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## The LUBAC participates in Lysophosphatidic Acid-induced NF- $\kappa$ B Activation

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### Abstract

The natural bioactive glycerophospholipid lysophosphatidic acid (LPA) binds to its cognate G protein-coupled receptors (GPCRs) on the cell surface to promote the activation of several transcription factors, including NF- $\kappa$ B. LPA-mediated activation of NF- $\kappa$ B relies on the formation of a signalosome that contains the scaffold CARMA3, the adaptor BCL10 and the paracaspase MALT1 (CBM complex). The CBM complex has been extensively studied in lymphocytes, where it links antigen receptors to NF- $\kappa$ B activation via the recruitment of the linear ubiquitin assembly complex (LUBAC), a tripartite complex of HOIP, HOIL1 and SHARPIN. Moreover, MALT1 cleaves the LUBAC subunit HOIL1 to further enhance NF- $\kappa$ B activation. However, the contribution of the LUBAC downstream of GPCRs has not been investigated. By using murine embryonic fibroblasts from mice deficient for HOIP, HOIL1 and SHARPIN, we report that the LUBAC is crucial for the activation of NF- $\kappa$ B in response to LPA. Further echoing the situation in lymphocytes, LPA unbridles the protease activity of MALT1, which cleaves HOIL1 at the Arginine 165. The expression of a MALT1-insensitive version of HOIL1 reveals that this processing is involved in the optimal production of the NF- $\kappa$ B target cytokine interleukin-6. Lastly, we provide evidence that the guanine exchange factor GEF-H1 favors MALT1-mediated cleavage of HOIL1 and NF- $\kappa$ B signaling in this context. Together, our results unveil a critical role for the LUBAC as a positive regulator of NF- $\kappa$ B signaling downstream of LPA receptors.

### 1. Introduction

Lysophosphatidic acid (LPA) is a natural serum phospholipid, which promotes growth-factor like activities through specific G-protein-coupled receptors (GPCR) on the cell surface [1]. Uncontrolled expression of this bioactive lipid or of its receptors has been linked to tumor growth and metastasis [1]. Stimulation with LPA culminates with the activation of several transcription factors, including NF- $\kappa$ B [2]. Although the molecular basis for GPCR-mediated

NF- $\kappa$ B activation continues to be elucidated, signal transduction was recently shown to share striking similarities with signaling through antigen receptors in lymphocytes. In both cases, NF- $\kappa$ B signaling requires the activation of apical Protein Kinase C (PKC) and the subsequent assembly of a large signaling complex of CARMA1/3, BCL10 and MALT1 coined CBM complex [3–8]. The CBM complex serves as a docking platform for the recruitment and activation of the I $\kappa$ B kinase complex (IKK), which phosphorylates the NF- $\kappa$ B inhibitors I $\kappa$ Bs, marking them for proteasomal degradation. NF- $\kappa$ B is then free to translocate into the nucleus and modulate the transcription of its target genes, driving lymphocyte activation and cytokines production [9].

In addition to its scaffolding function required for the activation of NF- $\kappa$ B, MALT1 is a paracaspase that cleaves a limited set of substrates to finely tune the immune response [10–13]. In the immune system, known MALT1 substrates count regulators of NF- $\kappa$ B, JNK/AP-1, mTOR, mRNA stability and of adhesion [9]. This enzymatic activity is crucial for the development of regulatory T cells, the differentiation of Th17 lymphocytes, and governs T-cell receptor (TCR)-driven proliferation and IL-2 production [10–12,14]. Interestingly, MALT1 protease activity was also found to modulate the integrity of the epithelial barrier in response to thrombin [4], therefore suggesting that MALT1 could be unleashed following GPCRs engagement.

