Lipin-1-derived diacylglycerol activates intracellular TRPC3 which is critical for inflammatory signaling

Javier Casas1,2 · Clara Meana1,3 · José Ramón López-López1,2 · Jesús Balsinde1,3 · María A. Balboa1,3

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Abstract
Exposure to Gram-negative bacterial LPS exacerbates host immune responses and may lead to sepsis, a life-threatening condition. Despite its high mortality and morbidity, no drugs specifically directed to treating sepsis are currently available. Using human cell genetic depletion, pharmacological inhibition, live-cell microscopy and organelle-targeted molecular sensors we present evidence that the channel TRPC3 is activated intracellularly during macrophage exposure to LPS and is essential for Ca2+ release from internal stores. In this manner, TRPC3 participates in cytosolic Ca2+ elevations, activation of the transcription factor NF-κB and cytokine upregulation. We also report that TRPC3 is activated by diacylglycerol generated by the phosphatidic acid phosphatase lipin-1. In accord with this, lipin-1-deficient cells exhibit reduced Ca2+ responses to LPS challenge. Finally, pharmacological inhibition of TRPC3 reduces systemic inflammation induced by LPS in mice. Collectively, our study unveils a central component of LPS-triggered Ca2+ signaling that involves intracellular sensing of lipin-1-derived DAG by TRPC3, and opens new opportunities for the development of strategies to treat LPS-driven inflammation.

Keywords TRPC3 · Lipin-1 · Macrophages · Inflammation · DAG · Ca2+ release

Introduction
Receptors on the surface of innate immune cells recognize multiple molecules derived from pathogens. These receptors initiate an inflammatory reaction that helps to eliminate the pathogen and repair the damaged area. TLR4, a prominent member of the Toll-like receptor family, is the canonical receptor for lipopolysaccharide (LPS) from Gram-negative bacteria [7]. LPS recognition by TLR4 initiates a cascade of events that culminates in the activation of the transcription factors AP-1, NF-κB, or IRF-3 through different signaling branches [7]. The final outcome is the transcriptional upregulation of cytokines, enzymes and proteins that help inactivate the invading microorganism. However, excessive TLR4 activation orchestrated by elevated concentrations of LPS could be detrimental, leading to sepsis, a life-threatening organ dysfunction caused by the dysregulated host response to infection. Hence, a finer knowledge of TLR4-mediated signaling is key to find helpful targets for the management of infections and inflammatory diseases.

It is now clearly established that complete TLR4 responses require cellular Ca2+ fluxes and [Ca2+]i elevations [10, 26, 40, 43, 49]. Ca2+ signaling in immune cells has traditionally been considered to be due to the release of Ca2+ from internal stores, such as the endoplasmic reticulum (ER), followed by Ca2+ influx through plasma membrane channels to restore homeostasis [12]. This mechanism is known as store-operated Ca2+ entry (SOCE). Regarding LPS-induced Ca2+ fluxes, it has been proposed that they are initiated by the activation of phospholipase Cγ2 (PLCγ2) at the plasma membrane, mediated by the TLR4 co-receptor CD14 [10, 49]. This model assumes that PLCγ2-derived inositol 1,4,5-trisphosphate (InsP3) interacts with receptors...
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Results

Involvement of TRPC3 channels in LPS-induced cytokine production

We began this study by analyzing the expression of DAG-regulated TRPC channels in human THP-1 macrophages using conventional semi-quantitative PCR. As shown in Fig. 1, TRPC3 is expressed in these cells, as well as TRPC6 and 7 (Fig. 1A). Interestingly, reduced expression levels of TRPC3 by siRNA or use of the TRPC3 cell-permeable selective blocker Pyr10 [41] strongly blunted the LPS-induced upregulation of a number of proinflammatory genes, including PTGS2, TNFA, IL1B, and IL6 (Fig. 1B–D). The increased expression of COX-2 protein (PTGS2) induced by LPS was also inhibited by Pyr10 (Fig. 1E). Consistent with these data, overexpression of the TRPC3 channel in HEK293 cells expressing the human sequences for TLR4/MD2/CD14 (HEK-TLR4 cells), markedly increased the expression levels of PTGS2, TNFA, and IL12B after treatment with LPS, as compared to control cells (Fig. 1F, G).

We investigated next whether DAG was required for TRPC3 to regulate the upregulation of inflammatory genes mediated by LPS. To this end, we took advantage of the recent finding that a single mutation in TRPC3, G652A, blunts its capacity to recognize DAG [27]. We transfected the HEK-TLR4 cells with the G652A-TRPC3 mutant and studied the capacity of the cells to produce inflammatory effectors. The results indicated a reduced capacity of G652A-TRPC3-transfected cells to upregulate PTGS2, TNFA, IL1B, and IL12B compared with cells transfected with the wild-type TRPC3 construct after LPS treatment (Fig. 1H, I). Collectively, these results suggest that DAG recognition by TRPC3 is key for LPS-activated inflammatory responses.
LPS-induced calcium signaling is mediated by TRPC3

