The Secretion and Uptake of Lysosomal Phospholipase A₂ by Alveolar Macrophages

Akira Abe, Robert Kelly, Jessica Kollmeyer, Miki Hiraoka, Ye Lu and James A. Shayman

J Immunol 2008; 181:7873-7881; doi: 10.4049/jimmunol.181.11.7873
http://www.jimmunol.org/content/181/11/7873

References
This article cites 24 articles, 13 of which you can access for free at:
http://www.jimmunol.org/content/181/11/7873.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Macrophages have long been known to secrete a Phospholipase A2 with an acidic pH optimum in response to phagocytic stimuli. However, the enzyme or enzymes responsible for this activity have not been identified. We report that mouse alveolar macrophages release lysosomal phospholipase A2 (LPLA2) into the medium of cultured cells following stimulation with zymosan. The release of the enzyme was detected by enzymatic activity assays as well as by Western blotting using an Ab against mouse LPLA2. LPLA2 is a high mannose type glycoprotein found in lysosomes, suggesting that the released enzyme might be reincorporated into alveolar macrophages via a mannose or mannannose phosphate receptor. Recombinant glycosylated mouse LPLA2 produced by HEK293 cells was applied to LPLA2-deficient (LPLA2−/−) mouse alveolar macrophages. The uptake of exogenous LPLA2 into LPLA2−/− alveolar macrophages occurred in a concentration-dependent manner. The LPLA2 taken into the alveolar macrophages colocalized with the lysosomal marker, Lamp-1. This uptake was significantly suppressed in the presence of α-methylmannoside but not in the presence of mannose 6-phosphate. Thus, the predominant pathway for uptake of exogenous LPLA2 is via the mannose receptor, with subsequent translocation into acidic, Lamp-1-associated compartments. LPLA2−/− alveolar macrophages are characterized by marked accumulation of phosphatidylcholine and phosphatidylethanolamine. Treatment with the recombinant LPLA2 rescued the LPLA2−/− alveolar macrophages by markedly decreasing the phospholipid accumulation. The application of a catalytically inactive LPLA2 revealed that the enzymatic activity of LPLA2 was required for the phospholipid reduction. These studies identify LPLA2 as a high m.w.-secreted Phospholipase A2.


Unlikely these sPLA2s, the biochemical characterization of the lysosomally secreted Phospholipase A2 is limited and its biological function unknown.

Our group previously reported the discovery of a novel enzyme, which has dual enzyme activities, short chain ceramide transacylation and Phospholipase A2, under acidic conditions (3). The preferred substrates for the enzyme are phosphatidylcholine and phosphatidylethanolamine. In the presence of a lipophilic alcohol such as ceramide as an acceptor for the fatty acyl group, the enzyme displays transacylase activity. In the presence of only water as an acceptor, the enzyme acts as a traditional Phospholipase A2. The enzyme was recovered in the soluble fraction obtained from cell homogenates and the lysosomal fraction of Madin-Darby canine kidney cells. Subsequently, we purified, cloned, and named the enzyme lysosomal Phospholipase A2 (LPLA2) (4, 5). Purified LPLA2 from bovine brain is a water-soluble glycoprotein consisting of a single polypeptide chain with a m.w. of 45 kDa. The protein exhibits acidic Ca2+-independent PLA and transacylase activities. LPLA2 is encoded by the same gene as lecithin-cholesterol acyl-transferase-like lysophospholipase (6). The primary structure of LPLA2 is highly preserved between mammalian and nonmammalian species. LPLA2 is post translationally modified both by a signal peptide cleavage and N-glycosylation. LPLA2 has 49% identity to lecithin-cholesterol acyl-transferase and belongs to the αβ-hydrolase superfamily. The catalytic triad and four cysteine residues conserved in LPLA2 are preserved in lecithin-cholesterol acyl-transferase (7).

LPLA2 is ubiquitously expressed in all tissues studied to date, but is most highly expressed in terminally differentiated alveolar macrophages (AMs) (8). Recently, LPLA2−/− mice were generated by systemic deletion of exon 5 of the LPLA2 gene, which codes the catalytic site (9). These mice displayed a systemic loss of...
LPLA2 activity. A marked accumulation of phospholipid was found in AM, peritoneal macrophages, and spleen in LPLA2−/− mice at an early stage time. Electron micrographs revealed extensive lamellar inclusion bodies, a hallmark of cellular phospholipidosis, in the LPLA2−/− mouse AM and PM. Therefore, LPLA2 is thought to play an important role in cellular phospholipid homeostasis in those cells.

LPLA2 activity has been found in the cell culture medium of LPLA2 overexpressed cells, PMA-treated THP-1 cells, and 1-phenyl-2-decanoylamino-3-morpholino-propanol treated Madin-Darby canine kidney cells, and bronchoalveolar lavage cell-free fluid (Our unpublished data). These observations indicate that LPLA2 is likely to be a secreted protein as well as a lysosomal protein, and suggests that the acidic Phospholipase A2 activity released from macrophages when exposed to phagocytic stimuli derives from LPLA2. In general, lysosomal enzymes contain oligosaccharide residues containing mannose and/or mannose 6-phosphate (10). Subsequently, secreted lysosomal enzymes are taken up by cells via a specific receptor mediated by carbohydrate recognition (11). Because LPLA2 is a high mannose-type glycoprotein, the LPLA2 released from the macrophages may be recovered by the cells via mannose or mannose 6-phosphate receptors.

