Lipin-1 Integrates Lipid Synthesis with Proinflammatory Responses during TLR Activation in Macrophages

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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 and activation of MAPKs and AP-1. Consequently, the generation of proinflammatory cytokines like IL-6, IL-12, IL-23, or enzymes like inducible NO synthase and cyclooxygenase 2, was reduced. In addition, 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. *The Journal of Immunology*, 2014, 193: 4614–4622.

Macrophages are highly plastic phagocytic cells responsible for the maintenance of physiological homeostasis owing 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 TLRs, specifically TLR4, by molecular patterns like LPS triggers a cascade of signaling events conducted by kinases and adaptor proteins that culminates in the phosphorylation and activation of the MAPK family of proteins and the family of kinases for the NF-κB transcription factor inhibitors IkB (2–4). MAPKs are responsible for the phosphorylation and activation of proteins that are part of the transcription factor AP-1, whereas the phosphorylation of IκB inhibitors promotes their proteasomal degradation, thereby releasing active NF-κB proteins. AP-1 and NF-κB are involved in the transcription of multiple proinflammatory genes, including IL-6, IL-12, IL-23, and TNF-α (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 diacylglycerol (DAG) generated via the Kennedy pathway of phospholipid synthesis (8). The enzymes that directly generate DAG, in the Kennedy pathway, display phosphatidic acid phosphatase activity and 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 in 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. In this study, 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 affecting MAPKs and AP-1 activation and, as a consequence, 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*^{fld}/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*$^{fld/+}$ genotype were

bred to generate *Lpin^{fld/fld}* (hereafter *fld*), *Lpin^{fld/+}*, 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). Sex-matched 12-week-old 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 Ab against β-actin were obtained from Sigma-Aldrich. Abs against IκBα, IκBβ, phospho-p38 MAPK (Thr[180]/Tyr[182]), p38 MAPK, phospho-p44/42 MAPK (Thr[202]/Tyr[204]), p44/42 MAPK, phospho-SAPK/JNK (Thr[183]/Tyr[185]), SAPK/JNK, and phospho–c-Jun were purchased from Cell Signaling. Abs against the nuclear protein p84 were obtained from Abcam. Abs against inducible NO synthase (iNOS) were obtained from BD Biosciences and anti–cyclooxygenase-2 (COX-2) from Cayman. The rabbit antiserum against lipin-1 was previously described (11). PE-conjugated Abs against CD11b, APC-conjugated Abs against F4/80, FITC-conjugated Abs against Gr1, and 7-amino-actinomycin D were purchased from eBioscience. Specific ON-TARGET*plus* small interfering RNAs (siRNAs) against murine mRNAs were obtained from Dharmacon (Thermo Scientific). Silencer 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 ice-cold PBS. Resident cells were centrifuged for 10 min at 300 × *g* and allowed to adhere to plastic for 18 h in RPMI 1640 medium containing 10% FBS. Nonadherent 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 PBS using a 25-gauge sterile needle. After filtration, bone marrow cells were centrifuged for 10 min at 300 × *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 d. The medium was changed at day 4, washing out the nonadherent cells, and differentiation was continued for a total of 7 d.

Neutrophils were isolated from bone marrow using a Percoll 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_2 for 2 h. Nonadherent cells containing mainly B and T cells were further subjected to nylon wool (Polysciences) purification, allowing them to adhere to the column at 37°C and 5% CO_2 in RPMI 1640 for 1 h. Nonadherent T cells were eluted with warm RPMI 1640, and B cells were also collected afterward 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 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 chloroform/methanol (2:1, v/v). The DAG fraction was transmethylated with 500 μl 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 *n*-hexane. Analysis of fatty acid methyl esters was carried out in an 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 the RNeasy Mini Kit (QIAGEN) and TRIzol reagent (Ambion), respectively. The cDNA templates were synthesized using M-MLW Reverse Transcriptase (Ambion) following the manufacturer's 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 database (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 performed by Laboratorios Echevarne (Barcelona, Spain).

