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GROUP VIA CALCIUM-INDEPENDENT PHOSPHOLIPASE A2 REGULATES BCL-XL PROTEIN LEVELS IN MICE LUNG

Sang-Jin Nam
Virginia Commonwealth University

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A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science at Virginia Commonwealth University.

by

SANG-JIN NAM
B.S., University of Massachusetts – Amherst, 2010
Certificate, Virginia Commonwealth University 2013

Directors: DANIEL H. CONRAD, PH.D.
PROFESSOR
MIRCOBIOLOGY AND IMMUNOLOGY

SUZANNE E. BARBOUR, PH.D.
PROFESSOR
DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

Virginia Commonwealth University
Richmond, Virginia
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Abstract

GROUP VIA CALCIUM-INDEPENDENT PHOSPHOLIPASE A_2 REGULATES BCL-XL PROTEIN LEVELS IN MICE LUNG

By Sang-Jin Nam, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science at Virginia Commonwealth University.

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Major Director: Daniel H. Conrad, Ph.D
Professor
Microbiology and Immunology

With previous indication of the Group VIA phospholipase A_2 ($iPLA_2\beta$) enzyme regulating ER-stress induced apoptosis in β-cells by regulating the anti-apoptotic protein Bcl-xL via alternative splicing, our lab postulated $iPLA_2\beta$ to be utilizing a similar mechanism to regulate apoptosis in mice lung. Our previous lab work has shown implications of lung function compromise in $iPLA_2\beta^{-/-}$ mice, and we speculated the cause to be due altered lung architecture stemming from the attenuation of apoptosis. The western blot analysis in this study suggested that $iPLA_2\beta$ is involved in the regulation of Bcl-xL, but the mRNA ratios of the splice variants suggested that alternative splicing is not the mechanism $iPLA_2\beta$ is utilizing for the regulation in our animal models. Additionally, the observation and assessment of the lung morphology of the $iPLA_2\beta^{-/-}$

and wild type mice suggested that iPLA$_2$β does not play an integral role in lung morphology.

Introduction

In multicellular organisms, part of survival is ironically dependent on death. Apoptosis, also known as programmed cell death, is a physiological process that occurs normally in multicellular organisms to maintain a balance in cellular proliferations by having an impact on a variety of biological events. These events range from embryogenesis and cellular homeostasis to disposal of harmful and damaged cells [1]. The term “apoptosis” was first used by John Kerr and colleagues in the early 1970’s to refer to the features of the unique morphological observation of cell death [2].

Some of the characteristics of cells going through apoptosis are loss of cellular contact with the matrix, nuclei condensation, plasma-membrane blebbing, cellular shrinkage, DNA fragmentation and disintegration of mitochondria [1]. Due to apoptosis having an integral role in many important biological events, abnormalities in the pathway of apoptosis can lead to various pathological conditions such as cancer, cardiovascular disease, autoimmune and neuronal disorders [3].

Apoptosis is triggered and regulated by a wide variety of factors and has conserved genetic and biochemical pathways as it is an important part of maintenance of homeostatic balance [4]. Some of the factors that induce apoptosis are death receptor ligands, such as Fas-ligands and tumor necrosis factor (TNF) [5]. Ultraviolet irradiation, bacteria, DNA damage, oxidative stress and ceramide treatment can also

trigger apoptosis, while various growth factors can regulate the process [6]. Apoptotic stimuli can lead to two pathways of apoptosis, which are the extrinsic pathway, also known as the death receptor pathway, and the intrinsic pathway, also known as the mitochondrial pathway [7]. A group of apoptosis regulating proteins, Bcl-2 related protein family, mediates the intrinsic pathway, while extracellular signals from other cells activate the extrinsic pathway [4].

Bcl-2 family

Evidence of physiological cell death tied to genetics was first observed in developmental studies in the 1980's of the invertebrate organism, *C. elegans*. Genetic studies lead to the discovery of genes necessary for regulation, execution and resolution of apoptosis during the development stages of *C. elegans* such as *egl-1*, *ced-3, ced-4* and *ced-9*. Proteins derived from *egl-1, ced-3* and *ced-4* caused programmed cell death, while the anti-apoptotic ced-9 protein prevented death of the cells destined to die during the developmental stages of *C. elegans*. Studies on the mechanism of programmed cell death flourished and mammalian homologue genes were discovered with the discovery of these genes. One of the key regulator proteins of apoptosis, Bcl-2 protein, was discovered soon after as the mammalian homologue of Ced-9 [8].

First observed in B-cell lymphomas of humans [9], the B-cell lymphoma/leukemia-2 (bcl-2) gene's anti-apoptotic effects demonstrated the correlation between the regulation of cell death and the development of tumors [10]. Over expression of Bcl-2 in cytokine dependent cell lines inhibited cell death caused by growth factor deprivation [6]. However, the promotion of cell proliferation was not observed in these cells suggesting that cell death and cytokine stimulated cell proliferation did not share a same mechanism [6]. These results suggested that cell death is regulated by a molecular mechanism separate from the mechanism of cytokine

stimulated cell proliferation, and for the first time demonstrated that abnormalities in the regulation of cell death could lead to cancer development [6].

With the discovery of Bcl-2, the importance of the regulation of apoptosis was demonstrated, which opened new doors and insight to cancer biology. The intense studies in this field lead to discoveries of many different Bcl-2 related proteins [6]. These apoptosis regulating proteins were soon categorized into one pro-survival subgroup and two pro-apoptotic subgroups [11]. It is the interactions of these three subgroups of the Bcl-2 related proteins on the mitochondrial outer membrane that subsequently regulate apoptosis. Due to the utilization of the three functionally and structurally distinct subgroups, the Bcl-2 protein family can be regarded as a tripartite apoptotic switch [12].

The factor that ties the members of the Bcl-2 related proteins into one family is the conserved BH, short for Bcl-2 homology, domains they possess. There are four distinct BH domains which correspond to the alpha-helical segments of the proteins and all subgroups possess the BH3 domain [13]. The protein members of the pro survival/anti apoptotic subgroup and the multi-domain effector proteins of the pro apoptotic subgroup possess all four BH domains and form globular structures. These structures are formed by surrounding one central hydrophobic core helix with a bundle of α helices, which produce hydrophobic surface grooves generated by the folding of helices. The tertiary structure provides the functionality of the proteins by allowing these grooves to be used as vital interfaces for the interaction with the BH3 domain of pro-apoptotic members [12].

Having their N-terminus face the cytosol, Bcl-2 related proteins carry their hydrophobic membrane insertion domain on their C-terminus. The membrane insertion

domain allows the proteins to integrate into the cytosolic side of the mitochondrial membrane, endoplasmic reticulum and nuclear envelope. The orientation of the proteins facing the cytosol allows the protein to interact with their associated proteins to function. The localization of the Bcl-2 related proteins can vary [13]. The variance of localization of the Bcl-2 related proteins can be due to their functions. For example, there are indications of BAX, an apoptotic Bcl-2 related protein, traveling back and forth from the cytosol to the outer mitochondrial membrane in healthy cells. They are suggested to be retro-translocated to the cytosol by Bcl-xL and other pro-survival Bcl-2 related proteins to prevent the accumulation of BAX thus preventing apoptosis [14].

Pro-apoptotic initiators (BH3-only)	**Pro-apoptotic mediators (Multi-domain)**	**Anti-apoptotic/Pro-survival**
Bik, Bad, Bid, Bim, Bmf, Hrk, Noxa, Puma, tBID	Bak, Bax, Bok	A1, Bcl-2, Bcl-B, Bcl-W, Bcl-xL, Mcl-1

Figure 1. Bcl-2 related protein family

The pro-apoptotic subgroups of Bcl-2 related proteins consists of the BH3-only proteins and the multi-domain pro-apoptotic proteins. Currently the BH3-only protein subgroup is consisted of members including Bik, Bad, Bid, Bim, Bmf, Hrk, Noxa, Puma and tBID, and the multi-domain pro-apoptotic protein subgroup members are composed of Bak, Bax and Bok [15].

Unlike the previously mentioned multi domain proteins, BH3-only proteins possess one BH domain. Once activated by a cytotoxic signal, the BH3-only proteins carry out their function by forming a complex with the pro-survival proteins by the formation of a salt bridge between the conserved Asp residue and the conserved Arg residue located in the BH1 domain of the pro-survival proteins [16]. While the BH3-only proteins can carry out apoptotic function by neutralizing the pro-survival proteins, there are also suggestions for a direct activation of the multi domain pro-apoptotic proteins by these proteins [17]. Regardless of the mechanism of the protein, the subsequent effect is the homo-oligomerization of the multi-domain pro-apoptotic proteins, BAK and BAX, which leads to formation of pores in the outer mitochondrial membrane [18]. The pores increase the permeability of the outer mitochondrial membrane leading to the release of Smac and cytochrome c into the cytosol, which induces a series of cascades leading to apoptosis [19].

