

# **Lipin-1 Integrates Lipid Synthesis with Proinflammatory Responses during TLR Activation in Macrophages<sup>1</sup>**

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## Abstract

Lipin-1 is a  $Mg^{2+}$ -dependent phosphatidic acid phosphatase involved in the *de novo* synthesis of phospholipids and triglycerides. Using macrophages from lipin-1-deficient animals and human macrophages deficient in the enzyme, we show in this work that this phosphatase acts as a proinflammatory mediator during TLR signaling and during the development of *in vivo* inflammatory processes. After TLR4 stimulation lipin-1 deficient macrophages showed a decreased production of diacylglycerol, activation of mitogen-activated protein kinases and AP-1. Consequently, the generation of proinflammatory cytokines like IL-6, IL-12, IL-23, or enzymes like iNOS and COX-2 was reduced. Also, animals lacking lipin-1 had a faster recovery from endotoxin administration concomitant with a reduced production of harmful molecules in spleen and liver. These findings demonstrate an unanticipated role for lipin-1 as a mediator of macrophage proinflammatory activation and support a critical link between lipid biosynthesis and systemic inflammatory responses.

## Introduction

Macrophages are highly plastic phagocytic cells responsible for the maintenance of physiological homeostasis due to their ability to clear pathogens and influence the behavior of other immune cells (1). They are activated by pathogen associated molecular patterns through specific receptors to generate an inflammatory response characterized by a cascade of cytokines and molecules that limit ongoing infection or tissue damage. Excessive macrophage activation responses, however, can lead to acute pathological diseases, best exemplified by sepsis, or chronic disorders such as arthritis, asthma, atherosclerosis or diabetes type 2 (1).

Engagement of Toll-like receptors (TLR), and specifically TLR4, by molecular patterns like lipopolysaccharide (LPS) triggers a cascade of signaling events conducted by kinases and adaptor proteins that culminates in the phosphorylation and activation of the mitogen activated protein kinase (MAPK)<sup>4</sup> family of proteins and the family of kinases for the NF- $\kappa$ B transcription factor inhibitors I $\kappa$ B (2-4). MAPKs are responsible for the phosphorylation and activation of proteins that are part of the transcription factor AP-1, while the phosphorylation of I $\kappa$ B inhibitors promotes their proteasomal degradation, thereby releasing active NF- $\kappa$ B proteins. AP-1 and NF- $\kappa$ B are involved in the transcription of multiple proinflammatory genes including IL-6, IL-12, IL-23, and TNF- $\alpha$  (5).

LPS-stimulated macrophages accumulate triacylglycerol (TAG) molecules, which are used to meet the increased demands of energy of these highly active cells (6, 7). TAG is formed by acylation of part of the DAG generated via the Kennedy pathway of phospholipid synthesis (8). The enzymes that directly generate diacylglycerol (DAG), in the Kennedy pathway, display phosphatidic acid phosphatase activity are a family known as the lipins (9, 10). We have previously shown that a member of this family of enzymes, lipin-1, is expressed in human macrophages, and regulates eicosanoid production and size and number of TAG-loaded lipid droplets (11). However, little is known about the role of lipin-1 during macrophage activation through TLRs. Questions on the possible involvement of lipin-1 on the production of DAG during TLR stimulation, the role of lipin-1 during TLR signaling, whether lipin-1 activity modulates proinflammatory gene upregulation, and whether lipin-1 influences inflammatory

conditions *in vivo* remain unanswered. Here we have used genetic and omics approaches to determine the role of lipin-1 during TLR activation of macrophages. We report that lipin-1 contributes positively to macrophage stimulation through TLR4, and other TLRs, by impacting on MAPKs and AP-1 activation and as a consequence, on the generation of proinflammatory factors during *in vitro* and *in vivo* models of inflammation. Thus, lipin-1 connects lipid synthesis with macrophage proinflammatory activation.

## Materials and Methods

### *Animals*

BALB/cByJ-*Lpin1*<sup>fld</sup>/J mice carrying a spontaneous mutation in the *Lpin1* gene (fatty liver dystrophy, *fld*) (9, 12) were purchased from The Jackson Laboratory and bred in the Service of Animal Research and Welfare of the University of Valladolid. Males and females of *Lpin*<sup>fld/+</sup> genotype were bred to generate *Lpin*<sup>fld/fld</sup> (hereinafter *fld*), *Lpin*<sup>fld/+</sup> and wild type (wt) sibling animals. Mice were housed in filter-top cages and were provided with sterile water and food ad libitum (Global diet 2014, Harlan). 12-Week old sex-matched animals were used for experimentation. All the protocols and procedures were approved by the Institutional Animal Care and Usage Committee and are in accordance with the Spanish and European Union guidelines for the use of experimental animals.

### *Reagents*

LPS from *Escherichia coli* 0111:B4, 1,2-diheptadecanoyl-sn-glycerol and the antibody against  $\beta$ -actin were obtained from Sigma-Aldrich. Antibodies against  $\text{I}\kappa\text{B}\alpha$ ,  $\text{I}\kappa\text{B}\beta$ , phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, phospho-p44/42 MAPK (Thr202/Tyr204), p44/42 MAPK, phospho-SAPK/JNK (Thr183/Tyr185), SAPK/JNK and phospho-c-Jun were purchased from Cell Signaling. Antibodies against the nuclear protein p84 were from Abcam. Antibodies against iNOS were from BD Biosciences and anti-COX-2 from Cayman. The rabbit antiserum against lipin-1 was previously described (11). PE-conjugated antibodies against CD11b, APC-conjugated antibodies against F4/80, FITC-conjugated antibodies against Gr1 and 7-amino-actinomycin D were purchased from ebiosciences. Specific *ON-Target plus* siRNAs against murine mRNAs were obtained from Dharmacon (Thermo Scientific). *Silencer<sup>®</sup> Select* siRNAs specific to decrease the expression of human lipin-1 mRNA and negative controls were purchased from Ambion.

### *Cells*

To obtain peritoneal macrophages, the peritoneal cavity was flushed twice with 5 ml of

ice-cold PBS. Resident cells were centrifuged for 10 min at 300 x g, and allowed to adhere to plastic for 18 h in RPMI 1640 medium containing 10% fetal bovine serum. Non-adherent cells were washed away, and attached cells were maintained in culture until use.

