1-1-2015

Characterization of the Relationship of Ros and Bcl-2 Family Members during Streptococcus Pneumoniae and Staphylococcus Aureus Challenge

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Characterization of the relationship of ROS and Bcl-2 family members during *Streptococcus pneumoniae* and *Staphylococcus aureus* challenge

By

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A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Biological Sciences
in the Department of Biological Sciences

Mississippi State, Mississippi

May 2015

Characterization of the relationship of ROS and Bcl-2 family members during

Streptococcus pneumoniae and *Staphylococcus aureus* challenge

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Title of Study: Characterization of the relationship of ROS and Bcl-2 family members during Streptococcus pneumoniae and Staphylococcus aureus challenge

Pages in Study: 58

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Apoptosis of innate immune is an important part of proper resolution of infection and inflammation. One major protein involved in apoptosis is p53 up-regulated mediator of apoptosis (PUMA). We hypothesize apoptosis induced by the p53/PUMA pathway is initially triggered by ROS thereby preventing neutrophils from defaulting to another form of cell death or remaining in a state of hyper-activation that is harmful to the host. HL-60 granulocytic cells were stimulated with the *Streptococcus pneumoniae* strain SpxB-. Despite inducing both ROS and DNA damage, PUMA transcription and subsequent apoptosis appeared to be independent of both factors. However, PUMA is still relevant in the terms of inflammation and infection as seen with the *Staphylococcus aureus* challenge. Mice lacking PUMA had less macrophages in tissue following challenge. In conclusion, while PUMA is important in the terms of resolving infectious diseases, PUMA-dependent apoptosis does not appear to be mediated by ROS and DNA damage.

DEDICATION

This project is dedicated to my amazing parents, Amy and Ronnie Heath. Without their support throughout my life, I would not be where I am today. I am thankful and grateful for everything that they have done for me.

ACKNOWLEDGEMENTS

First, I am grateful to my major professor, Dr. Justin Thornton, for his advice, encouragement, and his patience during my graduate school career. Through his guidance and knowledge, I have learned so much about this field and what it means to work hard. His encouragement kept me from getting discouraged during times when I wanted to give up. I am very thankful for all of the lessons and techniques I have learned during my time in his lab.

I have had the privilege of working with many great fellow students during my time at MSU. First I have to thank my "Club 21" friends, Lindsey Brown, Joseph Byrant, Gabe Posadas, Jessica Wilson, and Zachary Burcham. Not only have they provided me with encouragement, support, and someone to bounce ideas off of, but they have also provided me with some much needed laughs. I will always cherish our good times inside and outside of school. I also have to thank Katie Hill, our wonderful undergraduate student. She dedicated so much time and effort into this project and was the best undergraduate student I could have ever had for helping with this project.

I would like to thank the members of my committee, Dr. Janet Donaldson and Dr. Todd Pharr who have been instrumental in my success as a graduate student. Dr. James Stewart and his lab helped me with solve problems with those pesky Western blots. Additionally, Dr. Matthew Brown helped me through allowing me the privilege of using

his microscope. Dr. Tim Morgan was instrumentally in the histopathology of our mice tissue sections, and Dr. Seo provided much insight on all of our mice experiments.

Last but most definitely not least, I would like to thank Drew Mills, who has been my rock for these past two years. Without his encouragement and support, I would not have been able to accomplish so much.

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CHAPTER I

LITERATURE REVIEW

Host Immune Response

The innate immune system is critical in the clearance of bacteria during lung infections. In fact, depletion of key innate immune cells, such as alveolar macrophages, led to increased bacterial growth (Dockrell et al., 2003) and mortality (Knapp et al., 2003). One of the first responders to the site of infection is the neutrophil. Neutrophils must first recognize material as foreign, which is achieved through pathogen recognition receptors including toll-like receptors (Hayashi et al., 2003) and the peptidoglycan recognition receptor Nod1 (Clarke et al., 2010). This is followed by recruitment and migration of additional neutrophils through expression of adhesins, cytokines, and chemokines (Wagner and Roth, 2000), followed by activation. During this step, neutrophils phagocytose or engulf of the bacteria and initiate a bactericidal response involving the activity of cytoplasmic granules and reactive oxygen species (ROS). Reactive oxygen species, such as superoxide anion, hydroxyl radicals, hypochlorus acid, and hydrogen peroxide, are the products of NADPH reactions (Fridovich, 1998). The importance of ROS is illustrated in patients with chronic granulomatous disease (CGD). Patients with CGD are unable to utilize NADPH oxidase, which leads to recurrent bacterial infections due to inefficient bacterial killing (Holland, 2010).

While this activation stage can benefit the host through bacterial killing, it can also potentially be detrimental. Neutrophils can result in cytotoxic effects due to their ROS production and cytoplasmic granules. For instance, the incubation of neutrophils with alveolar epithelial cells led to increased DNA damage and decreased viability. Once antioxidants were added, the damage was reduced (Knaapen et al., 1999). Additionally, the same effect was seen in human endothelial cells that were incubated with phorbol myristate acetate (PMA)-stimulated neutrophils; however, cells from patients with CGD were lacking the damage present in normal cells, further showing the damaging effects of neutrophils (Weiss et al., 1981). Another potential detrimental effect is inflammation. Once an infection begins, neutrophils accumulate quickly. Tissues inundated with activated neutrophils in combination with ROS-produced damage can cause prolonged inflammation (Kobayashi et al., 2003b).

To prevent the damaging effects such as those listed above, neutrophils undergo a programmed cell death, termed apoptosis. Neutrophils immediately start to switch their chemical signals once entering infected tissue to stop recruitment and help resolve inflammation; however, once undergoing apoptosis, neutrophils are subsequently cleared by macrophages. This macrophage clearance of neutrophils then causes a surge in cytokines that work to decrease the inflammation (Serhan and Savill, 2005). Without apoptosis, the resolution of inflammation would not be complete. Alveolar macrophage apoptosis has been shown to be important in decreasing inflammation since mice with macrophages unable to undergo apoptosis have increased inflammation (Marriott et al., 2006); likewise, neutrophil apoptosis is key to the resolution of inflammation and infection (Nathan, 2006).

Apoptosis and the Bcl-2 Family Members

Once differentiated, neutrophils will not divide, leaving apoptosis as their eventual fate. This apoptosis can be triggered by a number of events (DNA damage, serum deprivation, "old age," etc.) and can be executed through numerous pathways (caspases, death receptors, etc.) (Geering and Simon, 2011). One factor that has a wide variety of effects on apoptosis is bacterial exposure. For instance, cytokines and bacterial products, such as lipopolysaccharide (LPS) and inactivated bacteria, can increase the survival time of a neutrophil by inhibiting apoptosis (Colotta et al., 1992). Exposure of neutrophils to *Mycobacterium tuberculosis* also rapidly induced ROS-dependent apoptosis (Perskvist et al., 2002). Additionally, apoptotic genes in neutrophils have been shown to be upregulated after phagocytosis of different pathogens, including *Streptococcus pyogenes* which changed the apoptotic pathway in a way that benefited bacterial survival (Kobayashi et al., 2003a). Thus, apoptosis is well established to be important in bacterial infection.

There are two main forms of apoptosis: extrinsic and intrinsic apoptosis. Extrinsic apoptosis involves the Fas (CD95)-Fas ligand system while the Bcl-2 family of proteins is an important part of intrinsic apoptosis regulation (Griffith et al., 1995). This Bcl-2 family consists of two groups: pro-apoptotic and anti-apoptotic proteins. The pro-apoptotic proteins include PUMA, Noxa, Bad, Bid, Bim, Bax, and Bak while the anti-apoptotic proteins include Mcl-1, Bcl-2, Bcl-X_L, and A1. These proteins exist in a delicate balance that allows cells to live and divide under normal environmental conditions. When some form of disruption (serum-starvation, DNA damage, etc.) occurs, that balance is tipped. The cell either goes into a state of growth arrest through the help

of anti-apoptotic proteins or goes into the initiation of apoptosis. Apoptosis usually begins with activation of the Bcl-2 family members by another molecule. This activation can be due to proteins including p53, p73, and Sp1 among others (Ming et al., 2008). These proteins then activate a variety of the pro-apoptotic proteins, which bind anti-apoptotic proteins using a BH3-only domain (Willis and Adams, 2005). This binding of the BH3-only domain is achieved due to structure of the amino acid chains (Petros et al., 2004). The BH3-only domain binding to anti-apoptotic proteins eventually allows for the activation of Bax or Bak. After translocation of these proteins to the mitochondrial membrane, the release of cytochrome c occurs as well as the formation of the apoptosome. Finally, a chain of caspases is activated, which leads to proteolytic breakdown and eventual dismantlement and death of the cell (Reed, 1998).

Changes in the anti-apoptotic members of the Bcl-2 family of proteins play an important role in apoptosis. One of the key players is the anti-apoptotic protein Mcl-1. Mcl-1 is partly regulated transcriptionally. Since the protein is short-lived, it must be transcribed continuously. If transcription stops, the balance will tip towards the pro-apoptotic proteins (Akgul et al., 2001). During bacterial infection, Mcl-1 levels first increase to help fight off infection and then decrease to help facilitate apoptosis in macrophages (Marriott et al., 2005). Additionally, after lysosomal membrane permeabilization and Bid cleavage, Mcl-1 levels decrease to facilitate apoptosis in neutrophils (Blomgran et al., 2007). Two other anti-apoptotic proteins, Bcl-2 and Bcl-X_L work to inhibit apoptosis by binding and interacting with the membrane protein Bax (Gross et al., 1999). While these anti-apoptotic proteins play an important role in regulating apoptosis, pro-apoptotic proteins must be present to initiate the cascade.

The importance of PUMA

One key pro-apoptotic protein is p53-upregulated mediator of apoptosis (PUMA). As its namesake, p53 is traditionally associated with PUMA. One mechanism of apoptosis for these two proteins is that cytoplasmic p53 enters the nucleus and upregulates the transcription of *PUMA*, which allows PUMA along with cytoplasmic p53 to bind with Bcl-X_L to free Bid (Vousden, 2005). Another mechanism is that nuclear p53 upregulates the transcription of *PUMA* allowing PUMA to bind Bcl-X_L. This binding then releases cytoplasmic p53 from Bcl-X_L and allows it to activate other downstream pro-apoptotic proteins (Chipuk et al., 2005). Additionally, PUMA can be activated by other proteins such as p73 to initiate the stepwise apoptotic breakdown of the cell (Melino et al., 2004). Regardless of which protein activates PUMA, it is seen as a critical protein in the mechanism of Bax activation and cytochrome c release (Kim et al., 2009).

The importance of PUMA is further illustrated due it being a determining factor in a wide variety of apoptotic events. For instance, when apoptosis has been induced in response to endoplasmic reticulum (ER) stress, mice lacking PUMA have a lower rate of apoptosis when compared to the wild type mice (Li et al., 2006). Additionally, when roscovitin, a compound that inhibits cyclin-dependent kinases and activates apoptosis, is added to neutrophils, the loss of PUMA along with Bim provided protection from apoptosis (Gautam et al., 2013). PUMA also is important in cancer and therefore is a target for novel cancer treatments. When treated with PUMA, cells were stimulated to undergo normal apoptosis, thereby preventing rapid tumor growth and allowing other treatments to be more effective. This effect was seen in lung (Yu et al., 2006), ovarian (Yuan et al., 2011), and colorectal (Yu et al., 2001) cancer cells.