Recently, we have generated purified recombinant mouse LPLA2, and developed mAbs against mouse LPLA2. In the present study, we explored whether LPLA2 is released from mouse AMs following treatment with zymosan, and whether extracellular LPLA2 is taken up to the AMs and trafficked back to the lysosome.

Materials and Methods

Reagents

2-Dioleoyl-sn-glycerol-3-phosphocholine was purchased from Avanti. N-acetylsphingosine (NAS) was obtained from Matreya. Endoglycosidase F1 was from Sigma-Aldrich. BCA protein assay reagent was from Pierce. HPTLC silica gel plates, 10 × 20 cm, were from Merck. Centriprep-10 (m.w. cut off 10,000) was from Amicon. The purified recombinant mouse LPLA2 tagged with polyhistidine was obtained from Proteins. Anti-Lamp1, anti-early endosomal Ag 1 (EEA1), anti-Rab7, anti-calregulin, and anti-translocase of the outer membrane of mitochondria (Tom20) rabbit polyclonal Abs were purchased from Santa Cruz Biotechnology. AlexaFluor 488 goat anti-rat IgG Ab, AlexaFluor 594 goat anti-rabbit IgG Ab, and Image-iT Signal Enhancer were obtained from Molecular Probes Invitrogen.

Preparation of cell homogenate from mouse alveolar macrophages

The cell homogenates of AMs were prepared as described (8). The adherent AMs (10^5/well) on 6-well culture plates were washed three times with 2 ml of cold PBS and then treated under various conditions as described. The treated cells were washed three times with 2 ml cold PBS, scraped with a small volume of PBS, and transferred into a 15 ml glass tube. The cell suspension was disrupted by a probe type sonicator for 10 s × 5 at 0°C to obtain the cell homogenate.

Transacylase activity of LPLA2

LPLA2 was originally discovered as a novel activity that transacylates short chain ceramides on the C-1 hydroxyl using sn-2 fatty acyl groups from phosphatidylcholine or phosphatidylethanolamine (3). Subsequent studies on the purified enzyme (4), recombinant enzyme (5), and knockout mouse tissues (9) have demonstrated that ceramide transacylation accounts for phospholipase A2 activity. Additionally, the phospholipid fatty acyl donor specificities (12) and lipophilic alcohol acceptor specificities (13) with the recombinant mouse LPLA2 used in the present study have recently been reported. The transacylation of C-2 ceramide (N-acetyl-sphingosine) therefore is a specific measurement of LPLA2 activity. The method for enzyme assay has been previously published in detail (14). In brief, the reaction mixture consisted of 40 mM sodium citrate (pH 4.5), 10 μM/ml BSA, 40 μM NAS incorporated into phospholipid liposomes (1, 2-Dioleoyl-sn-glycerol-3-phosphocholine/sulfatide/N-acetyl-sphingosine (10:1:3.2 in molar ratio)), and cell homogenate in a total volume of 500 μl. The reaction was initiated by adding the cell homogenate, incubated for 5 to 30 min at 37°C and terminated by adding 3 ml of chloroform/methanol (2/1) plus 0.3 ml of 0.9% (weight-to-volume ratio) NaCl. The mixture was centrifuged for 5 min at room temperature. The resultant lower organic layer was transferred into another glass tube and dried down under a stream of nitrogen gas. The dried lipid was dissolved in 40 μl of chloroform/methanol (2/1) and applied on an HPTLC plate or an argentation HPTLC plate and developed in a solvent system consisting of chloroform/acetic acid (9/1, v/v). The plate was dried and soaked in 8% (weight-to-volume ratio) CuSO4 5H2O. 6.8% (v/v) H3PO4, 32% (v/v) methanol. The uniformly wet plate was briefly dried by a hair dryer and charred for 15 min in a 150°C oven. The plate was scanned and the content of the product (1-O-acetyl-NAS) was estimated by NIH Image 1.63.

mAb production

Rat anti-mouse LPLA2 was generated as follows. Rats were immunized by injection with recombinant mouse LPLA2 protein. When sufficient Ab titer was achieved in the animal’s serum, the rat was euthanized and the splenocytes isolated and hybridomas were generated. Cell culture supernatants from hybridomas were screened against recombinant LPLA2, COS-7 cells transfected with either mouse or human LPLA2 constructs, and LPLA2 wild-type and knock-out alveolar macrophages. A hydridoma line that secreted a mAb that reacted strongly to the protein of interest was selected for use. The results from the mAb screen were compared with a polyclonal Ab described previously (9).

Identification of LPLA2

Immunoblotting

Cell homogenate and cell cultured medium were precipitated by the method of Benzodoun and Weinstein (15). The resultant pellet was dissolved with 30 μl of loading buffer plus 1.5 μl of 2 M Tris for SDS-PAGE. Proteins were separated using a 12% SDS polyacrylamide gel and transferred to a PVDF membrane using transfer buffer (20 mM Tris, 150 mM glycine in 20% methanol) at a constant voltage of 100 volts for 3 h at 4°C. The membrane was incubated with a monoclonal anti-mouse LPLA2 rat Ab; rat or monoclonal anti-polylphosphatidic acid mouse Ab. The Ag-Ab complex on the membrane was identified with an anti-rat IgG HRP-conjugated goat Ab or an anti-mouse IgG HRP-conjugated goat Ab using ECL reagent. Rat splenocytes cell culture medium from monoclonal anti-mouse LPLA2 Ab producing cell line was used as a monoclonal anti-mouse LPLA2 rat Ab.