LPS treatment of animals

Mice were i.p. injected with LPS at a lethal dose of 25 mg/kg or a sublethal dose of 1.5 mg/kg and monitored for 10 d. For the analysis of proinflammatory factors, some animals were sacrificed by ketamine (100 mg/kg): xylacine (10 mg/kg) administration and cervical dislocation 6 h 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.
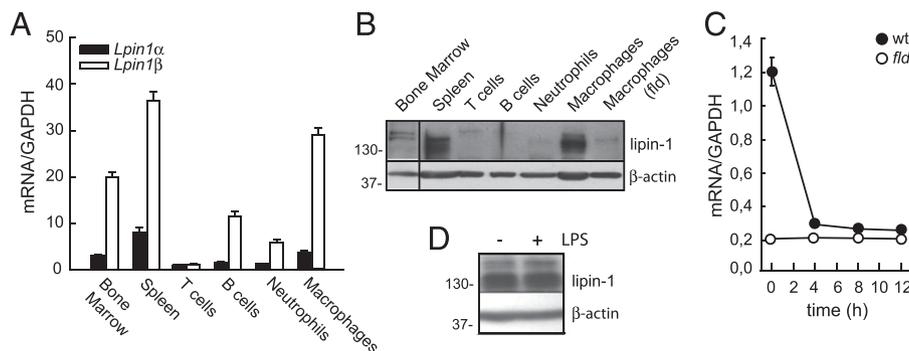
FIGURE 1. Lipin-1 expression in immune tissues and cells. (**A**) mRNA levels from bone marrow cells, whole spleen, T cells, B cells, neutrophils, and peritoneal macrophages were analyzed by quantitative PCR using specific primers for *Lpin1* spliced variants *Lpin1α* and *Lpin1β*. Levels of *Lpin1* mRNAs were normalized to GAPDH mRNA abundance. (**B**) Homogenates from tissues and cells as in (A) were analyzed by immunoblot using specific Abs against lipin-1 and β-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 quantitative PCR using specific primers that recognized both *Lpin1α* and β. 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 (B). Experiments were independently performed three times. Error bars represent the SEM.

Microarray gene expression

Peritoneal macrophages from four different control (*Lpin^{fld/+}*) and *fld* (*Lpin^{fld/fld}*) 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 4 × 44K, using the Agilent-recommended protocol. After extensive washing, fluorescence signals were detected using Agilent Microarray Scanner System (Agilent Technologies). The Agilent Feature Extraction Software was used to read out and process the microarray image files, and the Rosetta Resolver 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 ≥1.7-fold change up- or downregulated with an associated *p* value of 0.01 in relation to the reference. All microarray data have been deposited into the Gene Expression Omnibus database (accession number GSE54155; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54155).

Flow cytometry

Cells from whole-spleen or peritoneal lavage were incubated with Abs against CD16/CD32 (eBioscience) to block nonspecific Ab 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 (eBioscience). Isotype control Abs 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 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

Lipin1β-EGFP plasmid was constructed by introducing the cDNA sequence of the mouse *Lpin1b* (clone 4211202; Thermo Scientific) in the EGFP expression vector pEGFP-N3 (Clontech), using EcoRI and SalI restriction enzymes. The primers used were as follows: 5′-CACACA-GAATTCAATGAATTACGTGGGGCAGC-3′ and 5′-CACACAGTCGA-CAGCTGAGGCTGAATGCATGT-3. Confirmation of the correct insertion of the cDNA was performed by sequencing. Plasmids (EGFP or lipin1-EGFP) were transfected into BMDMs using the nucleofection method, and the kit specifications for murine macrophages were followed. A total of 3 μg plasmid was used, and the program was Y-001.

Statistical analysis

Data are represented as the mean ± SEM. Statistical significance was determined by the Student *t* test. A *p* value < 0.05 was considered statistically significant.