Elevations in intracellular calcium ([Ca$^{2+}$]i) during activation by LPS constitute an obligate signaling step [10, 26, 40, 43, 49]. In the next series of experiments, we used live-cell imaging to explore the involvement of TRPC3 in the regulation of Ca$^{2+}$ fluxes during LPS activation. Using the dynamic single-wavelength fluorescent Ca$^{2+}$ indicator Fluo-4, whose increase in fluorescence intensity reflects a rise in the cytoplasmic Ca$^{2+}$ level, we detected significant [Ca$^{2+}$]i increases after treating the human THP-1 macrophages with LPS (Fig. 2A, B), in agreement with previous reports [26, 40, 49]. Importantly, macrophages deficient in TRPC3 exhibited smaller [Ca$^{2+}$]i rises than control cells (Fig. 2B). Quantification of the areas under the curves demonstrated that, in the absence of TRPC3, cells experienced a reduction in [Ca$^{2+}$]i of ~60% during activation. To further assess the role of TRPC3 in intracellular Ca$^{2+}$ raises, we used several strategies, namely treating the macrophages with Pyr10, and overexpressing the G652A-TRPC3 mutant or an N-terminal dominant negative fragment of TRPC3 (N-ter-TRPC3) in HEK-TLR4 cells [38]. All these strategies were found to strongly reduce the LPS-induced [Ca$^{2+}$]i increases respect to control cells (Fig. 2C-E).
NF-κB is a transcription factor implicated in the upregulation of many of the genes described above, and its full activation depends on Ca^{2+} fluxes [40, 43]. Thus, we studied next the activation of this transcription factor by analyzing the translocation to the nucleus of one of its classical subunits, p65. LPS treatment of THP-1 macrophages induced a time-dependent translocation of p65 to the nuclear compartment. Such translocation reached a maximum at 30 min after LPS exposure, and was markedly reduced in TRPC3-deficient macrophages (Fig. 2F, G). Collectively, these data indicate that TRPC3 is involved in Ca^{2+} fluxes and in the activation of transcription factors that participate in the upregulation of inflammatory cytokines by LPS.

**Plasma membrane TRPC3 currents are not affected by LPS**

To investigate further the involvement of TRPC3 in signaling events during cellular treatment with LPS, we carried out whole-cell patch-clamp recordings. The advantage of this technology is that it can unequivocally measure the activation of channels located in the plasma membrane. Based on the studies measuring [Ca^{2+}]i (Fig. 2) and in the biology of the TRPC3 channel [44], we anticipated that a significant inward current during treatment of the cells with LPS would be detected, reflecting the activation of ion currents through the channel. However, we failed to detect such activation (Fig. 3A, B). As a positive control of the experiment, we treated the macrophages with the synthetic DAG 1-oleyl-2-acetyl-sn-glycerol (OAG) to activate the TRPCs that could be present at the plasma membrane [19]. Upon addition of OAG, we did detect the activation of a Pyr10-sensitive current (Fig. 3A). These results show that macrophages express channels whose currents can be activated by DAG (TRPCs), and inhibited by Pyr10 (TRPC3) but, interestingly, these channels are not sensitive to LPS, at least at the plasma membrane.

Although TRPC3 channels have been localized to the plasma membrane, they may also be present in intracellular membranes, particularly those of the ER and mitochondria [16, 28]. Therefore, because of the results obtained in the patch-clamp experiments, we performed immunofluorescence measurements in human macrophages to clearly establish the localization of TRPC3 in these cells. We validated commercially available TRPC3 antibodies that produced only marginal fluorescence in TRPC3-silenced macrophages (Fig. 3C). TRPC3 was mostly found in cytoplasmic membranes, being especially concentrated in membranes close to the nucleus. This was particularly evident when all the images obtained from single stacks were subjected to z-maximum projection (Fig. 3D). Co-localization experiments using antibodies against the ER membrane residing protein SERCA2 confirmed that most of the membranes where TRPC3 is located are part of the ER (Fig. 3E). In TRPC3-overexpressing HEK-TLR4 cells, the channel was also confirmed to be present in intracellular membranes resembling those of the ER (Fig. 1F).

**LPS increases DAG levels in the ER**

To assess whether TRPC3 could be activated by DAG in endomembranes, we analyzed physiological DAG dynamics. We used genetically encoded FRET-based DAG biosensors that can be targeted specifically to different biological membranes, namely Daglas-pm1 and Daglas-em1. Daglas-pm1 is directed to the plasma membrane through an 11-aminoacid sequence of N-Ras that allows its prenylation [39]. Daglas-em1, directed to ER membranes, is obtained by mutating the C181 of N-Ras to Ser [39]. Transfected biosensors localized as expected in both THP-1 macrophages and HEK-TLR4 cells (Fig. 4A, B). HEK-TLR4 cells expressing Daglas-pm1 immediately showed a substantial increase in the intramembrane FRET/CFP ratio when stimulated with...
the DAG analog phorbol 12-myristate 13-acetate (PMA) (Fig. 4A). However, when the cells were stimulated with LPS, no substantial effect on the fluorescence emission ratio was detected, suggesting that this particular sensor is not detecting changes in DAG levels at the plasma membrane under these conditions. In contrast, cells expressing the ER-directed Daglas-em1 sensor showed a significant increase in FRET/CFP ratio after LPS stimulation in both THP-1 macrophages and HEK-TLR4 cells (Fig. 4B).

To precisely identify the endomembranes where the relevant DAG pool accumulates during LPS activation, THP-1 macrophages were transfected with the Daglas-em1 biosensor, stimulated with LPS, and subsequently labeled with the ER-specific marker ER-Tracker-TR (Fig. 4C). Colocalization between DAG and ER was studied using the Manders’ colocalization index $M_1$ [31]. The colocalization index between FRET/CFP (representing DAG content) and ER-Tracker-TR (representing ER) was 0.58 ± 0.02. Given that
the colocalization index between CFP and YFP—which were engineered into the same molecule—was 0.78 ± 0.04, these data suggest that a substantial amount of the DAG generated during macrophage stimulation with LPS accumulates in the ER.

TRPC3 colocalizes with DAG at the ER and regulates [Ca\(^{2+}\)]_ER during TLR4 activation

We investigated next whether the DAG generated in endomembranes upon LPS treatment localized within the same intracellular structures as TRPC3. Macrophages expressing the sensor Daglas-em1 were stimulated with LPS, immunostained with antibodies against TRPC3, and the colocalization between DAG and TRPC3 was analyzed. A marked increase in the colocalization index in stimulated cells compared with control cells in areas adjacent to the nucleus, where the ER is located, was appreciated (Fig. 4D). These results suggest that a significant amount of TRPC3 is found at the same structures where DAG accumulates, i.e. ER membranes, during LPS treatment.

