PENTRAXIN 3 EXPRESSION IN THE LUNG AND NEUTROPHILS

A Thesis Submitted to the College of

Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of Master of Science

in the Department of Veterinary Biomedical Sciences

University of Saskatchewan

Saskatoon

By

Michelle Townsend

PERMISSION TO USE

In presenting this thesis in partial fulfilment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Veterinary Biomedical Sciences

Western College of Veterinary Medicine, University of Saskatchewan

52 Campus Drive, Saskatoon, Saskatchewan S7N 5B4

ABSTRACT

Respiratory diseases are a major cause of human morbidity and mortality and are a leading cause of economic loss to livestock producers. The respiratory tract is constantly in contact with dust, bacteria, fungi, viruses and other pathogenic agents that are found in the air. Normally, the body has the ability to clear these foreign particles. However, physiological and environmental stresses can impair airway defense mechanisms resulting in establishment of pulmonary infections. The microbes and their products engage various receptors in the lung to activate epithelium, endothelium, macrophages, neutrophils and other cells. The activation of inflammatory cascade in the lung results in recruitment of neutrophils, damage to air-blood barrier and development of edema. Although there have been significant advances in our understanding of mechanisms of lung inflammation, there have been a lack of any significant advances in the development of new therapeutics to manage lung disease, which may suggest that our understanding of the inflammatory mechanisms is still incomplete.

Pentraxin 3 (PTX3) is an innate immune protein which has been implicated in a diverse range of inflammatory processes, such as recruitment of cells and production of cytokines. PTX3 is an acute phase protein, with low or undetectable levels in the circulation of healthy humans and animals, and rapid, dramatic increase in inflammatory diseases. The expression and function of this protein has not been characterized in the lungs of domestic animal species. Because of potential implications of PTX3 in lung inflammation, I studied the expression of PTX3 in normal and inflamed lungs of calves, pigs, horses, foals and humans. Lungs from all of these species showed expression of PTX3 in airway epithelium, alveolar septa, vascular endothelium and inflammatory cells. Western blot performed on homogenates from normal and

inflamed lungs from calves and pigs show an increased expression of PTX3 in inflamed lungs ($P<0.05$).

Because protein function is influenced by its location in the cell, I clarified the subcellular expression of PTX3 with immuno-electron microscopy on normal and inflamed calf and horse lungs. PTX3 was localized on pulmonary intravascular macrophages, monocytes, neutrophils and, unexpectedly, platelets. PTX3 was also present in the nuclei of neutrophils, monocytes and pulmonary intravascular macrophages.

Neutrophils are critical regulators of acute lung inflammation. Having observed PTX3 in neutrophils, I investigated the effect of *E. coli* lipopolysaccharide-induced activation on PTX3 in neutrophils *in vitro*. Neutrophils challenged with *E. coli* LPS were examined at 30, 60, 90 and 120 minutes after the treatment. Normal peripheral blood neutrophils showed PTX3 expression. Neutrophils activated with LPS appeared ruffled and showed loss of PTX3 expression at 30 minutes followed by recovery of the expression. Western blots performed on normal and activated neutrophil homogenates did not show any differences ($P=0.05$).

Collectively, the data show PTX3 in normal and inflamed lungs across multiple species. PTX3 was also detected in normal and activated neutrophils. While the function of intriguing localization of PTX3 in the nuclei as well as in platelets is not known, the similarity of expression across the species suggest a role for PTX3 in lung inflammation.

ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Baljit Singh, my supervisor. He has a gift of spreading a positive outlook to all he encounters. He has a passion for teaching and a kind understanding. He is the reason I have made it to the end of my program. Thank you, Baljit, for your reassurance when I needed it. I have learned a lot through this process and gained confidence along the way.

I would like to thank my committee member, Dr. Heather Wilson, and my graduate chair, Dr. Jaswant Singh, for their keen attention to detail, thoughtful insight, encouragement and patience. Their ongoing support throughout my project is acknowledged and appreciated.

I received technical support from several people in the college. I would like to express gratitude to the staff and technicians that helped troubleshoot experiments or provide advice and technical assistance. Notably, I would like to thank Shanna Banman for assistance with confocal microscopy, Cathy Coghlin, Kim Tran, and the Animal Care Unit staff for drawing blood samples and Sarah Caldwell for her technical assistance with immuno-gold electron microscopy. Susan Fjeldstrom, Cheryl Hack and Sandra Rose are appreciated for their crucial roles in keeping our department operating smoothly.

Over the past three years, I have spent a lot of time with some people that I have much in common with, my lab mates. This group of people are not only my colleagues, they have become my friends. They have been my emotional support, my problem-solvers, my cheer-leaders and understand the challenges we have in common. Thank you for being a big part of my success. Stay positive and keep going. You can do it!

I appreciate my family and friends that have encouraged me throughout this journey. Through ups and downs they have been there for me to share my triumphs and to ease my woes. Though during my studies, I may have had less time to devote to my family and friends, they have stood by my side and supported me.

Last, but not least I would like to express my sincere gratitude to my husband, Dallas. We have been on this journey together. We have made sacrifices together and we will pursue this next step in our life together. Thank you for being there for me with love and support.

TABLE OF CONTENTS

LIST OF FIGURES

Figure Number and Name **Page**

LIST OF ABBREVIATIONS

ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
BALF	Broncho-alveolar lavage fluid
BRD	Bovine respiratory disease
BSA	Bovine serum albumin
CRP	C-reactive protein
H_2O_2	Hydrogen peroxide
IAD	Inflammatory airway disease
IHC	Immunohistochemistry
LBP	LPS-binding protein
LPS	Lipopolysaccharide
MD-2	Myeloid differentiation protein 2
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NET	Neutrophil extracellular trap
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen-associated molecular pattern
PIM	Pulmonary intravascular macrophage
PRR	Pattern recognition receptor
PTX	Pentraxin
PTX3	Pentraxin 3
RAO	Recurrent airway obstruction
RIG	Retinoic acid-inducible gene-1
SAP	Serum amyloid P component
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TSG-14	TNF-stimulated gene 14
vWF	von Willebrand Factor

CHAPTER 1: REVIEW OF LITERATURE

1.1 Introduction

The respiratory tract is constantly in contact with dust, bacteria, fungi, viruses and other pathogenic agents that are found in the air. Failure to clear the airway of these particles leads to inflammation of the airway or infection of the lower respiratory tract. Acute inflammation of the lungs causes significant mortality and morbidity, leading to enormous economic damage to the animal industry and costs billions of dollars in human health care. The first line of defense is the mechanical barrier that lines the respiratory tract. Ciliated bronchial epithelial cells sweep particulates towards the throat, where they are then expelled or swallowed. Mucous lining the respiratory tract helps to trap particulates before they enter the lower airways. If these mechanical barriers fail, the next line of defense is the innate immune system. Innate receptors can identify invading pathogens and mount an immune response with the goal of clearing the infection.

The innate immune system, which includes a variety of cells and receptors, plays a critical role in the initiation of the immune response in the lung. Pattern recognition receptors (PRRs) are programmed to recognize microbial structures unique to pathogens and mount an immune response[1]. Pentraxin 3 (PTX3) is a PRR that is expressed by various inflammatory cells upon stimulation by proinflammatory cytokines, and agonists such as endotoxins[2]. PTX3 recognizes and binds to many pathogens, activates the complement cascade, and plays a role in the clearance of apoptotic and necrotic cells[2].

Respiratory diseases such as bovine respiratory disease (BRD) and secondary bacterial infections are among the leading causes of economic loss in the livestock industry[3, 4].

Mortality is an obvious cause of economic loss due to disease, but morbidity can be even more costly due to the expenses associated with treatment, reduced production, and premature euthanasia of animals[5]. Several factors are involved in the onset of BRD, including infection, environmental factors, and immune deficiency[6]. Stress due to weaning, shipment and housing with unfamiliar animals in addition to pre-existing infection with viruses increase the likelihood of developing infections of opportunistic bacteria, often in the respiratory tract[7]. Bacterial infections can occur secondary to viral infections. *Mannheimia hemolytica* is of significant concern in cattle, causing a common and well-studied disease often referred to as Shipping Fever. *Pasteurella multocida* and *Haemophilus somnus* are other common causes of secondary bacterial pneumonia in cattle[4]. In horses, similar to cattle, infection with viruses can predispose the animal to bacterial respiratory infections. *Streptococcus equi zooepidemicus* is the most common opportunistic bacterial invader of the equine lung[8], although several others are cause for concern. *S. equi* causes strangles, a highly contagious upper respiratory tract infection in horses[9]. The complex biology of respiratory diseases is underscored by lack of any major therapeutic breakthroughs despite significant advances in our understanding of the inflammatory processes of these diseases.

In humans, inflammation of the lung accompanies infectious diseases similar to those found in animals, such as pneumonia, influenza and tuberculosis. Acute respiratory distress syndrome (ARDS) is a complex disease, characterized by hypoxemia, infiltration of neutrophils to the lung and excessive inflammation[10]. Chronic inflammation occurs due to persistent sensitivity such as in asthma, where inflammation of the bronchi and bronchioles is typical and the host immune system cannot eliminate the cause completely[11]. Chronic obstructive

pulmonary disease is a long-term inflammatory state of the lungs, often caused by smoking or exposure to noxious fumes. In chronic obstructive pulmonary disease, elastic recoil of the lung is diminished and airways narrow, resulting in the inability to completely exhale before the need to take the next breath[12]. These human diseases are extremely common and are of significant public health concern.

Treatment of respiratory diseases is currently focused on prevention and supportive care. Often, there is no single "cure" to treat these diseases, largely due to our incomplete understanding of the mechanisms of inflammation in the lung. The aim of this study is to characterize PTX3 in the airways of normal and inflamed animals and humans as well as to determine the role of neutrophils in the production of this protein.

1.2 Respiratory Diseases of Humans

Acute lung injury (ALI) is defined as "a syndrome of inflammation and increased permeability that is associated with a constellation of clinical, radiologic, and physiologic abnormalities that cannot be explained by, but may co-exist with, left atrial or pulmonary capillary hypertension"[13]. Acute respiratory distress syndrome (ARDS) meets the same criteria for diagnosis, but is a more severe form of ALI. These diseases are associated with high mortality and affect individuals of all ages. Estimates of the incidence of ALI and ARDS are often varied due to difficulty in reliability of diagnosis by clinicians[14]. The mortality rates between ALI and ARDS are similar, and are also highly variable, with one report of 35-40%[14], another estimated at 25-40%[10] and a third study reporting a range in the literature of 15-72%[15].

In 1993, the American-European Consensus Committee on ARDS established a criteria for diagnosis of ALI and ARDS[13]. These criteria facilitate studies of ALI and ARDS in making the criteria in defining these syndromes more consistent. For ALI, the criteria are: acute onset of hypoxemia ($PaO_2/FiO_2 \leq 300$ mmHg (regardless of positive end-expiratory pressure, the alveolar pressure above the atmospheric pressure that exists at the end of expiration; where PaO_2/FiO_2 is an index of arterial oxygenation efficiency that corresponds to ratio of partial pressure of arterial O_2 to the fraction of inspired O_2)) with bilateral pulmonary edema visible on a frontal chest radiograph and pulmonary artery wedge pressure ≤18 mmHg when measured or no clinical evidence of left atrial hypertension. The criteria for ARDS is the same as ALI, but oxygenation differs in that PaO_2/FiO_2 must be ≤ 200 mmHg (regardless of positive end-expiratory pressure) [10, 13].

Causes of ALI can be considered either direct or indirect. Some examples of direct injury include pneumonia, drowning or reperfusion. Examples of indirect injury include severe sepsis, transfusions, organ transplant, drugs, shock and pancreatitis[10, 16, 17]. ALI due to sepsis is associated with the highest incidence of mortality compared to other causes[16]. The onset of ALI can be caused by either activation of lung cells (such as neutrophils, macrophages, monocytes, and lymphocytes) or consequent to an acute systemic inflammatory response[13]. Pulmonary edema, the accumulation of protein-rich fluid in alveoli, occurs due to increased vascular permeability and results in neutrophil accumulation in the lung. The number of neutrophils in broncho-alveolar lavage fluid (BALF) sharply increase after intranasal lipopolysaccharide (LPS) instillation, an experimental model of ALI, in mice[18] and in clinical cases of ALI[19]. Due to the nature of neutrophils, they carry the potential for harming lung

tissue through production of reactive oxygen species, proteases and cytokines. Myeloperoxidase (MPO) activity, a surrogate for neutrophil accumulation, increases significantly in BALF of LPS-treated animals to suggest that recruited neutrophils are activated[18].

Lipopolysaccharide (LPS), also known as endotoxin, is often used as an experimental model of ALI because it induces hyper-inflammatory response. The ultimate goal of any experimental model is to replicate the clinical disease as closely as possible, but all models have their shortcomings. For example, mice are more resilient to the LPS-induced inflammation compared to humans[20]. The dose of LPS used in mouse studies that leads to a death rate of about 50% of the animals ranges from 1-25mg/kg. In humans 1/1,000,000 (2–4 ng/kg) of this dose would cause fever and production of cytokines, while 1/1,000 of the dose would lead to severe disease and shock in human subjects[20]. A second example of the limitations of the LPS model of ALI is that it may not induce all of the same mechanisms that occur during clinical ALI. For example, it is widely accepted that complement has a role in the development of clinical ALI/ARDS[21]. Yet, a study published in 2008 reports that LPS-induced ALI in mice occurs without complement activation[21]. Nonetheless, animal models have contributed greatly to our knowledge of the mechanisms of ALI. It is important to interpret results while keeping in mind the limitations of each model used.

Treatment of respiratory diseases usually consists of supportive care, especially treatment of the underlying cause and maintenance of oxygenation[10]. For example, patients with ALI as a result of bacterial pneumonia are treated with antibiotics[22]. In patients suffering from ALI due to aspiration or multiple transfusions, there is no direct treatment except support of physical symptoms[22]. Thus, mechanical ventilation of these patients is the main supportive measure.

The use of mechanical ventilation has lowered the mortality rate of ALI in recent years[23]. However, the use of artificial ventilation may result in further damage, exacerbating the disease[22]. In chronic obstructive pulmonary disease, management of the disease is the primary therapeutic measure. This may include relief of symptoms using pharmaceuticals, such as bronchodilators, disease prevention, such as smoking cessation and treatment of complications[12].

1.3 Respiratory Diseases of Domestic Animals

Bovine respiratory disease (BRD) complex is one of the leading causes of economic loss in the beef and dairy industry in North America[4]. Cattle are often afflicted with BRD when immune mechanisms are diminished following stress. Common infections associated with BRD include bovine rhinotracheitis virus, parainfluenza virus type 3, bovine respiratory syncytial virus or bovine viral diarrhea virus[4].

It is normal for some commensal bacteria to colonize the respiratory tract. Cilia in the respiratory tract constantly clear these bacteria and prevent infection. Only when these clearance mechanisms are inhibited do problems arise. Reduced mucociliary clearance of bacteria is common following stress associated with changes in weather, feed, housing or transport[4]. *Mannheimia hemolytica* colonizes the upper respiratory tract in cattle and sheep under normal conditions[24]. However, over-proliferation of *M. hemolytica* due to impaired clearance causes Shipping Fever, an aptly named disease that often occurs after transport of animals. It is reported that within the first year of life, about 25% of calves suffer from at least one episode of respiratory disease[24].

Horses are prone to similar diseases as cattle following transportation. Normal inhabitants of the equine respiratory tract include *Streptococcus equi* subsp. *zooepidemicus, Pasteurella* spp*., Escherichia coli, Actinomyces* spp., and *Streptococcus* spp.[25]. Viruses contribute to predisposition of horses to bacterial infection since they have the ability to replicate inside of ciliated epithelium, resulting in destruction of this anatomic barrier[25]. Horses undergoing surgical repair for intestinal problems, such as colic, suffer from endotoxemia[26], which also leads to lung dysfunction. Colic refers to pain and discomfort of the equine abdomen. There are diverse causes of colic including obstructions or strangulation of the gastrointestinal tract, internal parasites, change of diet, dietary management, housing conditions, lack of access to pasture and water, increased exercise and transport[27]. Yet in many (if not most) cases, the etiology is unknown[27-29].

Colic causing inflammation of the intestinal mucosa can lead to increased permeability and leakage of intestinal contents into the peritoneum. Bacteria then enter the circulation and cause serious endotoxemia. It is reported that endotoxin was found in the circulation of 25%-41% of horses with clinical colic[30]. Surgery for intestinal problems can also result in leakage of bacteria into the body cavity. A study that investigated the survival of horses after surgeries of the small intestine reports a death rate 51% during the post-operative period and a further 14% died after discharge from the hospital[31]. The most common complications were attributed to incisional drainage, dehiscence, or herniation (23%), peritonitis (18%), recurrent colic attributed to adhesions or peritonitis (18%) and postoperative ileus with persistent gastric reflux (16%)[31].

Presently, there are limited data on the incidence, mortality rate, causes, cost to industry and risk factors associated with colic in horses. Depending on the horse population studied, the incidence of colic ranges from 3.5 to 10.6 colic episodes per 100 horses per year[29]. Due to differences in horse populations as well as differences in outcomes of specific types of colic, it is estimated that fatality rates as a result of colic vary from 6.7% to 15.6%[29]. The economic loss associated with colic in USA is estimated to be $115.3 million in one year[28]. This estimate takes into account the loss of sales due to death (accounting for about 66% of the cost), veterinary services, drugs and additional care, and mean number of lost days.

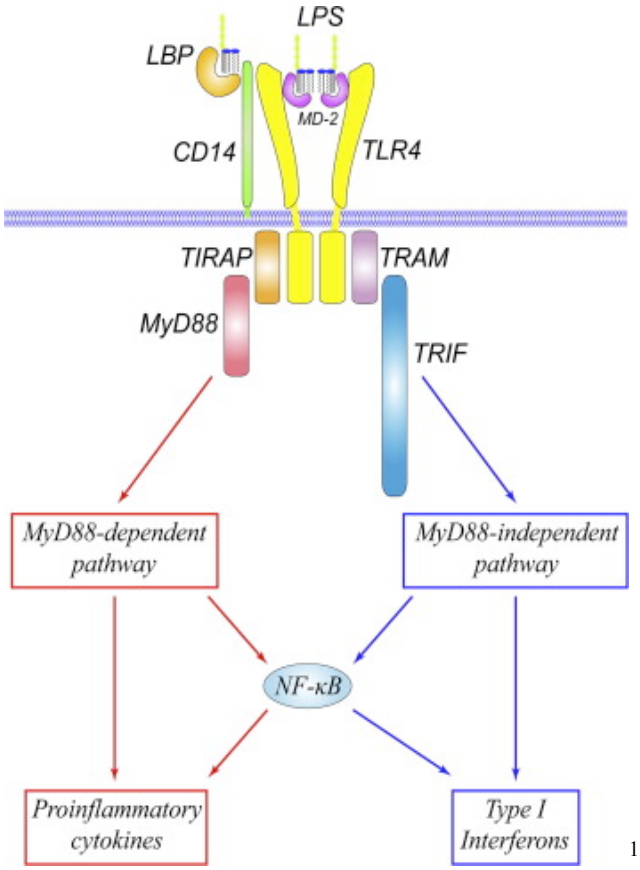
Foals between one and six months of age are susceptible to *Rhodococcus equi* infection, but adult horses rarely transmit the disease[32]. *R. equi* is a bacterial pathogen found in the soil that causes a potentially life-threatening pneumonia characterized by neutrophilic lung abscesses, intracellular bacterium in macrophages and ulcerative colitis[32]. The bacterium is taken up by macrophages, where it not only has the ability to survive, but also has the ability to cause granulomatous inflammation and destruction of the macrophage[32]. As a result, host suffers life-long infection and there is progressive formation of lung abscesses[33]. It appears that efficient signaling at earlier stage of infection with *R. equi* results in the pathogen gaining and maintaining a lead on the host immune responses. It is possible that a lack of effective expression of innate immune receptors such as PTX3 (described later) may underlie a failure to generate potent immune response against pathogens such as *R. equi*.

1.4 Innate Immune System

The innate immune system evolved as an early form of defense, providing protection for primitive multicellular organisms against infectious agents. As with the adaptive immune system, the innate immune system has both a cellular and a humoral arm[34]. The feature that distinguishes the innate immune system from the adaptive immune system is the presence of "germline-encoded receptors" in the innate immune system that recognize microbial pathogens[35] in a generally non-specific manner. These receptors, called pattern recognition receptors (PRRs) give the host organism the ability to recognize pathogen-associated molecular patterns (PAMPs)[35, 36]. The patterns that are targeted are most often molecular structures that are essential for a pathogen's survival and are likely to be evolutionarily conserved across phylogeny, making them an ideal target for the host's defense system[35]. PRRs are membrane-bound, cytoplasmic or secreted proteins, made up of many different molecular classes[35]. The humoral PRRs consist of collectins, ficolins and pentraxins[34]. Cellular PRRs include Toll-like receptors (TLRs), lectin receptors, G protein-coupled receptors, nucleotide-binding oligomerization domain (NOD)- and retinoic acid-inducible gene-1 (RIG)-like receptors, and the scavenger receptors[34, 37]. TLRs are central to the recognition of microbial molecules. The specificity of TLRs is demonstrated through examples such as recognition of LPS by TLR4[38] and lipoteichoic acid by TLR2[39, 40].

Gram-negative bacterial cell wall contains LPS, which is a potent inducer of inflammation and is recognized by TLR4[38, 41]. LPS is often used as an experimental model of ALI, as mentioned earlier. When LPS enters the host, the inflammatory response is initiated through binding to LPS-binding protein (LBP), CD14, myeloid differentiation protein 2 (MD-2)

and TLR4 (Figure 1.1). Membrane-bound or free LBP binds to LPS and catalyzes a reaction that transforms the polymers into monomers. The monomers are moved to CD14, which then transfers the LPS monomer to MD-2, resulting in the formation of LPS-MD-2-TLR4 trimer. A signaling cascade is activated and NF-κB initiates the transcription of specific genes, and proinflammatory cytokines are released[42].



[1]

Figure 1.1: TLR4 recognition of LPS is mediated by LPS-binding protein (LBP), CD-14 and MD-2, resulting in increased expression of proinflammatory cytokines.

The engagement of these receptors leads to activation of cell signaling pathways, nuclear translocation of molecules such as NF-κB and new transcription of inflammatory genes[42].

[1] Reprinted from Cytokine, Vol. 42, Yong-Chen Lu, Wen-Chen Yeh and Pamela S. Ohashi, LPS/TLR4 signal transduction pathway, 145-151, Copyright 2008, with permission from Elsevier.

Therefore, the PRR such as TLR4 and PTX3 are critical for the sensing of microbes and activation of immune response. Because of the topic of this thesis, I will restrict my review to only pentraxins (PTXs) and specifically, PTX3.

1.4.1 Neutrophils

1.4.1.1 Basic Origin

Neutrophils are white blood cells characterized by a multilobulated nucleus and a cytoplasm full of antimicrobial granules[43]. They are short-lived cells that are rapidly recruited to sites of inflammation within minutes after recognition of specific signals from danger-sensing cells such as macrophages and endothelial cells[44]. In healthy humans, the mean number of circulating neutrophils differs depending on ethnic decent. In Caucasian adults, there are 4.3-4.5x10^9 neutrophils/L and in African-American individuals, there are 3.5-3.8^9 neutrophils/ L[45]. In healthy horses, the percentage of white blood cells that are neutrophils is between 52-70%, correlating to a value of 2.9-8.5x10^9 neutrophils/L blood. In cattle, the percentage of neutrophils in total white blood cells is 15-33%, correlating to 0.6-4.0x10^9 neutrophils/L blood[46]. In inflammation, the number of leukocytes increases in the bloodstream and also in affected organs, such as the lung[47].

Neutrophils are produced in the bone marrow through a series of six developmental stages of stem cell differentiation to finally become mature neutrophils: first, the stem cell differentiates into a myeloblast, a relatively undifferentiated cell, followed by differentiation into promyelocytes, myelocytes, metamyelocytes, band cells, then finally the mature neutrophil is

released into the bloodstream where they circulate until they are recruited to inflammatory sites[43].

1.4.1.2 Neutrophil Granules

Each stage of neutrophil development differs in nuclear morphology and granule content[43]. The first granules appear during the promyelocyte phase of differentiation. They are positive for the detection of peroxidase[43], and are referred to as myeloperoxidase-positive granules, azurophil granules, or primary granules[48]. A second type of peroxidase-negative granule, called specific granules or secondary granules, appear at the myelocyte stage of differentiation[43]. Lastly, a third type of granule called gelatinase granules or tertiary granules[48], appear at the band cell and final neutrophil stages of maturation[49]. In addition to these granules, exocytic vesicles called secretory vesicles are present in mature neutrophils[49].

Although the contents of the different granules have been characterized, it has also been noted that there is some overlap of the proteins produced for each granule subset. For example, some proteins are shared between different granule types, such as lysozyme, while other proteins are used as a marker of a particular granule type[48]. Interestingly, it appears that each granule subtype provides unique function for the cell. Thus, certain granules are mobilized in a hierarchal manner at different stages of activation and migration[48]. For example, secretory vesicles are the first to be mobilized. Secretory vesicles are characterized by a membrane that is rich in receptors that fuses with the plasma membrane of the cell to enable the cell to integrate these receptors with the cell membrane[48]. Receptors found on secretory vesicles include β_2-integrin CD11b/CD18, complement receptor 1, formylmethionyl-leucyl-phenylalanine receptors,

LPS/lipoteichoic acid-receptor CD14, the Fc-gamma III receptor CD15 and the metalloprotease leukolysin[49]. Increasing the presence of adhesion receptors on the surface of neutrophils immediately after activation helps the neutrophil make contact with activated endothelium through binding of selectins[48]. Next, gelatinase granules are mobilized as the neutrophil begins its migration through the endothelium. The presence of matrix-degrading enzymes is thought to have a role in paving a pathway for the cell through basement membranes[49]. Specific granules that are full of antimicrobial peptides, such as lactoferrin, are more resistant to mobilization than gelatinase granules[49]. The last granules to be mobilized are the azurophil granules. Their primary function is to kill and inactivate microorganisms though reaction of myeloperoxidase (MPO) with hydrogen peroxide to form hypochlorous acid and other reactive oxygen species[49].

1.4.1.3. Recruitment, Migration, and Extravasation

The highly complex process of recruitment, migration and extravasation of neutrophils to the site of inflammation is well-characterized in organs such as liver but not in the lung. There are many receptors and mediators that are involved in the process and there are tissue-specific differences in the mechanism. A thorough description of the complexity of neutrophil biology is beyond the scope of this thesis. In summary, sentinel cells, such as macrophages, initiate neutrophil recruitment after sensing microbes or injury though release of chemokines and increased vascular permeability[44]. Activated endothelium increases its expression of adhesion receptors such as P-selectin, E-selectin and integrins[50]. P-selectin ligand 1 on the neutrophil membrane binds to P-selectin and E-selectin on the endothelium and enables the neutrophil to

slow its velocity in the bloodstream[50] and triggers mobilization of secretory vesicles[49]. Through binding, detachment and reattachment of these and other receptors, the neutrophil rolls along the surface of the endothelium[50]. Firm adhesion of the neutrophil to the endothelial wall is established through binding of integrins found on the surface of the neutrophil to their ligands on the surface of the endothelial cells[50]. The neutrophils then follow a chemotactic gradient and transmigrate between or through endothelial cells to exit the bloodstream and migrate into inflamed or damaged tissue[50].

1.4.1.4 Functions of Neutrophils

Once neutrophils have reached the site of inflammation, they release azurophil and specific granules[49], which contain proteolytic enzymes and antimicrobial proteins[51]. Certain stimuli, such as LPS, will prompt NADPH oxidase constituents to assemble and catalyze a series of chemical reactions collectively referred to as oxidative burst[51].

NADPH oxidase is an enzyme found in neutrophils among other phagocytes including eosinophils, monocytes and macrophages[52]. This enzyme catalyzes the reaction of oxygen and NADPH to produce superoxide (O_2^-)[52], a highly reactive oxygen species. In turn, superoxide forms hydrogen peroxide (H_2O_2) and reacts with MPO to form cytotoxic hypochlorous acid (HOCl)[52]. NADPH oxidase also participates in the formation of hydroxyl radicals and singlet oxygen, both of which are highly reactive species[52]. NADPH oxidase is inactive in resting neutrophils, but becomes functional upon activation of the cell[53].

Neutrophils are also efficient at microbial killing through NETosis, a unique form of neutrophil cell death[54]. This recently-discovered phenomenon results in the production of

neutrophil extracellular traps (NETs), composed of a fibrous DNA scaffold and proteins originating from primary and secondary granules[55]. This is an active process and is shown to be efficient at trapping Gram-negative and Gram-positive bacteria while proteases, such as neutrophil elastase that are associated with NETs, render the bacteria inert[55].

1.4.1.5 Fate of Neutrophils

When neutrophils reach the end of their lifespan, they will undergo apoptosis, necrosis or form NETs. Apoptosis, also known as "programmed cell death", is characterized by a set of well-known features: cell shrinkage, nuclear condensation, membrane blebbing, fragmentation into membrane bound apoptotic bodies, and changes to the surface of the cell membrane[56]. Apoptosis is a tightly regulated event that requires energy input[56]. Since neutrophils carry cytotoxic contents, it is important they are removed in a controlled manner and not to simply release their contents into healthy tissues. Neutrophils may undergo spontaneous apoptosis or pathogen-induced apoptosis. During the process of apoptosis, the plasma membrane remains intact[57] and prevents spillage of the intracellular contents. Signals are expressed on the cell membrane of neutrophils that alert macrophages to clear them from the environment by phagocytosis[57]. The clearance of neutrophils prevents further tissue damage and aids in resolution of inflammation and healing.

Necrosis occurs as a result of cell injury from toxins, ischemia or trauma[56]. A primary feature that sets necrosis apart from apoptosis is that necrosis occurs without the use of energy, where apoptosis requires energy in order to occur[56]. Other characteristics of necrosis include disruption of membranes and lysis of chromatin compared to the condensation of chromatin into

nuclear apoptotic bodies found in programmed cell death[56]. Whereas apoptosis is an organized removal of dead cells that aids in controlling unwanted inflammation, the process of necrosis usually results in increased inflammation[56]. Sometimes, when the number of apoptotic cells overwhelm the phagocytosis system, neutrophils may become necrotic and leak their inflammatory contents into the extracellular milieu. This process is referred to as bacterial secondary necrosis[58].

Alternatively, as mentioned above, neutrophils may undergo a form of cell death, called NETosis, that is unique from apoptosis and necrosis[54]. This form of cell death produces NETs and is known to have anti-microbial activity[55]. During the process of NETosis, the nucleus expands and becomes less lobulated, internal membranes disintegrate, and a network of DNA, chromatin and granule proteins emerge from the cell[54]. NETosis is dependent on activation by certain stimuli, such as IL-8, LPS, bacteria, fungi and platelets[59].