The pro-survival Bcl-2 related proteins, consists of members including A1, Bcl-2, Bcl-B, Bcl-W, Bcl-xL and Mcl-1. These proteins are antagonists of the BH3-only proteins that prevents the effects of various cytotoxic stimuli by neutralizing the apoptotic BH3-only proteins or by directly binding to the BH3 domain of activated BAK and BAX to inhibit their pro-apoptotic function [20]. The competition between the BH3-only proteins and the pro-survival proteins to regulate the multi domain pro-apoptotic proteins, BAK and BAX, suggests that the relative ratio of the two proteins dictate the sensitivity or resistance of the cell to apoptotic stimuli [21].

Alternative splicing of pre-mRNA is a common method of genes to encode for different variants of proteins having different functions. This method is utilized by the

Bcl-x pre-mRNA to produce two distinct isoforms, Bcl-xL and Bcl-xS [22]. Our target protein Bcl-xL is a longer isoform, 241 amino acid long in length, containing an internal region of 63 amino acids that shows the greatest homology to Bcl-2. This internal region is deleted in the shorter isoform Bcl-xS, which is 178 amino acid long, and shows to be the reason for Bcl-xS to not have the same function as Bcl-xL [18][23]. Functionally Bcl-xL prevents cell death, but Bcl-xS's role in apoptosis is still unclear [24].

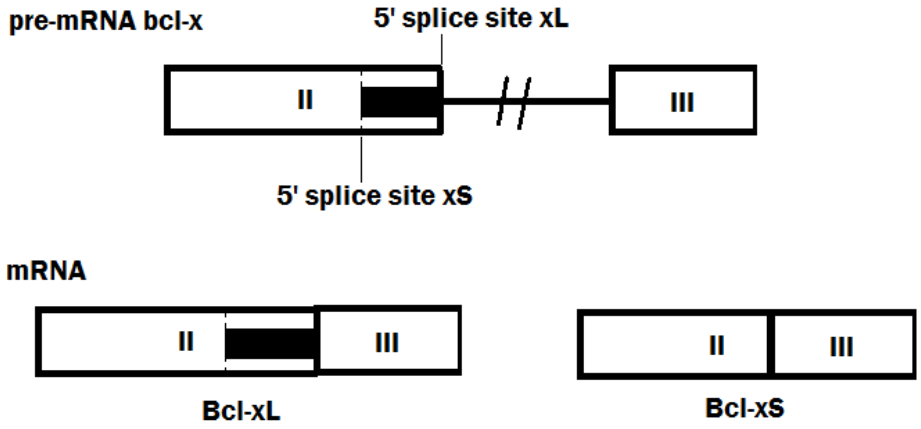


Figure 2. Bcl-x pre-mRNA is spliced into two splice variants, Bcl-xL (at downstream 5' splice site) and Bcl-xS (at upstream 5' splice site) from Wilhelm *et al.* with modification [25]

In most cases, the splice variant ratio of Bcl-xL to Bcl-xS is observed to be shifted in favor of Bcl-xL, which subsequently showed increased expression levels of Bcl-xL in a number of cancers and cancer cell lines. Observations of Bcl-xS splice

variant increase and the induction of apoptosis of cancer cells also existed when the 5'-splice sites of Bcl-xL were hindered with the treatment of antisense oligonucleotides [26]. There is also an indication of ceramide shifting the splice variant ratio in favor of Bcl-xS in human adenocarcinoma A549 cells [27]. The regulation and mechanism of Bcl-x alternative splicing is not clearly defined, but with the indication of ceramide having a potential role in this process an enzyme is postulated to be involved. That enzyme is thought to be iPLA$_2$β, a calcium-independent phospholipase A$_2$ enzyme which is observed in studies to cause apoptosis in β-cells using a novel mechanism involving ceramide generation [28][29][30].

Phospholipase A_2

Phospholipase A_2 (PLA_2) is a superfamily of enzymes involved in diverse lipid metabolism and disease progression [31][32][33]. In the late 19th century, the enzyme was first discovered as a major component of snake venom [32]. With the improvement of technology, researchers were able to purify different forms of PLA_2s from an assortment of sources ranging from old world and new world snake venoms to human pancreatic enzymes. Depending on the disulfide bond patterns and structural motifs, scientists were able to divide and expand the different PLA_2s into 15 distinct groups [32]. Furthermore, the groups were categorized into four types of PLA_2s depending on their characteristics such as cytosolic $cPLA_2$s, secreted $sPLA_2$s, calcium-independent $iPLA_2$s and platelet-activating factor acetylhydrolases [34]. Despite the diversity in structure and function, all the PLA_2 enzymes are 40 to 99 percent identical in their amino acid sequences [35].

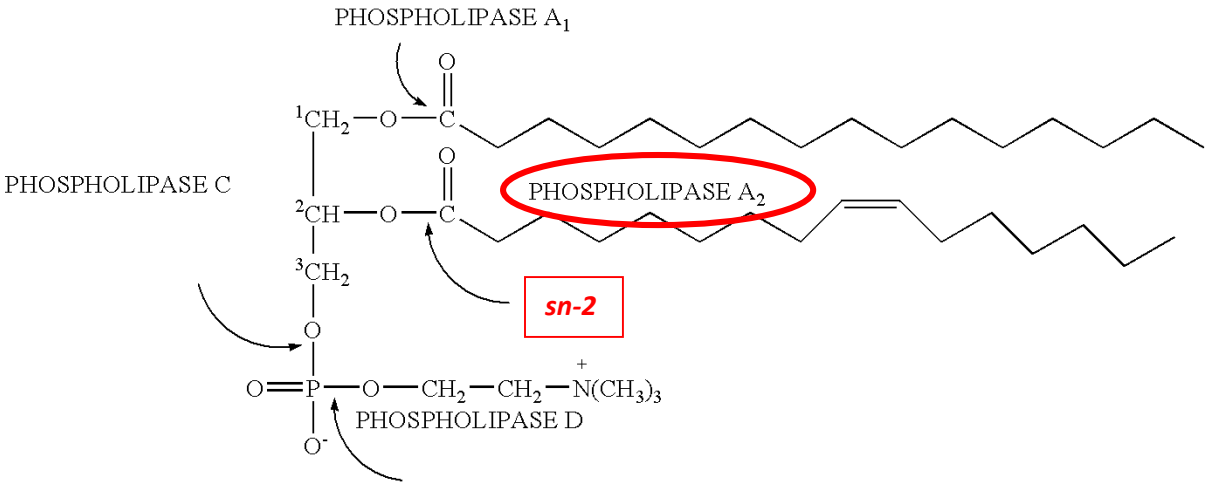


Figure 3. Phospholipase A_2 targets the *sn*-2 position of membrane glycerophospholipids [36]

PLA_2s target the *sn*-2 position of membrane glycerophospholipids, the second carbon group of the glycerol backbone, to catalyze the release of free fatty acids such as arachidonic acid and lysophospholipids, which play vital roles in various biological activities [37]. For mammalians, the group of enzymes' involvement can range from smooth muscle contraction to the biosynthesis of prostaglandins and leukotrienes, which plays an important role in signal transduction. In snake venom, the enzymes are toxic and are involved in the digestion and interference of normal physiological processes of their prey. Due to PLA_2 enzymes having great diversity in biological roles, they are the one of the most studied group among the phospholipases [38].

Group VI (GVI) PLA_2 enzymes were categorized as Ca^{2+}-independent PLA_2s ($iPLA_2$) due to their characteristics of not requiring calcium ions for catalytic activities unlike their related PLA_2 enzymes [39]. The subgroup of $iPLA_2$ enzymes, which are

classified into six distinct isoform groups, are the most recently described and the least well characterized subgroup of PLA_2 enzymes [40]. Out of the six isoforms, GVIA was the first member of the $iPLA_2$ family to be purified from macrophages in 1994 and is the best characterized $iPLA_2$ enzyme [41].

Group VIA PLA_2, also known as $iPLA_2\beta$, has a mass of 85 to 88 kDA and contains a linker region, a catalytic domain with a lipase motif (GXSXG sequence) and 7 to 8 ankyrin repeats [39][33], and is mainly found in the cytosol of resting cells [42]. Recent finding of $iPLA_2\beta$'s ability to translocate to the membranes of the endoplasmic reticulum and mitochondria suggest that the enzyme also possesses a targeting sequence, which has not been identified yet [40]. This enzyme is suspected to be regulated through a variety of mechanisms involving ATP binding, caspase cleavage, calmodulin, and quite possibly by protein aggregation utilizing its ankyrin repeats [43].

Possessing lysophospholipase activity along with transacylase activity, $iPLA_2\beta$'s catalytic activities are carried out by the serine residue in the lipase motif at the active site [33]. Like its related PLA_2 family members, $iPLA_2\beta$ cleaves the *sn*-2 position ester bond of membrane phospholipids, but exhibits no preference towards arachidonic acids in the *sn*-2 position [43]. The roles of $iPLA_2\beta$ are still to be defined, but with the recent generation of $iPLA_2\beta$ deficient mice allowing extensive research, recent studies including Dr. Barbour's work has indication that $iPLA_2\beta$ has a role in phospholipid remodeling, the maintenance of phosphatidylcholine mass, and signal transduction [31][40][34]. Having roles in these physiological activities, studies suggest that $iPLA_2\beta$ can have diverse roles depending on different cell types, subsequently having important roles in apoptosis, bone formation, insulin secretion and sperm motility [43]. Few

studies also suggest the enzyme's involvement in pathological conditions such as diabetes, Barth syndrome, ischemia and cancer [41].

