

Phospholipidomic Profile Variation on THP-1 Cells Exposed to Skin or Respiratory Sensitizers and Respiratory Irritant

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Occupational exposure to low molecular weight reactive chemicals often leads to development of allergic reactions such as allergic contact dermatitis and respiratory allergies. Further insights into the interaction of these chemicals with physiopathological relevant cellular models might provide the foundations for novel non-animal approaches to safety assessment. In this work we used the human THP-1 cell line to determine phospholipidome changes induced by the skin sensitizer 1-fluoro-2,4-dinitrobenzene (DNFB), the respiratory allergen hexamethylene diisocyanate (HDI), and the irritant methyl salicylate (MESA). We detected that these chemicals differently induce lipid peroxidation and modulate THP-1 IL-1 β , IL-12B, IL-8, CD86, and HMOX1 transcription. Decreased phosphatidylethanolamine content was detected in cells exposed to MESA, while profound alterations in the relative abundance of cardiolipin species were observed in cells exposed to DNFB. All chemicals tested induced a decrease in the relative abundance of plasmalogen phosphatidylcholine species PC (O-16:0e/18:1) and phosphatidylinositol species PI (34:1), while increasing PI (38:4). An increased abundance of oleic acid was observed in the phospholipids of cells exposed to DNFB while a decreased abundance of palmitic acid was detected in cells treated with MESA or DNFB. We conclude that both specific and common alterations at phospholipidome levels are triggered by the different chemicals, while not allowing a complete distinction between them using a Canonical Analysis of Principal Coordinates (CAP). The common effects observed at phospholipids level with all the chemicals tested might be related to unspecific cell cytotoxic mechanisms that nevertheless may contribute to the elicitation of specific immune responses.

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Allergic contact dermatitis (ACD) and respiratory allergies caused by low molecular weight (LMW) chemicals are highly disruptive pathologies and therefore have a significant socioeconomic impact. It is believed that identification of the sensitizing potential of chemicals and adoption of preventive and protective measures will mitigate the incidence of these types of allergies (Roggen, 2014). Although substantial progress has been achieved in the development of toxicity tests that can

identify contact sensitizers, establishing equivalent tests for respiratory chemical allergens has proved to be more problematic (Kimber et al., 2011; Roggen, 2014).

In contrast to their irritant counterparts that only trigger the innate immune system, both ACD and respiratory allergy to chemicals are adaptive immune responses. ACD is classically defined as a cell-mediated delayed-type hypersensitivity involving Th1 and cytotoxic T cells. LMW chemical respiratory

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allergy is considered a response relying on Th2 cells while the involvement of humoral immunity, dependent on B lymphocytes that produce antigen-specific IgE antibodies, remains controversial (Kimber et al., 2011). Several exceptions to this generalization have been reported, however (Kim et al., 2009) and, despite the significant achievements already made, it is believed that further insight into the pathophysiological mechanisms involved in LMW chemical contact and respiratory allergies is of the utmost importance (Kimber et al., 2011).

Dendritic cells (DC) are specialized innate immune antigen-presenting cells that link innate and adaptive immunity and hence are considered pivotal in allergic reactions (Kimber et al., 2013). It has been shown that depending on their maturation state and cytokine/chemokine profiles DC are able to polarize naïve T cells into their distinct effector populations (Steinman and Banchereau, 2007). The exact role played by different DC subsets in ACD and respiratory sensitization is not yet fully understood, although it is known that they are key elements in the physiopathology of these conditions (Kimber et al., 2013). As a result, some of the most promising *in vitro* approaches for evaluation of chemicals' contact sensitizing potential make use of DC-like cell lines such as MUTZ-3, U-937 and THP-1 (Roggen, 2014).

These tests mainly rely on analysis of alterations induced by LMW chemicals at protein level, especially induction of maturation markers such as CD83, CD86 or cytokines, like IL-8, and IL-18 (Mehling et al., 2012). However, it is plausible to consider that exposure of cells to chemicals also results in modification of their lipid content/profile and that these alterations can even contribute to DC functional abilities. In fact, a growing body of data shows that lipids play a pivotal role in immune responses. They are structurally important for the maintenance of skin and respiratory epithelium barrier function (Girod et al., 1992; Schürer, 2002) and contribute to the formation of lipid rafts that are crucial to the compartmentalization of membrane receptors and the formation of immune synapses (Dustin and Groves, 2012; Goddard and Watts, 2012). Functionally, some lipids behave as innate immune effectors with anti-microbial properties (Lee et al., 2010; Fischer et al., 2014). Lipids also serve as precursors of important signaling molecules such as phosphatidylinositol phosphates (PIPs) derivatives, nuclear receptors agonists (Schürer, 2002; Fowler et al., 2003), and extracellular signaling molecules eicosanoids and specialized pro-resolving mediators (Schumann, 2012). Several reports link dietary intake of fatty acids to a propensity for developing irritant and allergic skin reactions to chemicals (Wander et al., 1997; Barman et al., 2013). Moreover, an increased presence of glycolipids that have the capacity to stimulate invariant natural killer T cells has been observed in the liver of skin sensitized animals (Dey et al., 2011). Previous works from our group also showed that the skin sensitizer DNFB induces several alterations in keratinocytes' lipidome (Santinha et al., 2013) and that its evoked DC maturation is also associated with lipidome alterations (Santinha et al., 2012).

It is therefore plausible to consider that lipid alterations triggered by LMW reactive chemicals on DC membranes might have an important role in contact and respiratory allergies. We hypothesized that contact and respiratory sensitizers may evoke distinct phospholipidome profiles in DC that ultimately account for the different forms of allergic disease. To track those assumptions we determined phospholipidome changes induced by the extreme skin sensitizer DNFB, the respiratory sensitizer HDI, and the respiratory and skin irritant MESA (Ahmed and Bloese, 1983; Arts et al., 1997, 2008) on THP-1 monocytes. A phospholipidomic approach based on mass spectrometry was used for identification and relative quantification of individual phospholipid (PL) species within each PL class. Our results

show several lipidome alterations that are both specific and common to all tested chemicals.

Materials and Methods

Material

Fetal calf serum and trypsin were purchased from Invitrogen (Paisley, UK). Phospholipid internal standards (1,2-dimyristoyl-sn-glycero-3-phosphocholine (dMPC), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (dMPE), 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (dMPG), and 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine (dMPS) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Acetonitrile, chloroform, methanol and hexane (HPLC grade) were bought from Fisher scientific (Leicestershire, UK). TRIzol[®] reagent was purchased from Invitrogen (Barcelona, Spain). The iScript Select cDNA Synthesis Kit and SYBR green were obtained from BioRad (Hercules, CA). Primers were from MWG Biotech (Ebersberg, Germany). Unless otherwise stated, all other reagents were from Sigma Chemical Co. (St. Louis, MO) or Merck (Darmstadt, Germany) and were of the highest grade of purity commercially available. The water was of MilliQ purity filtered through a 0.22- μ m filter (Merck-Millipore, Darmstadt, Germany).

THP-1 cell culture

THP-1 human monocytic cell line (ATCC TIB-202, American Tissue Culture Collection, Manassas, VA) was cultured in RPMI 1640 supplemented with 10% of inactivated fetal bovine serum (FBS), 25 mM glucose, 10 mM Hepes, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.05 mM 2-mercaptoethanol. Cultures were maintained at a cell density of 0.2×10^6 to 1×10^6 cells/ml and were subcultured every 2–3 days. Cells were kept in culture for a maximum of 2 months.

Determination of THP-1 viability and chemical exposure

A certain level of cytotoxicity is required for dendritic cells to be effectively activated by chemical sensitizers (Hulet et al., 2005). DNFB, HDI, and MESA effects on THP-1 cells viability were determined using the resazurin assay (O'Brien et al., 2000). 0.2×10^6 cells/well in 300 μ l growth medium were plated in 96-well plates and exposed to different chemical concentrations for 24 h. Three hours before the end of this period, 50 μ M resazurin was added to each well. At the end of the exposure period absorbance was read at 570 and 620 nm in a standard spectrophotometer MultiSkan Go (Thermo Fisher Scientific, Waltham, MA), and cellular viability determined as a percentage of untreated cells (control) using Δ Abs(570–620). Data for DNFB has been previously published by our group (Luis et al., 2014) and results regarding HDI and MESA are presented in supplementary Figure S1.

For lipid and mRNA extraction, THP-1 cells were used at 0.6×10^6 cells/ml and incubated for 6 h or 24 h with chemical concentrations that induce 20–30% reduction in cell viability, specifically 8 μ M DNFB, 11.5 μ M HDI, or 600 μ M MESA. After the incubation time, cells were harvested by 5 min centrifugation at 250g at 4°C, washed with PBS, and stored dried or resuspended in TRIzol at –80°C for lipid and mRNA extraction, respectively. They were resuspended in 1 ml of MilliQ water immediately before lipid extraction.

RNA extraction and analysis of gene transcription by qPCR

Total RNA was isolated with TRIzol reagent according to the manufacturer's instructions, and the concentration was determined by measurement of the O.D. at 260 nm using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Samples

were stored in RNA Storage Solution (Ambion, Foster City, CA) at -80°C until they were used. Briefly, 1 μg of total RNA was reverse-transcribed using the iScript Select cDNA Synthesis Kit, and quantitative real-time PCR (qPCR) were performed, in duplicate for each sample, on a Bio-Rad MyCycler iQ5 as previously described (Neves et al., 2009). After amplification, a threshold was set for each gene and Ct values were calculated for all samples. Gene expression changes were analyzed using the built-in iQ5 Optical system software. The results were normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene. This gene was unaltered in the different treatment conditions used. Primers were designed using Beacon Designer version 7.7 (Premier Biosoft International, Palo Alto, CA) and thoroughly tested. Forward and reverse primer sequences were, respectively: GCTTGGTGATGTCTGGTC and GCTGTAGAGTGGGCTTATC for IL-1 β ; TGTCGTAGAATTGGATTGGTATC and AACCTCGCCTCCTTTGTG for IL-12B; CTTTCAGAGACAGCAGAG and CTAAGTTCCTTAGCACTCC for IL-8; GAACCTAAGAAGATGAGT and TCCAGAATACAGAAGATG for CD86; CCTGAGTTTCAAGTATCC and AACCAACAGAACACAACAA for HMOX1.

