EQUINE NEUTROPHIL APOPTOSIS IN INFLAMMATORY CONDITIONS

A Thesis Submitted to the College of

Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

In the Department of Veterinary Biomedical Sciences

University of Saskatchewan

Saskatoon

By

STACY ANDERSON

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
#52 Campus Drive, University of Saskatchewan
Saskatoon, Saskatchewan, S7N 5B4 Canada

Abstract

Horses are at high risk to develop systemic inflammation due to the release of bacterial endotoxin from an inflamed gastrointestinal tract. Neutrophils are critical for mounting an immune response to bacterial endotoxins. Neutrophil activation following engagement of bacterial endotoxin expands their lifespan through suppression of their constitutive apoptosis. The prolonged lifespan of neutrophils propagates acute inflammation and delays the resolution of inflammation. Since equine neutrophil lifespan has not been well-studied, I investigated the occurrence of equine neutrophil apoptosis *in vitro* and *in vivo*.

First, I investigated the effect of *Escherichia coli* lipopolysaccharide (LPS) treatment on the occurrence of equine neutrophil apoptosis *in vitro*. LPS treatment delayed *in vitro* equine neutrophil apoptosis in a dose-dependent manner at concentrations of 0.1-10 μg/ml through toll-like receptor (TLR)-4 signaling and down-regulation of the intrinsic pathway of apoptosis, specifically through reduced caspase-9 activity.

Next, I found that *ex vivo* neutrophil apoptosis was delayed in two models of intestinal inflammation, jejunal ischemia and reperfusion (IR) and oligofructose-induced colitis, through down-regulation of both the intrinsic and extrinsic apoptosis pathways via reduced caspase-3, -8, and -9 activities. Pulmonary intravascular macrophages (PIMs) depletion with systemic gadolinium chloride (GC) prevented the prolongation of *ex vivo* neutrophil lifespan in horses undergoing jejunal IR through modulation of caspase-3, -8 and -9 activities. PIM depletion in IR horses resulted in an earlier and greater increase in tumor necrosis factor-alpha and a concomitant decrease in interleukin-10 to suggest an enhanced systemic pro-inflammatory response.

I examined the effect of neutrophil concentration and co-incubation with aged, apoptotic neutrophils on the occurrence of neutrophil apoptosis *in vitro*. Neutrophil apoptosis was delayed with increasing concentrations of neutrophils *in vitro*, which may contribute to delayed neutrophil apoptosis in systemic inflammation. However, co-incubation with aged, apoptotic neutrophils did not alter *in vitro* neutrophil lifespan.

Taken together, the data show that LPS delays equine neutrophils apoptosis *in vitro* in a TLR4-dependent manner through inhibition of caspase-9. *Ex vivo* neutrophil apoptosis was also delayed with systemic inflammation via down-regulation of caspase activity. A novel finding of this work was the reversal of delayed neutrophil apoptosis by depletion of PIMs in horses experiencing intestinal IR.

Acknowledgements

There are many people who have contributed to this body of work, both directly and indirectly. First, I would like to thank my supervisor, Dr. Baljit Singh, for being a fantastic mentor throughout my PhD program by allowing me to focus on areas I was interested in and giving me the freedom to develop my own research plans, while also providing meaningful intellectual support.

I would like to thank my committee members for their contribution to my program: Drs. Jaswant Singh and Ali Honamorooz, graduate chairs; Dr. Hugh Townsend, statistical and experimental design; Dr. Tanya Duke, anesthetist and co-investigator; and Dr. Matthew Loewen, program development. I would like to thank the departmental staff of the Veterinary Biomedical Sciences Department for their assistance throughout my program and the Equine Health Research Fund and the Ryan/Dubé family for funding my projects.

I would like to thank all of my fellow lab mates: Yadu Balachandran, Le Nguyen Phuong Khanh, Nicole House, Laura Johnson, Kaitlin Merkowsky, Michelle Townsend, and Vanessa Vrolyk for their guidance and assistance in the laboratory. I would also like to thank the summer veterinary students who were under my supervision during my program, Leah Quanstrom, Alex Robinson, and Jacqueline Harrison, for their assistance with my various projects.

I would like to thank my parents, Brad and Eileen Thacker, for their sage advice and mentoring during this process. Lastly, I would like to thank my husband, Kevin, for being my voice of reason, pillar of support, and escape from the pressures of a graduate program.

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

Apaf-1	Apoptotic protease-activating factor 1
ATL	Aspirin-triggered lipoxin
Bad	Bcl-2-associated death promoter protein
Bak	Bcl-2 homologous antagonist/killer protein
BALF	Bronchoalveolar lavage fluid
Bax	Bcl-2 associated X protein
Bid	Bcl-2 homology-3-interacting domain death agonist protein
Bik	Bcl-2 killer protein
Bim	Bcl-2 interacting protein
Bcl	B cell lymphoma
C5a	Complement 5a
CDK	Cyclin-dependent kinase
CEL	Celiotomy
COX	Cyclooxygenase
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GA	Gambogic acid
GC	Gadolinium chloride
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFN	Interferon
IL	Interleukin
IR	Ischemia and reperfusion
IRGC	Ischemia and reperfusion plus administration of gadolinium chloride
LOX	Lipooxygenase

LPS	Lipopolysaccharide
LX	Lipoxin
MAPK	Mitogen-activated protein kinase
Mcl	Myeloid cell lymphoma
MNDA	Myeloid nuclear differentiation antigen
MODS	Multi-organ dysfunction syndrome
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
PBS	Phosphate buffered saline
PG	Prostaglandin
PIM	Pulmonary intravascular macrophage
PMA	Phorbol myristate acetate
PRR	Pathogen recognition receptor
RAO	Recurrent airway obstruction
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
SIRS	Systemic inflammatory response syndrome
Stauro	Staurosporine
TLR	Toll-like receptor
TNF	Tumor necrosis factor
XIAP	X-linked inhibitor of apoptosis

Chapter 1: Review of the Literature

1.1 Introduction

The innate immune response has evolved to effectively handle immediate threats, be it pathogenic or physical, to the body. Upon introduction of a foreign entity, immune cells within the vicinity recognize the threat using pattern-recognition receptors (PRR), such as Toll-like receptors (TLR), and initiate a coordinated response to neutralize the threat. Neutrophils serve as one of the primary effector cells of this immediate immune response. They may be activated by cytokines and chemokines released from resident macrophages, or primarily activated through PRRs. Upon activation, they are involved in neutralizing pathogens via phagocytosis and oxidative burst. They also potentiate the immune response by releasing cytokines and chemokines to attract more immune cells, primarily neutrophils, into the area.

As a sequela to activation by various stimuli, neutrophil life span is prolonged (Savill et al. 2002). Normally, neutrophils are short-lived cells, undergoing constitutive apoptosis at the end of their lives followed by their removal via phagocytosis by macrophages residing in the spleen, liver, and bone marrow (Akgul et al. 2001, Jia et al. 2008, Edwards et al. 2004, Tak et al. 2013). Prolongation of neutrophil lifespan conserves the neutrophil population in times of need, such as during sepsis, but at the same time can prove detrimental due to the non-specific method by which neutrophils neutralize threats through the release of histotoxic contents that induces further inflammatory cell infiltration (Colotta et al. 1992, Jia et al. 2008, Milot et al. 2012, Sano et al. 2005, Savill et al. 2002, Sweeny et al. 1998, Yamamoto et al. 1993). Additionally, the phagocytosis of apoptotic neutrophils causes the polarization of macrophage phenotype from an inflammatory to anti-inflammatory (Fadok et al. 1998, Huynh et al. 2002, Lucas et al. 2003).

Taken together, delayed neutrophil apoptosis contributes to the potentiation of an inflammatory response, possibly to the detriment of the body.

Targeting neutrophil apoptosis may prove to be an effective mechanism to control acute inflammation and promote healing during multiple disease processes; but before neutrophil apoptosis is used as a therapeutic target, the mechanisms by which neutrophil apoptosis is delayed in various situations must be understood.

1.2 Constitutive neutrophil apoptosis

Apoptosis is also defined as programmed cell death and involves various cellular pathways that ultimately cause nuclear DNA fragmentation and cessation of cellular processes without disturbing tissue homeostasis. There are two classical cellular pathways by which a cell may undergo apoptosis. The extrinsic pathway involves activation of membrane receptor proteins by an extracellular signal, such as tumor necrosis factor-alpha (TNF-α) or Fas ligand, that initiates a cascade of enzymatic reactions that are propagated by proteases called initiator caspases (e.g. caspase-8). Alternatively, the intrinsic pathway involves an intracellular signal, such as irreparable damage to DNA or severe cell stress, which changes the balance of the B cell lymphoma (Bcl)-2 family of proteins to increase mitochondrial permeabilization, allowing release of cytochrome *c* into the cytosol and activation of the apoptosome and initiator caspase-9. The extrinsic and intrinsic pathways are also linked through activation of the pro-apoptotic Bcl-2 homology-3-interacting domain death agonist protein (Bid) by caspase-8. As a final step in both pathways, the effector caspases, caspase-3, -6, and -7, enter the nucleus and cause DNA fragmentation leading to characteristic changes in nuclear morphology including karyorrhexis and pyknosis (Savill et al. 1989, Kamada et al. 2005). The effector caspases also activate

proteins that dissemble components of the cytoskeleton, such as actin, resulting in cell shrinkage and blebbing (Brown et al. 1997, Knepper-Nicolai et al. 1998).

Neutrophils undergo constitutive apoptosis via the intrinsic pathway at the end of their normal lifespan without the influence of extracellular signals (Akgul et al. 2001, Fox et al. 2010). Neutrophil lifespan is thought to be regulated by the balance between pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family (Figure 1.1), particularly myeloid cell lymphoma (Mcl)-1 (Fox et al. 2010, Edwards et al. 2004). In homeostatic conditions, there is a greater concentration of pro-apoptotic proteins compared to anti-apoptotic proteins to result in a shortened life span (Akgul et al. 2001). The phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signalling pathways are thought to play a role in regulation of Bcl-2 protein production through nuclear factor kappa (NFκ)-B activation, accounting for changes in neutrophil lifespan during inflammatory conditions (Akgul et al. 2001).

The Bcl-2 family of proteins is responsible for maintaining mitochondrial membrane potential. There are two arms to this protein family. One arm promotes apoptosis and includes Bid protein, Bcl-2 associated X protein (Bax), Bcl-2 homologous antagonist/killer protein (Bak), Bcl-2-associated death promoter protein (Bad), Bcl-2 interacting protein (Bim), and Bcl-2 killer protein (Bik). The other arm prevents apoptosis and includes Bcl-2, Bcl-xL, Mcl-1, and A1. In human neutrophils, Mcl-1 has been identified as the most important inhibitor of neutrophil apoptosis, especially in response to LPS treatment as discussed later in this review (Edwards et al. 2004, François et al. 2005, Wardle et al. 2011). The apoptotic state of a neutrophil relies on the balance of these proteins. In normal neutrophils, anti-apoptotic proteins have a rapid turnover compared to the pro-apoptotic proteins, which tends to keep the balance shifted toward apoptosis, making neutrophil lifespan relatively short (Geering and Simon 2011). With the

balance in favor of the pro-apoptotic members of the Bcl-2 family, mitochondrial membrane potential is lost leading to the release of cytochrome *c* into the cytosol and activation of the apoptosome, apoptotic protease-activating factor 1 (Apaf-1), and caspase-9 that initiates caspase-3 to induce apoptosis. Neutrophil mitochondria are unique in that they are primarily involved in death-mediated processes, and less so in energy production (Maianski et al. 2004). It has been demonstrated that neutrophils may undergo caspase-9 activation and subsequent apoptosis, independent of mitochondrial permeabilization and cytochrome *c* activation of the apoptosome as part of an alternative intrinsic pathway, which may also contribute to their relatively short lifespan compared to other leukocytes (Maianski et al. 2004).

Cell death by apoptosis provides a means by which a cell may die while maintaining the integrity of its membrane, thereby preventing the release of its cellular contents. Once a neutrophil has become apoptotic, its membrane undergoes changes, such as externalization of phosphatidylserine, to provide a signal to professional phagocytic cells, namely macrophages, to remove it from the circulation or tissue (Savill and Fadok 2000). Macrophages are then able to recognize apoptotic neutrophils and phagocytose them prior to the release of their histotoxic contents (Savill et al. 1989). Interestingly, CD14, a surface molecule traditionally involved in LPS activation of TLR4, is important for tethering non-inflamed apoptotic cells to macrophages during the course of phagocytosis (Devitt and Marshall 2011).

Concomitantly, neutrophil function is abrogated upon the induction of apoptosis. The neutrophil cytoskeleton is disabled as a result of apoptosis in order to maintain cell shape and prevent de-granulation and chemotaxis (Whyte et al. 1993, Brown et al. 1997). Respiratory burst and phagocytic functions are also impaired (Whyte et al. 1993). A hallmark of delayed

neutrophil apoptosis is the ability for neutrophils to maintain their capacity for effector functions, such as respiratory burst (Koedel et al. 2009, Watson et al.1997).

Another set of cytosolic proteases, called calpains, have also come under investigation for their role in constitutive neutrophil apoptosis. Calpains are regulated by intracellular calcium concentration and act as non-specific proteases to cleave target proteins that are involved with maintaining and regulating cytoskeleton structure (Knepper-Nicolai et al. 1998). Indeed, it has been shown that calpains are involved in multiple processes of neutrophil apoptosis including the processing of Bax, alteration of nuclear morphology, and phosphatidylserine exposure (Knepper-Nicolai et al. 1998, Geering and Simon 2011). Inhibition of calpain activity is associated with delayed neutrophil apoptosis (van Raam et al. 2008). Cathepsins are another type of protease, located in neutrophil granules, which are involved in apoptotic processes including the processing of caspase-8 and Bid that ultimately leads to activation of caspase-3 (Geering and Simon 2011). Recently, another cytosolic protease called serine protease proteinase 3 (PR3) was identified as playing a key role in caspase-3-dependent constitutive apoptosis of human neutrophils *in vitro* independent of caspase-8 or caspase-9 activation (Loison et al. 2014).

Neutrophils have other unique apoptotic pathways compared to other cells. Traditionally, tumor necrosis factor (TNF)-α initiates apoptosis via the extrinsic pathway. In neutrophils, this holds true for early or high concentration TNF-α exposure (Fox et al. 2010, Murray et al. 1997, Salamone et al. 2001). Additionally, exposure to high concentrations of TNF-α reduces Mcl-1 through increased turnover to induce apoptosis (Cross et al. 2008). However, late survival (after 12 h) is promoted simultaneously through activation of the NF-κB pathway and subsequent up-regulation of the anti-apoptotic members of the Bcl-2 family, particularly A1 (Cross et al. 2008). Another unique contribution to neutrophil apoptosis involves reactive oxygen species (ROS) that

are produced by the neutrophils themselves upon TNF-α stimulation. It is currently thought that most of the molecular pathways causing neutrophil apoptosis are dependent upon ROS generation; however, ROS can have alternative effects on neutrophils, such as interference with caspase activities causing delayed apoptosis or the initiation of cell necrosis rather than apoptosis (Fadeel et al. 1998, Geering and Simon 2011, Scheel-Toellner et al. 2004).

In conclusion, constitutive neutrophil apoptosis is not as straight-forward as other cell types, likely involving alternative pathways to the classically described pathways of apoptosis. Consequently, the process by which neutrophils undergo apoptosis is still under investigation in all species. Additionally, the process by which extravasated neutrophils undergo apoptosis in tissues remains largely unknown (Tak et al. 2013).

1.3 Models used to evaluate neutrophil apoptosis: *in vitro* and *in vivo*

Thus far, the most common method utilized to evaluate neutrophil apoptosis has been culturing isolated neutrophils *in vitro*. However, when evaluating neutrophil physiology *in vitro*, it is essential to create standard culture conditions due to the highly reactive nature of this cell type. One troublesome contaminant of neutrophil processing and culture is bacterial endotoxin, a substance that is ubiquitous in the environment and that requires additional steps to remove compared to traditional sterilization methods. The variability in results reported in the literature from studies on neutrophils is potentially due to unintentional activation with substances, such as LPS (Haslett et al. 1985). However, neutrophils can also become activated by seemingly innocuous stimuli, including changes in temperature, pipetting, and isolation technique (Freitas et al. 2008, Hannah et al. 1998, Haslett et al. 1985, Tennenberg et al. 1988, Watson et al. 1992).

Similar to other immune cells, functional *in vivo* studies on neutrophils are traditionally performed using knockout mice. Genetically modified mice have been used to evaluate the

contribution of various components of the known apoptosis pathways. For example, knockout mice for the various Bcl-2 family of proteins have been used to assess the relative importance and redundancy of each protein (Geering and Simon 2011). Though mice models are invaluable for studying cellular pathways, it must be understood that these models are substantially removed from the physiology of wild type mice or other species. Antibodies and silencing RNA may be used to selectively knockout proteins or genes in cells from non-laboratory animals to further study cellular pathways.

Studies have also been performed using humans and animals with known genetic defects of leukocytes, such as human chronic granulocytic leukemia and bovine leukocyte adhesion deficiency (Hasegawa et al. 2003, Naghata et al. 2004). Studies are also commonly performed using neutrophils isolated from humans and animals suffering from clinical diseases that involve neutrophil activation (Jimenez et al. 1997, Krista et al. 2013, McCracken and Allen 2014). Overall, models that use clinical disease are useful in evaluating neutrophil physiology and lifespan; however, the use of control neutrophils from normal humans and animals for comparison is less than ideal as there is significant variation among neutrophil response by individual (first author's unpublished observation).

1.4 Inflammatory mediators that delay neutrophil apoptosis *in vitro*

As primary effectors cells of the innate immune response, neutrophils are integral in neutralizing pathogenic and non-pathogenic threats to the body. Neutrophils may be activated by a variety of substances through PRRs, such as TLRs. PRR activation initiates intra-cellular signaling pathways involved in altering cellular processes, including apoptotic pathways, to neutralize the perceived threat. Alteration of apoptotic pathways may lead to prolongation of neutrophil lifespan via delayed apoptosis. This section will focus on inflammatory mediators that have been

identified in causing delayed neutrophil apoptosis *in vitro* and, if they are known, the proposed mechanisms by which these mediators act to delay neutrophil apoptosis.

Bacterial lipopolysaccharide (LPS) has been evaluated extensively for its effect on neutrophil lifespan both *in vitro*, and to a lesser extent, *in vivo*. It has been shown to consistently delay neutrophil apoptosis *in vitro* in multiple species, except the horse (Brazil et al. 2014, Colotta et al. 1992, Jia et al. 2008, Milot et al. 2012, Sweeny et al. 1998, Sano et al. 2005, Yamamoto et al. 1993). The mechanism of delayed neutrophil apoptosis involves a shifting of balance from pro-apoptotic to anti-apoptotic processes of the intrinsic pathway of apoptosis via TLR4 activation (Edwards et al. 2004, François et al. 2005, Figure 1.1). Upon TLR4 activation, two interrelated intracellular transcriptional pathways have been shown to be involved in LPS-delayed apoptosis including the NF-κB and PI3K pathways (François et al. 2005, Sweeney et al. 1998, Figure 1.1). These pathways are involved in regulating the balance of the Bcl-2 family of proteins at the transcriptional level. Specifically, the anti-apoptotic protein Mcl-1 has been shown to be up-regulated to tip the balance of the Bcl-2 family of proteins into an anti-apoptotic state (Edwards et al. 2004, François et al. 2005). Within canine neutrophils, Bcl-xL was identified to be highly expressed in neutrophils exhibiting LPS-delayed apoptosis (Sano et al. 2005).

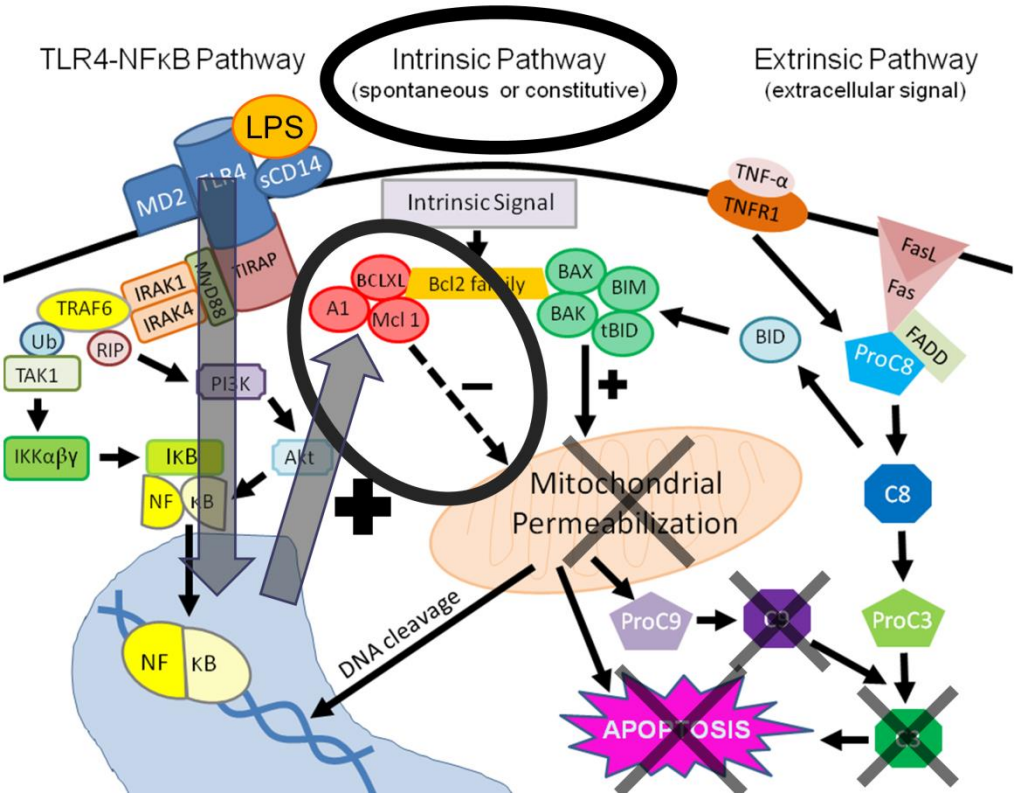


Figure 1.1 Potential mechanism of LPS-delayed neutrophil apoptosis

Other ligands of TLRs have also been implicated in causing delayed human neutrophil apoptosis *in vitro* (François et al., 2005, El Kebir et al. 2014, Sabroe et al. 2005). These include peptidoglycan (ligand for TLR2), flagellin (ligand for TLR5), R-848 (ligand for TLR7/8), and CpG DNA (ligand for TLR9, François et al., 2005, El Kebir et al. 2014, Salamone et al. 2010). Similar intracellular signalling pathways are activated compared to TLR4 signaling, resulting in increased concentrations of the anti-apoptotic Bcl-2 proteins Mcl-1 and A1 and phosphorylation of the pro-apoptotic Bcl-2 protein Bad (François et al., 2005, El Kebir et al. 2014).

Interleukin (IL)-1β is an important pro-inflammatory cytokine that is produced by granulocytes and mononuclear cells upon PRR activation. The production of IL-1β is a caspase-1-dependent process. In mononuclear cells, IL-1β production results in cell death via pyroptosis; but in neutrophils, IL-1β is produced without inducing pyroptosis (Chen et al. 2014). Additionally, IL-1β delays *in vitro* human neutrophil apoptosis via inhibition of Fas-mediated apoptosis by the IL-1β converting enzyme (caspase-1) (Colotta et al. 1992, Tatsuta et al. 1996, Watson et al. 1998).

Other cytokines and growth factors that delay *in vitro* neutrophil apoptosis include IL-2, IL-8, TNF-α, interferon (IFN)-γ, granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), and complement 5a (Colotta et al. 1992, Fox et al. 2010, Lee et al. 1993, Sakamoto et al. 2003). As discussed previously, TNF-α has variable effects on neutrophil apoptosis; however, it apparently increases the occurrence of apoptosis in the face of treatment with substances that typically delay neutrophil apoptosis, such as GM-CSF (Salamone et al. 2001). GM-CSF and G-CSF delay apoptosis through modulation of the intrinsic pathway of apoptosis as evidenced by reduced cytochrome *c* release and reduced caspase-3 and -9 activities (Molloy et al. 2005).

Adhesion molecules involved in neutrophil extravasation and activation also play a role in determining neutrophil lifespan. Cross-linking of adhesion molecules, such as β_2 integrin CD11b/CD18, activates neutrophils through outside-in signaling to produce and release ROS and myeloperoxidase (MPO). MPO, in turn, delays spontaneous neutrophil apoptosis via stabilization of mitochondria membrane potential and inhibition of the intrinsic pathway of apoptosis through ERK 1/2 and Akt signaling pathways (El Kebir et al. 2008), while ROS has variable effects on neutrophil apoptosis (Geering and Simon 2011). LPS also up-regulates β_2 integrins, contributing to delayed apoptosis, although cross-linking of β_2 integrins alone causes delayed apoptosis (Watson et al. 1997). Alternatively, apoptosis is delayed in neutrophils isolated from calves with bovine leukocyte adhesion deficiency that have CD18-deficient neutrophils and in CD18 knockout mice, both of which lack the ability to implement β2 integrin signaling pathways (Nagahata et al. 2004, Weinmann et al. 2003). In animals with dysfunctional CD18, neutrophils are unable to leave the bloodstream leading to a profound neutrophilia. It is likely that the increased concentration of neutrophils in this scenario is the cause of delayed apoptosis rather than a manifestation of the missing receptor (discussed later in this review). Another integrin, α9β1 integrin, also delays *in vitro* neutrophil apoptosis through the PI3K and MAPK pathways and NF-κB-dependent increased production of anti-apoptotic protein Bcl-xL (Saldanha-Gama et al. 2010). Vitronectin, a component of the extracellular matrix that modulates neutrophil adhesion and chemotaxis, also delays neutrophil apoptosis *in vitro* (Bae et al. 2012). Taken together, it is evident that activation of adhesion molecules leads to neutrophil activation and delayed apoptosis, which allows the prolongation of neutrophil lifespan as it enters into the inflammatory milieu outside of the circulation.

Pathogens themselves have a variable effect on human neutrophil apoptosis. The effect of bacteria on neutrophil apoptosis *in vitro* is dependent upon the bacterial species and strain, duration of exposure, multiplicity of infection, and the cell culture conditions (Fox et al. 2010). There are at least four possible outcomes following phagocytosis of bacteria by neutrophils. One outcome, which occurs after phagocytosis of most bacteria, results in phagocytosis-induced cell death and ROS production where neutrophils undergo apoptosis without releasing their cellular contents and are then cleared by macrophages (Kobayashi et al. 2003). A second outcome, which occurs after the phagocytosis of *Streptococcus pyogenes* by human neutrophils, results in rapid neutrophil death via necrosis with limited ROS production leading to pathogen release from the neutrophil and its survival (Kobayashi et al. 2003). A third outcome, which occurs after the phagocytosis of *Chlamydia pneumoniae* by human neutrophils, causes a temporary prolongation of neutrophil survival, likely mediated by LPS and IL-8, to allow the intracellular pathogen to multiply within the cell; after which time, the neutrophil undergoes apoptosis and an unsuspecting macrophage phagocytoses the apoptotic neutrophil with the hidden pathogen and polarizes to an anti-inflammatory phenotype (Fox et al. 2010). A fourth outcome has been described after phagocytosis of *Staphylococcus aureus*, where neutrophil lifespan may be prolonged or shortened based on the specific toxins carried by the bacteria (Fox et al. 2010). Taken together, it is obvious that neutrophil responses and lifespan may differ significantly by pathogen and may not relate directly to what occurs upon exposure to various components of a pathogen, such as LPS. Additionally, the behavior of neutrophils *in vivo* could potentially have even greater discrepancies than what has been observed *in vitro*.