Based on the findings that: (i) ion currents through TRPC3 are not activated at the plasma membrane by LPS, (ii) the channel mediates Ca\(^{2+}\) fluxes during activation, and (iii) the channel is present mainly in intracellular membranes in macrophages, we reasoned that TRPC3 could be involved in regulating the movement of Ca\(^{2+}\) from intracellular stores.

To investigate this possibility, we took advantage of a FRET-based calcium cameleon indicator engineered to reside in the lumen of the ER (\(\nu\)YC4er) [35]. This indicator detects changes in [Ca\(^{2+}\)]_ER during cellular stimulation [35]. HEK-TLR4 cells transfected with this indicator showed a reticular pattern of fluorescence characteristic of ER localization, and underwent a decrease in FRET/ECFP ratio when Ca\(^{2+}\) decreased in the organelle due to thapsigargin treatment (Fig. 4E). LPS treatment also reduced the FRET/ECFP ratio of the cameleon indicator (Fig. 4F-J). Interestingly, the effect was almost abolished when TRPC3-deficient cells were used (Fig. 4F, J) in the presence of Pyr10 (Fig. 4G), and also when the N-ter-TRPC3 dominant negative construct was overexpressed (Fig. 4H). Importantly, we corroborated these results in primary human monocyte-derived macrophages expressing the sensor Daglas-em1 biosensor (Fig. 5A). The LPS-stimulated increase in the FRET/CFP ratio was strongly inhibited by propranolol, both in THP-1 macrophages and HEK-TLR4 cells. Moreover, use of lipin-1-deficient cells by siRNA silencing [33] demonstrated no significant increases in ER DAG levels during the LPS challenge (Fig. 5A). In addition, high colocalization between the DAG present in the ER and lipin-1 was noted, as judged by the high M_1 index found between the FRET/CFP ratio from the Daglas-em1 biosensor, and the fluorescence from the chimera lipin-1-mCherry (M_1 = 0.72 ± 0.02) (Fig. 5B). A very high positive correlation (r = 0.9062) was also found between the levels of DAG in the ER (FRET/CFP ratio) and lipin-1-mCherry expression levels in transfected macrophages (Fig. 5C). These results demonstrate that lipin-1 and DAG are in close proximity in the ER—as it would be expected from an enzyme and its product—and that LPS-stimulated DAG production in the ER depends on lipin-1 expression.

Lipin-1 participates in Ca\(^{2+}\) Fluxes and TRPC3-dependent cytokine production during LPS stimulation

If, as the previous data suggested, lipin-1 is the enzyme that provides DAG for TRPC3 activation, inhibition or absence of the enzyme should affect the LPS-associated Ca\(^{2+}\) fluxes described above. We analyzed [Ca\(^{2+}\)]_i in macrophages treated with propranolol and found that the inhibitor prevented [Ca\(^{2+}\)]_i increases during cellular LPS treatment (Fig. 6A). The same effect was also observed in human macrophages silenced for LPIN1 (Fig. 6B), and in murine macrophages with a spontaneous mutation in the Lipin1 gene (fld animals), which abrogates lipin-1 expression [50] (Fig. 6C). Ca\(^{2+}\) responses of fld macrophages to LPS were not altered by Pyr10 treatment (Fig. 6C), supporting the idea that lipin-1 is upstream of TRPC3 activation in this setting.

ER enrichment with DAG during LPS activation depends on Lipin-1

Subsequent work was aimed to elucidate the molecular mechanisms by which DAG is generated in endomembranes during LPS stimulation. We have recently demonstrated that a member of the lipin family, called lipin-1, acts as a modulator of LPS-triggered signaling cascades [29, 33, 46]. Hence, we considered that lipin-1 could constitute a reasonable candidate to regulate DAG levels in the ER under those circumstances. As a first approach, we used propranolol, a well-established inhibitor of the enzymatic activity of lipins ([3, 4, 32] and estimated DAG production in the ER using the Daglas-em1 biosensor (Fig. 5A). The LPS-stimulated increase in the FRET/CFP ratio was strongly inhibited by propranolol, both in THP-1 macrophages and HEK-TLR4 cells. Moreover, use of lipin-1-deficient cells by siRNA silencing [33] demonstrated no significant increases in ER DAG levels during the LPS challenge (Fig. 5A). In addition, high colocalization between the DAG present in the ER and lipin-1 was noted, as judged by the high M_1 index found between the FRET/CFP ratio from the Daglas-em1 biosensor, and the fluorescence from the chimera lipin-1-mCherry (M_1 = 0.72 ± 0.02) (Fig. 5B). A very high positive correlation (r = 0.9062) was also found between the levels of DAG in the ER (FRET/CFP ratio) and lipin-1-mCherry expression levels in transfected macrophages (Fig. 5C). These results demonstrate that lipin-1 and DAG are in close proximity in the ER—as it would be expected from an enzyme and its product—and that LPS-stimulated DAG production in the ER depends on lipin-1 expression.