1.4.2 Pentraxins

Pentraxins are a superfamily of humoral pattern recognition receptors that are named for their pentameric molecular structure. Pentraxins can be divided into two main groups of molecules based on the length of their peptide sequences: short pentraxins and long pentraxins[34]. The members of the short pentraxins include C-reactive protein (CRP) and serum amyloid P component (SAP)[34]. Pentraxin 3 (PTX3), also called TNF-stimulated gene 14 (TSG-14), was the first long pentraxin identified followed by other long pentraxins including apexin (p50), neuronal pentraxin I (NP1; NPTX1), NP2 (Narp; NPII; NPTX2) and neuronal pentraxin receptor (NPR)[60]. All members of the pentraxin superfamily share a similar 200

amino acid sequence in the C-terminus of the protein, called the "pentraxin domain" with a series of 8 common amino acids, called the "pentraxin signature" (HxCxS/TWxS; where x is any amino acid)[34]. The pentraxin domain is a highly conserved sequence in the C-terminus of all pentraxins, found in lower vertebrates, arthropods, and mammals, but the N-terminus of the long pentraxins have revealed much diversity[34]. Pentraxin molecules can be divided into 5 main groups based on when they appeared in the evolutionary tree: short pentraxins, neuronal pentraxins, a group consisting of only pentraxin 3, pentraxin 4 and its orthologs, and finally the fifth group consisting of *Drosophila melanogaster* Swiss cheese protein[34].

1.4.2.1 Short Pentraxins

CRP was the first pentraxin to be identified as an acute phase protein in humans that reacted to a portion of pneumococci, called "fraction C"[61]. This protein has also been identified in other mammals and some species of fish[61, 62]. SAP is an acute phase protein in mice, and although it is present in humans, it does not react like an acute phase protein[63, 64]. Short pentraxins are produced almost exclusively by the liver[63, 65]. However, CRP is also produced in small quantities by lymphocytes, monocytes and macrophages[34]. The main inducer of CRP in humans is IL-6[66].

A characteristic structure of all the pentraxins is the arrangement of five or ten identical subunits in a symmetrical pentameric (or decameric) pattern[67]. SAP forms decamers of the protomer subunits, made up of two rings of pentamers, stacked face-to-face[68]. Each protomer consists of 204 amino acid residues[68]. CRP contains 187 amino acids in its sequence[69] and arranges in pentamers or sometimes is found in stacks to form decamers[70].

CRP and SAP are both found in humans and orthologs are found in other mammalian species[34]. They bind several ligands in a calcium-dependent manner[34, 62]. CRP binds microbes such as fungi, yeast and bacteria[34]. SAP also binds bacteria, such as *Streptococcus pyogens* and *Neisseria meningitidis,* viruses and microbial components, such as LPS[34]. Clinically, CRP is measured as a non-specific marker of inflammation and response to treatment[71].

The roles of CRP in the human host are diverse. CRP has a protective effect in some diseases, but is detrimental in others. CRP helps phagocytes recognize microbes through opsonization. For example, CRP increases the phagocytosis of several Gram-negative and Gram-positive bacteria, namely *Diplococcus pneumoniae, Staphylococcus aureus, Escherichia coli, Klebsiella aerogenes,* and *Aspergillus fumigatus*[34, 72]. Both CRP and SAP bind the classical complement component, C1q[34] and also opsonize apoptotic and necrotic cells, initiating their clearance by phagocytes[73]. Binding of CRP to apoptotic cells amplifies the activation of classical complement pathway[73], resulting in clearance of the cells in an anti-inflammatory manner. Although CRP can improve the health status of an individual in some states, CRP can cause adverse outcomes in others. In experimental acute myocardial infarction of the rat, human CRP exacerbates the extent of myocardial tissue damage[74]. SAP is observed in amyloid fibril deposits[75], a characteristic of Alzheimer's disease.

1.4.2.2 Pentraxin 3

Pentraxin 3 (PTX3), also called TNF-stimulated gene 14 (TSG-14), was the first long pentraxin identified. PTX3 was first discovered as a TNF-inducible gene in human

fibroblasts[76, 77], and at the same time, another laboratory identified PTX3 as a gene that is upregulated by human umbilical vein endothelial cells after stimulation with IL-1β[78]. The PTX3 protein is 381 amino acids long, has a carboxy-terminal sequence similar to the short pentraxins as well as the "pentraxin signature" mentioned earlier and exhibits two cysteines that are conserved in all pentraxins[77, 78]. The amino acid sequence of PTX3 in the amino-terminus is unlike any known proteins and is about twice the length of the short pentraxins[78]. PTX3 has one glycosylation site[77]. The glycosylated form of PTX3 has a molecular weight of ~42kD[77]. The human PTX3 gene has binding sites for proximal promoters such as Pu1, AP-1, NF-16-6, and NF-κB, which may have a role in LPS-stimulated production[79].

Unlike the short pentraxins that are produced almost exclusively in the liver, PTX3 is produced by a wide variety of cell types in response to inflammatory stimuli. IL-6, a major inducer of CRP and SAP, has little effect on the production of PTX3[78]. Several cell types have been tested in their ability to express PTX3 mRNA in resting and stimulated states. PTX3 mRNA is low or undetectable in unstimulated normal human monocytes and increased mRNA is detected after incubation with LPS, IL-1β and weakly by TNF-α[80]. The protein is further produced and released from the cells after stimulation[80]. Human endothelial cells produce PTX3 in response to certain stimuli, such as IL-1β[80] and TNF-α[81]. Myeloid dendritic cells are strong producers of PTX3 protein and mRNA in response to microbial components[82, 83].

There is consensus that PTX3 protein is found in mature neutrophils[84, 85]. However, opinions differ when it comes to mRNA in neutrophils. PTX3 mRNA was not found in mature human neutrophils in some reports[84], but other studies found mRNA in both unstimulated and stimulated neutrophils[85]. After stimulation with IL-8, PTX3 was increased in the supernatant

of human neutrophils[86]. Interestingly, the neutrophil precursors, promyelocytes and myelocytes/metamyelocytes express PTX3 mRNA which is further translated into the PTX3 protein[84]. In mature neutrophils, PTX3 protein is localized to the specific (secondary) granules[86, 87]. Since apoptosis is a controlled form of cell death where the membrane stays intact, it is not surprising that apoptotic neutrophils do not release PTX3[88]. Instead, during apoptosis of neutrophils, PTX3 is found on the surface of the cell membrane and increases phagocytosis by macrophages[88]. In NETosis, a unique form of neutrophil cell death, PTX3 is localized in extracellular networks of DNA and possibly promotes antimicrobial activity[86].

Similar to the short pentraxins, PTX3 is an acute-phase protein. The concentration of circulating PTX3 is low or undetectable in healthy humans and animals, but in the presence of pro-inflammatory molecules, the concentration drastically increases[89]. Studies of clinical cases have documented the elevation of PTX3 in the plasma of patients with severe inflammation, such as severe sepsis[90], acute myocardial infarction[91], heart failure[92], acute respiratory distress syndrome[93] and fever[94]. In many of these studies, a negative outcome is associated with persistently high levels of PTX3[90, 95]. In human patients with ulcerative colitis, PTX3 was found to be expressed in neutrophils, and to a lesser extent, in macrophages, of colon mucosal tissue. In ulcerative colitis, the severity of the disease correlated with increased PTX3 expression[86].

Similar results have been found in experimental inflammation in animals. For example, in ischemia-reperfusion injury in mice and different models of lung injury, PTX3 expression is increased in the lung and circulation and PTX3 deficiency is associated with more severe lung injury[81, 96-98]. Because PTX3 concentrations in BALF increased with increasing

concentrations of LPS challenge, it has been investigated as a potential biomarker of inflammatory states including acute lung injury[97].

Studies of PTX3 in domestic animals are scarce. There has been only one published study of PTX3 in horses suffering from recurrent airway obstruction (RAO)[99]. PTX3 was found in BALF macrophages and neutrophils from horses with RAO and healthy horses after exposure to dust[99]. In lung tissue from healthy and RAO-affected horses, immunohistochemistry revealed PTX3 in bronchial epithelial cells and inflammatory cells[99]. These data illustrate the activity of PTX3 in many forms of non-specific inflammation. Currently, the function of PTX3 in these diseases is not known. .

Both short and long pentraxins interact with components of the complement cascade. PTX3 binds to the globular head of C1q, a component of complement, and C1 complex consisting of C1q complexed with C1r and C1s[100]. This interaction occurs in the same manner as the binding of SAP and CRP with C1q[100]. Binding of C1 complex to PTX3 activates C4 and C3 downstream members of the classically-activated complement pathway[100]. Pre-incubation of apoptotic cells with PTX3 enhanced binding of C1q and C3[100]. Activation of C4 suggests that immobilized PTX3 is involved in activation of the classic complement pathway, but binding of PTX3 to C1q may inhibit activation of the classic complement pathway when in the fluid phase[100], suggesting PTX3 is a regulator for the activation of the complement pathway. PTX3 interacts with the globular head region of C1q[100]. TNF-α and bacterial endotoxin stimulate innate immune cells in inflamed tissues and fluids to release C1q and PTX3[101]. Upon activation of the complement, PTX3 aids in the clearance of microbes by facilitating recognition by phagocytes[102].

PTX3 also binds microbes such as bacteria, viruses, and fungi[37]. PTX3 binds to *Aspergillus fumigatus* conidia and increases their uptake by macrophages[103]. Neutrophils from bone marrow and peritoneum of $PTX3^{-/-}$ mice were unable to efficiently phagocytose conidia from *A. fumigatus* compared to cells from normal mice[84]. PTX3 recognizes and binds to viruses, with differences in efficiency depending on the virus[104].

PTX3 has been shown to play a role in the assembly of extracellular matrix and female fertility[105]. Female PTX3-deficient mice display a severe defect in fertility. However, heterozygous ($^{+/-}$) males and females and homozygous ($^{-/-}$) males are normal and fertile[106]. PTX3 localizes in the cumulus matrix and plays a crucial role in cumulus expansion[106].

Pentraxins are involved in the two main forms of cell death: apoptosis and necrosis[107]. Apoptosis is programmed cell death in which a stimulus causes activation of genes which produce enzymes that cause damage to the cell. The apoptotic cells are phagocytosed to prevent the release of the cell contents into the surrounding environment and to prevent damage in the neighboring tissue. Necrosis occurs in inflammation. In necrosis, cell contents are released, causing tissue damage surrounding the site of necrosis. C1q and PTX3 both bind to apoptotic cells in a dose-dependent manner, appearing to bind to different sites of the cells[108]. Regardless, they have very different effects on the phagocytosis of apoptotic cells. C1q increases ingestion of apoptotic cells by phagocytes, and PTX3 inhibits phagocytosis of apoptotic cells by dendritic cells and macrophages[108].

The roles of PTX3 in normal and diseased states are diverse. This is not an exhaustive review of every known detail of PTX3, but highlights some of the main findings from the literature that are relevant to this study. Because of the acute nature of PTX3 in inflammatory

states, and the fact it is released locally, and not only in hepatocytes, like the short pentraxins, there is likely a benefit for the expression of this protein locally, to the host. Furthermore, since neutrophils are a hallmark of lung inflammation and seem to be a significant, mobile producer of PTX3 I sought to characterize PTX3 in lung inflammation of humans and domestic species as well as neutrophils.

CHAPTER 2: HYPOTHESES AND OBJECTIVES

2.1 Hypotheses

1. PTX3 is expressed in the lungs and its expression will increase in inflamed lung tissue compared to normal lung tissue.
2. PTX3 is stored in neutrophils and is released upon activation with LPS.

2.2 Objectives

1. To characterize and compare the expression of PTX3 in normal and inflamed lungs of human, pig, horse and cattle.
2. To investigate the expression of PTX3 in normal and activated neutrophils.

2.3 Rationale

Cattle affected by BRD may be treated with broad spectrum antibiotics, but often do not regain the same healthy status as before the animal was infected because of the reduced feed conversion, weight gain and economic return. Vaccines have also been developed, but do not protect all animals. The most favorable scenario is prevention of the disease. Thus, defense mechanisms of the respiratory tract are being investigated.

The mechanisms underlying acute inflammatory diseases such as those of the lung in animals and humans are complex. The respiratory diseases of domestic animals such as cattle, horses and pigs inflict billions of dollars in damage to the respective industries. To develop new and effective therapies against pulmonary inflammatory diseases, we need to develop a more

detailed understanding of the mechanisms. The role of innate immune cells such as neutrophils and innate immune proteins such as PTX3 is not fully understood. Recently, PTX3 has been identified as an important receptor and regulator of various aspects of inflammation. Because PTX3 is expressed in neutrophils, it may also be a regulator of the biology of neutrophils, which have deep influence on the process of inflammation in the lung. Therefore, it is important to understand the expression of PTX3 in normal and inflamed lungs and as well as normal and activated neutrophils of domestic animal species. Because PTX3 is released by activated neutrophils and other cells, it may also be used as a marker of inflammatory disease in the host.

CHAPTER 3: PENTRAXIN 3 IN NORMAL AND INFLAMED LUNGS

3.1 Abstract

Bacterial lung diseases cause significant mortality and morbidity, causing enormous economic damage to the animal industry and cost billions of dollars in human health care. Few options exist for treatment of these diseases. Increasing our understanding of the mechanisms involved in the immune response will aid in the development of treatments for respiratory disease. The innate immune system plays a critical role in the initiation of immune response in the lung. Pattern recognition receptors (PRRs) are programmed to recognize microbial structures unique to pathogens and mount an immune response. Pentraxin 3 (PTX3) is a PRR that is produced at sites of inflammation by many cell types upon stimulation by proinflammatory cytokines, and agonists such as endotoxins. PTX3 recognizes and binds to many pathogens, activates the complement cascade, and has a role in the clearance of apoptotic and necrotic cells. Because there are very few data on the expression of PTX3 in the lungs, I examined PTX3 expression in normal and inflamed lungs of the horse, pig, human, and cattle using light and electron microscopic immunochemistry and Western blot. Through Western blotting, I show the presence of PTX3 in lungs of calves, pigs and horses, with a trend toward increased level of protein in inflamed lungs. Using immunohistochemistry, I show PTX3 staining in bronchial epithelial cells and vascular endothelium in normal and inflamed lungs. Alveolar macrophages and inflammatory cells recruited into the lungs of bacteria-infected animals stain intensely for PTX3. Immuno-gold electron microscopy on horse and calf lungs showed PTX3 in the nuclei, cytoplasm, and vesicular organelles of alveolar macrophages, endothelial cells and pulmonary

intravascular macrophages (PIMs). These data show PTX3 is expressed in normal lungs and its expression is altered in inflamed lungs.

3.2 Introduction

Respiratory diseases are some of the leading causes of morbidity and mortality in animals and humans. Bovine respiratory disease (BRD) and secondary bacterial infections are among the leading causes of economic loss in the livestock industry[3, 4]. As summarized in *Life and Breath*, the 2007 Respiratory Disease in Canada report, 6.5% of total human healthcare costs in Canada were related to respiratory illness (excluding lung cancer), which translates to a cost of $5.70 billion in direct costs of health care, such as hospitalization, physician consultations, research and medication, as well as an additional $6.72 billion for indirect expenses associated with disability and mortality[109].

The respiratory system is particularly susceptible to infection because it is in constant contact with the external environment through inhalation of air, which may also carry dust, bacteria and viruses. The lung may also be subjected to inflammatory agents through the circulatory system in conditions such as ischemia or sepsis[10, 110]. In addition to the entry of pathogens from the external environment, the upper respiratory tract is inhabited by a multitude of flora. For example, *Mannheimia hemolytica* is prominent in cattle and sheep[24], causing a disease called shipping fever, *Pasturella multocida* is found in cats, cattle and pigs, and *Bordetella bronchiseptica* is common in dogs and pigs[111]. *Pasteurella multocida* and *Haemophilus somnus* are other common causes of secondary bacterial pneumonia in cattle[4]. Normal inhabitants of the equine respiratory tract include *Streptococcus equi* subsp.

zooepidemicus, Pasteurella spp.*, Escherichia coli, Actinomyces* spp., and *Streptococcus* spp.[25]. *Streptococcus equi zooepidemicus* is the most common opportunistic bacterial invader of the equine lung[8] and *S. equi* causes strangles, a highly contagious upper respiratory tract infection in horses[9]. To counter these inflammatory threats, the lung has many protective cellular and molecular components.

When inflammation and infection occur in the respiratory system, the first line of defense is the innate immune system. The innate immune system is comprised of various proteins, such as Toll-like receptors, and cells, such as neutrophils and macrophages, and identifies non-self through recognition of specific molecular patterns found on foreign microbes, such as the molecule lipopolysaccharide (LPS) by pattern recognition receptors (PRRs)[35]. Pentraxin 3 (PTX3) is a PRR that is produced by several cellular sources upon stimulation by proinflammatory cytokines and agonists such as endotoxins. Endothelial cells, fibroblasts, monocytes, epithelial cells, and myeloid dendritic cells are among the many cells known to produce PTX3[77, 80, 82, 112, 113]. PTX3 first appeared in the evolutionary tree with the emergence of vertebrates and evolved directly from the first ancestor of the pentraxin superfamily, related to C-reactive protein and serum amyloid P component[34]. PTX3 is an acute phase protein[77, 78], with circulating low or undetectable levels in healthy individuals and rapid upregulation of plasma concentration in inflammatory states, such as sepsis[90], acute myocardial infarction[91] and acute respiratory distress syndrome[93]. PTX3 recognizes and binds to many pathogens[114], activates the complement cascade[100], and has a role in the clearance of apoptotic and necrotic cells[115]. PTX3 knockout mice suffered more severe LPS-induced lung injury, increased levels of pro-inflammatory cytokines in the lung and significantly

higher neutrophil infiltration into the lungs compared to wild-type controls[116]. PTX3-overexpressing transgenic mice were found to have increased survival following endotoxemia as well as cecal ligation and puncture-induced sepsis[117]. Interestingly, overexpression of PTX3 was found to be detrimental in survival of mice suffering from ischemia and reperfusion injury[118]. Collectively, these data show that PTX3 has important roles in host immune responses.

Currently, our knowledge of the molecular mechanisms that come into play during acute lung inflammation is incomplete. There are few options in terms of therapeutics for humans and livestock suffering from severe lung inflammation. Thus, there is a need to further characterize and understand the basic molecular biology of the lung in normal and inflamed states. To date, there are few data on the expression of PTX3 in pigs, foals and cattle, and none in the lungs of these species. Only one study has been published that investigates the expression of PTX3 in lungs of horses suffering from recurring airway obstruction[99]. There also is limited information on the expression of PTX3 in normal human lungs[103, 119] and there are no studies characterizing the expression of PTX3 in lungs from septic patients. Since PTX3 has a role in immune responses in rodent and human species, the aim of this study is to characterize comparative expression of PTX3 in normal and inflamed horse, foal, cattle, pig, and human lungs. I hypothesize that expression of PTX3 in inflamed lung tissue will be increased in comparison to normal lung tissue.

3.3 Materials and Methods

3.3.1 Lung Tissues

Calf lung tissues for immunohistochemistry were obtained during a previous student project in our lab[120]. The study involved infection of Holsein-Friesian calves (N=5; male; 4-6 weeks of age) with intratracheally-administered *M. hemolytica* (total dose $20x10^9$ *M. hemolytica*) to investigate the respiratory effects of this disease. These calves were anesthetized with xylazine (0.1 mg/kg intramuscular, Bayer) prior to *M. hemolytica* administration. Control lung tissues for immunohistochemistry were from the calves that had been administered normal saline, intravenously (N=5). Calves were euthanized with an overdose of pentobarbital sodium 24 hours after *M. hemolytica* infection. Separate calf tissues were obtained for Western blot.

Normal (N=5) and inflamed calf lung (N=5) tissue for Western blot was obtained from a study conducted by Dr. John Ellis[121]. This study involved neonatal Holstein calves infected with bovine respiratory syncytial virus (BSRV). The normal tissues were obtained from the same animal as the inflamed tissue by using lesions for the "inflamed" group and non-lesioned lung for the "normal" group. Each calf was inoculated on day 0 with ~10^5 pfu BRSV, using a nebulizer (Ultra- Neb 99, Devilbiss, Somerset, PA) equipped with a face mask. The BSRV was isolated from a calf with severe respiratory tract disease.

Inflamed pig lung was obtained from Dr. John Harding's laboratory. Pigs were infected with swine influenza and were euthanized between 24 and 96 hours post-infection (N=6; male; 6 weeks of age). On experimental day 0, all pigs were intratracheally inoculated with influenza A/ swine/Texas/4199-2/98 (H3N2)TX98 while anesthetized with a single intramuscular dose (up to 20 mg/kg) of ketamine (Ketalean®, Bimeda-MTC, Cambridge, ON, Canada) and 2 mg/kg

xylazine (Rompun®, Bayer HealthCare, Toronto, ON, Canada) after sedation with a single intramuscular dose (0.3 ml) of azaperone (Stresnil®, Merial, Baie D'Urfé, QC, Canada). Control pigs (N=5; female; 13 weeks of age) were euthanized via captive bolt by Dr. John Harding to investigate a gastrointestinal tract issue. Control animals did not undergo anesthesia.

Control and *E. coli* lipopolysaccharide-treated horse lung tissues were obtained during studies previously conducted in the laboratory[122, 123]. Quarter horses were administered 500 mL of endotoxin-free physiological saline intravenously. Forty-eight hours later, the endotoxin-treated horses (N=4; 1-2 years of age) were intravenously administered E. coli LPS (50 ng/kg body weight; B0128:12; Sigma Co, St. Louis, USA) in physiological saline over 20 min. Control horses (N=6; 1-2 years of age) were administered equal volumes of endotoxin-free saline instead of LPS treatment. Horses were euthanized with pentobarbital sodium (Euthenol®, Bimeda-MTS Animal Health Inc., Canada, 10 mL/50 kg body weight intravenously) up to two hours after LPS administration and 48 hours after saline administration for control horses.

Mixed breed draft-type foals up to 7-weeks of age were used in a study previously conducted in the laboratory to investigate a vaccine against *R. equi*[124]. The positive control foals from this study were used as the inflamed group for the present study (N=7). On day 1, foals were between 0-6 days of age and 10mL saline was delivered intramuscularly. On day 28, foals were given intrabronchial challenge with *R. equi* ($5x10^6$ CFU/ml) and were euthanized on day 49. Lung tissues from control foals (N=5) were obtained during a study conducted in our lab in the summer of 2012 (not yet published). The control foals were also considered a mixed draft-type breed. A maturity assessment[125] was performed at foaling and a physical exam including a complete blood count was performed by a large animal internist just prior to euthanasia via

pentobarbital overdose. The left lung was fixed *in situ* within 45 minutes of death. All control animals were 30 days of age.

Human lungs embedded in paraffin were obtained from the department of Pathology, the College of Medicine at the University of Saskatchewan. Lungs from patients who died from sepsis (N=5) and those died from non-respiratory causes (N=5) were examined in this study. The post-mortem interval for collection of the tissue ranges within 24-36 hours after death was pronounced. Patients were between 40 and 61 years of age with the exception of one sepsis case from a 6 month old female and a control case from a 20 year old male.

Tissues obtained for Western blot were harvested immediately post-euthanasia and frozen at -80°C until needed. Tissues obtained for staining techniques were processed as described below.

3.3.2 Tissue Fixation/Processing

The details of tissue fixation and embedding are provided in papers cited in respective sections for each of the animal species. All lung tissues except those from humans were placed in 4% paraformaldehyde overnight at 4°C. Human lung tissues were fixed in 10% buffered formaldehyde. Tissues were processed and embedded in paraffin prior to sectioning to 5µm and mounting on glass slides coated with poly-L-lysine for immunohistochemistry. Horse and calf lungs for immuno-electron microscopy were fixed using a suitable protocol as described previously[120, 122, 123]. Hematoxylin and eosin staining was performed to illustrate the level of inflammation in the different species.

3.3.3 Immunohistochemistry

The protocol for immunohistochemistry was previously standardized in our lab[120, 123, 126]. Tissue sections were deparaffinized in xylene for 30 minutes and rehydrated by placing for ten minutes in each of decreasing graded concentrations of ethanol (100%, 95%, 70%, 50%) followed by five minutes in deionized water and five minutes in PBS. Using a hydrophobic barrier pen, a circle was traced onto the slide to outline the tissue sections to contain the reagents within a defined space. The slides were placed into a humidified chamber and a solution of 0.5% H_2O_2 in methanol was placed on the tissue for 20 minutes in the dark to neutralize endogenous peroxidase. Slides were then placed into deionized water for five minutes followed by PBS for five minutes. The slides were returned to the humidified chamber and pepsin from porcine gastric mucosa (2mg/mL in 0.01N HCl), warmed to 37°C, was pipetted onto the tissue sections and left for 60 minutes in the dark. Blocking was achieved with 1% bovine serum albumin (BSA) in PBS for 30 minutes in the humidified chamber. Mouse anti-human PTX3 monoclonal antibody (PP-PPJ0069-00; R&D Systems, Minneapolis, MN, USA) was prepared by diluting to the appropriate concentration in 1% BSA and was pipetted onto the tissue sections. The concentrations used were as follows: 1:40 for human and pig lung tissue, 1:25 for foal, horse, and calf lung tissue. For tissue sections used as a negative control (secondary antibody only), 1% BSA was pipetted onto the tissue instead of primary antibody. For tissue sections used as a positive control, polyclonal rabbit anti-human von Willebrand Factor (vWF) antibody (P0448, Dako, Burlington , ON, Canada) was pipetted onto the tissue sections at a concentration of 1:100. vWF was used as a positive control because it is well-established that it is found in Weibel Palade bodies of endothelium. Primary antibody or BSA was left on the tissue overnight at 4°C.

Slides were then taken out of the humidified chamber and placed in three separate containers of PBS for five minutes in each container to wash excess primary antibody followed by incubation of sections with either polyclonal goat anti-mouse IgG's/HRP (1:100, P0447, Dako, Burlington, ON, Canada) or polyclonal goat anti-rabbit IgG/HRP (1:1000, Ab6721, Abcam, Toronto, ON, Canada) for 30 minutes. The slides were washed 3x5 minutes in PBS. Colour was developed using VECTOR VIP Peroxidase Substrate Kit (VECTOR Laboratories, Burlington, ON, Canada) for a period of 30 seconds to 2 minutes, depending upon the species. Following colour development, the slides were washed in gently running deionized water for five minutes and counter-stained with methyl green for 1 second to 5 minutes, washed in deionized water, quickly dipped in acetic acid (0.05% in acetone) four times, dehydrated in one minute in each of ascending concentrations of ethanol (50%, 70%, 95% and 100%) and xylene for 4 minutes, and coverslips were mounted. I also performed isotype-matched controls for each species by substituting the primary antibody with mouse IgG2a isotype control (R&D Systems).

3.3.4 Immuno-Histochemical Grading: Alveolar septum, airway epithelium, vascular endothelium and alveolar macrophages were assigned a grade between 0 and 3 to indicate the intensity of PTX3 staining. A grade of 0 indicated that staining was absent, 1 indicated low intensity staining, 2 indicated medium intensity staining, and 3 indicated high intensity staining. Ten fields of view for each animal were graded and a mean grade was calculated for each category for each species.

3.3.5 Immuno-Gold Electron Microscopy

Calf and horse lung tissues were stained for immuno-gold electron microscopy as previously described[120, 122, 123]. Briefly, tissues were sectioned to 90 nm and mounted on grids. Blocking of non-specific binding was achieved with goat/Tris blocking buffer for 3 x 10 minutes then incubated in mouse anti-human PTX3 monoclonal antibody (PP-PPJ0069-00; R&D Systems, Minneapolis, MN, USA) at a concentration of 1:10, diluted in blocking buffer for 1 hour at room temperature. The omission of primary antibody from the protocol served as a negative control. The grids were washed by passing each grid through a series of drops of Tris-buffered saline (TBS) then incubated in goat anti-mouse IgG (H+L) 15nm gold-conjugated secondary antibody (British Biocell International, Cardiff, UK) at a concentration of 1:100 for 1 hour, 20 minutes at room temperature. The grids were rinsed in TBS for 3 x 5 minutes and washed in water for 4 x 3 minutes. Staining of the grids was achieved placing them on 2% aqueous uranyl acetate with Triton X-100 for 20 minutes. Grids were washed 4 x 4 minutes in distilled water then placed in Renold's lead citrate for 10 minutes and quickly dipped in water then washed 5 x 4 minutes in water. Samples were allowed to dry and were examined at 120kV in the transmission electron microscope.

3.3.6 PTX3 Western Blots

Lung tissue (~0.05g) was placed in Fast Prep tubes with 1 mL T-PER tissue protein extraction reagent (Fisher Scientific, Canada) containing protease inhibitors (05892791001; Roche, Indianapolis, IN, USA) along with four stainless steel balls (7mm diameter; MP Biomedicals, Canada). The tissue was homogenized using Retsch Mixer Mill (MM 400) at a

frequency of 30 Hz for 8 minutes. The resulting homogenate was centrifuged at 10,000*g* for 10 minutes and protein content of the homogenate was quantified using the Bio-Rad DC Protein Assay (Bio-Rad, Mississauga, ON, Canada) according to the manufacturer's instructions.

SDS PAGE was performed according to the following protocol. 370µg of total protein from each sample was loaded into 12% gel after boiling the protein in 2x SDS loading buffer (1:1) for 5 minutes to denature the protein. Gel electrophoresis was performed at 120V. The gel was collected and arranged into a Western blot sandwich with nitrocellulose or PVDF membrane for protein transfer. The transfer of the gel to the membrane was performed using 100V for one hour. Following transfer, the membrane was incubated in blocking buffer (5% skim milk solution in PBS with 0.05% Tween-20) overnight at 4°C. For calf and pig lung tissue, mouse anti-human PTX3 antibody (R&D Systems) was diluted in blocking buffer to a final concentration of 1:1000. For horse tissue, rat anti-human PTX3 antibody (MNB1; Axxora, Enzo Life Sciences, Brockville, ON, Canada) was used at the same dilution factor. The membrane was incubated in the diluted antibody at room temperature for 1 hour on a rocking platform. Membranes were washed in PBS-T, then incubated in secondary polyclonal goat anti-mouse IgG/ HRP (Abcam) or polyclonal rabbit anti-rat IgG/HRP (P0450; Dako), diluted in blocking buffer to a final concentration of 1:1000 at room temperature for one hour on a rocking platform. Protein was detected by exposing X-ray film to membranes incubated in Amersham ECL Western Blotting Detection Reagents (RPN2108, GE Life Sciences, QC, Canada) as per manufacturer's instructions. Membranes were stripped using Blot Restore Membrane Rejuvenation kit (Millipore, Canada) according to manufacturer's instructions and detection protocol was repeated to ensure compete removal of antibodies. The membranes were then probed with anti-beta-actin

(1:5000; Ab8227; Abcam, Toronto, ON, Canada) and goat anti-rabbit/HRP (1:5000; Ab6721; Abcam, Toronto, ON, Canada). Densitometry was performed using ImageJ software (National Institute of Health) as described elsewhere[127]. Using ImageJ, each band was selected using the gel lane selecting tool. The density of each band was shown for the bands by selecting the "plot lanes" command and the area under the curve for the band was selected. Lane 1 was used as a reference point to which the relative change of the other lanes was compared (e.g. density of lane 2 was divided by density of lane 1). This process was calculated for PTX3, then β-actin, giving ratios referred to as "relative densities". Then, the relative density of PTX3 was divided by the relative density of β-actin for the same lane to calculate the ratio for each lane compared to β-actin, the loading control. Western blot was performed in triplicate for calf lung (N=5 for each healthy and inflamed) and once for pig lung (N=4 for each healthy and inflamed).