Moreover, mice that were doubly deficient in PUMA and Bim experienced many birth defects and had random generation of tumors due to unchecked cell proliferation and lack of apoptosis when compared to mice solely lacking Bim (Erlacher et al., 2006). PUMA also appears to be a main apoptotic factor in cells undergoing apoptosis due to γ-radiation and glucocorticoids (Erlacher et al., 2005) along with ionizing radiation, unregulated c-Myc expression, and cytokine removal (Jeffers et al., 2003). Even though there are few studies on the importance of PUMA during infectious disease, PUMA does seem to be important. When *PUMA*$^{-/-}$ mice were challenged with *Streptococcus pneumoniae*, mice developed high levels of bacteria in their blood and were rapidly killed by bacterial infection. The cell type responsible for this effect arose from bone marrow as determined by bone marrow transplantation and subsequent challenge (Garrison et al., 2010).

DNA Damage and ROS

Before the orchestrated events of apoptosis can begin, an event must trigger the cascade. One such event is DNA damage, a known activator of apoptosis (Roos and Kaina, 2006). In a previous mentioned study of *PUMA*$^{-/-}$ mice, it was also discovered that *p53*$^{-/-}$ and ataxia telangiectasia mutated knockout (*ATM*$^{-/-}$) mice also experienced increased mortality (Garrison et al., 2010). PUMA, p53, and ATM are all involved in DNA damage repair pathways. This illustrates the importance of DNA damage in surviving bacterial infections.

One trigger of the DNA damage is ROS. ROS causes breaks in the DNA of cells along with other detrimental effects (Buonocore et al., 2010). ROS has additionally been implicated as a trigger for apoptosis in several different studies. For instance, after

human lung epithelial cells were exposed to the metal Chromium VI, ROS, in particular hydrogen peroxide was produced by the cells. This ROS production activated p53 and caused apoptosis of the cells (Ye et al., 1999). The same effect was seen with exposure of mouse epidermal cells to the metal vanadate except in this case hydrogen peroxide was the specific species of ROS produced (Huang et al., 2000). Additionally, ROS has been shown to activate the extrinsic apoptotic pathway through the Fas-ligand system. Hydrogen peroxide in this study increased apoptotic cell numbers in neutrophils of CGD patients through the Fas-ligand system (Kasahara et al., 1997).

ROS is also shown to increase apoptosis through the p53/PUMA pathway. After exposure to hydrogen peroxide, rat neural cells had increased apoptosis with raised levels of p53 and upregulation of pro-apoptotic genes including *PUMA* (McNeill-Blue et al., 2006). Neutrophil apoptosis also is affected by ROS. After exposure of neutrophils to *Escherichia coli,* the cells experienced an increase in ROS and apoptosis that could be inhibited by blocking NADPH oxidase (Blomgran et al., 2004). Additionally, neutrophils treated with various oxidases that produce hydrogen peroxide showed increased apoptotic levels through the hydroxyl radical produced from hydrogen peroxide (Rollet-Labelle et al., 1998). Similarly, mice lacking NADPH oxidase had increased neutrophils in the lungs and lower levels of apoptosis after exposure to bacteria (Marriott et al., 2008). ROS and subsequent apoptosis in neutrophils were also found to be important in signaling for macrophage phagocytosis to occur (Arroyo et al., 2002). These studies demonstrate the importance of ROS in influencing not only apoptosis but also helping generate a healthy immune response.

Streptococcus pneumoniae

Streptococcus pneumoniae (pneumococcus) is a Gram positive, facultative anaerobic, extracellular bacterium commonly found in the upper respiratory tract. This bacterium is known to cause pneumonia, bacteremia, septicemia, meningitis, sinusitis, and otitis media (Musher, 1992). Part of this bacteria's potency is that it contains a large number of virulence factors, including the production of a polysaccharide capsule (Griffith, 1928), hydrogen peroxide (Duane et al., 1993), and pneumolysin (Rubins and Janoff, 1998). This bacterium also has the potential to wreak havoc on the immune system and surrounding cells in many ways. For instance, defects in the immune system, such as ROS and phagocytosis dysfunctions in neutrophils, are associated with secondary pnemococcal infections following primary influenza infections (McNamee and Harmsen, 2006). Competition among *Haemophilus influenzae* and pnemococcus for colonization also uses the activation of complement to target pnemococcus for clearance by neutrophils during the battle (Lysenko et al., 2005).

Streptococcus pneumoniae does not require a co-colonizer to cause damaging effects to the host. Through the product of the *spxB* gene, pnemococcus is able to survive in the presence of endogenous hydrogen peroxide production (Pericone et al., 2003). This fact allows pnemococcal produced pneumolysin and hydrogen peroxide to induce apoptosis in cells such as brain cells (Braun et al., 2002) and necrosis in neutrophils (Zysk et al., 2000). Additionally, pneumococcus can induce apoptosis in lung epithelial cells through a variety of mechanisms (Schmeck et al., 2004). As much as *Streptococcus pneumoniae* can damage the immune system, the immune system also plays a huge role in stopping pneumococcus. For instance, neutrophils will use serine proteases such as elastase and

cathepsin G to help kill *Streptococcus pneumoniae* (Standish and Weiser, 2009). Macrophages also work to phagocytose and kill bacteria before undergoing apoptosis (Dockrell et al., 2001). Either way, apoptosis is needed to fully resolve the infection and prevent inflammation.

Staphylococcus aureus in resolving skin infections

Staphylococcus aureus is a Gram positive, extracellular bacterium commonly found in the skin and respiratory tract. This bacterium can cause a wide variety of diseases including many different skin infections, pneumonia, meningitis, toxic shock syndrome, and sepsis. *S. aureus* possesses a wide number of virulence factors that aid in causing disease such as the cytotoxin Panton-Valentine Leukocidin (Lina et al., 1999), various exotoxins (Schlievert et al., 1981), and staphyloxanthin that helps prevent ROS stress for the bacterium (Clauditz et al., 2006). During *S. aureus* skin infections, neutrophils have been shown to be important in wound healing and clearance of the bacterium. In fact, mice lacking neutrophils had wounds that would not heal when compared to the resolving wound infections seen in normal mice (Molne et al., 2000). Additionally, when neutrophils were tagged with a green fluorescent protein, they were seen in high concentrations around and in the tissue that contained the *S. aureus* skin infection (Kim et al., 2008).

Significance and Purpose

Streptococcus pneumoniae is the leading cause of community-acquired pneumonia (Brown and Lerner, 1998). Additionally, it can also cause potentially devastating forms of disease. *Staphylococcus aureus* also causes a wide variety of

potentially deadly diseases. With these two bacteria, two major ways of prevention and treatment, vaccination for pneumococcus and antibiotics for both, have become less useful due to resistance and capsule serotype switching (Byington et al., 2005). This means that new ways to prevent and treat disease must be found. Naturally, the innate immune system, particularly neutrophils, plays an important role in stopping these diseases (Haslett, 1999). One part of this pathway that needs better characterization is how changes in Bcl-2 family member levels in response to inflammation affect the overall immune response. To better characterize this pathway, we must first better understand the relationship between ROS, DNA damage, and the Bcl-2 family members. These proposed experiments will examine and determine this relationship. Therefore, our study will improve our understanding of the Bcl-2 family members' role in bacterial infection. These findings could lead to development of a new set of treatment strategies that focus more on altering the host response rather than specifically attacking the pathogen.

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CHAPTER II

THE CONTRIBUTION OF ROS PRODUCTION TO DNA DAMAGE AND SUBSEQUENT CHANGES IN EXPRESSION OF BCL-2 PROTEINS FOLLOWING PNEUMOCOCCAL CHALLENGE

Abstract

Streptococcus pneumoniae (pneumococcus) is a Gram positive colonizer of the nasopharynx capable of causing pneumonia, bacteremia, septicemia, meningitis, and otitis media. Innate immune cells, including neutrophils and alveolar macrophages, are essential for effective clearance of this pathogen. Neutrophils are comparatively short lived cells that must undergo a process of organized cell death (apoptosis) following phagocytosis and bacterial killing. Our lab has previously demonstrated that hydrogen peroxide produced by pneumococcus causes DNA damage in neutrophils. The goal of this study was to determine the contribution of ROS produced by innate immune cells in causing DNA damage and assess expression of DNA damage-induced genes after *Streptococcus pneumoniae* exposure. We hypothesize that production of ROS triggers DNA damage in immune cells leading to altered expression of Bcl-2 family members and altered apoptosis. ROS production was quantified using the fluorescent probe DHR 123. Comet assays were used to assess DNA damage while western blot and qRT-PCR were used to assess changes in PUMA expression. Additionally, flow cytometry was used to quantify altered cell viability. Increased ROS and comet tail lengths were observed with

opsonized *Streptococcus pneumoniae* strain ΔSpxB compared to both the unopsonized form and the control. However, unopsonized ΔSpxB had higher levels of *PUMA* transcription and increased levels of apoptosis. These results contribute to our understanding of the role of DNA damage response genes in innate immunity.

Introduction

The innate immune system is essential in the clearance of bacterial pathogens during lung infections. Neutrophils, one of the first responders to infection, phagocytose bacteria and initiate a bactericidal response involving the activity of cytoplasmic granules and reactive oxygen species (ROS). Once this bacterial clearance begins, neutrophils must undergo a programmed cell death, termed apoptosis, and be subsequently cleared by macrophages to prevent damage to the host cells (Weiss et al., 1981). Without proper apoptosis, inflammation can occur and cause damage to host tissues (Kobayashi et al., 2003). Therefore, the initiation and abrogation of apoptosis is critical for both proper tissue maintenance and resolution of inflammation.

There are two main forms of apoptosis: extrinsic and intrinsic apoptosis. Extracellular apoptosis involves the Fas (CD95)-Fas ligand system while the Bcl-2 family of proteins is an important part of intracellular apoptosis regulation (Griffith et al., 1995). This Bcl-2 family consists of two groups, pro-apoptotic and anti-apoptotic proteins, that exist in a delicate balance. This balance is achieved by a complex interplay between members of both groups, thereby blocking the functions of one or the other using specific binding sites, such as the BH3 region (Petros et al., 2004). While the anti-apoptotic proteins are known to be important in maintaining survival of the innate

immune cells, the pro-apoptotic proteins are of particular interest in the case of infectious disease (Marriott et al., 2005). One important member of the pro-apoptotic proteins is the p53-upregulated mediator of apoptosis (PUMA), which is involved in p53 and serum deprivation apoptosis (Jeffers et al., 2003). Interestingly, previous research has shown that PUMA along with p53 and the atactic telangiectasis mutation (ATM) gene play a role in protecting against bacterial sepsis in a mouse model (Garrison et al., 2010). Not only does this illustrate the importance of PUMA and apoptosis in the context of disease, but this also indicates that the DNA damage response is important during infectious challenge. Other experiments have demonstrated that ROS induced DNA damage in differentiated HL-60 cells and can aid in the regulation of neutrophil recruitment and apoptosis (Takeuchi et al., 1996). In fact, both mice and human models lacking key oxidase enzymes, such as NADPH oxidase, have been shown to have decreased neutrophil apoptosis and increased inflammation (Marriott et al., 2008).

