Group V Secreted Phospholipase A2 Is Upregulated by IL-4 in Human Macrophages and Mediates Phagocytosis via Hydrolysis of Ethanolamine Phospholipids

Julio M. Rubio,* Juan P. Rodríguez,*,‡ Luis Gil-de-Gómez,*, Carlos Guijas,*,† Maria A. Balboa,*,† and Jesús Balsinde*†

Studies on the heterogeneity and plasticity of macrophage populations led to the identification of two major polarization states: classically activated macrophages or M1, induced by IFN-γ plus LPS, and alternatively activated macrophages, induced by IL-4. We studied the expression of multiple phospholipase A2 enzymes in human macrophages and the effect that polarization of the cells has on their levels. At least 11 phospholipase A2 genes were found at significant levels in human macrophages, as detected by quantitative PCR. None of these exhibited marked changes after treating the cells with IFN-γ plus LPS. However, macrophage treatment with IL-4 led to strong upregulation of the secreted group V phospholipase A2 (sPLA2-V), both at the mRNA and protein levels. In parallel with increasing sPLA2-V expression levels, IL-4–treated macrophages exhibited increased phagocytosis of yeast-derived zymosan and bacteria, and we show that both events are causally related, because cells deficient in sPLA2-V exhibited decreased phagocytosis, and cells overexpressing the enzyme manifested higher rates of phagocytosis. Mass spectrometry analyses of lipid changes in the IL-4–treated macrophages suggest that ethanolamine lysophospholipid (LPE) is an sPLA2-V–derived product that may be involved in regulating phagocytosis. Cellular levels of LPE are selectively maintained by sPLA2-V. By supplementing sPLA2-V–deficient cells with LPE, phagocytosis of zymosan or bacteria was fully restored in IL-4–treated cells. Collectively, our results show that sPLA2-V is required for efficient phagocytosis by IL-4–treated human macrophages and provide evidence that sPLA2-V–derived LPE is involved in the process. The Journal of Immunology, 2015, 194: 3327–3339.

Phospholipase A2 (PLA2) enzymes hydrolyze membrane phospholipids at the sn-2 position of the glycerol backbone to release a free fatty acid and a lysophospholipid (1). This reaction is especially important when the fatty acid liberated is arachidonic acid (AA), which can be converted into biologically active compounds called eicosanoids (2, 3). Free fatty acids also may act as intracellular signalers on their own (4), and lysophospholipids may initiate signaling through cell surface G protein–coupled receptors (5). The PLA2 superfamily includes 16 groups of enzymes, and most of these groups contain several subgroups (1). From a functional point of view, PLA2s can be classified into five major classes: Ca2+-dependent cytosolic enzymes, Ca2+-dependent secreted enzymes, Ca2+-independent cytosolic enzymes, platelet-activating factor acetyl hydrolases, and lysosomal PLA2s. Of these classes, the first two have repeatedly been implicated in AA mobilization and eicosanoid production in response to stimuli of innate immunity and inflammation. The Ca2+-dependent cytosolic group IVA PLA2 (cPLA2α) appears to be the critical enzyme in this process and, depending on cell type and stimulus, a secreted Ca2+-dependent PLA2 may cooperate as well by amplifying the cPLA2α-regulated response (1–3, 6, 7).

Macrophages participate in innate immunity reactions by mediatiing opsonic (IgG- and complement-mediated) or nonopsonic phagocytosis (via pattern-recognition receptors) of invading microorganisms (8–11). Phagocytosis of foreign material is usually accompanied by the secretion of large amounts of cytokines and AA-derived eicosanoids that, as time passes, contribute to modulate the progress and extent of the inflammatory process. Depending on changes in the microenvironment where the macrophages exert their functions, these cells may exhibit a continuum of activation states, ranging from proinflammatory and antitumor activities to tissue repair and resolution of inflammation. These two poles of the spectrum of states of macrophage activation are termed classic (M1) and alternative (M2) and are thought to play opposing roles during innate immune and inflammatory responses (12–15). Alternative activation of the macrophages induced by Th2 cytokines IL-4 and IL-13 is of particular interest because, in addition to modulating inflammatory repair, it may be implicated in a variety of unforeseen functions, such as glucose homeostasis (16), thermogenesis (17), and learning and memory (18). Alternative macrophage activation is...
sPLA2-V REGULATES PHAGOCYTOSIS IN IL-4–TREATED MACROPHAGES

Known to enhance the expression of a number of phagocytic receptors, such as MRC-1, and dectin-1, which results in increased endocytosis of mannosylated ligands and other receptor-mediated endocytic processes. These effects are likely intended to ameliorate inflammation via rapid clearance of foreign material and are clearly different from those induced by classic M1 activators, such as IFN-γ with or without LPS (14, 19, 20).