3.3.7 Statistics

The relative densities of normal and inflamed lungs were compared for each species using the Mann Whitney U test (GraphPad Prism, Version 5.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). The relative densities are reported as the mean +/- standard error. An asterisk denotes the mean of the inflamed group is different than the mean of the healthy group with $P<0.05$.

3.4 Results

3.4.1 Western blot in normal and inflamed lungs from horse, cow and pig.

I performed Western blot to detect PTX3 in the lungs of cattle, pig and horse as well as differences in expression of PTX3 in normal and inflamed lungs of cattle and pig. Bands were identified at 42 kD in calf (Figure 3.1A) , pig (Figure 3.1B), and horse lung (Figure 3.1C) homogenates. The PTX3 expression between the normal and the inflamed lungs was compared using densitometry against beta-actin, which was used a loading control (Figure 3.1D). The data showed an increase ($P<0.05$) in the amount of PTX3 in inflamed lungs from pigs and calves.

3.4.2 Hematoxylin and eosin staining in normal and inflamed lungs

Histopathology on hematoxylin and eosin-stained lung sections was done to confirm presence of signs of inflammation in lungs from foals, horses, calves, pigs and humans (Figure 3.2A-E). Because of the differences in the challenges used to induce inflammation, there were differences in pathology observed in the lungs. While low dose treatment with LPS caused minimal inflammation in lungs of the horse, *R. equi* challenge in the foals caused formation of granulomatous inflammation as well as many inflammatory cells in airways. Lungs from calves challenged with *M. hemolytica* caused thickening of alveolar septa and massive recruitment of inflammatory cells. Septic lungs from humans also showed numerous cells in alveoli and peribronchial areas compared to autopsied lungs from non-septic humans.

3.4.3 PTX3 staining in normal and inflamed lungs

Controls were implemented to ensure the validity of our IHC protocol. For tissue sections used as a negative control, omission of primary antibody resulted in lack of antibody staining (Figure 3.3A). Lung tissues stained for von Willebrand Factor (vWF) protein, which is present in Weibel-Palade bodies of endothelial cells, showed staining along the blood vessel endothelium but not on epithelium (Figure 3.3A). I also performed isotype-matched controls for each species by substituting the primary antibody with the appropriate isotype control.

Staining of lung sections (Figure 3.3B, D, F, H, J) with a PTX3 antibody showed staining in the alveolar septum, bronchial epithelium, vascular endothelium and inflammatory cells, such as neutrophils and macrophages. The bronchiolar epithelial cells have staining throughout their cytoplasm in normal animals, but after treatment with an inflammatory stimulus, the staining for PTX3 is most intense at the apical surface of the airway and very little at the base of the cells where they meet the basal membrane. There is an absence of staining in some of these cells. In addition, the alveolar septum shows a lesser degree of staining in inflamed animals compared to control. The vascular endothelium is positive in normal animals. The expression of PTX3 appears to decrease in vascular endothelium of inflamed lungs, especially in the calf tissues analyzed (Figure 3.3F).

3.4.4 Immuno-gold electron microscopy in normal and inflamed horse and calf lungs

Immuno-gold electron microscopy showed the presence of PTX3 in normal and inflamed lungs of horses and calves. Sections from normal horse lungs showed PTX3 staining in pulmonary intravascular macrophages (PIMs; Figure 3.4A-B), neutrophils (Figure 3.4C),

alveolar macrophage (Figure 3.4D), and the airway epithelium (Figure 3.4E). PIMs in control calves (Figure 3.4F) showed PTX3 staining which appeared to be lower than that observed in PIMs of calves challenged with M. hemolytica (Figure 3.4G). Similarly, PTX3 was found in monocytes (Figure 3.4H) and neutrophils (Figure 3.4I) in the lungs of calves infected with *M. hemolytica*. I also observed intense staining for PTX3 in platelets in inflamed lungs of calves (Figure 3.4J). The PTX3 staining was localized in the cytoplasm and nuclei of all of the inflammatory cells observed in this study. In addition to the inflammatory cells, capillary endothelial cells also showed expression of PTX3.

Figure 3.1A: Western blot of PTX3 from calf lung homogenates.

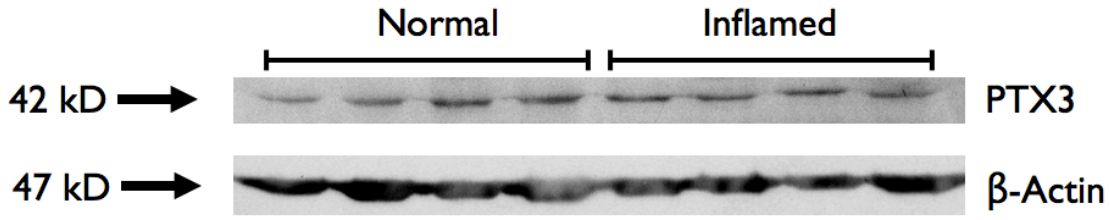


Figure 3.1B: Western blot of PTX3 from pig lung homogenates.

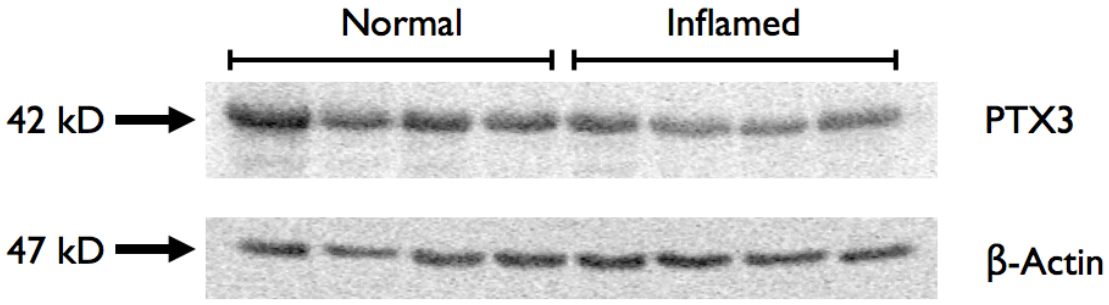


Figure 3.1C: Western blot of PTX3 from horse lung homogenates.

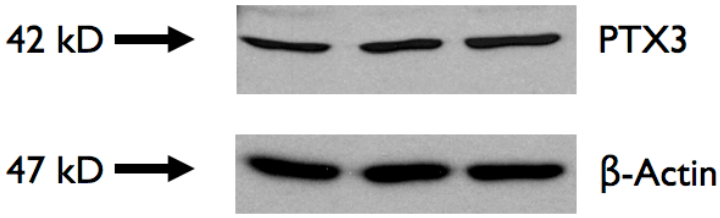


Figure 3.1D: Density of PTX3 relative to β-Actin for calf and pig lung Western blots.

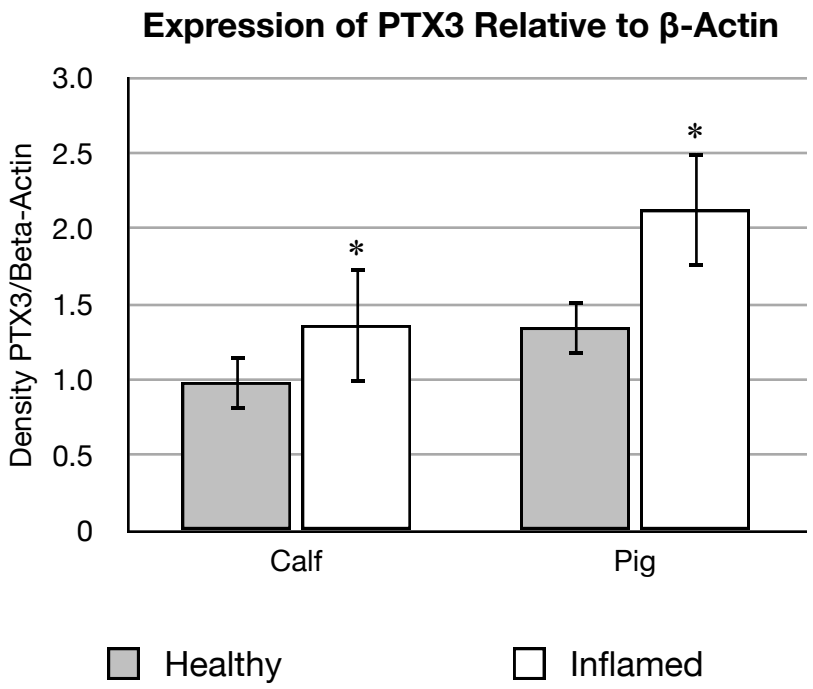


Figure 3.1: PTX3 Western blots in calf, pig, and horse lung homogenates. Western blot revealed bands at 42kD when pig, calf and horse lung tissue homogenates were probed for PTX3 (A, B, and C respectively). Only normal horse lung tissue was analyzed (C). Inflamed calf and pig lung tissue showed increased expression of PTX3 compared to control after normalizing data to β-actin loading control (D). Data are expressed as +/- standard error. Western blot was performed in triplicate for calf lung (N=5 for each healthy and inflamed) and once for pig lung (N=4 for each healthy and inflamed). The relative densities of normal and inflamed lungs were compared for each species using the Mann Whitney U test (GraphPad Prism, Version 5.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). The relative densities are reported as the mean +/- standard error. An asterisk denotes the mean of the inflamed group is different than the mean of the healthy group with $P<0.05$.

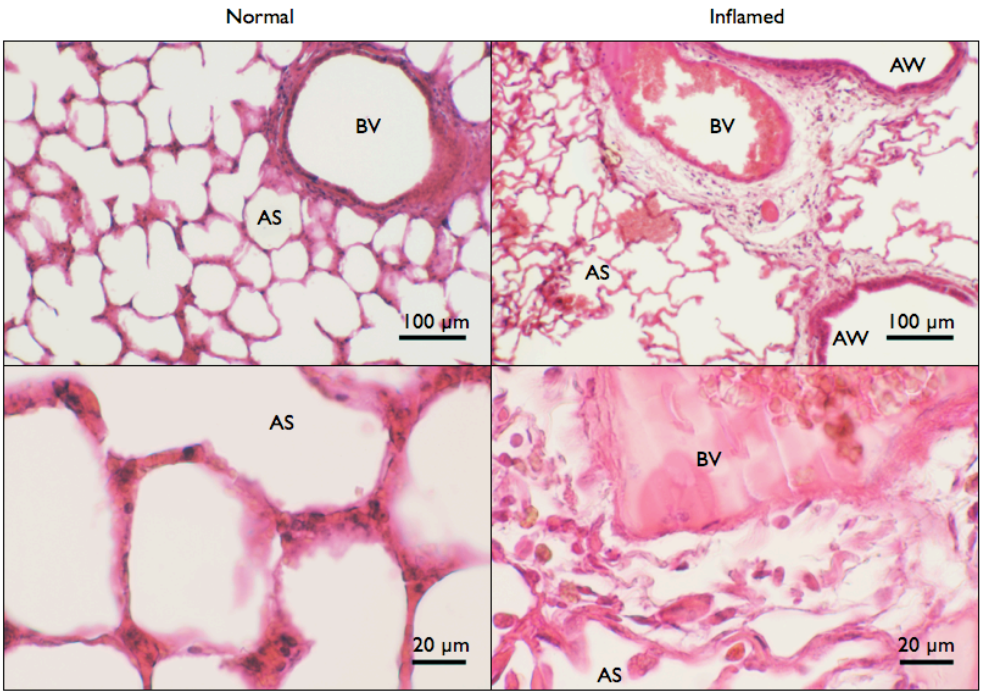


Figure 3.2A: H&E staining of horse lung. The lung sections from normal horse show normal alveolar septa and alveolar spaces compared to the inflamed lungs that show congestion and dilation of spaces around blood vessels (BV) and airways (AW).

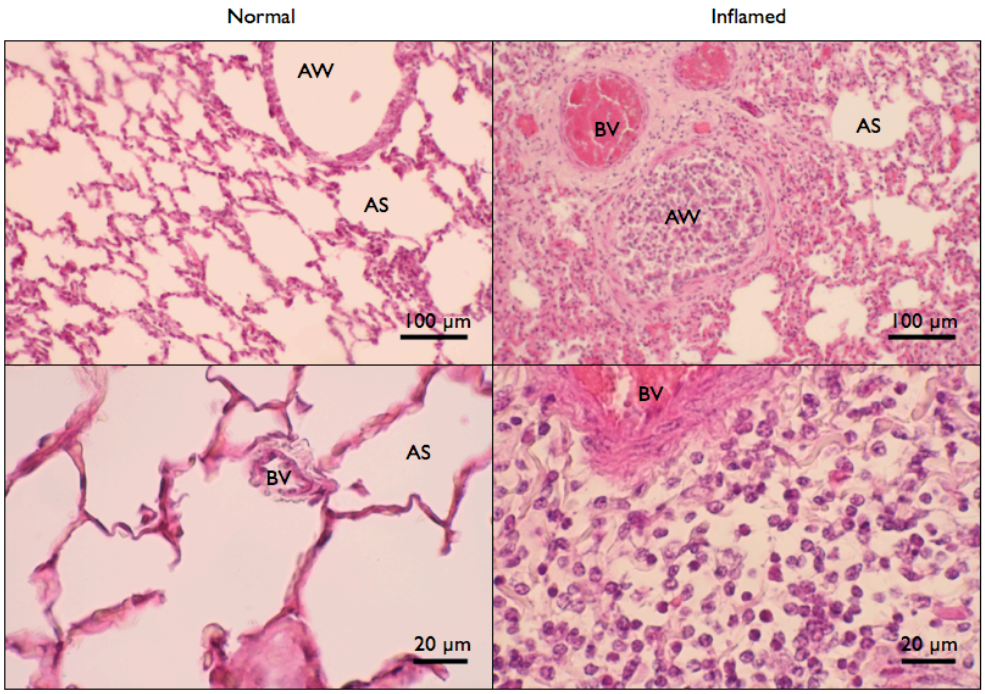


Figure 3.2B: H&E staining of foal lung. In comparison to the normal appearance of alveolar septa and alveolar spaces (AS), the inflamed airways (AW) are plugged with inflammatory cells and blood vessels (BV) are congested and surrounded by inflammatory cells.

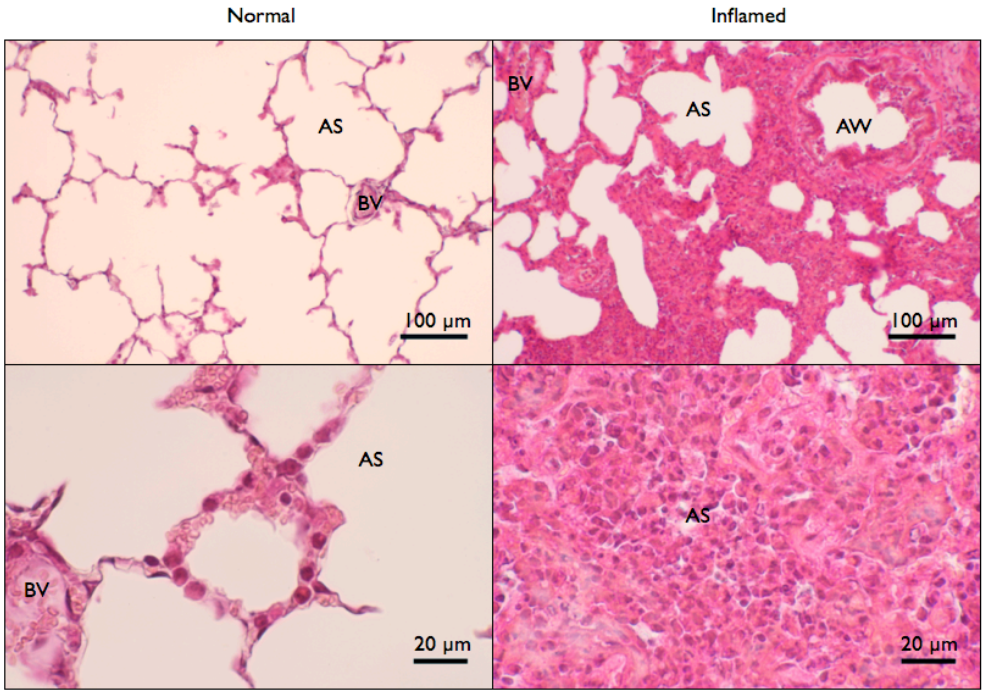


Figure 3.2 C: H&E staining of calf lung. Normal calf lungs show normal histology with thin alveolar septa and clear alveolar spaces (AS) along with presence of many septal cells which may be pulmonary intravascular macrophages (arrows). The lungs from M. hemolytica challenged calves are heavily inflamed as indicated by thickened septa, migration of inflammatory cells into alveolar spaces (AS) and infiltration of cells around airways (AW).

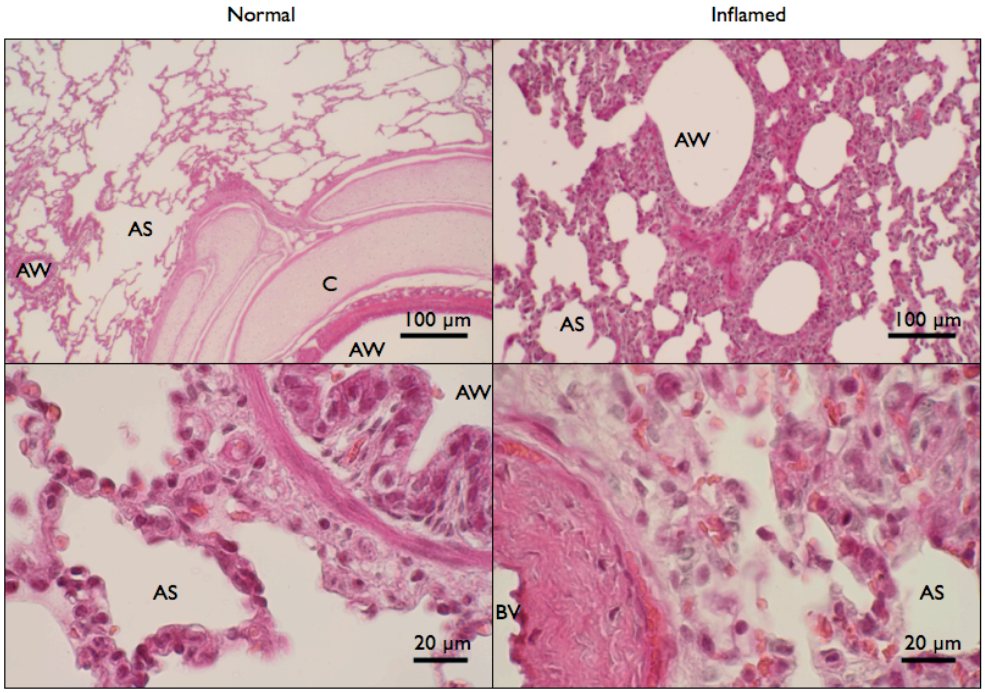


Figure 3.2D: H&E staining of pig lung. While the normal pig lung shows normal histology of the lung with clear alveolar spaces (AS) and lack of inflammation in airways (AW) surrounded by cartilages (C), the lungs from infected pigs are showing thickened alveolar septa and infiltrates around airways (AW) and blood vessels (BV).

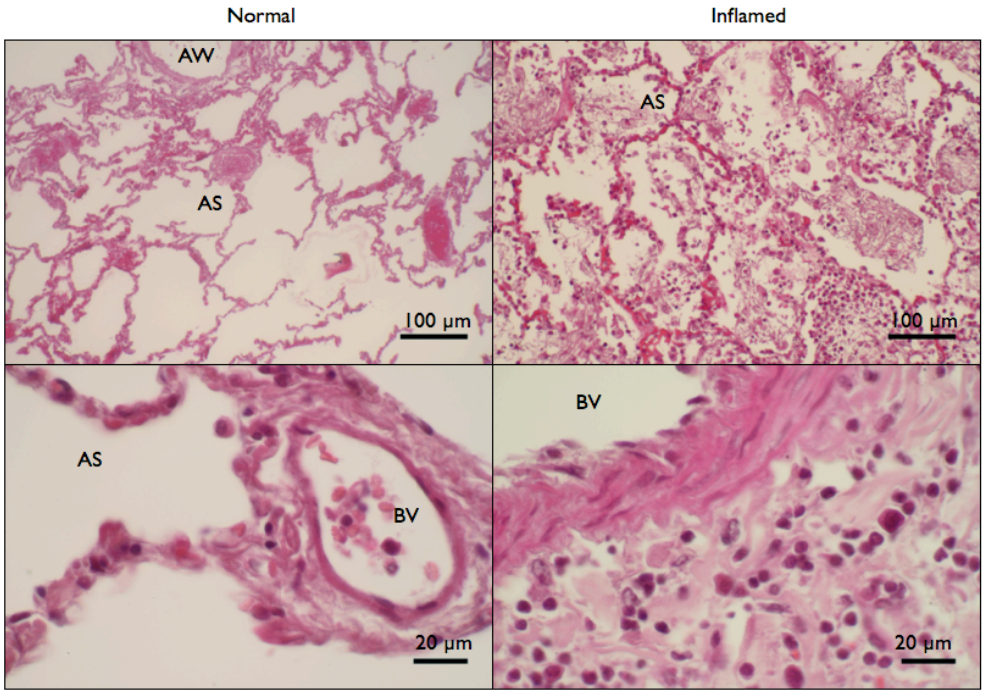


Figure 3.2E: H&E staining of human lung. The normal human lungs are not inflamed while those from septic patients show accumulation of inflammatory cells in alveolar spaces and around the blood vessels (BV).

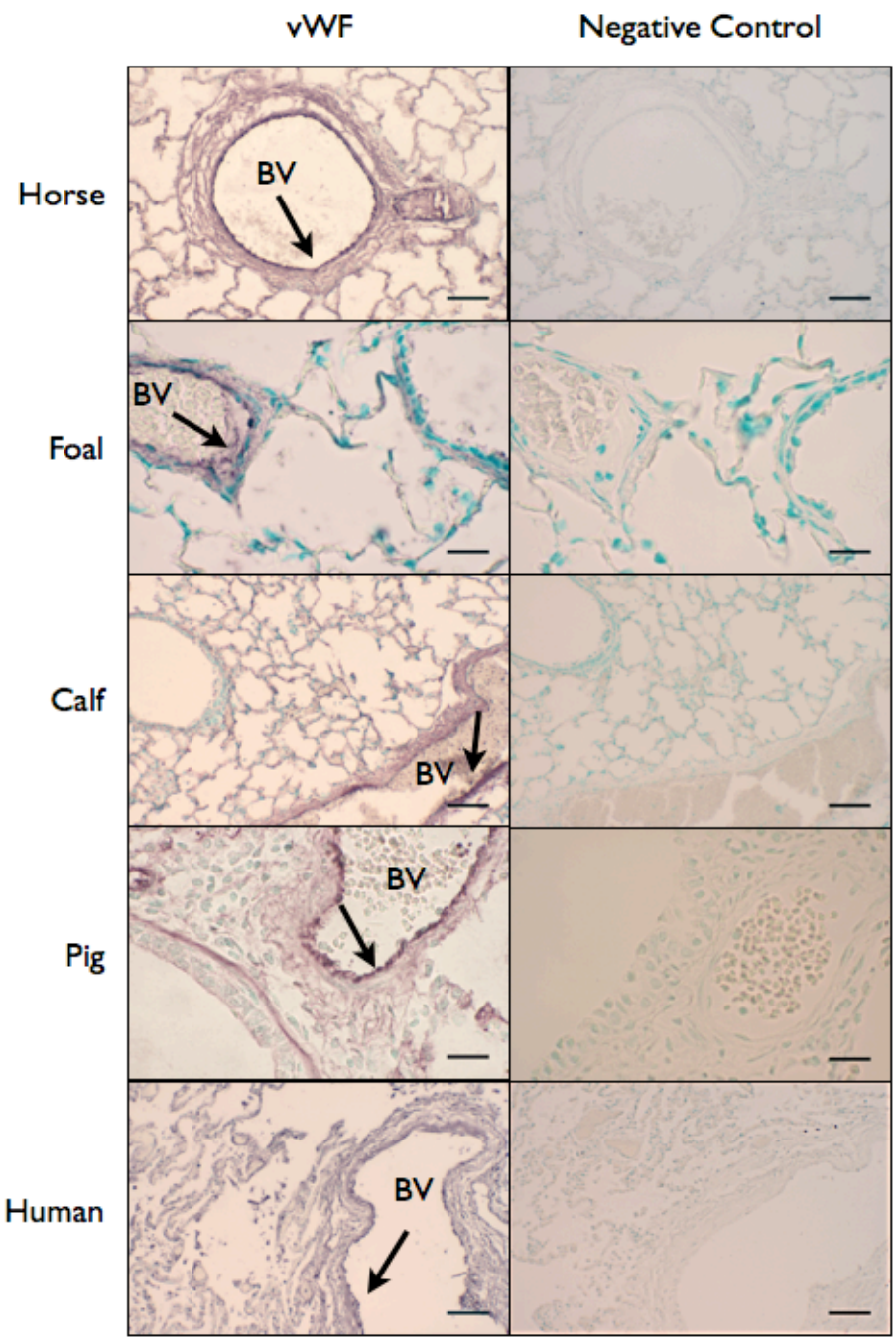


Figure 3.3A: Immunohistochemistry controls. Consecutive sections from the indicated species were stained with von Willebrand Factor antibody (vWF) which stained vascular endothelium of blood vessels (BV; arrows) but not airway epithelium (AW), and with only secondary antibody which did not cause any staining of the tissues. Scale bar = 20µm.

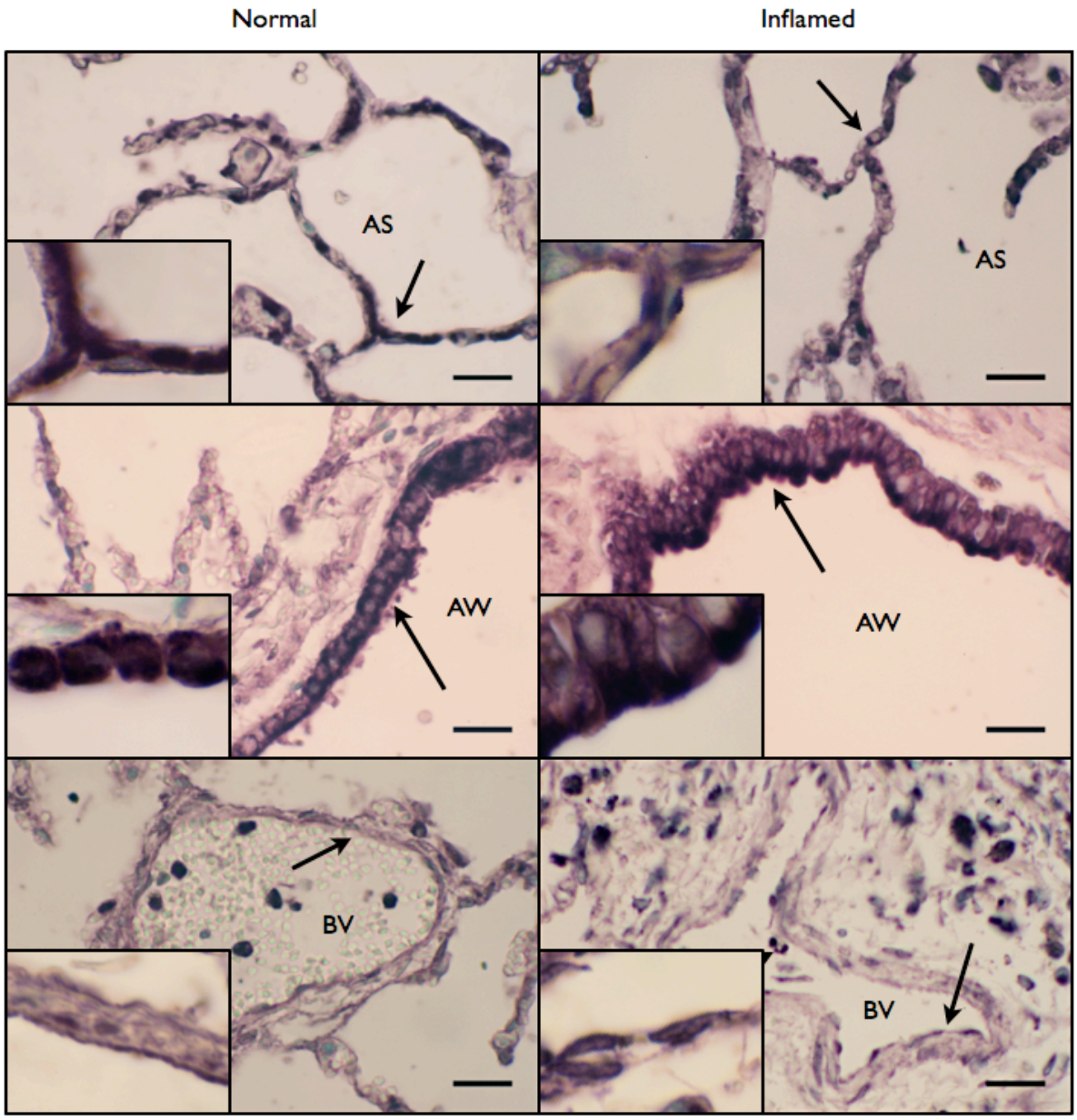
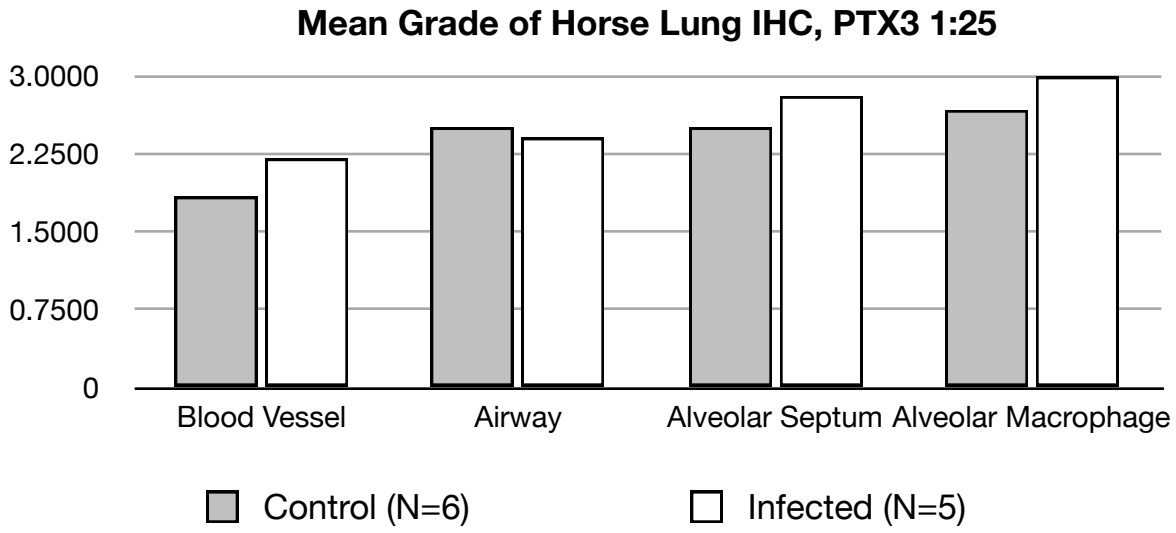


Figure 3.3B: Immunohistochemistry of normal and inflamed horse lung. The PTX3 staining (Arrows) is seen in alveolar septa lining the alveolar spaces (AS), epithelium of the airways (AW), endothelium of blood vessels (BV) and the vascular cells. Scale bar = 20µm in low magnification = 8 µm in high magnification inset.