1.5 Non-inflammatory causes of delayed neutrophil apoptosis

From the previous section, it is clear that exposure to inflammatory mediators and substances that promote neutrophil activation delay neutrophil apoptosis. However, the causes of delayed neutrophil apoptosis encompass even simpler mechanisms, including concentration- or density-dependent delayed neutrophil apoptosis, highlighting the highly reactive properties of neutrophils and their complex life and death balance.

A physiologic response to inflammation in the tissues or systemic inflammation is the accumulation of neutrophils through increased extravasation and increased release from the bone marrow, respectively. As neutrophils accumulate in tissues or the circulation, their concentration increases leading to their delayed apoptosis by a unique mechanism. This is in direct contrast to the majority of cell types that increase their apoptotic rate due to contact inhibition as their cell numbers increase. Overall, there has been very little research into concentration-dependent delayed neutrophil apoptosis. However, an elegant study performed by Attalah et al. (2012), demonstrated a clear relationship between increasing neutrophil concentration and delayed apoptosis *in vitro*. They identified the importance of cytosolic proteins S100A8 and S100A9 as having a clear role in delaying neutrophil apoptosis in highly concentrated cell cultures through CD11b/CD18 and TLR4 ligation and MAPK pathway activation without up-regulation of anti-apoptotic Bcl-2 proteins (Attalah et al. 2012).

Another group previously investigated the effect of neutrophil density and concentration on neutrophil lifespan *in vitro* (Hannah et al. 1998). This group determined that increased density was more influential than increased cell concentration on prolonging neutrophil lifespan *in vitro*, where the rate of apoptosis was inversely proportional to the number of cells per unit surface area. In addition, this group determined that density-delayed apoptosis was independent

of β2 integrin adhesion and that neutrophils did not secrete "survival factors" to delay apoptosis in a paracrine or autocrine fashion. They concluded that localized hypoxia and pH changes likely contributed to the effect of density-delayed apoptosis, although these parameters were not specifically tested (Hannah et al. 1998). The concept of concentration- or density-delayed neutrophil apoptosis should be considered in any disease process that causes an increase in circulating neutrophils, from acute inflammatory diseases to genetic leukocyte adhesion deficiency diseases.

A plethora of substances and culture conditions have been evaluated for their effect on neutrophil apoptosis *in vitro*, but only a selected few that are directly related to inflammation will be discussed herein. Hypoxia delays neutrophil apoptosis through up-regulation of Mcl-1 via the p38 MAPK and NF-κB signaling pathways through an unknown receptor (Hannah et al. 1995, Leuenroth et al. 2005, Walmsley et al. 2005). Additionally, neutrophils have a ferroprotein oxygen-sensing mechanism, comparable to the mechanism for erythropoietin regulation in kidney fibroblasts, which causes up-regulation of hypoxia inducible factor-1 to contribute to hypoxia-delayed apoptosis (Mecklenburgh et al. 2002). Most wounds are considered to be hypoxic due to disruption of blood supply. As first responders in the inflammatory response to a wound, neutrophils would be exposed to these hypoxic conditions in addition to inflammatory mediators, thus prolonging their lifespan and potentially prolonging the inflammatory stage of inflammation, which could delay healing. Therapeutic regimens, such as hyperbaric oxygen therapy, have been developed to address the hypoxic conditions within a wound or during inflammatory diseases with varying success indicating that the effects of hypoxia during inflammatory processes are complex (Kranke et al. 2015). A recent study reported that bovine neutrophil apoptosis was enhanced in neutrophils treated with hyperbaric oxygen compared to

cells cultured in normoxic and hypoxic conditions *in vitro* (Almzaiel et al. 2015). Wounds and inflammatory environments are also considered to be acidic and acidity (pH 6.5) has also been shown to delay neutrophil apoptosis (Negrotto et al. 2006). Alternatively, alkaline conditions promote rapid neutrophil apoptosis *in vitro* (Leblebicioglu et al. 1999).

Dexamethasone, a corticosteroid commonly used in veterinary practice, delays neutrophil apoptosis *in vitro* in multiple species via activation of the PI3K and MAPK pathways and up-regulation of Mcl-1 via NF-κB transcription (Barnes 2011, Brazil et al. 2014, Cox et al. 1997, Kettritz et al. 2006, Liles et al. 1995, Marwick et al. 2013, Saffar et al. 2008). However, the anti-apoptotic effect of dexamethasone on neutrophil apoptosis is abrogated in hypoxic conditions (<1% oxygen tension) to the point that it can rescue hypoxia- and GM-CSF-delayed neutrophil apoptosis (Marwick et al. 2013). This finding may have implications in ischemic disease processes that may be treated with glucocorticoids, but are characterized by extensive neutrophil accumulation. Additionally, the ability of glucocorticoids to prolong neutrophil lifespan has implications in acute inflammatory diseases where animals become stressed, further exacerbating delayed neutrophil apoptosis.

Similarly, non-steroidal anti-inflammatory drugs (NSAIDs) have also been shown to affect neutrophil apoptosis, although relatively few studies are available. Acetaminophen delayed human neutrophil apoptosis *in vitro* (Freitas et al. 2013); however, in another study, ibuprofen had no effect on LPS-delayed apoptosis, while aspirin and its metabolite, salicylate, rescued delayed neutrophil apoptosis due to multiple conditions including LPS and IL-1α treatment and hypoxia (Negrotto et al. 2006). Furthermore, humans who ingested aspirin had markedly reduced *ex vivo* LPS-delayed neutrophil apoptosis and mice pre-treated with aspirin had increased apoptotic neutrophils in their peritoneal cavity after a thioglycolate-induced peritonitis

model (Negrotto et al. 2006). Similarly, pre-treatment with aspirin reduced post-cardiopulmonary bypass delayed *ex vivo* neutrophil apoptosis (Bates et al. 2004). Another study evaluating neutrophil apoptosis after treatment with racemic mixtures of various NSAIDs demonstrated an increase in neutrophil apoptosis *in vitro* (Zielińska-Przyjemska et al. 2008).

Lastly, and a point of consideration when designing *in vivo* and *ex vivo* neutrophil experiments, a difference in the occurrence of neutrophil apoptosis has been observed between genders in humans (Molloy et al. 2003). Spontaneous neutrophil apoptosis was reduced in women compared to men mediated by estradiol and progesterone reducing cytochrome *c* release from the mitochondria (Molloy et al. 2003). Female sex hormones have been evaluated for their immunomodulatory effects due to the fact that women of reproductive age are less susceptible to sepsis and its accompanying complications; however, delayed neutrophils apoptosis would have a seemingly negative effect on septic process as discussed in a later section.

In conclusion, this section further highlights the complexities of neutrophil lifespan and demonstrates that the occurrence of neutrophil apoptosis in disease processes is affected by many factors. When evaluating neutrophil apoptosis in *in vivo* models, it is important to attempt to control for variables that might have a confounding effect on results.

1.6 Other immune cell involvement in neutrophil apoptosis

Macrophages play a critical role in managing circulating neutrophil concentration *in vivo*. As stated previously, macrophages in the spleen, liver, and bone marrow remove apoptotic neutrophils from the circulation. In a mouse model, where mice lacked macrophages in the spleen and bone marrow, the clearance of apoptotic neutrophils was delayed causing an increased production of G-CSF to drive granulopoiesis and delay neutrophil apoptosis (Gordy et al. 2011). Additionally, when macrophages fail to phagocytose neutrophils in CD18-deficient

mice that have delayed neutrophil apoptosis, they produce increased amounts of IL-23 that, in turn, drives IL-17-dependent granulopoiesis (Stark et al. 2005). These responses by macrophages could significantly contribute to a neutrophilic response during disease processes by increasing neutrophil concentration and further contributing to concentration-delayed neutrophil apoptosis. More work is needed to confirm these findings in wild type animals using *in vivo* inflammatory models.

Macrophages rely on the apoptosis of neutrophils to direct the resolution of inflammation (discussed below); however, they also play a key role in the induction of neutrophil apoptosis through the release of death receptor ligands, such as TNF-α and Fas-ligand (Brown and Savill 1999, Meszaros et al. 2000, Salamone et al. 2001). In a mouse wound model, macrophage-induced neutrophil apoptosis was dependent upon direct cell-to-cell contact via cell adhesion molecules, such as β3 integrin, and membrane-bound, not soluble, TNF-α (Meszaros et al. 2000). Similarly, in a mouse model of *Leishmania major* infection, *ex vivo* neutrophil apoptosis was positively correlated with the amount of co-cultured macrophages and was dependent on membrane-bound TNF (Allenbach et al. 2006).

Although not widely accepted, there is evidence that monocytes are necessary to facilitate neutrophils' responses to LPS, such as delayed apoptosis. One group reported a difference in *in vitro* LPS-delayed neutrophil apoptosis between isolated neutrophils cultured with and without contaminating monocytes (Sabroe et al. 2002). When monocytes were removed from neutrophil isolates via magnetic activated cell sorting, LPS failed to delay neutrophil apoptosis *in vitro*. The results of this study highlight an inherent problem when working with primary isolated cells. In general, purities of greater than 90% are considered to be acceptable when working with primary isolated neutrophils and the predominant contaminating cell types are other

granulocytes, such as eosinophils and basophils. Regardless, primary cell behavior may be altered by minute amounts of contaminating cell types unbeknownst to the investigator.

A unique type of macrophage, called the pulmonary intravascular macrophage (PIM), exists in the capillaries of the alveolar septal in select animals, including the horse (Brain et al. 1999, Aharonson and Singh 2010). Similar to Kupffer cells in the liver, PIMs are intimately associated with the endothelium of the alveolar capillaries where they phagocytose blood-borne foreign substances, pathogens, and apoptotic cells, including neutrophils (Brain et al. 1999). Because of their location, they are exposed to the entire blood volume as it passes through the alveolar capillaries for oxygen exchange. Therefore, PIMs are potentially influential cells in mounting a systemic inflammatory response (Aharonson and Singh 2010). *In vivo* studies in horses have demonstrated that significant pulmonary injury and inflammation occur in horses administered systemic lipopolysaccharide (LPS) and pulmonary intravascular macrophages (PIMs) are integral in potentiating these effects in the lung (Parbhakar et al. 2005). To the authors' knowledge, the involvement of PIMs in the regulation of neutrophil apoptosis during inflammatory conditions has not been investigated.

A recently identified pseudocytokine that is secreted and regulated by activated T lymphocytes, called pre-B cell colony enhancing factor, has also been identified for its role in inflammation-delayed neutrophil apoptosis (Jia et al. 2004, Sun et al. 2013). It has since been identified in a variety of tissues and cells, including the liver, lung, muscle, and leukocytes (Sun et al. 2013). In addition to its role in the development of B and T lymphocytes, PBEF interacts with multiple pro-inflammatory cytokines including IL-1β, IL-6, IL-8, and TNF-α. It acts on cellular processes through the PI3/MAPK pathway and NF-κB activation. In neutrophils, PBEF is synthesized and released upon activation and is associated with reduced caspase-3 and -8

activities, not caspase-9, in its role as an inhibitor of neutrophil apoptosis (Jia et al. 2004). Additionally, it is highly expressed in neutrophils isolated from septic human patients (Jia et al. 2004).

1.7 Apoptosis of neutrophils is required for the resolution of inflammation

One of the key elements of the inflammatory process is the ability for the immune system to switch from a pro-inflammatory state to and an anti-inflammatory state, thereby bringing about the resolution of inflammation and the initiation of healing. Macrophages are considered to be the orchestrators of this process. A fundamental signal for a macrophage to initiate the resolution of inflammation is to phagocytose apoptotic neutrophils. As discussed above, when an inflammatory process proceeds in an ideal manner, neutrophils undergo apoptosis after phagocytosing a pathogen or at the end of their short lifespan. Once neutrophils undergo apoptosis, they send out "eat me" signals or apoptotic cell-associated molecular patterns, attracting macrophages to them and initiating the process of efferocytosis (Savill et al. 1989). These signals involve changes in the neutrophil cell membrane, such as phosphatidylserine translocation to the outer leaflet of the plasma membrane, and may be modulated by charged glucose and amino acid molecules or pH (Fadok et al. 1992, Frasch et al. 2011, Huynh et al. 2002, Savill et al. 1989). A recent study identified the down-regulation of a surface receptor, CD47 as a signal for apoptotic neutrophil phagocytosis (Lawrence et al. 2009). Apoptosing neutrophils can express "find me" signals to attract macrophages, including lyophosphatidylserine, annexin-1, thrombospondin 1, and soluble IL-6R, or macrophages can secrete molecules to attract apoptosing neutrophils, such as MFG-8E (Silva 2011). The process by which apoptotic neutrophils and macrophages communicate to cause efferocytosis is still under investigation.

Macrophages bind to apoptotic neutrophils through bridging molecules, such as thrombospondin 1 via specific surface molecules, which have not been fully identified (Savill and Fadok 2000). A recent study identified the mechanism by which efferocytosis of apoptotic neutrophils is enhanced in macrophages (Frasch et al. 2011). Briefly, lysophophatidylserine, a form of phosphatidylserine produced by NADPH oxidase, signals to macrophages via a G-protein-coupled receptor, G2A, to enhance efferocytosis. G2A activation, in turn, enhances production of prostaglandin (PG) E_2 via a calcium-dependent cytosolic phospholipase A_2/cyclooxygenase (COX)-mediated mechanism within the macrophage. PGE_2 causes enhanced activity of Rac1 via adenyl cyclase and protein kinase A activation to increase macrophage efferocytosis of apoptotic neutrophils (Frasch et al. 2011).

Upon efferocytosis of apoptotic neutrophils, intracellular signaling blocks the release of pro-inflammatory mediators while up-regulating anti-inflammatory mediators to cause a change in macrophage phenotype from pro-inflammatory to anti-inflammatory (Fadok et al. 1998, Huynh et al. 2002). Specifically, the pro-inflammatory cytokines including IL-1β, IL-8, IL-10, GM-CSF, and TNF-α are inhibited while the robust anti-inflammatory cytokine TGF-β1 is maximized (Fadok et al. 1998). Additionally, it has been demonstrated that the cytosolic protein S100A9 is involved in suppression of the production of pro-inflammatory mediators nitric oxide (NO), hydrogen peroxide, and TNF-α and inhibition of macrophage phagocytic activity (De Lorenzo et al. 2010). Alternatively, a subset of macrophages may also undergo cell death by autophagy, apoptosis, or oncosis upon efferocytosis of apoptotic neutrophils; however the contribution of these processes to the resolution of inflammation is not well-understood (Wang et al. 2014).

Phagocytosis of neutrophils by macrophages, in the face of ongoing microbial infection, enhances macrophage killing abilities. Macrophages lack useful antimicrobial substances that neutrophils contain including cathelicidins, permeability-increasing protein, lactoferrin, and MPO (Silva 2010). Upon phagocytosis of apoptosing neutrophils, neutrophilic granules, or neutrophil apoptotic bodies, macrophages may adopt some portion of neutrophil antimicrobial function, thereby improving their destruction of invading microbes (Silva 2011).

In conclusion, the interaction of apoptotic neutrophils and macrophages is critical for orchestrating a concerted inflammatory response. In cases where these interactions are out of concert, asynchronous inflammation may ensue that could negatively affect the clearance of an infection and may contribute to an excessive inflammatory response that is detrimental to the body. Ultimately, the efferocytosis of apoptotic neutrophils shifts the balance of inflammation toward resolution and healing.

1.8 Neutrophil apoptosis associated with inflammatory disease

It has been well-established in human medicine that *ex vivo* neutrophil apoptosis is delayed in patients experiencing inflammatory diseases ranging from sepsis to inflammatory bowel disease (Hofman 2004). For this reason, modulating neutrophil apoptosis has become a focus of inflammation researchers, which will be discussed in a later section. However, before drugs can be developed to modulate neutrophil apoptosis, the mechanisms leading to delayed neutrophil apoptosis in the face of inflammatory conditions must be explicated, both in the circulating and extravasated neutrophil populations.

The systemic inflammatory response syndrome (SIRS) was a term developed by human practitioners to describe a set of clinical parameters they noticed in severely ill patients, including alterations in body temperature, heart rate, respiratory rate, white blood cell count, and

underlying pathologic mechanism (Bone et al. 1992). In general, patients must present with at least two abnormal parameters to be diagnosed with SIRS. The term SIRS has also been adopted by veterinary practitioners and is defined by the same criteria as for human patients. One of the most common causes of SIRS in humans and animals is sepsis, or in the case of horses, endotoxemia. It has come to the attention of the authors that the terms sepsis and endotoxemia are used to describe a patient's clinical presentation, where supporting diagnostics may be lacking. In these cases, SIRS is probably a more appropriate term. Nevertheless, it is well-established that neutrophil apoptosis is invariably delayed in patients experiencing SIRS (Fanning et al. 1999, Jimenez et al. 1997, Shang et al. 2007).

As stated previously, delayed neutrophil apoptosis has two major effects on inflammatory processes. First, an increase in number of neutrophils initiating an inflammatory response can cause collateral damage to nearby tissues due to their non-specific methods of killing resulting in further recruitment of neutrophils into a site of inflammation. Second, efferocytosis of apoptotic neutrophils by macrophages is a key component from shifting an inflammatory response from pro- to anti-inflammatory to initiate the resolution of inflammation. Taken together, delayed neutrophil apoptosis propagates an inflammatory response.

Delayed neutrophil apoptosis during SIRS is likely multifactorial due to the broad range of disease processes that can cause SIRS. Substances that have been identified in patients with SIRS and are also known inhibitors of neutrophil apoptosis include GM-CSF, IL-6, IL-1β, TNF-α, LPS, complement 5a (C5a), various pathogens, and Fas ligand (Fanning et al. 1999, Guo et al. 2006, Hofman 2004, Shang et al. 2007). Specifically, treatment with neutrophils isolated from healthy humans with plasma from patients with SIRS resulted in delayed *ex vivo* neutrophil apoptosis that was abrogated by co-incubation with a neutralizing antibody against GM-CSF

(Fanning et al. 1999). Interestingly, this group also demonstrated that neutrophil lifespan *in vitro* could be rescued by treating with recombinant IL-10 in the face of GM-CSF treatment via alteration of ROS generation (Fanning et al. 1999).

There has been a great deal of research evaluating neutrophil apoptosis in patients diagnosed with sepsis. C5a has been identified as playing a key role in delayed neutrophil apoptosis in septic rats and human patients through reduction of neutrophil C5a receptor (Guo et al. 2006, Unnewehr et al. 2013). C5a is a strong activator of neutrophils and has been shown to be up-regulated in multiple tissues in patients with sepsis (Riedemann et al. 2002). C5a has recently been evaluated as a potential biomarker for prognosticating the outcome of sepsis in human patients (Xu et al. 2015). C5a delays neutrophil apoptosis via the PI3K and MEK/ERK signaling pathways, Bad inactivation via phosphorylation, decreased Bim expression, increased Bcl-xL expression, and suppression of procaspase-3 and -9 cleavage into active caspase-3 and -9, respectively (Perianayagam et al. 2004, Guo et al. 2006). In addition, treatment of rats with anti-C5a antibody during a cecal-ligation-puncture model, abolished sepsis-delayed neutrophil apoptosis (Guo et al. 2006).

Another cause of delayed neutrophil apoptosis in septic patients involves the upregulation of Mcl-1 by myeloid nuclear differentiation antigen (MNDA), a protein that has been described as a "master regulator of monocytic and granulocytic lineages" (Milot et al. 2012). However, Paunel-Görgülü et al. (2009) demonstrated that *ex vivo* Fas stimulation can overcome Mcl-1-mediated delayed neutrophil apoptosis in neutrophils isolated from critically ill patients.

A hallmark of SIRS is alterations of white blood cell count, which can include leukopenia or leukocytosis with or without a left shift toward an increase in immature neutrophils. Immature neutrophils have a slightly retarded ability to perform normal functions in the face of

sepsis or SIRS, such as migration; however, they demonstrated the same capability to prolong their lifespan *ex vivo* following *in vivo* activation (Drifte et al. 2013). A trait that is not generally evaluated clinically in neutrophils from patients with SIRS is the presence of apoptotic neutrophils in circulation; however, a recent study demonstrated that in patients with severe trauma or hemorrhagic shock that required emergency surgery, increased neutrophil apoptosis in the early post-operative period was associated with a lower incidence of post-operative infection and, as expected, the occurrence of neutrophil apoptosis was inversely proportional to the severity of SIRS (Morrison et al. 2013). The authors of this study proposed that neutrophil apoptosis likely reduces early tissue injury, which then decreases the susceptibility to subsequent infection (Morrison et al. 2013). Taken together, neutrophil apoptosis, or lack thereof, plays a significant part in the development of SIRS and its associated sequelae during many disease processes.

1.9 The equine systemic inflammatory response syndrome

Similar to other species, equine SIRS is defined using clinical parameters with an underlying disease process including hyperthermia, tachycardia, tachypnea, leukocytosis or leukopenia with a left shift, with only two of these parameters needing to be present to make a diagnosis of SIRS (Epstein et al. 2011, Moore and Vandenplas 2014). Equine practitioners commonly use the terms "endotoxemia" and "SIRS" interchangeably, which has been brought to light by a recent review by Moore and Vandenplas (2014). Often time, horses are diagnosed as being "endotoxic," despite the fact that plasma endotoxin is rarely quantified in clinical cases. Indeed, in horses described clinically as being "endotoxemic," the sensitivity and specificity for the limulus amebocyte lysate assay, the assay of choice to identify plasma endotoxin, was only

58.4% and 87.5%, respectively (Senior et al. 2011). Therefore, we propose that the term SIRS should be used unless plasma endotoxin has been quantified.

Additional confusion in terminology involves the use of the term sepsis, which is defined by medical dictionaries as a bacterial infection in the tissues or bloodstream. In equine medicine, the term sepsis is used rigorously to define cases where bacteria have been cultured; whereas, the term sepsis appears to be used interchangeably with SIRS and endotoxemia in human medicine and basic research. Sepsis and endotoxemia both result in the development of SIRS, but SIRS can have other etiologies, such as extensive burns or other types of severe trauma independent of infection. A recent study identified activation of the MAPK pathway as a method to differentiate SIRS versus sepsis (West et al. 2007); however, we propose that the etiology of the disease needs to be considered when defining a disease. Ultimately, the discrepancy in terminology and evaluation of disease processes is important to realize and consider when assessing research on SIRS.

Equine SIRS can be caused by many disease processes; but by far, it is most commonly associated with gastrointestinal disease, otherwise known as colic. In horses admitted to a referral hospital for all types of colic, 28% had examination parameters meeting the SIRS criteria (Epstein et al. 2011). Traditionally, SIRS in colic patients is associated with the development of endotoxemia due to bacterial translocation after intestinal compromise from intestinal strangulation resulting in ischemia and reperfusion injury or from severe intestinal inflammation, such as acute colitis (Senior et al. 2011, Moore and Vandenplas 2014). However, it is likely that other factors come into play in the development of SIRS in these cases, one of which could include delayed neutrophil apoptosis. Regardless of the cause, the development of SIRS in

horses experiencing colic is associated with a significant increase in morbidity and mortality (Epstein et al. 2011, Moore and Vandenplas 2014).

The end organ affected in severe cases of SIRS that develops to multi-organ dysfunction (MODS) in horses tends to be the lamina within the hoof rather than internal organs, such as the lung that are affected in other species (Belknap et al. 2009, Suratt and Parsons 2006). Interestingly, horses do not develop laminitis from intravenous infusion of LPS alone, but only in cases where true gastrointestinal compromise has occurred, such as oligofructose-induced colitis (Belknap and Black 2012). The development of laminitis is one of the major causes of mortality in horses experiencing SIRS.

In conclusion, SIRS from multiple etiologies is a devastating process in the horse and requires further investigation into mechanisms that contribute to its development in order to develop an effective treatment regimen as currently utilized treatments are often ineffective. One method to investigate the innate immune response associated with the development of equine SIRS is to selectively deplete certain leukocytes. For example, it is possible to deplete intravascular macrophages using gadolinium chloride or deplete all types of macrophages using liposome-encapsulated bisphosphonates (Parbhakar et al. 2005, Van Rooijen and Sanders 1994). Experiments where selected leukocytes are depleted during established inflammatory models could contribute significantly to the advancement of knowledge of the innate immune response in horses, making the development of targeted therapeutics possible.

1.10 Equine neutrophil apoptosis

Neutrophil apoptosis in inflammatory conditions has recently gained attention by equine researchers (Brazil et al. 2014, Krista et al. 2013, Niedzwiedz et al. 2014, Turlej et al. 2001, Wauters et al. 2012). Brazil et al. (2014) evaluated constitutive equine neutrophil apoptosis *in*

vitro. As expected, neutrophils became apoptotic over time. Concurrently, neutrophils maintained their ability to generate respiratory burst upon phorbol myristate acetate (PMA), but not zymosan-activated serum (ZAS), a form of complement 5a. Expectedly, ZAS and dexamethasone inhibited apoptosis, but LPS unexpectedly increased the occurrence of neutrophil apoptosis in a dose-dependent manner. The authors of this study did not attempt to elucidate mechanisms of the various treatments for their effect on neutrophil apoptosis.

Neutrophil apoptosis has been evaluated in clinical scenarios in the horse. Wauters et al. (2012) demonstrated that neutrophils isolated from septic joints in clinical cases had greater viability than neutrophils isolated from healthy joints. Somewhat unexpectedly, neutrophils from septic joints had a higher percentage of apoptotic neutrophils while neutrophils from healthy joints had a higher percentage of dead neutrophils. An important consideration when interpreting the results of this study is that the septic joints were infected with multiple types of bacteria. It is known that microbial pathogens have different effects on neutrophil recruitment, activation, and lifespan depending on how they have adapted to evade the immune system (Fox et al. 2010, Kobayashi et al. 2003). In addition, it is important to distinguish between viable cells, where the cell membrane remains intact, but the stage of cell cycle may be unknown, compared to apoptotic cells, where the cell membrane is also intact, but with concomitant phosphatidylserine exposure indicating the induction of cell death via apoptotic pathways. These characteristics of cellular lifespan are discussed in the following section for their effect on the inflammatory process. Ideally, neutrophil apoptosis in septic joints should be evaluated using a more controlled *in vivo* model to identify the true pathophysiology of neutrophil lifespan in the face of sepsis.

Krista et al. (2013) examined the occurrence of *ex vivo* neutrophil apoptosis in horses undergoing elective orthopedic surgical procedures (little to no systemic inflammatory response) compared to horses undergoing abdominal exploratory surgery to correct colic (significant systemic inflammatory response). They further divided the colic surgery group into strangulating (high risk of endotoxemia) and non-strangulating (low risk of endotoxemia) groups. There was no difference in the *ex vivo* occurrence of neutrophil apoptosis between the elective orthopedic and colic surgery groups; however, horses with strangulating lesions had significantly delayed neutrophil apoptosis compared to horses with non-strangulating lesions. Again, mechanisms of apoptosis were not explored in this study. From the results of this study, it is important to recognize that even minor or local inflammation could have a global effect on circulating neutrophil lifespan.

