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Induction of Group IVC Phospholipase A2 in Allergic Asthma: Transcriptional Regulation by TNF- α in Bronchoepithelial Cells

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Abstract

Airway inflammation in allergen-induced asthma is associated with eicosanoid release. These bioactive lipids exhibit anti- and pro-inflammatory activities with relevance to pulmonary pathophysiology. We hypothesized that sensitization/challenge using an extract from the ubiquitous fungus, *Aspergillus fumigatus* (*Af*), in a mouse model of allergic asthma would result in altered phospholipase gene expression, thus modulating the downstream eicosanoid pathway. We observed the most significant induction in the group IVC phospholipase A2 (cPLA₂ γ or PLA₂G4C). Our results infer that *Af* extract can induce cPLA₂ γ levels directly in eosinophils while induction in lung epithelial cells is most likely a consequence of TNF- α secretion by *Af*-activated macrophages. The mechanism of TNF- α -dependent induction of cPLA₂ γ gene expression was elucidated through a combination of promoter deletions, ChIP and overexpression studies in human bronchoepithelial cells, leading to the identification of functionally relevant CRE, NF- κ B and E-box promoter elements. ChIP analysis demonstrated that RNA polymerase II, c-Jun/ATF-2, p65/p65 and USF1/USF2 complexes are recruited to the cPLA₂ γ enhancer/promoter in response to TNF- α with overexpression and dominant negative studies implying a strong level of cooperation and interplay between these factors. Overall, our data link cytokine-mediated alterations in cPLA₂ γ gene expression with allergic asthma and outline a complex regulatory mechanism.

Key words: Allergy, Lung, Inflammation, Transcription, PLA₂G4C, Promoter

Introduction

The initial physiological response to infection or tissue injury is inflammation. A defining event in this response is the liberation of bioactive lipid metabolites of arachidonic acid (AA), the eicosanoids. The eicosanoids play a pivotal role in the inflammatory response, ultimately mediating vasodilation, vascular permeability, bronchoconstriction, chemotaxis, and the transcription of pro-inflammatory enzymes, as well as being involved in cancer [1-3]. This extensive family of fatty acids includes the prostaglandins (PGs), prostacyclin (PGI), thromboxanes (TXs), leukotrienes (LTs), hydroperoxyeicosatetraenoic acids (HPETEs), hydroxyeicosatetraenoic acids (HETEs), lipoxins (LXs), resolvins and protectins [2, 3]. The eicosanoids arbitrate many general cellular processes, such as cell differentiation [4], apoptosis [5], lipid membrane integrity [6] and vascular homeostasis [7]. The precursor of these products is produced by hydrolysis of membrane phospholipids by the phospholipase A₂ (PLA₂) family of enzymes, yielding AA. The PLA₂ family is composed of multiple groups of secretory PLA₂s and two groups of cytosolic PLA₂s. The role of these enzymes in general cellular processes and, in particular, in inflammatory processes underscores the relevance of this pathway in driving the inflammatory and immune responses.

One such inflammatory and immune mediated pathology is allergen-induced asthma. Recently, studies have focused not only on the release of histamine and acetylcholine, but also the impact of AA metabolites on the inflammatory cascade in asthmatic airways. Therefore, we hypothesized that evaluation of transcriptional regulation of the phospholipases in an animal model that involves both cell-mediated (T_h1) and humoral immunity (T_h2) would provide insight into the early regulatory events that propagate the inflammatory response. We thus utilized a mouse model of allergic asthma in C57BL/6J mice which employs a crude extract from the ubiquitous fungus *Aspergillus fumigatus* (*Af*) as the sensitizing and challenging agent. The *Af* sensitized mice developed a T_h2-mediated allergic inflammatory response, including elevated levels of the T_h2 cytokines, IL-4, IL-5 and IL-13, increased total serum IgE, goblet cell hyperplasia and airway eosinophilia [8]. More specifically, an increase in *Af* specific IgE and IgG was found in the serum of sensitized mice, demonstrating an *Af* specific immune response [8]. To date, most asthma studies have focused primarily on secretory PLA₂s from sputum samples or cytosolic PLA₂s in circulating blood. We therefore evaluated the expression levels of a subset of phospholipases in lavaged whole lungs from these animals. Of these, group IVC phospholipase A2 [PLA₂G4C or cPLA₂γ] was identified as being significantly induced in the lungs of *Af* sensitized/challenged mice.

cPLA₂γ was first identified in 1998 from analysis of human brain ESTs [9]. Unlike the other members of the cPLA₂ family, cPLA₂γ is calcium independent lacking the C2 calcium binding domain and hence a requirement for micromolar concentrations of calcium for enzymatic activity [10, 11]. cPLA₂γ was analyzed and determined to possess phospholipase activity specific to the sn-2 position by *in vitro* assays and shown to increase arachidonic acid release from cells overexpressing cPLA₂γ [10, 12, 13]. Several independent studies have also helped to establish a biochemical and pathophysiological relevance for this unique calcium independent Group IV enzyme [14-17]. We also provide evidence, through studies with primary alveolar macrophages, that TNF-α (henceforth referred to as TNF) may serve as the underlying stimulus mediating cPLA₂γ gene regulation in our animal model. As a consequence, we have identified a TNF-responsive element in the proximal cPLA₂γ promoter region, residing within 114 bp upstream of the transcription start site which is associated with ATF-2/c-Jun, p65, and USF1/2 complexes.

Experimental

Animal Studies

C57BL/6J mice were housed in the SPF facility of the University of Florida according to NIH guidelines and all experiments were approved by the IACUC of the University of Florida. Animals were sensitized and challenged with *Aspergillus fumigatus* extract (*Af*) (Greer Laboratories) as previously described [8]. Briefly, C57BL/6J mice were sensitized to *Af* by intraperitoneal injections on days 0 and 14, aerosol challenged with *Af* extract on days 28, 29 and 30, and lungs were harvested for RNA isolation on day 32. Non-sensitized mice were mock sensitized with PBS and similarly challenged with *Af*.

Lung mRNA Extraction and Real-time RT-PCR

Whole lungs from mice were flash frozen in liquid nitrogen and total RNA was isolated [18]. cDNA was produced and real-time RT-PCR was performed on an ABI 7000 Sequence Detection System. Briefly, one μg of total RNA was reverse transcribed using the SuperScriptTM first strand synthesis kit (Invitrogen) and diluted to 100 μL . Two μL of the resulting cDNA was used for real-time PCR analysis using SYBR[®] Green Supermix with ROX (Bio-Rad) and analyzed by the $\Delta\Delta\text{C}_T$ method normalized to cyclophilin A as described previously [19]. Crossing threshold (CT) values from both cyclophilin A and respective target genes were used in the $\Delta\Delta\text{C}_T$ method to calculate relative fold inductions [19]. Analyzed genes are described in Supplementary Table 1 along with the mouse (MGI) and human (HGNC) gene symbols, common names and accession numbers, while primer sequences are provided in Supplementary Table 2.

Cytokine Measurements

Brochoalveolar lavage was used to collect primary alveolar macrophages from C57BL/6 mice. Macrophages were purified using magnetic selection and CD11b MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. 1×10^5 purified macrophages were stimulated with designated amounts of *Af* extract for 24 h, after which supernatants were collected and 14 cytokines and chemokines quantified using a Luminex multiplex kit (Millipore) according to the manufacturer's instructions.

Cell Culture

Human fetal lung fibroblasts (HFL-1), a human lung adenocarcinoma cell line (A549), or a human bronchial epithelial cell line (S9) [20], were maintained in Ham's F12K, the human eosinophilic leukemia cell line, EoL-1 [21], was maintained in RPMI-1640 medium. Cells were grown to 70-75% confluency before treatment. For human growth hormone (hGH) reporter assays, S9 cells were batch transfected at 60% confluency in 60 mm dishes with equimolar amounts of the indicated construct to control for transfection efficiency. For transfections, 1.5 μg of DNA was added to 4.5 μL of FuGene 6 (Roche) in serum free Ham's F12K medium to a final volume of 288 μL which was added to cells for 3 h, after which cells were rinsed with PBS and media was refreshed. In the batch transfection, cells were split into two plates 12 h post-transfection and allowed to recover for 12 h after which they were either left untreated or stimulated for 12 h as indicated below. Treatment concentrations, unless otherwise specified were 100 $\mu\text{g}/\text{mL}$ *Af*, 0.5 $\mu\text{g}/\text{mL}$ LPS, 5 ng/mL IFN γ , 10 ng/mL TNF, 2 ng/mL IL-1 β , 5 ng/mL IL-2, 5 ng/mL IL-3, 20 ng/mL IL-4, 10 ng/mL IL-6, 20 ng/mL IL-10, and 20 ng/mL IL-13.

Generation of Growth Hormone Reporter Gene Constructs

cPLA₂γ promoter fragments were amplified from bacterial artificial chromosome clones generously provided by the Wellcome Trust Sanger Institute using primers described in Supplementary Table 2. Amplified fragments were cloned into the TOPO-XL vector (Invitrogen), HindIII digested, and cloned into a HindIII digested promoterless growth hormone reporter plasmid (pØGH) [22]. The -114/-1 promoter was generated by site-directed deletion with the Quikchange® Site-Directed Mutagenesis Kit (Stratagene) from the -114/+174 fragment in pØGH according to the manufacturer's instructions. The heterologous viral thymidine kinase (TK) promoter was coupled to the hGH reporter gene to create a pTKGH vector as previously described [23]. pTKGH constructs were generated by inserting the -114/-1 fragment into a HindIII restriction site upstream of the TK promoter in the forward and reverse orientations. All constructs were verified by DNA sequencing.

Cell Culture RNA Isolation, Northern Analysis and Real-time RT-PCR

Total RNA was isolated as previously described [18] with the following modifications. Following guanidinium thiocyanate-phenol-chloroform extraction, cell lysates were precipitated at -20 °C for 30 min with isopropanol and the resulting pellets were resuspended in RNase-free water. LiCl was then added to a final concentration of 2 M. The tubes were again placed at -20 °C for 30 min, centrifuged for 20 min at 4 °C, rinsed with ethanol, dried and resuspended in RNase-free water prior to determination of concentration by UV spectrophotometry. Five µg of total RNA was fractionated on a 1% formaldehyde/agarose gel and electrotransferred to charged nylon membranes. The membranes were then probed for the indicated mRNA with a ³²P-radiolabeled probe and exposed to Hyperfilm™ MP (GE Healthcare) as previously described [24]. Densitometry was quantified from X-ray films using the ImageJ program (NIH). Data points are the means from at least three independent experiments. For real-time RT-PCR analysis, total RNA was isolated from transfected cells using an RNeasy Mini Kit (Qiagen), with reverse transcription and real-time PCR performed as described above.

Immunoblot Analysis

Protein was collected and subjected to immunoblot analysis as previously described [25]. Briefly, protein was isolated from treated cells in lysis buffer containing Complete Mini protease inhibitor (Roche) and the protein concentration was determined by a BCA assay (Pierce). 20 µg of protein was separated on a 7.5% Tris-HCl Ready-Gel (Bio-Rad) and electrotransferred to a Trans-Blot® (Bio-Rad) charged nitrocellulose membrane. Membranes were then stained with Ponceau S (Sigma/Aldrich) to verify even loading. After blocking, membranes were probed with antibodies to cPLA₂γ (kind gift from Christina Leslie) or β-actin. Probed membranes were subjected to ECL™ (GE Healthcare) and visualized by exposure to Hyperfilm™ MP (GE Healthcare). Densitometry was quantified from X-ray films using the ImageJ program (NIH).

