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ALTERATION IN THE ACTIVATION STATE OF NEW INFLAMMATION-ASSOCIATED TARGETS BY PHOSPHOLIPASE A₂-ACTIVATING PROTEIN (PLAA)

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Abstract

Phospholipase A₂ (PLA₂)-activating protein (PLAA) is a novel signaling molecule that regulates the production of prostaglandins (PGE₂) and tumor necrosis factor (TNF)-α. To characterize the function of native PLAA *in situ*, we generated HeLa (Tet-off) cells overexpressing *plaa* (*plaa*^{high}) and control (*plaa*^{low}) cells, with the *plaa* gene in opposite orientation in the latter construct. The *plaa*^{high} cells produced significantly more PGE₂ and interleukin (IL)-6 compared to *plaa*^{low} cells in response to TNF-α. There was increased activation and/or expression of cytosolic PLA₂, cyclooxygenase-2, and NF-κB after induction of *plaa*^{high} cells with TNF-α compared to the respective *plaa*^{low} cells. Microarray analysis of *plaa*^{high} cells followed by functional assays revealed increased production of proinflammatory cytokine IL-32 and a decrease in the production of annexin A4 and clusterin compared to *plaa*^{low} cells. We demonstrated the role of annexin A4 as an inhibitor of PLA₂ and showed that addition of exogenous clusterin limited the production of PGE₂ from *plaa*^{high} cells. To understand regulation of *plaa* gene expression, we used a luciferase reporter system in HeLa cells and identified one stimulatory element, with Sp1 binding sites, and one inhibitory element, in exon 1 of the *plaa* gene. By using decoy DNA oligonucleotides to Sp1 and competitive binding assays, we showed that Sp1 maintains basal expression of the *plaa* gene and binds to the above-mentioned stimulatory element. We demonstrated for the first time that the induction of native PLAA by TNF-α can perpetuate inflammation by enhancing activation of PLA₂ and NF-κB.

Keywords

Phospholipase A₂-activating protein; PGE₂; NF-κB; annexin A4; IL-32; clusterin

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1. Introduction

Inflammatory responses in the host are characterized by a variety of changes, including vasodilation, leukocyte extravasation, and an increase in vascular permeability. These changes are induced by inflammatory mediators, such as lipids (e.g., prostaglandins, leukotrienes and platelet-activating factor) [1,2], proinflammatory cytokines (e.g., tumor necrosis factor- α [TNF- α], interleukin [IL]-1 β and IL-6) and chemokines (e.g., IL-8 and macrophage chemoattractant protein [MCP]-1) [3]. Eicosanoids, including prostaglandins and leukotrienes, are formed from arachidonic acid (AA), which is hydrolyzed from membrane phospholipids by various forms of phospholipase A₂ (PLA₂), designated as secretory (sPLA₂), cytosolic (cPLA₂), and calcium-independent (iPLA₂) [4]. The substrate AA is then converted into prostaglandins and leukotrienes by the cyclooxygenase (e.g., *via* the COX-1 and COX-2) and lipoxygenase pathways, respectively [5]. The induction of PLA₂ and COX-2 is highly regulated through mitogen-activated protein kinases (MAPKs), nuclear factor-kappa B (NF- κ B), and other pathways [6], and can be stimulated by cytokines and trauma [5]. In turn, prostaglandins, especially prostaglandin E₂ (PGE₂), can modulate inflammatory responses by regulating cytokine production and leukocyte activation [1,7]. De-regulation of eicosanoid production has been implicated in many diseases, notably atherosclerosis [8,9], neurodegenerative diseases [9,10], sepsis [2], arthritis [11,12], inflammatory bowel disease (IBD) [13], and cancer [9,14].

PLAA is a novel activator of phospholipases that regulates the production of PGE₂, TNF- α , and IL-1 β [13,15,16]. The cDNA of murine PLAA was originally cloned from a smooth muscle-like cell line, BC₃H1, using antibodies to melittin, a peptide component (26-amino acid long) of bee venom known to activate PLA₂ [17,18]. Within murine PLAA, a stretch of amino acid residues between positions 503-538 exhibit 42% identity with melittin. Subsequently, a 28-kDa fragment of murine PLAA was partially purified from BC₃H1 cells by using anti-melittin antibody affinity chromatography, which was shown to stimulate PLA₂ activity in an *in vitro* assay [18]. Later, the cDNA encoding the rat *plaa* gene was cloned [19]. This led to our cloning and characterization of the human *plaa* gene from a macrophage/monocyte cell line U937 [20]. The full length human PLAA contains 738 amino acid residues with a predicted molecular mass of 82 kDa, sharing over 90% similarity at the amino acid level with murine and rat PLAA [20]. The gene encoding human PLAA is located in chromosome region 9p21 [21] and is expressed in the majority of body tissues at different levels [13,15,16].

Within amino acid residues 503-538, human PLAA also exhibits 39% identity with melittin [20]. Expression of the *plaa* gene in host cells (e.g., macrophages and intestinal epithelial cells) can be induced rapidly by bacterial products, such as cholera toxin (CT) [22] and lipopolysacchride (LPS) [13], as well as by proinflammatory cytokines, such as TNF- α and IL-1 β [13,23]. Our earlier studies indicated increased levels of PLAA in biopsy specimens of patients with IBD in concurrence with the induction of COX-2 and PLA₂ [13]. Likewise, monosodium urate crystals, the etiological agent of gout, also induced the expression of the *plaa* gene and activated PLA₂ [24]. Consistent with the induction of PLA₂ activity in patients with adult respiratory distress syndrome (ARDS) and atherosclerosis [25,26], LPS increases *plaa* mRNA abundance in murine and rat alveolar macrophages and human endothelial cells (unpublished results from our laboratory). In turn, induction of PLAA can modulate inflammatory responses. For example, injection of a 28-kDa murine PLAA polypeptide into rabbit knee joints resulted in acute inflammatory arthritis with increased eicosanoid production and synovial leukocyte count [27]. Treatment of human neutrophils with the same PLAA polypeptide increased neutrophil degranulation and production of lysosomal enzymes and superoxide anions [28]. In addition, the 28-kDa murine PLAA polypeptide, as well as the human PLAA synthetic peptide (36-amino acid long [position 503-538]) spanning the melittin homology domain, also induced the production of TNF- α and IL-1 β in human monocytes and

murine RAW 264.7 macrophages [15,29]. Conversely, blocking expression of the *plaa* gene by using antisense oligonucleotides reduced AA production in RAW 264.7 cells stimulated with LPS and CT [13,20]. The same antisense oligonucleotides reduced PGE₂ production in oxytocin (OT)-stimulated Chinese hamster ovary (CHO) cells transfected with the oxytocin receptor (CHO-OTR) (unpublished data). Oxytocin is known to induce PGE₂ production from CHO-OTR cells and is an important stimulator of uterus contraction during pregnancy [30].

Currently, the *in situ* function of PLAA is not understood, and there is a paucity of information in the following areas: 1) previous functional characterization of PLAA was conducted using either a 28-kDa murine PLAA polypeptide or PLAA synthetic peptides added exogenously to the cell culture. Therefore, the exact role of native full length PLAA within the host cell with respect to its capacity to activate PLA₂ and to regulate eicosanoid production is completely unknown; 2) the mechanism of mutual induction between PLAA and cytokines is not well understood; and 3) the regulation of *plaa* gene expression has not been illustrated. Therefore, the objectives of this study were as follows: 1) to determine whether native PLAA regulates eicosanoid production by overexpressing the *plaa* gene *in situ*; 2) to identify downstream effectors targeted by *plaa* gene induction using microarray analysis; 3) to delineate whether native PLAA regulates cytokine production by modulating NF-κB activation; and 4) to characterize the transcriptional regulation of the *plaa* gene and search for *cis*- and *trans*-acting regulatory elements in its 5' genomic region.

2. Materials and methods

2.1. Overexpression of the *plaa* gene in HeLa Tet-off cells

Using the following forward and reverse primers: 5' TGCACGCGTATGCACTGTATGAGCGGCCACTCCAAT 3' and 5' TATGCTAGCTACAGCAAATTTAGGATAAATCTACAGC 3', the human *plaa* gene was cloned into the pBI-EGFP vector (ClonTech Laboratories, Inc., Mountain View, CA) at the *MluI*/*NheI* restriction enzyme sites to form pBI-EGFP-PLAA recombinant plasmid. The underlined bases in the primer sequences represented restriction enzyme sites. The enhanced green fluorescent protein (EGFP) open reading frame is contained in the pBI-EGFP-PLAA plasmid as a reporter. HeLa Tet-off cells (G418 resistant, ClonTech) were co-transfected with plasmids pBI-EGFP-PLAA and pTK-Hyg. This HeLa cell line provides the transcriptional activator to bind the promoter in pBI-EGFP-PLAA, and the pTK-Hyg vector provides a hygromycin resistance selection marker. Three weeks after transfection, stable transfectants that were resistant to hygromycin (200 µg/ml) and G418 (200 µg/ml) (ClonTech) were individually selected based on intensity of green fluorescence and expanded. Five selected clones were screened by Northern blot analysis for the expression of the *plaa* gene. The clone that consistently showed the highest levels of expression of the *plaa* gene was designated as *plaa*^{high} and was used throughout these studies. A clone in which the *plaa* gene was inserted in the opposite orientation and exhibited the lowest level of *plaa* gene expression (representing expression of the native *plaa* gene in the HeLa cells) was referred to as *plaa*^{low}, and used as a negative control in all of the subsequent experiments.

2.2. Cell culture

The human cervical epithelial cell line HeLa was purchased from American Type Culture Collection (Manassas, VA.). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) supplemented with penicillin (100 U/ml)-streptomycin (100 µg/ml) (Invitrogen, Carlsbad, CA). HeLa Tet-off (*plaa*^{high} and *plaa*^{low}) cells were maintained in DMEM with 10% tetracycline-free FBS supplemented with hygromycin (100 µg/ml) and G418 (100 µg/ml). Both cell lines were cultured at 37°C with 5% CO₂. For

each experiment, cells were plated in either 25- to 75-cm² tissue culture flasks or 6-well tissue culture plates and allowed to attach overnight.

2.3. Northern blot analysis

Total RNA was isolated from HeLa cells using a RNAqueous kit (Ambion, Inc., Austin, TX) and then subjected to electrophoresis on 1.0% formaldehyde agarose gels, blotted onto nylon membranes, and probed with ³²P-labeled human *plaa* cDNA (10⁵ cpm/ng) [22]. The filters were subsequently prehybridized (using Quickhyb, Strategene, La Jolla, CA), hybridized, and washed using 2X SSC with 0.2% SDS at 68°C as recommended by the manufacturer. After washing, the filters were dried and exposed to X-ray film at -70°C for 12 hr. The ³²P-labeled *plaa* gene probe was subsequently stripped from the filters using 50% formamide (incubated at 68°C for 2 hr), and the latter re-probed with a ³²P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (ClonTech) as an internal control to normalize RNA loading onto each lane of the gel.

2.4. Western blot analysis

Host cell lysis was accomplished in RIPA buffer (Pierce, Rockford, IL). Western blot analysis was performed by established procedures [13] with slight modifications according to specifications of the antibody manufacturer. Polyclonal antibodies against COX-2, cPLA₂, β-tubulin, calnexin and annexin A4 were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Monoclonal antibody against the PLAA peptide was purchased from Eptomics, Burlingame, CA. Equal amounts of protein (30–100 μg) were separated on 4–20% gradient SDS-polyacrylamide gels. We used antibodies to β-tubulin and calnexin as loading controls for host cell lysates and membrane fractions, respectively. The blots were scanned using the Gel Doc 2000 scanner and Quantity One (Bio-Rad, Hercules, CA) software to calculate fold increase in antigen levels.