In previous work, we investigated the mechanisms of PL-A2-mediated phospholipid turnover in monocytes and macrophages responding to a variety of stimuli of innate immunity and inflammation (21–30). In these studies we took advantage of mass spectrometry– based lipidomic approaches to define, at a molecular species level, the phospholipid substrate specificities of the enzymes involved. In this study, we applied a similar approach to establish changes in the lipidome of human macrophages after polarization/activation to the M1 and M2 states and to relate such changes to the expression of particular PL-A2 forms in each of these states. Our goal was to define molecular “fingerprints” of each macrophage state that permit identification of specific traits of the immune response in terms of the lipid metabolic pathways involved. We show that, in human monocyte-derived macrophages, secreted group V PLA2 (sPLA2-V) is strongly upregulated by IL-4 but not by IFN-γ plus LPS. Thus, sPLA2-V constitutes a bona fide marker for human alternatively activated macrophages. We further show that the increased expression of sPLA2-V in IL-4–treated macrophages serves to regulate the cellular levels of ethanolamine lysophospholipids (LPEs) that are necessary to support the elevated phagocytic response that these cells exhibit.

Materials and Methods

Reagents

Ficoll-Paque Plus was purchased from GE Healthcare (Uppsala, Sweden). Gentamicin was from BioWhittaker (Walkersville, MD). The Amaxa Human Macrophage Nucleofector Kit was from Lonza (Basel, Switzerland). Macrophage serum-free medium and RPMI 1640 were from Life Technologies (Carlsbad, CA). Silencer Select small interfering RNAs (siRNAs) to decrease expression of human sPLA2-V mRNA and negative controls were from Ambion (Carlsbad, CA). IFN-γ and IL-4 were from ImmunoTools (Friesoythe, Germany). The plasmid containing human sPLA2-V (31, 32) was generously provided by Dr. Yasuhito Shirai (Kobe University, Kobe, Japan). Zymosan A labeled with Alexa Fluor 594 was from Molecular Probes (Carlsbad, CA). LPE (1-O-1,2-0ctadecenyl-sn-glycero-3-phosphoethanolamine) was from Avanti (Alabaster, AL). Oligonucleotides were from Eurofins MWG Operon (Hamburg, Germany). The sequences were obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank/).

Cells

Human monocytes were obtained from pooled buffy coats of healthy male volunteer donors from the Centro de Hemoterapia y Hemodonación de Castilla y León (Valladolid, Spain). Blood cells were diluted 1:1 with PBS, layered over a cushion of Ficoll-Paque, and centrifugated at 750 × g for 30 min. The mononuclear cell layer was recovered and washed three times with PBS, resuspended in RPMI 1640 supplemented with 2 mM t-glutamine and 40 μg/ml gentamicin, and allowed to adhere to plastic in sterile dishes for 2 h at 37°C. Nonadherent cells were removed by extensive washing with PBS. Cells of cells corresponding to five donors were used in all experiments, and replicates were performed with different pools of cells. Macrophage differentiation (95% monocytes) was achieved by incubating the adhered monocytes in RPMI 1640 supplemented with 2 mM L-glutamine and 40 μg/ml gentamicin, and 80 μM Na3VO4, 150 mM NaF, 1 mM PMSF, and 10 μM M pyrrophenone to completely inhibit cytosolic group IVA PLA2 activity (43) and 1 μM pyrrophene to completely inhibit cytosolic group IVA PLA2 activity (44). After lipid extraction, free [3H]AA was separated by thin-layer chromatography, using n-hexane/ethyl acetate/ethanol (70:30:1) as a mobile phase. These assay conditions were validated previously with regard to time, homogenate protein, and substrate concentration (43–47).

Phagocytosis

For these experiments, opsonized zymosan, labeled with the fluorophore Alexa Fluor 594, or opsonized Escherichia coli DH5α bacteria (American Type Culture Collection), transformed with an orange fluorescent protein plasmid (pmoOrange; Clontech, Mountain View, CA), was used as a stimuli. The zymosan particles and bacteria were opsonized with incubation with human serum for 20 min at 37°C, at a ratio of 1 ml serum: 3 mg particles or 9.5 × 10⁷ CFU (48). Zymosan was washed three times with PBS and sonicated in RPMI 1640 or HBSS for 15 min.

Macrophages were seeded over glass coverslips, allowed to adhere, washed with RPMI 1640, and resuspended in this medium. Cells were kept at 4°C for 5 min, and opsonized zymosan (five to seven particles/cell) or opsonized bacteria (10 bacteria/cell) was added. After a 15-min incubation at 37°C, coverslips were washed with PBS and transferred to plates with RPMI 1640 at 37°C, and phagocytosis was allowed to proceed for 15–30 min. Reactions were stopped by fixation with 4% paraformaldehyde and 3% sucrose for 15 min; paraformaldehyde was removed by washing the cells three times with PBS, DAPI staining was carried out by treating cells with this dye at a concentration of 1 μg/ml in PBS for 10 min. Coverslips were mounted on microscopy slides with 10 μl a polyvinyl alcohol solution until analysis by fluorescence microscopy. A Leica TCS SP5 X confocal microscope with white laser (470–670 nm) (Leica Microsystems, Wetzlar, Germany) was used for these studies. Images were analyzed with LAS AF v. 2.6.3 (Leica). The phagocytic index was calculated by dividing the number of phagosomes by the total number of cells in a field, which was multiplied by the percentage of phagocytosing cells, as described elsewhere (49, 50).