Immunofluorescence microscopic examination

The following procedures were conducted at room temperature. The cells in a slide culture were fixed with 4% paraformaldehyde in PBS for 30 min, washed twice with PBS, and permeabilized with 50 mM NH4Cl and 0.3% Triton-X 100 for 20 to 30 min. Then the cells were washed three times with PBS, covered with Image-IT 6 for 30 min, and washed three times with PBS. The cells were incubated with 2% BSA in PBS for 60 min and with 2% goat serum in PBS for another 60 min before the immunoreaction. In a routine study, each Ab was incubated for 90 min. After completing the whole immunoreaction, the cells were washed three times with PBS and the chambers were removed. The slide glass was covered with a slide cover with 2% long Gold antifade containing 4’,6-diamido-2-phenylindole (DAPI), allowed to fix/dry for 24–48 h and sealed with clear nail polish.

Image analysis was performed on an Olympus Fluoview 500 laser scanning confocal microscope mounted on an Olympus IX-71 inverted microscope. The apparatus was equipped with four lasers and one transmitted light source including blue violet diode excitation at 405 nm, multline argon blue excitation at 488 nm, helium neon green excitation at 543, and helium neon red excitation at 633 nm. The FV500 uses sequential scanning for reduced fluorescence cross talk. The focal plane of the alveolar macrophage used for the images included was determined by first focusing on the DAPI blue staining of the nuclei. The depth of the image in the Z plane was 0.31 microns. For immunofluorescent staining, each condition was studied in triplicate, and three images were captured for each sample. The figures shown are representative.

Results

Localization of LPLA2 in mouse AMs

Previously, we reported that in subcellular fractionation studies LPLA2 localizes to lysosomes (5). To substantiate the results of the subcellular fractionation studies in an intact cell, immunofluorescence microscopic examinations were conducted using mouse AMs. AMs prepared from LPLA2−/− mice and wild-type mice were treated with a mAb for LPLA2 and stained with DAPI. The AMs from LPLA2−/− mice showed no signal for LPLA2, although
**FIGURE 1.** Localization of LPLA₂ in mouse AMs. A. AMs obtained from LPLA₂−/− mice were fixed and incubated with a monoclonal anti-LPLA₂ and polyclonal anti-Lamp 1 Abs. The cells were treated with Abs against LPLA₂ (green) and stained with DAPI (blue). B. AMs from wild type mice were stained with DAPI and anti-LPLA₂, as in A and with anti-Lamp-1 Ab (red). The merged image demonstrates colocalization of LPLA₂ and Lamp-1. C. Merged images of wild type AMs stained for LPLA₂ and the organelle markers EEA1, Rab7, calregulin, and Tom20.

DAPI staining of the nuclei was readily apparent (Fig. 1A). A signal for LPLA₂ was present in the AMs prepared from wild-type mice (Fig. 1B). The LPLA₂ signal in wild-type AMs colocalized with the lysosomal marker Lamp-1 (red) (Fig. 1B). The overlap of the green LPLA₂ and red Lamp-1 signals resulted in a yellow hue (Fig. 1B). Consistent with the above observations, LPLA₂ colocalized with the early and late endosomal markers EEA1 and Rab7, but not with the mitochondrial marker Tom20 or endoplasmic reticular marker calregulin (Fig. 1C).

**Release of LPLA₂ activity from mouse AMs**

As noted above, previous papers have reported that phagocytic stimuli induce AMs to secrete lysosomal acidic enzymes. The release of an acidic Phospholipase A₂ activity was noted when the macrophages are treated with zymosan (1). According to this report, the total activity of acidic Phospholipase A₂ in the culture system was constant. Additionally, our recent studies using LPLA₂−/− mouse AMs demonstrated that >95% of the total activity of acidic Phospholipase A₂ in the cell is due to LPLA₂ (12). These data suggest that the acidic Phospholipase A₂ released from AMs treated by zymosan could be LPLA₂.

Mouse AMs were incubated with culture medium containing different concentrations of zymosan (Fig. 2, A and B). After 4-h incubation with zymosan, LPLA₂ activity in the cell homogenate and cell culture medium was measured. The transacylase activity at pH 4.5 was used to determine the LPLA₂ activity as described (14). Eighty-six percent of the total enzyme activity in the cell culture well was recovered in the cell homogenate when the AMs were treated with medium not containing zymosan. The enzyme activity was markedly reduced in the cell homogenate obtained from the cells treated with zymosan. The extent of the reduction was dependent on the concentration of zymosan. Approximately 40% of the total activity was recovered in AMs treated with 25 μg/ml zymosan. The total enzyme activity in each well was comparable (Fig. 2B). On the basis of these results, 50 μg/ml zymosan was chosen as the routine dose of incubation in subsequent experiments.

AMs were then treated with zymosan for different time periods (Fig. 2, C and D). The enzyme activity found in the cultured medium increased with time. The enzyme activity in the cell homogenate decreased in parallel with time. The total enzyme activity of LPLA₂ in each well was constant. These results show that the reduction of the enzyme activity in the cell homogenate and the increase of the enzyme activity in the culture medium are dependent on the time of exposure to zymosan.

Immunoblotting using a mAb against mouse LPLA₂ revealed that LPLA₂ was detected in the cell homogenate and the cell cultured medium when the cells were treated for 1 h with 50 μg/ml zymosan (Fig. 2, E and F). The difference in molecular weights between the endogenous cellular LPLA₂ and the LPLA₂ standard reflects the presence of the presence of the polyhistidine tag on the recombinant LPLA₂. The reduction in cell homogenate transacylase activity (Fig. 2F) is not apparent in the immunoblots (Fig. 2E) due to a difference in protein loading as described in the figure legend. The reduction of LPLA₂ from mouse AMs treated with zymosan was confirmed with immunofluorescence microscopy. The LPLA₂ signal (green) in the AMs decreased as the AMs ingested more zymosan in a time dependent manner (Fig. 2G). These immunofluorescence studies demonstrate an inverse relationship between LPLA₂ signal and time of treatment with zymosan.

