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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,*† María 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 mediating 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

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The online version of this article contains supplemental material.

Abbreviations used in this article: AA, arachidonic acid; cPLA2α, Ca2+-dependent cytosolic group IVA PLA2; LPC, choline lysophospholipid; LPE, ethanolamine lysophospholipid; LPI, lysophosphatidylinoisotol; PC, choline glycerophospholipid; PE, ethanolamine glycerophospholipid; PI, phosphatidylinositol; PLA2, phospholipase A2; qPCR, quantitative PCR; siRNA, small interfering RNA; sPLA2-V, secreted group V PLA2.

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known to enhance the expression of a number of phagocytic receptors, such as MRC-1, and dectin-1, which results in increased endocytosis of mannansylated 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 PL2α-mediated phospholipid turnover in monocytes and macrophages responding to a variety of stimuli of innate immunity and inflammation (21–30). In those 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 PL2α 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 PL2α (sPL2α-V) is strongly upregulated by IL-4 but not by IFN-γ plus LPS. Thus, sPL2α-V constitutes a bona fide marker for human alternatively activated macrophages. We further show that the increased expression of sPL2α-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 Amaza 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 sPL2α-V mRNA and negative controls were from Ambion (Carlsbad, CA). IFN-γ and IL-4 were from ImmunoTools (Friesoythe, Germany). The plasmid containing human sPL2α-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 (1-0-1-octadecenyl-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/).

All other reagents were from Sigma-Aldrich.

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 centrifuged at 750 × g for 30 min to separate the mononuclear cell layer, which was recovered and resuspended in RPMI 1640 supplemented with 2 mM t-glutamate 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. Pools of cells corresponding to five donors were sterile plated for 2 h at 37°C. Nonadherent cells were removed by extensive washing with PBS. Pools of cells corresponding to five donors were sterile plated for 2 h at 37°C. Nonadherent cells were removed by extensive washing with PBS. Pools of cells corresponding to five donors were sterile plated for 2 h at 37°C. Nonadherent cells were removed by extensive washing with PBS. 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Nonadherent cells were removed by extensive washing with PBS. Pools of cells corresponding to five donors were sterile plated for 2 h at 37°C. Nonadherent ce...
m2/cm². Membranes were incubated with the corresponding primary Abs. Mouse anti-PLA2-V IgG (C-4; sc-393606; Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:100, and mouse anti-ß-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 exposures within the linear response defined by Quantity One software (version 4.5.2; Bio-Rad).

Liquid chromatography/mass spectrometry analyses of macrophage glycerophospholipids

A cell extract corresponding to 10⁷ cells was used for these analyses. The following internal standards were added—600 pmol each 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, 1,2-dilauroyl-sn-glycero-3-phosphoethanolamine, and 1,2-dipalmitoyl-sn-glycero-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 Supelguard LC-Si guard cartridge (Sigma-Aldrich). Mobile phase was a gradient of solvent A (methanol/water/32% ammonium hydroxide, 87.5:10.5:1.5; by volume) and solvent B (methanol/32% ammonium hydroxide, 87.5:120.5; 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 ion-trap mass spectrometer (Bruker Dalteonic, 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 365°C. Ethanolamine glycerophospholipids (PE) and phosphatidylinositol 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 exposures within the linear response defined by Quantity One software (version 4.5.2; Bio-Rad).