Because qPCR results are presented as ratios of chemically treated samples to untreated cells (control), a two-base logarithmic transformation was used to make observations symmetric and closer to a normal distribution. If x represents the fold change of a gene in one sample, then the two-base logarithmic transformation $[\log_2(x)]$ is $\ln(x)/\ln(2)$. Therefore, fold changes of 2 and 0.5 correspond to mean $\log_2$ values of 1 and -1 , respectively.

Lipid extraction

Lipids were extracted from cells with the Bligh and Dyer method (Bligh and Dyer, 1959). In brief, 3.75 ml of chloroform-methanol 1:2 (v/v) were added to each sample, vortexed, and incubated on ice for 30 min. After the incubation period, 1.25 ml of chloroform plus 1.25 ml of doubly distilled water were added, and the samples were vortexed and centrifuged at 153g for 5 min (Santinha et al., 2013; Maciel et al., 2014). The lipid extracts recovered from the organic phase were dried using a nitrogen flow and stored at -20°C until used.

Quantification of phospholipids by phosphorus assay

Quantification of phospholipids in the total lipid extracts and in the spots separated by TLC was performed according to Bartlett and Lewis (Bartlett and Lewis, 1970). Concentrated perchloric acid (0.65 ml, 70% m/v) was added to the samples in acid-washed glass tubes and the mixture was incubated for 60 min at 180°C in a heating block (Block Heater SBH200D/3, Stuart[®], Bibby Scientific Ltd., Stone, UK). After incubation, 3.3 ml of water, 0.5 ml of 2.5% ammonium molybdate, and 125 μl of freshly prepared 10% ascorbic acid were sequentially added to the mixtures, with vortex homogenization between each addition, and incubated for 10 min at 100°C in a water bath (Precistern, JP Selecta S.A., Barcelona, Spain). Phosphate standards with concentrations ranging from 0.2 to 1.5 μg of phosphate were prepared from a standard solution of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ with 100 μg of phosphorus per ml and were processed as the samples. The absorbance of standards and samples was measured at 800 nm, at room temperature, in a microplate UV-vis spectrophotometer (Multiskan GO, Thermo Scientific, Hudson, NH). An average phospholipid MW of 775 was considered to calculate the phospholipid mass of the samples.

Fatty acid analysis

The FA content of the total lipid extracts was assessed by measuring the fatty acid methyl esters through gas chromatography (GC), according to a previously described method (Aued-Pimentel et al., 2004). Briefly, a methanolic solution of potassium hydroxide (2 M) and a saturated solution of NaCl

were added to lipid extracts (20 μg). The solution was then vortexed and centrifuged for 5 min at 613g to obtain a two-phase system. The FA methyl esters recovered from the bottom phase were dried using a nitrogen flow and resuspended in hexane. Methyl esters of fatty acids were analyzed in a PerkinElmer Clarus 400 gas chromatograph (Waltham, MA) equipped with a flame ionization detector (FID) and a DB-1 column 30 m in length, 0.25 mm in internal diameter, and 0.1 μm of film thickness (J&W Scientific, Folsom, CA). The oven temperature was programmed from an initial temperature of 50°C , staying at this temperature for 3 min; a linear increase at $25^{\circ}\text{C}/\text{min}$ to 180°C , staying at this temperature for 6 min; followed by a linear increase at $40^{\circ}\text{C}/\text{min}$ to 260°C , staying at this temperature for 3 min. The injector and detector temperatures were 220 and 230°C , respectively. Hydrogen was used as a carrier gas at a flow rate of 1.7 ml/min.

Separation of phospholipid classes by thin layer chromatography

Separation of PL classes by TLC from the total lipid extract was performed using plates of silica gel 60 with concentration zone 2.5×20 cm. Before TLC separation, the plates were washed in $\text{CHCl}_3:\text{MeOH}$ (1:1 v/v) and treated with boric acid in ethanol (2.3% w/v). The plates with spots containing about 30 μg of sample were developed in a mixture of solvents $\text{CHCl}_3:\text{MeOH}:\text{H}_2\text{O}$: triethylamine (30:35:7:35, v/v/v/v). To expose the phospholipid spots, the TLC plates were sprinkled with a primuline solution (50 $\mu\text{g}/100$ ml acetone:water, 80/20, v/v), and viewed with a UV lamp ($\lambda = 254$ nm). After identification of the phospholipid spots, by comparison with phospholipid standards, the spots were scraped off from the plates and quantified using the phosphorus assay. The relative abundance of each phospholipid class was calculated by the relation of the amount of phosphorus in each spot to the phosphorous amount of the total lipid extract in the sample applied in the TLC spot.

Characterization of the phospholipid profile

The individual molecular species of phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), sphingomyelin (SM), and cardiolipin (CL) classes were characterized by mass spectrometry (MS), using a linear ion trap (LXQ; Thermo Finnigan, San Jose, CA) mass spectrometer after separation by liquid chromatography (LC). Hydrophilic interaction chromatography (HILIC) was performed in a Waters Alliance 2690 HPLC system with an Ascentis[®]Si column (15 cm $\times$ 1 mm, 3 μm) and a precolumn split (Acurate, LC Packings, Dionex, Thermo Fisher, MA) in order to obtain a flow rate of 60 $\mu\text{l}/\text{min}$. The solvent system consisted of two mobile phases as follows: mobile phase A (acetonitrile:methanol:water; 50:25:25 (v/v/v) with 1 mM ammonium acetate) and mobile phase B (acetonitrile:methanol 60:40 (v/v) with 1 mM ammonium acetate). Initially, 0% of mobile phase A was held isocratically for 8 min followed by linear increase to 60% of A within 7 min and maintained for 22 min. The LXQ was operated in the negative mode (electrospray voltage -4.7 kV), the capillary temperature was 275°C and the sheath gas flow was 8 U. Normalized collision energyTM (CE) ranged between 20 and 27 (arbitrary units) for MS/MS experiments. Data acquisition and analysis was carried out in an Xcalibur data system V2.0 (ThermoFinnigan, San Jose, CA) (Ferreira et al., 2013). Lipid species were manually identified considering the HPLC retention time (supplementary Fig. S3). The putative lipid species for each m/z value were determined using MS analysis tools from Lipid MAPS: lipid metabolites and pathways strategy website (<http://www.lipidmaps.org/>) (Fahy et al., 2007), a research project from the San Diego supercomputer center, and their presence confirmed by manual analysis of MS/MS spectra considering the characteristic fragmentation pattern of each lipid

class (Murphy et al., 2001). Relative quantitation of individual phospholipid species was performed using Xcalibur QuanBrowser (Thermo) software and determined by the ratio between the area of reconstructed ion chromatogram of a given m/z value against the area of the reconstructed ion chromatogram of the respective class (Simões et al., 2013).

Quantification of lipid hydroperoxides using the FOX II assay

In order to quantify lipid hydroperoxides, 50 μ l of the total lipid extracts (2 mg lipid/ml) were added to 950 μ l of the FOX reagent solution (100 μ M xylenol orange, 250 μ M Fe^{2+} , 25 mM H_2SO_4 , and 4 mM BHT in 90% (v/v) methanol) in microtubes, homogenized in a vortex mixer, and incubated for 30 min at room temperature in the dark. After incubation, the absorbance of samples and H_2O_2 standards with concentrations ranging from 0.0–0.4 mM (H_2O_2 1 mM, FOX2 reagent and water) was read at 560 nm. The FOX reagent (100 ml) was prepared as follows: 250 μ M $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ (9.8 mg) and 25 mM H_2SO_4 (139 μ l) were dissolved in 5 ml of water, mixed with 4 mM 2,6-di-tert-butyl-p-hydroxytoluene (BHT) (88.2 mg), 100 μ M xylenol orange (7.2 mg) and 45 ml of methanol. Then, other 45 ml of methanol and 5 ml of water were added (Jiang et al., 1991).

Statistical analysis

Significant differences between samples were determined by two-way analysis of variance (ANOVA) using Dunnett's post-test. A multivariate analysis was performed with HILIC-LC-MS generated data. Prior to this analysis, the resemblance matrix between samples was obtained with the Bray–Curtis similarity coefficient, following a $\text{Log}(x + 1)$ transformation of the original data. A Canonical Analysis of Principal Coordinates (CAP) (Anderson and Willis, 2003) was used to test if sample identity could be predicted by the corresponding phospholipid species profile variations determined by HILIC-LC-MS. CAP is a constrained ordination tool that discriminates between groups defined a priori. This approach identifies how well the chemical treatments applied to THP-1 cells can be correctly identified by phospholipid profile variations. To

determine which phospholipids contributed to the separation of the different chemical treatments, the Spearman correlation ($|r| > 0.70$) of all phospholipid with the CAP axes was considered and displayed in the CAP plot. ANOVAs were performed using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA), while multivariate analysis (CAP) was employed using PRIMER v6 with the add-on PERMANOVA+ (Anderson et al., 2008).

Results

Determination of mRNA profile by qPCR

Thorough this work we used concentrations of DNFB, HDI, and MESA that decrease THP-1 cells viability approximately 30% after an exposure of 24 h. To test whether these incubation conditions significantly affect THP-1 cells maturation status, we have determined the transcription profile of several genes commonly associated with DC maturation after exposure to immunostimulants (Fig. 1). Using qPCR we observed increased mRNA levels of the pro-inflammatory cytokine IL-1 β , chemokine IL-8, and inducible heme oxygenase-1 enzyme (HMOX1) in cells treated with DNFB and HDI comparing to untreated cells. mRNA levels of the 40 kDa subunit of interleukin 12 (IL-12B) were also increased in cells exposed to DNFB. The co-stimulatory protein CD86 mRNA levels were increased in cells treated with DNFB, HDI, or MESA for 6 h. However, after 24 h of exposure CD86 mRNA levels remained increased in cells exposed to DNFB while in cells treated with HDI or MESA they were decreased.