**Incorporation and translocation of exogenous LPLA₂ in LPLA₂−/− mouse AMs**

Many lysosomal enzymes are highly glycosylated proteins and are taken up by cells via a carbohydrate binding receptor. Previous studies showed that LPLA₂ is bound to a Con A-conjugated resins, released from the resins with the buffer containing high concentration (500 mM) of mannose or α-methyl mannrose, and degraded by endoglycosidase F1 to a core peptide (4, 5). Additionally, depending on the species studied, three or four N-glycolation
sites are estimated to be present based on the primary structure of LPLA2S (5). These findings indicate that LPLA2 is a high mannose type glycoprotein. AMs express mannose and mannose 6-phosphate receptors on their plasma membrane. Thus, LPLA2 released from AMs could potentially be incorporated into the cells via mannose or mannose 6-phosphate receptors.

**FIGURE 2.** Release of LPLA2 from mouse AMs by treatment with zymosan. A–D and F, Transacylase activity. AMs isolated from wild-type mice were seeded in 6-well plate (10⁶ cells/well). The adherent cells on the plate were washed with PBS and treated with 0, 25, 50, and 75 μg/ml zymosan for 4 h at 37°C (A and B). C and D, The cells were treated with 50 μg/ml zymosan for 0.5, 1, and 2 h at 37°C. After the treatment, the cell homogenate (3.3 μg of protein) prepared from the treated AMs was incubated for 30 min at 37°C in citrate buffer (pH 4.5) with 40 μM NAS incorporated into phospholipid liposomes (PC/sulfatide/NAS (10:1:3.2 in molar ratio). The reaction product by the transacylase activity of LPLA2, 1-O-acyl-NAS, was detected by TLC. The plate was developed in a solvent system consisting of chloroform/acetic acid (9:1, v/v) (A and C). E and F, The cells were treated with or without 50 μg/ml zymosan for 1 h. After the treatment, the transacylase activities in each cell homogenate (H) and medium (M) were determined as described above.

E, Western blotting. Twenty and twenty-five micrograms of protein of cell homogenate obtained from the cells treated with 0 and 50 μg/ml zymosan, respectively, were separated in a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. For the medium, 50% of total volume was used for the electrophoresis. The membrane was incubated with a monoclonal anti-mouse LPLA2 rat Ab. The Ag-Ab complexes on the membrane were visualized with an anti-rat IgG HRP-conjugated goat Ab using ECL reagents. The band intensity for the medium of the zymosan treated cells was weaker than that as expected from the transacylase activity (F). However, this was due to absorption of LPLA2 to the membrane of Centriprep that was used for reducing the volume of collected medium. G, The ingestion of zymosan particles and the location of LPLA2 of the AMs. The cells were treated with 50 μg/ml zymosan for 1, 2, and 4 h at 37°C. After the treatment, the cells were fixed and incubated with a monoclonal anti-LPLA2 Ab, treated Ab against LPLA2 (green) and stained with DAPI (blue).
LPLA2 into AMs. The recombinant mouse LPLA2 used is glycosylated and has a poly-histidine C-terminal extension (13). LPLA2−/− mouse AMs were incubated for 1 day with culture medium containing various concentrations of the purified recombinant LPLA2. The adherent AMs were washed extensively before analysis. A concentration dependent increase of the LPLA2 enzyme activity recovered in the cell homogenate was observed (Fig. 3A, left panel). The LPLA2 in the cell homogenate was measured by immunoblotting using a monoclonal anti-LPLA2 Ab (Fig. 3A, right panel). An increase of the intensity of LPLA2 protein bands detected by the mAb was accompanied with an increase of the exogenous recombinant enzyme (Fig. 3A, right panel), indicating that the recombinant LPLA2 was effectively incorporated into cultured LPLA2−/− mouse AMs in a concentration dependent manner.