Immunoblot

The cells were lysed with 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 0.5% Triton X-100, 1 mM Na3VO4, 150 mM NaF, 1 mM PMSF, and a protease inhibitor mixture (Sigma) at 4°C. Homogenates were centrifuged at 12,000 rpm for 10 min. Protein from the supernatants was quantified by using a Bradford protein assay kit (Bio-Rad), and 75 μg total protein was separated by standard 15% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) using a Trans-Blot SD semidy transfer system (Bio-Rad) for 20 min and 2.5
mA/cm². Membranes were incubated with the corresponding primary Abs. Mouse anti–PLA 2-V IgF (C-4; sc-393606; Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:100, and mouse anti-b-actin IgG (GE Healthcare) was used at 1:20,000, followed by HRP-conjugated anti-IgG mouse secondary Abs (GE Healthcare). Immunoreactive bands were detected by ECL (Amersham Biosciences, Piscataway, NJ) using Amersham Hyperfilm ECL (GE Healthcare, Amersham, Buckinghamshire, U.K.) and were digitalized with a GS-800 Scanner (Bio-Rad). The resulting digital images were analyzed for quantitative band densitometry at different time points within the linear response defined by Quantity One software (version 4.5.2; Bio-Rad).

Liquid chromatography/mass spectrometry analyses of macrophage glycerolphospholipids

A cell extract corresponding to 10⁷ cells was used for these analyses. The following internal standards were added—600 pmol each 1,2-dipentadecanoyl-sn-glycerol-3-phosphocholine, 1,2-dilauroyl-sn-glycerol-3-phosphoethanolamine, and 1,2-dipalmitoyl-sn-glycerol-3-phosphoinositol—before lipid extraction, according to the method of Bligh and Dyer (51). After evaporation of the organic solvent under vacuum, the lipids were redissolved in 100 μl methanol/water (9:1, v/v) and injected into a high-performance liquid chromatograph equipped with a binary pump Hitachi LaChrom Elite L-2130 and a Hitachi Autosampler L-2200 (Merck). The column was a SUPELCOSIL LC-18 (5-μm particle size, 250 × 2.1 mm) protected by a SUPELCOSIL LC-18 Supelguard (20 × 2.1 mm) Cartridge (both from Sigma-Aldrich). Mobile phase was a gradient of solvent A (methanol/water/hexane/52% ammonium hydroxide, 87.5:10.5:1:5.0, by volume) and solvent B (methanol/hexane/32% ammonium hydroxide, 87.5:12.0:5.0, by volume). The gradient was started at 100% solvent A; it was decreased linearly to 65% solvent A, 35% solvent B in 20 min, to 10% solvent A, 90% solvent B in 5 min, and to 0% solvent A, 100% solvent B in an additional 5 min. Flow rate was 0.5 ml/min, and 80 μl lipid extract was injected. The liquid chromatography system was coupled online to a Bruker esquire6000 quadrupole time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany). The total flow rate into the column was split, and 0.2 ml/min entered the electrospray interface of the mass spectrometer. Nebulizer gas was set to 30 pounds per square inch, dry gas was set to 8 l/min, and dry temperature was set to 380°C. Ethanolamine and inositol lysophospholipids were detected in negative ion mode with the capillary current set at −3500 V as [M-H]⁻ ions. Choline glycerolphospholipid (PC) species were detected over the elution interval from 25 to 35 min in positive ion mode, as [M+H+]⁺ ions, with the capillary current set at −3500 V.

PE and PI molecular species were identified by multiple reaction monitoring experiments on chromatographic effluent by comparison with previously published data (24, 26–30, 52–54). Cutoff parameter was set at m/z 150, and fragmentation amplitude was set at one arbitrary unit. Because of the lability of vinyl ether linkages in acid media, plasmalogen (1-alkyl) and plasmalogen (1-alk-1’-enyl) glycerophospholipids were distinguished by acidifying the samples before lipid extraction. For the identification of acyl chains of PC species, ionization was carried out in negative mode with postcolumn addition of acetic acid at a flow rate of 100 μl/h as [M+CH₂COO⁻]⁻ adducts. Stereospecific assignment of fatty acyl chains was carried out by comparing the relative intensities of the 1-lysophospholipid and 2-lyso phospholipid compounds arising in the fragmentation experiments (the signal of the latter predominates over that of the former in ion-trap mass spectrometry) (24, 26–30, 52–54).

Gas chromatography/mass spectrometry analysis of fatty acid methyl esters

After incubations, the cells were washed twice with PBS, and a cell extract corresponding to 10⁷ cells was scraped in ice-cold water and sonicated in a tip homogenizer twice for 15 s. Before extraction and separation of lipid classes, internal standards were added. For total phospholipids, 10 nmol 1,2-diheptadecanoyl-sn-glycerol-3-phosphocholine was added; for triacyl-glycerolphospholipids, 10 nmol 1,2-diheptadecanoyl-sn-glycerol-3-phosphoethanolamine, 20 nmol cholesteryl erucate was added. Total lipids were extracted according to Bligh and Dyer (51), and the resulting lipid extract was separated by thin-layer chromatography using n-hexane/diethyl ether/acetetic acid (70:30:1, by volume) as the mobile phase. Spots corresponding to phospholipids were detected under ultraviolet light (254 nm) and scraped in ice-cold water and sonicated in 100 μl methanol (2:1, v/v) for 1 min. Phospholipids were transmethylelated with 500 μl 0.5 M KOH in methanol for 30 min at 37°C, and 500 μl 0.5 M HCl was added to neutralize. Extraction of fatty acid methyl esters was carried out in 1 ml n-hexane twice. Analysis of fatty acid methyl esters was carried out in a Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass-selective detector operated in electron impact mode (70 eV) equipped with an Agilent 7693 Autosampler and an Agilent DB23 column (60 μm length × 250 μm internal diameter × 0.15 μm film thickness) under the conditions described previously (35, 53). Data analysis was carried out with Agilent G1701EA MSD Productivity Chemstation software, revision E.02.00.