To obtain bone marrow-derived macrophages (BMDMs), intact femurs and tibias were aseptically dislocated from the hind legs of the mice. The marrow was flushed with 5 ml of PBS using a 25 g sterile needle. After filtration, bone marrow cells were centrifuged for 10 min at 300 x g, cultured and differentiated according to the procedure described by Johnson *et al.* (13). Briefly, the cells were cultured in growth medium supplemented with 20% supernatant of the mouse L929 cell (conditioned medium) and cultured for 7 days. The medium was changed at day 4, washing out the non-adherent cells and differentiation was continued for a total of 7 days.

Neutrophils were isolated from bone marrow using a Percoll<sup>®</sup> gradient as described (14). T and B lymphocyte populations were isolated from spleen using nylon wool columns (15). Briefly, splenocytes were depleted of erythrocytes by ammonium chloride-mediated lysis. Cells were then incubated in plastic plates at 37°C and 5%CO<sub>2</sub> for 2 h. Non-adherent cells containing mainly B and T cells were further subjected to nylon wool (Polysciences, Inc.) purification, allowing them to adhere to the column at 37°C and 5%CO<sub>2</sub> in RPMI for 1 hour. Non adherent T cells were eluted with warm RPMI, and B cells were also collected afterwards by adding cold media, knocking the column to dislodge binding cells. The cell surface markers of the resulting populations were routinely monitored.

Human macrophages were obtained from blood monocytes and transfected using the nucleofection technique (Amaxa), as previously described (11, 16-18).

#### *Measurement of DAG mass*

A cell extract corresponding to 1 mg of protein was used and, before the extraction and separation of lipid classes, 1,2-diheptadecanoyl-sn-glycerol was added as an internal standard. Total lipids were extracted according to Bligh and Dyer (19), and the resulting lipid extract was separated by thin-layer chromatography using n-

hexane/diethyl ether/acetic acid (70:30:1, by vol.) as the mobile phase. Spots corresponding to the various lipid classes were scraped, and DAG was extracted from the silica with 1 ml chloroform/methanol (1:1, v/v) followed by 1 ml of chloroform/methanol (2:1, v/v). The DAG fraction was transmethylated with 500 µl of 0.5 M KOH in methanol for 45 min at 37°C and a volume of 0.5 M HCl was added before extracting twice with 1 ml of n-hexane. 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 m length × 250 µm internal diameter × 0.15 µm film thickness) under the conditions established previously (20-22). The amount of DAG mass in each sample was calculated by adding the molar masses of all fatty acids measured and dividing by 2. Data analysis was carried out with the Agilent G1701EA MSD Productivity Chemstation software, revision E.02.00.

#### *Real time PCR*

Total RNA from mouse tissues and cells was extracted using RNeasy Mini Kit (Quiagen) and Trizol reagent (Ambion) respectively. The cDNA templates were synthesized using M-MLV Reverse Transcriptase (Ambion) following the manufacturer instructions. Quantitative real time (RT)-PCR analysis was performed in a LightCycler® 480 (Roche) as previously described (18) using specific primers obtained from the Primer Bank data base (23).

#### *Cytokine determination and serum analysis*

Supernatants from activated cells or serum from LPS-treated animals were used for quantification of IL-6, IL12p40, IL2p70 and IL23p19 by specific ELISA kits (eBioscience) following the manufacturer's instructions. Urea, blood urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) measurements in serum were kindly performed by Laboratorios Echevarne (Barcelona, Spain).

### *LPS treatment of animals*

Mice were intraperitoneally injected with LPS at a lethal dose of 25 mg/kg or a sublethal dose of 1.5 mg/kg and monitored for 10 days. For the analysis of proinflammatory factors some animals were sacrificed by ketamine (100 mg/kg): xylazine (10 mg/kg) administration and cervical dislocation 6 hours after LPS treatment. Blood was collected through cardiac puncture or from the facial vein. Livers and spleens were collected in RNAlater (Ambion) for further analysis by real-time PCR.

### *Microarray gene expression*

Peritoneal macrophages from four different control (*Lpin<sup>fld/+</sup>*) and *fld* (*Lpin<sup>fld/fld</sup>*) male animals were separately stimulated with 100 ng/ml LPS for 5 h and RNA was isolated using Trizol reagent (Ambion). Labeled RNA was hybridized overnight (17 h, 65°C) to Agilent Whole Mouse Genome Oligo Microarrays 4x44K using Agilent recommended protocol. After extensive washing, fluorescence signals were detected using Agilent Microarray Scanner System (Agilent Technologies). The Agilent Feature Extraction Software (FES) was used to read out and process the microarray image files and the Rosetta Resolver<sup>®</sup> gene expression data analysis system (Rosetta Biosoftware) was used for further analysis. The ratios represent comparisons to a common artificial reference in which all untreated samples are included (control and *fld*). For selection genes were required to be at least 1.7-fold change up- or downregulated with an associated p-value of 0.01 in relation with the reference. All microarray data have been deposited into the Gene Expression Omnibus (GEO) database (accession number GSE54155).

### *Flow cytometry*

Cells from whole spleen or peritoneal lavage were incubated with antibodies against CD16/CD32 (eBiosciences) to block nonspecific antibody binding to Fc receptors. Cells were then stained with PE-conjugated rat anti-mouse CD11b IgG2b, APC-conjugated rat anti-mouse F4/80 IgG2a and FITC-conjugated rat anti-mouse Gr1 IgG2b (ebiosciences). Isotype control antibodies were used to subtract background staining. Staining with 7-amino-actinomycin D was also performed to exclude nonviable cells

during the analysis. Data collection was performed in a Beckman Coulter Gallios flow cytometer and data analyses were performed using the Kaluza software.

#### *DNA Binding Assays*

The DNA binding activity of nuclear c-Jun was assayed by a commercial kit (Active Motif) following the manufacturer's instructions.

#### *Constructs and Transfections*

Lipin1 $\beta$ -EGFP plasmid was constructed by introducing the cDNA sequence of the mouse *Lpin1b* (Thermo Scientific, clone 4211202) in the EGFP expression vector pEGFP-N3 (Clontech) by using EcoRI and Sall restriction enzymes. The primers used were as follows: 5'-CACACAGAATTCAATGAATTACGTGGGGCAGC-3' and 5'-CACACAGTCGACAGCTGAGGCTGAATGCATGT-3. Confirmation of the correct insertion of the cDNA was performed by sequencing. Plasmids (EGFP or lipin1-EGFP) were transfected into BMDMs by using the Nucleofection method, and the kit specifications for murine macrophages were followed. 3  $\mu$ g of plasmid was used and the program was Y-001.