2.5. Microarray analysis

Total RNA (50 μg) was processed and hybridized to human GeneChip arrays in the Molecular Genomics Core at the University of Texas Medical Branch, Galveston. Briefly, cDNA synthesis, *in vitro* transcription, labeling and fragmentation of the oligonucleotide probes were performed as instructed by the GeneChip manufacturer (Affymetrix, Santa Clara, CA). The probes were hybridized to the GeneChip human genome HG-U133 Plus 2 using the GeneChip Hybridization Oven 640. The chips were washed in a GeneChip Fluidics Station 400 (Affymetrix) and the results visualized with a Gene Array scanner using the Affymetrix software. Data from four replicates were analyzed using GeneSifter (VizX Labs, Seattle, WA), Significance Analysis of Microarrays (SAM; Stanford, CA) and Spotfire DecisionSite 9.0 (Spotfire Inc., Somerville MA). The data discussed in this publication will be deposited in NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and will be accessible through GEO series accession number prior to the publication of this manuscript.

2.6. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA samples for Real Time PCR Analysis were quantified using a Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE) and qualified by analysis on an RNA Nano chip using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Synthesis of cDNA was performed using 1 μg of total RNA in a 20 μl reaction using the reagents in the Taqman Reverse Transcription Reagents Kit from Applied Biosystems Inc., Foster City, CA. The reaction conditions were as follows: 25°C, 10 min, 48°C, 30 min, and 95°C, 5 min. Real-time RT-PCR amplifications (in triplicate) were performed using 2 μl of cDNA in a total volume of 25 μl using the SYBR Green PCR Master Mix (Applied Biosystems) as specified by the manufacturer. The final concentration of the cDNA was 250 nM and of the primers was

900 nM. Real-time RT-PCR assays were performed with 18S RNA as a normalizer. All PCR assays were run in the ABI Prism 7000 Sequence Detection System and the conditions were as follows: 50°C, 2 min, 95°C, 10 min; and then 40 cycles of the following: 95°C, 15 sec, and 60°C, 1 min. Primers used in gene amplification were synthesized by Integrated DNA Technologies (Coralville, IA) and are listed in Supplemental data (Table I).

2.7. Measurement of PGE₂

HeLa Tet-off cells (*plaa^{high}* and *plaa^{low}*) were treated with 30 ng/ml of TNF- α for 8 hr and the cell culture supernatants collected. When indicated, cPLA₂ inhibitor methyl arachidonyl fluorophosphonate (MAFP) [4,31] (Cayman, Ann Arbor, MI) was added to the cells at indicated concentrations 1 hr prior to TNF- α stimulation, and was present throughout the 8 hours' stimulation. Dimethyl sulfoxide (DMSO) was used to dissolve MAFP before addition to cells and was used as a vehicle control for the treatment. For pre-treatment of cells with clusterin antibody or recombinant clusterin (Biovendor, Candler, NC), increasing amounts were added to cells 4 hr before TNF- α stimulation (30 ng/ml) and were present throughout the 8 hours' stimulation. The final concentrations of clusterin antibody in the supernatants were 31.25, 62.5, 125, 250, and 500 ng/ml. The final concentrations of recombinant clusterin in the supernatants were 5, 10, 20, 40, 80, 160, and 320 ng/ml. PGE₂ levels in the supernatant were determined using a commercially available competitive ELISA kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's instructions. PGE₂ standards were provided in the kit and were used in each assay.

2.8. Preparation of membrane fractions

Membrane fractions from HeLa Tet-off cells (*plaa^{high}* and *plaa^{low}*) were isolated by using established procedures [32]. Briefly, cells were washed and suspended in ice-cold homogenization buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM EGTA, 20% glycerol, freshly added 1 mM DTT, and Protease Inhibitor Cocktail [Roche, Mannheim, Germany] at 1:100 dilution). Cell suspensions were sonicated on ice using a Branson sonicator (15 second cycle for 4 cycles at 30% output) and sonicates centrifuged at 1000 $\times$ g for 5 min at 4°C to remove unbroken cells and debris. The low speed supernatant, designated as crude homogenate, was further centrifuged at 100,000 $\times$ g for 1 hr at 4°C. The high speed supernatant was designated as the cytosolic fraction. The high speed pellet, designated as the membrane fraction, was washed 3 times with homogenization buffer to remove cytosolic protein contaminants and was suspended in 400 μ l of ice-cold homogenization buffer containing 0.1% Triton X-100. The pellet was then sonicated on ice for 4 cycles of 15 s each. The cytosolic and membrane fractions were aliquoted and stored at -80 °C until used for PLA₂ assays or Western blot analysis. Protein concentrations in various fractions were determined using the Bradford Protein Reagent (Bio-Rad, Hercules, CA).

2.9. Measurement of PLA₂ activity

PLA₂ activity in the membrane fractions of HeLa cells was determined using a commercially available PLA₂ activity kit (Cayman, Ann Arbor, MI) according to the manufacturer's instructions [33]. Equal amounts of membrane proteins (20–40 μ g) were used for each sample. When inhibitors were used, they were dissolved in DMSO and incubated with membrane samples at indicated final concentrations for 30 min at 25°C. DMSO was used as a vehicle control for the treatment. Included inhibitors (Cayman) were: cPLA₂ inhibitor MAFP (used at concentrations of 1.2 μ M, 0.6 μ M, and 0.3 μ M), iPLA₂ inhibitor Bromoenol lactone (BEL) (used at concentrations of 140 nM, 70 nM, and 35 nM) [4,34], cPLA₂ and sPLA₂ inhibitor 7,7-dimethyl-5,8-Eicosadienoic acid (DMEDA, used at a concentration of 35 μ M) [35] and sPLA₂ inhibitor 1-Palmitylthio-2-palmitoylamido-1,2-dideoxy-sn-glycero-3-phosphorylcholine (TPC, used at a concentration of 5 μ M) [36]. Bee venom PLA₂ (Cayman)

was used as a positive control. To measure inhibition of PLA₂ activity by annexin A4, membrane proteins were pre-incubated with increasing amounts of recombinant annexin A4 (Novus Biologicals, Littleton, CO) for 30 min at 25°C. The final concentrations of annexin A4 in the PLA₂ activity reaction mixtures were 31.25, 62.5, 125, 250, 500, 1000, 2000 ng/ml. Bovine serum albumin (BSA) was used as a negative control. The units of PLA₂ activity in the membrane fractions were expressed as $\mu\text{mole}/\text{min}/\text{mg}$. One unit of PLA₂ enzyme hydrolyzes one μmole of the substrate arachidonoyl thio-phosphatidylcholine per minute at 25°C.

2.10. Phosphorylation profiles of signaling proteins

Bio-Plex phosphoprotein assays (Bio-Rad) were performed at the Gastrointestinal Immunology Core of the University of Texas Medical Branch at Galveston. Included human signaling targets were: p65, I- κ B α , ERK-1, p38 MAPK, JNK, c-jun, CREB, Akt, GSK 3 α & β , TrkA, HSP27, and histone 3. Equal amounts of protein samples were used in each measurement and phosphorylated protein standards were employed in the assay.

2.11. Measurement of nuclear translocation of NF- κ B p65 by confocal immunofluorescence

The nuclear translocation of NF- κ B p65 induced by TNF- α was detected by established procedures [37] with slight modifications. Briefly, HeLa Tet-off cells (*plaa^{high}* and *plaa^{low}*) were treated with 30 ng/ml of TNF- α for 0, 15 or 60 min before fixation. Subsequently, 4% freshly prepared paraformaldehyde and 0.5% Triton X-100 were used to fix and permeabilize the cells, respectively. Goat anti-p65 C-20 antibody (Santa Cruz Biotechnology) and Alexa Fluor 488 (green) donkey anti-goat antibody (Molecular Probes, Carlsbad, CA) were used as primary and secondary antibodies, respectively. Samples stained with secondary antibody alone were used as negative controls. Mounting medium with DAPI (Vector Laboratories, Burlingame, CA) and Alexa Fluor 568 (red) phalloidin (Molecular Probes) was used to stain the nucleus and actin, respectively.

2.12. Measurement of DNA binding activity of NF- κ B p50

HeLa Tet-off cells (*plaa^{high}* and *plaa^{low}*) were treated with 30 ng/ml of TNF- α for 0, 15 or 60 min before nuclear isolation using the NE-PER™ kit (Pierce, Rockford, IL). Nuclear extracts were assayed for DNA binding activity using the TransAM™ p50 kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Briefly, DNA oligonucleotides containing p50 consensus sites were used to pre-coat plate wells and to capture p50 from the samples. Primary antibody against human p50 and secondary antibody conjugated with horse-radish peroxidase (HRP) were employed for the detection of p50. DNA oligonucleotides containing either wild type (WT) or mutated (MT) p50 consensus sites were used as competitors to ensure specificity of the assay. WT oligonucleotides were: 5'-AGTTGAGGGGACTTTCCAGGC-3' (forward sequence) and 5'-GCCTGGGAAAGTCCCCTCAACT-3' (reverse sequence). MT oligonucleotides were: 5'-AGTTGAGGCCACTTTCCAGGC-3' (forward sequence) and 5'-GCCTGGGAAAGTGGCCTCAACT-3' (reverse sequence). Stimulated Jurkat (TPA+CI) cell nuclear extract was used as a positive control. The reaction mixture without nuclear extract was used as a negative control.

2.13. Cytokine profiles

Bio-Plex cytokine assays (Bio-Rad) were performed at the Gastrointestinal Immunology Core of the University of Texas Medical Branch at Galveston as described by the manufacturer. Included human cytokine targets were: IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, TNF- α , MCP-1, MIP-1 β , G-CSF, GM-CSF, and IFN- γ . Cytokine standards were used in the assay.

2.14. Measurement of clusterin concentration

Clusterin concentration in culture supernatants and in cellular and nuclear fractions of HeLa Tet-off cells (*plaa^{high}* and *plaa^{low}*) was measured using a commercially available sandwich ELISA kit (Biovendor), according to the manufacturer's instructions. Recombinant clusterin protein was used to generate a standard curve.

2.15. Measurement of IL-32 concentration

A sandwich ELISA method was used to measure the concentration of IL-32 in cellular lysates and supernatants of HeLa Tet-off cells (*plaa^{high}* and *plaa^{low}*). Briefly, mouse antiserum against IL-32 (CIM Antibody Core, Arizona State University, Tempe, AZ) was diluted 1:200 in binding solution (0.1 M Na₂CO₃, pH 9.0) and incubated in a high-binding microtiter plate overnight at 4°C. An aliquot (10 µg) of total cellular proteins were diluted in phosphate-buffered saline (PBS) and subsequently incubated in the microtiter plate overnight at 4°C. Wells were blocked with freshly made 1% BSA in PBS and washed in PBS-Tween 20 (0.05%). The plate was then blocked with detection antibody (developed in rabbits) against IL-32 (ProSci, Poway, CA) at 10 µg/ml for 3 hr at room temperature. Subsequently, HRP-conjugated goat anti-rabbit antibody (Santa Cruz) and TMB (3,3',5,5'-tetramethylbenzidine) substrate were applied. The color reaction was stopped with 50 µl of 1 M H₂SO₄ and read with a microtiter ELISA plate reader (Molecular Devices Corp., Sunnyvale, CA) at 450 nm. BSA was used as a negative control. A 15-amino acid long peptide from the carboxyl terminal of IL-32 (ProSci) was serially diluted (100 to 6400 pg/ml) and used to generate a standard curve.

2.16. Construction of luciferase reporter plasmids for mapping the 5' genomic region of the *plaa* gene

We mapped the DNA sequence ranging from 4008 base pairs (–4008 bp) upstream to the 1st ATG (0 bp) of exon I in the *plaa* gene. This 4008 bp sequence was progressively truncated into smaller fragments using a PCR-based strategy and the latter inserted in front of the firefly luciferase gene in the pGL3-Basic vector (Promega, Madison, WI). The pGL3-Basic vector without insert was used as a negative control in the luciferase reporter assay. The pGL3-Control vector, which contains a SV40 promoter and SV40 enhancer in addition to the backbone structure of pGL3-Basic, was used as a positive control. All of the plasmid constructs were prepared in the Molecular Genomics Core facility, University of Texas Medical Branch, Galveston. All of the plasmids were purified using cesium chloride density gradient centrifugation and sterilized (through 0.22 µm filters) prior to transfection of HeLa cells.

