TRANSCRIPTIONAL REGULATION OF GROUP IVC PHOSPHOLIPASE A2 BY TNF-ALPHA

By

JUSTIN SCOTT BICKFORD

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To my grandparents

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LIST OF ABBREVIATIONS

ATF	Activating transcription factor
bp	basepair
ChIP	Chromatin Immunoprecipitation
COX	Cyclooxygenase
DN	Dominant negative
HETE	Hydroxyeicosatetraenoic acid
hGH	Human growth hormone
hnRNA	Heterogeneous nuclear RNA
HPETE	Hydroperoxyeicosatetraenoic acid
IFN	Interferon
IL	Interleukin
kb	kilo-basepair
LO	Lipoxygenase
LT	Leukotriene
LX	Lipoxin
mRNA	Messenger RNA
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Prostaglandin
PLA_2	Phospholipase A2
PO	Phosphodiester
PS	Phosphorothioate
RT-PCR	Reverse-transcription PCR
TF	Transcription factor

TNF	Tumor necrosis factor
TSS	Transcriptional start site
TX	Thromboxane
USF	Upstream stimulating factor

Abstract of Dissertation Presented to the Graduate School
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Justin Scott Bickford

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Lipid metabolites are an integral part of normal physiology as well as the inflammatory cascade. At the apex of this cascade is the release of arachidonic acid from membrane phospholipids. Physiologically relevant lipid mediators are derived from arachidonate through the activity of various enzymes, most relevantly the cyclooxygenase and lipoxygenase pathways. This study uniquely identifies group IVC phospholipase A2 (PLA2G4C/cPLA$_2$γ) as a potentially important player in the inflammatory response. In a mouse model of allergic asthma, cPLA$_2$γ was found to be induced at the mRNA level in the lungs of sensitized and challenged mice compared to control animals. From these results, this study goes on to characterize the molecular regulation of the cPLA$_2$γ gene in cell culture. It was hypothesized that the pro-inflammatory cytokines most likely responsible for the induction of cPLA$_2$γ in the mouse model were TNF-α and IL-1β. Utilizing promoter deletion analyses, the first 114 bp upstream of the cPLA$_2$γ transcriptional start site was identified as a minimal promoter element able to confer TNF-α responsiveness. Tethering this fragment to a heterologous promoter confirmed that this fragment also possesses cytokine-specific enhancer-like properties mediated through three potential transcription factor binding

sites. These sites were initially identified by *in silico* analysis, and their functional relevance was verified by mutagenesis studies through specific site deletions. Based on chromatin immunoprecipitation analyses, the cognate transcription factors binding to these sites within the cPLA$_2$γ enhancer/promoter were found to be ATF-2/c-Jun, p65, and USF1/USF2. Overexpression studies demonstrate the importance and cooperation of these factors in both basal and stimulated gene expression. Additionally, a unique response to extracellular DNA resulting in increased cPLA$_2$γ expression has been identified and will be a focus of future studies. Overall, the data presented here demonstrate that cPLA$_2$γ is an important member of the inflammatory cascade controlled by transcriptional regulation.

CHAPTER 1
INTRODUCTION

Arachidonic Acid Metabolism

Arachidonic acid is a 20-carbon omega-6 fatty acid which resides in the tails of membrane phospholipids (Figure 1-1). Free arachidonic acid within the cell comes from either cleavage from membrane phospholipids by the phospholipases or synthesis from the 18-carbon linoleic acid. Once released, arachidonic acid is further metabolized by the cyclooxygenases or lipoxygenases into downstream signaling molecules. The roles of eicosanoids and their downstream metabolites have been studied extensively [1]. The phospholipase A_2s (PLA_2s) release arachidonic acid (AA), its precursor linoleic acid, or platelet activating factor (PAF), from membrane phospholipids through specific cleavage at the sn-2 position [2]. AA is then further metabolized by downstream pathways involving either the cyclooxygenases or lipoxygenases as shown in Figure 1-2.

Metabolism by the Cyclooxygenases

The cyclooxygenase (COX) family of enzymes produce prostaglandin H2 (PGH_2), and thus are also known as PGH_2 synthase enzymes (PGHS). The products of this pathway are illustrated in Figure 1-1. COX enzymes incorporate a single covalent bond between carbons 8 and 12 in the arachidonic acid backbone, a peroxy bridge between carbons 9 and 11, as well as an additional OOH group at carbon 15. The –OOH group is then converted to an –OH to yield PGH_2, which is then used by all subsequent prostanoid synthases. Prostacyclin synthase opens up the peroxy bridge, resulting in a hydroxyl group at carbon 11, whilst forming an ether bond between carbons 6 and 9 to form PGI_2, or prostacyclin. Splitting open the peroxy bridge of PGH_2 by their respective

prostaglandin synthases yields the remainder of the prostaglandins, with PGD_2, PGE_2, and PGF_2 differing in the final state of these two oxygens. PGD_2 contains a hydroxyl and ketone at carbons 9 and 11, respectively, while PGF_2 contains two hydroxyl groups and PGE_2 contains a ketone and a hydroxyl at these two sites. Repositioning by thromboxane A2 synthase (TXAS) of an oxygen from the peroxy bridge into an ether bond in the cyclopentane ring between carbons 11 and 12 results in the production of TXA_2.

Metabolism by the Lipoxygenases

The lipoxygenase family of enzymes incorporate an oxygen into the carbon backbone of linoleic or arachidonic acid to create the leukotrienes and lipoxins. Lipoxygenases are named based on the position within arachidonic acid to which an oxygen is added via reduction of a hydroperoxy intermediate, hydroperoxyeicosatetraenoic acid (HPETE) (Figure 1-1). Initially, metabolism by 5-LO forms an oxygen bridge between carbons at positions 5 and 6 to form the leukotrienes. The result of this first reaction is the formation of the leukotriene A4 (LTA_4) via a 5-HPETE intermediate. LTB_4 is formed by the addition of a water molecule to LTA_4 resulting in the addition of a second oxygen at carbon 12. Further metabolism of LTA_4 by addition of the sulphur atom of glutathione (a tripeptide of L-cysteine, L-glutamic acid and glycine) to carbon 6 results in the formation of the cysteinyl leukotrienes, initially LTC_4. LTD_4, LTE_4 are formed by the removal of portions of the glutathione. Removal of the glutamic acid portion results in the formation of LTD_4 and further removal of the glycine creates LTE_4. In all three of these cysteinyl leukotrienes, the presence of the central cysteine from the glutathione remains bound to carbon 6. Lipoxins, or lipoxygenase interaction products (LXs), were first identified by Serhan et al. in 1984 [3].

The lipoxins are synthesized through the action of 5-LO on the 15-LO product 15-HPETE resulting in the trihydroxytetraene structure of LXA_4 with hydroxyl groups at carbons 5, 6 and 15 and double bonds at carbons 7, 9, 11 and 13 (Figure 1-1). The lipoxins possess anti-inflammatory properties in opposition to the leukotrienes by stimulating vasodilation [4, 5]; they also participate in the resolution phase of inflammation and can be synthesized by COX-2 in response to aspirin [6].

Eicosanoids and Inflammation

As a result of metabolism by the cyclooxygenases, COX-1, -2 or -3, the prostaglandins (PGs) and thromboxanes (TXs) are produced. The main functions of thromboxanes are facilitation of vasoconstriction and platelet aggregation while the prostaglandins have the opposite effect and mediate vasodilation along with other tissue-specific physiological activities [7]. This metabolic pathway has been the major focus in the treatment of pain and inflammation and is blocked at the level of the COX enzymes by non-steroidal anti-inflammatory drugs, or NSAIDs, such as aspirin and Vioxx [8]. While the prostanoids are one class of targets for inflammation, several asthma medications have targeted either the lipoxygenases or the leukotriene receptors.

Prostaglandins and Thromboxanes

The prostaglandins are comprised of a complex family of bioactive eicosanoids as shown in Figure 1-2, each of which have varying effects on inflammation and, given the focus of this proposal, will be discussed predominantly in relation to the lung. PGD_2 can induce sleep, decrease body temperature, and modulate odor and pain responses and, more relevant to this proposal, both PGD_2 and $PGF_{2\alpha}$ have been shown to act as bronchoconstrictors [9, 10]. PGE_2 has the opposite effect and acts to reduce

inflammation by relieving bronchoconstriction [11]. Finally, PGI_2 acts as a bronchodilator and exists in a delicate balance with TXA_2, which induces platelet clumping and bronchoconstriction [12].

Leukotrienes and Lipoxins

The second major branch point in eicosanoid metabolism results from the action of the lipoxygenases on arachidonic acid producing the leukotrienes (LTs) (Figure 1). The leukotrienes are also important in both initiating and maintaining the inflammatory response [13]. Their physiological relevance is best illustrated by the action of leukotriene antagonists as effective preventative measures in the treatment of asthma by relieving bronchoconstriction [14, 15]. LTB_4 acts primarily as a chemotactic substance to recruit eosinophils to the lung while the cysteinyl leukotrienes (LTC_4, LTD_4 and LTE_4) act as powerful stimulators of bronchoconstriction, with each one being less potent than the previous [16, 17]. Lipoxins (LXs), such as LXA_4, promote downregulation of PGE_2, cell clearance and resolution of inflammation [18]. Inhibition of this pathway by cysLT receptor antagonists or 5-LO inhibitors, Singulair or Zyflo, has been used for treatment of asthma [19, 20].

15-LO converts arachidonic acid to 15-HPETE. 15-HPETE is converted to LXA_4 by 5-LO and epoxide hydrolase [3]. The activation of 5-LO reduces further production of the leukotrienes, breaking the cyclical nature of the inflammatory cascade. The lipoxins can be produced by the action of 12-LO on LTA_4 as well, also diminishing the pool of leukotriene precursors. The lipoxins are rapidly produced and involved in the resolution phase of inflammation and possess anti-inflammatory properties. The production of these signaling molecules prevents further damage from the inflammatory response.

Phospholipase A2s

As described, the eicosanoids play a pivotal role in inflammation, ultimately mediating vasoconstriction and dilation [21, 22] and possessing pro- and anti-inflammatory properties [23]. Although this metabolism leads to the biosynthesis of the bioactive lipids including the prostaglandins, thromboxanes, leukotrienes and lipoxins, lipid mediators are initially metabolized from membrane phospholipids, yielding the ubiquitous fatty acid, arachidonic acid. At the apex of this pathway are the phospholipases, which liberate arachidonate, the sole substrate for the downstream enzymatic cascade. The phospholipase superfamily is present in all organisms and is comprised of ten groups of secretory phospholipases ($sPLA_2$s), with group XIV being recently identified in prokaryotes [24, 25], two groups of platelet activating factor acyl hydrolases (groups VII and VIII) [26] and two cytosolic groups (groups IV and VI). Of the cytosolic groups, one contains calcium independent phospholipases (group VI, $iPLA_2$s) whilst the other is almost exclusively composed of calcium dependent phospholipases (group IV, $cPLA_2$s).

Prior to formation of the eicosanoids, phospholipases first cleave arachidonic acid from membrane phospholipids, providing the substrate for the downstream metabolic pathway. The phospholipases were first identified in snake venom in the late 1800s and, since then, have been characterized as an important family of enzymes across species [27]. PLA_2 enzymes catalyze the cleavage of fatty acids, such as arachidonic acid, from phospholipids and are distinguished from the PLA_1 enzymes by their propensity to catalyze an sn-2 dependent cleavage reaction. Several PLA_2 enzymes have been identified in mammals and are categorized into groups I, II, III, V, VI, X, and XII [25, 28]. Group XIII phospholipase A_2 has subsequently been reclassified as a group XII

phospholipase isozyme. These enzymes are further grouped based on sequence homology, localization and calcium requirements as secretory ($sPLA_2$s) and cytosolic calcium-dependent ($cPLA_2$s) and calcium-independent ($iPLA_2$s). The $sPLA_2$s are small proteins (~14 kDa), have little fatty acid specificity and all require millimolar concentrations of calcium, while the $iPLA_2$ are intracellular, have a wide range of specificities and lack any calcium requirement for enzymatic activity [29-31].

Secretory Phospholipase A2s ($sPLA_2$s)

The most diverse group of phospholipases are members of the low-molecular weight category of secreted phospholipases, $sPLA_2$s. All of the $sPLA_2$s are calcium dependent, and, with the exception of group III, they are all smaller than 20 kDa. The secretory phospholipases also have very little preference for arachidonic acid over any other fatty acid. The impact of secretory phospholipases varies greatly from breaking down low-density lipoprotein complexes to possessing antibacterial properties [32, 33].

Intracellular Calcium-Independent Phospholipase A2s (group VI or $iPLA_2$s)

The group VI calcium-independent phospholipase A2s, $iPLA_2$s, are grouped together based on their lack of regulation by calcium, although they share the distinction of being intracellular with the $cPLA_2$ family, to be discussed later. While this group contains six members, only one has been studied extensively, $iPLA_2\alpha$. Despite this family's independence from calcium, one member, $iPLA_2\beta$, is partially regulated by direct interactions with calmodulin [34]. This family of phospholipases share a catalytic serine, but are grouped together based on their calcium independence rather than sequence homology. While the $iPLA_2$s do cleave fatty acids from the sn-2 position of phospholipids, they have very low substrate specificity.

Cytosolic Phospholipase A2s (group IV or cPLA$_2$s)

Of specific relevance to the current studies are the recently identified cytosolic phospholipase A2s (cPLA$_2$), of which six isozymes have been identified in mammals beginning with cPLA$_2$α and, more recently, β, γ, δ, ε and ζ [35-37]. The human isozymes are labeled as group IVA through IVF and also designated with the corresponding Greek letters as cPLA$_2$α, β, γ, δ, ε and ζ. The majority of the cPLA$_2$s contain a C2 calcium binding domain and hence require micromolar concentrations of calcium for activity with the exception of cPLA$_2$γ, which lacks both the regulatory phosphorylation sites and the calcium binding C2 domain, thus having no requirement for Ca^{2+} [38, 39]. However, cPLA$_2$γ is included in this family based on conservation, most importantly, of the conserved catalytic triad, Arg54, Ser82, Asp385 [36]. An analysis of the six cPLA$_2$ isoforms by amino acid alignment using Clustal software (ClustalW v1.83, European Bioinformatics Institute, www.ebi.ac.uk/Tools/clustalw) along with a phylogenetic tree (as determined by a BLOSUM62 matrx) illustrate the clustering of the β, δ, ε and ζ isoforms in a more highly conserved group versus the alpha and gamma isoforms (Figures 1-3 and 1-4). By far, cPLA$_2$α has been the most widely studied of the group IV phospholipases since its discovery and cloning in 1990 [40, 41]. Current studies are also focusing on acquiring a better understanding of the remaining group members [28, 42, 43]. As illustrated in Figure 1-4, cPLA$_2$γ is also the smallest member of the group at 61 kDa, due to the exclusion of the C2 domain, while cPLA$_2$α, β, δ, ε and ζ are 85, 114 and 92, 100 and 96 kDa, respectively [39, 43, 44].

cPLA$_2$γ was first identified in 1998 from analysis of human brain expressed sequence tags (ESTs) and found to be highly expressed in heart and skeletal muscle [42]. Simultaneously, cPLA$_2$γ was being studied by Underwood and colleagues in much

greater detail and found to be localized to the same tissues [38]. In the paper by Underwood, et al., cPLA$_2$γ was analyzed and determined to possess phospholipase activity specific to the sn-2 position by *in* vitro assays. According to these investigators, the presence of the C-terminal prenylation domain was not required for association with the membrane fraction. Since its discovery, approximately fourteen papers have studied cPLA$_2$γ at the RNA or protein levels in the past nine years.

One of the first papers following the discovery of cPLA$_2$γ briefly addressed its transcriptional regulation. The first study to reveal cPLA$_2$γ regulation was by Lindbom, et al., in 2001 [45]. This study identified a number of expressed phospholipases in human nasal mucosa, including cPLA$_2$γ. In 2002, the same group went on to investigate the regulation of cPLA$_2$γ in nasal epithelial cells (RPMI 2650) as well as bronchoepithelial cells (BEAS-2B) [46]. Of the panel of 19 phospholipases in this study, it was found that cPLA$_2$γ was substantially induced at the mRNA level by TNF-α in the two airway epithelial cells lines. These studies revealed that, in response to TNF-α, a proinflammatory cytokine, both cPLA$_2$α and cPLA$_2$γ were induced at the mRNA level.

Also in 2002, the Leslie laboratory published a study on the proposed enzymatic activity of purified cPLA$_2$γ [47]. They confirmed that isolated cPLA$_2$γ possesses the proposed PLA$_2$ activity, but will also subsequently hydrolyze the fatty acid in the sn-1 position with little preference for any fatty acid over another. This observation was also confirmed in a mouse cell line derived from cPLA$_2$α knockout mice.

In early 2003, the Shimizu lab studied the role of cPLA$_2$γ in a human embryonic kidney cell line, HEK293, overexpressing cPLA$_2$γ [48]. When cPLA$_2$γ was conjugated to GFP in a pcDNA vector, it was found to be localized to the ER and Golgi. Furthermore,

they found that, by mass spectrometry, the relative abundance of phospholipids containing ethanolamine and 16-carbon fatty acid chains was reduced in these cPLA$_2$γ overexpressing cells relative to their control cells. Lastly, they also observed an increase in free arachidonic acid following hydrogen peroxide exposure in cPLA$_2$γ overexpressing cells. Based on these studies, Asai, et al. proposed a role for cPLA$_2$γ in membrane remodeling, particularly under oxidative stress [48].

Later in 2003, Murakami, et al. also utilized HEK293 cells stably expressing cPLA$_2$γ [49]. They observed that the addition of either IL-1β or serum to the media of these cells were able to increase arachidonic acid release relative to control cells. They also observed that the increase in arachidonic acid was accompanied by an increase in PGE_2 via a COX-2 dependent pathway. In addition, Murakami, et al. also confirmed the requirement of the catalytic serine, S82, on enzymatic activity of cPLA$_2$γ by mutagenesis. They found that in HEK293 cells expressing this mutant, the levels of arachidonic acid release were equivalent to the levels seen in control cells. These studies also confirmed that cPLA$_2$γ is strongly associated with membrane fractions in these cells and localized to the ER, as previously demonstrated [48]. Despite being associated with membrane fractions in multiple studies, neither this association nor the enzymatic activity was found to be dependent on C-terminal prenylation. These results did not fully exempt the C-terminal prenylation from contributing to membrane association due to the fact that phospholipase activity was observed in both the isolated cytosolic and membrane fractions at levels well above those containing the wild-type protein. Most significantly, Murakami et al. have strongly argued that cPLA$_2$γ is second only to cPLA$_2$α in the generation of spontaneous and stimulus induced arachidonic acid

release [49], thus providing evidence for the importance of this isoform to the inflammatory response as implied in the present study.

Although the C-terminal prenylation motif (-CCLA) was found to affect the localization of phospholipase activity to the membrane fraction, the details of this moiety were not studied until later in 2003. That year, Jenkins, et al. performed experiments on purified cPLA$_2$γ to analyze this modification [50]. Prenylation occurs by the covalent addition of a farnesyl or geranyl-geranyl group to the protein. Therefore, by utilizing radiolabeled farnesyl or geranyl-geranyl precursors, they determined that cPLA$_2$γ did contain a farnesyl moiety. They next proceeded to more accurately determine the nature of this modification by mass spectrometry and found that, within the –CCLA motif, the first of these two cysteines (C538) contains the farnesyl modification while C539 is cleaved from the modified protein. These results were corroborated by two studies in 2005 investigating post-translational prenylation on cPLA$_2$γ and the effect on its activity [51, 52]. Utilizing a different methodology, the studies by Tucker et al. confirmed the presence of a C-terminal farnesyl group by HPLC (high-performance liquid chromatography) [51]. Both of these subsequent studies found that the prenylation is neither required for the activity nor the very tight membrane association of the protein, which is retained even in the presence of a high salt concentration.

It has been shown that the appetite-regulating hormone, leptin, can increase leukotriene synthesis in macrophages from leptin-deficient mice [53]. Following up on this data, in 2004 Mancuso et al. investigated the effects of leptin on cPLA$_2$γ protein levels [54]. They found that, in isolated mouse alveolar macrophages, leptin was able to induce cPLA$_2$γ protein expression by 80%. Because the concomitant increase in

leukotriene synthesis was calcium independent, they proposed that the increase in cPLA$_2$γ protein contributed to downstream production of leukotrienes in the mouse alveolar macrophage cells, although a direct link between the two events was not shown.

Also in 2005, Vitale et al. cloned the mouse homolog of cPLA$_2$γ [55]. Despite being 56% identical to the human cDNA sequence for cPLA$_2$γ, the mouse form lacked any clear C-terminal prenylation motif. Interestingly, when investigating the tissue specificity of the cPLA$_2$γ protein, they did not observe any detectable expression in heart, skeletal muscle or lung, as had been observed for the human protein. The mouse cPLA$_2$γ protein appeared to be specific only to the ovary and embryo out of the tissues which they examined. Unlike previous studies investigating cellular localization of overexpressed and tagged cPLA$_2$γ, this study examined the endogenous protein by immunofluorescence and found it to be distributed in the cytoplasm and targeted to the nuclear envelope, perhaps participating in germinal vesicle breakdown. Worth noting, however, is that the difference in cellular localization between the overexpressed human protein and the endogenous mouse protein may be an effect of the C-terminal prenylation or an artifact of overexpression.

In 2007, Tithof et al. undertook a study to determine which phospholipase was contributing to downstream prostaglandin production in bovine endometrial cells [56]. It has been known that prostaglandins PGE$_2$ and PGF$_{2\alpha}$ participate in the regulation of estrous and that, in cattle, the downregulation of PGF$_{2\alpha}$ in response to interferon tau (IFNτ) is required to maintain pregnancy. In these studies, they observed that, of the calcium independent phospholipase A2s, only cPLA$_2$γ was substantially increased in

response to IFNτ. Concomitantly, there was an increase in PGE_2 production relative to $PGF_{2\alpha}$, implying that arachidonic acid produced by $cPLA_2\gamma$ preferentially produces PGE_2.

In 2008, the first studies were performed in which $cPLA_2\gamma$ was investigated in a physiological disease state. In this study, Brown et al. infected mice with the parasitic nematode, *Trichinella spiralis*, and found that $cPLA_2\gamma$ protein levels in the intestinal epithelium were increased substantially such that it became one of the most abundant cellular proteins [57]. It was also shown that $cPLA_2\gamma$ is highly sensitive to degradation by the extracellular β-chymase, Mcpt-1. It is proposed that this sensitivity may be beneficial in rapidly degrading $cPLA_2\gamma$ protein if it is released from cells or presented on the cellular surfaces. Subsequent to these studies, in 2009, $cPLA_2\gamma$ mRNA was found to be elevated in mouse intestines, as well as lungs, in response to IL-9 in an IL-13 dependent manner [58].

Again, in 2009, studies were performed to investigate the subcellular localization of $cPLA_2\gamma$. The Yamashita laboratory revisited this topic from their 2005 paper [52] in order to better define the localization as well as to contribute to the knowledge about the enzymatic activities of this protein [59]. By utilizing a C-terminal FLAG-tagged $cPLA_2\gamma$, which would disrupt any possible C-terminal prenylation, or an N-terminal FLAG-tag, Yamashita et al. further confirmed that the C-terminal processing of the $cPLA_2\gamma$ protein is not required for its subcellular localization. With each of these modifications, $cPLA_2\gamma$ was found to be localized to the ER membrane. Upon further investigation, they found that a portion of this protein was localized to the mitochondria. The most substantial observation in this manuscript was not the contribution to previous bodies of work

detailing the membrane association of cPLA$_2$γ, but the enzymatic studies involving the transacylase activity of cPLA$_2$γ. Purified cPLA$_2$γ has previously been shown to have specificity for arachidonic acid, although Yamashita et al. utilized shorter acyl chains of 8-18 carbons. cPLA$_2$γ was found to preferentially cleave these fatty acids from phospholipids containing choline or ethanolamine head-groups, suggesting that cPLA$_2$γ has specificity not only for fatty acids, but also for the head-groups of phospholipids as seen by the Shimizu laboratory in 2003 [48].

In addition to the studies detailed here, cPLA$_2$γ was briefly investigated in 2005 due to an association of this locus with schizophrenia by the Wei lab in a series of studies involving Chinese schizophrenia patients [60-63]. The chromosomal region of 19q13.3 is also a common deletion in human gliomas and thus the lack of cPLA$_2$γ has been suspected of contributing to this form of cancer [64]. It has since been dismissed as a contributing factor, but this region is still a useful marker for tumor aggressiveness [65].

Gene regulation

Promoter Elements

Transcription can be driven by *cis*-regions of DNA in close proximity the transcriptional start site (TSS), referred to as promoters. The best studied of these is the TATA box which lies 35 bp upstream of a small fraction of promoters and is a recognition site for the TATA binding protein of the TFIID complex. Genes lacking a TATA box often contain downstream promoter elements (DPEs) or pyrimidine-rich initiator elements (INRs). Any number of these elements are capable of directing the binding of transcription factors such as TFIID and, subsequently, the rest of the pre-initiation complex (PIC). Although most genes contain some combination of these

elements, some genes lack any known promoter element and it is not well characterized as to what defines the specific start sites of these genes. It has been established that a competent PIC can form at other transcription factor binding sites, such as those involving NF-κB, and subsequently be transferred to a TATA-less promoter for transcription initiation [66]. Regardless of the particular components present in a promoter, it always contains the TSS and is bound by the general transcription factors.

Defining an Enhancer Element and Promoter Interactions

Promoters are often regulated by the activity of enhancer elements. Enhancer elements can be found upstream, downstream or within a gene as a *cis*-regulatory element, or they may be found on a different chromosome as a *trans*-regulatory element. Enhancer elements are defined as being both position and orientation independent as well as being able to function with a heterologous promoter. Because these regions must be accessible to various transcription factors when the gene is in an active state, these regions of open chromatin structure may be identified by DNase I hypersensitivity assays. The transcription factors that are known to bind DNA also have specific consensus sequences to which they bind as well as cofactors that they may bind to. Although some enhancer elements are proximal to their respective promoters and may interact directly, many lay hundreds or even several thousand base-pairs from their promoters. There are a number of ways in which a distal enhancer can interact with a promoter including tracking, reeling, or looping [67-69]. Several other proteins are often involved in helping to arrange these regions so that the bound proteins may interact or even transfer between the enhancer and promoter elements.

The Process of Transcription and Transcription Factors

Two of the first steps required for transcription to occur at the gene level are the recruitment of chromatin modifying proteins and the opening of the chromatin structure. This process is also directed by other factors which bind to the enhancer or promoter and act to direct this process. Once the DNA at the promoter is accessible, TATA-binding protein (TBP) binds upstream of the TSS and the TFIID complex assembles. This is followed by assembly of the remaining factors of the pre-initiation complex (PIC) at the promoter. The c-terminal domain (CTD) of RNA Polymerase II is phosphorylated at serine-5 when the PIC is primed and this mark disappears shortly after transcription initiation. As transcription initiation begins, serine-2 of the RNA Pol II CTD becomes phosphorylated and synthesis of the new mRNA transcript begins.

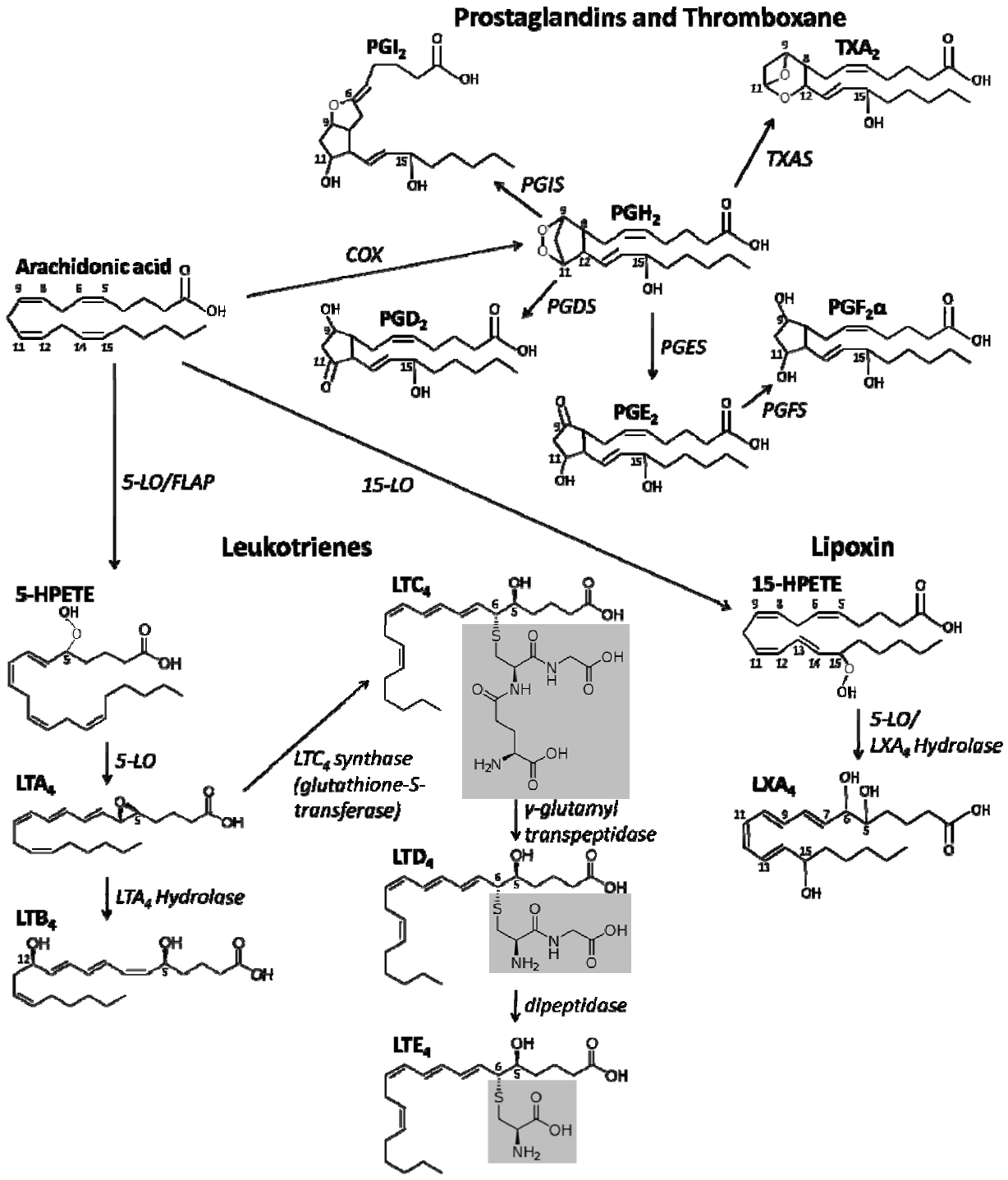

Figure 1-1. Chemistry of arachidonic acid metabolism. Illustrated here are arachidonic acid and three downstream metabolic pathways via COX (prostaglandins and thromboxanes), 5-LO (leukotrienes), and 15-LO (lipoxins). Eicosanoid products are listed in bold while enzymes are listed in italics. The glutathione moiety and subsequent products are presented with a grey background.

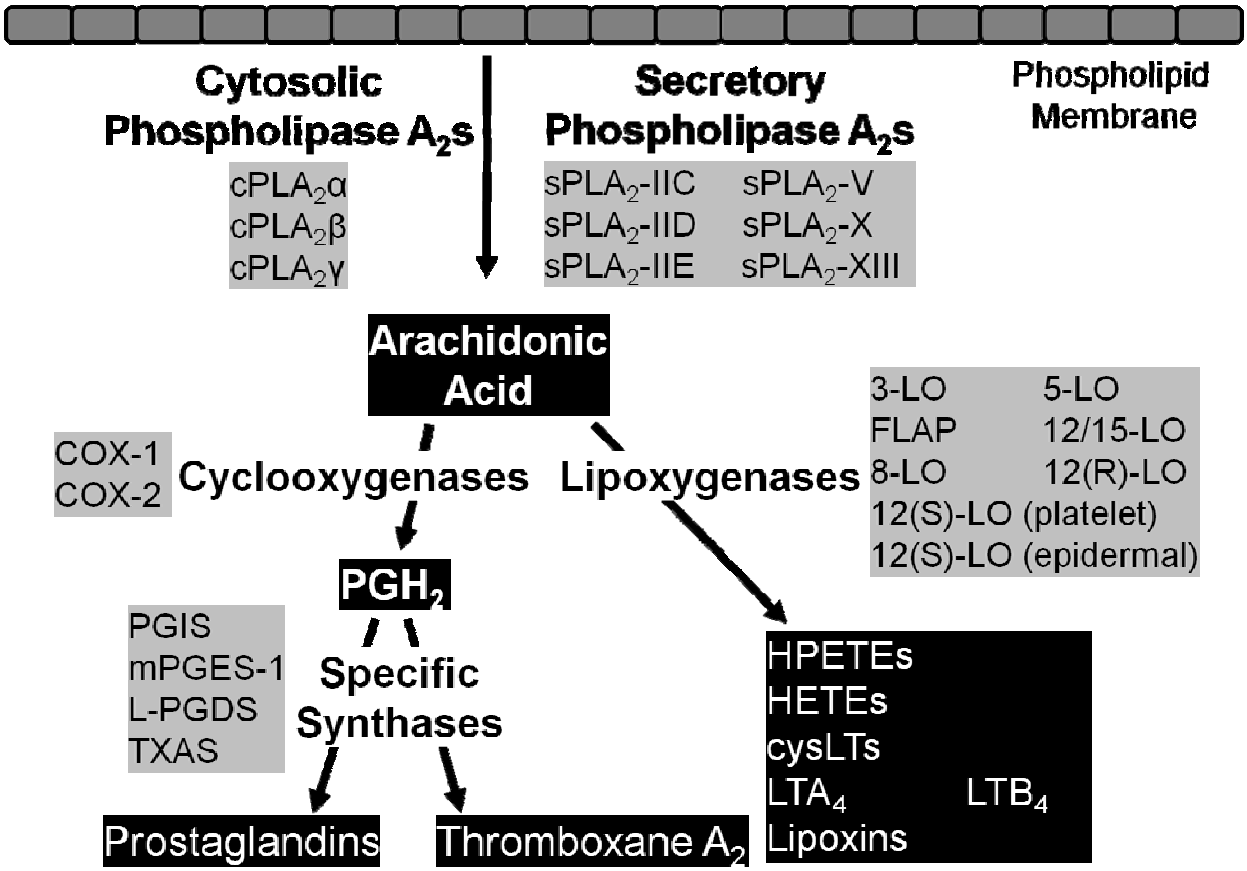

Figure 1-2. Diagram of eicosanoid metabolism. The enzyme families within the arachidonic acid pathway are shown in bold with the corresponding specific enzymes (which were evaluated in this study by real-time PCR) in the shaded gray boxes with the products produced from these reactions shown in the black boxes. AA is liberated from the phospholipid membrane through the action of the phospholipase A2 enzymes (both cytosolic and secretory). AA is then utilized by either the cyclooxygenase or lipoxygenase enzymes. The cyclooxygenases produce PGH2 which is converted by specific synthases to prostaglandins or thromboxane A2; the lipoxygenases produce HPETEs, HETEs, cysLTs, LTA4, LTB4, and lipoxins.