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[Ca\(^{2+}\)]\text{er} was studied next using the calcium cameleon indicator vYC4er. The reduction in [Ca\(^{2+}\)]\text{er} experienced by the cells during LPS treatment was significantly prevented in \(LPIN1\)-silenced cells (Fig. 6D). Further evidence for the implication of lipin-1 in TRPC3 activation was obtained when we assessed the upregulation of inflammatory genes in HEK-TLR4 cells that overexpressed TRPC3 and exhibited reduced levels of lipin-1 by gene silencing. The absence
TRPC3 inhibition ameliorates systemic inflammation in vivo

We evaluated the pathophysiological relevance of TRPC3 in vivo by analyzing the effects of the inhibitor Pyr10 during an acute systemic inflammatory response induced by treating mice with LPS (Fig. 7). The animals were pretreated i.p. with Pyr10 (1mg/kg) for 1 h. Afterward, LPS was injected i.p. (5 mg/kg) for 3 h (Fig. 7A). Measurement of cytokine levels in blood serum showed decreased levels of TNF-α (Fig. 7B). COX-2 protein levels were assayed by immunoblot in liver, where LPS has a very potent effect. The results showed that the enzyme was less expressed in the Pyr10-pretreated group, implying reduced production of inflammatory eicosanoids under these conditions (Fig. 7C). Also, analysis of mRNA levels for different inflammatory genes showed that the drug strongly prevented the upregulation of Ptgs, Tnfa, Il1b, and Il6 mRNA levels in the liver (Fig. 7D). These results demonstrate that inhibition of TRPC3 with Pyr10 reduces the inflammatory response induced by LPS in animals.

Discussion

The involvement of Ca²⁺ fluxes in LPS signaling in macrophages and their relevance to mount a full inflammatory reaction have long been recognized [26]. However, the molecular players regulating these processes remain poorly defined. In the present work, we describe a new key effector for LPS-triggered Ca²⁺ signaling, the ion channel TRPC3. Our data support a scenario during macrophage activation by LPS wherein lipin-1 hydrolyzes the PA pools present in ER membranes, generating DAG (Fig. 7E). The ER-associated TRPC3 pools that are close to lipin-1-derived DAG thus become activated, thereby opening a pore that allows Ca²⁺ release from the organelle to the cytosol. In this manner, TRPC3 participates in the release of Ca²⁺ from intracellular stores and triggers a cascade of events that impact on NF-κB translocation to the nucleus and upregulation of inflammatory genes. Since the development of new pharmacological strategies that use TRPC3 as a target are well advanced, and we have observed that selective inhibition of TRPC3 reduces inflammation in a murine sepsis model, our study provides the foundation for new therapies against inflammatory-based diseases using drugs targeting this channel.

A striking feature of TRPC3 channels is their dependence on DAG. Regulated increases in DAG levels provide an efficient means to turn on the activity of TRPC3 in accordance with the activation state of the cells [15, 27], facilitating its role in modulating cellular Ca²⁺ fluxes. It is generally accepted that phosphoinositide-specific phospholipase C, by producing inositol 1,4,5-trisphosphate (InsP₃) of lipin-1 clearly prevented TRPC3 from being involved in LPS-induced upregulation of Ptgs2 and Tnfa (Fig. 6E).

The substrate of lipins, PA, can be produced in activated cells by the direct action of phospholipase D on membrane glycerophospholipids [2, 6]. We treated macrophages with the specific phospholipase D inhibitor FIP1 [42], and found that it affected the rise in [Ca²⁺]i during LPS treatment (Fig. 6A), consistent with a scenario whereby phospholipase D provides phosphatidic acid for DAG production and TRPC3 activation. Collectively, these results highlight a key role for lipin-1 in regulating Ca²⁺ mobilization from the ER and TRPC3-mediated inflammation during LPS activation.
and DAG, impacts as a double-edge sword on intracellular Ca$^{2+}$ signaling [19]. InsP$_3$ interacts with specific receptors in the ER favoring Ca$^{2+}$ release, and DAG activates TRPCs at the plasma membrane, allowing Ca$^{2+}$ entry from the extracellular medium [19]. However, the unanticipated findings that Pyr10-sensitive ion currents are not altered by LPS at the plasma membrane level, and that TRPC3 is mainly present in intracellular membranes in macrophages, makes it difficult to envision a mechanism for channel activation coupled to phosphoinositide hydrolysis at the plasma membrane. Rather, our results are consistent with the notion that intracellular TRPC3 is activated by DAG generated close to the channel, which allows the release of Ca$^{2+}$ from internal stores.

Considering possible candidate enzymes that could be involved in the regulation of intracellular DAG levels during TLR4 challenge, lipin-1 fulfills key requirements. For example, although lipin-1 does not reside permanently in the