All of this information led us to believe that the chain of events leading to induction of apoptosis plays an important role in context of infection. We hypothesized that apoptosis induced by the p53/Puma pathway is initially triggered by ROS thereby preventing neutrophils from defaulting to another form of cell death or remaining in a state of hyper-activation that is harmful to the host. Our lab has previously shown that challenging bone marrow neutrophils with *Streptococcus pneumoniae* wild type strain T4R causes DNA-damage (unpublished results). Therefore, we used the *Streptococcus pneumoniae* strain ΔSpxB, which lacks the ability to produce hydrogen peroxide, to determine the levels of ROS produced by neutrophils themselves. To test this hypothesis,

we quantitated ROS and PUMA in innate immune cells after exposure to *Streptococcus pneumoniae* or pro-inflammatory stimuli such as PMA.

Materials and Methods

HL-60 cell treatments

Human promyelocytic leukemia (HL-60) cells were differentiated in Iscove's Modified Dulbecco's Medium (IMDM) with 1.5% Dimethyl sulfoxide (DMSO) containing 0.1 mg/mL Penicillin-Streptomycin (pen/strep) for six days. HL-60 cells were then washed four times with the buffer 1x Hank's Balanced Salt Solution (HBSS) (without Ca and Mg) and 0.1% Gelatin. The cells were then diluted to a volume of $1x10^7$ cell/mL using O Buffer (1xHBSS with calcium and magnesium, 0.1% Gelatin). The cells were then exposed to a 1:1 ratio of *Streptococcus pneumoniae* strain ΔSpxB with or without baby rabbit complement for one hour in a rotator. Cytochalasin D (0.02 mg/mL) was added to cells to prevent actin polymerization and phagocytosis. After the one hour, dilutions were plated for bacterial counts to assess phagocytosis efficiency.

ROS quantification with DHR 123

DMSO-differentiated HL-60 cells were pre-incubated with DHR 123, a fluorescent ROS probe, at 37^oC for 20 minutes. DHR 123 was then removed via centrifugation, and the cells were resuspended in O Buffer. A microtiter plate was utilized to test the production of ROS in HL-60 cells. Cells were seeded in the microtiter plate at $8x10^5$ cells per well. Cells were then incubated with O Buffer only, 500nM PMA, opsonized ΔSpx at a 1:1 ratio, unopsonized ΔSpx at 1:1 ratio, complement only, opsonized or unopsonized ΔSpx at 1:1 ratio with cytochalasin D, and a DHR 123 blank.

Additionally, n-acetylcysteine (NAC) was added to inhibit ROS to samples receiving PMA or bacteria. Each well then had an additional 100µL of DHR 123 added. A BioTek Synergy plate reader was used for measuring fluorescence (ex 485/ em 528) for four and a half hours. After three repetitions, fluorescence was then compared among samples to determine ROS production.

Comet Assay

The comet assay was performed on all of the samples from the DHR 123 experiments in addition to a sample of HL-60 cells receiving 2µM H_2O_2 as a positive control. The samples were incubated while rotating with stimuli for two hours at 37^{o}C with the exception of one sample receiving 2µM H_2O_2 for 30 minutes. After two hours, samples were placed on ice and diluted 1:8 in PBS. Another 1:10 dilution in 1% low melting agarose was performed, and 50µL of that dilution was transferred to a slide. Agar was allowed to solidify on the slides at 4^{o}C for 30 minutes, at which point chilled lysis buffer was added for another 35 minutes at 4^{o}C. Lysis buffer was removed, and a fresh alkaline solution (50% NaOH, 200mM EDTA, distilled water) was added and incubated for 35 minutes in the dark at room temperature. Afterwards, electrophoresis was performed with chilled alkaline buffer at 17V for 35 minutes. Slides were then washed twice with distilled water for 10 minutes apiece and 70% ethanol for 5 minutes apiece. Slides were then dried at 37^{o}C for 30 minutes. SyberGreen dye was added for 5 minutes at 4^{o}C, and excess was removed. An AxioSkop 2 Plus_Canon 650D microscope was used to analyze the images.

Western Blot of HL-60 cells

HL-60 cells were differentiated in IMDM media with 1.5% DMSO containing pen/strep for six days. HL-60 cells were then washed four times with 1xHBSS (without Ca and Mg) and 0.1% Gelatin. The cells were then diluted to a volume of $1x10^7$ cell/mL using O Buffer (1xHBSS with calcium and magnesium, 0.1% Gelatin). The cells were then exposed to various stimuli mentioned in previous HL-60 cell experiment for two hours or six hours. The two hour time point cells were lysed as discussed below while the six hour time point cells were washed with 1 mL of PBS twice and then resuspended in media with antibiotics for the remaining four hours.

After the two and six hour time points, cells were washed with PBS and lysed with 150 µl of modified hunter's buffer (10mM Hepes, 150mM NaCl, 1.5mM $MgCl_2$, 1mM EDTA, 10mM Na-pyrophosphate, 10mM NaF, 0.1mM Na_3VO_4, 1% deoxycholic acid, 1% Triton X 100, 0.1% SDS) for 5 minutes at room temperature. Cells were then sonicated three times at 3 second pulses and centrifuged at 10,000 rpm for 5 minutes. Supernatant was then transferred to a new 1.5 mL Eppendorf tube. The BCA protein assay kit (Pierce) was used to determine the protein concentration. Proteins were then separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a handcast 10% gel and then transferred to a polyvinylidene fluoride (PVDF) membrane using semi dry electrophoresis. The membrane was then blocked using 5% milk in 1xTTBS (50mM Tris pH 8.0, 138mM NaCl, 2mM KCl, 0.05% Tween 20) for one hour. The membrane was then incubated with the desired antibody overnight at 4°C. The next day, the membranes were washed four times for 5 minutes each with 1xTTBS (before and after secondary) and probed for one hour with the appropriate secondary antibody.

Immunoreactivity was then detected with either Luminata Forte or Pierce ECL Western Blotting Substrate.

qRT-PCR

After differentiation in IMDM media with 1.5% DMSO containing pen/strep for six days, HL-60 cells were washed four times with 1xHBSS (-Ca –Mg) and 0.1% Gelatin. The cells were then diluted to a volume of $1x10^7$ cells/mL using O Buffer (1xHBSS with calcium and magnesium, 0.1% Gelatin). The cells were then exposed to a various stimuli mentioned in previous HL-60 cell experiment for one hour or three hours. At each time point, the cells were centrifuged at 2000 rpm for four minutes and then washed with 1 mL of nuclease-free PBS. RNA from the cells was then extracted using the RNeasy Mini Kit from Qiagen with the following procedure. Briefly, the cells were resuspended in one volume of Buffer RLT and one volume of 70% ethanol. The sample was then transferred to an RNeasy Mini spin column and centrifuged for 15 seconds at 8000 x g. After discarding of the supernatant, the samples were washed with one volume of Buffer RW1. The samples were then incubated with DNase for 30 minutes. After another RW1 Buffer wash, 500 µL of Buffer RPE was added to the samples, and they were centrifuged at 8000 x g for 15 seconds. Buffer RPE was added again, and the samples were centrifuged at 8000 x g for 2 minutes. The column was then placed in a new collection tube, and the RNA was eluted from the column using 50 µl of nuclease-free water and subsequently stored at -80^{o}C overnight.

To determine the amount of RNA present along with any potential DNA contamination, a Qubit spectrophotometer was used. A Quitbit RNA HS Assay Kit

(#Q32852) and Quibit dsDNA HS Assay Kit (#Q32854) were used for detecting RNA and DNA. The working solution was then made using Qubit Buffer and Qubit reagent for both DNA and RNA. After adding working reagent and the correct sample or standard, the samples were incubated with the working reagent for two minutes and then analyzed with the Qubit 2.0 Fluorometer. After confirmation of no DNA contamination, the RNA samples were then diluted to 50ng and prepared for cDNA synthesis. The Maxima cDNA synthesis enzyme mix and reaction mix along with RNA from samples and nuclease-free water were mixed together at pre-determined amounts. The cDNA synthesis reaction was allowed to run for 30 minutes per manufacturer's instructions.

For qRT-PCR, Thermo Scientific Luminaris Color HiGreen High ROX qPCR Master Mix was used. The cDNA was diluted 1:10 and added to qRT-PCR reactions for a total of 10 µL. The samples were then run on Applied Biosystems StepOne Plus Real-Time PCR System using the following cycles: holding cycle (95^{o}C for 10 minutes), cycling cycle (95^{o}C for 15 seconds and 60^{o}C for 1 minute, repeated forty times), and melting curve cycle (95^{o}C for 15 seconds, 60^{o}C for 1 minute, and 95^{o}C for 15 seconds). PUMA and ß-Actin primers used are listed in Table 2.1 below.

Table 2.1 qRT-PCR primers utilized

Sequence Name	Sequence (5'-3')
PUMA-F	TCA CCA GCC CAG CAG CAC TTA GA
PUMA-R	TGT CGA TGC TGC TCT TCT TGT CTC
ActB-F	GTC CTA TCC CTG TAC GCC TC
ActB-R	CCA GGA AGG AAG GCT GGA AG

Flow Cytometry

After differentiation in IMDM media with 1.5% DMSO containing penicillin and streptomycin for six days, HL-60 cells were then washed four times with 1xHBSS (without Ca and Mg) and 0.1% Gelatin. The cells were then diluted to a volume of $1x10^7$ cell/mL using O Buffer (1xHBSS with calcium and magnesium, 0.1% Gelatin) prior to exposure. The cells were then exposed to various stimuli mentioned in previous HL-60 cell experiment for one hour. At the one hour time point, cells were centrifuged at 2000 rpm for 4 minutes. The supernatant was vacuumed off, and the cells were washed with 1 mL of PBS. After this wash, the cells were then resuspended in IMDM media with 10 µg/mL penicillin G and 250 µg/mL gentamycin and incubated at 37^oC for another three or seven hours.

At the end of these time points, the cells were centrifuged again at 2000 rpm for four minutes. The supernatant was vacuumed off, and the cells were washed with 1 mL of PBS. The cells were then resuspended in FITC Buffer containing Annexin V and

7AAD. The cells were incubated with these stains for ten minutes and then analyzed via flow cytometer for cell death or apoptosis.

Results and Discussion

The Bcl-2 family of proteins plays an important role in determining the fate of innate immune cells during infection. The first responder innate immune cells, neutrophils, flood into the infected tissue to phagocytose and kill the pathogen. These neutrophils must then undergo an orchestrated apoptosis and be cleared by macrophages to prevent inflammation or other damage to the host cells (Kobayashi et al., 2003).

While the role of the pro-apoptotic members of bcl-2, namely PUMA, have been shown to be important in protecting against lethal bacterial sepsis, the chain of events that lead to the apoptosis is less characterized (Garrison et al., 2010). We sought to determine if phagocytosis was essential to initiate this process through the induction of ROS. *Streptococcus pneumoniae* is mainly cleared due to opsonin-dependent phagocytosis usually require serotype specific antibodies. Our unencapsulated ΔSpxB strain was found to be phagocytosed readily when opsonized with complement (Figure 2.1). Our opsonized bacteria were phagocytosed significantly better than cells without complement or opsonized baceteria in the presence of cytochalasin D. DHR 123 experiments were performed to determine the relative ROS production among the different samples (Figure 2.2). We found that the addition of cytochalasin D did not inhibit ROS production in the samples with opsonized ΔSpxB. Moreover, the opsonized ΔSpxB had high levels of ROS when compared to the control or the unopsonized ΔSpxB. Additionally, these levels of ROS are probably not due to phagocytosis of the bacteria. Killing of

pnemococcus has been shown to be independent of ROS and instead dependent on degranulation and serine proteases (Standish and Weiser, 2009). This ROS appears to be being produced independent of phagocytosis, potentially through cellular activation via molecular pattern receptors such as toll-like receptors. The fact that the presence of cytochalasin D does not alter the ROS production illustrates that phagocytosis is not essential and that a receptor exposed to the outside of the cell may be initiating the ROS production.