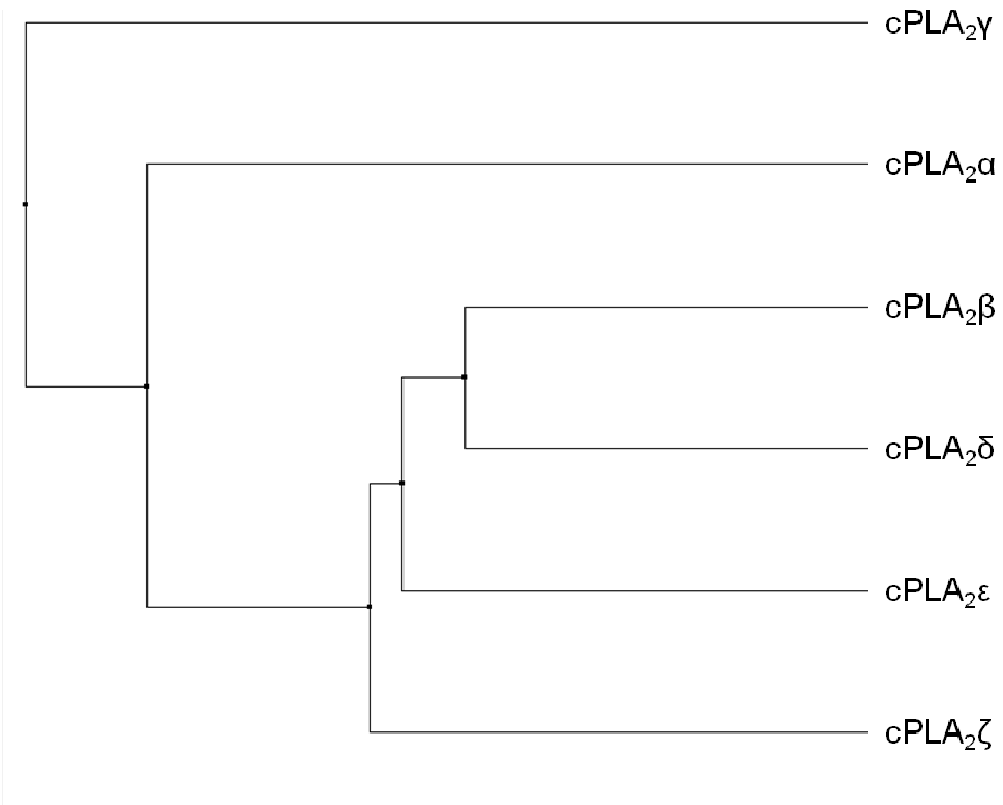

Figure 1-3. Phylogenetic tree of group IV phospholipase family members.

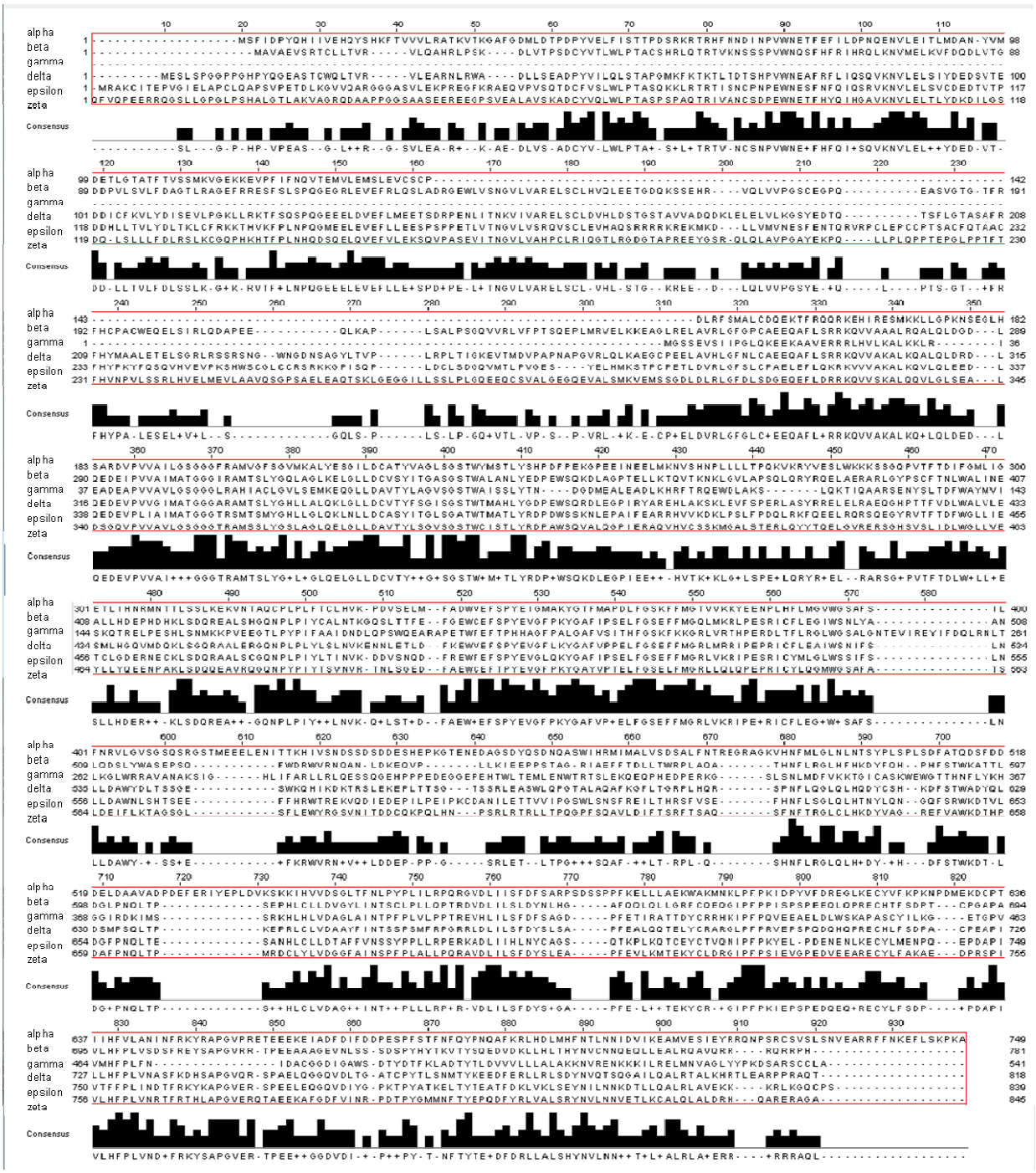

Figure 1-4. Amino acid sequence alignment of group IV phospholipase family members. Beneath the sequences for this family of phospholipases is a consensus score at each amino acid residue.

CHAPTER 2
METHODS

Mouse model of Allergic Asthma

All animal experiments were done in accordance with the IACUC at the University of Florida. Mouse sensitization and challenge experiments were carried out as described by Müller et al. [70]. Briefly, C57BL/6J mice were sensitized to 200 µg of *Aspergillus fumigatus* (*Af*) extract (Greer Laboratories) in phosphate buffered saline (PBS), ovalbumin (OVA), or mock sensitized with PBS alone by intraperitoneal injection on days 0 and 14. OVA sensitized mice were then exposed to aerosolized OVA, while all other mice were exposed to aerosolized *Af* extract for 20 min on days 28, 29 and 30. Lungs were subsequently harvested on day 32 and used for RNA isolation.

RNA Isolation and Purification

RNA used for northern analyses and animal studies was isolated from cells or lungs as described by the Chomczynski and Sacchi with modifications [71]. For lung tissue, the lungs were flash frozen in liquid nitrogen followed by pulverization in a liquid nitrogen cooled mortar and pestle prior to lysis. For cultured cells, the media was aspirated and the dishes were washed once with phosphate-buffered saline (PBS). All samples were subsequently lysed in 500 µL of guanidinium thiocyonate (GTC) denaturing solution consisting of 4 M GTC, 25 mM sodium citrate at pH 7.0, 0.5% sarcosyl and 0.1 M β-mercaptoethanol. After vortexing briefly to help lyse the cells, 50 µL of 2 M sodium acetate at pH 4.0, and 500 µL of water-saturated phenol was added. The solution was then vortexed and incubated at room temperature for 5 min. 110 µL of a 49:1 chloroform:isoamyl alcohol mixture was added to the lysate, vortexed vigorously and centrifuged at 12,000 g at room temperature for 20 min. The aqueous phase was

then transferred to a clean tube, an equal amount of isopropanol was added, and this solution was incubated at -20 °C for 30 min. The RNA was then pelleted by centrifugation at 12,000 g at 4 °C for 15 min, the supernatant was decanted, and the remaining pellet was resuspended in 75 µL of diethyl pyrocarbonate (DEPC)-treated water. Following resuspension of the pellet for 10 min at 50 °C, 25 µL of 8 M LiCl was added and incubated at -20 °C for 30 min. The RNA was again pelleted by centrifugation at 12,000 g at 4 °C for 20 min and the supernatant was decanted. The remaining pellet was rinsed with 100 µL of 70% ethanol followed by the addition of 200 µL of 70% ethanol prior to centrifugation at 12,000 g at 4 °C for 10 min. The ethanol was then decanted and the remaining pellet completely dried for 1 min under vacuum. The purified RNA pellet was finally resuspended by addition of 100 µL of DEPC-treated water and incubated at 50 °C for 5 min. RNA concentrations were determined in a spectrophotometer by absorbance at 260 nm.

Total RNA from cell culture studies used for real-time RT-PCR analysis was purified with an RNeasy Mini Kit from Qiagen as per manufacturer's instructions. Additionally, this RNA was treated with RNase-free DNase (Qiagen) to insure that there was neither genomic nor plasmid contamination. RNA concentrations were determined in a spectrophotometer by absorbance at 260 nm.

Northern Blot Analysis

10-20 µg of total RNA was lyophilized, resuspended in 30 µL loading buffer (500 µL deinozided formamide, 302 µL H_2O, 175 µL formaldehyde, 2 µL sodium acetate, 1 µL EDTA), fractionated on a 1% agarose, 6% formaldehyde gel, electrotransferred to a Zetabind membrane (Bio-Rad) and UV cross-linked. Membranes were then incubated

for 1 h in a prehybridization buffer consisting of 0.45 M sodium phosphate, 6% sodium dodecyl sulfate (SDS), 1 mM EDTA, and 1% bovine serum albumin (BSA) [72]. The membranes were then incubated overnight at 60°C in the same hybridization buffer with a ^{32}P-radiolabeled gene specific probe for cPLA$_2\gamma$ or L7a, generated by random primer extension. The membrane was then washed three times for 5 min each at 65 °C in a high stringency buffer composed of 0.04 M sodium phosphate, 2 mM EDTA and 1% SDS, placed in a cassette with autoradiographic film and the film was subsequently developed and used for densitometry. Densitometry was performed using ImageJ software imaging software, distributed by the NIH.

Real-Time Reverse-Transcription PCR

One µg of total RNA was used to synthesize first strand cDNA by reverse transcriptase PCR using the Superscript™ First-Strand Synthesis System for RT-PCR from Invitrogen and the final product was diluted to 100 µL. Two µL of the newly synthesized cDNA were added to the following in duplicate in a 96-well tray: 1.5 µL of 5 mM stocks of each forward and reverse gene specific primers, 12.5 µL of SYBR Green master mix (Invitrogen), and 7.5 µL water for a total volume of 25 µL. Primers for cyclophilin A were used as a loading control and the subsequent real-time PCR was carried out in an ABI Prism 7000 Sequence Detection System. Crossing threshold (CT) values were defined as the cycle number at which amplification crossed a designated threshold level within the exponential amplification range of the samples. Normalized CT values (ΔCT) were obtained by subtracting cyclophilin A CT values from CT values of the indicated genes. ΔΔCT values were obtained by subtracting the ΔCT value of the

control sample from the ΔCT value of the target sample for the indicated experiment. Finally, fold induction values were defined as $2^{-\Delta\Delta CT}$ as per the ΔΔCT method [73].

Cell Culture

EoL-1 (a human eosinophilic leukemia cell line [74]) and THP-1 cells (a human monocytic cell line [75]), were maintained in RPMI-1640 medium supplemented with 25 mM NaHCO3, 4 mM glutamine, antibiotic/antimycotic (ABAM) and 10% FBS at 37°C with 5% CO_2. A549 (a human epithelial-like lung adenocarcinoma cell line), HFL-1 (a human fetal lung fibroblast cell line [76]), as well as S9 cells (a human cystic fibrosis transmembrane conductance regulator (CFTR)-corrected cell line [77]) were maintained in Ham's F12K medium with the same supplements. S9 cells are derived from IB3.1 cells [78], but they express a functional CFTR which was inserted by adeno-associated viral transduction. IB3.1 cells are a bronchoepithelial cell line derived from a cystic fibrosis patient containing both a Δ508 deletion, resulting in a misfolded CFTR protein, and a W1282X mutation, resulting in a premature stop codon; these cells were immortalized with SV40 T-antigen. HEK293 (a human embryonic kidney cell line) and HEK293 cells stably expressing Toll-like receptor 9 (HEK-TLR9, a kind gift from Dr. Golenbock, University of Massachusetts) were grown similarly in Eagle's Minimum Essential Medium (MEM) while J774A.1 cells (a mouse monocytic cell line [79]) were grown in Dulbecco's Modified Eagle's Medium. The cells were grown to ~50% confluency in 10 cm dishes and treated with the indicated compounds. After 12 h of treatment (unless otherwise indicated) cells were collected and gene or protein expression was determined by real-time RT-PCR, northern or immunoblot analyses. Treatment concentrations, unless otherwise specified, were as follows: *Af*, 100 μg/mL;

LPS, 0.5 μg/mL; IFNγ, 5 ng/mL; TNF-α, 10 ng/mL; IL-1β, 2 ng/mL; IL-2, 5 ng/mL; IL-3, 5 ng/mL; IL-4, 20 ng/mL; IL-6 10 ng/mL; IL-10, 20 ng/mL; or IL-13, 20 ng/mL.

Cloning the cPLA$_2$γ Promoter and Human Growth Hormone (hGH) Reporter Constructs for Promoter Deletion Analysis

A promoterless human growth hormone reporter vector, pØGH, was utilized for the analysis of promoter fragments. This vector contains the five-exon genomic sequence for human growth hormone (hGH) immediately downstream of a multicloning site with no upstream regulatory sequences [80]. To obtain the cPLA$_2$γ promoter fragments to be cloned into pØGH, primers which include HindIII restriction sites were made in the forward direction to position -3776, -1177 and in the reverse direction beginning at +174 relative to the cPLA$_2$γ transcription initiation site at +1 as listed in Table 2-5. These primers were used to clone the 3.9 kb and 1.3 kb promoters from a BAC clone containing the cPLA$_2$γ gene. Once the 3.9 kb and 1.3 kb promoters were obtained by PCR and verified by gel electrophoresis, they were gel purified and cloned into TOPO-XL using the TOPO cloning kit. From this point, they were cut and isolated from the TOPO-XL vector using the flanking HindIII restriction enzyme sites and ligated into the HindIII site of pØGH lying immediately upstream of the hGH sequence. Subsequent promoter fragments were amplified from the 1.3 kb pGH construct using the appropriate 5’ primers for promoter fragments 490 and 288 and the 3’ primers within the pØGH backbone immediately after the previously inserted fragment with primers listed in Table 2-5. Each of these were similarly cloned into TOPO-XL before being cleaved by HindIII and cloned into the HindIII site of pØGH. The 762 bp promoter was obtained by a restriction digest of the 1.3 kb promoter in pØGH at unique KpnI sites at -588 bp and immediately upstream of the 5’ HindIII cloning site. The -114/-1 fragment was generated

by quick-change mutagenesis of the -114/+174 construct to remove the sequence of DNA from +1 to +174 with the primer set in Table 2-6 labeled as “114.”

hGH Reporter Constructs for Site-Directed Deletion Analysis

These constructs were all made by QuikChange site-directed mutagenesis (Agilent, formerly Stratagene) of the -114/-1 cPLA$_2$γ promoter construct in pGH and verified by sequencing. The primers used are listed in Table 2-6. Forward and Reverse primers were made to be complimentary to each other and to remove the following elements at sequences illustrated in Figure 4-12: CRE (del I), NF-B (del II), or E-Box (del III).

Transient Transfection of Promoter Constructs

Cells were grown to ~50% confluency on 60 mm dishes prior to transfection with the specified plasmid. Reporter plasmids were transfected using the FuGENE 6 transfection reagent (Roche). For promoter deletion studies, 2 µg of the empty vector was used or an equimolar amount of a plasmid containing one of the cPLA$_2$γ promoter constructs. Six µL of FuGENE 6 was used with the empty vector and this amount was adjusted for each plasmid to maintain the recommended ratio of µg of plasmid to µL of FuGENE at 1:3. Initially, an amount of medium lacking any supplements was added to each tube necessary to obtain a final volume of 288 µL. Next, the appropriate amount of FuGENE 6 was carefully added and the mixture left to stand. After 5 min, the DNA was added and gently mixed. Following a 15 min incubation, this mixture was added to 3.5 mL of medium on a 60 mm dish of cells to be transfected. After leaving the transfection mixture on the cells for three hours, the cells were rinsed with PBS and fresh media was added. The following day, each plate of cells was split 1:2 and left to grow overnight. After an additional day, cells were treated with TNF-α for the designated amounts of

time and total RNA was collected for subsequent northern or real-time RT-PCR analysis.

Immunoblot Analysis

Cells were incubated in media with or without TNF-α for the specified amounts of time, washed twice with ice cold PBS and lysed with a 50 µL of a buffer containing a proteinase inhibitor mini-tablet (Roche), 500 µL of 1 M Tris at pH 7.5, 200 µL of 5 M NaCl, 100 µL of 0.5 M EDTA at pH 8 and 100 µL of Triton X-100 with H_2O to a final volume of 10 mL. Protein concentrations were then determined with a bicinchoninic acid (BCA) assay [81]. 20-50 µg of total cellular protein were separated on a 10% SDS/polyacrylamide gel and transferred to a Hybond™ ECL nitrocellulose membrane (GE Healthcare). The membrane was then blocked for 1 hour with 7.5% Carnation instant non-fat dry milk powder dissolved in Tris-Buffered Saline Tween-20 (TBST) at room temperature. The membrane was then incubated overnight with rabbit anti-cPLA$_2$γ polyclonal antibody (a kind gift from Dr. Christina Leslie, University of Colorado [47]), diluted 1:1,000 in 7.5% bovine serum albumin (BSA) in TBST, washed three times with TBST, incubated with a goat anti-rabbit horseradish peroxidase conjugated antibody diluted 1:10,000 in 7.5% non-fat/TBST for 1 h, washed again three times, subjected to enzymatic chemiluminescence (ECL, GE Healthcare) and used to expose autoradiographic film.

Chromatin Immunopreciptiation Analysis

Cells used for ChIP were grown in two 150 mm dishes for each condition. Following TNF-α treatment, formaldehyde was added at a final concentration of 1% to crosslink the DNA and proteins, and the plates were shaken at room temperature for 10 min. 0.125 M of glycine was added to stop the crosslinking and the plates were shaken

for an additional 5 min. The cells were then scraped into 50 mL tubes and either flash frozen in liquid nitrogen for overnight storage or immediately centrifuged at 3000 rpm for 10 minutes before removing the medium and rinsing twice with PBS. The remaining pellet was resuspended in 1 mL of nuclei swelling buffer (5 mM PIPES, 85 mM KCl, 0.5% NP-40) and transferred to a 1.5 mL tube. The tubes were then centrifuged at 5000 rpm for 5 min at 4 °C and the pellet was resuspended in 1 mL of SDS lysis buffer (1% SDS, 10 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0). This solution was transferred to 4 mL conical tubes and incubated on ice. The chilled tubes were sonicated in ice five times each for 25 s with two min rests in between. The samples were then transferred to 1.5 mL tubes and centrifuged at 13000 rpm for 10 min and then transferred to 15 mL tubes. 100 µL of this solution was reverse crosslinked by addition of 4 µL of 5 mM NaCl and incubated at 65 °C for 2 hours prior to a phenol/chloroform extraction and analysis on a 1.6% agarose gel to verify that the samples were sonicated to an average of 500 bp. The remaining sample was diluted to 5 mL in ChIP dilution buffer (0.01% SDS, 1.1 % Triton X-100, 1.2 mM EDTA, 20 mM Tris-HCl pH 8.0), 50 µL of protein A-Sepharose beads were added for each mL of ChIP dilution buffer and the solution was precleared by rotating at 4 °C for 2 h. This solution was then centrifuged at 1000 rpm for 10 min and 1 mL of the supernatant was aliquoted into 1.5 mL tubes for each immunoprecipitation to be performed. 2 mg/mL of antibody to the indicated protein was added to the lysates and incubated overnight at 4 °C. Antibodies to the following human proteins were purchased from Santa Cruz with the organism of origin listed for each: rabbit c-Jun (sc-1694), rabbit ATF-2 (sc-187), rabbit p65 (sc-372), mouse p50 (sc-8414), rabbit RNA Polymerase II (sc-899), rabbit USF1 (sc-229), rabbit USF2 (sc-862),

mouse IgG (sc-2025), rabbit IgG (sc-2027). 60 µL of protein A-sepharose beads, blocked in bovine serum albumin (BSA) the night before, were added to the lysate using a wide-boar pipette tip and rotated at 4 °C for 2 h. The beads were pelleted by centrifugation at 1000 rpm for 2 min and supernatants other than the no-antibody control were discarded. The no-antibody control supernatant was kept as input. The beads were then washed with 1 mL of a low salt wash (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl) followed by a high salt wash (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl) and rotated at 4 °C for 5 min. The beads were subsequently washed with a LiCl wash (250 mM LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl), spun at 1000 rpm for 1 min and rinsed twice with Tris-EDTA. The protein-DNA complexes were obtained by eluting with 275 µL ChIP elution buffer (0.1% SDS, 0.1 M $NaHCO_3$), vortexing, shaking at 37°C for 15 min, centrifugation at 1000 rpm for 1 min and collection of the supernatant. At this point, 20 µL of 5 M NaCl was added to each tube, including the 500 µL of input taken as supernatant at an earlier step, and reverse crosslinked at 65 °C for 5 h. The samples were then treated with proteinase K (Qiagen) by addition of 10 µL 0.5 M EDTA, 20 µL 1 M Tris-HCl and 1 µL of 20 mg/mL proteinase K to each tube and incubated at 4 °C for 1 h. Finally, using a Qiaprep Spin Miniprep Kit (Qiagen), samples were diluted with 2 mL buffer PB, put through a column, washed with buffer PE and eluted in 100 µL Tris-EDTA. For the input sample, only 100 µL of the sample was used as opposed to the 500 µL of each of the others. The purified DNA was subjected to real-time PCR using primers specific to the enhancer/promoter of an intergenic region located 5’ of the $cPLA_2\gamma$ gene with primers specified in Table 2-4.

Overexpression of Transcription Factors

Transient transfection was performed similarly as above with modifications. 0.25 µg of each indicated transcription factor expression plasmid and 0.1 µg of the -114/-1 promoter/reporter construct were added to pcDNA-3.1 for a total amount of 2 µg of plasmid for each transfection. Overexpression of transcription factor proteins or their respective dominant negative forms was performed using a mammalian expression plasmid (pcDNA3.1) containing the coding region for the following transcription factors or their respective dominant negative forms: c-Jun or Tam67 [82], p65 or ΔTA, ATF-2 (kind gift from Dt. Alt Zantema, Leiden University Medical Center, Netherlands [83]) or A-ATF2 (kind gift from Dr. Charles Vinson, NIH [84]), or USF1 (kind gift from Dr. Jörg Bungert, University of Florida [85]) or A-USF (Dr. Vinson [86]). After the cells were rinsed and supplied with fresh medium, they were allowed to grow for 48 h prior to harvesting of total RNA for subsequent analyses. Overexpression of each transcription factor was also verified by immunoblot analysis.

Interferon Stimulating DNA

Oligodeoxynucleotides (ODNs) were synthesized based on the human TLR9-specific ODN, termed ODN-2006, with the sequence 5'-TCGTCGTTTTGTCGTTTTGTCGTT-3'. This sequence constructed with normal deoxyribonucleotides joined with a normal phosphodiester backbone is designated PO-2006. When synthesized with a phosphorothioated backbone, this sequence was designated as PS-2006. When the CG dinucleotides in this sequence were replaced by GC dinucleotides, the resulting oligonucleotides were designated as PO-GC or PS-GC. The indicated ODNs were added to the cells at 10 ng/mL and RNA was collected after 48 h of treatment.

Statistical Analyses

All graphs are plotted as the mean of the indicated number of experiments with error bars representing +/- SEM. A "*" is used to denote $p \leq .05$ as determined by a Student's t-test. For TNF-α treated samples, a one-tailed t-test was used to determine induction, while a two-tailed t-test was used in all other cases. A paired t-test was used for experiments in which samples were paired, such as with batch transfections and time-courses, while unequal variance was assumed for all other analyses.

Table 2-1. List of mouse primers for cytokines, chemokines, growth factors and cyclophilin.

Gene	Sequence
IL-4	GCG ACA AAA ATC ACT TGA GAG AG
	GCA CCT TGG AAG CCC TAC AG
IL-13	TTG CTT GCC TTG GTG GTC TC
	CCT CTG GGT CCT GTA GAT GGC
IL-1β	GCC TGT GTT TTC CTC CTT GC
	CAG TGC GGG CTA TGA CCA A
IL-2	CAC CCT TGC TAA TCA CTC CTC A
	CTG CTG TGC TTC CGC TGT AG
IL-3	TCC TGA TGC TCT TCC ACC TG
	CCA CTT CTC CTT GGC TTT CC
IL-6	TGG GAC TGA TGC TGG TGA CA
	TCA TTT CCA CGA TTT CCC AGA G
IL-10	CCA GTA CAG CCG GGA AGA CA
	TTT CTG GGC CAT GCT TCT CT
EOTAXIN	GCT CAC GGT CAC TTC CTT CAC
	GTG CTT TGT GGC ATC CTG G
KC	GCA CCC AAA CCG AAG TCA TAG
	CAG ACA GGT GCC ATC AGA GC
GM-CSF	CAA GTT ACC ACC TAT GCG GAT TT
	CAT TAC GCA GGC ACA AAA GC
TGF-β	AGA CAG CAA AGA TAA CAA ACT CCA C
	GCC GCA CAC AGC AGT TCT T
Cyclophillin A	GCG GCA GGT CCA TCT ACG
	GCC ATC CAG CCA TTC AGT CT

All sequences are listed in the 5'-> 3' direction for each primer with the forward strand primer listed first followed by the reverse strand primer.

Table 2-2. List of mouse primers for cyclooxygenase and lipoxygenase pathways.

Gene	Sequence
COX-1	GCT CAC AGG AGA GAA GGA GAT G
	GGA GCC CCC ATC TCT ATC ATA C
COX-2	GGC TTC GGG AGC ACA ACA
	CAA TGC GGT TCT GAT ACT GGA
mPGES-1	TTA GAG GTG GGC AGG TCA GAG
	CCA CTC GGG CTA AGT GAG AC
TXAS	AAG AGG AAG TAT CCC CAG AAC C
	TGT CCA GAT ACG GCA GAC CTT
L-PGDS	GTC AGT CAG AGG GCT GGT CAC
	GGA CTC TTA TCC TTC TCC TCA CG
PGIS	TTC CAT CCC TAT GCC ATC TTC
	TGA GCA GGG CGT GTA GGA
3-LO	CGG TTC CCA GAG TTG TCA TCC
	AAG CCC GCC AAG AAT GTT ATC
5-LO	TGT TCC CAT TGC CAT CCA G
	CAC CTC AGA CAC CAG ATG CG
FLAP	ATC AAG AGG CTG TGG GCA AC
	TAG ACC CGC TCA AAG GCA AG
Platelet 12(S)-LO	TGA CGA TGG AGA CCG TGA TG
	GCT TTG GTC CTT GGG TCT GA
12(R)-LO	GCA CTT TGG TCC TGA TGG C
	CCT CGT GGC TGT AGA ACT CC
Epidermal 12(S)-LO	CCT TTT TCC CCT GCT ACA GTT G
	CCC CAC CGA TAC ACA TTC CT
12/15-LO	AAA GGC ACT CTG TTT GAA GCG
	CAC CAA GTG TCC CCT CAG AAG
8-LO	CCT GCC CAG CGA TGA CAC
	CCG AAT GTG AGG AAT CAA TAG C

All sequences are listed in the 5'-> 3' direction for each primer with the forward strand primer listed first followed by the reverse strand primer.

Table 2-3. List of mouse primers for phospholipase A2s.

Gene	Sequence
PLA2G2C	CCA ACC CAT CTT GAA TGC CTA CC
	GGA GTT TGT CCC TGC CAC A
PLA2G2D	AAC CTG AAC AAG ATG GTC ACA CAC
	GGT GGG CAT AGC AAC AAT CAT
PLA2G2E	CAG TGG ACG AGA CGG ATT GG
	CAG GTT GTG GCG AAA GCA G
PLA2G5	CTA CTG CCT GCC GAG AAA CC
	ACA CAT CAG GAA TAC AGC AGA GG
PLA2G10	GAA ATA CCT CTT CTT CCC CTC C
	CAG GTG GCT TTA GCA CTT GG
PLA2G13	GGT GCC TCC ACT CAA TCT GC
	GCT GCC CGC TGA CTG TTC
PLA2G4A	CAG CCA CAA CCC TCT CTT ACT TC
	CGG CAT TGA CCT TTT CCT TC
PLA2G4B	GCA CAA GGA CCA CTA TGA GAA TC
	ACC ACC CTA AAA GTG CCC TC
PLA2G4C	CAC AAA CGC AGT CCC AAG G
	AGA CCC CTG CGA GGT ATG TG

All sequences are listed in the 5'-> 3' direction for each primer with the forward strand primer listed first followed by the reverse strand primer.

Table 2-4. List of human primers for real-time PCR.

Gene	Sequence
Cyclophilin A	CAT CCT AAA GCA TAC GGG TCC
	GCT GGT CTT GCC ATT CCT G
PLA2G4C hnRNA	TAC CCT TCT TCT TGT TCC CAC C
	ATC CAG AGA CCC CTG CGA G
PLA2G4C	CAC CTG GCT GAC TGA GAT GCT
	CGC AAA TGC CTG TTT TCT TC
hGH	GAA CCC CCA GAC CTC CCT
	CAT CTT CCA GCC TCC CCA T
ChIP enhancer	CTG GTC TCG GGT GCC TAA TG
	TCT GTG GTC CTC CTG CTT TCC
ChIP 3'UTR	TTG ACA CCA CCA TAA CTT CAC ACC
	TGG AGG ATT AGA GCA GAC GGC

All sequences are listed in the 5'-> 3' direction for each primer with the forward strand primer listed first followed by the reverse strand primer.

Table 2-5. List of primers used for promoter deletion analysis of the human cPLA$_2$γ enhancer/promoter.

Construct	Sequence
3.9	aag ctt TGG CTT CTT CCT CCG TCC
	aag ctt GCA GGA GGA CCA CAG AAG C
1.3	aag ctt TGG TGA TAC TCC TGC CTT GG
	aag ctt GCA GGA GGA CCA CAG AAG C
490	aag ctt ATC CGC CTG CCT CAG CCT CC
	aag ctt GGA AAG CAG GAG GAC CAC AG
288	aag ctt TGA ATA CTG GTC TCG GGT GCC TAA TG
	aag ctt GGA AAG CAG GAG GAC CAC AGA AGC

Letters in lower-case denote HindIII restriction sites. All sequences are listed in the 5'-> 3' direction for each primer with the forward strand primer listed first followed by the reverse strand primer.