#### *Statistical analysis*

Data are represented as means  $\pm$  standard error of the mean (SEM). Statistical significance was determined by Student's t test.  $p < 0.05$  was considered statistically significant.

## Results

### *Lipin-1 deficient macrophages have a decreased inflammatory gene expression after TLR4 stimulation*

We began this study by analyzing the expression levels of lipin-1 in immune cells and tissues. The highest levels of lipin-1 were present in peritoneal macrophages and spleen, both at the mRNA and protein levels (Figs. 1A and 1B). The absence of lipin-1 altered neither the overall percentage of macrophages in peritoneal cells and spleen nor the percentage of cells in the blood of *fld* animals (Supplemental Fig. 1). Next, we evaluated whether the expression of lipin-1 was affected by TLR activation in macrophages. Figure 1C shows that although the mRNA levels rapidly decreased within the first 4 h after TLR4 activation by LPS, lipin-1 protein levels were still maintained after 24 h of treatment. Protein levels did not change between 0 and 12 h, nor did mRNA levels change between 12 and 24 h.

To assess whether lipin-1 is involved in macrophage responses to TLR4, peritoneal cells from wt and *fld* animals were stimulated with LPS, and mRNA abundance of the proinflammatory factors *Il6*, *Il12b* (p40), *Nos2* and *Cox2* were analyzed by quantitative PCR. The results showed that *fld* macrophages increased the mRNA levels for all those factors to a lesser extent than wt macrophages (Fig. 2A). To rule out the possibility that the diminished response of *fld* macrophages to TLR4 stimulation occurs because of alterations in the *in vivo* differentiation process due to the absence of lipin-1, experiments were also conducted using the macrophage-like cell line RAW 264.7. In these cells, lipin-1 was knocked down by siRNA technology. Again, the absence of lipin-1 led to reduced responses to LPS (Fig. 2B). A more exhaustive analysis in bone marrow-derived macrophages (BMDMs) indicated that many inflammation-related genes were affected in *fld* derived cells, including *Il12a* (p35), *Il23a* (p19), *Tnfa*, and *Ifng* (Fig. 2C). These results were also corroborated by analysis of protein levels in the supernatants or cell homogenates of TLR4-activated cells (Fig. 2D and E). The observed responses were not due to a lower expression of TLR4 in the *fld* macrophages, as the latter cells expressed TLR4 at levels identical to wt cells (data not shown). Also, no differences in macrophage cell surface markers were found between wt and *fld* BMDMs, suggesting no differences in their level of

differentiation (Supplemental Fig. 2). Collectively, these results suggest that lipin-1 is centrally involved in the inflammatory response to TLR4 occupancy in macrophages.

*Lipin-1 deficient macrophages show a distinctive gene expression pattern after TLR4 stimulation.*

To obtain a broader view of lipin-1 regulated genes during LPS activation, gene expression was analyzed by RNA microarrays in BMDMs stimulated with LPS during 5 h (Fig. 3). A total of 1640 genes were upregulated by LPS in control macrophages while 1519 genes were upregulated in *fld* cells. Of note, only 943 genes were upregulated in both types of cells indicating a qualitative differential response between control and *fld* macrophages to LPS. The same behavior was observed on examination of downregulated genes, where only 1009 genes were downregulated in both types of cells, while 1044 genes were downregulated exclusively in control macrophages and 780 genes in *fld* macrophages. Genes differentially regulated in *fld* macrophages are involved in processes such as receptor signaling, G-protein signaling, transcription, lipid metabolism, cell adhesion, and extracellular matrix, among others (Fig. 3 and Supplemental Table 1), which indicates that lipin-1 might be relevant not only for the proinflammatory response, but also for other processes during the macrophage response to pathogens.

*Lipin-1 deficient macrophages have altered responses to other TLRs*

To assess whether signaling through other TLRs is also affected in *fld* macrophages, experiments were conducted using the TLR3 ligand poly(I:C) and the TLR1/2 ligand Pam3CSK4. Evaluation of the expression of proinflammatory genes showed that mRNA levels for *Il6* and *Il13a* were lower in poly(I:C)-stimulated *fld* macrophages while *Nos2* and *Cox2* were not affected (Fig. 4A). Also, *fld* cells stimulated with Pam3CSK4 exhibited lower mRNA levels for *Il6*, *Il23a* and *Nos2* than wt cells (Fig. 4B). We also examined the effects of non classical TLR4 activators such as the saturated fatty acid palmitic acid (24). In line with our findings with LPS, the effects of palmitic acid were diminished in *fld* cells (Fig. 4C). Thus, these results suggest that the diminished inflammatory response of *fld* macrophages is not restricted to a particular TLR but rather works at a more general level during cell activation.

### *Lipin-1 deficient macrophages exhibit altered TLR4 signaling*

We next evaluated whether TLR4 signaling pathways are affected in lipin-1 deficient cells. As illustrated in Fig. 5, *fld* macrophages responding to LPS showed a reduced phosphorylation of the MAPK family members p44/p42 ERKs, JNK and p38 (Fig. 5A, B). Of note, phosphorylation and nuclear activity of the downstream protein c-jun, which forms part of the transcription factor AP-1, was also diminished in LPS-stimulated macrophages from *fld* mice (Fig. 5C, D). We also examined the degradation of the NF- $\kappa$ B inhibitors, I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  in *fld* macrophages, and found no significant differences when compared to wt cells (Fig. 5E). Hence, these data suggest that diminished activation of MAPK/AP-1, but not NF- $\kappa$ B, is responsible for the reduced proinflammatory behavior observed in *fld* macrophages. Therefore, not all TLR4-mediated signaling seems to be affected by lipin-1 depletion, but only the branch that is downstream to MAPK activation.

Because the enzymatic activity of lipin-1 produces DAG, experiments were conducted next to evaluate the involvement of lipin-1 in DAG generation during TLR4 activation. DAG production by LPS in *fld* and wt macrophages was analyzed by mass spectrometry. In wt macrophages, LPS stimulation promoted a time-dependent increase in total cellular DAG mass (Fig. 5F). Importantly, in *fld* cells the opposite situation occurred, and DAG levels not only did not increase but actually decreased after LPS activation, thus suggesting that in the absence of lipin-1 the degradation of DAG and/or its conversion to other products predominates over its accumulation (Fig. 5F). Collectively, these data are consistent with lipin-1 being a major mediator of early DAG generation in LPS activated macrophages.