2.17. Transfection of HeLa cells with luciferase reporter plasmids and measurement of luciferase activity

The luciferase reporter plasmids were transfected into HeLa cells using Transfast™ reagent (Promega), according to the manufacturer's instructions. The charge ratio of transfection reagent to DNA was 1:1. A pRL-TK vector expressing the *Renilla* luciferase reporter was transfected together with the firefly luciferase plasmid as a control for luciferase activity, transfection efficiency, and cell viability. Twenty-four hr after transfection, the HeLa cells were lysed and assayed for luciferase activity using the Dual-Luciferase Reporter Assay kit (Promega), according to the manufacturer's instructions. Luciferase activities of various constructs were measured by luminometer LB 953 (Berthold, Australia). Individual firefly luciferase activity was normalized against the *Renilla* luciferase activity.

2.18. Generation of Sp1 decoy oligonucleotides and transfection

Double-stranded DNA oligonucleotide containing the consensus Sp1 binding sequence (underlined) [38] was designated "Wild Type (WT) Sp1 decoy" and synthesized by Bio-Synthesis (Lewisville, TX). Mutated (MT) Sp1 decoy was altered at two positions (shown as

lower case letters) in the WT Sp1 decoy. WT Sp1 decoy was (complementary strand not shown), 5'-ATTACCGGGCGGGCGGGCTAC-3' and MT-Sp1 decoy was 5'-ATTACCGGtaGGtaGGGCTAC-3'. No binding sites for other known transcription factors were detected in these sequences [38,39]. The decoy oligonucleotides were HPLC purified and

3. Results

3.1. Overexpression of the *plaa* gene in HeLa Tet-off cells

The expression of the *plaa* gene is rapidly induced by TNF- α , LPS and CT in murine RAW 264.7 macrophages and human colonic epithelial contained three pairs of phosphorothioate bonds at both ends. The decoy oligonucleotides were transfected together with the firefly luciferase plasmids at a molar ratio of 50:1 to compete for Sp1 binding. Resulting luciferase activities were measured as described above.

2.19. Generation of the core stimulatory element of the *plaa* gene and competitive binding assay

The double-stranded DNA oligonucleotide, which resides between 255 to 277 bp upstream of the ATG start codon in exon I of the *plaa* gene, was designated "Wild Type (WT) element" and synthesized by Bio-Synthesis. Mutated (MT) element was altered at five positions (shown as lower case letters) in the WT element. The WT element (complementary strand not shown) had the following sequence: 5'-CCGCATCGGCGTCGGCGGTTGGG-3' and MT-Sp1 decoy had the following sequence: 5'-CCaCATCGaCGTCGtCaGTTGGa-3'. The competitive binding assay was performed using a Sp1 ELISA kit (Panomics, Fremont, CA) according to manufacturer's instruction. Briefly, a biotinylated oligonucleotide containing Sp1 consensus binding site (labeled Sp1 probe) was used to bind and immobilized recombinant Sp1 protein to streptavidin coated wells. The bound Sp1 protein was then detected by primary antibody against Sp1 and HRP-conjugated secondary antibody. When indicated, the WT or MT element was used to compete with the Sp1 probe for Sp1 binding. A Sp1 probe without biotin label (unlabeled Sp1 probe) was used as a positive control for the competitive binding assay.

2.20. Statistical analysis

The data were analyzed using Student's t test, with a p value of < 0.05 considered significant. Caco-2 cells [13,22]; however, PLAA-induced functional changes in host cells are unknown.

To address this question, we established HeLa Tet-off cells that overexpressed the *plaa* gene using a bidirectional promoter in vector pBI which also expresses EGFP as a reporter (methods section). Individual stable transfectants were selected based upon the intensity of green fluorescence and expanded. Five cell clones were screened for *plaa* gene expression by Northern blot analysis. As shown in Fig. 1A, the clone F13 designated as *plaa*^{high} had the highest level of *plaa* expression with a 7.5-fold higher abundance of the *plaa* transcript as compared to the control clone R19 (designated as *plaa*^{low}) in which the *plaa* gene inserted in the opposite orientation was expressed from the pBI vector. We used expression of GAPDH as a loading control. This induction in the expression of the *plaa* gene was confirmed by mRNA microarray analysis (10.1-fold increase, Table 1) and real-time RT-PCR (2.2-fold increase, Table 2).

In addition, Western blot analysis confirmed an increasing trend of the *plaa* gene expression when unstimulated *plaa*^{high} and *plaa*^{low} cells were compared, with a 2 fold increase in the PLAA level in the former cells (Fig. 1B). This trend intensified after stimulation with 30 ng/ml of TNF- α for 8 hr, with *plaa*^{high} cells producing 4–5-fold more PLAA than *plaa*^{low} cells, based on Western blot analysis (Fig. 1B). The host cells were stimulated with TNF- α as the latter is known to induce the expression of native *plaa* [13]. Normal HeLa cells expressed similar amounts of *plaa* as the *plaa*^{low} cells with or without TNF- α stimulation (data not

shown). The growth and survival properties of *plaa^{high}* and *plaa^{low}* cells were comparable to that of the normal HeLa cells (data not shown). The expression level of *plaa* in *plaa^{high}* cells is physiologically relevant, as a similar level of PLAA is seen in murine RAW 264.7 macrophages. These macrophages not only produce a high level of PLAA but also other proinflammatory mediators.

3.2. Global transcriptional profile and real-time RT-PCR analysis of *plaa^{high}* cells

In order to confirm the differential expression of *plaa* and to identify downstream proteins altered by *plaa* overexpression, we compared the transcriptional profiles of *plaa^{high}* and *plaa^{low}* cells using microarray analysis. Resting *plaa^{high}* and *plaa^{low}* cells were grown without TNF- α stimulation for 8 and 16 hr, and their RNA were isolated and compared. We did not stimulate these cells with TNF- α to prevent having many variables, which would make data analysis and interpretation more cumbersome. Each experiment was performed in duplicate, yielding a total of 8 arrays. Data were analyzed by MAS 5.0, and a stringent data analysis approach was subsequently employed in order to minimize the incidence of false positives. Pairwise comparisons and Student's *t* test were performed using GeneSifter, and genes that were not differentially expressed at least 1.5-fold between *plaa^{low}* and *plaa^{high}* cells with a *p* value less than 0.05 were eliminated from further analyses. SAM was also used to perform a two-class unpaired comparison between *plaa^{low}* and *plaa^{high}* cells, and only genes that resulted in an FDR (False Detection Ratio) of 5% or less were considered as significantly differentially expressed. Finally, Spotfire DecisionSite was used to perform all 28 possible individual pairwise comparisons between the 8 different samples. The fold-change between any *plaa^{low}* sample and any *plaa^{high}* sample was expected to be at least 1.5-fold (up- or down-regulated). Genes that were differentially expressed between early and late time points (e.g., *plaa^{low}* grown for 8 hr versus 16 hr) were eliminated, as were any genes that differed (1.5-fold or greater) between any replicates (e.g., comparison of *plaa^{low}* cells grown for 8 hr on different days). This resulted in a highly robust set of 64 probe sets representing 54 different genes that were consistently differentially expressed between *plaa^{low}* and *plaa^{high}* cells (Table 1). As expected, the fluorescence signal intensity for the probe set representing the *plaa* gene was much higher (10.1-fold) in *plaa^{high}* cells, compared to *plaa^{low}* cells (Table 1). In addition to *plaa*, 11 altered genes were selected and verified by real-time RT-PCR (Table 2).

Differentially expressed genes were grouped by function based on information available in Medline, NCBI, and Stanford SOURCE databases. Functional categories included cell adhesion and migration (20 probes sets representing 13 different genes), cell cycle arrest and apoptosis (4 genes), inflammation and immune response (7 genes), signal transduction (9 genes), and transcription regulation (5 genes). Differentially expressed immune-related molecules included annexin A4 and clusterin (down-regulated 1.7-fold and 2.0-fold, respectively, in *plaa^{high}* cells compared to *plaa^{low}* cells) and interleukin-32 (IL-32; up-regulated 1.9-fold in *plaa^{high}* cells). The trend of differential expression of annexin A4 (Fig. 2A), IL-32 (Fig. 6) and clusterin (Fig. 9A) were not changed by TNF- α stimulation.

3.3. Overexpression of the *plaa* gene decreases annexin A4 expression in *plaa^{high}* cells

Based on our microarray data (Table 1), the mRNA level of annexin A4 was 1.6-fold lower in *plaa^{high}* cells compared to *plaa^{low}* cells. The annexin A4 protein is known to inhibit PLA₂ [40], possibly by binding and shielding the phospholipid surface [41]. According to our Western blot analysis data (Fig. 2A), *plaa^{high}* cells expressed 3–4-fold less annexin A4 antigen than *plaa^{low}* cells, and this trend of differential expression was not altered by TNF- α stimulation. To determine whether PLA₂ activity in *plaa^{high}* and *plaa^{low}* cells was altered by differential expression of annexin A4, we performed an *in vitro* enzymatic assay using membrane-bound PLA₂ [33] from cells. TNF- α was used to stimulate cells because it is known to induce PLA₂ activation [42,43]. PLA₂ activities were comparable in unstimulated

plaa^{high} and *plaa^{low}* cells but were differentially induced by TNF- α (30 ng/ml) to 3.6- and 2.3-fold of the unstimulated level, respectively, after 8 hr (Fig 2B). Importantly, addition of annexin A4 exogenously dose-dependently inhibited PLA₂ activity in both *plaa^{high}* and *plaa^{low}* cells stimulated with TNF- α , confirming its specificity (Fig. 2B). We used BSA as a negative control in this assay, and it did not elicit this effect. Since *plaa^{low}* cells contained comparable PLA₂ activity to normal HeLa cells in response to TNF- α stimulation (data not shown), they were used as treatment control cells in the following experiments.

3.4. Overexpression of the *plaa* gene increases PGE₂ production and induces cPLA₂ and sPLA₂ activation by TNF- α in HeLa Tet-off cells

Differential activation of PLA₂ in TNF- α -stimulated *plaa^{high}* and *plaa^{low}* cells led us to compare their production of PGE₂ in response to TNF- α (30 ng/ml for 8 hr). Although unstimulated *plaa^{high}* and *plaa^{low}* cells produced comparable amounts of PGE₂, stimulation with TNF- α differentially increased PGE₂ production in *plaa^{high}* and *plaa^{low}* cell to 5- and 2.5-fold, respectively, as determined by ELISA (Fig. 3A) [44]. MAFP, a specific cPLA₂ inhibitor [31], dose-dependently reduced PGE₂ production from stimulated *plaa^{high}* and the *plaa^{low}* cells. A 4 μ M concentration of MAFP essentially reduced PGE₂ to basal levels in *plaa^{high}* and *plaa^{low}* cells (Fig. 3B).

We next determined which PLA₂ subtype was responsible for increased activity in *plaa^{high}* and *plaa^{low}* cells. The cPLA₂ inhibitor MAFP [4,31] (1.2 μ M), the iPLA₂ inhibitor BEL [34] (0.14 μ M) and DMEDA (an inhibitor for both cPLA₂ and sPLA₂, at 35 μ M) [35] were able to significantly reduce PLA₂ activity in both *plaa^{high}* and *plaa^{low}* cells. However, the sPLA₂ inhibitor thioetheramide-PC (TPC) [36] (5 μ M) did not alter PLA₂ activity in these TNF- α -induced *plaa^{high}* and *plaa^{low}* cells, when compared to cells exposed to only TNF- α (Fig. 3C), which suggests that cPLA₂ and iPLA₂ and not the sPLA₂ are induced by *plaa* gene overexpression and TNF- α stimulation. In addition, MAFP and BEL dose-dependently inhibited PLA₂ activity in stimulated *plaa^{high}* and *plaa^{low}* cells, confirming their specificities (Fig. 3D). A 1.2 μ M (2*IC₅₀) of MAFP reduced PLA₂ activity close to that of the unstimulated control, whereas 0.14 μ M (2*IC₅₀) of BEL was less potent in inhibiting iPLA₂ activity. These results indicated a greater induction of cPLA₂ than iPLA₂ by *plaa* overexpression and TNF- α stimulation.

