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Anti-inflammatory Actions of Acanthoic Acid-Related Diterpenes Involve Activation of the PI3K p110γ/δ Subunits and Inhibition of NF-κB

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SUMMARY

The effect of acanthoic acid analogs on the response to proinflammatory challenge was investigated. Some pimarane diterpenes are known activators of the LXRαβ nuclear receptors, but we show here that they also exert a rapid, potent, and selective activation of the p110γ and p110δ subunits of PI3K. Combination of these effects results in an important attenuation of the global transcriptional response to LPS in macrophages. PI3K/Akt activation leads to inhibition of the LPS-dependent stimulation of Ikk/ NF-κB and p38 and ERK MAPKs. Macrophages from LXRαβ-deficient mice exhibited an inhibition of these pathways similar to the corresponding wild-type cells. Silencing or inhibition of p110γ/δ suppressed the effect of these diterpenes (DTPs) on Ikk/NF-κB and MAPKs signaling. Taken together, these data show a multitarget anti-inflammatory mechanism by these DTPs including a selective activation of PI3K isoenzymes.

INTRODUCTION

Macrophages are key players in the innate immune response (Gordon, 2007). During the inflammatory process, the early stages are dominated by macrophages displaying inflammatory and tissue-destructive activities followed by tissue repair (Gordon and Martinez, 2010; Mantovani et al., 2004). To carry out these functions, macrophages have membrane receptors, which recognize antigens (such as Toll-like receptor [TLR] ligands or pathogen-associated molecular patterns); cytokines; and chemokines, which trigger the specificity of the innate immune response (Garantziotis et al., 2008; Nathan and Ding, 2010; Pasare and Medzhitov, 2004). TLRs exert essential functions in the activation of innate immunity as sensors of microbi-al infection (Garantziotis et al., 2008; Trinchieri and Sher, 2007), leading to the killing and clearance of pathogens. Signaling downstream from TLRs involves the recruitment of distinct Toll/interleukin-1 receptor (TIR)-domain-containing adaptor molecules including the myeloid differentiation factor 88 (MyD88) and the TIR-domain-containing adaptor-inducing interferon β (IFNβ) (TRIF) (Akira and Takeda, 2004; Jin and Lee, 2008; Kawai and Akira, 2011; Kumar et al., 2009; O’Neill and Bowie, 2007). Most TLRs signal through MyD88-dependent pathway, whereas TLR3, the receptor recognizing double-stranded RNA (dsRNA), is coupled to TRIF. TRIF, the receptor for lipopolysaccharide (LPS), is the only receptor that engages both MyD88- and TRIF-dependent pathways. MyD88- and TRIF-dependent pathways result in the activation of the transcription factor nuclear factor kappa B (NF-κB). TLRs also activate phosphatidylinositols 3-kinase (PI3K)/Akt pathway, which plays an important role in cell migration, phagocytosis, and apoptosis (Diaz-Guerra et al., 1999; Fukao and Koyasu, 2003; Katso et al., 2001; Troutman et al., 2012) and regulates MyD88- and TRIF-dependent events (Arbibe et al., 2000; Hazeki et al., 2007; Sarkar et al., 2004). PI3K converts phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol (3,4,5)-trisphosphate, which acts as second messenger to activate downstream protein kinases, including Akt (Franke et al., 1995; Guha and Mackman, 2002). Indeed, PI3K activation in macrophages contributes to the negative regulation of the NF-κB pathway, decreasing the inflammatory response (Aksoy et al., 2005, 2012; Chaurasia et al., 2010; Diaz-Guerra et al., 1999; Guha and Mackman, 2002; Medina et al., 2010; van Dop et al., 2010).