Chromatin Immunoprecipitation (ChIP)

ChIP analysis was performed as previously described [25]. Briefly, S9 cells were treated with 10 ng/mL of TNF for 12 h prior to formaldehyde crosslinking. Immunoprecipitation without antibody (noAb) or with nonspecific IgG were used as controls alongside antibodies for c-Jun, ATF-2, p65, p50, USF1, USF2, or RNA Polymerase II (Santa Cruz). Following immunoprecipitation and purification, samples were subjected to real-time PCR analysis using primers for the cPLA₂γ promoter/enhancer and a nonspecific control region for the cPLA₂γ 3'UTR (Supplementary Table 2). All values are graphed as a

fraction of the total isolated chromosomal DNA (input) prior to immunoprecipitation. Only values above those of baseline IgG were considered relevant.

Transcription Factor Overexpression

Overexpression of transcription factor proteins or their respective dominant negative forms was performed using a mammalian expression plasmid containing the coding region for the following transcription factors: c-Jun, p65 (kind gift from Dr. Mary E. Law), ATF-2 (kind gift from Dr. Alt Zantema), or USF1 (kind gift from Dr. Jörg Bungert) or the dominant negative forms: c-Jun/Tam67, p65/ Δ TA (kind gift from Dr. Mary E. Law), ATF-2/A-ATF2 (kind gift from Dr. Charles Vinson) [26], or USF/A-USF (kind gift from Dr. Vinson) [27]. These plasmids were cotransfected, with the cPLA₂ γ -114/-1 fragment in p \emptyset GH, as described above. 48 h post-transfection, RNA was isolated, DNase I treated and subjected to real-time RT-PCR analysis as described above. The fold induction was calculated relative to cells transfected with the cPLA₂ γ enhancer/promoter hGH reporter construct and an empty mammalian expression vector (pcDNA3.1) without transcription factors (no TF) normalized to 1.

Statistical Analysis

Statistical analyses were determined by an unpaired Student's t-test to a p value ≤ 0.05 for all experiments. A two-tailed Student's t-test was performed for the animal and cell culture overexpression studies.

Results

Gene Expression of the Phospholipase A₂s

Our previous report demonstrated that *Af*-sensitized mice develop a T_H2 mediated response typical of asthma as indicated by increases in total serum IgE, goblet cell hyperplasia and airway eosinophilia [8]. Having established a physiologically and environmentally relevant asthmatic model, we proceeded to evaluate changes in phospholipase gene expression in tissue from lavaged lungs as a consequence of *Af*-induced asthma. The gene expression levels for a subset of the sPLA₂s were evaluated by real-time RT-PCR (Fig. 1A). Only *Pla2g2e* and *Pla2g5* levels were increased in the *Af* sensitized/challenged animals. In contrast, *Af* resulted in a ~50% reduction in the basal expression of *Pla2g12b*. We next evaluated the expression levels of three members of the PLA₂ group IV family, *Pla2g4a*, *Pla2g4b*, and *Pla2g4c*. Interestingly, the expression levels for *Pla2g4a* and *Pla2g4b* remained unchanged, while the relative expression of *Pla2g4c* increased dramatically (~35 fold) in *Af* sensitized/challenged mice (Fig. 1B).

Evaluation of Pla2g4c/cPLA₂γ Expression in Immune and Parenchymal Lung Cells

We therefore sought to further evaluate the molecular mechanisms regulating *Pla2g4c*, which will be henceforth referred to as cPLA₂γ, in relationship to our allergen-induced asthma model. We previously noted that eosinophils composed ~70% of cells in the BALs in sensitized/ challenged C57BL/6J mice [8]. Therefore, we first analyzed the expression of cPLA₂γ in a human eosinophilic leukemia cell line, EoL-1 [21]. Additionally, several components of *Af* are ligands for the toll-like receptors [28], many of which are constitutively expressed in eosinophils. Figure 2A illustrates that cPLA₂γ expression levels are increased up to 15 fold in response to increasing concentrations of *Af* extract in EoL-1 cells. In contrast, cPLA₂γ mRNA levels were unchanged after *Af* exposure in a human bronchial epithelial-derived cell line, S9 [20] (Fig. 2A).

To determine which other cell types or downstream signaling events in the lung could be contributing to the overall induction seen in whole lungs from our mouse model, primary mouse alveolar macrophages were exposed to *Af* extract. Interestingly, primary alveolar macrophages exposed to *Af* extract did not exhibit an increase in cPLA₂γ mRNA levels (data not shown), however we did observe changes in secreted levels of specific cytokines and chemokines (Fig. 2B). IFNγ, TNF, IL-6, IL-10, and the immune cell-specific chemokines, MCP1 and MIP-1α, were elevated, providing potential insight into the robust induction of cPLA₂γ seen in whole lungs. Based on this finding, cPLA₂γ mRNA levels were then evaluated in S9 cells treated with LPS, IFNγ, TNF and a variety of interleukins (Fig. 2C). Only TNF and IL-1β were capable of inducing cPLA₂γ mRNA in this parenchymal cell line, with the most potent activation elicited by TNF (>6 fold) (Fig. 2C). We also investigated this response in two additional human lung-derived cell lines, A549 (epithelial-like adenocarcinoma) and HFL-1 (human fetal lung fibroblast), with cPLA₂γ mRNA induced in each of these cell lines, although the largest induction was still observed in the S9 cells (Fig. 2D). Furthermore, Figure 2E illustrates a representative immunoblot (top) and corresponding densitometry (bottom), confirming that cPLA₂γ protein levels are commensurately elevated by 3 fold, 24 h following TNF exposure in S9 cells.

It has previously been demonstrated that TNF plays a critical role in asthma progression in response to ovalbumin in mice [29]. Lindbom et al. [30] have demonstrated an increase in cPLA₂γ mRNA by TNF in lung epithelial cells. Based on these studies, we believe that macrophage derived TNF may be the common link between observations in the animal model of *Af*-induced asthma and the induction of cPLA₂γ in the S9 bronchoepithelial cells. To further characterize the temporal pattern of this induction, human S9 cells were exposed to TNF for increasing durations, displaying a statistically significant induction of the steady-state mRNA by 4 h (Fig. 2F). An increase in steady-state mRNA levels may be a result of *de novo* transcription of the gene or due to increased stability of the mRNA

transcript. Analogous to a nuclear run-on, we utilized real-time RT-PCR of heterogeneous nuclear RNA (hnRNA) [24] to determine the level of contribution of *de novo* transcription. These results demonstrate that hnRNA levels are rapidly induced within 30 min and become maximal at 1 h (~9 fold) (Fig. 2F).

Identification of a TNF responsive region controlling cPLA₂ γ gene expression

We next sought to identify the regulatory sequences responsible for TNF-mediated induction. The structure of the human cPLA₂ γ gene derived from the Wellcome Trust Sanger Institute (www.sanger.ac.uk) is displayed in Figure 3A. The human mRNA is 2581 bp in length and comprises 17 exons spanning over 60,000 bp. The promoter region of the cPLA₂ γ gene is expanded in Figure 3A depicting the fragments utilized for promoter deletion analyses from a human cPLA₂ γ BAC clone and subcloned into a human growth hormone (hGH) reporter plasmid [22]. To evaluate the functionality and cytokine-responsiveness of these promoters, each construct was transiently transfected into S9 cells. To control for transfection efficiency, S9 cells were batch transfected with each construct, exposed to TNF and hGH expression evaluated by northern analysis. Following incubation with IL-1 β or TNF, total cellular RNA was extracted and hGH reporter gene expression evaluated by northern analysis and densitometry (Fig. 3A). It was determined that cytokine-responsive sequences reside within the 1.3 kb promoter fragment and that TNF clearly elicits the most significant induction (Fig. 3B). The TNF response requires sequences from -114 to +174 relative to the transcriptional start site (Fig. 3C). To determine the relevance of the 174 bp exonic sequence, we also studied a fragment from -114/-1, which demonstrated a lower but significant TNF-dependent induction (Fig. 3D), with no statistically significant difference between the two constructs. In addition to the ability of this region to function as a promoter, we tested this fragment's function as a stimulus-dependent enhancer. Constructs were generated by coupling the -114/-1 fragment in the forward and reverse orientation to a minimal viral thymidine kinase (TK) promoter that exhibits low level expression in most cell types. This fragment was able to drive the heterologous TK promoter in an orientation-independent manner, satisfying the definition of a stimulus-specific enhancer element (Fig. 3E).

We next sought to identify potential regulatory factor binding sequences within the -114/-1 enhancer/promoter fragment. The consensus sites for CRE, NF- κ B and a potential E-box were identified using the TESS software (www.cbil.upenn.edu/cgi-bin/tess/tess) based on the TRASFAC database [31]. The potential regulatory factor consensus binding sites are underlined in Figure 4A along with the sites which were subsequently deleted in black italicized text. Northern and real-time RT-PCR analyses demonstrate that deletion of the respective binding sites inhibits the TNF-mediated induction to varying degrees, with the deletions of the CRE and NF- κ B sites having the most pronounced effect (Fig. 4B, C). ***ATF-2, c-Jun, p65/RelA, USF1 and USF2 as Cognate Regulatory Factors Binding the cPLA₂ γ Enhancer/Promoter by ChIP Analysis***

To determine the cognate transcription factors in the endogenous chromatin environment that may bind the respective enhancer/promoter consensus binding elements, we employed chromatin immunoprecipitation (ChIP) analysis. Two structurally and functionally related protein families have been associated with CRE sites, Jun and ATF [32]. ATF-2/c-Jun heterodimers have been identified previously, such as in the well-studied IFN- β [33] and TNF enhancers, the latter of which also responds to TNF [34]. We next evaluated the potential interaction of c-Jun and ATF-2 with primers flanking the -114/-1 enhancer/promoter in response to 12 h of TNF treatment by ChIP analysis (Fig. 5A). We utilized three controls in our ChIP studies to ensure the specificity of the results: no antibody (noAb), nonspecific IgG (IgG) and ChIP analysis of a downstream 3' untranslated region (3'UTR) of the cPLA₂ γ gene. The data demonstrate the inducible association of both ATF-2 and c-Jun with the enhancer/promoter region as compared to the 3'UTR or other control samples (Fig. 5A). The NF- κ B consensus element can be occupied by a family of transcription factors, including p50, p52, p65 (RelA),

c-Rel and RelB [35]. The results with p65/RelA-specific ChIP clearly demonstrate that p65 inducibly binds to the human cPLA₂γ enhancer/promoter region following 12 h of TNF treatment (Fig. 5B). The E-box sequence (5'-CANNTG-3') is bound by a repertoire of cognate factors, including the upstream stimulating factors (USF1 and 2). USF1 and 2 proteins are ubiquitously expressed and function as transcriptional activators, through interaction with members of the pre-initiation complex as well as in the recruitment of chromatin remodeling enzymes [36]. ChIP analysis demonstrated specific interaction of USF1 and 2 with the cPLA₂γ enhancer/promoter region following 12 h of TNF stimulation (Fig. 5C). It should be noted that USF1 and USF2 function almost exclusively as a heterodimeric complex [37], which is supported by our ChIP results.

We next sought to determine if there was a temporal ordered recruitment of these factors following stimulation. We performed similar ChIP analyses at 15, 30 and 60 min (Fig. 6). RNA Polymerase II is recruited to the enhancer/promoter within 15 min of stimulation with TNF, as were p65 and USF1 (Figs. 6A-C). We also tested p50, the prominent binding partner for p65, and found no detectable association of p50 with the enhancer/promoter up to 60 min after stimulation (Fig. 6B). Interestingly, neither ATF-2 nor c-Jun showed a statistically significantly inducible association with the enhancer/promoter region up to two hours, although there does appear to be an upward trend of ~2 fold above baseline IgG levels 1-2 h following TNF stimulation (Fig. 6D).