Analysis of lipid classes by thin layer chromatography

Total lipid extracts were fractionated by thin layer chromatography (TLC) (Fig. 2A) and the spots corresponding to each class were identified by comparison with pure phospholipid standards applied on the same TLC plate. Relative abundance (RA) of each PL class was determined by quantification of phosphate content for each PL spot. This approach allowed the separation of seven phospholipid classes: lysophosphatidylcholine (LPC), phosphatidylcholine (PC),

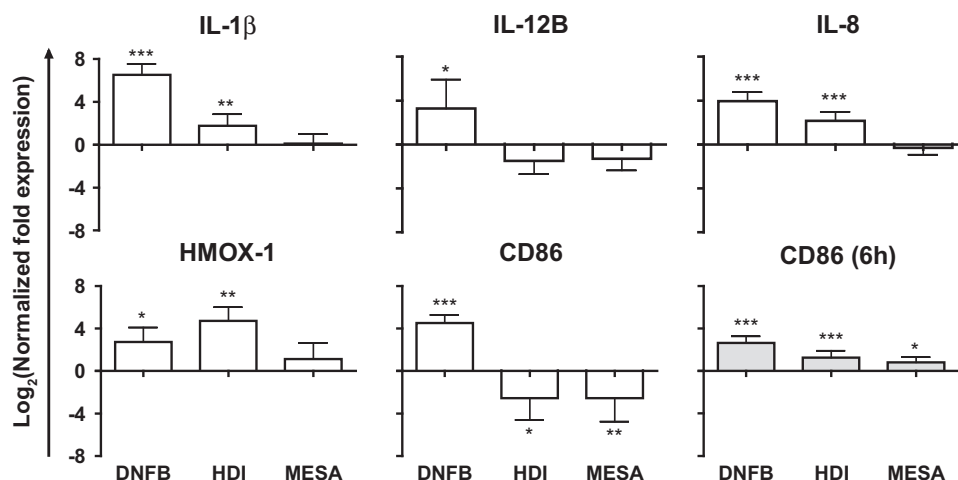


Fig. 1. Alterations induced by DNFB, HDI, and MESA in THP-1 mRNA levels of relevant genes. THP-1 cells were incubated with 8 μ M DNFB, 11.5 μ M HDI, or 600 μ M MESA for 6 h (gray bars) or 24 h (white bars). The total RNA was extracted and reverse transcribed as described in the material and methods. The mRNA levels of the cytokines IL-1 β and IL-12B, chemokine IL-8, maturation marker CD86 and electrophilic stress response enzyme HMOX1 were determined by qPCR. Results are presented as $\log_2$ of the normalized fold expression using untreated cells as control and GAPDH as reference gene. Mean values and standard deviation of at least three independent experiments are shown. One way ANOVA with Dunnett post-test was used to compare the results relatively to the control. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

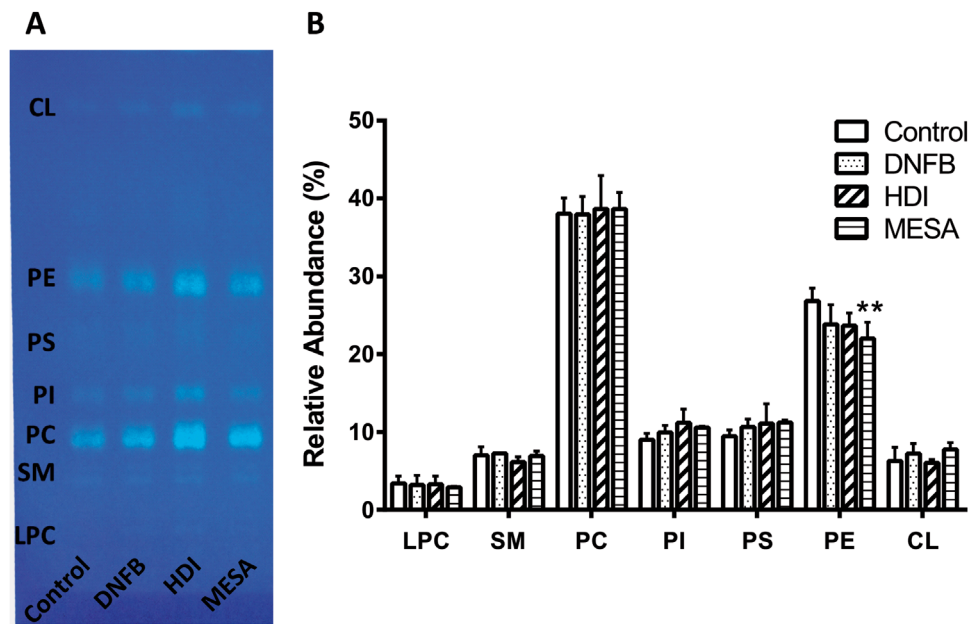


Fig. 2. Effects of the sensitizers DNFB and HDI and of the irritant MESA in the relative abundance of phospholipids (PLs) on THP-1 cells. (A) THP-1 cells PLs extracted from untreated or treated cells during 24 h with 8 μ M DNFB, 11.5 μ M HDI, or 600 μ M MESA (from left to right) were separated by thin layer chromatography and identified using phospholipid standards (not shown). (B) The phosphate content of each PL spot was quantified and is expressed as a % of the phosphate content in the respective total lipid extract. CL, cardiolipin; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; SM, sphingomyelin; LPC, lysophosphatidylcholine. Mean values $\pm$ standard deviation from three independent experiments carried out in duplicates are shown. Statistical treatment was performed using two-way ANOVA followed by Dunnett's post-test for control versus DNFB, HDI, and MESA. ** $P < 0.01$.

sphingomyelin (SM), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), and cardiolipin (CL) (Fig. 2A). The most abundant PL class was PC followed by PE, PS, PI, CL, SM, and LPC (Fig. 2B). The relative PL classes' abundance was not affected either by the sensitizers or the irritant. However, compared to untreated cells, it was possible to observe a decreased PE class content in THP-1 cells exposed to all the chemicals tested, although only statistically significant in cells treated with the irritant MESA (Fig. 2B).

Analysis of fatty acid profile

In order to determine the fatty acid content of THP-1 cells, total lipid extracts were subjected to alkaline hydrolysis and the esterified fatty acids analyzed by GC-FID (Table I). The results obtained showed palmitic (FA16:0) and oleic acid (FA18:1) as the most abundant THP-1 fatty acids regardless of the cell treatment applied. Stimulation of THP-1 cells with HDI did not affect the cells' fatty acid profile. However, DNFB stimulation induced a significant increase in FA18:1 and a decrease in

TABLE I. The effect of the sensitizers DNFB and HDI and of the irritant MESA in THP-1 cells fatty acid content

Fatty acids	Control		DNFB		HDI		MESA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
SFA	48.67	2.49	46.94	4.35	49.27	3.60	47.30	2.85
14:0	3.03	0.23	2.13	0.23	2.70	0.10	2.67	0.74
16:0	33.30	1.75	30.97	2.08	33.63	2.37	30.30**	1.13
18:0	11.83	0.31	12.97	1.48	12.07	0.84	13.80	0.78
24:0	0.50	0.20	0.87	0.55	0.87	0.29	0.53	0.21
MUFA	34.70	1.15	36.33	2.80	36.30	2.10	36.50	1.72
16:1	7.33	0.74	6.87	0.98	7.93	1.26	7.80	0.96
18:1 cis	21.87	0.15	24.53**	0.91	23.20	0.46	23.57	0.60
18:1 trans	5.50	0.26	4.93	0.91	5.17	0.38	5.13	0.15
PUFA	16.50	2.19	16.73	5.31	14.40	1.76	16.07	2.58
18:2	2.23	0.25	2.33	0.29	2.07	0.35	1.93	0.31
20:4n3	6.67	1.29	6.80	1.57	6.37	0.15	6.57	0.78
20:3	1.40	0.10	1.43	0.40	1.27	0.15	1.50	0.17
22:6n3	3.57	0.40	2.90	0.89	2.83	0.50	3.57	0.81
22:5	2.63	0.15	3.27	2.15	1.87	0.60	2.50	0.52

Relative abundance of fatty acids were observed by GC-FID in untreated cells (control) and cells exposed 24 h to 7.5 μ M DNFB, 11.5 μ M HDI, or 600 μ M MESA. Values are expressed as mean and standard deviation of three independent experiments. Statistical treatment was performed using two-way ANOVA followed by Dunnett's post-test for control versus DNFB, HDI, and MESA.

SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids.

* $P < 0.05$.

** $P < 0.01$.

FA16:0 content. Treatment with MESA only induced a decrease of FA 16:0.