The ability of natural and chemically modified diterpenes (DTPs) and polyphenols to impair inflammatory responses has been evaluated in the past years (Brenner and Heinrich, 2002; de las Heras et al., 2003; Palladino et al., 2003). Most of the studies on terpenes have focused on NF-κB pathway as a common target in the action of these compounds, explaining their anti-inflammatory and immunomodulatory...
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Acanthoic Diterpenes Activate p110γ/δ

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**A**

Acanthoic DTP: 1, 2, 3, 4, 5

**B**

LPS DEGs=5075

DPT1+LPS DEGs=2456

**C**

FatiScan analysis of KEGG pathways:

- Sphingolipids metabolism
- Ribosome
- Oxidative phosphorylation
- MAPK signaling pathway
- Cytokine-receptor interaction
- Leukocyte migration
- Jak-STAT signaling pathway
- Focal adhesion
- FcRII signaling pathway
- TLR signaling pathway
- Adherens junction
- Cell adhesion molecules

**D**

<table>
<thead>
<tr>
<th>DTP:</th>
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**E**

![Graph showing NOx levels](image)

**F**

![Graph showing apoptosis](image)

**G**

![Graphs showing cytokine levels](image)

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Acanthoic Diterpenes Activate p110γ/δ

Figure 1. Effect of Acanthoic Acid-Related DTPs on Inflammatory-Mediators Expression

(A) Chemical structure of the five acanthoic acid C4 derivatives that were synthesized.

(B) Transcriptional response to LPS and DTP1 + LPS measured by gene expression microarrays. Peritoneal macrophages were pretreated with 10 μM DTP1 or the vehicle (DMSO) and then activated with 250 ng/ml of LPS for 4 hr. The number of significant DEGs (FDR ≤ 0.05) calculated after DTP1 + LPS is about half the number of DEGs after LPS + vehicle (left). The global fold changes (indicated in the heat map as log2) of LPS up- and downregulated genes were dramatically

(C) Functional analysis of KEGG pathways using FatiScan resulted in 13 significantly enriched (FDR ≤ 0.05) pathways: three of them were enriched in genes upregulated (red) and the other ten were enriched in genes downregulated (blue) by DTP1.

(D-F) After 18 hr of incubation, the protein levels of NOS-2 and COX-2 (D) and the accumulation of NOx in the culture medium (E) were determined. Macrophages were incubated with staurosporine (200 ng/ml) or DTPs (10 μM) for 18 hr. Apoptosis was determined using flow cytometry using annexin-V labeling (F).

(G and H) Accumulation (after 18 hr) in the culture medium of IL-1β, CXCL-1 (KC), CXCL-10 (IP-10), IFN-γ, IL-6, RANTES, IL-10, and IL-12 (G). The intracellular levels of IL-1β, CXCL-1, and CXCL-10 were also determined at 18 hr (H).

Results show a representative blot out of three (D and H) and the mean ± SD (n = 4; E–G). *p < 0.05, **p < 0.005 versus the vehicle condition (E and F); *p < 0.05 versus the LPS condition (G).
(Figure 1E). DTP1, DTP3, and DTP5 exhibited an inhibition higher than 60% at 10 μM. To ensure that the inhibitory effects of these compounds were not due to cytotoxicity, we analyzed the percentage of apoptotic cells using staurosporine as positive control (Figure 1F). In agreement with the anti-inflammatory activity of DTP1 and DTP5, the accumulation in the culture medium of IL-1β, chemokine (C-X-C motif) ligand 1 (CXCL-1), CXCL-10, interferon γ (IFNγ), IL-6, chemokine ligand 5 (CCL5 or RANTES), IL-12, and the anti-inflammatory cytokine IL-10 were decreased in the presence of the DTPs (Figure 1G). The intracellular protein levels of some of these cytokines and chemokines were measured at 18 hr after LPS stimulation (Figure 1H).