Functional relevance of ATF-2, c-Jun, p65, USF: Overexpression and Dominant Negative Analyses

To establish the functional relevance of these transcription factors to human cPLA₂γ gene regulation, we cotransfected S9 cells with each individual transcription factor and the cPLA₂γ enhancer/promoter hGH reporter construct (Fig. 7A). Real-time RT-PCR demonstrates the ability of c-Jun, p65 or USF1 alone to drive expression from the cPLA₂γ enhancer/promoter, with no statistically significant response to ATF-2 (Fig. 7A). To further investigate the roles of these factors, we next utilized dominant negative forms of each factor lacking activation domains: c-Jun/Tam67, ATF-2/A-ATF2, p65/ΔTA, and USF/A-USF. The dominant negative USF/A-USF has been shown to inhibit function of both USF1 and USF2 [27]. We observed a small but statistically significant inhibition of TNF stimulation with c-Jun/Tam67 and USF/A-USF (1.6 and 1.8 fold, respectively), relative to the 3.6 fold observed in the control with no transcription factor transfection (Fig. 7B). However, p65/ΔTA completely blocked TNF induction mediated from the cPLA₂γ enhancer/promoter (Fig. 7B). Interestingly, only ATF-2/A-ATF2 was capable of inhibiting basal activity (>50%), while c-Jun/Tam67 showed an ~2.5 fold increase in basal activity. Our repeated attempts to further delineate the roles of these transcription factors utilizing combinations or individual siRNAs were unsuccessful despite >50% knockdown of the endogenous protein levels (data not shown). This may be a consequence of transcription factor cooperativity/interdependence and/or the close proximity of the CRE, NF-κB and E-box binding sites (Fig. 4A).

To address the cooperation between the subset of factors involved in transcriptional activation, c-Jun, p65, and USF1 (Fig. 7A) were coexpressed with specific dominant negatives. We chose to exclude further studies with c-Jun/Tam67 due to its ability to activate transcription in our studies as well as others (Fig. 7B) [38]. Overexpression of ATF-2/A-ATF2 and USF/A-USF in combination with p65 caused a >50% inhibition relative to p65 alone (Fig. 7C). Similarly, c-Jun overexpression results in a ~3.5 fold induction which is inhibited >50% by coexpression with a dominant negative to its putative heterodimeric binding partner, ATF-2/A-ATF2 (Fig. 7C).

Discussion

Given the recent studies which have linked the ubiquitous fungus, *Aspergillus fumigatus* (*Af*), with an increased prevalence in asthmatics [39, 40], we employed a mouse model which could mimic the pathology of allergen-induced asthma using an *Af* extract for sensitization/challenge. The metabolic flux through the eicosanoid pathway initially requires the release of AA from membrane phospholipids, so we examined a subset of phospholipase A₂s in our mouse model (Fig. 1A). Our observed induction of *Pla2g5* correlates with recent studies by the Balestrieri laboratory linking PLA₂G5 with the innate response demonstrating its importance in the macrophage response to the phagocytosis of zymosan and *Candida albicans* [41] as well as in antigen processing and dendritic cell maturation during pulmonary inflammation and the immune response to the house dust mite *Dermatophagoides farinae* [42].

To date, most asthma studies have focused primarily on secretory PLA₂s from sputum samples or cytosolic PLA₂s in circulating blood. Therefore, we examined RNA from lavaged whole lung for members of the cytosolic group IV PLA₂s, which have been designated IVA through IVF with the corresponding Greek letters as cPLA₂α, β, γ, δ, ε and ζ, respectively. These enzymes have been directly linked to the cytosolic liberation of AA as a consequence of the inflammatory response, with the vast majority of studies focused on PLA₂G4A [43]. The only phospholipase for which we observed a significant induction was *Pla2g4c/cPLA₂γ* (~35 fold) (Fig. 1B).

In addition to the present data associating cPLA₂γ with an allergic T_H2-type effector response, Brown et al. [14] previously demonstrated an analogous induction of this enzyme in the infection of intestinal epithelium by the helminth, *Trichinella spiralis*. These investigators proposed a major role for cPLA₂γ in the initiation and propagation of parasite induced allergic inflammation. Our observation that this gene is elevated in an asthma model is novel, with the only other documented connection coming from a study on the secreted group X phospholipase in asthmatic patients where the investigators simply noted the presence of *PLA2G4C* in sputum cells [44].

Given the connection of cPLA₂γ with *Af* sensitization/challenge and in parasitic infection by *Trichinella spiralis* [14], we felt it was physiologically relevant to further evaluate the molecular mechanisms mediating cPLA₂γ gene regulation in the lung. Our analyses (Fig. 2A-E) of potential cell types and stimuli in the lung argue that *Af* extract may induce cPLA₂γ levels directly in eosinophils and through activation of macrophages leading to TNF secretion, ultimately inducing cPLA₂γ gene expression in lung fibroblasts and epithelial cells (Fig. 2D). Furthermore, we also confirmed the relevance of TNF-dependent induction at both the protein and transcriptional levels (Fig. 2E and 2F). Similarly, Lindbom et al. [30] showed induction of cPLA₂γ mRNA levels by TNF in RPMI 2650 nasal epithelial cells as well as BEAS-2B bronchoepithelial cells. This observation is consistent with the significant role of TNF in the etiology of severe or refractory asthma with the increased presence of TNF in the airway and peripheral blood mononuclear cells from severe asthmatics [45]. Furthermore, inhalation of recombinant TNF in normal subjects leads to the development of airway hyperresponsiveness and airway neutrophilia [46], while TNF blockade has been associated with improvement in severe asthma in both mice [47] and humans [48].

Therefore, to address the importance of TNF-mediated cPLA₂γ induction, we identified a TNF-responsive regulatory region within 114 bp of the cPLA₂γ transcriptional initiation site (Fig. 3A-D) in bronchoepithelial cells. We also demonstrated that this region could function as a stimulus-dependent enhancer based on its function with a heterologous promoter in an orientation independent fashion (Fig. 3E). Three separate transcription factor consensus sites within the enhancer/promoter, CRE, NF-κB and E-box, identified by *in silico* analysis, were functionally verified through deletion studies (Fig. 4) and shown to interact with ATF-2/c-Jun, p65 and USF1/USF2, respectively, through CHIP analysis (Fig. 5). Analogously, p65, ATF-2 and c-Jun have been previously associated with TNF-mediated responses [34,

49, 50]. In fact, the best characterized examples of ATF-2/c-Jun heterodimer interaction and activation occur within the IFN β and TNF- α promoters [33, 51].

A temporal analysis of regulatory factor occupancy within the enhancer/promoter demonstrated that RNA polymerase II, p65 and USF are found associated with this region within 15 min of TNF stimulation (Fig. 6A-C), whereas ATF-2/c-Jun may arrive within two hours (Fig. 6D). Interestingly, we found no evidence of p50 interaction with the cPLA $_2\gamma$ enhancer/promoter (Fig. 6B), in contrast to the classical NF- κ B complex, p65/p50. We therefore hypothesize that the interaction with the NF- κ B consensus sequence involves a p65 homodimer complex. Our overexpression studies further functionally link c-Jun, USF1, and, most significantly, p65, with induction of cPLA $_2\gamma$ enhancer/promoter activity (Fig. 7A), while the expression of dominant negative proteins established the central importance of p65 in TNF induction and ATF-2 in basal expression (Fig 7B). The functional interdependence of these transcription factors results from four independent observations: 1) our inability to affect the cytokine induction by individual siRNA knockdown, 2) the close proximity of each of the respective consensus sequences (Fig. 4A), 3) the interaction of six proteins (ATF-2/c-Jun, p65/p65, and USF1/USF2) within ~40 bp or four DNA helical turns, and 4) most relevantly, the coexpression of activators with functional dominant negative proteins (Fig. 7C). Our current thoughts are that the function of c-Jun is dependent on the presence of ATF-2, given the significant inhibition of c-Jun-dependent induction by the dominant negative ATF-2/A-ATF2 (Fig. 7C). Figure 7C also establishes that the function of p65 requires an strong interdependence with both ATF-2 and USF. This is further supported by the close proximity of the flanking ATF-2/c-Jun and USF1/USF2 binding sites to the centrally localized p65 consensus sequence (Fig. 4A). Interestingly, p65/ Δ TA has little to no effect on either the induction by c-Jun or USF1, implying that p65 is dependent on co-habitation with these factors with no reciprocity.

In summary, our results are the first to establish the induction of cPLA $_2\gamma$ in a model of allergic asthma. This is a unique finding given the limited number of studies which have addressed this specific group IV phospholipase. We hypothesize that allergen sensitization/challenge mediates the induction of cPLA $_2\gamma$ directly in eosinophils and indirectly in pulmonary epithelial and fibroblast cells via TNF elicited through the activation of alveolar macrophages. Our molecular analyses of cPLA $_2\gamma$ gene expression have revealed the existence of a TNF-responsive proximal enhancer/promoter element. We have demonstrated inducible association of ATF-2/c-Jun, p65, and USF1/2 complexes to this region along with a cooperative interaction between these transcription factors, thus providing the first comprehensive evaluation of cPLA $_2\gamma$ gene regulation in a pathophysiologically relevant context.

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Figure Legends

Figure 1.

Identification of transcriptionally regulated phospholipases in a mouse model of allergic asthma:

C57BL/6J mice were sensitized to *Af* by intraperitoneal injections on days 0 and 14, aerosol challenged with *Af* extract on days 28, 29 and 30, and lungs were harvested for RNA isolation on day 32. Non-sensitized mice were mock sensitized with PBS and similarly challenged with *Af*. **(A)** Real time RT-PCR of sPLA_{2s}, *Pla2g2c*, *Pla2g2d*, *Pla2g2e*, *Pla2g5*, *Pla2g10*, *Pla2g12b*, and **(B)** group IV cPLA_{2s}, *Pla2g4a*, *Pla2g4b*, and *Pla2g4c*. Data points represent the mean of $2^{-\Delta\Delta CT}$ values \pm SEM ($7 \leq n \leq 10$), and * indicates $p \leq 0.05$ as compared to PBS sensitized animals. A list of all mouse and orthologous human phospholipase genes used in the study appear in Supplementary Table 1 with accompanying accession numbers. Primer sets for each gene are listed in Supplementary Table 2.

Figure 2.

Determination of physiologically relevant stimuli and cell types associated with cPLA_{2γ} expression:

(A) Cells were treated with the indicated amounts of *Af* extract (0, 1, or 100 μ g/mL) and steady state mRNA levels were analyzed. Real-time RT-PCR of cPLA_{2γ} mRNA in response to *Af* extract in the human eosinophil cell line, EoL-1 (left) or the human bronchopulmonary epithelial cell line, S9 (right). Data points represent means of $2^{-\Delta\Delta CT}$ values (EoL-1 $n = 2$, $n = 3$ S9). **(B)** Mouse alveolar macrophages were purified from bronchoalveolar lavage and exposed to the indicated amounts of *Af* extract. Levels of IFN γ , TNF, IL-6, IL-10, MIP-1 α and MCP1 were measured in supernatants by ELISA. Data points represent the mean \pm SEM ($n = 3$). * indicates $p \leq 0.05$ as compared to respective controls. **(C)** Real-time RT-PCR of cPLA_{2γ} mRNA in S9 cells in response to the indicated cytokines: 100 μ g/mL *Af*, 0.5 μ g/mL LPS, 5 ng/mL IFN γ , 10 ng/mL TNF, 2 ng/mL IL-1 β , 5 ng/mL IL-2, 5 ng/mL IL-3, 20 ng/mL IL-4, 10 ng/mL IL-6, 20 ng/mL IL-10, and 20 ng/mL IL-13. **(D)** Real-time RT-PCR of cPLA_{2γ} mRNA in various human lung cell lines, A549, HFL-1 and S9, following treatment with 10 ng/mL of TNF for 12 or 24 h. **(E)** A representative immunoblot (top) and corresponding densitometry (bottom) of cPLA_{2γ} protein levels in response to 10 ng/mL of TNF in S9 cells. **(F)** A time-course of cPLA_{2γ} mRNA and heterogeneous nuclear RNA (hnRNA) by real-time RT-PCR in S9 cells treated with 10 ng/mL of TNF for the indicated amounts of time. All real-time RT-PCR data points represent mean of $2^{-\Delta\Delta CT}$ values \pm SEM ($n = 3$, unless otherwise indicated), and * indicates $p \leq 0.05$ as compared to the respective untreated controls.