The correlation of iPLA$_2$β and apoptosis first surfaced from Dr. Kenneth Polonsky's group's study [44]. The study indicated that in mouse insulinoma cell line MIN-6, ER stress induced by inhibiting sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) triggered apoptosis due to the depletion of Ca^{2+} in the intracellular Ca^{2+} stores. The ER stress induced apoptosis was triggered independent of the change in the intracellular free Ca^{2+} concentration. However the trigger of apoptosis was dependent on a signal caused by the depletion of ER Ca^{2+} stores that utilized a lipoxygenase pathway of arachidonic acid generation, which implied that a PLA$_2$ enzyme was involved [40][44]. A different study involving Fas-induced apoptosis of human leukemic U937 cells by Dr. Ichiro Kudo and colleagues [45] also implied the correlation of iPLA$_2$β and apoptosis [40]. Reports from this study indicated that Fas-related arachidonic acid release related to Fas-induced apoptosis was dependent on iPLA$_2$, and that the inhibition of iPLA$_2$β delayed Fas-induced apoptosis while the inhibition of cPLA$_2$ and sPLA$_2$ had no effect [45].

Recent studies with INS-1 cells by Dr. Ramanadham and colleagues [28][29][30][40] indicated a correlation of iPLA$_2$β and apoptosis. In these studies, causing ER stress with thapsigargin was shown to promote the release of apoptogenic factors such as cytochrome c and Smac from the mitochondira, and the accumulation of these factors were observed to be amplified in iPLA$_2$β-overexpressing cells [40]. The correlation of the accumulation of factors known to trigger the intrinsic apoptotic pathway and the expression levels of iPLA$_2$β suggested that iPLA$_2$β is likely to be

involved in the activation of the ER stress-induced apoptosis [40]. In these studies, the inhibition or knockdown of iPLA$_2$β in β-cells reduced the induction of Neutral Sphingomyelinase-2 (NSMase2) which subsequently prevented ceramide accumulation. After observing a prevention of cytochrome c release following the inhibition of iPLA$_2$β and NSMase2, ceramide generation which is induced by iPLA$_2$β, was suggested to cause apoptosis in the INS-1 cells [28][29][30][40].

In the study with A549 cells [27], ceramide was shown to promote apoptosis by shifting the alternative splicing of Bcl-x pre-mRNA in favor of Bcl-xS at the expense of decreasing the levels of Bcl-xL. This observation and the observation made in the previously mentioned study with INS-1 cells [28][29][30][40] prompted our lab to postulate that iPLA$_2$β might be regulating apoptosis via regulating Bcl-xL with ceramide. In previous studies, our lab was able to obtain preliminary data from a study which the manuscript is submitted to support this postulation. INS-1 cells treated with thapsigargin to induce ER-stress exhibited significantly lower levels of Bcl-xL protein, and also showed a shift in alternative splicing towards Bcl-xS. Further investigation of ceramide's effect on Bcl-x alternative splicing in INS-1 cells suggested that iPLA$_2$β regulates Bcl-x splicing through both a ceramide dependent mechanism and a ceramide-independent mechanisms that utilizes the arachidonic acid or its metabolites. These observations further supported that iPLA$_2$β regulates apoptosis in β-cells.

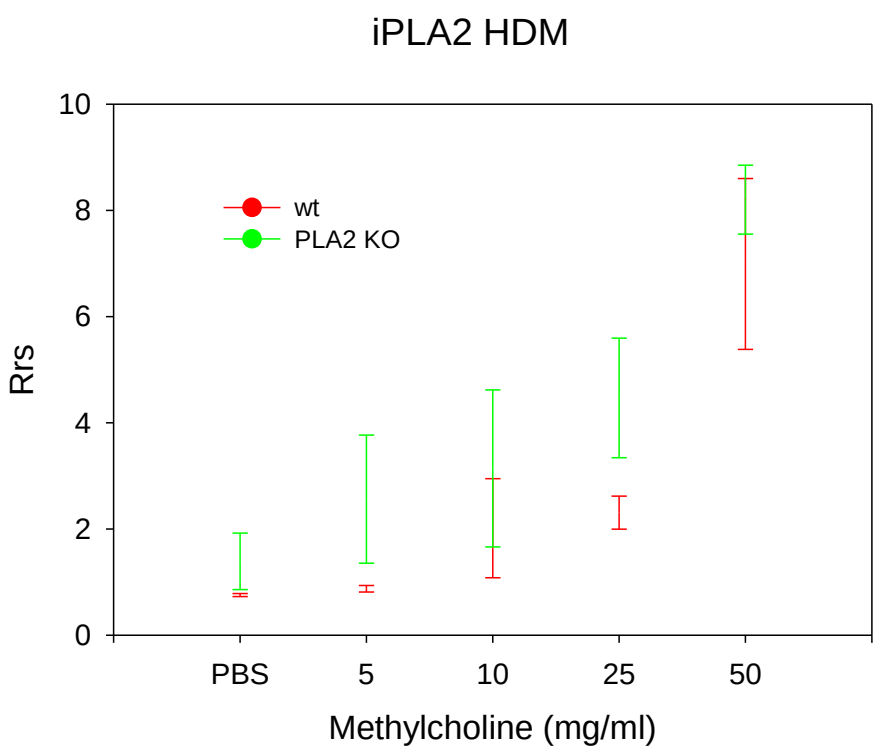


Figure 4. Airway resistance assay for iPLA$_2$β$^{-/-}$ and wild type mice

After the investigations from our previous studies, our lab postulated that iPLA$_2$β might be regulating apoptosis in mice lung with the similar mechanism observed in β-cell. This hypothesis was postulated due to previous unpublished data [Fig 4] from Dr. Conrad's and our lab's study with iPLA$_2$β$^{-/-}$ mice. An unexpected observation was made during an airway resistance assay in the experiment observing the effects of house dust mite induced asthma inflammation in the lungs of iPLA$_2$β$^{-/-}$ and wild type mice. There was an observation of lung function compromise in the iPLA$_2$β$^{-/-}$ mice compared to the wild type mice prior to the inhalation of methylcholine during the airway resistance assay [Fig 4]. The compromised lung function in iPLA$_2$β$^{-/-}$ mice was thought to be secondary to altered lung architecture due to changes in remodeling, which is a process

dependent on apoptosis [47]. With indication of iPLA$_2$β being involved in inducing apoptosis in β-cells as well as other cells [40], our lab speculated that the reduced apoptosis due to lack of iPLA$_2$β is causing altered remodeling and architecture of the lung which subsequently compromises the lung function in our iPLA$_2$β$^{-/-}$ mice.

Hypothesis

Based on the observation of lung function compromise in $iPLA_2\beta^{-/-}$ mice from our lab's previous studies, we hypothesize that GVIA Phospholipase A_2 ($iPLA_2\beta$) is involved in inducing apoptosis by regulating the expression of Bcl-xL in mice lung via alternative splicing.

Materials and Methods

Animals and Tissue processing

Our experimental protocol was performed in accordance with regulations of the Institutional Animal Care and Use Committee of Virginia Commonwealth University. Wild type C57BL/6 mice (n = 5) were received from The Jackson Laboratory (Bar Harbor, Maine), and the $iPLA_2\beta^{-/-}$ mice (n = 4) were from the generated and maintained colony at Virginia Commonwealth University originating from Dr. John Turk's lab at Washington University. The animal models used in this experiment were gender and age matched, and were fed ad libitum laboratory chow. With the help of Dr. Norbert F. Voelkel's lab, one-half of the fresh lung tissue was snap frozen for protein and RNA analysis, and the other one-half was fixed by intratracheal infusion of 4% phosphate-buffered formalin at a pressure of 23 cmH_2O for histological examination.

Identification of knockout mice

For the identification of the knockout mice, genotyping of the alleles were done with the isolated genomic DNA of the mice tail. Tail tissue samples size of 0.5 cm from each mouse were used with the DirectPCR Lysis Reagent kit (Viagen Biotech). The samples used for the polymerase chain reaction (PCR) amplification were prepared in accordance to the manufacturer's protocols. For the PCR analysis of the genomic DNA,

the 1400 bp wild type iPLA$_2$β allele was assessed using a primer mix containing Neo primer (5' TCG CCT TCT ATC GCC TTC TTG AC 3') and wild type-antisense primer (5' CTC CGC TTC TCG TCC CTC ATG GA 3'). The 400 bp knockout allele was assessed using primer mix containing the wild type sense primer (5' AGC TTC AGG ATC TCA TGC CCA TC 3') and MaEx-antisense primer (5' GGG GCC TCA GAC TGG GAA TC 3'). The prepared samples were put through 35 cycles of PCR amplification after being incubated at 95^{o}C for 2 minutes for complete denaturing and activation of the DNA polymerase. Each cycle consisted of 30 seconds of denaturing of the template strands at 95^{o}C, 30 seconds of annealing of the DNA primer at 56.5^{o}C and 3 minute of elongation of the copy strands at 72^{o}C. The PCR samples were separated and visualized by an agarose gel electrophoresis method. SYBR® Gold Nucleic Acid Gel Stain (Invitrogen) was used for the staining of the separated bands, and the bands at 1400 bp and 400 bp were used to identify the wild type allele and knockout allele respectively.