Liquid chromatography/mass spectrometry analyses of eicosanoids

A small amount of butylated hydroxytoluene in methanol (0.01%, w/v) was added to the supernatants to prevent eicosanoid degradation. Deuterated PG E₂ and leukotriene B₄ (200 pmol each) were added as internal standards before lipid extraction. Eicosanoids were separated by high-performance liquid chromatography system was coupled online to a Bruker esquire6000 quadrupole time-of-flight mass spectrometer equipped with an Agilent G1311C quaternary pump and an Agilent G1329B Autosampler. The column was a SUPELCOSIL LC-18 (250 × 2.1 mm, 5-μm particle size) protected by a LC-18 Supelguard (20 × 2.1 mm) Cartridge (Sigma-Aldrich). The mobile phase consisted of a gradient of solvent A (water/acetonitrile/acetetic acid, 70:30:0.2, by volume) and solvent B (acetonitrile/isopropanol, 50:50, by volume). The gradient was started at 100% solvent A, which was decreased linearly to 75% at 3 min, 55% at 11 min, 40% at 13 min, 25% at 18 min, and 10% at 18.5 min. The last solvent mixture was held for an additional 1.5-min period; finally, the column was re-equilibrated with 100% solvent A for 10 min before the next sample injection (30). The flow rate through the column was fixed at 0.6 ml/min, and this flow entered into the electrospray interface of an API2000 triple quadrupole mass spectrometer (Applied Biosystems). The parameters of the source were set as follows: ion spray voltage, −4500 V; curtain gas, 25 pounds per square inch; nebulizer gas, 40 pounds per square inch; de- solvation gas, 80 pounds per square inch; and desolvation gas temperature, 525°C. The analyzer mode was set to scheduled multiple-reaction monitoring with negative ionization, defining for each analyte the m/z of the parent ion as Q1 mass and the m/z of its daughter ion fragment (transition) as Q3 mass and associating with the chromatographic retention time to improve the number of analytes collected in a single chromatographic run. The retention time window was set to 120 s. The declustering potential and collision energy for each analyte were optimized by the use of analytical standards. Other parameters were fixed for all analytes: entrance potential, −10 V; focusing potential, −350 V; and collision cell exit potential, −10 V. Quantification was carried out by integrating the chromatographic peaks of each species and comparing with an external calibration curve made with analytical standards (30).

Liquid chromatography/mass spectrometry analyses of lysophospholipids

A cell extract corresponding to 10⁷ cells was used for these analyses. The following internal standards were added—200 pmol each 1-tridecanoyl-sn-glycerol-3-phosphocholine and 1-mirosteryl-sn-glycerol-3-phosphoethanolamine—before lysophospholipid extraction with n-butanol. After evaporation of the organic solvent under vacuum, the lipids were redissolved in 100 μl methanol/water (9:1, v/v) and injected into a high-performance liquid chromatograph equipped with a binary pump Hitachi LaChrom Elite L-2130 and a Hitachi Autosampler L-2200 (Merck). The column was a Supelcosil LC-Si (150 × 3 mm, 3 μm particle size) protected by a Supelguard LC-Si guard cartridge (Sigma-Aldrich). Mobile phase was a gradient of solvent A (chloroform/methanol/water/32% ammonium hydroxide, 75:24:5:0.5, by volume) and solvent B (chloroform/methanol/water/10% ammonium hydroxide, 55:39:5:5.5, by volume). The gradient was started at 100% solvent A; it was decreased linearly to 50% solvent A in 2 min, it was maintained for 8 min, and finally it was decreased to 0% solvent A in 2 min. Flow rate was 0.5 ml/min, and 50 μl the lipid extract was injected. The liquid chromatography system was coupled online to a Bruker esquire6000 ion-trap mass spectrometer (Bruker Daltonics). The trap was refreshed with the nitrogen gas at 3.5 l/min, and 0.2 μl/min entered into the electrospray interface of the mass spectrometer. Nebulizer gas was set to 30 pounds per square inch, dry gas was set to 8 l/min, and dry temperature was set to 365°C. Ethanolamine and inositol lysophospholipids were detected in negative ion mode with the capillary current set at +3500 V as...
[M-H]− ions. Choline lysophospholipids (LPCs) were detected in positive ion mode as [M+H]+ ions, with the capillary current set at −4000 V. Measurements correspond to the intensity of each species divided by the intensity of the internal standards corresponding to that particular headgroup. No internal standards were available for the lysocephatidylinositol (LPI) subclass, so the intensity of each species was divided by the total LPI intensity in this case. The amount of internal standard added to each sample was always identical. The relative intensity values were normalized to the measured quantity of protein present in each cell, so the intensity of each species was divided by the intensity of the internal standards corresponding to that particular headgroup. No internal standards were available for the lysophosphatidylinositol subclass, so the intensity of each species was divided by the total LPI intensity in this case. The amount of internal standard added to each sample was always identical. The relative intensity values were normalized to the measured quantity of protein present in each cell preparation following treatment or not with IL-4 for each condition.