Results

Lipin-1–deficient macrophages have a decreased inflammatory gene expression after TLR4 stimulation
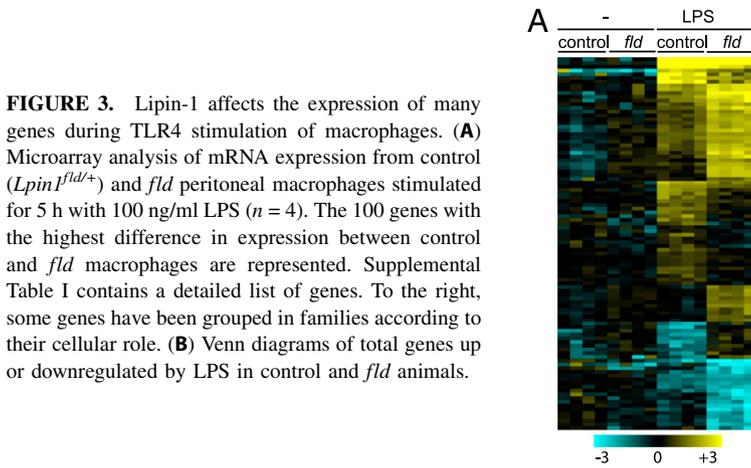
We began this study by analyzing the expression levels of lipin-1 in immune cells and tissues. The highest levels of lipin-1 were present in peritoneal macrophages and spleen, at both the mRNA and the protein levels (Fig. 1A, 1B). The absence of lipin-1 altered neither the overall percentage of macrophages 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. Fig. 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* was 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 owing 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 ex-
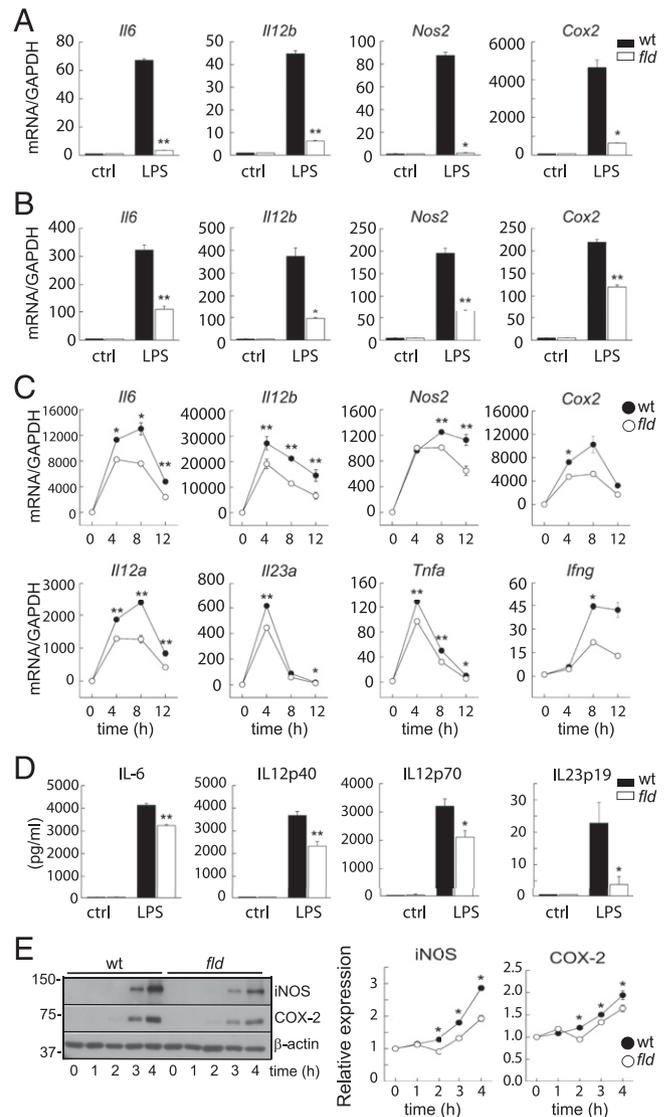
FIGURE 2. Lipin-1 mediates production of proinflammatory factors after TLR4 stimulation of macrophages. (**A**) mRNA abundance of proinflamatory genes (as indicated) in peritoneal macrophages from wt or *fld* animals treated or not with 100 ng/ml LPS for 8 h was analyzed by quantitative PCR. mRNA levels 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 (A). (**C**) BMDMs 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 (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 with respect to β-actin. Experiments were independently performed three times in triplicate (*n* = 6). Error bars represent the SEM. The *p* values by Student *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 (*Lpin1fld/+*) and *fld* peritoneal macrophages stimulated for 5 h with 100 ng/ml LPS (*n* = 4). The 100 genes with the highest difference in expression between control and *fld* macrophages are represented. Supplemental Table I 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 downregulated by LPS in control and *fld* animals.