Fig. 5 Lipin-1 mediates DAG increases in the ER during LPS activation. A THP-1 macrophages (left panel) and HEK-TLR4 cells (right panel) were transfected with the Daglas-em1 sensor. Fluorescence was analyzed by confocal microscopy before and after treating the cells with LPS (100 ng/ml and 1 μg/ml LPS, respectively), in cells treated or not with 200 μM propranolol, or silenced for LPIN1, as indicated. Changes in nFRET/CFP ratio are shown. Multiple t test was applied. ***, $p<0.001$, ****, $p<0.0001$. B THP-1 macrophages transfected with the Daglas-em1 sensor and lipin-1-mCherry were stimulated with 100 ng/ml LPS for 10 min and counterstained with Hoechst 33342. Fluorescence from CFP (cyan), YFP (yellow), nFRET/CFP (DAG, green), lipin-1-mCherry (red) Hoechst 33342 (nuclei, blue), was analyzed by confocal microscopy and images, including the colocalization mask between DAG and lipin-1-mCherry fluorescences (white), are shown. Scale bar represents 10 μm. Manders’ colocalization indexes ($M_1$) were calculated and are shown (lower panel). Error bars represent SEM (n = 22). C Cells expressing lipin-1-mCherry were individually analyzed for DAG content (nFRET/CFP fluorescence) and lipin-1-mCherry content (red fluorescence) and plotted against each other to obtain a regression index ($r = 0.9062$, $n = 45$ cells, left panel). Representative fluorescence images are shown in the right panel.
Fig. 6 Lipin-1 participates in Ca\(^{2+}\) fluxes during LPS stimulation. A THP-1 macrophages were labeled with Fluo-4, and fluorescence was recorded before and after 100 ng/ml LPS treatment, in the absence or presence of 200 μM propranolol or 1 μM FIPI, as indicated. Representative fluorescence images (left panel) and quantification of the areas under the curves (arbitrary units, a.u.; right panel) are shown. Error bars represent SEM (n = 25). ***, p < 0.001, by Student's t test. B THP-1 macrophages treated with siRNA control or against LPIN1 were labeled with Fluo-4, and fluorescence was recorded before and after treating the cells with 100 ng/ml LPS, as indicated (middle panel). Quantification of the area under the curve is shown in arbitrary units (a.u., right panel). Error bars represent SEM (n = 25). mRNA levels for LPIN1 analyzed by qPCR are also shown (left panel). Error bars represent SEM (n = 3). **, p < 0.01, Student’s t test. C Peritoneal macrophages from wt or lipin-1-deficient animals (fld) were used for [Ca\(^{2+}\)]\(_{i}\) quantification as in B. Error bars represent SEM (n = 650). ***, p < 0.001, Student’s t test. D HEK-TLR4 cells were silenced with siRNA control, or against LPIN1, transfected with the vYC4er sensor, and changes in [Ca\(^{2+}\)]\(_{er}\) were analyzed before and after treating the cells with 1 μg/ml LPS, as indicated. Mean nFRET/eCFP ratio ± SEM is represented (n = 40 cells). Multiple t test was applied. ***, p < 0.001. E HEK-TLR4 cells were transfected with TRPC3, silenced with siRNA control or against LPIN1 and stimulated with 1 μg/ml LPS for 3 h. mRNA levels for the indicated genes were analyzed by qPCR. mRNA levels for LPIN1 analyzed by qPCR is also shown (left panel). Error bars represent SEM (n = 3). **, p < 0.01; ***, p < 0.001, by Student’s t test.
ER, it is able to translocate to ER membranes via a polybasic domain that facilitates binding to its PA substrate [14]. Importantly, lipin-1 has previously been found to regulate cellular DAG levels during macrophage activation by LPS and, in this manner, impact on many cellular and biological processes of activated macrophages [5, 33]. However, the molecular mechanisms governing these effects were not identified. The discovery that lipin-1 regulates TRPC3 activation and associated Ca\(^{2+}\) suggests that this phosphatase plays a wider role than anticipated during macrophage activation. From a signaling point of view, lipin-1 resembles the effects triggered by phospholipase C at the plasma membrane, i.e., promoting the accumulation of DAG and initiating Ca\(^{2+}\) fluxes. Thus, our data support the view that LPS triggers similar processes at two different cellular locations, i.e., the plasma membrane and at the ER, generating DAG.
to activate signaling effectors and, by different mechanisms, regulating the release of Ca^{2+} from the organelle.

Consistent with the above scenario, ER lipidomics performed in TLR4-activated macrophages has revealed that the organelle undergoes a profound lipid remodeling [1]. Interestingly, a few molecular species of PA increase in this compartment, namely PA(32:1), PA(34:1), PA(36:0), PA(40:3), and PA (40:6). It is clear that some of these species may serve as substrates for phospholipid synthesis—a process that is known to occur during TLR4 activation—but others could participate in signaling either directly or via conversion to other signaling lipids such as DAG [13]. We have shown here that PLD inhibition decreases Ca^{2+} fluxes during LPS stimulation. Macrofrophages express two phospholipase D forms, called PLD1 and PLD2, and both have been found to be activated by LPS ([21, 24, 42]). While PLD1 is present at the plasma membrane and intracellular vesicles, PLD2 seems to localize at perinuclear regions [21]. Importantly, it has recently been described that during activation of macrophages by LPS, PLD1, but not PLD2, associates with TLR4 and MyD88, increasing its activity [20]. It is thus plausible that the TLR4/MyD88/PLD1 axis mediates increases in PA pools that serve as substrates for lipin-1 during the events of macrophage proinflammatory activation and sepsis development described in this study.

The participation of TRPC3 in the release of Ca^{2+} during LPS challenge does not preclude the involvement of InsP3 receptors as well, as proposed by previous work [10]. It should be noted that the InsP3 antagonist utilized in those studies, 2-aminoethoxydiphenyl borate, has also been found to block TRPC3 [8], which makes it difficult to estimate the actual participation of InsP3 receptors to the LPS response. Based on our studies using ER-tagged cameleon genetic probes where TRPC3 was silenced or inhibited, we have shown that during activation of macrophages by LPS, PLD1, but not PLD2, associates with TLR4 and MyD88, increasing its activity [20]. It is thus plausible that the TLR4/MyD88/PLD1 axis mediates increases in PA pools that serve as substrates for lipin-1 during the events of macrophage proinflammatory activation and sepsis development described in this study.

To conclude, we have uncovered the ion channel TRPC3 as a new early player in LPS-stimulated responses in human macrophages. Our work introduces the concept that intracellular, lipid-activated TRPC3 participates in Ca^{2+} signaling during LPS-driven inflammation and sepsis development. Furthermore, we have presented evidence that lipin-1 provides the DAG used for TRPC3 activation in this context. These findings help fill some gaps in our perception on how innate responses are mounted. Also, due to the recent development of photopharmacology and optochemical genetics of TRPC3 that could be the foundation for novel therapeutics [25], these discoveries highlight pharmacological targeting of TRPC3 as an attractive new strategy for the treatment of sepsis and inflammatory diseases.