Lastly, Niedzwiedz et al. (2014) evaluated percentages of apoptotic neutrophils in bronchoalveolar lavage fluid (BALF) from healthy horses compared to horses afflicted with recurrent airway obstruction (RAO). RAO horses had reduced amounts of apoptotic neutrophils compared to healthy horses. This is an expected finding based on the fact that horses with RAO have inflamed airways with increased neutrophil concentrations that would likely result in neutrophil activation and prolongation of neutrophil lifespan. Additionally, GM-CSF concentrations are increased in BALF from horses with RAO, which would further delay local neutrophil apoptosis (Turlej et al. 2001).

Taken together, there is paucity of data on the occurrence of neutrophil apoptosis and associated mechanisms in equine inflammatory diseases. Considering the role of neutrophils in inflammatory diseases and the impact of many inflammatory diseases on the horse, it is critical to undertake studies to explore the biology of neutrophil apoptosis. Data generated from such

studies could also lead to the development of the horse as model for studying neutrophil biology as horses are subject to many of the same inflammatory responses as humans and can provide a large number of cells for experiments.

1.11 The induction of neutrophil apoptosis as a future therapeutic target

Many acute inflammatory diseases are characterized by neutrophil infiltration, particularly those involving extracellular pathogens. One reason that neutrophils are so effective at neutralizing pathogens is due to their shear increase in number that allows them to overwhelm the pathogen. However, within the process of neutralizing a pathogen, neutrophils cause collateral damage to tissues through the release of their cytotoxic granules. So while they may effectively neutralize pathogens, they also potentiate an inflammatory response through damage to neighboring cells that then send out danger signals to recruit additional inflammatory cells. Additionally, in the face of neutrophil activation and accumulation, the prolongation of neutrophil lifespan has a significant effect on the overall prolongation of the inflammatory response due to inhibition of macrophage polarization from a pro- to anti-inflammatory phenotype. Therefore, the modulation of neutrophil apoptosis is an attractive alternative to reducing inflammation in neutrophil-driven inflammatory processes, such as sepsis and SIRS. Modulation of neutrophil apoptosis to reduce inflammation can potentially be mediated through two separate mechanisms. One mechanism would involve the induction of neutrophil apoptosis at the specific site(s) of inflammation. A second mechanism would be to introduce apoptotic neutrophils into the site of inflammation to influence macrophage polarization and, potentially, neutrophil lifespan.

Within the context of inducing neutrophil death to stimulate an anti-inflammatory response, it is important to differentiate apoptosis from necrosis. Necrosis is characterized as a passive, accidental death from environmental perturbations that disrupt tissue homeostasis, while

apoptosis is characterized as an active, purposeful death that is beneficial to tissue homeostasis (Fink and Cookson 2005). The induction of neutrophil death by necrosis is associated with the propagation of inflammation primarily through the lack of membrane integrity causing the release of cytotoxic contents; whereas, the induction of neutrophil death by apoptosis is associated with the reduction of inflammation through maintenance of membrane integrity and subsequent macrophage efferocytosis.

The timing of inducing neutrophil apoptosis as part of a therapeutic regimen must be developed in order to allow enough time for neutrophils to neutralize the pathogen, but before they have accumulated to such a degree as to generate an inappropriate or excessive inflammatory response. It is well-documented that neutropenia, both congenital and iatrogenic, leads to increased risk for bacterial and fungal infections (Geering and Simon 2011). Therefore, it is critical that neutrophils are available in the immediate inflammatory period to do their intended duties, while later on it may be advantageous to induce apoptosis as a method to promote the resolution of inflammation.

The induction of neutrophil apoptosis has been initiated using various pharmacologic agents that have been used with success *in vitro* and, to a lesser extent, *in vivo* in laboratory animal models (Duffin et al. 2010, Hallet et al. 2008). The ideal pharmacologic agent should alter a pathway involved in initiating neutrophil apoptosis without causing neutrophil necrosis or affecting non-target cells. As discussed earlier in this review, neutrophil apoptosis is a complex process that may utilize multiple pathways to promote or delay apoptosis, including NF-κB, MAPK, and PI3K, that ultimately regulate the Bcl2 family of proteins (Hallet et al. 2008). Indeed, inhibitors of these pathways have been evaluated, at least *in vitro*, for their ability to modulate neutrophil apoptosis (Hallet et al. 2008). Thus far, only modulation of NF-κB and the

Bcl2 family of proteins have demonstrated promising results in *in vivo* rodent models of inflammation.

A physiological inhibitor of NF-κB has been identified as prostaglandin (PG)D_2 and its metabolite PGJ_2 (Hallet et al. 2008, Lawrence et al. 2002). These metabolites of the arachidonic acid pathway have been shown to inhibit NF-κB activation and induce caspase-dependent apoptosis in neutrophils *in vitro* (Ward et al. 2004). Additionally, pre-treatment with PGJ_2 reduced LPS-induced acute lung injury in a rat model (Liu et al. 2014) and post-treatment with PGJ_2 dramatically improved survival and reduced neutrophil infiltration into tissues in an endotoxic shock mouse model (Kaplan et al. 2005). Products of the arachidonic acid pathway, specifically those produced by cyclooxygenases (COX), have been historic targets of drugs designed to reduce inflammation, namely corticosteroids, such as dexamethasone, and non-steroidal anti-inflammatory drugs (NSAIDs). Indeed, both classes of drugs are commonly used to treat inflammatory conditions in veterinary patients. However, a well-known consequence of altering COX function in order to reduce inflammatory metabolites, such as PGE*2*, is interference with production of anti-inflammatory metabolites, including PGD_2. This highlights the need to develop more targeted therapeutics in order to circumvent unintentional side effects of currently used drugs.

Related products of arachidonic metabolism produced through the lipoxygenase (LOX) arm of the pathway, called lipoxins (LX), have also been identified as potent anti-inflammatory mediators, in part through their ability to modulate neutrophil apoptosis and promote macrophage efferocytosis (Hallet et al. 2008, Lawrence et al. 2002). Specifically, aspirin-triggered lipoxins (ATL) induce neutrophil apoptosis via activation of Annexin-1, which concurrently enhances macrophage efferocytosis to promote the resolution of inflammation

(Hallet et al. 2008). Treatment with aspirin increased concentrations of ATL, which in turn reduced platelet-neutrophil aggregation and lung injury in a mouse model of LPS-induced lung injury (Ortiz-Muñoz et al. 2014).

Perhaps the most promising substance being evaluated for modulation of neutrophil apoptosis is R-roscovitine, a cyclin-dependent kinase (CDK). R-roscovitine has been historically investigated as a chemotherapeutic drug in treating cancer and is currently undergoing human clinical trials for this purpose. However, in multiple *in vitro* studies R-roscovitine induced neutrophil apoptosis by down-regulating the anti-apoptotic member of the Bcl2 family Mcl-1, even being able to override delayed neutrophil apoptosis caused by GM-CSF and LPS (Gautam et al. 2013, Leitch et al. 2010, Rossi et al. 2006). R-roscovitine has been further evaluated in rodent models with promising results by enhancing the resolution of established inflammation through the promotion of neutrophil apoptosis (Rossi et al. 2006, Koedel et al. 2009).

The second mechanism by which disease process may be modulated by neutrophil apoptosis involves the administration of apoptotic neutrophils as a therapy in itself. The ability of apoptotic neutrophils to polarize macrophage phenotype from pro-inflammatory to anti-inflammatory has been discussed above, and so it follows that investigators have exploited this effect to promote the resolution of inflammation in various rodent models (Miles et al. 2009, Ren et al. 2008). Miles et al. (2009) discovered that apoptotic and necrotic neutrophils released α–defensins from their azurophilic granules, which, in addition to being antibacterial, reduce production of pro-inflammatory cytokines and NO from macrophages. Intra-peritoneal injection of necrotic neutrophils containing α-defensins, but not whole apoptotic neutrophils, reduced inflammatory cell influx in a mouse thioglycolate model of peritonitis. (Miles et al. 2009).

In a study by Ren et al. (2008), the administration of apoptotic neutrophils up to 24 h after inducing endotoxic shock in mice reduced circulating pro-inflammatory cytokines, neutrophil infiltration in target organs, and serum LPS concentration. The positive effects of administering apoptotic neutrophils in this model are likely multimodal due to non-functional apoptotic neutrophils binding and neutralizing LPS and the efferocytosis of apoptotic neutrophils driving macrophages toward an anti-inflammatory phenotype. Esmann et al. (2010) demonstrated that neutrophils themselves phagocytose apoptotic neutrophils *in vitro* leading to an inhibition of pro-inflammatory neutrophil functions, such as respiratory burst. Taken together, the administration of apoptotic neutrophils could serve as a simple, yet useful treatment modality in treating neutrophilic inflammatory diseases.

In conclusion, the modulation of neutrophil lifespan as a therapeutic target is in its infancy of investigation. In the future, targeted modulation of an inflammatory response will hopefully comprise targeted therapeutic regimens, moving away from the currently used nonspecific and generally ineffective methods to reduce severe, life-threatening inflammation.

1.12 Summary

Despite having a relatively short lifespan compared to other leukocytes, the ability of neutrophils to alter their lifespan to suit the situation at hand is unique and involves a complex process involving multiple intracellular signaling pathways. Within the scope of an inflammatory response, the ability to modulate neutrophil lifespan is an attractive option to better coordinate an acute inflammatory response and promote the resolution of inflammation as required.

Neutrophil apoptosis has received limited attention by equine researchers; however, the horse could serve as an ideal model to study neutrophil lifespan in inflammatory conditions. The following chapters of this thesis form the groundwork for confirming that equine neutrophil

lifespan is prolonged in various inflammatory scenarios with the goal of providing a place from which further detail of the mechanisms of inflammation-delayed neutrophil may be elucidated and then manipulated as part of a multimodal therapeutic regimen for the treatment of inflammatory conditions.

CHAPTER 2: HYPOTHESES AND OBJECTIVES

2.1 Hypotheses

1. Lipopolysaccharide will delay equine neutrophil apoptosis *in vitro* via down-regulation of the intrinsic pathway of apoptosis.
2. *Ex vivo* equine neutrophil apoptosis will be delayed following intestinal ischemia and reperfusion.
3. Treatment with parenteral gadolinium chloride to deplete pulmonary intravascular macrophages will modulate inflammation associated with intestinal ischemia and reperfusion in horses.
4. Ex vivo equine neutrophil apoptosis will be delayed following the induction of colitis with oligofructose overdose.
5. Treatment with apoptotic equine neutrophils *in vitro* will rescue LPS- and systemic inflammation-delayed neutrophil apoptosis.

2.2 Objectives

1. Determine if *Escherichia coli* lipopolysaccharide delays equine neutrophil apoptosis *in vitro* in a dose-dependent manner.
2. Investigate mechanisms by which lipopolysaccharide delays equine neutrophil apoptosis *in vitro*.
3. Determine if *ex vivo* neutrophil apoptosis is delayed following intestinal ischemia and reperfusion.
4. Determine if depletion of pulmonary intravascular macrophages affects systemic inflammation and *ex vivo* neutrophil apoptosis during an equine intestinal ischemia and reperfusion model

5. Determine if *ex vivo* neutrophil apoptosis is delayed following the induction of colitis with oligofructose overdose.
6. Determine if co-incubation of apoptotic neutrophils with freshly isolated neutrophils will affect the occurrence of apoptosis *in vitro* and rescue lipopolysaccharide- and systemic inflammation-delayed apoptosis *in vitro*.

2.3 Rationale

Neutrophils are one of the primary cells involved in mounting an acute inflammatory response; however, their non-specific methods of neutralizing threats may propagate an overzealous inflammatory response, often to the detriment of the body. It has been shown in humans and laboratory animals that neutrophil lifespan is prolonged during inflammatory conditions through alteration of apoptotic pathways. The prolongation of neutrophil lifespan delays the resolution of inflammation and may contribute to a prolonged, counter-productive, inflammatory state. Therefore, the modulation of neutrophil lifespan has come under investigation as a tool to control inflammatory conditions.

Neutrophil lifespan in inflammatory conditions has received limited attention by equine researchers, despite the propensity for horses to develop inappropriate inflammatory responses resuling in increased morbidity and mortality. Lipopolysaccharide entering the bloodstream (endotoxemia) is one of the most significant contributors to the development of an inappropriate inflammatory response in horses. The most common cause of equine endotoxemia is from gastrointestinal barrier compromise due to infectious or non-infectious intestinal inflammation. The focus of the work presented herein is to confirm that equine neutrophils experience prolongation of lifespan in the face of inflammatory conditions, specifically those associated with endotoxin, and to examine the mechanisms of such an alteration of their lifespan.

CHAPTER 3: LIPOPOLYSACCHARIDE DELAYS EQUINE NEUTROPHIL APOPTOSIS IN VITRO

3.1 Abstract

Horses are particularly susceptible to lipopolysaccharide (LPS) endotoxin, generating a robust systemic inflammatory response resulting in serious, often life threatening, consequences. Delayed neutrophil apoptosis has been implicated, in part, as a contributing factor to the development of an inappropriate systemic inflammatory response to LPS in other species, but has not been well-studied in horses. Our objective was to evaluate equine neutrophil apoptosis in response to LPS treatment *in vitro*. Neutrophil apoptosis was assessed with cytology; Annexin V and propidium iodide staining quantified with flow cytometry; caspase-3, -8, and -9 activities; and western blotting for NF-κB and A1 proteins in neutrophil lysates. *In vitro* treatment with LPS resulted in delayed apoptosis after 12 and 24 h incubation in neutrophils isolated from 44 horses on 79 occasions (12 h: $P = 0.008$, 24 h: $p < 0.001$). There was a significant correlation between increasing LPS dose and decreasing occurrence of apoptosis after 24 h incubation ($r = -0.67$, $P < 0.0001$). Caspase-9 activity was significantly reduced in LPS-treated neutrophils after 12 h incubation ($P = 0.006$). Treatment with an inhibitor of toll-like receptor 4 (TLR4) and inducers of apoptosis rescued LPS-delayed apoptosis. We conclude that *in vitro* LPS treatment delays equine neutrophil apoptosis for up to 24 h in a dose-dependent manner, and the delay is dependent upon TLR4 signaling and alteration of the intrinsic pathway of apoptosis.

This work has been accepted for presentation at the American College of Veterinary Surgeon Surgical Summit. Nashville, TN, USA. October 22-24, 2015.

3.2 Introduction

Horses are extremely sensitive to endotoxin in the blood, generating a robust systemic inflammatory response to as little as 20 ng/kg of lipopolysaccharide (LPS) endotoxin administered systemically (Moore and Vandenplas 2014, Tadros et al 2013). Horses may become endotoxemic from various disease processes, the most common being from lesions affecting the gastrointestinal tract including colitis and strangulating intestinal lesions (Moore and Vandenplas 2014, Senior et al 2011). Endotoxemia results from the compromise of the intestinal epithelial barrier and subsequent bacterial translocation, ultimately leading to a systemic inflammatory response (Moore and Vandenplas 2014, Senior et al 2011, Werners et al 2005). Neutrophils are important for the propagation of an inappropriate systemic inflammatory response to endotoxin because of their non-specific methods of antigen neutralization, often leading to collateral damage to nearby cells and tissues (Werners et al 2005).

There are two classically described pathways by which a cell may undergo apoptosis. The extrinsic pathway involves activation of cell membrane receptors by specific ligands, such as tumor necrosis alpha (TNF-α), followed by activation of a cascade of intracellular proteases, called caspases, that initiate apoptosis (Fox et al. 2010). The intrinsic pathway involves a shifting of a balance of the B cell lymphoma 2 (Bcl2) family of proteins that maintain mitochondrial membrane potential. An intracellular signal, such as cell stress, activates various intracellular signaling pathways, such as mitogen-activated protein kinases (MAPK), that ultimately change the balance of the Bcl2 family through changes in specific protein production to pro-life or pro-death depending on the situation at hand (Fox et al. 2010).

Although not specifically studied in the horse, in other species it is understood that neutrophils have a relatively short circulatory lifespan (6-8 h half-life in circulation), which ends

with the induction of apoptosis via the intrinsic pathway of apoptosis (Edwards et al 2004, Jia et al 2008, Tak et al 2013). The abbreviated life span of neutrophils may be a mechanism by which the body removes a potentially activated cell population, thereby having a role in anti-inflammatory processes (Savill et al 2002). Indeed, the phagocytosis of apoptotic neutrophils by macrophages results in release of anti-inflammatory cytokines such as TGF-β and the inhibition of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-8, and tumor necrosis factor (TNF)-α (Fadok et al 1998, Huynh et al 2002, Lucas et al 2003). In addition, neutrophil apoptosis is the primary signal for their removal from the blood by professional phagocytic cells, especially macrophages, located in the spleen, liver, and bone marrow (Hofman 2004, Tak et al 2013).

Delayed neutrophil apoptosis has been implicated in the development of an inappropriate systemic inflammatory response to endotoxin and the prevention of the resolution of inflammation (Hofman 2004, Milot et al 2012, O'Brien and Kirby 2008, Serhan and Savill 2005). It is well-accepted that bacterial LPS or sepsis delays neutrophil apoptosis in laboratory mammals and humans *in vitro* and *in vivo* (Colotta et al 1992, Jia et al 2008, Milot et al 2012, Sano et al 2005, Sweeny et al 1998, Yamamoto et al 1993). The proposed mechanism of delayed neutrophil apoptosis in response to LPS is influenced by activation of the nuclear factor kappa B (NF-κB) and phosphoinositide 3-kinase (PI3) pathways via toll-like receptor (TLR) 4 signaling leading to an increase in anti-apoptotic proteins, such as B-cell lymphoma (Bcl)-2, myeloid leukemia cell (Mcl)-1, and A1, that down-regulate the intrinsic pathway of apoptosis (Edwards, et al., 2004, François et al., 2005).

To the authors' knowledge, there is only a single study in the literature evaluating the effect of LPS treatment on equine neutrophil lifespan *in vitro* (Brazil et al. 2014). This group

demonstrated an increase in neutrophil apoptosis upon LPS treatment, which is in direct contrast to results from all other species. Because horses are highly susceptible to the effects of endotoxin, further evaluation of the effect of LPS on neutrophil lifespan was needed. The objective of this study was to investigate the effect of LPS treatment on the occurrence of apoptosis in equine neutrophils *in vitro*. We hypothesized that LPS would delay apoptosis in equine neutrophils in a dose-dependent manner via down-regulation of the intrinsic pathway of apoptosis.

3.3 Materials and Methods

3.3.1 Animals

Healthy adult horses were used for all experiments. All procedures and experimental protocols were approved by the University of Saskatchewan Committee on Animal Care and Supply and the University of Saskatchewan Animal Research Ethics Board.

3.3.2 Neutrophil isolation

Neutrophils were isolated using a previously reported protocol with minor modification (Cook et al. 2009). Blood was collected aseptically from a jugular vein into vacuum tubes containing ethylenediaminetetraacetic acid (EDTA, Vacutainer™, BD, Mississauga, ON, Canada). Isolation was performed at room temperature (20-22°C) immediately after blood collection. The blood was allowed to separate into plasma and red cell fractions for 30-45 min at room temperature. Plasma was layered onto 10 ml Ficoll-Paque™ Plus (GE Healthcare, Mississauga, ON, Canada) and centrifuged for 30 min at 400 x *g*. The subsequent pellet, containing erythrocytes and granulocytes, was retained after aspirating the supernatant and washed with Hank's Buffered Salt Solution (HBSS) without phenol, magnesium, or calcium for 10 min at 200 x *g*. Erythrocytes were lysed with 2 ml sterile distilled water with pH of 7.4 for 25 s and restored to normotonicity

with an equal volume of hypertonic (2x) HBSS. Cells were washed 3 times with HBSS at 200 x *g* for 10 min, suspended in culture medium (Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 50 μg/ml streptomycin, and 25 mM Hepes, Lonza BioWhittaker®, VWR, Edmonton, AB), assessed for viability using Trypan blue staining, and counted with a hemocytometer. Cell purity was determined on cytology preparations made with a cytocentrifuge (Shandon Cytospin 4, Thermo Scientific, Waltham, MA, USA) at 1000 rpm for 4 min with medium acceleration and stained with a modified Giemsa stain (Hemacolor®, EMD chemical, Gibbstown, NJ). Experiments proceeded if viability was greater than 98% and purity was greater than 90%.

3.3.3 Neutrophil culture conditions

Neutrophils were suspended in culture medium in plastic 24-well cell culture plates at a concentration of 2 x 10^6 cells/ml. Cells were left untreated or were treated with various concentrations of LPS (*Escherichia coli* 055:B5, Sigma-Aldrich Canada, Oakville, ON, Canada). For mechanistic experiments, neutrophils were cultured with LPS and inducers of apoptosis gambogic acid (GA, 2 μg/ml, apoptosis inducer via inhibition of anti-apoptotic Bcl2 proteins, Enzo, Farmingdale, NY, USA) or staurosporine (Stauro, 2 μM, apoptosis inducer via pan-caspase activation, Biovision, Milpitas, CA, USA); with TLR4 inhibitor CLI-095 (1 μg/ml, Invivogen, San Diego, CA, USA); or with the dissolving agent dimethyl sulfoxide (1 μl/ml DMSO, Sigma-Aldrich Canada, Oakville, ON, Canada). For all experiments, cells were incubated at 37°C in 5% CO_2 for various times depending on experiment. Prior to performing apoptosis assays, neutrophils were taken out of culture, pelleted, and washed twice with HBSS at 400 x *g* for 8 min.

3.3.4 Apoptosis assays

3.3.4.1 Cytology

Cytology preparations were prepared as for assessment of sample purity after neutrophil isolation. Neutrophil nuclear morphology was evaluated by the first author, who was blinded to treatment, with light microscopy at 100X magnification. Neutrophils were classified as apoptotic if they were karyorrhectic or pyknotic (Savill et al., 1989). Five hundred neutrophils were counted in multiple random fields to provide a percentage of apoptotic neutrophils out of normal neutrophils.

3.3.4.2 Flow cytometry

Neutrophils were stained with CF™488-conjugated Annexin V (AV) and propidium iodide (PI) per manufacturer's instructions (CF™488A-Annexin V and PI Apoptosis Assay Kit, Biotium, Hayward, CA, USA). Staining controls included unstained untreated cells, untreated cells stained with AV only, and untreated stained with PI only. A flow cytometer (CyFlow®, Partec, Swedesboro, NJ, USA) was used to quantify negatively stained (live cells), AV positively stained (apoptotic cells), or AV and PI positively stained (dead) cells. Flow cytometry data were analyzed with commercial software (FlowMax Software © Version 2.6, Quantum Analysis GmbH, Münster, Germany). The gated population was determined with unstained cells based on size (forward scatter) and granularity (side scatter). Quadrants to set threshold of fluorescence intensity for both Annexin V and PI were determined using untreated cells stained with only AV or PI. Data were acquired on at least 10,000 gated events.

3.3.4.3 Caspase activity assay

Caspase-3, -8, and -9 activities were indirectly measured using a commercially available kit per manufacturer's instructions (Caspase-3, -8, or -9 Colorimetric Assay Kit, Biovision, Milpitas,

CA, USA). Caspase-3 activity was measured to determine overall apoptotic activity, caspase-8 activity was measured to assess the contribution of the extrinsic pathway, and caspase-9 activity was measured to assess the contribution of the intrinsic pathway on the occurrence of neutrophil apoptosis. Neutrophils were lysed using the manufacturer's lysis buffer following removal from culture and washing as described above. Cell lysates were stored at -80°C until processing. Upon thawing, protein concentration in cell lysates was quantified using a commercial kit (DC™ Protein Assay, Bio-Rad Laboratories, Mississauga, ON, Canada). Cell lysates were incubated with a manufacturer's reaction buffer containing dithiothreitol (DTT) and each caspase's substrate: DEVD-pNA (caspase-3), IETD-pNA (caspase-8), or LEHD-pNA (caspase-9) in 96-well plates for 2 h at 37°C. Immediately after incubation, free pNA was quantified by measuring absorbance at 405 nm in a microtiter plate reader. Levels of free pNA were corrected for protein concentration in cell lysates.

3.3.4.4 Immunoblotting for inflammatory and apoptotic proteins

Western blots were performed on neutrophils lysed with radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich Canada, Oakville, ON, Canada) containing protease inhibitor (cOmplete protease inhibitor cocktail tablets, Roche, Mississauga, ON, Canada). Protein concentrations in cell lysates were quantified using the DC™ Protein Assay (Bio-Rad Laboratories, Mississauga, ON, Canada) and cell lysates were diluted to a standard protein concentration using RIPA buffer. Lysates were diluted and boiled with Laemmli sample buffer (Bio-Rad Laboratories, Mississauga, ON, Canada) containing 10% 2-mercaptoethanol (Sigma-Aldrich Canada, Oakville, ON, Canada) at 95°C for 5 min, run on a 12% SDS-PAGE gel, transferred to a low-fluorescent polyvinylidene fluoride (PVDF) membrane (Amersham Hybond™-LFP, GE Healthcare, Mississauga, ON, Canada), and immunoblotted with antibodies against anti-apoptotic protein A1

(Santa Cruz Biotechnologies, Dallas, TX, USA), nuclear factor kappa B (NFκB, Abcam Inc, Toronto, ON, Canada), and housekeeping protein beta-actin (Abcam Inc, Toronto, ON, Canada). Species- and immunoglobulin-appropriate fluorescent-labeled secondary antibodies were used to label primary antibodies (Santa Cruz Biotechnologies, Dallas, TX, USA). Blots were developed using a fluorescent membrane reader (Typhoon 9400, GE Healthcare, Mississauga, ON, Canada). Densitometry was performed by comparing relative fluorescence of protein of interest to the housekeeping protein using imaging software (ImageJ, National Institutes of Health, USA).

3.3.5 Statistical analysis

Data were analyzed with a commercial software package (Graphpad Prism, Graphpad Software, Inc. La Jolla, CA, USA). For all comparisons, a *P*-value of < 0.05 was considered significant. A Shapiro-Wilk test of normality was performed on all dependent variables. A Spearman's rank correlation was performed to assess correlation of flow cytometry data with light microscopy. Linear regression and a Spearman's rank correlation were used to assess the effect of LPS concentration on the occurrence of neutrophil apoptosis. For normal data, multiple comparisons among continuous variable were assessed with one-way ANOVA with Bonferroni's post-hoc analysis and comparisons between two continuous variables were assessed with a Student's *t*-test. Data are reported as mean with standard deviation (SD). For non-normal data, multiple comparisons among continuous variables were assessed with a Kruskal-Wallis rank test with a Dunn's multiple comparisons test and comparisons between two continuous variables were assessed with a Mann-Whitney U test. Data are represented by median and range.