Figure 3.

Identification of a TNF responsive region controlling cPLA_{2γ} gene expression.

(A) The cPLA_{2γ} genomic structure with an enlargement of the promoter region. Promoter deletion fragments used in subsequent studies are numbered relative to the transcription start site, +1, on the right with total sizes of each fragment listed on the left. **(B)** S9 cells were batch transfected with human growth hormone (hGH) reporter constructs containing the indicated promoter. Northern analysis was performed and membranes were probed with hGH, and reprobed with the internal control L7a, as described in the Materials and Methods. Controls include cells alone with no transfection (NT) or cells transfected with a promoterless hGH construct (pØGH). Representative northern analysis of hGH and the loading control, L7a, in S9 cells transfected with the indicated promoter fragment (1.3 or 3.9 kb) and treated with the indicated cytokine (2 ng/mL of IL-1 β or 10 ng/mL of TNF) for 12 h. **(C)** Representative northern analyses of hGH mRNA from S9 cells transiently transfected with hGH reporter constructs containing the -588/+174, -316/+174 and -114/+174 cPLA_{2γ} promoter fragments (top). Samples were untreated (control) or treated

with 10 ng/mL of TNF for 12 h with corresponding densitometric data (bottom). **(D)** Representative northern analyses of hGH mRNA from S9 cells transiently transfected with hGH reporter constructs harboring either the -114/+174 or -114/-1 cPLA₂γ promoter fragments (top). Samples were untreated (control) or treated with 10 ng/mL of TNF for 12 h. Of note, the size of the transcript from the -114/-1 promoter appears shorter than that of the -114/+174 due to the removal of sequence from exon one. Corresponding densitometric data shown below. **(E)** Representative northern analysis of hGH and a loading control, L7a, in S9 cells analyzing the enhancer properties of the -114/-1 cPLA₂γ promoter driving the heterologous viral thymidine kinase (TK) promoter versus the hGH reporter construct containing the TK promoter alone (pTKGH). The -114/-1 promoter fragment was inserted upstream of the TK promoter in an hGH reporter construct in the forward and reverse directions and treated with 10 ng/mL of TNF for 12 h. Densitometric data for all northern analyses are represented as mean ± SEM ($n = 3$), and * indicates $p \leq 0.05$ as compared to respective control cells.

Figure 4.

Identification of functional transcription factor binding sites within the proximal cPLA₂γ enhancer/promoter. **(A)** The sequence of the proximal cPLA₂γ enhancer/promoter is presented with *in silico* derived transcription factor consensus sites underlined and deleted regions illustrated in black italicized text as follows: ΔCRE (-82/71), ΔNF-κB (-58/-49), ΔE-Box (-41/-36). **(B)** A representative northern analysis of hGH mRNA from untransfected cells (NT) and cells transfected with the empty hGH vector (pØGH), the WT -114/-1 enhancer/promoter and the respective site deletions with or without 12 h of 10 ng/mL of TNF. **(C)** Real-time RT-PCR analysis identical experiments outlined in **(B)**. Real-time RT-PCR data points represent mean of $2^{-\Delta\Delta CT}$ values ± SEM ($n = 3$). The values above each TNF data point indicates the relative fold induction as compared to the respective untreated cells (Note: all TNF bars were statistically significant with a $p \leq 0.05$). The * denote inhibition of basal levels with statistical significance with a $p \leq 0.05$ relative to the WT -114/-1 in untreated cells. The (†) denotes a statistically significant reduction compared to the 9.53x fold induction observed for the WT -114/-1 construct with a $p \leq 0.05$.

Figure 5.

Chromatin immunoprecipitation (ChIP) analysis of the proximal cPLA₂γ enhancer/promoter at 12 h post stimulation. Samples were collected from cells with 10 ng/mL of TNF for 12 h and immunoprecipitated with antibodies to **(A)** ATF-2 or c-Jun ($n = 4$), **(B)** p-65 ($n = 5$) or **(C)** USF1 or USF2 ($n = 3$) and analyzed by real-time PCR with primers specific to the enhancer/promoter region as described in the Materials and Methods. Controls include samples which were not immunoprecipitated (noAb), samples immunoprecipitated with nonspecific IgG (IgG) and real-time PCR of a nonspecific 3' untranslated region of the cPLA₂γ gene (3'UTR). All values are graphed as a fraction of the total isolated chromosomal DNA (input) prior to immunoprecipitation. Data points represent the mean of $2^{-\Delta\Delta CT}$ values ± SEM and * denotes statistical significance with a p value ≤ 0.05 as compared to respective control cells.

Figure 6.

Temporal ChIP analysis of the proximal cPLA₂γ enhancer/promoter. Samples were collected from cells treated with 10 ng/mL of TNF for the indicated amounts of time (0, 15, 30, 60 or 120 min) and immunoprecipitated with antibodies to **(A)** RNA polymerase II (PolII), **(B)** p65 or p50, **(C)** USF1, or **(D)** ATF-2 or c-Jun and analyzed by real-time PCR with primers specific to the enhancer/promoter and the control 3'UTR regions as described in the Materials and Methods and Figure 5. All values are

graphed as a fraction of the total isolated chromosomal DNA (input) prior to immunoprecipitation. Data points represent the mean of $2^{-\Delta\Delta CT}$ values \pm SEM ($n = 3$) and * denotes statistical significance with a p value ≤ 0.05 as compared to respective control cells.

Figure 7.

Effect of individual and combined transcription factor and dominant negative overexpression on cPLA₂ γ enhancer/promoter activity. (A) S9 cells were cotransfected with an hGH reporter construct containing the proximal cPLA₂ γ enhancer/promoter (-114/-1) and expression vectors for the indicated transcription factors: c-Jun, ATF-2, USF1 or p65. The fold induction was determined relative to cells with no transfected transcription factor (no TF). Data points represent the mean of $2^{-\Delta\Delta CT}$ values \pm SEM ($n = 3$) and * denotes statistical significance with a p value ≤ 0.05 as compared to "no TF". (B) S9 cells were cotransfected with an hGH reporter construct containing the proximal cPLA₂ γ enhancer/promoter (-114/-1) and the indicated dominant negative transcription factors. S9 cells were then exposed to 10 ng/mL of TNF for 12 h and hGH levels were analyzed by real-time RT-PCR. Data points represent the mean of $2^{-\Delta\Delta CT}$ values \pm SEM ($n = 3$). Relative fold inductions are printed above the TNF bars and statistical significance for TNF induction with $p \leq 0.05$ is denoted by §. The * for TNF treatment in the case of c-Jun/Tam67, p65/ Δ TA, and USF/A-USF denotes statistical significance with a p value ≤ 0.05 as compared to the fold induction seen in the respective "no TF" bars. The † for the unstimulated/basal in the case of c-Jun/Tam67 (2.6x) and ATF2/A-ATF2 (46%) denotes $p \leq 0.05$ as compared to the "no TF" respective unstimulated/basal control. (C) S9 cells were cotransfected with an hGH reporter construct containing the proximal cPLA₂ γ enhancer/promoter (-114/-1) along with p65, c-Jun, or USF1 in combination with the indicated dominant negatives and analyzed by real-time RT-PCR. Data points represent of $2^{-\Delta\Delta CT}$ values mean \pm SEM relative to no TF ($n = 3$) and * denotes statistical significance with a p value ≤ 0.05 as compared to the respective wild-type transcription factor alone (no DN).