Results
Expression of PLA2 forms in human macrophages

Cells involved in inflammatory reactions are known to express multiple PLA2 forms. Thus, the challenge is to delineate the role that each of these enzymes plays in cell functioning. We began the current study by determining, by qPCR, the expression level of PLA2 genes (Table I). Of these, only the PLA2-V forms were expressed at significant levels by the cells (fluorescence signal in reactions with specific primers detected below 50 cycles): PLA2-IID, -IVA, -IVB, -IVC, -VIA, -VIB, -VIC, -VID, -VIE, -VIF, -XIIA, -XV, and -XVI (Supplemental Fig. 1A). In the next series of experiments, we treated the macrophages with LPS/IFN-γ or IL-4 to induce polarization/activation of the macrophages to M1 or M2 phenotypes, respectively, and changes in the expression levels of the various PLA2s were studied. Control measurements indicated that these treatments induced the expected macrophage polarizations to either M1 or M2 phenotypes, as assessed by specific marker analysis (TNF-α, IL-12α, IL-12β, and IL-6 for M1; ARG1, MRC1, and CCL13 for M2) (Fig. 1). Treating the cells with LPS/IFN-γ induced little or no change in the levels of the various PLA2s expressed by the cells (Supplemental Fig. 1B). Unexpectedly, IL-4 induced a strong increase in the expression level of sPLA2-V (150-fold increase at 24 h) (Supplemental Fig. 1B). This is a surprising finding because, in murine macrophages, sPLA2-V was demonstrated to be upregulated by LPS (55–59) and to function primarily to exacerbate inflammatory reactions (60). Immunoblot analyses of sPLA2-V

Table I. Oligonucleotide primers used for detection of PLA2 genes from human macrophage cDNA

<table>
<thead>
<tr>
<th>PLA2 Group</th>
<th>Primer Sequence (5′→3′)</th>
<th>Amplification Size (bp)</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA2-IIB</td>
<td>Sense</td>
<td>TGGCAGACACATGACAACTG</td>
<td>97</td>
</tr>
<tr>
<td>PLA2-IIA</td>
<td>Sense</td>
<td>ACAGATATGGAATGTTGTTG</td>
<td>110</td>
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<tr>
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<td>Sense</td>
<td>ATGAGACCTTCTCGTTGTG</td>
<td>110</td>
</tr>
<tr>
<td>PLA2-IIE</td>
<td>Sense</td>
<td>CGAGCGAACCAGAAAAGA</td>
<td>68</td>
</tr>
<tr>
<td>PLA2-IIF</td>
<td>Sense</td>
<td>TGGCAGTGAGCCCAAAGA</td>
<td>68</td>
</tr>
<tr>
<td>PLA2-IIH</td>
<td>Sense</td>
<td>GCCGCCACAAAAAAATG</td>
<td>122</td>
</tr>
<tr>
<td>PLA2-IIJ</td>
<td>Sense</td>
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</tr>
<tr>
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<td>Sense</td>
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<tr>
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content in homogenates from IL-4–treated cells indicated a 5–6-fold increase in the expression of the protein at 24 h (Fig. 2A). To assess whether the effect of IL-4 on sPLA2-V was stimulus specific or also was observed with other model stimuli of M2 polarization, we tested the effects of M-CSF and IL-10. The results (Fig. 2B, 2C) indicated that these two stimuli increased sPLA2-V protein expression to levels similar to those found with IL-4, suggesting that elevated expression of sPLA2-V is a general feature of the M2 phenotype.

**sPLA2-V regulates phagocytosis in IL-4–treated cells**

In agreement with previous observations (13, 14), treating the macrophages with IL-4 increases their capacity to phagocytose yeast-derived zymosan particles. A possible involvement of sPLA2-V in this response was investigated initially by inhibiting expression of the enzyme by siRNA. Using this technology, an ~90% inhibition of sPLA2-V mRNA levels was achieved in IL-4–treated cells, as judged by qPCR (Supplemental Fig. 2A), and in vitro activity assays confirmed a decrease of similar magnitude in total cellular sPLA2 activity, as measured using a natural membrane–based assay (Supplemental Fig. 2B). Importantly, the sPLA2-V–deficient cells exhibited a marked decrease in their ability to phagocytose zymosan particles after IL-4 treatment (Fig. 3A).

To complement the above data, in the next series of experiments we prepared macrophages overexpressing a plasmid containing human sPLA2-V. The cells showed a 15–20-fold increase in mRNA for this enzyme at 24 h, as measured by qPCR.
blots were quantified from three different experiments (mean ± SEM), and the quantifications are shown (lower panels).
decreases in any of the other species measured, either of the LPC or LPI class, highlighting a specific effect on a particular class of phospholipids. Because the levels of lysophospholipids in activated cells represent a balance between the opposing actions of activated PLA2s versus activation of CoA-dependent reacylation and CoA-independent transacylation reactions (2, 3), the significant decrease in LPE in sPLA2-deficient cells constitutes unambiguous evidence that the levels of these particular phospholipid species are maintained by sPLA2-V during IL-4 stimulation of the macrophages. By inference, the data suggest that sPLA2-V-mediated PE hydrolysis leading to LPE formation may constitute an important step in IL-4–induced events in human macrophages.