Experiments were also conducted to assess whether expressing lipin-1 in *fld* macrophages restores the proinflammatory responses of these cells. *Fld* BMDMs were transiently transfected with a plasmid encoding for lipin-1, and ERK-1/2 phosphorylation and iNOS expression was analyzed by immunoblot. The results, as shown in Fig. 6, clearly show that lipin-1 expression restored these two responses.

Analysis of LPS-stimulated DAG production in the moreover, transfecting the cells with lipin-1 also restored the DAG response to LPS

t Thus, transfecting in lipin-1 also restores DAG synthesis and the DAG response to LPS

#### *Lipin-1 deficient mice have a quicker recovery from endotoxin treatment*

To define the *in vivo* implication of the above findings, the effect of an i.p. administration of LPS was evaluated in animals. At high doses of LPS (25 mg/Kg) there were not significant differences in mortality rates between wt and *fld* animals (Fig. 7A). However, at lower LPS doses (1.5 mg/Kg), which did not provoke death, *fld* animals had a faster weight recovery than wt animals (Fig. 7B). While *fld* animals returned to their original weight after 6 days of treatment, wt animals needed longer than 10 days to recover, suggesting that *fld* animals resolve the injury faster. Furthermore, analyses of urea, BUN, creatinine, AST and ALT in serum from *fld* versus wt animals showed reduced levels in the former, thus suggesting a less pronounced organ damage (Fig. 7C).

Analysis of the blood content of proinflammatory cytokines in LPS-treated animals showed a substantial decrease in the content IL-6, IL-12p40 and IL-12p70 in *fld* animals compared with control ones (Fig. 8A). A more exhaustive investigation of proinflammatory gene expression performed in the liver and the spleen of treated animals showed that many of those genes were less up-regulated in *fld* animals (Fig. 8B, C). Significantly decreased levels of the proinflammatory protein iNOS, were also detected in peritoneal cells of LPS-exposed *fld* animals (Fig. 8D).

At the cellular level, we noticed that animals without treatment showed no differences in the percentage of total CD11b<sup>+</sup> cells in spleen. However, we found that the absence of lipin-1 had opposite effects on splenic CD11<sup>+</sup>F4/80<sup>-</sup>Gr1<sup>+</sup> and CD11b<sup>+</sup>F480<sup>+</sup>Gr1<sup>+</sup> populations, decreasing the former (neutrophils) and increasing the later (inflammatory monocytes) (Fig. 9A). On the other hand, spleens from LPS-treated animals showed an increase in CD11b<sup>+</sup> cell populations that was significantly higher in *fld* animals (Fig. 9A) than in wt animals. A more detailed analysis showed that the behavior of F4/80 and Gr1 expressing cells was very similar in wt and *fld* animals, except for the CD11b<sup>+</sup>F4/80<sup>+</sup>Gr1<sup>+</sup> cells, whose recruitment was significantly lower in the spleens of *fld* animals (Fig. 9A and Supplemental Fig. 3). Macrophages (CD11b<sup>+</sup>F4/80<sup>+</sup>Gr1<sup>-</sup>) decreased their presence in LPS-treated spleens in the same manner in wt and *fld* animals (Fig. 9A and Supplemental Fig. 3). These observations are

in agreement with a reduced recruitment of inflammatory monocytes to the spleen during LPS treatment in the absence of lipin-1.

The recruitment of immune cells to the peritoneal cavity of non-treated and LPS-treated animals was also studied and no significant differences were found in the total number or type of cells recruited in *fld* versus wt animals (Fig. 9B and C). Overall these results suggest that *fld* animals experience a reduced proinflammatory response during endotoxin treatment that protects them from excessive damage.

#### *Lipin-1 has a proinflammatory role in human macrophages*

To address whether the proinflammatory effects of lipin-1 in murine macrophages also occur in human macrophages, experiments were conducted in blood monocyte-derived human macrophages that were made deficient in lipin-1 by nucleofection of a siRNA against lipin-1 (Fig. 10A). We observed that, after LPS-treatment, the increases in mRNA levels for *IL6*, *IL12a*, *IL12b* and *IL23a* in control cells were less prominent in the lipin-1 deficient macrophages. These data are in accordance with our previous results in mice.

## Discussion

The execution of immune responses by macrophages requires an exquisite balance between effector and regulatory pathways, and perturbation of this network can result in chronic inflammation or persistent infection. Thus it is important to define the effectors that positively and negatively modulate these responses to open new avenues for the control of inflammation-related conditions. Our studies demonstrate that lipin-1 plays a key regulatory role in the generation of proinflammatory factors by mediating the activation of downstream pathways during TLRs activation by microbial components. This conclusion is supported *by in vitro* data using primary human and murine macrophages and cell lines as well as by *in vivo* observations obtained from LPS-treated mouse models. Our key findings can be summarized as follows: (i) in macrophages, lipin-1 mediates responses to LPS treatment, regulates cellular DAG levels, and mediates activation of MAPKs and proteins of the transcription factor AP-1 that ultimately coordinate the expression of proinflammatory genes (Figure 11); (ii) mRNA expression microarray analyses suggest that lipin-1 also affects other processes like G-protein signaling, transcription, cell adhesion, etc. in TLR4 stimulated macrophages; (iii) lipin-1 delays the recovery of animals to endotoxin treatment by limiting the expression of detrimental mediators; (iv) the expression levels of lipin-1 also impact on other TLRs in addition to TLR4, namely TLR3 and TLR1/2; and (v) the proinflammatory role of lipin-1 is also detected in human macrophages.