Analysis of phospholipids' molecular species profile by HILIC-LC-MS

Although the quantification by phosphorus assay showed only a decrease in PE class, the variation in PL composition at molecular level was analyzed by HILIC-LC-MS. LC-MS is a technique that allows the separation of phospholipid classes and, at the same time, the identification and quantification of molecular species within each class by tandem mass

TABLE 2. Identification of ions $[M + CH_3COO]^-$ observed in the LC-MS spectra for PC, LPC, and SM and of ions $[M-H]^-$ observed in the LC-MS spectra for PE, PS, PI, and CL

m/z	Fatty acyl chains	Molecular species
Phosphatidylcholines (PC)		
764	14:0/16:0	30:0
776	O-16:1 e/16:0	e32:1
	O-16:0 e/16:1	
790	16:0/16:1	32:1
	14:0/18:1	
804	O-18:1 e/16:0	e34:1
	O-16:0 e/18:1	
	O-18:0 e/16:1	
816	16:0/18:2	34:2
818	16:0/18:1	34:1
844	18:0/18:2	36:2
	18:1/18:1	
850	O-16:1 e/22:5	e38:6
	O-16:0 e/22:6	
866	18:1/20:4	38:5
	18:0/20:5	
	16:0/22:5	
Lysophosphatidylcholines (LPC)		
526	14:0	
540	O-16:0	
554	16:0	
568	O-18:0	
580	18:1	
582	18:0	
Sphingomyelins (SM)		
761	d18:1/16:0	
Phosphatidylethanolamines (PE)		
700	(O-16:0 e/18:2)	e34:2
	(O-16:0 p/18:1)	p34:1
714	(16:0/18:2)	34:2
	(16:1/18:1)	
716	(16:1/18:0)	34:1
	(16:0/18:1)	
722	(O-16:0 p/20:4)	p36:4
	(O-16:0 p/20:5)	p36:5
724	(O-16:0 p/20:3)	p36:3
	(O-16:0 e/20:4)	e36:4
742	(20:2/16:0)	36:2
	(18:0/18:2)	
	(18:1/18:1)	
744	(18:0/18:1)	36:1
746	(18:0/18:0)	36:0
	(O-16:0 p/22:6)	p38:6
748	(O-16:0 p/22:5)	p38:5
	(O-18:0 p/20:5)	
	(O-18:1 p/20:4)	
	(O-16:0 e/22:6)	e38:6
750	(O-18:0 p/20:4)	p38:4
	(O-16:0 p/22:4)	
	(O-18:0 e/20:5)	e38:5
	(O-16:0 e/22:5)	
762	(16:0/22:6)	38:6
	(18:1/20:5)	
	(18:2/20:4)	
766	(22:4/16:0)	38:4
	(18:0/20:4)	
	(18:1/20:3)	
774	(O-18:0 p/22:6)	p40:6
	(O-18:1 p/22:5)	
	(18:0/20:0)	38:0
776	(O-18:0 p/22:5)	p40:5
	(O-18:0 e/22:6)	e40:6

spectrometry (MS/MS). The MS data is presented as the mean % of the relative abundance of each molecular species per phospholipid class. Molecular species were identified in the mass spectra obtained in the negative ion mode as $[M-H]^-$ ions in the case of PE, PS, PI, and CL, or as $[M + CH_3COO]^-$ ions in the case of LPC, PC, and SM. LC-MS/MS was performed for each ion to identify and confirm the respective structures, according to the typical fragmentation pathways (Pulfer and Murphy, 2003). The identified molecular species for PC, LPC, SM, PE, PS, PI, and CL are presented in Table 2.

Phosphatidylcholine profile. PC is the most abundant phospholipid class in THP-I cells, similarly to other mammalian

TABLE 2. (Continued)

m/z	Fatty acyl chains	Molecular species
Phosphatidylserines (PS)		
758.4	16:0/18:2	34:2
	16:1/18:1	
760.4	16:0/18:1	34:1
	16:1/18:0	
786	18:0/18:2	36:2
	18:1/18:1	
788	18:0/18:1	36:1
810	18:0/20:4	38:4
	18:1/20:3	
812	18:0/20:3	38:3
	18:1/20:2	
834	18:0/22:6	40:6
	18:1/22:5	
836	18:0/22:5	40:5
	18:1/22:4	
Phosphatidylinositols (PI)		
809	14:0/18:0	32:0
	16:0/16:0	
835	16:0/18:1	34:1
	16:1/18:0	
857	16:0/20:4	36:4
	16:1/20:3	
861	16:0/20:2	36:2
	18:1/20:1	
	18:0/18:2	
	18:1/18:1	
863	16:0/20:1	36:1
	18:0/18:1	
883	18:0/20:5	38:5
	18:1/20:4	
	18:2/20:3	
885	16:0/22:4	38:4
	18:0/20:4	
	18:1/20:3	
887	16:0/22:3	38:3
	18:0/20:3	
	18:1/20:2	
911	18:1/22:4	40:5
	18:0/22:5	
	20:1/20:4	
Cardiolipins (CL)		
1399	16:1/16:0/18:1/18:2	68:4
	14:0/20:2/(18:1) ₂	
1397	14:0/(18:2) ₂ /18:1	68:5
	(16:1) ₂ /18:1/18:2	
1423	n.d.	70:6
1425	16:1/16:0/20:3/18:1	70:5
	16:1/18:2/(18:1) ₂	
1427	16:0/18:0/(18:2) ₂	70:4
	16:0/(18:1) ₂ /8:2	
	16:1/18:0/18:1/18:2	
	16:1/(18:1) ₃	
1447	n.d.	72:8
1449	n.d.	72:7
1451	(18:1) ₂ /(18:2) ₂	72:6
	(16:1) ₂ /20:3/20:1	
	16:1/(18:1) ₂ /20:3	
	16:1/18:1/18:2/20:2	
	16:1/(18:2) ₂ /20:1	
1453	n.d.	72:5
1455	n.d.	72:4

The attribution of the fatty acyl composition of each PL molecular species was done accordingly to the interpretation of the corresponding MS/MS spectra. Plasmalogen and plasmalogen species are noted with "e" and "p" respectively.

cells in which they are major structural components of cell membranes. The MS spectra obtained in the negative mode showed PC species as $[M + CH_3COO]^-$ ions. PC molecular species were identified and analyzed in detail by LC-MS/MS and the results are summarized in Table 2. Several alkyl ether PC species (1-O-alkyl-2-acyl glycerophospholipids) were identified and these plasmalogen lipids are identified as e in Table 2.

Analysis of the data revealed that PC (16:0/18:1) at m/z 818.3 and plasmalogen PC (O-16:0e/18:1) at m/z 804.3 are the major PC molecular species in THP-1 cells (Fig. 3). The same PC molecular species were observed in all conditions; however, changes in their RA were determined between different chemical treatments (Fig. 3). Significant differences occurred in major molecular species. In particular a decrease in PC (O-16:0e/18:1) was observed with all tested chemicals. Cells treated with DNFB showed additional alterations of some PC species not observed with the other chemicals. Both PC (16:0/18:1) and PC (18:0/18:2)/(18:1/18:1) species had higher RA while decreased abundances of plasmalogen PC (e32:1) and PC (32:1) species were determined in DNFB exposed cells (Fig. 3).

Phosphatidylethanolamine profile. PE is one of the main phospholipid classes of THP-1 cells. An analysis of total lipid extracts by LC-MS identified the PE molecular species summarized in Table 2. Both alkyl and alkenyl ether PE species were identified. The plasmalogen (1-O-alkyl-2-acyl glycerophospholipids; indicated as e) and plasmenyl (1-O-alk-1'-enyl-2-acyl glycerophospholipids; indicated as p) species are difficult to distinguish by LC-MS/MS with the used lipid extraction method. The identification of these species was based on observation of the carboxylate anion at the sn-2 position. Since the relative abundance of these lipids was not altered by the chemical treatments used (supplementary Fig. S2), no further experiments were performed to distinguish them and both plasmalogen and plasmenyl species are proposed (Table 2).

The most abundant PE species were the plasmalogens PE (p34:1)/PE (e34:2), PE (p36:4), PE (p38:6)/PE (36:0), and PE (p38:5)/PE (e38:6), which were identified as $[M-H]^-$ ions at m/z 700.4, 722.4, 746.4, and 748.4, respectively. The diacyl PE (34:1) and PE (36:1) were also identified as other major PE molecular species. Analysis of PE class showed a high number of