Upon activation, cPLA₂ is known to translocate from the cytosol to the nuclear envelope, endoplasmic reticulum (ER), and Golgi apparatus, where arachidonic acid is produced and presented to cyclooxygenases [5,32]. Therefore, the membrane localization of cPLA₂ in *plaa^{high}* and *plaa^{low}* cells was quantified by Western blot analysis (Fig. 4). Consistently more cPLA₂ was present in the membrane fractions of *plaa^{high}* cells than *plaa^{low}* cells, irrespective of the TNF- α stimulation time. Without TNF- α stimulation, *plaa^{high}* cells contained 3–4-fold more cPLA₂ antigen in their membrane fraction than *plaa^{low}* cells (Fig. 4). After stimulation with 30 ng/ml of TNF- α for 8 hr, *plaa^{high}* cells contained 3–5-fold more cPLA₂ antigen in their membrane fraction than *plaa^{low}* cells. We used antibodies to membrane-associated calnexin as a loading control for these Western blots [45].

We also examined expression of COX-2, a major contributor to inducible PGE₂ production [5] (Fig. 5). Although unstimulated *plaa^{high}* and *plaa^{low}* cells did not express COX-2 antigen, based on Western blot analysis, stimulation of cells with TNF- α differentially increased COX-2 expression in *plaa^{high}* and *plaa^{low}* cells. As shown in Fig. 5, expression of COX-2 was consistently higher in *plaa^{high}* cells than in *plaa^{low}* cells. After stimulation with 30 ng/ml of TNF- α for 8 hr, *plaa^{high}* cells expressed 2–3-fold more COX-2 antigen than *plaa^{low}* cells. Antibodies to β -tubulin were used for Western blot analysis to ensure equal protein load onto the gel.

3.5. Overexpression of the *plaa* gene increases IL-32 expression in *plaa*^{high} cells

Our microarray (Table 1) and real-time RT-PCR data (Table 2) revealed a significant increase (1.9-fold to 2.1-fold) in IL-32 mRNA in *plaa*^{high} cells, compared to that of *plaa*^{low} cells. IL-32 is a novel proinflammatory cytokine [46] implicated in rheumatoid arthritis [47] and known to induce PGE₂ production [47] and NF-κB activation [46]. ELISA assay confirmed a significantly higher level of IL-32 protein in the cytoplasm of resting *plaa*^{high} cells than resting *plaa*^{low} cells (Fig. 6). TNF-α (100 ng/ml) stimulation significantly increased the expression of IL-32 in both cells, with consistently higher levels detected in *plaa*^{high} cells. Consistent with the observations of Goda *et al.* [48], we found that the majority of IL-32 antigen resided in the cytoplasm, rather than the supernatant. Serial dilutions of the cell lysates were also performed in the ELISA to show specificity of the reaction (data not shown).

3.6. Overexpression of the *plaa* gene enhances TNF-α-induced NF-κB activation in HeLa Tet-off cells

The increased expression of COX-2 [15] (Fig. 5) and IL-32 (Fig. 6) promoted by PLAA led us to investigate whether overexpression of the *plaa* gene affected the activation of NF-κB. We stimulated *plaa*^{high} and *plaa*^{low} cells with 30 ng/ml of TNF-α for up to 60 min, and compared the levels of NF-κB activation using a Bioplex phosphoprotein assay (I-κBα and p65), confocal immunofluorescence (p65), and ELISA (p50). To compare signaling profiles between *plaa*^{high} and *plaa*^{low} cells, we performed the Bioplex assay to analyze the phosphorylation levels of NF-κB (p65 and I-κBα) and other signaling proteins including ERK-1, p38 MAPK, JNK, c-jun, CREB, Akt, GSK 3α and β, TrkA, HSP27, and histone 3. We found a significant and consistent difference only for NF-κB (p65 and I-κBα). There were slightly more phosphorylated forms of I-κBα (Fig. 7A-I) and p65 (Fig. 7B) in unstimulated *plaa*^{high} cells than in *plaa*^{low} cells. These same differences became significant after 15 min (for phospho-IκBα, Fig. 7A-I) and 5 min (for phospho-p65, Fig. 7B) of TNF-α stimulation, respectively, and were sustained throughout the rest of the stimulation (60 min). The amount of total IκBα remained comparable between *plaa*^{high} and *plaa*^{low} cells, with an overall decreasing trend throughout the stimulation (Fig. 7A-II).

Confocal immunofluorescence was then performed to trace the translocation of p65 from the cytoplasm to the nucleus. Unstimulated *plaa*^{high} and *plaa*^{low} cells exhibited comparable fluorescence staining of p65 in the cytoplasm only, with no nuclear staining (Fig. 7D-I and II). On the contrary, stimulated (with TNF-α for 60 min) *plaa*^{high} and *plaa*^{low} cells showed localized staining mainly in the nucleus, with significantly more staining in *plaa*^{high} cells than in *plaa*^{low} cells (Fig. 7D-III and IV). We noted a time-dependent increase of p65 in the nucleus after TNF-α stimulation (from 5–60 min [only data at 60 min were shown]).

To confirm the nuclear translocation and DNA binding of the NF-κB p50 subunit, we isolated nuclear proteins from *plaa*^{high} and *plaa*^{low} cells and performed the Trans^{AM} p50 assay, which is an ELISA equivalent of the electrophoretic mobility shift assay (EMSA) [33]. There were comparable levels of nuclear protein binding to p50-specific oligonucleotides from unstimulated *plaa*^{high} and *plaa*^{low} cells; however, a significantly higher level of p50-specific binding occurred in stimulated *plaa*^{high} cells (with TNF-α for 5 or 60 min) compared to stimulated *plaa*^{low} cells (Fig 7C). To confirm the specificity of the Trans^{AM} p50 assay, we performed parallel competition assays using competitor oligonucleotides containing either the specific p50-binding consensus sequence (wild-type [WT] competitor) or the mutated sequence (MT). The WT competitor oligonucleotides significantly decreased p50 binding for both *plaa*^{high} and *plaa*^{low} cells, whereas the MT competitor did not (Fig 7C).

3.7. Overexpression of the *plaa* gene increases IL-6 production in TNF- α -stimulated HeLa Tet-off cells

Since NF- κ B regulates the expression of many cytokine genes, we performed the Bioplex cytokine assay to compare the cytokine production profiles between *plaa*^{high} and *plaa*^{low} cells. The *plaa*^{high} and *plaa*^{low} cells were stimulated with 30 ng/ml of TNF- α for 8 hr, and then their supernatants were collected and analyzed using the Bioplex cytokine assay. We found a significant and consistent difference only in the case of IL-6. IL-6 is a proinflammatory cytokine involved in both acute (e.g., sepsis) and chronic inflammation (e.g., rheumatoid arthritis) [49]. Unstimulated *plaa*^{high} cells produced approximately 8 fold more IL-6 than unstimulated *plaa*^{low} cells (Fig. 8). After stimulation with TNF- α , IL-6 production from *plaa*^{high} cells was consistently 2.5-fold higher than that of *plaa*^{low} cells. Compared to their unstimulated states, *plaa*^{high} and *plaa*^{low} cells produced 46- and 150-fold more IL-6, respectively.

3.8. Overexpression of the *plaa* gene decreases clusterin expression in *plaa*^{high} cells

Our microarray (Table 1) and real-time RT-PCR data (Table 2) revealed a significant decrease (1.6-fold to 2-fold) of clusterin mRNA in *plaa*^{high} cells, compared to that of *plaa*^{low} cells. Decreased expression of clusterin is involved in rheumatoid arthritis [50]. Disruption of the clusterin gene promotes NF- κ B activation [50,51] and IL-6 production [50]. A sandwich ELISA assay confirmed the microarray and real-time RT-PCR results. The *plaa*^{high} cells expressed significantly less secreted clusterin (sCLU) antigen than *plaa*^{low} cells, both in the cell culture supernatant and in the cytoplasm (Fig 9A). TNF- α stimulation did not change this trend of differential expression. Recombinant clusterin was employed to generate a standard curve and to determine the specificity of the assay (data not shown). Slightly lower nuclear clusterin (nCLU) levels were noted in the nucleus of *plaa*^{high} cells than that of *plaa*^{low} cells.

Interestingly, sCLU is involved in lipid transport [52] and can be induced by nefiracetam, a drug that inhibits canine renal prostaglandin synthesis and COX-2 expression through an unknown mechanism [53,54]. We therefore examined whether reduction in extracellular levels of sCLU in *plaa*^{high} cells contributed to the induction of PGE₂ production. Indeed, after antibody against clusterin was supplied to the cell culture medium, a dose-dependent induction of PGE₂ was observed for both *plaa*^{high} and *plaa*^{low} cells stimulated with TNF- α (Fig. 9B). An antibody against β -tubulin did not induce any PGE₂ production, which confirmed the specificity of the assay. Conversely, we examined whether extracellularly supplied clusterin is sufficient to reduce PGE₂ production stimulated by TNF- α . As shown in Fig. 9C, addition of exogenous clusterin partially reduced PGE₂ production from both stimulated *plaa*^{high} and *plaa*^{low} cells in a dose-dependent manner. Saturation points were reached when 20–40 ng/ml of clusterin was supplied to the culture medium. As a control, BSA did not exert this effect (Fig. 9C). Neither clusterin antibody nor clusterin protein interfered with the results of the PGE₂ ELISA (data not shown).

3.9. A Sp1-binding stimulatory element and an inhibitory element are responsible for the transcriptional regulation of the *plaa* gene

To search for *cis*- and *trans*-acting regulatory elements of the *plaa* gene, we performed luciferase reporter assays on progressive deletion mutants spanning the 5' genomic region of the *plaa* gene. The luciferase activities correlated with the ability of different elements from the *plaa* gene to stimulate transcription. In HeLa cells, we identified two important regulatory elements (Fig. 10A); one inhibitory element located between -901 to -1204 bp (upstream of the ATG start codon in exon I of the *plaa* gene) and a stimulatory element located between -252 to -418 bp. The position -252 bp denoted 252 bp remaining upstream to the initiation codon (0 bp) in the *plaa* gene. Luciferase assays with the same constructs performed in murine RAW 264.7 macrophages produced similar results (data not shown).

Deletion of the inhibitory element between -901 to -1204 bp resulted in ~33% increase in luciferase activity (Fig. 10A). Fusion of the inhibitory element (-901 to -1204 bp) in front of the -488 bp construct created a new recombinant clone which had ~67% less luciferase activity than that of the -488 bp construct. An even greater reduction in luciferase activity was observed when the inhibitory element was fused in front of the -545 bp construct. These results suggested that the inhibitory element is both sufficient and necessary to inhibit the expression of the *plaa* gene. In this inhibitory element (-901 to -1204 bp), 32 potential transcription factor binding sites were predicted by *in silico* analysis, using the online transcription factor binding site prediction tool, "AliBaba2" [39] (data not shown).

Serial deletions within the stimulatory element (-252 to -418 bp) of the *plaa* gene defined a core promoter between -252 to -293 bp (Fig. 10A). Deletion of the element between -252 to -293 bp resulted in ~80% reduction in luciferase activity (Fig. 10A). Using *in silico* analysis, we identified one potential binding site for Sp1 between -252 to -293 bp and six other Sp1 sites between -294 to -418 bp (Supplemental data Fig. I). Sp1 is a transcription factor known to regulate the activities of proteins involved in eicosanoid production, such as cPLA₂ [55], sPLA₂ [56], platelet-activating factor acetylhydrolase (PAF-AH) [57], and 12(S)-lipoxygenase [58].