DNA Damage and Bcl-2 Protein Analysis

To determine if exposure to ROS induced DNA damage, HL-60 cells were incubated with opsonized ΔSpxB and assessed for DNA breaks using the comet assay. The results for the comet assay showed that HL-60 cells receiving the opsonized ΔSpxB had, on average, longer comet tails when compared to the unopsonized ΔSpxB or any of the controls (Figure 2.3). This indicates that the production of ROS by HL-60 cells exposed to opsonized bacteria had an increase in DNA damage in the form of DNA breaks. Additionally, HL-60 cells exposed to opsonized ΔSpxB with cytochalasin D did not have as many DNA breaks as the opsonized ΔSpxB. Since the ROS production from these two samples was not significantly different (Figure 2.2), this indicates that not all of the DNA damage was produced due to the direct phagocytosis. Additionally, the presence of NAC did not lessen DNA breaks seen in the comet assay when compared to the relevant sample. Therefore we suggest that DNA damage may be independent of ROS and may be formed due to other stimuli.

To determine the role of the Bcl family of proteins after ROS production and DNA damage, we performed a number of analyses including western blots to evaluate protein levels and qRT-PCR to evaluate gene expression. Western blot analysis appeared to show an effect on PUMA via complement (Appendix B.1). The levels of PUMA in samples containing complement at the six hour time point appeared to be substantially higher when compared to samples lacking complement. Further probing of the blots led to the discovery of cross-reactivity of the secondary antibodies. Due to this complement dependent effect, we decided to performed qRT-PCR to quantitate gene expression levels. The qRT-PCR data at the three hour time point showed that in the samples with unopsonized ΔSpxB, *PUMA* was upregulated two fold higher than the opsonized ΔSpxB (Figure 2.4). *PUMA* gene transcription was downregulated in sample containing cells and complement. This indicates that complement is prolonging the life of the cell. These results with *PUMA* expression match the subsequent apoptosis results discussed below.

We then utilized flow cytometry to assess cell viability and apoptosis progression in HL-60 neutrophils exposed to ΔSpxB with and without complement. HL-60 cells were exposed to the stimuli for one hour and then resuspended in media containing antibiotics for either three or seven additional hours. At the four hour time point, HL-60 cells were observed to have decreased levels of apoptosis (Figure 2.5). However, at the eight hour time point, nearly 96% of the HL-60 cells exposed to unopsonized ΔSpxB were undergoing apoptosis, which is an increase in comparison to cells that received the opsonized ΔSpxB. Additionally, the addition of NAC added little to no protection while the addition of cytochalasin D provided protection for the HL-60 cells exposed to opsonized ΔSpxB, but not to unopsonized ΔSpxB. The eight hour viability results match

closely the results observed when activated neutrophils isolated from the peritoneum of mice receive the same stimuli (Figure 2.6).

In summary, one mechanism by which neutrophils undergo apoptosis involves the pro-apoptotic protein PUMA; however, the induction of PUMA does not appear to correlate specifically with ROS or DNA damage. The flow cytometry results and qRT-PCR results suggest that complement is protecting the cells from apoptosis to a greater extent than cells just exposed to bacteria. In addition, we suggest that unopsonized bacteria are potentially interacting with receptors on the neutrophils or producing a chemical that is inducing cell death, which then leads to an increase in *PUMA* expression and subsequent dismantlement and death. The activation could also be related to a serine protease release event to help kill the pathogen. Additionally, the complement dependent pathway of killing bacterial pathogens could be activating apoptosis through another pathway. The determination of this pathway could then lead to many new treatments for bacterial infections.

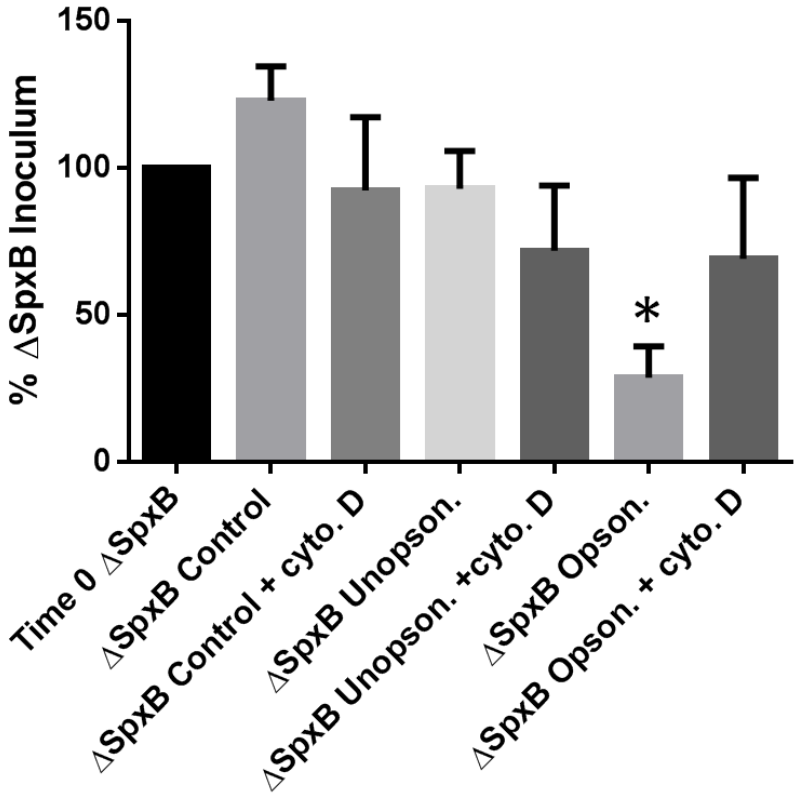

Figure 2.1 Phagocytosis Assay of ΔSpxB

HL-60 neutrophils were exposed to the stimuli for one hour and then plated on blood agar plates and incubated at 37^{o}C for 24 hours. Viable CFUs were quantitated by colony enumeration. Samples receiving no neutrophils served as a control. The sample (ΔSpxB opsonized) coated in rabbit complement and antibody was phagocytosed at a significantly higher rate than compared to the control or neutrophils that were just given unopsonized ΔSpxB (* $p<0.05$).

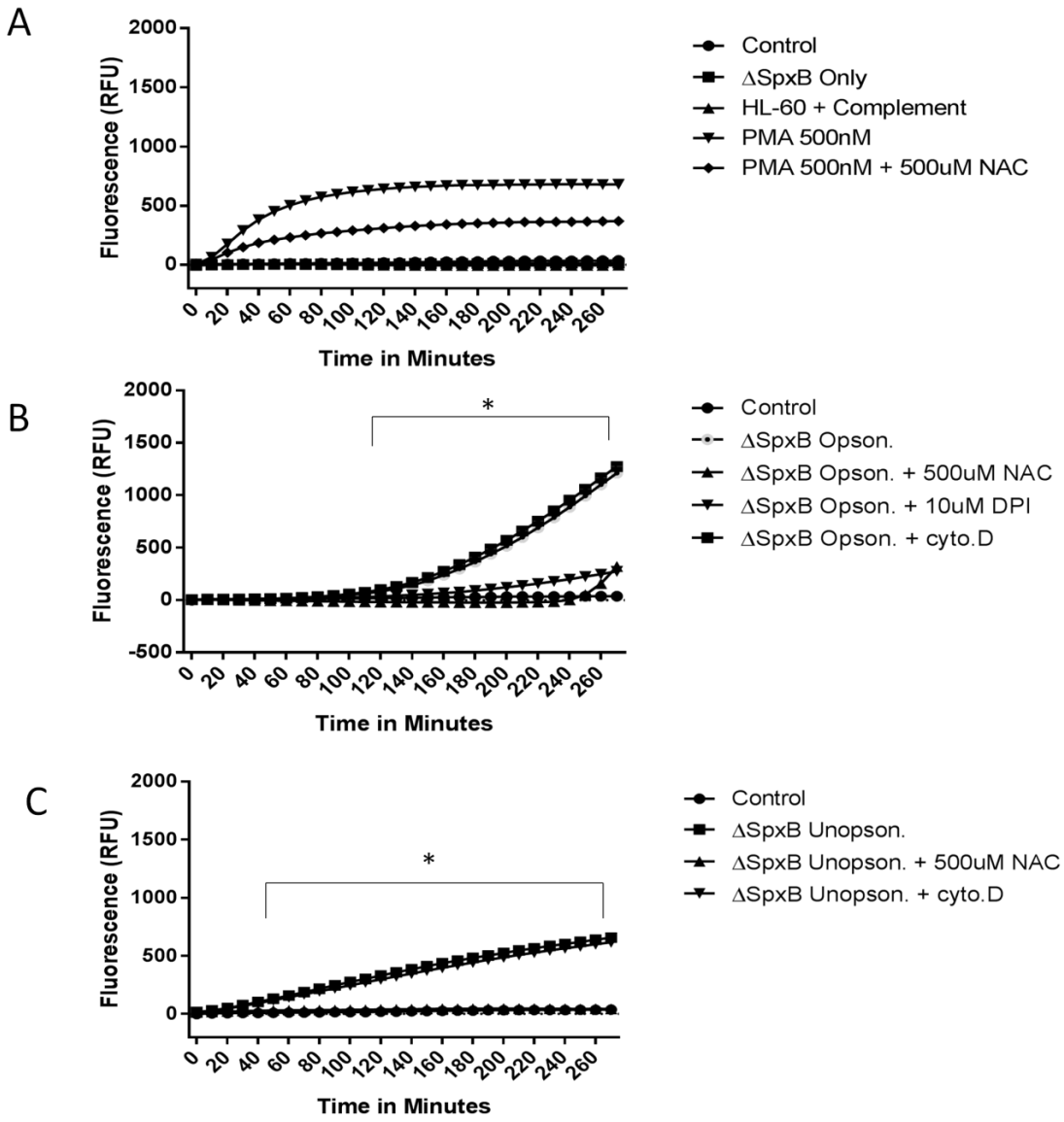

Figure 2.2 DHR 123 Fluorescence of Samples

HL-60 cells were pre-incubated with DHR 123 for 20 minutes prior to exposure to various stimuli in the presence of DHR 123 for 4.5 hours. A BioTek Synergy plate reader was used for measuring fluorescence (ex 485/ em 528). The HL-60 cells given the opsonized ΔSpxB produced a reactive oxygen species burst around the two and half hour mark. This ROS production was significant compared to the control (* $p<0.05$) (B). The cells given the unopsonized ΔSpxB had a steady increase in ROS that was still lower than the opsonized ΔSpxB. The unopsonized ΔSpxB had a significant increase in ROS when compared to the control (* $p<0.05$) (C). PMA served as a control (A).

