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Protein Kinase Cδ Mediates Lysophosphatidic Acid - Induced NF-κB Activation and Interleukin-8 Secretion in Human Bronchial Epithelial Cells

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Short Title: Protein Kinase Cδ in LPA-induced NF-κB activation and IL-8 secretion.

Key words: Lysophosphatidic acid; Interleukin-8; NFκB; Protein Kinase Cδ; neutrophils infiltration; human bronchial epithelial cells.

The abbreviations used are: BAL, bronchoalveolar lavage; BEAS-2B, human bronchial epithelial cell line; ECL, enhanced chemiluminescence; EDG, endothelial differentiation gene; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; HBEpCs, human bronchial epithelial cells; HBSS, hanks balanced salt solution; GAPDH, glyceraldehyde phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cell; IL-8, interleukin-8; IκB, inhibitory kappa B; LPA, lysophosphatidic acid; MIP-2, macrophage inhibitory protein-2; MOI, multiplicity of infection; NF-κB, nuclear factor kappa B; PDGF, platelet derived growth factor; PKC, protein kinase C; PLD, phospholipase D; PT_X, pertussis toxin; S1P, sphingosine-1-phosphate; TNF-α, tumor necrosis factor-α.

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ABSTRACT

Lysophosphatidic acid (LPA), a potent bioactive lipid, elicits many of its biological actions via specific G-protein coupled receptors, LPA_1, LPA_2, LPA_3 and LPA_4. Recently, we have shown that LPA induced transactivation of platelet derived growth factor receptor-β is regulated by phospholipase D2 in human bronchial epithelial cells (HBEpCs). Here, we report that protein kinase C (PKC)δ mediates LPA-induced NF-κB transcription and interleukin-8 (IL-8) secretion in HBEpCs. Treatment of HBEpCs with LPA increased both IL-8 gene and protein expression, which was coupled to G_i and $G_{12/13}$ proteins. LPA caused a marked activation of NF-κB in HBEpCs as determined by phosphorylation of IκB, of NF-κB nuclear translocation and strong induction of NF-κB promoter-mediated luciferase activity. Furthermore, LPA activated PKCδ and the LPA-mediated activation of NF-κB and IL-8 production was attenuated by overexpression of dominant negative PKCδ and rottlerin. Intratracheal administration of LPA in mice resulted in elevated levels of macrophage inflammatory protein-2, a murine homolog of IL-8, and an influx of neutrophils in the bronchoalveolar lavage fluid. These results demonstrate for the first time that LPA is a potent stimulator of IL-8 production in HBEpCs, which involves PKC δ/ NF-κB signaling pathways.

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INTRODUCTION

The respiratory epithelium, a complex physical and biochemical barrier, has the capacity both to initiate inflammatory responses and to participate in repair. The balance between the protective, physiologic inflammatory response and injurious, pathological inflammation involves coordinating the production of chemokines, cytokines and growth factors (1). The innate immune response is of relatively short duration and involves increased vascular transudation, interstitial edema and infiltration of inflammatory cells, predominantly of neutrophils. Initiation and maintenance of this leukocyte recruitment requires intercellular communication between infiltrating neutrophils, the endothelium, and respiratory epithelial cells. In this light, the airway epithelium plays a pivotal role in this response through its reaction to and secretion of necessary intercellular regulatory molecules. One such paracrine messenger, interleukin-8 (IL-8), the major chemoattractant and activator for neutrophils in the lung, is a key component of the innate immune response (2). Studies supporting the involvement of IL-8 in pulmonary inflammatory disorders have revealed elevated levels of IL-8 in the bronchoalveolar lavage (BAL) fluid of patients with chronic obstructive lung disease, asthma, idiopathic pulmonary fibrosis, pulmonary sarcoidosis, pneumonia, bronchitis's obliterans syndrome, and acute lung injury/acute respiratory distress syndrome (3-8). Cultured airway epithelial cells also secrete significant amounts of IL-8 when exposed to tobacco smoke, diesel exhaust particles, ozone, viral infection, and hyperoxia as well as various endogenous mediators such as TNFα and IL-1 (9-14).

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Derived from membrane glycerophospholipids and sphingolipids, lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) are released from activated platelets and multiple other cells in response to a wide array of inflammatory stimuli (15). Both LPA and S1P are constitutively found in human serum (~1-20 μM) bound to lipoproteins (16), albumin (17) and gelsolin (18). At target cells, extracellular LPA binds with high affinity and specificity to three of the nine G protein-coupled lysophospholipid receptors belonging to the endothelial differentiation gene (EDG) family (19), LPA_1, LPA_2 and LPA_3. The LPA receptor isoforms display heterogeneous tissue expression patterns, but we have detected the native expression of all three in primary human bronchial epithelial cells (HBEpC) (20).

Possessing the growth-related effects of proliferation, cell survival and differentiation as well as the cytoskeletal-related effects of aggregation, chemotaxis, adhesion and secretion, LPA and S1P have recently emerged as novel immuno-modulating factors (15). Studies in our laboratory have revealed that S1P stimulates a significant production and secretion of interleukin-8 (IL-8) in a human bronchial epithelial cell line (BEAS-2B) (21) and that LPA is capable of transactivating the platelet-derived growth factor (PDGF) receptor-β in HBEpCs (20). In guinea pigs, administration of exogenous LPA into the airway enhances the response to acetylcholine (22) and infiltration of neutrophils (23). Other reports have shown that LPA induces airway smooth muscle contractility (24),

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proliferation (25), and transcription factor activation (26). In ovarian cancer cells, LPA induces both IL-6 and IL-8 expression (27, 28).

Protein kinase C (PKC) belongs to a homologous serine/threonine kinase family that includes three classes of isoenzymes in which the functional specificity is determined by their subcellular localization. The groups consist of the calcium-, phorbol ester- and phosphatidylserine-activated classical isoforms (α, β, and γ), the calcium-insensitive, phorbol ester- and phosphatidylserine-activated novel isoforms (δ, ε, η, θ, and μ), and the phosphatidylserine-activated, calcium- and phorbol ester-insensitive atypical isoforms (ζ and ι/λ). It has recently been shown that PKCδ regulates ICAM-1 expression via NF-κB activation in HUVECs (29) as well as NF-κB-dependent (and IL-8 promoter-dependent) gene expression in a human bronchial epithelial cell line (30).

These earlier reports support the notion that LPA plays a role in the innate immune response. In this study, we utilized a primary HBEpC culture system to analyze LPA-induced IL-8 secretion. In addition to potent and specific production of IL-8, extracellular LPA stimulated IκB phosphorylation, NF-κB-dependent gene expression and NF-κB nuclear translocation. The overexpression of dominant negative PKCδ significantly attenuated LPA-mediated IκB phosphorylation, NF-κB translocation, and IL-8 secretion. We also supplemented our cell culture experiments with *in vivo* studies. Intratracheally delivered LPA in C57BL/6J mice resulted in significant elevations of macrophage inflammatory protein-2 (MIP-2)

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and a neutrophilic influx in the BAL fluid from these animals. Together, these data indicate that PKCδ plays a key role in the regulation of LPA-mediated IL-8 production via NF-κB activation in primary HBEpCs and that extracellular LPA produces an analogous inflammatory response *in vivo*.

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EXPERIMENTAL PROCEDURES

Materials: LPA and S1P were obtained from BIOMOL research Labs (Plymouth, PA). Bovine serum albumin (BSA) was obtained from the Sigma Chemical Company (St. Louis, MO). Bisindolylmaleimide (BIM) and pertussis toxin (PT_X) were purchased from EMD Biosciences, Inc. (San Diego, CA). FuGene-6 Transfection Reagent was obtained from Roche (Indianapolis, IA). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse was purchased from Molecular Probes (Eugene, OR). Enhanced chemiluminescence (ECL) kit for the detection of proteins by Western blotting was obtained from Amersham (Arlington Heights, IL). All other reagents were of analytical grade.

Cell Culture: Primary human bronchial epithelial cells (HBEpCs) were isolated from the lungs of healthy organ donors purchased from Tissue Transformation Technologies (Edison, N.J.). The use of anonymous lung tissues obtained in this manner was considered by the Committee of Human Research of the Bloomberg School of Public Health, Johns Hopkins University to be exempt from human subjects review. Cell isolation was carried out in the laboratory of Dr. E. William Spannhake (Johns Hopkins University) by a modification of the method described by Schroth et al. (31) and Wu et al. (32). Briefly, airway specimens were rinsed with Hanks balanced saline solution (HBSS) and then placed in dissociation solution consisting of F12 media supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml), and pronase (1 mg/ml; Boehringer Mannheim, Indianapolis, IN) for 48 h at 4° C. After incubation, fetal bovine serum (FBS) was

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added to a final concentration of 20%, and epithelial cells were detached from the stroma by gentle agitation. The cells were collected by centrifugation at 1,000 x g for 10 min, washed, and suspended in serum-free hormonally supplemented basal essential growth media (BEGM; BioWhittaker, Walkersville, MD). All primary cell cultures were grown in BEGM in 35 or 60 mm dishes at 37° C in 5% CO_2 / 95% air. Experiments with HBEpCs were conducted between 0 and 4 passages at 70-90% confluence.

Measurement of IL-8 Secretion: HBEpCs were pretreated in BEGM with or without PT_X (100 ng/ml) or rottlerin (5 μM) or BIM (1 μM) for 1 h prior to stimulation. The pretreatment media were removed and the cells were treated in BEGM containing 0.1% BSA with or without LPA at the indicated concentrations for the specified lengths of time. Cell supernatants were removed, centrifuged at 5,000 x g for 5 min at 4° C, and frozen at –80° C for later analysis for IL-8 by ELISA, which was performed according to the manufacturer's instructions (Biosource International, Camarillo, CA).

Immunofluoresence microscopy: HBEpCs grown on coverslips to ~ 50% confluence were infected with vector control or dn-PKCδ (25 MOI) for 48 h prior to challenge with LPA (1 μM) for 15min. In some experiments without infection with adenoviral constructs, ~80% confluence cells were used for LPA challenge. Cells were rinsed twice with PBS (37°C) and fixed with 3.7% formaldehyde in Tris-buffered saline containing 0.01% Tween 20 (TBST) for 15 min at room temperature. The cells were rinsed twice with PBS and incubated for 5 min in

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PBS containing 0.25% Triton X-100. The cells were stained for 20 min with 1: 200 dilutions of either PKCδ or NF-κB subunit (p65Rel A) antibody in TBST containing 1% BSA. The cells were thoroughly rinsed with TBST and stained for 30 min with a 1:200 dilution of Alexa Flour 488 in TBST containing 1%BSA. Cells were examined by Nikon Eclipse TE 2000-S immunofluorescent microscope with Hamamatsu digital camera using a X 60 oil immersion objective and Meta Vue software (Universal Imaging).

Cytokine Gene Expression by cDNA Array Analysis: Total cellular RNA was isolated from HBEpCs using RNEasy kit (QIAGEN, Los Angeles, CA) following vehicle or LPA treatment. Aliquots of total RNA (10 μg) were used to analyze the differential gene expression of common cytokines using Nonrad-GEArray technology (Super Array Inc., Bethesda, Maryland) according to the manufacturer's instructions. Briefly, biotinylated cDNA probes were prepared using total RNA as a template by reverse transcription with MMLV reverse transcriptase. Following denaturation, the cDNA probes were hybridized to a positively charged nylon membrane containing the arrayed DNA. After blocking and incubation with AP-streptavidin and chemiluminescent substrate, the membranes were exposed to X-ray film. Gene expression was quantified by scanning densitometry and normalized based on the intensity of hybridization signals to the housekeeping genes, β-actin and GAPDH. The experiment was performed three times to ensure reproducibility of results.