To examine whether Sp1 binds to the stimulatory element and maintains basal transcription of the *plaa* gene, we employed decoy DNA oligos to compete for Sp1 binding with the stimulatory element (-252 to -418 bp) of the *plaa* gene in our luciferase assay. As shown in Fig. 10B, the WT Sp1 decoy significantly reduced the luciferase activity associated with the stimulatory element. The luciferase activity from this element was reduced to 20% of its original value and was similar to that of the -252 construct (which did not contain the element between -252 to -418 bp). A scrambled (MT) decoy, on the other hand, had no effect. To prove the specific binding between Sp1 and the core promoter element (-252 to -293 bp) of the *plaa* gene, we performed a competitive binding assay using a 23 bp fragment of the core promoter element (-255 to -277 bp). As shown in Fig. 10C, the WT fragment of the core promoter element (WT element) dose-dependently inhibited the binding between Sp1 and the probe containing Sp1 site (biotin-labeled Sp1 probe). The inhibitory potency of the WT element was similar to that of the unlabeled Sp1 probe. Conversely, the MT element, whose Sp1 binding site was mutated, could not inhibit the binding between Sp1 and the labeled Sp1 probe. These results suggest that Sp1 maintains basal expression of the *plaa* gene and binds to the stimulatory element.

4. Discussion

Induction of systemic proinflammatory cytokines, such as TNF- α , contributes to the development of acute and chronic inflammatory conditions (e.g., trauma, infection, and rheumatoid arthritis). The effect of TNF- α is partially mediated by PLA₂ and COX-2, which promote a shift towards a proinflammatory lipid profile (e.g., leukotriene B₄ and PGE₂) to further perpetuate inflammatory responses [6,43]. The induction of PLA₂ and COX-2 can be mediated through NF- κ B and MAPK signaling in response to TNF- α . Full activation of cPLA₂ involves phosphorylation by MAPK and translocation of cPLA₂ from the cytosol to the plasma membrane, where it hydrolyzes phospholipids to generate AA [6,43,59]. The transcriptional induction of COX-2 is regulated by NF- κ B and activating protein-1 (AP-1) transcription factors (e.g., Jun and Fos). We previously reported that expression of the *plaa* gene is required for AA production since antisense *plaa* significantly reduced AA production from LPS-stimulated macrophages [13,20]. In this study, we explored the functional consequences of *plaa* gene induction and the transcriptional regulation of the *plaa* gene.

We performed microarray analysis to identify downstream effectors targeted by *plaa* gene induction to better understand cell signaling initiated by PLAA. Overexpression of the native

plaa gene decreased expression of annexin A4 and clusterin and up-regulated IL-32 expression (Table. 1 and 2). Interestingly, in other microarray studies of synovial tissues from rheumatoid arthritis patients and rat model of arthritis, the expression of genes encoding PLAA [60,61], clusterin [60] and IL-32 [62,63] were also found to be altered. Annexin A4 belongs to the annexin family of proteins, which all possess calcium-dependent phospholipid binding activities and participate in membrane organization and trafficking [41]. Several members of the annexin family, such as annexin A4/PP4-X/PAP-II [40], annexin A1/lipocortin I [64], and annexin A5/lipocortin V/PP4/PAP-I [40], are known to inhibit PLA₂ possibly by interfering its membrane binding activity [41]. We confirmed that *plaa*^{high} cells expressed less annexin A4 antigen than *plaa*^{low} cells and showed that *plaa*^{high} cells contained more PLA₂ activity in response to TNF- α stimulation (Fig. 2). The greater level of TNF- α -induced PLA₂ membrane binding in *plaa*^{high} cells, compared to *plaa*^{low} cells (Fig. 4), may at least partially result from less annexin A4 expression and hence more membrane exposure to PLA₂ in *plaa*^{high} cells.

We demonstrated for the first time that overexpression of the native *plaa* gene increases the production of PGE₂ through induction of cPLA₂, iPLA₂ and COX-2 (Fig. 3, 4, and 5). We revealed that with TNF- α stimulation, the induction of PLAA is sufficient to increase PGE₂ production. Following TNF- α stimulation, *plaa*^{high} cells showed increased activation and membrane translocation of cPLA₂ than did control *plaa*^{low} cells. Interestingly, PLAA contains G protein beta domain (G β) (amino acid residues 1- 250) [16], a known activator of phospholipase C [65], in its amino terminal region. It is possible that the G β domain in PLAA may contribute to the activation/membrane binding of cPLA₂ in a similar fashion. Furthermore, human PLAA contains a domain (amino acid residues 503-508) with 39% identity to melittin, a known activator of PLA₂ and phospholipase D [15]. This melittin homology domain itself, when used to stimulate macrophages, can induce the expression of TNF- α and COX-2. Therefore, PLAA might also activate PLA₂ directly through the melittin domain.

We revealed a novel link between the induction of PLAA and IL-32. Our results from microarray, real-time RT-PCR (Table 2) and ELISA (Fig. 6) demonstrated that overexpression of the native *plaa* gene is sufficient to induce the expression of IL-32. Stimuli such as TNF- α [66] and LPS [67], have been shown to induce IL-32 expression in various cell types. In turn, IL-32 can perpetuate inflammatory responses and activate NF- κ B and p38 MAP kinase [46], and induce the production of IL-6 [68], PGE₂ [47], TNF- α [46] and IL-8 [46]. Since overexpression of the *plaa* gene enhanced the activation of NF- κ B (Fig. 7) and production of PGE₂ (Fig. 3) and IL-6 (Fig. 8) induced by TNF- α , the induction of IL-32 may contribute to these effects.

Overexpression of *plaa* promoted NF- κ B activation induced by TNF- α (Fig. 7). Indeed, PLA₂ [69], COX-2 [70] and metabolites [70] from the eicosanoid production pathway are known to modulate NF- κ B activation. Interestingly, murine PLAA was reported to interact with valosin containing protein (VCP, AKA cdc48p) [71], whose human homolog modulates ubiquitin-dependent degradation of I- κ B α in B lymphocytes [72]. Furthermore, UFD3/DOA1 (which is 715-amino acid long), the yeast homolog of PLAA [71], also binds directly to cdc48p/VCP and modulates the degradation of yeast NF- κ B-related transcription factor Spt23 [73]. It is appealing to expect that PLAA could modulate NF- κ B activity through ubiquitin signaling. However, caution must be taken since the homology between PLAA and UFD3/DOA1 is limited to only two regions in PLAA; region I resides within amino acid residues 10-407 which exhibits 58% overall similarity and region II resides within amino acid residues 479-706 which shows 43% overall similarity [74]. Domains in PLAA outside these two regions, and small domains inside the regions, such as the melittin homology domain (amino acid residues 503-538, 39% identity to melittin) [15], do not exist in UFD3/DOA1. The melittin homology domain, which has been shown to induce the production of TNF- α and IL-1 β from macrophages [15], may modulate NF- κ B activation.

Studies using mutated PLAA proteins and PLAA^{-/-} knockout mice could shed light on its functions including influence on NF-κB activity. Our initial attempt to create PLAA^{-/-} mice failed due to the infertility of heterozygous (PLAA^{+/-}) mice. Interestingly, defects in reproduction have been observed in cPLA₂^{-/-} mice [75], COX-2^{-/-} mice [76] and PGE₂ receptor EP4^{-/-} mice [77].

Overexpression of the *plaa* gene in HeLa cells specifically enhances IL-6 production induced by TNF-α (Fig. 8). Since NF-κB activation is also promoted by *plaa* overexpression, it is interesting that only IL-6, rather than other cytokines, was differentially expressed in *plaa*^{high} cells. These data suggested that additional signaling in *plaa*^{high} cells may contribute to IL-6 production. Indeed, an autocrine/paracrine loop of the IL-6/Stat3 and cPLA₂/COX-2/PGE₂ signaling pathways has been shown in human cholangiocarcinoma cells [78]. PGE₂ can bind EP4 receptor and mediate production of IL-6, which in turn enhances PGE₂ signaling through MAP kinase-mediated phosphorylation and activation of cPLA₂. Our data may represent a similar situation, since PGE₂ production in *plaa*^{high} cells was also promoted by *plaa* overexpression (Fig. 3).

We demonstrated for the first time that induction of PLAA reduced the expression of clusterin, a novel regulator of PGE₂ production (Fig. 9). Extracellular clusterin is a lipid binding chaperone that protects cells against stress [52]. Clusterin may act as a stress sensor and protects cells by binding proinflammatory lipids (e.g., PGE₂) for clusterin receptor (e.g., megalin) uptake [52] and degradation. Exogenous clusterin antibody promoted PGE₂ production induced by TNF-α (Fig. 9B), mostly by blocking the interaction between clusterin and its receptor. Conversely, exogenous sCLU dose-dependently decreased the PGE₂ production induced by TNF-α (Fig. 9C). Once in the cytoplasm, clusterin may further reduce inflammation by inhibiting NF-κB activation [50,51], thus limiting the expression of COX-2 [53,54] and proinflammatory cytokines [50]. Indeed, disruption of the clusterin gene in cells promotes NF-κB activation [50,51] and IL-6 production [50]. The induction of NF-κB activation (Fig. 7) and IL-6 production (Fig. 8) observed in *plaa*^{high} cells may partially be due to a reduction in the expression of clusterin.

De novo synthesis of the *plaa* gene is required for the production of PGE₂ and AA [13,20]. Actinomycin-D blocked PGE₂ production from smooth muscle-like cells [13,20], and antisense *plaa* inhibited AA production from macrophages [13,20]. Further, the expression of the *plaa* gene is highly controlled and can be induced in macrophages within 10 min by LPS or CT [13,20]. In order to characterize transcriptional regulation of the *plaa* gene, we mapped its 5' genomic region (Fig. 10). An inhibitory element, located between -901 bp to -1204 bp (upstream of the ATG start codon in exon I of the *plaa* gene), was found to be both sufficient and necessary to reduce the expression of the *plaa* gene. Further characterization is needed to identify regulatory transcription factors that bind to this inhibitory element, and such studies will be performed in our future research.

A stimulatory element, located between -252 to -418 bp (core promoter element resides between -252 to -293 bp), maintains basal transcription of the *plaa* gene and binds specifically with a Sp1 transcription factor (Fig. 10). Sp1 is a ubiquitously expressed general transcription factor which binds GC-rich elements in DNA [79]. Consistent with our results, other proteins involved in eicosanoid production, such as cPLA₂, sPLA₂, and 12(S)-lipoxygenase, are also transcriptionally regulated by Sp1 [55–58]. By *in silico* mapping of the stimulatory element (-252 to -418 bp) in the *plaa* gene, we identified a potential NF-κB binding site near one Sp1 binding site in the core promoter element (-252 to -418 bp) (Supplemental data Fig. I). Interestingly, binding sites for NF-κB and Sp1 are also adjacent in the HIV-1 long terminal repeat, where they can cooperatively activate transcription [80]. In the absence of activated nuclear NF-κB, Sp1 can maintain basal expression of NF-κB-dependent genes [81]. The

expression of the *plaa* gene probably resembles such an arrangement, since it can be induced by TNF- α and its basal expression is maintained by Sp1.

In summary, we demonstrated for the first time that induction of native PLAA promotes the production of PGE₂ and IL-6 and activation of NF- κ B induced by TNF- α (Fig. 11). Previous studies from our laboratory revealed that the expression of the *plaa* gene can be induced by stimuli such as TNF- α and LPS [13,20]. However, the functional consequences of native PLAA induction have been elusive until our current study. Our microarray and functional studies revealed new inflammation-associated genes altered by the induction of PLAA.

Overexpression of the *plaa* gene induces the expression of the proinflammatory cytokine IL-32 and reduces the expression of annexin A4 and clusterin. We confirmed the role of annexin A4 as an inhibitor of PLA₂ and demonstrated for the first time that extracellular clusterin limits the production of PGE₂. We showed that upon TNF- α stimulation, HeLa cells overexpressing the *plaa* gene revealed enhanced activation of cPLA₂ and iPLA₂, as well as increased expression of COX-2. Induction of PLA₂ and COX-2 resulted in increased production of PGE₂. Furthermore, we found a novel link between the induction of PLAA and the activation of NF- κ B. Enhanced activation of NF- κ B leads to induction of COX-2 and IL-6 [82]. Figure 11 summarizes the role of PLAA in inducing inflammation-associated targets demonstrated in this study. Moreover, we found that Sp1 maintains basal transcription of the *plaa* gene, whereas an inhibitory *cis*-acting element may limit its overexpression. Given the importance of PGE₂ and IL-6 in inflammatory signaling, these findings may provide knowledge for developing therapies against both acute and chronic inflammatory diseases.