Figure 3.3C: Grading of PTX3 immunohistochemistry in horse lung on a range of 1 to 3.



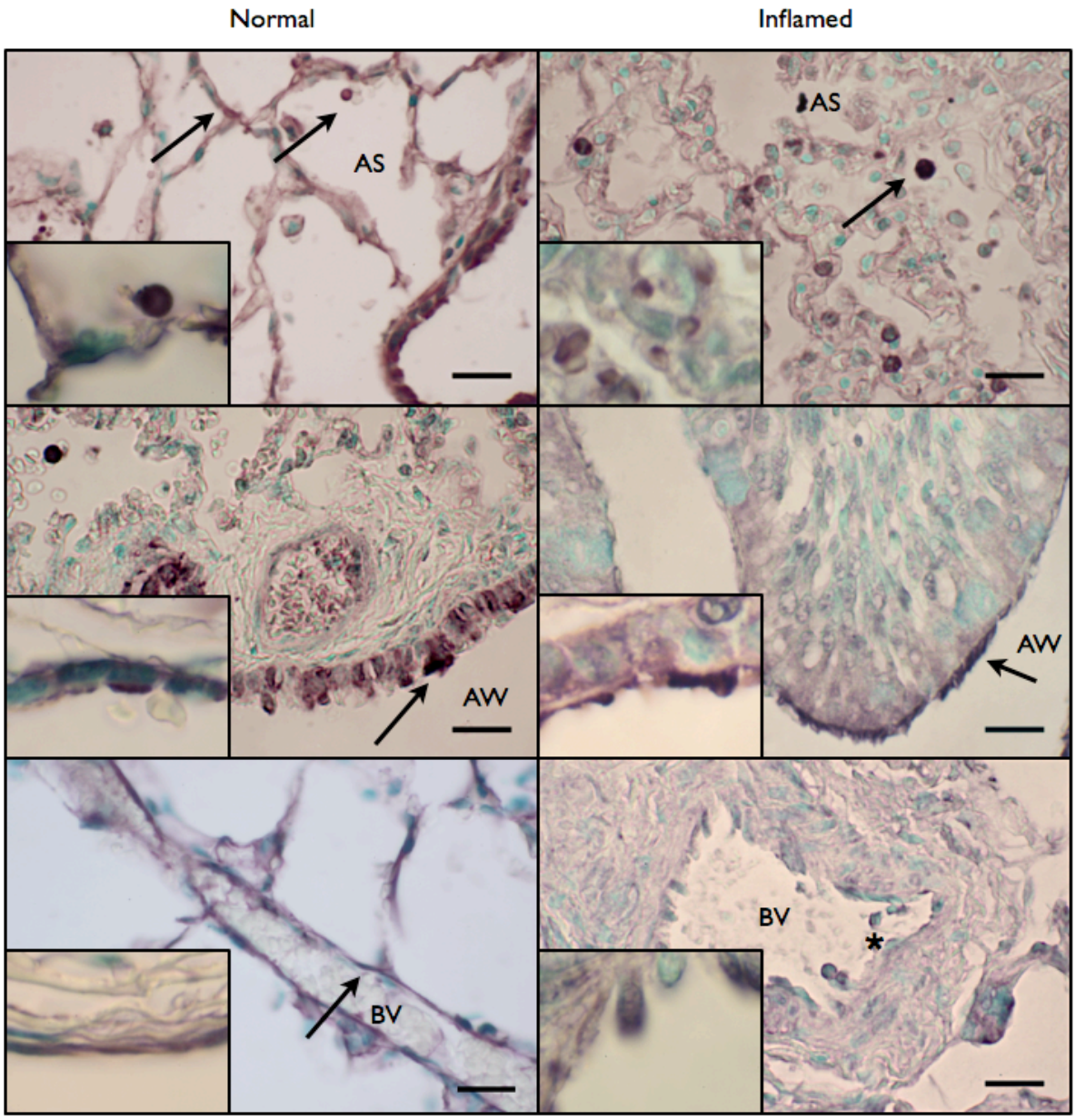
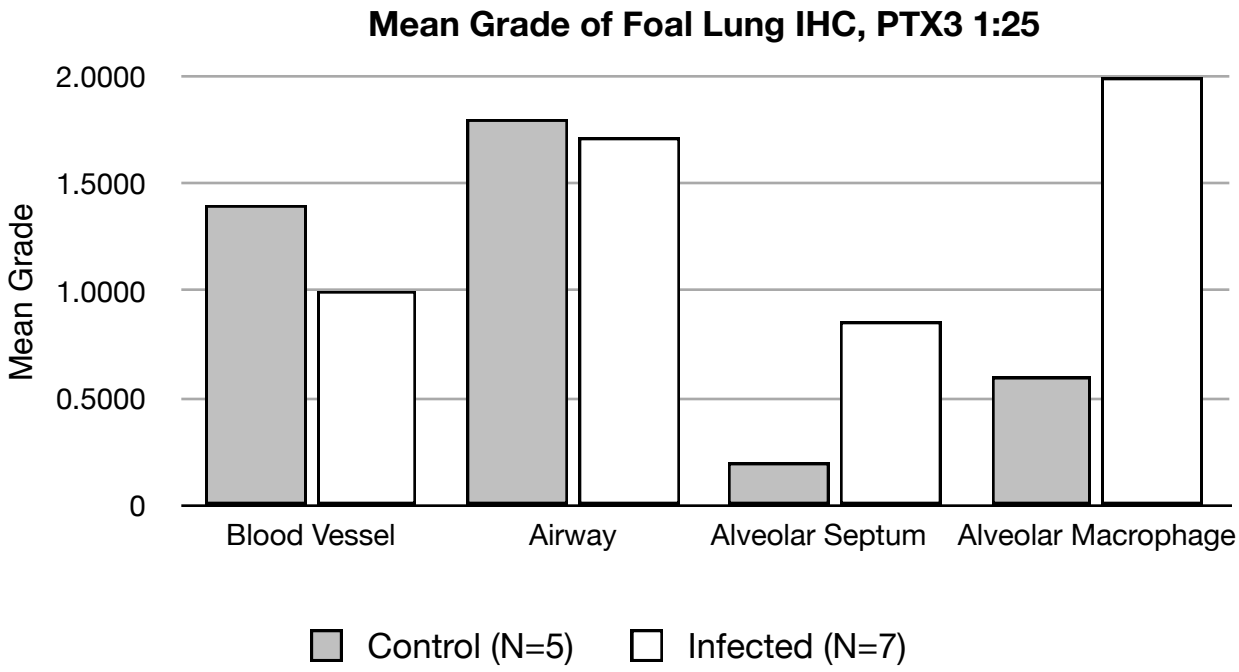


Figure 3.3D: Immunohistochemistry of normal and inflamed foal lung. The PTX3 staining (Arrows) is seen in alveolar septa lining the alveolar spaces (AS), epithelium of the airways (AW), endothelium of blood vessels (BV) and the vascular cells. An absence of staining was noticed in some blood vessels of inflamed foal lungs (*). Scale bar = 20µm in low magnification = 8 µm in high magnification inset.

Figure 3.3E: Grading of PTX3 immunohistochemistry in foal lung on a range of 1 to 3.



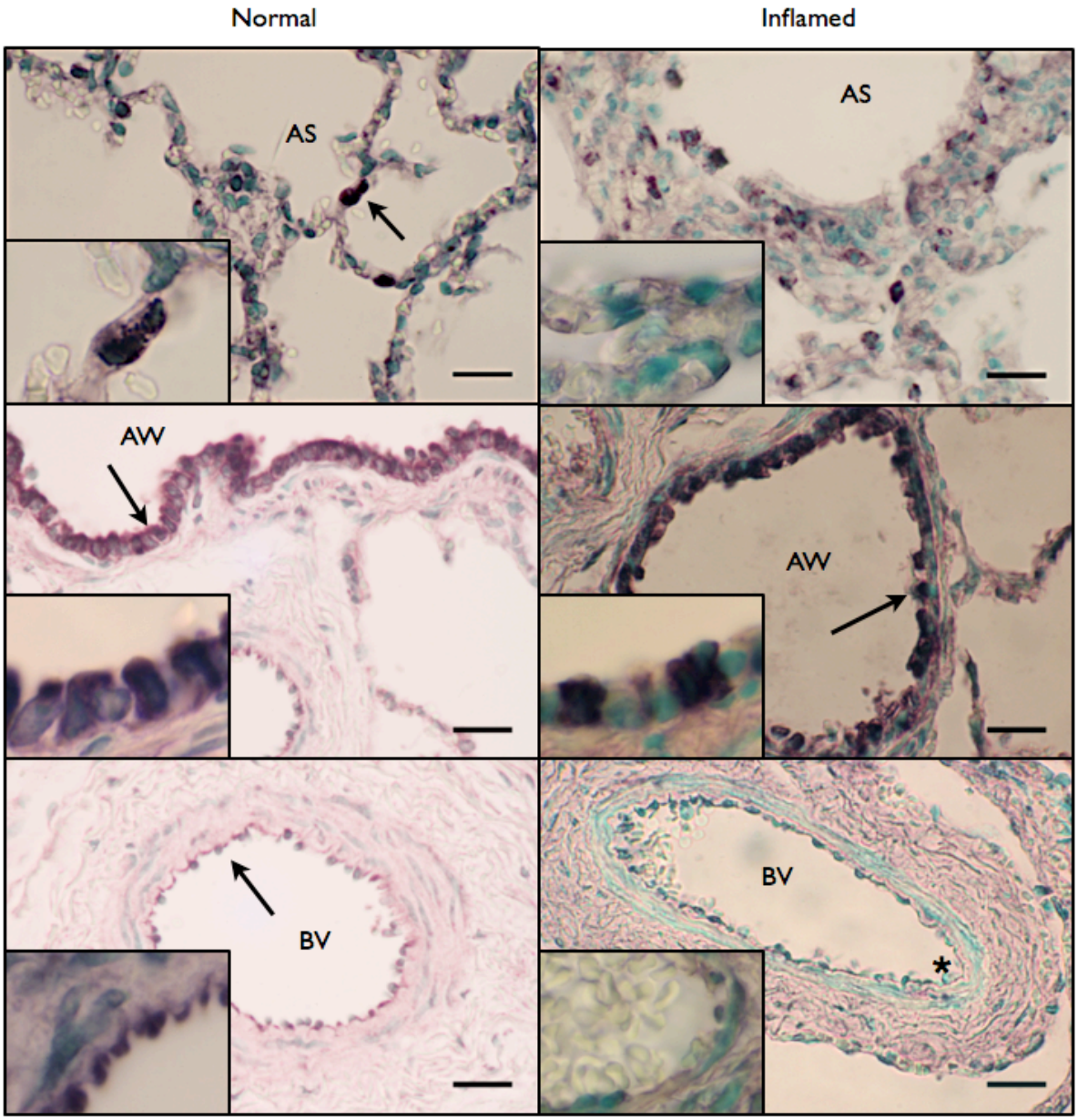
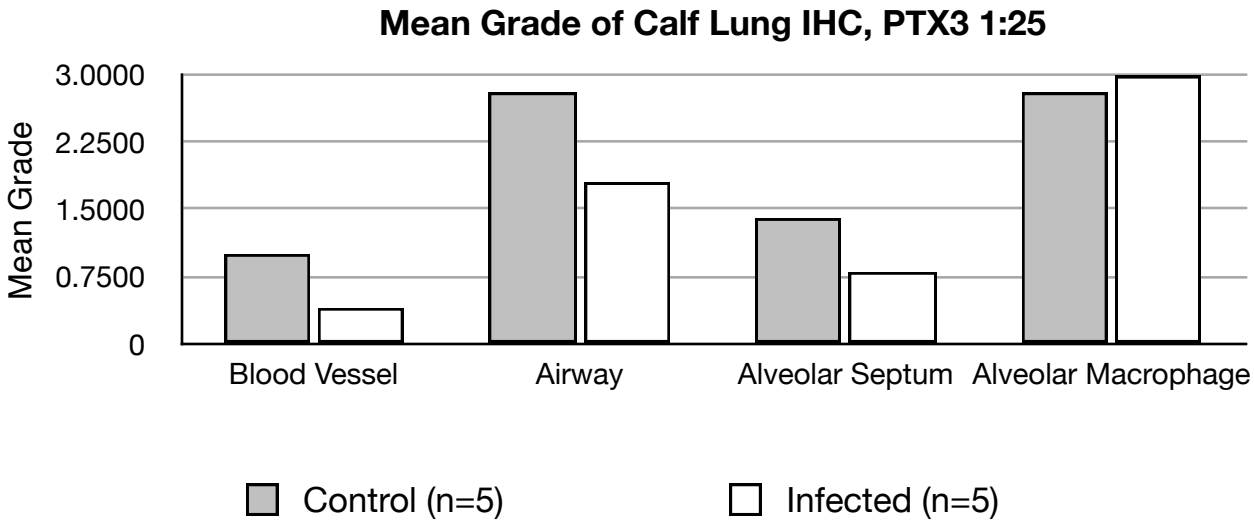


Figure 3.3F: Immunohistochemistry of normal and inflamed calf lung. The PTX3 staining (Arrows) is seen only few cells of the alveolar septa lining the alveolar spaces (AS), epithelium of the airways (AW), endothelium of blood vessels (BV) and the vascular cells. An absence of staining was noticed in some blood vessels of inflamed calf lungs (*). Scale bar = 20µm in low magnification = 8 µm in high magnification inset.

Figure 3.3G: Grading of PTX3 immunohistochemistry in calf lung on a range of 1 to 3.



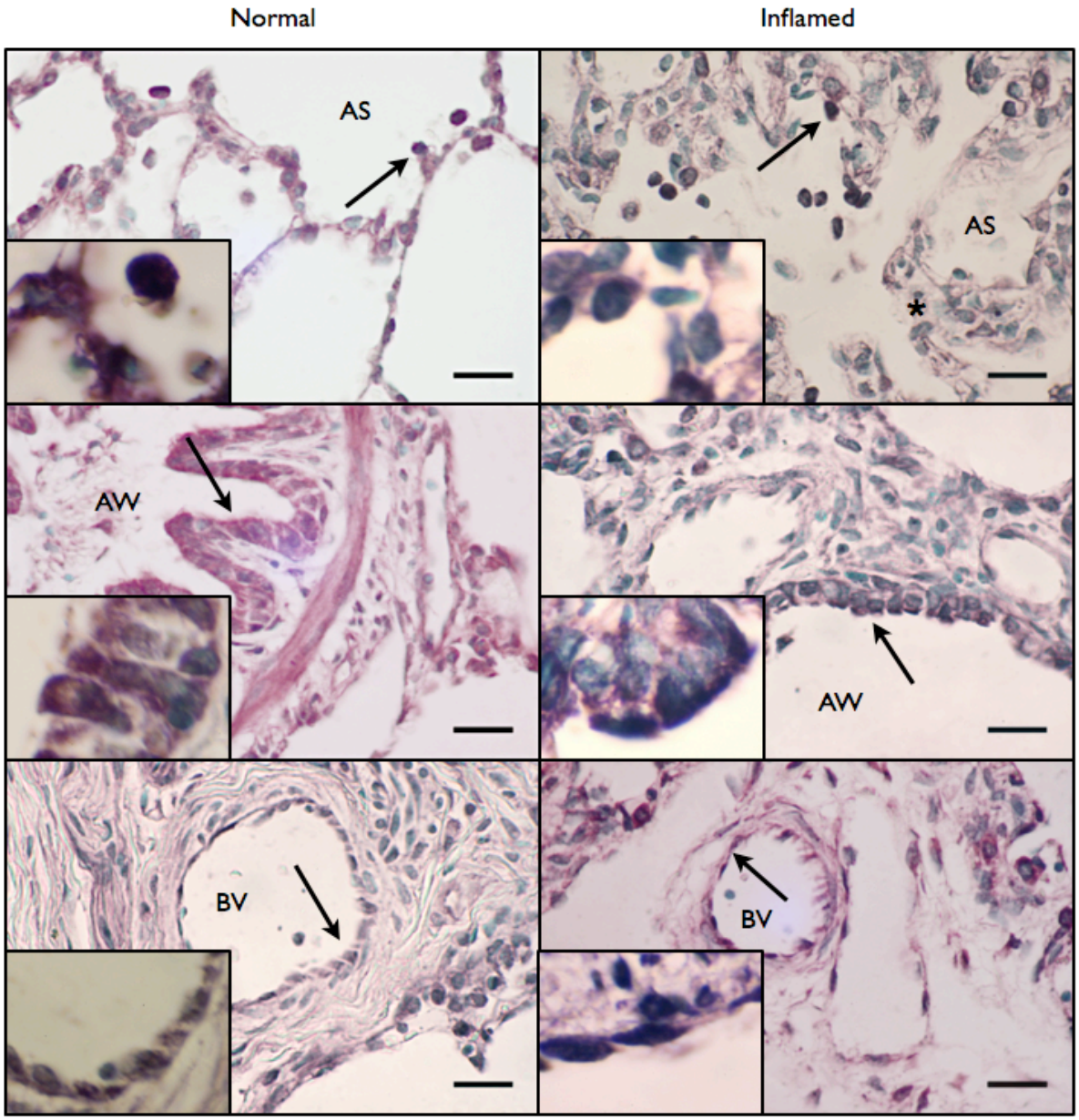
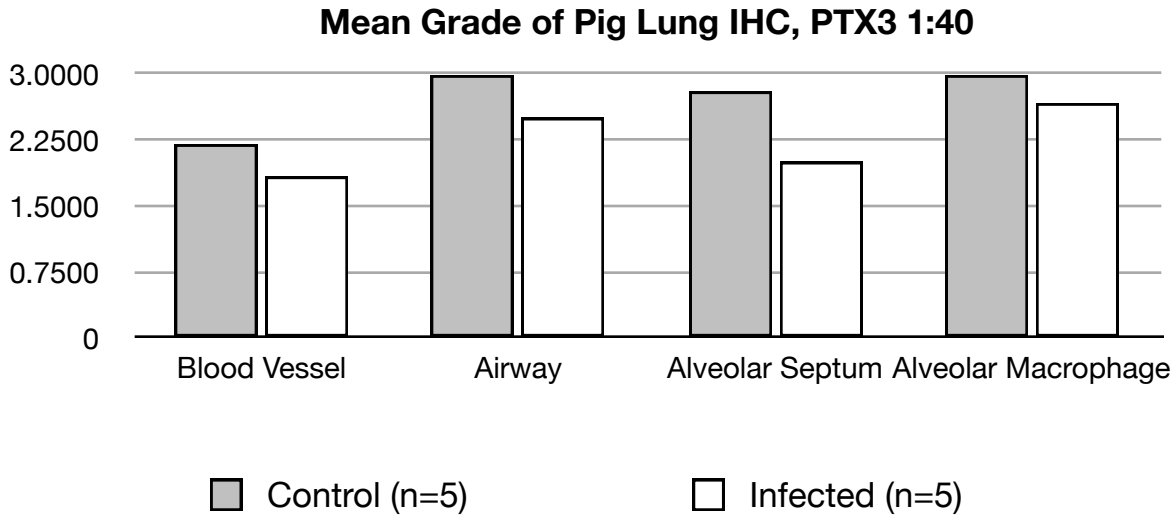


Figure 3.3H: Immunohistochemistry of normal and inflamed pig lung. The PTX3 staining (Arrows) is seen in alveolar septa lining the alveolar spaces (AS), epithelium of the airways (AW), endothelium of blood vessels (BV) and the vascular cells. Scale bar = 20μm in low magnification = 8 μm in high magnification inset.

Figure 3.3I: Grading of PTX3 immunohistochemistry in pig lung on a range of 1 to 3.



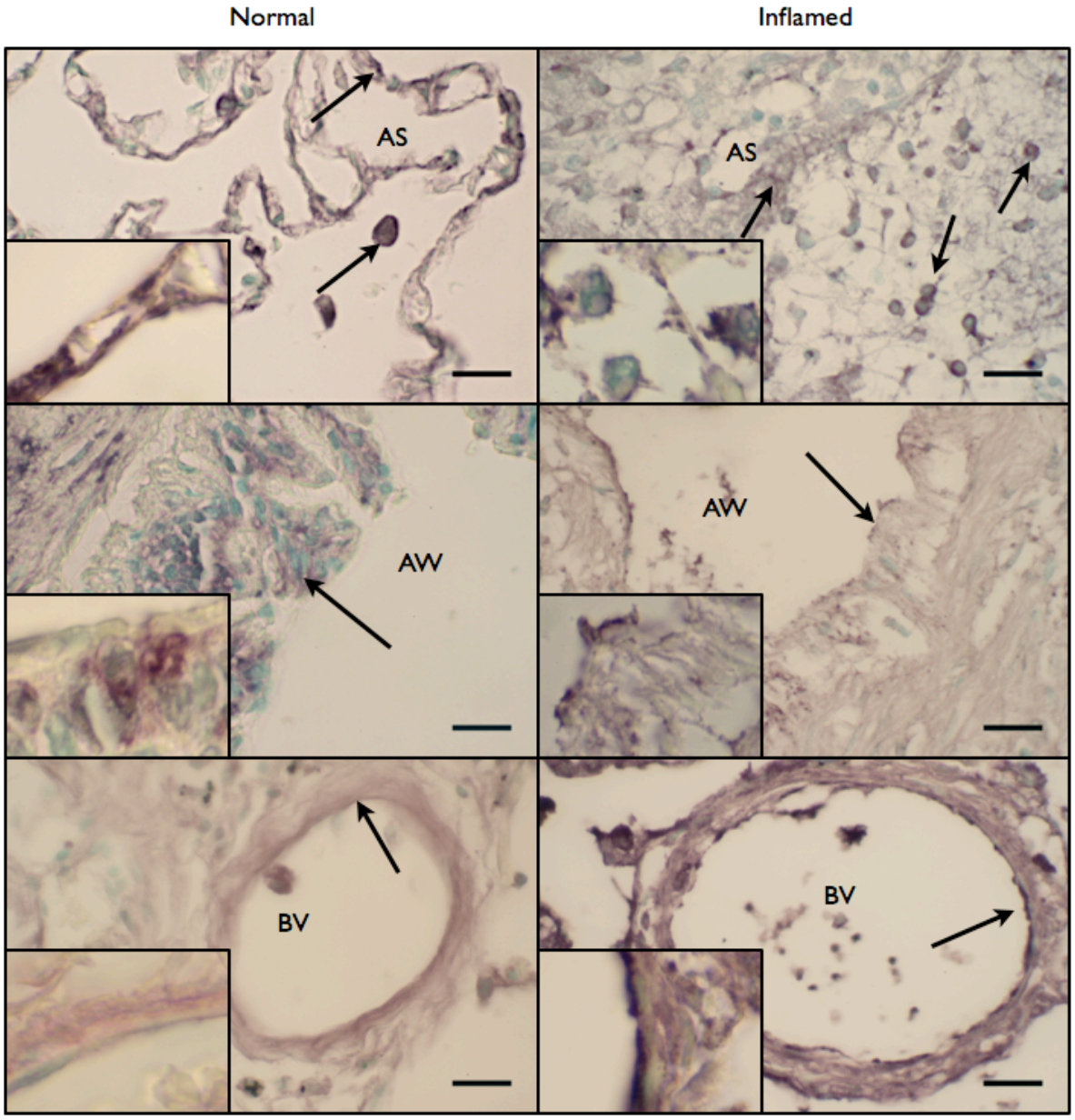


Figure 3.3J: Immunohistochemistry of normal and inflamed human lung. The PTX3 staining (Arrows) is seen in alveolar septa lining the alveolar spaces (AS), epithelium of the airways (AW), endothelium of blood vessels (BV) and the vascular cells. Scale bar = 20μm in low magnification = 8 μm in high magnification inset.

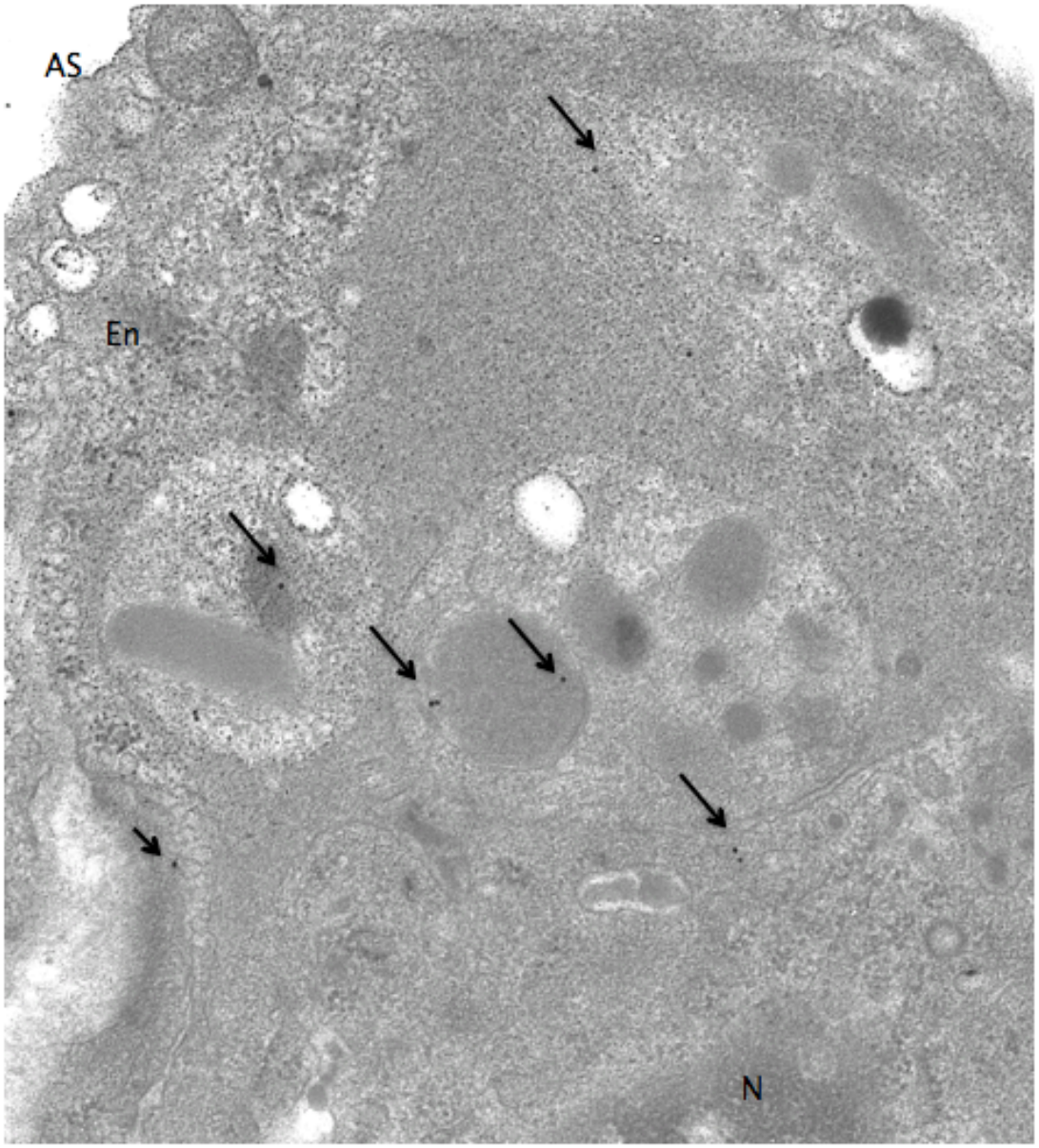


Figure 3.4A: Immuno-gold electron microscopy for PTX3. Pulmonary intravascular macrophage in the lung of a normal horse shows PTX3 (arrows). En: Endothelium; AS: alveolar space. Original magnification: X13,000.

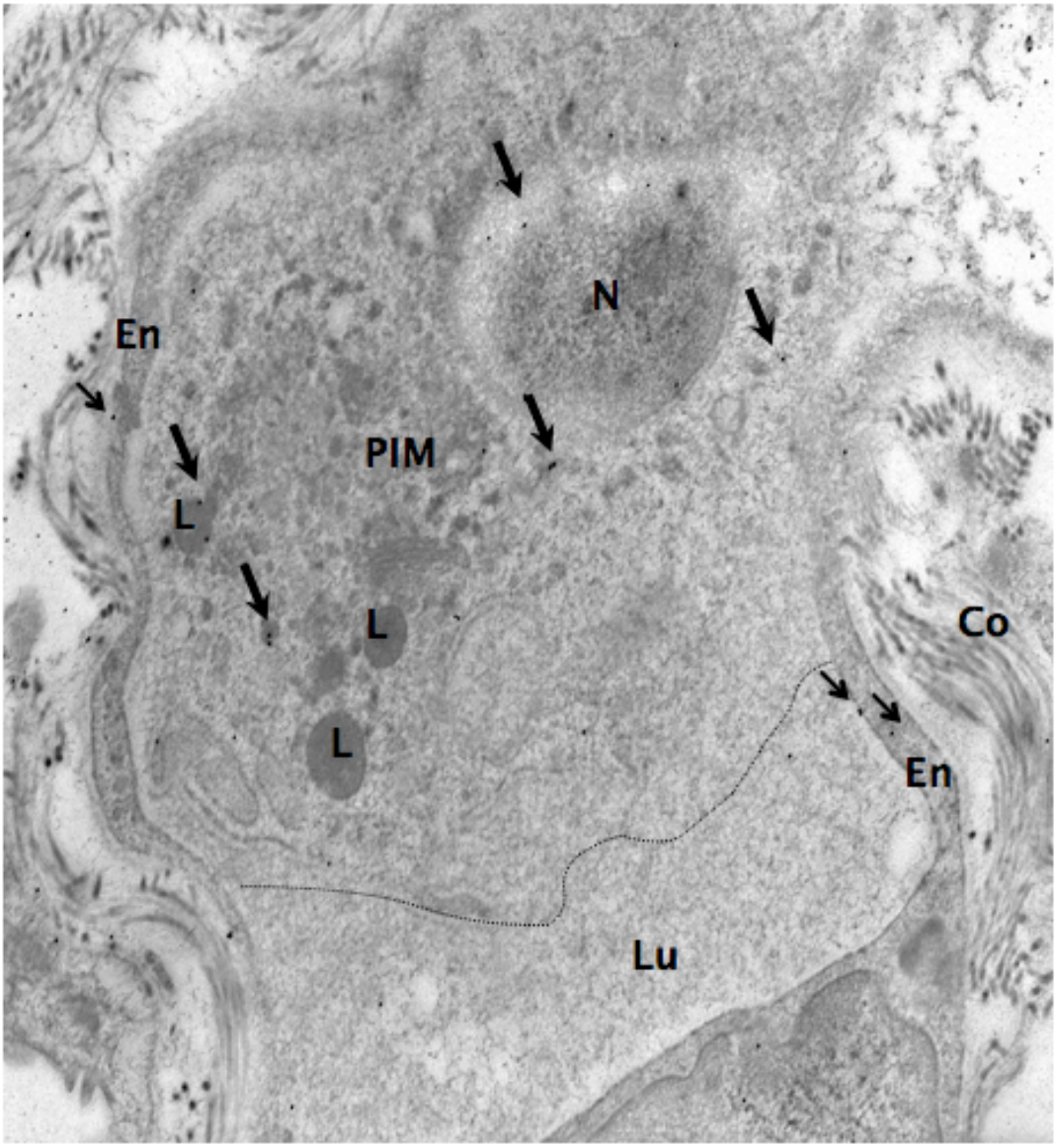


Figure 3.4B: Immuno-gold electron microscopy for PTX3. Pulmonary intravascular macrophage (PIM) in the lung of an LPS-treated horse shows PTX3 (arrows) and many lysosomes (L). Endothelium (En) also shows labeling for PTX3 (short arrows). N: nucleus; Co: collagen; Lu: lumen of blood vessel; dotted line marks boundary of PIM in blood vessel. Original magnification: X13,000.