Figure 1A

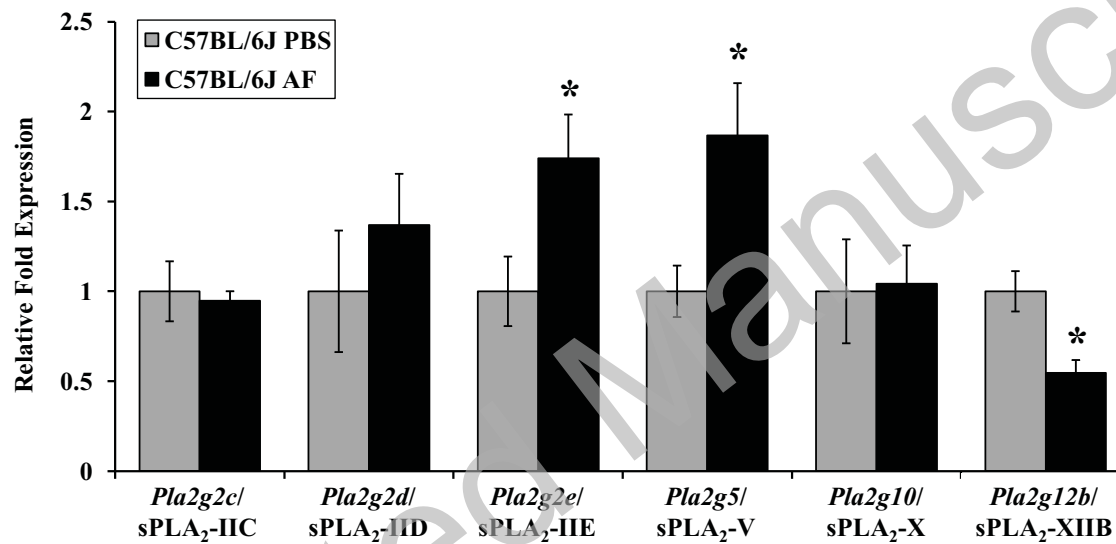


Figure 1B

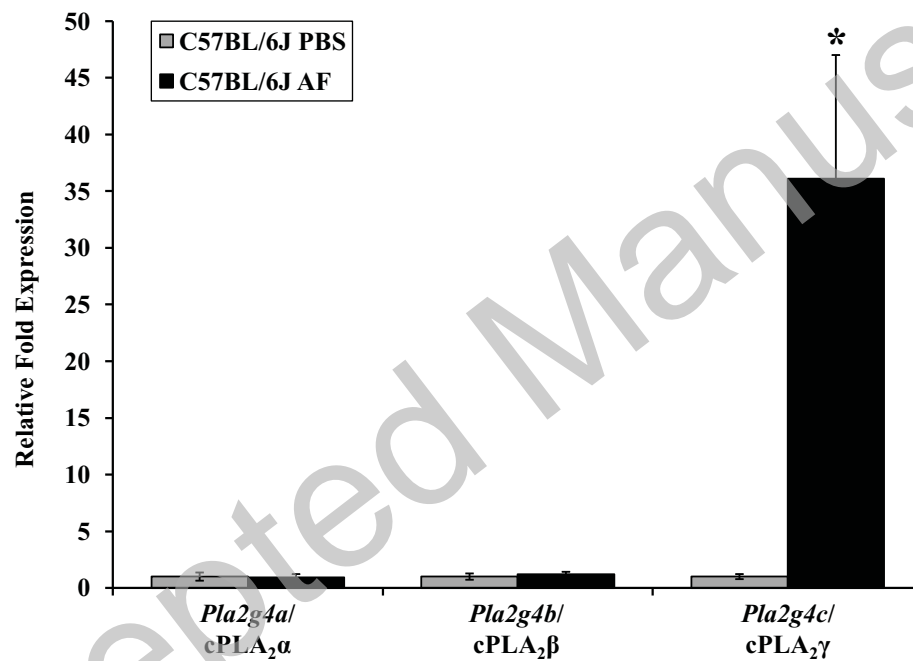


Figure 2A

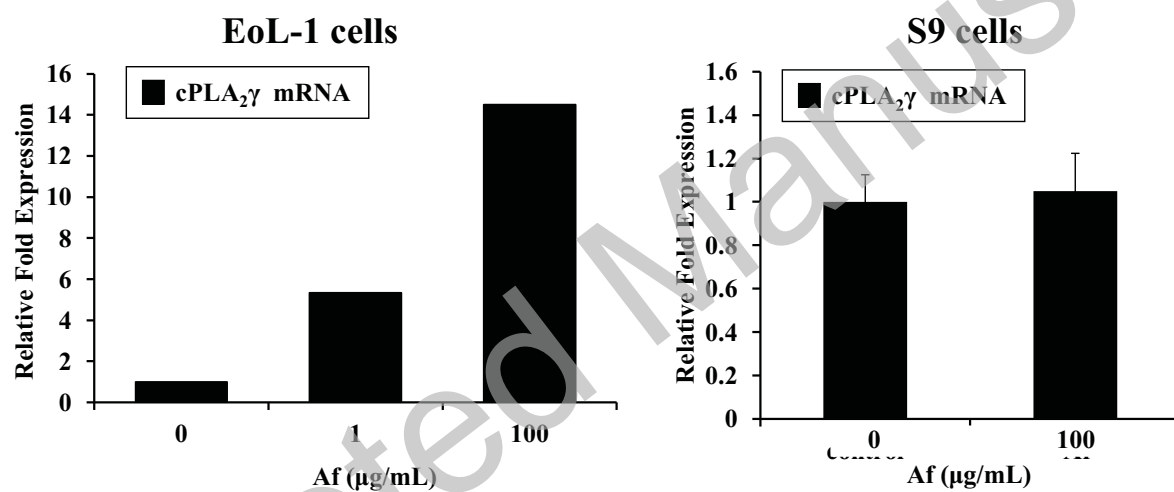


Figure 2B

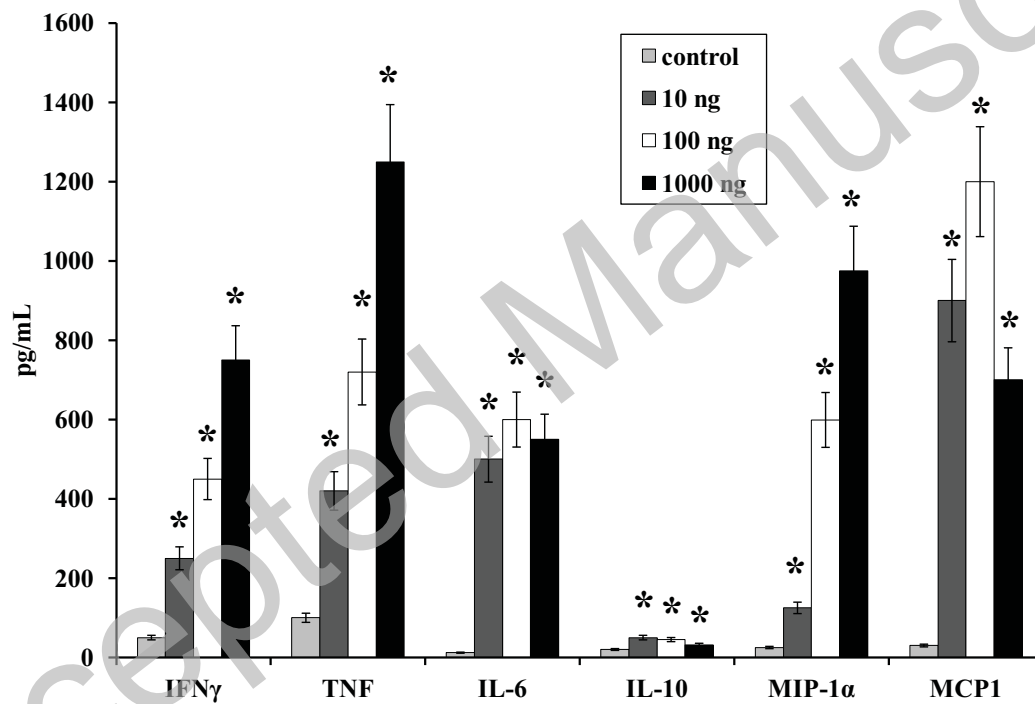


Figure 2C

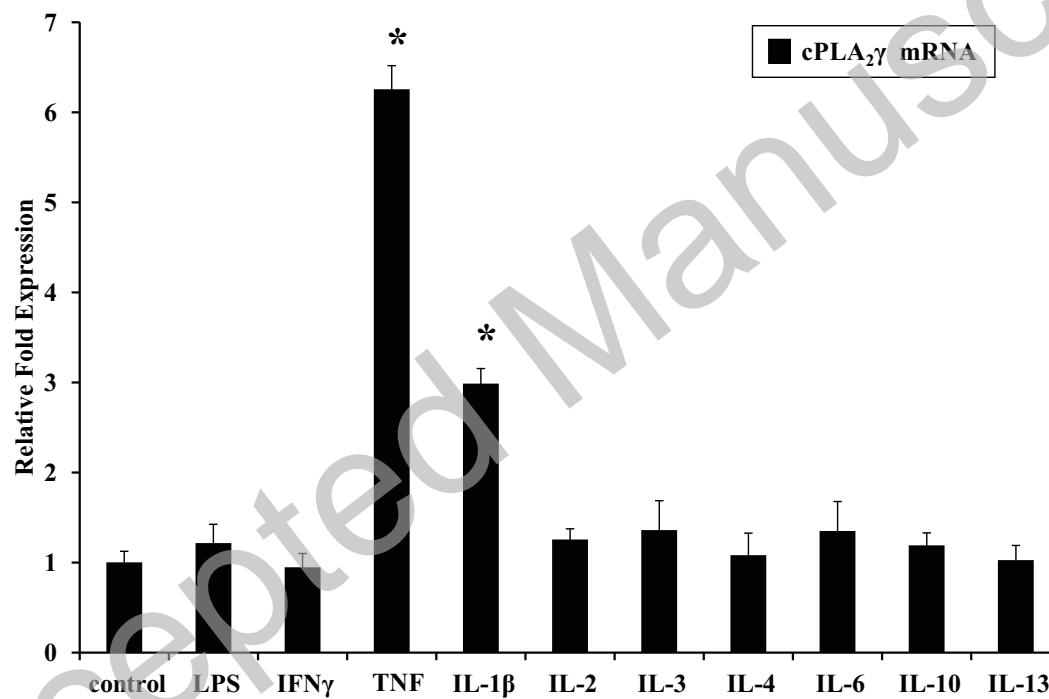


Figure 2D

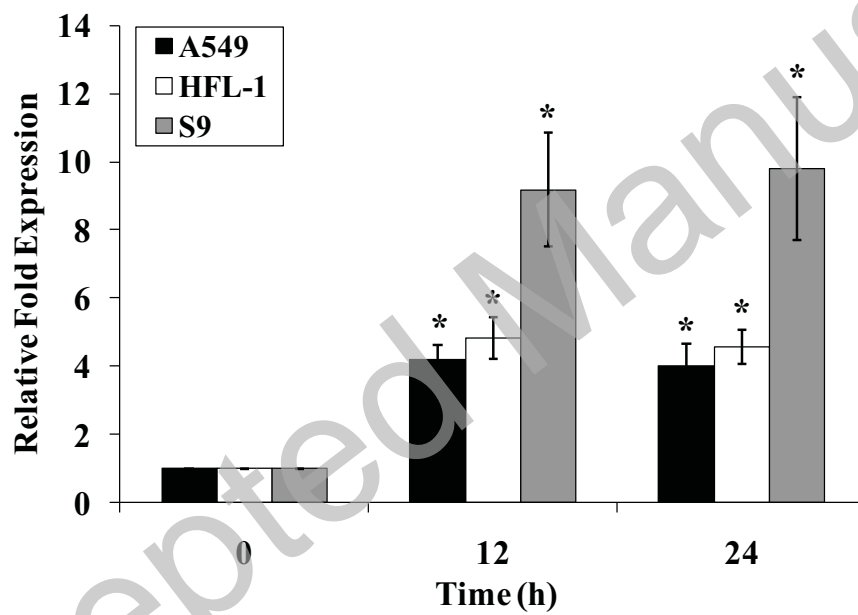


Figure 2E

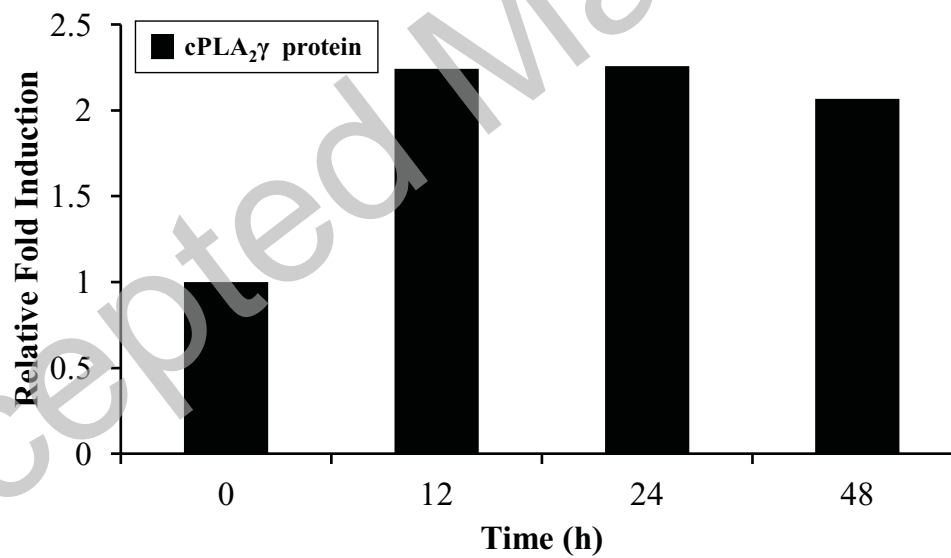


Figure 2F