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Preparation of Cell Lysates and Western Blotting: HBEpCs were rinsed two times with ice-cold PBS, scraped in 1 ml of lysis buffer containing 20 mM Tris-HCl (pH, 7.4), 150 mM NaCl, 2 mM EGTA, 5 mM glycerophosphate, 1 mM $MgCl_2$, 1% Triton X-100, 1 mM sodium orthovanadate, 10 µg/ml protease inhibitors, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin, incubated at 4°C for 20 min, and cleared by centrifugation in a microfuge at 10,000 *g* for 5 min at 4°C. After determination of the total protein in the lysates by the bicinchoninic acid method, 6X Laemmli sample buffer was added to cell lysates and the lysates were boiled for 5 min. Equally loaded proteins were separated on 12% gels by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and subjected to immunoblotting with either anti-PKCα (1:1,000 dilution), anti-PKCδ (1:1,000 dilution), anti-PKCε (1:1,000 dilution) (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), anti-PKCζ (1:1,000 dilution), anti-PKCθ (1:500 dilution), anti-PKCι / λ (1:500 dilution) (BD/Transduction Labs, San Diego, CA) overnight at 4°C. The membranes were washed at least three times with Tris-buffered saline containing 0.1% Tween 20 (TBST) and were incubated for 2 to 4 h at room temperature in horseradish peroxidase-conjugated goat anti-rabbit (1:1,000 dilution in TBST containing 5% BSA) or goat anti-mouse secondary antibodies (1:1,000 dilution in TBST containing 5% nonfat milk). The immunoblots were developed with ECL according to the manufacturer's recommendation.

Preparation of Nuclear Extracts: Nuclear extracts were prepared from HBEpCs according to manufacturer’s instructions (Active Motif North America, Carlsbad,

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CA). Briefly, cells were collected by scraping in ice-cold PBS with phosphatase inhibitors and pelleted by centrifuging at 1000 x g for 5 min. The pellet was resuspended in 500 µl of 1 x Hypotonic Buffer, incubated on ice for 15 min, followed by addition of 25 µl of detergent and high speed vortexing for 30 seconds as per manufacturer's recommendation (Active Motif North America, Carlsbad, CA). The suspension was centrifuged at 14,000 x g for 20 min in a microcentrifuge at 4 ^{o} C; the nuclear pellet was resuspended in 50 µl of complete lysis buffer and incubated on ice for 15 min. This suspension was centrifuged for 10 min at 14,000 x g in a microcentrifuge, the supernatant (nuclear extract) was aliquoted and stored at – 80 ^{o} C for further analysis. Protein in the nuclear extract was quantified by BCA protein assay.

Electrophoretic Mobility Shift Assay: The probe for electrophoretic mobility shift assay (EMSA) was a 22-bp double-stranded construct of NF-κB consensus binding sequence (5$^{'}$ –AGTTGAGGGGACTTTCCCAGGC – 3$^{'}$). End labeling of the probe was performed using T4 kinase and [γ-^{32}P]ATP. EMSAs were performed using nuclear extracts (4 µg) from vector control or dominant negative PKCδ adenovirus infected cells with or without LPA treatment and binding buffer containing 10 mM Tris-HCl (pH 7.5), 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 50 µg of the non-specific blocker poly (dI-dC) and 20,000 dpm of [γ-^{32}P] labeled probe at 37 ^{o} C for 30 min. Specific competition was performed by adding 200 ng of unlabeled double-stranded 22-bp probe. The protein-DNA complexes were analyzed by electrophoresis on a 4% non-

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denaturing polyacrylamide gel. The gels were vacuum- dried and subjected to autoradiography.

Transfection and Viral Infection: For transient transfection, HBEpCs grown in 6-well plates (~60% confluence) were transfected with G protein minigene vectors (Cue Biotech, Chicago, IL). The minigene vectors contain oligonucleotides ligated into pcDNA3.1 that encode a unique peptide that specifically blocks the receptor-G protein interface. The minigene plasmids (1 µg of cDNA) was mixed with 3 µl of FuGene 6 per well in 1mL BMGM medium and transfections were performed for 4 h. At the end of 4 h, the transfection medium was aspirated and regular BEGM medium (2mL) was added and cells incubated for additional 24 h. Infection of HBEpCs (~60% confluence) in 35 or 60 mm dishes was carried out with purified kinase negative adenoviral vectors of PKC isoforms as described previously (33). Following infection of 50 p.f.u /cell for in 1mL BEGM for 24h, the virus containing medium was replaced with complete BEGM and experiments performed.

PKCδ Kinase Assay: HBEpCs infected with vector or dominant negative (dn-) PKCδ adenoviruses (50 MOI) for 24 h were challenged with medium alone or medium containing LPA (1 µM) for 15min prior to harvesting. Cells were lysed in lysis buffer [20 mM Tris-HCl (pH 7.5), 150mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and protease inhibitors from complete, EDTA

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free cocktail tablets (Roche Applied Science)]. Lysates (500 μg protein) were immunoprecipitated with anti-PKCδ antibody (10 μl, Santa Cruz) at 4 ^{o} C overnight, followed by addition of protein A/G (40 μl) and incubated for an additional 2 h at 4 ^{o} C. The immunoprecipitates were washed three times with the lysis buffer and two times with kinase buffer [20 mM HEPES (pH 7.4), 25 mM β-glycerophosphate, 1 mM EGTA, 1mM sodium orthovanadate and 1mM dithiothreitol] and resuspended in 200 μl of kinase buffer. The activity of PKCδ was measured in 100 μl of kinase buffer containing 25 μg of myelin basic protein as an exogenous substrate to which 5 μC_i of [γ-^{32}P]ATP containing cold ATP (10 μM; final specific activity = 10,000 dpm/pmole), 2 μg DAG, 12 μg phosphatidylserine and 25 μl of immunoprecipitate were added. Incubations were carried out for 15 min at 37 ^{o} C and terminated by the addition of 100 μl SDS sample buffer. Aliquots (25 -50 μl) of the reaction mixture were spotted onto P81 filter paper, washed three times with 1 % phosphoric acid, once with acetone and radioactivity measured by scintillation spectroscopy.

LPA Instillation and Bronchoalveolar Lavage: In procedures approved by this institution's Animal Care and Use Committee, C57BL/6J wild type mice were anesthetized by intraperitoneal injection of 30 μl of 10:1 ketamine (100mg/kg): xylazine (10 mg/kg). LPA (10 μM) was administered intranasally after sonication in physiological saline containing tissue culture grade 0.1%BSA while the control mice received saline plus 0.1%BSA. BAL was performed 3 or 6 h following vehicle or LPA instillation. Following euthanasia with an overdose of urethane (3

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g/kg body weight) intraperitoneally, the trachea was cannulated with an 18 gauge intracath and the lungs were lavaged with three 1ml aliquots of physiological saline (3 ml total). BAL fluids were centrifuged at 200 x g for 10 min and pellet was immediately resuspended in physiological saline for total cell counts. Differential cell counts were made from the centrifuged cells after staining with Wright-Giemsa. The supernatant were frozen at -80°C for later measurements of MIP-2 and total protein.

Statistical Analysis of Data: Standard deviation (SD) for each data point was calculated from triplicate samples. Unless otherwise stated, data were subjected to one-way analysis of variance and pair-wise multiple comparison was done by Dunnett's method with P<0.05.

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RESULTS

LPA Activates IL-8 in Primary Human Bronchial Epithelial Cells – We have recently reported that the bioactive sphingolipid, S1P, stimulates IL-8 production in BEAS-2B bronchial epithelial cells (21). Extracellular LPA has been shown to be an immunologic regulator by activating NF-κB and increasing levels of mRNA transcripts encoding E-Selectin, intercellular adhesion molecule-1, IL-8 and monocytes chemoattractant protein-1 in endothelial cells (17). Additionally, LPA has been demonstrated to activate NF-κB in fibroblasts (10). We examined the ability of both S1P and LPA to modulate IL-8 secretion in HBEpCs. As shown in **Fig. 1**, treatment of HBEpCs with S1P (1 μM) resulted in significant increases in IL-8 secretion at both 3 and 24 h (2.6- and 1.8-fold over control, respectively). Interestingly, LPA treatment (1 μM) produced an even greater induction of IL-8 secretion of 4.8-fold at 3 h and 2.2-fold at 24 h (**Fig. 1**).

Treatment of HBEpCs with LPA for 3 h resulted in a dose-dependent secretion of IL-8 (**Fig. 2A**) with significant production at 10 nM (1.4-fold over control) and 4.1-fold over control at 1 μM. These concentrations are within reported physiological concentrations of LPA found in human serum (34). The release of IL-8 into the medium by HBEpCs upon stimulation with LPA (1 μM) significantly increased after 3 h (3.4-fold) and reached a maximum at 12 h (**Fig. 2B**).

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LPA-Induced IL-8 Gene Expression - In order to compare the effect of LPA treatment on IL-8 production to other cytokines, we utilized cytokine-specific cDNA technology. Total RNA from vehicle- versus LPA-treated (1 μM for 3 h) HBEpCs was isolated and analyzed for differential cytokine gene expression. As shown in **Fig. 3A and 3B**, among the 23 different cytokines examined, only the expression of IL-8 was increased (11.5 fold) relative to control cells. HBEpCs produced basal amounts of IL-1α (spot pair #1) and TNFα (spot pair #22), which were not affected by LPA treatment. This striking data indicate that LPA specifically up-regulates IL-8 gene expression in HBEpCs.

G Protein-Coupling Involved in LPA-Induced IL-8 Secretion - We have previously identified the expression of LPA_{1-3} receptors in HBEpCs using RT-PCR, Western blotting and immunocytochemistry (20). To further examine which member(s) of the heterotrimeric G-proteins is responsible for LPA-mediated IL-8 secretion, we investigated the effects of inhibition of LPA-receptor/G-protein coupled signaling. Treatment of HBEpCs with PTx (100 ng/ml for 3 h), which uncouples G-protein coupled receptors for G_i, significantly prevented LPA-induced IL-8 secretion (60% inhibition), but had no effect on basal IL-8 secretion (**Fig. 4A**). In addition to PTx, we also investigated the involvement of G_i, G_q and $G_{12/13}$ by transiently transfecting minigenes that encode peptides that specifically block the respective G-protein/receptor interface. As shown in **Fig. 4B**, expression of $G_{\alpha i}$ and $G_{12/13}$ but not G_q resulted in attenuation of LPA-mediated IL-8 secretion ($G_{\alpha i}$ 30% and $G_{12/13}$ 40% inhibition). These results indicate that

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endogenously expressed $G_{\alpha i}$ and $G_{12/13}$ are responsible for the initial mediating signals that stimulate IL-8 secretion by LPA.