Table 2-6. List of primers used for quick-change mutagenesis of the human cPLA$_2$γ enhancer/promoter.

Construct	Sequence
114	TAGCTCCGGGTGAGCTCTGG/AAGCTTGGGCTGCAGGTCGA
	TCGACCTGCAGCCCAAGCTT/CCAGAGCTCACCCGGAGCTA
del I	TGCCTAATGACAGAA/TAAGGAAGCCTGGAA
	TTCCAGGCTTCCTTA/TTCTGTCATTAGGCA
del II	TCACTAAGGAAGCCT/AGCCCTCCACGTGAT
	ATCACGTGGAGGGCT/AGGCTTCCTTAGTGA
del III	AAAGTCCCAGCCCTC/ATCCCACGGATGAAA
	TTTCATCCGTGGGAT/GAGGGCTGGGACTTT

The region that was removed by quick-change mutagenesis has been replaced with "/" in each of these primer sequences. All sequences are listed in the 5'-> 3' direction for each primer with the forward strand primer listed first followed by the reverse strand primer.

CHAPTER 3
MOUSE MODEL OF ALLERGIC ASTHMA

Introduction

Allergic Asthma

Asthma is a disease of chronic lung inflammation that affects roughly 5% of the world's population. Asthma can be divided into several groups including, but not limited to, allergen induced (extrinsic), exercise induced and occupational asthma [87]. Regardless of the type of asthma, an attack is characterized by airway hyper-responsiveness, bronchoconstriction and difficulty breathing. More specifically, proinflammatory cytokines are released in response to allergen or stress which cause inflammation of the bronchi and restricted airflow. This disease has predominantly been associated with a strong Th2 response involving cytokines such as interleukins 4 and 13 (IL-4 and IL-13). However, more recent studies have also associated chronic asthma with a Th1 response via tumor necrosis factor-alpha (TNF-α) release [88, 89]. This has been specifically linked to TNF-α release from alveolar macrophages as a result of allergen exposure as well as induced release in mast cells of the asthmatic lung [90]. Asthma sufferers also exhibit an upregulation of cyclooxygenase-2 (COX-2) and downstream production of prostaglandin E2 (PGE_2) [11, 91]. The upregulation of prostaglandins is predominantly associated with the early phase of asthma, whereas increased production of lipoxins corresponds with a decrease of prostaglandins and resolution of inflammation [18, 92, 93].

Characteristics of an allergic response

One of the primary risk factors for asthma is exposure to allergens that can trigger an allergic asthmatic response. In this context, the allergic response is characterized by

elevated serum IgE and an increase in eosinophils both circulating and in the lung [94, 95]. Initially, an allergen comes into contact with antigen presenting cells. The antigen presenting cells stimulate T cells, which in turn activate B cells to produce IgE. Once the IgE attaches to mast cells, they are primed for activation upon reexposure to the antigen. Reexposure to the antigen triggers an allergic response and subsequent inflammatory cascades are similar regardless of the stimulus, although they may vary in their severity.

Cytokine pathways leading to the production of lipid mediators

Cytokine effects range from proliferation or death at the cellular level to inflammation and fever on the organismal scale. Cytokines are categorized based on their role in the cell-mediated Th1 response versus the humoral Th2 response [96, 97]. Th1 cytokines include the tumor necrosis factors (TNFs), interferons (IFNs) and interleukin-2 (IL-2) and this is the primary response pathway for viruses, intracellular pathogens and cancer, while Th2 cytokines include IL-4, IL-10 and IL-13 and act in response to extracellular bacteria, parasites or other toxins [98]. The Th1 and Th2 pathways are not distinct from each other and, in fact, modulate each other in order to maintain a balance between them [99]. An imbalance between these two pathways in either direction can be harmful. A prolonged Th1 response and exposure to Th1 cytokines leads to tissue damage whereas the Th2 response is implicated in increased IgE and allergic reactions such as asthma [100, 101].

Inflammation

The initial physiological response to infection or tissue injury is inflammation. A defining event in this response is the liberation of eicosanoids, the bioactive lipid metabolites of arachidonic acid (AA). The eicosanoids play a pivotal role in

inflammation, ultimately mediating vasodilation, vascular permeability, bronchoconstriction, chemotaxis, and the transcription of pro-inflammatory enzymes as well as being involved in cancer [21-23, 102, 103]. This extensive family of fatty acids includes the prostaglandins (PGs), prostacyclin, thromboxanes, leukotrienes (LTs), hydroperoxyeicosatetranoic acids (HPETEs), hydroxyeicosatetraenoic acids (HETEs), lipoxins, resolvins and protectins [102, 103].

The hydrolysis of membrane phospholipids by the phospholipase A_2 (PLA_2) family of enzymes yields both lysophospholipids and AA which in turn produce platelet activating factor (PAF) and the eicosanoids, respectively [35, 104]. AA is further metabolized into various bioactive lipids by specific downstream enzymes. In addition to their roles as inflammatory mediators, the eicosanoids arbitrate many general cellular processes, such as cell differentiation [104], apoptosis [105, 106], lipid membrane integrity [107] and vascular homeostasis [108]. Therefore, their important role in general cellular processes and, in particular, in the inflammatory process underscores the relevance of this pathway and importance of these metabolites in driving the inflammatory and immune responses.

Airway inflammation is an important underlying factor in the pathogenesis of allergic bronchopulmonary aspergillosis (ABPA) [109-111] and asthma [112, 113]. These pathophysiological events are also characterized by the elevated production of eicosanoids, with the LTs and prostanoids regulating many aspects of airway inflammation and reactivity [114]. Recently, studies have focused not only on the release of histamine and acetylcholine, but also the impact of AA metabolites on the inflammatory cascade that predominates in asthmatic airways. Prostaglandins normally

maintain a balance in airway responsiveness; both PGD_2 and thromboxane A_2 are bronchoconstrictors, while PGE_2 and prostacyclin serve in bronchoprotection. On the other hand, the action of LTs appears to be principally pro-inflammatory, with LTB_4 possessing potent chemoattractive activity for neutrophils and eosinophils in the airways [115]. Furthermore, the cysteinyl leukotrienes (cysLTs), LTC_4, LTD_4 and LTE_4, elicit bronchoconstriction and increased endothelial membrane permeability leading to airway edema and enhanced mucosal secretion, which are also physiological hallmarks of asthma [116]. As a consequence, pharmacologic agents potentially important for the management of inflammatory diseases have been designed to target the eicosanoid-mediated elements of the inflammatory cascade; these include cysLT antagonists, zafirlukast, montelukast/Singulair and pranlukast, as well as the 5-lipoxygenase (5-LO) inhibitor, zileuton/Zyflof [19, 20]. However, even with their widespread use as a treatment regimen in the different forms of asthma, the effectiveness of anti-LT pharmaceuticals in the treatment and management of asthma remains controversial [117].

Therefore, we hypothesized that a more detailed evaluation of expression levels for the eicosanoid pathway enzymes in an animal model that involves both cell mediated (Th1) and humoral immunity (Th2) would be valuable in predicting other points in eicosanoid metabolism relevant to inflammation. In collaboration with the laboratory of Dr. Terrence Flotte, a model of allergic asthma was recently developed that employs crude *Aspergillus fumigatus* (*Af*) extract as the sensitizing and challenging agent evaluated in C57BL/6J mice. The *Af* sensitized mice develop a Th2 mediated allergic inflammatory response, including elevated levels of the Th2 cytokines IL-4, IL-5

and IL-13, increased total serum IgE, goblet cell hyperplasia and airway eosinophilia [70]. More specifically, an increase in *Af* specific IgE and IgG are found in the serum of sensitized mice, demonstrating an *Af* specific immune response [70]. In the present study, the gene expression levels of many of the enzymes involved in eicosanoid metabolism are analyzed by real-time RT-PCR after sensitization and challenge to the *Af* extract in C57BL/6J mice. These data demonstrate alterations in gene expression for a unique subset of eicosanoid pathway enzymes which may provide relevant alternative targets in the development of therapeutic regimens for allergic asthma and ABPA.

Results

Cytokine and Chemokine Induction in Mice Treated with Ovalbumin (OVA) or *Apsergillus fumigatus (Af)* Extract

Af crude extract was recently evaluated in normal C57BL/6J mice as an allergen in an animal model of allergic asthma or ABPA [70]. As a comparison to the more commonly used sensitizing antigen, OVA [118, 119], mice were either mock sensitized or sensitized to OVA or *Af* on days 0 and 14 and then, on days 28-30, airway challenged with the relevant antigen or PBS. Adjuvant was included with the OVA sensitization as an immune stimulant, as compared to *Af* extract alone, which contains natural immune activating epitopes. Previous reports demonstrate that *Af* sensitized mice develop a Th2 mediated response typical of asthma as indicated by increases in total serum IgE, goblet cell hyperplasia and airway eosinophilia [70]. Therefore, the gene expression of relevant Th2 cytokines was evaluated by comparing OVA and *Af* sensitized and challenged mice. First the gene expression of two Th2 cytokines, IL-4 and IL-13, was determined. Although a portion of this data was previously reported showing only the response to *Af* [70], it is repeated here for comparison to treatment

with OVA as well. Figure 3-1A demonstrates that the expression of IL-4 and IL-13 was significantly more pronounced in the animals treated with *Af* (IL-4, ~42 fold; IL-13, ~124 fold) than OVA (IL-4, ~5 fold; IL-13, ~25 fold). As the previous reports have shown, the *Af* extract elicits a stronger Th2 response, potentially functioning as a more potent natural stimulus for a model of allergic asthma.

The mRNA of several other cytokines (IL-1β, IL-2, IL-3, IL-6 and IL-10) (Figure 3-1B), growth factors (GM-CSF and TGF-β) and chemokines (eotaxin-1 and KC) (Figure 3-2) thought to be involved in the asthmatic response was also evaluated. The increases in many of these factors had been previously shown to occur following *Af* treatment [70], but included in Figures 3-1 and 3-2 are the comparisons to treatment with OVA as well as the other factors not previously reported such as IL-3, IL-6, GM-CSF and TGF-β. Increased mRNA levels in IL-4, IL-13, IL-6, IL-10, eotaxin-1, KC, GM-CSF and TGF-β were observed in *Af* sensitized mice as compared to the mock sensitized (PBS) animals. The mRNA induction in response to *Af* was significantly higher than in animals exposed to OVA for the IL-6 and eotaxin-1, whereas IL-10, KC, GM-CSF and TGF-β responded similarly to both sensitizing agents. Primer sequences for these experiments can be found in Table 2-1.

Steady State mRNA Levels of the Cyclooxygenase Family in *Af* Treated Mice

Having established a physiologically and environmentally relevant allergen, *Af*, changes in eicosanoid gene expression in the lungs of C57BL/6J mice with *Af*-induced asthma were evaluated next. Eicosanoid biosynthesis following the liberation of AA from membrane phospholipids bifurcates into two pathways (Figure 1-2). As illustrated, the left branch involves the metabolism of AA by cyclooxygenases (COXs) and specific

downstream synthases into prostaglandins, prostacyclin and thromboxane A2. The relative expression levels of COX-1 and COX-2, microsomal prostaglandin E synthase (mPGES-1), thromboxane A2 synthase (TXAS), lipocalin-type prostaglandin D synthase (L-PGDS) and prostaglandin I2 synthase (PGIS) involved in the metabolism of their respective prostanoids were analyzed (Figure 3-3 with primers in Table 2-2). Although there were specific upward trends observed for mPGES-1 as well as a downward trend for L-PGDS, none of these data reach statistical significance. These results imply that although there may be a significant increase in AA levels potentially derived from cPLA$_2$γ in response to *Af* sensitization/challenge, the flux of this metabolite is not likely to follow down the cyclooxygenase branch.

Steady State mRNA Levels of the Lipoxygenase Family in *Af* Treated Mice

The alternate fate of AA is peroxidation by the lipoxygenase (LO) family of enzymes leading to the production of HPETEs, which naturally breakdown to HETEs. The specific HPETEs produced depend on the LOs present in the cell at the time of AA release. 5-LO requires the cofactor 5-LO activating protein (FLAP), which is believed to aid in the delivery of AA to 5-LO [120]. The 5-LO metabolite, 5-HPETE, can be further metabolized to the potent inflammatory lipid mediators called leukotrienes (LTs), LTB$_4$ and the cysLTs (LTC$_4$, LTD$_4$) [114, 121]. The LTs have physiological roles in innate immune responses in the lung and in the pathology of inflammatory diseases, such as asthma, allergic rhinitis and atherosclerosis [114, 121]. To this end, several anti-LT drugs have been developed that target either 5-LO/FLAP or the cysLTs including zileuton or montelukast, zafirlukast and pranlukast, respectively [20]. Therefore, the intuitive hypothesis would be that 5-LO or FLAP might show altered gene expression in

our model of allergen sensitization and challenge. However, as Figure 3-4A illustrates, the gene expression levels for 3-LO, 5-LO and FLAP are not elevated in these animals.

Other members of the LO family were also evaluated and are listed with gene names with common names in parentheses [122, 123]: Alox12 (platelet 12(S)-LO) [124], Alox12e (epidermal 12(S)-LO) [125], Alox12b (12(R)-LO) [126], Alox15 (12/15-LO) with a human ortholog of ALOX15 (15-LO-1) [127], and Alox8 (8-LO) with a human ortholog of ALOX15B (15-LO-2) [128] (Figure 3-4B with primers in Table 2-2). Uniquely in mice, the epidermal-derived 12(S)-LO (e-12(S)-LO) is functionally expressed [125] while the only homologous human ortholog exists as an expressed pseudogene designated ALOX12P2 [129]. 12(S)-LO was induced 20 fold in the *Af* challenged mice (Figure 3-4B with primers listed in Table 2-2). There was also a significant increase (31 fold) in the expression of 12/15-LO as a result of *Af* sensitization/challenge and a 5 fold induction of 8-LO. Immunohistochemistry for 12/15-LO demonstrated that alveolar macrophages in mice stain positively for this enzyme with an increase in staining following antigen challenge (data not shown). This increase in staining of 12/15-LO in alveolar macrophages may be due, in part, to increased mRNA levels, as has been previously reported in mouse alveolar macrophages and human lung epithelial cells in response to IL-4 [130].

Steady State mRNA Levels of Phospholipase A2s

Secretory phospholipase A_2s (groups II, V, X, and XIII)

The aforementioned lipid metabolites and the PLA_2 family of enzymes which are responsible for the initial release of the primary metabolite, arachidonic acid (AA), all have important roles in inflammation and lung disease. Metabolically upstream of the previously measured enzymes lie the phospholipases (PLA_2s). The PLA_2 family is

composed of several groups of secretory PLA_2s and two groups of cytosolic PLA_2s, calcium-dependent group IV ($cPLA_2$s) and calcium-independent group VI ($iPLA_2$s). Figure 1-2 illustrates the eicosanoid pathway with the relevant PLA_2s as well as the remaining downstream enzymes whose gene expression levels were previously evaluated.

The secretory PLA_2s ($sPLA_2$s) are low-molecular-weight enzymes that require Ca^{2+} and have been shown to have roles in inflammation, host defense and atherosclerosis [131]. The gene expression levels evaluated by real-time RT-PCR for the $sPLA_2$s are illustrated in Figure 3-5. Only $sPLA_2$-IIE and $sPLA_2$-V levels were increased in the *Af* sensitized and challenged animals (Figure 3-5 with primers listed in Table 2-3). In contrast, *Af* resulted in a ~45% reduction in the basal expression of $sPLA_2$-XIII. The alteration of expression for these $sPLA_2$s may be of significant importance to future studies of *Af* as a complicating factor in ABPA [109-111] and asthma [112, 113].

Cytosolic phospholipase A_2s (group IV)

We next evaluated the expression levels of three members of the $cPLA_2$ family, $cPLA_2\alpha$, $cPLA_2\beta$ and $cPLA_2\gamma$ (Figure 3-6). We hypothesized that an increase in the expression of $cPLA_2\alpha$ would coincide with the development of allergic asthma. This hypothesis was based on data from Uozumi et al. [132], which showed $cPLA_2\alpha$-/- mice sensitized and challenged with OVA had a significant reduction of anaphylactic responses and bronchial reactivity to methacholine. Furthermore, the transcriptional activation of $cPLA_2\alpha$ mRNA expression has also been shown to be increased in response to other pro-inflammatory stimuli [133]. Interestingly, the expression of neither $cPLA_2\alpha$ nor $cPLA_2\beta$ increased; however, the relative expression of $cPLA_2\gamma$ did increase

significantly in *Af* sensitized and challenged mice and this result lead to the further studies of this gene (Figure 3-6 with primers listed in Table 2-3) as described in subsequent chapters.

Discussion

Eicosanoids constitute a diverse family of physiologically active fatty acids that play important roles in regulating airway inflammation and reactivity and are linked to the pathophysiology of asthma [114, 134]. The two major subsets of these bioactive lipids are the cyclooxygenase metabolites or prostanoids [134], including prostaglandins (PGs) and thromboxane A_2 (TXA_2), and the 5-LO-derived LTs [114], including LTB_4 and cysLTs. Both sets of these AA metabolites have been implicated in the inflammatory cascade that occurs in allergic responses and the asthmatic airways, representing both pro- and anti-inflammatory activities.

The goal of this study was to determine if enzymes in the eicosanoid pathway display distinct alterations in gene expression in an allergen sensitization/challenge model of asthma or ABPA, potentially highlighting novel therapeutic targets. To achieve this, two approaches were employed, the use of quantitative real-time RT-PCR to address reproducible changes in gene expression levels and an allergen model where the sensitizing agent was more indicative of a naturally encountered allergen. While gene array analyses would allow for a broader analysis of many more genes, the use of real-time RT-PCR has distinct advantages in that logical assumptions for relevant genes are made upfront and this analysis affords the opportunity to immediately generate reproducible and statistically significant results.

Given the recent studies which have linked the ubiquitous fungus, *Aspergillus fumigatus* (*Af*), with an increased prevalence in asthmatics [113, 135], we employed a

mouse model which could mimic the pathology of allergen-induced asthma or ABPA using an *Af* extract in both sensitization and challenge. We first evaluated the responses in gene expression of a small subset of cytokines, growth factors and chemokines between sensitization/challenge with the complex *Af* extract and the classically employed allergen, OVA [118, 119]. We observed a significant difference in the expression of the T_h2 cytokines IL-4, IL-13, the acute phase cytokine IL-6 and the CC chemokine, eotaxin-1 (CCL11) in the *Af* animals as compared to OVA (Figures 3-1 and 3-2). These data further establish the relevancy of the *Af* model and demonstrate that the enhanced responses from critical mediators of asthma pathology like IL-4, IL-13 and eotaxin-1 may be more reflective of the human pathology [136] than sensitization with a non-clinical, simple protein allergen.

Another interesting yet conflicting result is the lack of any response from the enzymes on the COX branch of AA metabolism (Figure 3-3) which, at least at the transcriptional level, implies that the prostanoids may have a limited role in this allergy model. These data directed attention to the LO branch where the literature [114] and the significant pharmaceutical investment in anti-LT therapies would argue that alterations in the expression of either 5-LO or FLAP in response to *Af* could be expected. As with the prostanoid enzymes, no changes were observed with these enzymes or in the levels of 3-LO (Figure 3-4A).

Af sensitization/challenge did, however, cause a significant increase for the 12/15-LO, 8-LO and the epidermal-derived 12(S)-LO, which is unique to the mouse (Figure 3-4B). 12/15-LO has previously been shown to be induced in response to IL-4 [137], hence it is unsurprising that this gene is upregulated in this model. The mouse data for

12/15-LO induction are also in line with recent studies by Andersson et al. who demonstrated that 12/15-LO-/- mice in a systemic OVA sensitization model had impaired airway inflammation, reduced levels of eosinophils, lymphocytes and macrophages in BAL fluid along with lower levels of the T_h2 cytokines (IL-4, IL-5 and IL-13) [138]. The presented data and that of Andersson et al. [138] would imply that 12/15-LO (mouse homolog of human 15-LO-1) inhibition may provide an alternative therapeutic target for asthma and/or ABPA patients. This would also be consistent with studies that have demonstrated an increased level of 15-LO metabolites in asthmatics, where 15(S)-HETE levels in BAL fluid were elevated and associated with tissue eosinophil numbers, sub-membrane thickness and the observation that severe asthmatics presenting with persistent airway eosinophils exhibit high levels of 15(S)-HETE in BALF [139, 140].

8-LO is the mouse ortholog of human 15-LO-2 with 78% protein identity, while another ortholog was recently crystallized from coral [141]. Evidence exists implicating both enzymes as potential tumor suppressors [128], with the most convincing evidence in prostate cancer where this gene's expression is decreased or lost in high-grade prostate intraepithelial neoplasia and prostate cancer [142]. It has also been demonstrated that the 15-LO-2 gene may be negatively regulated by its own product [143], while being upregulated by aldosterone [144]. The significance of the induction of 8-LO seen in these mice is not clear, although it has been postulated that human 15-LO-1 and 15-LO-2 (or mouse 12/15-LO and 8-LO by association) may have opposing effects on inflammation by metabolizing linoleic and arachidonic acids respectively [145].

It is also interesting to note that the mouse specific, epidermal 12(S)-LO was significantly induced in the asthmatic mice. Although this gene does not exist as a functional enzyme in humans, its role here could be postulated based on it possessing a function similar to that of other 12(S)-LOs. The cells of the lung may be expected to preferentially express the epidermal form over the platelet form, thus explaining why this particular 12(S)-LO is induced while the platelet form is not. It is also possible that, in this instance, the downstream effect is specific to mice and thus it would be of interest to determine the effect on functional 12(S)-LOs in humans and human cell lines under similar conditions.

In addition to its role in the synthesis of pro-inflammatory products, 15-LO also participates, through transcellular biosynthesis, in the production of anti-inflammatory bioactive lipid mediators of resolution, the lipoxins [146, 147]. Lipoxins, LXA_4 and LXB_4 being the main components, are lipid mediators generated from AA that act to reduce inflammation and promote resolution. Lipoxins are generated through the combined action of 5-LO and 15-LO during cell-cell interactions.

The metabolic flux through the eicosanoid pathway initially requires the release of AA from membrane phospholipids, and the examination of the PLA_2s has provided new insights into the role these enzymes may play in ABPA and asthma. The data in Figure 3-5 have defined $sPLA_2$-IIE, $sPLA_2$-V and $sPLA_2$-XIII as secretory phospholipases with intriguing potential roles in allergen sensitization and challenge. Examined next were the cytosolic group IV PLA_2s, which have been directly linked to the liberation of AA as a consequence of the inflammatory response [36]. The vast majority of studies have focused on $cPLA_2\alpha$ [148], which responds to a variety of stimuli and is regulated both at

the transcriptional and post-translational levels. Most surprisingly, as shown in Figure 3-6, a significant induction of cPLA$_2$γ was observed with no effect on cPLA$_2$α or cPLA$_2$β. To date, the physiological roles for cPLA$_2$γ have not been concretely elucidated; this group IV family member was first identified by searching the EST database for orthologs to cPLA$_2$α, to which it has ~30% overall sequence identity [42, 48, 52, 54, 55]. This is the second observation of an increase in the expression of cPLA$_2$γ in a disease, with the first being an increase in response to a parasitic infection in mice [57]. In addition to its PLA$_2$ activities, Yamashita et al. [52] have also reported that this enzyme displays coenzyme A (CoA)–independent transacylation and lysophospholipid (LPL) dismutase (LPLase/transacylase) activities and have suggested a possible role in fatty acid remodeling of phospholipids and the clearance of toxic lysophospholipids.

Based on this evidence, it is hypothesized that the coupled induction of both cPLA$_2$γ and 15-LO in this mouse model of allergic asthma or ABPA provide for the generation of both arachidonic acid and 15-HETE as potent mediators in the pathology of asthma and/or ABPA. With this rationale, it was important to further evaluate the regulation of cPLA$_2$γ, as shown in the subsequent chapter. An important component missing from the animal studies using whole lung is the knowledge of the cells expressing cPLA$_2$γ during the development of the allergic response. Unfortunately, attempts to localize the expression of cPLA$_2$γ in the lung by immunohistochemistry and *in situ* hybridization were unsuccessful.

In summary, efforts were designed to demonstrate the effectiveness of an *Af* sensitization/challenge mouse model in combination with real-time RT-PCR in the identification of unique genes with altered mRNA expression. To this end, a number of

genes in the eicosanoid pathway have been identified that display altered gene expression that may be associated with allergic asthma and ABPA. The results have also helped to highlight a cPLA$_2\gamma$➔15-LO axis where the downstream metabolites can act as potentially important mediators in the inflammatory response in these diseases.

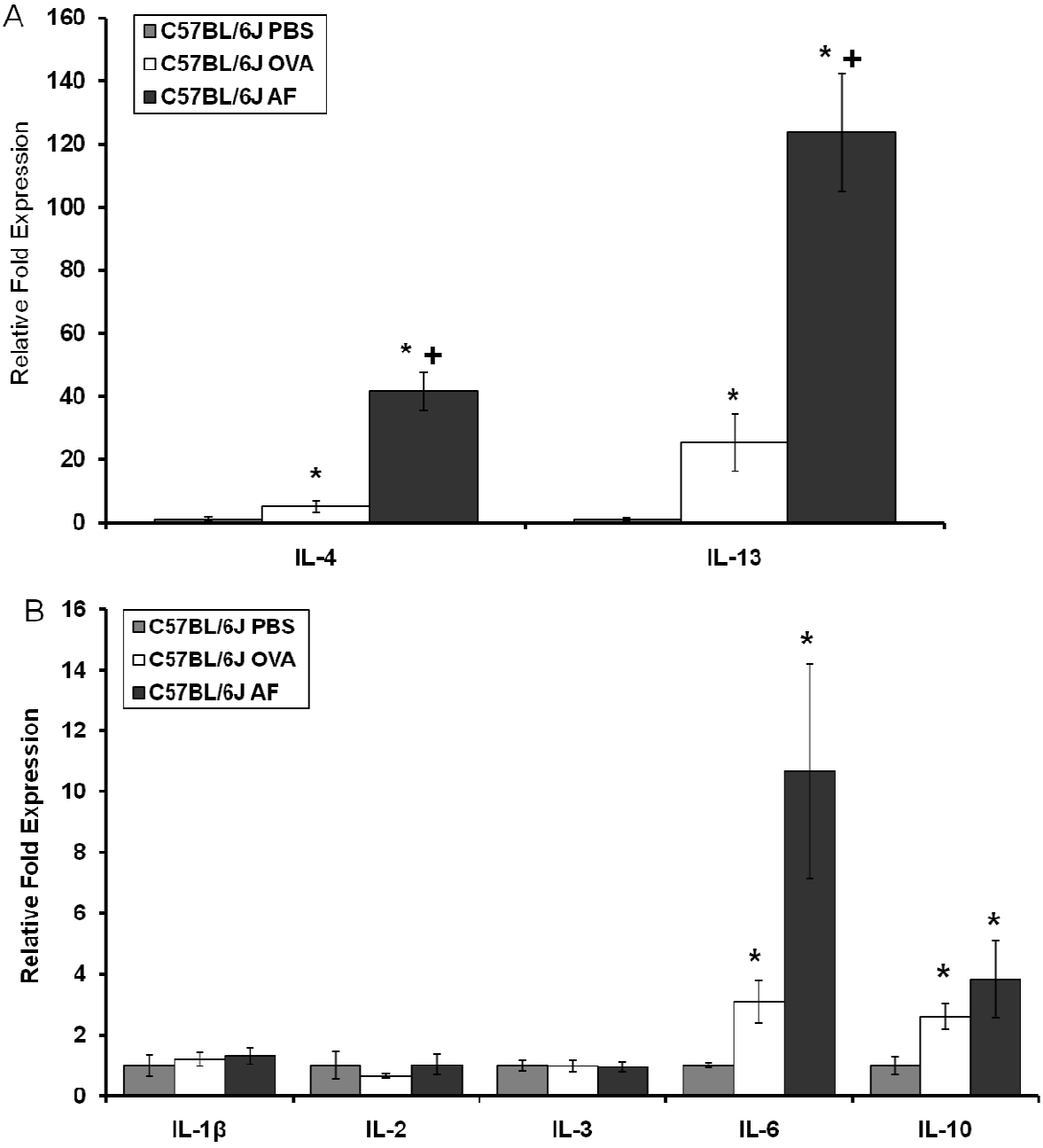

Figure 3-1. Comparative cytokine analysis in OVA (OVA) and *Aspergillus fumigatus* (*Af*) sensitization and challenge in C57BL/6J mice. Gene specific real-time RT-PCR analysis was performed on RNA extracted from whole lungs. Graphs represent steady state mRNA levels of A) IL-4 and IL-13 and B) IL-1β, IL-2, IL-3, IL-6 and IL-10, with cyclophilin A used as an internal control. Data points represent the means of ΔΔCT +/- SEM ($n \geq 3$). * and + indicate $p \leq 0.05$ as compared to PBS and OVA sensitized and challenged mice, respectively.

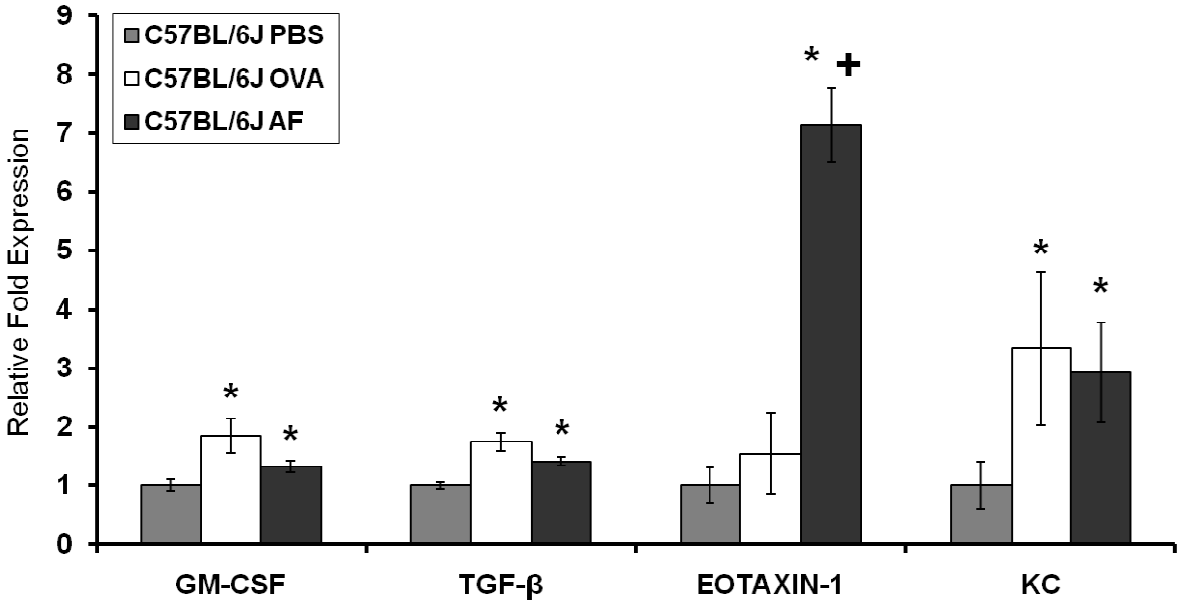

Figure 3-2. Comparative growth factor and chemokine analysis in OVA (OVA) and *Aspergillus fumigatus* (*Af*) sensitization and challenge in C57BL/6J mice. Gene specific real-time RT-PCR analysis was performed on RNA extracted from whole lungs. Graphs represent steady state mRNA levels of growth factors, GM-CSF and TGF-β, and chemokines, eotaxin-1 and KC, with cyclophilin A used as an internal control. Data points are the means of ΔΔCT +/- SEM ($n \geq 3$). * and + indicate $p \leq 0.05$ as compared to PBS and OVA sensitized and challenged mice, respectively.