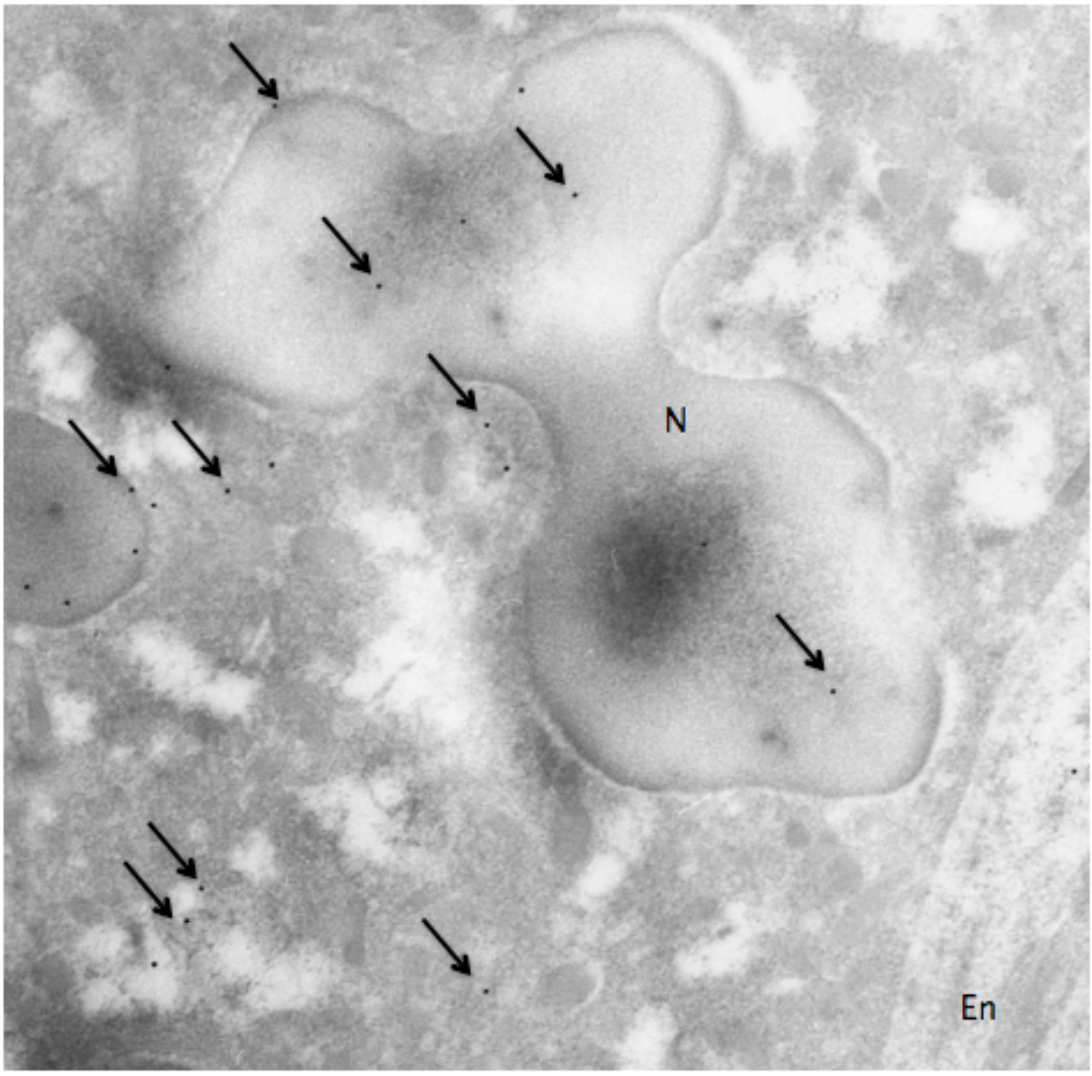


Figure 3.4C: Immuno-gold electron microscopy for PTX3. Neutrophil from a horse treated with *E. coli* LPS shows PTX3 (arrows) in cytoplasm and nucleus (N). En: Endothelium. Original magnification: X13,000.

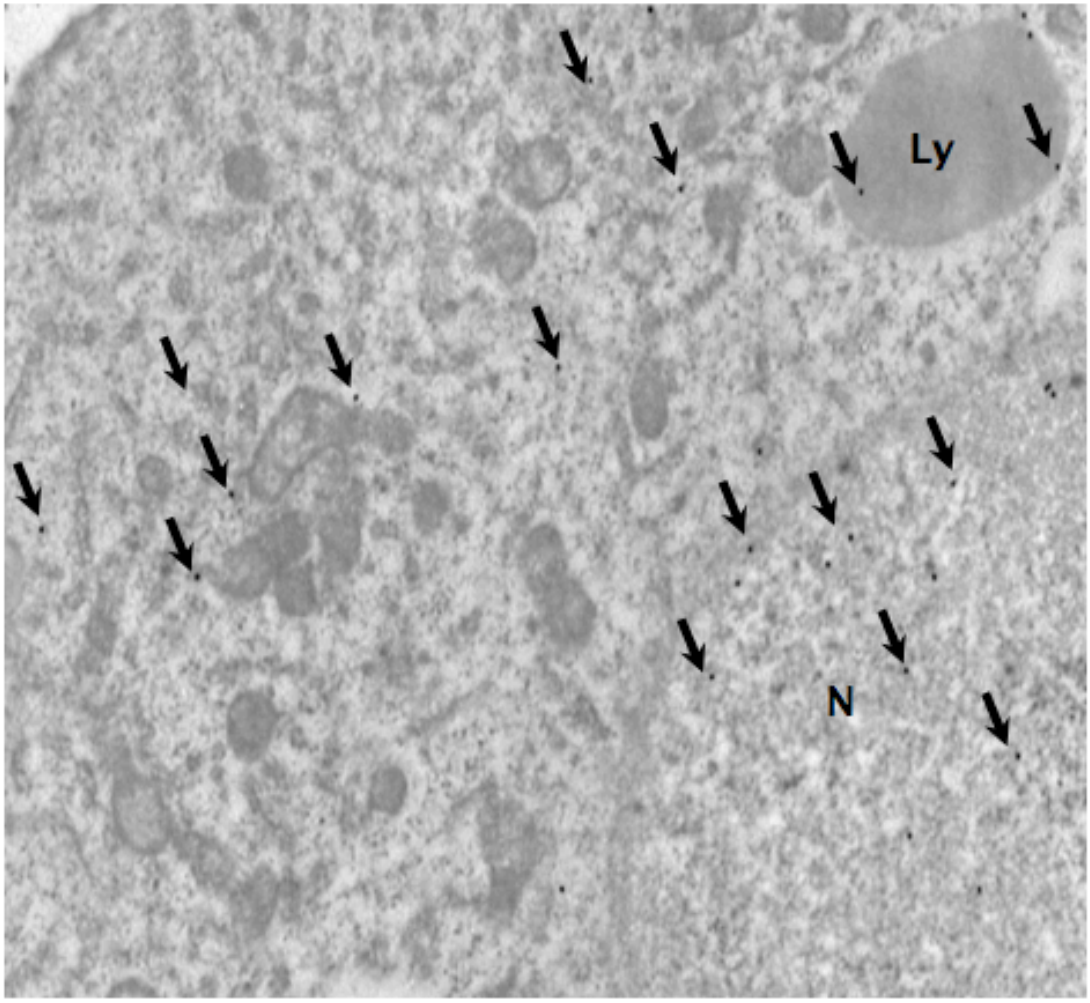


Figure 3.4D: Immuno-gold electron microscopy for PTX3. This equine alveolar macrophage shows staining for PTX3 (arrows) in the nucleus (N), lysosomes (Ly) and cytoplasm. Original magnification X13,000.

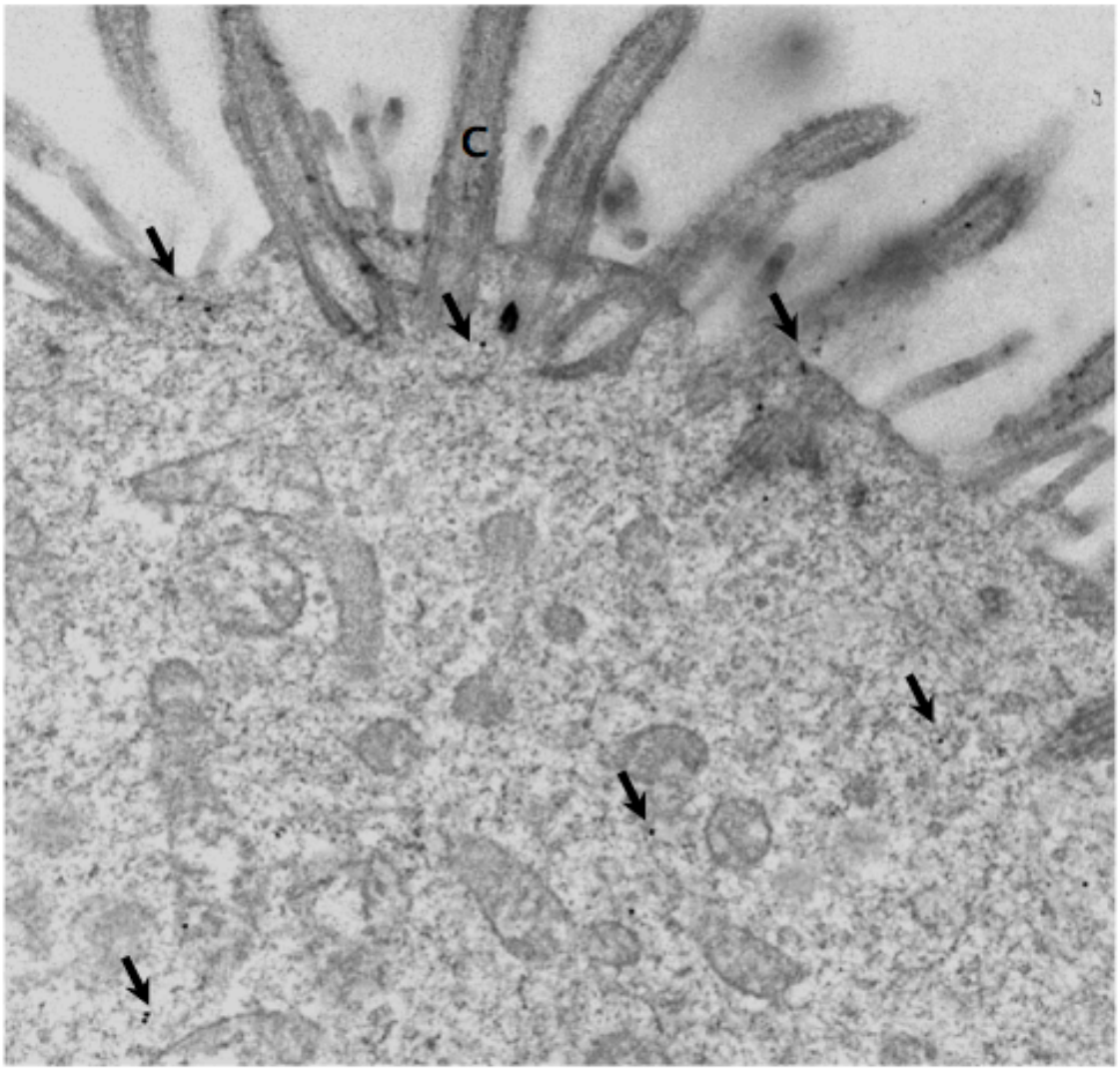


Figure 3.4E: Immuno-gold electron microscopy for PTX3. A bronchiolar epithelial cell with cilia (C) from a horse lung shows apical and and cytoplasmic staining for PTX3 (arrows). Original magnification: X13000.

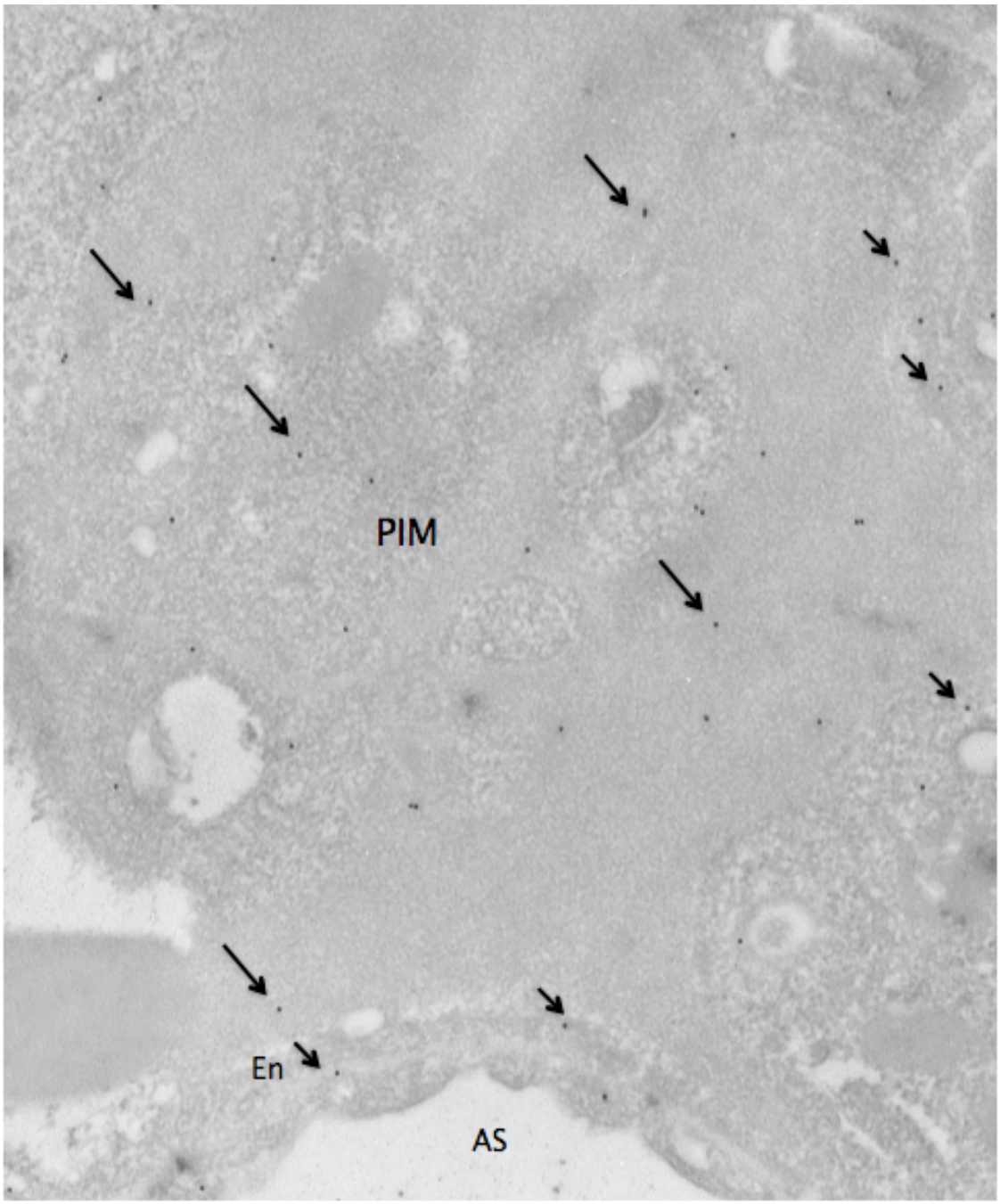


Figure 3.4F: Immuno-gold electron microscopy for PTX3. Pulmonary intravascular macrophage (PIM) from a calf shows PTX3 (arrows). Endothelium (En) also shows PTX3 staining (short arrows); AS: alveolar space. Original magnification: X13,000.

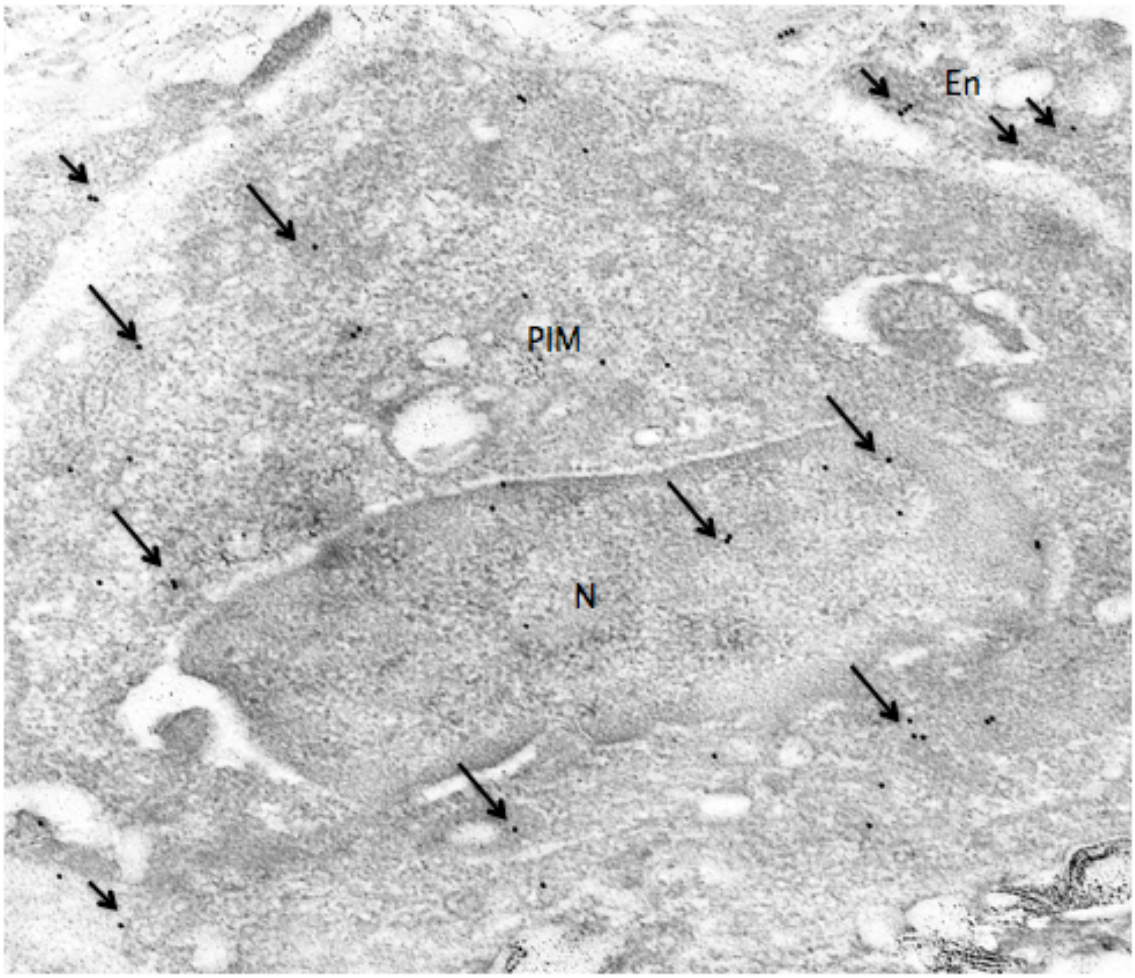


Figure 3.4G: Immuno-gold electron microscopy for PTX3. Pulmonary intravascular macrophage (PIM) of calf treated with *Mannheimia hemolytica* shows PTX3 (arrows) in cytoplasm and nucleus (N). Endothelial cell (En) also shows labeling for PTX3 (short arrows). Original magnification: X13,000.

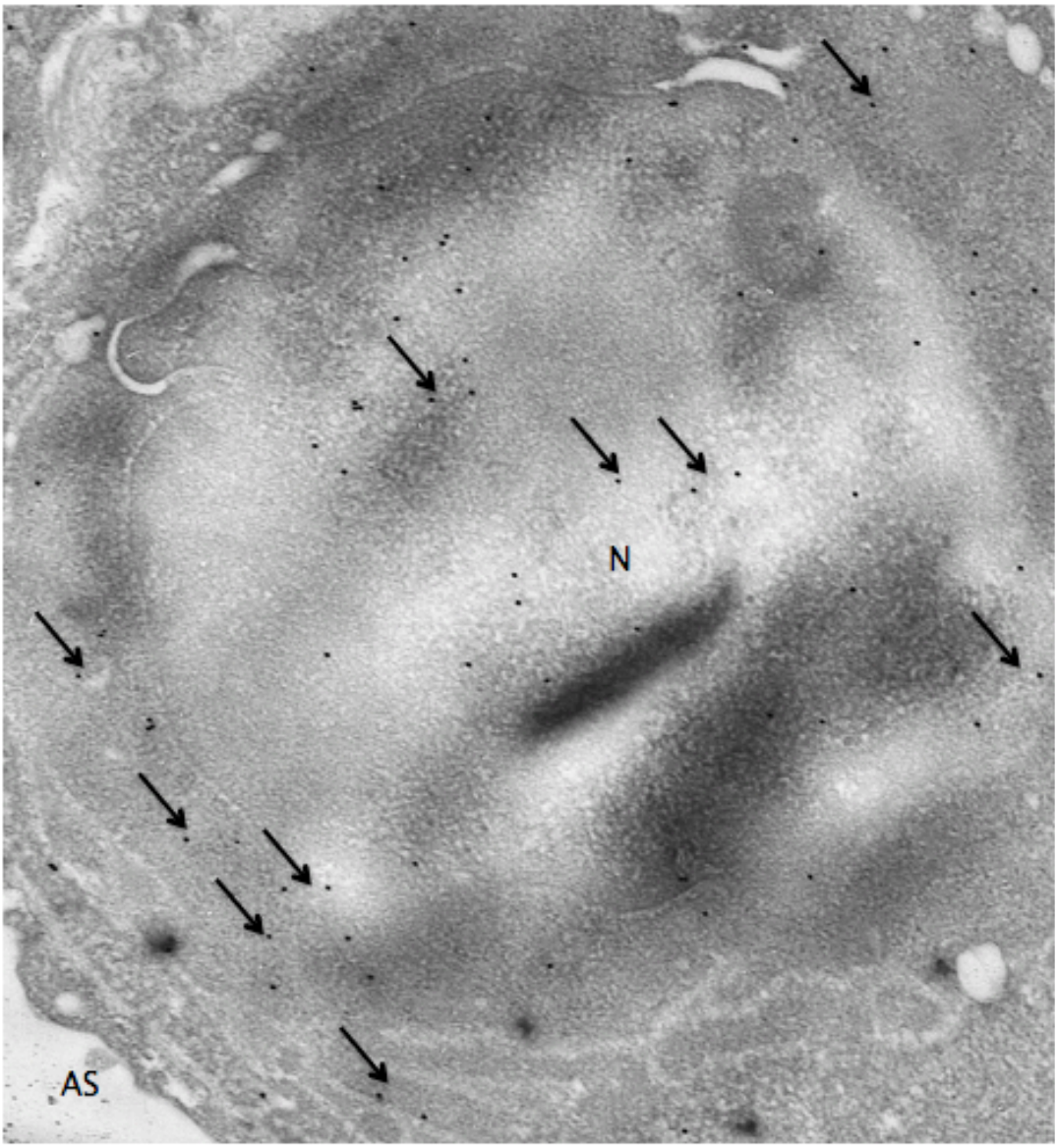


Figure 3.4H: Immuno-gold electron microscopy for PTX3. Pulmonary intravascular monocyte the lung of a calf infected with *Mannheimia hemolytica* shows PTX3 (arrows). N: Nucleus; AS: alveolar space. Original magnification: X13,000.

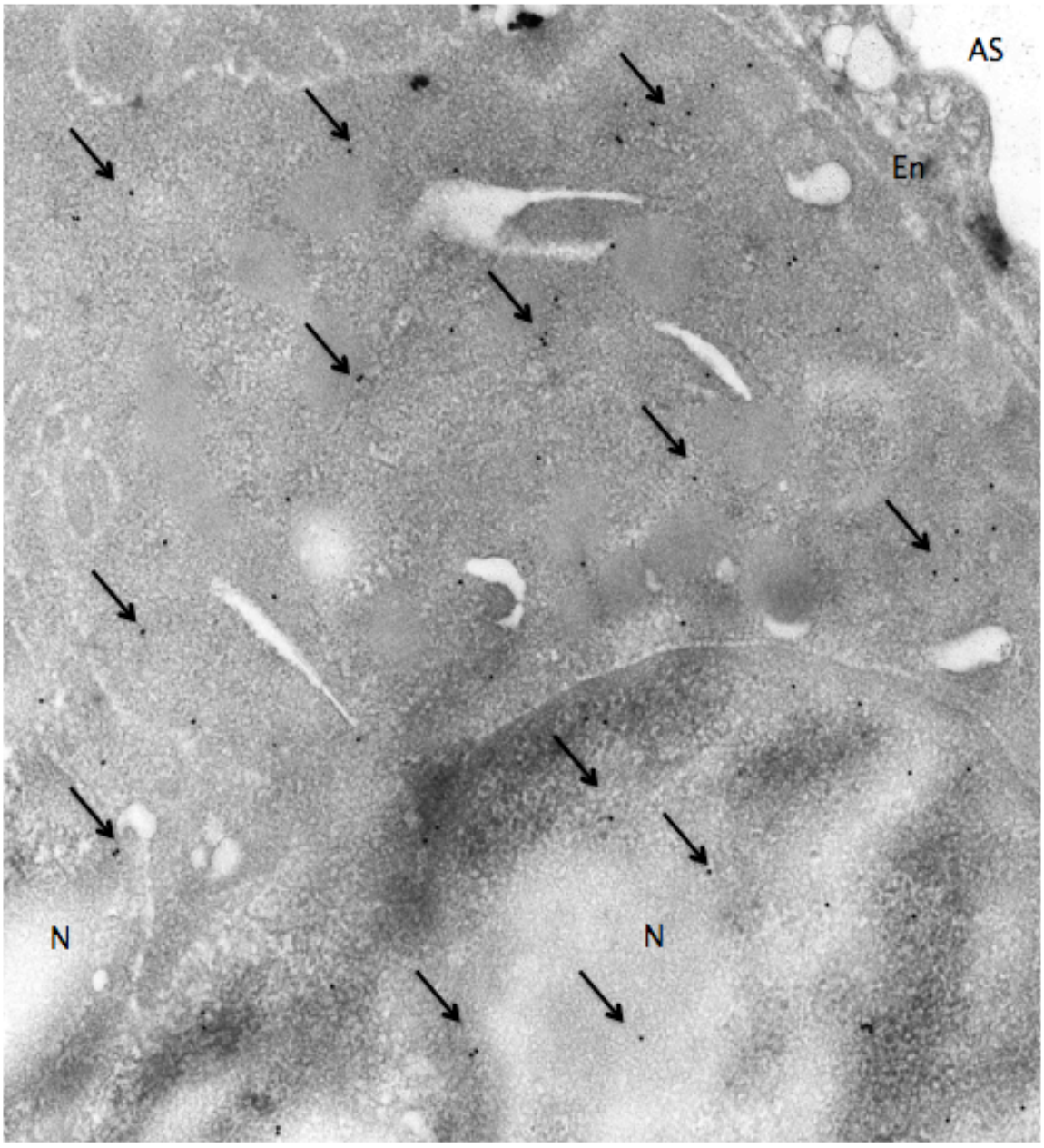


Figure 3.4I: Immuno-gold electron microscopy for PTX3. Neutrophil from a calf treated with *Mannheimia hemolytica* shows PTX3 (arrows) in cytoplasm and nucleus (N). En: Endothelium; AS: alveolar space. Original magnification: X13,000.

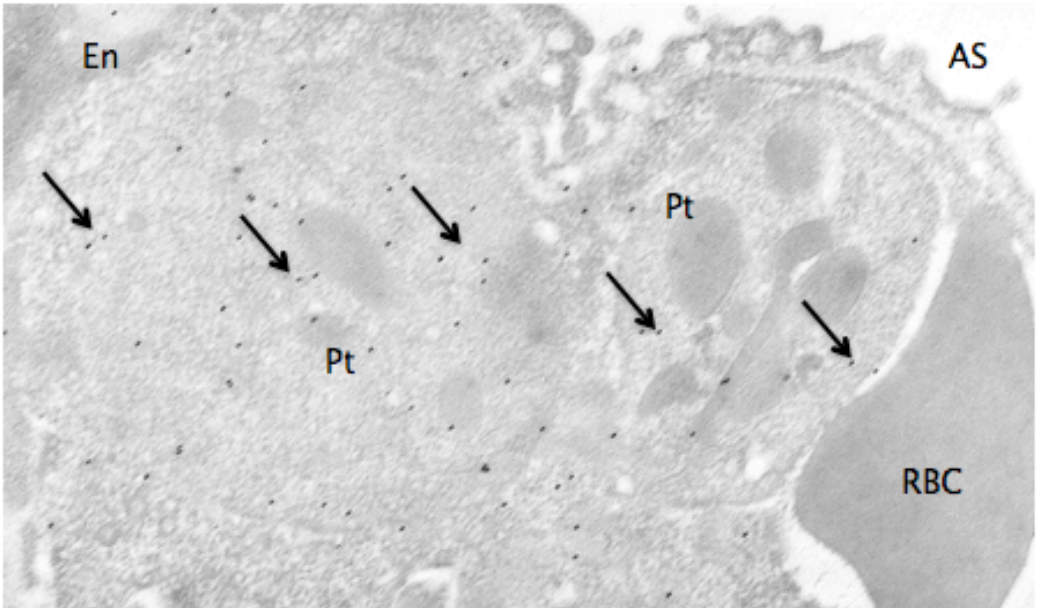


Figure 3.4J: Immuno-gold electron microscopy for PTX3. Platelets (Pt) in a lung capillary of calf treated with *Mannheimia hemolytica* show PTX3 (arrows) in cytoplasm. RBC: red blood cell; En: Endothelium; AS: alveolar space. Original magnification: X13,000.