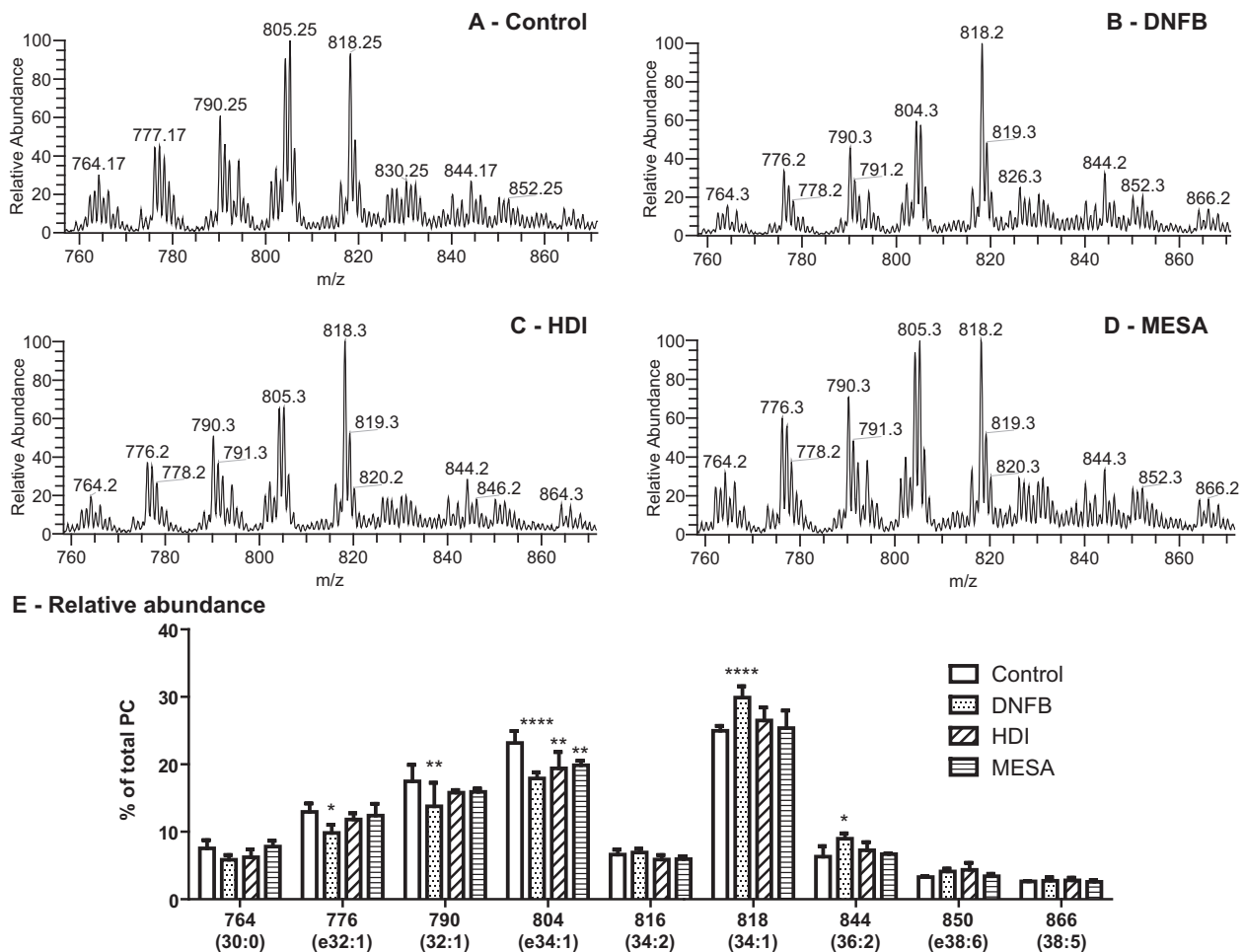


Fig. 3. Typical LC-MS spectra of phosphatidylcholines (PC) analyzed in negative mode from total lipid extracts of untreated THP-1 cells (control) (A) and cells exposed for 24 h to 8 μ M DNFB (B), 11.5 μ M HDI (C), or 600 μ M MESA (D). Relative abundance of $[M + CH_3COO]^-$ ions of PC molecular species observed in the LC-ESI-MS spectra of THP-1 total lipid extracts (E). Values are expressed as mean $\pm$ standard deviation of three independent experiments. Between brackets are presented the PC molecular species corresponding to each $[M + CH_3COO]^-$ ion and plasmalogen species are noted with "e." Statistical treatment was performed using two-way ANOVA followed by Dunnett's post-test for control versus DNFB, HDI, and MESA. **** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$.

plasmalogen species but, contrary PC, they did not undergo alterations in their relative content in cells treated with either the sensitizers or the irritant chemicals (supplementary Fig. S2). Similarly, no changes were observed in PE diacyl species' relative content. However, the quantification results of the phosphorus assay showed a decreased content of PE class in THP-I cells treated with the respiratory irritant MESA, with a similar tendency in DNFB and HDI treated cells (Fig. 2B).

Phosphatidylinositol profile. Despite the low abundance compared with PC and PE, PI class has an important role in cell signaling and is essential in metabolic processes, making this class a very important group of PL. Although no significant differences were observed on PI content (Fig. 2B), a detailed analysis by LC-MS and LC-MS/MS (Fig. 4) revealed differences between untreated and chemical exposed THP-I cells. Both sensitizers DNFB and HDI influenced the relative abundance of the molecular species PI (34:1) at m/z 835.4 by decreasing it. PI (38:4) at m/z 885.4, the main PI molecular species, had an increased RA in THP-I exposed to every chemical tested.

Cardiolipin profile. The sensitizers and the irritant used in this work did not affect the content of mitochondrial phospholipid CL (Fig. 2B). Cardiolipins are complex dimeric phospholipids that contain two phosphatidic acids linked

together through an additional glycerol molecule and thus bearing four fatty acyl chains. CL molecular species are found mainly in the inner mitochondrial membrane and are associated with different proteins of the complexes in the respiratory chain, conferring a central role in mitochondrial processes and cellular metabolism to CL. The cardiolipin profile was determined by identifying the negative ions $[M-H]^-$ and $[M-2H]^{2-}$ and by MS/MS analysis of the double charge ions in order to identify the molecular species, which are summarized in Table 2.

The most abundant molecular species were CL (68:4) at m/z 1399.5, CL (70:5) at m/z 1425.5, and CL (72:6) at m/z 1451.5 (Fig. 5). At m/z 1399.5 and 1425.5 two different species were identified for the same m/z CL-(16:1)(16:0)(18:1)(18:2) and CL-(14:0)(20:2)(18:1)₂ and CL-(16:1)(16:0)(20:3)(18:1) and CL-(16:1)(18:2)(18:1)₂, respectively. At m/z 1451.5 five different species were identified: CL-(18:1)₂(18:2)₂, CL-(16:1)₂(20:3)(20:1), CL-(16:1)(18:1)₂(20:3), CL-(16:1)(18:1)(18:2)(20:2), and CL-(16:1)(18:2)(20:1). Although the quantification by phosphorus assay did not reveal changes in the CL content of THP-I cells exposed to the sensitizers and irritant used in our study, analysis by LC-MS of lipid extracts from DNFB treated cells showed alterations in RA of some

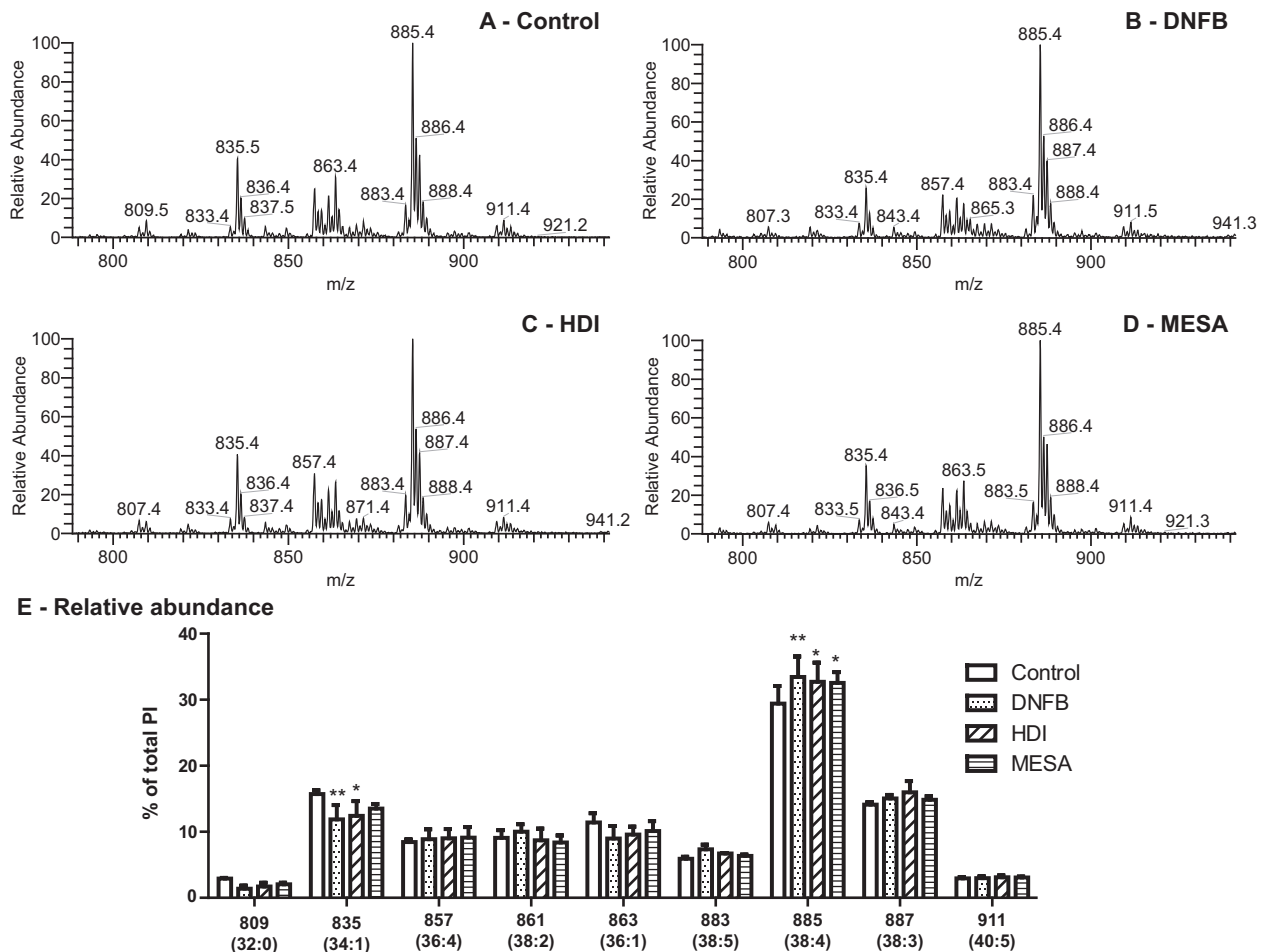


Fig. 4. Typical LC-MS spectra of phosphatidylinositols (PI) analyzed in negative mode from total lipid extracts of untreated THP-I cells (control) (A) and cells exposed for 24 h to 8 μ M DNFB (B), 11.5 μ M HDI (C), or 600 μ M MESA (D). Relative abundance of $[M-H]^-$ ions of PI molecular species observed in the LC-ESI-MS spectra of THP-I total lipid extracts (E). Values are expressed as mean $\pm$ standard deviation of three independent experiments. Below m/z values are presented the PI molecular species corresponding to each $[M-H]^-$ ion. Statistical treatment was performed using two-way ANOVA followed by Dunnett's post-test for control versus DNFB, HDI, and MESA. ** $P < 0.01$; * $P < 0.05$.

