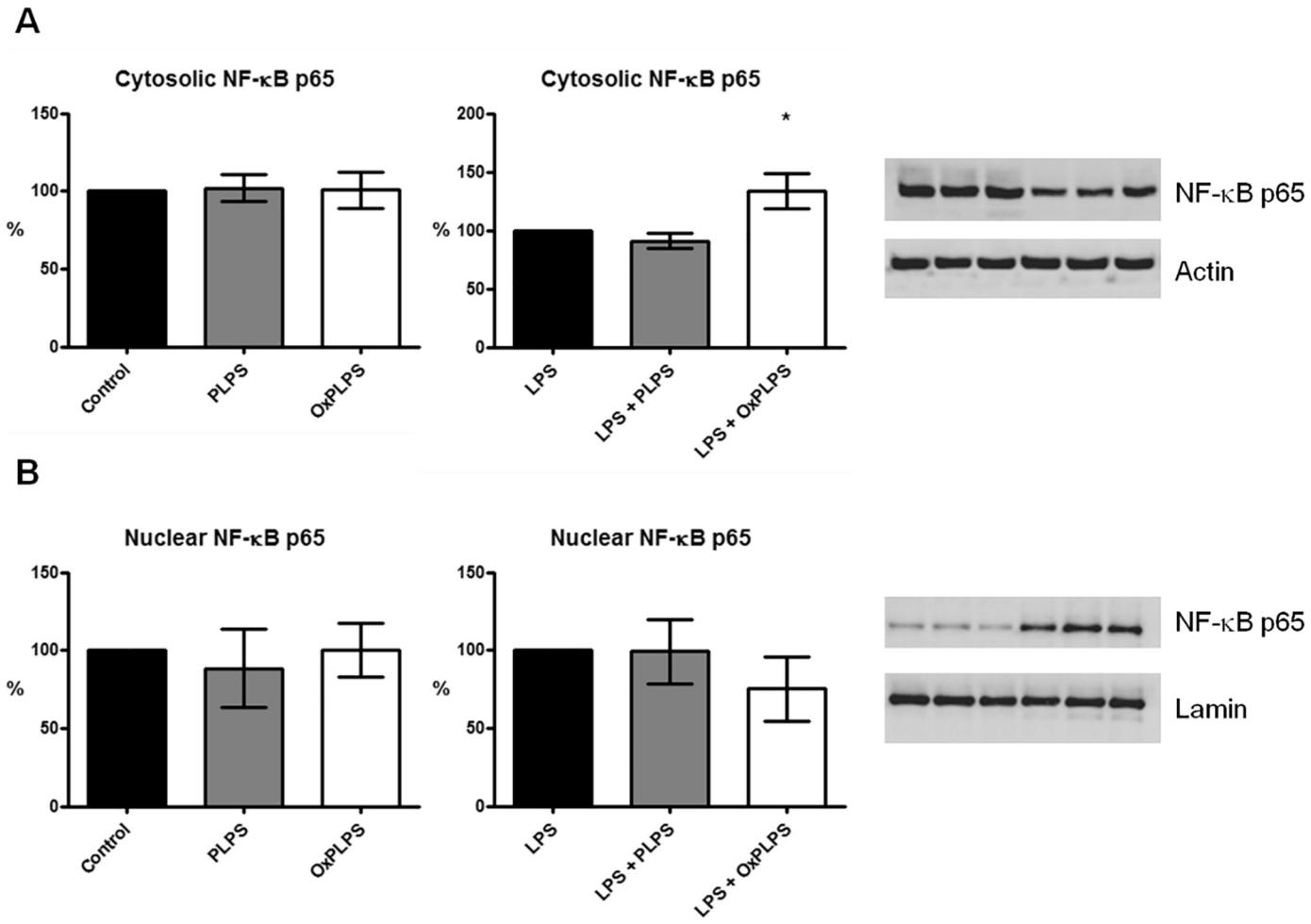

Fig. 4. Effect of PLPS and oxPLPS on cytosolic (A) and nuclear (B) p65 protein levels of macrophages cultured in the absence (left) or in the presence (right) of LPS. The translocation of NF-κB p65 to the nucleus was analyzed by western blot analysis of cytoplasmic (A) and nuclear (B) protein extracts. Equal protein loading was controlled using antibodies against actin and lamin in cytosolic and nuclear extracts respectively. A representative blot is shown, and each value represents the mean ± SD of 3 to 5 independent experiments. * $p < .05$.

interfere with the nuclear translocation of p65 NF-κB.