The predominant bands detected by a monoclonal anti-LPLA2 Ab were smaller molecular size (45 kDa) than the intact recombinant LPLA2 band (in Fig. 3A, right panel) and were not detected by a monoclonal anti-poly-histidine Ab (Fig. 3A, middle panel). The recombinant LPLA2 bands tagged with a poly-histidine at the C terminus were detected in the samples obtained from 3 and 10 μg/ml LPLA2−/− mouse AMs were incubated with 0, 0.3, 1, 3, or 10 μg/ml recombinant mouse LPLA2 for 1 day. After the treatment, the transacylase activity in cell homogenate (3.3 μg of protein) prepared from the treated AMs was determined as described in Fig. 2. Western blotting (Fig. 3A, right panel). Protein (70 μg) from each homogenate in A was separated in a 12% SDS-polyacrylamide gel and transferred to a polyvinylidine difluoride membrane. The membrane was incubated with either monoclonal anti-poly-histidine Ab or a monoclonal anti-mouse LPLA2 rat Ab. The Ag-Ab complexes on the membrane were visualized as described in Fig. 2. Purified recombinant LPLA2 was applied to the far right lane and is denoted by an asterisk (3A middle and right-handed panels). B, LPLA2 activity was measured as both the formation of 1-O-oleoyl-N-acetylsphingosine and the release of free fatty acid. C, Incorporation of LPLA2 incorporated into LPLA2−/− mouse AMs. LPLA2−/− mouse AMs were treated with or without 1 μg/ml recombinant mouse LPLA2 for 1 day. After the treatment, the cells were fixed with paraformaldehyde and incubated with a monoclonal anti-LPLA2 and D, Colocalization of exogenously added LPLA2 and Lamp-1. LPLA2−/− mouse AMs were treated with or without 1 μg/ml recombinant mouse LPLA2 for 1 day. After the treatment, the cells were fixed with paraformaldehyde and incubated with a monoclonal anti-LPLA2 and polyclonal anti-Lamp 1 Abs. The cells were then treated with Abs against LPLA2 (green) and Lamp 1(red) and stained with DAPI (blue).
FIGURE 4. Effect of mannose derivatives on uptake of LPLA2 into mouse AMs. A, Transacylase activity. LPLA2−/− mouse AMs were preincubated in the presence of or absence of 10 mM α-methyl mannoside (Me-Man) or 10 mM mannose-6-phosphate (Man-6-P) for 30 min. LPLA2−/− mouse AMs were treated with or without 1 ug/ml recombinant mouse LPLA2 for 1 day. After the treatment, the transacylase activity in cell homogenate (4.0 µg of protein) measured from the treated AMs was determined as described in Fig. 2. B, Western blotting. Protein (70 µg) from each homogenate in A was separated in a 12% SDS-polyacrylamide gel and transferred to a polyvinylidine difluoride membrane. The membrane was incubated with a monoclonal anti-LPLA2 Ab. Then the cells were fixed with paraformaldehyde and incubated with a monoclonal anti-LPLA2 Ab (right). The Ag-Ab complexes on the polyvinylidine difluoride membrane. The membrane was incubated with a monoclonal anti-mouse LPLA2 Ab (right). The Ag-Ab complexes on the membrane were visualized as described in Fig. 2. C, The uptake of LPLA2 into LPLA2−/− mouse AMs. The cells were treated with 1 µg/ml recombinant mouse LPLA2 for 1 day in the presence or absence of 10 mM Me-Man or Man-6-P. After the treatment, the cells were fixed with paraformaldehyde and incubated with a monoclonal anti-LPLA2 Ab. Then the cells were treated with an Ab against LPLA2 (green) and stained with DAPI (blue). The parallel panels reflect the results of two independent experiments.

To investigate cellular trafficking of the LPLA2 taken into LPLA2−/− mouse AMs, cells were incubated for 24 h with the recombinant LPLA2 and stained with a mAb against LPLA2 (Fig. 3B). A robust signal for LPLA2 was detected with a staining pattern that was similar to the signal for endogenous LPLA2 observed in wild-type AMs. The LPLA2 incorporated into LPLA2−/− mouse AMs colocalized with the lysosomal marker Lamp-1 producing a readily detectable yellow color caused by the overlapping green and red signals of the LPLA2 and Lamp-1 respectively (Fig. 3, C and D).

**Effect of mannose derivatives on uptake of exogenous LPLA2 into LPLA2−/− mouse AMs**

To evaluate whether the mannose receptor or mannose 6-phosphate receptor is responsible for the LPLA2 uptake by LPLA2−/− mouse AMs, the LPLA2−/− mouse AMs were treated with recombinant LPLA2 in the presence of α-methyl mannoside or mannose 6-phosphate. The enzymatic activity of LPLA2 in the cell homogenate from LPLA2−/− mouse AMs treated in the presence of 10 mM α-methyl mannoside was significantly reduced, 69 ± 5% reduction compared with the control AMs (n = 3) (Fig. 4A). Additionally, the cell homogenate from LPLA2−/−/− mouse AMs treated in the presence of mannose showed the comparable enzyme activity as the α-methyl mannoside (data not shown). Western blot analysis showed a similar reduction in LPLA2 when LPLA2−/− mouse AMs were treated in the presence of α-methyl mannoside (Fig. 4B). When LPLA2−/− mouse AMs were treated in the presence of mannose 6-phosphate, no reduction was seen in enzymatic activity of LPLA2 or the presence of LPLA2, as shown in Fig. 4, A and B, respectively.

Additionally, immunofluorescence examinations of LPLA2−/− mouse AMs in the presence of 10 mM α-methyl mannoside or mannose 6-phosphate showed a parallel effect. Incorporation of the recombinant LPLA2 by LPLA2−/− mouse AMs was noticeably reduced in the presence of α-methyl mannoside but not mannose 6-phosphate as shown in two independent experiments (Fig. 4C). These results indicate that either mannose or α-methyl mannoside in the culture medium specifically inhibited the uptake of recombinant mouse LPLA2 into LPLA2−/− mouse AMs.

**Reduction of phospholipid in LPLA2−/− AMs by recombinant LPLA2**

The results above show that mouse AMs have secretion and re-capture pathways of LPLA2, and the LPLA2 taken in the AMs could biologically and physiologically function. Hence, we considered that phospholipidosis of mouse AMs induced by a deficiency of LPLA2 could be rescued by enzyme replacement treatment with the recombinant LPLA2.

In 3-mo-old mice, the total phospholipid content of the LPLA2−/− mouse AMs (593 nmol of phospholipid/mg of protein in Fig. 5A) was more than twice higher as compared with that of the wild-type mouse AMs (256 nmol of phospholipid/mg of protein in Fig. 5A). A marked accumulation of phospholipids, in particular phosphatidylethanolamine and phosphatidylethanolamine, except for sphingomyelin, was found in LPLA2−/− mouse AMs (Fig. 5A). Phosphatidylethanolamine and phosphatidylcholine levels in the LPLA2−/− mouse AMs were four and two times higher, respectively, than those of the wild-type mouse AMs. Electron micrographs in the LPLA2−/− mouse AMs revealed the appearance of foam cells with lamellar inclusion bodies, a hallmark of cellular phospholipidosis (Fig. 5B).