Materials and methods

Cells

Human blood monocyte-derived macrophages were prepared from buffy coats of healthy volunteer donors (Centro de Hemoterapia y Hemodonación de Castilla y León, Valladolid, Spain) as previously described [9, 29]. Non-adherent mononuclear cells were differentiated to macrophages by culture in RPMI 1640 supplemented with 2 mM L-glutamine, 100 μM penicillin, 100 μg/ml streptomycin, and heat-inactivated 5% human serum without exogenous cytokines for 2 weeks [9, 29]. The human promonocytic cell line THP-1 was maintained in RPMI 1640 medium supplemented with 10 mM HEPES, 10% FBS, 100 μM penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1% sodium pyruvate, 1% non-essential amino acids solution and 50 μM β-mercaptoethanol at 37 °C in a 5% CO2 humidified incubator. For differentiation to a macrophage phenotype, the cells were treated with 25 ng/ml PMA for 24 h, after which they were left to rest for 48 h.

Mouse peritoneal macrophages were obtained as previously described [33]. Briefly, the peritoneal cavity was flushed with 5 ml ice-cold PBS. Cells were then centrifuged at 300 g for 10 min and allowed to adhere to glass coverslips overnight. Non-adherent cells were washed away with PBS.

HEK293 cells expressing human TLR4/MD2/CD14 (HEK-TLR4, Invivogen, Catalog #293-hl4rmd2cd14) were cultured in DMEM supplemented with 2 mM glutamine, 10% fetal bovine serum (FBS), 1% sodium pyruvate, 1% non-essential amino acids solution, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml blasticidin and 25 μg/ml hygromycin B at 37°C in a 5% CO2 humidified incubator. The cells were passaged twice a week by detachment with 1 mM EDTA in PBS.

Animals

BALB/cJ mice were obtained from the University of Valladolid Animal House (10–12-week-old males). The mice were intraperitoneally injected with the TRPC3 specific inhibitor Pyr10 (1 mg/kg) (Sigma-Aldrich) for 1h and then treated with LPS at a sublethal dose of 5 mg/kg (i.p.). For the analysis of proinflammatory factors, animals were sacrificed by ketamine (100 mg/kg) / xylazine (10 mg/kg) administration and cervical dislocation 3 h after LPS treatment. Blood was collected through cardiac puncture. Livers were collected in...
RNAlater (Ambion) for further analysis by real-time PCR or in protein lysis buffer for western blot analysis. Serum from animals was used for quantification of TNF-α by specific ELISAs (Thermo) following the manufacturer’s instructions. BALB/cByJ- Lpin1fld/J mice carrying a spontaneous mutation in the Lpin1 gene (fatty liver dystrophy, fld) were also used [33]. All procedures involving animals were carried out under the supervision of the Institutional Committee of Animal Care and Usage of the University of Valladolid (Approval No. 7406000), and are in accordance with the guidelines established by the Spanish Ministry of Agriculture, Food, and Environment, and the European Union.

**Plasmids and mutagenesis**

Human TRPC3 (eYFP-hTRPC3) and the N-terminal fragment of hTRPC3 (hTRPC3(1-321), eYFP-N-TRPC3) expression plasmids were kindly provided by Dr. K. Groeschner (University of Graz, Austria). Wild-type hTRPC3 was mutagenized within the transmembrane pore domain (S6 helix) to modify its DAG discrimination capacity [27] by replacing Gly652 with Ala (G652A) using the Quick-Change XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and the oligonucleotides described in Supplemental Table 1. Mutagenesis was confirmed by sequencing. The plasmids pcDNA3-Daglas-pm1 and pcDNA3-Daglas-em1 were provided by Dr. M.J. Caloca from our institute (originally from Dr. Y. Umezawa, University of Tokyo, Japan). Daglas-em1 was amplified by PCR adding 5′Xba-I and Sal-I 3′specific restriction sites and which removes copGFP gene in the plasmid inserted into lentiviral vector pCDH-CMV-MCS-EF1α-copGFP. pBudCE4.1-vYC4er (vYC4er cameleon) was provided by Dr. M.T. Alonso from our institute (originally from Dr. W. Graier, University of Graz, Austria). Wild-type hTRPC3 was mutagenized within the transmembrane pore domain (S6 helix) to modify its DAG discrimination capacity [27] by replacing Gly652 with Ala (G652A) using the Quick-Change XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and the oligonucleotides described in Supplemental Table 1. Mutagenesis was confirmed by sequencing. The plasmids pcDNA3-Daglas-pm1 and pcDNA3-Daglas-em1 were provided by Dr. M.J. Caloca from our institute (originally from Dr. Y. Umezawa, University of Tokyo, Japan). Daglas-em1 was amplified by PCR adding 5′Xba-I and Sal-I 3′specific restriction sites and which removes copGFP gene in the plasmid inserted into lentiviral vector pCDH-CMV-MCS-EF1α-copGFP. (System Biosciences) after removing an internal Sal-I restriction site by mutagenesis.

**Gene silencing and plasmid transfection**

Gene silencing and plasmid transfection (vYC4er cameleon) of human monocyte-derived macrophages and THP-1 macrophages was achieved using a Nucleofector device (Amaxa), following the specifications of the manufacturer. Briefly, the human macrophages were harvested by treatment with 1 mM EDTA in PBS for 30 min followed by trypsin treatment for 20 min and then by gentle scraping. The THP-1 macrophages were detached by accutase treatment (Gibco) for 30 min. Cells were then washed and resuspended in 100 μl Nucleofector solution plus 1 μg plasmid or 20 nM siRNAs (Sigma). Nucleofection was carried out using the program Y-010, and the cells were resuspended in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 5% heat-inactivated human serum. Cells were used for experimentation 48 h later.