Lipin-1 affected genes	
Receptor signaling	*Folr1, Trpc2, Prlhr, V1ra6, Galr2, Amhr2, Thrb, Ak034299, Unc5c, V1rd19, Agrp, Chrna6, Sstr4*
G-protein signaling	*Plekhk1, Centd1, Chn2, Gpr153, Kndc1, Gnao1*
Cellular Import/Export	*Accn4, Accn1, Cacna1c, Kcnh4, Slc38a4, Gja5*
Transcription	*Pcbd1, Nkx2-6, Myod1, Foxd1, Plagl1, Tef, Alx3, Nkx2-4, Barx-2, Zbtb10, Gsx2, Zfp74*
Protein folding modification	*St6galnac2, Msrb3, Prima1, St6gal1*
Cell adhesion	*Pcdha6, Cldn14, Itgb3bp, Pcdha9, Nlgn3, Vcam1*
Kinase/Phosphatase signaling	*Ppp1r1a, Braf, Mapk15, Jakmip1, x9938X, Tssk6*
Nucleotide metabolism	*Dnase1, Uox, Smyd3, Ttc5, Cad, AF288221*
Extracellular matrix	*Adam19, Timp1, Emid1, Col4a1, Col1a1*
Lipid metabolism	*Cpt1b, Cln8, Fads3, Hsd3b2*
Response to toxins	*Adh1, Hspb1*
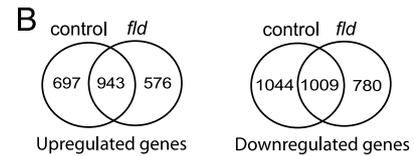
haustive analysis in BMDMs indicated that many inflammation-related genes were affected in *fld*-derived cells, including *Il12a* (p35), *Il23a* (p19), *Tnfa*, and *Ifng* (Fig. 2C). These results were also corroborated by analysis of protein levels in the supernatants or cell homogenates of TLR4-activated cells (Fig. 2D, 2E). 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 those in wt cells (data not shown). In addition, 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, whereas 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; only 1009 genes were downregulated in both types of cells, whereas 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, Supplemental Table I), 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 polyinosinic-polycytidylic acid [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, whereas *Nos2* and *Cox2* were not affected (Fig. 4A). In addition, *fld* cells stimulated with Pam3CSK4 exhibited lower mRNA levels for *Il6*, *Il23a*, and *Nos2* than did wt cells (Fig. 4B). We also examined the effects of nonclassical 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, 5B). 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
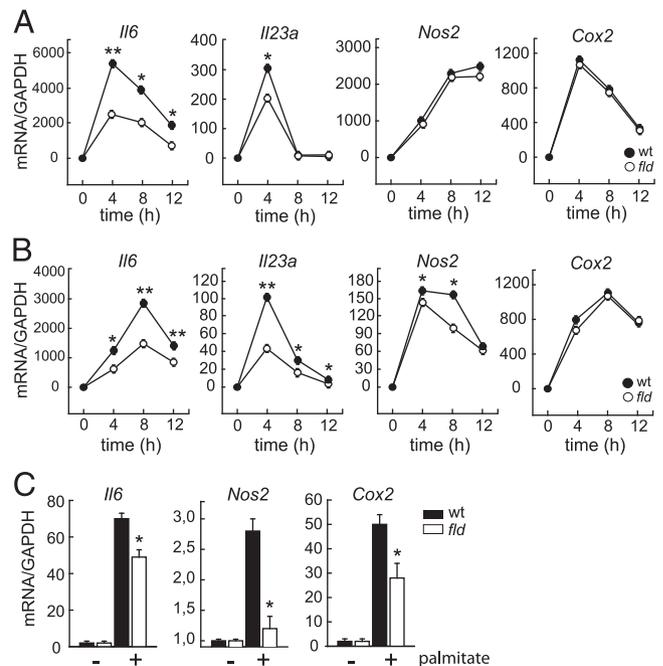
FIGURE 4. Lipin-1 effects on TLR3 and TLR1/2 activation in macrophages. (**A**) BMDMs from wt or *fld* animals were treated with 50 μg/ml poly(I:C) for the indicated time points. mRNA levels for the indicated proinflammatory genes were assessed by quantitative PCR and normalized to GAPDH mRNA abundance. (**B**) BMDMs were stimulated with 1 μg/ml Pam3CSK4 for the indicated time points and analyzed as in (A). (**C**) BMDMs were stimulated with 300 μM palmitic acid complexed to albumin (2:1 molar ratio) and analyzed as in (A). Experiments were independently performed three times in triplicate (*n* = 6). Error bars represent the SEM. The *p* values by Student *t* test are as indicated: *$p < 0.05$, **$p < 0.01$.
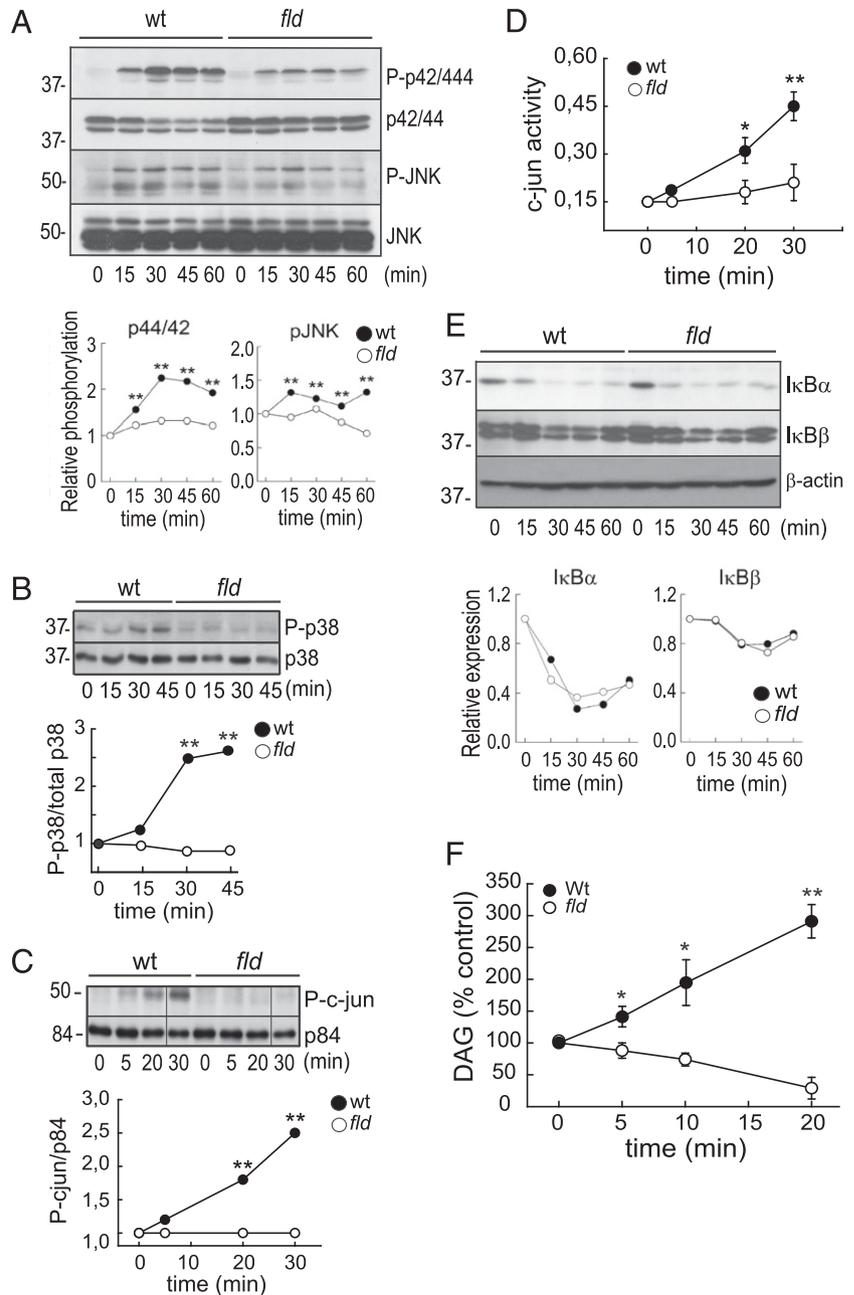
FIGURE 5. Lipin-1 is involved in TLR4-dependent signaling in macrophages. (**A**) BMDMs from wt or *fld* animals were stimulated with 100 ng/ml LPS for the indicated periods of time. Homogenates were then evaluated by immunoblot for the presence of total and phosphorylated MAPK family members ERKs 1/2 and JNK. (**B**) Homogenates from cells treated as in (A) were analyzed for total and phosphorylated p38. (**C**) Nuclear extracts from cells treated as in (A) were analyzed by immunoblot for the presence of phosphorylated c-Jun. The nuclear protein p84 was used as loading control. *Lower graphs* in (A)–(C) represent relative quantification of the phosphoproteins against total protein or a loading control. (**D**) Nuclear extracts from cells treated as (A) were assayed for c-Jun activity using a commercially available kit. (**E**) Homogenates from cells treated as in (A) were analyzed by immunoblot for expression of the NF-κB inhibitors IKKα and IKKβ. *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. The *p* values by Student *t* test are as indicated: *$p < 0.05$, ** $p < 0.01$.