FIGURE 3. sPLA2-V is involved in the phagocytosis of zymosan by IL-4–treated macrophages. Human macrophages, either left untreated or treated with 1000 U/ml IL-4 for 24 h, as indicated, were analyzed for phagocytosis of fluorescent zymosan particles by confocal microscopy (red, middle columns). (A) Effect of sPLA2-V depletion by siRNA. The cells were treated with siRNA control or siRNA for sPLA2-V, as indicated. (B) Effect of sPLA-V overexpression. The cells were transfected with an empty plasmid (control) or a plasmid containing human sPLA2-V, as indicated. DAPI (1 μg/ml) was used to mark the nuclei (blue; left columns). Nomarski images are also shown (right columns). The average of three independent experiments with triplicate determinations (mean ± SEM) are shown (bottom panels). Original magnification ×40.
Exogenous LPE restores phagocytosis in sPLA₂-V–deficient cells

The above results would be compatible with the possibility that LPE acts as a lipid metabolite that mediates some of the actions of sPLA₂-V during stimulation of the cells with IL-4. To examine this possibility, we designed a phagocytosis experiment to assess whether exogenous supplementation of LPE to cells lacking sPLA₂-V restores phagocytosis. After the siRNA treatments aimed at blocking sPLA₂-V expression, the cells, treated or not with IL-4, were exposed to LPE before phagocytosis was measured; LPC and LPI were used as controls. The lysophospholipids were used at 5 μM, a concentration well below their critical micellar...
achieved by IL-4, as long as the higher levels of sPLA2-V are mined by liquid chromatography/mass spectrometry. Data are mean ± SEM of three independent experiments with duplicate determinations. 14,15-DHET, 15-hydroxyeicosatetraenoic acid; TXB2, thromboxane B2.

To make our study more physiologically relevant, we also used a more physiological model of phagocytosis: bacteria. We used E. coli expressing orange fluorescent protein, which allowed monitoring of phagocytosis by confocal microscopy. The results (Fig. 8) were wholly comparable to those found for zymosan phagocytosis (Fig. 3); E. coli ingestion by IL-4-treated macrophages was markedly diminished by sPLA2-V depletion, and addition of LPE restored the response.

Discussion

Much evidence has been obtained in recent years to support a role for sPLA2-V in regulating innate immune responses. sPLA2-V is present in the secretory granules of WBCs and macrophages, and it is released in response to a large number of innate immune stimuli (60, 61). Part of the enzyme that has been secreted to the extracellular medium may reassociate with the secreting cell and/or be interiorized via several mechanisms to exert its function in an intracellular compartment. Most results on the role of sPLA2-V in pathophysiology have come from studies in mice, and the availability of the sPLA2-V knockout mouse model has provided valuable insights (66, 67). The full eicosanoid response of murine peritoneal macrophages and mast cells to innate immunity stimuli appears to depend on sPLA2-V, which acts to amplify the cPLA2α-initiated response (66–68). In these cells, the enzyme translocates to the phagosome after ingestion of zymosan and regulates phagocytosis by mechanisms that may or may not depend on eicosanoid synthesis, and macrophages from sPLA2-V-null mice exhibit impaired phagocytosis and killing of fungal particles and bacterial clearance (50, 69).

In contrast with the wealth of data in murine cells, little is known about the biological functioning of sPLA2-V in human cells. Studies by Rubin and coworkers (70) demonstrated that the enzyme is secreted by neutrophils and participates in the killing of Gram-negative bacteria, with a limited role in eicosanoid production. Other studies showed that sPLA2-V triggers leukotriene biosynthesis by human neutrophils through activating cPLA2α (71). However, in human eosinophils, the enzyme was reported to directly hydrolyze phospholipids at the plasma membrane and later at the nuclear envelope in close proximity to eicosanoid biosynthetic enzymes (72). In this study, we sought to determine whether the role of sPLA2-V in innate immunity extends to regulating human macrophage polarization to either M1 or M2 phenotypes. Our data provide evidence that sPLA2-V is important for zymosan phagocytosis by human macrophages and that the regulatory role that the enzyme plays in this process involves generation of LPE, which is produced by the hydrolysis of membrane PE. We found that, in human macrophages, in contrast to murine macrophages, sPLA2-V is not upregulated by cell exposure to the proinflammatory stimuli IFN-γ plus LPS, which polarize the cell to a proinflammatory M1 phenotype. Instead, sPLA2-V is strongly upregulated by IL-4, which polarizes macrophages to an anti-inflammatory M2 state. Although these data could suggest that the biological functioning of sPLA2-V in humans is fundamentally different from that in murine cells, we note that, in both instances, the regulation of phagocytosis by sPLA2-V in human and murine cells might lead to similar outcomes (sPLA2-V regulating phagocytosis in a “positive manner,” in that its absence slows phagocytosis). The anti-inflammatory implications of this type of regulation in human cells exposed to IL-4 would be evident, because rapid elimination of foreign material by phagocytosis should accelerate repair mechanisms and the return to homeostasis. A similar purpose could be served as well under classical activation of murine macrophages, because rapid clearance of foreign material would also help to limit the
deleterious effects of inflammation. Thus, in murine, but not in human, cells, sPLA₂-V could function as a bifaceted enzyme, augmenting the early stages of acute inflammation (50, 66) and, in contrast, accelerating the clearance of pathogens (69, 73). A recent report showed that sPLA₂-V expression also can be upregulated by IL-4 in murine lung cells (74).