LPLA2−/− mouse AMs were treated with different concentrations of the recombinant mouse LPLA2 for 1 day or 3 days (Fig. 5, C and D). Although the level of phospholipid in the AMs treated without the LPLA2 decreased gradually during the treatment, the
reduction of that in the AMs was markedly accelerated by treatment with the LPLA2 (Fig. 5C). After the treatment for 1 day, the phospholipid level of the LPLA2-treated cells was nearly reduced to that of wild type (Fig. 5, C and D). Interestingly, the phospholipid level in the LPLA2-treated cells declined substantially after one day of treatment, but little further change occurred with continued incubation for up to 3 days (Fig. 5C). Additionally, it was confirmed that the recombinant LPLA2 was incorporated into wild-type mouse AMs as observed in LPLA2−/− mouse AMs and that the level of phospholipid of the wild-type mouse AMs was almost unchanged by the LPLA2 treatment (data not shown). Furthermore, thin layer chromatography revealed that all accumulated phospholipids in LPLA2−/− mouse AMs were diminished by treatment with the recombinant LPLA2 (Fig. 5E). In all LPLA2-treated cases, the profile of phospholipids is quite similar to that of wild type (Fig. 5A).

The active catalytic site of LPLA2 is necessary for a decrease in accumulated phospholipids in LPLA2−/− mouse AMs

We sought to determine whether the enzymatic activity of LPLA2 incorporated is required to reduce the accumulated phospholipids in LPLA2−/− mouse AMs. The LPLA2-deficient cells were treated with recombinant mouse LPLA2 with or without a site directed mutation at the catalytic site. In the mutated LPLA2, the catalytic serine residue was converted to alanine (7). As reported previously, the wild-type mouse LPLA2 and the mutated mouse LPLA2 can be transiently expressed in transfected COS-7 cells. The soluble fraction prepared from the transfected cells was used for the treatment. Before initiating the enzyme treatment, immunoblotting was used to confirm that the expression level of LPLA2 protein found in the soluble fraction prepared from wild-type LPLA2 transfectants was very similar to that observed in the mutated LPLA2 transfectants (data not shown). LPLA2−/− mouse AMs were treated for 1 day with the same amount of protein of soluble fraction. A statistically significant decrease of phospholipid was measured in the AMs treated with the wild-type LPLA2 compared with vector alone (p = 0.0048) or to no treatment (p = 0.007, Fig. 6A).

![FIGURE 5. Enzyme replacement treatment with recombinant LPLA2 for LPLA2 deficient mouse AMs. A, Phospholipid accumulation in AMs obtained from 3-mo-old wild-type and LPLA2−/− mice. The homogenates (30 μg of protein) prepared from AMs of 3-mo-old wild-type and LPLA2−/− mice were used for lipid extraction. Lipid extracts were applied to an HPTLC plate and separated in a solvent system consisting of chloroform/methanol/acetic acid/water (40/15/15/10, v/v). BMP, PG, PE, PS, PC, PI, and SM indicate bis(monoacylglycerol)-phosphate, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, phosphatidylinositol, and sphingomyelin, respectively. B, Electron micrographs of AMs obtained from 3-mo-old wild-type and LPLA2−/− mice. C, LPLA2−/− mouse AMs were seeded into 6-well plates (106 cells/well) and treated with or without recombinant mouse LPLA2 for 1 and 3 days. After the treatment, the cells were collected and cellular lipid extraction was conducted. D, LPLA2−/− mouse AMs seeded as described in C were treated with or without 1, 3 and 10 μg/ml recombinant mouse LPLA2 for 1 day. The homogenates (30 μg of protein) prepared from each treated-AMs were used for lipid extraction. E, Lipid extracts were applied to an HPTLC plate and separated in a solvent system consisting of chloroform/methanol/acetic acid/water (45/15/15/10, v/v). BMP, PG, PE, PS, PC, PI, and SM indicate bis(monoacylglycerol)-phosphate, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, phosphatidylinositol, and sphingomyelin, respectively.](http://www.jimmunol.org/content/181/6/7879/F5)

![FIGURE 6. Active catalytic site of LPLA2 is essential for reducing the accumulated phospholipid in LPLA2−/− mouse AMs. A, LPLA2-deficient mouse AMs were treated with or without the same amount of protein of the soluble fraction (16 μg protein/ml) obtained from COS-7 cells transfected with pcDNA3 (Vector), pcDNA3-tagged-mouse LPLA2 (LPLA2), or pcDNA3-tagged-mutated mouse LPLA2 (LPLA2, mut) for 1 day. In the LPLA2, mut, a putative catalytic serine residue was replaced with an alanine residue. The expression level of LPLA2 was similar to that of the mutated mouse LPLA2. After the treatment, the cells were collected and cellular lipids were extracted. B, Transacylase activity. The cell homogenate (12 μg of protein) prepared from the AMs treated in A was incubated for 60 min at 37°C in citrate buffer (pH 4.5) with 40 μM NAS incorporated into phospholipid liposomes (PC/sulfatide phosphate/NAS (10/1/3.2 in molar ratio).](http://www.jimmunol.org/content/181/6/7879/F6)
The soluble fraction obtained from the mutated LPLA₂ transfec-
tants failed to decrease the accumulated phospholipid in the AMs (Fig. 6A). Additionally, a sizable enzymatic activity of LPLA₂ was
detected in the homogenate prepared from the AMs treated with
the soluble fraction from wild-type LPLA₂ transfecteds (Fig. 6B).