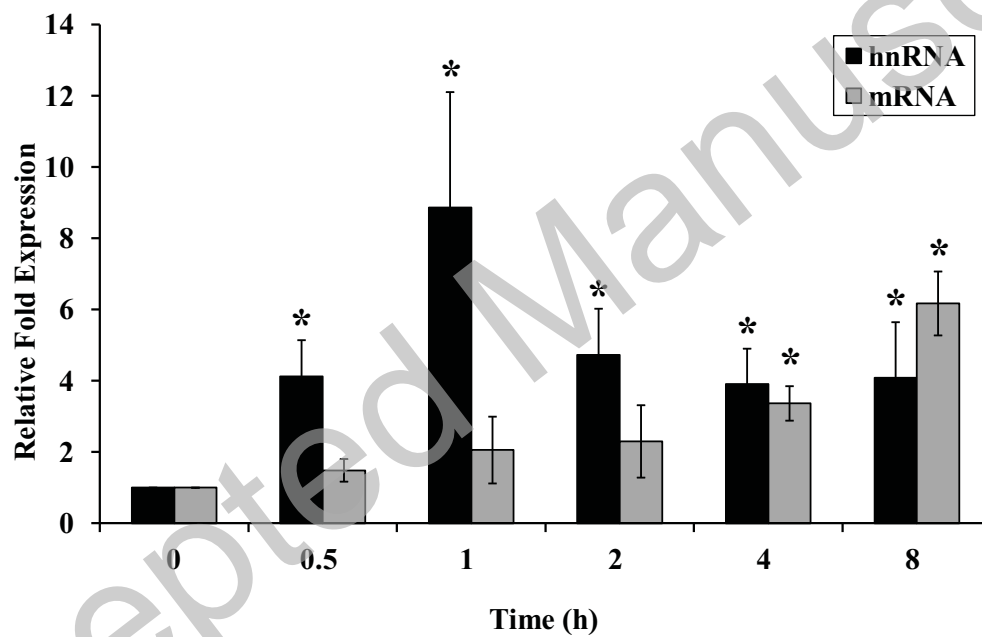


Figure 3A

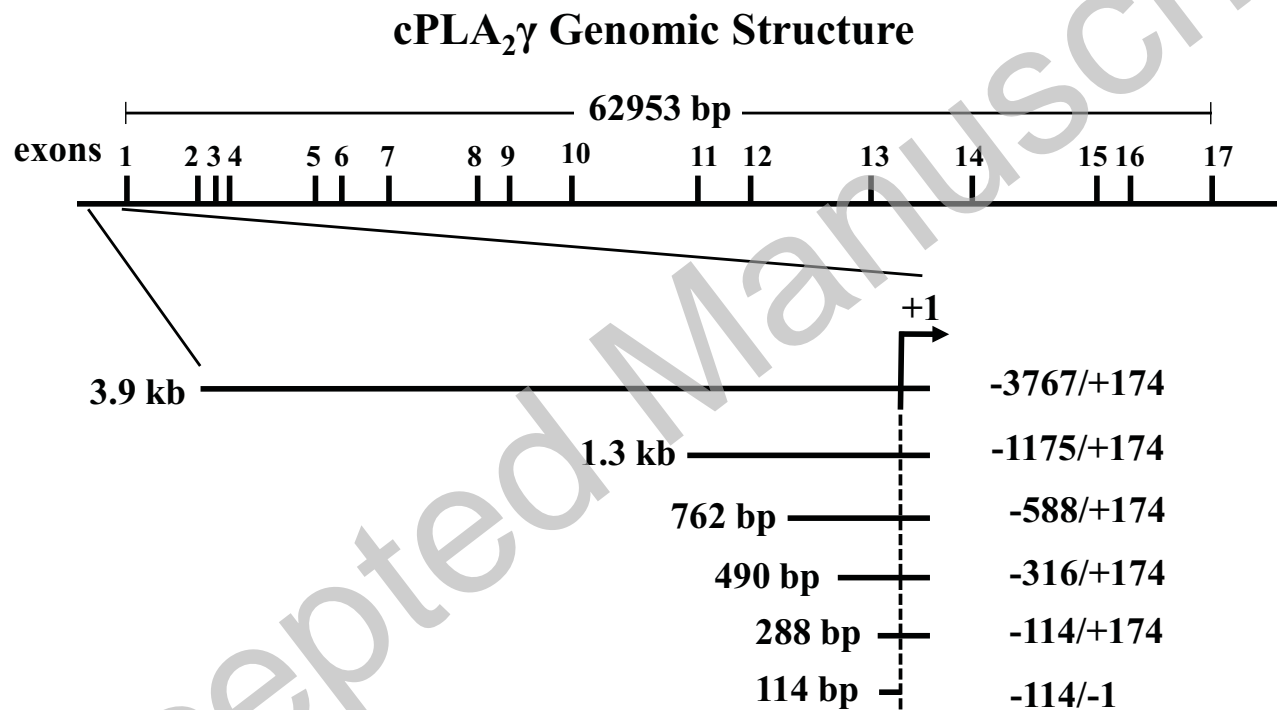
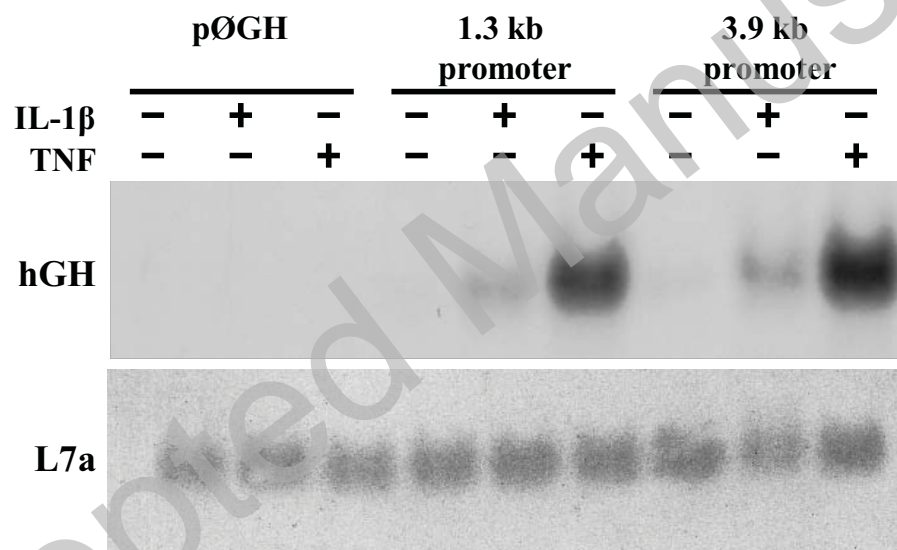


Figure 3B



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20111269

Figure 3C

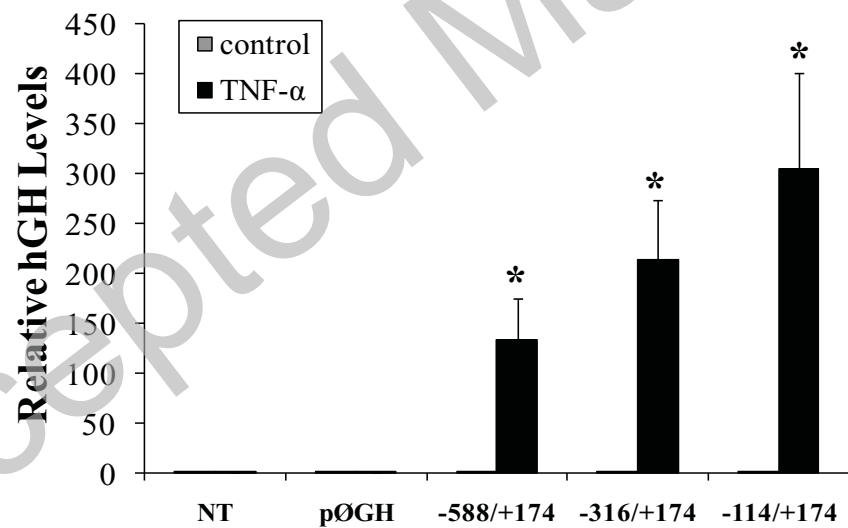
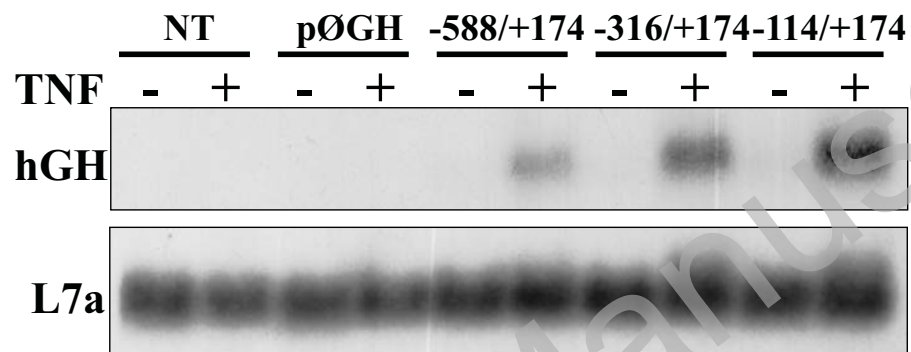


Figure 3D

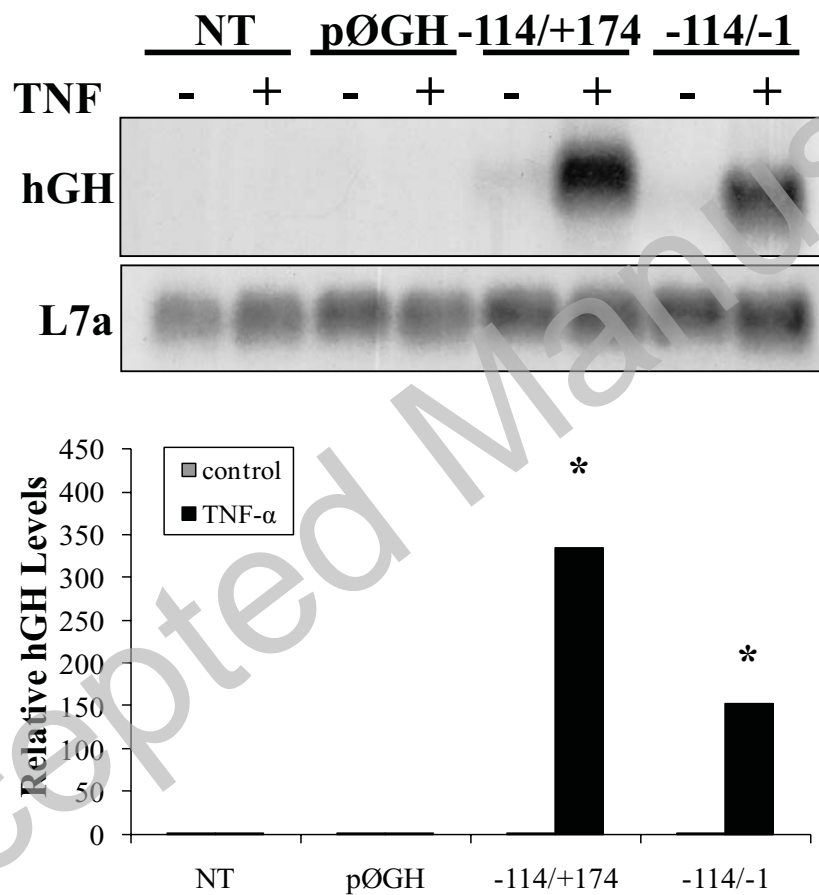
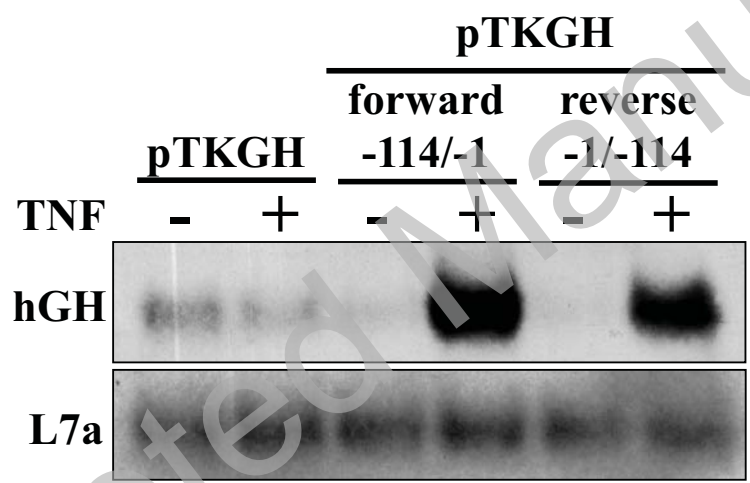