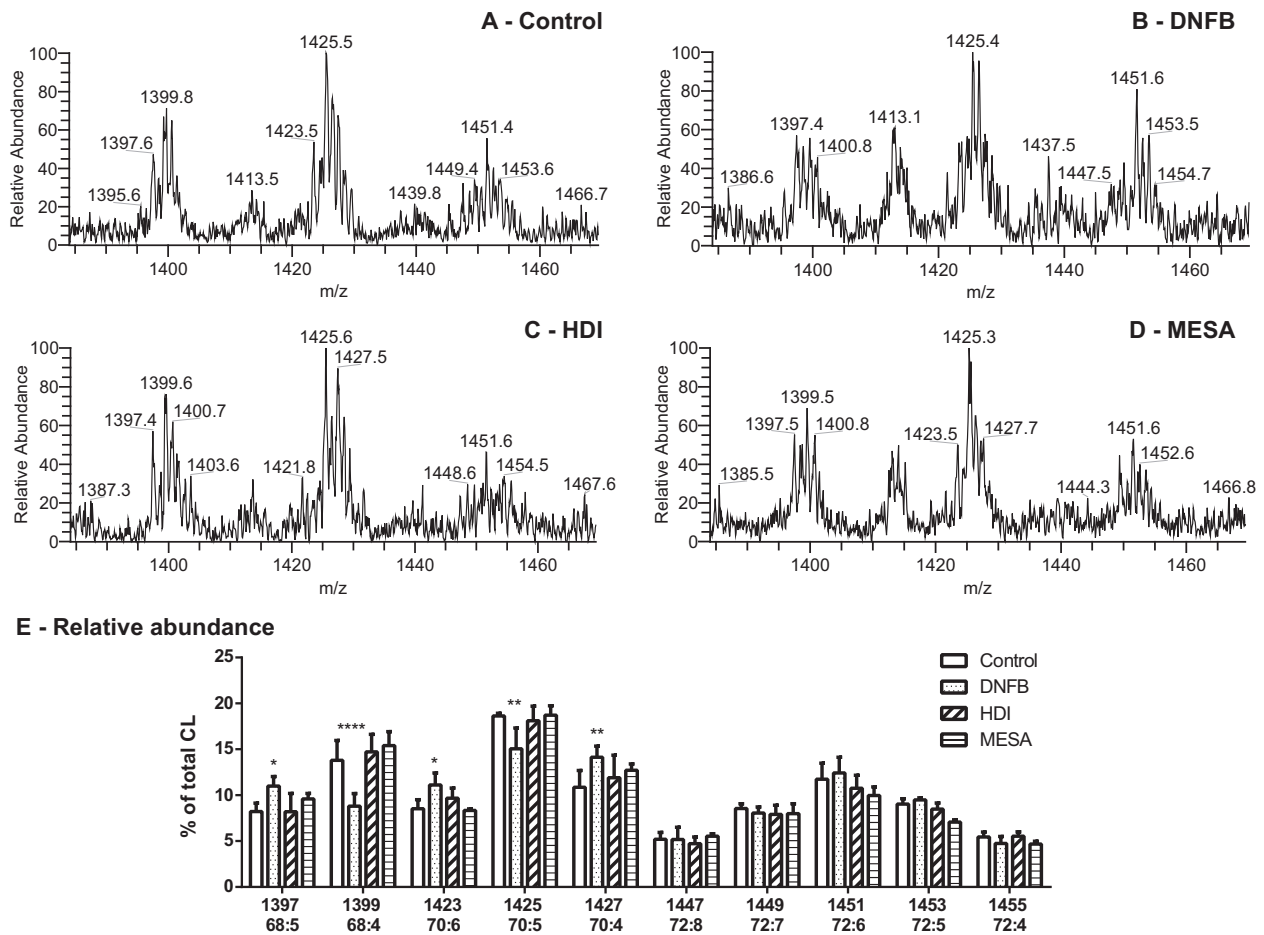


Fig. 5. Typical LC-MS spectra of cardiolipins (CL) analyzed in negative mode from total lipid extracts of untreated THP-I cells (control) (A) and cells exposed for 24 h to 8 μ M DNFB (B), 11.5 μ M HDI (C), or 600 μ M MESA (D). Relative abundance of $[M-H]^-$ ions of CL molecular species observed in the LC-ESI-MS spectra of THP-I total lipid extracts (E). Values are expressed as mean $\pm$ standard deviation of three independent experiments. Below m/z values are presented the CL molecular species corresponding to each $[M-H]^-$ ion. Statistical treatment was performed using two-way ANOVA followed by Dunnett's post-test for control versus DNFB, HDI, and MESA. **** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$.

molecular species (Fig. 5). Species at m/z 1399.5 and 1425.5 decreased while species at m/z 1397.5, 1423.5, and 1427.5 increased with DNFB treatment. THP-I CL molecular profile was not affected by exposure to HDI or MESA.

Quantification of lipid hydroperoxides

The quantification of lipid hydroperoxides was performed by the FOX2 assay in the total lipid extracts from untreated cells (control) and cells exposed to DNFB, HDI or MESA for 24h (Fig. 6). There was a significant increase in the concentration of lipid hydroperoxides in cells exposed to DNFB (mean of 0.0499 ± 0.0045 (SD) μ g OOH/ μ g of phospholipid), which is 53.5% higher than the one found in the control. No differences were observed between the total lipid extracts from cells exposed to HDI or MESA and untreated cells.

Canonical analysis of principal coordinates of HILIC-LC-MS results

The relative abundance of phospholipid species determined by HILIC-LC-MS in THP-I cells was analyzed using CAP. A good graphical separation of the different chemical treatments was achieved by vector overlay of Spearman correlations of

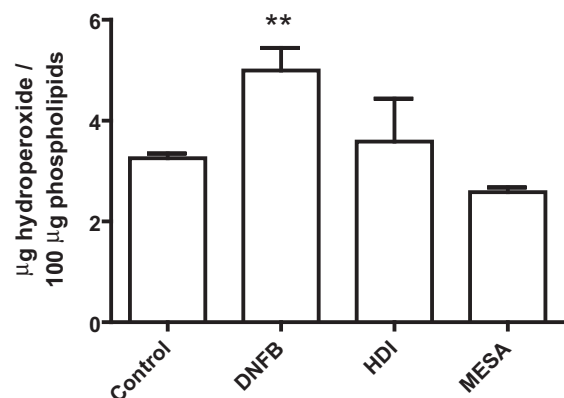


Fig. 6. Hydroperoxides concentration determined by FOX2 assay in the total lipid extracts of untreated cells (control) and cells exposed for 24 h to 8 μ M DNFB, 11.5 μ M HDI or 600 μ M MESA. Mean values and standard deviation of three independent experiments are shown. One-way ANOVA with Dunnett's post-test was used to compare the results relatively to the control. ** $P < 0.01$.

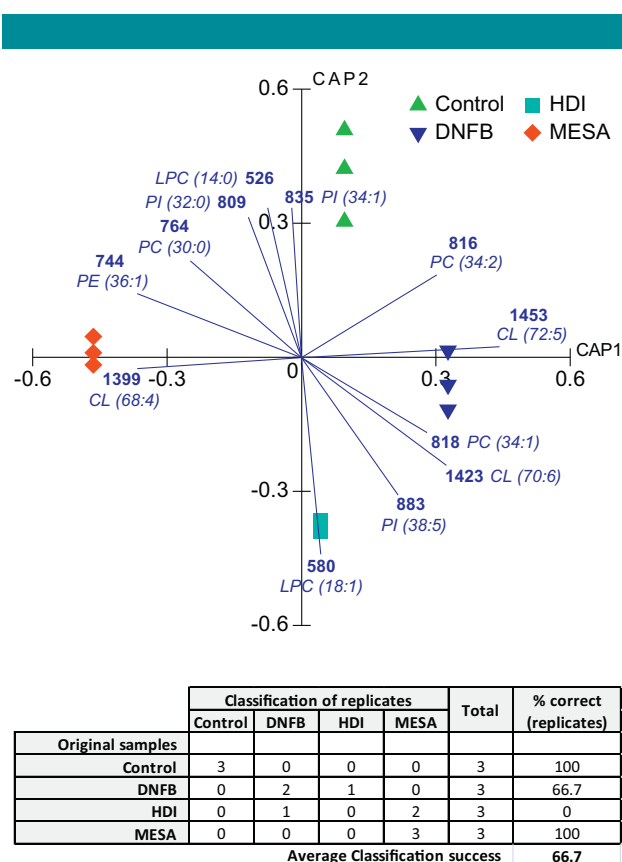


Fig. 7. Canonical analysis of principal coordinates (CAP) of the phospholipids relative abundance determined by HILIC-LC-MS in untreated THP-I cells (control) and cells exposed during 24 h to 8 μ M DNFB, 11.5 μ M HDI or 600 μ M MESA. In the graphical representation axes are drawn to maximize discrimination among the different chemical treatments and vector overlay Spearman correlations of phospholipids with canonical axes are shown if $|r| > 0.70$. In the table are presented the results of the “leave-one-out” cross-validation performed in the CAP analysis, which shows the number of correct/incorrect classifications of the different biological replicates of each chemical treatment based on the determined phospholipidomic alterations.

HILIC-LC-MS results with CAP axes (Fig. 7). However, the leave-one-out procedure resulted in an average CAP classification of 66.67%. Based on the phospholipid profile of HDI exposed cells, none of the replicates performed was correctly classified as HDI by CAP, and one DNFB replicate was misclassified as HDI (Fig. 7).

Discussion

In this study, we used THP-I cells to explore a possible correlation between phospholipidome changes in DC and different immune responses triggered by chemicals. THP-I is a leukemia cell line of the monocytic lineage, which differentiates into macrophage-like cells after phorbol-esters (such as phorbol-12-myristate-13-acetate; PMA) or vitamin D₃ cell treatment. Compared to MUTZ-3, THP-I cells have a limited capacity to generate functional immature DC (Santegoets et al., 2008). However, they have the advantages of presenting growth factor-independent proliferation and are equally able to differentiate into a mature DC-phenotype (Santegoets et al., 2008). Several differences exist between leukemia and normal cells, namely at the lipid level (Agatha et al., 2001; Leidl et al.,

2008), and therefore extrapolation of results obtained with leukemia cell lines to normal cells as to be done with caution (Qin, 2012). Still, THP-I cells are extensively used and considered a good model for studying chemical induced DC maturation and distinguishing skin allergens from irritants (Ashikaga et al., 2006; Kimber et al., 2013; Roggen, 2014).