During LPS stimulation of macrophages there is an increase in intracellular DAG content that does not occur in cells lacking lipin-1, suggesting that lipin-1 is a major enzyme involved in the short-term generation of DAG in LPS-treated cells. A recent exhaustive state-of-the-art lipidomic study performed in RAW 264.7 macrophages has also demonstrated increased DAG levels during TLR4 stimulation (6). DAG may serve different roles in cells. From a lipid viewpoint, its classical role is to serve as a biosynthetic precursor of different species of phospholipids, which are important for membrane organization and signaling, and also of TAG, the main energy storage of cells. LPS-treated cells display a very robust membrane rearrangement (25, 26), hence they require high energy supply to accomplish all the remodeling that activation

encompasses, including the upregulation of many genes and proteins. In fact, TAG production is increased in activated macrophages (6, 27-29), and its hydrolysis is an absolute requirement for efficient ATP supply and macrophage functioning (30). Thus, the possibility exists that in the absence of lipin-1 macrophages may not be able to meet the necessary energy levels to fulfill all their activation requirements, resulting in the whole cell reprogramming being altered. In such a scenario, a reduction of the whole activated transcriptional program would be expected. However, the wide analysis of gene expression performed in *fld* macrophages indicates that in these macrophages the gene transcription footprint is quite different than in wt cells, and is not the result of a mere reduction of transcriptional events. On the other hand, the activation of transcriptional effectors such as NF- $\kappa$ B does not seem to be altered in the absence of lipin-1, and this is a process that also needs ATP for the phosphorylation and degradation of the NF- $\kappa$ B inhibitor, I $\kappa$ B. Thus it could be deduced from these data that the effects observed for lipin-1 may not necessarily be related with reduced energy availability, at least during the early phases of activation.

From an intracellular signaling viewpoint, DAG may bind and change the activation/localization state of many enzymes in the cell (31). Several DAG-activated enzymes are known to be required for full downstream responses during LPS activation. For example, enzymes from the protein kinase C (PKC) family such as PKC $\epsilon$  and PKC $\delta$  can associate with different adaptor proteins that are recruited to TLR4 (32-33). Activation of PKCs requires phosphorylation and enhanced levels of DAG, and the action of these kinases impacts on TLR4 downstream pathways such as MAPKs activation, which ensures full production of inflammatory factors (34). Our studies support a scenario whereby lipin-1, by regulating DAG levels and the activation of upstream effectors like PKC, regulates TLR4 downstream signaling. In this regard, we have previously shown that Mg<sup>2+</sup>-dependent phosphatidate phosphatase is involved in arachidonic acid mobilization, cyclooxygenase expression and eicosanoid formation when WISH cells are activated through PKC (35,36) and when macrophages are stimulated with lipopolysaccharide (37). Although the mechanisms for these actions are not known these results highlight a role for lipin-1 in cell signaling through

modulating PA and DAG levels. Studies are being carried out in our laboratory to further explore these possibilities.

Excessive activation of LPS-promoted responses results in sepsis and septic shock. These are systemic inflammatory conditions that constitute a major cause of morbidity and mortality in hospitalized patients. It is clear that amelioration of the exacerbated proinflammatory response would be a good strategy for their treatment. The studies presented here suggest that lipin-1 participates in the development of these acute conditions. The absence of lipin-1 clearly promotes an earlier recovery of animals treated with a low-dose of LPS. The effect seems to be related with a lower expression of harmful proinflammatory mediators, which may favor reduced tissue damage and shortens healing time. Furthermore, upregulation of enzymes that generate key factors for the development of sepsis such as iNOS and COX-2 are also decreased in *fld* animals. In this sense, we have previously reported that in human macrophages decreased expression of lipin-1 reduces the activation of group IVA cytosolic phospholipase A<sub>2</sub>, the enzyme that controls the release of arachidonic acid from phospholipids (11). Availability of free arachidonic acid is well described to constitute a limiting factor for eicosanoid production via various pathways including COX-2 (38, 39). Collectively, these results suggest that lipin-1 may impact on sepsis not only by regulating the expression of enzymes such as COX-2, but also by impacting on the levels of their substrates.

Due to the diminished inflammatory response of *fld* animals, a lower death rate during treatment with high LPS doses would be expected. However, animals die within 24 h of treatment and no differences between wt or *fld* groups are appreciated. It should be noted in this regard that recent work has shown that *fld* mice exhibit cardiac dysfunction *in vivo* (40). Such defect, together with the well-described cardiac dysfunction induced by high levels of LPS, could explain why *fld* mice are not protected against high LPS doses (40, 41).

To conclude, in this work we have unveiled a hitherto unrecognized role for lipin-1, an enzyme of lipid metabolism, in macrophage signaling and animal responses to bacterial components. The data presented here support the idea that reducing lipin-

1 levels would limit the inflammatory response and the damage that exacerbated responses during TLR activation could produce. Whether targeted modulation of lipin-1 can provide therapeutic benefits for the control of inflammatory-related conditions should be the focus of future research.

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## References

1. Wynn, T.A., A. Chawla, and J. W. Pollard. 2013. Macrophage biology in development, homeostasis and disease. *Nature* 496: 445-455.
2. Medzhitov, R., P. Preston-Hurlburt, and C. A. Janeway, Jr. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 388: 394-397.
3. Dong, C., R. J. Davis, and R. A. Flavell. 2002. MAP kinases in the immune response. *Annu. Rev. Immunol.* 20: 55-72.
4. Kawai, T., and S. Akira. 2006. TLR signaling. *Cell Death Differ.* 13: 816-825.
5. Mackman, N., K. Brand, and T. S. Edgington. 1991. Lipopolysaccharide-mediated transcriptional activation of the human tissue factor gene in THP-1 monocytic cells requires both activator protein 1 and nuclear factor kappa B binding sites. *J. Exp. Med.* 174(6):1517-1526.
6. Dennis, E. A., R. A. Deems, R. Harkewicz, O. Quehenberger, H. A. Brown, S. B. Milne, D. S. Myers, C. K. Glass, G. Hardiman, D. Reichart, A. H. Merrill, Jr., M. C. Sullards, E. Wang, R. C. Murphy, C. R. Raetz, T. A. Garrett, Z. Guan, A. C. Ryan, D. W. Russell, J. G. McDonald, B. M. Thompson, W. A. Shaw, M. Sud, Y. Zhao, S. Gupta, M. R. Maurya, E. Fahy, and S. Subramaniam. 2010. A mouse macrophage lipidome. *J. Biol. Chem.* 285: 39976-39985.
7. Huang, Y. L., J. Morales-Rosado, J. Ray, T. G. Myers, T. Kho, M. Lu, and R. S. Munford. 2013. Toll-like receptor agonists promote prolonged triglyceride storage in macrophages. *J. Biol. Chem.* 289: 3001-3012.
8. Kennedy, E. P. 1961. Biosynthesis of complex lipids. *Fed. Proc.* 20: 934-940.
9. Péterfy, M., J. Phan, P. Xu, and K. Reue. 2001. Lipodystrophy in the fld mouse results from mutation of a new gene encoding a nuclear protein, lipin. *Nat. Genet.* 27: 121-124.