PKCδ Isoform is involved in LPA-induced IL-8 Secretion - Recent reports suggest that PKCδ plays a key role in the regulation of NFκB-dependent gene expression in airway epithelial cell line (30). To gain further insight into signaling pathways mediated by LPA in enhancing IL-8 secretion in HBEpCs, we investigated the role of PKC isoforms using pharmacological inhibitors and dominant negative isoforms of PKC. Analysis of the total cell lysates by Western blotting with PKC specific antibodies revealed the expression of PKC α, δ, ι/λ and ζ as the major isotypes present in HBEpCs (**Fig. 5A**). We then pretreated HBEpCs with PKC inhibitors, bisindolylmaleimide (BIM), a general inhibitor of PKC, and rottlerin, a specific inhibitor of PKCδ, for 1 h prior to LPA challenge. Bisindolylmaleimide (1 μM) significantly attenuated LPA-induced IL-8 production by 55% without altering the basal values (**Fig. 5B**). Similarly, rottlerin (5 μM) blocked LPA-mediated generation of IL-8 by 75% (**Fig. 5C**). In addition to the pharmacological inhibitors, we also tested the effect of the expression of dominant negative (dn-) PKC α, δ, λ, and ζ adenoviral vectors on LPA-induced IL-8 production. Infection of HBEpCs with varying MOI of the dn- PKC isoforms for 12, 24 and 48 h resulted in overexpression of the protein (**Fig. 6A**). Based on these expression profiles, HBEpCs were infected with the dn-PKC α, δ, λ, and ζ adenoviral vectors (25 MOI) for 24 h before challenging the cells with LPA. As shown in Figure 5B, overexpression of dn-PKCδ significantly blocked LPA-

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mediated IL-8 secretion. Also, a small but significant attenuation in IL-8 production was seen with dn-PKC λ overexpression (**Fig. 6B**). Overexpression of dn-PKCα had no effect on LPA-mediated IL-8 generation; however, overexpression of dn-PKCζ appeared to slightly enhance the effect of LPA on IL-8 secretion.

Having established a role for PKCδ in LPA-induced IL-8 secretion, we investigated the activation of PKCδ by LPA. HBEpCs were infected with vector control or dn-PKCδ dominant (25 MOI, 48 h) and cell lysates subjected to immunoprecipitation with PKCδ antibody. Immunoprecipitates were analyzed for PKC activity using [γ-^{32}P]ATP and myelin basic protein (MBP) as substrate. Treatment of cells with LPA (1 μM for 15 min) stimulated PKCδ activity by 2.5 fold compared to control cells. Overexpression of dn-PKCδ blocked the phosphorylation of MBP mediated by LPA in HBEpCs (**Fig. 7A**). In these experiments, the phosphorylation of MBP by PKCδ was normalized to total ERK in the supernatants after immunoprecipitation of PKCδ. In addition, LPA stimulation of HBEpCs also increased phosphorylation of PKCδ as evidenced by Western blotting with anti-phospho PKCδ antibody (**Fig. 7B**). As seen in the immunocytochemical analysis in **Fig. 7C**, LPA treatment stimulated translocation of PKCδ to the plasma membrane when compared to control cells. These results suggest that LPA activates the PKCδ isoform in HBEpCs, which subsequently regulates LPA-mediated IL-8 production.

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PKCδ Participates in LPA-mediated NF-κB Activation - Activation of transcriptional factors such as NF-κB and activation-protein 1 (AP-1) by proinflammatory cytokines and agonists via G-protein coupled receptors (GPCRs) has been reported (28-30). In most mammalian cells, NF-κB exists as an inactive heterodimer comprising of p50 and p65 (Rel A) units through its interaction with inhibitory-κB (IκB) proteins of which there are three major isoforms, IκBα, IκBβ and IκBε. Signal-induced phosphorylation of IκB by IKK results in ubiquitination and subsequent degradation of IκB by the 26S proteosome. This releases NF-κB heterodimers from the inactive complex and allows its translocation to the nucleus for interaction with cognate DNA sequences (35). We examined LPA-mediated activation of NF-κB utilizing various techniques. As shown in **Fig. 8A**, LPA treatment (1 μM for 15 min) increased phosphorylation of IκB in HBEpCs. The LPA-induced phosphorylation of IκB-α by LPA was time-dependent, reaching a maximum at 15 min and decreased to thereafter to near basal levels (data not shown). The role of PKCδ in LPA-dependent activation of NF-κB was investigated by infecting HBEpCs with adenoviral constructs containing dn-PKCδ. The LPA-induced phosphorylation of IκB was attenuated by overexpression of dn-PKCδ (**Fig. 8A**). Next, we investigated the ability of LPA to promote migration of NF-κB to the nucleus. Using an antibody specific to the p65 subunit of human NF-κB, immunohistochemistry was carried out in control and LPA treated cells. In control cells, the immunoreactivity of p65 appeared to be in the cytosol. On the other hand, LPA treatment (1 μM for 15 min) induced a rapid and significant

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accumulation of p65 into the nucleus (**Fig. 8B**). Furthermore, the LPA-induced translocation of NF-κB to nucleus was attenuated by overexpression of dominant negative PKCδ or by rottlerin, suggesting a role for PKCδ in NF-κB nuclear translocation (**Fig. 8B**). However, dn- PKCζ overexpression did not affect the LPA-mediated translocation of NF-κB to the nucleus indicating the specificity of PKCδ in regulating the translocation (**Fig. 8B**). A direct evaluation of NFκB activation was carried out by measuring the transcriptional activating potential of an NFκB-driven luciferase reporter plasmid by LPA. Luciferase activity was measured in HBEpCs transfected with pNF-κB-Luc and pCMV-β-gal treated with LPA (1 μM for 3 h). **Fig. 8C** shows that LPA produced a 2-fold enhancement of NF-κB driven luciferase activity, which was completely blocked with pretreatment of the cells with rottlerin (5 μM for 1 h). To further confirm the role of PKCδ in LPA-induced NF-κB, HBEpCs were infected with either vector control or PKCδ dominant negative adenovirus for 24 h, cells were treated with medium alone or medium containing LPA (1 μM) for 1 h and nuclear extracts were prepared for EMSAs. LPA treatment increased binding of nuclear proteins to the NF-κB oligonucleotides as well as induced super shift of the DNA binding complex (**Fig. 8D**). In cells over-expressing PKCδ dominant negative protein, LPA did not induce binding of nuclear proteins to the NF-κB oligonucleotides and super shift of the DNA binding complex (**Fig. 8D)**. Together, these results reveal that LPA-induced IL-8 secretion is mediated via PKCδ / NF-κB dependent signaling processes in HBEpCs.

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Intratracheally Administered LPA Stimulates Airway Neutrophil Infiltration and MIP-2 Secretion in Mice - To examine the *in vivo* effects of LPA as a pro-inflammatory lipid mediator, we carried out experiments in C57BL/6J mice. Following routine anesthetization, mice were treated intratracheally with 50 μl of saline with 0.1% bovine serum albumin (BSA) or LPA (10 μM) in this same vehicle. The mice were sacrificed and BAL was performed at the indicated time points. Measurements of MIP-2 level, the murine homolog of IL-8, total cell count with differential and total protein concentrations were analyzed from the BAL fluid. As shown in **Fig. 9A**, intratracheal instillation of LPA stimulated a significant infiltration of neutrophils into the airways (12-fold) 6 h following administration. MIP-2 levels were 40-fold higher 3 h post treatment in the LPA treated mice (**Fig. 9B**). Protein concentration, a surrogate of measuring permeability, was similar in vehicle and LPA treated mice at 6 h (**Fig. 9C**). These findings in an animal model parallel our findings in the HBEpC culture system and strongly suggest that extracellular LPA is a mediator of the innate immune response by inducing IL-8 production and subsequent airway neutrophils infiltration.

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DISCUSSION

Increased expression and release of IL-8, a potent chemo attractant of neutrophils, has been described in bronchial epithelial cells in response to TNF-α, IL-1, exposure to tobacco smoke, diesel exhaust particles, ozone, viral infection and hyperoxia (3-8). The data presented here show for the first time enhanced expression of IL-8 gene as well as its secretion by LPA in primary cultures of HBEpCs. Our results also suggest that LPA-induced expression and secretion of IL-8 is transcriptionally regulated by NF-κB. Furthermore, evidence is provided that LPA-mediated activation of PKCδ is involved in the activation of IκB and translocation of NF-κB to the nucleus.

LPA is a potent bioactive lipid that mediates several cellular responses such as proliferation, differentiation, suppression of apoptosis, tumor metastasis and cytoskeletal reorganization (15, 21-28). LPA mediates the broad range of cellular responses through GPCRs, LPA_1/Edg-2, LPA_2/Edg-4 and LPA_4/Edg-7 (19). Recently, a fourth LPA receptor, LPA_4/$p2y_9$-GPR23 which shares about 20-24% homology with LPA_{1-3} was identified from an analysis of the expressed sequence tag (EST) data base and cloned from human genomic DNA (36). Although, LPA_4 is also GPCR, only very limited information is available regarding its biological functions. LPA binds with high affinity to LPA_{1-3} that is coupled to heterotrimeric G proteins, G_i, G_q/G_{11}, and $G_{12/13}$. Earlier studies showed that LPA-induced transactivation of PDGF-Rβ and ERK activation were sensitive to PT_X,

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suggesting role for G_i dependent signaling in HBEpCs (20). In human aortic smooth muscle cells, ERK activation by LPA was attenuated by PT_X (37), while in fibroblasts LPA-mediated cell growth was PTx sensitive (18). Our present results demonstrate that LPA-induced IL-8 secretion is PT_X sensitive suggesting coupling of LPA-Rs to G_i. Additionally, transfecting cells with minigenes for G_q and $G_{12/13}$ also demonstrated possible involvement of $G_{12/13}$ but not G_q in IL-8 release mediated by LPA in HBEpCs. LPA_{1-3} are present in the lung and HBEpCs (20). It is unclear which LPA-R is coupled to G_i and/or $G_{12/13}$ resulting in downstream activation of NFκB and IL-8 secretion in HBEpCs. Studies using siRNA to differentially block individual LPA-Rs will be necessary to dissect out exact coupling between LPA-Rs and heterotrimeric G protein signaling.

The present study demonstrates the LPA-elicited PKCδ signaling is coupled to IL-8 expression and secretion in primary cultures of human bronchial epithelial cells. The role of PKC in S1P-induced IL-8 production has been previously reported (21); however, little information is available on the PKC isoform(s) and downstream target(s) of PKC regulating IL-8 generation. The PKC family of serine/threonine kinases includes three major types of isoenzymes. The classical isoforms (α, β_1, β2, and γ) are activated by diacylglycerol, calcium and phosphatidylserine, whereas the novel isoforms (δ, ε, η, θ, and μ) are calcium insensitive but are activated by diacylglycerol and phosphatidylserine. The atypical isoforms (ζ and ι/λ) are insensitive to calcium and diacylglycerol but dependent on phosphatidylserine. In agreement with published results (38), we

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detected the presence of PKC α, δ, ζ, θ, ι/λ but not β, γ, ε, and η in HBEpCs (**Fig.5**). By infecting cells with dn- adenoviral constructs of PKC α, δ, ζ, and ι / λ, we observed that the LPA-mediated IL-8 secretion was dependent on PKC δ and to a much lesser degree on PKC ι/λ isoforms (**Fig. 6**). Furthermore, using myelin basic protein as a substrate for PKCδ, it was shown that LPA activated PKCδ in HBEpCs. Experiments with rottlerin also suggest a role for PKC δ in LPA-mediated IL-8 production. Besides the role of PKCδ signaling, our data also show the significance of NF-κB activation by LPA in IL-8 expression and secretion in HBEpCs. This work concurs with other studies suggesting regulation of epithelial cell cytokine secretion by NF-κB (30). In cells of the A549 respiratory line, the rhinovirus-, TNF-α-, and LPS- mediated IL-8 generation was dependent on NF-κB activation and translocation to the nucleus (13, 39). In unstimulated cells, NF-κB exists as p50/p65 heterodimers and is sequestered in the cytoplasm by IκB family of kinases, of which IκBα is the best characterized. Upon stimulation, Iκκs phosphorylate IκB with subsequent ubiquitination and degradation leading to the release of NF-κB and its translocation to the nucleus. The experiments presented here show that LPA stimulates phosphorylation of IκB, enhances NF-κB luciferase reporter gene expression, and translocation of NF-κB to the nucleus. These data demonstrate very clearly a role for NF-κB transcriptional regulation of IL-8 secretion by LPA in HBEpCs.