Preparation of the lung protein samples

Frozen lung tissues from each mouse were cut into a size of 0.5 cm with fresh razor blades. Each tissue samples were solubilized in separate test tubes filled with CelLytic^{TM} MT buffer (Sigma-Aldrich) for 45 minutes on ice. Once the tissue was homogenized in the buffer, the homogenates were centrifuged at 1000 x g for 20 minutes in a 4^{o}C room. The retrieved protein samples were stored in a -80^{o}C freezer. The protein concentration of each lung sample was obtained using a protein assay kit (Bio-Rad), and the sample protein size of 150 µg was assessed to be used for the

western blot analysis. Before the loading of the samples on to the SDS-PAGE gel, 150 µg of each sample was added into 4X SDS-PAGE sample buffer and was denatured by heating the samples for 10 minutes at 95^{o}C.

Western blot analysis

The purified and reduced proteins were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The reducing SDS-PAGE gel consisted of a 6% acrylamide stacking gel on top of a 10% acrylamide separating gel. The prepared proteins were separated against the Kaleidoscope^{TM} (Bio-Rad) protein ladder in 1X Tris/Glycine/SDS Electrophoresis running gel buffer (Bio-Rad) consisting 25 mM Tris-HCl, pH8.3, 200 mM glycine, 0.1% (w/v) SDS and water. The separated proteins on the SDS-PAGE gel were transferred via method of electroblotting onto nitrocellulose membrane. The protein transferred membrane was treated with a blocking buffer consisting of 5% diluted non-fat dry milk in Tris-buffered saline (TBS) 0.05% tween solution for 1 hour with gentle rocking at room temperature. The non-specific binding blocked membranes were immunoblotted with primary antibodies overnight in a 4^{o}C room followed by incubation of horseradish peroxidase (HRP) linked secondary antibodies for 1 hour at room temperature. Bcl-xL protein was probed with rabbit primary antibody (BD Pharmingen^{TM}) diluted to 1:1000 in 5% non-milk solution, and α-tubulin protein was probed with mouse monoclonal IgM (Santa Cruz) diluted to 1:200 in 5% non-milk solution. Bcl-xL was detected with HRP-conjugated Goat anti-rabbit (Pierce) antibody diluted to 1:5000, and α-tubulin was detected with HRP-conjugated rabbit anti-mouse IgM antibody (Pierce) diluted to 1:10,000. The immunoblotted proteins

were then visualized using the SuperSignal West Femto Chemiluminescent Substrate (Pierce™), and the band intensities were analyzed using ChemiDoc™ XRS+ System (Bio-Rad) and Image Lab™ software (Bio-Rad).

RNA extraction, PCR and RNA analysis

TRIZOL® LS Reagent (Invitrogen) was used for RNA extraction from lung tissue samples. The protocol was done in accordance to the manufacturer's instruction of homogenization of the tissue, phase separation of the homogenized samples, RNA precipitation and RNA washing. The obtained mRNA samples were quantified using a spectrophotometer instrument (NanoDrop) by getting the absorbance ratio of 260 nm/280 nm. With the ratio's being above 1.8 for every samples, the purity of each samples were accepted. The quantification of each sample determined the mRNA sample size of 0.08 µg to be used for the reverse transcriptase polymerase chain reaction (RT-PCR). The ThermoScript™ Reverse Transcriptase kit (Invitrogen) was used for the RT-PCR to produce a cDNA of the obtained mRNA, and the protocol was carried out according to the manufacturer's instructions. In the end, the non-specific endonuclease (RNase H) was added and incubated at 37°C for 20 minutes to remove RNA complementary to the cDNA. Bcl-x forward primer (5' CCA GCT TCA CAT AAC CCC AG 3') and Bcl-x reverse primer (5' CCG TAG AGA TCC ACA AAA GTG TC 3') were used for the RT-PCR. The samples of cDNA were then stored in a -80°C freezer.

The cDNA samples from each mouse were used for the PCR amplification for the quantification of Bcl-xL and Bcl-xS expression levels in each lung sample via

polyacrylamide gel electrophoresis. AccuPrime™ *Taq* DNA Polymerase kit (Invitrogen) was used for the amplification of the cDNA in accordance to the manufacturer's protocol. The prepared samples were put through 35 cycles of PCR amplification after being incubated at 94°C for 2 minutes for complete denaturing and activation of the DNA polymerase. Each cycle consisted of 30 seconds of denaturing of the template strands at 94°C, 30 seconds of annealing of the DNA primer at 54°C and 1 minute of elongation of the copy strands at 68°C.

SDS-PAGE method was utilized for the quantification of Bcl-xL and Bcl-xS expression levels. 8% polyacrylamide gel was used for the separation of 5 µL of the PCR products which was ran against a 100 bp DNA ladder (New England BioLabs) at 110 volts in 0.5X Tris/Borate/EDTA (TBE) buffer solution. SYBR® Gold Nucleic Acid Gel Stain (Invitrogen) diluted in TBE solution was used for the staining of the separated bands, and the band intensities were analyzed using ChemiDoc™ XRS+ System (Bio-Rad) and Image Lab™ software (Bio-Rad).

Morphological assessment

With the assistance of Dr. Voelkel's lab, the lungs were fixed by intratracheal infusion of 4% phosphate-buffered formalin at a pressure of 23 cmH_2O. The tissues were then sectioned at a thickness of 2 µm after being embedded in paraffin, and stained with hematoxylin and eosin. The lung sections were from 4 $iPLA_2\beta$ knockout mice and 4 wild type mice for morphological observation under light microscopy. Dr. Voelkel's lab was gracious for granting access to their lab's Axio Imager A1 (Zeiss) and

AxioVision (Zeiss) microscopy software for obtaining lung field images of the tissue sections.

The Mean Linear Intercept (MLI) of the lung sections were obtained in reference to the method of Mizuno et al [48]. Ten random images of lung fields per tissue section were captured at magnification of X400. 2 diagonal lines connecting each corner of the lung field image were drawn using the ImageJ software to measure the line intercepts of septal wall of the lung. This process was done for every lung field image, and the number of Intercepts for the 2 diagonal lines were averaged for every individual lung tissue sections. The MLI of each lung tissue section was calculated by dividing the length of the diagonals by the mean septum intercept count. The length of the diagonal lines were measured by the ImageJ software.

Statistical analysis

Results are expressed as means ± standard error. The numerical data were analyzed in a type 2 two tailed t-test in Microsoft Excel. It was determined that at p-value less than 0.05 to be significant.

Results

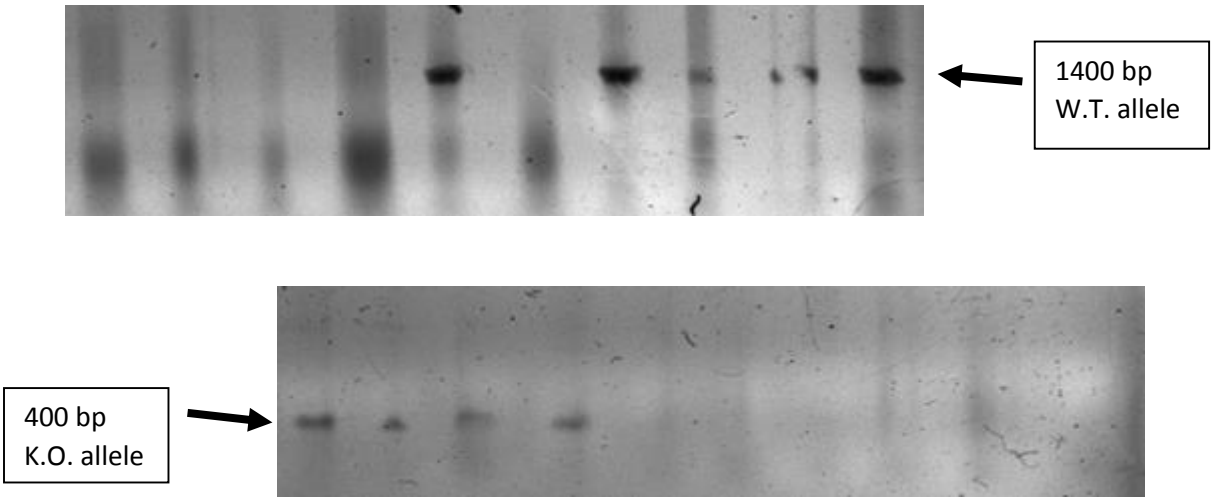
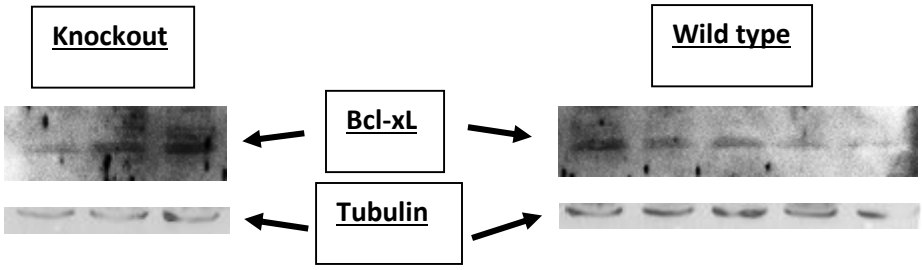