3.4 Results

3.4.1 LPS treatment delays equine neutrophil apoptosis in vitro in a dose-dependent manner

For determination of dose dependency of the effect of LPS on apoptosis, neutrophils were isolated for 3 separate experiments using 3 horses. Neutrophils were treated with 0.001 μg/ml, 0.01 μg/ml, 0.1 μg/ml, 1 μg/ml, or 10 μg/ml LPS or left untreated and cultured for 24 h. Neutrophil apoptosis, as determined by Annexin V and PI staining, was reduced by LPS treatment in a dose-dependent manner where increasing dose of LPS was significantly correlated with reduced apoptosis ($r = -0.97$, $P = 0.003$, Figure 3.1). Treatment with LPS concentrations of 0.1, 1, and 10 μg/ml significantly reduced apoptosis in neutrophils after 24 h in culture compared to untreated cells (0.01 μg/ml LPS: $P = 0.037$, 0.1 μg/ml LPS: $P = 0.003$, 1 μg/ml LPS: $P < 0.001$, 10 μg/ml LPS: $P < 0.001$, Figure 3.1).

Neutrophils were isolated from 44 horses on 79 occasions. Following isolation, neutrophil viability was greater than 99% and purity averaged 93.5% (SD: 2.3%) with eosinophils serving as the primary contaminating cell type. Neutrophils were cultured for 12 or 24 h with or without 1 μg/ml LPS. There was a significant reduction in the occurrence of apoptosis as measured by Annexin V positive and PI negative staining at both time points for cells treated with LPS compared to untreated cells (12 h: $P = 0.008$, 24 h: $P < 0.001$, Figure 3.2). There was no difference in percentages of live cells (Annexin V negative and PI negative) or dead cells (Annexin V positive and PI positive) after 12 h of culture; but after 24 h, there were significantly more live cells with LPS treatment ($P < 0.001$, Figure 3.2).

Nuclear morphology was concurrently assessed in neutrophils isolated from 8 horses following culture for 12 or 24 h with or without 1 μg/ml LPS. There was a significant reduction in apoptotic neutrophils as measured by altered neutrophil nuclear morphology at both time

points. (Figure 3.3, 12 h: $P = 0.0008$, 24 h: $P = 0.0002$). Light microscopy results were highly correlated with flow cytometry results at 12 h ($r = 0.66$, $P = 0.006$) and 24 h ($r = 0.90$, $P < 0.001$).

3.4.2 LPS treatment modifies the intrinsic pathway of equine neutrophil apoptosis via TLR4 signaling

Evaluation of caspase activity was used to identify the involvement of the intrinsic and extrinsic pathways. Caspase-8 and -9, both initiator caspases, were chosen to identify activation of the extrinsic and intrinsic pathways, respectively. Caspase-3 activity was used to identify overall apoptotic activity as it is an effector caspase for both the extrinsic and intrinsic pathways. When caspase activity was compared between untreated and LPS-treated cells isolated from 16 horses after 12 h of incubation, there was no difference in caspase-3 or caspase-8 activity between untreated and LPS-treated cells, but there was a significant reduction in caspase-9 in LPS-treated compared untreated cells ($P = 0.012$, Figure 3.4). After 12 h of incubation, treatment with LPS resulted in increased total NF-κB and decreased A1protein expression, although the difference was not statistically significant (NF-κB: $P = 0.24$, A1: $P = 0.18$, Figure 3.5).

Co-incubation of neutrophils with CLI-095, a chemical that blocks the intracellular domain of TLR4, and LPS for 24 h reduced LPS-delayed neutrophils apoptosis (1 experiment in 7 horses, $P < 0.05$, Figure 3.6).

In neutrophils isolated from 6 horses and cultured for 12 h with pan-caspase activator staurosporine or inhibitor of the anti-apoptotic Bcl2 proteins gambogic acid and LPS increased apoptosis compared to LPS treated cells, as measured by Annexin V and PI staining and flow cytometric quantification ($P < 0.05$, Figure 3.7).

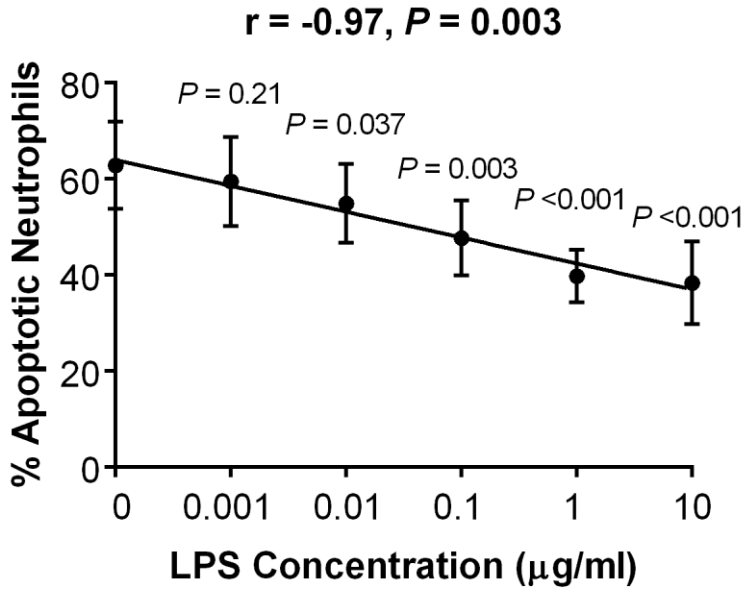


Figure 3.1 Apoptosis is reduced in equine neutrophils by *in vitro* LPS treatment in a dose-dependent manner. Data pointes represent the mean and standard deviation from 3 separate experiments in 3 horses. Data were analyzed with linear regression. *P*-values represent difference in the occurrence of apoptosis from untreated neutrophils.

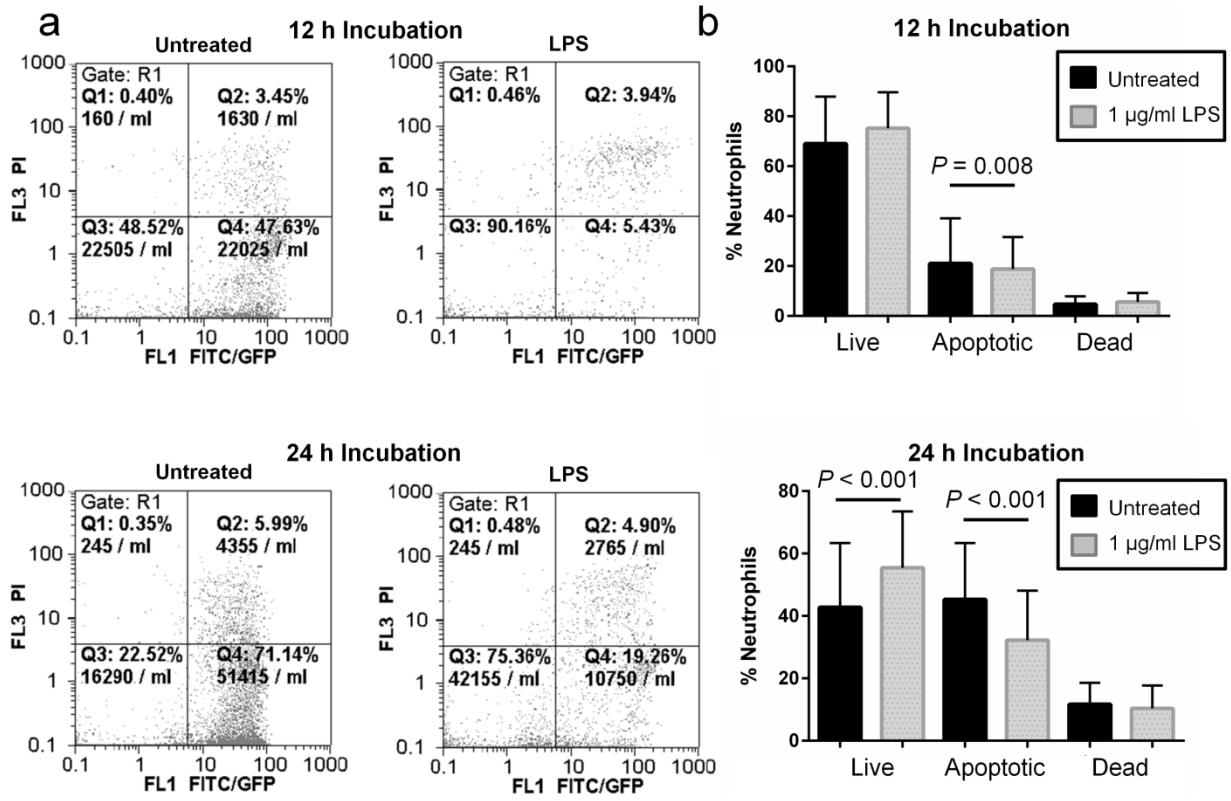


Figure 3.2 LPS delays equine neutrophil apoptosis *in vitro*. Incubation of equine neutrophils for 12 or 24 h with or without 1 µg/ml LPS. a) Representative results for flow cytometric quantification of Annexin V and PI staining. Live neutrophils are Annexin V and PI negative (Q3), apoptotic neutrophils are Annexin V positive and PI negative (Q4), and dead neutrophils are Annexin V positive and PI positive (Q2). b) Graphic representation of flow cytometric quantification of neutrophil apoptosis. Data represents mean ± SD from 79 neutrophil isolations from 44 horses. Comparison between untreated and LPS treated cells for each neutrophil outcome was assessed with a Student's *t*-test.

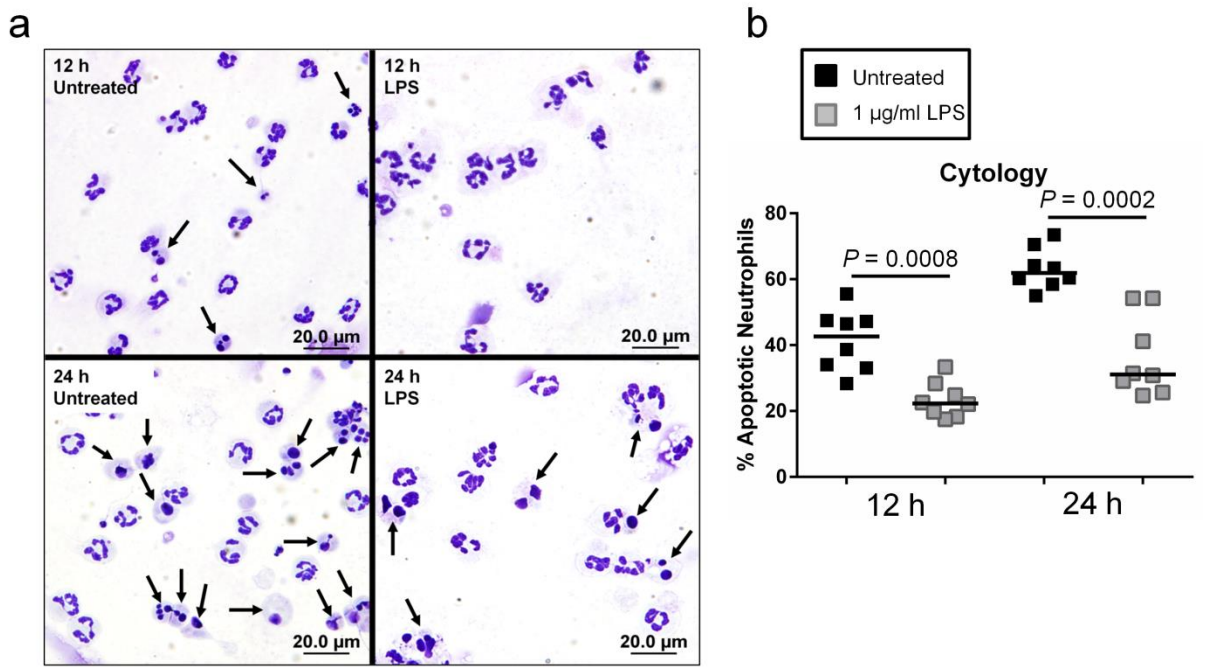


Figure 3.3 a) Representative cytology preparations evaluating neutrophil nuclear morphology after 12 and 24 h incubation. Arrows indicate apoptotic neutrophils. b) Graphic representation of cytology data. Bar represents median and data represents 1 experiment on neutrophils isolated from 8 horses. Comparisons between treatments for each time point were assessed with a Mann-Whitney U test.

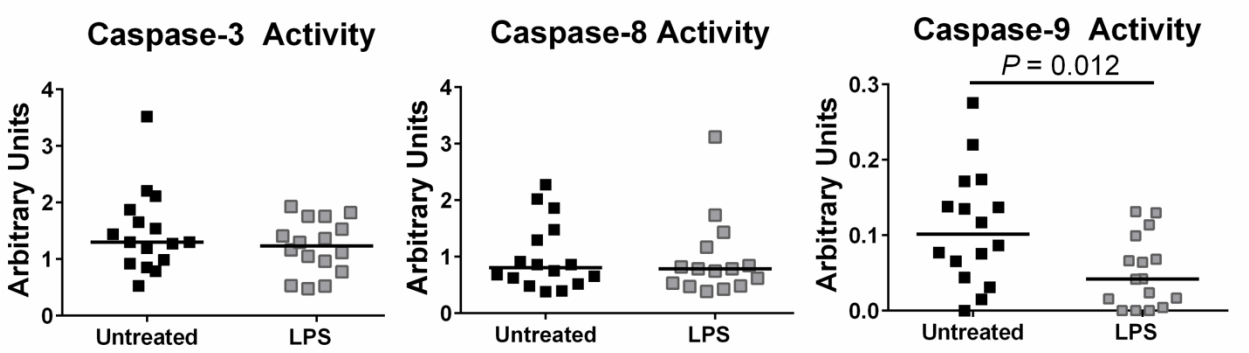


Figure 3.4 Caspase-9, but not Caspase-3 or Caspase-8, activity was reduced in equine neutrophils treated with LPS (1 μg/ml) for 12 h. Bar represents median. Data represents 1 experiment on neutrophils isolated from 16 horses. Comparison between treatments was analyzed with a Mann-Whitney U test.

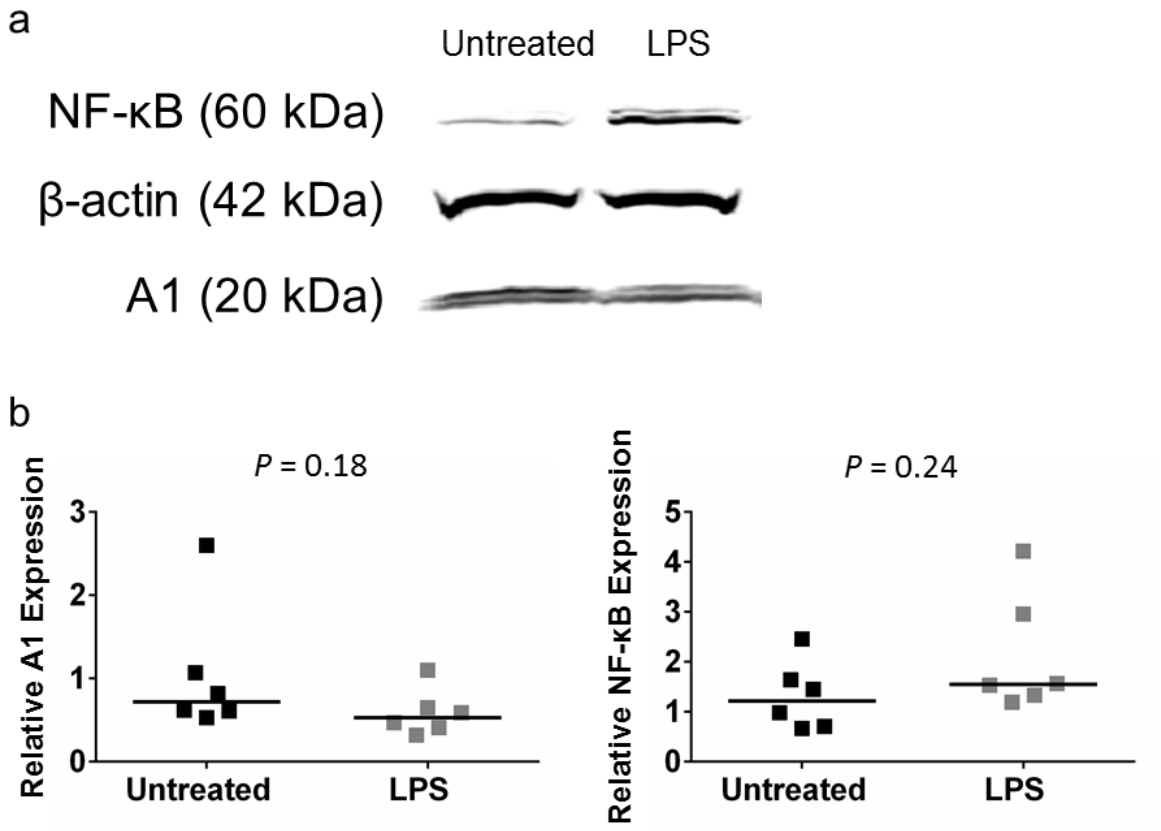


Figure 3.5 There was no difference in NF-κB and A1 protein expression in equine neutrophils treated with LPS for 12 h in culture compared to untreated cells. a) Representative immunoblots from equine neutrophil lysates after 12 h of incubation. b) Relative expression of NF-κB and A1 based on densitometry. Data are corrected with density of the corresponding β-actin band. Data represent 1 experiment in 6 horses. Bar represents the median. Data were analyzed with the Mann-Whitney U test.

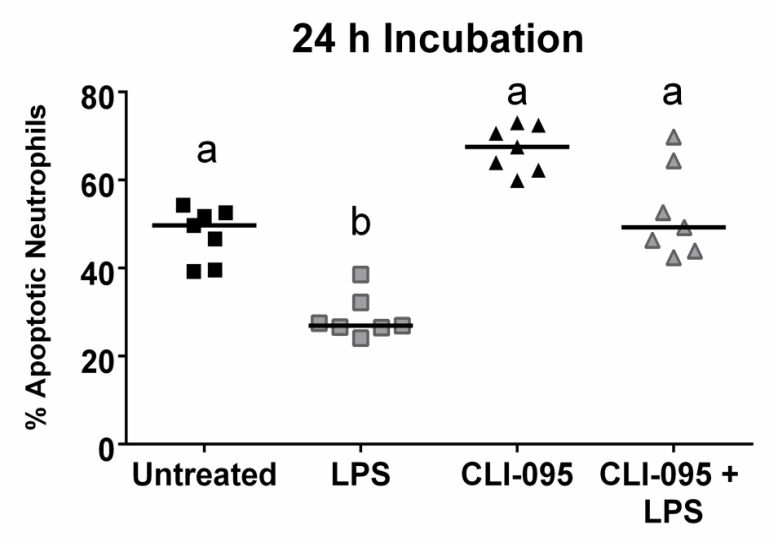


Figure 3.6 LPS-delayed neutrophil apoptosis is dependent upon TLR4 signaling. Treatment with CLI-095 inhibited LPS-delayed neutrophil apoptosis during 24 h in culture. Bar represents median. Data represents 1 experiment on neutrophils isolated from 7 horses. Data were analyzed with a Kruskal-Wallis rank test with a Dunn's multiple comparisons test. Difference in letters denotes significant difference among groups ($P < 0.05$).

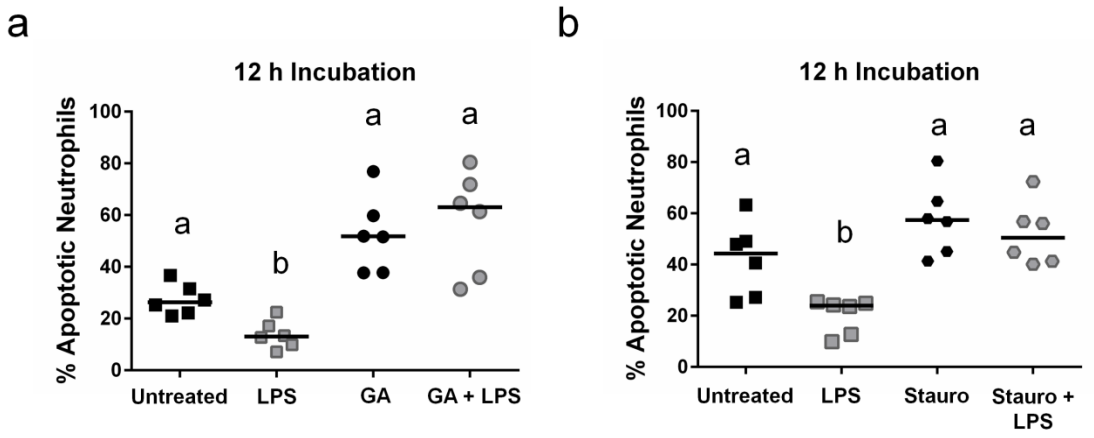


Figure 3.7 Inducers of apoptosis effectively rescued LPS-delayed neutrophil apoptosis. a) Treatment with 2 μg/ml gambogic acid (GA), 2 μg/ml gambogic acid and 1 μg/ml LPS (GA + LPS). Data represent 1 experiment in 6 horses. b) Treatment with 2 μM staurosporine (Stauro), or 2 μM staurosporine and 1 μg/ml LPS (Stauro + LPS). Data represent 1 experiment in 6 horses. Bars represent medians for each treatment. Data were analyzed with a Kruskal-Wallis rank test with a Dunn's multiple comparisons test. Difference in letters denotes significant difference among groups ($P < 0.05$).

3.5 Discussion

The results of our study support the hypothesis that LPS delays apoptosis in a dose-dependent manner in equine neutrophils *in vitro*. Though the mechanism by which LPS delays apoptosis in equine neutrophils was not fully elucidated, our results suggest that LPS treatment interferes with the intrinsic pathway of apoptosis through reduced caspase-9 activity and is dependent on TLR4 signaling.

Recently, Brazil et al. (2014) reported that treatment of equine neutrophils with multiple types of LPS *in vitro* promoted neutrophil apoptosis, which is in direct contrast to our results and findings in other species, including humans (Colotta et al. 2012, François et al. 2005, Jia et al. 2008, Milot et al. 2012, Sano et al. 2005, Sweeny et al. 1998, Yamamoto et al. 1993). It must be stated that there was substantial inter-horse and, to a lesser extent, inter-experiment variability, in the effect of LPS treatment on equine neutrophil apoptosis in our study. Therefore, it is possible that a lower number of experimental units (especially biologic units represented by number of horses) could lead to the opposite result, such as reported by Brazil et al. who reported results for triplicate experiments in 10 horses. In select horses, we performed up to six replicates of the experiment to assess the occurrence of apoptosis after 12 and 24 h of incubation using Annexin V and propidium iodide.

The discrepancy in our results compared to Brazil et al. could be due to the challenges in working with neutrophils. Neutrophils are highly reactive cells, so small differences in the health status of a horse, neutrophil isolation procedures, contamination, type of LPS, and culture conditions could have a profound effect on their short life span in culture (Attalah et al. 2012, Freitas et al. 2008, Hannah et al. 1998, Haslett et al. 1985, Sabroe et al., 2002, Tennenberg et al. 1988, Watson et al. 1992). Activation, or lack thereof, may have an effect on neutrophil's

responsiveness to LPS leading to differential responses to the *in vitro* occurrence of apoptosis. To the authors' knowledge, there are no studies that have specifically addressed the occurrence of neutrophil apoptosis *in vitro* after pre-stimulation with an activating substance, such as LPS.

In this study, neutrophil apoptosis was delayed in a dose-dependent manner, which has been previously demonstrated in human neutrophils (Sweeney et al. 1998). However, the concentration of LPS (1 μg/ml) to which neutrophils were exposed to in prolonged culture conditions to reliably delay apoptosis in this study would be difficult to achieve in a horse systemically. First, LPS is rapidly removed from the equine blood compartment after intravenous infusion (Fessler et al. 1989); and second, if such a concentration was achieved in the blood, it would be approaching the lethal dose of LPS for a horse (150-175 μg/kg or ~2 μg/ml blood volume, Burrows 1981). However, it is possible that activation of a small population of neutrophils by a relatively high concentration of LPS could lead to a global effect on neutrophil lifespan *in vivo* through intercellular signaling via cytokine production and mononuclear cell activation. Further investigation is required to evaluate *ex vivo* neutrophil lifespan following *in vivo* LPS challenge.

We further explored the mechanisms of LPS-delayed neutrophil apoptosis. As expected based on known pathways of LPS activation, delayed equine neutrophil apoptosis was dependent upon TLR4 signaling. These results support the current mechanism proposed in other species for LPS-delayed neutrophil apoptosis (Figure 3.8). We used a TLR4 inhibitor that is a cyclohexene derivative, called CLI-095 or TAK-242. This substance was first identified for its ability to modulate sepsis and later it was found to suppress TLR4 signaling by blocking its intracellular domain (Kawamoto et al. 2008). To our knowledge, CLI-095 has not been used to evaluate

neutrophil apoptosis, but it has been used to evaluate other neutrophil signaling pathways involving TLR4 (Lefebvre et al. 2011).

One of the end pathways of neutrophil TLR4 activation involves NF-κB formation to up-regulate protein transcription; however in our study, the results of western blot analysis for anti-apoptotic protein A1 and NF-κB are equivocal. This result is somewhat unexpected based on known pathways of LPS-delayed apoptosis in human neutrophils where both NF-κB and A1 expression have been shown to be elevated (Chuang et al. 1998, François et al. 2005, Figure 3.8). NF-κB is considered to be the major regulator of production of the Bcl2 family of proteins, which are vital for determining pro-life vs pro-death in neutrophil survival (Fox et al. 2010). For example, A1 protein expression is considered to be dependent upon NF-κB translocation, so it is difficult to explain the tendency for an opposite level of expression in our results (Vogler 2012). However, A1 is an extremely unstable protein with a short half-life of approximately 3 h (Moulding et al. 2001). Therefore, the discrepancy in these results is more likely due to the choice of sampling time in the continuum of *in vitro* neutrophil apoptosis. The data presented for these proteins represents one point in time. It is possible that if the expression of these proteins was evaluated over time, a difference in expression with LPS treatment would become evident. Ideally, more proteins of the Bcl2 family would have been evaluated, however we struggled to find antibodies that were compatible with equine proteins of interest.

The reduction in caspase-9 activity and increased occurrence of apoptosis upon concurrent LPS and gambogic acid treatment suggests that the intrinsic pathway is affected by LPS treatment as has been previously demonstrated in other species (Edwards et al. 1992, François et al. 2005, Figure 3.8). Caspase-9 is the main initiator caspase for the intrinsic pathway of apoptosis. It has previously been reported that caspase-9 activity was reduced in

neutrophils isolated from septic human patients supporting the importance of its role in LPS-delayed neutrophil apoptosis (Taneja et al. 2004). However, to the authors' knowledge, this is the first study that has demonstrated reduced caspase-9 activity upon treatment of neutrophils with LPS *in vitro*. In a human melanoma cell line, gambogic acid induces apoptosis through regulation of the pro-apoptotic Bcl2 protein Bax and the anti-apoptotic protein Bcl2 (Xu et al. 2009); although to the authors' knowledge, there are no studies evaluating the effect of gambogic acid treatment on neutrophil apoptosis *in vitro*. Further investigation is required to identify the effect of gambogic acid on Bcl2 family protein expression in equine neutrophils.

It is becoming apparent that neutrophil apoptosis or delayed apoptosis occurs via alternative pathways in the face of various substances. For example granulocyte colony-stimulating factor delays neutrophil apoptosis independently of the Bcl-2 family of proteins by inhibiting calcium-dependent cysteine proteases called calpains while stabilizing X-linked inhibitor of apoptosis that, in turn, inhibits caspase-3 and -9 in human neutrophils (van Raam et al. 2008). It is possible that an alternative method of apoptosis inhibition occurred due to LPS treatment in equine neutrophils. Further experiments need to be performed to further investigate apoptosis in equine neutrophils, including the evaluation of additional proteins and signaling pathways that might be involved.