3.5 Discussion

This study has identified the presence of PTX3 in normal and inflamed lungs of four species: human, horse, cattle and pig and compared the expression of PTX3 in lungs of young foals to adult horses. The expression of PTX3 is consistent among species and IHC has demonstrated concentrated staining in the bronchiolar epithelium, vascular endothelium and inflammatory cells, such as macrophages and neutrophils. The expression of PTX3 appears to be altered in inflamed lungs compared to control lungs. Using Western blot, I showed that there is an increased level of PTX3 in inflamed lungs of cattle and pig compared to control and have identified the presence of PTX3 at the sub-cellular level using immuno-gold electron microscopy. Immuno-gold electron microscopy demonstrated PTX3 staining in the nucleus, cytoplasm and lysosomes of equine alveolar, pulmonary intravascular macrophages, neutrophils and airway epithelium. PTX3 was also found in the cilia of ciliated airway epithelial cells. In calf lungs, immuno-gold electron microscopy demonstrated positive staining for PTX3 in pulmonary intravascular macrophages and monocytes, neutrophils and platelets.

I used multiple methods to investigate the *in situ* expression of PTX3 in normal and inflamed lungs. While isolated cells are amenable to many mechanistic studies, it is important to understand the cell specific expression of proteins in an intact organ because inter-cellular communication is critical to the organ-specific physiological responses. The overall expression of proteins was analyzed in frozen tissues with Western blots to get a picture of global expression of PTX3. While Western blots provide information in tissue homogenates, *in situ* methods are essential to characterize cell specific expression of PTX3, which was obtained with immunohistochemistry. Immunohistochemistry and Western blots allow semi-quantification of

the protein expression for comparing various treatments but don't provide fine details of localization of PTX3 in cells. I performed immuno-gold electron microscopy on normal and inflamed lungs from calves and horses to obtain organelle specific data on the localization of PTX3. Taken these methods together, I was able to obtain a rather comprehensive view of the expression of PTX3 in lungs of four species. Obviously it would have been useful to do analyses of mRNA expression, but I remained focused on the protein analyses because proteins are the functional end point of gene transcription

The data showed localization of immune cells such as macrophages, neutrophils and platelets as well as endothelial and epithelial cells in the normal and inflamed lungs. The presence of PTX3 in cells such as airway epithelium and alveolar macrophages suggests a role for the protein in protecting the lung against inhaled pathogens and dust particles. PTX3 has been shown to recognize and bind to certain pathogens, opsonizing them for uptake by phagocytes. Alveolar macrophages reside in the lungs, surveying the environment for invading pathogens. Upon recognition of pathogens, they release pro-inflammatory cytokines and chemokines. Furthermore, macrophages produce and release PTX3 after LPS stimulation[85] and are known to be a potent source of PTX3 in response to the opportunistic fungal pathogen, *Aspergillus fumigatus* conidia[103]. It is possible that the role of PTX3 in alveolar macrophages is to provide a local source of PTX3 at the initial site of pathogen recognition. This would allow the host to quickly opsonize bacteria in the surrounding environment, making recognition and phagocytosis more efficient and quicker to help eliminate the insult. The apparent increase observed in the expression of PTX3 in inflamed lungs in our studies is consistent with the results of other studies investigating the change in PTX3 levels in the whole lung. For example, PTX3

mRNA and protein were significantly increased in lungs from rats challenged with intravenously-administered LPS[81] and mice challenged with intratracheally-administered LPS[97, 116]. The increase in PTX3 protein in inflamed lungs may be due to the release of PTX3 protein from activated epithelium, endothelium and migrating inflammatory cells, and also upregulated gene transcription under the effects of inflammatory cytokines. In a study investigating the effect of *in vitro* TNF-α and LPS stimulation on the production of PTX3 in human alveolar epithelial A549 cells, human bronchial epithelial BEAS-2B cells, human primary alveolar type II cells, human U937 monocytes and HMEC-1 endothelial cells, TNF-α caused a significant increase in the amount of PTX3 protein released from all of these cells[81]. Interestingly, the fold-increase of PTX3 protein was the highest in A549 cells in response to TNF-α. Upon stimulation with LPS, PTX3 could be detected in only U937 cells and HMEC-1, and U937 responded more strongly to LPS than TNF-α. The results from this study suggest that alveolar epithelial cells may be a significant source of PTX3 in the inflamed lung. Further, the expression of PTX3 in each cell type appears to be stimulus-dependent.

The intense positive staining of inflammatory cells in the lung may also indicate another significant source of PTX3. During inflammation, the numbers of macrophages and neutrophils increases in inflamed lungs due to their migration in response to release of chemotactic factors such as IL-8 and activation of endothelium by inflammatory cytokines. Since neutrophils infiltrate in high numbers in inflamed organs, it is possible that the increase in PTX3 is due to higher numbers of these cells in the lung. It is reported that neutrophils store PTX3 in specific granules and PTX3 is released upon stimulation with microbial components and pro-inflammatory stimuli[84-86]. In addition, *in vivo* data show positive PTX3 staining in

neutrophils in lesions of ulcerative colitis[86]. Macrophages produce and release PTX3 after LPS stimulation, but not after TNF-α[85]. Macrophages are known to be a potent source of PTX3 in response to the opportunistic fungal pathogen, *Aspergillus fumigatus* conidia[103].

I made an interesting observation on the localization of PTX3 in platelets that were aggregated in the inflamed lungs of calves. The localization of inflammatory proteins in platelets has not received as much attention as neutrophils and macrophages. Platelets appear to play important roles in inflammation including that in the lungs through their ability to promote recruitments of neutrophils[120]. It was previously reported that platelets carry IL-8 in their granules in addition to vWF and P-selectin, which they release at the site of injury thus creating a molecular environment suitable for the recruitment of neutrophils. Because PTX3 has a role in recruitment of inflammatory cells and platelets are one of the very first responders to reach inflamed organs, I wonder if PTX3 in platelets plays a critical role in the genesis of inflammation in the lungs.

To my knowledge, this is the first study investigating PTX3 expression in lungs of cattle, foals and pigs and there are limited studies investigating the expression of PTX3 in intact human lung tissue. One study shows the expression of PTX3 in the lungs one patient with invasive pulmonary aspergillosis via immunohistochemistry[103]. In this study, IHC illustrated cytoplasmic staining in alveolar macrophages and circulating mononuclear cells, but lack of staining in epithelial and stromal cells. A second study shows the expression of PTX3 in airway smooth muscle cells, airway epithelium and inflammatory cells of the bronchial mucosa in human lungs with allergic asthmatic asthma[119]. The differences in staining in these studies

compared to my results may be due to the differences in expression in different pulmonary disease.

There has been only one published study that investigates PTX3 in horses[99]. PTX3 was found in BALF macrophages and neutrophils in horses suffering from recurrent airway obstruction (RAO) and healthy horses after exposure to dust[99]. In lung tissue from healthy and RAO-affected horses, immunohistochemistry revealed PTX3 in bronchial epithelial cells and inflammatory cells, similar to my findings[99]. The authors of this study noted that the staining of the bronchial epithelial cells was "localized at the apex of the cells just behind the ciliated process and the staining was more intense in horses with RAO"[99], similar to my findings. With the use of low dose intravenous LPS to induce lung inflammation in the horse, considerable PTX3 staining was observed in vascular endothelium and alveolar septum of normal and inflamed lungs. My observation is in disagreement with those of Ramery et. al[99]. The differences may be due to the different model of inflammation between the two studies.

In this study, the control tissues used were from "normal" animals. This means that the animals used were euthanized without any evidence of respiratory disease. Domestic animals typically spend their life outdoors in a dusty environment. The control human lung tissues were obtained from individuals that died from non-respiratory illness. Similar to animals, human lungs are constantly in contact with dusts, aerosols, fumes, cigarette smoke, etc. Therefore, a low level of inflammation in both the animal and human lungs may be present in the "normal" tissues. Furthermore, individuals that died from non-respiratory illness would likely have a high level of systemic inflammation. Therefore, some of the variability in the expression of PTX3 may be due to variability in clinical cases from where the tissues were collected.

Taken together, the data show constitutive expression of PTX3 in normal lungs and the expression appears to be increased in inflamed lungs. The presence of PTX3 in the nuclei of various cells is intriguing and requires *in vitro* experiments to understand the dynamics of the protein between the various cell compartments. Also, it would be useful to study whether PTX3 has any specific functions in the nucleus. The *in vitro* studies will also be useful to determine the kinetics of expression of PTX3 in cells such as neutrophils and alveolar macrophages in response to endotoxins.

3.6 Brief Introduction to Chapter 4

Inflammation is a highly complex process, involving the coordination of cellular and soluble mediators. To fully understand the mechanisms involved in inflammation, one must pull apart the complex web to study the individual parts, then put the pieces of the puzzle back together to understand the big picture. Since the influx of neutrophils is considered a hallmark of acute lung injury and inflammation in general, I investigated the expression of PTX3 in isolated neutrophils to further characterize the expression profile of PTX3 in these cells. I used cells from cattle and horses since these domestic species are of economic value and often suffer from respiratory disease. Furthermore, there is little data on the expression of PTX3 in these species, warranting this investigation.

CHAPTER 4: PENTRAXIN 3 IN BOVINE AND EQUINE NEUTROPHILS

4.1 Abstract

Neutrophils are the first responders in inflammation. Their job is to find pathogens and to destroy them. Neutrophils are indispensable in their role in host defense, and without functioning neutrophils, the host is often immunodeficient. However, when neutrophils are recruited to the site of inflammation, they are recruited in high numbers and release their proteolytic enzymes into the extracellular milieu. Thus, increasing unwanted tissue damage in the host. Recently, PTX3 has been reported in human and mouse neutrophils, localized in the specific granules. Few data exist on the expression of PTX3 in neutrophils from cattle and horses. I have used Western blot and immunocytochemical analyses to show the expression of PTX3 in bovine and equine neutrophils. Immunohistochemical staining for PTX3 in neutrophils shows an altered staining pattern in neutrophils stimulated with LPS. These data suggest that neutrophils may be a mobile form of PTX3 that is readily shuttled to the site of inflammation, where it can be released and involved in host defense.

4.2 Introduction

Neutrophils, also known as polymorphonuclear granulocytes (PMN), are a type of white blood cell with a multilobed nucleus and a cytoplasm full of granules[51]. They are often called the first responders in inflammation and their main role is in host defense through phagocytosis of pathogens and release of their intracellular granules to kill microbes[51]. They are recruited by tissue-resident sentinel cells, such as macrophages, that detect infectious agents and release chemotactic agents such as IL-8 and MIP-1[44]. In controlled inflammation, neutrophils are

beneficial in helping remove the infectious agent. However, when neutrophils are alerted to the presence of tissue damage or infection, they release their protease-filled granules, causing liquefaction of tissues and cell damage[128].

Neutrophils are recruited in inflammatory diseases, such as bacterial infections of the lung and nonseptic diseases. Inflammatory diseases cause significant morbidity and mortality in the livestock industry. Respiratory diseases such as bovine respiratory disease (BRD) and secondary bacterial infections are among the leading causes of economic loss in the livestock industry[3, 4]. Infection with *Mannheimia hemolytica* in cattle is common, causing a prevalent and severe disease called Shipping Fever, characterized by dense, neutrophilic infiltrate and exudate consisting of fibrin, seroproteinaceous fluid, and blood within the lung bronchi, bronchioles and alveoli[129]. *Pasteurella multocida* and *Haemophilus somnus* are other common causes of bacterial pneumonia in cattle[4]. In addition to bacterial pneumonias, equine respiratory disease is typically caused by hypersensitivity to environmental agents, colic, and endotoxemia[130]. Horses are prone to environmental hypersensitivities, such as inflammatory airway disease (IAD) and chronic lung inflammation, such as recurrent airway obstruction (RAO)[131]. Both of these diseases have unique pathogenesis, but are characterized by nonseptic inflammation, mild to severe neutrophilia of broncho-alveolar lavage fluid and poor performance[131]. Endotoxemia in the horse is characterized by systemic inflammation, often caused by colic[130]. It is generally believed that secretion of chemokines by activated airway epithelium and alveolar macrophages plays a role in the migration of neutrophils[132]. Therefore, neutrophil activation and their migration into inflamed tissues in cattle and horses is a critical part of the host immune response.

Pentraxins (PTX) are a superfamily of humoral pattern recognition receptors[133, 134]. Pattern recognition receptors are responsible for recognition of foreign molecules to alert the host of an invasion[1]. Two sub-families of pentraxins exist: short pentraxins, such as C-reactive protein and serum amyloid P component, and long pentraxins, such as pentraxin 3 (PTX3)[134]. Short and long pentraxins share similarities in their peptide sequences at the carboxy-terminus, but have a distinct sequence at the amino-terminus[78]. PTX3 is an acute phase protein with low circulating concentrations in healthy individuals, but high concentrations in individuals with sepsis[90], acute myocardial infarction[91], and acute respiratory distress syndrome[93]. PTX3 is produced in many cell types such as endothelial cells, mononuclear phagocytes, fibroblasts and dendritic cells in response to pro-inflammatory cytokines and microbial moieties[77, 78, 80, 82]. Recently, PTX3 was found to be stored in the specific granules of human and mouse neutrophils[84, 85, 87] and in neutrophil extracellular traps[84], a unique form of neutrophil activation where the DNA is expelled from the cell along with antimicrobial peptides[55]. Neutrophils from PTX3-deficient mice exhibited defective phagocytosis of *Aspergillus fumigatus* conidia compared to wild-type cells *in vivo* and *in vitro*[84]. Additionally, PTX3 has been shown to dampen neutrophil recruitment due to its ability to bind P-selectin in three models of P-selectin-dependent inflammation in mice: pleurisy, acid-induced acute respiratory distress syndrome and mesenteric inflammation[135]. These findings show that PTX3 may modulate the recruitment and function of neutrophils in inflamed organs.

To date, PTX3 expression in normal and activated neutrophils of domestic animals has not been characterized. Since neutrophils are recruited in high numbers to inflammatory sites and release their granules in many diseases of the cattle and the horse, it is important to

understand the expression of PTX3 in neutrophils from these animal species. I hypothesized that cattle and horse neutrophils express PTX3 and PTX3 will be released from neutrophils upon stimulation with LPS.

4.3 Materials and Methods

4.3.1 Isolation of Equine and Bovine Neutrophils

The protocol for neutrophil isolation from bovine and equine blood was modified from methods published elsewhere[136, 137]. During the isolation of equine and bovine neutrophils, sterile conditions were maintained throughout the procedures and temperature was maintained at room temperature (~ 25°C) unless otherwise noted. For equine neutrophils, blood was collected from healthy horses in EDTA tubes and pooled in 50mL polypropylene centrifuge tubes and left at room temperature for 30 minutes to allow for red blood cell rouleaux formation and sedimentation to occur. The leukocyte-rich plasma was collected and 12mL was layered over 10mL Ficoll Paque PLUS (GE Healthcare Life Sciences, Baie d'Urfe, QC, Canada) in a new 50mL centrifuge tube. The tubes were centrifuged at 400*g* for 30 minutes at 20°C with brake off. The upper layers consisting of plasma, lymphocytes, monocytes, platelets and Ficoll Paque PLUS were carefully removed and discarded. The remaining pellet consisting of erythrocytes and granulocytes was washed by addition of sterile 1X HBSS (pH 7.4; no Mg^{2+}, Ca^{2+}, or phenol red; Gibco, Life Technologies, Burlington, ON, Canada) and centrifugation at 200*g* for 10 minutes with low brake. Residual red blood cells were lysed by addition of 5mL sterile water (pH 7.4) and tonicity was restored by adding an equal volume of 2X HBSS. The cells were

pelleted by centrifugation at 200*g* for 10 minutes and the red blood cell lysis step was repeated. The remaining neutrophils were pelleted by centrifugation and washed twice with 1X HBSS.

For the isolation of bovine neutrophils, blood was collected from healthy cows in heparin. Blood was mixed with an equal volume of sterile PBS (pH 7.4) in a 50mL polypropylene centrifuge tube. Twelve mL of the diluted blood was then carefully layered over 10mL Ficoll Paque PLUS in a new 50mL centrifuge tube, then centrifuged for 30 minutes at 400*g* at 20°C with the brake off. The remaining steps are identical to equine neutrophil isolation above with the exception of using 500*g* for pelleting of cells instead of 200*g*.

Following neutrophil isolation, cells were resuspended in 1X HBSS, counted with a hemocytometer and cell concentration was adjusted to desired density and used immediately. Cytospins were prepared using a concentration of $1x10^6$ cells/mL. The purity of the fraction was measured by manually counting the neutrophils present in a cytospin preparation stained with a HemaColor kit (Harleco, EMD Millipore, Billerica, MA, USA).

4.3.2 Seeding of Neutrophils on Cover Slips and Staining for Immunocytochemisty

The protocol used for immunocytochemistry was modified from a protocol for immunofluorescence, previously published elsewhere[138]. In brief, 12mm round glass coverslips were acid-washed, rinsed thoroughly and stored in 70% ethanol to remove residues from the manufacturing process. Immediately before use, coverslips were dipped in 70% ethanol and passed through a flame before placing into a sterile 24-well tissue culture plate. Coverslips were coated with 50µl fibrinogen (1mg/ml in PBS; Sigma-Aldrich, Oakville, ON, Canada) and

placed into a 30°C incubator for at least 30 minutes. Excess fibrinogen was washed from the coverslips with sterile PBS. One mL neutrophil cell suspension was added to each well at a concentration of $1x10^6$ cells/mL along with 12.61mM calcium chloride and 4.93mM magnesium chloride. For treated cells, LPS (*Escherichia coli* 055:B5; Sigma-Aldrich, Oakville, ON, Canada) was added to each well to achieve a concentration 1µg/mL LPS. Cells were incubated for 30, 60, 90 or 120 minutes in the 37°C incubator with, or without, LPS. Non-adhered neutrophils along with cell media were removed from the plates after the treatment time. Cells were fixed by addition of 4% paraformaldehyde was added to each well for 15 minutes at room temperature and cells were washed thrice with PBS, 5 minutes each on a rocking platform. PBS was removed and cells were permeabilized by the addition of 0.1% Triton X-100 (Sigma-Aldrich, Oakville, ON, Canada) in PBS for 10 minutes at room temperature. Endogenous peroxidase was quenched with 3% H_2O_2 (Sigma-Aldrich) in PBS for 20 minutes in the dark. Cells were washed thrice with PBS as described above. Blocking was achieved with 1% bovine serum albumin (BSA; Sigma-Aldrich, Oakville, ON, Canada) in PBS at room temperature for 60 minutes. Blocking buffer was removed and coverslips were carefully removed from the 24-well plates using fine forceps and placed cell-side up on Parafilm atop moist paper towel in an airtight chamber. One hundred µL rat anti-human PTX3 antibody (MNB1; Axxora, Enzo Life Sciences, Brockville, ON, Canada), diluted to 1:75 in blocking buffer was pipetted onto horse neutrophils and mouse anti-human PTX3 monoclonal antibody (PP-PPJ0069-00; R&D Systems, Minneapolis, MN, USA), diluted 1:25 in blocking buffer was pipetted onto cattle neutrophils and incubated for one hour at room temperature. Negative controls were also prepared by using blocking buffer in place of antibody. To wash unbound antibody, coverslips were floated on PBS

three times, 5 minutes each. Secondary antibody was prepared by diluting in blocking buffer to a concentration of 1:100 (equine: polyclonal rabbit anti-rat IgG/HRP; bovine: polyclonal goat anti-mouse IgG/HRP; Dako, Burlington , ON, Canada). One hundred µL secondary antibody was added to each coverslip and incubated for 30 min at room temperature. After 30 minutes, unbound secondary antibody was washed by floating coverslips on PBS three times, five minutes each. To counterstain cells, cover slips were dipped in methyl green for ten seconds, rinsed by dipping in three different beakers of distilled water, allowed to air dry and mounted onto glass slides.

4.3.3 PTX3 Western Blots

One mL of neutrophils suspended in HBSS was aliquoted into 1.5mL centrifuge tubes corresponding to each of the different treatment groups: No LPS (time=0), LPS 30 minutes, LPS 60 minutes, LPS 90 minutes, LPS 120 minutes and no LPS (time=120 minutes). Calcium chloride was added to achieve a final concentration of 12.61mM and magnesium chloride was added to achieve a final concentration of 4.93mM. LPS was added to the appropriate treatment tubes to achieve a final concentration of 1µg/mL. Cells were incubated at room temperature for the appropriate time periods. Then, tubes were centrifuged at 1000*g* for 10 minutes to pellet cells. The supernatant was removed and the cells were resuspended in cold RIPA buffer (Sigma-Aldrich, Oakville, ON, Canada), with 1 tablet protease inhibitors (05892791001; Roche, Indianapolis, IN, USA) for every 7mL RIPA buffer. The cells were vortexed briefly and cells were incubated on ice for 5 minutes. The cells were then subjected to one quick burst of the sonicator then centrifuged at 10,000*g* for 10 minutes at 4°C to pellet cellular debris. The

resulting lysate was transferred to a new centrifuge tube and stored at -80°C until needed. To estimate the quantity of total protein in the cell lysates, I performed the Bio-Rad DC Protein Assay (Bio-Rad, Mississauga, ON, Canada) according to the manufacturer's instructions.

I performed SDS-PAGE according to the following protocol. Sixty µg of protein from bovine lysates and 40µg of protein from equine lysates were loaded into 12% gel after boiling the protein in 4x SDS loading buffer for 5 minutes to denature the protein. Total protein was separated by performing gel electrophoresis at 120V. The gel was collected and arranged into a Western blot sandwich with nitrocellulose or PVDF membrane for protein transfer. The transfer of the gel to the membrane was performed at 100V for one hour. Following transfer, the membrane was incubated in blocking buffer (5% skim milk solution in PBS with 0.05% Tween-20) overnight at 4°C. For bovine neutrophil lysate, mouse anti-human PTX3 antibody (PP-PPJ0069-00; R&D Systems, Minneapolis, MN, USA) was diluted in blocking buffer to a final concentration of 1:1000. For horse neutrophil lysate, rat anti-human PTX3 antibody (MNB1; Axxora, Enzo Life Sciences, Brockville, ON, Canada) was used at the same dilution factor. The membrane was incubated in the diluted antibody at room temperature for 1 hour on a rocking platform. Membranes were washed in PBS-T, then incubated in secondary polyclonal goat anti-mouse IgG/HRP (Abcam, Toronto, ON, Canada) or polyclonal rabbit anti-rat IgG/HRP (Dako, Burlington , ON, Canada)), diluted in blocking buffer to a final concentration of 1:1000 at room temperature for one hour on a rocking platform. Protein was detected by exposing X-ray film to membranes incubated in Amersham ECL Western Blotting Detection Reagents (GE Life Sciences, QC, Canada) as per manufacturer's instructions. Membranes were stripped using Blot Restore Membrane Rejuvenation kit (EMD Millipore, Billerica, MA, USA) according to

manufacturer's instructions and detection protocol was repeated to ensure compete removal of antibodies. The membranes were then probed with anti-β-actin (1:5000; Abcam, Toronto, ON, Canada) and goat anti-rabbit/HRP (1:5000; Abcam, Toronto, ON, Canada). Densitometry was performed using ImageJ software (National Institute of Health) as described elsewhere[127]. Using ImageJ, each band was selected using the gel lane selecting tool. The density of each band was shown for the bands by selecting the "plot lanes" command and the area under the curve for the band was selected. Lane 1 was used as a reference point to which the relative change of the other lanes was compared (e.g. density of lane 2 was divided by density of lane 1). This process was calculated for PTX3, then β-actin, giving ratios referred to as "relative densities". Then, the relative density of PTX3 was divided by the relative density of β-actin for the same lane to calculate the ratio for each lane compared to β-actin, the loading control. Each experiment was performed in triplicate with samples from a different animal each time.

4.3.4 Statistics

The relative densities of normal and inflamed lungs were compared for each species using the Kruskal-Wallis test (non-parametric one-way ANOVA; P=0.05; GraphPad Prism, Version 5.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). The relative densities are reported as the mean +/- standard error.

4.4 Results

4.4.1 Western blot in equine and bovine neutrophil homogenates

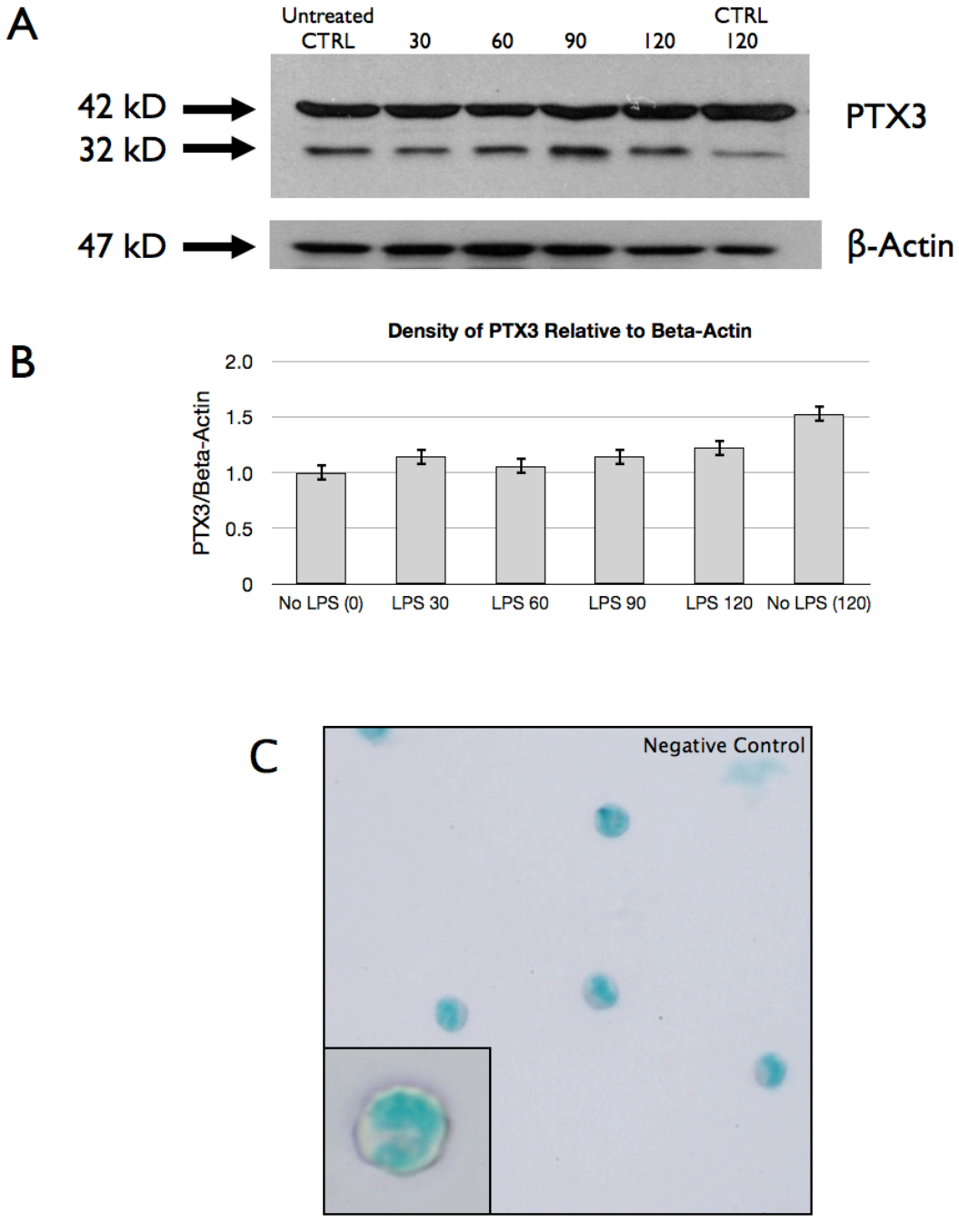
Lysates from stimulated and unstimulated equine and bovine neutrophils showed a distinct band at 42kD weight when probed with anti-PTX3 antibody (Figure 4.1A and 4.2A). The equine neutrophil lysates showed an additional band at 32kD in addition to the expected 42kD band (Figure 4.1A). After normalizing the values as a ratio of PTX3 density to β-Actin density, there were no statistical differences between groups. In the bovine neutrophils, intracellular protein increases nearly 1.6-fold after 30 minutes of LPS stimulation and decreases slightly after 60 minutes to 120 minutes. The unstimulated cells showed an increase of nearly 1.6-fold after 120 minutes (Figure 4.2A). Equine neutrophils did not show apparent increase in stimulated neutrophil lysates at any of the observed time points. However, unstimulated equine neutrophils had 1.5-fold increase in PTX3 after 120 minutes in culture (Figure 4.1A).

4.4.2 PTX3 immunocytochemistry of equine and bovine neutrophils

Normal, untreated equine and bovine neutrophils express PTX3 as shown by immunostaining isolated cells. The cytoplasm of unstimulated neutrophils stain intensely for presence of PTX3 (Figure 4.1D and 4.2D). Upon activation with LPS, the staining profile of PTX3 changes. After 30 minutes of LPS stimulation, many cells appear ruffled, empty and staining for PTX3 appears to be concentrated to one side of the cell. The cell membrane produces projections and the cell appears polarized. After 60 minutes of stimulation with LPS, many of the cells seem to recover and appeared less activated. Some cells still hold PTX3 and it is localized to one side of the cell which appears to be the leading edge of the cells. After

stimulation with LPS for 90 minutes, the cytoplasm appears to be more uniformly stained. When viewing the cells under the light microscope, intensely stained granules were visible when focused on the different *z*-planes of the cell. Due to the limitations in the resolution of the light microscope and digital camera, it was difficult to photograph this feature.

Figure 4.1: Expression of PTX3 in isolated equine neutrophils.