**DTP1 and DTP5 Exert In Vivo Anti-inflammatory Activity**

The anti-inflammatory action of DTP1, DTP2, and DTP5 was evaluated in a murine ear edema model. As Figure 2A shows, the myeloperoxidase (MPO) activity and edema due to infiltrated neutrophils after the topical application of tetradecanoylphorbol acetate (TPA) were significantly attenuated when animals received DTP1 and DTP5, but not after DTP2 (indomethacin was used as the control). In addition, TNF-α production by whole blood cells obtained from animals treated orally with DTP1 and DTP5 (not with DTP2) was decreased after LPS challenge (Figure 2B). The anti-inflammatory activity was also evaluated using the β-galactosamine (D-GalN)-sensitized LPS-dependent toxicity. As Figure 2C shows, only DTP1 and DTP5 exerted a significant protection against the lethality induced by LPS/D-GalN, both in LXRα/β−/− and LXRα/β−/− mice (p < 0.001 versus the treatment with DTP1 or DTP5 in both cases). In agreement with this, a dose-dependent inhibition of plasma TNF-α levels (1 hr after LPS) was observed when animals were injected intraperitoneally (i.p.) with DTPs and 30 min later were injected i.p. with LPS (1 mg/kg; Figure 2D). Also, inhibition of MPO activity was observed in vivo using bioluminescence recording after DTP5 (30 mg/kg) i.p. injection followed 1 hr later by zymosan (10 mg/kg) administration (Figure 2E).

**DTPs Inhibit the IKK/NF-κB and MAPKs Activation in Response to Different Proinflammatory Stimuli**

DTP1, DTP3, and DTP5 activate LXRα/β, nuclear receptors that counteract NF-κB-dependent responses (Traves et al., 2007). Therefore, we designed a series of experiments to evaluate the anti-inflammatory effects mediated by DTPs independently of LXR activation. Figure 3A shows a potent inhibitory effect of DTP5 on NOS-2 and COX-2 protein levels regardless of the absence of LXRα/β. The dose-dependent effect of all DTPs on
NO\textsubscript{x} accumulation after LPS treatment in LXR\textsubscript{ab}\textsuperscript{+/+} and LXR\textsubscript{ab}\textsuperscript{−/−} macrophages reflected a significant inhibition by the active DTPs (Figure S3A). The effect of DTP1 on the time course of NOS-2 and COX-2 expression was evaluated using LXR\textsubscript{ab}\textsuperscript{+/+}/C0/C0 macrophages (Figure S3B). Furthermore, DTP5 impaired I\textkappa\textB{a} degradation in LPS-treated cells, regardless the presence of LXRs. Nevertheless, the pharmacological activation of LXR with T1317 was not able to modify the I\textkappa\textB{a} degradation induced by LPS (Figure 3A), which agrees with previous work (Castrillo et al., 2003a). These results suggest that DTPs are also interfering with early signaling events after LPS challenge, independent of the LXRs activation. In addition to LPS, the response of macrophages to the TLR3 ligand polyriboinosinic:polyribocytidylic acid (polyI:C) and to the TLR2 ligand lipoteichoic acid (LTA) was analyzed. As Figure 3B shows, LPS, polyI:C, and LTA promoted the expression of NOS-2 and COX-2, an effect that was attenuated in the presence of DTP5. Moreover, the accumulation of NO\textsubscript{x} in the culture medium induced by these stimuli was also inhibited by DTP5 (Figure 3C). The time course of NOS-2 and COX-2 mRNA levels after LPS challenge showed an inhibitory effect by DTP1 and DTP5 (Figure 3D). Figure 3E shows the inhibitory effect of DTP5 on the I\textkappa\textB{a} and IL-6 mRNA levels after the activation of TLR2, TLR3, and TLR4 with LTA, polyI:C, and LPS, respectively. When MyD88-deficient mice were used, DTP1 and DTP5 inhibited the accumulation of CXCL-10 in LPS and polyI:C-stimulated macrophages, regardless of the presence of MyD88 (Figure 3F). This suggests, together with the data from LTA treatment, that DTPs are effective in both the MyD88- and TRIF-dependent pathways.

Our data point to a possible effect of the DTPs at early signaling events after proinflammatory challenge; thus, to study the effect of these DTPs on IKK activation, we immunoprecipitated this enzyme from cells pretreated with the indicated DTPs (30 min) and then with LPS (10 min). The IKK activity was determined in vitro using purified I\textkappa\textB{a} as the substrate and measuring the phospho-Ser32-I\textkappa\textB{a} levels using specific antibodies.
antibodies. Figure 4A shows a decreased activation of IKK in cells pretreated with DTP1, DTP3 and DTP5. Figure 4B shows the effect of DTP5 on the in vivo phosphorylation of IκBα in Ser32, analyzed after proteasome inhibition with MG132, in macrophages challenged with LPS or polyI:C. Furthermore, the in vitro addition of DTPs to immunoprecipitated IKK from LPS-treated cells did not affect the activity of this enzyme (Figure 4C).