Figure 5. Genotyping of the alleles confirms the proper knockout of iPLA$_2$β in the proposed knockout mice. Identification of the knockout and wild type mice was done from the genomic DNA's from tail biopsies of the animal models. Genotyping was performed by PCR analysis, and the 1400 bp wild type allele was detected in 5 mice while the 400 bp knockout allele was detected in 4 mice. This control experiment allowed us to confirm and work with the proper animal models. (iPLA$_2$β$^{-/-}$ mice, n = 4, control mice, n = 5)

a.



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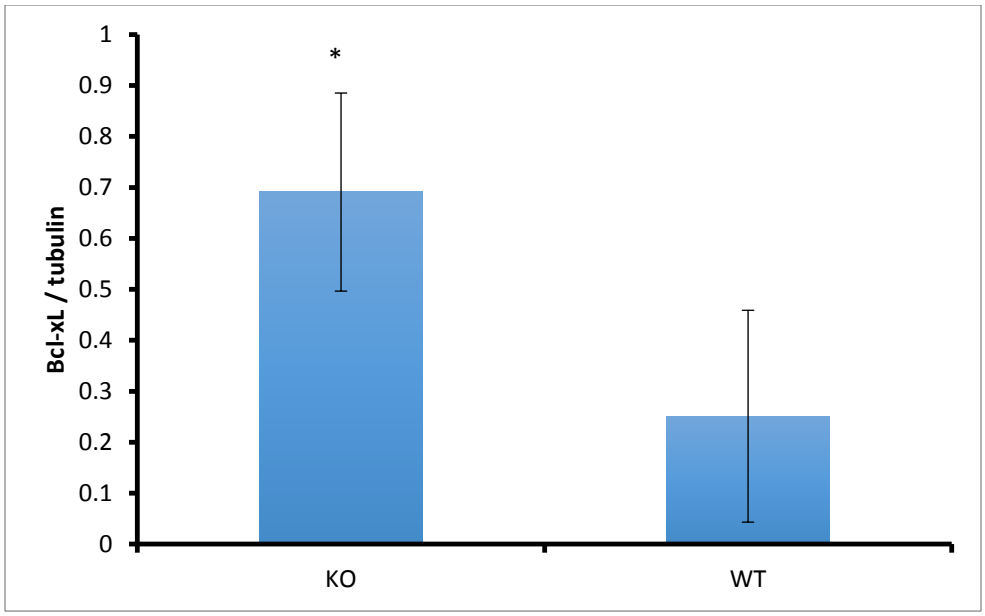


Figure 6. Bcl-xL to tubulin ratios show that Bcl-xL protein levels are higher in the iPLA$_2$β$^{-/-}$ mice than wild type mice. (a) 150 ug of protein from each mice lung sample was used for western blot analysis. (b) The ratios of Bcl-xL to tubulin protein from the western blot analysis represent the protein levels of Bcl-xL in the knockout and wild type mice. Protein analysis was the first step in finding a correlation of iPLA$_2$β and Bcl-xL protein levels, and Bcl-xL protein levels were found to be higher in the iPLA$_2$β$^{-/-}$ mice lung compared to the wild type mice lung. KO = iPLA$_2$β$^{-/-}$ mice group, n = 3; WT = wild type mice group, n= 4. Values are expressed as the means ± standard error. *, $p < 0.05$ (p-value = 0.036).

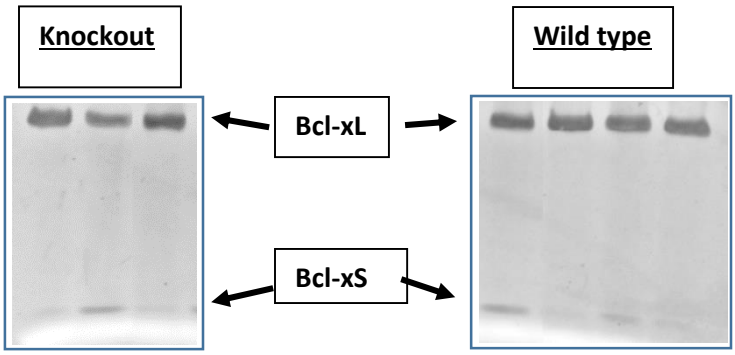
iPLA$_2$β regulates the protein level of Bcl-xL in mice lung

The protein levels of Bcl-xL in each mouse lung were quantified by western blot analysis utilizing SDS-PAGE separation and ELISA assay. The Bcl-xL expression levels are observed due to postulation of iPLA$_2$β regulating Bcl-xL via alternative splicing. The postulation was prompted due to the observation made in previous studies with iPLA$_2$β overexpressing INS-1 cells indicating the involvement of iPLA$_2$β in β-cell apoptosis by inducing the generation of ceramide [28]. Ceramide having implication for lowering the ratio of Bcl-xL to Bcl-xS by affecting the alternative splicing of Bcl-x in A549 lung adenocarcinoma cells [27] prompted us to hypothesize that iPLA$_2$β regulates Bcl-xL via alternative splicing pre-mRNA through a novel mechanism possibly utilizing ceramide in mice lung. In order to support this statement, it was essential for an initial measurement of Bcl-xL protein levels in the tissue. The Bcl-xL levels in the iPLA$_2$β knockout and wild type mice were normalized to the levels of α-tubulin, a structural protein which protein levels should be consistent in all samples.

Higher protein levels of Bcl-xL in the knockout mice compared to the wild type mice were observed which supports our hypothesis. The mean ratio of Bcl-xL to α-tubulin protein levels from the 3 knockout mice lung tissue was 0.691 ± 0.195, while the mean ratio of the 4 wild type mice were observed to be lower at 0.251 ± 0.208 [Fig 6b]. These ratios directly reflected the protein levels of Bcl-xL in the mice lung, and at the 0.05 level of significance, the data rejected the null hypothesis with a p-value of 0.036. This statistically significant data indicating a 3.3 times higher expression levels of Bcl-xL protein in the iPLA$_2$β knockout mice lung compared to the wild type mice lung supported the first step of our hypothesis postulating that the presence of iPLA$_2$β promotes

apoptosis by reducing the Bcl-xL protein levels. In the present study, we further investigate our hypothesis by looking at the alternative splicing of Bcl-x pre-mRNA and lung morphology to get a better idea of how iPLA$_2$β is regulating Bcl-xL and to see if it is has a correlation to levels of apoptosis in the mice lung.

a.



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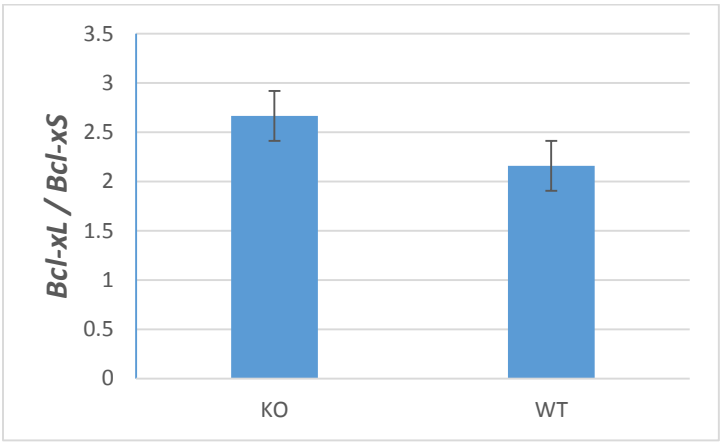

Figure 7. No significant difference in Bcl-x pre-mRNA alternative splicing were found in iPLA$_2$β$^{-/-}$ and wild type mice lungs. (a) The mRNA of Bcl-xL and Bcl-xS expressed in the iPLA$_2$β$^{-/-}$ and wild type mice were purified from each lung tissue samples, and their cDNA were amplified via PCR and separated via SDS-PAGE. (b) The quantification of the ratio of Bcl-xL to the Bcl-xS was to give a measurement of the shift in Bcl-x pre-mRNA alternative splicing. RNA analysis was used to observe correlation in the mice lung to the patterns observed in our lab's unpublished work showing iPLA$_2$β regulating Bcl-xL protein levels via alternative splicing in β-cells. KO = iPLA$_2$β$^{-/-}$ mice group, n = 3; WT = wild type mice group, n= 4. Values are expressed as the means ± standard error. (p-value = 0.41).