(Fig. 5C, 5D). We also examined the degradation of the NF-κB inhibitors IκBα and IκBβ in *fld* macrophages and found no significant differences when compared with wt cells (Fig. 5E). Hence, these data suggest that diminished activation of MAPK/AP-1, but not NF-κB, 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 also 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 pre-

dominates 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. The *fld* BMDMs were transiently transfected with a plasmid encoding for lipin-1, and DAG production, ERK-1/2 phosphorylation, and iNOS expression were analyzed after LPS stimulation of the cells. The results, shown in Fig. 6, clearly indicate that lipin-1 expression restored all these responses.

Lipin-1–deficient mice have a quicker recovery from endotoxin treatment
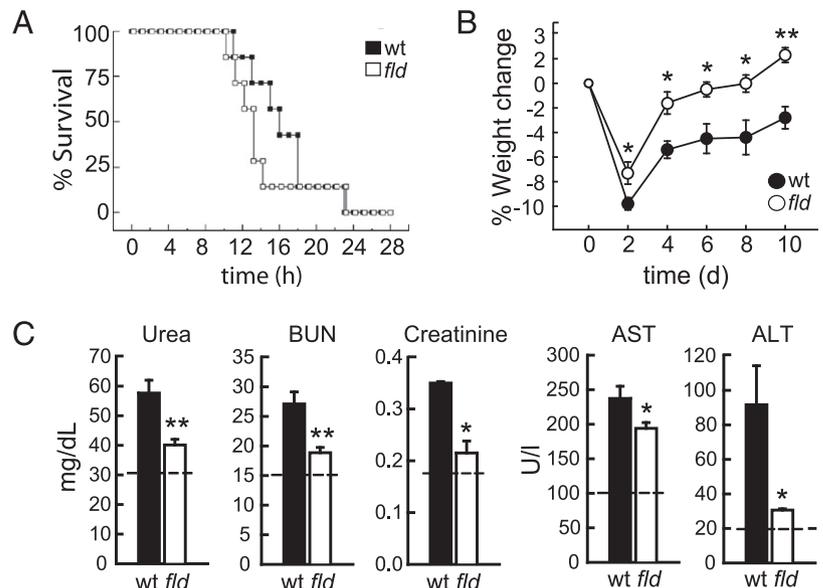
To define the in vivo implication of the above findings, the effect of an i.p. administration of LPS was evaluated in animals. At high doses of LPS (25 mg/kg), no significant differences were found in mortality rates between wt and *fld* animals (Fig. 7A). However, at lower LPS doses (1.5 mg/kg), which did not provoke death, *fld*
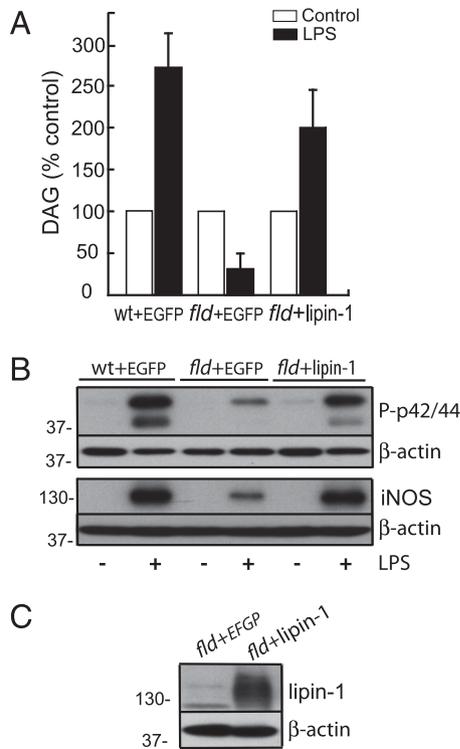
FIGURE 6. Lipin-1 restores LPS-driven responses in *fld* macrophages. BMDMs from wt and *fld* animals were transfected with plasmids encoding for EGFP as a control or Lipin-1β–EGFP. At 6 h after transfection, the cells were stimulated with 100 ng/ml LPS for 30 min [for analysis of DAG, (**A**), or ERK phosphorylation, (**B**)] or 24 h [for iNOS expression analysis, (**B**)]. Homogenates were analyzed by immunoblot using specific Abs against ERKs 1/2, iNOS, or β-actin. Homogenates were also analyzed by immunoblot with an Ab against lipin-1, and β-actin was used as a loading control (**C**). Experiments were independently performed twice.