Liquid chromatography/mass spectrometry analyses of eicosanoids

A small amount of butylated hydroxytoluene in methanol (0.01%, v/v) was added to the supernatants to prevent eicosanoid degradation. Deuterated PGE₂ and leukotriene B₄ (200 pmol each) were added as internal standards before lipid extraction. Eicosanoids were separated on a reversed-phase column using a Bruker esquire6000 ion-trap 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/acidic 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; desolvation 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-miristoyl-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 LC-18 Supelguard LC-Si guard cartridge (Sigma-Aldrich). Mobile phase was a gradient of solvent A (chlorform/methanol/water/32% ammonium hydroxide, 75:24:3:5.5:0.5; by volume) and solvent B (chlorform/methanol/ water/32% ammonium hydroxide, 75:24:3:5.5:0.5; by volume). The gradient was started at 100% solvent A, which 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 Dalteonic). The before-treatment into the column was suspended using Bond Elut PLD 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 the
[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 lysophosphatidylinositol (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 sample. 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 mammalian PLA2s in human monocyte-derived macrophages. All of the PLA2s assessed are listed in Table I. Of these, only the following were expressed at significant levels by the cells (fluorescence signal in reactions with specific primers detected below 30 cycles): PLA2-IID, -IVA, -IVB, -IVC, -V, -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 up-regulated 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 TGGCCAGCACTATGCAACTG</td>
<td>97</td>
<td>Yes</td>
</tr>
<tr>
<td>PLA2-IIA</td>
<td>Sense ACTGGAGATGAGGATTCTG</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>PLA2-IID</td>
<td>Sense GCACGGGCAAGAGGATAAG</td>
<td>68</td>
<td>Yes</td>
</tr>
<tr>
<td>PLA2-IIIE</td>
<td>Sense TGGGCTGGAGGGCAAATA</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>PLA2-IIIF</td>
<td>Sense ACCAGAGAGGATGGAGAGA</td>
<td>122</td>
<td></td>
</tr>
<tr>
<td>PLA2-IIIG</td>
<td>Sense GTGGGGAGGTGAGGAGA</td>
<td>132</td>
<td></td>
</tr>
<tr>
<td>PLA2-IIH</td>
<td>Sense ATATGCGAGATGCTATGAC</td>
<td>146</td>
<td>Yes</td>
</tr>
<tr>
<td>PLA2-IIID</td>
<td>Sense AGGGCCGAGCTGGACGTG</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>PLA2-IJE</td>
<td>Sense AGTGGGCTGATACGAGCAGT</td>
<td>109</td>
<td></td>
</tr>
<tr>
<td>PLA2-IVF</td>
<td>Sense GGGCGGGAACATCGGAGAC</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>PLA2-V</td>
<td>Sense CCTGTGCCCAGATGTGTG</td>
<td>89</td>
<td>Yes</td>
</tr>
<tr>
<td>PLA2-VIA</td>
<td>Sense TACGACAGTACCGTCTTAC</td>
<td>80</td>
<td>Yes</td>
</tr>
<tr>
<td>PLA2-VIB</td>
<td>Sense CAGCCGAGAAAGATATCGCAG</td>
<td>89</td>
<td>Yes</td>
</tr>
<tr>
<td>PLA2-VIC</td>
<td>Sense TGGTGTGTTCTTCTGGGAG</td>
<td>125</td>
<td>Yes</td>
</tr>
<tr>
<td>PLA2-VID</td>
<td>Sense GCCACCCCTTCTCCAGCAG</td>
<td>132</td>
<td>Yes</td>
</tr>
<tr>
<td>PLA2-VIE</td>
<td>Sense GGGCGGGAACATCGGAGAC</td>
<td>68</td>
<td>Yes</td>
</tr>
<tr>
<td>PLA2-VIF</td>
<td>Sense ACGAAGCTGGACGACGAC</td>
<td>112</td>
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<td>PLA2-X</td>
<td>Sense ATCAATATGCGAGACGAC</td>
<td>133</td>
<td>Yes</td>
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<td>PLA2-XIIA</td>
<td>Sense ATGCGAGATGCGAGACGAC</td>
<td>54</td>
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</tr>
<tr>
<td>PLA2-XIIB</td>
<td>Sense CGCCCAACATAGTGCGAGAC</td>
<td>250</td>
<td>Yes</td>
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<tr>
<td>PLA2-XV</td>
<td>Sense CGCCCAACATAGTGCGAGAC</td>
<td>150</td>
<td>Yes</td>
</tr>
</tbody>
</table>
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.
FIGURE 2. Induction of sPLA2-V protein during human macrophage polarization to M2. The cells were either left untreated (zero time) or treated with 1000 U/ml IL-4 (A), 50 ng/ml M-CSF (B), or 50 ng/ml IL-10 (C) for the indicated times. sPLA2-V protein was analyzed by immunoblot (upper panels). The blots were quantified from three different experiments (mean ± SEM), and the quantifications are shown (lower panels).