Recently, we and others reported that the linear ubiquitin assembly chain complex (LUBAC) is dynamically recruited to the CBM following antigen receptor ligation and participates in IKK/NF- $\kappa$ B activation [15–18]. This triad composed of the E3 ligases HOIP (also known as RNF31), HOIL1 (also known as RCBK1) and the adaptor SHARPIN catalyzes linear ubiquitin chains and conveys NF- $\kappa$ B signaling downstream a variety of immunoreceptors [19]. In addition, HOIL1 was subsequently found to be cleaved and inactivated by MALT1 at the residue Arginine in position 165 [20–22]. Nevertheless, the contribution of the LUBAC to LPA-mediated NF- $\kappa$ B activation in non-immune cells has not been addressed. Here, by using embryonic fibroblasts derived from mice knockout for the LUBAC subunits, we unveiled a pivotal role for this complex during NF- $\kappa$ B activation in response to LPA. We also show that, paralleling the situation in lymphocytes, LPA triggers MALT1 protease activity and that the paracaspase cleaves HOIL1 at the Arginine 165. Although dispensable for IKK activation, this processing participates in the optimal production of the CBM target cytokine interleukin-6 (IL-6). Lastly, we report that the guanine exchange factor GEF-H1, which is activated by LPA [23], promotes MALT1 enzymatic activity and NF- $\kappa$ B activation in non-immune cells.

## **2. Materials and Methods**

### **2.1. Cell culture and Reagents**

HEK293T cells were purchased from the American Type Culture Collection (CRL-1573). MEFs deficient for HOIP (*Tnf<sup>-/-</sup> Hoip<sup>-/-</sup>*) and HOIL1 (*Tnf<sup>-/-</sup> Hoil1<sup>-/-</sup>*) as well as their wild-type counterparts were kindly provided by H. Walczak (University College London, London,

United Kingdom) [24,25]. MEFs deficient for SHARPIN (chronic proliferative dermatitis, *Cpdm*) and their wild-type counterparts were a generous gift by J. Ivaska (University of Turku, Turku, Finland) [26]. MEFs deficient for GEFH1 and their wild-type littermates were previously described [23,27]. All cell lines were cultured in Dulbecco's modified Eagle's media (DMEM, Life Technologies) supplemented with 10% FBS and Penicillin/Streptomycin (Life Technologies). Cells were stimulated with 10  $\mu\text{g.mL}^{-1}$  of LPA (Santa Cruz), with a mixture of 50  $\text{ng.mL}^{-1}$  PMA (Sigma) and 100  $\text{ng.mL}^{-1}$  Ionomycin (Sigma), or with 10  $\text{ng.mL}^{-1}$  TNF $\alpha$  (R&D Systems). The protease activity of MALT1 was blocked with 20  $\mu\text{M}$  of Mepazine (Chembridge) or 75  $\mu\text{M}$  of the tetrapeptide inhibitor zVRPR.fmk (Enzo Life Sciences). Mouse IL-6 was detected from cell culture supernatants by ELISA, according to the manufacturer's instructions (R&D Systems).

## 2.2. Western Blotting Analysis and Antibodies

Cells were washed with ice-cold PBS and lysed with TNT buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% Igepal, 2 mM EDTA] supplemented with protease inhibitors (Thermo Fisher Scientific) for 30 min on ice. Samples were cleared by centrifugation at 9,000g and protein concentration determined by a BCA assay (Thermo Fisher Scientific). 5-10  $\mu\text{g}$  denatured proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare). Antibodies against HOIL1 (H-1, 1:1,000), GAPDH (6C5, 1:20,000) were from Santa Cruz. Antibodies to phosphorylated I $\kappa$ B $\alpha$  (5A5, 1:5,000), and GEF-H1 (55B6, 1:1,000) were from Cell Signaling Technology. Antibodies against SHARPIN (A303-559A, 1:5,000) from Bethyl Laboratories and against FLAG (M2, 1:5,000) from Sigma were also used. Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Southern Biotechnology. Densitometric analyses were performed using ImageJ software (National Institutes of Health).