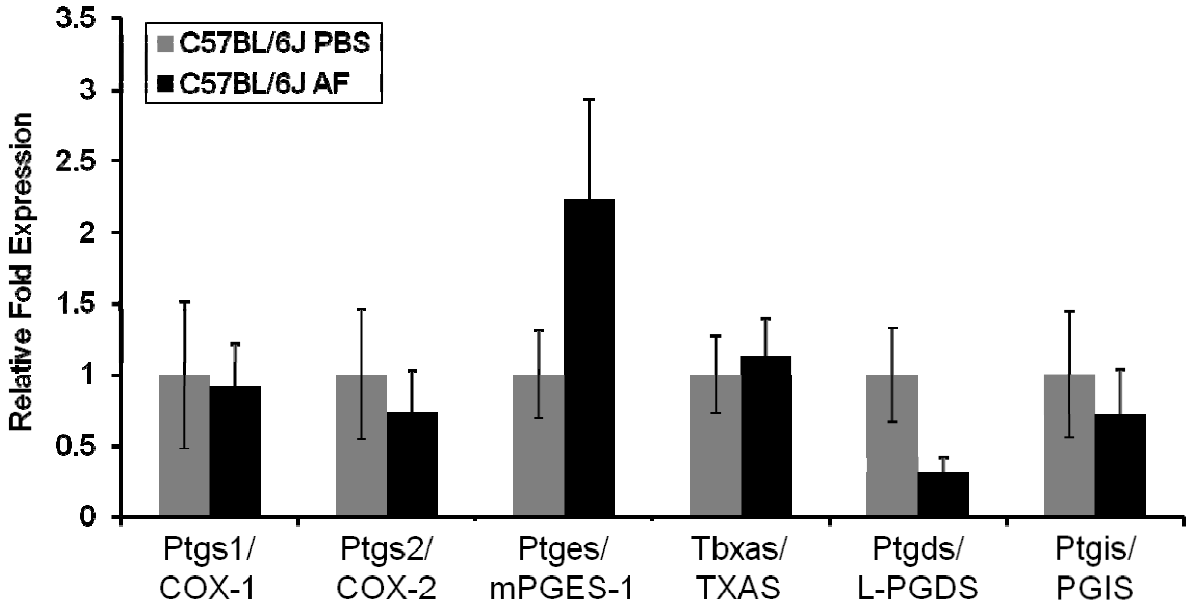

Figure 3-3. Gene expression analysis of enzymes involved in prostanoid synthesis in *Af* sensitized and challenged C57BL/6J mice. C57BL/6J mice were sensitized and challenged with PBS or *Af* as described in Methods and RNA was extracted from whole lungs. The graph represents steady-state mRNA levels of COX-1, COX-2 and mPGES, thromboxane A_2 synthase (TXAS), L-PDGS and prostacyclin synthase (PGIS) as determined by real-time RT-PCR. Genes are denoted by mouse and human homologs (mouse/human). Data points are the means of ΔΔCT +/- SEM ($7 \leq n \leq 10$).

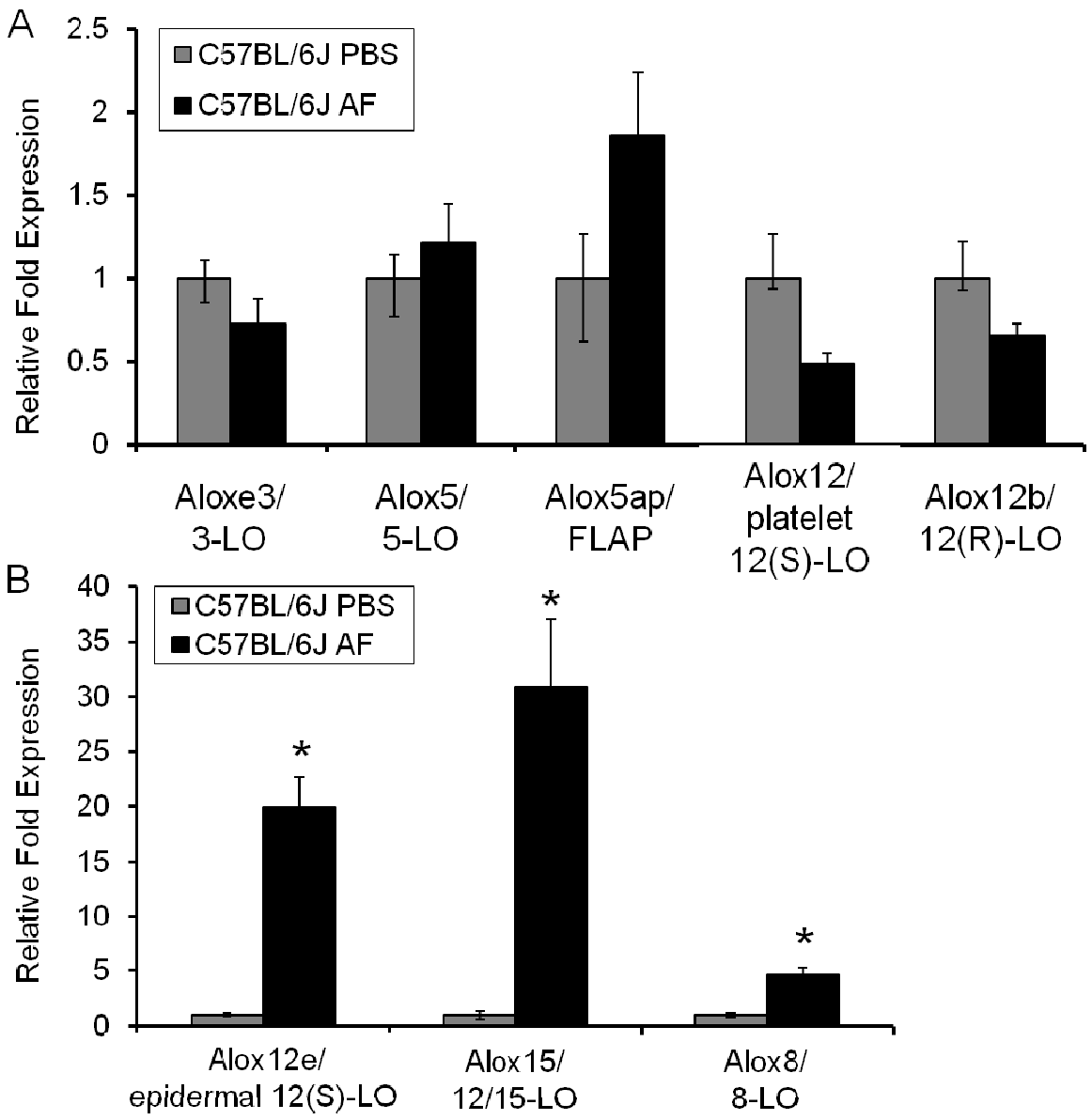

Figure 3-4. Gene expression analysis of lipoxygenase family members in sensitized and challenged C57BL/6J mice. C57BL/6J mice were sensitized and challenged with *Af* as described in Methods and RNA was extracted from whole lungs. A) The graphs represent steady state mRNA levels of 3-LO, 5-LO and FLAP as determined by real-time RT-PCR. Data points are the means of ΔΔCT +/- SEM (n=7-10). B) Steady state mRNA levels of platelet 12(S)-LO, epidermal 12(S)-LO, 12(R)-LO, 12/15-LO and 8-LO. Genes are denoted by mouse and human homologs (mouse/human). Data points are the means of ΔΔCT +/- SEM ($7 \leq n \leq 10$). * indicates $p \leq 0.05$ as compared to PBS.

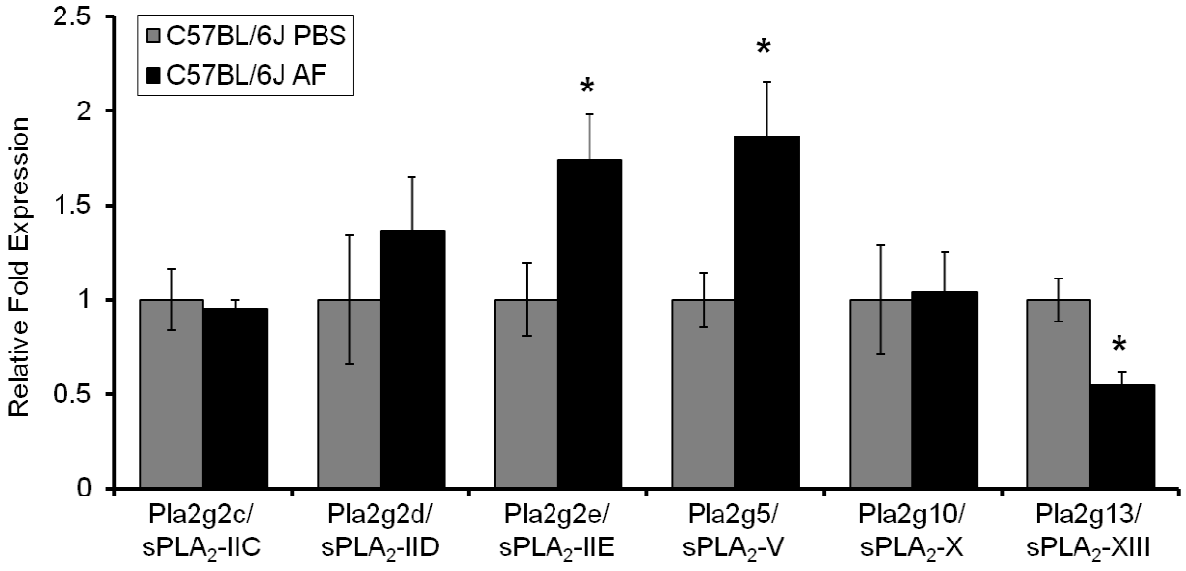

Figure 3-5. Secretory phospholipase A_2 gene expression levels in sensitized and challenged C57BL/6J mice. C57BL/6J mice were sensitized and challenged with PBS or *Af* as described in Methods and RNA was extracted from whole lungs. The graph represents steady-state mRNA levels of group II PLA_2s, $sPLA_2$-IIC, $sPLA_2$-IID and $sPLA_2$-IIE, as well as group V, X and XIII $sPLA_2$s as determined by real-time RT-PCR. Genes are denoted by mouse and human homologs (mouse/human). Data points are the means of ΔΔCT +/- SEM ($7 \leq n \leq 10$). * indicates $p \leq 0.05$ as compared to PBS.

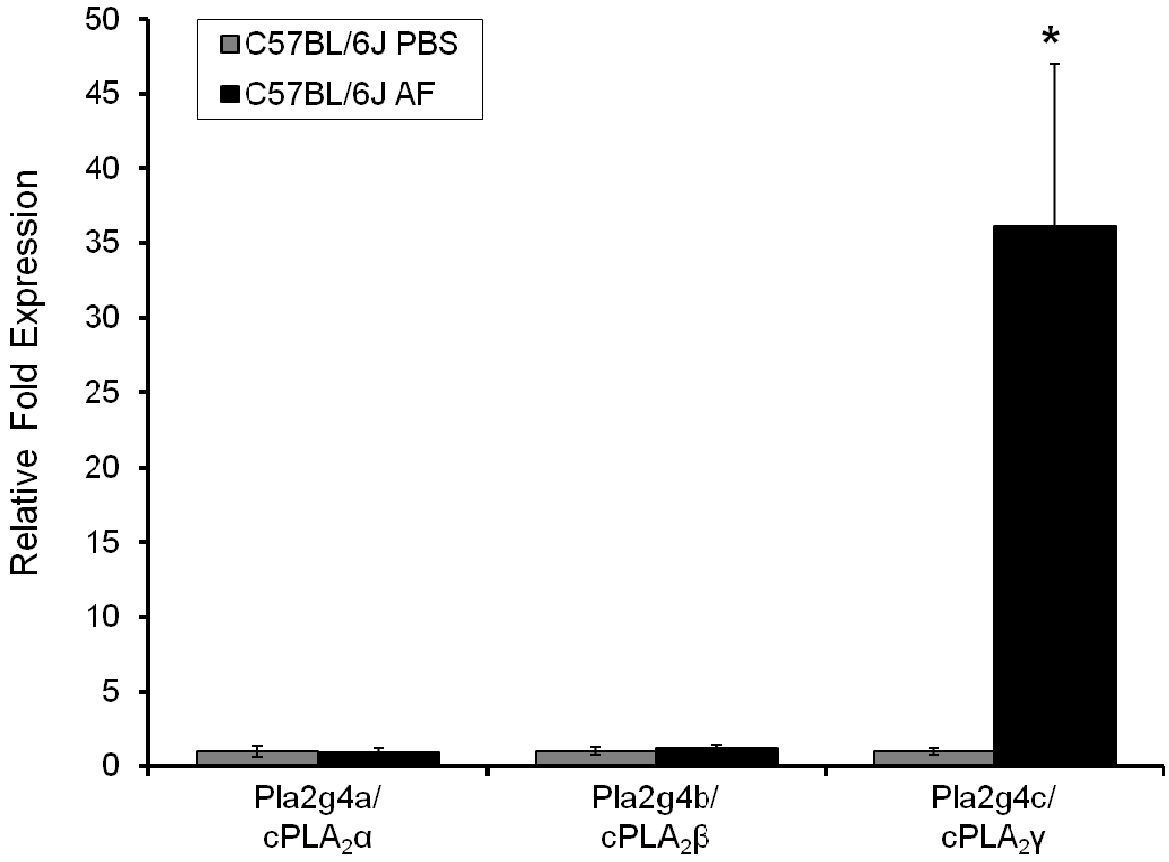

Figure 3-6. Cytosolic phospholipase A2 gene expression levels in sensitized and challenged C57BL/6J mice. C57BL/6J mice were sensitized and challenged with PBS or *Af* as described in Methods and RNA was extracted from whole lungs. The graph represents steady-state mRNA levels of group IV PLA_2s, $cPLA_2\alpha$, $cPLA_2\beta$ and $cPLA_2\gamma$ as determined by real-time RT-PCR. Genes are denoted by mouse and human homologs (mouse/human). Data points are the means of ΔΔCT +/- SEM ($7 \leq n \leq 10$). * indicates $p \leq 0.05$ as compared to PBS.

CHAPTER 4
CYTOSOLIC PHOSPHOLIPASE A2 GAMMA

Introduction

Regarding the regulation of group IVC phospholipase A_2 (cPLA$_2$γ), Lindbom et al. [46] first showed that, amongst a survey of 19 other phospholipases, cPLA$_2$γ was selectively induced by tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) in human bronchial epithelial (BEAS-2B) and nasal epithelial (RPMI 2650) cells. Additionally, Mancuso et al. showed a significant increase in cPLA$_2$γ protein in response to leptin in rat alveolar macrophages [54]. More recent studies have shown that, while cPLA$_2$γ is normally undetectable in normal mouse jejunal epithelium, parasitic infection by *Trichinella spiralis* in the intestine causes a significant increase in the expression of this phospholipase [57]. Experiments in Chapter 3 have also demonstrated that this phospholipase is significantly induced in an animal model of allergic asthma induced by sensitization and challenge with the ubiquitous fungus, *Aspergillus fumigatus*, in the lung. Additionally, it has been demonstrated by Nakae, et al. that TNF is a critical component of a mouse model of allergic mice involving ovalbumin [149]. It is hypothesized that the observed *in vivo* increases in cPLA$_2$γ expression are mediated by associated increases of TNF-α or other cytokines in the lung. This hypothesis is based on the known role of TNF-α in inflammation as well as the studies by Lindbom et al. [46].

Therefore, because increasing evidence points to a potentially critical role for this phospholipase in the inflammatory response as well as in the control of membrane phospholipid composition, the regulation of cPLA$_2$γ has been further characterized here. In the present studies, the cytokines responsible for cPLA$_2$γ gene activation are

analyzed, followed by a promoter deletion analysis to identify the cytokine responsive elements in the proximal promoter region. Subsequently, this region is used for computer analysis coupled with deletion of putative transcription factor consensus sequences to demonstrate the importance of potential transcription factor binding sites. Lastly, ChIP and over-expression are utilized to verify the specific transcription factors in a functionally relevant manner. These experiments demonstrate that TNF-α-dependent $cPLA_2\gamma$ gene regulation is mediated by complexes of activating transcription factor 2 (ATF-2) and c-Jun, p65/RelA, and upstream stimulatory factors 1 and 2 (USF1/USF2) each binding to a proximal enhancer/promoter region in close juxtaposition to each other to allow for transcriptional activation of this gene.

Tumor Necrosis Factor-Alpha (TNF-α)

TNF-α has been shown to be both pro- and anti-inflammatory. Studies in both wild-type and TNF-α knockout mice have strongly implicated TNF-α in the allergic asthma in OVA treated mice [149]. Following binding of TNF-α to the TNF receptor, several potential signaling pathways are activated. Activation of TNF receptor-associated death domains may lead to apoptosis [150], while activation of MAP/ERK kinases (MEK) or IκB kinase may lead to activation of gene transcription [151]. Activation of MEK, and subsequently, JNK or p38 MAPK can lead to phosphorylation and activation of transcription factors such as c-Jun and ATF-2 [152, 153]. Activation of IκB kinase (IKK) leads to phosphorylation and degradation of IκB, which in turn releases the NF-κB complex into the nucleus to drive transcription.

Transcription Factors (ATF-2/c-Jun, p65, and USF)

Several transcription factors have been found to be activated and interact in response to TNF-α, including p65, ATF-2 and c-Jun [154, 155]. Many examples of ATF-

2/c-Jun activation and interactions exist, with the best characterized occurring at the IFNβ and TNF-α promoters [156, 157]. c-Jun and ATF-2 can be activated by stimulation with TNF-α, which causes phosphorylation of c-Jun and ATF-2 kinases, JNK and p38 MAPK. These activated proteins heterodimerize and bind to CRE (5'-TGACGTCA-3') or AP-1 (5'-TGACTCA-3') sites on the DNA. In this case, the identified binding site (5'-TGACATCA-3') is identical to a CRE consensus sequence with the exception of one of the less conserved central bases. Based on thermodynamics, it has been recently postulated that the dimerization of these proteins may occur after DNA binding as monomers [158], although this finding is still novel and not thoroughly studied. The AP-1 family of transcription factors consists of the Jun and Fos subfamilies. Typically, the Jun subfamily can heterodimerize with either the Fos subfamily to bind AP-1 sequences or select ATF family members to bind CRE sequences. Each of these transcription factor families contain a basic zipper (bZIP) domain which binds DNA and a helix-loop-helix domain for dimerization.

The NF-κB family is made up of five proteins, p50, p52, p65/RelA, RelB and cRel, each containing an N-terminal Rel homology domain, which bind to a 10 bp κB consensus binding site (5'-GGGA/GNA/TC/TC/TCC-3'). In this case, the identified sequence (5'-GGAAAGTCCC-3') differs from the consensus in only the third and sixth base-pair. p65, cRel and RelB are the only family members to contain a C-terminal activation domain. Similar to the AP-1/CRE families of proteins, the NF-κB family can also be activated by TNF-α, but in a different manner. The NF-κB complex is retained in the cytosol by the IκB protein until IκB kinase (IKK) is activated by a stimulus such as

TNF-α. This results in phosphorylation of IκB, causing its degradation and release of NF-κB which can then traffic to the nucleus to bind DNA and regulate gene expression.

The upstream stimulating factor (USF) genes produce the ubiquitously expressed basic helix-loop-helix leucine zipper (bHLH-LZ) proteins USF1 and USF2, which bind to E-Box consensus sequences, 5'-CANNTG-3'. In this case, the identified sequence (5'-CACGTG-3') is a perfect match. The USF proteins are very highly conserved with nearly 60% homology between orthologs (USF1 and USF2) and 90-99% conservation between species at the DNA and protein levels for USF1 [159, 160]. USFs have also been implicated in the immune response and regulation of tumor suppressing genes, although the action of TNF-α has not been previously linked to the activity of the USF transcription factors.

cPLA$_2$γ Gene, Transcript and Protein Structure

The cPLA$_2$γ gene consists of 17 exons spanning 63 kb of chromosome 19 at position 19q13.33 and is illustrated in the top portion of Figure 4-7. The TSS and surrounding promoter region fully lack any TATA element, INR, DPE or other well defined promoter elements. The fully spliced mRNA transcript is 2541 bp with a 1624 bp coding region resulting in a protein of 541 amino acids. To date, no phosphorylation sites have been identified on the resulting protein, although lipid modification sites do exist. The cPLA$_2$γ protein has been shown to be farnesylated at the C-terminal end of the protein and it is associated with membrane fractions of cells. Even in the absence of this modification, the association of cPLA$_2$γ protein with the membrane fractions of cell lysates is resistant to high salt concentrations, implying that this modification is not wholly responsible for the membrane association [38, 48, 51, 52].

Results

cPLA$_2$γ Expression in Response to the antigen *Aspergillus fumigatus* (*Af*)

To date, very little has been identified about the transcriptional induction and regulation of cPLA$_2$γ gene expression [36]. The data from Chapter 3 demonstrate that cPLA$_2$γ can be induced at the mRNA level in mice sensitized and challenged with *Af*. What is not clear from this result is precisely which cells in the lung are causing this induction. Due to the fact that one of the major changes in the lungs of these mice was the infiltration of eosinophils into the lungs [70], a human eosinophilic leukemia cell line (EoL-1) was employed. As shown in Figure 4-1, when EoL-1 cells are treated with increasing concentrations of the same crude *Af* extract, cPLA$_2$γ mRNA levels are induced up to 15 fold by real-time RT-PCR.

cPLA$_2$γ Expression in Response to a Panel of Cytokines and Chemokines

Going a step beyond basal expression in the initial studies of cPLA$_2$γ, Lindbom et al. [46] first showed the induction of cPLA$_2$γ mRNA levels by TNF-α in human bronchial epithelial (BEAS-2B) and nasal epithelial (RPMI 2650) cells from a screen of 19 different PLA$_2$ types. Also within the respiratory system, the studies in a mouse model of allergic asthma in C57BL/6J mice have physiologically demonstrated that allergen sensitization and challenge can cause a significant induction of cPLA$_2$γ mRNA levels in intact lung (Figure 3-6).

Based on our research and the studies by Lindbom in bronchial epithelial cells, a human bronchial epithelial cell line, S9, was employed. This cell line is derived from IB3.1 cells which are SV40 T-antigen transformed cells from a cystic fibrosis patient and hence deficient for a functional cystic fibrosis transmembrane conductance regulator (CFTR) gene [78]. S9 cells were subsequently derived from the IB3.1 cells by AAV

mediated reintroduction of the wild type CFTR gene and so therefore are representative of a normal human bronchial epithelial cell [77]. S9 cells were exposed to *Af*, LPS, and a panel of cytokines. The data in Figure 4-2 demonstrate that *Af* is unable to induce cPLA$_2$γ in S9 cells, although IL-1β does induce the mRNA of cPLA$_2$γ (~3 fold) with TNF-α being the most potent stimulus (>6 fold). These data also show that cPLA$_2$γ does not respond to LPS in these cells. These data indicate that, although immune cells may be inducing cPLA$_2$γ in response to the allergen, the levels of this gene are also increased in epithelial cells as part of the downstream inflammatory response. Specifically, the TNF-α possibly originating from *Af*-mediated activation of resident alveolar macrophages would then mediate the induction of cPLA$_2$γ in the epithelial cells of the lung.

To further confirm this response within lung cells, two other cells lines were also evaluated. A human lung adenocarcinoma epithelial-like cell line (A549), a human fetal lung fibroblast cell line (HFL-1), and S9 cells were all treated with TNF-α for 12 or 24 hours and cPLA$_2$γ mRNA levels were analyzed by real-time RT-PCR. As shown in Figure 3-2, following 12 and 24 hours of TNF-α exposure cPLA$_2$γ mRNA levels are significantly increased in each of these cell lines by ~4-5 fold in A549 and HFL-1 cells and ~9-10 fold in S9 cells at each time point. In order to verify that this induction of mRNA was physiologically significant and corresponded to an increase in protein levels, A549 cells were treated with TNF-α for up to 48 hours. Figure 4-4 illustrates a representative immunoblot and corresponding densitometry confirming that cPLA$_2$γ protein levels are indeed elevated by ~3 fold by 24 hours following TNF-α exposure in A549 cells.

To characterize the temporal pattern of this induction, human S9 cells were exposed to TNF-α for increasing durations and changes in cPLA$_2$γ mRNA levels were analyzed by real-time RT-PCR (Figure 4-5). cPLA$_2$γ mRNA levels displayed a statistically significant induction by four hours and, by eight hours had reached six fold, implying that the 9 fold induction seen at 12 hours (Figure 4-3) was the maximal induction of cPLA$_2$γ mRNA by TNF-α. Based on this observation, a 12 hour treatment of S9 cells with TNF-α was used for many of the remaining studies.

An increase in mRNA steady state levels as measured by real-time RT-PCR may be a result of *de novo* transcription of the gene or due to increased stability of the mRNA transcript. To help elucidate the mechanism underlying the increase in cPLA$_2$γ mRNA levels, cPLA$_2$γ heterogeneous nuclear RNA (hnRNA) levels were measured [161]. Analogous to a nuclear run-on study, this method assesses the levels of intron-containing precursor mRNA by real-time RT-PCR as a direct evaluation of the level of *de novo* transcription. This was accomplished by using PCR primers within an exon and an adjoining intron that span the exon-intron boundary, thus making the amplicon specific for the hnRNA species (Table 2-4). These results (Figure 4-6) demonstrate that hnRNA levels are rapidly induced within 30 min and become maximal at 1 h with a ~9 fold induction. These data demonstrate that TNF-α mediated induction of the human cPLA$_2$γ gene occurs primarily due to *de novo* transcription.

Promoter Deletion Analysis of cPLA$_2$γ

Figure 4-7 illustrates the genomic structure of human cPLA$_2$γ on chromosome 19 as predicted from a comparison of the human cDNA sequence [38] comprised of 2541 bp (Accession NM_003706) to the human genome. This gene structure was derived from Ensembl (www.ensembl.org). The predicted transcriptional initiation site was

based on RACE analysis by Pickard et al. [42]. It is important to note that the cPLA$_2$γ promoter lacks a CAAT or TATA box and does not have a GC rich island in the promoter, often indicative of housekeeping genes. In addition, the lack of these canonical sites is sometimes associated with Initiator (INR) [162] and/or downstream promoter element (DPE) [163] sites, however neither of these exist in the cPLA$_2$γ locus. The cPLA$_2$γ locus contains 17 exons spanning ~63 kb, encoding a protein of 541 amino acids with a predicted molecular mass of 60.9 kDa.

Figure 4-7 also illustrates the various promoter constructs that were generated to identify the TNF-α response element. These elements were coupled to a human growth hormone (hGH) in an hGH reporter construct [161] at the HindIII cloning site immediately upstream of the transcription start site for hGH. These fragments were amplified by PCR using the primers listed in Table 2-5 with the 3.9 and 1.3 kb constructs being amplified directly from a BAC clone and the 490 and 288 bp constructs being amplified later from the 1.3 kb promoter fragment in the hGH plasmid. The 762 bp construct was made by a KpnI digestion of a restriction site immediately upstream of the cloning site and the other lying 588 bp upstream of the cPLA$_2$γ transcription start site. All of these fragments were confirmed by sequence analysis and the 3' ends of each fragment extends 174 bp into exon 1. Initially, the -3.9 and -1.3 kb promoter fragments were analyzed by northern analysis in response to both IL-1β and TNF-α with L7a used as a loading control (Figure 4-8). Both of these fragments were found to contain sequences that responded to cytokine treatment thus potentially constituting a cPLA$_2$γ inducible promoter. Therefore, three smaller fragments were constructed starting at -588, -316 and -114 bp relative to the cPLA$_2$γ transcription start site, also referred to by

their total bp sizes, including the first 174 bp of exon 1, as 762, 490, and 288, respectively. These remaining promoter deletion fragments were also confirmed by sequence analysis and evaluated following transient transfection in S9 cells. Figure 4-9 (top) depicts a representative northern blot analysis. To eliminate problems of transfection efficiency, each individual vector construct was batch transfected into cells, split 12 h later into plates for control and stimulus treatment, and then allowed to recover for 12 h followed by TNF-α exposure for 12 h. Total RNA was isolated from cells and the RNA was then fractionated on 1% formaldehyde/agarose gels, electrotransferred to a nylon membrane and then hybridized to ^{32}P-radiolabeled probes specific to hGH or the large ribosomal RNA subunit, L7a, as a loading control. These results demonstrate that the smallest construct, spanning from -114 to +174, retains TNF-α responsiveness. Densitometric data from three independent experiments are shown in Figure 4-9 (bottom), where the fold induction is reported relative to that of the untreated control cells normalized to a value of 1. This was necessary since the basal level of expression from each of these promoter deletion fragments is undetectable in S9 cells as well as other pulmonary cells that we have tested.

All of these promoter fragments included 174 bp of exonic sequence, so the next step was to determine whether this region contained important regulatory sequences by comparing the expression of the -114/+174 fragment to a fragment where this region had been deleted. The -114/-1 fragment of the cPLA$_2$γ promoter lacks any transcribed portion of the cPLA$_2$γ gene and was created by quick-change mutagenesis of the -114/+174 fragment with the primers listed in Table 2-6. A representative northern blot is provided in Figure 4-10 (top) with the corresponding densitometric data from three

independent experiments shown in Figure 4-10 (bottom). These data confirm that the minimal promoter construct from -114 to -1 retains the majority of the TNF-α-dependent transcriptional induction. Given our inability to accurately detect the basal expression, we are not at this time confident that there is any statistical difference between these two constructs (Figure 4-10), thus our further analyses focuses on the sequences 5' to the transcriptional initiation site utilizing the -114/-1 cPLA$_2$γ promoter fragment.

Characterization of the Proximal Promoter of cPLA$_2$γ Containing Enhancer Activity

To further characterize the exact nature of this proximal promoter sequence, we tested its ability to function as a stimulus-dependent enhancer. True enhancer elements, by definition, should be able to drive a heterologous promoter and function in both an orientation and position independent manner. We therefore generated constructs coupling the -114/-1 fragment in the forward and reverse orientation to a minimal viral thymidine kinase (TK) promoter that has low level expression in most cell types. S9 cells were batch transfected with each construct, exposed to TNF-α and evaluated by northern analysis for hGH expression. As shown in Figure 4-11, this fragment is able to drive the heterologous TK promoter and do so in an orientation-independent manner. Due to the fact that the plasmid is circular DNA, the element is essentially located before and after the promoter which is essentially a "circular argument" for position independence. We therefore conclude that this proximal promoter region essentially satisfies the definition of a stimulus-specific enhancer element.

Identification of Functional Transcription Factor Binding Sites within the cPLA$_2$γ Enhancer/Promoter

Potential consensus binding sites within the -114/-1 cPLA$_2$γ enhancer/promoter fragment for CRE, NF-κB and an E-Box were identified using the TESS software (www.cbil.upenn.edu/cgi-bin/tess/tess) designed to identify putative transcription factor consensus elements based on the TRASFAC database. Figure 4-12 illustrates the sequence of the -114/-1 cPLA$_2$γ enhancer/promoter fragment with potential regulatory factor consensus binding sites underlined and the sequences that were sequentially deleted in italicized bold text. Each of the respective cognate elements were deleted from the -114/-1 enhancer/promoter fragment by quick-change mutagenesis with the primers listed in Table 2-6 and evaluated following transient transfection in S9 cells by northern analysis (Figure 4-13). These results illustrate that deletion of the respective protein binding sites inhibits the TNF-α mediated induction to varying degrees. The potential effects of these deletions may be a reduction in the relative fold induction conferred by TNF-α treatment, or simply a broad reduction in total levels of transcription. Due to the relative lack of detectable basal activity by northern analysis, these potential effects could not be determined, therefore these fragments were also analyzed by real-time RT-PCR. Figure 4-14 is a real-time RT-PCR analysis of three independent experiments with the -114/-1 cPLA$_2$γ enhancer/promoter fragment illustrating that deletion of either the CRE or NF-κB sites results in a statistically significant reduction of both basal levels as well as relative fold inductions as compared to the wild type -114/-1 enhancer fragment. Although deletion of the E-Box (del III) does not affect the relative induction conferred by TNF-α (9.53 versus 9.18 fold), deletion of this site does have a

statistically significant impact on the basal levels of transcription from the cPLA$_2$γ enhancer/promoter conferring a 34% reduction in basal activity.

Chromatin Immunoprecipitation Analysis of the cPLA$_2$γ Enhancer/Promoter Region

To test the hypothesis that each of the respective consensus binding elements within the stimulus-dependent enhancer interacts specifically with a cognate transcription factor in the endogenous chromatin environment, we employed chromatin immunoprecipitation (ChIP) analysis. As identified prior, the -114/-1 region contains a cAMP response element (CRE), TGACATCA, nearly identical to the perfect consensus CRE sequence, TGACGTCA, with the only difference being the fifth base-pair substitution of an adenine for guanidine while maintaining the critical palindromic TGANNTCA. ATF-2/c-Jun heterodimers have been identified previously, with one of the most studied of these occurring in the IFN-β promoter [156] as well as in the TNF-α promoter [157]. This heterodimerization and binding to CRE sites have also been previously documented in response to TNF-α [157, 164]. Therefore the potential interaction of c-Jun and ATF-2 in response to TNF-α with the -114/-1 enhancer/promoter region by ChIP was evaluated followed by quantification by real-time PCR (Figure 4-15A). Three controls were utilized in the ChIP analyses to ensure specificity of the results: no primary antibody (noAb), nonspecific IgG (IgG) and ChIP analysis in an unrelated 3' untranslated region (3'UTR) of the cPLA$_2$γ gene with primers listed in Table 2-4. These ChIP results demonstrate the inducible interaction of both ATF-2 and c-Jun (~4 fold each relative to respective untreated controls) with the enhancer element as compared to the 3'UTR and the other control samples after 12 hours of TNF-α exposure. This data, along with the aforementioned studies regarding

ATF-2/c-Jun heterodimers strongly indicate that the CRE site in the cPLA$_2$γ promoter is most likely occupied and stimulated by such a heterodimer.

The NF-κB consensus element can potentially be occupied by a family of transcription factors, including p50, p52, p65 (RelA), c-Rel and RelB, that have a shared N-terminal Rel homology domain (RHD) involved in DNA binding and homo- and heterodimerization [165, 166]. Combinations of these family members are believed to bind as dimers to κB consensus sites in promoters and enhancers of target genes. Gene activation is mediated through the transcriptional activation domain which is a region present only in p65, c-Rel, and RelB. Therefore the -114/-1 enhancer/promoter region was analyzed by ChIP with a p65/RelA specific antibody with real-time PCR (Figure 4-15B). The results with p65/RelA-specific ChIP clearly demonstrate that p65 is inducibly associated with the human cPLA$_2$γ enhancer/promoter region following 12 hours of TNF-α stimulation (~21 fold relative to untreated control).