animals had a faster weight recovery than did wt animals (Fig. 7B). Although *fld* animals returned to their original weight after 6 d of treatment, wt animals needed >10 d 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 less pronounced organ damage (Fig. 7C).
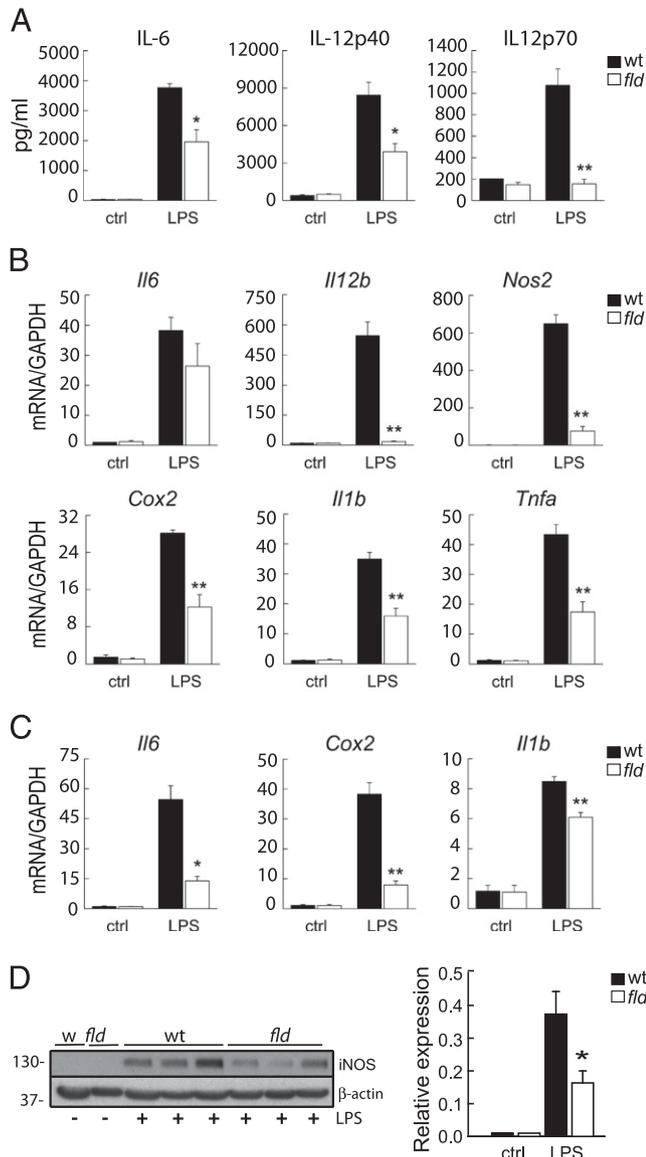
Analysis of the blood content of proinflammatory cytokines in LPS-treated animals showed a substantial decrease in the content of IL-6, IL-12p40, and IL-12p70 in *fld* animals compared with control ones (Fig. 8A). A more exhaustive investigation of proinflamatory gene expression performed in the liver and the spleen of treated animals revealed that many of those genes were less upregulated in *fld* animals (Fig. 8B, 8C). 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^{+} cells in spleen. However, we found that the absence of lipin-1 had opposite effects on splenic CD11^{+}F4/80^{−}Gr1^{+} and CD11b^{+}F480^{+} Gr1^{+} populations, decreasing the former (neutrophils) and increasing the latter (inflammatory monocytes) (Fig. 9A). In contrast, spleens from LPS-treated animals showed an increase in CD11b^{+} 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^{+}F4/80^{+} Gr1^{+} cells, whose recruitment was significantly lower in the spleens of *fld* animals (Fig. 9A, Supplemental Fig. 3). Macrophages (CD11b^{+}F4/80^{+}Gr1^{−}) decreased their presence in LPS-treated spleens in the same manner in wt and *fld* animals (Fig. 9A, 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 nontreated 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, 9C). 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*,