## 2.3. Expression Plasmids and Transfections

Plasmids encoding for G $_q$  $\alpha$ , its constitutively active mutant G $_q$  $\alpha_{\text{QL}}$  and vGPCR were a gift from J.S. Gutkind [28]. HOIL1-WT and HOIL1-R165G in a pCDH1-MSCV-EF1 $\alpha$ -GreenPuro vector were previously described [20]. GEF-H1 (pCMV-ARHGEF2, Origene) was cloned into a pCDH1-MSCV-EF1 $\alpha$ -GreenPuro plasmid (SBI). HEK293T cells were transfected using a standard calcium phosphate protocol, as previously described [29,30]. NF- $\kappa$ B luciferase gene reporter assays were performed according to the manufacturer's instruction (Promega), as previously described [20]. For infections, HEK293T were transfected with plasmids encoding for HOIL1-WT, HOIL1-R165G or an empty vector together with pVSV-G and psPAX2 plasmids. Cell culture supernatants containing lentiviral particles were collected after 48 hours and added on *Tnf<sup>f/-</sup> Hoil1<sup>-/-</sup>* MEFs with 8  $\mu\text{g.mL}^{-1}$  polybrene (Santa Cruz) during a 2,500 x *rpm* centrifugation for 90 minutes. 24 hours post-infection, cells were selected with 1 medium containing 1  $\mu\text{g.mL}^{-1}$  of puromycin.

## 2.4. Statistical Analysis

Statistical analyses were performed using GraphPad Prism 7 software using two-way ANOVA and *P* values are indicated in the figure legends.

### 3. Results and Discussion

The treatment of mouse embryonic fibroblasts (MEFs) with LPA, or with phorbol 12-myristate 13-acetate (PMA) plus ionomycin (PI) to mimic GPCR engagement, activates NF- $\kappa$ B in a CBM-dependent manner (Fig. 1A) [3,5,7,8]. To gain insights into the contribution of the LUBAC, we first used MEFs knockout for the catalytic subunit HOIP (*Tnf<sup>-/-</sup> Hoip<sup>-/-</sup>*) and control *Tnf<sup>-/-</sup> Hoip<sup>+/-</sup>* cells [24]. Although LPA and PMA plus ionomycin led to a robust activation of NF- $\kappa$ B in *Tnf<sup>-/-</sup> Hoip<sup>+/-</sup>* cells, as demonstrated by the phosphorylation of the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$ , this was not the case with *Tnf<sup>-/-</sup> Hoip<sup>-/-</sup>* cells, suggesting a defect in NF- $\kappa$ B activation (Fig. 1B). In line with this, the secretion of the CBM-dependent cytokine IL-6 was impaired without HOIP (Fig. 1C). Importantly, the genetic ablation of SHARPIN (chronic proliferative dermatitis, *Cpdm*, [26]) and HOIL1 (*Tnf<sup>-/-</sup> Hoil1<sup>-/-</sup>*, [25]) also precluded the activation of NF- $\kappa$ B (Fig. 1D and 1E). Taken together, these data suggest that the LUBAC is required for NF- $\kappa$ B activation by LPA.