Discussion

LPLA₂ was originally identified by subtraction cloning in a model
of foam cell formation in a macrophage cell line (6). The enzyme,
originally termed LYPLA₃, was characterized as a lysospho-
lipid lipase. We independently identified the protein and gene as a
ceramide acyltransferase with an acidic pH optimum (3, 5). In the
absence of ceramide or lipophilic alcohol, the enzyme behaved as
an acid Phospholipase A₂. Because the Phospholipase A₂ activity
of the gene product is several orders of magnitude greater than the
lysospholipid lipase, we have argued that the more appropriate
appellation for the enzyme is lysosomal Phospholipase A₂.

Among the Phospholipase A₂, LPLA₂ has been assigned as
the first member of a new group among this superfam-
ily, group XV (16). LPLA₂ is distinct from the fourteen other groups of
Phospholipase A₂ in having an acidic pH optimum and a lysosomal
intracellular localization. However, the transacylase activity ob-
served in LPLA₂ is a characteristic of the group IV and VI Phos-
pholipase A₃, as well, although the acceptor specificity differs.

Several secreted Phospholipase A₂ have been identified. These
enzymes are primarily characterized by small molecular masses
(13–19 kDa) and by the presence of five to eight disulfide bonds.
These enzymes require millimolar levels of calcium for catalysis
and use an active site histidine. A lone exception in terms of the
molecular mass is the human and murine group III secreted
Phospholipase A₂ (17). This 55 kDa protein contains a group III
domain. The enzyme is characterized by a pH optimum of 8, a
substrate preference for phosphatidylglycerol, and calcium
dependence.

In the present study, we report that LPLA₂ is also a secreted
high m.w. Phospholipase A₂, but of lysosomal origin. Four previ-
ously published observations lend credence to this finding. First,
an early study reported that peritoneal macroages secrete a pho-
spholipase A₂ activity in response to zymosan characterized by an
acidic pH optimum (1). Second, recent studies on the mannose-6-
phosphate plasma and urine glycoproteomes reported that LPLA₂
is recovered in both types of biological fluids (18, 19). Third,
LPLA₂ was identified in the Secreted Protein Discovery Initiative
to be secreted (20). Fourth and finally, in the original paper de-
scribing the cloning of LYPLA₃, protein was detected in human
plasma in association with HDL lipoprotein (6). Based on these
reports and the observation that 1-O-acylceramide transacylase
activity was easily recovered in the cell medium of LPLA₂
transfected cells, we sought to determine whether macroages secreted
LPLA₂ in a regulated fashion.

We have previously observed that Phospholipase A₂ transacy-
lase activity can be measured in the medium of LPLA₂ transfected
COS-7 cells, THP-1 cells treated with PMA, and lung bronchoal-
veolar lavage cell-free fluid. The Phospholipase A₂ transacylase
activity that is detected under acidic conditions is specific for
LPLA₂ because LPLA₂ mouse tissues, organs and macro-
phages lack this particular Phospholipase A₂ transacylase activity
(9). In addition, in mouse AMs >95% of the total acid Phospho-
lipase A₂ activity is attributable to LPLA₂ (12). Therefore, we
postulated that the acid Phospholipase A₂ released from AMs by
phagocytic stimuli is LPLA₂.

In the present study we applied the ceramide transacylase ac-
tivity assay, immunohistochemistry, and immunoblotting using a
specific Ab against mouse LPLA₂ to identify intracellular and ex-
tracellular LPLA₂. These experiments confirmed that LPLA₂ in
mouse AMs localizes to endosomes as well as lysosomes and is
secreted when stimulated with zymosan. The enzyme assay dem-
strated that LPLA₂ activity is lost from AMs treated with zy-
mosan and is quantitatively recovered in the cultured medium.
This indicates that LPLA₂ is unlikely to be either transcriptionally
or translationally regulated during the treatment with zymosan.
Collectively, these data support the conclusion that the lysosomal
acid Phospholipase A₂ secreted from the zymosan-treated AMs is
LPLA₂.

The physiological role of LPLA₂ released from the AMs is spec-
ulative. The creation and phenotyping of an LPLA₂ null mouse by
two groups provides some potential clues. The LPLA₂ knockout
mouse reported by our group was characterized by the onset within
3 mo of a foam cell phenotype in the alveolar macrophages and a
modest accumulation of pulmonary surfactant (9). These findings
are consistent with a role for LPLA₂ in the catabolism of pulmo-
nary surfactant, a substance containing high quantities of phos-
phatidicholine and phosphatidylethanolamine, the primary sub-
strates for LPLA₂. Additionally, the lungs demonstrated a
mononuclear cell associated perivascular and peribronchial inflam-
mation. In mice older than 12 mo of age, splenomegaly and
hepatomegaly was observed in association with the conversion of
resident macrophages to foam cells within these organs. These data
suggest that LPLA₂ may have a role in long term immuno-
regulation. Taniyama and coworkers also described an LPLA₂ knockout
mouse (21). These mice, when bred on an apoE null background
and fed a high fat diet, displayed an increase in the area of the
aortic tree associated with atherosclerosis.