The E-Box sequence (5'-CANNTG-3') is found in the transcriptional regulatory region of a number of genes with a repertoire of cognate factors that share the evolutionary conserved basic-Helix-Loop-Helix-Leucine Zipper (b-HLH-LZ) motif [167]. This motif and the ability to interact with the E-Box element is common to the Myc family, including Mad, Max, and Mxi1, as well as the upstream stimulating factors, USF-1 and -2. USF-1 and -2 proteins are thought to be ubiquitously expressed and can function as transcriptional activators, through interaction with other co-activators and members of the pre-initiation complex as well as in the recruitment of chromatin remodeling enzymes [167]. The basic regions of USF-1 and -2 comprise the DNA binding domain whilst the HLH and LZ domains participate in homo- and

heterodimerization between USF-1 and -2, as well as other partners. We next evaluated the interaction of USF-1 and -2 with the cPLA$_2$γ enhancer by ChIP and found that both of these factors associated with the element in an inducible manner (Figure 4-15C, ~14 fold each relative to respective untreated controls). This data, along with supporting evidence of USF1/USF2 heterodimers in the literature strongly imply that this region is associated with such a dimer.

Due to the combined apparent presence of all of these factors at the enhancer/promoter region of cPLA$_2$γ following 12 hours of stimulation, this phenomenon was next evaluated immediately following stimulation in order to determine a potential temporal association of these factors. Firstly, the association of RNA polymerase II (Pol II) was analyzed at 15, 30, and 60 minutes following TNF-α treatment. Due to the rapid induction of cPLA$_2$γ transcription by 30 minutes as indicated by hnRNA data in Figure 4-6, it was expected that Pol II would rapidly bind to this element. The data in Figure 4-16 confirms this hypothesis with Pol II binding inducibly within 15 minutes of TNF-α exposure. This timing was also tested with p65 as well as its potential binding partner p50. The data in Figure 4-17 demonstrates that p65 is also inducibly associated with this region while it would appear that it is not partnered with p50. Next, the same time points were used to analyze USF1 association by ChIP and, as shown in Figure 4-18, USF1 is also found in this region within 15 minutes of exposure to TNF-α. It is assumed that the USF1 found here is similarly associated with USF2 as demonstrated in Figure 4-15. Finally, ATF-2 and c-Jun were analyzed at these earlier time points. Interestingly, no statistically significant binding of either of these factors was observed following up to one hour of TNF-α treatment (Figure 4-19). Despite this observation, when an additional

time point was added at two hours, there does appear to be an upward trend in the association with the promoter for both of these factors.

Effects of Overexpression of ATF-2/c-Jun, p65, and USF1 on the Proximal cPLA$_2$γ Enhancer/Promoter

To further demonstrate the functional relevance of c-Jun, ATF-2, p65, and the USFs to the regulation of the human cPLA$_2$γ enhancer/promoter region, each of these factors have been over-expressed in combination with the hGH reporter construct driven by the cPLA$_2$γ enhancer/promoter. Total RNA was isolated and treated with DNase I to remove potential plasmid and genomic contamination and samples were analyzed for hGH expression by real-time RT-PCR. The data in Figure 4-20 shows that overexpression of c-Jun (~4 fold), USF1 (~8 fold), or p65 (~30 fold) individually are sufficient to activate transcription from the cPLA$_2$γ enhancer/promoter with p65 being the most potent activator. The fold induction was calculated relative to cells transfected with an empty mammalian expression vector (pcDNA3.1) and no transcription factors (no TF) which was normalized to 1.

In order to further define specific roles for each of these factors, dominant negative (DN) constructs for each of these factors were utilized to determine their effects on both the uninduced levels of transcription as well as their impact on the TNF-α induction (Figure 4-21). The DN forms of c-Jun and p65, Tam67 and ΔTA, lack the activation and DNA binding domains, whereas the DN forms of ATF-2 and USF1/2, A-ATF2 and A-USF, lack not only the activation domains but have also had their basic DNA binding domains replaced with acidic residues. The USF DN, A-USF functions as a DN for both USF1 and USF2. Studies on the ATF-2 DN, A-ATF2, were performed separately from the other DN experiments and thus are shown with its own control in triplicate. The data

presented in Figure 4-21B demonstrate that only the DN for ATF-2, A-ATF2, is capable of reducing the basal level of transcription from the $cPLA_2\gamma$ enhancer/promoter (54% reduction). The only other DN that had an effect on basal transcription is the c-Jun DN, Tam67 (Figure 4-21A, 2.6 fold induction). It is hypothesized that the activation seen here is a result of Tam67 recruiting the endogenous heterodimerization partner, ATF-2, to the enhancer/promoter, which contains an activation domain capable of showing increased expression. Interestingly, A-ATF2 is also the only DN to have no effect on the observed relative fold induction by TNF-α. Each of the other DN proteins diminish the effect of TNF-α over basal transcription by at least half, with the p65 DN, ΔTA, completely blocking any effect of TNF-α on the ability of the $cPLA_2\gamma$ enhancer/promoter to activate transcription (Figure 4-21A,C,D).

Delineating Interplay between Transcription Factor Binding Sites by Co-overexpression

Having established c-Jun, p65 and USF1 as activators of the $cPLA_2\gamma$ enhancer/promoter and the DNs A-ATF2, Tam67, ΔTA and A-USF as potential repressors of either basal or induced activity, potential interplay between these factors was analyzed. Because ATF-2 was not confirmed to be able to activate transcription by itself, it was included as having possible inhibitory properties for these studies. Firstly, p65 was co-overexpressed with ATF-2, A-ATF2, Tam67, A-USF, or p50 (Figure 4-22). As seen here, the DNs for both ATF-2 and USF1 are capable of interfering with the induction seen by p65 overexpression. p50 was included here because the lack of observed association by ChIP does not completely rule out the possibility of a potential role for this protein. As shown p50 does not seem to provide any additive effect on the observed activation by p65, further indicating, in conjunction with the ChIP data that p50

does not play a role in this activation. Due to the weak activator properties of Tam67 (Figure 4-21A), it was unclear whether it would have any effect on the induction caused by p65 and, as shown, co-expression of p65 and Tam67 was not different than p65 alone.

Next, c-Jun was overexpressed with the factors ATF-2, A-ATF2, ΔTA and A-USF. As predicted, A-ATF2 was able to reduce the induction seen by c-Jun alone. This is less surprising than the effect of A-ATF2 on p65 due to the known interactions between ATF-2 and c-Jun. Also of note is that the induction seen by c-Jun is not dependent on USF as the DN, A-USF, has no effect on the induction imparted by c-Jun. Lastly, the induction seen by USF1 does not seem to be dependent on any of the other factors utilized in these studies.

Knockdown of ATF-2/c-Jun, p65, and USF1 by siRNA

Due to the established relevance of ATF-2/c-Jun, p65, and USF1 to the regulation of $cPLA_2\gamma$, attempts were made to individually knock down each of these factors. Despite attempts utilizing various transfection reagents (DharmaFECT from Dharmacon and HiPerFect from Qiagen) as well as siRNAs from each of these sources, no effect on $cPLA_2\gamma$ mRNA levels was observed. The efficiency of knockdown of transcription factors was measured by both real-time RT-PCR and immunoblot demonstrating a decrease in the targeted transcription factors at both the mRNA and protein levels. It is now believed that the efficacy of these siRNA experiments was likely hindered due to each factor contributing to a portion of the $cPLA_2\gamma$ transcriptional regulation. This cooperation in the transcriptional regulation is supported by overexpression studies in Figure 4-22.

Discussion

Group IVC phospholipase A2 ($cPLA_2\gamma$) has been implicated in cellular functions ranging from alteration of membrane composition to a possible critical role in the liberation of bioactive lipids that mediate the inflammatory response. Furthermore, recent studies have associated either SNPs or chromosomal deletions of $cPLA_2\gamma$ with schizophrenia [60, 63] or human gliomas [64, 168], respectively. With regards to membranes, the $cPLA_2\gamma$ protein is found to be membrane associated with either the ER/Golgi [48, 49] or mitochondrial membranes [51] in human cells. The constitutive association of $cPLA_2\gamma$ to membrane phospholipids coupled to the protein's ability to catabolically alter membrane composition may play an important role in the regulation of integral membrane protein occupancy, membrane fluidity and the formation of lipid rafts [169].

As shown in Figure 4-1, $cPLA_2\gamma$ mRNA can be induced in human eosinophils-like cells by a physiologically relevant antigen, *Af*. In addition to this direct immune response, immune cells produce an array of cytokines, some of which were examined in Figure 4-2. As demonstrated, the levels of $cPLA_2\gamma$ mRNA can also be induced in response to pro-inflammatory stimuli, such as TNF-α and IL-1β, thus further connecting the physiological role of $cPLA_2\gamma$, through its effects on downstream eicosanoid production, to inflammation. Additionally, to date $cPLA_2\gamma$ has been associated with two pathologies with relevant immunological responses. This phospholipase is induced in response to the parasite *Trichinella spiralis* in mouse jejunal epithelium [57] and from our studies in mouse lungs in response to airway sensitization/challenge by the allergen *Aspergillus fumigatus* (Figure 3-6). In each of these conditions, the induction of $cPLA_2\gamma$ occurred in association with an increase in cytokine levels such as TNF-α. These data

further imply that the regulation of the cPLA$_2$γ gene may be closely associated with the inflammatory response. Therefore, it was hypothesized that the TNF-α-dependent induction of this phospholipase and thus the underlying regulatory mechanisms will be of significant importance to events leading to tissue inflammation. The examination of other pulmonary cells lines in Figure 4-3, as well as studies in nasal epithelia by Lindbom, et al. and intestinal epithelium by Brown, et al. help to substantiate the importance of this gene across tissues with different inflammatory stimuli [46, 57]. The data in Figures 4-4, -5, and -6 also demonstrate that this induction is both transcriptional and results in a concomitant increase in cPLA$_2$γ protein levels.

The subsequent efforts shown here have focused on the identification of regulatory elements in the proximal promoter that mediate cytokine induction. The relevant sequences have been narrowed down to 114 base pairs immediately upstream of the cPLA$_2$γ transcription initiation site (Figures 4-8, -9, -10) and it has been demonstrated that this region can function as a stimulus-dependent enhancer element (Figure 4-11). Three separate putative consensus sites have been identified, CRE, NF-κB and E-Box, by computational analysis (Figure 4-12) and their functional relevance has been verified through mutagenesis (Figures 4-13, -14). The transcription factor occupancy of this region has been determined by ChIP analyses, demonstrating the TNF-α-dependent inducible association of ATF-2/c-Jun, p65/RelA and USF1/USF2 (Figure 4-15). While p65, USF1 and RNA Pol II are all recruited to this region within 15 minutes of TNF-α treatment (Figs. 4-16, -17, -18), c-Jun and ATF-2 may be arriving slightly slower to participate in this induction (Fig. 4-19). Many of these factors have previously been shown to be involved in TNF-α signaling pathways. ATF-2 and c-Jun

are known to heterodimerize and bind DNA to activate transcription of genes such as IFNβ and TNF-α, while the NF-κB pathway is the prominent target of TNF-α. The involvement of USF proteins is not well characterized in response to TNF-α, although, due to the ubiquitous nature of this family, it is not particularly surprising.

Furthermore, the functional importance of these transcription factors to the induction of the $cPLA_2\gamma$ enhancer/promoter was substantiated by over-expression. The data in Figure 4-20 show that overexpression of select transcription factors can activate the minimal $cPLA_2\gamma$ enhancer/promoter. The one exception to this is overexpression of ATF-2. It has been argued that the overepxression of ATF-2 is only mildly effective as an analytical tool due to a requirement of phosphorylation by JNK and p38 MAPK [170], therefore this may explain why no additional activity was seen with overexpressed wild-type ATF-2.

Additionally, the requirement for each of these factors in the TNF-α induction of the $cPLA_2\gamma$ enhancer/promoter was analyzed by overexpression of dominant negative forms of these transcription factors. The data in Figure 4-21 demonstrate that, although c-Jun and USF1/2 are contributing to the induction by TNF-α, p65 appears to be essential for this induction to occur. Surprisingly, only ATF-2 appears to be required for normal basal transcription to occur through the $cPLA_2\gamma$ enhancer/promoter. The involvement of ATF-2 in this basal activity is supported by the fact that only the c-Jun dominant negative can increase basal activity, presumably by acting as an activator by recruiting endogenous ATF-2 to the $cPLA_2\gamma$ enhancer/promoter. The potential for Tam67 to function as an activator has been previously documented [171].

Lastly, these transcription factors were analyzed in relation to each other to determine possible interactive effects of potential activating factors with potential inhibitory factors (Figure 4-22). It is believed that the increased activity of the cPLA$_2$γ enhancer/promoter by transcription factor overexpression is not identical to the effect of TNF-α. The impact of p65 on the cPLA$_2$γ enhancer/promoter appears to be at least partially dependent on the functional activity of the neighboring transcription factors as demonstrated by co-expression of DNs for ATF-2 and USF1 and the resulting ~50% reduction in the p65 dependent increase in cPLA$_2$γ enhancer/promoter activity. Whereas, the effect of c-Jun appears to be only dependent on its binding partner, ATF-2. Finally, the action of USF is independent of the other factors based on the observation that this induction cannot be blocked by any dominant negative proteins even though A-USF can reduce p65 induction, as already noted.

Based on the above data, it is clear that TNF-α can induce the cPLA$_2$γ gene via an enhancer/promoter element which dependent on the interplay of c-Jun, p65, and USF1/2. These data also support the idea that ATF-2 may be involved in basal transcription from the identified enhancer/promoter element, although this hypothesis needs to be investigated further. USF1/2 have previously been implicated in chromatin remodeling and it is believed that this may explain their tacit interactions with the other members of the cPLA$_2$γ regulatory machinery [172, 173].

Overall, the regulatory data presented here are important to the understanding of this unique phospholipase's role in the inflammatory response. This data will be crucial to understanding this enzyme's function in both the generation of bioactive lipids and its effects on membrane composition. The similarities between the transcriptional

regulation of cPLA$_2$γ and many other components of the inflammatory cascade, such as IFNβ and TNF-α [174-176], further support the importance of this gene in this cascade. It is our hope that the newly established transcriptional regulation of cPLA$_2$γ will lead to a new target for therapeutics for inflammatory disease and mitigation.

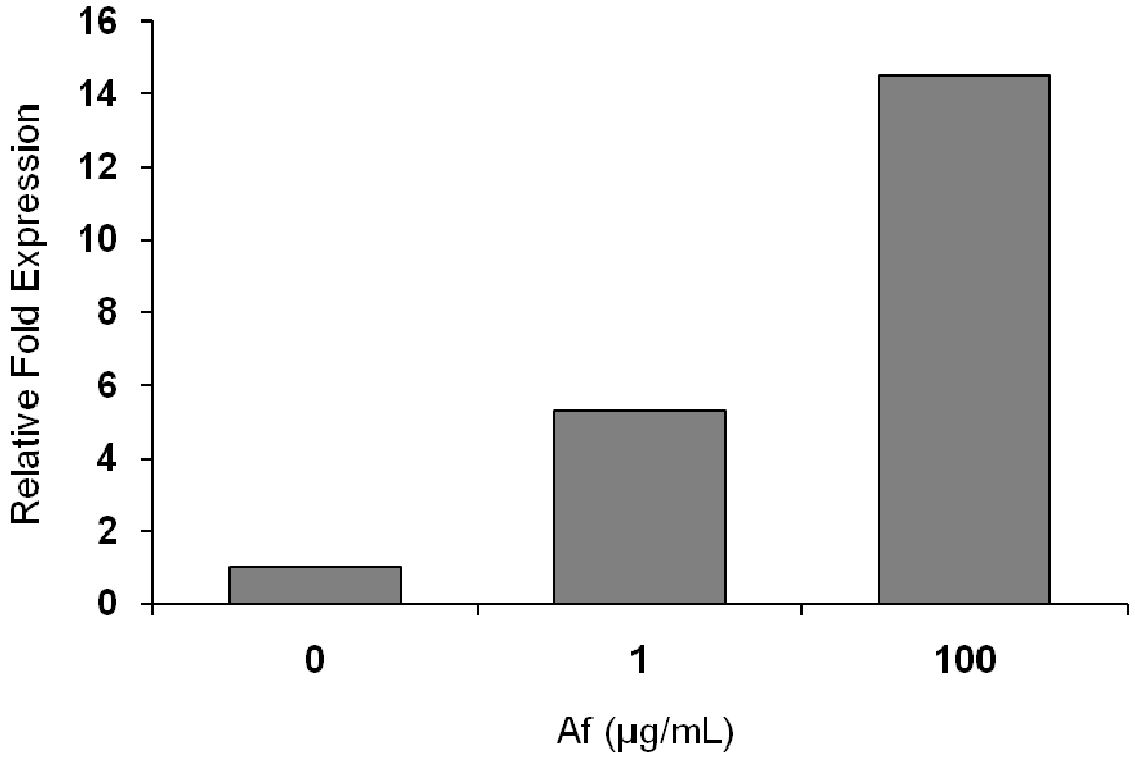

Figure 4-1. Evaluation of steady state mRNA levels of $cPLA_2\gamma$ in human eosinophils. Real-time PCR of $cPLA_2\gamma$ in response to Af in the eosinophil cell line, EoL-1. EoL-1 cells were treated with the indicated amounts of Af and steady state mRNA levels were analyzed by real-time RT-PCR. Data points are the means of ΔΔCT.

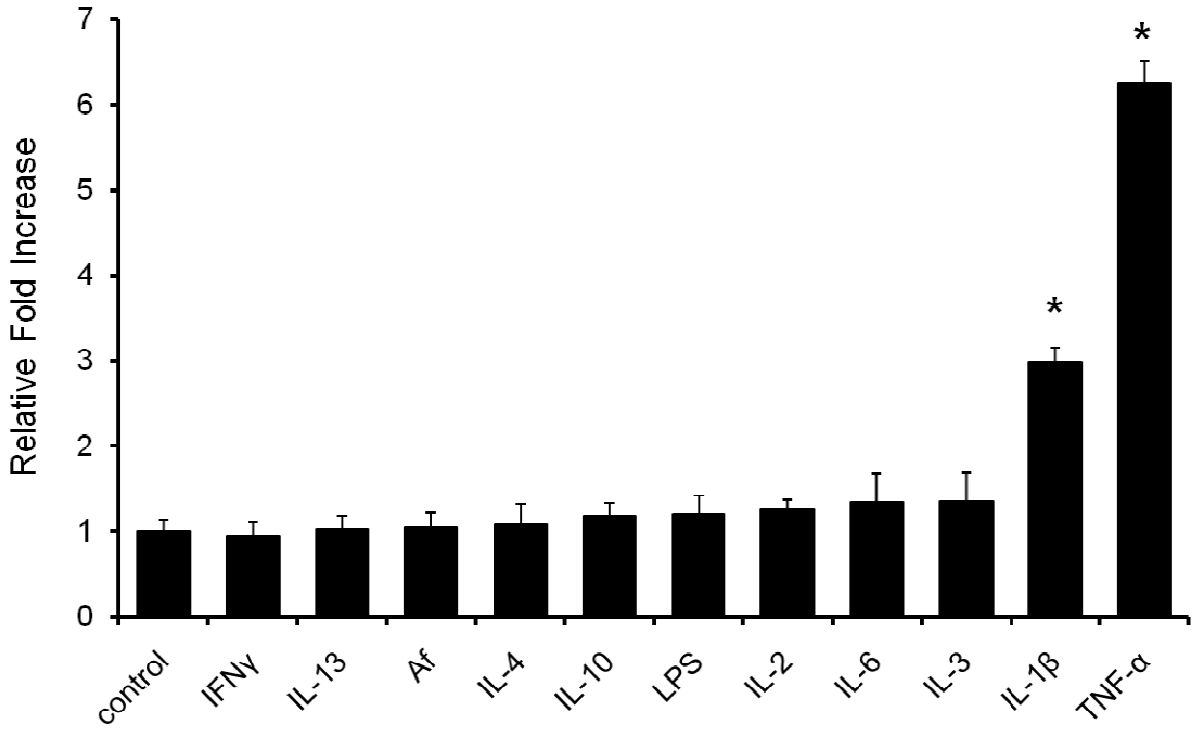

Figure 4-2. Evaluation of steady state mRNA levels of $cPLA_2\gamma$ in pulmonary cells in response to a panel of cytokines. Real-time RT-PCR of $cPLA_2\gamma$ in S9 cells in response to the indicated cytokines. Data points are the means of ΔΔCT +/- SEM (n=3). * indicates $p \leq 0.05$ as compared to the untreated control.

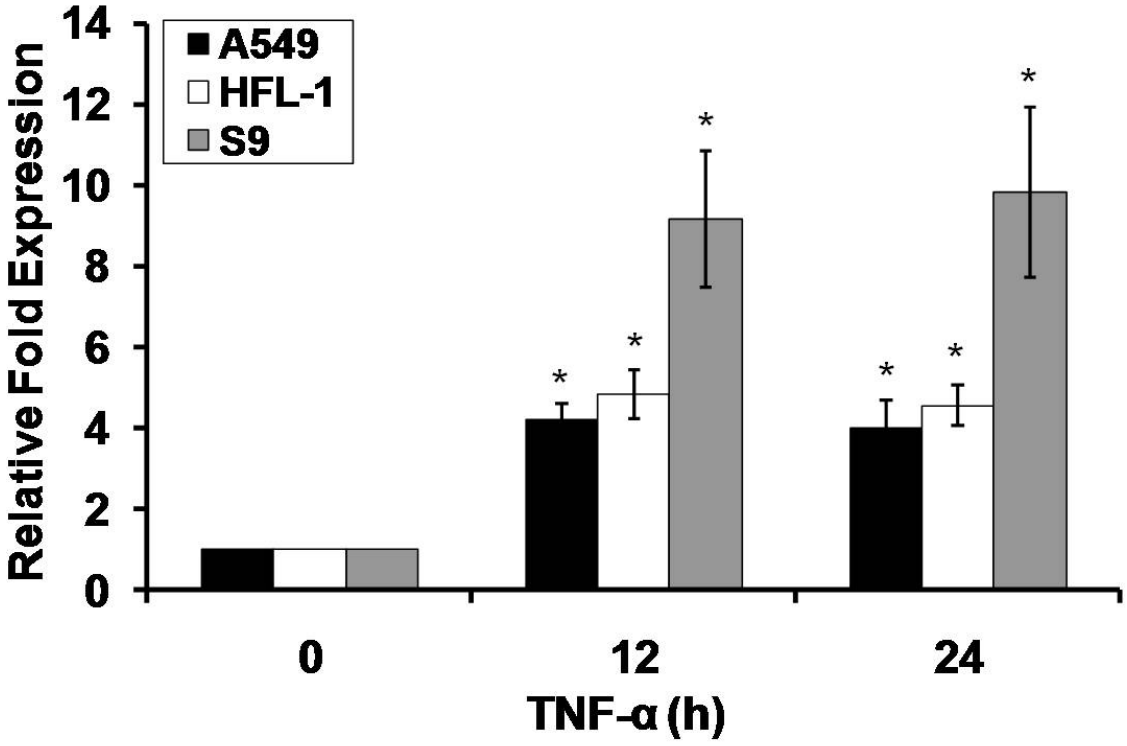

Figure 4-3. Evaluation of steady state mRNA levels of $cPLA_2\gamma$ in various human pulmonary cells. Real-time RT-PCR of $cPLA_2\gamma$ in A549, HFL-1 and S9 cells following treatment of TNF-α for the indicated amounts of time. Data points are the means of ΔΔCT +/- SEM (n = 3). * indicates $p \leq 0.05$ as compared to their respective untreated controls.

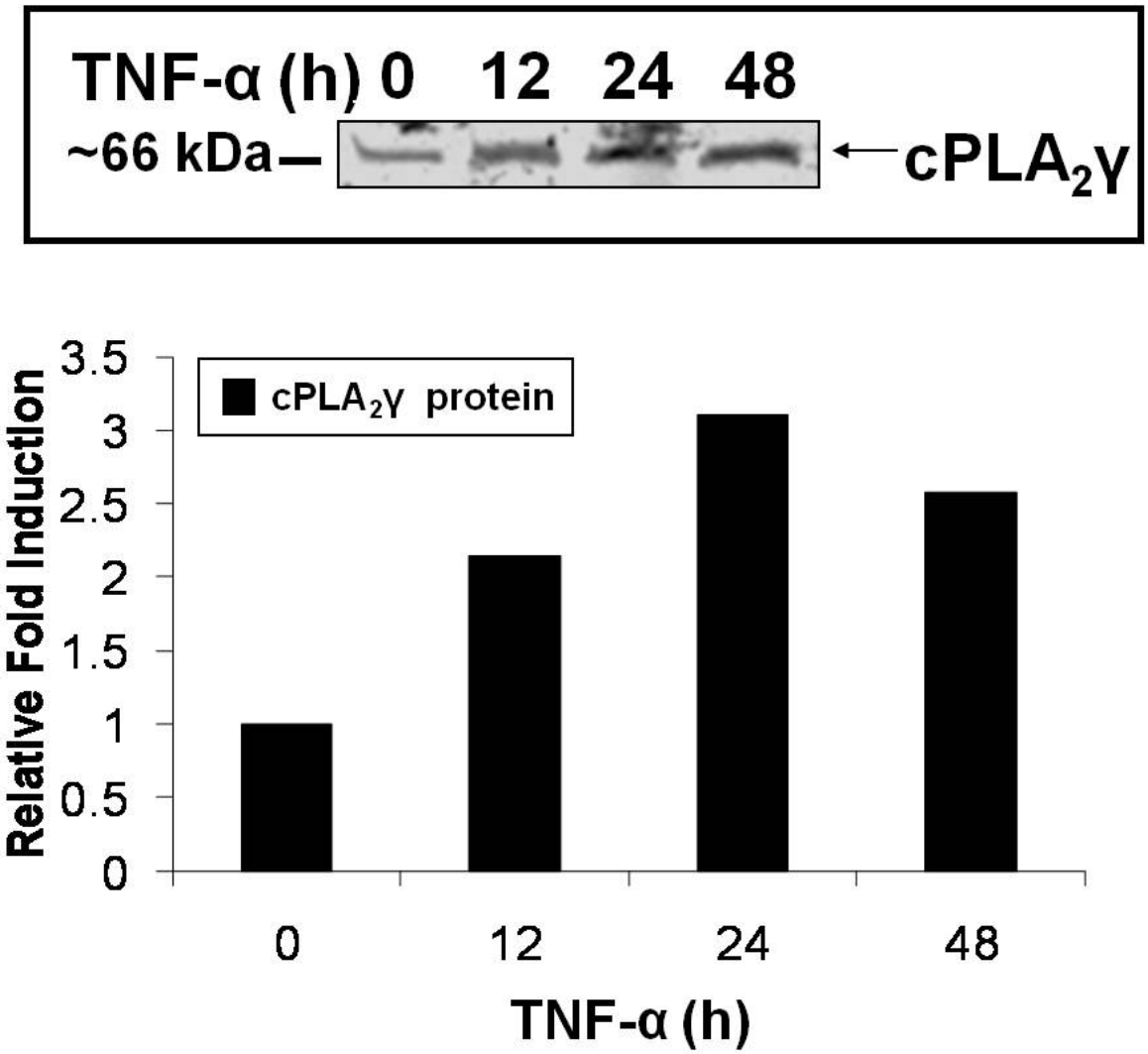

Figure 4-4. Immunoblot analysis of $cPLA_2\gamma$ in A549 cells. A549 cells were treated with TNF-a for the indicated amounts of time and protein was harvested and for an immunoblot analysis with an antibody to $cPLA_2\gamma$ (top). The bottom panel illustrates densitometry of the aforementioned immunoblot.

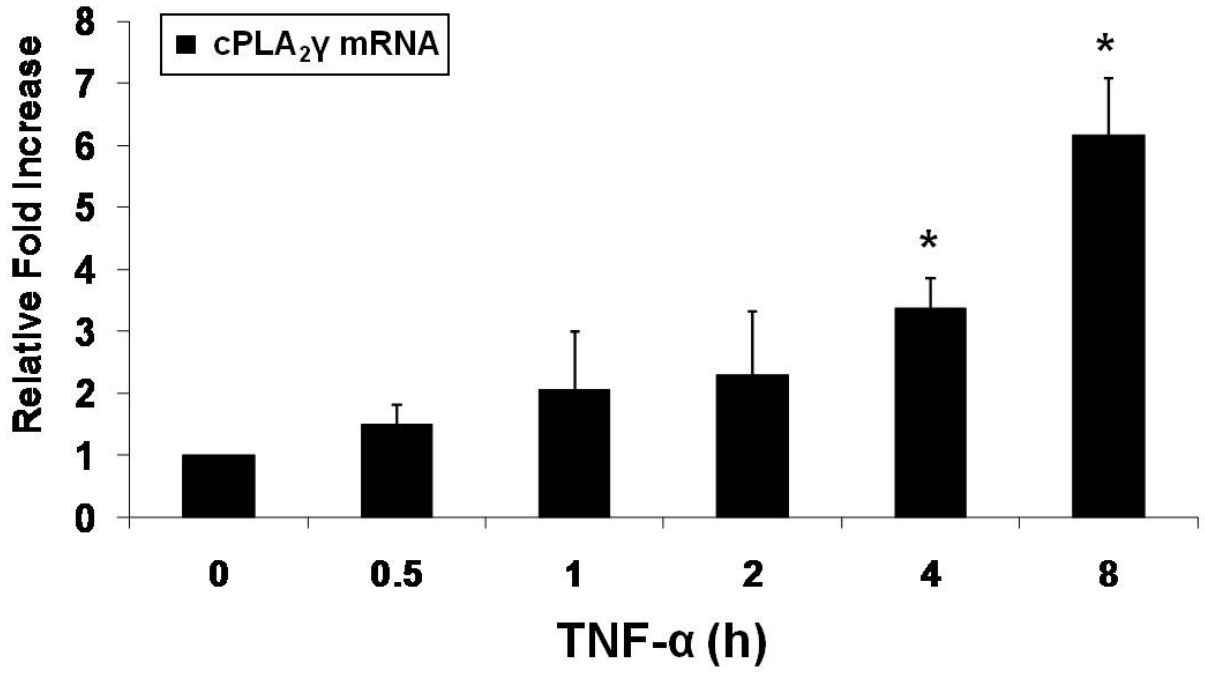

Figure 4-5. Steady-state mRNA levels of $cPLA_2\gamma$ in S9 cells up to eight hours. S9 cells were treated with TNF-a for the indicated periods of time and total RNA was collected and used for real-time RT-PCR. Data points are the means of ΔΔCT +/- SEM (n = 3). * indicates $p \leq 0.05$ as compared to the untreated (0 h) sample.

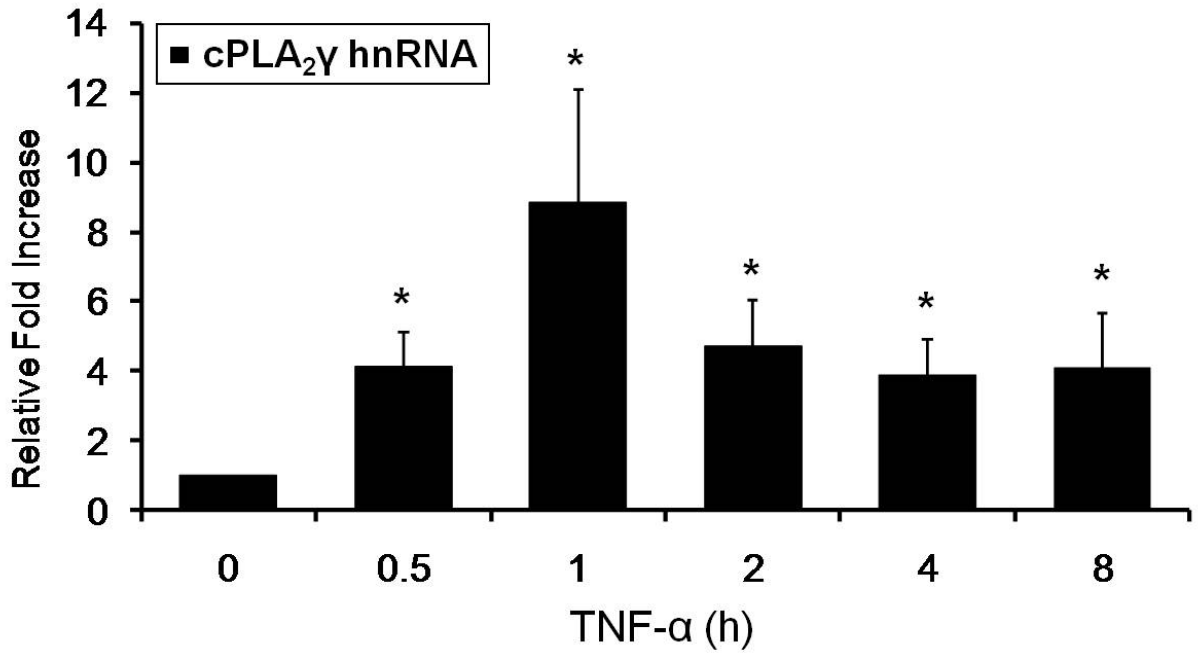

Figure 4-6. Heterogeneous nuclear RNA (hnRNA) levels of $cPLA_2\gamma$ in S9 cells up to eight hours. S9 cells were treated with TNF-a for the indicated periods of time and total RNA was collected, DNase-treated, and used for real-time RT-PCR. Data points are the means of ΔΔCT +/- SEM (n = 3). * indicates $p \leq 0.05$ as compared to the untreated (0 h) sample.