In lymphocytes, a portion of HOIL1 is rapidly cleaved by the paracaspase MALT1 after the arginine residue in position 165 [20–22]. Because MALT1 proteolytic activity was also proposed to be unleashed upon activation of the thrombin PAR1 GPCR in epithelial cells [4], we next assessed the status of HOIL1 in cells treated with LPA. We found that HOIL1 was partially cleaved in cells exposed to LPA or to PMA plus ionomycin (Fig. 2A). By contrast, stimulation with tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), a potent NF- $\kappa$ B activator that operates independently of MALT1, had no overt effect on HOIL1 (Fig. 2A). The treatment of cells with the MALT1 inhibitors Mepazine and zVRPR.fmk [31–33] showed a reduction in HOIL1 processing, which was more pronounced with Mepazine (Fig. 2B). GPCRs promote the activation of several signaling transduction pathways such as MAPK, Akt or NF- $\kappa$ B via the stimulation of guanine nucleotide exchange by heterotrimeric G proteins [34,35]. For instance, the ectopic expression of a constitutively active mutant of G $_q\alpha$  (G $_q\alpha_{QL}$ ) is sufficient to activate NF- $\kappa$ B in a BCL10-dependent manner [8]. We found that such mutant also drove the processing of FLAG-tagged HOIL1 when overexpressed in HEK293T cells (Fig. 2C). This was however not the case when the Arginine in position 165 on HOIL1 was substituted by a Glycine (HOIL1-R165G), hinting that the cleavage site of MALT1 is conserved upon GPCR engagement (Fig. 2C). Of note, similar results were obtained when the Kaposi Sarcoma-associated herpes virus (KSHV) GPCR ( $\nu$ GPCR), which encodes for constitutively active GPCR [36], was overexpressed. Altogether, our results suggest that MALT1 cleaves HOIL1 upon GPCR stimulation.

Because HOIL1 can negatively regulate antigen receptor-mediated NF- $\kappa$ B activation independently of IKK, unless cleaved by MALT1 [20], we next assessed the impact of HOIL1 cleavage in fibroblasts. To this end, *Tnf<sup>-/-</sup> Hoil1<sup>-/-</sup>* MEFs were retrovirally reconstituted with plasmids encoding for an empty vector (EV), HOIL1-WT or MALT1-resistant HOIL1-R165G (Fig. 2D). Both HOIL1-WT and HOIL1-R165G were expressed at levels comparable to parental cells, and restored SHARPIN levels supporting previous reports [37–39]. As expected, HOIL1 was partially cleaved upon PMA plus ionomycin treatment in HOIL1-WT,

and not HOIL1-R165G expressing cells. Interestingly, the defect in I $\kappa$ B $\alpha$  phosphorylation observed without HOIL1 upon LPA or PMA plus ionomycin treatment was overcome in cells expressing HOIL1-WT or HOIL1-R165G, hinting at a normal activation of IKK (Fig. 2D). By contrast, HOIL1-R165G expressing cells did not secrete as much as IL-6 when compared to MEFs reconstituted with HOIL1-WT upon PMA plus ionomycin treatment (Fig. 2E), suggesting that HOIL1 processing contributes to optimal IL-6 production. Of note, paralleling our observation with a MALT1-resistant mutant, treatment with Mepazine also led to a reduced production of IL-6 (Fig. S1). Together, our findings suggest a crucial role for the LUBAC in NF- $\kappa$ B activation by LPA. Echoing the situation in lymphocytes, MALT1 protease activity is unleashed and the paracaspase cleaves HOIL1 at the arginine 165. Although dispensable for IKK activation, HOIL1 processing appears required for the optimal secretion of the target IL-6.

LPA has been shown to drive the activation of the guanine exchange factor GEF-H1 (also called ARGHEF2) by releasing it from microtubules [23]. GEF-H1 exerts its varied functions through the activation of Rho GTPases. For instance, GEF-H1-driven activation of RhoA promotes actin polymerization, modulating cell contractility or endothelial barrier permeability [40–42]. Notably, GEF-H1 participates in the activation of NF- $\kappa$ B during intracellular pathogen recognition [43,44] or in response to microtubule destabilization [45]. Supporting previous works [43,44], we observed that the overexpression of GEF-H1 in HEK293T was sufficient to promote the transcriptional activity of NF- $\kappa$ B (Fig. 3A). Interestingly, we found that ectopic GEF-H1 also led to a robust cleavage of HOIL1 (Fig. 3B). Consistent with a contribution for GEF-H1 in MALT1 activity, MALT1-resistant HOIL1-R165G remained intact (Fig. 3B). In addition, MEFs deficient for GEF-H1 displayed reduced phosphorylation of I $\kappa$ B $\alpha$  and a partial defect in cleavage of HOIL1 in response to LPA and PMA plus ionomycin stimulations (Fig. 3C). In addition, PMA and ionomycin-mediated secretion of IL-6 was blunted (Fig. 3D). Collectively, these results suggest that GEF-H1 contributes to the CBM-driven activation of NF- $\kappa$ B and MALT1.