10. Kok, B. P., G. Venkatraman, D. Capatos, and D. N. Brindley. 2012. Unlike two peas in a pod: lipid phosphate phosphatases and phosphatidate phosphatases. *Chem. Rev.* 112: 5121-5146.
11. Valdearcos, M., E. Esquinas, C. Meana, L. Gil-de-Gómez, C. Guijas, J. Balsinde, and M. A. Balboa. 2011. Subcellular localization and role of lipin-1 in human macrophages. *J. Immunol.* 186: 6004-6013.
12. Langner, C. A., E. H. Birkenmeier, O. Ben-Zeev, M. C. Schotz, H. O. Sweet, M. T. Davisson, and J. I. Gordon. 1989. The fatty liver dystrophy (*fld*) mutation. A new mutant mouse with a developmental abnormality in triglyceride metabolism and associated tissue-specific defects in lipoprotein lipase and hepatic lipase activities. *J. Biol. Chem.* 264: 7994-8003.
13. Johnson, C. R., D. Kitz, and J. R. Little. 1983. A method for the derivation and continuous propagation of cloned murine bone marrow macrophages. *J. Immunol. Methods* 65: 319-332.
14. Boxio, R., C. Bossenmeyer-Pourié, N. Steinckwich, C. Dournon, and O. Nüße. 2004. Mouse bone marrow contains large numbers of functionally competent neutrophils. *J. Leukoc. Biol.* 75: 604-611.
15. Julius, M. H., E. Simpson and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* 3: 645–649.
16. Casas, J., C. Meana, E. Esquinas, M. Valdearcos, J. Pindado, J. Balsinde, and M. A. Balboa. 2009. Requirement of JNK-mediated phosphorylation for translocation of group IVA phospholipase A<sub>2</sub> to phagosomes in human macrophages. *J. Immunol.* 183: 2767–2774.
17. Casas, J., M. Valdearcos, J. Pindado, J. Balsinde, and M. A. Balboa. 2010. The cationic cluster of group IVA phospholipase A<sub>2</sub> (Lys488/Lys541/Lys543/Lys544) is involved in translocation of the enzyme to phagosomes in human macrophages. *J. Lipid Res.* 51: 388-399.

18. Valdearcos, M., E. Esquinas, C. Meana, L. Gil-de-Gómez, C. Guijas, J. Balsinde, and M. A. Balboa. 2012. Lipin-2 reduces proinflammatory signaling induced by saturated fatty acids in macrophages. *J. Biol. Chem.* 287: 10894-10904.
19. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37: 911–917.
20. Astudillo, A. M., G. Pérez-Chacón, D. Balgoma, L. Gil-de-Gómez, V. Ruipérez, C. Guijas, M. A. Balboa, and J. Balsinde. 2011. Influence of cellular arachidonic acid levels on phospholipid remodeling and CoA-independent transacylase activity in human monocytes and U937 cells. *Biochim. Biophys. Acta.* 1811: 97–103.
21. Astudillo, A. M., G. Pérez-Chacón, C. Meana, D. Balgoma, A. Pol, M. A. del Pozo, M. A. Balboa, and J. Balsinde. 2011. Altered arachidonate distribution in macrophages from caveolin-1 null mice leading to reduced eicosanoid synthesis. *J. Biol. Chem.* 286: 35299-35307.
22. Guijas, C., G. Pérez-Chacón, A. M. Astudillo, J. M. Rubio, L. Gil-de-Gómez, M. A. Balboa, and J. Balsinde. 2012. Simultaneous activation of p38 and JNK by arachidonic acid stimulates the cytosolic phospholipase A<sub>2</sub>-dependent synthesis of lipid droplets in human monocytes. *J. Lipid Res.* 53: 2343–2354.
23. Wang, X., A. Spandidos, H. Wang, and B. Seed. 2012. PrimerBank: a PCR primer database for quantitative gene expression analysis, 2012 update. *Nucl. Acids Res.* 40: D1144-D1149.
24. Lee, J. Y., K. H. Sohn, S. H. Rhee, and D. Hwang. 2001. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. *J. Biol. Chem.* 276: 16683–16689.
25. Peppelenbosch, M.P., M. DeSmedt, T. ten Hove, S. J. van Deventer, and J. Grooten. 1999. Lipopolysaccharide regulates macrophage fluid phase pinocytosis via CD14-dependent and CD14-independent pathways. *Blood* 93: 4011-4018.

26. Jain, V., A. Halle, K. A. Halmen, E. Lien, M. Charrel-Dennis, S. Ram, D. T. Golenbock, and A. Visintin. 2008. Phagocytosis and intracellular killing of MD-2 opsonized gram-negative bacteria depend on TLR4 signaling. *Blood* 111: 4637-4645.
27. Kazemi, M.R., C. M. McDonald, J. K. Shigenaga, C. Grunfeld, and K. R. Feingold. 2005. Adipocyte fatty acid-binding protein expression and lipid accumulation are increased during activation of murine macrophages by toll-like receptor agonists. *Arterioscler. Thromb. Vasc. Biol.* 25: 1220-1224.
28. Posokhova, E. N., O. M. Khoshchenko, M. I. Chasovskikh, E. N. Pivovarova, and M. I. Dushkin. 2008. Lipid synthesis in macrophages during inflammation in vivo: effect of agonists of peroxisome proliferator activated receptors alpha and gamma and of retinoid X receptors. *Biochemistry (Moscow)* 73: 296-304.
29. Tannahill, G. M., A. M. Curtis, J. Adamik, E. M. Palsson-McDermott, A. F. McGettrick, G. Goel, C. Frezza, N. J. Bernard, B. Kelly, N. H. Foley, L. Zheng, A. Gardet, Z. Tong, S. S. Jany, S. C. Corr, M. Haneklaus, B. E. Caffrey, K. Pierce, S. Walmsley, F. C. Beasley, E. Cummins, V. Nizet, M. Whyte, C. T. Taylor, H. Lin, S. L. Masters, E. Gottlieb, V. P. Kelly, C. Clish, P. E. Auron, R. J. Xavier, and L. A. O'Neill. 2013. Succinate is an inflammatory signal that induces IL-1 $\beta$  through HIF-1 $\alpha$ . *Nature* 496: 238-242.
30. Chandak. P. G., B. Radovic, E. Aflaki, D. Kolb, M. Buchebner, E. Fröhlich, C. Magnes, F. Sinner, G. Haemmerle, R. Zechner, I. Tabas, S. Levak-Frank, and D. Kratky. 2010. Efficient phagocytosis requires triacylglycerol hydrolysis by adipose triglyceride lipase. *J. Biol. Chem.* 285: 20192-20201.
31. Colón-González, F., and M. G. Kazanietz. 2006. C1 domains exposed: from diacylglycerol binding to protein-protein interactions. *Biochim. Biophys. Acta* 1761: 827-837.
32. Kubo-Murai, M., K. Hazeki, N. Sukenobu, K. Yoshikawa, K. Nigorikawa, K. Inoue, T. Yamamoto, M. Matsumoto, T. Seya, N. Inoue, and O. Hazeki. 2007. Protein kinase C delta binds TIRAP/Mal to participate in TLR signaling. *Mol. Immunol.* 44: 2257-2264.