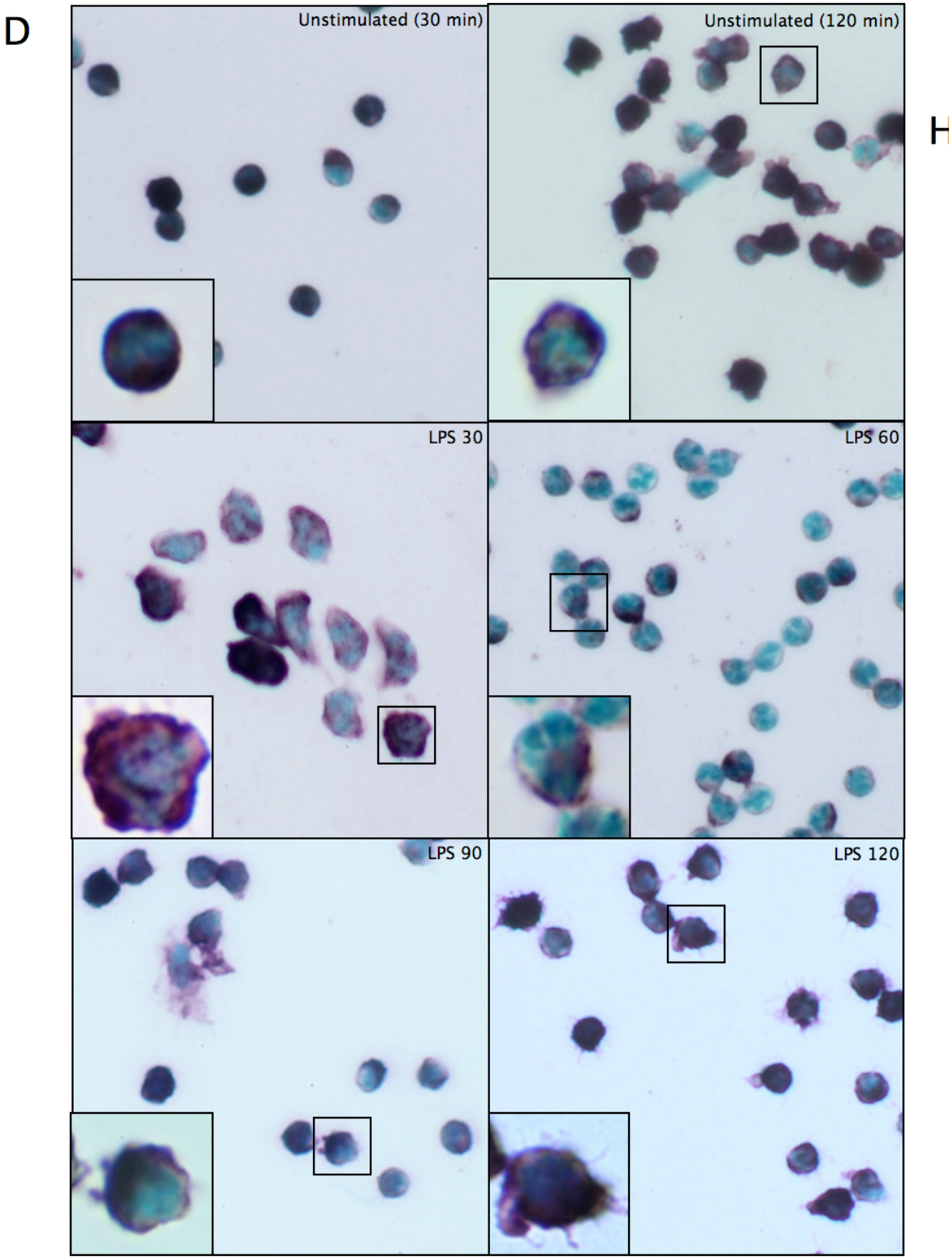
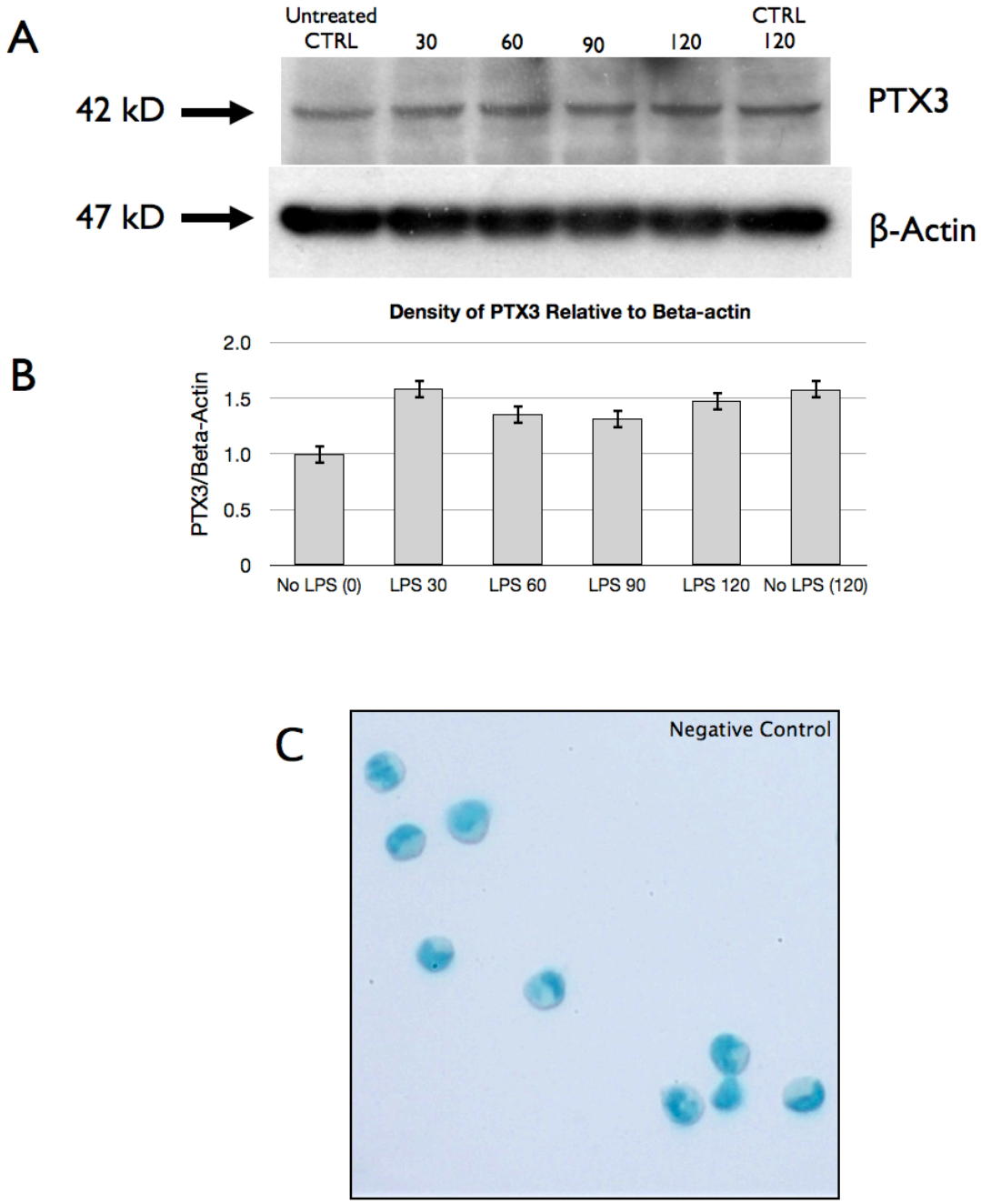

D
Unstimulated (30 min)
Unstimulated (120 min)
H
LPS 30
LPS 60
LPS 90
LPS 120

Figure 4.1: Expression of PTX3 in isolated equine neutrophils. I performed Western blot on neutrophil homogenates either non-stimulated, or stimulated with LPS to characterize the expression profile following activation. A band was observed at the expected 42kD weight in addition to a second band at the 32kD weight (A). The relative density of PTX3/β-actin is shown (B). Isolated neutrophils were cultured on fibrinogen-coated coverslips with or without LPS stimulation and were immunostained for PTX3 at different time points (C-D). The immunocytochemistry shows PTX3 in unstimulated neutrophils at 0 and 120 minutes. It appears that PTX3 was localized to the leading edges of activated neutrophils at 30 and 60 minutes of the treatment, and cytoplasmic staining appeared to be reduced at 60 minutes of LPS-treatment. Original magnification = 400x; inset = 1000x. The relative densities of normal and inflamed lungs were compared for each species using the Kruskal-Wallis test (non-parametric one-way ANOVA; P=0.05; GraphPad Prism, Version 5.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). The relative densities are reported as the mean +/- standard error.

Figure 4.2: Expression of PTX3 in isolated bovine neutrophils.



D

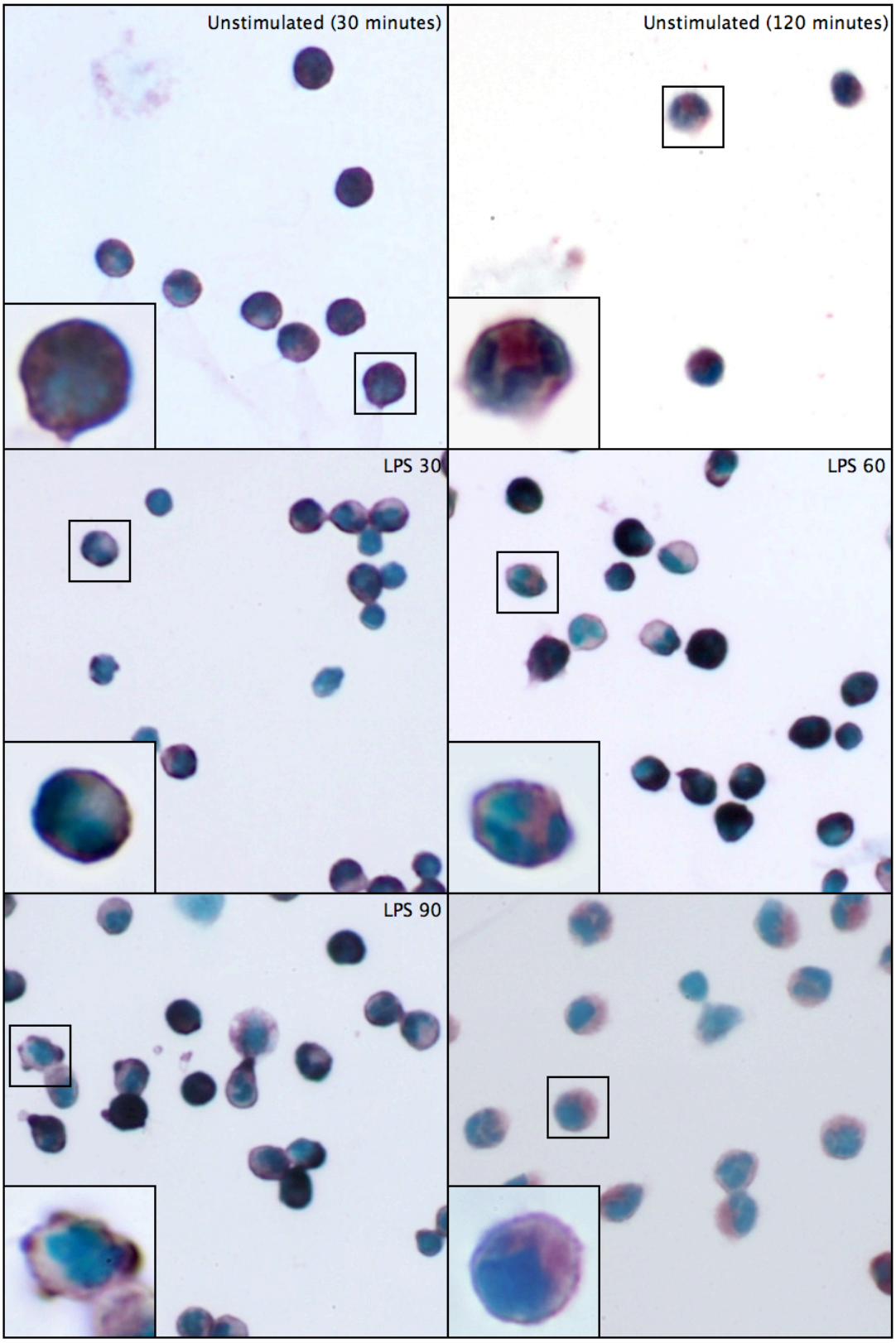

Unstimulated (30 minutes)
Unstimulated (120 minutes)
LPS 30
LPS 60
LPS 90

Figure 4.2: Expression of PTX3 in isolated bovine neutrophils. Isolated bovine neutrophils express PTX3 in a similar manner to equine neutrophils. I performed Western blot on neutrophil homogenates either non-stimulated, or stimulated with LPS to characterize the expression profile following activation. A band was observed at the expected 42kD(A). The relative density of PTX3/β-actin is shown (B). Isolated neutrophils were cultured on fibrinogen-coated coverslips with or without LPS stimulation and were immunostained for PTX3 at different time points (D). The PTX3 staining was altered spatially as that at 90 minutes there was more localization to the plasma membranes and also on ruffled areas of the plasma membrane. Original magnification = 400x; inset = 1000x. The relative densities of normal and inflamed lungs were compared for each species using the Kruskal-Wallis test (non-parametric one-way ANOVA; P=0.05; GraphPad Prism, Version 5.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). The relative densities are reported as the mean +/- standard error.

4.5 Discussion

I report the first immunohistochemical and Western blot data on the expression of PTX3 in freshly isolated, non-stimulated and stimulated neutrophils from horses and cattle. The data show the presence of PTX3 in non-stimulated neutrophils and altered expression in LPS-activated neutrophils from these species. Freshly isolated, unstimulated neutrophils from both species show strong immunostaining for PTX3 in the cytoplasm. After stimulation with LPS, many cells show concentrated, polarized staining for PTX3 and others show a complete loss of PTX3 staining. I had difficulty staining cytospins and alternatively used a method of immunostaining previously developed for immunofluorescence and confocal microscopy[138]. This method required the adherence of neutrophils to fibrinogen-coated glass coverslips as described elsewhere[138]. Fibrinogen was the preferred coating agent since, as described by Allen[138], it still allows the cell to perform normal functions, such as phagocytosis, in contrast poly-lysine, a common coating agent, which is more “sticky”. In contrast to using the cytospin method, this protocol allowed us to visualize morphological changes in the neutrophil after LPS stimulation as well as differences in localization of intracellular PTX3 protein. I observed the presence of PTX3 concentrated on the edges of activated cells. This feature may not have been observed using cytospins.

Western blotting of neutrophil lysates revealed the presence of intracellular PTX3, with a band at ~42kD, the expected molecular weight for PTX3. Horse neutrophils express a strong signal at ~32kD in addition to the 42kD band. These experiments don't provide the reasons for the detection of two separate bands in equine neutrophil lysates with the PTX3 antibody. It is possible that the smaller molecular weight band is a fragment of PTX3 or the antibody is cross-

reacting with another protein. Consistent with my findings, Ramery, et al.[99] also found a 32kD band upon Western blotting and attributed this lower-than-expected molecular weight to be due to alternative splicing. My Western blot data show that the expression profile of PTX3 in neutrophils is subtly altered after LPS stimulation.

The presence of PTX3 in whole neutrophil lysates is altered after LPS treatment as evidenced by Western blot analyses. The increase in PTX3 protein in bovine neutrophils after 30 minutes of LPS stimulation suggests there is an upregulation in the production of PTX3 protein. Since the level of PTX3 protein remains higher than baseline at the 60-120 minute time points but less than the initial surge of PTX3 at 30 minutes, it is possible that the cells begin to release PTX3. LPS-simulated equine neutrophils did not show change of intracellular PTX3. There are several possible explanations for this observation: (1) It is possible that LPS alone was not sufficient to activate the equine neutrophils to produce PTX3; (2) equine neutrophils do not respond as quickly to LPS stimulation as cattle neutrophils and require more time after LPS stimulation to increase intracellular PTX3; (3) equine neutrophils may produce new PTX3 protein, but release significant quantities so that differences in intracellular PTX3 was not apparent using Western blot; (4) equine neutrophils do not produce PTX3 at the mature stage of differentiation; or (5) a different mechanism occurs in equine innate immunity. Unstimulated neutrophils from both species showed increased intracellular PTX3 protein after 120 minutes in culture, suggesting neutrophils upregulate the production of PTX3 protein without LPS stimulation, but this protein was not released from the cell. IHC confirms the presence of PTX3 in the neutrophils.

The findings from this study are important because these are the first data to show the expression of PTX3 in equine and bovine neutrophils at the very early time points in response to LPS stimulation, confirming that these cells do indeed have intracellular PTX3 protein as found with Western blotting. The observation that, after LPS-stimulation, several neutrophils showed a difference in the staining pattern of PTX3 suggests that the intracellular PTX3 is localized to different regions of the cell. In some LPS-stimulated cells, complete loss of PTX3 staining was observed, indicating that the protein was released from the cell. It is unknown why PTX3 may localize to edges of the cell, but may be a snapshot of the cell before the protein is released.

Similar to my observations, Savchenko, *et al.*[86] reported after treatment with IL-8, some neutrophils exhibited strong positive staining for PTX3 in the cytoplasm, while others were only slightly positive or were completely negative for PTX3, likely due to release of PTX3. The data from other studies show an increase of PTX3 in cell media of neutrophils cultured with different agents. PTX3 was increased in the media of human neutrophils stimulated with LPS after 6 and 24 hours[85]. *E. coli, S. aureus*, zymosan, PMA, ionomycin, TNF-α, and Toll-like receptor agonists stimulation for 16 hours induced neutrophils to release PTX3 into the supernatant in a dose-dependent manner[84]. The intracellular concentration of PTX3 was measured in the cells stimulated by *E. coli* and *S. aureus* and was found to have decreased, indicating extracellular release of the protein. Furthermore, PMA and *S. aureus* resulted in a time-dependent release of PTX3 by neutrophils and suggests that the protein is released over time[84]. The concentration of PTX3 released by the neutrophils was significant at 1 hour and continued to climb until 16 hours, the last time point analyzed[84]. Another study reports that PTX3 is released from human neutrophils after IL-8 stimulation[86]. These studies indicate that

appropriate stimulation will induce release of PTX3 from human neutrophils. However, most of these studies observed neutrophils at a minimum of 2 hours after stimulation. One study found that only after 1 hour of stimulation and not earlier, was the release of PTX3 significant[84]. In my study, I did not see apparent increase or decrease of PTX3 in the cell homogenates between 30 minutes and 2 hours of LPS stimulation. This is possibly due to a low level of extracellular release by the cells at these early time points. Yet, from these studies we can expect to find a release of PTX3 from equine and bovine neutrophils with appropriate stimulation which requires quantification of PTX3 in the supernatant to precisely address this issue.

Currently there is not a consensus regarding PTX3 mRNA expression in human neutrophils. One study showed the presence of PTX3 mRNA in freshly-isolated neutrophils and increasing mRNA in response to several hours in culture and LPS-stimulation[85], but other studies did not find mRNA expressed in freshly isolated or stimulated human neutrophils[80, 84, 139]. I did not assess the expression of mRNA in equine and bovine neutrophils. Thus, further clarification of this issue is required in these species and would complement the data I have presented here.

The use of PTX3 knockout mice by other researchers has increased our knowledge of PTX3 in neutrophil functions. PTX3-deficient mice exhibited neutrophil accumulation after induction of pleural inflammation compared to wild-type mice. But, when PTX3-deficient mice were given wild-type bone marrow or exogenous PTX3, neutrophil accumulation was similar to wild-type mice, indicating that neutrophils are the main source of PTX3, which negatively affects the recruitment of neutrophils in inflammatory conditions[135]. Furthermore, PTX3-deficient mice show increased neutrophil accumulation in the lungs and more severe lung

damage after acid-induced acute lung injury[135]. PTX3-deficient mouse neutrophils do not phagocytose conidia from *A. fumigatus* as efficiently as wild-type mice[84]. It appears that PTX3 may be a fine regulator of neutrophil migration and activation.

During the recruitment of neutrophils in inflammation, P-selectin and E-selectin are upregulated on the surface of activated endothelium, receptors which bind to PSGL-1 on the surface of neutrophils and cause them to slow down in the bloodstream[50]. PTX3 binds P-selectin and functions as a competitive inhibitor of the P-selectin-PSGL-1 interaction[135]. With this knowledge, my observation that PTX3 localizing to the margins of the cells may indicate that the alteration of PTX3 expression in neutrophils during the very early stages of neutrophil recruitment has a role in regulating neutrophil recruitment whether or not the protein is sequestered or released from the cell.

Because PTX3 is found to localize in specific granules of neutrophils of human and mouse[87, 88], it was expected I would find a similar expression in neutrophils from horse and cattle. Yet, in my study, I encountered species differences, validating the need to characterize the protein in these species. The PTX3 antibody clone PP-PPJ0069-00 (R&D Systems) did not react with equine PTX3 (not shown), suggesting the binding site for this antibody is unique on the equine PTX3 protein.

In conclusion, I have shown the presence of PTX3 in cattle and horse neutrophils. The conserved presence of PTX3 in multiple species and the lack of any documented natural cases of PTX3 deficiency is a testament to its crucial role in host survival. The alteration in expression of PTX3 in neutrophils of these domestic species is intriguing and, given what is currently known

about PTX3, may indicate roles for PTX3 in neutrophil migration, antimicrobial activity, and host defense.

CHAPTER 5: GENERAL DISCUSSION AND FUTURE DIRECTIONS

I have characterized the expression of PTX3 in normal and inflamed lungs of humans, horses, foals, cattle and pigs. To my knowledge, the expression of PTX3 has not been investigated in lungs of pigs, cattle, and foals prior to this study. However, the presence of PTX3 in lungs of horses affected by heaves has been reported[99]. The results from my study show similarities in the expression of PTX3 in the lungs of these species. For instance, in all species, PTX3 was observed the airway epithelium, alveolar septum, vascular endothelium and inflammatory cells, as shown by immunohistochemical staining. Since PTX3 is highly conserved from mouse to humans, it is not surprising that the expression profile was consistent across these species. However, some differences were observed. In the inflamed calf and foal lung, there appeared to be a decrease in the amount of positive staining in the vascular endothelium compared to control, an observation that was not found in the other species. Also, the amount of positive staining in normal 30 day old foal lung appeared much less than in other species or in inflamed foal lungs.

The apparent lower expression of PTX3 in lungs of normal 30-day old foals by immunohistochemistry is intriguing. Lack of Western blot or other quantitative data on lungs from foals does not permit me to conclude that there were differences in the amount of PTX3 produced in foal lungs. However, foals have long been characterized as immunodeficient, especially in regard to respiratory disease as evidenced by their extreme susceptibility to *Rhodococcus equi*[140]. If a lower basal expression of PTX3 exists, this could be a connected with the phenomenon of higher susceptibility to certain diseases as found in PTX3 knockout mice. PTX3-knockout mice exhibit worse lung injury after LPS administration[116] and more

susceptibility to *Aspergillus fumigatus* infection[103]. Alternatively, the lower basal expression could be due to a relatively naive lung in the very young animal. An older animal may have experienced more contact with environmental pathogens, dust and microbes compared to the foals, leading to an increased expression of PTX3 in the "normal" state.

To examine the sub-cellular localization of PTX3, I performed immuno-gold electron microscopy on normal and inflamed horse and cattle lungs. In normal horse lungs, I observed the presence of PTX3 in pulmonary intravascular macrophages, neutrophils, alveolar macrophages, and the airway epithelium. Similarly, PIMs in normal calf lungs and neutrophils and monocytes in lungs from calves infected with *M. hemolytica* also stained positively for the presence of PTX3. Endothelium from both species showed expression of PTX3. In the above-mentioned cells, the PTX3 was mainly localized to the cytoplasm and nuclei. Surprisingly, these experiments also illustrated the expression of PTX3 in platelets from a calf treated with *M. hemolytica*. Binding of PTX3 to the membrane of thrombin-activated platelets via P-selectin has previously been shown[135], but the intracellular expression of PTX3 observed in platelets is unexpected. The observation of PTX3 present in the platelets may be due to PTX3 binding to the surface of the platelets, but this is unlikely since the tissue has been sectioned, cutting through the cell membrane. It seems more likely that the presence of PTX3 is intracellular. Though they are often underestimated, platelets are critical regulators in inflammation. For instance, they migrate to sites of infection, express other pattern recognition receptors, such as TLRs that recognize microbial components, contain microbicidal molecules and generate reactive oxygen species[141]. Platelets also have the ability to internalize circulating proteins[142] and microorganisms[143]. The observation that PTX3 is found in platelets may be

a result of constitutive expression of PTX3 by platelets or a species-specific expression. Alternatively, it is possible that they have internalized circulating PTX3. If this is the case, other researchers that have studied the expression of PTX3 in platelets may have missed this observation if platelets were isolated from healthy individuals, with low levels of circulating PTX3.

Another interesting feature observed as a result of the immuno-gold EM was the presence of PTX3 in the nucleus of several cells. No previously published studies have noted this characteristic or speculated its significance. The related pentraxins, CRP and SAP, are known to translocate to the nucleus of cells[144]. The nuclear pore allows proteins 40kD and smaller into the nucleus, but larger proteins require the presence of a nuclear translocation signal to permit their entry[144]. A nuclear translocation signal has been described for CRP and SAP[144], but not yet for PTX3. Because PTX3 shares its ability to bind DNA, chromatin and histones with CRP and SAP in addition to the finding of PTX3 in the nucleus, it is possible a similar nuclear localization signal exists for PTX3. Previous studies by our lab have shown the localization of other pattern recognition receptors, TLR4 and TLR9, to the nucleus following LPS insult[145-147].

Because neutrophils are important inflammatory cells and their recruitment into lungs is a hallmark of pulmonary disease, I studied the expression of PTX3 in normal and activated neutrophils in two species, cattle and horses. Cattle and horses are often afflicted with respiratory diseases that are difficult to treat, and the expression of PTX3 has not been characterized in these species. Thus, these species were relevant to my study. I show the expression of PTX3 in cattle and horse neutrophils with Western blotting and

immunohistochemical staining. The methods used are subjective and semi-quantitative. However, I observed an apparent alteration in the expression of PTX3 in cattle and horse neutrophils upon stimulation with LPS. Methods to quantify the change in expression must be performed. ELISA could be utilized to measure the intracellular concentration of PTX3 in neutrophil lysates or extracellular release in neutrophil supernatants. Quantitative real-time reverse transcriptase PCR would allow us to identify the presence of mRNA and, if present, to measure changes in the regulation of mRNA.

A limitation of this study is the lack of quantitative data. Although informative of the expression of PTX3 in isolated cells and tissues, Western blot is only semi quantitative, allowing the comparison of "fold changes" in expression of the protein in relation to other groups. Next, I anticipated a greater difference in the expression of PTX3 in isolate cattle and horse neutrophils. The methods used were not sensitive enough to show changes in the expression of PTX3.

Although our understanding of PTX3 and its roles in inflammation has greatly broadened over the past two decades, there are still questions that remain to be answered. My research has uncovered some unexpected results such as the presence of PTX3 in platelets. Research on the involvement of platelets in inflammatory diseases is gaining momentum. As mentioned above, they are no longer considered unspecialized cells only involved in the coagulation cascade.

Neutrophils are short-lived cells that are easily activated and are difficult to culture. Manipulations for *in vitro* studies often result in changes in the activation and metabolic state of these cells. Thus, results from *in vitro* studies of neutrophils tend to be inconsistent. Since there is not a consensus on the expression of PTX3 mRNA and protein by neutrophils, more research needs to be completed to resolve these issues.

In summary, I report the expression of PTX3 in alveolar and airway epithelium, vascular endothelium and inflammatory cells of normal and inflamed lungs of human, horse, foal, calf and pig. *In vitro* studies of alveolar epithelial cells have shown that they secrete PTX3 after stimulation with pro-inflammatory cytokines[81]. I postulate that, in the lung during acute inflammation, airway and alveolar epithelial cells secrete PTX3 and are a significant source of circulating PTX3. With the lung being a significant source of soluble PTX3, this would allow PTX3 to quickly reach the entire circulation since the pulmonary circulation reaches the rest of the body. Fluid-phase PTX3 may then go on to to bind the fluid-phase complement component C1q, resulting in an inhibition of C1q hemolytic activity or immobilized C1q (such is C1q bound to a microbial surface), activating the classical complement pathway.

Release of PTX3 by endothelium may result in binding of PTX3 to P-selectin, dampening the recruitment of neutrophils. Since neutrophils migrate rapidly to the site of inflammation and are a vessel for PTX3, I hypothesize that the role of PTX3 in neutrophils is to provide a mobile form of PTX3 that can be shuttled to the site of inflammation, where it can be discharged to opsonize bacteria, facilitating their uptake by macrophages. It is likely that PTX3 is released at the site of inflammation since it resides in specific granules, which are usually released after extravasation. In addition, PTX3 on the surface of apoptotic neutrophils increases the phagocytosis of these cells, aiding in the resolution of inflammation. Although I did not show release of PTX3 from neutrophils using these methods, it is likely that since I have shown PTX3 to be present in these cells, they will follow similar trends as other published studies.

Macrophages present in tissues release PTX3 in response to microbial recognition. Thus, opsonizing any microbes in the vicinity to aid in the recognition and phagocytosis by other phagocytes.

In conclusion, these cellular sources of PTX3 work together in acute lung inflammation to give rise to the primary functions of PTX3: complement activation, opsonization, agglutination and neutralization, clearance of cellular debris and apoptotic cells, regulation of inflammation and resolution of inflammation. There are so many pathways implicated in the pathogenesis of acute lung injury, that there is no doubt that modulating one aspect of these pathways will not be a cure for the disease. When one target is altered, other pathways come into play. However, increasing our knowledge of these pathways as a whole helps us to understand the complexity of these diseases and will aid in the development of potential therapeutics in the future.

LIST OF REFERENCES

1. Medzhitov R, Charles Janeway J: **Innate immune recognition: mechanisms and pathways**. *Immunological Reviews* 2000, **174**:89-97.
2. Bottazzi B, Bastone A, Doni A, Garlanda C, Valentino S, Deban L, Maina V, Cotena A, Moalli F, Vago L, Salustri A, Romani L, Mantovani A: **The long pentraxin PTX3 as a link among innate immunity, inflammation and female fertility**. *Journal of Leukocyte Biology* 2006, **79**:909-912.
3. Miles DG: **Overview of the North American beef cattle industry and the incidence of bovine respiratory disease (BRD)**. *American Health Research Reviews* 2009(10): 101-103.
4. Hodgins DC, Conlon JA, Shewen PE: **Respiratory Viruses and Bacteria in Cattle**. In: *Polymicrobial Diseases.* Edited by KA B, JM G. Washington, DC: ASM Press; 2002.
5. Smith RA: **Impact of disease on feedlot performance: A review**. *American Society of Animal Science* 1998(76):272-274.
6. Bowland SL, Shewen PE: **Bovine respiratory disease: Commercial vaccines currently available in Canada**. *Canadian Veterinary Journal* 2000(41):33-48.
7. Panciera RJ, Confer AW: **Pathogenesis and pathology of bovine pneumonia**. *Veterinary Clinics of North America: Food Animal Practice* 2010(26):191-214.
8. Rush BR, Mair T: **Equine Respiratory Diseases**. Oxford, UK: Blackwell Science Ltd.; 2004.
9. Timoney JF: **The pathogenic equine streptococci**. *Veterinary Research* 2004(35): 397-409.

10. Wheeler AP, Bernard GR: **Acute lung injury and the acute respiratory distress syndrome: a clinical review**. *Lancet* 2007, **369**:1553-1565.

11. Medzhitov R: **Origin and physiological roles of inflammation**. *Nature* 2008, **454**:428-435.

12. Fromer L, Cooper CB: **A review of the GOLD guidelines for the diagnosis and treatment of patients with COPD**. *International Journal of Clinical Practice* 2008, **62**(8):1219-1236.

13. Bernard GR, Artigas A, Brigham KL, Carlet J, Falke K, Hudson L, Lamy M, LeGall JR, Morris A, Spragg R: **Report of the American-European consensus conference on ARDS: defintions, mechanisms, relevant outcomes and clinical trial coordination**. *Intensive Care Medicine* 1993, **20**:225-232.