No significant evidence of iPLA$_2$β regulating the alternative splicing of Bcl-x in mice lung

Observation of the expression levels of Bcl-xL protein could only provide supporting evidence for iPLA$_2$β regulating Bcl-xL protein expression, but could not provide evidence for the postulated mechanism of regulating via alternative splicing. Few studies indicated that a treatment with splice-switching oligonucleotides at the 5' splice site of Bcl-xL pre-mRNA increases the splicing of the Bcl-xS isoform at the expense of reducing the levels of Bcl-xL mRNA [27][49]. Furthermore the previously mentioned study with A549 cells [27] demonstrated that ceramide can be responsible for this process. Indications of iPLA$_2$β being involved in inducing ceramide generation via induction of NSMase2 in β-cells [28][29][30][40] draw a correlation of a possible involvement of iPLA$_2$β in regulating Bcl-x pre-mRNA alternative splicing. This suggests the shifting of Bcl-xL alternative splicing to Bcl-xS to be a possible method of iPLA$_2$β for regulating Bcl-xL protein levels in the mice lung. Therefore quantifying the mRNA levels of the two splice variants of Bcl-x in respect to each other was necessary for a clearer indication. The mRNA of Bcl-xL and Bcl-xS obtained from lung tissue samples were converted into cDNA and amplified through PCR. The PCR products were separated by polyacrylamide gel electrophoresis. The band intensities were quantified to assess the mRNA levels of Bcl-xL and Bcl-xS in each animal model lung tissue. The comparison of the ratios of Bcl-xL to Bcl-xS levels of the knockout mice to the wild type mice represented a measurement of the shift in alternative splicing of Bcl-x pre-mRNA.

The knockout mice showed a ratio of 2.666 ± 0.882 compared to the wild type mice ratio of 2.160 ± 0.612 [Fig 7b], and at the significance level of 0.05 the data could not reject the null hypothesis with a p-value of 0.41. These results could not support our

hypothesis of a possible involvement of iPLA$_2$β in regulating Bcl-x pre-mRNA alternative splicing, suggesting iPLA$_2$β regulating Bcl-xL via a different mechanism.

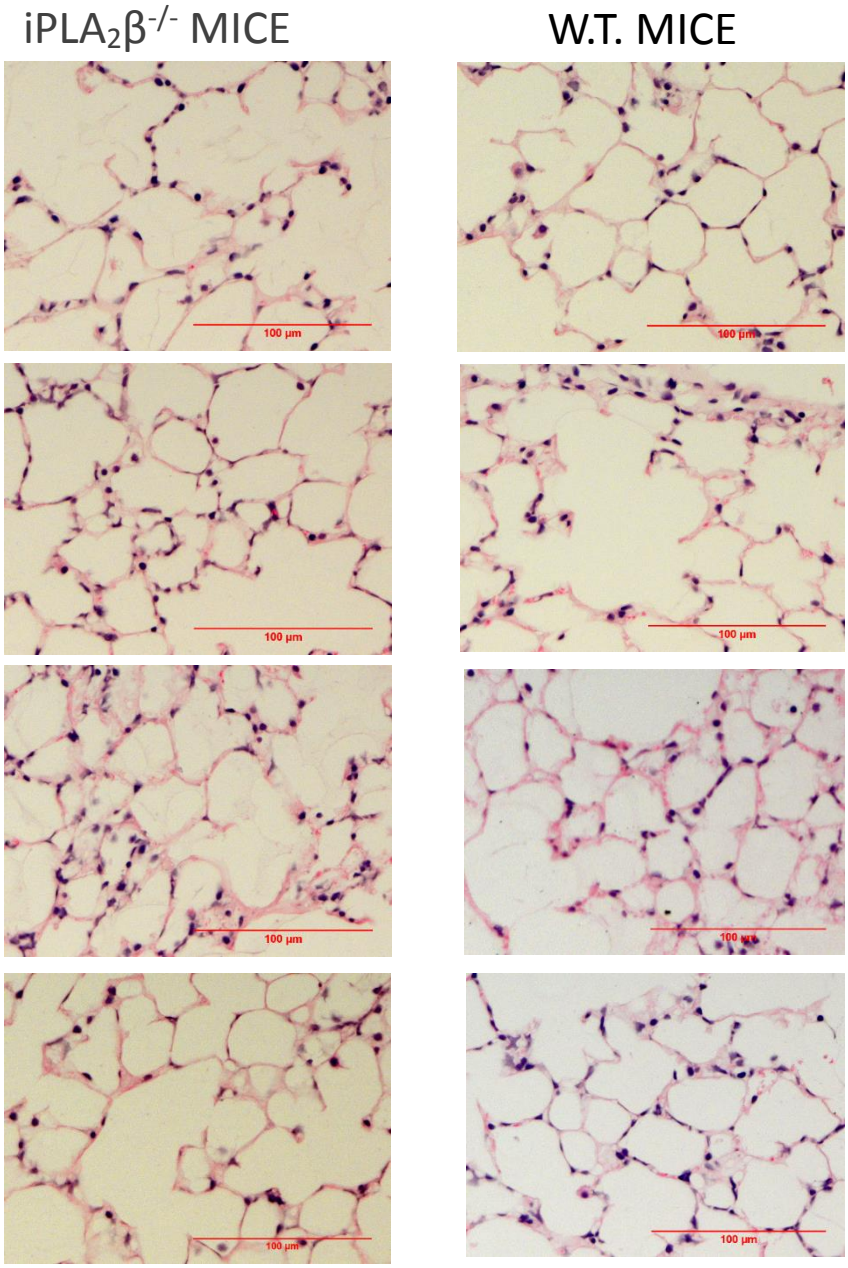

Figure 8. H&E stained lung sections from iPLA$_2$β knockout and wild type mice show no significant difference in lung morphology. Fixated tissues were embedded in paraffin then sectioned (2 um). The sections were stained with hematoxylin and eosin. Images shown are one of the 10 lung field of each animal model that best represents the animal model's MLI. Original magnification, X400.

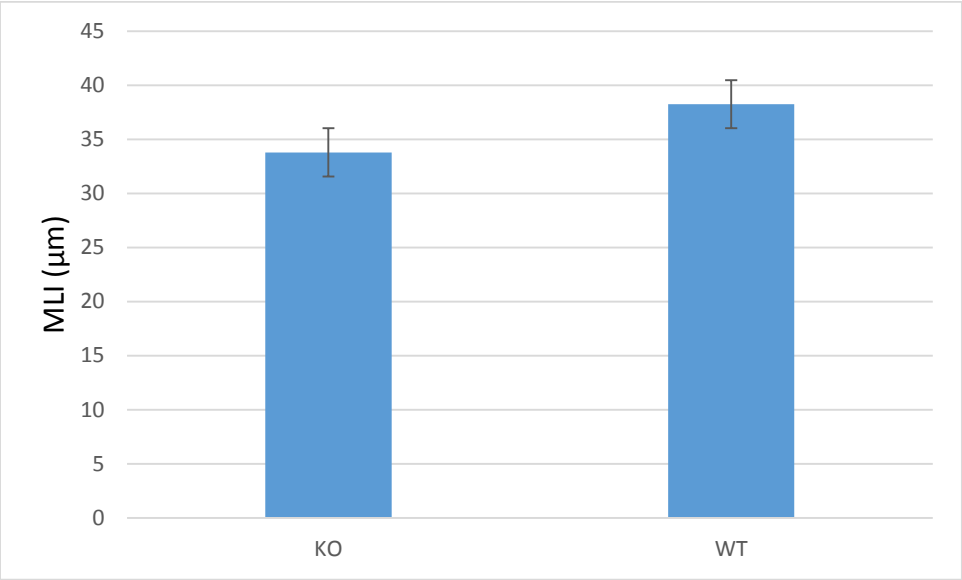

Figure 9. Quantification of the mean linear intercept of the lung sections from iPLA$_2$β knockout and wild type mice show no significant difference in lung morphology. Lung septum walls were counted for the measurement of MLI in the lung, which gave an assessment for the average alveolar space sizes of the knockout and wild type mice. Alveolar space sizes were assessed to see if there was a difference in the lung morphology between the iPLA$_2$β$^{-/-}$ and wild type mice lung. KO = iPLA$_2$β$^{-/-}$ mice group, n = 4; WT = wild type mice group, n= 4. Values are expressed as the means ± standard error. (p-value = 0.093).

No strong evidence for iPLA$_2$β involved in maintenance of mice lung morphology

Indication of iPLA$_2$β regulating the expression of Bcl-xL protein from our western blot analysis lead to an investigation of iPLA$_2$β's effect on mice lung morphology. With a phenotypic observation of lung function compromise in iPLA$_2$β$^{-/-}$ mice [Fig 4], we postulated a correlation of iPLA$_2$β's regulation of Bcl-xL to the maintenance of mice lung morphology. Lung function is suggested to be correlated to the maintenance of homeostasis and the structure of the lung [50][51], thus a possible alteration of structure in the knockout lung was postulated be observed.