33. Faisal, A., A. Saurin, B. Gregory, B. Foxwell, and P. J. Parker. 2008. The scaffold MyD88 acts to couple protein kinase Cepsilon to Toll-like receptors. *J. Biol. Chem.* 283: 18591-18600.
34. Loegering, D. J., and M. R. Lennartz. 2011. Protein kinase C and toll-like receptor signaling. *Enzyme Res.* 2011: 537821.
35. Balboa, M. A., J. Balsinde, and E. A. Dennis. 1998. Involvement of phosphatidate phosphohydrolase in arachidonic acid mobilization in human amnionic WISH cells. *J. Biol. Chem.* 273: 7684-7690.
36. Johnson, C. A., M. A. Balboa, J. Balsinde, and E. A. Dennis. 1999. Regulation of cyclooxygenase-2 expression by phosphatidate phosphohydrolase in human amnionic WISH cells. *J. Biol. Chem.* 274: 27689–27693.
37. Grkovich, A., C. A. Johnson, M. W. Buczynski, and E. A. Dennis. 2006. Lipopolysaccharide-induced cyclooxygenase-2 expression in human U937 macrophages is phosphatidic acid phosphohydrolase-1-dependent. *J. Biol. Chem.* 281: 32978–32987.
38. Pérez-Chacón, G., A. M. Astudillo, D. Balgoma, M. A. Balboa, and J. Balsinde. 2009. Control of free arachidonic acid levels by phospholipases A<sub>2</sub> and lysophospholipid acyltransferases. *Biochim. Biophys. Acta* 1791: 1103-1113.
39. Astudillo, A. M., D. Balgoma, M.A. Balboa, and J. Balsinde. 2012. Dynamics of arachidonic acid mobilization by inflammatory cells. *Biochim. Biophys. Acta* 1821: 249-256.
40. Kok, B.P, P. C. Kienesberger, J. R. Dyck, and D. N. Brindley. 2012. Relationship of glucose and oleate metabolism to cardiac function in lipin-1 deficient (*fld*) mice. *J. Lipid Res.* 53: 105-118.
41. Tavener, S.A., E. M. Long, S. M. Robbins, K. M. McRae, H. Van Remmen, and P. Kubes. 2004. Immune cell Toll-like receptor 4 is required for cardiac myocyte impairment during endotoxemia. *Circ. Res.* 95: 700-707.

## Footnotes to the Text

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<sup>4</sup>Abbreviations used in this article: DAG, diacylglycerol; TAG, triacylglycerol; MAPK, mitogen-activated protein kinase.

## Figure Legends

**Figure 1.** Lipin-1 expression in immune tissues and cells. (A) mRNA levels from bone marrow cells, whole spleen, T cells, B cells, neutrophils and peritoneal macrophages were analyzed by qPCR using specific primers for *Lpin1* spliced variants *Lpin1 $\alpha$*  and *Lpin1 $\beta$* . Levels of *Lpin1* mRNAs were normalized to GAPDH mRNA abundance. (B) Homogenates from tissues and cells as in panel A were analyzed by immunoblot using specific antibodies against lipin-1 and  $\beta$ -actin. Peritoneal macrophages from *fld* mice were also included. A representative experiment from three performed is shown. (C) BMDMs from wt and *fld* animals were stimulated with 100 ng/ml LPS for the indicated time points and mRNA was extracted and analyzed by qPCR using specific primers that recognized both *Lpin1 $\alpha$*  and  *$\beta$* . Results were normalized by GAPDH mRNA abundance. (D) Homogenates from BMDMs treated or not with 100 ng/ml LPS for 24 h were analyzed as in panel B. Experiments were independently performed three times. Error bars represent the SEM.

**Figure 2.** Lipin-1 mediates production of proinflammatory factors after TLR4 stimulation of macrophages. (A) mRNA abundance of proinflammatory genes (as indicated) in peritoneal macrophages from wt or *fld* animals treated or not with 100 ng/ml LPS for 8 h was analyzed by qPCR. mRNA levels were normalized to GAPDH mRNA levels. (B) mRNA abundance of the indicated proinflammatory genes in RAW 264.7 cells treated with control siRNA or siRNA against *lpin1* and then with LPS as in panel A. (C) BMDM from wt or *fld* animals were treated with 100 ng/ml LPS for the indicated time points and mRNA abundance for the indicated genes was assessed as in panel A. (D) Protein levels of the indicated factors were quantified by specific ELISAs in BMDM supernatants from wt and *fld* animals after 24 h of stimulation with 100 ng/ml LPS. (E) Analysis by immunoblot of iNOS and COX-2 levels in BMDMs from wt and *fld* mice after stimulation with 100 ng/ml LPS for the indicated time points. Right graphs represent relative quantification of iNOS and COX-2 bands respect to  $\beta$ -actin. Experiments were independently performed three times in triplicates (n=6). Error bars represent the SEM. P-values by Student's t test are as indicated (\*, p <0.05; \*\* p<0.01).

**Figure 3.** Lipin-1 affects the expression of many genes during TLR4 stimulation of macrophages. (A) Microarray analysis of mRNA expression from control (*Lpin1*<sup>fl<sup>d</sup>/+</sup>) and *fld* peritoneal macrophages stimulated for 5 hours with 100 ng/ml LPS (n=4). The 100 genes with the highest difference in expression between control and *fld* macrophages are represented. Table S1 contains a detailed list of genes. To the right, some genes have been grouped in families according to their cellular role. (B) Venn diagrams of total genes up or down-regulated by LPS in control and *fld* animals.