Gene silencing of HEK-TLR4 cells, was carried out using Lipofectamine RNAiMAX (Thermo Fisher Scientific) as specified by the manufacturer, and experiments were conducted 48 h later. Plasmid delivery (eYFP-TRPC3, eYFP-N-ter-TRPC3, eYFP-G652A-TRPC3, and vYC4er cameleon) in HEK293-TLR4 cells was done using linear polyethylenimine 25K (Polysciences) complexed with DNA in a 2:1 ratio, respectively, for 24–48 h. Stable transfections of eYFP-TRPC3 were carried out by culturing the cells in the presence of 0.5 mg/ml G418.

**Lentiviral transduction**

VSV-G–pseudotyped lentiviral particles were produced by cotransfection of 293FT cells with transfer constructs (Daglas-em1 and lipin-1-Cherry) and the compatible packaging plasmids pMD2.G and psPAX2 in the presence of PEI 25K. Viruses were harvested at 48 and 72 h after transfection. Lentiviral transduction of primary human macrophages THP-1 macrophages and HEK-TLR4 cells, were carried out using concentrated lentiviral particles in the presence of 8 μg/ml Polybrene (Sigma-Aldrich), and infected cells were selected by FACS sorting and subjected to imaging experiments.

**Cytosolic Ca²⁺ measurement**

Cells were loaded with 3 μM Fluo-4-AM for 20 min in culture medium at 37 °C in a 5% CO₂ incubator. Cells were then washed in indicator-free medium to remove any dye that was nonspecifically associated with the cell surface, and then incubated for a further 20-min period to allow for complete hydrolysis of the acetoxymethyl esters. Live cell fluorescence was monitored by confocal microscopy (TCS SP5X, Leica) with a HCX Plan Apo CS 40X/1.25 NA oil or a HCS Plan Apo CS 63X/1.4 NA oil immersion lenses, using 488 nm laser excitation and an emission window of 500–560 nm, with the pinhole fully open. Before imaging started, medium was replaced by HBSS supplemented with 10 mM HEPES, with 1.3 mM CaCl₂ and 1.3 mM MgCl₂. We recorded a 5 min baseline before adding LPS acquiring images each 15 s. In some experiments, calibration of Ca²⁺ levels was achieved at the end of each experiment as previously described by Kao et al. [22]. Cells were treated with MnCl₂ at a final concentration of 2 mM in the presence of 2.5 μM A23187, and then cells were lysed with 0.05% digitonin to obtain the fluorescence background signal. Fluorescence
data were analyzed using a combination routine of ImageJ and Cell Profiler semiautomated custom pipeline.

**Live cell FRET imaging**

Cells expressing FRET-based sensors (Daglas or vYC4er) were plated together with a small proportion of single-color control expressing cells on glass-bottom culture dishes (MatTek) coated with poly-L-lysine (Sigma) when improved adhesion was required. The culture medium was replaced with HBSS supplemented with 10 mM HEPES, pH 7.4, 1.3 mM CaCl₂ and 1.3 mM MgCl₂ for confocal imaging. Cells were imaged at 37 °C on a Leica confocal system TCS SP5X, with a HCS Plan Apo CS 63X/1.4 NA oil immersion lens, correct membrane/endomembranes localization of the Daglas sensor was confirmed by YFP imaging. For ratio imaging, CFP fluorescence was obtained by 458 nm excitation and 465–510 nm emission, YFP by 515 nm excitation and 525–580 nm emission, and uncorrected FRET by 458 nm excitation and 465–510 nm emission, YFP by 515 nm excitation and 525–580 nm emission. For ratio calculation, CFP and FRET were simultaneously acquired while YFP was acquired alternately. ImageJ software with customized plugins was used to correct the background from raw images and to create ratio images and export raw data to Microsoft Excel for further ratio and correction calculations. To correct for any laser power illumination inconsistency, the FRET/CFP ratio values were divided by single-color YFP expressing cells values [37], then divided by the baseline normalized FRET/CFP ratio according to the following equation:

\[
\text{nFRET Ratio} = \frac{\left( \frac{\text{FRET}_{\text{t}}}{\text{YFP}_{\text{t}}} \right)}{\left( \frac{\text{FRET}_{\text{0}}}{\text{YFP}_{\text{0}}} \right)}
\]

**Patch-clamp electrophysiology**

Mouse peritoneal macrophages were plated on 12-mm coverslips, previously treated with poly-L-lysine (0.01 mg/ml), for 30 min. Patch micropipettes were made from borosilicate glass (2.0 mm OD; WPI) and double pulled (Narishige) to resistances ranging from 5 to 10 MΩ when filled with the internal solution containing: 125 mM KCl, 4 mM MgCl₂, 10 mM HEPES, 10 mM EGTA, 5 mM MgATP, pH 7.2 with KOH. Cells were bathed in an external solution containing: 141 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl₂, 1.8 mM CaCl₂, 10 mM glucose, and 10 mM HEPES, pH 7.4 with NaOH. Immediately after starting the recording of whole-cell currents, the external solution was switched to a solution containing: 131 mM NaCl, 10 mM TEACl, 4.7 mM KCl, 1.2 mM MgCl₂, 1.8 mM CaCl₂, 10 mM glucose, 100 μM BaCl₂, 100 μM niflumic acid and 10 mM HEPES, pH 7.4 with NaOH. This solution allows to record in isolation currents mediated by TRP like channels, blocking currents through voltage-dependent K⁺ channels, inward rectifier channels and chloride channels. Cells were voltage-clamped at −40 mV. Ionic currents were recorded at room temperature using the whole-cell configuration of the patch-clamp technique. Current–voltage relationships were obtained with a 1 s ramp protocol from −100 mV to +100 mV applied every 10 s. Whole-cell currents were recorded using an Axopatch 200A patch-clamp amplifier (Axon Instruments) filtered at 2 kHz (−3 dB, 4-pole Bessel filter) and sampled at 10 kHz. Recordings were digitized with a Digidata 1200, driven by CLAMPEX 10.2 software (Molecular Devices). Electrophysiological data analyses were performed using Clampfit subroutine of the pClamp software (Axon Instruments) and with Origin 7 software (OriginLab Corporation). Current amplitude was corrected for cell size variations and expressed as current density (pA/pF), by dividing for cell capacitance values.