In conclusion, equine neutrophil apoptosis was delayed *in vitro* after treatment with >1 μg/ml LPS from *Escherichia coli* 055:B5 through reduced capase-9 activity, dependent upon TLR4 signaling. Further work is necessary to fully elucidate the mechanisms involved in LPS-delayed apoptosis in equine neutrophils. From the results presented here, it is conceivable that alteration of the classical intrinsic apoptotic pathway is not the only cause of LPS-delayed apoptosis in neutrophils.

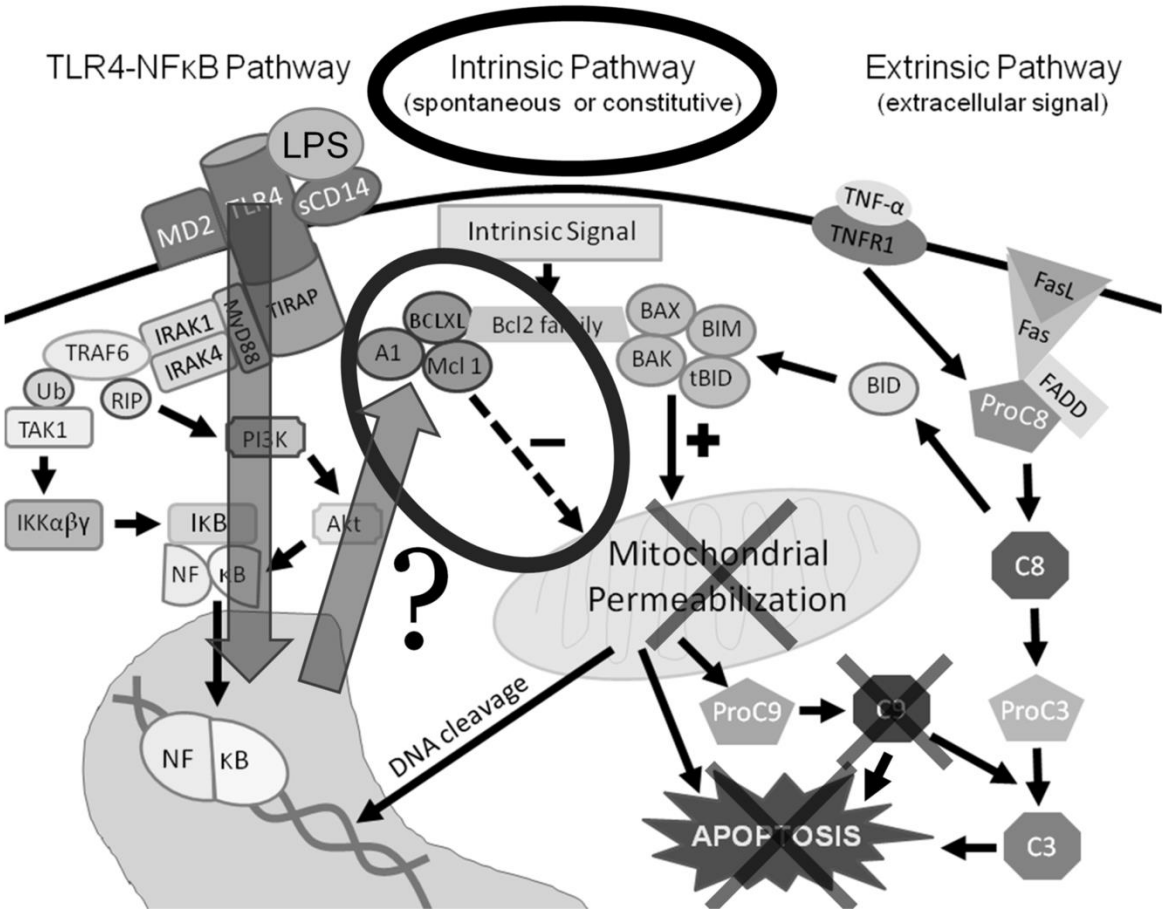


Figure 3.8. Proposed mechanism of LPS-delayed apoptosis in equine neutrophils.

3.6 *Contineo* to *in vivo* models reported in Chapters 4 and 5

After verifying that treatment with LPS, albeit at a relatively high dose, did in fact delay equine neutrophil apoptosis *in vitro*, the next step was to assess neutrophil apoptosis in equine models that are associated with "endotoxemia." Chapter 4 contains data on the occurrence of *ex vivo* neutrophil apoptosis associated with intestinal ischemia and reperfusion, a commonly used model of intestinal inflammation and a model that has reported measurable concentrations of endotoxin in the blood (Moore et al. 1981). Chapter 5 has data on the occurrence of *ex vivo* neutrophil apoptosis associated with an oligofructose-induced colitis, a model that creates significant signs of systemic inflammation that are conventionally attributed to endotoxemia.

We chose these models of gastrointestinal inflammation over an LPS-infusion model for 2 reasons. First, equine LPS-infusion models do not mimic a true clinical scenario as has been demonstrated by the fact that horses do not experience laminitis, a complication of clinical cases of endotoxemia. Horses demonstrate a multitude of clinical signs including tachycardia, tachypnea, colic, hyperthermia, and neutropenia followed by rebound neutrophilia that are consistent with SIRS. However, all of these signs are transient and horses rapidly recover following LPS infusion, which does not mimic the clinical scenario of a horse diagnosed as having endotoxemia, most commonly from underlying gastrointestinal disease.

Second, we were interested in evaluating the role of pulmonary intravascular macrophages (PIMs) in systemic inflammation associated with intestinal ischemia and reperfusion injury. PIMs are a unique type of macrophage that are attached intimately to the endothelium of the alveolar capillaries. Similar to Kupffer cell, they are exposed to the entire blood volume as it passes through the lung for oxygenation. Therefore, they may play a significant role in modulating an inflammatory response, including neutrophil apoptosis.

CHAPTER 4: EQUINE NEUTROPHIL LIFESPAN IS PROLONGED IN HORSES EXPERIENCING JEJUNAL ISCHEMIA AND REPERFUSION

4.1 Abstract

Dysregulation of neutrophil apoptosis may contribute to the development of an excessive systemic inflammatory response in horses as in other species, but requires further investigation especially because horses are highly sensitive to systemic endotoxemia. The objective of this study was to determine the effect of systemic inflammation, using an experimental model of intestinal ischemia and reperfusion injury, on *ex vivo* neutrophil apoptosis in horses. Neutrophils were isolated before and after surgery from horses that were randomized to 3 treatment groups: sham celiotomy (CEL, n=4), intestinal ischemia and reperfusion (IR, n=6), intestinal ischemia and reperfusion with gadolinium chloride treatment to deplete pulmonary intravascular macrophages (IRGC, n=6). Neutrophil apoptosis was assessed with Annexin V and propidium iodide staining quantified with flow cytometry and caspase-3, -8, and -9 activities in neutrophil lysates. All horses experienced a systemic inflammatory response following surgery without detectable plasma endotoxin concentrations 2 h following reperfusion (<0.06 EU/ml). Caspase-3 activity was reduced in freshly isolated neutrophils from IR horses ($P = 0.03$). Following surgery, neutrophil apoptosis was significantly delayed after 12 or 24 h in culture, except in IRGC horses (12 h: CEL: $P = 0.03$, IR: $P = 0.05$, IRGC: $P = 0.2$; 24 h: CEL: $P = 0.001$, IR: $P = 0.004$, IRGC: $P = 0.3$). Caspase-3, -8, and -9 activities were significantly reduced in neutrophils isolated after surgery and cultured for 12 h in IR horses, but not IRGC horses (IR caspase-3: $P = 0.002$, IR caspase-8: 0.002 $P =$, IR caspase-9: $P = 0.04$). Following surgery, *ex vivo* equine neutrophil apoptosis was delayed via down-regulation of caspase activity, which could have implications for the development and progression of systemic inflammatory responses.

Additionally, PIM depletion may rescue neutrophil apoptosis through the modulation of plasma cytokines and reduced down-regulation of caspase activity.

This work has been presented as a poster at Experimental Biology, Boston, MA, USA, April 1, 2015 and the American College of Veterinary Surgeon Surgical Summit, Nashville, TN, USA, October 22-24, 2015.

4.2 Introduction

Neutrophils serve as one of the primary effector cells of the innate immune response triggered by danger- or pathogen-associated molecular patterns. Upon neutrophil activation with substances, such as lipopolysaccharide (LPS), there is a concomitant prolongation of lifespan through delayed constitutive apoptosis, although this has not been fully established in horses (Brazil et al. 2014, Colotta et al. 1992, Lee et al. 1993). Delayed neutrophil apoptosis has a direct effect on the magnitude and duration of the innate immune response as a whole, which may contribute to the development of a highly exuberant innate immune response, also known as the systemic inflammatory response syndrome (SIRS), in two ways (Fanning et al. 1999, Jimenez et al. 1997, Shang et al. 2007). First, neutrophils are histotoxic due to their non-specific method of neutralizing threats through de-granulation, which may result in further inflammation with additional neutrophil activation and recruitment (Wright et al. 2010). Second, the resolution of inflammation begins, in large part, with the efferocytosis of apoptotic neutrophils by macrophages causing their phenotype to change from an inflammatory M1 phenotype to an anti-inflammatory M2 phenotype (Fadok et al. 1998, Hofman 2004, Lucas et al. 2003). Therefore, the delay in neutrophil apoptosis slows the onset of macrophage-driven resolution of inflammation (McCracken and Allen, 2014).

One of the most common causes of SIRS in horses is endotoxemia from gastrointestinal compromise (Moore and Vandenplas 2014). In the case of small intestinal strangulation,

endotoxin (bacterial LPS) enters the circulation through a compromised intestinal epithelial barrier (Moore et al. 1981). The inflammatory response to endotoxemia in the horse is profound and carries increased morbidity and mortality in horses with gastrointestinal disease. In a recent study, horses with strangulating intestinal lesions had delayed *ex vivo* neutrophil apoptosis compared to horses with non-strangulating lesions, however the mechanisms as to why neutrophil apoptosis was delayed were not fully explored (Krista et al. 2013).

Compared to the study on the role of extravascular tissue macrophages in inflammatory processes, including delayed neutrophil apoptosis, the biology of pulmonary intravascular macrophages (PIMs) has not received the same attention for their contribution to a systemic inflammatory response in any species (Ahronson-Raz et al. 2010). Equine PIMs up-regulate their expression of toll-like receptors (TLR)2, 4 and 9, and cytokines interleukin (IL)-1β and tumor necrosis factor alpha (TNF-α) during intravenous *Escherichia coli* LPS challenge (Parbhakar et al. 2005, Schneberger et al. 2009, Suri et al. 2006); but, parenteral treatment with gadolinium chloride (GC), a chemical used to induce apoptosis in PIMs, reduces IL-1β and TNF-α expression by PIMs, as well as total lung TLR4 and TLR9 expression, in this model (Parbhakar et al. 2005). Depletion of PIMs reduces migration of neutrophils into the alveoli of horses with recurrent airway obstruction (Aharonson-Raz et al. 2012). However, there are no data on the role of PIMs in modulating neutrophil apoptosis in SIRS.

We hypothesized that *ex vivo* neutrophil apoptosis would be delayed in horses following intestinal ischemia and reperfusion (IR) and that treatment with GC would reduce the systemic inflammatory effects associated with intestinal IR, including delayed neutrophil apoptosis. Therefore, our objective was to evaluate the effect of intestinal inflammation, using a model of equine intestinal ischemia and reperfusion (IR) that has previously been shown to cause

endotoxemia (Moore et al. 1981), on *ex vivo* neutrophil apoptosis. In addition, we wanted to determine the effect of depleting PIMs using GC on the systemic inflammatory response following intestinal IR, including *ex vivo* neutrophil apoptosis.

4.3 Materials and Methods

4.3.1 Animals

Adult horses, quarantined for at least three weeks and determined to be healthy by physical examination and complete blood cell count, were used for the study. Throughout the study period, horses were offered hay and water *ad libitum*. At least 2 weeks prior to the study, horses were de-wormed with ivermectin (Eqvalan, Merial, Baie-d'Urfé, QC, Canada). Three days prior to surgery, horses were hospitalized to become acclimatized to their environment. A physical examination was performed daily while the horses were hospitalized prior to surgery. Horses were randomized to one of three groups using an on-line random number generator program (http://www.randomizer.org/form.htm). One group received only a ventral midline celiotomy (n = 4, CEL), a second group received intestinal ischemia and reperfusion (n = 6, IR), and a third group received intestinal ischemia and reperfusion and were administered gadolinium chloride (GC) to deplete intravascular macrophages (n = 6, IRGC). All procedures and experimental protocols were approved by the University of Saskatchewan Committee on Animal Care and Supply and the University of Saskatchewan Animal Research Ethics Board.

4.3.2 Intestinal ischemia and reperfusion model and sample collection

Forty-eight hours prior to surgery, a catheter was placed into the right jugular vein. Blood was collected for "pre" neutrophil isolation, measurement of plasma endotoxin and cytokines, and complete blood cell count. Samples for plasma endotoxin quantification were prepared by aseptically collecting 6-ml of blood into endotoxin free tubes containing

ethylenediaminetetraacetic acid (Sarsdet, Montreal, QC, Canada), separating the plasma by centrifugation (100 x *g* for 10 min), diluting the plasma 1:10 with endotoxin free water (Lonza, Allendale, NJ, USA), and heat inactivating for 60°C for 15 min. Presence of endotoxin in the plasma was detected at 0.06 EU/ml or 0.25 EU/ml using a commercially available limulus amebocyte assay (Lonza, Allendale, NJ, USA) using manufacturer's directions. Gadolinium chloride (Sigma Aldrich Canada, Oaksville, ON, Canada) in 60 ml of 0.9% sodium chloride was administered twice to IRGC horses (10 mg/kg bwt i.v.), while CEL and IR groups received 60 ml of 0.9% sodium chloride, 24 and 48 h pre-operatively.

Horses were anesthetized, placed in dorsal recumbency on an equine surgical table, and the ventral abdomen was clipped and prepared for aseptic surgery. A 25-cm incision was made along the ventral midline through the *linea alba*. The peritoneal cavity was entered using blunt dissection. For IR and IRGC horses, a 3-m section of jejunum was isolated 9-m oral to insertion of the ileocecal band on the proximal ileum. Sterile 0.9% sodium chloride solution was used throughout the procedure to moisten the intestine. Doyen intestinal clamps were placed across the lumen at each end of the isolated segment. Mesenteric vessels were occluded with temporary ligatures of latex penrose drains and hemostatic forceps to induce ischemia. After 2 h, the clamps and ligatures were removed. Celiotomies were closed routinely in 3 layers using various sutures sizes in a simple continuous pattern. Horses were allowed to recover unassisted from anesthesia. Following recovery from anesthesia, horses received butorphanol (Torbugesic,10 mg IM, Zoetis Canada, Kirkland, QC, Canada) every 4-6 h for pain management. Horses exhibiting high levels of pain received a single dose of detomidine (Dormosedan,1 μg/kg bwt IV, Zoetis Canada, Kirkland, QC, Canada).

A physical examination was performed every 6 h post-operatively. Blood was collected at 2 h and 6 h after the start of reperfusion for plasma endotoxin quantification and neutrophil isolation, respectively. Plasma was collected at 2, 6, 12, and 18 h after the start of reperfusion and stored at -80°C until quantification of plasma cytokines including equine interleukin (IL)-1 beta, equine tumor necrosis factor (TNF) alpha, equine IL-10, and human transforming growth factor-beta 1 (TGF-β1), was performed using commercially available enzyme-linked immunosorbent assays (ELISA) as per manufacturer directions (Duoset ELISA, R & D Systems, Minneapolis, MN, USA). At 18 h after ischemia, a post-operative complete blood cell count was performed and horses were euthanized with an overdose of pentobarbital.

Lung samples were collected and fixed as previously described (Johnson et al. 2014). Briefly, the left lung was fixed *in situ* by instilling 0.1% glutaraldehyde, 4% paraformaldehyde in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) into the left main bronchus, clamping the bronchus, and submerging the lung in an air-tight plastic bag. After 24 h of fixation at 4ºC, the left lung was sectioned by a randomized fractionation method to achieve appropriate sized tissue for paraffin embedding. Samples of the right lung and intestine were fixed by submersion in 4% paraformaldehyde in phosphate buffered saline (PBS). After 24 h of fixation at 4ºC, tissues were trimmed, dehydrated, and embedded in paraffin. Tissues were sectioned at a thickness of 7 μm. Additional samples of lung and intestine, from lesion and non-lesion areas of jejunum, were snap frozen on dry ice and stored at -80ºC.

4.3.3 Assessment of pulmonary inflammation and presence of pulmonary intravascular macrophages

4.3.3.1 Lung histology

Multiple lung sections from the cranial and caudal lobes of the left and right lungs were stained routinely with hematoxylin and eosin (H and E). A pathologist, blinded to treatment group, examined all sections by light microscopy. Lung sections were graded on a scale of 0-3 (0 = absent, 1 = mild, 2 = moderate, 3 = severe) for degree of inflammation based on amount of edema, septal thickening, and cellular infiltration.

4.3.3.2 Myeloperoxidase activity in lung samples

Myeloperoxidase (MPO) activity was assessed in fresh frozen lung tissues. Briefly, 500 mg of tissue was homogenized in 50 mM HEPES (Sigma-Aldrich Canada, Oakville, ON) using stainless steel beads (Qiagen Sciences, Germantown, MD). Tissues were re-homogenized to lyse cells in 0.5% cetyltrimethylammoniumchloride (Sigma-Aldrich Canada, Oakville, ON). Supernatants were diluted 1:50 in phosphate citrate buffer and protein concentration in supernatants was quantified using a commercial kit (DC™ Protein Assay, Bio-Rad Laboratories, Mississauga, ON, Canada). Diluted samples were incubated for two minutes with substrate solution containing resorcinol (Fischer Scientific Company, Ottawa, ON), tetramethylbenzidine (Sigma-Aldrich Canada, Oakville, ON), and hydrogen peroxide (Sigma-Aldrich Canada, Oakville, ON) in distilled water in a 96-well plate. The reaction was stopped with sulfuric acid and the color change was measured at an absorbance of 405 nm using a microtiter plate reader. Results for MPO activity was compared to a standard of known MPO concentration and corrected for protein concentration in the sample.

4.3.3.3 Immunostaining with anti-macrophage antibody

Lung sections were deparaffinized and fixed with ethanol. Endogenous peroxidase activity was inhibited (0.5% hydrogen peroxide in methanol) before incubating in pepsin to unmask antigens (2mg/ml pepsin in 0.01N HCl). Tissues were incubated in pepsin at room temperature for 60 minutes. Non-specific binding was blocked using bovine serum albumin (1% in PBS buffer) for 30 minutes prior to application of primary antibodies. Lung sections were evaluated for the primary antibody anti-human macrophage IgG_1 antibody (MAC387, AbD Serotec®, Raleigh, NC, USA), an antibody that binds to calprotectin, a protein present in many inflammatory cells including macrophages and neutrophils. This antibody has previously been used to identify equine pulmonary intravascular macrophages (Parbhakar et al. 2005, Schneberger et al. 2012). Following incubation with the primary antibody overnight at 4^{o}C, horseradish peroxidase (HRP)-conjugated secondary antibodies were applied and incubated at room temperature for 30 minutes. Color was developed using a substrate peroxidase kit (Vector Laboratories, Ontario, Canada), washed, and counterstained with methyl green (Vector Laboratories, Ontario, Canada). Two negative controls were prepared by omitting the primary or secondary antibody and an isotype control was prepared for the primary antibody. The primary antibody concentration was standardized using three serial concentrations. Mononuclear cells stained positive for MAC387 were counted by an observer blinded to treatment in 10 fields at 60x magnification in at least 3-4 different lung sections. Cells were counted only within the alveolar septum and mononuclear cells were differentiated from polymorphonuclear cells by nuclear morphology.

4.3.3.4 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

The TUNEL assay was performed using a commercially available kit per manufacturer's directions (Roche Applied Science, Laval, QC, Canada) to determine the extent of apoptosis in

PIMs. Briefly, lung sections were deparaffinized and fixed with ethanol. Tissues were permeabilized with proteinase K (20 μg/ml in 10 mM TrisHcl, Sigma-Aldrich Canada, Oakville, ON) for 30 minutes at 37°C. The TUNEL enzyme solution was diluted to 1:10 with label solution. Tissues were incubated with the diluted enzyme solution for one hour at 37°C. The slides were washed and the fluorescent signal was converted to a color signal using manufacturer's directions. A negative control was prepared by eliminating the enzyme solution. TUNEL stained cells were counted by an observer blinded to treatment in 10 fields at 60x magnification in at least 3-4 different lung sections. Only cells contained within the alveolar septum were counted.

4.3.4 Assessment of intestinal inflammation

4.3.4.1 Intestine histology

Intestinal sections were stained routinely for hematoxylin and eosin (H and E). A pathologist, blinded to treatment group, examined all sections by light microscopy. Intestinal sections were graded on a scale of 0-3 (0 = absent, 1 = mild, 2 = moderate, 3 = severe) for degree of inflammation. The presence of villous atrophy, epithelial degeneration, and hemorrhage were evaluated with a yes/no score. Predominate infiltrating cell type was also recorded.

4.3.4.2 Myeloperoxidase activity in intestine samples

MPO activity was measured in intestinal samples using the same protocol as for lung samples.

4.3.5 Neutrophil isolation and culture

Neutrophils were isolated using a previously reported protocol with minor modification (Cook et al. 2009). Blood was collected aseptically from a jugular vein into vacuum tubes containing EDTA (EDTA, Vacutainer™, BD, Mississauga, ON, Canada). Isolation was performed at room temperature (20-22°C) immediately after blood collection. The blood was allowed to separate

into plasma and red cell fractions for 30-45 min at room temperature. Plasma was layered onto 10 ml Ficoll-Paque Plus (GE Healthcare, Mississauga, ON, Canada) and centrifuged for 30 min at 400 x *g*. The subsequent pellet, containing erythrocytes and granulocytes, was retained after aspirating the supernatant and washed with Hank's Buffered Salt Solution (pH 7.4, HBSS) without phenol, magnesium, or calcium for 10 min at 200 x *g*. Erythrocytes were lysed with sterile distilled water for 25 s and restored to normotonicity with an equal volume hypertonic (2x) HBSS. Cells were washed 3 times with HBSS at 200 x *g* for 10 min, suspended in culture medium (Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 50 μg/ml streptomycin, and 25 mM HEPES, Lonza BioWhittaker®, VWR, Edmonton, AB, Canada) assessed for viability using trypan blue staining, and counted with a hemocytometer. Cell purity was determined on cytology preparations made with a cytocentrifuge (Shandon Cytospin 4, Thermo Scientific, Waltham, MA, USA) at 1000 rpm for 4 min with medium acceleration and stained with a modified Giemsa stain (Hemacolor® EMD chemical, Gibbstown, NJ).

Neutrophils were suspended in culture medium in plastic multi-well cell culture plates at a standard concentration of 2×10^6 cells/ml and cells were incubated at 37°C in 5% CO_2. To assess the effect of gadolinium chloride on neutrophil apoptosis, cells were treated in culture with 270 μM gadolinium chloride or were left untreated for 24 h. For *ex vivo* experiments, neutrophils isolated before and after surgery were cultured for 12 or 24 h. Prior to performing apoptosis assays, neutrophils were taken out of culture, pelleted, and washed twice with HBSS at 300 x *g* for 8 min.

4.3.6 Apoptosis assays

4.3.6.1 Flow cytometry

Neutrophils (1 x 10^6 cells/ml) were stained with CF488-conjugated Annexin V (AV) and propidium iodide (PI) apoptosis kit per manufacturer's instructions (CF™488A-Annexin V and PI Apoptosis Assay Kit, Biotium, Hayward, CA, USA). Staining controls included unstained untreated cells, untreated cells stained with AV only, and untreated stained with PI only. A flow cytometer (CyFlow®, Partec, Swedesboro, NJ, USA) was used to count negatively stained (live cells), AV positively stained (apoptotic cells), or AV and PI positively stained (dead) cells. Flow cytometry data were analyzed with commercial software (FlowMax Software© Version 2.6, Quantum Analysis GmbH, Münster, Germany). The gated population was determined with unstained cells based on size (forward scatter) and granularity (side scatter). Quadrants were determined using untreated cells stained with only AV or PI. Data were acquired on at least 10,000 gated events.

4.3.6.2 Caspase activity assay

Caspase-3, -8, and -9 activities were indirectly measured using a commercially available colorimetric assay kit per manufacturer's instructions (Caspase-3, -8, or -9 Colorimetric Assay Kit, Biovision, Milpitas, CA, USA). Caspase-3 activity was measured to determine overall apoptotic activity. Caspase-8 activity was measured to assess the contribution of the extrinsic pathway and Caspase-9 activity was measured to assess the contribution of the intrinsic pathway on the occurrence of neutrophil apoptosis. At least 1 x 10^6 neutrophils were lysed using the manufacturer's lysis buffer following removal from culture and washing as described above. Cell lysates were stored at -80°C until processing. Upon thawing, protein concentration in cell lysates was quantified using a commercial kit (DC™ Protein Assay, Bio-Rad Laboratories,

Mississauga, ON, Canada). Cell lysates were incubated with the manufacturer's reaction buffer containing dithiothreitol (DTT) and each Caspase's substrate: DEVD-pNA (Caspase-3), IETD-pNA (Caspase-8), or LEHD-pNA (Caspase-9) in 96-well plates for 2 h at 37°C. Immediately after incubation, free pNA was quantified by measuring absorbance at 405 nm in a microtiter plate reader. Levels of free pNA were corrected for protein concentration in cell lysates.

4.3.7 Statistical analysis

Data were analyzed with commercial software packages (StataIC 13, StataCorp, College Station, TX, USA and Graphpad Prism, Graphpad Software, Inc. La Jolla, CA, USA). For all comparisons, a *P*-value of < 0.05 was considered significant. Differences in physical examination parameters and plasma cytokine concentrations over time were compared among groups using a repeated-measure ANOVA. Differences in physical examination parameters and plasma cytokine concentrations from baseline within a group and differences in physical examination parameters and plasma cytokine concentrations among groups at each time point were analyzed using a Kruskal-Wallis rank test with a Dunn's multiple comparisons test. Difference in complete white cell and neutrophil counts before and after surgery was assessed for each group by comparing the difference in the means before and after surgery using a one-sided *t*-test. Difference in complete white cell and neutrophil counts among groups was assessed using a Kruskal-Wallis rank test with a Dunn's multiple comparisons test. A Kruskal-Wallis rank test with a Dunn's multiple comparisons test was used to assess differences in histology scores, MPO activity, MAC387 staining, and TUNEL staining among groups. A Wilcoxon rank sum test was used to compare inflammation scores and MPO activity between lesion and non-lesion intestinal samples within the IR and IRGC groups. To assess differences in *ex vivo* neutrophil apoptosis and caspase activity before and after surgery within a group, difference in the means were

calculated from before and after surgery, then a one-sided *t*-test was performed. Differences in *ex vivo* neutrophil apoptosis and caspase activity among groups, before or after surgery, were assessed with a Kruskal-Wallis rank test with a Dunn's multiple comparisons test. A Mann-Whitney U test was used to assess the effect of treatment with gadolinium chloride on *in vitro* neutrophil apoptosis.