A major finding of this study is the PKCδ dependent activation of NF-κB and IL-8 secretion by LPA in HBEpCs. Earlier reports have implicated several atypical (ζ

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and ι/λ) and novel PKC isoforms (δ, ε and θ) to stimulate NF-κB signaling in mammalian cells. In NIH 3T3 cells, PKCζ directly activates IKKβ *in vitro* and regulates the transcriptional activity of NF-κB (40). A role for PKCζ-NF-κB signaling in TNF-α mediated ICAM-expression was shown in endothelial cells (41), while targeted disruption of PKCζ attenuates TNF-α and IL-1 dependent phosphorylation of p65 and NF-κB transcriptional activity in mice (42). In contrast to the above studies, we observed that over-expression of dominant negative PKCζ in HBEpCs had no effect on LPA-induced NF-κB translocation to the nucleus and slightly increased IL-8 production (**Fig. 6B**). In the present study, selective inhibition of PKCδ effectively blocked LPA dependent phosphorylation of IκB, NF-κB translocation to the nucleus and luciferase reporter gene activity. Consistent with our results in primary cultures of HBEpCs, a recent report suggest a key role for PKCδ in the regulation of TNF-α and phorbol ester mediated transcription of GM-CSF, RANTES, ICAM-1 and IL-8 in the16HBE14$_0$ human airway epithelial cell line (30). The mechanism(s) by which PKC δ activates NFκB is unclear. In 16HBE14$_0$ cells, over-expression of a dominant negative mutant of Iκκβ (Iκκβ-AA) attenuated PKC δ-CAT mediated NF-κB transactivation, suggesting regulation via Iκκ signaling (30). However, in thrombin-mediated ICAM-1 gene expression, transactivation of NF-κB was dependent on PKCδ / p38 MAPK pathway (29). A similar regulation by p38 MAPK in thrombin-induced NFκB stimulation and VCAM-1 expression was observed in HUVECs (43). Alternatively, PKCδ may directly phosphorylate NF-κB resulting in enhanced DNA binding and transcriptional activity. Recent studies

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suggest that endogenous PKCδ translocates to the nucleus after etoposide treatment in C5 cells suggesting regulation of nuclear event(s) by nuclear import of PKCδ (44). Although our results indicate that LPA-induced IκB phosphorylation and NF-κB activation are dependent on PKCδ, it is unclear whether PKCδ activates IκB directly or via the Iκκ kinase mediated pathway. Furthermore, expression of a dominant negative mutant of IκκB (IκκB-AA) did not completely attenuate bryostatin 1 or PKCδ-CAT-induced NF-κB activation in 16HBE14o bronchial epithelial cells, suggesting that PKCδ also activates NF-κB via IκκB-independent pathways (30). Therefore, further studies are necessary to delineate the signaling pathways of LPA-induced NF-κB activation by PKCδ in HBEpCs.

IL-8 is a potent chemo-attractant for neutrophils as well as eosinophils, basophils and T lymphocytes in the airways. In airway diseases, severity of inflammation has been correlated with levels of IL-8 in the BAL fluid of various respiratory diseases. We have recently observed that LPA levels in BAL fluids from segmental allergen challenged asthmatics to be significantly higher compared to non-segmental challenged asthmatics and further the elevation of LPA levels correlated with higher eosinophils in the BAL fluid (Natarajan, et al. manuscript in preparation). In the present study, increased influx of neutrophils in the alveolar space within the first 6 hrs of instillation of LPA into the mouse trachea was observed. Interestingly, the neutrophils infiltration was preceded by an elevation of MIP-2, the analog of human IL-8, in BAL fluid within 3 h of LPA instillation. At later time periods (>12 h), the levels of MIP-2 and neutrophils returned to near

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normal values. These results suggest that accumulation of LPA in the airway induces accumulation of neutrophils and other inflammatory cells at local sites of injury and inflammation. LPA (1-10 μg/ml) instilled into guinea pig airways substantially increased eosinophils and neutrophils in BAL fluid after 4 h of inhalation which was blocked by Y-27632, an inhibitor of Rho-associated protein kinase (23). The *in vivo* studies in murine and guinea pig models of airway inflammation indicate that generation of LPA at or near the site of injury can contribute to the activation of inflammatory cells as well the initial process of inflammation. Further studies in our laboratory are in progress to address the source, pathway(s) and amounts of LPA generated in the airway during allergic responses or bronchial asthma.

In summary, our results provide a definitive link between LPA and secretion of the pro-inflammatory cytokine IL-8 in HBEpCs and which are reproducible in a murine model of lung injury. For the first time, we demonstrate that LPA-induced IL-8 secretion in HBEpCs is dependent on PKCδ mediated activation of NF-κB. The importance of LPA as a pro-inflammatory lipid mediator is evidenced by instillation of LPA into the trachea and demonstrating increased influx of neutrophils and higher MIP-2 levels in the BAL fluid. These results suggest that LPA may be potent lipid mediator inducing secretion of IL-8 from the bronchial epithelial cells followed by chemotaxis of neutrophils and other inflammatory cells into the airway. Better understanding of the mechanisms of LPA production by

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different inflammatory cells in the airway may be relevant to treatment of asthma and other airway inflammatory diseases.

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FIGURE LEGENDS

Figure 1: Stimulation of IL-8 secretion by LPA and S1P- HBEpCs (~80% confluence in 35-mm dishes) were treated with BEGM with 0.1% BSA (vehicle) or BEGM with 0.1% BSA plus LPA (1 μM) or S1P (1 μM) for indicated time (3 and 24 h). IL-8 secreted into the medium was quantified by ELISA as described under "Experimental Procedures". Data are means $\pm$ S.D. of triplicate determinations of three independent experiments. *, Values are significantly different from vehicle control ($p < 0.01$); **, Values are significantly different from vehicle control ($p < 0.05$).

Figure 2: Dose response and time course of LPA-induced IL-8 secretion - HBEpCs were grown in 35-mm dishes to ~80% confluence in BEGM. **A,** cells were treated for 3 h in BEGM with 0.1% BSA (vehicle) or BEGM with 0.1% BSA containing indicated concentrations of LPA. **B,** cells were treated in BEGM with 0.1% BSA (vehicle) or BEGM with 0.1% BSA plus LPA (1 μM) for indicated times (1-24 h). ELISA as described under "Experimental Procedures" quantified IL-8 secreted into the medium. Data are means $\pm$ S.D. of triplicate determinations of three independent experiments. *, Values are significantly different from vehicle controls ($p < 0.05$).

Figure 3: Expression profile of cytokine genes by cDNA expression array system in HBEpCs – A, Total RNA was isolated from HBEpCs (2 x 10^6 cells) after 3 h incubation with or without LPA (1 μM). Total RNA was reverse-transcribed and labeled with biotin, and gene expression was detected using

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Nonrad-GEArray cytokine series kit according to manufacturer's instructions. The numbers on the membrane correspond to: 1, IL-1α; 2, IL-β; 3, IL-2; 4, IL-3; 5, IL-4; 6, IL-5; 7, IL-6; 8, IL-7; 9, IL-8; 10, IL-9; 11, IL-10; 12, IL-11; 13, IL-12A; 14, IL-12B; 15, IL-13; 16, IL-14; 17, 1L-15; 18, IL-16; 19, IL-17; 20, IL-18; 21, IFN-γ; 22, TNF-α; 23, TNF-β (Lt-α). **B,** IL-8 gene expression was quantified by scanning densitometry and intensity normalized to housekeeping genes β-actin and GAPDH. Values are average of three independent GE Array expression array systems.

Figure 4: PT_X and G-protein minigenes attenuate LPA-induced IL-8 secretion – A, HBEpCs (~80% confluence in 35 mm dishes) were treated with PT_X (100 ng/ml) for 3h. Cells were challenged with BEGM containing 0.1%BSA (vehicle control) or BEGM with 0.1%BSA containing LPA (1 μM) for an additional 3 h. IL-8 secreted into the medium was quantified by ELISA as described under "Experimental Procedures". Data are means ± S.D. of three independent determinations. * Values are significantly different from control ($p < 0.05$); ** values significantly different from LPA challenged cells without PT_x treatment ($p < 0.05$). **B,** HBEpCs (~50% confluence in 35 mm dishes) were transfected with G_i , G_q , G_{12} and G_{13} minigene plasmids as described in "Experimental Procedures" for 24 h. Cells were treated with BEGM with 0.1%BSA or BEGM with 0.1%BSA containing LPA (1 μM) for 3 h. The medium was collected and analyzed for IL-8 by ELISA. Data are means ± S.D. of three independent experiments in triplicate. * Values are significantly different compared to vehicle ($p < 0.05$); **, values are

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significantly different compared to LPA challenge in the absence of minigenes $G_{i,}$ $G_{12/13}$ ($p < 0.05$).

Figure 5: Expression of PKC isoforms and effects of bisindolylmaleimide and rottlerin on LPA-mediated IL-8 secretion – A, Cell lysates (20 µg protein) from HBEpCs were subjected to SDS-PAGE and Western blotting with PKC isoform specific antibodies. **B,** HBEpCs (~80% confluence in 35 mm dishes) were treated with bisindolylmaleimide (1 µM) for 1h prior to LPA stimulation in BEGM plus 0.1%BSA for 3 h. **C,** HBEpCs (~80% confluence in 35 mm dishes) were treated with rottlerin (5 µM) for 30 min prior to LPA challenge in BEGM plus 0.1%BSA for 3 h. The medium was collected and analyzed for IL-8 by ELISA as described in "Experimental Procedures". Data are means ± S.D. of three independent experiments in triplicate. *, Values are significantly different from vehicle treated cells ($p < 0.05$); **, Values are significantly different from LPA challenged cells without BIM or rottlerin ($p < 0.05$).

Figure 6: Effects of overexpression of dominant negative PKC isoforms on LPA-induced IL-8 secretion - A, HBEpCs (~50% confluence in 35 mm dishes) were infected with vector or each of the PKC dominant negative isoforms in adenoviral constructs at an MOI of 25 for varying time periods (12, 24 and 48 h). Cell lysates from vector control or PKC dominant negative infected cells were subjected to SDS-PAGE and Western blotting as described under "Experimental Procedures". Shown is a representative blot of three independent experiments. **B,** HBEpCs (~50% confluence in 35 mm dishes) were infected with dominant negative PKC α, δ, ζ, and λ adenoviral constructs (25 MOI) for 48 h. Cells were

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then treated with BEGM plus 0.1%BSA (vehicle) or BEGM with 0.1%BSA plus LPA (1 μM) for 3 h. IL-8 secreted into the medium was quantified by ELISA as described under "Experimental Procedures". Data are means ± S.D. of three independent experiments in triplicate. * Values are significantly different from vehicle controls ($p < 0.05$); **, Values are significantly different from LPA treated cells ($p < 0.05$); ***, Values not significantly different from LPA treatment ($p < 0.05$).