The mechanisms underlying these phenotypic changes are un-
known. Although the knockout study supports a role for LPLA₂ in
the intracellular degradation of phospholipids, it remains possible
that LPLA₂ may have a role in the extracellular catabolism of
phospholipids as well. Although the pH of most extracellular fluids
is in the neutral range, extracellular pH may become acidic during
ischemia or inflammation, such as with a consolidating pneumo-
nia. Under normal physiological conditions, the extracellular pH
may also decrease with the selective secretion of protons into a
restricted pericellular compartment. Under such conditions,
LPLA₂ might degrade phosphatidyicholine or phosphatidylethano-
lamine forming the respective lysophospholipids and free fatty
acid. Lyso-phosphatidicholine, in particular, is recognized as an
important ligand for G protein coupled receptors (22). Free fatty
acids, notably arachidonic acid, may be biologically important as
well. An older study on the activation of cytosolic and lysosomal
Phospholipase A₂ suggests that the latter may be important in zy-
mosan-stimulated arachidonic and eicosanoid formation (23).
With the identification of LPLA₂ as the zymosan-stimulated, ly-
sosome-associated Phospholipase A₂, these findings should be
reevaluated.

Most lysosomal enzymes are mannose-conjugated glycoproteins
that are recovered via mannose and/or mannose 6-phosphate re-
ceptors on the cells. For example, α-galactosidase and acid spin-
gomylase are incorporated into cultured cells and organs via
mannose 6-phosphate receptors on the plasma membrane (11). Be-
cause LPLA₂ is a high mannose type glycoprotein, the binding and
reincorporation of secreted LPLA₂ by AMs via such carbohydrate
binding receptors was not surprising.

In the present study, recombinant mouse LPLA₂ was produced
by HEK-293 cells and confirmed to be an N-glycoprotein by en-
doglycosidase F1 digestion (data not shown). This recombinant
mouse LPLA₂ was efficiently taken up to LPLA₂ mouse AMs
and significant fraction of the LPLA₂ taken to the cells displayed
limited degradation at the C-terminal region. The truncated LPLA₂.
was associated with a m.w. of 45 kDa and retained enzymatic activity. Immunofluorescent staining revealed that the distribution of LPLA₂ incorporated into LPLA\textsubscript{2-/-} mouse AMs was similar to that of endogenous LPLA₂ in the wild-type AMs. Additionally, we observed that the LPLA₂ incorporated into the cells colocalized with Lamp-1, which localizes to late endosomes as well as lysosomes in AMs. The LPLA₂ not associated with Lamp-1 may also localize with early endosomes since the colocalization of LPLA₂ with an early endosome marker, EEA1, was observed in the wild-type AMs. These data indicate that the extracellular LPLA₂ is internalized via carbohydrate receptor mediated endocytotic pathway and targeted to acidic compartments including lysosomes and late endosomes.

As expected, the uptake of extracellular LPLA\textsubscript{2} by LPLA\textsubscript{2-/-} mouse AMs was significantly inhibited by mannose and α-methyl-mannoside but not mannose 6-phosphate. The 10 mM concentration used for the competition study was insufficient in completely blocking uptake, suggesting that the affinity between LPLA₂ and the mannose receptor is very high. Because AMs express mannose receptors, which recognize mannose-terminated glycoproteins, the predominant uptake pathway for extracellular LPLA₂ in AMs is likely via these receptors.

In lung, 20–30% of the pulmonary surfactant pool is taken up by AMs, sorted to lysosomes, and catabolized (24). Therefore, the defect of this catabolic pathway associated with loss of LPLA₂ activity results in phospholipidosis in those cells. Because LPLA\textsubscript{2-/-} mouse AMs demonstrate an early and robust foam cell phenotype, we explored whether recombinant LPLA₂ could rescue this phenotype in LPLA\textsubscript{2-/-} mouse AMs, LPLA\textsubscript{2-/-} mouse AMs treated with the recombinant mouse LPLA₂ showed a marked reduction of the accumulated cellular. Interestingly, the accumulated phospholipids in the LPLA\textsubscript{2-/-} mouse AMs gradually decreased with time even without any treatments. This observation possibly means that other phospholipases besides LPLA₂ are involved in catabolism for the accumulated phospholipids in the LPLA\textsubscript{2-/-} mouse AMs. Alternatively, AMs in culture might clear lysosomal phospholipids through exocytosis.

The phospholipid levels of the LPLA\textsubscript{2-/-} mouse AMs reached that of wild-type AMs by 24 h of exposure to recombinant LPLA₂. These levels did not change further after 24 h. A similar effect was observed when lower concentrations of the extracellular LPLA₂ (0.1–0.3 μg/ml in medium, data not shown) were used. These data suggest that the LPLA₂, taken up by the AMs degrades accumulated phospholipids, but does not result in the degradation of non-lysosomal associated phospholipid. Additionally, the rescue study using a catalytically inactive LPLA₂ demonstrated that enzymatically active LPLA₂ is essential for the reduction of the accumulated phospholipids. Thus, the extracellular LPLA₂ incorporated into AMs was translocated to the lysosome and retained its enzymatic activity.

The present study confirms that LPLA₂ in AMs is both a secreted enzyme as well as a lysosomal enzyme. LPLA₂ is released from AMs in response to zymosan. Additionally, once present in the extracellular compartment, LPLA₂ is incorporated into cells via a mannose receptor, internalized and translocated into acidic compartments, including endosomes and lysosomes, by a receptor-mediated endocytotic pathway. Finally, the enzyme rescue study indicates that the enzyme taken to the cell retains its catalytic function.

**Disclosures**

The authors have no financial conflict of interest.

**References**