Figure 3E



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20111269

Figure 4A



Figure 4B

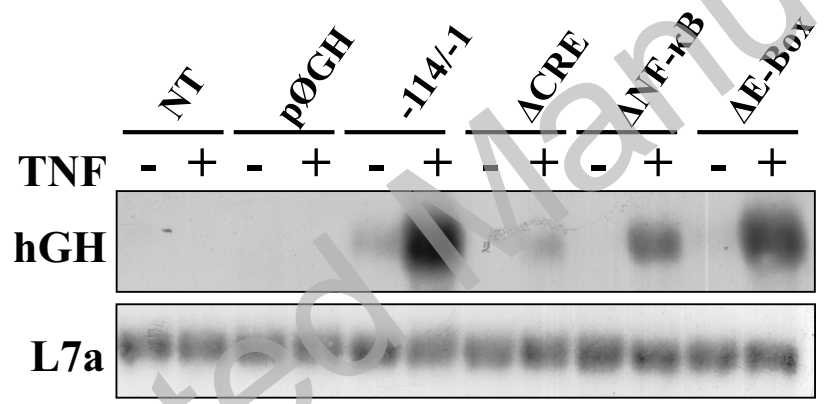


Figure 4C

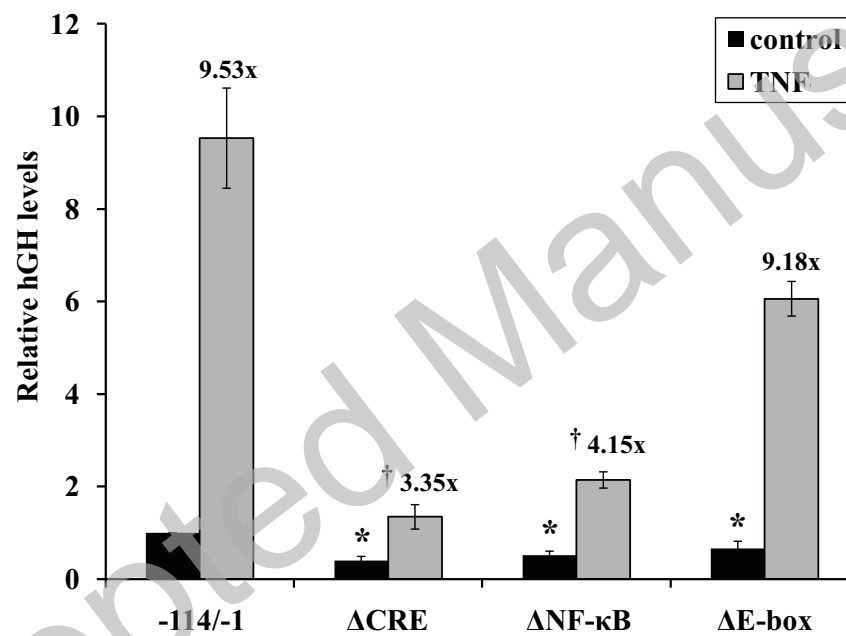


Figure 5A

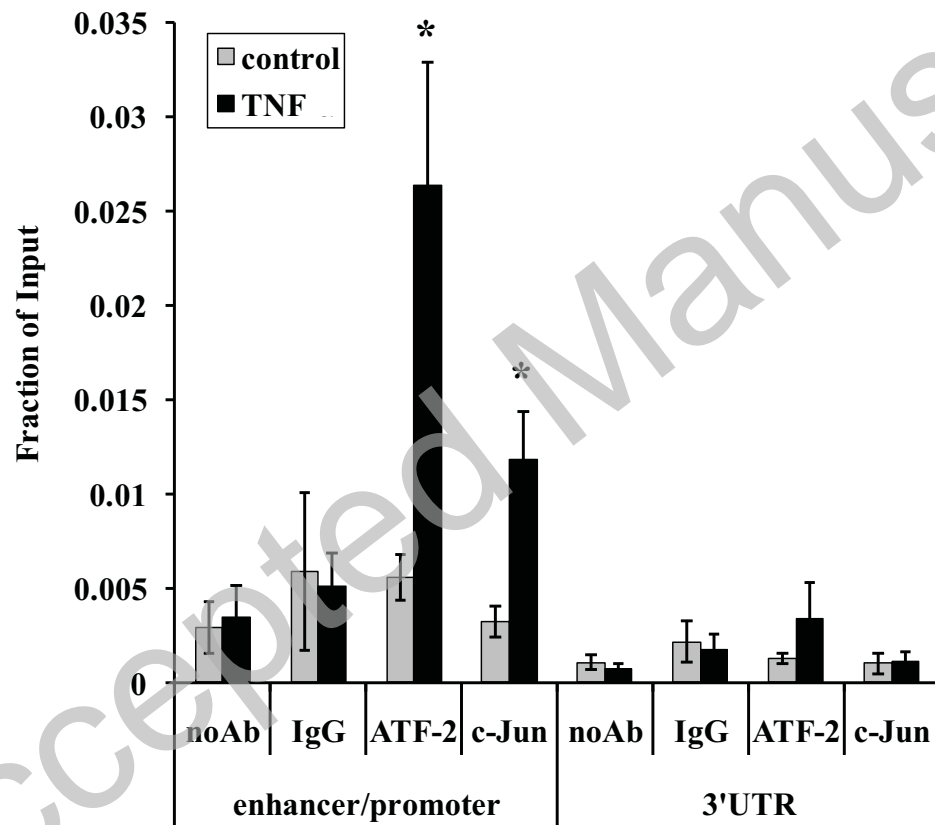


Figure 5B

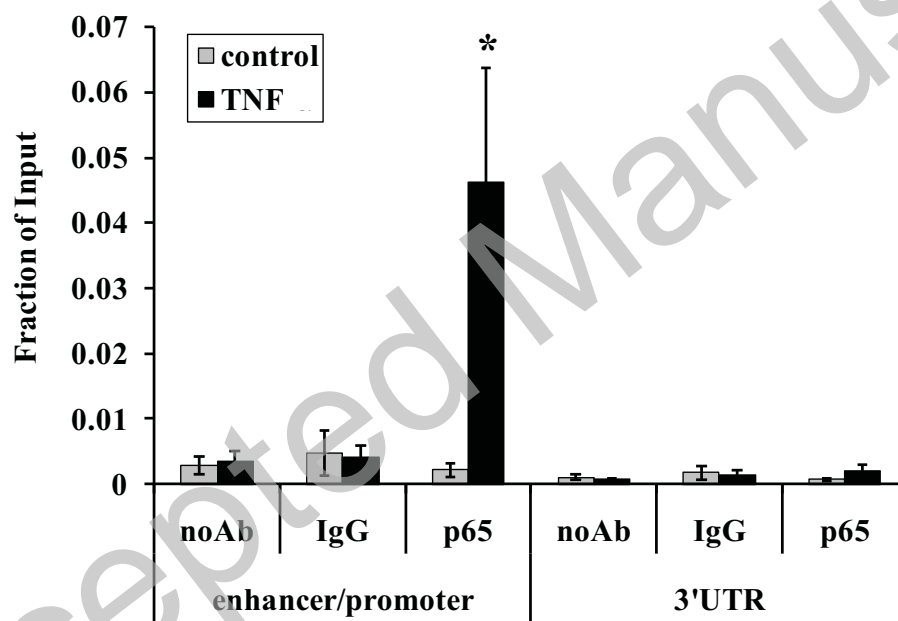


Figure 5C

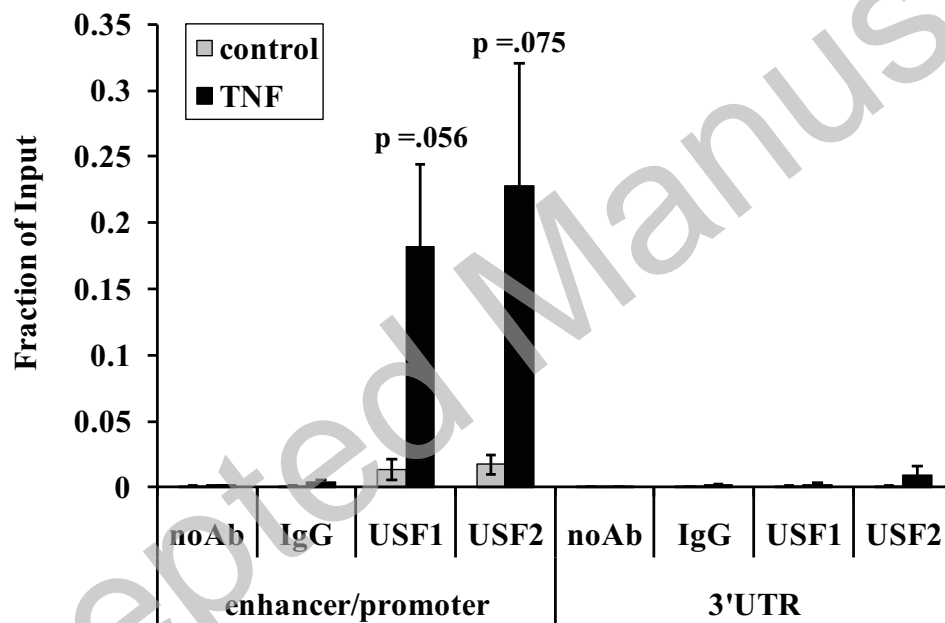