Supplementary Material

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Abbreviations

PLAA	phospholipase A ₂ -activating protein
cPLA₂	cytosolic phospholipase A ₂
TNF-α	tumor necrosis factor α
PGE₂	prostaglandins E ₂
COX-2	cyclooxygenase-2
NF-κB	

nuclear factor kappaB

CT

cholera toxin

References

1. Tilley SL, Coffman TM, Koller BH. *J Clin Invest* 2001;108(1):15–23. [PubMed: 11435451]
2. Cook JA. *Crit Care Med* 2005;33(12 Suppl):S488–491. [PubMed: 16340430]
3. Steinke J, Borish L. *J Allergy Clin Immunol* 2006;117(2 Suppl MiniPrimer):S441–445. [PubMed: 16455343]
4. Schaloske RH, Dennis EA. *Biochim Biophys Acta* 2006;1761(11):1246–1259. [PubMed: 16973413]
5. Funk CD. *Science* 2001;294(5548):1871–1875. [PubMed: 11729303]
6. Lindstrom T, Bennett P. *Prostaglandins Leukot Essent Fatty Acids* 2004;70(2):115–135. [PubMed: 14683688]
7. Williams JA, Shacter E. *J Biol Chem* 1997;272(41):25693–25699. [PubMed: 9325293]
8. Oestvang J, Johansen B. *Biochim Biophys Acta* 2006;1761(11):1309–1316. [PubMed: 16904370]
9. FitzGerald GA. *Nat Rev Drug Discov* 2003;2(11):879–890. [PubMed: 14668809]
10. Bazan NG, Colangelo V, Lukiw WJ. *Prostaglandins Other. Lipid Mediat* 2002;68–69:197–210.
11. Bonventre J. *Trends Immunol* 2004;25(3):116–119. [PubMed: 15049290]
12. Martel-Pelletier J, Pelletier JP, Fahmi H. *Semin Arthritis Rheum* 2003;33(3):155–167. [PubMed: 14671726]
13. Ribardo DA, Crowe SE, Kuhl KR, Peterson JW, Chopra AK. *J Biol Chem* 2001;276(8):5467–5475. [PubMed: 11094054]
14. Wang D, Dubois RN. *Gut* 2006;55(1):115–122. [PubMed: 16118353]
15. Ribardo DA, Kuhl KR, Peterson JW, Chopra AK. *Toxicon* 2002;40(5):519–526. [PubMed: 11821123]
16. Ribardo DA, Peterson JW, Chopra AK. *Indian J Exp Biol* 2002;40(2):129–138. [PubMed: 12622174]
17. Clark MA, Ozgur LE, Conway TM, Dispoto J, Crooke ST, Bomalaski JS. *Proc Natl Acad Sci U S A* 1991;88(12):5418–5422. [PubMed: 2052621]
18. Clark MA, Conway TM, Shorr RG, Crooke ST. *J Biol Chem* 1987;262(9):4402–4406. [PubMed: 3549734]
19. Wang H, Lemasters J, Herman B. *Gene* 1995;161(2):237–241. [PubMed: 7665086]
20. Chopra AK, Ribardo DA, Wood TG, Prusak DJ, Xu XJ, Peterson JW. *Biochim Biophys Acta* 1999;1444(1):125–130. [PubMed: 9931468]
21. Beatty B, Qi S, Pienkowska M, Herbrick J, Scheidl T, Zhang Z, Kola I, Scherer S, Seth A. *Genomics* 1999;62(3):529–532. [PubMed: 10644453]
22. Peterson JW, Saini SS, Dickey WD, Klimpel GR, Bomalaski JS, Clark MA, Xu XJ, Chopra AK. *Infect Immun* 1996;64(6):2137–2143. [PubMed: 8675318]
23. Homaidan FR, Zhao L, Burakoff R. *Am J Physiol* 1997;272(6 Pt 1):G1338–1346. [PubMed: 9227468]
24. Bomalaski JS, Baker DG, Brophy LM, Clark MA. *J Immunol* 1990;145(10):3391–3397. [PubMed: 2230125]
25. Kim DK, Fukuda T, Thompson BT, Cockrill B, Hales C, Bonventre JV. *Am J Physiol* 1995;269(1 Pt 1):L109–118. [PubMed: 7631805]
26. Schiering A, Menschikowski M, Mueller E, Jaross W. *Atherosclerosis* 1999;144(1):73–78. [PubMed: 10381280]
27. Bomalaski JS, Fallon M, Turner RA, Crooke ST, Meunier PC, Clark MA. *J Lab Clin Med* 1990;116(6):814–825. [PubMed: 2246557]
28. Bomalaski JS, Baker DG, Brophy L, Resurreccion NV, Spilberg I, Muniain M, Clark MA. *J Immunol* 1989;142(11):3957–3962. [PubMed: 2541202]
29. Bomalaski JS, Ford T, Hudson AP, Clark MA. *J Immunol* 1995;154(8):4027–4031. [PubMed: 7706741]

30. Strakova Z, Copland J, Lolait S, Soloff M. *Am J Physiol* 1998;274(4 Pt 1):E634–641. [PubMed: 9575824]
31. Lio YC, Reynolds LJ, Balsinde J, Dennis EA. *Biochim Biophys Acta* 1996;1302(1):55–60. [PubMed: 8695655]
32. Liu T, Zaman W, Kaphalia BS, Ansari GA, Garofalo RP, Casola A. *Virology* 2005;343(1):12–24. [PubMed: 16153673]
33. Choi SH, Langenbach R, Bosetti F. *J Neurochem* 2006;98(3):801–811. [PubMed: 16787416]
34. Ackermann EJ, Conde-Frieboes K, Dennis EA. *J Biol Chem* 1995;270(1):445–450. [PubMed: 7814408]
35. Lister MD, Glaser KB, Ulevitch RJ, Dennis EA. *J Biol Chem* 1989;264(15):8520–8528. [PubMed: 2498315]
36. Plesniak LA, Boegeman SC, Segelke BW, Dennis EA. *Biochemistry* 1993;32(19):5009–5016. [PubMed: 8494876]
37. Ding GJ, Fischer PA, Boltz RC, Schmidt JA, Colaianne JJ, Gough A, Rubin RA, Miller DK. *J Biol Chem* 1998;273(44):28897–28905. [PubMed: 9786892]
38. Ishibashi H, Nakagawa K, Onimaru M, Castellanous EJ, Kaneda Y, Nakashima Y, Shirasuna K, Sueishi K. *Cancer Res* 2000;60(22):6531–6536. [PubMed: 11103824]
39. Grabe N. *In Silico Biol* 2002;2(1):S1–15. [PubMed: 11808873]
40. Romisch J, Grote M, Weithmann KU, Heimbürger N, Amann E. *Biochem J* 1990;272(1):223–229. [PubMed: 2148260]
41. Gerke V, Creutz CE, Moss SE. *Nat Rev Mol Cell Biol* 2005;6(6):449–461. [PubMed: 15928709]
42. Kuwata H, Nakatani Y, Murakami M, Kudo I. *J Biol Chem* 1998;273(3):1733–1740. [PubMed: 9430720]
43. Kronke M, Adam-Klages S. *FEBS Lett* 2002;531(1):18–22. [PubMed: 12401196]
44. Fadl AA, Galindo CL, Sha J, Zhang F, Garner HR, Wang HQ, Chopra AK. *Microb Pathog* 2007;42(5–6):193–203. [PubMed: 17368824]
45. Bergeron J, Brenner M, Thomas D, Williams D. *Trends Biochem Sci* 1994;19(3):124–128. [PubMed: 8203019]
46. Kim SH, Han SY, Azam T, Yoon DY, Dinarello CA. *Immunity* 2005;22(1):131–142. [PubMed: 15664165]
47. Joosten LA, Netea MG, Kim SH, Yoon DY, Oppers-Walgreen B, Radstake TR, Barrera P, van de Loo FA, Dinarello CA, van den Berg WB. *Proc Natl Acad Sci U S A* 2006;103(9):3298–3303. [PubMed: 16492735]
48. Goda C, Kanaji T, Kanaji S, Tanaka G, Arima K, Ohno S, Izuhara K. *Int Immunol* 2006;18(2):233–240. [PubMed: 16410314]
49. Mastorakos G, Ilias I. *Ann N Y Acad Sci* 2006;1088:373–381. [PubMed: 17192581]
50. Devauchelle V, Essabbani A, De Pinieux G, Germain S, Tourneur L, Mistou S, Margottin-Goguet F, Anract P, Migaud H, Le Nen D, Lequerre T, Saraux A, Dougados M, Breban M, Fournier C, Chiochia G. *J Immunol* 2006;177(9):6471–6479. [PubMed: 17056579]
51. Santilli G, Aronow BJ, Sala A. *J Biol Chem* 2003;278(40):38214–38219. [PubMed: 12882985]
52. Trougakos IP, Gonos ES. *Free Radic Res* 2006;40(12):1324–1334. [PubMed: 17090421]
53. Tsuchiya Y, Yabe K, Takada S, Ishii Y, Jindo T, Furuhashi K, Suzuki KT. *Toxicol Pathol* 2005;33(5):561–569. [PubMed: 16105799]
54. Tsuchiya Y, Tominaga Y, Matsubayashi K, Jindo T, Furuhashi K, Suzuki KT. *Arch Toxicol* 2005;79(9):500–507. [PubMed: 16007418]
55. Blaine SA, Wick M, Dessev C, Nemenoff RA. *J Biol Chem* 2001;276(46):42737–42743. [PubMed: 11559711]
56. Antonio V, Brouillet A, Janvier B, Monne C, Bereziat G, Andreani M, Raymondjean M. *Biochem J* 2002;368(Pt 2):415–424. [PubMed: 12188923]
57. Wu X, Zimmerman GA, Prescott SM, Stafforini DM. *J Biol Chem* 2004;279(34):36158–36165. [PubMed: 15215249]
58. Chang WC, Chen BK. *Biochem Biophys Res Commun* 2005;338(1):117–121. [PubMed: 16122700]