We found no evidence for such a functional plasticity of sPLA₂-V in human cells, where the enzyme appears to serve an anti-inflammatory role by regulating the clearance of phagocytosed material. In this regard, it is striking that sPLA2-V translocates to the forming phagosome in zymosan-stimulated murine macrophages (50, 66) but not in zymosan-stimulated human macrophages (33, 34). These data suggest that, at least in humans, the regulatory actions of the enzyme on the phagocytosis process itself occur at a level distinct from that of the phagosome, probably at the plasma membrane level. From that location, the enzyme could promote membrane modifications via phospholipid hydrolysis and the corresponding accumulation of lysophospholipids that allow lateral movement of phagocytic receptors and/or regulatory components. A scenario such as this would be fully consistent with the large body of literature indicating that, after secretion of sPLA₂-V to the extracellular medium, the enzyme reassociates with the outer leaflet of the plasma membrane to hydrolyze phospholipids and, in this manner, regulates specific cellular responses (60–62). Thus, sPLA₂-V may act in an autocrine or paracrine fashion at different subcellular locations in the cell, depending on cell type and the nature of the activating stimulus.

Formation of LPE in IL-4–treated human macrophages, dependent on sPLA₂-V, is the molecular event that we identify in this study as key in the regulation of phagocytosis of both yeast-derived zymosan particles and bacteria, because addition of exogenous LPE fully restores phagocytosis in sPLA₂-V–deficient cells. Lysophospholipids have been observed to induce a wide array of effects in a cell-specific manner. Although many of these effects have been attributed to interaction with surface receptors, a number of receptor-independent effects also have been appreciated: partitioning into the lipid bilayer and altering the properties of cell membranes or directly binding to nonreceptor proteins, such as ion channels (65). The latter are of special relevance for LPE because, unlike other lysophospholipids, specific receptors for LPE have not been described (5). Pertinent to the results of this

FIGURE 7. LPE restores phagocytosis of zymosan particles in IL-4–treated, sPLA₂-V–deficient cells. Human macrophages, either untreated or treated with 1000 U/ml IL-4 for 24 h, as indicated, were analyzed for phagocytosis of fluorescent zymosan particles by confocal microscopy (red color, middle columns). The cells were treated with siRNA control or siRNA for sPLA₂-V, as indicated. LPE (5 μM) was added, where indicated. DAPI (1 μg/ml) was used to mark the nuclei (blue; left columns). Nomarski images are also shown (right columns). The average of three independent experiments with triplicate determinations is shown (mean ± SEM) (bottom panel). Original magnification ×20. **p < 0.01.
study, recent studies in neutrophils showed that LPE can induce Ca\textsuperscript{2+}-mediated signaling in neutrophils in a manner that involves participation of the G2A receptor. Importantly, the lysophospholipid effects reported were not due to interaction with the receptor but, rather, occurred via alteration of the structure of the cell membrane (75). In analogy with these results, we speculate that accumulation of LPE, due to increased sPLA\textsubscript{2}-V activity, may favor oligomerization/interaction of phagocytic receptors at the plasma membrane that enables efficient subsequent signaling. Further, LPE could regulate signaling by altering the structure and fluidity of a variety of microdomains, including lipid rafts. These are specialized microdomains of the plasma that act as docking platforms for receptors and signaling effectors to interact to initiate intracellular responses (76). Interestingly, a variety of receptors that may mediate phagocytosis have been localized to lipid rafts (77–79). Because lipid rafts are enriched in cholesterol and sphingomyelin, as well as in ethanolamine plasmalogen (80, 81), accumulation of LPE at these particular microdomains is a possibility that deserves further consideration. Alternatively, the possibility that loss of function in sPLA\textsubscript{2}-V–depleted macrophages may, in part, be unrelated to lysophospholipids cannot be ruled out if the enzyme is also exerting noncatalytical functions in the cells. In this regard, recent data showed that cPLA\textsubscript{2}\textsubscript{a}, the first rate-limiting enzyme for eicosanoid synthesis, was found to translocate to the phagosome to regulate phagocytosis at various steps, some of which may depend on eicosanoid synthesis, whereas others may not (49, 82).