14. Rubenfeld GD, Herridge MS: **Epidemiology and outcomes of acute lung injury**. *Chest* 2007(131):554-562.

15. Zambon M, Vincent J-L: **Mortality rates for patients with acute lung injury/ARDS have decreased over time**. *Chest* 2008(133):1120-1127.

16. Doyle RL, Szaflarski N, Modin GW, Weiner-Kronish JP, Matthay MA: **Identification of patients with acute lung injury: Predictors of mortality**. *American Journal of Respiratory and Critical Care Medicine* 1995, **152**:1818-1824.

17. Rubin DB, Wiener-Kronish JP, Murray JF, Green DR, Turner J, Luce JM, Montgomery AB, Marks JD, Matthay MA: **Elevated von Willebrand Factor antigen is an early plasma predictor of acute lung injury in nonpulmonary sepsis syndrome**. *Journal of Clinical Investigation* 1990, **86**:474-480.

18. Chignard M, Balloy V: **Neutrophil recruitmment and increased permeability during acute lung injury induced by lipopolysaccharide**. *American Journal of Physiology - Lung Cellular and Molecular Physiology* 2000(279):L1083-L1090.

19. Abraham E: **Neutrophils and acute lung injury**. *Critical Care Medicine* 2003, **31**:S195-S199.

20. Warren HS, Fitting C, Hoff E, Adib-Conquy M, Beasley-Topliffe L, Tesini B, Liang X, Valentine C, Hellman J, Hayden D, Cavaillon J-M: **Resilience to bacterial infection: difference between species could be due to proteins in serum**. *Journal of Infectious Diseases* 2010, **201**:223-232.

21. Rittirsch D, Flierl MA, Day DE, Nadeau BA, McGuire SR, Hoesel LM, Ipaktchi K, Zetoune FS, Sarma JV, Leng L, Huber-Lang MS, Neff TA, Bucala R, Ward PA: **Acute lung injury induced by lipopolysaccharide is independent of complement activation**. *Journal of Immunology* 2008, **180**:7664-7672.

22. Brower RG, Ware LB, Berthiaume Y, Matthay MA: **Treatment of ARDS**. *Chest* 2001, **120**:1347-1367.

23. Matthay MA, Zimmerman GA: **Acute lung injury and the acute respiratory distress syndrome: four decades of inquiry into pathogenesis and rational management**. *American Journal of Respiratory Cell and Molecular Biology* 2005, **33**:319-327.

24. Zecchinon L, Fett T, Desmecht D: **How *Mannheimia haemolytica* defeats host defence through a kiss of death mechanism**. *Veterinary Research* 2005, **36**:133-156.

25. Davis EG: **Respiratory Infections**. In: *Equine Infectious Diseases. Volume 1*. Edited by Sellon DC, Long M. St. Louis, Missouri: Saunders Elsevier; 2007.

26. Proudman CJ, Edwards GB, Barnes J, French NP: **Factors affecting long-term survival of horses recovering from surgery of the small intestine**. *Equine Veterinary Journal* 2005, **37**:360-365.

27. Jassim RA, Andrews FM: **The bacterial community of the horse gastrointestinal tract and its relation to fermentative acidosis, laminitis, colic and stomach ulcers**. *Veterinary Clinics of North America: Equine Practice* 2009, **25**:199-215.

28. Traub-Dargatz JL, Kopral CA, Seitzinger AH, Garber LP, Forde K, White NA: **Estimate of the national incidence of and operation-level risk factors for colic among horses in the United States, spring 1998 to spring 1999**. *Journal of the American Veterinary Association* 2001, **219**:67-71.

29. Archer DC, Proudman CG: **Epidemiological clues to preventing colic**. *The Veterinary Journal* 2006, **172**:29-39.

30. Werners AH, Bull S, Fink-Gremmels J: **Endotoxaemia: a review with implications for the horse**. *Equine Veterinary Journal* 2005, **37**:371-383.

31. MacDonald MH, Pascoe JR, Stover SM, Meagher DM: **Survival after small intestine resection and anastomosis in horses**. *Veterinary Surgery* 1989, **18**:415-423.

32. Hondalus MK: **Pathogenesis and virulence of *Rhodococcus equi***. *Veterinary Microbiology* 1997, **56**:257-268.

33. Prescott JF: ***Rhodococcus equi*: an animal and human pathogen**. *Clinical Microbiology Reviews* 1991, **4**(1):20-34.

34. Garlanda C, Bottazzi B, Bastone A, Mantovani A: **Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility**. *Annual Review of Immunology* 2005, **23**:337-366.

35. Medzhitov R, Charles Janeway J: **Innate immunity: the virtues of a nonclonal system of recognition**. *Cell* 1997, **91**:295-298.

36. Medzhitov R: **Toll-like receptors and innate immunity**. *Nature Reviews Immunology* 2001, **1**:135-145.

37. Bottazzi B, Doni A, Garlanda C, Mantovani A: **An integrated view of humoral innate immunity: pentraxins as a paradigm**. *Annual Review of Immunology* 2010, **28**:157-183.

38. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S: **Cutting Edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: Evidence for TLR4 as the *Lps* gene product**. *Journal of Immunology* 1999, **162**:3749-3752.

39. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S: **Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components**. *Immunity* 1999, **11**:443-451.

40. Schwander R, Dziarski R, Wesche H, Rothe M, Kirschning CJ: **Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2**. *Journal of Biological Chemistry* 1999, **274**(June 18):17406-17409.

41. Poltorak A, He X, Smirnova I, Liu M-y, Huffel CV, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B: **Defective LPS**

signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in the *Tlr4* gene. *Science* 1998, **282**:2085-2088.

42. Jerala R: **Structural biology of the LPS recognition**. *International Journal of Medical Microbiology* 2007, **297**:353-363.

43. Bainton DF, Ullyot JL, Farquhar MG: **The development of neutrophilic polymorphonuclear leukocytes in human bone marrow**. *Journal of Experimental Medicine* 1971, **134**:907-934.

44. Kolaczkowska E, Kubes P: **Neutrophil recruitment and function in health and inflammation**. *Nature Reviews Immunology* 2013, **13**:159-175.

45. Vietinghoff Sv, Ley K: **Homeostatic regulation of blood neutrophil counts**. *Journal of Immunology* 2008, **181**:5183-5188.

46. **The Merck Veterinary Manual**. Whitehouse Station, N.J., U.S.A.: Merck Sharp & Dohme Corp.; 2012.

47. Brown KA, Brain SD, Pearson JD, Edgeworth JD, Lewis SM, Treacher DF: **Neutrophils in development of multiple organ failure in sepsis**. *Lancet* 2006, **368**:157-169.

48. Borregaard N, Cowland JB: **Granules of the human neutrophilic polymorphonuclear leukocyte**. *Blood* 1997, **89**:3503-3521.

49. Faurschou M, Borregaard N: **Neutrophil granules and secretory vesicles in inflammation**. *Microbes and Infection* 2003, **5**:1317-1327.

50. Borregaard N: **Neutrophils, from marrow to microbes**. *Immunity* 2010, **33**:657-670.

51. Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A: **Neutrophil function: from mechanisms to disease**. *Annual Review of Immunology* 2012, **30**:459-489.

52. Babior BM: **NADPH oxidase**. *Current Opinion in Immunology* 2004, **16**:42-47.

53. Babior BM, Lambeth JD, Nauseef W: **The neutrophil NADPH oxidase**. *Archives of Biochemistry and Biophysics* 2002, **397**:342-344.

54. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, Weinrauch Y, Brinkmann V, Zychlinsky A: **Novel cell death program leads to neutrophil extracellular traps**. *Journal of Cell Biology* 2007, **176**(2):231-241.

55. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A: **Neutrophil extracellular traps kill bacteria**. *Science* 2004, **303**:1532-1535.

56. Saikumar P, Mikhailov V, Denton M, Weinberg JM, Venkatachalam MA: **Apoptosis: definition, mechanisms, and relevance to disease**. *American Journal of Medicine* 1999, **107**:489-506.

57. Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM, Haslett C: **Macrophage phagocytosis of aging neutrophils in inflammation: programmed cell death in the neutrophil leads to its recognition my macrophages**. *Journal of Clinical Investigation* 1989, **83**:865-875.

58. Silva MT: **Bacteria-induced phagocyte secondary necrosis as a pathogenicity mechanism**. *Journal of Leukocyte Biology* 2010, **88**:885-896.

59. Brinkmann V, Zychlinsky A: **Beneficial suicide: why neutrophils die to make NETs**. *Nature Reviews Microbiology* 2007, **5**:577-582.

60. Goodman A, Cardozo T, Abagyan R, Altmeyer A, Wisniewski H, Vilcekt J: **Long pentraxins: an emerging group of proteins with diverse functions**. *Cytokine and Growth Factor Reviews* 1996, **7**(2):191-202.

61. Tillett WS, Thomas Francis J: **Serological reactions in pneumonia with a non-protein somatic fraction of pneumococcus**. *Journal of Experimental Medicine* 1930:561-571.

62. Gotschlich EC: **C-reactive protein: a historical overview**. *Annals New York Academy of Sciences* 1989, **557**:9-18.

63. Zahedi K, Whitehead AS: **Acute phase induction of mouse serum amyloid P component: correlation with other parameters of inflammation**. *Journal of Immunology* 1989, **143**(9):2880-2886.

64. Pepys MB, Baltz M, Gomer K, Davies AJS, Doenhoff M: **Serum amyloid P-component is an acute-phase reactant in the mouse**. *Nature* 1978, **278**:259-261.

65. Kushner I, Feldmann G: **Control of the acute phase response: demonstration of C-reactive protein synthesis and secretion by hepatocytes during acute inflammation in the rabbit**. *Journal of Experimental Medicine* 1978, **148**(2):466-477.

66. Castell J, Gómez-Lechón MJ, David M, Andus T, Geiger T, Trullenque R, Fabra R, Heinrich PC: **Interleukin-6 is the major regulator of acute phase protein synthesis in adult human hepatocytes**. *Federation of European Biochemical Societies Letters* 1989, **242**(2):237-239.

67. Gewurz H, Zhang H, Lint T: **Structure and function of the pentraxins**. *Current Opinion in Immunology* 1995, **7**:54-64.

68. Emsley J, White HE, O'Hara BP, Oliva G, Srinivasan N, Tickle IJ, Blundell TL, Pepys MB, Wood SP: **Structure of pentameric human serum amyloid P componene**. *Nature* 1994, **367**:338-345.

69. Oliveira EB, Gotschlich EC, Liu T-y: **Primary structure of human C-reactive protein**. *Journal of Biological Chemistry* 1979, **254**:489-502.

70. Osmand AP, Friedenson B, Gewurz H, Painter RH, Hofmann T, Shelton E: **Characterization of C-reactive protein and the complement subcomponent C1t as homologous proteins displaying cyclic pentameric symmetry (penraxins)**. *Proceedings of the National Academy of Sciences of the United States of America* 1977, **74**(2):739-743.

71. Clos TWD: **Function of C-reactive protein**. *Annals of Medicine* 2000, **32**(4):274-278.

72. Kindmark C-O: **Stimulating effect of the C-reative protein on phagocytosis of various species of pathogenic bacteria**. *Clinical Experimental Immunology* 1971, **8**:941-948.

73. Gershov D, Kim S, Brot N, Elkon KB: **C-reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains and antiinflammatory innate immune response: implications for systemic autoimmunity**. *Journal of Experimental Medicine* 2000, **192**(9):1353-1363.

74. Griselli M, Herbert J, Hutchinson WL, Taylor KM, Sohail M, Krausz T, Pepys MB: **C-reactive protein and complement are important mediators of tissue damage in acute myocardial infarction**. *Journal of Experimental Medicine* 1999, **190**(12):1733-1739.

75. Pepys MB, Dyck RF, Beer FCD, Skinner M, Cohen AS: **Binding of serum amyloid P-component (SAP) by amyloid fibrils**. *Clinical Experimental Immunology* 1979, **38**:284-293.

76. Lee TH, Lee GW, Ziff EB, Vilček J: **Isolation and characterization of eight tumor necrosis factor-induced gene sequences from human fibroblasts**. *Molecular and Cellular Biology* 1990, **10**(5):1982-1988.

77. Lee GW, Lee TH, Vilček J: **TSG-14, a tumor necrosis factor- and IL-1-inducible protein, is a novel member of the pentraxin family of acute phase proteins**. *Journal of Immunology* 1993, **150**:1804-1812.

78. Breviario F, d'Aniello EM, Golay J, Peri G, Bottazzi B, Bairoch A, Saccone S, Marzella R, Predazzi V, Rocchi M, Valle GD, Dejana E, Mantovani A, Introna M: **Interleukin-1-inducible genes in endothelial cells. Cloning of a new gene related to C-reactive protein and serum amyloid P component.** *Journal of Biological Chemistry* 1992, **267**(31):22190-22197.

79. Basile A, Sica A, d'Aniello E, Breviario F, Garrido G, Castellano M, Mantovani A, Martino I: **Characterization of the promotor for Human PTX3: Role of NF-{kappa}B in tumor necrosis factor-{alpha} and interleukin-1{beta} regulation**. *Journal of Biological Chemistry* 1997, **272**(13):8172-8178.

80. Alles V, Bottazzi B, Peri G, Golay J, Introna M, Mantovani A: **Inducible expression of PTX3, a new member of the pentraxin family, in human mononuclear phagocytes**. *Blood* 1994, **84**(10):3483-3493.

81. Han B, Mura M, Andrade C, Okutani D, Lodyga M, dos Santos C, Keshavjee S, Matthay M, Liu M: **TNFalpha-induced long pentraxin PTX3 expression in human lung epithelial cells via JNK**. *Journal of Immunology* 2005, **175**:8303-8311.

82. Doni A, Peri G, Chieppa M, Allavena P, Pasqualini F, Vago L, Romani L, Garlanda C, Mantovani A: **Production of the soluble pattern recognition receptor PTX3 by myeloid, but not plasmacytoid, dendritic cells**. *European Journal of Immunology* 2003, **33**(10):2886-2893.

83. Doni A, Michela M, Bottazzi B, Peri G, Valentino S, Polentarutti N, Garlanda C, Mantovani A: **Regulation of PTX3, a key component of humoral innate immunity in human dendritic cells: stimulation by IL-10 and inhibition by IFN-gamma**. *Journal of Leukocyte Biology* 2006, **79**(4):797-802.

84. Jaillon S, Peri G, Delneste Y, Fremaux I, Doni A, Moalli F, Garlanda C, Romani L, Gascan H, Bellocchio S, Bozza S, Cassatella MA, Jeannin P, Mantovani A: **The humoral pattern recognition receptor PTX3 is stored in neutrophil granules and localizes in extracellular traps**. *Journal of Experimental Medicine* 2007, **204**(4):793-804.

85. Imamura M, Kawasaki T, Savchenko AS, Ohashi R, Jiang S, Miyamoto K, Ito Y, Iwanari H, Sagara M, Tanaka T, Hamakubo T, Kodama T, Uchiyama M, Naito M: **Lipopolysaccharide induced expression of pentraxin 3 in human neutrophils and monocyte-derived macrophages**. *Cellular Immunology* 2007, **248**:86-94.

86. Savchenko AS, Inoue A, Ohashi R, Jiang S, Hasegawa G, Tanaka T, Hamakubo T, Kodama T, Aoyagi Y, Ushiki T, Naito M: **Long pentraxin 3 (PTX3) expression and**

release by neutrophils *in vitro* and in ulcerative colitis. *Pathology International* 2011, **61**:290-297.

87. Lominadze G, Powell DW, Luerman GC, Links AJ, Ward RA, McLeish KR: **Proteomic analysis of human neutrophil granules**. *Molecular and Cellular Proteomics* 2005, **4**:1503-1521.

88. Jaillon S, Jeannin P, Hamon Y, Fremaux I, Doni A, Bottazzi B, Blanchard S, Subra JF, Chevailler A, Mantovani A, Delneste Y: **Endogenous PTX3 translocates at the membrane of late apoptotic human neutrophils and is involved in their engulfment by macrophages**. *Cell Death and Differentiation* 2009, **16**(3):465-474.

89. Lee GW, Goodman AR, Lee TH, Vilček J: **Relationship of TSG-14 protein to the pentraxin family of major acute phase proteins**. *Journal of Immunology* 1994, **153**:3700-3707.

90. Mauri T, Bellani G, Patroniti N, Coppadoro A, Peri G, Cuccovillo I, Cugno M, Lapichino G, Gattinoni L, Pesenti A, Mantovani A: **Persisting high levels of plasma pentraxin over the first days after severe sepsis and septic shock onset are associated with mortality**. *Intensive Care Medicine* 2010, **36**(4):621-629.

91. Peri G, Introna M, Corradi D, Iacuitti G, Signorini S, Avanzini F, Pizzetti F, Maggioni AP, Moccetti T, Metra M, Dei Cas L, Ghezzi P, Sipe JD, Re G, Oliveti G, Mantovani A, Latini R: **PTX3, a prototypical long pentraxin, is an early indicator of acute myocardial infarction in humans**. *Circulation* 2000, **102**:636-641.

92. Suzuki S, Takeishi Y, Niizeki T, Koyama Y, Kitahara T, Sasaki T, Sagara M, Kubota I: **Pentraxin 3, a new marker for vascular inflammation, predicts adverse clinical outcomes in patients with heart failure**. *American Heart Journal* 2008, **155**(75-81):75.

93. Mauri T, Coppadoro A, Bellani G, Bombino M, Patroniti N, Peri G, Mantovani A, Pesenti A: **Pentraxin 3 in acute respiratory distress syndrome: an early marker of severity**. *Critical Care Medicine* 2008, **36**(8):2302-2308.

94. de Kruif MD, Limper M, Sierhuis K, Wagenaar JF, Spek CA, Garlanda C, Cotena A, Mantovani A, ten Cate H, Reitsma PH, van Gorp EC: **PTX3 predicts severe disease in febrile patients at the emergency department**. *Journal of Infection* 2010, **60**(2): 122-127.

95. Muller B, Peri G, Doni A, Torri V, Landmann R, Bottazzi B, Mantovani A: **Circulating levels of the long pentraxin PTX3 correlate with severity of infection in critically ill patients**. *Critical Care Medicine* 2001, **29**(7):1404-1407.

96. Han B, Ma X, Zhang J, Zhang Y, Bai X, Hwang DM, Keshavjee S, Levy GA, McGilvray I, Liu M: **Protective effects of long pentraxin PTX3 on lung injury in a severe acute respiratory syndrome model in mice**. *Laboratory Investigation* 2012, **92**:1285-1296.

97. He X, Han B, Bai X, Zhang Y, Cypel M, Mura M, Keshavjee S, Liu M: **PTX3 as a potential biomarker of acute lung injury: supporting evidence from animal experimentation**. *Intensive Care Medicine* 2010, **36**(2):356-364.

98. Souza DG, Amaral FA, Fagundes CT, Coelho FM, Arantes RM, Sousa LP, Matzuk MM, Garlanda C, Mantovani A, Dias AA, Teixeira MM: **The long pentraxin PTX3 is crucial**

for tissue inflammation after intestinal ischemia and reperfusion in mice. *American Journal of Pathology* 2009, **174**(4):1309-1318.

99. Ramery E, Fievez L, Fraipont A, Bureau F, Lekeux P: **Characterization of pentraxin 3 in the horse and its expression in airways**. *Veterinary Research* 2010, **41**(2):18.

100. Nauta A, Bottazzi B, Mantovani A, Salvatori G, Kishore U, Schwaeble WJ, Gingras AR, Tzima S, Vivanco F, Egido J, Tijsma O, Hack EC, Daha MR, Roos A: **Biochemical and functional characterization of the interaction between pentraxin 3 and C1q**. *European Journal of Immunology* 2003, **33**:465-473.

101. Manfredi AA, Rovere-Querini P, Bottazzi B, Garlanda C, Mantovani A: **Pentraxins, humoral innate immunity and tissue injury**. *Current Opinion in Immunology* 2008, **20**(5):538-544.

102. Salio M, Chimenti S, Angelis ND, Molla F, Maina V, Nebuloni M, Pasqualini F, Latini R, Garlanda C, Mantovani A: **Cardioprotective function of the long pentraxin PTX3 in acute myocardial infarction**. *Circulation* 2008, **117**(8):1055-1064.

103. Garlanda C, Hirsch E, Bozza S, Salustri A, De Acetis M, Nota R, Maccagno A: **Non-redundant role of the long pentraxin PTX3 in anti-fungal innate immune response**. *Nature* 2002, **420**:182-186.

104. Reading PC, Bozza S, Gilbertson B, Tate M, Moretti S, Job ER, Crouch EC, Brooks AG, Brown LE, Bottazzi B, Romani L, Mantovani A: **Antiviral activity of the long chain pentraxin PTX3 against influenza viruses**. *Journal of Immunology* 2008, **180**:3391-3398.

105. Varani S, Elvin JA, Yan C, DeMayo J, DeMayo FJ, Horton HF, Byrne MC, Matzuk MM: **Knockout of pentraxin 3, a downstream target of growth differentiation factor-9, causes female subfertility**. *Molecular Endocrinology* 2002, **16**:1154-1167.

106. Salustri A, Garlanda C, Hirsch E, De Acetis M, Maccagno A, Bottazzi B, Doni A, Bastone A, Mantovani G, Beck Peccoz P, Salvatori G, Mahoney DJ, Day AJ, Siracusa G, Romani L, Mantovani A: **PTX3 plays a key role in the organization of the cumulus oophorus extracellular matrix and in in vivo fertilization**. *Development* 2004, **131**(7): 1577-1586.

107. Gaipl US, Franz S, Voll RE, Sheriff A, Kalden JR, Herrmann M: **Defects in the disposal of dying cells leads to autoimmunity**. *Current Rheumatology Reports* 2004, **6**:401-407.

108. Baruah P, Dumitriu IE, Peri G, Russo V, Mantovani A, Manfredi AA, Rovere-Querini P: **The tissue pentraxin PTX3 limits C1q-mediated complement activation and phagocytosis of apoptotic cells by dendritic cells**. *Journal of Leukocyte Biology* 2006, **80**(1):87-95.

109. **Life and Breath: Respiratory Disease in Canada**. In: *Government of Canada Publications.* Ottawa: Public Health Agency of Canada; 2007.

110. Perrot M, Liu M, Waddell TK, Keshavjee S: **Ischemia-reperfusion-induced lung injury**. *American Journal of Respiratory and Critical Care Medicine* 2003, **167**:490-511.

111. López A: **Respiratory System, Mediastinum, and Pleurae**. In: *Pathologic Basis of Veterinary Disease.* 5th. Edited by Zachary JF, McGavin MD. St. Louis, Missouri: Elsevier; 2012.

112. Introna M, Alles VV, Castellano M, Picardi G, De Gioia L, Bottazzi B, Peri G, Breviario F, Salmona M, De Gregorio L, Dragani TA, Srinivasan N, Blundell TL, Hamilton TA, Mantovani A: **Cloning of mouse PTX3, a new member of the pentraxin gene family expressed at extrahepatic sites**. *Blood* 1996, **87**:1862-1872.

113. Nauta A, de Haij S, Bottazzi B, Mantovani A, Borrias M, Aten J, Rastaldi M, Daha MR, van Kooten C, Roos A: **Human renal epithelial cells produce the long pentraxin PTX3**. *Kidney International* 2005, **67**:543-553.

114. Moalli F, Jaillon S, Inforzato A, Sironi M, Bottazzi B, Mantovani A, Garlanda C: **Pathogen recognition by the long pentraxin PTX3**. *Journal of Biomedicine and Biotechnology* 2011:1-6.

115. Rovere P, Peri G, Fazzini F, Bottazzi B, Doni A, Bondanza A, Zimmermann V, Garlanda C, Fascio U, Grazia Sabbadini M, Rugarli C, Mantovani A, Manfredi AA: **The long pentraxin PTX3 binds to apoptotic cells and regulates their clearance by antigen-presenting dendritic cells**. *Blood* 2000, **96**(13):4300-4306.

116. Han B, Haitsma JJ, Zhang Y, Bai X, Rubacha M, Keshavjee S, Zhang H, Liu M: **Long pentraxin PTX3 deficiency worsens LPS-induced acute lung injury**. *Intensive Care Medicine* 2011, **37**(2):334-342.

117. Dias AAM, Goodman AR, Santos JLD, Gomes RN, Altmeyer A, Bozza PT, Horta MdF, Vilcek J, Reis LFL: **TSG-14 transgenic mice have improved survival to endotoxemia and to CLP-induced sepsis**. *Journal of Leukocyte Biology* 2001, **69**:928-936.

118. Souza DG, Soares AC, Pinho V, Torloni H, Reis LF, Martins MT, Dias AA: **Increased mortality and inflammation in tumor necrosis factor-stimulated gene-14 transgenic**

mice after ischemia and reperfusion injury. *American Journal of Pathology* 2002, **160**(5):1755-1765.

119. Zhang J, Shan L, Koussih L, Redhu NS, Halayko AJ, Chakir J, Gounni AS: **Pentraxin 3 (PTX3) expression in allergic asthmatic airways: Role in airway smooth muscle migration and chemokine production**. *PLOS ONE* 2012, **7**(4).

120. Singh B, Pearce JW, Gamage LN, Janardhan K, Caldwell S: **Depletion of pulmonary intravascular macrophages inhibits acute lung inflammation**. *American Journal of Physiology - Lung Cellular and Molecular Physiology* 2004, **286**:L363-:372.

121. Ellis JA, Gow SP, Goji N: **Response to experimentally induced infection with bovine respiratory syncytial virus following intranasal vaccination of seropositive and seronegative calves**. *Journal of the American Veterinary Association* 2010, **236**:991-999.

122. Parbhakar OP, Duke T, Townsend HGG, Singh B: **Immunophenotypic characterization and depletion of pulmonary intravascular macrophages of horses**. *Veterinary Research* 2004, **2004**(35):39-51.

123. Parbhakar OP, Duke T, Townsend HGG, Singh B: **Depletion of pulmonary intravascular macrophages partially inhibits lipopolysaccharide-induced lung inflammation in horses**. *Veterinary Research* 2005, **36**:557-569.

124. Kaur N: **Immune response in *Rhodococcus equi* infected foals**. Saskatoon: University of Saskatchewan; 2010.

125. Wilkins PA: **High-Risk Pregnancy**. In: *Equine Neonatal Medicine.* Edited by Paradis MR. Philadelphia, PA: Elsevier Inc.; 2006: 13-29.

126. Wassef A, Janardhan K, Pearce J, Singh B: **Toll-like receptor 4 in normal and inflamed lungs and other organs of pig, dog and cattle**. *Histology and Histopathology* 2004, **4**:1201-1208.

127. **Analyzing gels and Western blots with ImageJ** [http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/]

128. Nathan C: **Neutrophils and immunity: challenges and opportunities**. *Nature Reviews Immunology* 2006, **6**:173-182.

129. Ackermann MR, Brogden KA: **Response of the ruminant respiratory tract to *Mannheimia (Pasteurella) haemolytica***. *Microbes and Infection* 2000, **2**:1079-1088.

130. Morris DD: **Endotoxemia in horses: a review of cellular and humoral mediators involved in its pathogenesis**. *Journal of Veterinary Internal Medicine* 1991, **5**(3): 167-181.

131. Couëtil LL, Hoffman AM, Hodgson J, Buechner-Maxwell V, Viel L, Wood JLN, Lavoie J-P: **Inflammatory airway diseases of horses**. *Journal of Veterinary Internal Medicine* 2007, **21**:356-361.

132. Martin TR, Frevert CW: **Innate immunity in the lungs**. *Proceedings of the Amercan Thoracic Society* 2005, **2**:403-411.

133. Agrawal A, Singh PP, Bottazzi B, Garlanda C, Mantovani A: **Pattern recognition by pentraxins**. *Advances in Experimental Medicine and Biology* 2009, **653**(98-116):98.

134. Mantovani A, Valentino S, Gentile S, Inforzato A, Bottazzi B, Garlanda C: **The long pentraxin PTX3: a paradigm for humoral recognition molecules**. *Annals New York Academy of Sciences* 2013, **1285**:1-14.

135. Deban L, Russo RC, Sironi M, Moalli F, Scanziani M, Zambelli V, Cuccovillo I, Bastone A, Gobbi M, Valentino S, Doni A, Garlanda C, Danese S, Salvatori G, Sassano M, Evangelista V, Rossi B, Zenaro E, Constantin G, Laudanna C, Bottazzi B, Mantovani A: **Regulation of leukocyte recruitment by the long pentraxin PTX3**. *Nature Immunology* 2010, **11**(4):328-334.

136. Cook VL, Neuder LE, Blikslager AT, Jones SL: **The effect of lidocaine on *in vitro* adhesion and migration of equine neutrophils**. *Veterinary Immunology and Immunopathology* 2009, **129**:137-142.

137. Yamamori T, Inanami O, Nagahata H, Cui Y-D, Kuwabara M: **Roles of p38 MAPK, PKC and PI3-K in the signaling pathways of NADPH oxidase activation and phagocytosis in bovine polymorphonuclear leukocytes**. *Federation of European Biochemical Societies* 2000, **467**:253-258.

138. Allen L-AH: **Immunofluorescence and Confocal Microscopy of Neutrophils**. In: *Neutrophil Methods and Protocols.* Edited by Quinn MT, DeLeo FR, Bokoch GM, vol. 412: Humana Press; 2007: 273-287.

139. Maina V, Cotena A, Doni A, Nebuloni M, Pasqualini F, Milner CM, Day AJ, Mantovani A, Garlanda C: **Coregulation in human luekocytes of the long pentraxin PTX3 and TSG-6**. *Journal of Leukocyte Biology* 2009, **86**:123-132.

140. Yager JA: **The pathogenesis of *Rhodococcus equi* pneumonia in foals**. *Veterinary Microbiology* 1987, **14**:225-232.

141. Yeaman MR: **Platelets in defense against bacterial pathogens**. *Cellular and Molecular Life Sciences* 2010, **67**:525-544.

142. Handagma PJ, George JN, Shuman MA, McEver RP, Bainton DF: **Incorporation of a circulating protein into megakaryocyte and platelet granules**. *Proceedings of the National Academy of Sciences of the United States of America* 1987, **84**:861-865.

143. Yeaman MR: **The role of platelets in antimicrobial host defense**. *Clinical Infectious Diseases* 1997, **25**:951-970.

144. Clos TWD, Mold C, Stump RE: **Identification of a polypeptide sequence that mediates nuclear localization of the acute phase protein C-reactive protein**. *Journal of Immunology* 1990, **145**:3869-3875.

145. Schneberger D, Caldwell S, Suri SS, Singh B: **Expression of toll-like receptor 9 in horse lungs**. *The Anatomical Record* 2009, **292**:1068-1077.

146. Janardhan KS, McIsaac M, Fowlie J, Shrivastav A, Caldwell S, Sharma RK, Singh B: **Toll like receptor-4 expression in lipopolysaccharide induced lung inflammation**. *Histology and Histopathology* 2006, **21**:687-696.

147. Schneberger D, Caldwell S, Kanthan R, Singh B: **Expression of toll-like receptor 9 in mouse and human lungs**. *Journal of Anatomy* 2013, **222**:495-503.