4.4 Results

4.4.1 Clinical data

All horses exhibited various clinical signs of a systemic inflammatory response, including depression, tachycardia, hyperthermia, and to a lesser extent tachypnea at some point post-operatively. There was no difference in body temperature, heart rate, or respiratory rate among groups at any single time point or over time after surgery (Figure 4.1). Body temperature was significantly elevated above baseline for IR and IRGC horses at 12 h post-reperfusion (IR: $P < 0.001$, IRGC: $P = 0.002$, Figure 4.1). Heart rate was significantly elevated from baseline in CEL and IR horses at 12 h post-reperfusion (CEL: $P = 0.008$, IR: $P = 0.002$, Figure 4.1). Respiratory rate was not different from baseline in any group (Figure 4.1). Horses in the CEL and IRGC groups had a significant increase in total white blood cell count after surgery (CEL: $P = 0.02$, IRGC: $P = 0.008$, Figure 4.2), but all groups had a significant increase in total circulating neutrophils after surgery (CEL: $P = 0.007$, IR: $P = 0.01$, IRGC: $P = 0.0006$, Figure 4.2). There was no difference in total white cell counts or neutrophils counts among groups before or after surgery. There was no difference over time for IL-1β, TNF-α, IL-10, or TGF-β concentrations among groups. IRGC horses had greater plasma IL-1β concentration compared to IR horses 18 h after surgery ($P = 0.03$, Figure 4.3) and greater TNF-α concentrations compared to IR horses 2 and 6 h after surgery (2 h: $P = 0.03$, 6 h: $P = 0.03$, Figure 4.3). In IRGC horses, TNF-α was

significantly increased from baseline at post-reperfusion hours 6, 12, and 18 and IL-10 was significantly decreased from baseline at post-reperfusion hours 12 and 18 h ($P < 0.05$, Figure 4.3). No horses had measurable endotoxin (>0.06 EU/ml) in their plasma before surgery or 2 h after the start of reperfusion.

4.4.2 Intestinal ischemia and reperfusion failed to induce significant intestinal inflammation or remote lung injury

There was no difference among groups for total pulmonary inflammatory scores or lung MPO activity (Figure 4.4a). There was no difference among groups for total intestinal inflammation scores or intestine MPO activity (Figure 4.4b). Additionally, there was no difference in total intestinal inflammation scores or MPO activity between lesion and non-lesion areas of jejunum with IR and IRGC groups. The primary infiltrating cell type in areas of the IR lesion consisted of lymphocyte plasma cells.

4.4.3 Treatment with gadolinium chloride reduces MAC387 positive mononuclear cell infiltration associated with intestinal ischemia and reperfusion

There was no difference in histologic grades of lung inflammation among groups and, overall, horses had very mild pulmonary inflammation (Figure 4.5a). There were significantly fewer alveolar septal mononuclear cells in pulmonary sections from IRGC compared with IR horses following intestinal ischemia and reperfusion ($P = 0.02$, Figure 4.5b). There was no difference in TUNEL positive cells within the alveolar septum among groups (Figure 4.5c).

4.4.4 Ex vivo neutrophil apoptosis is reduced following surgery due to reduced caspase activity except in horses treated with gadolinium chloride

There was no difference among percentage of apoptotic neutrophils as measured by flow cytometry immediately after isolation before and after surgery for each group (Figure 4.6).

However, there was a significant reduction in caspase-3 activity in freshly isolated (0 h incubation) neutrophils in the IR group, but not the CEL and IRGC groups post-operatively (P = 0.03, Figure 4.7).

Neutrophil apoptosis was significantly delayed *ex vivo* after 12 or 24 h of culture in neutrophils isolated from horses after the induction of systemic inflammation compared to baseline for all groups, except IRGC (12 h: CEL: P = 0.03, IR: P = 0.05, IRGC: P =0.2; 24 h: CEL: P = 0.001, IR: P = 0.004, IRGC: P = 0.3, Figure 4.6). There was no difference in the occurrence among groups pre- or post-operatively; however when IR and IRGC groups were compared without CEL group, IRGC horses had greater percentage of apoptotic neutrophils in post-operative samples incubated for 24 h (P = 0.04, Figure 4.6).

Caspases are important proteases involved in both the classical intrinsic and extrinsic pathways of apoptosis. Caspase-3 is the end effector caspase for both pathways, caspase-8 is an initiator caspase involved in the extrinsic pathway, and caspase-9 is an initiator caspase involved in the intrinsic pathway. Caspase-3, -8, and -9 activities were reduced in neutrophils isolated from IR horses after surgery compared to before surgery (caspase-3: P = 0.002, caspase-8: P = 0.002, caspase-9, P = 0.04, Figure 4.7). Horses in the CEL group only had reduced caspase-3 activity after surgery as compared to before surgery (P = 0.04, Figure 4.7), while caspase activity was not reduced in IRGC horses.

4.4.5 Gadolinium chloride does not affect the occurrence of neutrophil apoptosis in vitro

When neutrophils were incubated with concentrations of gadolinium up to 270 μM for 24 hours, there was no effect on the occurrence of apoptosis compared to untreated cells (P = 0.39, Figure 4.8).

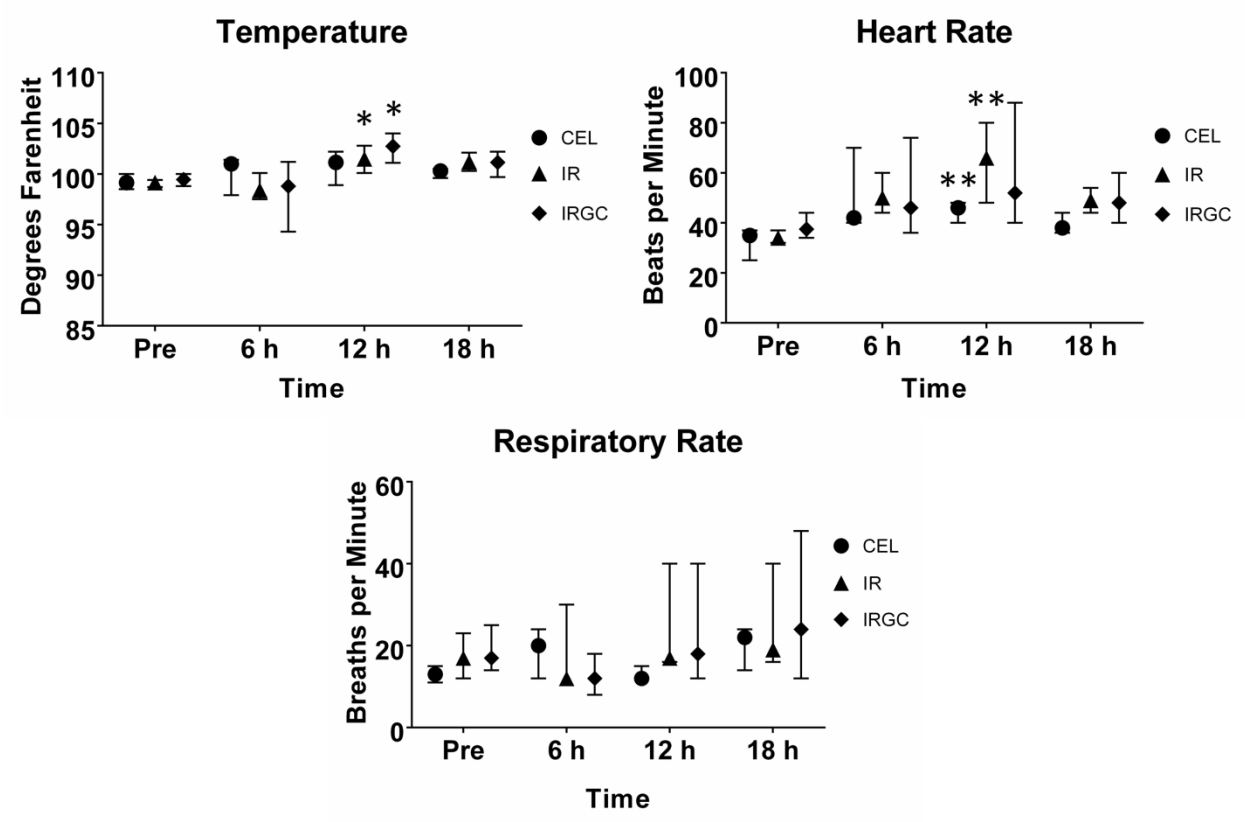


Figure 4.1. Physical examination parameters recorded following surgery for sham celiotomy (CEL) or intestinal ischemia and reperfusion (IR) or IR with administration of gadolinium chloride (IRGC). Data represents the median with range. Differences for each group's parameters for each time point compared to baseline and difference among groups at each time point was assessed with a Kruskal-Wallis rank test with a Dunn's multiple comparisons test. Difference among groups over time for each physical examination parameter was analyzed with a repeated-measure ANOVA. Stars represent difference from baseline (*P<0.05, **P<0.01).

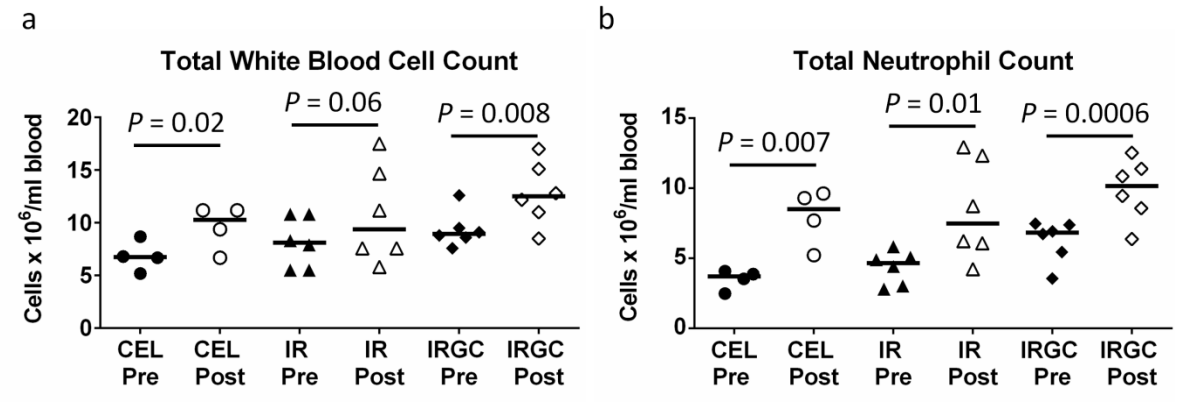


Figure 4.2. Total white blood cell counts and total neutrophil counts for horses receiving sham celiotomy (CEL) or intestinal ischemia and reperfusion without (IR) or with administration of gadolinium chloride (IRGC) before and after surgery. Bar represents the median. Difference in the means before and after surgery was assessed using a one-sided t-test. Difference groups before and after surgery was assessed using a Kruskal-Wallis rank test with a Dunn's multiple comparisons test.

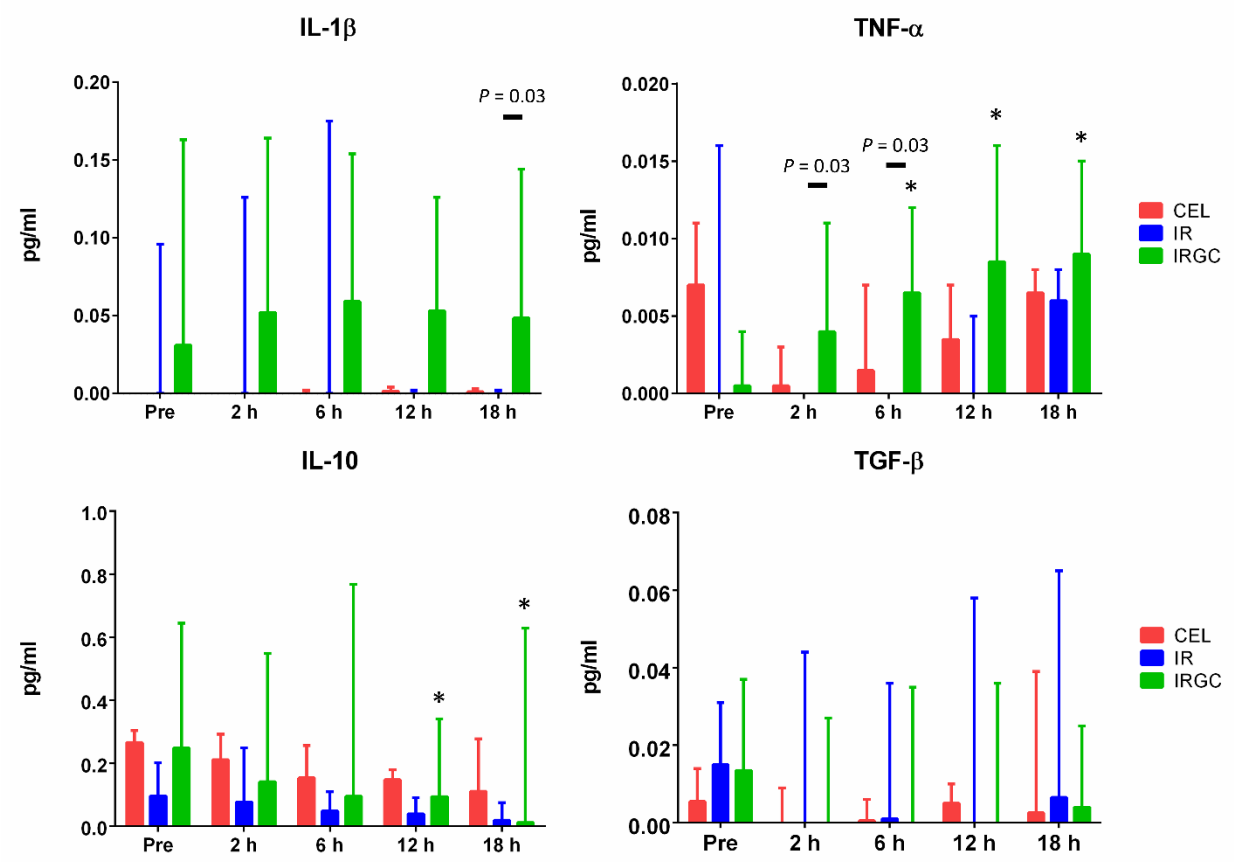
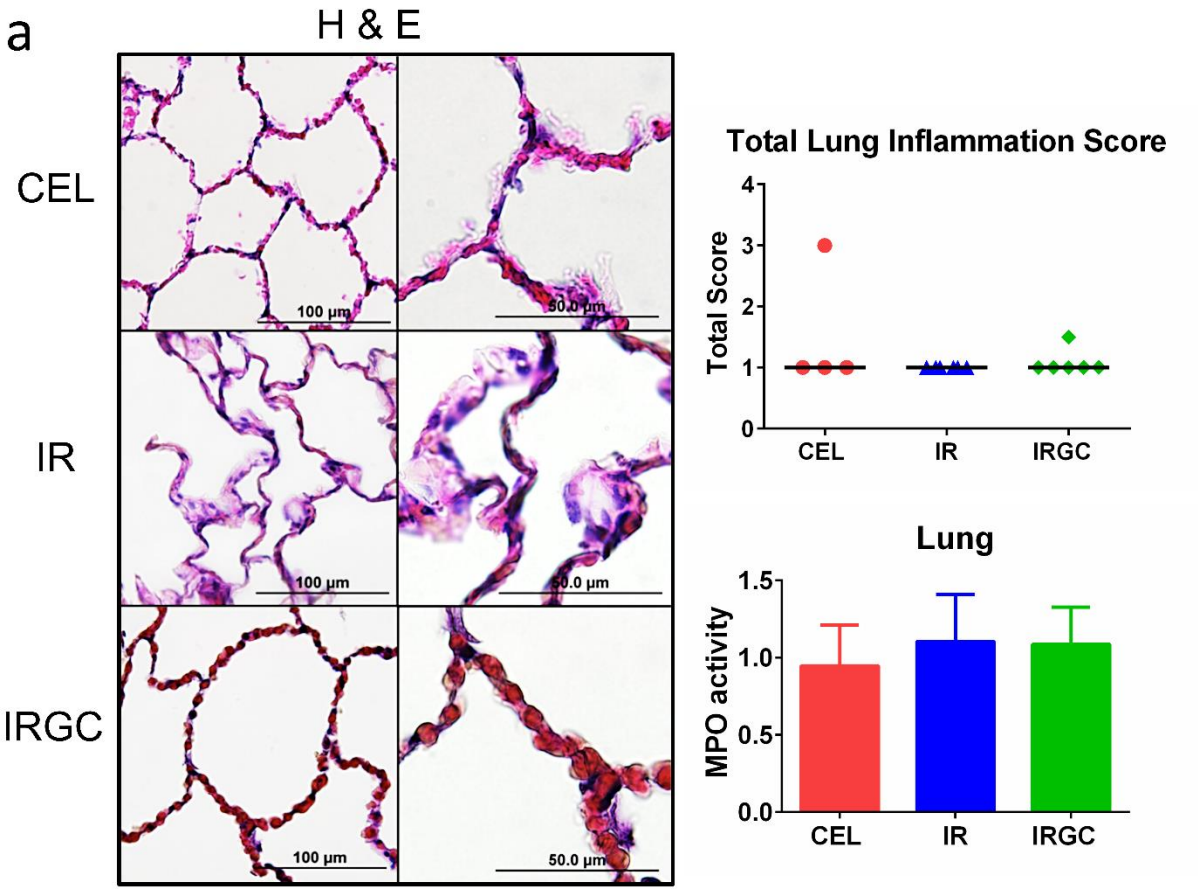


Figure 4.3. Plasma cytokine concentrations for horses receiving sham celiotomy (CEL) or intestinal ischemia and reperfusion without (IR) or with administration of gadolinium chloride (IRGC) before and after surgery. Difference among groups at each time point and difference within a group among time points were assessed with a Kruskal-Wallis rank test with a Dunn's multiple comparisons test. Difference among groups over time for each cytokine was analyzed with a repeated-measure ANOVA. Data represent median and range. Stars represent significance from pre-operative values ($P < 0.05$).


a
H & E
CEL
IR
IRGC
100 µm
50.0 µm
Total Lung Inflammation Score
Total Score
0
1
2
3
4
CEL
IR
IRGC
Lung
MPO activity
0.0
0.5
1.0
1.5
CEL
IR
IRGC

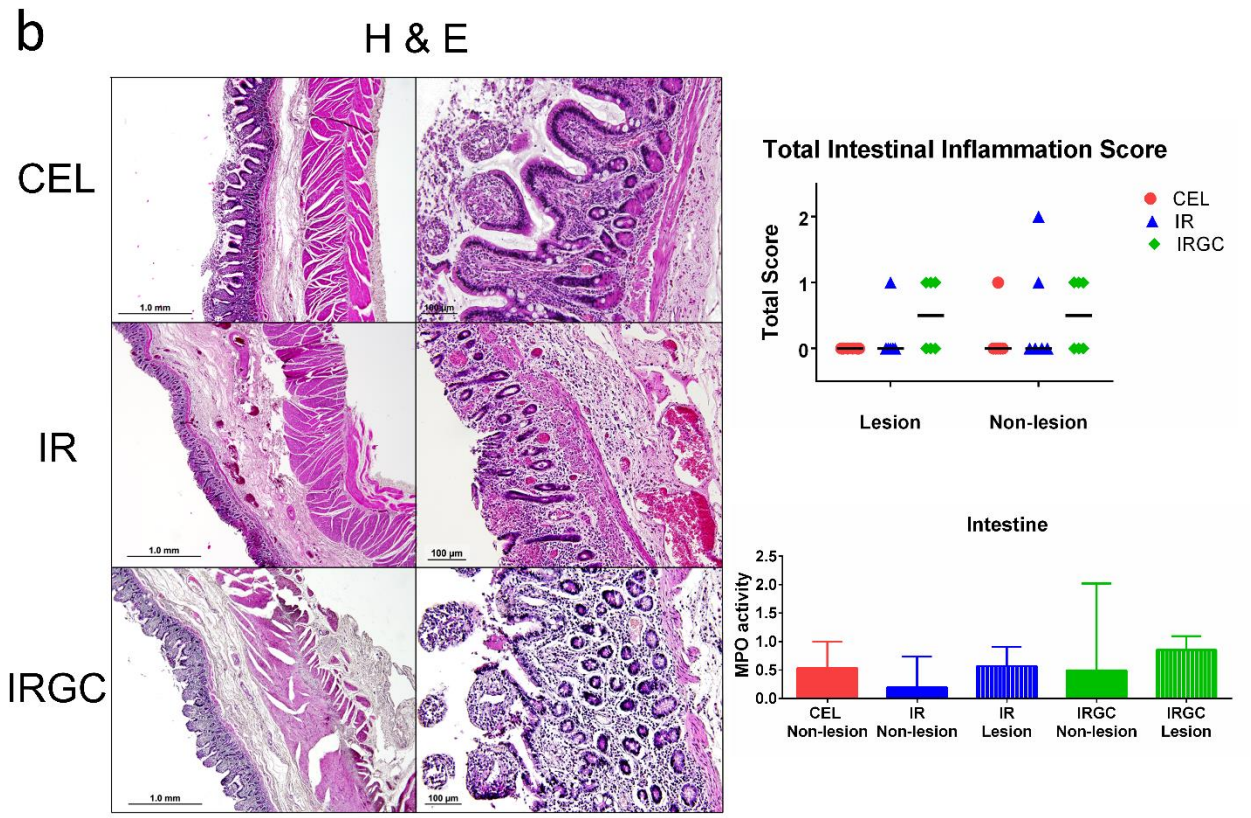
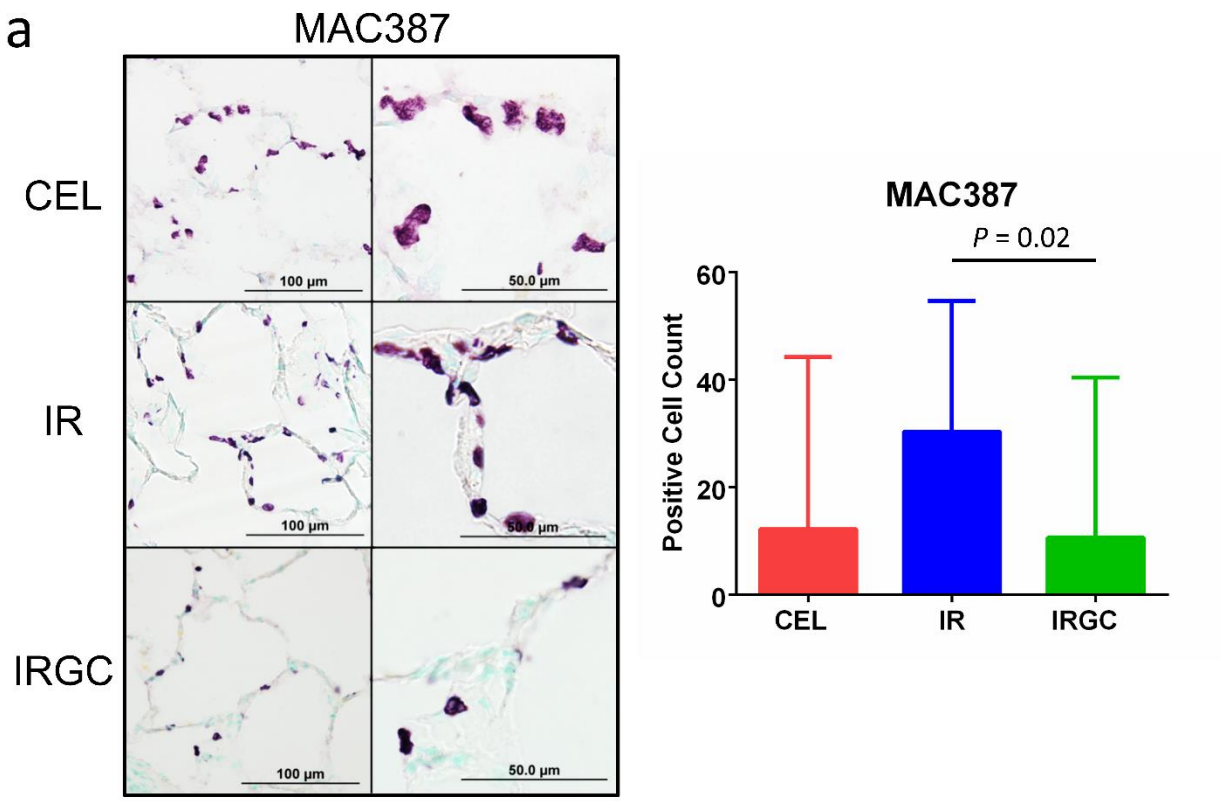


Figure 4.4. Assessment of pulmonary and intestinal inflammation. a) Representative photomicrographs of pulmonary H & E stained slides, total lung inflammation scores, and lung MPO activity. b) Representative photomicrographs of intestinal H & E stained slides, total intestinal inflammation scores, and intestinal MPO activity. Difference among groups was assessed with Kruskal-Wallis rank test with a Dunn's multiple comparisons test. Difference between lesion and non-lesion inflammation scores were compared for each group using a Wilcoxon Rank Sum Test. Data are represented by the median and range. Bar represents the median.


a
MAC387
CEL
IR
IRGC
100 µm
50.0 µm
MAC387
P = 0.02
Positive Cell Count
60
40
20
0
CEL
IR
IRGC

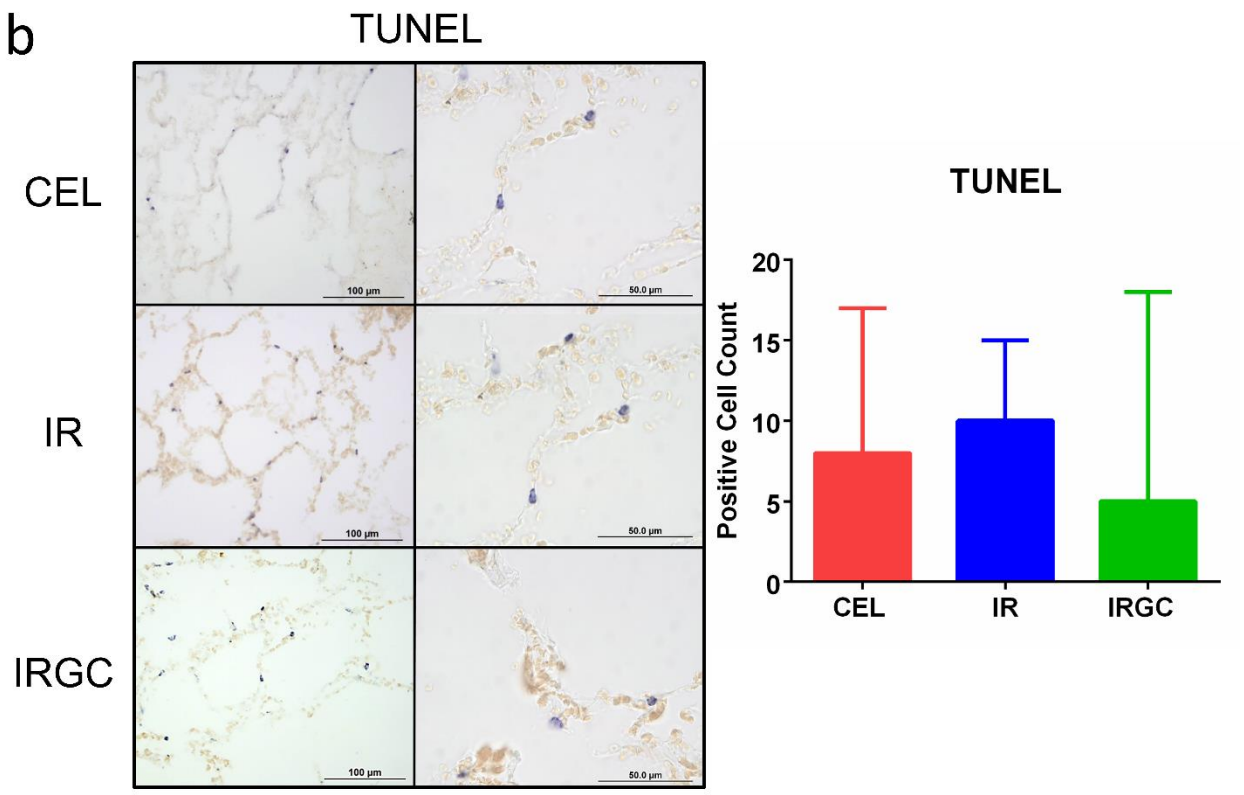


Figure 4.5 Parenteral administration of gadolinium chloride reduces mononuclear cell infiltration associated with intestinal ischemia and reperfusion. Photomicrographs of representative pulmonary sections stained with a) calprotectin (MAC387) or against b) TUNEL with corresponding positive cell counts in 10 fields counted at 60x. Data are represented by the median and range. Data were analyzed using a Kruskal-Wallis rank test with a Dunn's multiple comparisons test.