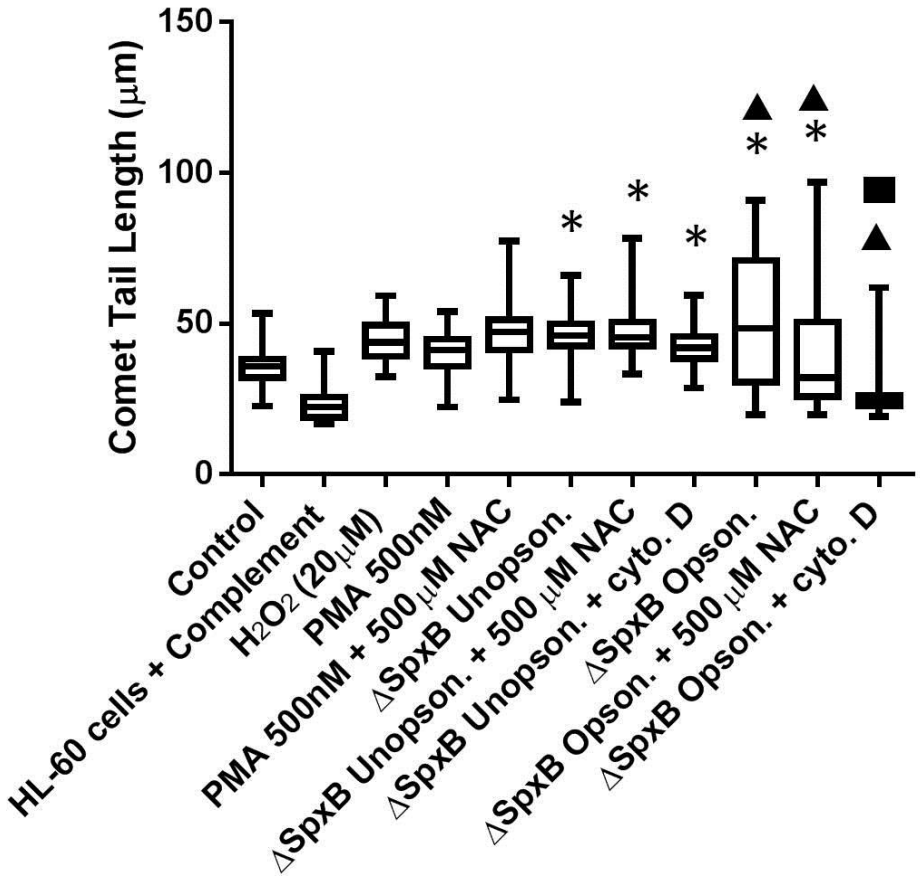

Figure 2.3 Quantitation of DNA damage using a comet assay

Comet assays were used to quantitate DNA damage. Cells were exposed to stimuli for two hours. Cells were then adhered to slides and immersed in lysis buffer. Single cell electrophoresis was then performed. Slides were then washed and stained with SybrGreen and DNA tail lengths were measured; longer lengths indicated an increase in DNA breaks. HL-60 cells with unopsonized ΔSpxB and opsonized ΔSpxB had significantly longer comet tails when compared to their respective controls (* $p<0.05$). Additionally, HL-60 cells with opsonizedΔSpxB had significantly longer comet tails when compared to their unopsonized counterparts (▲ $p<0.05$). HL-60 cells receiving opsonized ΔSpxB and cytochalasin D had significantly shorter comet tails than compared to the cells receiving opsonized ΔSpxB alone (■ $p<0.05$). Hydrogen peroxide served as a control.

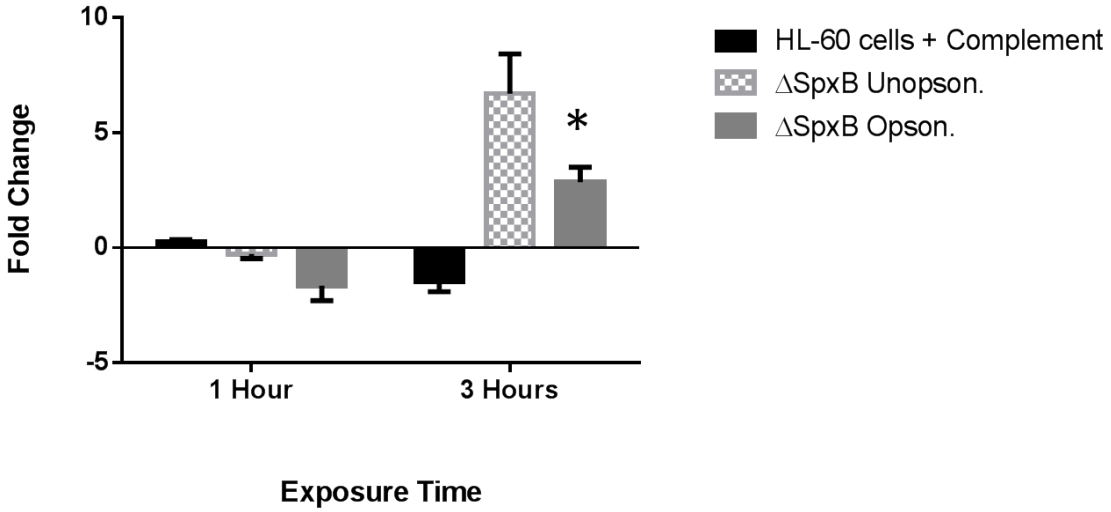

Figure 2.4 qRT-PCR of HL-60 cells

Differentiated HL-60 cells were exposed to various stimuli for one or three hours. RNA was then extracted, quantified, and converted to cDNA. The qRT-PCR was then performed on the cDNA using primers for the housekeeping gene and *PUMA*. Fold changes were calculated using the ddCT. *PUMA* gene expression significantly increased in the sample containing unopsonized ΔSpxB when compared to the sample containing opsonized ΔSpxB (* $p<0.05$).

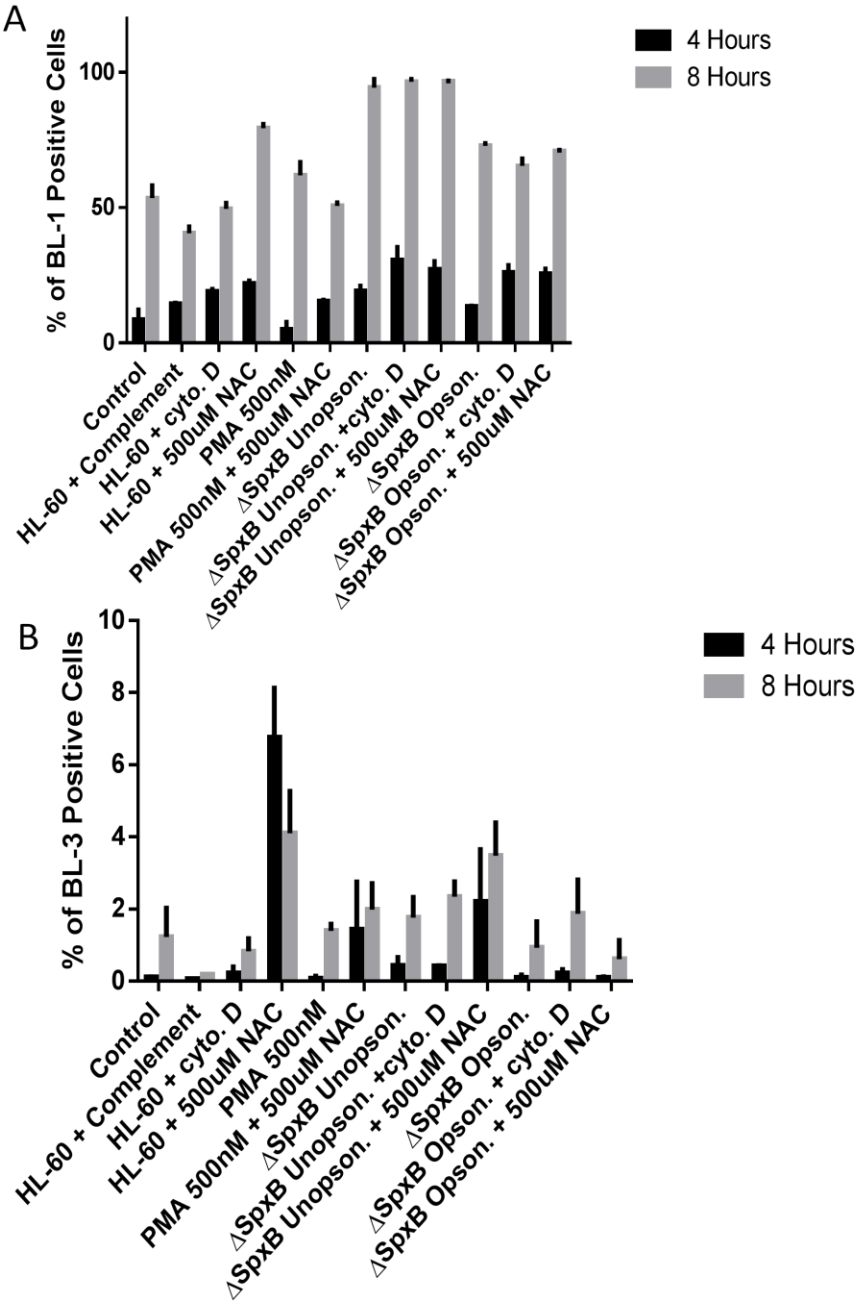

Figure 2.5 Flow cytometry of HL-60 cells

Differentiated HL-60 cells were exposed to various stimuli for four or eight hours. Cells were then washed and stained with Annexin V and 7AAD. Apoptotic cells were higher in cells containing unopsonized ΔSpxB when compared to opsonized ΔSpxB samples (A). Additionally, only a low percentage of cells stained positive for 7AAD (B).

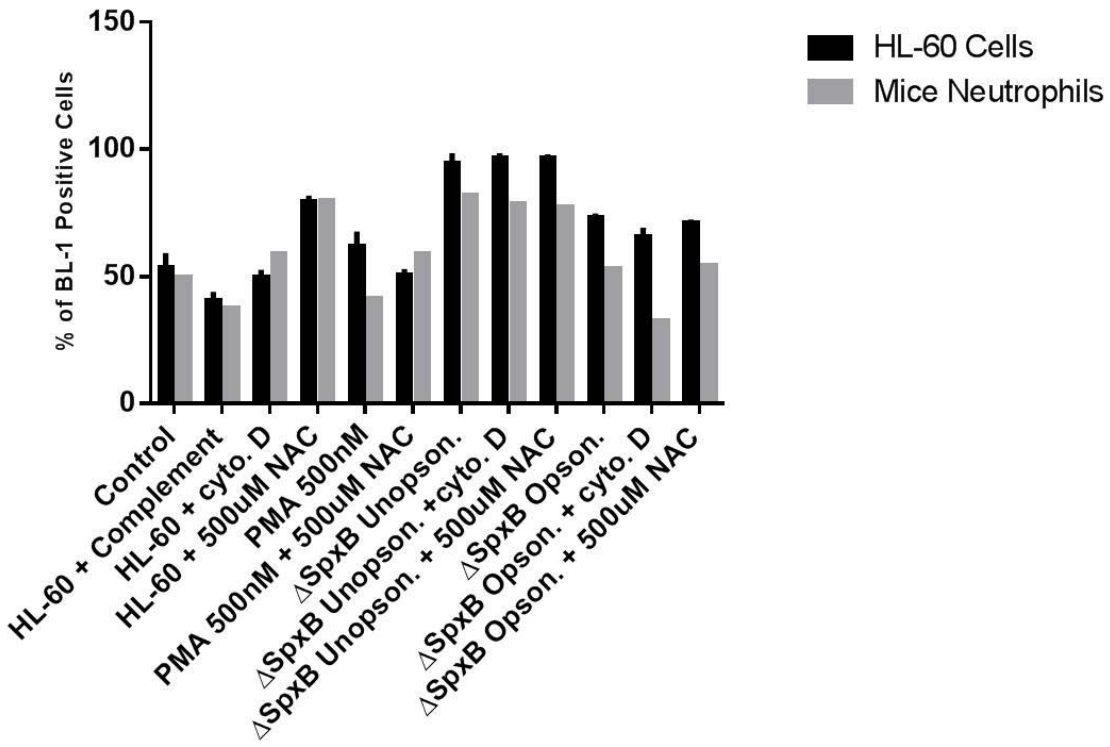

Figure 2.6 HL-60 Cell Apoptosis versus Mice Neutrophil Apoptosis

Mouse neutrophils were exposed to the same set of stimuli as the HL-60 cells for eight hours. Cells were then washed and stained with Annexin V and 7AAD. The mouse neutrophils and HL-60 cells at the eight hour time point showed a similar pattern of cell apoptosis.