59. Chakraborti S. *Cell Signal* 2003;15(7):637–665. [PubMed: 12742226]
60. Devauchelle V, Marion S, Cagnard N, Mistou S, Falgarone G, Breban M, Letourneur F, Pitaval A, Alibert O, Lucchesi C, Anract P, Hamadouche M, Ayrat X, Dougados M, Gidrol X, Fournier C, Chiochia G. *Genes Immun* 2004;5(8):597–608. [PubMed: 15496955]
61. Rioja I, Clayton CL, Graham SJ, Life PF, Dickson MC. *Arthritis Res Ther* 2005;7(1):R101–117. [PubMed: 15642130]
62. Cagnard N, Letourneur F, Essabbani A, Devauchelle V, Mistou S, Rapinat A, Decraene C, Fournier C, Chiochia G. *Eur Cytokine Netw* 2005;16(4):289–292. [PubMed: 16464743]
63. van der Pouw Kraan TC, van Gaalen FA, Kasperkovitz PV, Verbeet NL, Smeets TJ, Kraan MC, Fero M, Tak PP, Huizinga TW, Pieterman E, Breedveld FC, Alizadeh AA, Verweij CL. *Arthritis Rheum* 2003;48(8):2132–2145. [PubMed: 12905466]
64. Parente L, Solito E. *Inflamm Res* 2004;53(4):125–132. [PubMed: 15060718]
65. Drin G, Scarlata S. *Cell Signal* 2007;19(7):1383–1392. [PubMed: 17524618]
66. Shioya M, Nishida A, Yagi Y, Ogawa A, Tsujikawa T, Kim-Mitsuyama S, Takayanagi A, Shimizu N, Fujiyama Y, Andoh A. *Clin Exp Immunol*. 2007
67. Netea M, Azam T, Lewis E, Joosten L, Wang M, Langenberg D, Meng X, Chan E, Yoon D, Ottenhoff T, Kim S, Dinarello C. *PLoS Med* 2006;3(8):e277. [PubMed: 16903774]
68. Netea MG, Azam T, Ferwerda G, Girardin SE, Walsh M, Park JS, Abraham E, Kim JM, Yoon DY, Dinarello CA, Kim SH. *Proc Natl Acad Sci U S A* 2005;102(45):16309–16314. [PubMed: 16260731]
69. Anthonen MW, Solhaug A, Johansen B. *J Biol Chem* 2001;276(32):30527–30536. [PubMed: 11390371]
70. Poligone B, Baldwin AS. *J Biol Chem* 2001;276(42):38658–38664. [PubMed: 11509575]
71. Seigneurin-Berny D, Verdel A, Curtet S, Lemercier C, Garin J, Rousseaux S, Khochbin S. *Mol Cell Biol* 2001;21(23):8035–8044. [PubMed: 11689694]
72. Dai RM, Chen E, Longo DL, Gorbea CM, Li CC. *J Biol Chem* 1998;273(6):3562–3573. [PubMed: 9452483]
73. Rumpf S, Jentsch S. *Mol Cell* 2006;21(2):261–269. [PubMed: 16427015]
74. Tatusova T, Madden T. *FEMS Microbiol Lett* 1999;174(2):247–250. [PubMed: 10339815]
75. Bonventre J, Huang Z, Taheri M, O'Leary E, Li E, Moskowitz M, Sapirstein A. *Nature* 1997;390(6660):622–625. [PubMed: 9403693]
76. Lim H, Paria B, Das S, Dinchuk J, Langenbach R, Trzaskos J, Dey S. *Cell* 1997;91(2):197–208. [PubMed: 9346237]
77. Challis J. *Nat Med* 1997;3(12):1326–1327. [PubMed: 9396601]
78. Han C, Demetris AJ, Stolz DB, Xu L, Lim K, Wu T. *J Biol Chem* 2006;281(34):24831–24846. [PubMed: 16790433]
79. Bouwman P, Philipsen S. *Mol Cell Endocrinol* 2002;195(1–2):27–38. [PubMed: 12354670]
80. Perkins ND, Edwards NL, Duckett CS, Agranoff AB, Schmid RM, Nabel GJ. *Embo J* 1993;12(9):3551–3558. [PubMed: 8253080]
81. Hirano F, Tanaka H, Hirano Y, Hiramoto M, Handa H, Makino I, Scheidereit C. *Mol Cell Biol* 1998;18(3):1266–1274. [PubMed: 9488441]
82. Kracht M, Saklatvala J. *Cytokine* 2002;20(3):91–106. [PubMed: 12453467]

Figure 1. Overexpression of the *plaa* gene in HeLa Tet-off cells.

(A) Northern blot analysis on clones for *plaa* gene expression. HeLa Tet-off cell clones were cultured for 8 hr before RNA isolation. The RNA samples were run on 1.0% formaldehyde agarose gels, transferred to nylon membranes, and the membranes then probed with ^{32}P -labeled human *plaa* cDNA. Lane 1 to 5 represent RNA samples from clones overexpressing the *plaa* gene. These clones were designated as F13, F4, F12, F17, and F22, respectively. The F13 clone was designated as *plaa*^{high} and used in all of the experiments. Lane 6 represents the RNA from the control clone R19 in which the *plaa* gene was inserted in the opposite orientation. The R19 clone was designated as *plaa*^{low} and used in all of the experiments. A ^{32}P -labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe was used to normalize RNA loading. (B) Western blot analysis of the PLAA protein from *plaa*^{high} and *plaa*^{low} cells stimulated with TNF- α (30 ng/ml) or remained untreated. Aliquots (80 μg) of total proteins were subjected to 4–20% SDS-PAGE and the membranes probed with monoclonal antibody against PLAA. The same amount of proteins were run on a second gel, subjected to Western blot analysis, and the membranes probed with a polyclonal antibody against β -tubulin to normalize protein loading. The blots presented are representative of at least three independent repetitions.

Figure 2. Overexpression of the *plaa* gene decreases annexin A4 expression in *plaa*^{high} cells
 The *plaa*^{high} and *plaa*^{low} cells were stimulated with TNF- α (30 ng/ml) or left untreated for 8 hr. **(A)** Western blot analysis of annexin A4 from cells. Equal amounts of whole cell lysate (50 μ g) were subjected to 4–20% SDS-PAGE and probed with polyclonal antibody against annexin A4. An antibody against β -tubulin was used to normalize protein loading. The blots presented are representative of at least three independent repetitions. **(B)** Recombinant annexin A4 inhibits PLA₂ activity from *plaa*^{high} and *plaa*^{low} cells. Membrane fractions from stimulated cells were isolated and incubated with the indicated amounts of recombinant annexin A4 (ANXA4) for 30 min prior to performing the PLA₂ enzyme activity assay. BSA was used as a negative treatment control. PLA₂ activity was expressed as μ mol/min/mg, and values were plotted as the mean $\pm$ standard deviation of four independent repetitions. *a denotes statistically significant values ($p < 0.05$) compared to that of the *plaa*^{low} cells. *b denotes statistically significant values ($p < 0.05$) compared to untreated membrane fractions from TNF- α -stimulated *plaa*^{high} cells. *c denotes statistically significant values ($p < 0.05$) compared to untreated membrane fractions from TNF- α -stimulated *plaa*^{low} cells.

Figure 3. Overexpression of the *plaa* gene increases PGE₂ production and PLA₂ activation by TNF- α in HeLa Tet-off cells

Both *plaa*^{high} and *plaa*^{low} cells were stimulated with TNF- α (30 ng/ml) or left untreated for 8 hr. (A) PGE₂ levels were measured in cell culture supernatants. The PGE₂ values were normalized to the amount of protein in the cell lysates and the data expressed as mean $\pm$ standard of four independent repetitions. (B) cPLA₂ inhibitor MAFP dose-dependently reduced PGE₂ production from stimulated *plaa*^{high} and *plaa*^{low} cells. MAFP was added to the cells at indicated concentrations 1 hr prior to TNF- α stimulation and was present throughout the 8 hours' stimulation. DMSO was used to dissolve MAFP before addition to cells and was used as a vehicle control for the treatment. (C) cPLA₂ inhibitor (MAFP) and iPLA₂ inhibitor (BEL) reduced PLA₂ activity from stimulated *plaa*^{high} and *plaa*^{low} cells. Equal amounts of membrane proteins of stimulated cells were incubated with inhibitors for 30 min prior to PLA₂ enzyme activity assay. DMSO was used as a vehicle control for the treatment. Included inhibitors were: cPLA₂ inhibitor MAFP (1.2 μ M), iPLA₂ inhibitor BEL (0.14 μ M), sPLA₂ inhibitor TPC (5 μ M), and inhibitor against both cPLA₂ and sPLA₂ (DMEDA, 35 μ M). Titration of the inhibitors MAFP and BEL was also performed to confirm their specificities (D). PLA₂ activity was expressed as μ mol/min/mg, and values were plotted as the mean $\pm$ standard deviation of four independent repetitions. *a denotes statistically significant values ($p < 0.05$) compared to that of the control (*plaa*^{low}) cells. *b denotes statistically significant values ($p < 0.05$) compared to that of the DMSO (0 μ M) and TNF- α -treated *plaa*^{high} cells. *c denotes statistically significant values ($p < 0.05$) compared to that of the DMSO (0 μ M) and TNF- α -treated *plaa*^{low} cells.

Figure 4. Overexpression of the *plaa* gene increases cPLA₂ membrane translocation by TNF- α in HeLa Tet-off cells

Western blot analysis of cPLA₂ protein from stimulated *plaa*^{high} and *plaa*^{low} cells. The *plaa*^{high} and *plaa*^{low} cells were stimulated with TNF- α (30 ng/ml) or left untreated for 8 hr. Membrane proteins of cells were prepared, and equal amounts of protein (60 μ g) were subjected to 4–20% SDS-PAGE and probed with polyclonal antibody against cPLA₂. A polyclonal antibody against calnexin (specific membrane bound host protein) was used to normalize protein loading. The Western blot figure presented is representative of three independent blots.

Figure 5. Overexpression of the *plaa* gene increases COX-2 expression by TNF- α in HeLa Tet-off cells

Western blot analysis of COX-2 protein from stimulated *plaa*^{high} and *plaa*^{low} cells. The *plaa*^{high} and *plaa*^{low} cells were stimulated with TNF- α (30 ng/ml) or left untreated for up to 20 hr. An aliquot (40 μ g) of total proteins was subjected to 4–20% SDS-PAGE and probed with polyclonal antibody against COX-2. An antibody against β -tubulin was used to normalize protein loading. The Western blot figure presented is representative of three independent blots.

Figure 6. Overexpression of the *plaa* gene increases IL-32 expression in *plaa*^{high} cells

The *plaa*^{high} and *plaa*^{low} cells were stimulated with TNF- α (100 ng/ml) or left untreated for 8 hr. A sandwich ELISA method was used to measure the concentration of IL-32 in cellular lysates and supernatants. BSA was used as a negative control. A 15 amino acid-long peptide from the carboxyl terminal of IL-32 was serial-diluted (100 to 6400 pg/ml) and used to generate a standard curve. Values were given as pg/ml and expressed as the mean $\pm$ standard deviation of three independent repetitions. *a denotes statistically significant values ($p < 0.05$) compared to that of the *plaa*^{low} cells.

Figure 7. Overexpression of the *plaa* gene enhances TNF- α -induced NF- κ B activation in HeLa Tet-off cells

The *plaa*^{high} and *plaa*^{low} cells were stimulated with TNF- α (30 ng/ml) or left untreated for up to one hr. (A) **I.** Phosphorylation analysis of I κ B α . A total of 200 μ g of proteins from cells was prepared and phosphorylation of protein targets in these samples was measured using the Bio-plex phosphoprotein assay. Phosphorylated protein standards were used in the assay. The amount of total I κ B α (**II**) and phosphorylated p65 (**B**) was also measured in the same samples using Bio-plex phosphoprotein assay. Values were given as relative fluorescence units, and bars represent means of duplicates $\pm$ standard deviation of three independent repetitions. *a denotes statistically significant values ($p < 0.05$) compared to that of the *plaa*^{low} cells. (C)

Measurement of DNA binding activity of p50. Nuclear fractions from stimulated *plad^{high}* and *plad^{low}* cells were prepared and used in similar amounts (1 µg each well) and subjected to DNA binding assay. DNA oligonucleotides containing either wild type (WT) or mutated (MT) p50 consensus sites were used as competitors to ensure specificity of the assay (for sequences see “Materials and methods section”). Values are presented as the mean optical density at $A_{450\text{ nm}}$ (OD A_{450}) and expressed as the mean $\pm$ standard deviation of three independent repetitions. *a denotes statistically significant values ($p < 0.05$) compared to that of the *plad^{low}* cells. **(D)** Immunofluorescence staining was used to visualize nuclear translocation of p65 by confocal microscopy. An Alexa Fluor 488-conjugated primary antibody was used to stain p65 (green). The nuclei were stained blue using DAPI. The actin filaments were stained red using Alexa Fluor 568-conjugated phalloidin. The images showing the staining of p65, nuclei and actin filaments were merged and shown in the lower right panels. The images are representative of one experiment, although three independent experiments were performed.

Figure 8. Overexpression of the *plaa* gene increases IL-6 production in TNF- α -stimulated HeLa Tet-off cells

IL-6 levels were measured using Bio-plex cytokine assay in culture supernatants of *plaa*^{high} and *plaa*^{low} cells stimulated with TNF- α (30 ng/ml) or left untreated for 8 hr. IL-6 values were normalized with amounts of protein in the cell lysates and expressed as the mean $\pm$ standard deviation of four independent repetitions. *a denotes statistically significant values ($p < 0.05$) compared to that of the *plaa*^{low} cells.