**Figure 4.** Lipin-1 effects on TLR3 and TLR1/2 activation in macrophages. (A) BMDM from wt or *fld* animals were treated with 50 µg/ml poly (I:C) for the indicated time points. mRNA levels for the indicated proinflammatory genes were assessed by qPCR and normalized to GAPDH mRNA abundance. (B) BMDMs were stimulated with 1 µg/ml Pam3CSK4 for the indicated time points and analyzed as in panel A. (C) BMDMs were stimulated with 300 µM palmitic acid complexed to albumin (2:1 molar ratio), and analyzed as in panel A. Experiments were independently performed three times in triplicates (n=6). Error bars represent the SEM. P-values by Student's t test are as indicated (\*, p <0.05; \*\* p<0.01).

**Figure 5.** Lipin-1 is involved in TLR4-dependent signaling in macrophages. (A) BMDMs from wt or *fld* animals were stimulated with 100 ng/ml LPS for the indicated periods of time. Homogenates were then evaluated for the presence of total and phosphorylated MAPKs family members ERKs 1/2 and JNK by Immunoblot. (B) Homogenates from cells treated as in panel A were analyzed for total and phosphorylated p38. (C) Nuclear extracts from cells treated as in panel A were analyzed for the presence of phosphorylated c-Jun by Immunoblot. The nuclear protein p84 was used as loading control. Lower graphs in panels A-C represent relative quantification of the phosphoproteins against total protein or a loading control. (D) Nuclear extracts from cells treated as in panel A were assayed for c-jun activity using a commercially available kit. (E) Homogenates from cells treated as in panel A were analyzed for expression of the NF-κB inhibitors IKKα and IKKβ by Immunoblot. Lower graphs represent relative quantifications against β-actin. Experiments were independently performed three times (n=6). (F) Total content of DAG was analyzed by mass spectrometry in BMDMs from wt or *fld* mice treated with 100 ng/ml LPS for the

indicated time points. Data are from a representative experiment from four independent ones with similar results (n=10). Error bars represent the SEM. P-values by Student's t test are as indicated (\*,  $p < 0.05$ ; \*\*  $p < 0.01$ ).

**Figure 6.** Lipin-1 restores LPS-driven responses in *fld* macrophages. (A) BMDMs from wt and *fld* animals were transfected with plasmids encoding for EGFP as a control or Lipin-1 $\beta$ -EGFP. Six hours after transfection the cells were stimulated with 100 ng/ml LPS for 30 min (for ERK phosphorylation analysis) or 24 h (for iNOS expression analysis). Homogenates were analyzed by immunoblot using specific antibodies against ERKs 1/2, iNOS or  $\beta$ -actin. (B) Homogenates used in panel A were also analyzed by immunoblot with an antibody against lipin-1, and  $\beta$ -actin was used as a loading control. Experiments were independently performed twice.

**Figure 7.** Lipin-1 delays recovery after endotoxin treatment in mice. (A) Wt and *fld* animals were treated with 25 mg/kg LPS and survival rates were analyzed. Data represents two independent experiments (n=7). (B) Weight changes were evaluated in wt or *fld* animals after an i.p. injection of 1.5 mg/Kg LPS at the indicated time points. A representative experiment from 5 different ones is shown (n=12). (\*,  $p < 0.05$ ; \*\*  $p < 0.01$ ). (C) 24 h after 1.5 mg/Kg LPS administration, serum concentrations of urea, BUN, creatinine, AST and ALT were quantified. Discontinuous lines represent basal values. Experiments were performed twice (n=9). Error bars represent the SEM. P-values by Student's t test are as indicated (\*,  $p < 0.05$ ; \*\*  $p < 0.01$ ).

**Figure 8.** Lipin-1 contributes to the proinflammatory response that follows endotoxin treatment in mice. (A) Analysis by specific ELISAs of proinflammatory factors in the blood of wt or *fld* mice after an i.p. injection of 1.5 mg/Kg LPS for 6 h. (B,C) mRNA levels of different proinflammatory genes analyzed by qPCR in livers (B) or spleens (C) from wt or *fld* mice treated as in panel A. mRNA levels were normalized to GAPDH. Experiments were independently performed twice (n=4) (D) Peritoneal cell homogenates from

animals treated with 1.5 mg/ml for 24 h were analyzed by Immunoblot using antibodies against iNOS and  $\beta$ -actin. The graph on the right shows the relative quantification of iNOS against  $\beta$ -actin. Responses from three different animals are shown. Experiments were repeated three times. Error bars represent the SEM. P-values by Student's t test are as indicated (\*,  $p < 0.05$ ; \*\*  $p < 0.01$ ).

**Figure 9.** Lipin-1 effects on CD11<sup>+</sup> cell populations during endotoxin treatment. (A) Splenocytes from wt or *fld* animals treated or not with 1.5 mg/Kg LPS for 24 h (i.p. injection) were characterized by flow cytometry using antibodies against CD11, F4/80 and Gr-1. Gated CD11<sup>+</sup> cells were analyzed for F4/80 and Gr-1 expression. (B) Total peritoneal cells from mice treated as in panel A were counted and (C) analyzed by flow cytometry. Percentage of gated CD11<sup>+</sup> cells expressing F4/80 or Gr-1 is shown. Experiments were independently performed three times (n=9). Error bars represent the SEM. P-values by Student's t test are as indicated (\*,  $p < 0.05$ ; \*\*  $p < 0.01$ ).

**Figure 10.** Lipin-1 mediates proinflammatory activation in human macrophages. Blood monocyte-derived macrophages were nucleofected with 20 nM control siRNA or siRNA against *Lpin1* and stimulated with 100 ng/ml LPS for 8 h. (A) Cellular homogenates were analyzed by immunoblot using antibodies against lipin-1 and  $\beta$ -actin. (B) mRNA levels were analyzed by qPCR using cyclophilin for normalization. Experiments were independently performed three times in triplicate. Error bars represent the SEM. P-values by Student's t test are as indicated (\*,  $p < 0.05$ ; \*\*  $p < 0.01$ ).

**Figure 11.** Lipin-1 impacts on TLR4 signaling pathways.