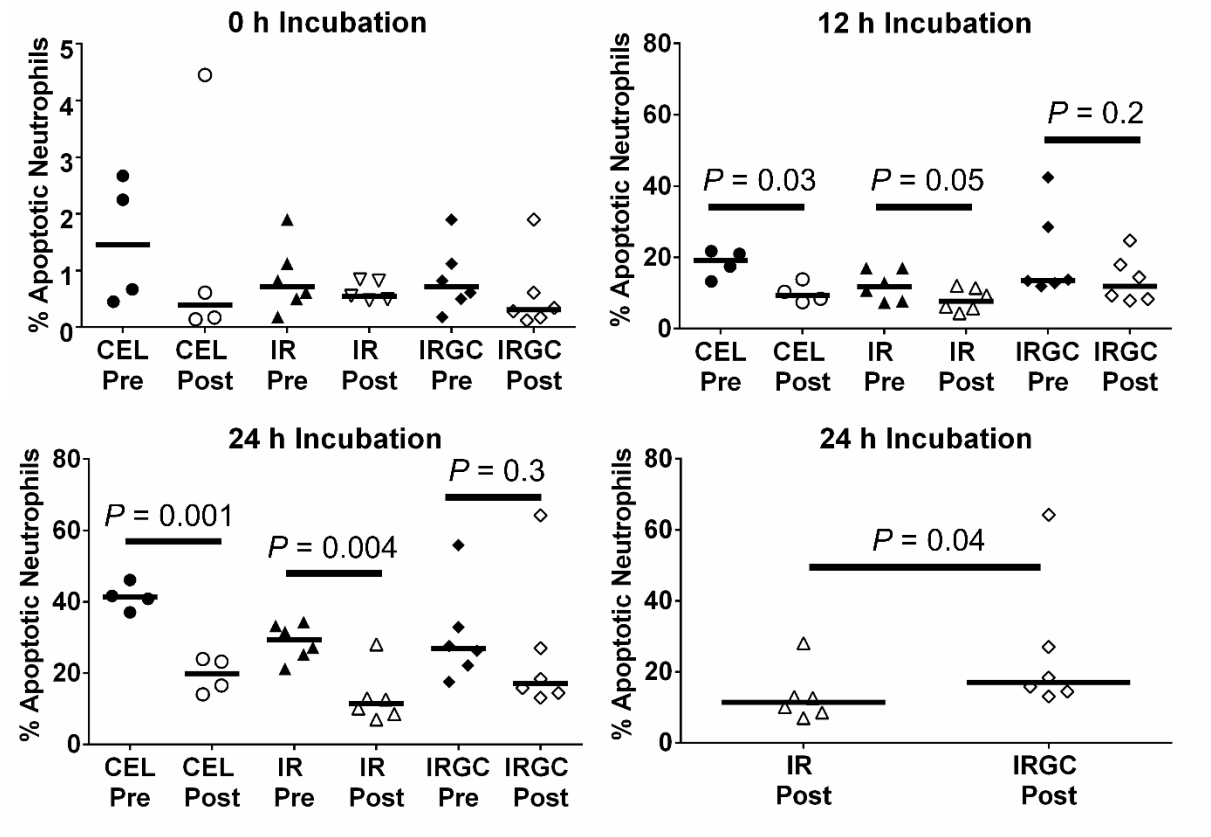


Figure 4.6 *Ex vivo* neutrophil apoptosis is delayed following surgery, except in horses administered parenteral gadolinium chloride. Differences in mean occurrence of apoptosis before and after surgery were calculated then a one-sided *t*-test was performed to assess difference before and after surgery within a group. A Kruskal-Wallis rank test with a Dunn's multiple comparisons test was used to assess difference among groups in the occurrence of neutrophil apoptosis before or after surgery. For 24 h incubation, IR and IRGC groups were compared without CEL group using a Mann-Whitney U test. Bar represents the median.

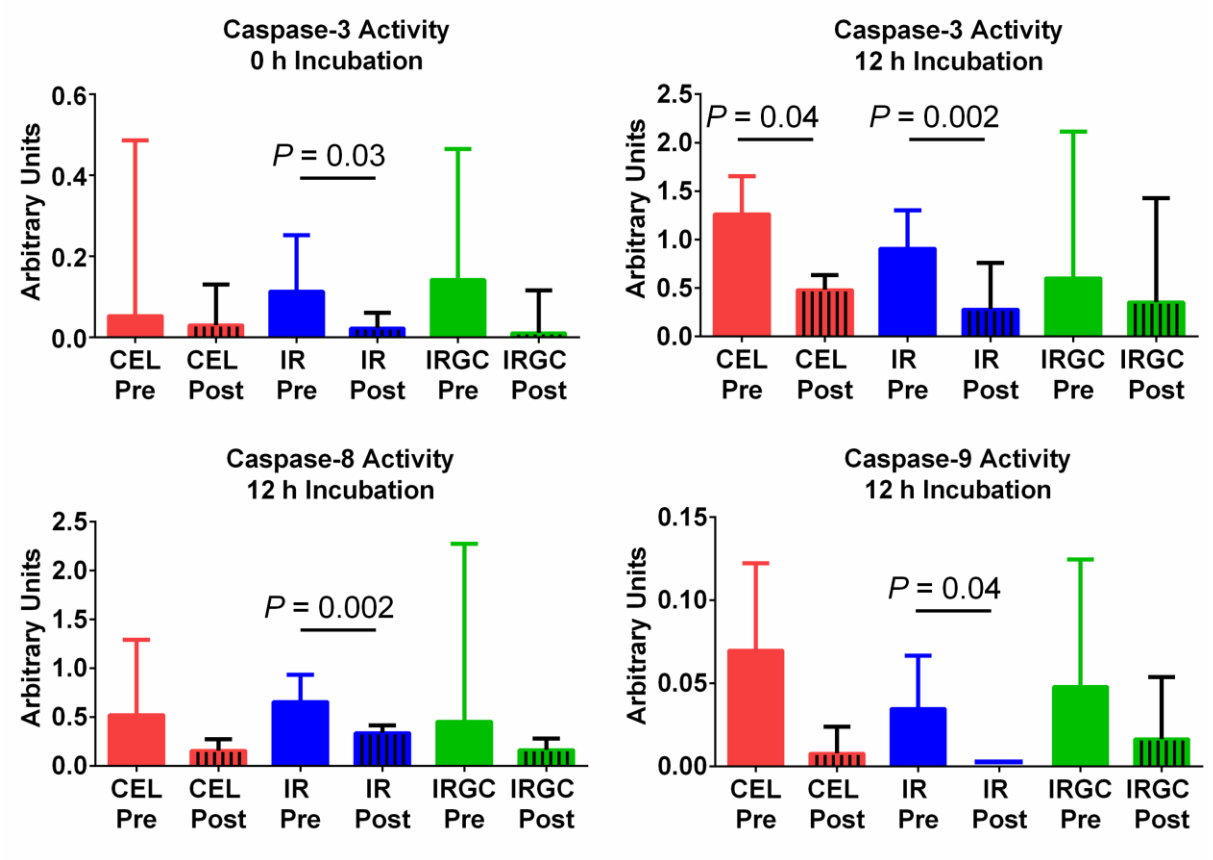


Figure 4.7 Neutrophil caspase activities are reduced in horses undergoing celiotomy and intestinal ischemia and reperfusion, but not in horses that received parenteral gadolinium chloride. Differences in mean occurrence of apoptosis before and after surgery were calculated then a one-sided *t*-test was performed to assess difference before and after surgery within a group. A Kruskal-Wallis rank test with a Dunn's multiple comparisons test was used to assess difference among groups in caspase activity. Data are represented by the median and range.

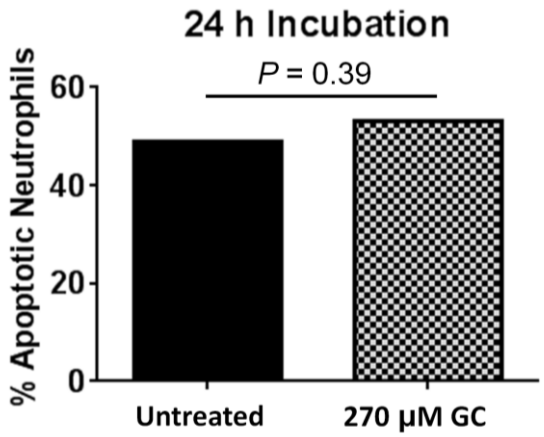


Figure 4.8 Gadolinium chloride treatment has no effect on the occurrence of neutrophil apoptosis *in vitro*. Data were analyzed with a Mann-Whitney U test and is represented by the median (n = 6 horses).

4.5 Discussion

This study supports our hypothesis that *ex vivo* neutrophil apoptosis is delayed following intestinal IR due to reduced caspase activity and parenteral administration of GC reduced delayed *ex vivo* neutrophil apoptosis and reduced pulmonary mononuclear infiltration into the lung following intestinal IR; however, treatment with GC did not alter the measured parameters of systemic inflammation. In addition, control horses undergoing a sham celiotomy unexpectedly experienced a mild systemic inflammatory response and delayed *ex vivo* neutrophil apoptosis following surgery.

Gadolinium chloride is a chemical that may be used to preferentially inactivate and deplete intravascular macrophages, specifically equine PIMs, with the intent to study the contribution of these cells to inflammatory processes in horses (Parbhakar et al. 2005). GC causes mononuclear cell apoptosis in a dose- and time-dependent manner starting 8 h after administration with near complete depletion occurring by 72 h (Mizgerd et al. 1996, Parbhakar et al. 2005). In our horses, treatment with GC significantly reduced mononuclear cell counts in lungs of horses that underwent IR, representing the first time this has been observed in an equine systemic inflammatory model. The reduction in mononuclear cell counts could be due to depletion of constitutive PIMs and reduced infiltration of new cells following induction of IR. However, there was no difference in the number of apoptotic septal cells within the lung as demonstrated by TUNEL staining. This finding could be explained by the fact that PIMs were already dead, having been depleted by two doses of GC administered 66 and 42 hours prior to euthanasia and lung fixation, rather than apoptotic, which is required for positive TUNEL staining. Nevertheless, the use of GC resulted in significant depletion of PIMs in IR horses.

Contrary to another study published by our research group, horses in this study did not develop significant pulmonary inflammation following intestinal ischemia and reperfusion or anesthesia (Montgomery et al. 2014). One explanation for this difference is that samples in the previous report were taken one hour after the start of reperfusion; whereas, the samples in the current study were taken 18 hours following reperfusion, potentially giving the lung time to "recover" from the effects of anesthesia and remote injury. Even so, the majority of lungs had evidence of dorsal bruising at necropsy, which would be considered a normal sequela to undergoing prolonged anesthesia in dorsal recumbency in the horse.

In addition to the lack of pulmonary inflammation, there was an unexpected lack of inflammation on histologic examination in areas of the jejunum that experienced ischemia and reperfusion despite grossly visible lesions observed at necropsy. In addition, the primary infiltrating cell types consisted of lymphocytes and plasma cells, which is different than the previously reported primary infiltrating cell type of neutrophils (Cook et al. 2009, Little et al. 2005). Despite the lack of local inflammation observed in intestinal and pulmonary tissues, horses did develop a systemic inflammatory response following surgery that met the criteria of SIRS.

It has been demonstrated in multiple models and clinical cases, that SIRS delays neutrophil apoptosis in humans and laboratory mammals (Fanning et al. 1999, Jimenez et al. 1997, Shang et al. 2007). Though all horses in this study experienced only a mild systemic inflammatory response with minimal local intestinal inflammation, they would have met the classification for SIRS (having 2 of the following: tachycardia, tachypnea, fever, leukopenia or leukocytosis with or without a left shift) between12-18 h post-operatively. Concomitantly, there was a significant delay in *ex vivo* neutrophil apoptosis in neutrophils isolated following surgery

in CEL and IR horses, but not IRGC horses, due to reduced intrinsic and extrinsic caspase activity.

Despite failing to identify the precise mechanism of delayed *ex vivo* neutrophil apoptosis following intestinal IR, we were able to determine that delayed *ex vivo* neutrophil apoptosis following the induction of systemic inflammation from intestinal IR involved reduction in both intrinsic (caspase-9) and extrinsic (caspase -8) initiator caspase activities that likely culminated in a significant reduction in effector caspase-3 activity based on known pathways of apoptosis (Figure 4.9). These data suggest that both the intrinsic and extrinsic pathways of apoptosis are affected in neutrophils isolated from horses experiencing intestinal IR. Similarly, in humans, delayed *ex vivo* neutrophil apoptosis in neutrophils isolated from septic patients, who are invariably experiencing concurrent SIRS, was associated with reduced caspase-3 and -9 activities (Taneja et al. 2004).

This is the first report investigating the role of PIMs in the modulation of neutrophil apoptosis. The parenteral administration of GC rescued *ex vivo* delayed neutrophil apoptosis, apparently through less suppression of caspase-3, -8, and -9 activities allowing neutrophils to undergo apoptosis *in vitro* at a similar rate as before surgery. However, GC had no effect on the occurrence of *in vitro* neutrophil apoptosis or *in vitro* neutrophil caspase activity (data not shown). Further investigation is warranted to characterize the role of PIMs in modulating neutrophil lifespan.

We have demonstrated that delayed *ex vivo* equine neutrophil apoptosis is associated with the development of SIRS; and due to the multifactorial etiology of SIRS the exact cause of delayed *ex vivo* neutrophil apoptosis cannot be surmised from the data collected in this study; however, certain causes may be ruled out or postulated. First, it is possible that delayed *ex vivo*

neutrophil apoptosis was independent of endotoxemia as our horses did not have measurable endotoxin 2 h after the start of reperfusion. It is also possible that the development of endotoxemia could have been missed due to incorrect time of sampling. However, we chose the time point based on work performed by Moore et al. (1981), where they quantified endotoxin at 60 and 120 minutes following the start of intestinal reperfusion after 50 or 180 minute period of ischemia. In a clinical study, the sensitivity and specificity of detecting endotoxin in the plasma of horses that were clinically classified as being endotoxic was only 58.4% and 87.5%, respectively, suggesting that plasma endotoxin quantification is not straightforward or the term endotoxemia is improperly used in clinical cases where horse are experiencing SIRS rather than endotoxemia (Moore and Vandenplas 2014, Senior et al. 2011). Endotoxemia is transient as LPS is rapidly removed from the equine blood compartment (Fessler et al. 1989), so ideally multiple samples should have been collected over time to more accurately measure the presence of endotoxin in the plasma in the horses in this study. Furthermore, there may other microbial molecules in the blood of horses experiencing IR that may activate neutrophils, similarly to LPS.

Second, the cytokine profile was opposite of what was expected given the *ex vivo* neutrophil apoptosis results; namely, IRGC horses had greater concentrations of IL-1β and TNF-α compared to IR horses post-operatively with a concomitant reduction in IL-10 concentration from baseline. Unfortunately, there is a distinct void in the equine literature on the plasma cytokine profile of horses undergoing experimental intestinal ischemia and reperfusion, so we cannot relate our results to previous studies. In a rat model of intestinal ischemia and reperfusion, plasma concentrations of IL-1β and TNF-α peaked at 1 h and 3 h, respectively, which is much sooner than what we measured in horses undergoing intestinal ischemia and

reperfusion, but is similar in the fact that IL-1β peaks prior to TNF-α (Guzma´n-de la Garza et al. 2013).

Inflammatory cytokines, including IL-1β and TNF-α, have been shown to delay neutrophil apoptosis *in vitro* (Colotta et al. 1992). However, we have failed to delay equine neutrophil apoptosis with treatment of recombinant equine IL-1β *in vitro* (unpublished data) and TNF-α has been shown to have variable effects on neutrophil apoptosis depending on concentration and time of exposure (Cross et al. 2008, Fox et al. 2010, Murray et al. 1997, Salamone et al. 2001). In fact, early exposure to low concentrations of TNF-α induces neutrophil apoptosis; and when neutrophils are pre-treated with TNF-α, apoptosis is accelerated when exposed to substances that commonly delay neutrophil apoptosis, such as *Escherichia coli* (Salamone et al. 2001). In IRGC horses, TNF-α concentrations increased from 2 h up to the last time point measured at 18 h post-reperfusion; whereas TNF-α concentrations started to increase in IR horses at 12 h post-reperfusion. Given that neutrophils were isolated 6 h post-reperfusion, when TNF-α was beginning to increase in IRGC horses and not measurable in IR horses, variable effects of TNF-α on neutrophil lifespan would be expected. In IRGC horses, neutrophils were being exposed to a low level of TNF-α during the early inflammatory phase, which may have contributed to the observed difference in *ex vivo* neutrophil apoptosis. Additionally, IL-1β and TNF-α activate equine neutrophils to promote reactive oxygen species (ROS) production (Benbarek et al. 2008). It is currently thought that most of the molecular pathways causing neutrophil apoptosis are dependent upon ROS generation; however, ROS can have alternative effects on neutrophils, such as interference with caspase activities causing delayed apoptosis or the initiation of cell necrosis rather than apoptosis (Fadeel et al. 1998,

Geering and Simon 2011, Scheel-Toellner et al. 2004). Based on our results, the effect of TNF-α and ROS production on equine neutrophil apoptosis deserves further evaluation.

It has previously been shown by our research group that depletion of PIMs reduces the expression of IL-1β and TNF-α in lung cells following parenteral endotoxin challenge; however, circulating cytokines were not measured (Parbhakar et al. 2005). Our findings suggest that PIMs are not a primary source of circulating cytokines during inflammatory processes. However, it is possible that by depleting PIMs a shift toward resolution of inflammation may have been impaired within the scope of the inflammatory response, which could explain why IRGC horses experienced similar signs of systemic inflammation as IR horses and why the inflammatory cytokines, TNF-α and IL-1β, were elevated and the anti-inflammatory cytokine, IL-10, was reduced in IRGC. For example, in mice depleted of intravascular macrophages and subjected to intestinal ischemia and reperfusion, TNF was increased and IL-10 was decreased compared to non-depleted mice (Ellet et al. 2010). Additionally, because PIMs are important for removal of foreign entities, such as LPS, from the bloodstream, their depletion could result in a greater inflammatory response. Further work is needed to elucidate the plasma cytokine profile, both pro- and anti-inflammatory, in horses undergoing inflammatory models to provide a basis from which modulation of the innate immune response can be assessed.

Third, another contributor to delayed neutrophil apoptosis, which has received limited investigation, has been coined the "community effect," whereby neutrophil apoptosis is delayed by increased concentration and density *in vitro* (Attalah et al. 2012, Hannah et al. 1998). To the authors' knowledge, concentration- and density-delayed neutrophil apoptosis have not been studied *in vivo* nor have they been investigated in equine neutrophils; however, the results of our study, where horses experienced a significant increase in circulating neutrophil concentrations

after surgery and concomitant delayed *ex vivo* apoptosis without measurable plasma endotoxin, support this mechanism of delayed neutrophil apoptosis. Indeed, we have supporting data that demonstrate the occurrence of equine neutrophil apoptosis is concentration dependent *in vitro* (Chapter 5).

Finally, there are other inflammatory mediators that were not measured in this study that might have contributed to the observed delay in *ex vivo* neutrophil apoptosis in CEL and IR horses. Granulocyte macrophage-colony stimulating factor (GM-CSF) is a powerful inhibitor of neutrophil apoptosis and has been shown to play a significant role in SIRS-delayed neutrophil apoptosis *in vivo* (Fanning et al. 1999). Also, complement 5a (C5a) has been identified as a key mediator of delayed neutrophil apoptosis in septic rats and human patients (Guo et al. 2006, Unnewehr et al. 2013).

It is important to notice that horses in the CEL group also experienced a significant increase in systemic neutrophils and delayed *ex vivo* neutrophil apoptosis, even though they were considered to be controls. This observation serves two purposes, first it highlights the need for appropriate controls in inflammatory models. To the authors' knowledge, this is the first study that has included a sham celiotomy group in an equine intestinal IR model. Secondly, it is important to realize that performing any type of surgery will lead to some amount of systemic inflammatory response, which could explain, in part, why Krista et al. failed to show a difference in the occurrence of *ex vivo* neutrophil apoptosis between horses undergoing elective orthopedic procedures and abdominal exploratory surgery (Krista et al. 2013).

In conclusion, the induction of systemic inflammation in horses undergoing celiotomy and intestinal ischemia and reperfusion resulted in delayed *ex vivo* neutrophil apoptosis via down-regulation of caspase activity. Ultimately, delayed neutrophil apoptosis may have a

significant impact on the progression of many inflammatory disease processes due to the potential for inadvertent tissue damage to occur during neutrophil activation and the reduction of apoptotic neutrophils that are necessary for macrophage-driven resolution of inflammation. Therefore, the selective induction of neutrophil apoptosis could prove to be a potential therapeutic target to treat diseases associated with excessive neutrophil activation and accumulation, such as SIRS.

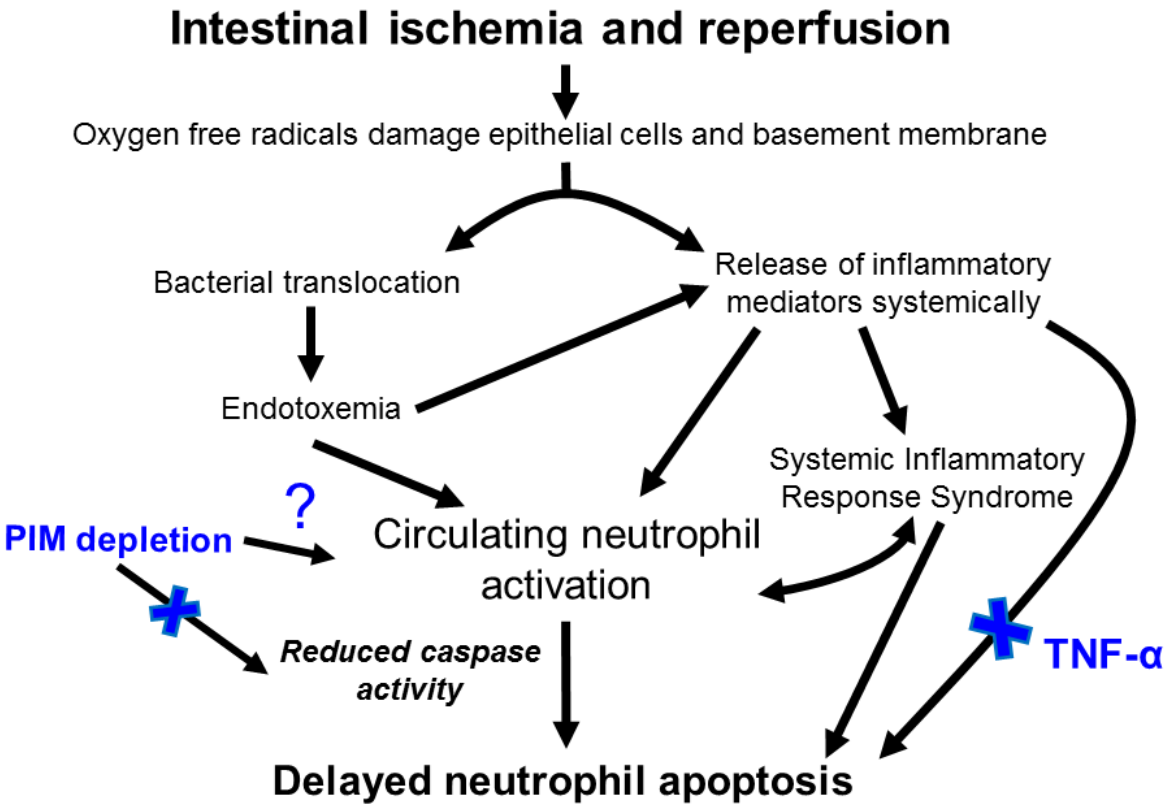


Figure 4.9 Potential mechanism for delayed *ex vivo* equine neutrophil apoptosis following intestinal ischemia and reperfusion.

CHAPTER 5: EX VIVO NEUTROPHIL APOPTOSIS IS DELAYED IN AN EQUINE COLITIS MODEL

5.1 Abstract

Horses that develop colitis invariably exhibit signs of a systemic inflammatory response syndrome (SIRS). A significant contributor to the development of SIRS in humans is delayed neutrophil apoptosis, but this has not been specifically studied in horses. The objective of this study was to evaluate the occurrence of *ex vivo* neutrophil apoptosis upon the SIRS from an equine colitis model. Neutrophils were isolated before and after the induction of colitis using an oligofructose-overdose model, placed into culture for 12 or 24 h with or without lipopolysaccharide (LPS), and then assessed for the occurrence of apoptosis using Annexin V and propidium iodide staining with flow cytometric quantification. Caspase-3, -8, and -9 activities were measured after 12 h of incubation. *Ex vivo* neutrophil apoptosis was significantly delayed in neutrophils isolated after the induction of colitis (12 h incubation: $P = 0.004$, 24 h incubation: $P = 0.003$) with concomitant reduction in caspase-3, -8, and -9 activities (caspase-3: $P = 0.004$, caspase-8: $P = 0.02$, caspase-9: $P = 0.02$). The data show that neutrophil apoptosis is delayed in horses with induced colitis, which may contribute to the development of SIRS.