The CBM complex is an essential gateway for transducing NF- $\kappa$ B signaling downstream of antigen receptors and GPCRs [9]. In T and B lymphocytes, the CBM complex recruits the LUBAC to facilitate the optimal activation of NF- $\kappa$ B [15–18]. However, the situation in non-immune cells remains unknown. We now report that the LUBAC also contributes to LPA-induced NF- $\kappa$ B activation. This signaling node may therefore emerge as an appealing therapeutic target to jugulate this GPCR signaling. The LUBAC has been reported to decorate key components of the antigen receptor-mediated NF- $\kappa$ B signaling pathway such as BCL10 and the IKK subunit NEMO with linear ubiquitin chains, and can also serve as an adaptor [15,17,18,46,47]. It will therefore be of clear interest to define the role of the catalytic activity of the LUBAC following LPA treatment. Our results also unveil a dual role for HOIL1. Whereas HOIL1 positively conveys LPA-induced NF- $\kappa$ B activation, it lessens NF- $\kappa$ B signaling independently of IKK unless cleaved by the paracaspase MALT1 through a molecular mechanism that remains unclear. We previously showed that HOIL1 COOH-terminal fragment, which contains its E3 ligase domain, is released from the LUBAC upon

MALT1 processing [20]. It will therefore be interesting to define the impact of HOIL1 enzymatic activity in this context and how MALT1-mediated processing modifies it [48]. Interestingly, akin to observations made in lymphocyte stimulated through the antigen receptor [20], only a limited portion of HOIL1 is processed upon LPA treatment. This may reflect the fact that a selective part of the LUBAC participates in the CBM complex. Future work is also required to investigate whether MALT1-mediated proteolysis of HOIL1 exerts additional function. Lastly, we provide evidence that the guanine exchange factor GEF-H1 contributes to the activation of MALT1 and NF- $\kappa$ B. GEF-H1 participation appears however partial, suggesting a role in the fine-tuning of the CBM activity. How exactly GPCRs are connected to the CBM continues to be elucidated. For instance,  $\beta$ -arrestin2 was shown to physically interact with CARMA3 and to promote NF- $\kappa$ B activation in response to LPA [7]. Whether GEF-H1 also associates with the CBM is unknown. Our future work will therefore be aimed at defining precisely how GEF-H1 activates MALT1, and whether its GEF activity is required. In endothelial cells, the activation of the GPCR PAR1 disrupts microtubules stability via MALT1-mediated CYLD processing and may therefore assist GEF-H1 activation [4]. In that case, it is tempting to speculate that GEF-H1 engages a positive feedback loop on the CBM complex. Of note, our ongoing work shows that, when overexpressed in HEK293T cells, active mutants of RhoA promote MALT1 activation. Hence, our future investigation may provide a molecular framework to explain how the small GTPase RhoA, which can be activated by GEF-H1, drives NF- $\kappa$ B activation [49].

### **Author Contributions**

Conceptualization, TD and NB; Methodology, TD and NB; Investigation, TD, SC and NB; Resources, RR; Supervision, JG; Writing-Original Draft, TD and NB; Writing-Review & Editing, TD, JG, and NB; Funding Acquisition, JG and NB.

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### **Conflict of Interest**

The authors declare no conflict of interest.