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CHAPTER III

PUMA'S CONTRIBUTION TO INNATE DEFENSE IN RESPONSE TO DIFFERENT STIMULI

Abstract

Apoptosis is capable of limiting inflammation caused by bacterial infection. While there are many proteins responsible for regulating apoptosis, one important pro-apoptotic protein is p53-upregulated mediator of apoptosis (PUMA). Typically activated by p53, PUMA can perform one of two mechanisms. It can bind to anti-apoptotic proteins freeing further downstream pro-apoptotic proteins (Chipuk et al., 2005). Additionally, PUMA can directly bind Bax and activate apoptosis (Gallenne et al., 2009). While PUMA has been shown to be important in protecting against lethal bacterial sepsis, limited information is known in regards to the extent of this proteins importance in bacterial infections (Garrison et al., 2010). The goal of this study is to determine the effect of PUMA in response to *Staphylococcus aureus* and *Streptococcus pneumoniae*. We hypothesized that mice deficit in PUMA will have prolonged wound healing when subcutaneously challenged with *Staphylococcus aureus* compared to the wild type mice. To test this, we challenged *PUMA*$^{-/-}$ mice with *Staphylococcus aureus*, which causes a resolving skin abscess. While there was difference in wound severity or healing between the wild type and *PUMA*$^{-/-}$ mice over the course of our study, we found that *PUMA*$^{-/-}$ mice had fewer macrophages in the infected tissue when compared to the wild type mice.

This led us to evaluate PUMA protein levels in macrophages after bacterial exposure using western blotting. PUMA was slightly upregulated at the 16 hour time point in cells exposed to *Streptococcus pneumoniae* strain ΔSpxB and to a lesser degree the wild type bacteria T4R. These results further our understanding of PUMA during bacterial infection.

Introduction

During an infection, immune cells are recruited to the site of infection and attempt to eliminate the pathogen. After this stage is accomplished, cells must die and be removed from the area; otherwise, there is a risk of tissue damage due to prolonged inflammation (Weiss et al., 1981). To prevent dangerous contents of immune cells from spilling into the host tissues, the immune cell undergoes an organized cell death known as apoptosis (Serhan and Savill, 2005). One key protein in certain apoptotic pathways is the p53-upregulated mediator of apoptosis (PUMA). One study found *PUMA*$^{-/-}$ mice have prolonged survival when compared to the wild type (Garrison et al., 2012). This protein also plays an important role in infectious disease. For instance, when *PUMA*$^{-/-}$ mice were challenged with *Streptococcus pneumoniae*, mice rapidly died due to the bacterial infection and had a significant increase in bacteremia (Garrison et al., 2010). Another study has shown that bacteria such *Chlamydia trachomatis* target PUMA for degradation in order to prolong bacterial survival within the host (Dong et al., 2005).

Two bacterial infections that could illustrate the importance of PUMA are that of the Gram positive, extracellular pathogens *Streptococcus pneumoniae* and *Staphylococcus aureus*. Both bacteria can cause a wide variety of disease states such as pneumonia, meningitis, and sepsis while *Staphylococcus aureus* is also known for its skin

infections. Additionally, both can produce a wide variety of toxins to damage host tissue including pneumolysin from *Streptococcus pneumoniae* (Rubins and Janoff, 1998) and Panton-Valentine Leukocidin from *Staphylococcus aureus* (Lina et al., 1999). The immune system is shown to play a critical role in infections involving both of these bacteria. Neutrophils kill *Streptococcus pneumoniae* through various serine proteases (Standish and Weiser, 2009) while macrophages phagocytose and also kill the bacteria (Dockrell et al., 2001). Mice with wounds caused by *Staphylococcus aureus* are not able to properly heal without neutrophils (Molne et al., 2000). Once these immune cells have mediated bacterial clearance, they must also be removed from the initial site of infection.

All of this information led us to believe that PUMA plays an important role in the context of infection and subsequent resolution. We hypothesize that mice lacking the *PUMA* gene would not be able to easily clear bacterial infections and would have poor wound healing. To test this hypothesis, we challenged $Puma^{-/-}$ mice with *Staphylococcus aureus* subcutaneously. One study that led us to use *Staphylococcus aureus* was the study by Garrison and coworkers (2010) mentioned previously. We wanted to determine whether resolution of inflammation would occur in a non-lethal wound healing model such as that provided by a *S. aureus* subcutaneous infection.

Materials and Methods

Subcutaneous challenge

$Puma^{+/+}$ and $Puma^{-/-}$ mice were shaved and had rest of their hair removed with hair removal cream. The following day mice were injected with 50µL ($1x10^7$cfu) *S. aureus* strain USA300 or the LAC mutant lacking the leukotoxins on one flank. The mice were weighed every day and wounds were measured and imaged. At the end of the

challenge, abscess sites were biopsied and tissue was sectioned for histopathology. Tissue sections were stained with hematoxylin and eosin (H&E) stain and histopathology assessments were made by a pathologist.

Preparation of J774.1 macrophage cells

Cells were diluted to $1x10^6$ cell/mL in Dulbecco's Modified Eagle Meidum (DMEM) containing no antibiotics and 1 mL of cells was transferred to two 12-well plates. The cells were then incuabated at 37°C in a CO_2 incubator overnight to allow adherence to the bottom of the wells. The next day cells were exposed to a 1:1 bacteria to cell ratio along with a positive control sample of etoposide. T4R and ΔSpxB were used as the bacterial samples. The plates were incubated at 37°C with 5% CO_2 for one hour. After the one hour, the media was vacuumed off, and the cells were washed with 1 mL of PBS twice and resuspended in DMEM containing 0.1 mg/mL of Penicillin-Streptomycin. The cells were then placed in 37°C CO_2 incubator for 16 hours and 24 hours

Western blots (J774.1 cells)

After the 16 and 24 hour time points, cells were washed with DPBS +Ca +Mg and lysed with 300 µl of modified hunter's buffer (10mM Hepes, 150mM NaCl, 1.5mM $MgCl_2$, 1mM EDTA, 10mM Na-pyrophosphate, 10mM NaF, 0.1mM Na_3VO_4, 1% deoxycholic acid, 1% Triton X 100, 0.1% SDS) for 5 minutes at room temperature. Cells were then sonicated three times at 3 second pulses and centrifuged at 10,000 rpm for 5 minutes. The supernatant was then transferred to a new 1.5 mL tube. The BCA protein assay kit (Pierce) was used to determine the protein concentration. Proteins were then

separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a handcast 10% gel and then transferred to a polyvinylidene fluoride (PVDF) membrane using semi dry electrophoresis. The membrane was then blocked using 5% milk in 1xTTBS (50mM Tris pH 8.0, 138mM NaCl, 2mM KCl, 0.05% Tween 20) for one hour. The membrane was then incubated with the desired antibody overnight at 4°C. The next day, membranes were washed four times for 5 minutes each with 1xTTBS (before and after secondary) and probed for one hour with the appropriate secondary antibody. Immunoreactivity was then detected with either Luminata Forte or Pierce ECL Western Blotting Substrate.

Results and Discussion

One of the goals of this study was to determine the importance of PUMA in a non-lethal, resolving wound infection. We used *Staphylococcus aureus* as our infecting agent and *PUMA*$^{-/-}$ and wild type mice. The wound measurements that were taken daily did not differ between wild type and *PUMA*$^{-/-}$ mice in appearance (Figure 3.1A) or surface area (Figure 3.1B). Additionally, the wounds from both groups of mice healed approximately at the same rate. However, histopathology revealed that wounds of the *PUMA*$^{-/-}$ mice had high levels of neutrophils packed into the tissue when compared to the wild type mice (Figure 3.2). Additionally, there were far fewer macrophages present in the tissues of *PUMA*$^{-/-}$ mice; whereas, the wild type mice had typical histiocytic influx. This led us to theorize that the neutrophils are not undergoing apoptosis efficiently, which is potentially leading to a lack of signaling for macrophages to enter the infected tissue.

This scenario could lead to later negative effects within the wound, such as a lack of resolution or a susceptibility to secondary infections. Typically, macrophages engulf

apoptotic neutrophils, removing them and their reactive granules from the infected tissue (Cox et al., 1995). These macrophages are able to switch from pro-inflammatory to anti-inflammatory using their activation phenotypes (Porcheray et al., 2005). At this point, macrophages release anti-inflammatory cytokines, such as transforming growth factor, and exit the infected site, which allows for complete inflammation resolution (Serhan and Savill, 2005). Since the sites of infection contain few macrophages, the release of cytokines that promote resolution of inflammation may be on a smaller scale and may cause a lack of full healing.

The lack of macrophages in the *PUMA*$^{-/-}$ mice led us to question whether PUMA expression is altered in these cells following bacterial exposure. Macrophages were exposed to both the wild type and ΔSpxB *Streptococcus pneumoniae*, and then a western blot was performed to see the effect of the bacteria on PUMA protein expression. PUMA was slightly upregulated at the 16 hour time point in cells exposed to ΔSpxB and to a lesser degree T4R; whereas, pro-apoptotic protein Bad had no change in expression across time points and samples (Appendix B.2). The anti-apoptotic protein Bcl-X_L was upregulated at the 16 hour time point as compared to the 24 hour time point while Bcl-2 and Mcl-1 had no change in expression across samples and time points. This suggests that PUMA may not be as important of a factor in macrophages following *Streptococcus pneumoniae* bacterial exposure.

In summary, the importance of PUMA during infection was confirmed in the case of bacterial infections due to the changes in the immune response to the pathogen. Due to the lack of macrophages seen in *PUMA*$^{-/-}$ mice, the complete resolution of inflammation may be difficult to obtain due to the potential lack of reparative cytokines.

The exposure of *Streptococcus pneumoniae* demonstrates that macrophages do not follow the same pattern of PUMA as the neutrophils seen in the previous chapter.

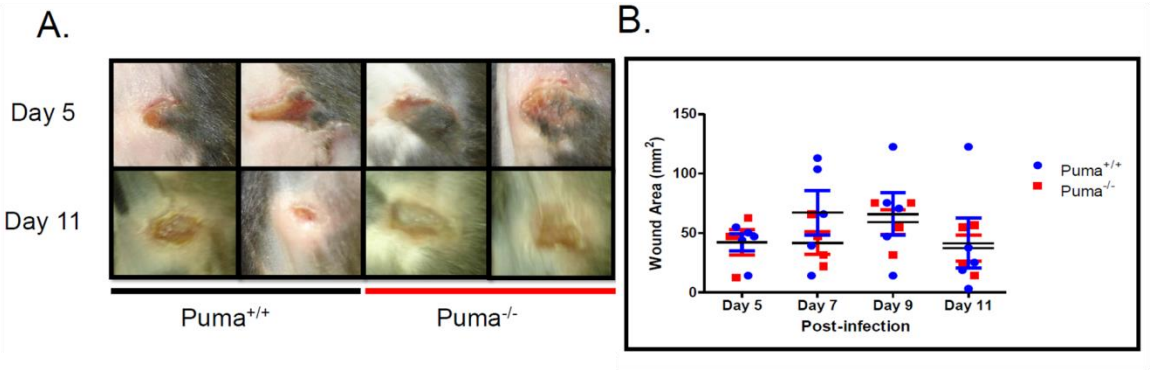

Figure 3.1 Wound Healing in *PUMA*$^{-/-}$ Mice infected with *S. aureus*

Groups of seven week old C57BL/6 wild type or *PUMA*$^{-/-}$ mice were challenged subcutaneously along the right flank with 1.3x10^{7} cfu of *Staphylococcus aureus* LAC (USA300) in 50µl. Mice were monitored for fifteen days by weighing, imaging wounds (A, representative images), and measuring wound sizes (B). All animal work was done in compliance with MSU Institutional Care and Use Committee (IACUC protocol #14-016).