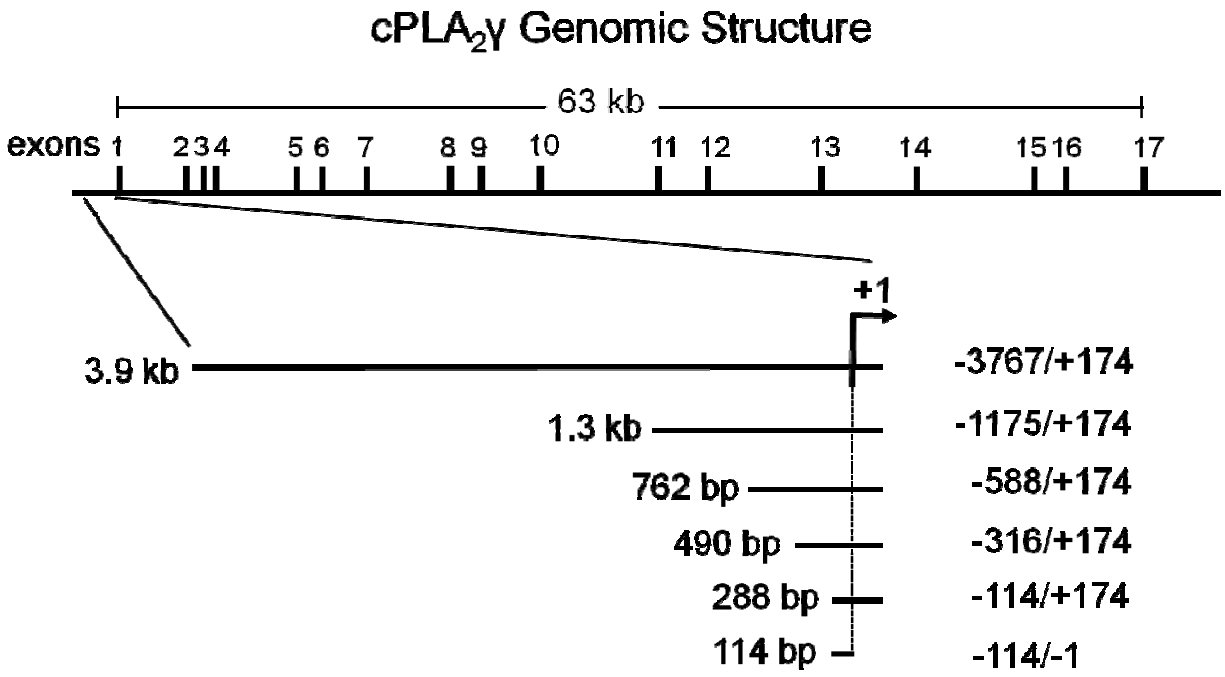

Figure 4-7. Diagram of the cPLA$_2$γ gene structure and subsequent promoter constructs. The 63 kb, 17-exon cPLA$_2$γ gene is illustrated at top with the 3.9 kb promoter blown up on bottom. The transcription start site is designated as "+1," and other promoter fragments to be cloned into the promoterless growth hormone reporter plasmid are also illustrated below.

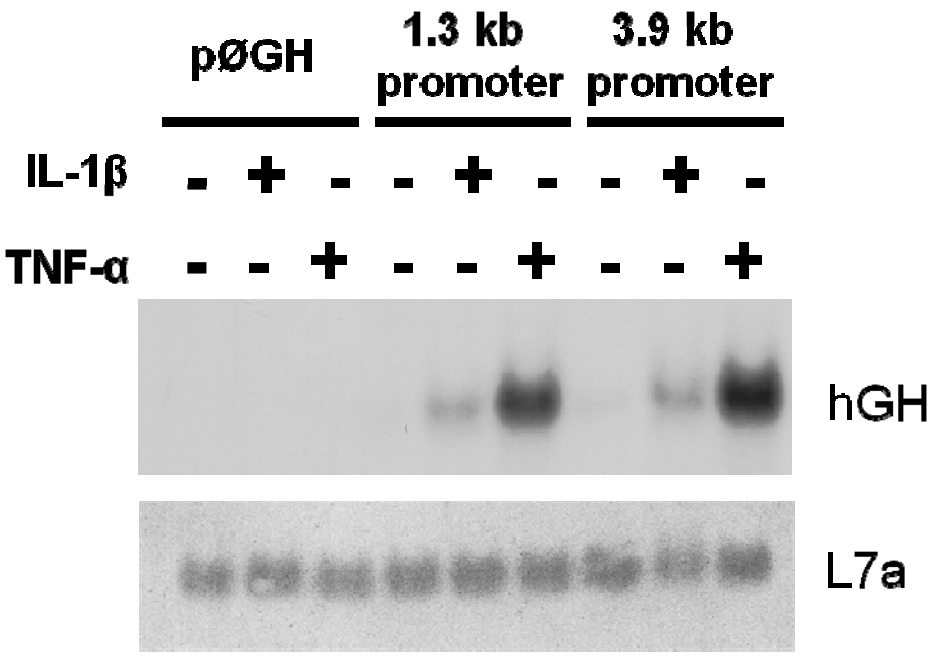

Figure 4-8. Determination of the IL-1β and TNF-α responsive cPLA$_2$γ promoter. S9 cells were transfected with the indicated hGH reporter constructs and the collected RNA was subjected to northern analysis for hGH with L7a being utilized as a loading control.

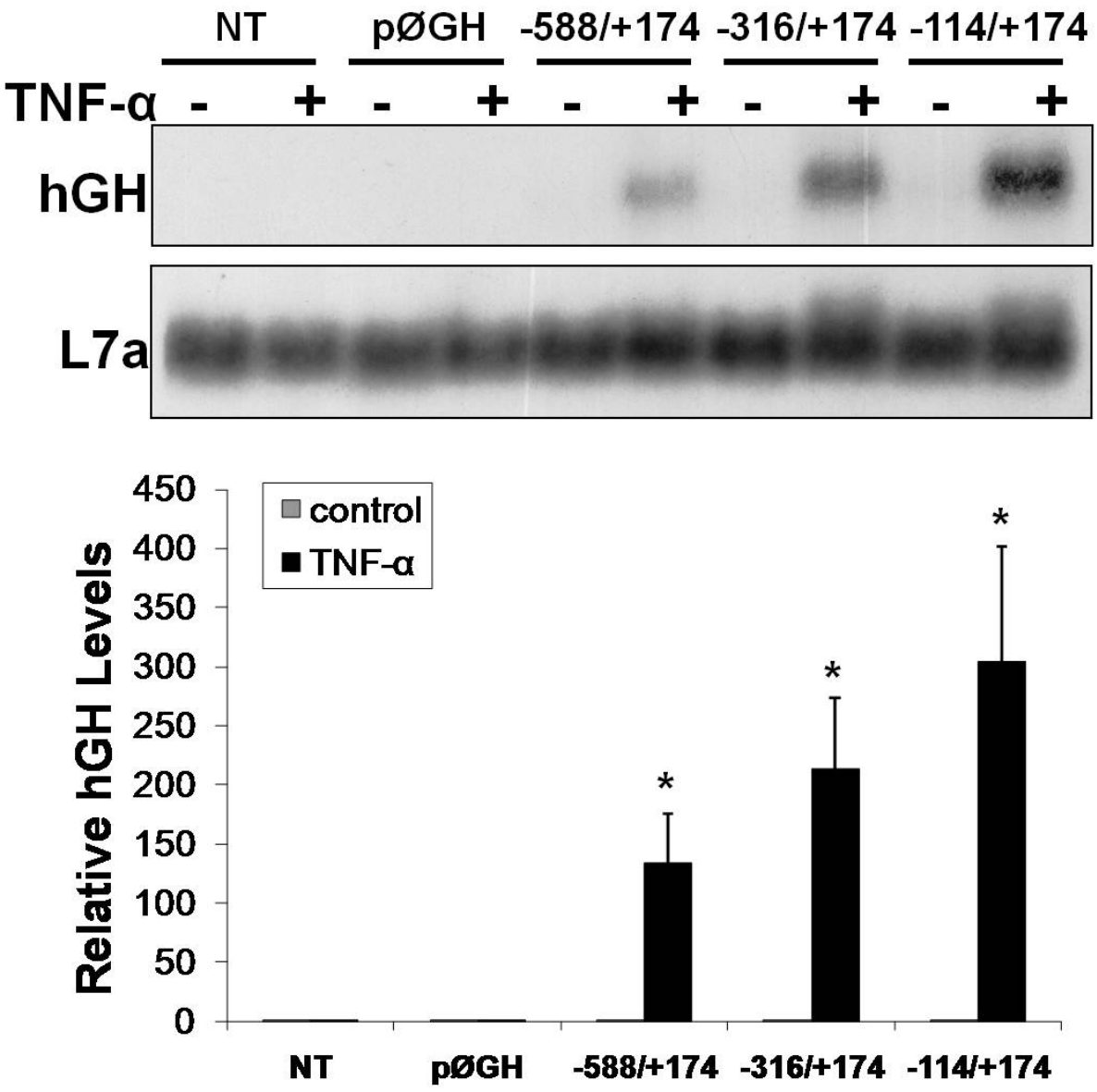

Figure 4-9. Determination of the TNF-α responsive cPLA$_2$γ promoter. S9 cells were transfected with the indicated cPLA$_2$γ promoter tethered to the hGH reporter construct and the collected RNA was subjected to northern analysis for hGH with L7a being utilized as a loading control. A representative northern (top) and corresponding densitometric data (bottom) are shown. Data points are represented as means +/- SEM (n = 3) and * signifies $p \leq 0.05$ as compared to their respective controls.

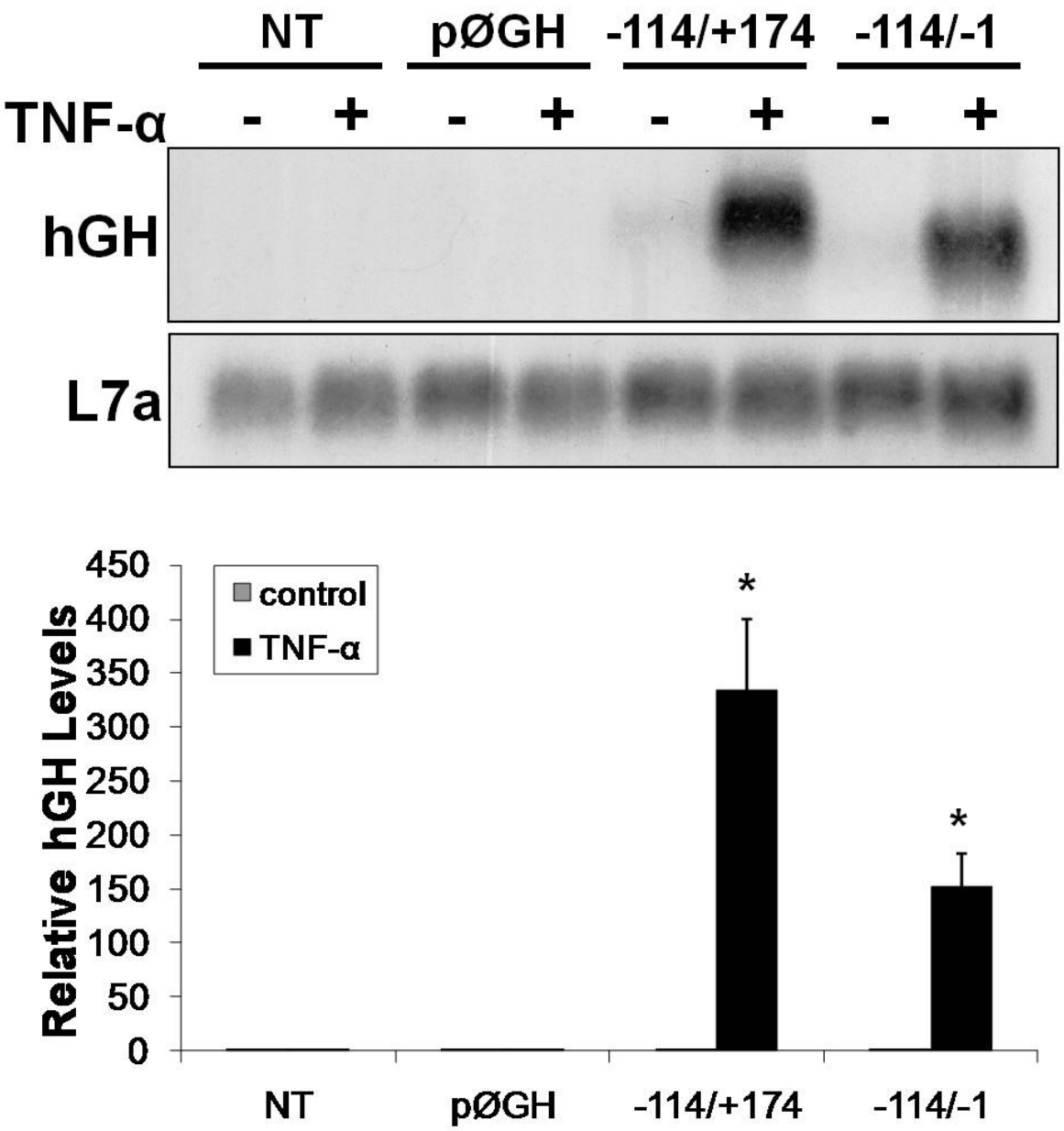

Figure 4-10. Determination of the minimal TNF-α responsive cPLA$_2$γ promoter. S9 cells were transfected with the indicated cPLA$_2$γ promoters tethered to the hGH reporter construct and the collected RNA was subjected to northern analysis for hGH with L7a being utilized as a loading control. A representative northern (top) and corresponding densitometric data (bottom) are shown. Data points are represented as means +/- SEM (n = 3) and * signifies $p \leq 0.05$ as compared to their respective controls.

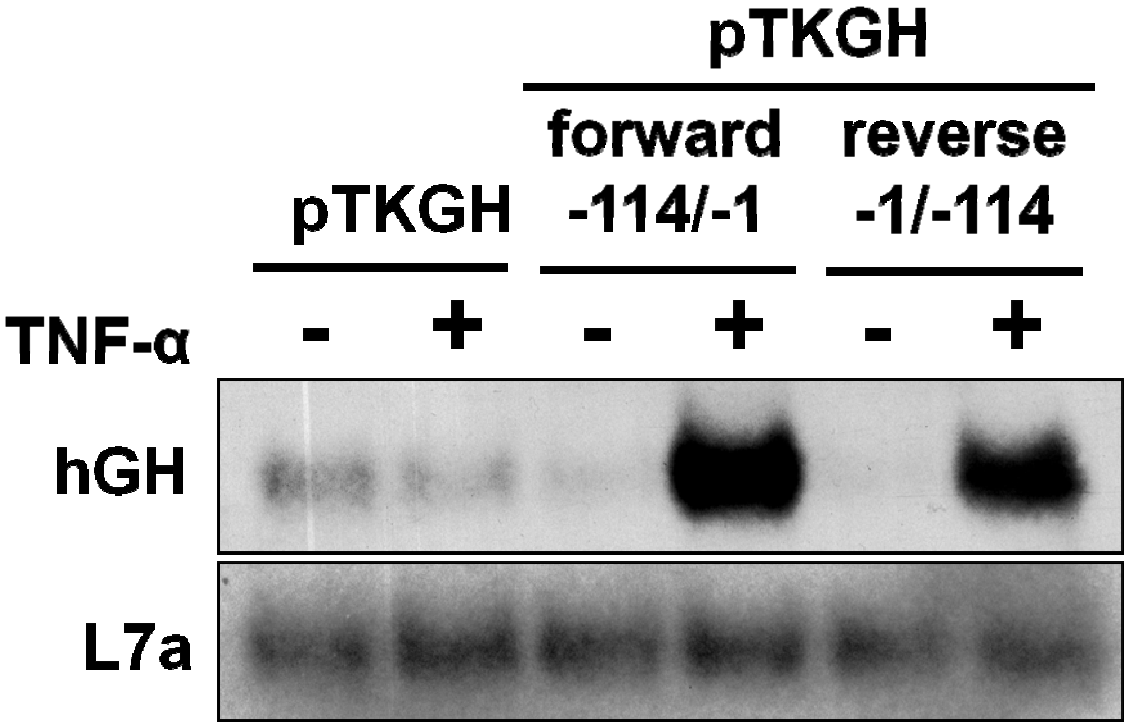

Figure 4-11. Determination of enhancer activity within the minimal TNF-α responsive $cPLA_2\gamma$ promoter. S9 cells were transfected with the indicated $cPLA_2\gamma$ promoter driving the minimal viral thymidine kinase promoter (TK) tethered to the hGH reporter construct (pTKGH) and the collected RNA was subjected to northern analysis for hGH with L7a being utilized as a loading control.

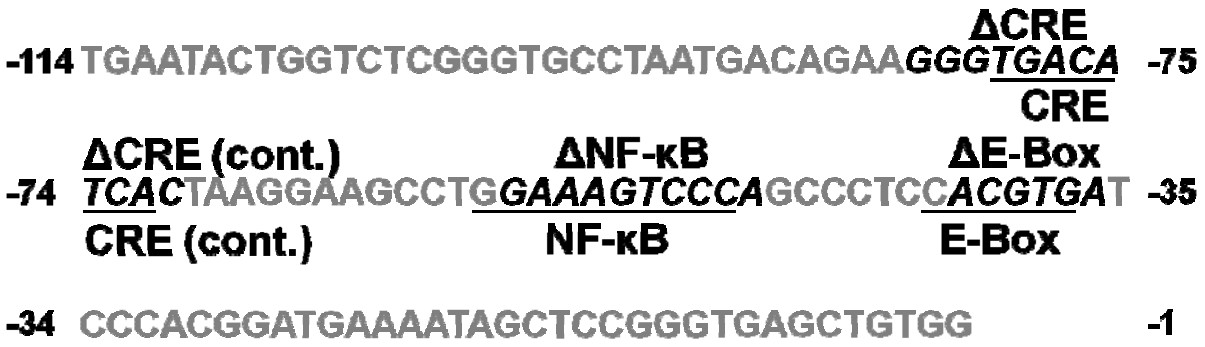

Figure 4-12. Site deletions of the cPLA$_2$γ enhancer/promoter. The sequence of the 114 bp cPLA$_2$γ enhancer/promoter is shown with consensus sites for the CRE, NF-kB and an E-box sites underlined and labeled under the sequence. The italicized letters have been deleted by mutagenesis for subsequent studies and the constructs are named with the labels written above the sequence.

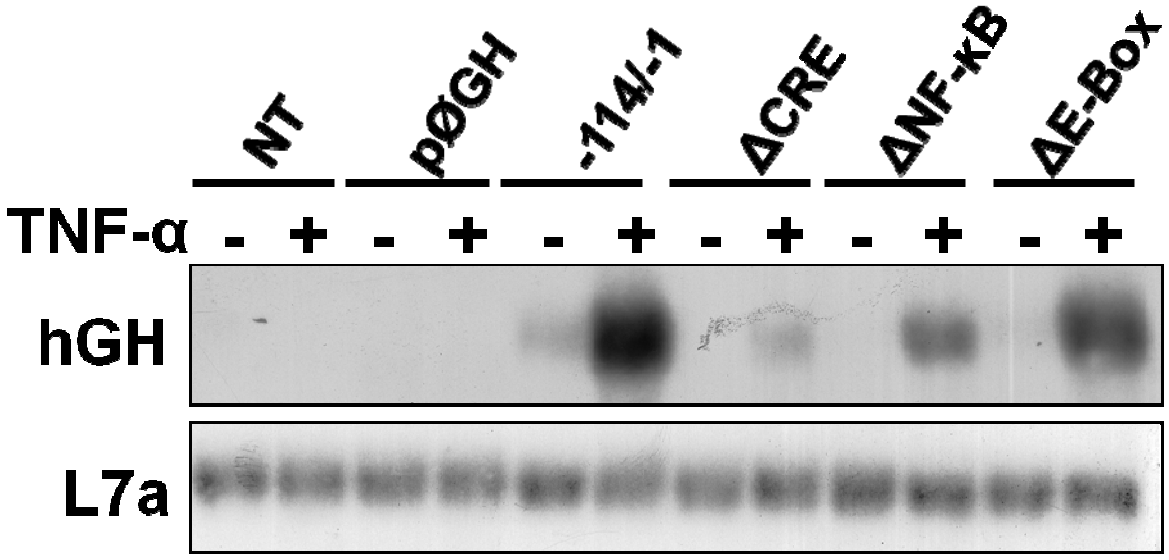

Figure 4-13. Northern blot analysis of cPLA$_2$γ enhancer/promoter site deletions. S9 cells were transfected with the indicated -114/-1 enhancer/promoter constructs tethered to the hGH reporter gene.

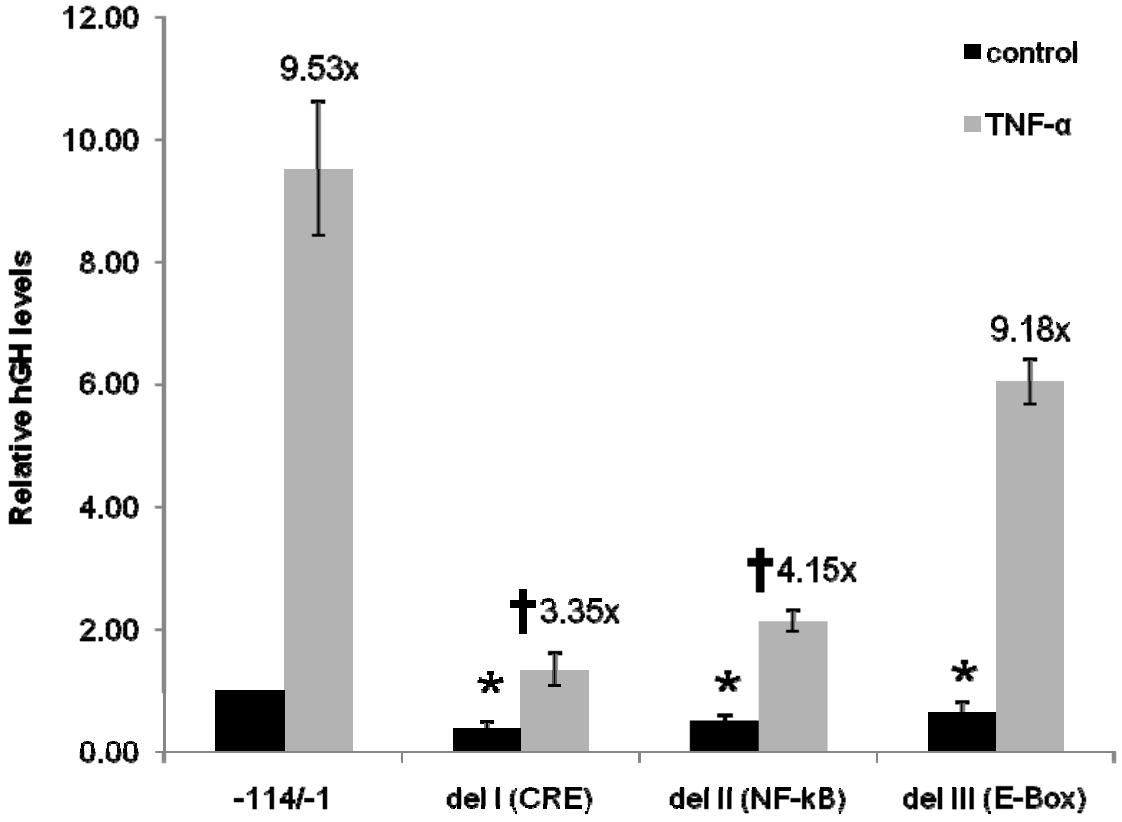

Figure 4-14. Real-time RT-PCR analysis of $cPLA_2\gamma$ enhancer/promoter site deletions. S9 cells were transfected with the indicated -114/-1 enhancer/promoter constructs tethered to the hGH reporter gene. Relative fold inductions of each construct are listed above each TNF-α data point. Data points are represented as means +/- SEM (n = 3) and * signifies $p \leq 0.05$ as compared to untreated -114/-1 control, while † signifies $p \leq 0.05$ as compared to the level of TNF-α induction seen in the -114/-1 construct.

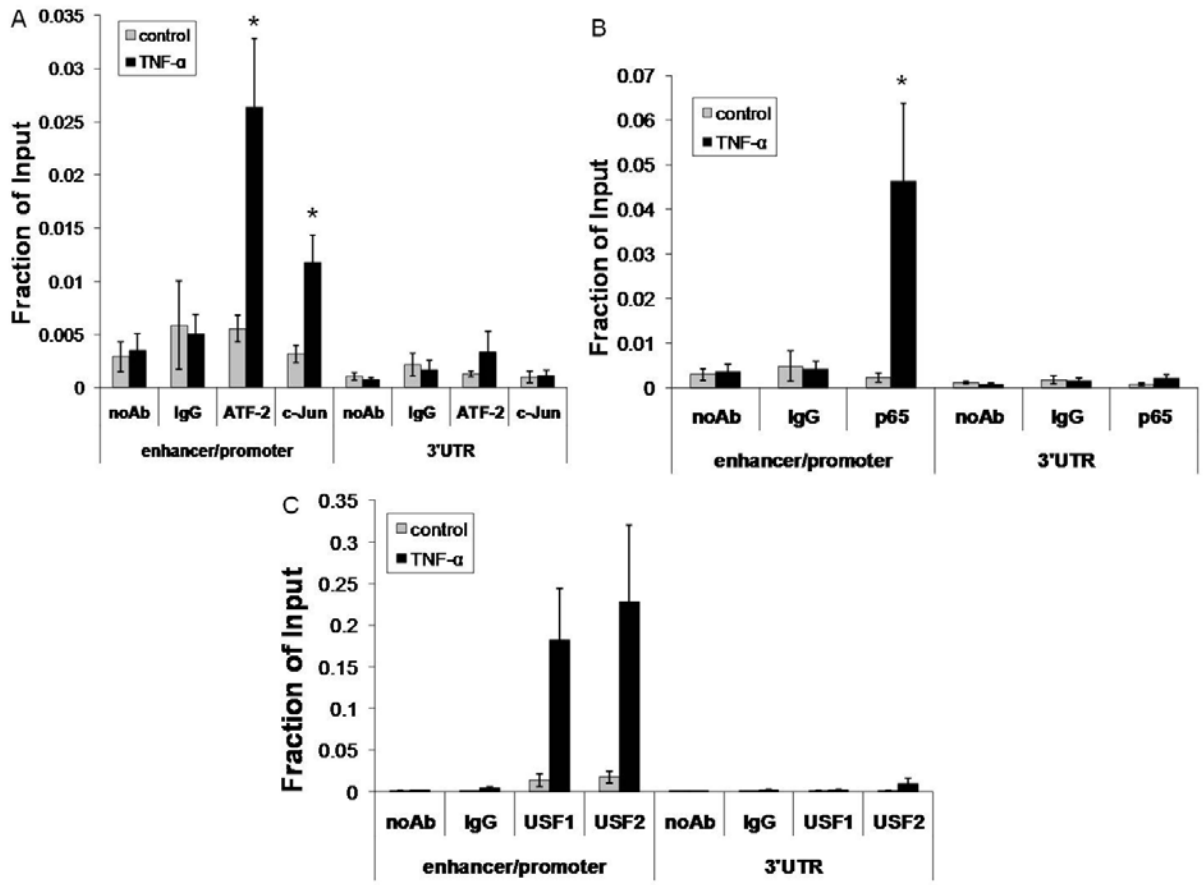

Figure 4-15. ChIP of CRE, NF-κB and E-Box specific transcription factors after 12 hours of TNF-α treatment. ChIP was performed with antibodies to IgG and A) ATF2 or c-Jun, B) p65, or C) USF1 or USF2. Association of these factors was analyzed by real-time PCR at the cPLA$_2$γ enhancer/promoter as well as a nonspecific region of the cPLA$_2$γ 3'UTR. In addition to IgG, a no-antibody (noAb) control was also evaluated. Data points are represented as means +/- SEM (n = 3) and * signifies $p \leq 0.05$ as compared to the respective untreated control.

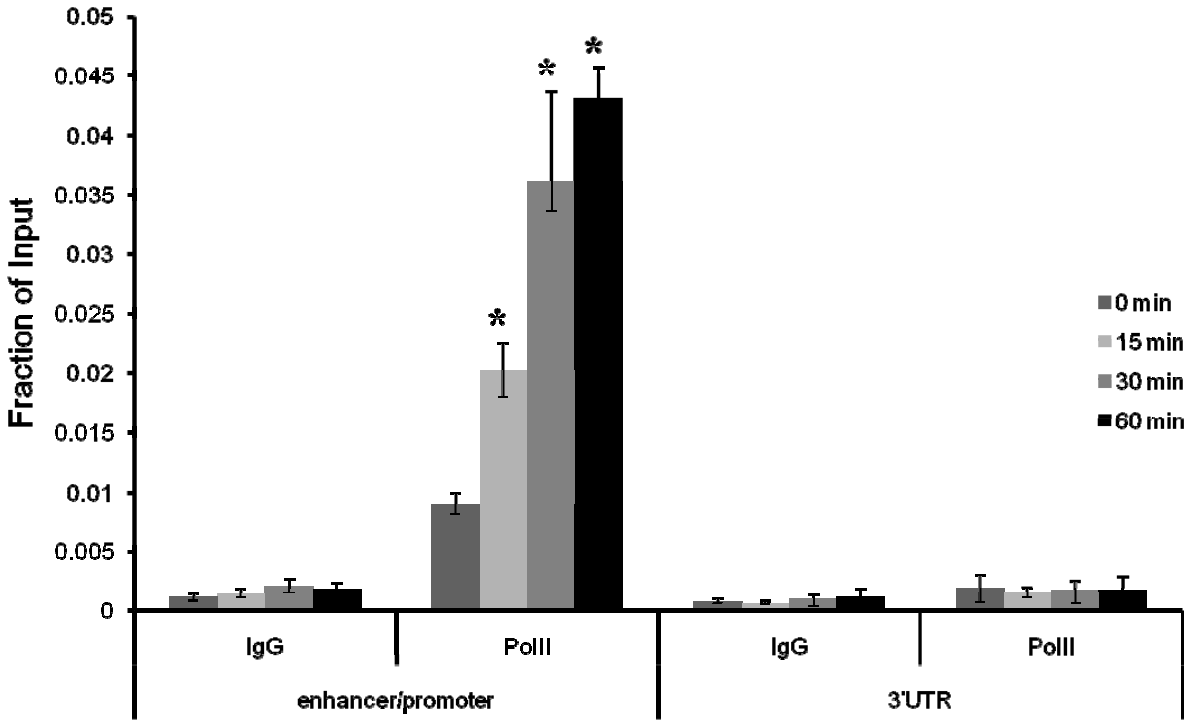

Figure 4-16. ChIP of RNA Polymerase II within one hour of TNF-α stimulation. Association of RNA Pol II was analyzed by real-time PCR at the $cPLA_2\gamma$ enhancer/promoter as well as a nonspecific region of the $cPLA_2\gamma$ 3'UTR. In addition to IgG, a no-antibody (noAb) control was also evaluated. Data points are represented as means +/- SEM (n = 3) and * signifies $p \leq 0.05$ as compared to the respective 0 min time point.

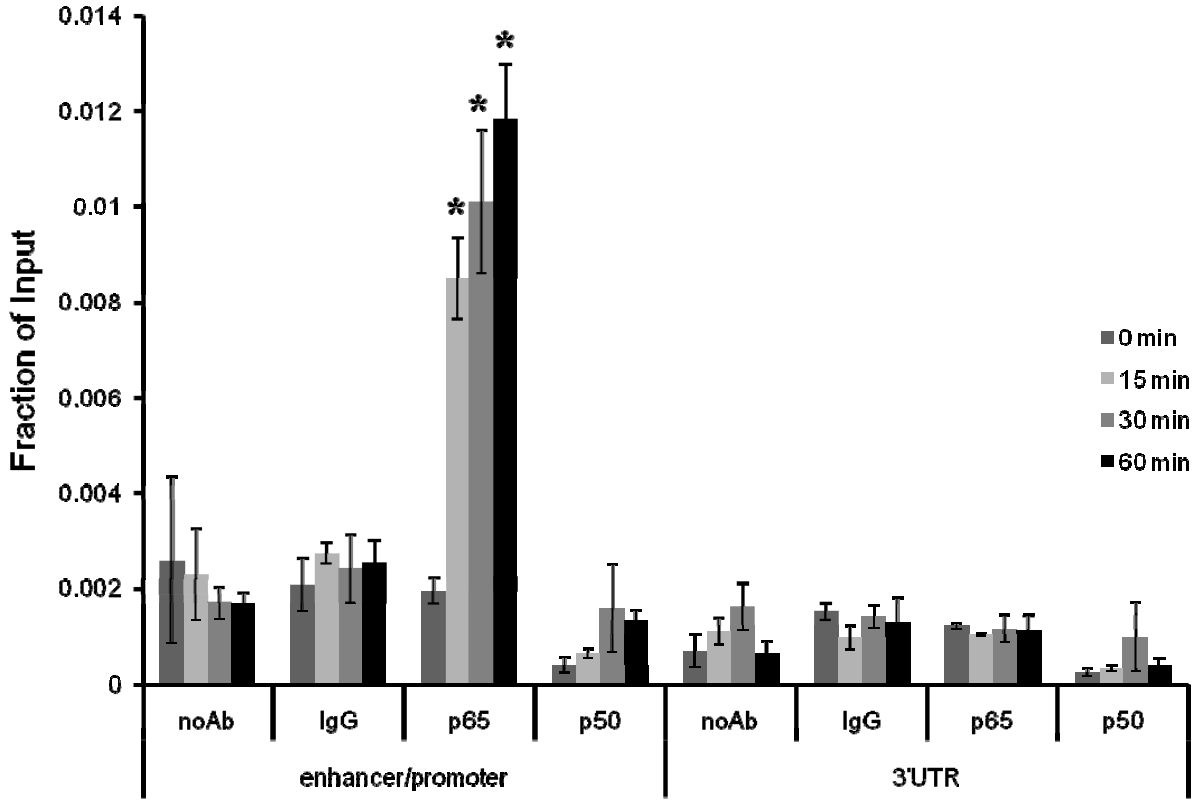

Figure 4-17. ChIP of p65 and p50 within one hour of TNF-α stimulation. Association of these factors was analyzed by real-time PCR at the cPLA$_2$γ enhancer/promoter as well as a nonspecific region of the cPLA$_2$γ 3'UTR. In addition to IgG, a no-antibody (noAb) control was also evaluated. Data points are represented as means +/- SEM (n = 3) and * signifies $p \leq 0.05$ as compared to the respective 0 min time point.