The direct effects of the DTPs on IKK were evaluated using recombinant IKKβ (that is constitutively active) and adding the DTPs to an in vitro homogeneous time-resolved fluorescence (HTRF) assay. As Figure 4D shows, the activity remained unaffected by the DTPs when assayed up to 100 μM. As expected, treatment with staurosporine, which was used as a positive control, inhibited IKK. The activation and translocation to the nucleus of NF-κB, evaluated using electrophoretic mobility shift assays (EMSAs), were significantly impaired when cells were incubated with DTP5 (Figure 4E) and DTP1 (not shown), which agrees with an LXR-independent effect. Figure 4F shows the time-dependent changes in IKK phosphorylation, which were less in the presence of DTP5, and the levels of IκBα and IκBβ, which showed a delayed degradation (for IκBα) and a more rapid resynthesis (for IκBβ). Under the same conditions, the LPS-dependent activation of the MAPKs ERK and p38 was inhibited by DTP5, whereas c-Jun N-terminal kinase (JNK) activation was less affected (Figure 4G). Consistent with these data, the phosphorylation of dual-specificity mitogen-activated protein kinase kinase 3 and 6 (MKK3/6), an upstream kinase of p38 (Risco et al., 2012), was reduced in LPS-activated macrophages incubated with DTP5 (Figure 4H). In addition, activation with polyI:C or LTA of DTP5-pretreated macrophages showed a decreased IKK and p38 phosphorylation, but without noticeable effects on ERK and JNK phosphorylation (Figure 5).

Taken together, these data pointed to early steps of IKK/NF-κB and MAPKs signaling as the main targets in the LXR-independent mechanism of action of DTP5.

**DTP1, DTP3, and DTP5 Activate the PI3K/Akt Pathway**

Previous reports indicate that the PI3K/Akt pathway plays a negative role on NF-κB activation in macrophages (Diaz-Guerra et al., 1999; Fukao and Koyasu, 2003). In view of our results presented here, we reasoned that this pathway was one likely candidate to mediate the effects of the DTPs. DTP1, DTP3, and DTP5, but not DTP2 and DTP4, promote the phosphorylation of Akt in the TPA-induced ear edema response to DTP5 (Figure 6D). Furthermore, Akt was phosphorylated in vivo after the administration of DTP1, DTP3, and DTP5, but not with DTP2 and DTP4, in the TPA-induced ear edema
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Figure 5. Effect of DTP5 on IKK and MAPKs Activation in Macrophages Treated with poly IC or LTA.

Discussion

Few studies have evaluated and molecularly characterized the anti-inflammatory effects of acanthoic acid, first isolated from the root bark of Acanthopanax koreanum Nakai, which has been used in traditional Korean medicine for the treatment of rheumatism and other chronic inflammatory diseases (Kang et al., 1996; Kim et al., 2004; Park et al., 2004), and now identified in other plants, including a bush native to California, Coleonema puchellum. Previous work using a series of synthetic analogs of this molecule identified the main structural motifs essential for their anti-inflammatory properties (Kang et al., 1996; Kim et al., 2004). As we described previously, some of these DTPs are potent activators of LXRβ, which might explain in part their anti-inflammatory effects (Traves et al., 2007); however, these DTPs retain part of their anti-inflammatory activity when assayed in LXRβ−/− mice. We found that the chemical modifications introduced in the DTPs, mainly positional modifications of the R-group at C4 of ring A, render molecules that have different quantitative effects on the expression of typical proinflammatory genes. Most of the studies in this report have been performed using DTP1 and DTP5 because these molecules exhibited greater effects in models of inflammation (effects persistent even in the absence of LXRβ) that were chemically more stable than the other potent derivatives, DTP3.