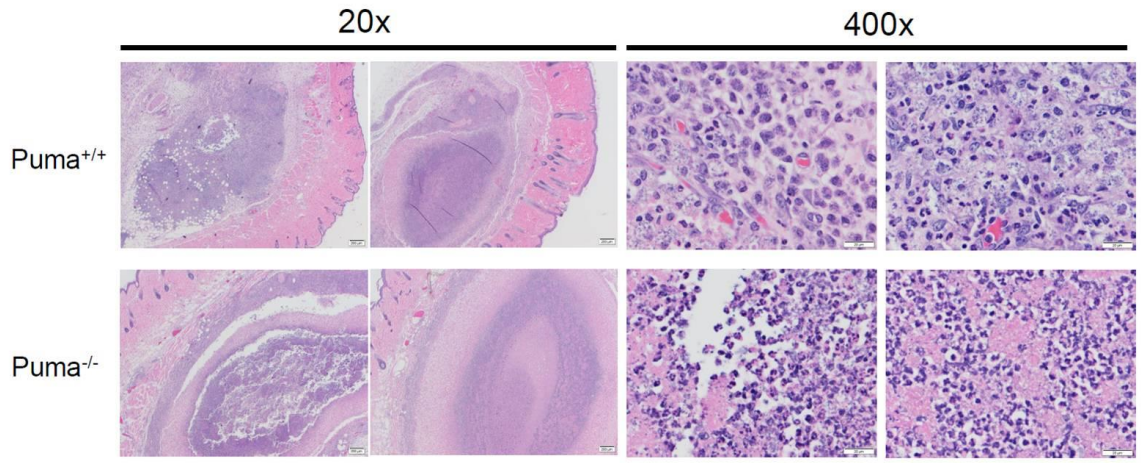

Figure 3.2 Differences in Inflammation in *PUMA*$^{-/-}$ Mice

PUMA$^{+/+}$ and *PUMA*$^{-/-}$ mice (8 weeks old) were challenged subcutaneously with 4x10^{7} cfu of *S. aureus* LAC (cytotoxin deletion mutant) in 50µl. Mice were humanely euthanized day seven post-challenge and skin snips containing abscesses were removed, fixed in 10% formalin, embedded in paraffin, and sectioned for H&E staining. Shown are 20x and 40x sections from representative animals.

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CHAPTER IV

SUMMARY

Streptococcus pneumoniae and *Staphylococcus aureus* are two potentially harmful bacteria that cause many forms of disease including pneumonia, sepsis, and meningitis. These two bacteria are significant causes of morbidity and mortality worldwide. Pneumococcal disease was responsible for 136,000 deaths of children in 2005 in India alone (Liu et al., 2012). Treatments for these bacteria, such as antibiotics, are becoming less effective due to increasing resistance. The body, however, has its own form of defense that we could potentially use to our advantage. As previously stated, proper regulation of apoptosis is critical for clearance of microbes and minimization of detrimental effects on the host (Kobayashi et al., 2003). The studies presented here built upon previous results demonstrating a role for DNA-damage and PUMA in the proper resolution of infection and inflammation. While it is known that PUMA is important in preventing lethal bacterial sepsis in mice (Garrison et al., 2010) and ROS can induce DNA damage (Takeuchi et al., 1996), little is known concerning the series of events leading to PUMA activation and apoptosis following bacterial infection. We initially hypothesized that apoptosis induced by the p53/Puma pathway is initially triggered by ROS thereby preventing neutrophils from defaulting to another form of cell death or remaining in a state of hyper-activation that is harmful to the host.

Interestingly, our results indicate that levels of PUMA gene transcription in neutrophils is not dependent on ROS, DNA damage, or phagocytosis. In fact, cells exposed to bacteria that had the lower percentages of ROS and DNA damage; whereas, cells phagocytosing the bacteria had higher levels of PUMA transcription and higher levels of apoptosis. It was surprisingly to see that the increased DNA damage did not lead to increased PUMA transcription in the opsonized ΔSpxB sample. Additionally, we wanted to determine the *in vivo* relevance of PUMA during bacterial infection. We were able to test with *Staphylococcus aureus* and discovered that while wound appearance and size did not change among the wild type and knockout mice the composition of immune cells in the infected tissue did. Interestingly, the infected tissues in *PUMA*$^{-/-}$ mice contained fewer macrophages compared to that seen with the wild type mice. Increased numbers of neutrophils were found in tissues from PUMA$^{-/-}$ mice. This could potentially lead to prolonged inflammation and supports the importance of PUMA during bacterial infection.

Our current proposed model of PUMA and apoptosis activation during infection is a complex one. Our hypothesized model involves TNF-α and NF-κB. TNF-α acts in two ways in that it can both induce and suppress apoptosis. During extended exposure, TNF-α has been shown to prevent neutrophil apoptosis through the activation of cytokine IL-8 (Dunican et al., 2000). On the other side, TNF-α can lead to a transcriptional activation of PUMA through the transcription factor NF-κB, which has led to subsequent cell apoptosis (Wang et al., 2009). For neutrophils being exposed to opsonized bacteria, we believe that it is possible that TNF-α is prolonging the life of the neutrophil. While the neutrophil has indicators of future apoptosis, such as DNA damage, the pro-survival

effects of that TNF-α are limiting the apoptosis to an extent. As for neutrophils being exposed to unopsonized bacteria, TNF-α may be activating NF-κB causing increased PUMA transcription and apoptosis. This mechanism could potentially explain the differences observed in DNA damage and subsequent PUMA levels since this method is independent of ROS and DNA damage.

In conclusion, we found PUMA-dependent apoptosis may not be induced directly by ROS and DNA damage. Additionally, we found that mice lacking PUMA have an altered innate immune cell prescence. These organisms potentially risk prolonged inflammation and less efficient bacterial clearance. Further studies need to be done on cytokines such as TNF-α and proteins such as NF-κB to determine their role in driving PUMA-dependent apoptosis. Additionally, more *in vivo* PUMA experiments with different bacteria need to be performed to see the breadth of PUMA's importance. All of these experiments will lead to a better understanding of how our immune system responds to bacterial infection.

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APPENDIX A

CELL VIABILITY AND ROS PRODUCTION

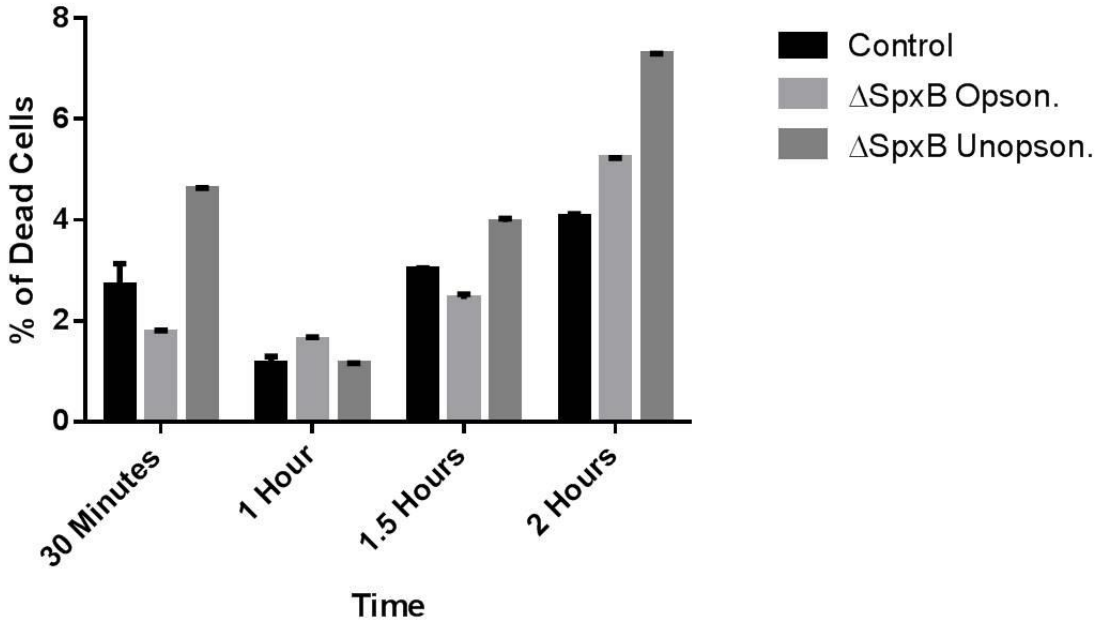

Figure A.1 Viability of HL-60 cells over Time after ΔSpxB Exposure

HL-60 neutrophils were exposed to a 1:1 ration of ΔSpxB for 30 minutes, 1 hour, 1.5 hours, and 2 hours. They were then stained with Trypan Blue and counted for cell viability. Cell death remained at low percentage for all samples across all time points.

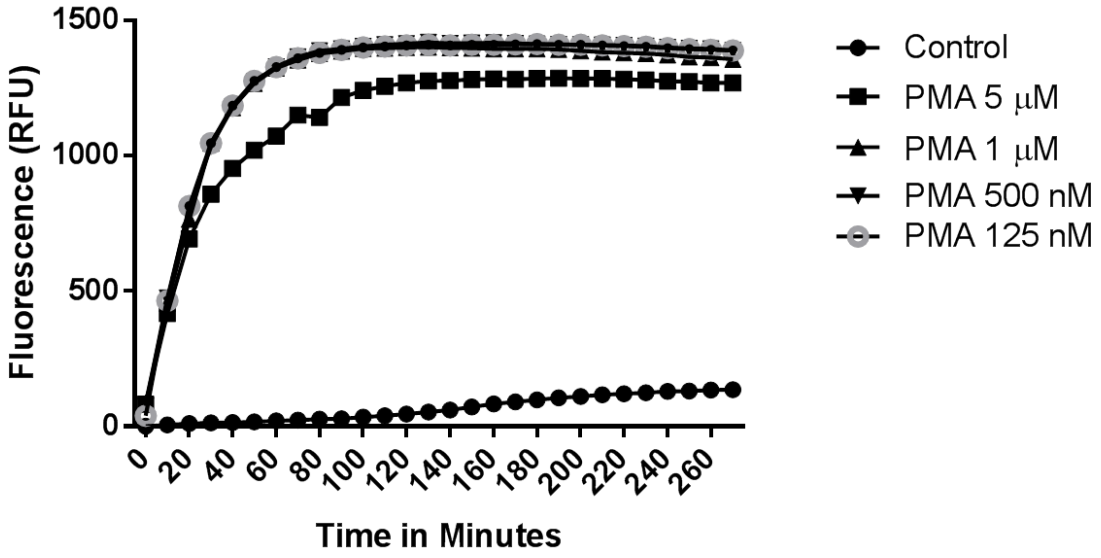

Figure A.2 DHR 123 Fluorescence of HL-60 cells exposed to PMA

HL-60 neutrophils were exposed to different concentrations of PMA for 4.5 hours. ROS production was measured by the fluorescent probe DHR 123. The PMA concentration of 500nM was later picked for experimental use due to this data and the Trypan Blue staining data shown below.