Unexpectedly, a visual assessment provided no obvious observable morphological changes [Fig 8], therefore a quantitative assessment was necessary. As a preliminary assessment for any structural changes, a simple quantification of alveolar space sizes was adapted from Mizuno et al [48]. A widely used method of measuring the changes in alveolar sizes is assessing the MLI of two groups of lung samples [48][52][53][54]. MLI is a measurement of the mean distance between interalveolar septal walls, and could be utilized to assess the alveolar space sizes of the mice lungs [48]. The iPLA$_2$β knockout lungs had an average MLI measurement of 33.786 ± 3.331 and the wild type lungs had an average measurement of 38.243 ± 3.251 [Fig 9], which were not statistically significant. At a statistical significance of 0.05 could not be observed to reject the null hypothesis with a p-value of 0.093.

Discussion

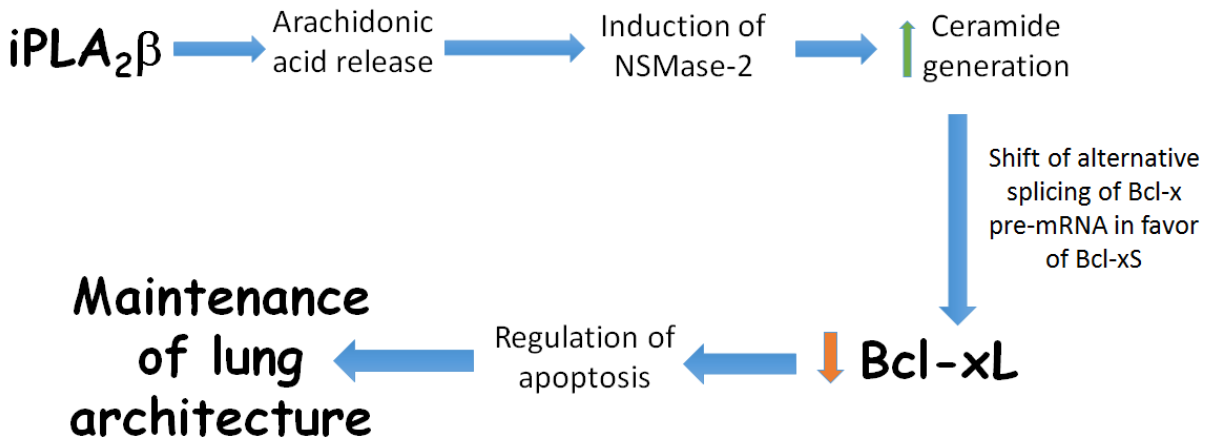

Figure 10. Model of hypothesized mechanism of iPLA$_2$β regulating Bcl-xL levels

The hypothesis postulated for this experiment was that iPLA$_2$β subsequently regulates apoptosis in the mice lungs by regulating the expression levels of Bcl-xL via alternative splicing [Fig 10]. The hypothesis stemmed from an unexpected observation of compromised lung function in the iPLA$_2$β knockout mice during a collaborative project of Dr. Barbour and Dr. Conrad [Fig 4]. The lung compromise was thought to be due to the changes in lung morphology of the iPLA$_2$β knockout mice lung due to attenuated apoptosis in the lung cells. Furthermore, the correlation of iPLA$_2$β and Bcl-x alternative splicing was postulated by our lab due to the observation made by Ramanadham et al including Dr. Barbour [28][29][30] of ER stress induced prolonged activation of iPLA$_2$β in

INS-1 β-cells triggered mitochondrial abnormalities which subsequently caused apoptosis. The β-cell apoptosis is suggested to be carried out by iPLA$_2$β's induction of a novel mechanism involving sphingomyelin hydrolysis leading to ceramide generation [28]. The observation of β-cell apoptosis and the iPLA$_2$β-dependent generation of ceramide is important in linking iPLA$_2$β, Bcl-x splicing and apoptosis, because of the implication of ceramide causing a shift in alternative splicing of Bcl-x pre-mRNA in A549 lung adenocarcinoma cells [27]. The treatment of ceramide eventually lead to an overexpression of Bcl-xS mRNA at the cost of reducing Bcl-xL mRNA subsequently increasing apoptosis in the A549 cells [27], which is a similar mechanism we are postulating iPLA$_2$β to be utilizing in our animal models' lung tissue to regulate Bcl-xL.

The results obtained through this experiment displayed patterns indicating our hypothesis to be considered partially true. Our western blot analysis indicated an increase in Bcl-xL protein levels in the iPLA$_2$β$^{-/-}$ mice lung compared to the wild type mice lung [Fig 6b], which implied iPLA$_2$β's involvement in down-regulating Bcl-xL. This was the most significant observation made in our experiment. In the previously mentioned study of ceramide-induced apoptosis with A549 cells [27], it was observed that the decreased levels of the splice variant Bcl-xL mRNA via ceramide treatment translated into decreased levels of Bcl-xL protein levels. Thus the decrease in levels of Bcl-xL protein observed in our iPLA$_2$β$^{-/-}$ mice suggested that iPLA$_2$β might be regulating Bcl-x alternative splicing via ceramide generation as we proposed. However, the results from our mRNA ratio analysis of Bcl-xL to Bcl-xS [Fig 7b] did not support the postulated mechanism for Bcl-xL regulation of our hypothesis. Our data from the MLI analysis [Fig 9] also did not support our proposal of iPLA$_2$β to be involved in the maintenance of mice

lung morphology which could have possibly indicated a correlation of iPLA$_2$β and apoptosis regulation in mice lung.

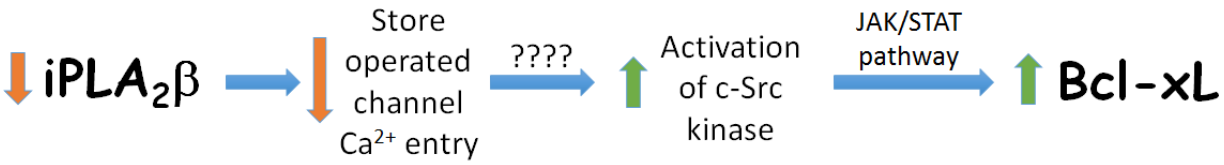

Figure 11. Alternative model of iPLA$_2$β effecting Bcl-xL protein levels

After rejecting the proposed mechanism of iPLA$_2$β regulating Bcl-xL via alternative splicing, the lab postulated a different probable mechanism [Fig 11]. Observing the levels of the non-receptor tyrosine kinase c-Src could provide a better understanding of how iPLA$_2$β regulates the Bcl-xL levels. Past studies [55][56][57][58] show iPLA$_2$ to be involved in the regulation of Ca^{2+} influx by activating store operated Ca^{2+} channels (SOC) during Ca^{2+} store depletion in various cell types, including smooth muscle cells. It is a possibility that in the absence of iPLA$_2$β, cells activate and upregulate different enzymes to respond to the depletion of intracellular Ca^{2+} stores. In a study with mouse embryo fibroblast [59] it was shown that Ca^{2+} influx induced by Ca^{2+} store depletion in Src^- fibroblasts were significantly lower than the wild type fibroblasts. This suggests that c-Src could also be a possible downstream activator of SOCs during the depletion of internal Ca^{2+} stores. We postulate that c-Src could be upregulated in the absence of iPLA$_2$β as an alternative to cause Ca^{2+} influx during the depletion of intracellular Ca^{2+} stores.

The c-Src kinase could be the link in explaining the increased levels of Bcl-xL protein in our iPLA$_2$β$^{-/-}$ animal models. In a study with DHER14 and CSH12 cell lines [60], treating the cells with a Src family kinase inhibitor and repressing c-Src expression with Src antisense RNA down-regulated the Bcl-xL levels in addition to causing apoptosis. Similar to these observations, we propose that c-Src kinase could possibly be up-regulated and activated in our iPLA$_2$β$^{-/-}$ mice lungs which is subsequently leading an increased protein level of Bcl-xL in our knockout mice lungs. There have been studies that demonstrated the importance of transcription factor families such as STATs, NF-kB, Ets families for the regulation of the *BCL2L1* gene [61], the gene of Bcl-x. In our case STAT-3, a possible downstream target protein of Src kinase [60], may be involved in regulating Bcl-xL levels [62]. In the next steps of investigating the possible mechanism of iPLA$_2$β regulating Bcl-xL, our lab could compare the protein levels of pp60$^{c\text{-}Src}$ in the wild type and iPLA$_2$β$^{-/-}$ mice lung. Confirming whether iPLA$_2$β has an effect on c-Src kinase can have an implication of iPLA$_2$β involving a JAK/STAT pathway to regulate the expression levels of Bcl-xL in the mice lung.