Our data show that DTPs negatively regulate inflammatory responses triggered by different TLRs, both in vitro and in vivo, decreasing the expression of cytokines, chemokines, and pro-inflammatory mediators. These DTPs significantly inhibit the IKK/NF-κB pathway and the ERK/p38 MAPKs activated in response to LPS. These results are consistent with previous studies in which DTPs with kaurene structure, in addition to the NF-κB pathway, also inhibited these MAPKs (Castillo et al., 2001a). LPS activates TLR4, which is the only receptor able to signal through both MyD88-dependent and TRIF-dependent pathways (Aksoy et al., 2005; Gordon, 2007; Jin and Lee, 2008; O’Neill and Bowie, 2007). Similar results were obtained when macrophages were activated with TLR2 (MyD88-dependent) or TLR3 (TRIF-dependent) ligands. Interestingly, the absence of MyD88 (using CXCL-10 as the expression target) did not affect the inhibitory actions of DTPs upon challenge with LPS or polyIC; All together, these data suggest the presence of a shared target for DTPs, common to the different signaling pathways analyzed and independent of MyD88 or TRIF. Moreover, these inhibitory effects persisted when the compounds were assayed in macrophages from LXRβ−/− mice, pointing to an upstream nuclear receptor-independent mechanism. In this regard, the EMSAs demonstrated that DTPs reduced NF-κB translocation to the nucleus in addition to the transrepression effects described for the LXR inhibition of NF-κB activity (Castillo and Tontonoz, 2004; Traves et al., 2007). In a previous work, the action of these DTPs was assayed in LXRβ WT and KO mice after 24–48 hr of treatment with the DTPs (Traves et al., 2007); however, because LXR-deficient (GFP-AktPH) as a molecular propidium iodide (PI)-sensor, we show that DTP1 promoted an increase in the fluorescence at the time that the probe aggregated in the perimembrane domain, suggesting a rapid and direct action of this DTP on PI3K activity (Figure S4F).
mice lack these proteins in all tissues and immunological alterations have been described (A-Gonzalez et al., 2013), we preferred to use WT animals and to evaluate the effects of DTPs for the short times analyzed in the present report. Studies in vivo with DTP1 and DTP5 showed a significant anti-inflammatory action in all the models assayed. The synthesis of TNF-α by whole blood collected from animals treated orally with these DTPs was inhibited; systemic i.p. administration significantly delayed the rate of animal death in a model of LPS/D-GalN-induced hepatotoxicity (even in the absence of LXRα), decreased the LPS-induced TNF-α plasma levels, and reduced the zymosan-induced peritoneal inflammation; and finally, the development of TPA-induced edema was also prevented to levels similar to those elicited by indomethacin.

When the inhibition of the NF-κB early signaling was investigated, we were unable to observe a direct effect of the DTPs on the activity of the IKK complex despite IKKβ phosphorylation being abrogated. Various reports show that PI3K contributes to the fine-tuning of the innate immune response to diverse microbial pathogens (Fukao and Koyasu, 2003; Hazeki et al., 2007; Ruse and Knaus, 2006). In dendritic cells and macrophages, the activation of PI3K/Akt was found to limit LPS-induced production of cytokines and chemokines and the expression of NOS-2 through both MyD88-dependent and -independent pathways (Aksoy et al., 2005). In this study, we have shown that DTP1, DTP3, and DTP5 induce a persistent Akt phosphorylation in vivo (ear edema and liver) and ex vivo (macrophages) at the two major sites, Thr308 and Ser473. We confirmed that this Akt activation was dependent on PI3K activity as far as it was suppressed by PI3K inhibitors. Furthermore, the phosphorylation of Akt in Ser473 was blocked by the expression of a kinase-deficient isoform of p110. In the same way, the involvement of the DTP-dependent activation of PI3K/Akt in the negative regulation of LPS-induced IKK, p38, and ERK phosphorylation was confirmed by the suppression of the DTPs effects after full PI3K inhibition (LY294002) or using selective p110γ/δ inhibitors and siRNAs. Taken together, these data contribute to a better understanding of the mechanisms of action of these DTPs in innate immunity. Finally, the dual action of DTP1, DTP3, and DTP5 on the PI3K/Akt/MAPK/NF-κB axis, on the one hand, and the selective activation of LXR, on the other, offers the possibility of modifying key cellular responses involved in many physiopathological processes in which macrophages play an active role in the onset of the disease, including inflammatory diseases, diabetes, and perhaps cancer.
SIGNIFICANCE