Figure 6A

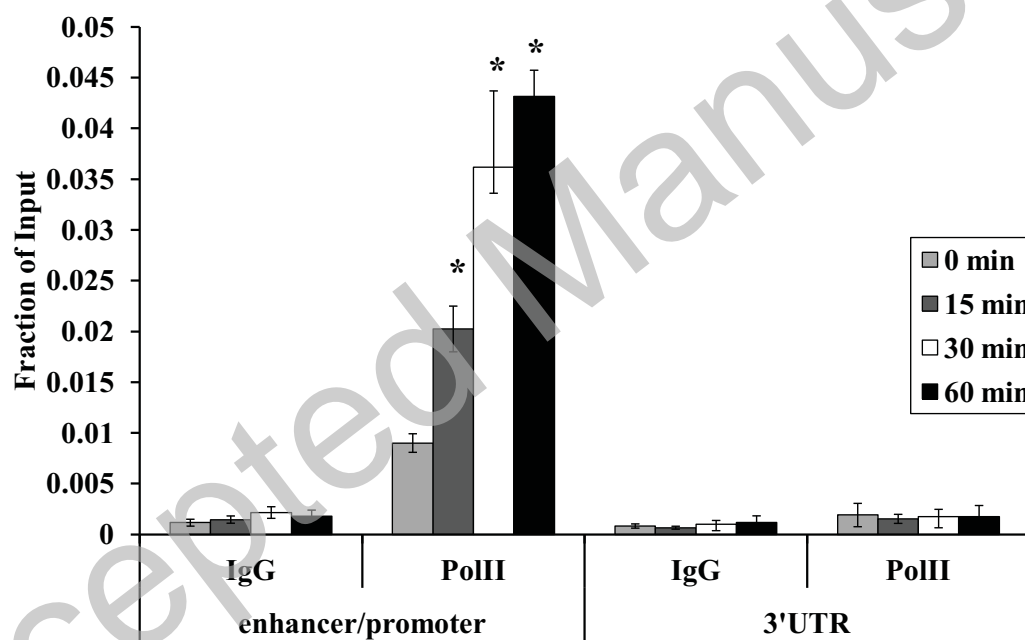


Figure 6B

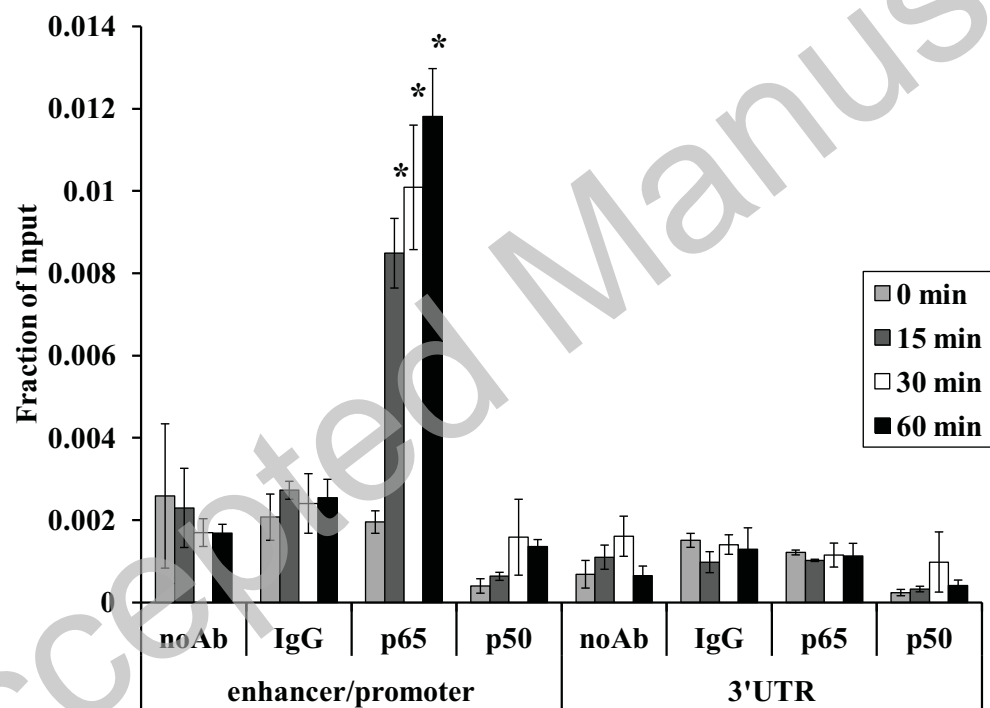


Figure 6C

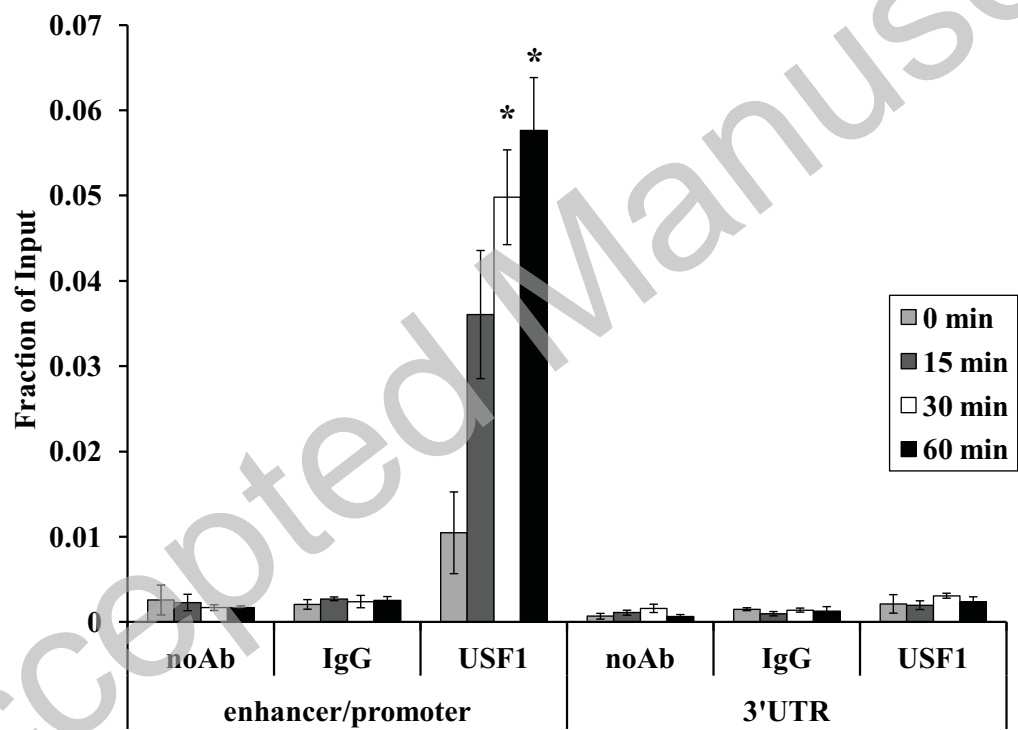


Figure 6D

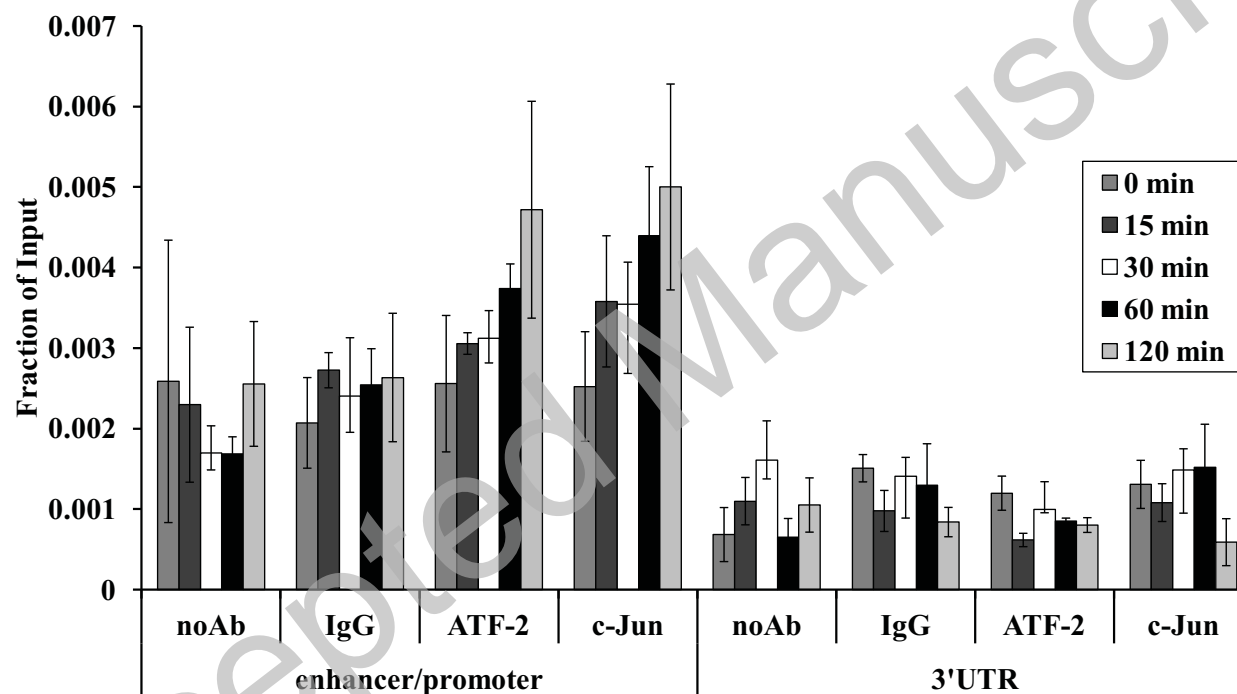


Figure 7A

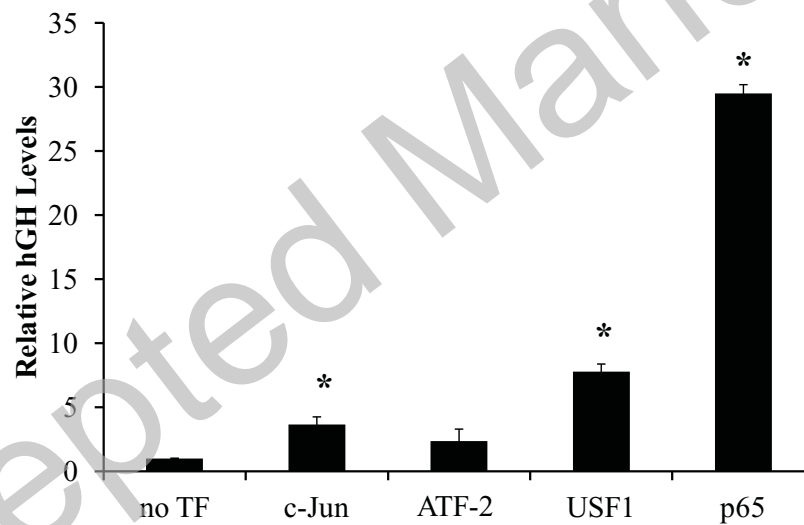


Figure 7B

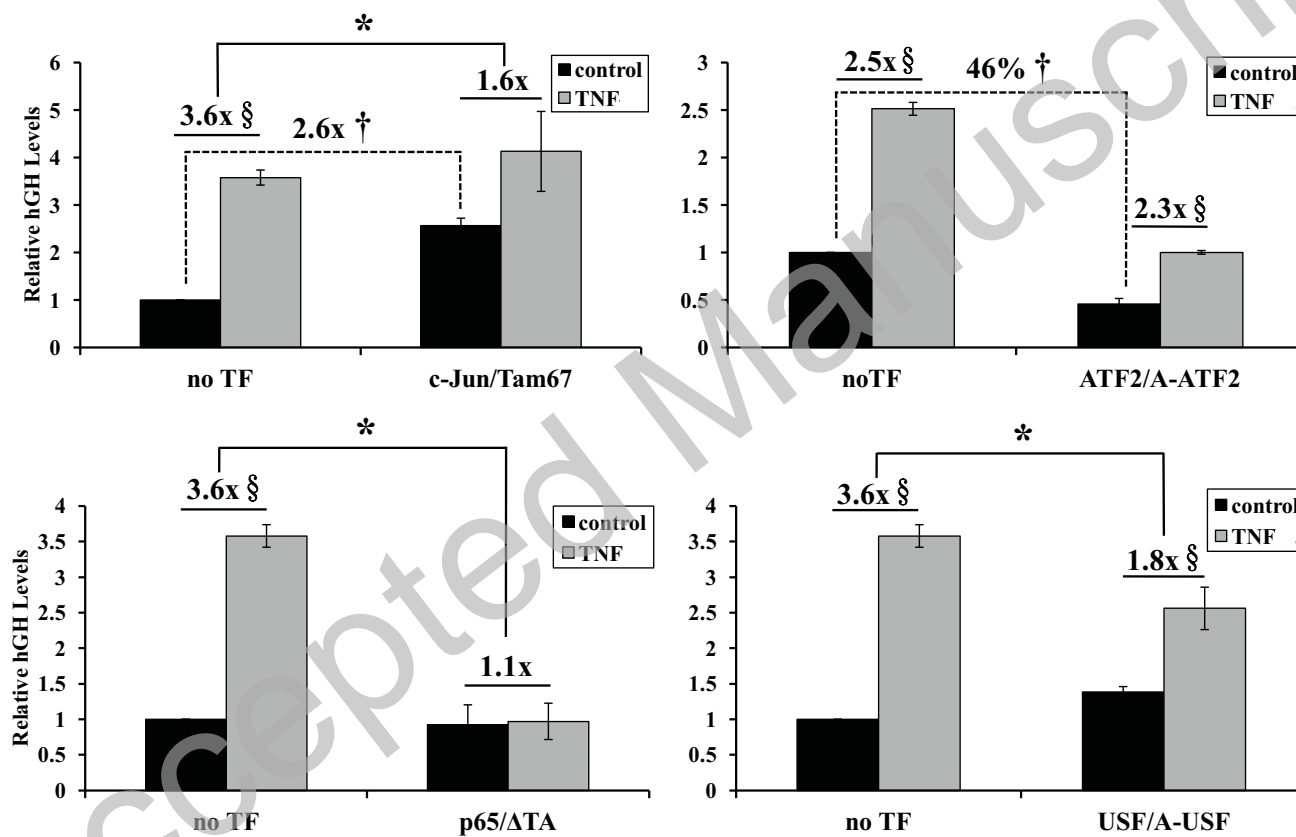


Figure 7C