Our results for the phospholipidome of THP-I monocytes are in agreement with previous reports describing PC and PE as their major glycerophospholipid classes (Surette and Chilton, 1998) and we also identified a small percentage of trans fatty acids in THP-I cells (Ferreri et al., 2005). All chemicals tested produced significant alterations in the phospholipidome of THP-I cells and these effects were distinct between the respiratory sensitizer, skin sensitizer, and irritant. Accordingly, the transcription pattern of several genes usually associated with DC maturation and immune response onset was also dependent on the chemical treatment applied. The respiratory irritant MESA was the only chemical that significantly decreased THP-I PE content. The contact allergen DNFB and the respiratory allergen HDI significantly decreased PI (34:1) species and induced the transcription of the inflammatory cytokine IL-1 β , chemokine IL-8, and enzyme HMOX1. IL-12B mRNA levels were increased exclusively in cells exposed to DNFB. All chemicals tested induced the transcription of the maturation marker CD86 when cells were exposed for 6 h. However, with 24 h incubation periods only cells treated with DNFB showed increased CD86 mRNA levels, while HDI and MESA treated cells had slightly decreased levels of CD86 mRNA compared to untreated cells. Both DNFB and MESA induced a reduction in THP-I palmitic acid (16:0) levels while HDI failed to do so. The most striking differences between the chemicals' effects in the phospholipidome were observed with the contact sensitizer DNFB at the level of cardiolipins.

The cardiolipin species profile of THP-I cells was drastically affected by DNFB, suggesting that mitochondria are an important intracellular target of this chemical. Accordingly, a direct interaction between DNFB and the respiratory chain cytochrome bc1 complex with consequent inhibition of electron transport and proton translocation had previously been reported (Lorusso et al., 1986). Moreover, DNFB inhibits the mitochondrial creatine kinase (Wallimann et al., 2011), an enzyme with an important antioxidant activity in the skin (Lenz et al., 2005). CL and PE are important to the formation and stabilization of mitochondrial respiratory supercomplexes (Pfeiffer et al., 2003; Böttinger et al., 2012) and fatty acid alterations of CL may induce cytochrome c to act as a CL specific peroxidase (Kagan et al., 2009). Furthermore, the observed inability of HDI to induce CL alterations corroborates the generation of ROS by this allergen being mainly dependent on cytosolic superoxide dismutase inhibition (Silva et al., 2014). Our results showing increased lipid hydroperoxides in cells exposed to DNFB demonstrate that its capacity to induce oxidative stress (Luis et al., 2014) is also observed at the phospholipidome level. The remodeling of cardiolipin lipids may be an important mechanism by which this allergen causes redox imbalance.

Our results show that THP-I PC and PE are differently affected by all chemicals tested. While a decreasing effect in PC was exclusively observed in alkyl ether species, all PE species were equally affected. This observation may be a consequence of the sub-compartmentalization of the metabolic pathways involved in lipid homeostasis (Chu, 1992; Bohdanowicz and Grinstein, 2013), or may also be caused by the asymmetrical distribution of lipids within cells. Membrane “rafts” are enriched in vinyl-ether lipids (Braverman and Moser, 2012) and are considered essential to the proper formation and function of immune synapses and thus DC priming of T-cells (Dustin and Groves, 2012; Goddard and Watts, 2012). Additionally, plasmalogens, PE, and CL are lipids with a propensity to form

non-lamellar structures which, among other features, are important to membrane fusion and fission events (Seddon, 1990; Jahn and Südhof, 1999; Van Meer et al., 2008; Braverman and Moser, 2012; Santucci et al., 2014). Therefore, it is possible that the observed effect of chemicals on THP-1 lipidome interferes with vesicle trafficking and alters important inter-organellar interactions, such as mitochondria-endoplasmic reticulum communication. Plasmalogens are also thought to play an important role in discontinuing the propagation of lipid peroxidation reactions (Braverman and Moser, 2012). The deeper decrease in alkyl ether phosphatidylcholines observed in THP-1 cells exposed to DNFB comparatively to those treated with HDI and MESA is probably related to DNFB's ability to generate lipid hydroperoxides.

We also found that the tested chemicals affect fatty acid content of cells differently. DNFB increases THP-1 content in oleic acid (18:1), which is in accordance with previous results reported in HaCaT keratinocytes (Santinha et al., 2013). In DNFB-treated THP-1 cells, we observed a tendency for the remodeling of PC, PI, and CL species containing palmitic acid (16:0) towards species with an increased content in the MUFA oleic acid (18:1) and possibly palmitoleic acid (16:1). We can speculate that such remodeling of the glycerophospholipid profile is related to structural adaptations towards more fluid lipid bilayers. Indeed, increased membrane fluidity has been previously reported in A431 keratinocytes exposed to DNFB

(Mehrotra et al., 2005). Interestingly, mice fed with a high-fat diet enriched in oleic acid present impaired contact hypersensitivity reactions when exposed to skin sensitizers DNCB and FITC (Katagiri et al., 2007; Kendall and Nicolaou, 2013).

The decrease in palmitic acid (16:0) observed in DNFB and MESA-treated cells might be due to increased palmitoylation of cellular proteins. G-protein coupled receptors and caveolin are an example of proteins whose function is regulated in such a way (Van Meer et al., 2008; Goddard and Watts, 2012) and that are crucial to antigen uptake and DC maturation. The formation of N-acyl PE (NAPE) is another possibility to explain the observed lower levels of palmitic acid.

Palmitoylethanolamide (PEA) is a NAPE breakdown product whose levels are increased in mice sensitized with DNFB (Petrosino et al., 2010). PEA attenuation of DNFB induced mice hypersensitivity seems to involve transient receptor potential vanilloid type-1 (TRPV1) channels (Petrosino et al., 2010) which are known to be regulated by phosphatidylinositol-4,5-bisphosphate (Cao et al., 2013). We found that all chemicals tested induced an increase in the abundance of PI with *m/z* 885 while lower levels of PI *m/z* 835 were induced by the allergens DNFB and HDI. PIs are the precursors of several glycosylated and phosphorylated phosphoinositides with important signaling functions. It is therefore plausible to hypothesize that the observed alterations in PI species are related to processes

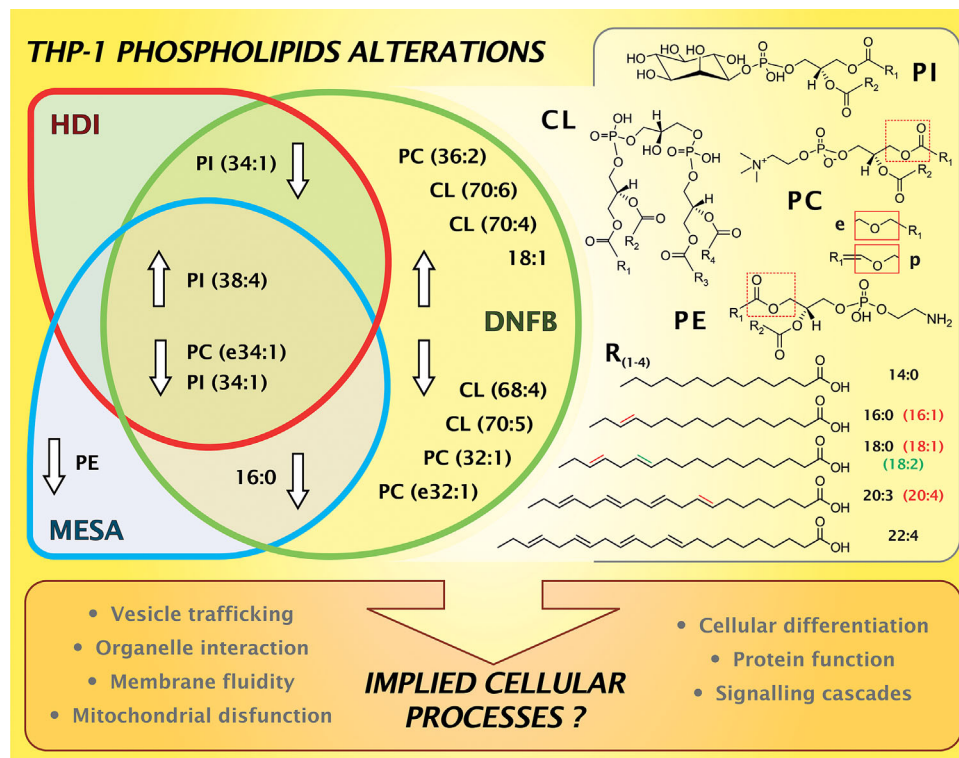


Fig. 8. The phospholipidomic alterations determined in THP-1 cells exposed to DNFB, HDI, or MESA are summarized in a Venn diagram (top left) that allows the rapid identification of phospholipid alterations that are unique or shared by the different chemical treatments used. The structure of the hydrophilic moieties of these phospholipids is shown (top right): PI, phosphatidylinositol; CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine. The ester bond substitution by alkyl- and alkenyl ether bonds characteristic of plasmalogen (e) and plasmalogen (p) lipids, respectively, observed in PC and PE is represented in small boxes. The hydrophobic moieties of altered lipids are fatty acids (Table 2) denoted by their number of carbons:number of unsaturations. To reduce the space occupied by the figure unsaturations were represented in the less frequently occurring trans configuration and more unsaturated fatty acids are represented together with their more saturated counterparts with different shadings in the additional double bonds. Several cellular processes (discussed in the main text) that are known to be simultaneously altered in dendritic cells exposed to chemicals and related to some of the mentioned phospholipids are highlighted (bottom).

crucial to DC maturation and allergic reactions such as the aforementioned regulation of TRPV1 channels (Petrosino et al., 2010; Cao et al., 2013), pattern recognition receptors (PRRs) signaling (van Bergenhenegouwen et al., 2013), vesicle trafficking (Van Meer et al., 2008), and CD86 and IL-8 expression (Corsini et al., 2014), among many others.