Figure 7: Effects of infection of dominant negative PKCδ on LPA-induced activation of native PKCδ – HBEpCs (~50% confluence) were infected with either vector control or PKCδ dominant negative adenoviral constructs (25 MOI) for 48 h prior to LPA (1 μM) challenge for 15 min. **A,** cell lysates from vector control or dominant negative PKCδ adenoviral infected cells were subjected to immunoprecipitation with PKCδ antibody as described under "Experimental Procedures" and immunoprecipitates were tested for activity using myelin basic protein (MBP) as a substrate. Data are expressed as relative counts per minute (CPM) in the phosphorylated MBP. **B,** cell lysates (20 μg protein) from vector control or dominant negative PKCδ infected cells were stimulated with LPA (1 μM) for varying time periods (0-15 min). Total cell lysates were subjected to SDS-PAGE and Western blotting with phospho PKCδ and pan PKCδ antibodies. **C,** HBEpCs were grown on coverslips to ~70% confluence and infected with vector control or dominant negative PKCδ adenoviral construct (25 MOI) for 48 h prior to challenge with LPA (1 μM) for 15min. Cells were then subjected to immunostaining with anti PKCδ antibody and examined by fluorescent

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microscopy as described under “Experimental Procedures”. The immunofluorescence image is representative of the monolayer visualized in three independent experiments.

Figure 8: Effects of Dominant negative PKCδ and rottlerin on LPA-induced NF-κB activation – A, HBEpCs (~50% confluence in 35 mm dishes) were infected with vector or Dominant negative PKCδ (25 MOI) adenoviral construct for 48 h. Cells were stimulated with BEGM plus 0.1% BSA (vehicle) or BEGM plus 0.1% BSA containing LPA (1 μM) for 15 min. Cell lysates (20 μg protein) were subjected to SDS-PAGE and Western blotting with phospho-IκB or pan ERK1/2 antibodies as described under “Experimental Procedures”. Shown is a representative Western blot of three independent experiments. The extent of IκB phosphorylation was quantified by image analysis and normalized to total ERK. Data are means ± S.D. of three independent experiments in triplicate. *, Values are significantly different from vehicle ($p < 0.05$); **, Values are significantly different from LPA challenged cells without dn PKCδ overexpression. **B,** HBEpCs (~50% confluence) grown on glass coverslips were infected with vector or dominant negative PKCδ or PKCζ isoform (25 MOI) for 48 h. Cells were pretreated with BEGM plus 0.1%BSA or BEGM with 0.1%BSA containing rottlerin (5 μM) for 30min prior to stimulation with LPA (1 μM) for 15 min. The cells were rinsed in PBS, fixed in 3.7% formaldehyde, immunostained with anti NF-κB antibody and examined by fluorescent microscopy as described under “Experimental Procedures”. **C,** HBEpCs were grown in 35 mm dishes to ~50% confluence and transiently transfected with a NF-κB promoter driven luciferase

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reporter plasmid. Eighteen to twenty four h after transfection, cells were incubated for 3 h with BEGM plus 0.1%BSA (vehicle) or BEGM plus 0.1%BSA containing LPA (1 μM). Luciferase activity was measured using a commercial kit and values were normalized to Renilla luciferase. Data are means ± S.D. of three independent experiments in triplicate. *, Values are significantly different from vehicle controls ($p< 0.05$); **, Values are significantly different from LPA stimulated cells without rottlerin treatment ($p < 0.05$). **D,** EMSA showing the effect of PKCδ dominant negative overexpression on LPA-induced binding of nuclear proteins to the NF-κB oligonucleotides and super shift of the DNA binding complex. These data are representative of three separate experiments.

Figure 9: Intratracheal instillation of LPA stimulates secretion of MIP-2 and influx of neutrophils into bronchoalveolar lavage- C57BL/6J mice, following routine anesthetization, were instilled with either 50 μl of 0.9% saline plus 0.1% BSA or 0.9% saline with 0.1%BSA containing 10 μM LPA. **A,** Bronchoalveolar lavage was performed 6 h after LPA instillation and cell pellet was obtained immediately as described under "Experimental Procedures". Differential cell counts were made from centrifuged preparations stained with Wright-Giesma. **B,** Bronchoalveolar lavage was performed 3 h after LPA instillation and centrifuged to pellet the cells. The supernatants were stored at – 80 ^{o} C and analyzed for MIP-2 by ELISA as described under "Experimental Procedures". **C,** Total protein in bronchoalveolar lavage obtained from A was determined with a Pierce protein assay kit. Data are means ± S.D. of three independent experiments in triplicate. *, Values are significantly different from vehicle instilled group ($p < 0.05$).

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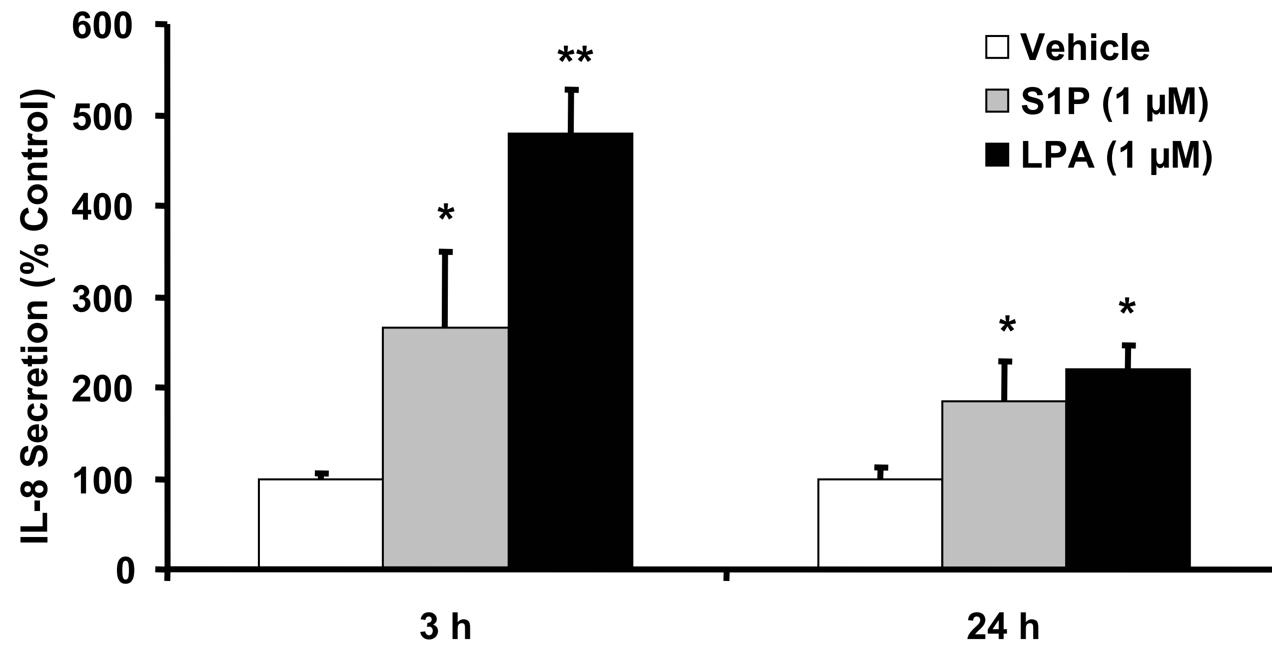

Fig 1. Cummings, R., et al.

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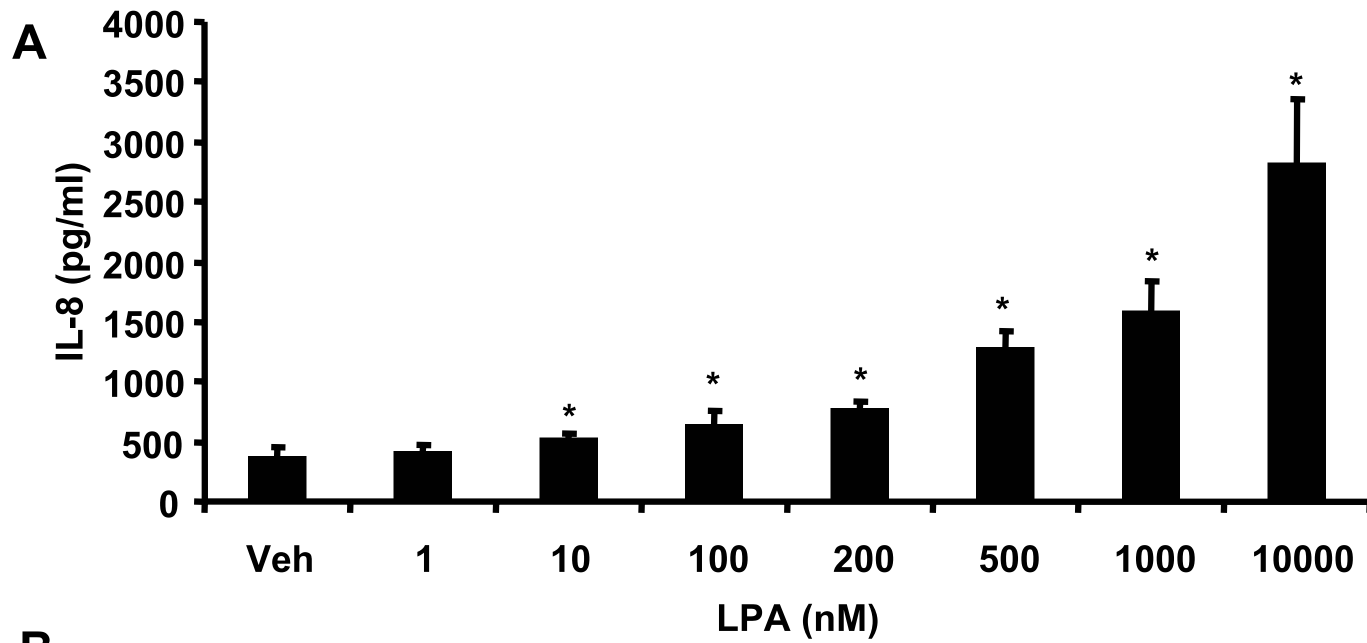