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## Figure Legends

**Figure 1. LPA-mediated NF- $\kappa$ B Activation requires the LUBAC.** **A**, Graphical representation of NF- $\kappa$ B activation in response to antigen receptor engagement and to LPA stimulation. **B**, *Tnf<sup>-/-</sup> Hoip<sup>-/-</sup>* MEFs and their wild type counterparts *Tnf<sup>-/-</sup> Hoip<sup>+/-</sup>* were stimulated with 10  $\mu\text{g.mL}^{-1}$  LPA or with 50  $\text{ng.mL}^{-1}$  PMA plus 100  $\text{ng.mL}^{-1}$  Ionomycin (PI), as indicated. Cell lysates were prepared and subjected to Western blotting analysis with antibodies specific for the indicated proteins. Molecular weight markers  $M_r$  (kDa) are shown. **C**, Abundance of IL-6, measured by ELISA in the supernatant of cells as in (B) stimulated for 16 hours (means  $\pm$  SEM,  $n=3$ , \*\*\*\* $P<0.0001$ , ANOVA). **D and E**, *Cpdm* (D), *Tnf<sup>-/-</sup> Hoil1<sup>-/-</sup>* (E) and their wild type counterparts were treated as in (B). Lysates were prepared and subjected to Western blotting analysis as indicated. Data are representative of three independent experiments.

**Figure 2. HOIL1 is cleaved by MALT1 following LPA Stimulation.**

**A**, MEFs were stimulated with 10  $\mu\text{g.mL}^{-1}$  LPA, with 50  $\text{ng.mL}^{-1}$  PMA plus 100  $\text{ng.mL}^{-1}$  Ionomycin (PI), or with 10  $\text{ng.mL}^{-1}$  TNF $\alpha$  as indicated. Lysates were subjected to Western blotting analysis with antibodies specific for the indicated proteins. White and red arrowheads show full-length and cleaved HOIL1, respectively. Molecular weight markers ( $M_r$ ) are shown. **B**, Western blotting analysis of MEFs pretreated with 20  $\mu\text{M}$  of Mepazine or 75  $\mu\text{M}$  of zVRPR for 90 minutes and stimulated as in (A). **C**, HEK293T cells were transfected with an empty vector (EV), or with plasmids encoding for G $_q\alpha$ , G $_q\alpha_{\text{QL}}$  or vGPCR together with FLAG-tagged HOIL1-WT or HOIL1-R165G. Lysates were prepared and Western blotting analysis was performed as indicated. **D**, *Tnf<sup>-/-</sup> Hoil1<sup>-/-</sup>* MEFs were retrovirally reconstituted with plasmids encoding for EV, HOIL1-WT or HOIL1-R165G, and stimulated as in (A). Cell lysates were prepared and subjected to Western blotting analysis, as indicated. **E**, Cells as in (D) were stimulated for 3 hrs. IL-6 secretion was analyzed by ELISA (means  $\pm$  SEM.  $n=9$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ , \*\*\*\* $P<0.0001$ , ANOVA). Data are representative of three independent experiments.

**Figure 3. GEF-H1 participates in NF- $\kappa$ B activation and MALT1 Cleavage in response to LPA.**

**A**, HEK293T were transfected with a vector encoding for GEF-H1 or with an empty vector (EV) together with an NF- $\kappa$ B reporter plasmid. Shown is the mean  $\pm$  SEM of triplicate experiments. \*\*\*\* $P<0.0001$  (ANOVA). **B**, HEK293T cells were transfected with an empty vector (EV), or with a GEF-H1 plasmid together with FLAG-tagged HOIL1-WT or HOIL1-R165G. Cell lysates were subjected to Western blotting analysis with antibodies specific to the indicated proteins. White and red arrowheads show full-length and cleaved HOIL1, respectively. Molecular weight markers ( $M_r$ ) are shown. **C**, MEFs knockout for GEF-H1 (*GEF-H1<sup>-/-</sup>*) or control MEFs (*GEF-H1<sup>+/+</sup>*) were stimulated with 10  $\mu\text{g.mL}^{-1}$  LPA or with 50  $\text{ng.mL}^{-1}$  PMA plus 100  $\text{ng.mL}^{-1}$  Ionomycin (PI) as indicated. Lysates were prepared and Western blotting analysis was performed as indicated. The intensities of bands were quantified, and normalized with those of GAPDH. Heatmap shows the mean of three biological repeats. **D**, Abundance of IL-6, measured by ELISA in the supernatant of cells as in

(C) stimulated for 3 hours (means  $\pm$  SEM, n=3, \*\*\*\* $P$ <0.0001, ANOVA). Data are representative of three independent experiments.