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 represent 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 five different ones is shown (*n* = 12). *$p < 0.05$, **$p < 0.01$. (**C**) At 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. The *p* values by Student *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 proinflamatory factors in the blood of wt or *fld* mice after an i.p. injection of 1.5 mg/kg LPS for 6 h. (**B** and **C**) mRNA levels of different proinflammatory genes analyzed by quantitative PCR in livers (B) or spleens (C) from wt or *fld* mice treated as in (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 Abs against iNOS and β-actin. The graph on the *right* shows the relative quantification of iNOS against β-actin. Responses from three different animals are shown. Experiments were repeated three times. Error bars represent the SEM. The *p* values by Student *t* test are as indicated: *$p < 0.05$, ** $p < 0.01$.

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 TLR 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: 1) 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; 2) mRNA expression microarray analyses suggest that lipin-1 also affects other processes like G-protein signaling, transcription, cell adhesion, and so forth, in TLR4-stimulated macrophages; 3) lipin-1 delays the recovery of animals to endotoxin treatment by limiting the expression of detrimental mediators; 4) the expression levels of lipin-1 also affect other TLRs in addition to TLR4, namely, TLR3 and TLR1/2; and 5) 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 a 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 alteration of the whole cell reprograming. 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 from that in wt cells and is not the result of a mere reduction of transcriptional events. In contrast, the activation of transcriptional effectors such as NF-κB 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-κB inhibitor, IkB. Thus it could be deduced from these data that the effects observed for lipin-1 may not necessarily be related to 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 PKCε and PKCδ, 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 affects TLR4 downstream pathways such as MAPK 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
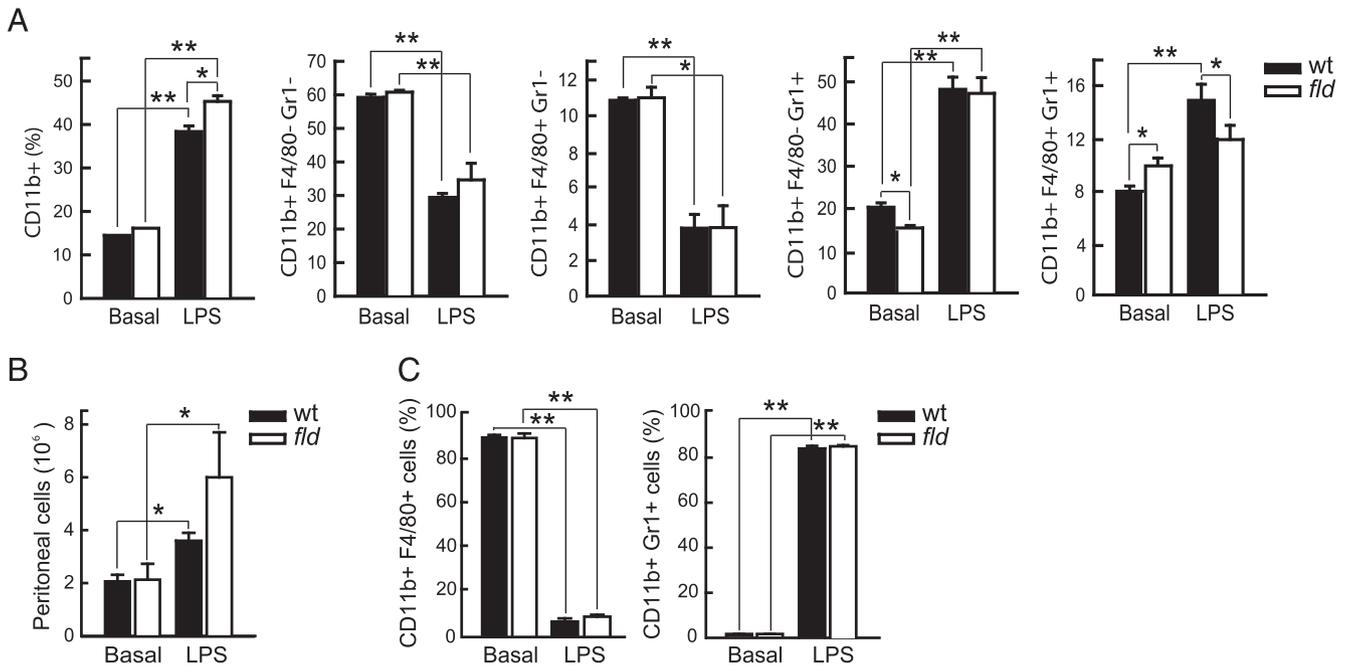
FIGURE 9. Lipin-1 effects on CD11⁺ cell populations during endotoxin treatment. (**A**) Splenocytes from wt or *fld* animals treated or not with 1.5 mg/kg LPS for 24 h (i.p. injection) were characterized by flow cytometry using Abs against CD11, F4/80, and Gr-1. Gated CD11⁺ cells were analyzed for F4/80 and Gr-1 expression. (**B**) Total peritoneal cells from mice treated as in (A) were counted and (**C**) analyzed by flow cytometry. Percentage of gated CD11⁺ cells expressing F4/80 or Gr-1 is shown. Experiments were independently performed three times ($n = 9$). Error bars represent the SEM. The p values by Student t test are as indicated: *$p < 0.05$, **$p < 0.01$.