Our results demonstrate that these DTPs negatively regulate IKK/NF-κB and MAPKs (p38 and ERK) pathways by increasing p110γ/d activity and, consequently, Akt phosphorylation. The ability of our compounds to selectively activate some isoforms of PI3K opens new prospects in the clinical development of anti-inflammatory molecules and points to the use of these acanthoic acid-related DTPs as anti-inflammatory and possibly anticancer drugs with potential therapeutic activity in inflammatory pathologies targeting simultaneously the PI3K/Akt, LXRα, and NF-κB pathways.

EXPERIMENTAL PROCEDURES

Chemicals

Reagents were obtained from Sigma-Aldrich, Roche, Amersham-GE Healthcare, Merck, Bio-Rad, R&D Systems, and Promega. DTPs were synthesized by Prof. E. Theodorakis (University of California, San Diego) and were dissolved in DMSO before being used in cell culture (Ling et al., 2000, 2001).

Animals

Male mice were housed and bred in our pathogen-free facility and were maintained on standard chow under pathogen-free conditions. BALB/c were from Charles River Laboratories. LXRα−/− and LXRβ−/− mice (mixed Sv129/C57BL/6 background) were obtained as previously described (Traves et al., 2007). MyD88+/+ (WT) and MyD88−/− (KO) were obtained from a collaboration with Dr. T. Miethke (Institute for Medical Microbiology Immunology and Hygiene, Technical University of Munich). The study was conducted following the guidelines of the Spanish Animal Care and Use Committee, according to the guidelines for ethical care of experimental animals of the European Union (2010/63/EU).

Peritoneal Macrophage Isolation

Animals were used at ages 8–12 weeks, as indicated in the online Supplemental Information.

Cell Death Detection

Cells were collected and washed in cold PBS. After centrifugation for 5 min at 4°C and 1,000 × g, cells were resuspended in annexin V binding buffer (10 mM HEPES; pH 7.4, 140 mM NaCl, 2.5 mM CaCl2) and labeled with annexin V–FITC (BD/PharMingen) and/or propidium iodide (PI; 100 μg/ml) for 15 min at room temperature (RT) in the dark, following a previous protocol (Hortelano et al., 2003). Analysis was carried out using a FC 500 BD FACScan flow cytometer.
**Determination of NO Synthesis**

NO release was measured as the accumulation of nitrite and nitrate in the incubation medium (phenol-red free). Nitrate was reduced to nitrite with nitrate reductase and was determined spectrophotometrically with Griess reagent; the absorbance at 548 nm was compared with a NaNO₂ standard.

**Cytokine and Chemokine Assays**

Cytokines production by cultured macrophages and its accumulation in the incubation medium were quantified using ELISA kits from PeproTech according to the manufacturer’s instructions. Serum TNF-α was measured using cytoket ELISA kits (BioSource International). Levels of CXCL-1 and CXCL-10 were assayed using the Bio-Rad protein reagent. All cell fractionation protocols were performed as indicated in the online Supplemental Information.

**Preparation of Total Protein Cell Extracts**

Cells were homogenized in a buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM EGTA, 10% glycerol, 0.5% CHAPS, 1 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonylfluoride (PMSF), and a protease and phosphatase inhibitor cocktail (Sigma-Aldrich). The extracts were vortexed for 30 min at 4°C, and after centrifuging for 15 min at 13,000 × g, the supernatants were stored at −20°C. Protein levels were determined using the Bio-Rad reagent.