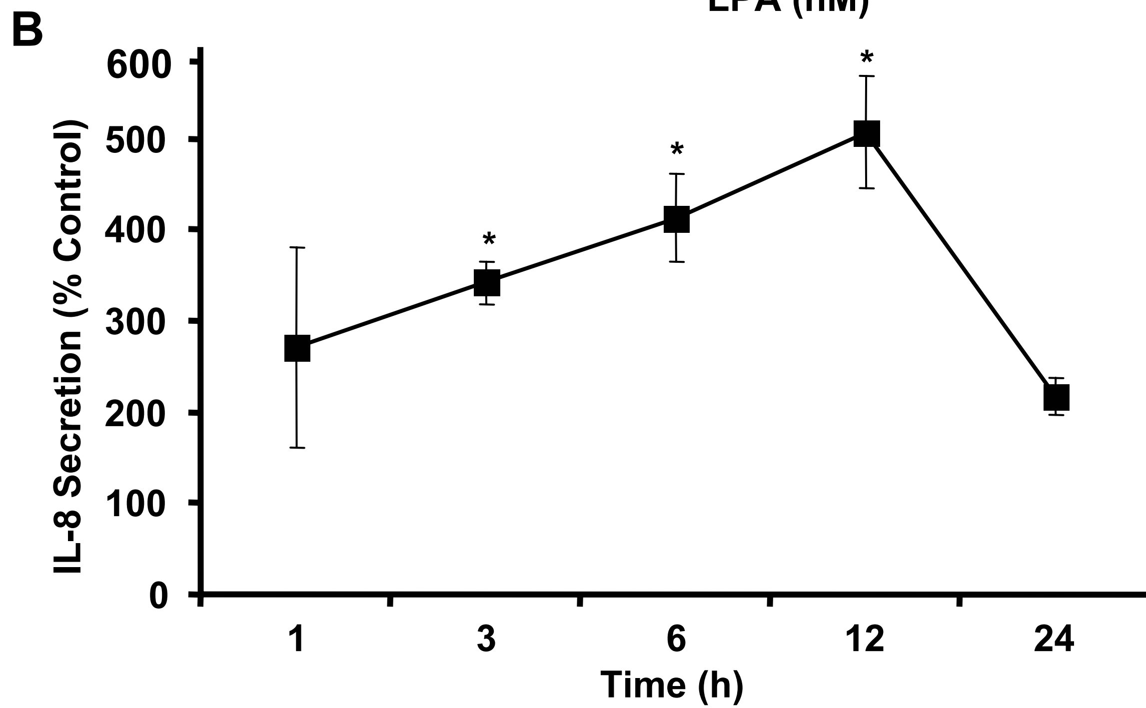

Fig 2. Cummings, R., et al.

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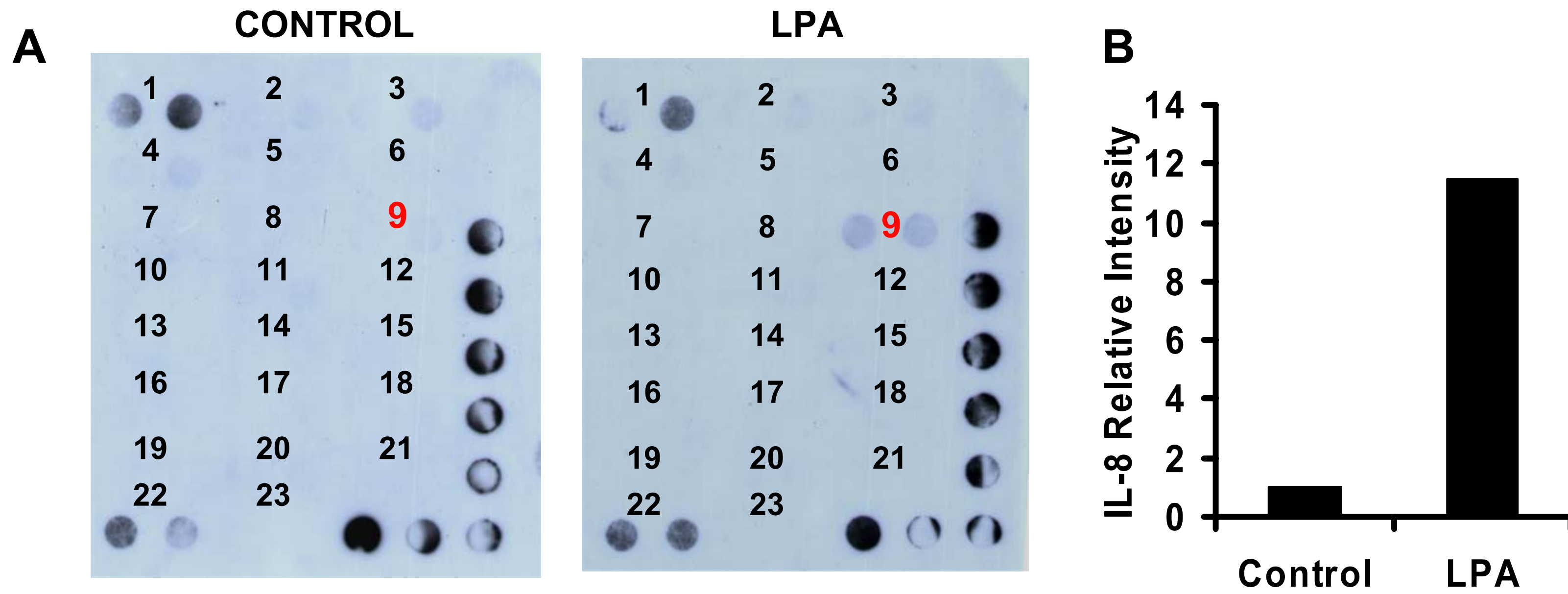

Fig 3. Cummings, R., et al.

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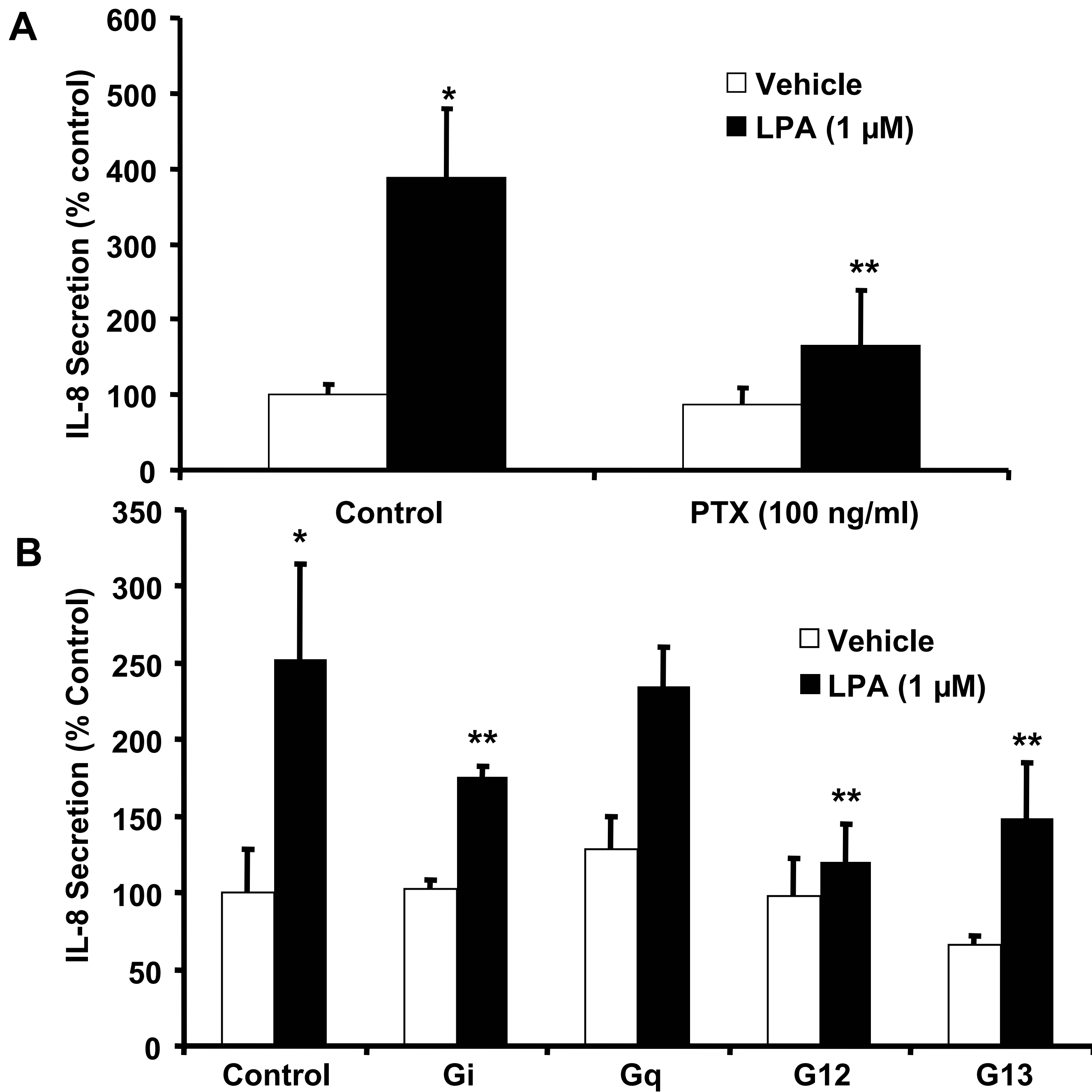

Fig 4. Cummings, R., et al.

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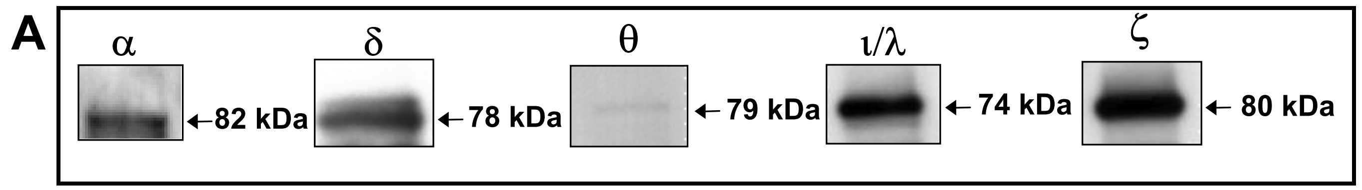