(Supplemental Fig. 2A), which translated into a 6–11-fold increase in cellular sPLA2 activity, as measured in a natural membrane-based assay (Supplemental Fig. 2B). Fig. 3B shows that simply overexpressing sPLA2-V into the macrophages was sufficient to produce a significant augmentation of the phagocytic capacity of the cells, which was comparable to that induced by IL-4. Treating sPLA2-V-overexpressing cells with IL-4 did not increase the phagocytic capacity of the cells beyond those levels already attained with either treatment alone (Fig. 3B). The latter finding was not unexpected because the increase in sPLA2-V levels in the overexpressing cells was comparable to that observed in IL-4-treated cells, as judged by activity assay (Supplemental Fig. 2).

To study whether the key role for sPLA2-V in phagocytosis in IL-4–treated cells could be extended to other models of M2 polarization, experiments also were conducted with M-CSF, which, as previously shown in Fig. 2, also increases sPLA2-V to levels comparable to that of IL-4. In keeping with IL-4–treated cells, sPLA2-V–depleted cells treated with M-CSF also showed a significant inhibition of their ability to phagocytose zymosan particles (Supplemental Fig. 3). Collectively, these results highlight a key role for sPLA2-V in regulating phagocytic events in human macrophages polarized to an M2 state.

sPLA2-V–dependent changes in lipid metabolism in IL-4–treated cells

Unlike other sPLA2 family members, such as sPLA2-IIA, most, if not all, biological effects attributed to sPLA2-V are thought to be due to its hydrolytic activity on cell membranes (60–62). Therefore, it was logical to hypothesize that a metabolite produced by sPLA2-V is responsible for its biological effects on IL-4–induced phagocytosis. To identify such a metabolite, we conducted liquid chromatography/mass spectrometry–based lipidomic analyses of major glycerophospholipids to detect changes in the levels of these lipids that may arise as a consequence of cell activation with IL-4 (Fig. 4A–D).

Phospholipid species within each class are given in abbreviated form (number of carbon atoms and double bonds of the two lateral chains together) because, in most cases, fragmentation of the m/z peaks yielded fragments corresponding to various species, and it was not possible to unequivocally assign structures to these m/z peaks. A designation of O- before the first fatty chain indicates that the sn-1 position is ether linked, whereas a P- designation indicates a plasmalogon form (sn-1 vinyl ether linkage) (63). In general, no significant alterations were detected in the levels of major phospholipid classes after treating the cells with IL-4 for 24 h, nor was any change detected when sPLA2-V–deficient versus normal cells were used. It should be noted that human macrophages are relatively large cells (diameter > 30 μm) (33, 34); hence, their membrane phospholipid content is quite high (35). Thus, if the hydrolytic action of IL-4–induced sPLA2-V on membrane phospholipids is limited, the small changes involved easily could be obscured by the high phospholipid amount present in the cells. In accord with this, analysis by gas chromatography/mass spectrometry of total phospholipid fatty acids revealed no significant changes in any of the fatty acids measured (Fig. 4E).

In some cases, there seemed to be a tendency for the IL-4–treated cells to exhibit mild decreases in certain fatty acids (i.e., 16:0, 18:0 principally, but also 20:4), which were not appreciated when sPLA2-V–deficient cells were used. However, these changes were too small to reach significance.

Liquid chromatography/mass spectrometry analyses of AA–derived eicosanoid metabolites in supernatants revealed that resting human macrophages primarily produced thromboxane B2, PGE2, and 14,15-dihydroxyeicosatetraenoic acid, as well as smaller amounts of 15-hydroxyeicosatetraenoic acid, after 24 h in culture (Fig. 5). Production of these eicosanoids was increased modestly by treating the cells with IL-4 (Fig. 5). Use of sPLA2-V–deficient macrophages did not appreciably change the eicosanoid profiles observed in resting or IL-4–treated cells (Fig. 5), suggesting that this limited eicosanoid response likely proceeds without the participation of sPLA2-V.