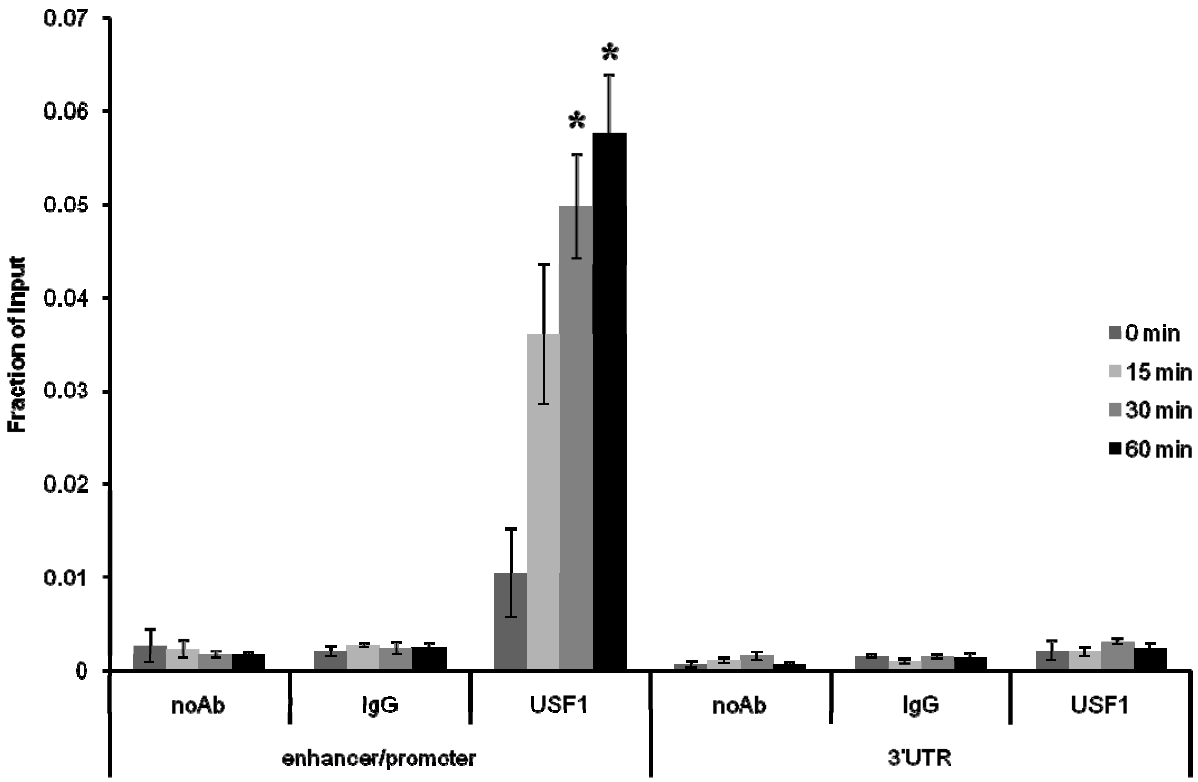

Figure 4-18. ChIP of USF1 within one hour of TNF-α stimulation. Association of USF1 was analyzed by real-time PCR at the cPLA$_2$γ enhancer/promoter as well as a nonspecific region of the cPLA$_2$γ 3'UTR. In addition to IgG, a no-antibody (noAb) control was also evaluated. Data points are represented as means +/- SEM (n = 3) and * signifies $p \leq 0.05$ as compared to the respective 0 min time point.

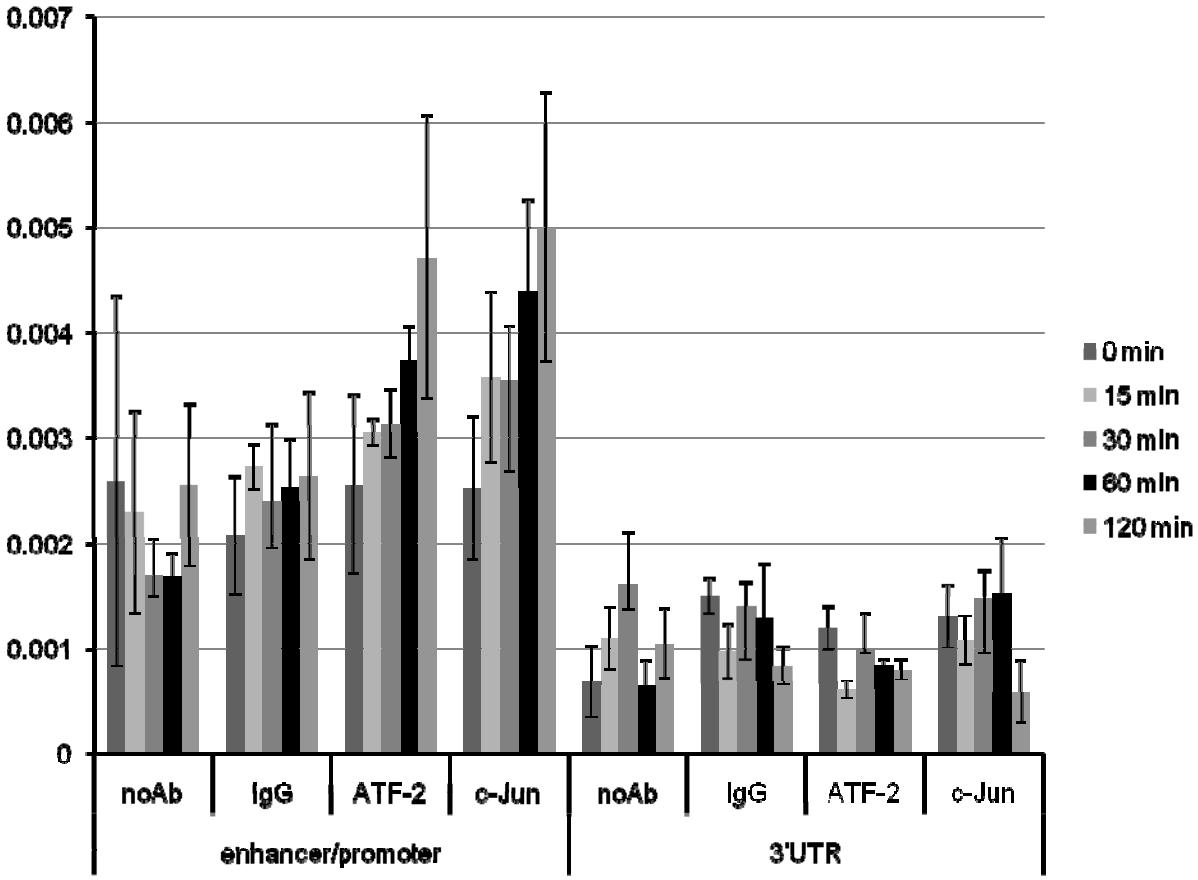

Figure 4-19. ChIP of ATF-2 and c-Jun within two hours of TNF-α stimulation. Association of these factors was analyzed by real-time PCR at the cPLA$_2$γ enhancer/promoter as well as a nonspecific region of the cPLA$_2$γ 3'UTR. In addition to IgG, a no-antibody (noAb) control was also evaluated. Data points are represented as means +/- SEM (n = 3).

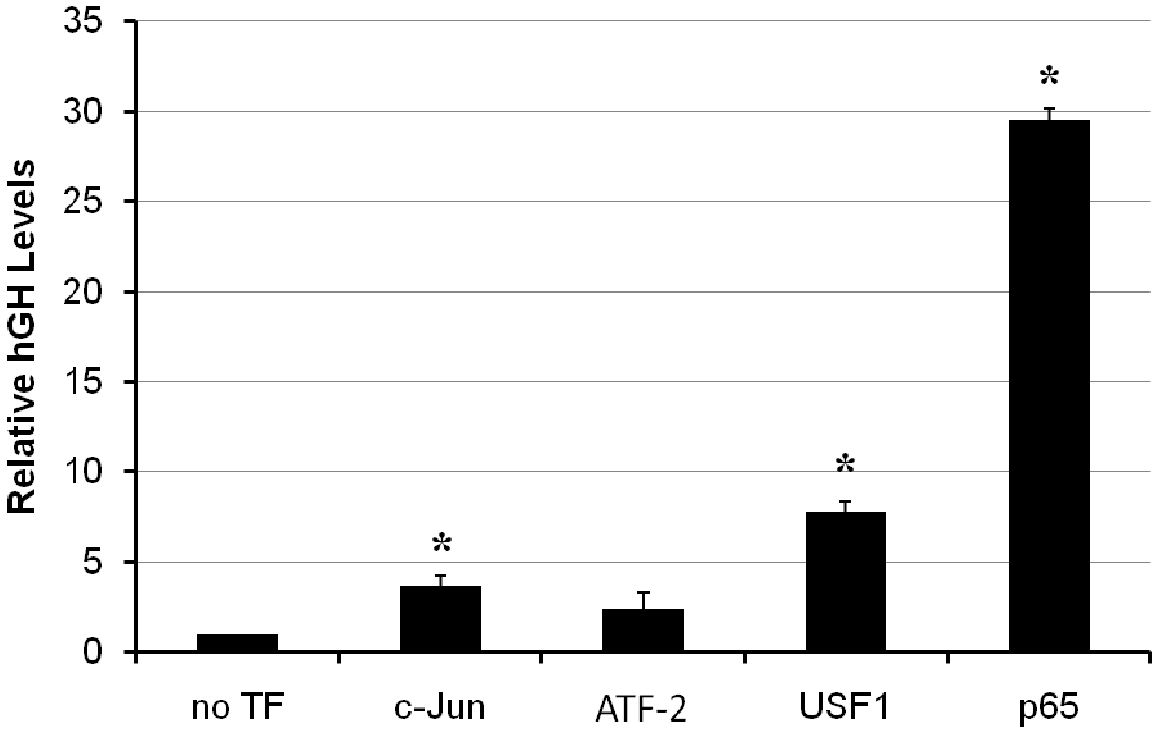

Figure 4-20. Effect of overexpression of wild-type transcription factors on the activity of the $cPLA_2\gamma$ enhancer/promoter. S9 cells were transfected with the -114/-1 hGH construct as well as the indicated transcription factors, hGH levels were analyzed by real-time RT-PCR and the resulting data were normalized to the no transcription factor control (no TF). Cyclophilin A was used as a loading control. Data points are represented as means +/- SEM (n = 3) and * signifies $p \leq 0.05$ as compared to their respective controls.

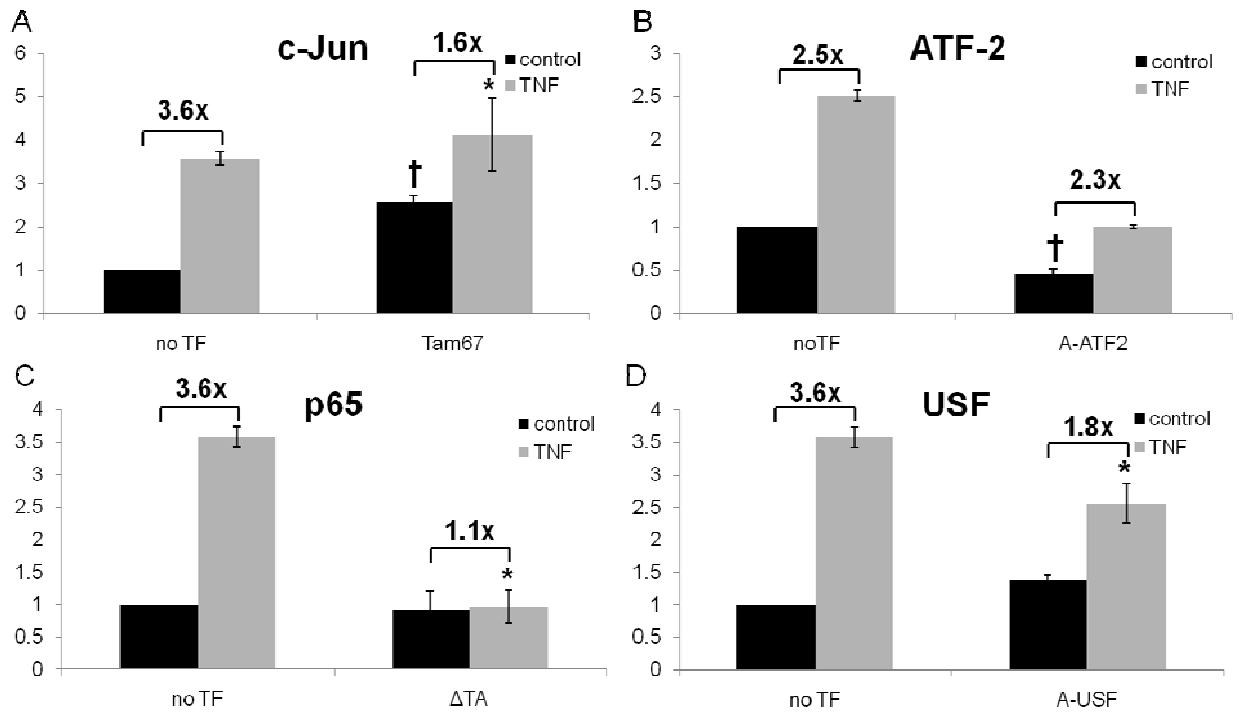

Figure 4-21. Effect of overexpression of dominant negative (DN) forms of transcription factors on the activity of the cPLA$_2$γ enhancer/promoter. S9 cells were transfected with the -114/-1 hGH construct as well as the indicated dominant negative transcription factors as follows: A) DN for c-Jun, Tam67; B) DN for ATF-2, A-ATF2; C) DN for p65, ΔTA; D) DN for USF1/2, A-USF. hGH levels were analyzed by real-time RT-PCR and the resulting data were normalized to the no transcription factor control (noTF). Cyclophilin A was used as a loading control. Data points are represented as means +/- SEM (n = 3) and † signifies $p \leq 0.05$ as compared to the untreated noTF while * signifies $p \leq 0.05$ relative to the fold induction of the noTF measurements.

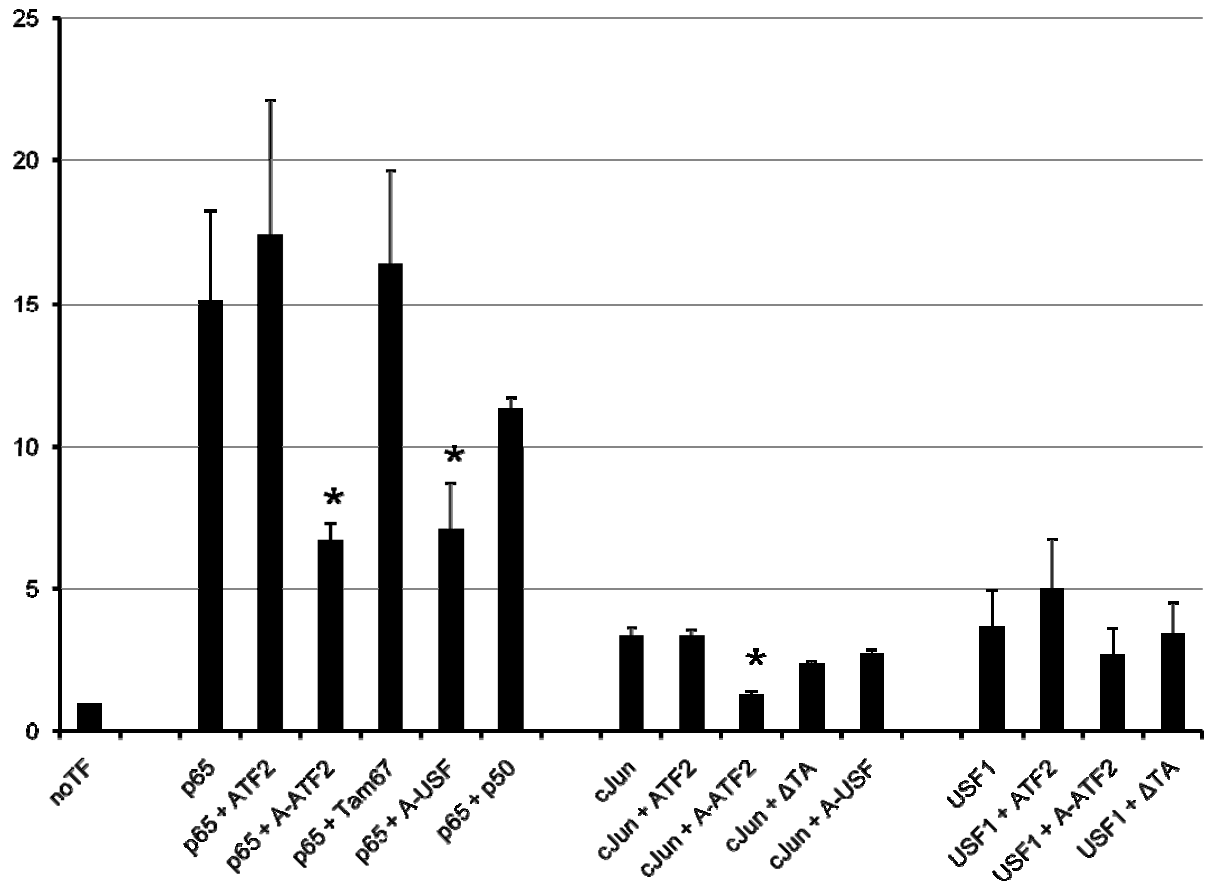

Figure 4-22. Effect of co-overexpression of wild-type transcription factors on the activity of the cPLA$_2$γ enhancer/promoter. S9 cells were transfected with the -114/-1 hGH construct as well as the indicated transcription factors, hGH levels were analyzed by real-time RT-PCR and the resulting data were normalized to the no transcription factor control (no TF). Cyclophilin A was used as a loading control. Data points are represented as means +/- SEM (n = 3) and * signifies $p \leq 0.05$ as compared to the respective transcription factor alone.

CHAPTER 5
INDUCTION OF CPLA2 GAMMA BY EXPOSURE TO EXTRACELLULAR DNA

Potential Pathways for a Transcriptional Response to Extracellular DNA

There are several DNA sensing pathways that exist in non-immune cells. Most of these pathways rely on DNA being taken into a cell and recognized by an internal receptor. One of the better studied receptors is Toll-like receptor 9 (TLR9). This receptor exists endosomally and recognizes unmethylated CG dinucleotides on DNA that has been endocytosed. The optimal human consensus sequence for this intracellular endosomal receptor has been identified as GTCGTT by stimulation of an 6xNF-κB promoter construct in HEK-293 cells stably expressing human or mouse TLR9 [177]. Although this data is the basis for many TLR9 related studies, this information is still disputed and it is not clear whether the observed effects are purely due to the DNA sequence or the fact that the DNA used for these studies had a synthetic phosphorothioated backbone [178, 179]. Upon activation, TLR9 signals through MyD88 and TNF-receptor associated factors (TRAFs) to activate NF-κB and interferon regulatory factors, IRFs, resulting in the activation of type I interferons. Although TLR9 is the only known endosomal DNA sensor, many cytosolic DNA sensing pathways have been recently identified.

The AIM2 inflammasome was characterized in 2009, is composed of the caspase-1-activating adaptor protein ASC, and is also referred to as a pyroptosome due to the requirement of an active pyrin domain within the interferon-inducible AIM2 [180, 181]. Activation of the AIM2 inflammasome by cytosolic DNA results in the activation of caspase-1, which cleaves pro-IL-1β and pro-IL-18 into the active forms of IL-1β and IL-18, respectively. This results in proinflammatory programmed cell death similar to that of

apoptosis; this method of cell death is caspase-1-dependent and referred to as pyroptosis [182].

Newly designated DAI, formerly known as DLM-1 or ZBP1, was first identified in 1999, but in 2007, the Taniguchi lab defined the cytosolic DNA sensing properties of this gene [183, 184]. Activation of DAI initiates a signaling cascade through TRAF family member-associated NF-κB kinase, or TBK1. As with TLR9, the end result of the activation of DAI is the activation of type I interferons.

The RNA sensor RIG-I has also been controversially referred to as a DNA sensor, but recent studies have attempted to explain away this controversy. The new models implicating RIG-I as a DNA sensor are focused around the fact that RNA Polymerase III has long been known to exist in the cytoplasm for unknown reasons [185]. It was recently found that cytosolic RNA Pol III can transcribe cytosolic DNA, and the resulting small RNAs can be recognized by RIG-I RNA sensing domains [186]. Due to the lack of other RNA processing enzymes in the cytosol, the RNA transcribed by cytosolic RNA Pol III contains a 5' triphosphate, which is a requirement for IFN-stimulating RNA as well as detection by RIG-I.

Results

Induction of cPLA$_2$γ Following Plasmid Transfection

During the course of attempting to overexpress transcription factors in the regulation studies detailed in Chapter 4, it was found that endogenous cPLA$_2$γ mRNA levels were elevated even in the presence of empty plasmid vectors. This observation led to the hypothesis that plasmid transfection was capable of inducing the cPLA$_2$γ gene. To test this hypothesis, S9 cells were transfected with pcDNA3.1 for 24, 48 or 72 hours. After the indicated amount of time, total RNA was collected and used to assay

cPLA$_2$γ mRNA levels by real-time RT-PCR. As shown in Figure 5-1, the cPLA$_2$γ message is elevated 48 hours post-transfection. A single TNF-α treatment was performed as a positive control for cPLA$_2$γ induction. None of the transfection reagents tested (FuGene, HiPerFECT, or Lipofectamine) were capable of inducing cPLA$_2$γ expression in the absence of transfected DNA (data not shown).

Effect of Interferon-Stimulating DNA on cPLA$_2$γ Expression

One of the primary ways in which foreign DNA is recognized by the cell is by pattern recognition receptors. Of these, Toll-like receptor 9 (TLR9) is capable of recognizing unmethylated CGs. The commonly used oligodeoxynucleotide (ODN) used to stimulate human TLR9 is referred to as ODN-2006, which contains the human specific recognition sequence flanking the CG dinucleotide. Therefore, this ODN was used to determine if cPLA$_2$γ was being induced via a TLR9-dependent pathway. Due to the ability of cells to rapidly degrade small linear DNA fragments, ODN-2006 was constructed with both a normal phosphodiester backbone (PO-2006) as well as a more stable phosphorothioated backbone (PS-2006). For negative controls, each of these ODNs were also constructed with each of the CGs replaced with GCs. As shown in Figure 5-2A, none of these ODNs were capable of stimulating cPLA$_2$γ in S9 cells as determined by real-time RT-PCR. It would stand to reason that an immune cell would be more responsive to foreign DNA and that a stronger response could be elicited from such cells. Unfortunately, neither murine monocytes, J774, nor human monocytes, THP-1, responded to the ODNs with an increase in cPLA$_2$γ expression (Figure 5-2B,C). Although the sequence used is human-specific, mice still respond, albeit to a lesser extent.

Finally, HEK293s were utilized due to the fact that they have been established to respond to this treatment. In addition, many researchers in the TLR9 field have employed an HEK293 cell line stably expressing TLR9 which has been clearly shown to demonstrate the involvement of this DNA receptor [187]. As with the previously tested cells, neither parental HEK293 cell line or the TLR9 overexpressing cells seemed to induce cPLA$_2$γ in response to the TLR9 specific sequences (Figure 5-3).

In order to verify that the previously observed phenomenon was not specific to the S9 cell line, HEK293 cells were also transiently transfected. Within this experiment, the cells were also treated with FuGENE alone to determine if perhaps the aforementioned response was simply due to the presence of the transfection reagent. Figure 5-3 shows that the effect seen in S9 cells is not cell specific and that HEK293 cells can respond similarly to transiently transfected plasmid DNA. Inclusion of the wild-type HEK293 as well as the HEK293 cells stably expressing TLR9 strongly implies that this observation is not based on TLR9 since the plasmid sequence contains multiple CG dinucleotides which are identical to the human TLR9 recognition sequence.

Implication of the RNA Sensing Pathway in Transfection-Dependent cPLA$_2$γ Induction

RIG-I and MDA5 have long been considered cytosolic RNA sensors with a questionable tie to cytosolic DNA sensing [188, 189]. In 2009, it was reported by two groups that this elusive connection was via transcription of cytosolic DNA to RNA by RNA Polymerase III [186, 190]. To determine if this novel sensing pathway was involved, S9 cells were transfected with the pcDNA3.1 plasmid and mRNA levels were assayed by real-time RT-PCR. As shown in Figure 5-4, both RIG-I and MDA5 were induced nine and eight fold, respectively, in response to transfection. Additionally, the

prominent downstream target of this pathway is transcription of IFNβ, therefore, the levels of this gene were also analyzed. The nearly 1500 fold induction of IFNβ confirms that this pathway is indeed intact in these cells. Interestingly, a transcriptional regulator of IFNβ, IRF7, was not found to be induced at the mRNA level despite this transcription factor being regulated at the mRNA level [191]. The next step in this pathway is binding of IFNβ to cell surface receptors to activate IFN-specific genes such as Mx1. Hence, levels of this IFNβ target gene were also analyzed by real-time RT-PCR and, as shown in Figure 5-4, it was also induced by 35 fold. The data compiled in Figure 5-4 firmly supports the function of the entirety of this particular DNA-sensing pathways in S9 cells.

IFNβ-Independence of Transcriptional Activation

Having established the RIG-I/MDA5 pathway as being both responsive and functional in these cells, it was next determined whether this was the pathway responsible for $cPLA_2\gamma$ activation in response to plasmid transfection. The obvious mode of action for this pathway would be to induce genes via IFNβ. Therefore, S9 cells were treated with IFNβ at both low (10 U/mL) and high (1000 U/mL) concentrations with TNF-α being used as a positive control for $cPLA_2\gamma$ gene induction. As shown in Figure 5-5A, IFNβ has no effect on $cPLA_2\gamma$ levels even at (1000 U/mL). A positive control for IFNβ treatment, Mx1, was included in Figure 5-5B. The data for Mx1 confirms that these cells did in fact respond to IFNβ treatment in a dose-dependent manner. These results imply that the activation of $cPLA_2\gamma$ by transient transfection is occurring concomitantly with the induction of IFNβ or secondarily by another activated pathway.

Defining the Specific Stimulus Involved in the DNA-Dependent Induction of cPLA$_2$γ

Within the realm of cytosolic DNA and RNA sensors, some specific stimuli exist. One such compound is an activator of the RIG- and MDA5 pathways and is a synthetic RNA, poly(I:C), constructed by annealing a strand of polyinositol to polycytidine. Therefore, HFL-1 cells were treated with poly(I:C) as well as IFNβ. As shown in Figure 5-6, poly(I:C) is incapable of inducing cPLA$_2$γ in these cells, however, the RIG-I is also not induced. Therefore, studies with poly(I:C) will require additional experimentation to unravel the role of the RNA sensing pathway in response to extracellular DNA. Additionally, HFL-1 cells were transfected with bacterial and mammalian DNA from E. coli and genomic DNA isolated from HFL-1 cells. Bacterial DNA appears to induce RIG-I in these cells (~13 fold) without affecting cPLA$_2$γ expression while mammalian DNA seems to only modestly (2 fold) induce both cPLA$_2$γ and RIG-I.

To determine the specificity of the response to extracellular DNA, the effects on the other prominent cPLA$_2$ family member, cPLA$_2$α, were investigated. To identify potentially broader effect on cPLA$_2$ inducibility, another time course was performed and cPLA$_2$γ was analyzed alongside cPLA$_2$α. As observed before, cPLA$_2$γ is induced 4-5 old at the mRNA level in response to transfected plasmid DNA, and is also induced two fold in response to bacterial or mammalian genomic DNA (Figure 5-7). Most significantly, cPLA$_2$α is not affected by transfection of plasmid, bacterial or mammalian DNA, implying that cPLA$_2$γ may be a dominant phospholipase in this unique inflammatory response.

Having confirmed that plasmid transfection is the most potent DNA inducer of cPLA$_2$γ and that this response also induces IFNβ, it was possible that priming the cells

with a pretreatment of IFNβ would elicit a more potent response to transfected plasmid DNA. In order to test this hypothesis, S9 cells were treated with IFNβ for 12 hours prior to transfection with plasmid DNA and collected 24, 48 or 72 hours after DNA transfection. As illustrated in Figure 5-8, pretreatment with IFNβ did not enhance the $cPLA_2\gamma$ induction seen by plasmid transfection. At this point, it has been identified that $cPLA_2\gamma$ can respond uniquely to extracellular DNA without the central involvement of IFNβ. Therefore future studies are necessary to complete the understanding and physiological relevance of this response.

Discussion

These DNA sensing pathways provide a great deal of variety for recognition of foreign DNA in cells. The data presented here demonstrate that $cPLA_2\gamma$ mRNA levels are elevated in response to DNA in a TLR9 and IFNβ-independent response. It has also been shown here that transient transfection concomitantly induces the RIG-I and MDA5 pathways leading to production of IFNβ and a subsequent autocrine response to the newly secreted IFNβ, confirmed by elevated levels of Mx1. These results indicate that $cPLA_2\gamma$ may be induced directly by a DNA sensing pathway such as RIG-I, MDA5 or DAI, while it does not rule out the possibility that the observed induction is due to a secondary response, for example, to the secretion of a different type I interferon.

Although these data demonstrate that transfection of plasmid DNA definitively activates the RIG-I pathway as well as IFNβ, it does not rule out the DAI or even AIM2 pathways. Potential future directions regarding these studies are described in more detail in Chapter 6. In summary, the AIM2, DAI and RIG-I pathways are still considered possible pathways for $cPLA_2\gamma$ gene induction by cytosolic DNA. Irrespective of the exact

mechanism of cPLA$_2$γ induction observed here, it is clear that cPLA$_2$γ has as of yet undiscovered roles in this response.

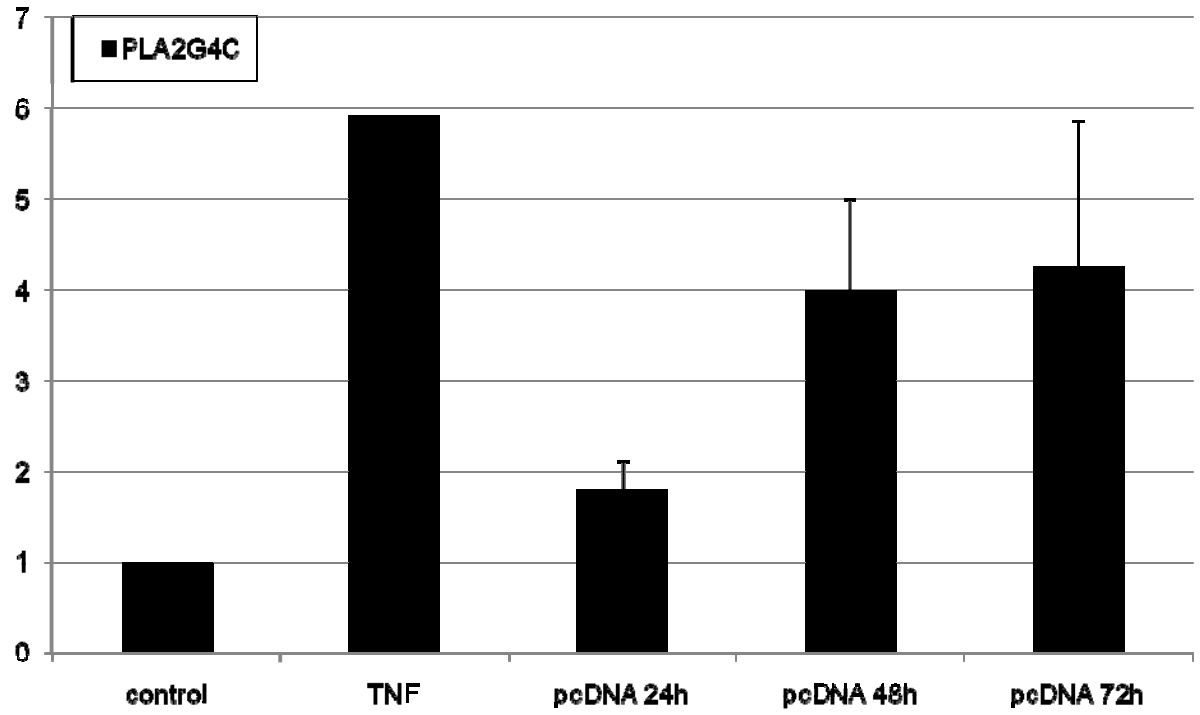

Figure 5-1. Endogenous cPLA$_2$γ expression in S9 cells transfected with pcDNA3.1. Cells were treated with TNF-α as a positive control or transiently transfected with pcDNA3.1. Transfected cells were allowed to rest for 24, 48, or 72 hours prior to collection of RNA. Total RNA was collected and used to measure cPLA$_2$γ levels by real-time RT-PCR compared to cyclophilin A as an internal control. Data points are represented as means +/- SEM (n = 3).