4. Discussion

Cell apoptosis and subsequent 'silent' removal represents an important check-point for the resolution of inflammation. Failure in apoptotic cell clearance results in secondary necrosis-driven tissue damage and has been implicated in chronic inflammation and autoimmune diseases [3]. Apoptotic cells undergo profound biological changes, such as PS externalization, an event that warrant efficient recognition and uptake by macrophages before fading to secondary necrosis. However, the nature of the "eat-me" signals and receptors that lead to cell recognition, engulfment and concomitantly silencing of pro-inflammatory pathological pathways are still under discussion. The recognition of apoptotic cells via CD36 was shown to occur via membrane associated oxidized phospholipids such as oxPS and to a lesser extent oxPC, but not by non-oxidized phospholipid species [16]. Bochkov and colleagues showed the crucial role of oxidized phospholipids as biological active compounds (as reviewed in [25,27]). Therefore, in this study, we sought to evaluate whether PS oxidation products may modulate pro-inflammatory events in macrophages. For this, we compared the effects of PLPS and oxPLPS on LPS-stimulated RAW 264.7 macrophages. We found that both PLPS and oxPLPS are deprived of intrinsic pro-inflammatory activity. Exquisitely, our results indicate that oxPLPS, beside of their role as a "eat-me" signal, may actively contribute to restrain inflammatory events in macrophages during apoptotic cell engulfment. More specifically, oxPLPS in contrast to PLPS, inhibits LPS-induced NO production. This was in part due to a strong impairment of *Nos2* transcription and to a decrease of L-arginine caused by an increase expression of Arginase 1. The availability of L-arginine is a major determinant for NO synthesis in activated macrophage, and therefore the competition between Arginase 1 and iNOS for this common substrate strongly limit NO production [46]. We also observed that only oxidized PLPS species where able to effectively counteract LPS-triggered *Il1b* transcription. IL-1β is a pleiotropic pro-inflammatory cytokine canonically associated with M1 activated macrophages [47]. These data reinforce the notion of an oxidation-dependent role of PLPS in tempering inflammation during apoptotic cell engulfment. The dissimilar performance of non-oxidized versus oxidized species has also been reported for other phospholipid classes. Friedl and co-workers, demonstrated that only the oxidized forms of PC, inhibit iNOS expression in macrophages [48] and oxPS and oxPC, but not their native species, were shown to participate in macrophage recognition of apoptotic cells via CD36 [16]. Seyerl and collaborators have also verified that oxidized phospholipids, including PS, but not their native forms, are specific regulators of T cell activation [49]. Over the last three years, several new anti-inflammatory activities were disclosed strictly for oxidized phospholipids, based on the ability of these molecules to antagonize TLR-2 mediated activation in innate immune cells [50], or to covalently bind redox sensitive proteins as Keap-1 and

consequently allow an Nrf-2 mediated transcription of pro-resolving genes [30,31]. However, contradictory results exist in literature, and several studies also ascribed to non-oxidized PS anti-inflammatory properties such as the inhibition of LPS-induced NO production [51–53]. These conflicting results may be partially explained by different experimental conditions, namely the concentrations of PS used as well as their oxidation levels and molecular species.

It is well-known that oxidation of phospholipids can generate a plethora of oxidation products with different structural features and also different properties [25,27,54]. A recent work from our group highlighted the ability of oxidized 1-palmitoyl-2-arachidonoyl-PS derivatives to contrast the proliferation and the production of inflammatory cytokines in peripheral blood immune cells activated with LPS [55]. However PS, upon oxidation, can generate not only distinct products with oxygenated fatty acyl chain, but also products resulting from PS polar head oxidation [41]. Recently, it was reported that several isolated oxidation products of 1-palmitoyl-2-oleoyl-PS did not induce cytokine production in monocytes, while PS oxidation products with a terminal hydroperoxyacetaldehyde in the polar head showed pro-inflammatory properties, increasing the expression of IL-1β, IL-6, IL-8, MIP-1β and TNF-α [56]. Furthermore, it was also demonstrated that the polar groups of oxidized phospholipids affected the LPS-triggered dysfunction of endothelial cells in vitro and in vivo, by attenuating RHO GTPases activation [57]. This particular observation indicated that anti-inflammatory activities ascribed to oxidized phospholipid species may result from their direct modulation of intracellular signaling pathways.

Due to their potential deleterious effect over tissues, the expression of inflammatory mediators by immune cells is tightly regulated by a complex network of intracellular signaling pathways and transcription factors such as MAPKs, PKC, NF-κB, NFAT and AP1 [58,59]. Therefore we further addressed if the anti-inflammatory effects of oxPLPS in LPS-stimulated macrophages were due to modulation of MAPKs and transcription factor NF-κB. We observed that, in the experimental conditions used, PS species independent of their oxidation status, do not activate these canonical pro-inflammatory signaling cascades. However, oxPLPS significantly mitigate LPS-induced JNK 1/2 phosphorylation and NF-κB subunit p65 nuclear translocation. These findings suggest that the observed down-regulation of *Nos2* and *Il1b* genes results, at least in part, from an inhibitory effect of oxPLPS over TLR-induced NF-κB and JNK signaling cascades. Although we do not identify the exact point where oxPLPS is modulating the cascades, it is unlikely to be by decreasing LPS-TLR4 interaction or by interfering with early signaling adaptors such Myd88 and TRIF. This because, from our results, not all TLR4 downstream pathways are affected, for instance the LPS-dependent activation of ERK and p38 is not decreased by oxPLPS. Additionally, we observed that oxPLPS also decreases the activation of the JNK pathway elicited by TNF-α, confirming that it was not an effect exclusive of TLR-ligand interaction. We hypothesize therefore that oxPLPS may be directly interfering with intermediary signaling effectors of the NF-kB and JNK pathways, restraining their activation upon pro-inflammatory stimulation.

Overall, we demonstrated that oxidation of phosphatidylserines confer them modulatory characteristics that may be important in the containment of inflammatory signals during apoptosis. In addition to their role marking apoptotic cells for engulfment, oxPLPS appear to actively modulate in phagocytic cells important pro-inflammatory signaling cascades such as JNK 1/2 and NF-κB. Given that “silent” clearance of apoptotic cells is critical for restoration of tissue function, we hypothesize that oxPLPS play a key role in promoting timely resolution of inflammation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.04.015.

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