Figure 9. Overexpression of the *plaa* gene decreases clusterin expression in *plaa*^{high} cells
 The *plaa*^{high} and *plaa*^{low} cells were stimulated with TNF- α (30 ng/ml) or left untreated for 8 hr. (A) ELISA analysis of clusterin concentration from cells. Supernatants, cytoplasmic fractions, and nuclear fractions from cells were isolated and assayed for clusterin concentration. Values were expressed as the mean $\pm$ standard deviation of three independent repetitions. To determine the effect of clusterin on PGE₂ production from stimulated cells, antibodies against clusterin (α -CLU) (B) and exogenous clusterin (CLU) (C) were supplied to the culture supernatant after 4 hr of TNF- α stimulation and were present throughout the 8 hours' stimulation. Supernatants from cells were then assayed for PGE₂ levels. Antibodies against β -tubulin (α -TUB) (B) and BSA (C) were used as negative treatment controls. Values were expressed as the mean $\pm$ standard deviation of four independent repetitions. *a denotes statistically significant values ($p < 0.05$) compared to that of the *plaa*^{low} cells. *b denotes statistically significant values ($p < 0.05$) compared to that of the TNF- α -stimulated *plaa*^{high} cells. *c denotes statistically significant values ($p < 0.05$) compared to that of the TNF- α -stimulated *plaa*^{low} cells.

Fig 10. An Sp1-binding stimulatory element and an inhibitory element are responsible for the transcriptional regulation of the *plaa* gene.

(A) Mapping of the 5' genomic region of the *plaa* gene using luciferase assays. -4008 denotes 4008 base pairs remaining upstream to the 1st ATG codon (0 bp) of exon I in the *plaa* gene, while the rest of the DNA sequence (upstream of 4008 bp) was deleted. This 4008 bp-sequence was progressively truncated into smaller DNA fragments and the latter inserted in front of the firefly luciferase gene in the pGL3-Basic vector. The region colored in red represents sequence between -901 to -1204. The red element was moved forward and fused with the -545 or -488 element to determine whether spacing affects its inhibitory activity. A pRL-TK vector expressing the *Renilla* luciferase reporter was transfected together with the firefly luciferase plasmids into HeLa cells as a control for luciferase activity, transfection efficiency, and cell viability. Individual firefly luciferase activity from transfected cells was normalized against the *Renilla* luciferase activity. Values were given in arbitrary luciferase units (one unit equals approximately 10⁵ RLU) and expressed as the mean $\pm$ standard deviation of four independent repetitions. *a denotes statistically significant values ($p < 0.05$) compared to that of the -4008

construct. *b denotes statistically significant values ($p < 0.05$) compared to that of the -545 construct. *c denotes statistically significant values ($p < 0.05$) compared to that of the -488 construct. *d denotes statistically significant values ($p < 0.05$) compared to that of the -418 construct. **(B)** Sp1 decoy oligonucleotides reduced transcription from the element between -252 to -418 bp. Double-stranded DNA oligonucleotides containing the consensus Sp1 binding sequence (underlined) were designated “Wild Type (WT) Sp1 decoys” and synthesized. Mutated (MT) Sp1 decoys were mutated at two positions in the WT Sp1 decoys (for sequences see “Materials and methods section”). The decoy oligonucleotides were transfected together with the firefly luciferase plasmids at a molar ratio of 50:1 to compete for Sp1 binding. Luciferase activities values were given in arbitrary luciferase units (one unit approximately equals 10^5 RLU) and expressed as the mean $\pm$ standard deviation of three independent repetitions. *d denotes statistically significant values ($p < 0.05$) compared to that of the -418 construct. **(C)** Sp1 specifically binds to the element between -255 to -277 bp. The double-stranded DNA oligonucleotide, which resides between 255 to 277 bp upstream of the ATG start codon in exon I of the *plaa* gene, was designated “Wild Type (WT) element” and synthesized. Mutated (MT) element was altered at five positions in the WT element (for sequences see “Materials and methods section”). The competitive binding assay was performed using a Sp1 ELISA kit. When indicated, the WT or MT element was used to compete with the Sp1 probe for Sp1 binding. A Sp1 probe without biotin label (unlabeled Sp1 probe) was used as positive control for the competitive binding assay. Values were shown as OD A_{450} and expressed as mean $\pm$ standard deviation of three independent repetitions. * denotes statistically significant values ($p < 0.05$) compared to that of the Sp1 binding without using competitor oligos.

Fig 11.
Schematic diagram summarizing the role of PLAA during inflammation.

Table 1
Genes differentially expressed between *plaa^{low}* and *plaa^{high}* cells, based on microarrays analysis.

GenBank ID	Gene Name	FC
<i>Cell Adhesion and Migration</i>		
BE875786	Cathepsin B (CTSB)	-1.9
NM_001908	Cathepsin B (CTSB)	-1.6
NM_001908	Cathepsin B (CTSB)	-1.5
AJ224869	Chemokine (C-X-C motif) receptor 4 (CXCR4)	-1.8
AF130082	Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant) (COL3A1)	-1.9
AU144167	Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant) (COL3A1)	-1.6
AI983428	Collagen, type V, alpha 1 (COL5A1)	-1.9
AI130969	Collagen, type V, alpha 1 (COL5A1)	-1.5
NM_014333	Immunoglobulin superfamily, member 4 (IGSF4)	-1.8
AI924630	Melanoma antigen family D, 2 (MAGED2)	-1.5
NM_017459	Microfibrillar-associated protein 2	-2.1
NM_004772	Neuronal protein 3.1 (P311)	-2.2
D86862	Paxillin (PXN)	1.6
NM_014459	Protocadherin 17 (PDCH17)	-1.6
D13989	Rho GDP dissociation inhibitor (GDI) alpha (ARHGDIA)	1.7
AI571798	Rho GDP dissociation inhibitor (GDI) alpha (ARHGDIA)	1.9
BF059159	Roundabout, axon guidance receptor, homolog 1 (ROBO1)	-2.0
AF091395	Triple functional domain (PTPRF interacting) (TRIO)	1.8
NM_007118	Triple functional domain (PTPRF interacting) (TRIO)	1.9
AF091395	Triple functional domain (PTPRF interacting) (TRIO)	2.0
<i>Cell Cycle Arrest and Apoptosis</i>		
NM_021197	WAP four-disulfide core domain 1 (WFDC1)	-2.6
NM_014782	Armadillo repeat protein ALEX2 (ARMCX2)	-1.6
AK021947	Mitofusin 2 (MFN2)	1.7
AF074717	RAD1 (S. pombe) homolog (RAD1)	1.6
<i>DNA Packaging</i>		
AA451996	Histone 2, H2aa3 (HIST2H2AA3)	1.6
<i>Energy Production</i>		
NM_001976	Enolase 3, (beta, muscle) (ENO3)	-1.8
<i>Inflammation and Immune Response</i>		
BC000373	Amyloid beta (A4) precursor-like protein 2 (APLP2)	-1.6

GenBank ID	Gene Name	FC
NM_001153	Annexin A4 (ANXA4)	-1.7
AI982754	Clusterin (CLU)	-2.0
BC000055	Follistatin-like 1 (FSTL1)	-1.7
X56210	H factor (complement)-like 2 (CFHR1)	-1.7
AF200348	Melanoma associated gene (PXDN)	-1.8
AF200348	Melanoma associated gene (PXDN)	-1.7
NM_004221	Natural killer cell transcript 4 (IL32)	1.9
<i>Lipid Metabolism</i>		
AB032261	Stearoyl-CoA desaturase (delta-9-desaturase) (SCD)	-1.7
AF145020	Phospholipase A2-activating protein (PLAA)	10.1
<i>Protein Processing</i>		
M55575	Branched chain keto acid dehydrogenase E1, beta polypeptide (maple syrup urine disease) (BCKDHB)	-1.7
D84294	Tetratricopeptide repeat domain 3 (TTC3)	-1.7
D83077	Tetratricopeptide repeat domain 3 (TTC3)	-1.6
NM_017423	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 7 (GALNT7)	-1.6
NM_012228	Methionine sulfoxide reductase B2 (MSRB2)	-1.5
<i>Signal Transduction</i>		
NM_024087	Ankyrin repeat and SOCS box-containing 9 (ASB9)	-1.8
AI817041	Chemokine orphan receptor 1 (CMKOR1)	-2.3
L37882	Frizzled (Drosophila) homolog 2 (FZD2)	-1.8
NM_002195	Insulin-like 4 (INSL4)	-1.6
NM_017648	Mucin 13, cell surface associated (MUC13)	1.8
NM_005019	Phosphodiesterase 1A, calmodulin-dependent	-2.1
AL535113	Phospholipase C, beta 4 (PLCB4)	-1.8
NM_016240	Scavenger receptor class A, member 3 (SCARA3)	-1.7
NM_003022	SH3 domain binding glutamic acid-rich protein like (SH3BGRL)	-1.8
<i>Transcription Regulation</i>		
NM_006602	Transcription factor-like 5 (basic helix-loop-helix) (TCFL5)	-1.7
AI951185	Nuclear receptor subfamily 2, group F, member 1 (NR2F1)	-1.7
AI700518	Nuclear factor I/B (NFIB)	-1.5
AB030824	Kruppel-like factor 5 (KLF5)	1.5
NM_006470	Tripartite motif-containing 16 (TRIM16)	1.6
<i>Transport</i>		
AL050002	Olfactomedin-like 2A (OLFML2A)	-1.9

GenBank ID	Gene Name	FC
AI630178	Solute carrier family 43, member 3 (SLC43A3)	-2.8
BC003163	Solute carrier family 43, member 3 (SLC43A3)	-1.5
NM_016354	Solute carrier family 21 (organic anion transporter), member 12 (SLC04A1)	1.6
<i>Unknown</i>		
AK025298	Autism susceptibility candidate 2 (AUTS2)	-2.5
NM_024717	Multiple C2 domains, transmembrane 1 (MCTP1)	-2.1
AL049949	Chromosome 10 open reading frame 56 (C10orf56)	-1.6
AL049233	Chromosome 10 open reading frame 18 (C10orf18)	1.6
AW006290	RIO kinase 3 (RIOK3)	1.7

The *plaa^{high}* and *plaa^{low}* cells were cultured for 8 or 16 hr before RNA isolation. Total RNA (25µg) was processed and hybridized to human GeneChip arrays HG-U133 Plus 2 and the data analyzed using GeneSifter, Significance Analysis of Microarrays and Spotfire DecisionSite 9.0. Genes differentially expressed between *plaa^{high}* and *plaa^{low}* cells (8 or 16 hr) are shown. FC = fold-change. Genes listed more than once were represented by more than one probe set, each of which was separately determined to be differentially expressed between *plaa^{low}* and *plaa^{high}* cells. Negative (–) sign before fold-change indicates down-regulation of gene in *plaa^{high}* cells, compared to *plaa^{low}* cells.

Table 2

Verification of microarray analysis results by real-time RT-PCR.

Gene Name	Real-time	Microarray
	<i>FC</i>	
PLAA	2.2	10.1
Interleukin 32 (IL32)	2.1	1.9
Microfibrillar-associated protein 2 (MFAP2)	-2.6	-2.1
Phosphodiesterase 1A calmodulin dependent (PDE1A)	-1.5	-2.1
Phospholipase C beta 4 (PLCB4)	-2.2	-1.8
DNA repair exonuclease (RAD1)	1.7	1.6
Solute carrier family 43 member 3 (SLC43A3)	-22.6	-2.8
Clusterin (CLU)	-1.6	-2.0
Cathepsin B (CTSB)	-1.6	-1.6
Chemokine receptor 4 (CXCR4)	-1.3	-1.8
Kruppel-like factor 5 (KLF5)	1.9	1.5
Nuclear receptor subfamily 2 group F member 1 (NR2F1 or COUP TF1)	-1.4	-1.7

Experiments were performed in parallel, and the fold-change values were calculated using the comparative threshold method, after normalization of each gene to an internal control (16S rRNA). FC = fold-change (expression level of gene in *plaa^{high}* cells compared to *plaa^{low}* cells). Negative (-) sign before fold-change indicates down-regulation of gene in *plaa^{high}* cells, compared to *plaa^{low}* cells.