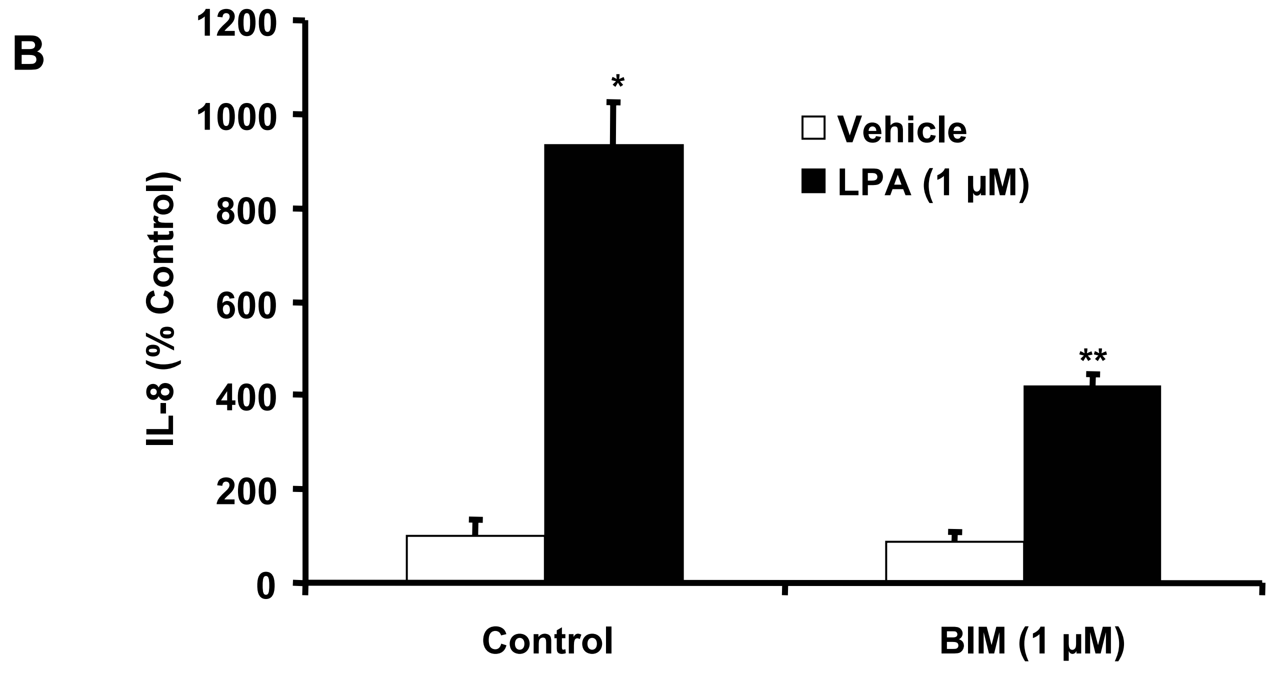

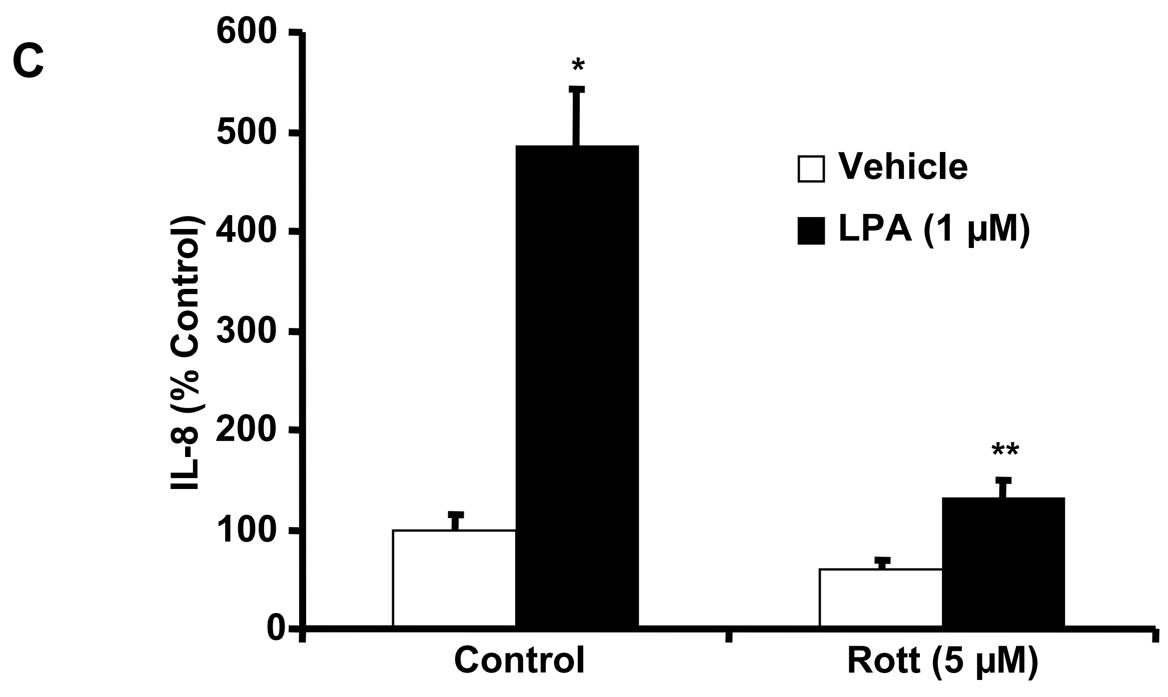

Fig 5. Cummings, R., et al.

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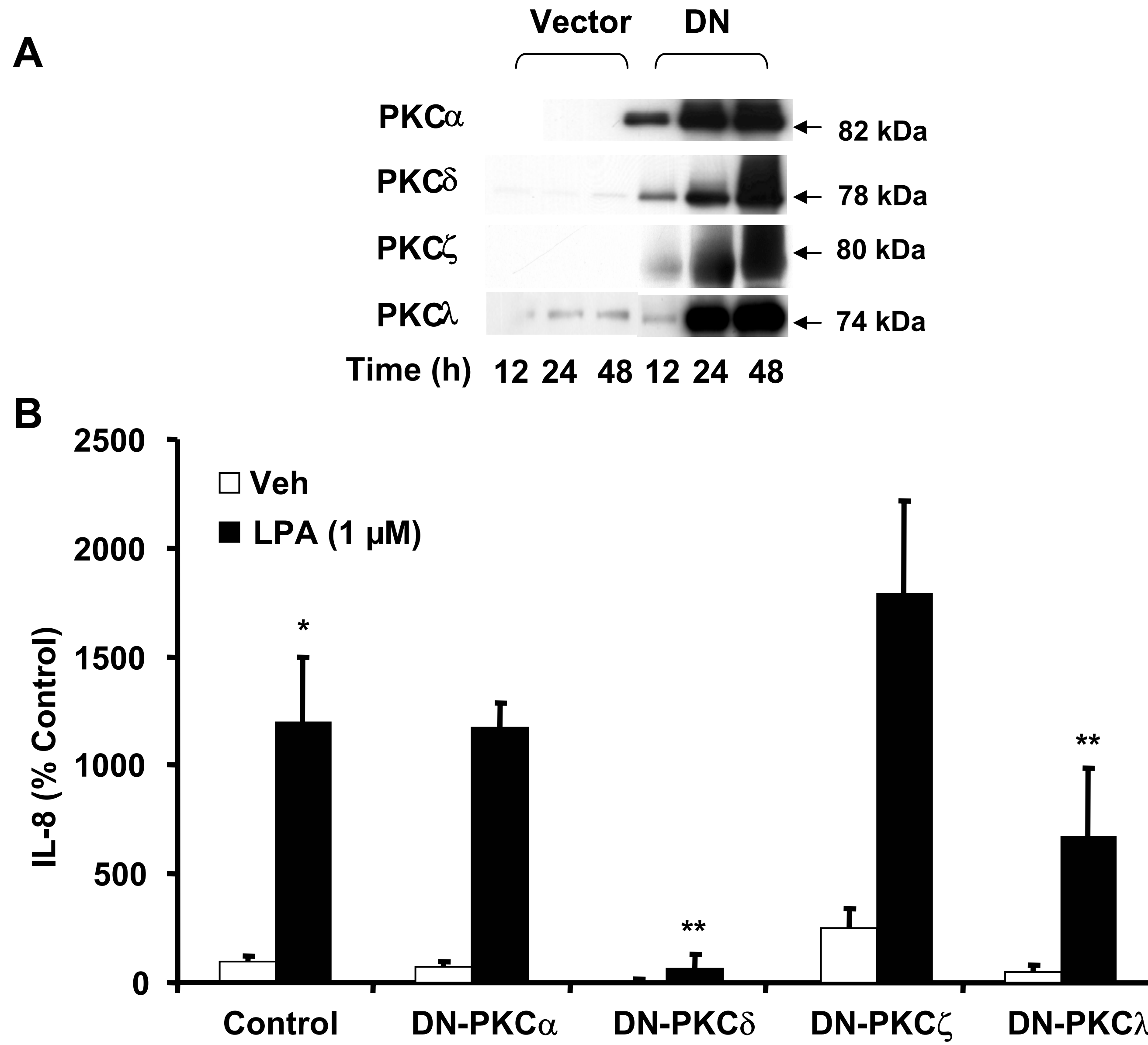

Fig 6. Cummings, R., et al.

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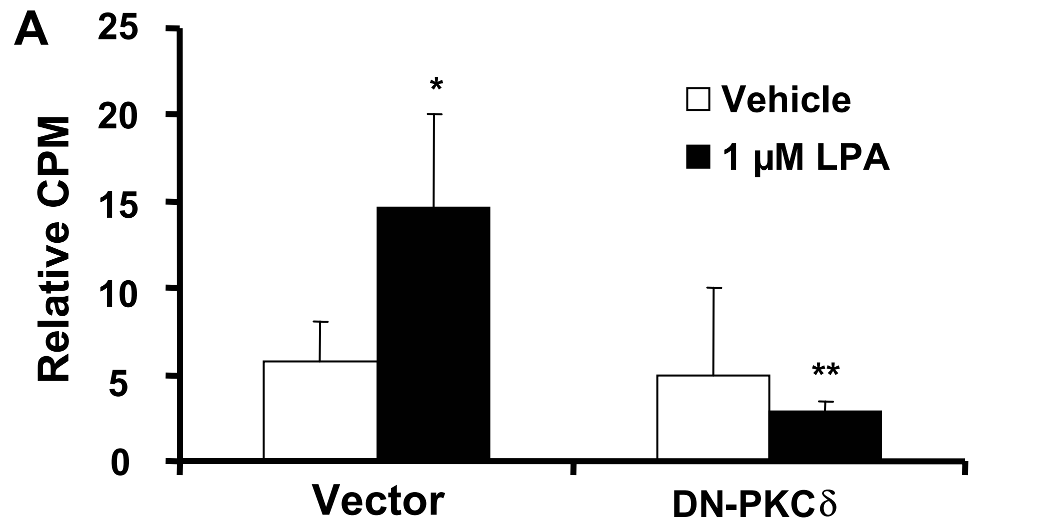

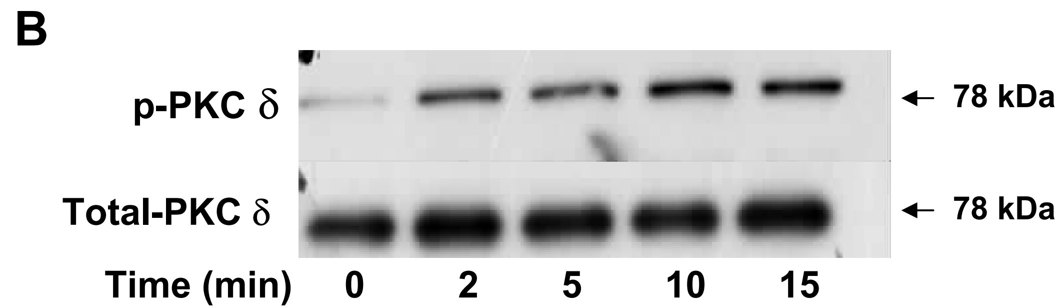

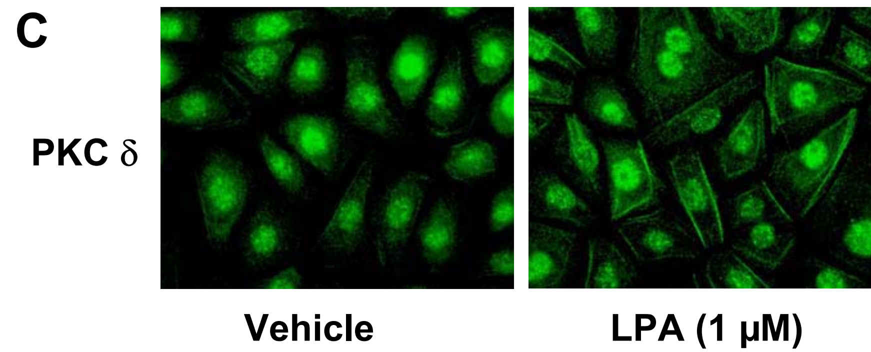

Fig 7. Cummings, R., et al.