Figure 1

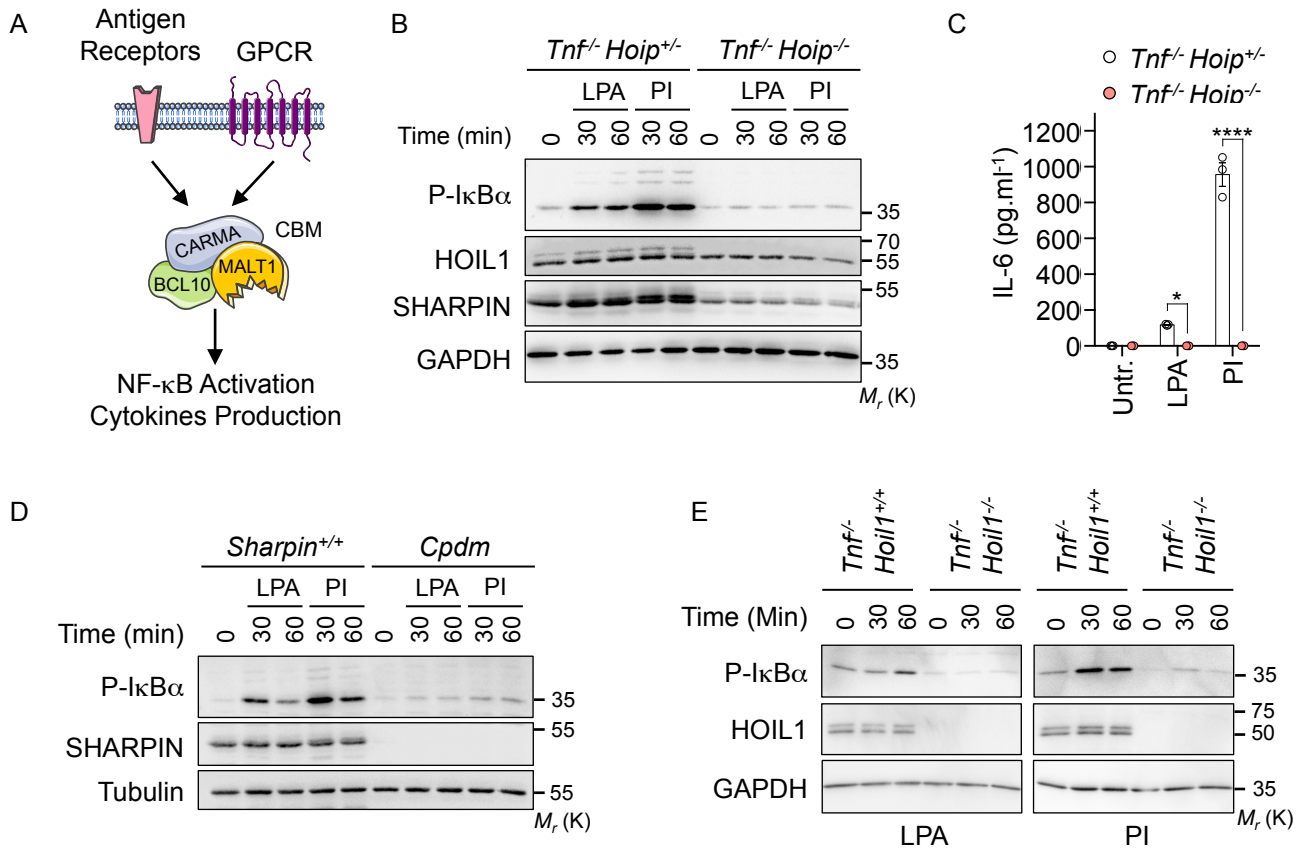