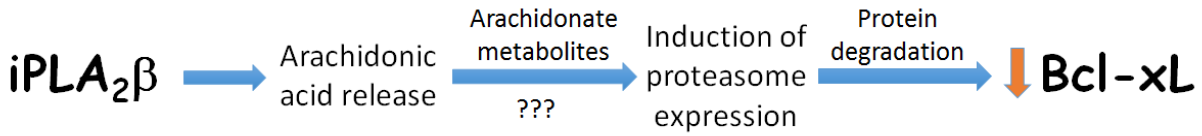

Figure 12. Alternative model of iPLA$_2$β regulating Bcl-xL protein levels utilizing ubiquitin-proteasome system

Another possible method of iPLA$_2$β to be affecting the levels of Bcl-xL protein is by targeting the turnover rate of the protein [Fig 12]. Observation of a difference in Bcl-xL protein levels without a shift in alternative splicing suggests that iPLA$_2$β to be affecting the levels of Bcl-xL at the post translational stage of Bcl-xL. The ubiquitin-proteasome system (UPS) is known to be involved in the regulation of wide variety of cellular processes by degrading variety of regulatory proteins [63][64][65][66]. Among the many cellular processes that UPS is involved, UPS is known to be involved in the degradation of proteins that regulate apoptosis [63][64][65]. Involvement of iPLA$_2$β in UPS could be a possible mechanism in regulating the protein levels of Bcl-xL in mice lung. In muscle cells it is suggested that arachidonic acid metabolites are involved in inducing the expression of proteasomes and causing protein degradation [67]. Furthermore it is shown that inhibitors of PLA$_2$ enzymes attenuate expressions of proteasome which suggests that PLA$_2$ enzymes along with other enzymes are essential in inducing the ubiquitin proteasome system [67]. Arachidonic acid released by iPLA$_2$β could possibly be targeting proteasomes to degrade Bcl-xL proteins to subsequently down-regulate Bcl-xL. The increased Bcl-xL protein levels observed in our knockout mice lungs could possibly be due to the decrease in degradation of Bcl-xL protein caused by the absence of iPLA$_2$β.

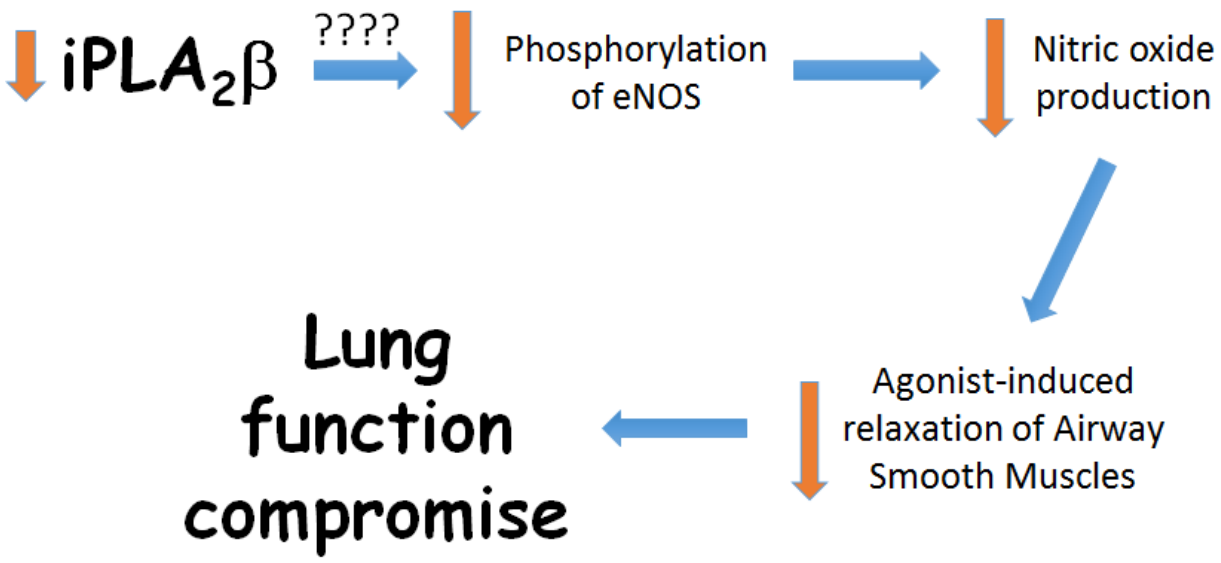

Figure 13. Alternative model of iPLA$_2$β affecting lung function in mice

With our data suggesting no morphological changes to be observed in the iPLA$_2$β$^{-/-}$ mice lung to explain the lung function compromise, we postulate that iPLA$_2$β is affecting the lungs at the physiological levels rather than the morphological level [Fig 13]. An experiment with iPLA$_2$β$^{-/-}$ mice [68] suggested that iPLA$_2$β plays an important role in agonist-induced constriction and dilation in vascular smooth muscles and endothelium by being involved in intracellular Ca^{2+} homeostasis. It was shown that basal state of vessels displayed no significant difference in diameters between the iPLA$_2$β$^{-/-}$ and wild type mice, but iPLA$_2$β$^{-/-}$ vessels displayed an increase sensitivity to phenylephrine induced constriction displaying smaller diameter at maximum constriction at a steady state while being insensitive to acetylcholine induced dilation [68]. This suggests that our iPLA$_2$β$^{-/-}$ animal model might be experiencing lung function compromise due to the agonist-induced hypercontractility and attenuated endothelium-

dependent relaxation in airway smooth muscles of the lung. Hypercontractility and abnormalities in relaxation of airway smooth muscles have been associated with asthma [69] which causes lung function compromise in patients, and could be the case for our iPLA$_2$β$^{-/-}$ mice. This could explain the reason for the iPLA$_2$β$^{-/-}$ house dust mite mice model experiencing lung function compromise compared to the wild type even before the treatment with the lung airway constricting methylcholine during the airway resistance assay [Fig 4] of our previous study. This postulation is further supported by the observation of the exacerbated difference in airway resistance between the iPLA$_2$β$^{-/-}$ and wild type mice after the treatment of methylcholine. In order to assess the possibility of abnormalities in relaxation of the iPLA$_2$β$^{-/-}$ airway smooth muscles, further investigation of vasopressin or nitric oxide (NO) production in the mice lung is necessary. In studies [70][71] NO is found to induce relaxation in airway smooth muscle cells, and looking into phosphorylation of NO synthases (NOS) can be a good indication of a possible malfunction in NO production in the endothelium cells associated with airway smooth muscle cells causing insensitivity to agonist-induced relaxation just as it was proposed in the study with vessels of iPLA$_2$β$^{-/-}$ mice [68]. In the study with iPLA$_2$β$^{-/-}$ vessels [68] they observed an inhibition of NO production and endothelial NOS (eNOS) phosphorylation by an iPLA$_2$ inhibitor. Western blot analysis of phospho-eNOS at Ser1177 was used in their study [68], and a similar analysis in our animal model lungs can give us an indication of any attenuated NO production levels to cause lung function compromise in an iPLA$_2$β knockout condition.

There is a possibility that our data was underestimating iPLA$_2$β's effect on Bcl-x pre-mRNA alternative splicing due to a broad sample pool of mRNA which was taken

from the lung tissue as a whole. Since we are postulating that iPLA$_2$β is possibly involved in the functionality of airway smooth muscles and endothelial cells, it will be worth repeating the mRNA quantification of Bcl-x isoforms with these specific lung cells. The quantification with the specific lung cells relevant to lung functionality and investigating c-Src kinase activity could clarify the mechanism of Bcl-xL regulation and possibly pave pathways to further our current study.

In summary, our data from the present study suggest that iPLA$_2$β regulates protein levels of Bcl-xL in mice lung, and the data further confirms that iPLA$_2$β regulating alternative splicing of Bcl-x via ceramide is possibly a β-cell specific mechanism that is not found be true in mice lung tissue. Although there was no evidence in our data of iPLA$_2$β regulating alternative splicing of Bcl-x pre-mRNA in mice lung as a whole, further investigation is needed to discern if it is also the case for specific cells and tissue within the lung such as airway smooth muscle and endothelial cells. Understanding the mechanism utilized by iPLA$_2$β in regulating Bcl-xL in cells and tissue that is relevant to lung function, and iPLA$_2$β's effects on the physiology of the mice lung could possibly lead to therapeutic targets for asthma and lung function compromise related diseases.

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Vita

Sang-Jin Nam was born on September 23rd, 1986 in the city of Seoul, Republic of Korea. He became a naturalized U.S. citizen in Richmond, Virginia in 2014 during his process of completing his Master's degree. He graduated from Longmeadow High School in 2005, then went on to graduate in Winter 2010 with a Bachelor of Science degree in Biochemistry at University of Massachusetts - Amherst. In Fall 2012, he earned a Pre-Medical Basic Health Science Certificate at Virginia Commonwealth University, and later joined Dr. Barbour's laboratory in Fall 2013. He completed his research thesis for the Master of Science in Biochemistry degree in Summer 2014, and continued on with his education at University at Buffalo School of Dental Medicine.