PI with m/z 885 was identified as both PI (16:0/22:4) and PI (18:0/20:4). However, if we consider the intensity of the fatty acid peaks in the MS/MS spectra (data not shown), the latter species prevails. It seems, therefore, that the tested chemicals have a tendency to increase arachidonic acid incorporation into PI species. Increases in arachidonic acid incorporation into PC and PI species were proposed as maturation markers in human primary monocytes stimulated with the toll-like receptor (TLR) two agonist zymosan, PMA and platelet activating factor (PAF) (Balgoma et al., 2010). Thus, our results may also be a reflection of the THP-1 maturation process. Our observations, however, are clearly distinct from the lipid alterations reported in THP-1 cells and in the murine epidermal DC cell line FSDC exposed to the TLR4 agonist lipopolysaccharide (LPS) (Santinha et al., 2012) or in HaCaT cells exposed to DNFB (Santinha et al., 2013). It is therefore evident that phospholipidome alterations are dependent on the maturation stimulus and cellular model used, and it is interesting to speculate as to what extent differences in the phagocytic activity of cells are related to lipidome changes and vice versa.

Despite the differences observed between cells exposed to MESA, DNFB, or HDI, several phospholipidome alterations were common to all these chemicals (Fig. 8). These observations evidence shared effects between allergens and irritants at the phospholipidome level. Not surprisingly, modest success was achieved in the identification of samples using a statistical model based on phospholipidomic data. Distinction between the two allergens studied, DNFB and HDI, and between the respiratory allergen HDI and the irritant MESA was not totally achieved. It is currently believed that the "irritancy" or "autoadjuvanticity" of chemicals and hence their capacity to generate danger-associated molecular patterns (DAMPs) is crucial to their ability to evoke allergic reactions (Martin, 2012). Indeed, a certain level of irritancy that translates into cytotoxicity was shown to be required for chemicals to trigger ACD (Hulette et al., 2005). Thus, although elicited immune responses are completely distinct, allergens and irritants may share unspecific cell cytotoxic mechanisms, with consequences at protein and also at lipid level.

Overall, our results indicate that, besides some common alterations, skin sensitizers, respiratory sensitizers, and irritants induce distinct phospholipidome profiles in DC. These distinct profiles can condition DC immunostimulatory abilities and, as a result, the characteristics of elicited immune responses, establishing a direct relationship between lipid alterations and the physiopathology of allergic reactions. As our knowledge on the real extension to which lipid alterations influence immune cell functions is scarce, further investigation on this new field is required.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

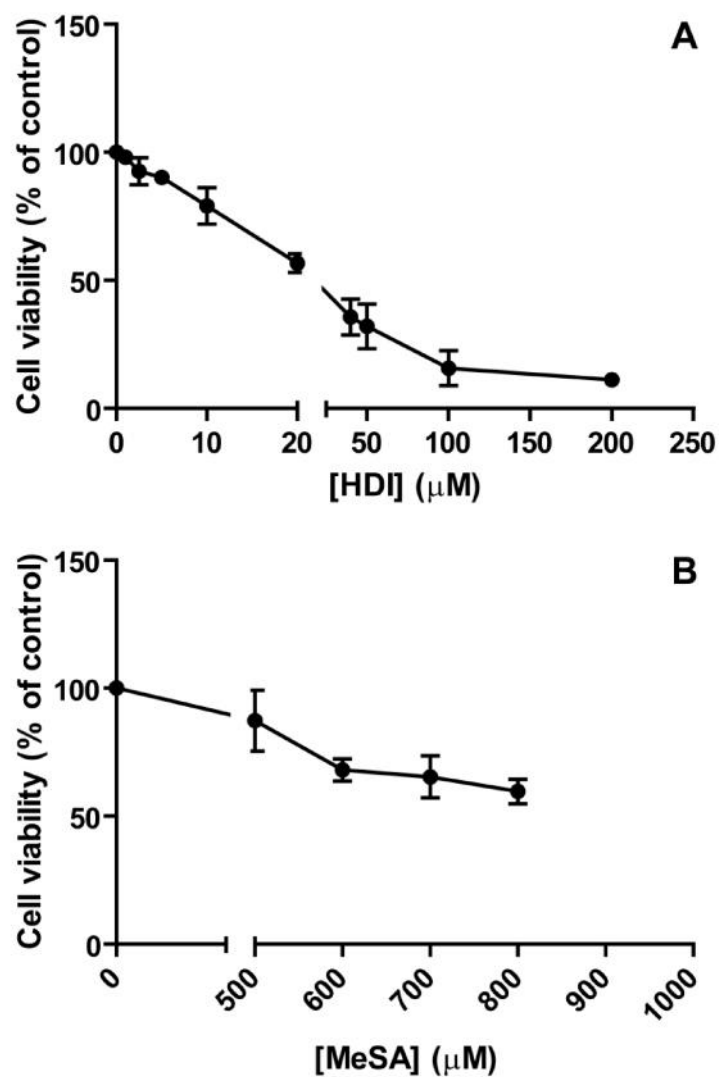


Figure S1: Viability of THP-1 cells exposed for 24h to different concentrations of hexamethylene diisocyanate (HDI) (A) and methyl salicylate (MeSA) (B) determined by the alamar blue assay. Mean $\pm$ standard deviation of the % cell viability relatively to untreated cells from 3 independent experiments is shown.

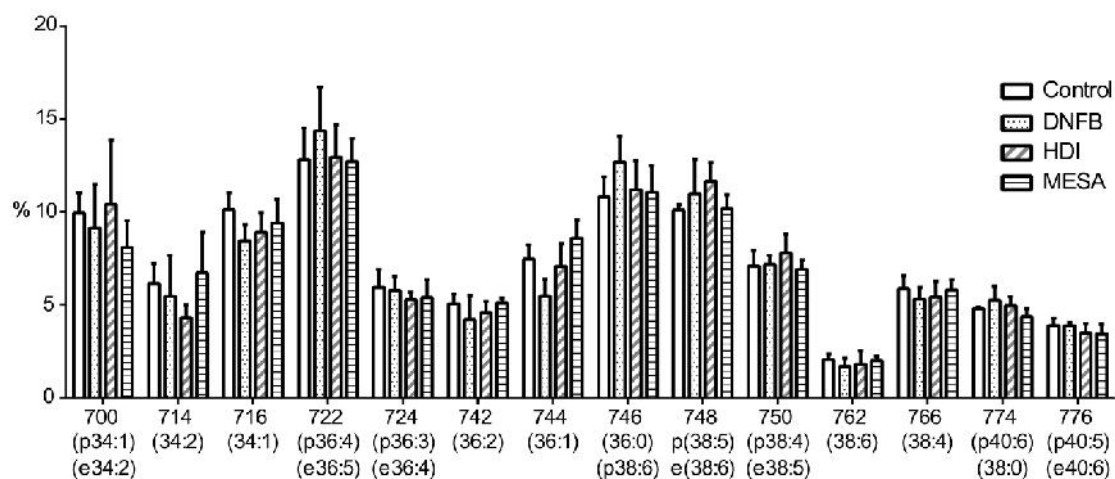


Figure S2: Relative abundance of $[M-H]^-$ ions of PE molecular species observed in the LC-ESI-MS spectra of untreated THP-1 cells (control) and cells exposed for 24h to 8 μ M DNFB , 11.5 μ M HDI or 600 μ M MESA. Values are expressed as mean $\pm$ standard deviation of 3 replicates. Between brackets are presented the PE molecular species corresponding to each $[M-H]^-$ ion. Plasmalogen species are noted with an 'e' and plasmalogen species with a "p". Statistical treatment was performed using two-way ANOVA followed by Dunnett's post-test for control versus DNFB, HDI and MESA.

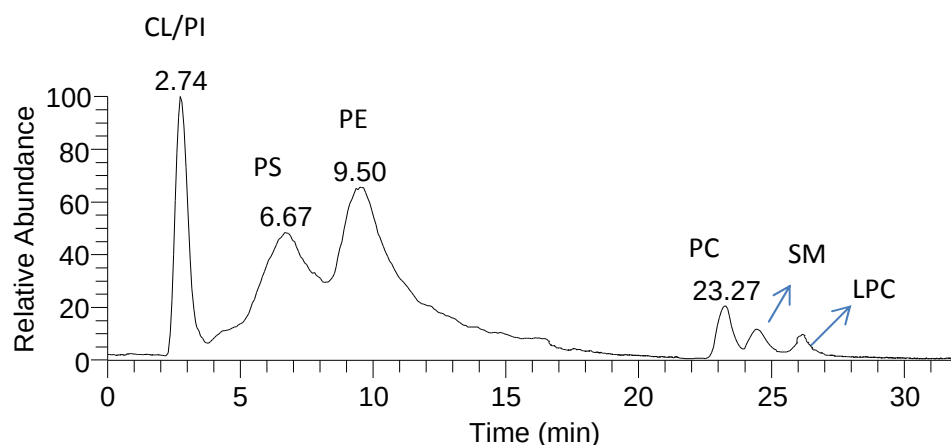


Figure S3: HILIC-ESI-MS chromatogram of a mixture of lipid standards obtained with running conditions similar to those used in the present study and that shows the temporal separation of the different lipid classes. The lipid standards used were: dimyristoyl phosphatidylcholine (DMPC) (PC), phosphatidylethanolamine (DMPE) (PE) and phosphatidylserine (DMPS) (PS); 1-tridecanoyl lysophosphatidylcholine (LPC); dipalmitoyl phosphatidylinositol (PI); tetramyristoyl cardiolipin (CL); and N-heptadecanoyl sphingomyelin (SM).