**Preparation of Cytosolic and Nuclear Extracts**

Cells were collected into ice-cold PBS, resuspended in 200 μl of cytosolic buffer (10 mM HEPES; pH 8, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, and 0.5% Nonidet P-40) and left to swell on ice for 15 min. The tubes were gently vortexed for 30 s, and the nuclei were collected using centrifugation at 13,000 × g for 10 min (Castillo et al., 2001a, 2003b). The supernatants were stored at −20°C (cytosolic extracts); the pellets were resuspended in 50 μl of nuclear buffer (20 mM HEPES; pH 8; 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, and 20% glycerol) and gently shaken for 30 min at 4°C. Nuclear protein extracts were obtained using centrifugation at 13,000 × g for 10 min, and the supernatants were transferred to a fresh tube and stored at −80°C. Protein content was assayed using the Bio-Rad protein reagent. All cell fractionation steps were carried out at 4°C. All buffers contained protease and phosphatase-inhibitor cocktail (Sigma-Aldrich) (Prieto et al., 2010).

**Characterization of Proteins Using Immunoblot**

Protein extracts were boiled in loading buffer (250 mM Tris-HCl; pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol) and size separated using 10%–15% SDS-PAGE gels, as indicated in the online Supplemental Information.

**EMSAs**

The sequence 5'-TGTAGGGGATTTCCCTCTGCTG-3', corresponding to the consensus NF-κB binding site (nucleotides –978 to –952) of the murine NOS-2 promoter (Castillo et al., 2001a), was used and assayed as indicated in the online Supplemental Information.

**Measurement of IKKβ Activity**

Cells (10⁶) were homogenized in cytosolic buffer and centrifuged at 13,000 × g for 15 min. The supernatant (1 ml) was precleared, and IKKβ was immunoprecipitated with 1 μg of anti-IKKβ (Castillo et al., 2001b). After the immunoprecipitate was extensively washed with cytosolic buffer, the pellet was resuspended and assayed as indicated in the online Supplemental Information.

**Measurement of PI3K Activity**

PI3K activity was measured in the anti-p85 Ab immunoprecipitates using in vitro phosphorylation of PIP2, as previously described (Díaz-Guerra et al., 1999).

**In Vitro Translation of p110α and p110δ and Measurement of PI3K Activity**

The vectors encoding p110α, p110δ, and p110δ were kindly provided by Dr. Julian Downward (Cancer UK, London), Dr. Bart Vanhaesebroeck (Queen Mary University, London) and Dr. Ana C. Carrera (CNB, Madrid). The p110α and p110δ were subcloned in a PGSS vector (Agilent Technologies) and transcribed and translated in vitro using the Megascript T7 kit for transcription (Life Technologies) and the TNT Quick Translation System (Promega) for translation and incorporating the transcribed p85α in the translation reaction. The PI3K complexes were recovered after immunoprecipitation (IP) with anti-p85 Ab and the kinase activity was measured as previously described (Díaz-Guerra et al., 1999).

**Confocal Microscopy**

Peritoneal macrophages (1 × 10⁶) were grown on coverslips and incubated with the indicated stimulus. After the covers were washed with PBS, the cells were fixed with paraformaldehyde (4%; pH 7.2) for 15 min at RT and washed again. The macrophages were permeabilized with cold methanol for 10 min at RT. After incubation with anti-phospho-S473Akt (1:500) overnight at 4°C, the cells were washed with PBS, followed by incubation with anti-rabbit secondary Ab (Alexa-Fluor 488, 1:500) for 2 hr at RT. Nuclei were revealed by staining with DAPI (1:3,000). Coverslips were mounted in Prolong Gold antifade reagent (Molecular Probes) and analyzed using an Espectral Leica TCS SP5 confocal microscope. Fluorescence intensity measurements were performed using Image J software (NIH).

**Transient Transfections**

NIH 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing antibiotics and 10% FCS. Before experiments were conducted, the medium was challenged to 1% FCS. Transient transfections were performed using FuGene HD transfection reagent (Roche), with a FuGene:DNA ratio of 8:2 and following the indications of the manufacturer (Roche). Transfection experiments were performed using vectors encoding p110α wild-type (p110WT) and p110α kinase deficient (p110KD) (Díaz-Guerra et al., 1999). Phosphorylation of Akt was analyzed using immunoblot as a marker of PI3K activity. In addition to this, a GFP-tagged-Akt pleckstrin homology domain (GFP-AktPH) vector kindly provided by Dr. O. Weiner (University of California, San Francisco) was used as a molecular sensor of PI3K activation upon transfection in NIH 3T3 cells, following a previously described protocol (Servat et al., 2000; Toettcher et al., 2011).