Figure 2

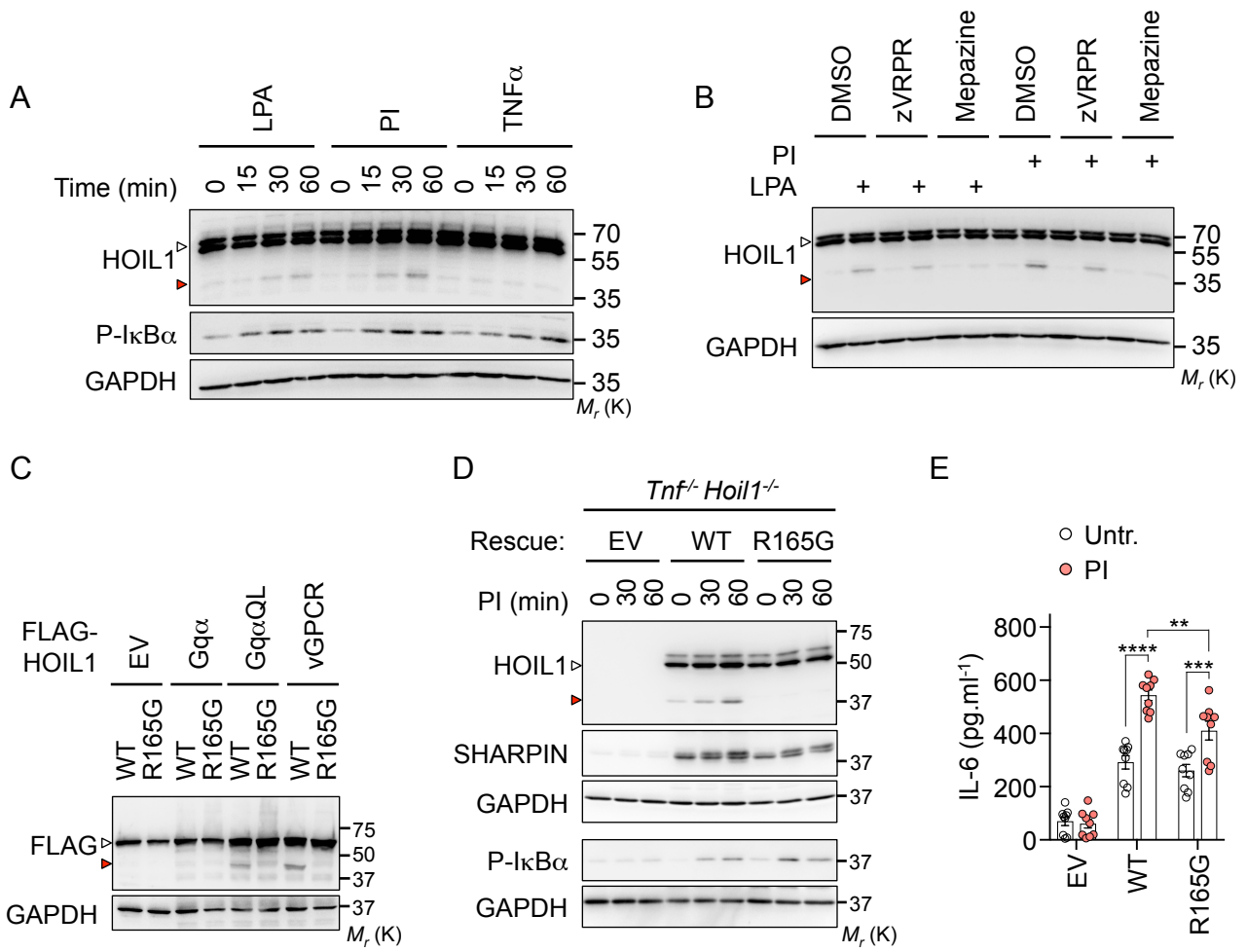


Figure 3