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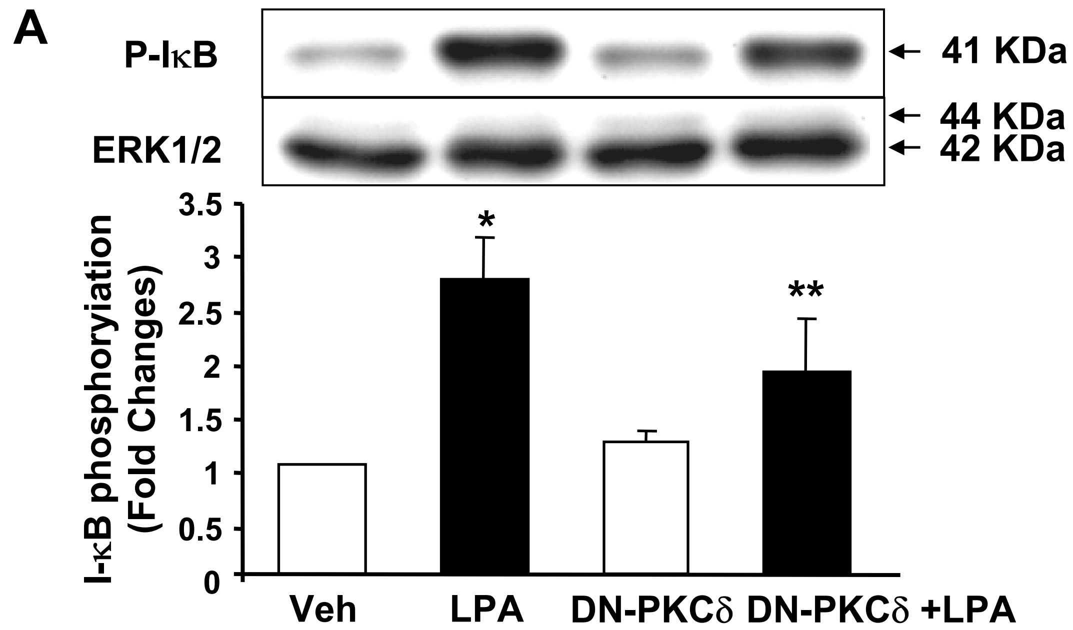

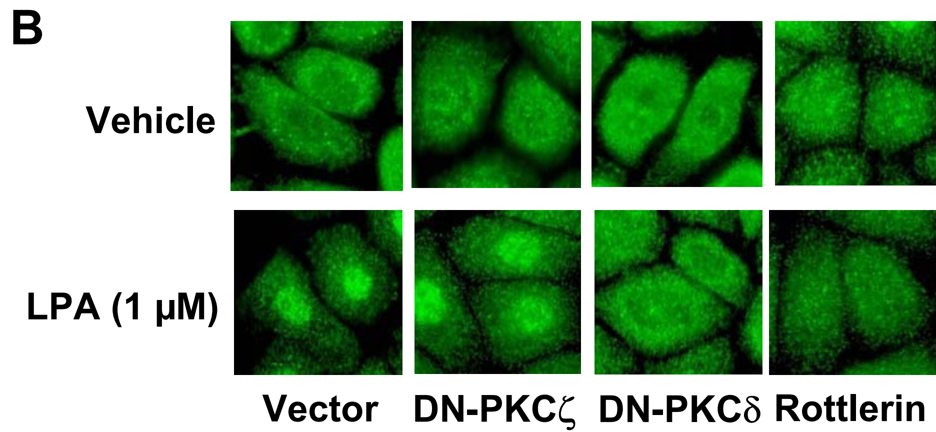

Fig 8. Cummings, R., et al.

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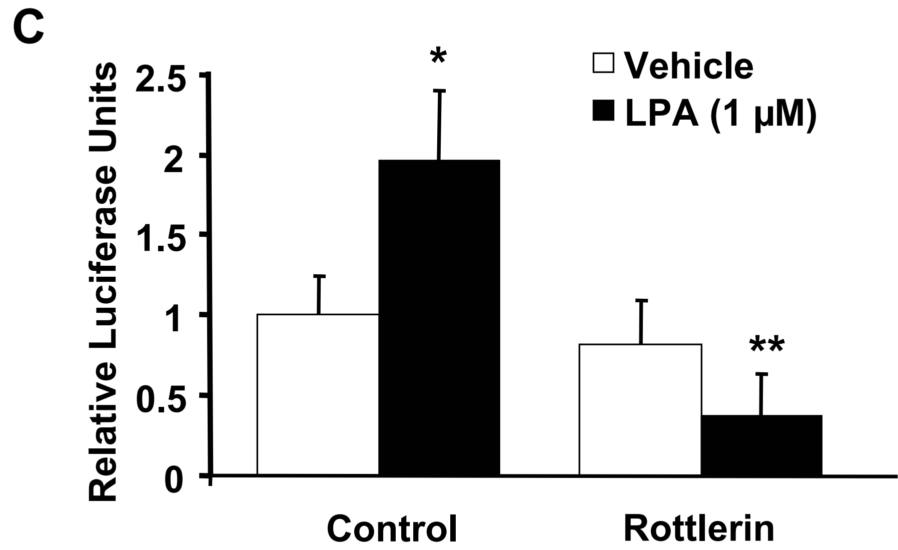

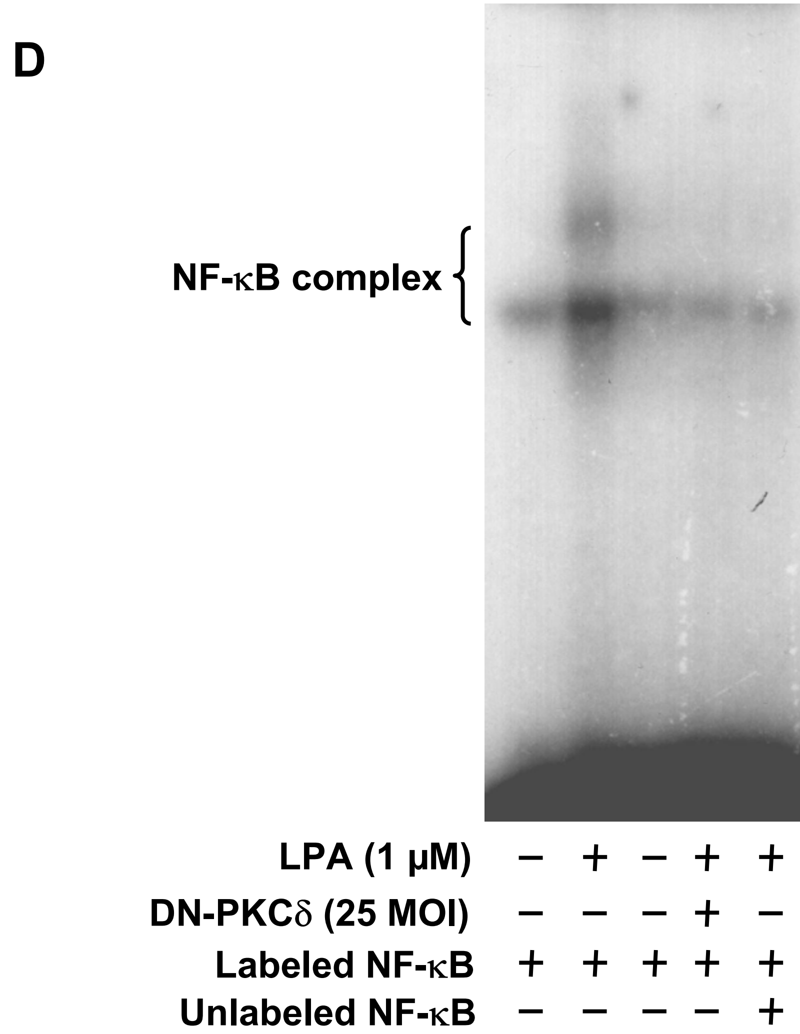

Fig 8. Cummings, R., et al.

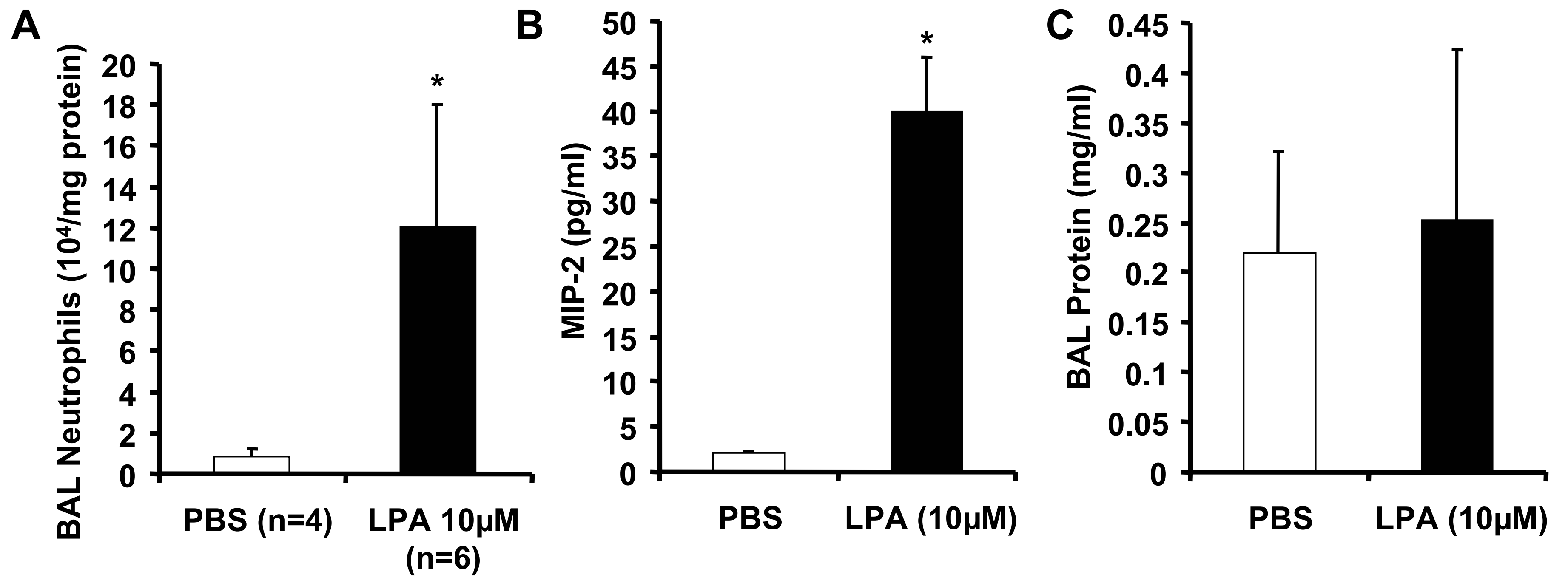

Fig 9. Cummings, R., et al.

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Protein kinase Cd mediates lysophosphatidic acid -induced NF-kB activation and interleukin-8 secretion in human bronchial Epithelial cells

Rhett Cummings, Yutong Zhao, David Jacoby, E. William Spannhake, Motoi Ohba, Joe G. N. Garcia, Tonya Watkins, DongHong He, Bahman Saatian and Viswanathan Natarajan

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