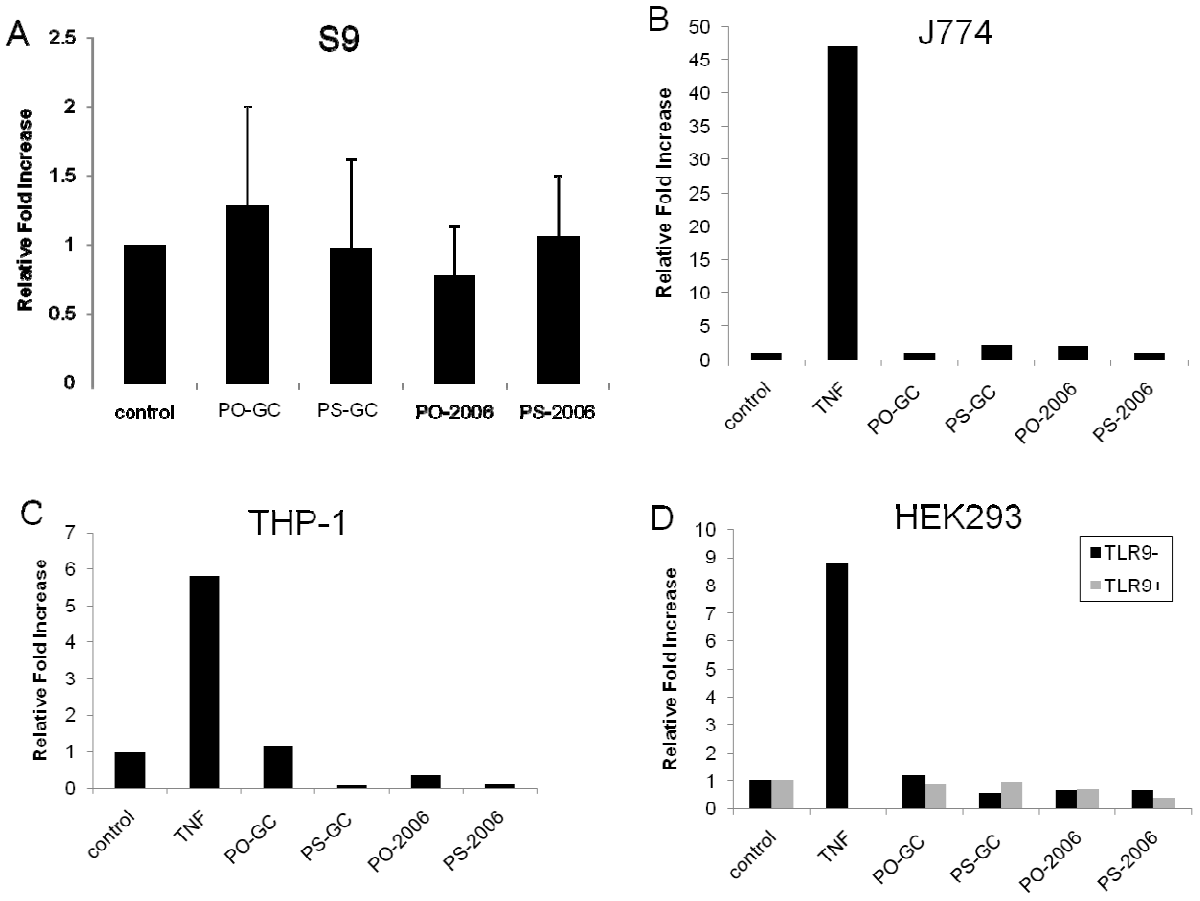

Figure 5-2. Endogenous cPLA$_2$γ expression in indicated cell lines exposed to interferon-stimulating oligonucleotides. A) Bronchoepithelial cells (S9), B) murine monocytes (J774A.1), C) human monocytes (THP-1), or D) HEK293 cells were treated with indicated oligonucleotides and cPLA$_2$γ expression was analyzed by real-time RT-PCR. Wild-type HEK293 cells contain minimal expression of TLR9 (TLR9-) while an HEK293 cell line overexpressing TLR9 (TLR9+) has also been utilized. Oligonucleotides are labeled as follows: PO, contains a phosphodiester backbone; PS, contains a stable phosphorothioate backbone; 2006 contains the human TLR9 recognition sequence; GC is similar to 2006 although all of the CG dinucleotides have been replaced with GC. Cells were also treated with TNF as a positive control for cPLA$_2$γ expression. Total RNA was collected and used to measure cPLA$_2$γ levels by real-time RT-PCR compared to cyclophilin A as an internal control.

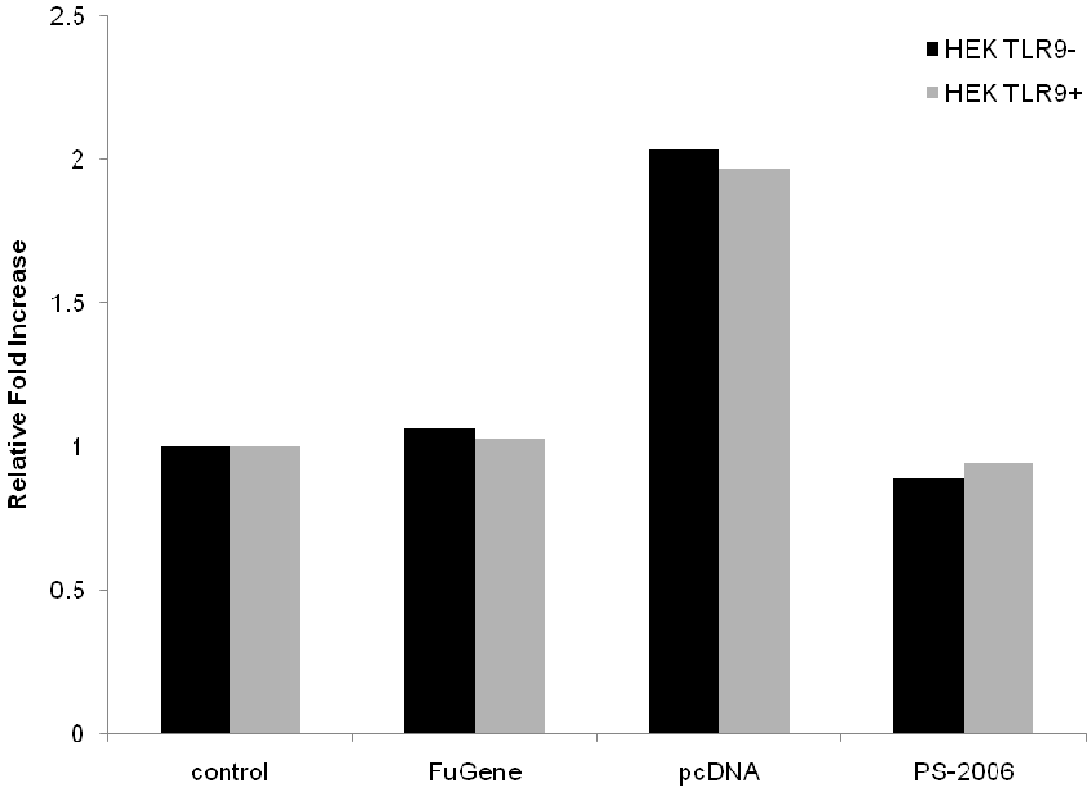

Figure 5-3. $cPLA_2\gamma$ induction by transfection reagent alone, transfected plasmid, or PS-2006 in HEK293 cells with and without TLR9 expression. Wild-type HEK293 cells contain minimal expression of TLR9 (TLR9-) while an HEK293 cell line overexpressing TLR9 (TLR9+) has also been utilized. Cells were treated with transfection reagent alone (FuGENE), transiently transfected with plasmid (pcDNA), or exposed to PS-2006. Total RNA was collected and used to measure $cPLA_2\gamma$ levels by real-time RT-PCR compared to cyclophilin A as an internal control.

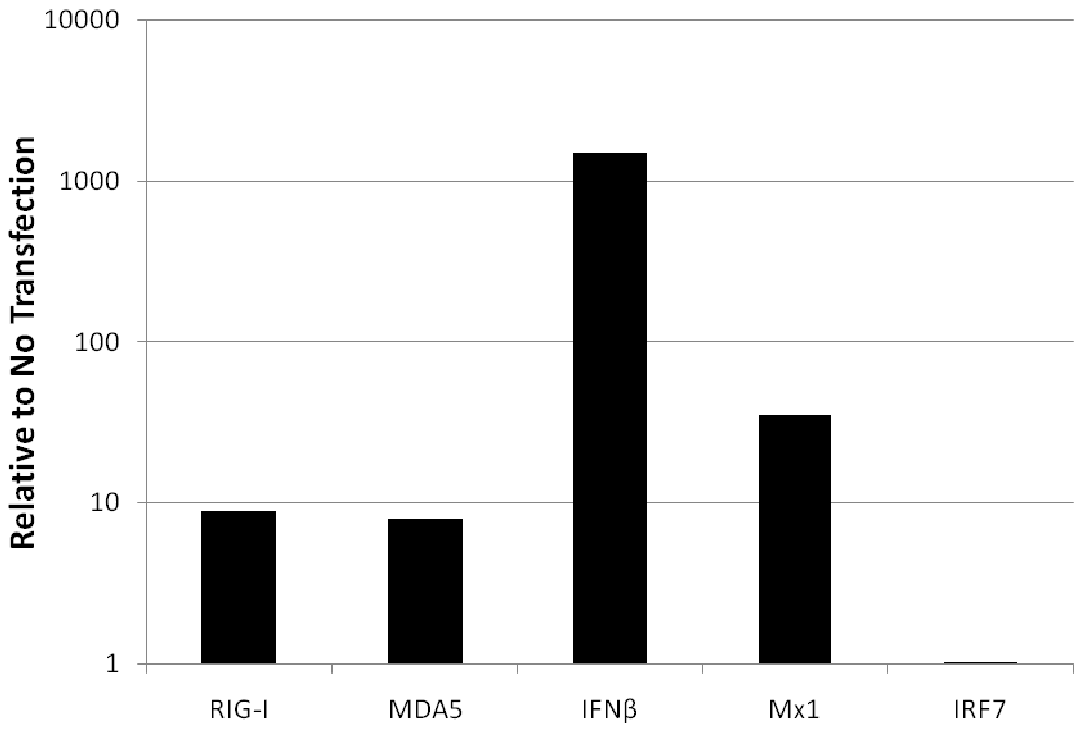

Figure 5-4. Expression of genes associated with the RNA sensing pathway in response to plasmid transfection in S9 cells. Total RNA was collected from S9 cells and used to measure the indicated genes levels by real-time RT-PCR compared to cyclophilin A as an internal control and graphed on a logarithmic scale.

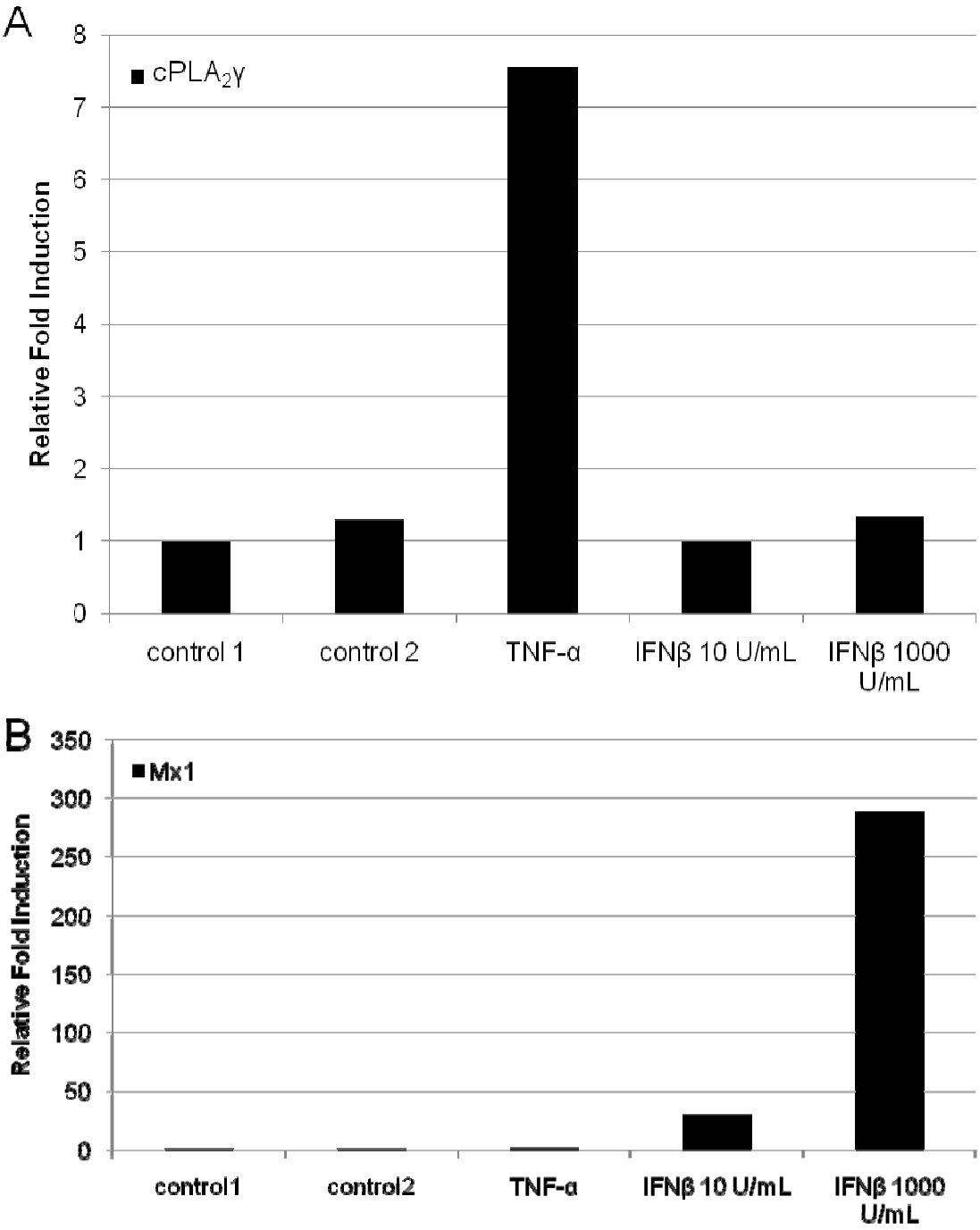

Figure 5-5. $cPLA_2\gamma$ expression in S9 cells following treatment with TNF-α or IFN-β. S9 cells were untreated (control 1 or control 2), treated with TNF-α, or treated with IFNβ at two different concentrations as indicated. Total RNA was collected and used to measure A) $cPLA_2\gamma$ or B) Mx1 mRNA levels by real-time RT-PCR compared to cyclophilin A as an internal control. Mx1 is included as a positive control for IFNβ treatment.

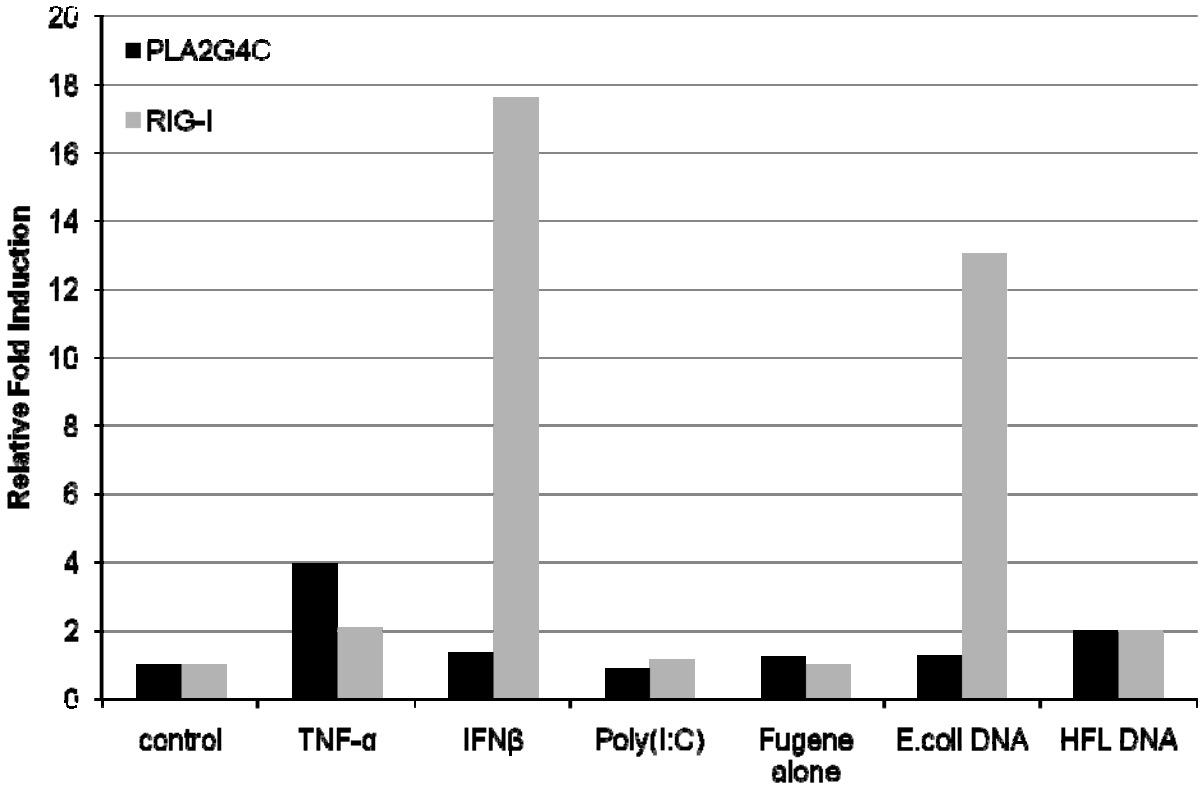

Figure 5-6. Real-time RT-PCR analysis of cPLA$_2$γ and RIG-I in HFL-1 cells with various treatments. HFL-1 cells were treated with TNF-α, IFNβ, poly(I:C) or FuGENE transfection reagent for 24 hours, or cells were transfected with 6 or 10 μg/mL of E. coli or mammalian genomic DNA isolated from HFL-1 cells for 24 hours. Total RNA was collected and cPLA$_2$γ or RIG-I, a cytosolic RNA sensor, were analyzed compared to cyclophilin A as an internal control.

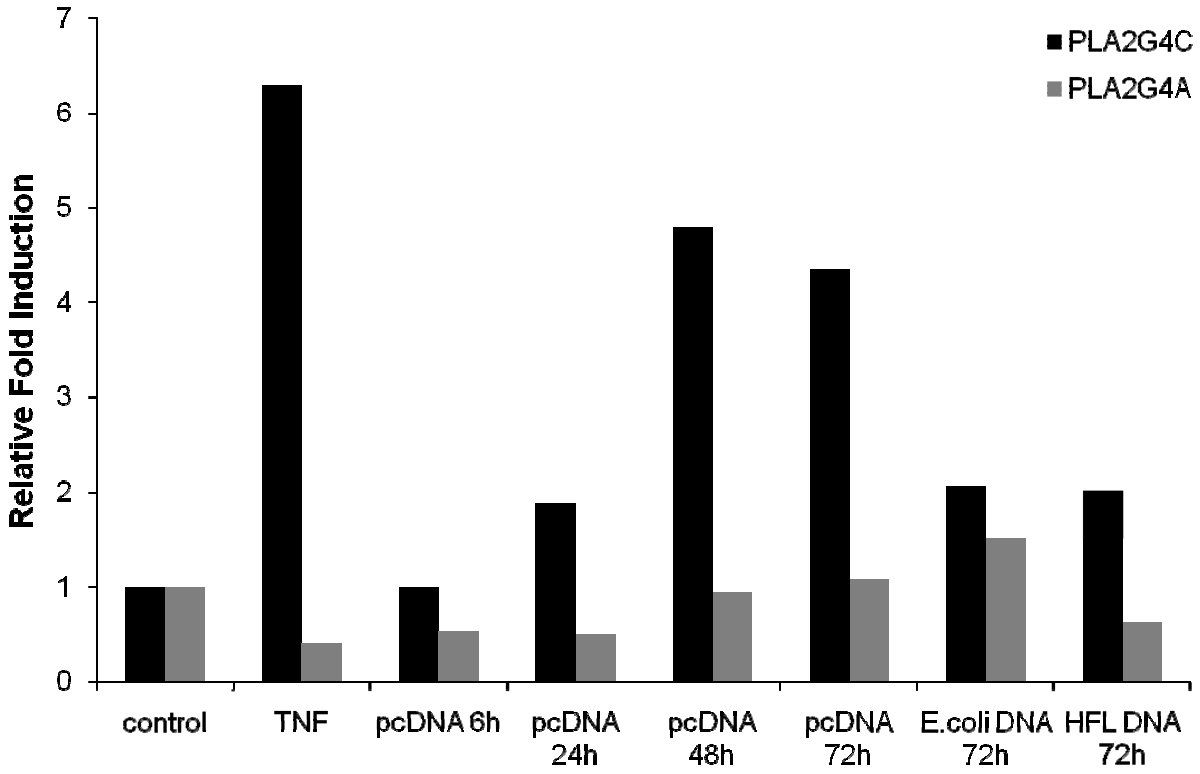

Figure 5-7. Real-time RT-PCR analysis of cPLA$_2$γ and cPLA$_2$α in response to various DNA treatments in S9 cells. S9 cells were treated with TNF-α or transfected with pcDNA, E. coli DNA, or mammalian (HFL) DNA for the indicated amounts of time. Following treatment, total RNA was collected and used for real-time RT-PCR analysis of cPLA$_2$γ and cPLA$_2$α compared to cyclophilin A as an internal control.

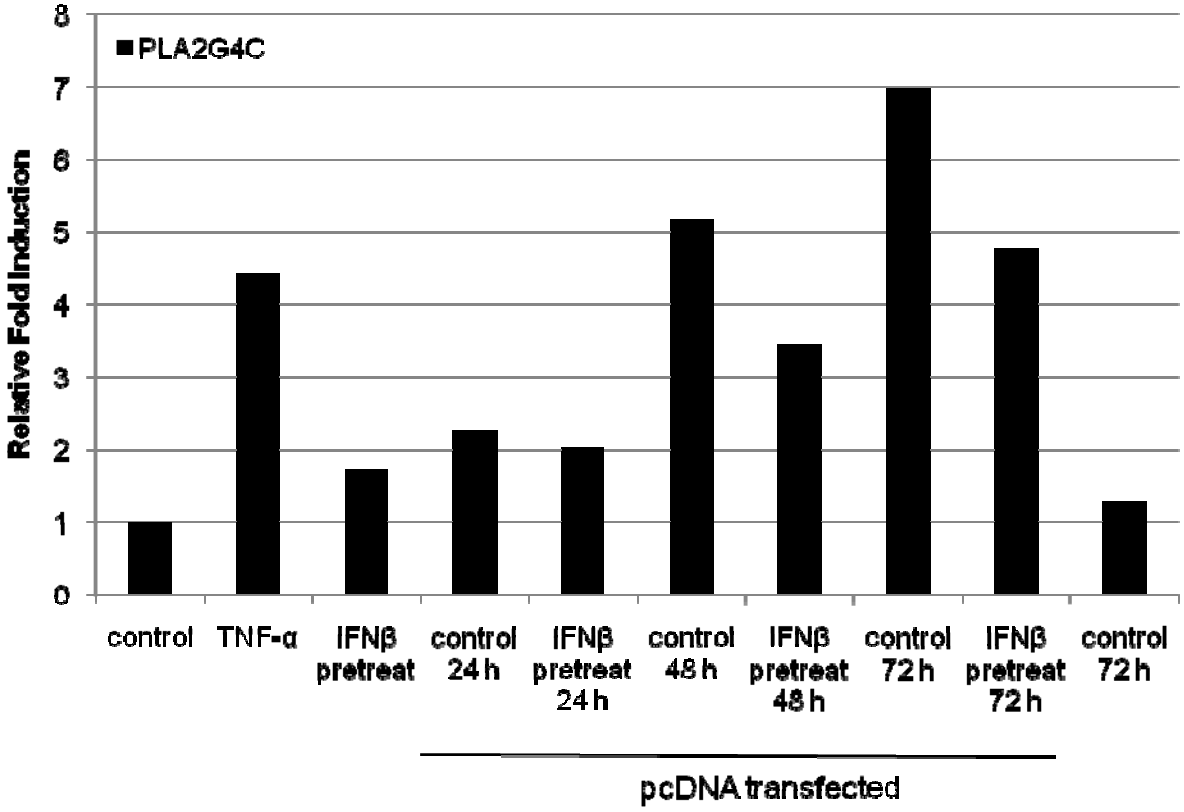

Figure 5-8. Real-time RT-PCR analysis of cPLA$_2$γ in S9 cells primed with IFNβ and transfected with pcDNA. S9 cells were untreated (control) or treated TNF-α or IFNβ for 4 hours prior to transfected with pcDNA3.1 for the indicated amounts of time. Following transfection, cells were given fresh media and collected after the indicated amounts of time for analysis of cPLA$_2$γ compared to cyclophilin A as an internal control.

CHAPTER 6
FUTURE DIRECTIONS

Role of cPLA$_2$γ in the Inflammatory Response

Although the data in Chapter 3 imply that the 15-LO pathway may be associated with cPLA$_2$γ gene induction, the downstream effects of this gene's induction is unclear. Previously published data have not thoroughly investigated any possible connection to 15-LO, but they have implied that cPLA$_2$γ leads to downstream production of prostaglandins, specifically PGE$_2$ [56]. These uncertainties leave open the possibility that cPLA$_2$γ may be contributing to the resolution phase of inflammation or to the acute phase. Due to this lack of understanding, it still needs to be determined whether the function of this gene is beneficial or harmful to the organism in the context of the inflammatory response.

In order to fully analyze the downstream effects of cPLA$_2$γ, complete knockdown of the gene must be obtained, ideally through the generation of a cPLA$_2$γ knockout mouse line. At the time of writing, cPLA$_2$γ knockout mice are in the final stages of production by the Knockout Mouse Project (KOMP, current progress available at www.sanger.ac.uk/htgt/report/project_gene_report?project_id=40524). Utilizing cells derived from knockout mice alongside the a parental control cell line, prostanoid and leukotriene synthesis must be measured. These two cell lines should be analyzed in the absence and presence of TNF-α to stimulate endogenous gene transcription, along with low and high serum levels, 1% and 10% to effect enzymatic activity [49]. Enzyme-linked immunoassays (ELISAs) can then be performed to identify the relative abundances of released downstream eicosanoid products. If it is determined that cPLA$_2$γ has a positive influence on anti-inflammatory eicosanoid products, such as lipoxins, then increasing

$cPLA_2\gamma$ activity relative to other phospholipases may prove to be beneficial in controlling inflammation. If the opposite is found to be true, then methods of inhibition of $cPLA_2\gamma$ need to be determined, potentially utilizing knowledge of how the $cPLA_2\gamma$ is transcriptionally regulated.

Figure 4-2 demonstrated that $cPLA_2\gamma$ mRNA induction observed in Chapter 3 was not a direct response to *Aspergillus fumigatus* in lung epithelial cells. It remains likely that immune cells that have responded to the *Af* extract secrete a cytokine, likely TNF-α, which is responsible for the induction of $cPLA_2\gamma$ mRNA seen in epithelial cells. Currently, studies are being pursued in collaboration with Dr. Shannon Wallet at the University of Florida to determine the cytokine or cytokines being produced by mouse alveolar macrophages which is eliciting the response seen in parenchymal cells.

Therapeutic Control of Inflammation by Regulating the Expression of $cPLA_2\gamma$

In order to determine the impact that this gene has on the inflammatory cascade and inflammation in general, $cPLA_2\gamma$ needs to be studied in an animal model. Because a mouse model of allergic asthma has already been established, it would be reasonable to use this model as the starting point. Either knockout mice would be utilized (when available), or mice would be treated with inhaled siRNA specific to $cPLA_2\gamma$. In order to induce allergic asthma and an inflammatory response, mice would be similarly sensitized and challenged with *Af* and indicators of inflammation would be measured as before [70]. Based on the downstream mediators that have been determined by ELISAs in the previously described experiments, either an exacerbation or ablation of inflammation would be expected when $cPLA_2\gamma$ is knocked down or absent from the lungs of these mice. The results of such a study would demonstrate the impact that

cPLA$_2$γ has on the inflammatory cascade and thus define the direction to take to utilize induction or repression of cPLA$_2$γ for potential therapies targeting inflammation.

The Role of cPLA$_2$γ at the Phospholipid Membrane

Although attempts have been made to determine specificity of cPLA$_2$γ for both head-groups and acyl chains of phospholipids, all of these experiments have been performed in *in vitro* assays which do not recapitulate the endogenous environment of the cPLA$_2$γ protein [48, 59]. In order to gain a true understanding of the role that this enzyme has on membrane composition, and perhaps membrane remodeling, phospholipid content would have to be analyzed in the presence and absence of endogenous cPLA$_2$γ activity in whole cells. It has previously been shown that specific phospholipid species can be identified by MALDI IMS (matrix-assisted laser desorption/ionization imaging mass spectrometry) and their distribution can be determined in several day old mouse embryos [192]. Knowing the precise phospholipid species and how their relative abundances are affected by cPLA$_2$γ expression would provide the most accurate representation of both head-group and acyl chain specificity to date. Additionally, if this imaging can be refined to visualize molecules on subcellular levels, then local changes in phospholipid species could be determined, this would serve to codify which membranes cPLA$_2$γ acts upon, whether it is endoplasmic reticulum, Golgi, mitochondrial membranes, or, in the case of mouse oocytes, the nuclear envelope.

Role of cPLA$_2$γ in the Innate Immune Response

The studies provided here are preliminary in regards to identifying the role that cPLA$_2$γ plays as a target of the DNA sensing pathway. This is a critical pathway in the innate response to DNA based viruses and is present in all cell types. Upon infection,

viral DNA is released into the cytoplasm which is then sensed by this part of the innate immune response. In order to determine if cPLA$_2$γ is involved in a physiological response upon infection with a DNA virus, collaboration will be pursued with Dr. David Bloom at the University of Florida. Currently, he has offered mouse brain tissue from mice that have been infected with HSV, a DNA based virus.

The possibility still exists that the response seen by the induction of cPLA$_2$γ could be due to other type I interferons, such as any of the various IFNα subtypes. In order to eliminate some of the remaining pathways from the possible causes of cPLA$_2$γ gene induction, an RNA Polymerase III inhibitor, such as ML-60218, will be employed. If treatment with such an inhibitor blocks cPLA$_2$γ induction upon plasmid transfection, then the DAI and AIM2 pathways can be eliminated since an RNA Pol III inhibitor would not affect the direct sensors of DNA. Although no interferon-sensitive response element (ISRE) was found within 2 kb up- or downstream of the cPLA$_2$γ start site by *in silico* analysis, identification of such a site would be useful for codifying cPLA$_2$γ as a primary response to cytosolic DNA and not a secondary response to something such an IFNα. Additionally, the AIM2 pathway needs to be investigated further because IL-1β is a prominent end-product of this response, and, as shown in Figure 4-2, IL-1β is an inducer of cPLA$_2$γ. This pathway can be eliminated or codified by treatment with an IL-1β receptor antagonist or IL-1β antibodies to prevent a possible autocrine response of IL-1β following DNA transfection. Also, inhibitors or siRNA can be used to specifically inhibit these pathways, while other antibodies or receptor antagonists can be used to analyze potential autocrine responses if cPLA$_2$γ is a secondary product of cytosolic-

DNA sensing. Regardless of the precise method of induction of $cPLA_2\gamma$, it is clear that this gene may have roles in the inflammatory response as a first-responder to infection.

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BIOGRAPHICAL SKETCH

Justin was born to Alan and Cindy Bickford in South Florida in the winter of 1980. He grew up in the subtropical climate with an early interest in science starting with paleontology, then progressing to marine biology. In high school, he followed every available avenue for science and theatre. While in college, Justin undertook various leadership positions and founded an improvisational theatre troupe and, in his senior year, he finally settled on pursuing a degree in chemistry while working as an engineer for BellSouth. After two summers as an engineer, he opted for a laboratory technician position in a research laboratory and found himself working for Dr. Amy Hearn in the laboratory of Dr. Harry Nick. Upon the realization that a Ph.D. program did not require a 30 mortgage on his education, he chose to pursue a research Ph.D. in biomedical science.