**Gene Silencing with siRNAs**

Peritoneal macrophages were transfected with Pikh3a, Pikh3c, Pikh3g, Pikh3d, and nontargeting siRNAs (Thermo Scientific Dharmacon), using Lipofectamine 2,000 transfection reagent (in vitro) and following the manufacturer’s instructions. After 48 hr, the medium was changed to RPMI medium 1,640 with 2% FCS, and the cells were stimulated for the indicated time periods for protein or RNA expression analysis.

**LPS-Induced D-GalN-Dependent Lethality Studies**

An endotoxic shock was induced in male LXRα−/− and LXRβ−/− mice ages 8–10 weeks old. The lethal injury was produced by i.p. injection of LPS (10 μg/kg) in combination with D-GaIN (800 mg/kg). The DTPs (30 mg/kg) were administered i.p. in 0.5 ml Solutol 1 hr prior to challenge with D-GaIN/LPS. Solutol was given to the control animals. The lethality was monitored until 25 hr after the administration of LPS/D-GaIN.

**12-O-Tetradecanoylphorbol Acetate-Induced Mouse Ear Edema and Myeloperoxidase Activity Assay**

A solution of 2 μg TPA dissolved in 20 μl DMSO was applied to both surfaces of the right ear of each mouse. The left ear (control) received the vehicle (DMSO). The DTPs were administered topically (500 ng per ear in 20 μl DMSO) simultaneously with the TPA application. The reference drug, indomethacin, was administered at the same doses. After 4 hr, the animals were killed by cervical dislocation and a 6 mm diameter disc from each ear was removed using a metal punch and weighed. Ear edema was calculated by subtracting the weight of the left ear (vehicle) from the right ear (treatment). Ear sections were homogenized in 750 μl of saline. After centrifugation at 10,000 × g for 15 min at 4°C, MPO activity was measured in supernatants, as described before (Navarro et al., 1997). In addition to this, ear samples obtained at 30 min after TPA + DTP administration were homogenized and processed using western blot to evaluate the phosphorylation of Akt.
In Vivo Administration of DTP and Ex Vivo Whole-Blood Assay
C57BL/6 mice were starved overnight and orally administered 25 mg/kg of DTP1 and DTP5 prepared in 40% Solutol HS15 (BASF). After 90 min, whole blood was collected, as indicated in the online Supplemental Information.

Bilirubin Imaging of Myeloperoxidase Activity In Vivo
MPO activity was measured in the whole animal, as described previously (Gross et al., 2009). Briefly, BALB/c mice were pretreated with the DTP5 (30 mg/kg), and after 1 hr zymosan (10 mg/kg) was administered by i.p. injection. At the times indicated, luminol (5 mg in 200 µl Sigma) was injected i.p. and the bioluminescence was recorded in an IVIS Lumina (Caliper Life Sciences).

Microarray Analysis
RNA samples were labeled and hybridized in Agilent 4x44K one-color microarrays following the manufacturer’s protocol and after two independent experiments. The slides were scanned and images were converted to raw signal text files using Feature Extraction software (Agilent). Data preprocessing and normalization were performed using Codelink and Limma and Bioconductor packages (Genteman et al., 2004). Differential expression was analyzed using the Limma paired t test (Smyth, 2005), as implemented in Pomelo II (Morrissey and Diaz-Uriarte, 2009). Gene-set-enrichment analysis was done using the FatScans tool in Babelomics v3 (Al-Shahrour et al., 2006).

Statistical Analysis
Unless otherwise stated, data are expressed as mean ± standard deviation (SD). To compare means between two independent samples, we used the Mann-Whitney rank sum test. The results were considered significant at p < 0.05. Data were analyzed using the SPSS for Windows statistical package v.21.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2014.06.005.

AUTHOR CONTRIBUTIONS
P.G.T., M.A.P., and L.B. designed the research; P.G.T. and M.P.S. developed the study in animal models and the analysis of PI3K activity, P.G.T. and N.R. performed the experiments. Nucleic Acids Res. 23, 1196–1208.

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