previously shown that a Mg^{2+}-dependent phosphatidate phosphatase activity is involved in arachidonic acid mobilization, COX expression, and eicosanoid formation when WISH cells are activated through PKC (35, 36) and when macrophages are stimulated with LPS (37). Although the mechanisms for these actions are not known, these results highlight a role for lipin-1 in cell signaling through modulating phosphatidic acid 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 alleviation of the exacerbated proinflammatory response would be a good strategy for their treatment. The studies presented in this article 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 to 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_2, 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 affect sepsis not only by regulating the expression of enzymes such as COX-2 but also by affecting the levels of their substrates.

Owing to the diminished inflammatory response of *fld* animals, a lower death rate during treatment with high LPS doses would be
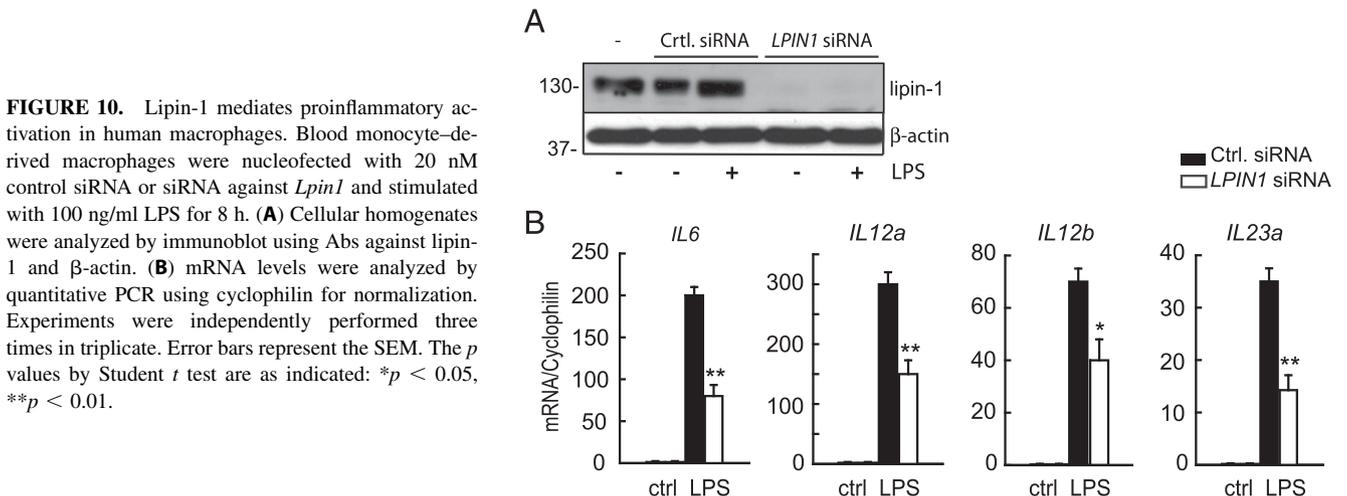
FIGURE 10. Lipin-1 mediates proinflammatory activation in human macrophages. Blood monocyte–derived macrophages were nucleofected with 20 nM control siRNA or siRNA against *Lpin1* and stimulated with 100 ng/ml LPS for 8 h. (**A**) Cellular homogenates were analyzed by immunoblot using Abs against lipin-1 and β-actin. (**B**) mRNA levels were analyzed by quantitative PCR using cyclophilin for normalization. Experiments were independently performed three times in triplicate. Error bars represent the SEM. The p values by Student t test are as indicated: *$p < 0.05$, **$p < 0.01$.

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 a 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 in this article 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.

Acknowledgments

Disclosures

The authors have no financial conflicts of interest.

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