**Semi-quantitative PCR and qPCR**

Total RNA was extracted using TRizol reagent (Ambion). cDNA was obtained using Verso cDNA kit Reverse Transcription for RT-PCR (Thermo Fisher Scientific), following the manufacturer’s instructions. For semi-quantitative PCR forward and reverse primers specific to human TRPC3, 6, 7 and β-actin were used (see Supplemental Table S1). PCR reactions were carried out on an Eppendorf Mastercycler Personal thermal cycler using Taq polymerase (BioTools) and the following parameters: denaturation at 94°C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 40 s. A total of 40 cycles were performed followed by a final extension at 72 °C for 5 min. PCR products were analyzed by electrophoresis with 1.5% agarose gel and visualized by GelRed staining. Bands were intensity quantified with Image Studio Software (LiCor).

Quantitative PCR analysis was performed with an ABI7500 machine (Applied Biosystems) using specific primers, 20 ng cDNA and the PowerUp SYBR Green Master Mix (Applied biosystems). Relative mRNA expression was obtained using the ΔΔCt method using ACTB or Gapdh as reference genes [47].

**Colocalization analyses**

Confocal z-stacks images were deconvolved using an ImageJ parallel iterative plugin (Wiener Filter Preconditioned Landweber (WPL) method) after calculating the experimental setup point spread function (PSF) using sub resolution (0.17 μm) fluorescent beads. Colocalization was performed with JACoP plugin in ImageJ. Background levels were
obtained by measuring the mean intensity of each signal outside the cells and were subtracted; negative pixel values were clipped to zero. Positive values were selected by Costes automatic thresholding, removing the bias of visual interpretation [11]. Colocalization index Manders’ $M_1$ and $M_2$, were calculated [31]. Manders’ split coefficients are based on the Pearson’s correlation coefficient but avoid issues relating to absolute intensities of the signal, since they are normalized to total pixel intensity [31]. These coefficients vary from 0 (non-overlapping) to 1 (100% colocalization). The index $M_1$ is defined as the percentage of above-background pixels from the first channel (green) that overlap above-background pixels from the second channel (red). This index is sensitive to changes in the background but not to differences in the intensity of overlapped pixels and is suitable to apply in images with a high and clear signal to background ratio.

**Immunocytochemistry analyses**

For intracellular staining of endogenous TRPC3 or NFkB-p65, cells were fixed with 4% paraformaldehyde for 20 min in LabTek II chambers and then permeabilized with 0.1% Triton X-100 for 20 min at room temperature. Cells were treated with 5% goat serum for 30 min and incubated with antibodies against TRPC3 (Alomone Labs, validated in TRPC3-deficient cells), NFkB-p65 (Cell Signaling), or SERCA (Santa Cruz) for 1 h. After washing, the cells were incubated with spectrally appropriate Alexa Fluor-conjugated Fab fragments against primary species antibodies (Molecular Probes) for 1 h. After intracellular staining, nuclei were counterstained with the DNA-binding dye Hoechst 33342 (Invitrogen). All images were captured with a Leica confocal system TCS SP5X inverted microscope with a HCS Plan Apo CS 63X/1.4 NA oil immersion lens. Leica Application Suite Advanced Fluorescence software was used for the capture, and ImageJ was used for deconvolution and image presentation. Translocation was analyzed using Cell Profiler software (Broad Institute) with a custom pipeline (available upon request) that automatically detects cell boundaries and calculates the ratio between cytoplasm and nuclei fluorescence median.

**Immunoblotting**

Cells or tissues were lysed in a buffer 1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM Na$_4$P$_2$O$_7$, 50 mM sodium β-glycerol phosphate, 270 mM sucrose, 1 mM Na$_3$VO$_4$, 10 mM NaF, 1 mM phenylmethyl sulphon fluoride and a protease inhibitor cocktail (Sigma). Cell protein was separated by 10% reducing SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated with specific primary antibodies followed by incubation with HRP-conjugated secondary antibodies and detected and quantified with a LiCor Odyssey Fc infrared imaging system.

**Quantification and statistical analysis**

Statistical details of experiments are indicated in the figure legends. All data analyses were performed with Prism software (GraphPad). All data are presented as means ± standard error of the mean (SEM), indicating individual biological replicates. No statistical analysis was used to predetermine sample size. For in vivo experiments, animals were randomized in different treatment groups. All datasets were analyzed by unpaired two-tailed Student’s t test. Welch’s correction was performed when the variances were significantly different. Some data required multiple t tests, with a false discovery rate of 1% based on two stage step up by Benjamini, Krieger and Yekutieli.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00018-021-03999-0.

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**Data availability** Available upon request.

**Declarations**

**Conflict of interest** The authors declare no competing financial interests and that no conflict of interest exists.

**Ethical approval** The present study was approved by the Ethics and Bioethics Committee of the Spanish National Research Council (CSIC) and meets the standards of the Declaration of Helsinki in its revised version of 1975 and its amendments of 1983, 1989, and 1996.

**Consent to participate** The written informed consent of all healthy donor subjects was obtained at Centro de Hemoterapia y Hemo-
donación de Castilla y León Biobank. The researchers received the samples in an anonymous way.

Consent for publication Not applicable.

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