The process of phagocytosis is accompanied by the rapid generation of AA-derived eicosanoids that promote acute inflammatory responses (83) and may even act to regulate the phagocytic process itself, although the mechanisms involved have not been clearly established. In this regard, cPLA\textsubscript{2}\textsubscript{a}, the first rate-limiting enzyme for eicosanoid synthesis, was found to translocate to the phagosome to regulate phagocytosis at various steps, some of which may depend on eicosanoid synthesis, whereas others may not (49, 82). We detected a rather modest production of eicosanoids in IL-4–treated cells that did not change whether sPLA\textsubscript{2}-V–deficient or normal cells were used. Although cPLA\textsubscript{2}\textsubscript{a} does translocate to the phagosome in human macrophages (33, 34), the lack of involvement of eicosanoids in regulating phagocytosis in human macrophages could constitute another striking difference between human and murine systems, in the light of previous studies showing that alveolar macrophages from 5-lipoxygenase–

**FIGURE 8.** LPE restores phagocytosis of bacteria in IL-4–treated, sPLA\textsubscript{2}-V–deficient cells. Human macrophages, either untreated or treated with 1000 U/ml IL-4 for 24 h, as indicated, were analyzed for phagocytosis of fluorescent bacteria by confocal microscopy (red color, middle columns). The cells were treated with siRNA control or siRNA for sPLA\textsubscript{2}-V, as indicated. LPE (5 μM) was added, where indicated. DAPI (1 μg/ml) was used to mark the nuclei (blue; left columns). Nomarski images are also shown (right columns). The average of three independent experiments with triplicate determinations is shown (mean ± SEM) (bottom panel). Original magnification ×40. **p < 0.01.
null mice have impaired phagocytosis and killing of bacteria (83). However, it should be noted that these effects only were observed for phagocytosis of IgG-opsonized bacteria and not for complement-coated or unopsonized bacteria (83), suggesting that the regulation of phagocytosis by eicosanoids takes place only under specific conditions.

In summary, we provide novel data to indicate that sPLA2-V is required for efficient phagocytosis of zymosan particles and bacteria by IL-4-treated human macrophages and offer evidence that this requirement may involve generation of LPE, likely at the plasma membrane. In addition to its multiple roles in innate immunity and inflammation, sPLA2-V was suggested to participate in regulating arachidonic acid mobilization by IL-4-treated human macrophages and offer evidence that sPLA2-IIA that also can block, at least in part, sPLA2-V, could compromise innate immune responses to microorganism infection and, hence, delay resolution of inflammation.

Acknowledgments
We thank Monse Duque and Yolanda Noriega for expert technical help. Centro de Investigación Biomédica en Red de Diabetes e Enfermedades Metabólicas Asociadas is an initiative of Instituto de Salud Carlos III.

Disclosures
The authors have no financial conflicts of interest.

References


**Supplemental Figure 1** - A) Expression of PLA$_2$ forms in human macrophages, as determined by qPCR. B) Effect of a 24-h treatment with 500 U/ml IFN$_\gamma$ plus 10 ng/ml LPS (green bars) or 1,000 U/ml IL-4 (orange bars) on the expression level of PLA$_2$ forms, as determined by qPCR. Data are average of three independent experiments with triplicate determinations (mean ± S.E.M.).
**Supplemental Figure 2**  
A) qPCR analysis of sPLA₂-V levels. Left panel: cells were transfected with control siRNA (closed bar) or siRNA against sPLA₂-V (open bar), and after a 24-h treatment with 1,000 U/ml IL-4, the levels of mRNA expression for sPLA₂-V were analyzed by qPCR. Right panel: mRNA expression levels for sPLA₂-V were analyzed in macrophages overexpressing an empty plasmid (light gray bar) or a plasmid containing human sPLA₂-V (dark gray bar). Data are representative of at least three independent experiments. Error bars represent ± SEM. (n=3). B) sPLA₂ activity of human macrophage homogenates. Left panel: homogenates were prepared from untreated cells without (open bar) or with (gray bar) siRNA to sPLA₂-V, or cells treated with 1,000 U/ml IL-4 without (black bar) or with (dark gray bar) siRNA to sPLA₂-V, as indicated. Right panel: homogenates were prepared from cells overexpressing a plasmid containing sPLA₂-V (gray bar) or an empty vector (open bar). PLA₂ activity in the homogenates was assayed using a [³H]AA-labeled natural membrane substrate, as detailed in Materials and Methods. Data are shown as means ± SEM of three independent determinations from different homogenate samples.
Supplemental Figure 3 - sPLA₂-V is involved in the phagocytosis of zymosan by M-CSF-treated macrophages. Human macrophages, either untreated (control) or treated with 50 ng/ml M-CSF for 24 h, and treated with siRNA control or siRNA for sPLA₂-V, as indicated, were analyzed for phagocytosis of fluorescent zymosan particles by confocal microscopy, and a phagocytic index was calculated by dividing the number of phagosomes by the total number of cells in a field, which was multiplied by the percentage of phagocytosing cells. Data are shown as means ± SEM of three independent experiments.
Supplemental Figure 4 – Effect of various lysophospholipids on the phagocytosis of zymosan by IL-4-treated macrophages. Human macrophages, either untreated or treated with 1,000 U/ml IL-4 for 24 h, as indicated, were analyzed for phagocytosis of zymosan particles. The cells were treated with siRNA control or siRNA for sPLA2-V as indicated. Where indicated, LPE, LPC or LPI (5 μM) were added. A phagocytic index was calculated by dividing the number of phagosomes by the total number of cells in a field, which was multiplied by the percentage of phagocytosing cells. The average of three independent experiments with triplicate determinations is shown (mean ± S.E.M.).