Lysophospholipids are the other products of PLA2 action on membrane phospholipids. Because of their toxic nature, lysophospholipids accumulate in cells at much lower levels than those of their precursor phospholipids. Given the experiments above, suggesting that the action of IL-4 on phospholipid hydrolysis is, at best, limited, we reasoned that lipid changes probably could be detected in the lysophospholipid fraction because of its lower amount. Using liquid chromatography coupled to mass spectrometry, measurement of lysophospholipid species was carried out, and the effect of depleting the cells of sPLA2-V by siRNA was studied as well. Fig. 6 shows the major lysophospholipid species detected in resting and IL-4–treated human macrophages. IL-4 failed to induce measurable increases in the levels of any of the species analyzed, confirming the limited extent of action of this cytokine on lipid metabolism. However, when sPLA2-V–deficient cells were examined, a very striking change was appreciated: the levels of all LPE species were significantly decreased following exposure to IL-4 (Fig. 6). This decrease was not observed in the otherwise untreated macrophages, implying that it was related to the activation state of the cells. Moreover, the decrease in LPE species was not mirrored by...
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 during IL-4 stimulation of the macrophages. By inference, the data suggest that sPLA2-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 measured by liquid chromatography/mass spectrometry. Data are mean ± SEM of three independent experiments with duplicate determinations. 14,15-DHET, 14,15-dihydroxyeicosatrienoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid; TXB2, thromboxane B2.

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 phospholipid 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 formation of vesicles of varying sizes that could interact with the cells in a nonspecific manner (64, 65). The data are shown in Fig. 7. As noted in Fig. 3, the increased phagocytic response of the macrophages to IL-4 was not observed if sPLA2-V–deficient cells were used; however, adding LPE to the sPLA2-V–deficient cells fully restored the IL-4 response (Fig. 7). Importantly, LPE did not increase the effect of IL-4 on cells expressing normal sPLA2-V levels, indicating that the signals generated by LPE are already achieved by IL-4, as long as the higher levels of sPLA2-V are preserved. When added in the absence of IL-4, LPE did not exert any effect on its own, as manifested by the finding that phagocytosis of zymosan in control cells was the same as that found in cells treated only with LPE, both under normal and reduced sPLA2-V expression levels. Neither LPC nor LPI, added at the same concentrations as LPE and under the same conditions, exerted any effect, providing support to the specificity of action of LPE (Supplemental Fig. 4). Together, the results shown in Fig. 7 suggest that generation of LPE is a requisite step for IL-4–induced phagocytosis and that this LPE is produced selectively by sPLA2-V.

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.
deleterious effects of inflammation. Thus, in murine, but not in human, cells, sPLA\(_2\)-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 sPLA\(_2\)-V expression also can be upregulated by IL-4 in murine lung cells (74).

We found no evidence for such a functional plasticity of sPLA\(_2\)-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 sPLA\(_2\)-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 sPLA\(_2\)-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, sPLA\(_2\)-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 sPLA\(_2\)-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 sPLA\(_2\)-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, sPLA\(_2\)-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 zymosan particles by confocal microscopy (red color, middle columns). The cells were treated with siRNA control or siRNA for sPLA\(_2\)-V, as indicated. LPE (5 \(\mu\)M) was added, where indicated. DAPI (1 \(\mu\)g/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 \(\pm\) SEM) (bottom panel). Original magnification \(\times 20\). **p < 0.01.
study, recent studies in neutrophils showed that LPE can induce Ca2+-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 sPLA2-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 plasmalogens (80, 81), accumulation of LPE at these particular microdomains is a possibility that deserves further consideration. Alternatively, the possibility that loss of function in sPLA2-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 cPLA2α translocation to nascent phagosomes in murine macrophages takes place in a manner that is independent of its enzymatic activity (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, cPLA2α, 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 sPLA2-V–deficient or normal cells were used. Although cPLA2α 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–

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**FIGURE 8.** LPE restores phagocytosis of bacteria 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 bacteria 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 ×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 the progression of atherosclerosis (84). sPLA2–V–modified low-density lipoproteins promote foam cell formation, and the enzyme has been found in human atherosclerotic lesions. Thus, inhibition of the enzyme is contemplated as a possibly valid therapeutic approach to treating cardiovascular disease. Although recent trials testing the effect of varespladib, an inhibitor of the closely related enzyme sPLA2–IIA that also can block, at least in part, sPLA2–V, provided discouraging results (85, 86), our results implicating enzyme sPLA2–IIA that also can block, at least in part, sPLA2–V and testing the effect of varespladib, an inhibitor of the closely related group IVA phospholipase A2s. Regulation of TLR-mediated arachidonic acid mobilization in macrophages by group IVA and group V phospholipase A2s. Ann. Rev. Immunol. 27: 451–483.