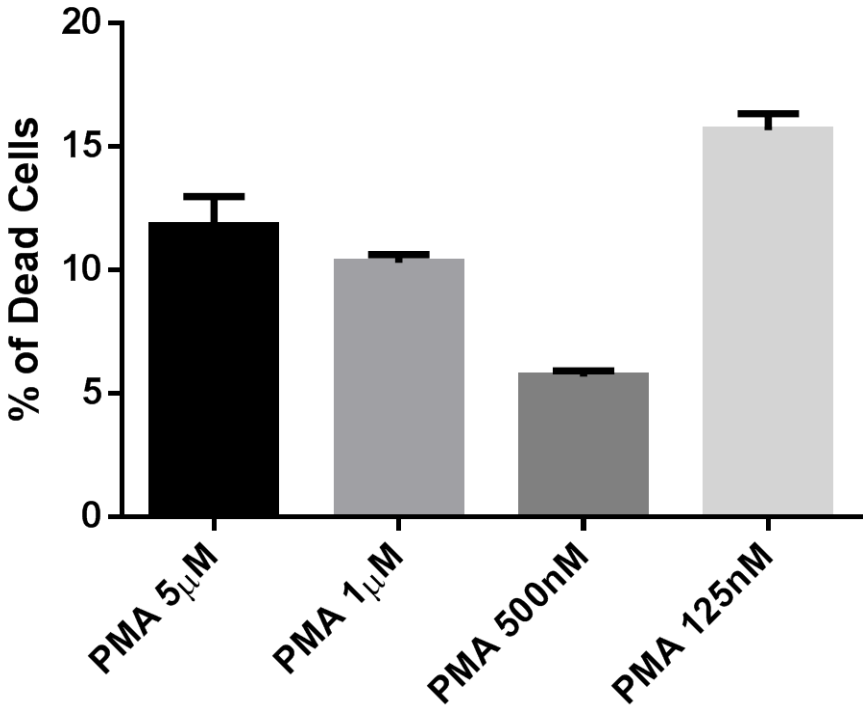

Figure A.3 Viability of HL-60 cells over Time after PMA Exposure

HL-60 neutrophils were exposed to different concentrations of PMA for 3 hours. All samples had less than 20% cell death with the 500nM concentration having the lowest. The PMA concentration of 500nM was then chosen for experimental use due to this data and the ROS production data shown above.

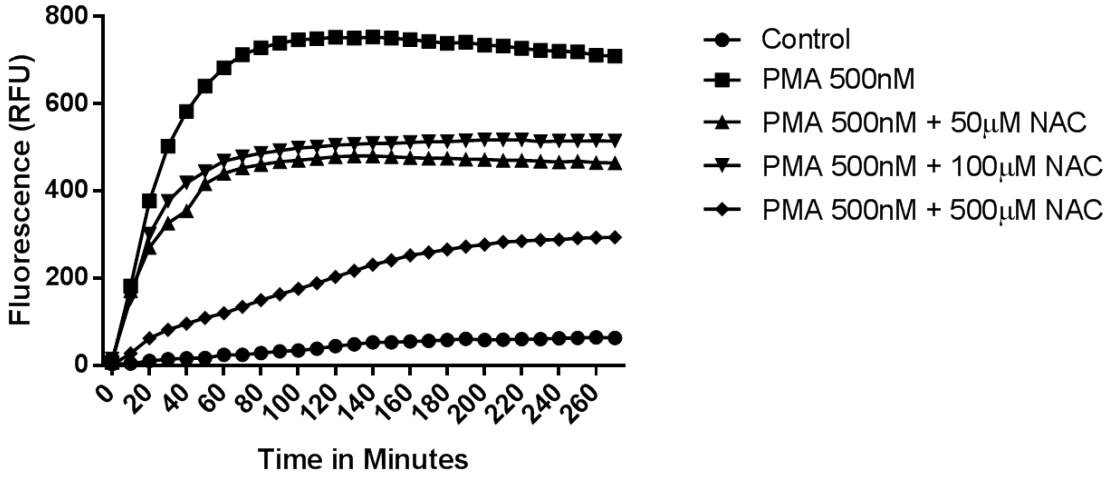

Figure A.4 DHR 123 Fluorescence of HL-60 cells during PMA and NAC exposure

HL-60 neutrophils were exposed to 500 nM PMA and different concentrations of NAC for 4.5 hours. ROS production was measured by the fluorescent probe DHR 123. The NAC concentration of 500μM was then chosen for experimental use based on this data.

APPENDIX B

WESTERN BLOTS

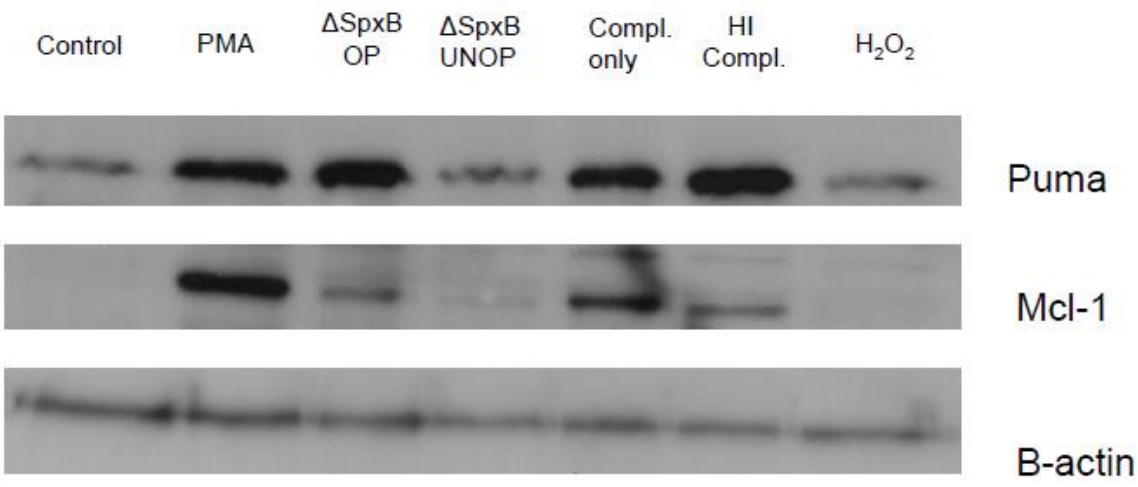

Figure B.1 Western Blot of HL-60 cells exposed to ΔSpxB

Differentiated HL-60 cells were exposed to various stimuli for two hours, washed, and incubated at 37^{o}C for four additional hours. Cells were then washed with PBS and lysed using Modified Hunter's Buffer. Sonication followed by centrifuged was then performed. Protein concentrations were determined by BCA assay and equal concentrations of protein were loaded onto SDS-PAGE gels, blotted, and probed with commercially available antibodies. The blot shows that the presence of complement may be effecting the Bcl-2 family proteins.

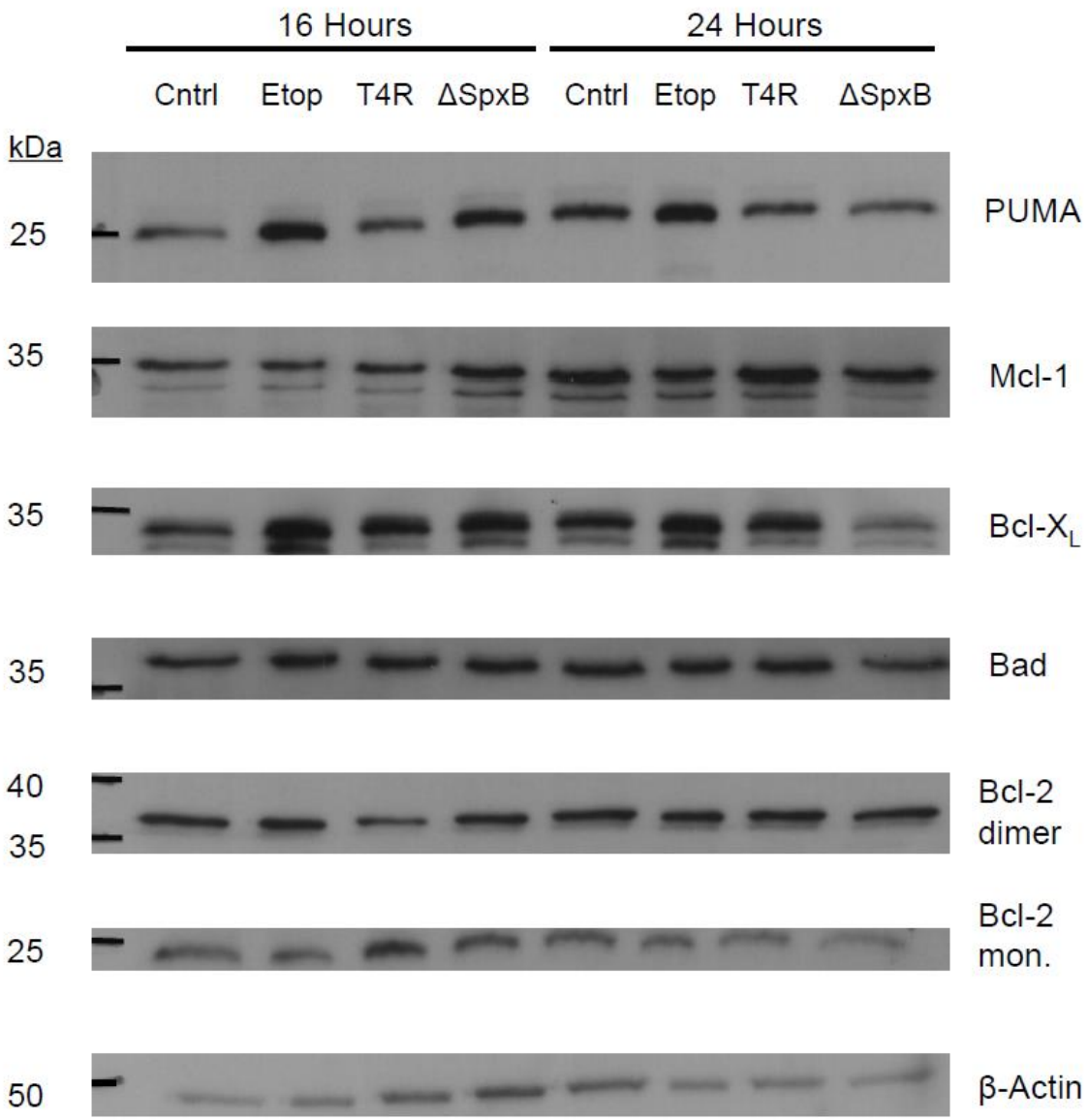

Figure B.2 Western blot of J774.1 exposed to T4R and ΔSpxB

Puma is slightly upregulated at the 16 hour time point in cells exposed to ΔSpxB and to a lesser degree wild type T4R. Bcl-X_L was upregulated at the 16 hour time point as compared to the 24 hour time point. Bad, Bcl-2, and Mcl-1 had equal regulation across samples and time points.