5.2 Introduction

Horses experiencing colitis are at increased risk of developing a systemic inflammatory response syndrome (SIRS), an overzealous inflammatory response that is associated with increased morbidity and mortality (Moore and Vandenplas 2014). SIRS in adult horses is defined using clinical parameters including hyperthermia, tachycardia, tachypnea, leukocytosis or leukopenia with a left shift, with only two of these parameters needing to be present to make a diagnosis of SIRS (Epstein et al. 2011, Moore and Vandenplas 2014). In cases of colitis, SIRS is thought to develop from endotoxin (bacterial lipopolysaccharide, LPS) entering the vascular compartment

from a compromised intestinal epithelial barrier (Senior et al. 2011). Horses with colitis generally exhibit profound clinical signs of SIRS that may progress to multiple organ dysfunction syndrome (MODS), which in the horse is evidenced by the development of laminitis (Belknap 2009). Indeed, models used to study laminitis include models of colitis, such as inducing colitis by oligofructose-overdose (Kalck et al. 2009, Nourian et al. 2007).

In humans, circulating neutrophil apoptosis is delayed in patients experiencing SIRS and is thought to contribute to an inappropriate inflammatory response (Fanning et al. 1999, Jimenez et al. 1997, Paunel-Görgülü et al. 2012, Shang et al. 2007). The increased life span of activated neutrophils is associated with exacerbation of inflammation and tissue damage caused by cytotoxic molecules released by the neutrophils. Furthermore, delayed efferocytosis of apoptotic neutrophils delays the initiation of macrophage-orchestrated resolution of inflammation (Fadok et al. 1998, Hofman 2004, Lucas et al. 2003, Wright et al. 2010).

The cause of delayed neutrophil apoptosis in cases of SIRS may be from any number of substances that activate neutrophils, thereby prolonging their lifespan. One substance that has been evaluated extensively *in vitro* for its effect on neutrophil lifespan and has been identified as a cause of SIRS *in vivo* is LPS (Colotta et al. 1992, Hofman et al. 2004, Sweeny et al. 1998, Sano et al. 2005). The mechanism of LPS-delayed neutrophil apoptosis has been studied in humans and involves a shifting of balance from pro-apoptotic to anti-apoptotic processes of the intrinsic pathway of apoptosis via TLR4 activation (Edwards et al. 2004, François et al. 2005). It is thought that LPS plays a significant role in delaying neutrophil apoptosis in human patients with sepsis and endotoxemia, both of which are also associated with the development of SIRS (Hofman 2004); however, this has not been demonstrated in the horse.

To the authors' knowledge, *ex vivo* neutrophil apoptosis has not been examined in an equine model of colitis. We hypothesized that *ex vivo* apoptosis would be delayed in neutrophils isolated from horses following the induction of colitis by oligofructose overdose due to the development of SIRS and endotoxemia. Therefore, our goal was to study the occurrence of *ex vivo* neutrophil apoptosis in horses before and after the induction of oligofructose-overdose colitis. Additionally, we evaluated the effect of *in vitro* LPS treatment in neutrophils isolated before and after the induction of colitis and the effect of *in vitro* neutrophil concentration on the occurrence of apoptosis.

5.3 Materials and methods

5.3.1 Animals

Adult horses, determined to be healthy by physical examination or complete blood cell count, were used for the study. Throughout the study period, horses were offered hay and water *ad libitum*. Horses served as their own controls by comparing the occurrence of *ex vivo* apoptosis in neutrophils isolated before and after the induction of colitis. All procedures and experimental protocols were approved by the University of Saskatchewan Committee on Animal Care and Supply and the University of Saskatchewan Animal Research Ethics Board.

5.3.2 Oligofructose-induced colitis model

Blood was collected for neutrophil isolation immediately prior to sedation with xylazine (0.5 mg/kg IV). Horses were administered an overdose of oligofructose (10 g/kg bwt) dissolved in water via nasogastric tube. Colitis developed over the next 12-15 hours. Blood was collected 18-20 h after oligofructose administration for neutrophil isolation. In 6 horses, blood was collected for complete blood count and plasma endotoxin quantification before and 18-20 h after oligofructose overdose. For plasma endotoxin quantification blood was collected aseptically

from a jugular vein into endotoxin free tubes containing ethylenediaminetetraacetic acid (EDTA, Sarsdet, Montreal, QC, Canada). Plasma was separated by centrifugation (100 x *g* for 10 min), diluted 1:10 with endotoxin free water (Sigma Aldrich Canada, Oaksville, ON, Canada), and heat inactivated for 60°C for 15 min. Presence of endotoxin in the plasma was detected at 0.25 EU/ml concentration using a commercially available limulus amebocyte assay using manufacturer's directions (Lonza, Allendale, NJ, USA).

5.3.3 Neutrophil isolation

Neutrophils were isolated using a previously reported protocol with minor modification (Cook et al. 2009). Blood was collected aseptically from a jugular vein into vacuum tubes containing EDTA (Vacutainer™, BD, Mississauga, ON, Canada). Neutrophil isolation was performed at room temperature (20-22°C) immediately after blood collection. The blood was allowed to separate into plasma and red cell fractions for 30-45 min at room temperature. Plasma was layered onto 10 ml Ficoll-Paque™ Plus (GE Healthcare, Mississauga, ON, Canada) and centrifuged for 30 min at 400 x *g*. The subsequent pellet, containing erythrocytes and granulocytes, was retained after aspirating the supernatant and washed with Hank's Buffered Salt Solution (pH 7.4, HBSS) without phenol, magnesium, or calcium for 10 min at 200 x *g*. Erythrocytes were lysed with 2 ml sterile hypotonic distilled water with pH of 7.4 for 25 s and restored to normotonicity with an equal volume hypertonic (2x) HBSS. Cells were washed 3 times with HBSS at 200 x *g* for 10 min, suspended in culture medium (Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 50 μg/ml streptomycin, and 25 mM Hepes, Lonza BioWhittaker®, VWR, Edmonton, AB, Canada), assessed for viability using Trypan blue staining, and counted with a hemocytometer. Cell purity was determined on cytology preparations made with a

cytocentrifuge (Shandon Cytospin 4, Thermo Scientific, Waltham, MA, USA) at 1000 rpm for 4 min with medium acceleration and stained with a modified Giemsa stain (Hemacolor® EMD chemical, Gibbstown, NJ, USA).

5.3.4 Neutrophil culture

For *ex vivo* experiments, neutrophils were suspended in culture medium in plastic multi-well cell culture plates at a concentration of 2×10^6 cells/ml with or without lipopolysaccharide from *Escherichia coli* (LPS, 1 μg/ml, *Escherichia coli* 055:B5, Sigma-Aldrich Canada, Oakville, ON, Canada) for 12 or 24 h at 37°C in 5% CO_2. For *in vitro* experiments to assess the effect of neutrophil concentration on the occurrence of neutrophil apoptosis, neutrophils were cultured at concentrations of 2, 5, and 8×10^6 cells/ml for 12 and 24 h. For all experiments, neutrophils were taken out of culture, pelleted, and washed twice with HBSS at 300 x *g* for 8 min. prior to performing assays.

5.3.5 Apoptosis assays

5.3.5.1 Flow cytometry

Neutrophils were stained with CF™488-conjugated Annexin V (AV) and propidium iodide (PI) per manufacturer's instructions (CF™488A-Annexin V and PI Apoptosis Assay Kit, Biotium, Hayward, CA, USA). Staining controls included unstained untreated cells, untreated cells stained with AV only, and untreated stained with PI only. A flow cytometer (CyFlow®, Partec, Swedesboro, NJ, USA) was used to count negatively stained (live) cells, AV stained (apoptotic) cells, or AV and PI stained (dead) cells. Flow cytometry data were analyzed with commercial software (FlowMax Software © Version 2.6, Quantum Analysis GmbH, Münster, Germany). The gated population was determined with unstained cells based on size (forward scatter) and

granularity (side scatter). Quadrants were determined using untreated cells stained with only AV or PI. Data were acquired on at least 10,000 gated events.

5.3.5.2 Caspase activity assay

Caspase-3, -8, and -9 activities were indirectly measured using a commercially available kit per manufacturer's instructions (Caspase-3, -8, or -9 Colorimetric Assay Kit, Biovision, Milpitas, CA, USA). Caspase-3 activity was measured to determine overall apoptotic activity. Caspase-8 activity was measured to assess the contribution of the extrinsic pathway and Caspase-9 activity was measured to assess the contribution of the intrinsic pathway on the occurrence of neutrophil apoptosis. One million neutrophils were lysed using the manufacturer's lysis buffer following removal from culture and washed as described above. Cell lysates were stored at -80°C until processing. Upon thawing, protein concentration in cell lysates was quantified using a commercial kit (DC™ Protein Assay, Bio-Rad Laboratories, Mississauga, ON, Canada). Cell lysates were incubated with a manufacturer's reaction buffer containing dithiothreitol (DTT) and each Caspase's substrate: DEVD-pNA (Caspase-3), IETD-pNA (Caspase-8), or LEHD-pNA (Caspase-9) in 96-well plates for 2 h at 37°C. Immediately after incubation, free pNA was quantified by measuring absorbance at 405 nm in a microtiter plate reader. Levels of free pNA were corrected for total protein concentration in cell lysates.

5.3.6 Statistical analysis

Data were analyzed with a commercial software package (Graphpad Prism, Graphpad Software, Inc. La Jolla, CA, USA) using non-parametric methods. For all comparisons, a *P*-value of < 0.05 was considered significant. The Wilcoxon matched-pairs signed rank test was used to compare data before and after the induction of colitis. The Mann-Whitney U test was used to compare flow cytometry data between untreated and LPS-treated cells. The Kruskal-Wallis rank test with

a Dunn's multiple comparisons test was used to compare difference among *in vitro* treatment groups for LPS experiments. The effect of neutrophil concentration on the occurrence of apoptosis was assessed with a Spearman's rank correlation.

5.4 Results

5.4.1 SIRS developed in horses following the induction of oligofructose-induced colitis

All horses developed severe diarrhea within 12 h of oligofructose administration. In the six horses evaluated, heart rate and total white cell count were significantly increased from baseline values 18 h after the administration of oligofructose (heart rate: $P = 0.03$, total white cell count: $P = 0.03$, Figure 5.1). There was a significant increase in the absolute number of neutrophils isolated per ml of blood after the induction of colitis compared to baseline in all 16 horses ($P < 0.0001$, Figure 5.2). None of the horses had measurable endotoxin (>0.25 EU/ml) before or 18 h after the induction of colitis.

5.4.2 Ex vivo neutrophil apoptosis is delayed following the induction of colitis through reduced caspase activity

Ex vivo neutrophil apoptosis was significantly reduced after 12 and 24 h in culture in neutrophils isolated after the induction of colitis compared to those isolated before the induction of colitis (12 h (n=10): $P = 0.004$, 24 h (n=16): $P = 0.003$, Figure 5.3). Concomitantly, caspase-3, -8, and -9 activities were reduced after 12 h in culture in neutrophils isolated after the induction of colitis compared to those isolated before the induction of colitis (n =10, caspase-3: $P = 0.004$, caspase-8: $P = 0.02$, caspase-9: $P = 0.02$, Figure 5.4).

5.4.3 LPS-delayed apoptosis is abrogated in neutrophils isolated following the induction of colitis

After 24 h incubation, treatment with LPS *in vitro* significantly reduced the occurrence of apoptosis in neutrophils isolated from healthy horses prior to the induction of colitis (P <0.0001, Figure 5.5). However, *in vitro* LPS treatment failed to delay apoptosis in neutrophils isolated following the induction of colitis compared to untreated neutrophils (P = 0.83, Figure 5.5). Additionally, there was an increased occurrence of apoptosis in LPS-treated neutrophils isolated after the induction of colitis compared to LPS-treated neutrophils isolated before the induction of colitis (P = 0.013, Figure 5.5).

5.4.4 Equine neutrophil apoptosis is delayed by increased concentration in vitro

Increasing neutrophil concentration was significantly correlated with a reduced occurrence of apoptosis *in vitro* after 12 or 24 h in culture (12 h: r = -0.67, P < 0.0001, 24 h: r = -0.54, P = 0.004, Figure 5.6).

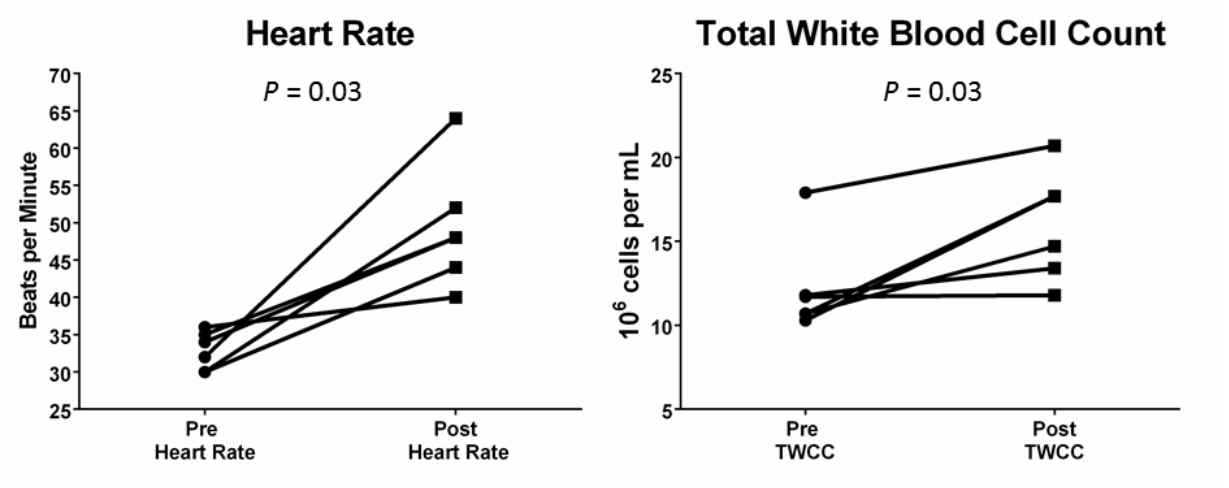


Figure 5.1. Heart rate and total white blood cell count for 6 horses before and 18 h after the administration of oligofructose.

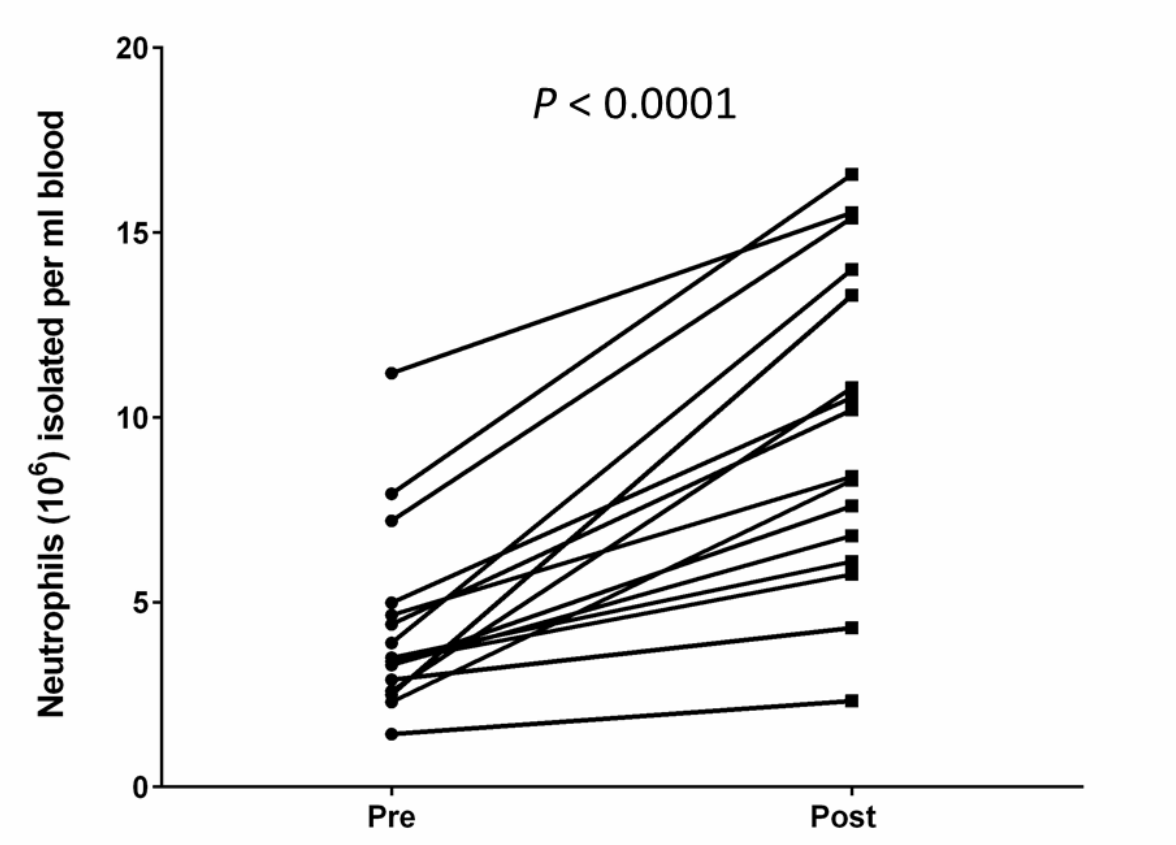


Figure 5.2 Total neutrophil count increased following the induction of colitis. Blood was collected for neutrophil isolation immediately before and 18 h after the administration of oligofructose. Data were analyzed with a Wilcoxon matched-pairs signed rank test (n = 16).

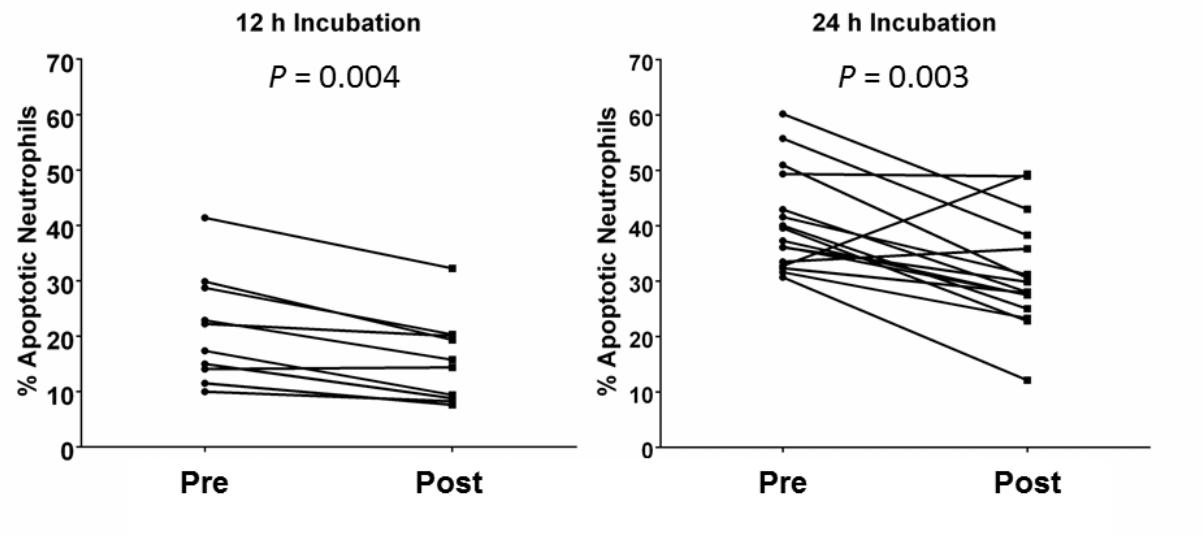


Figure 5.3 *Ex vivo* neutrophil apoptosis was delayed following the induction of colitis. . Blood was collected for neutrophil isolation immediately before and 18 h after the administration of oligofructose. Data were analyzed with the Wilcoxon matched-pairs signed rank test (12 h: n = 10, 24 h: n = 16).

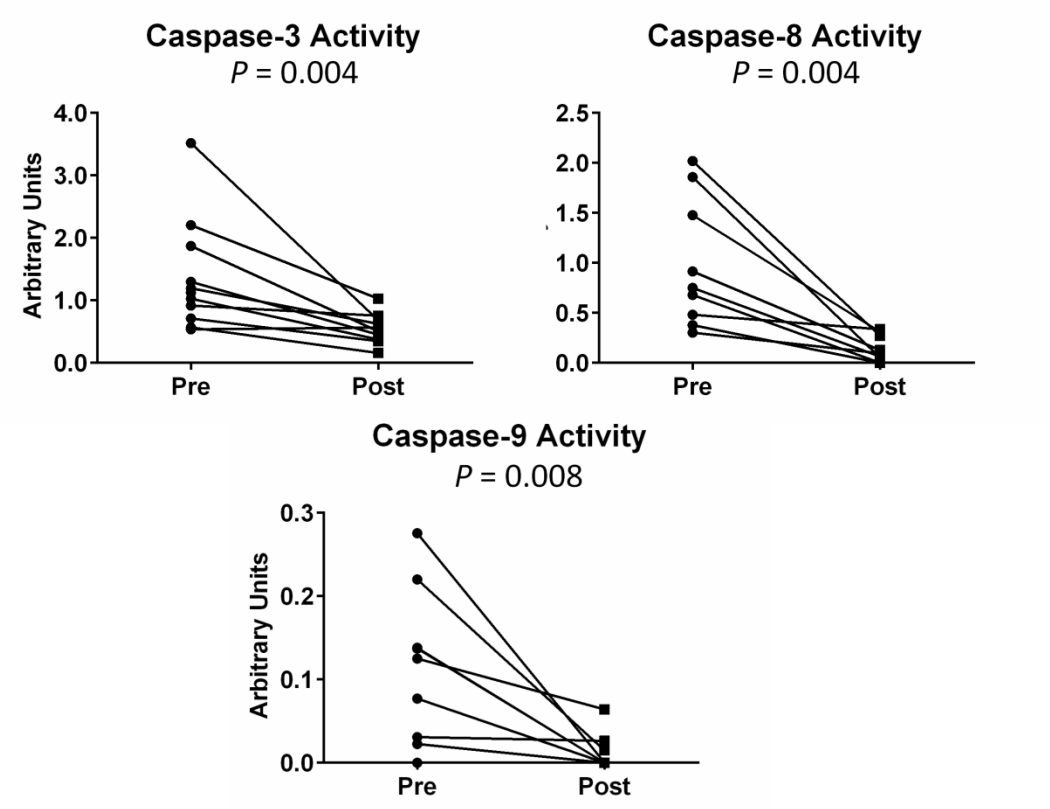


Figure 5.4 Caspase-3, -8, and -9 activities were reduced in neutrophils isolated following the induction of colitis and 12 h incubation. . Blood was collected for neutrophil isolation immediately before and 18 h after the administration of oligofructose. Data were analyzed with the Wilcoxon matched-pairs signed rank test (n = 10).

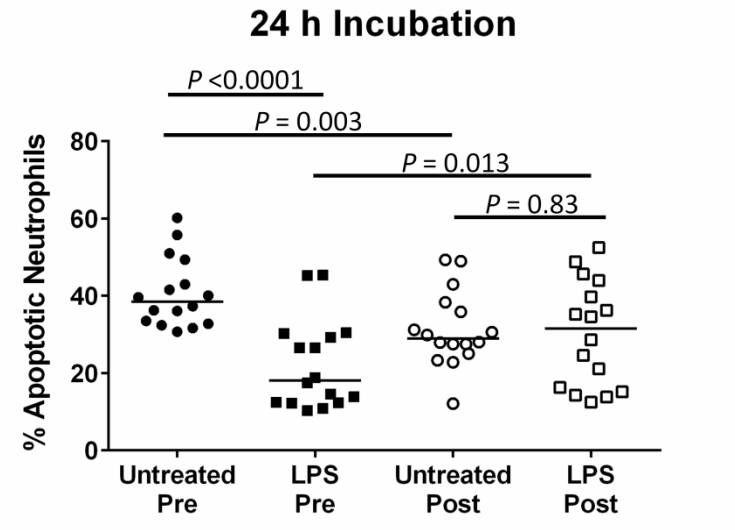


Figure 5.5 LPS-delayed apoptosis was abolished in neutrophils isolated after the induction of colitis. Data were analyzed with Kruskal-Wallis rank test with a Dunn's multiple comparisons test (n = 16).

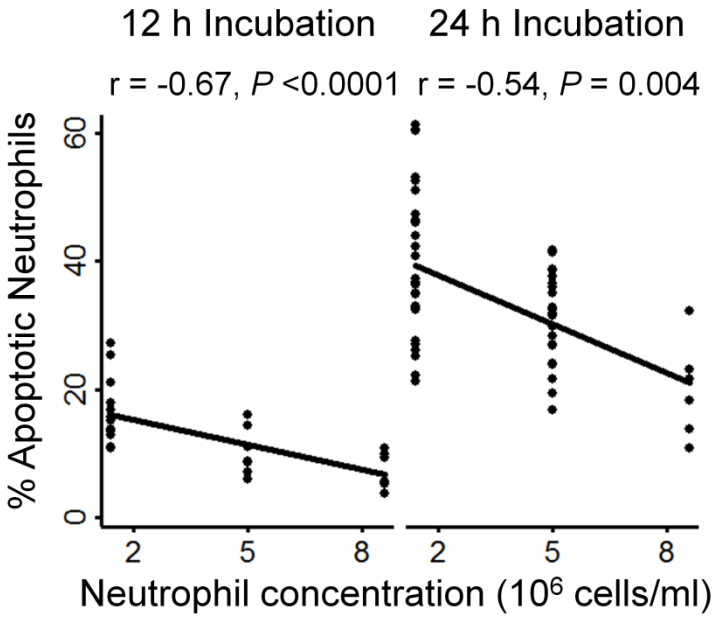


Figure 5.6 Neutrophil apoptosis is dependent upon concentration *in vitro*. Data were analyzed with a Spearman's rank correlation.

5.5 Discussion

In support of our hypothesis, *ex vivo* neutrophil apoptosis was significantly delayed following the induction of colitis by oligofructose overdose with a concomitant reduction in neutrophil caspase activity (Figure 5.6). In the horses evaluated, SIRS developed, but plasma endotoxin was not detected. We demonstrate that LPS treatment delays neutrophil apoptosis *in vitro*; but for the first time, we demonstrate that the effect of *in vitro* LPS treatment was abolished in neutrophils isolated after the induction of colitis. In addition, we demonstrate that the occurrence of neutrophil apoptosis is dependent on concentration *in vitro*.

Ex vivo neutrophil apoptosis was clearly delayed following the induction of colitis and SIRS, which is similar to what has been reported in the human literature (Fanning et al. 1999, Jimenez et al. 1997, Paunel-Görgülü et al. 2009, Shang et al. 2007). The cause of SIRS-related delayed neutrophil apoptosis may be multifactorial and is still under investigation. However, sepsis and exposure to endotoxin is a well-established cause of delayed neutrophil apoptosis in species other than the horse (Brazil et al. 2014, Colotta et al. 1992, Milot et al. 2012, Paunel-Görgülü et al. 2009, Sano et al. 2005). Sepsis in humans results in a significant systemic inflammatory response, similar to what occurs in horses with oligofructose-induced colitis. Horses that are overdosed with oligofructose demonstrate signs of systemic inflammation in addition to severe colitis, which are generally attributed to endotoxemia and bacterial translocation through a compromised intestinal epithelial barrier (Senior et al. 2011).

In this study, all horses, where parameters were evaluated, developed SIRS; however, measurable endotoxin was not detected in the same horses. We cannot rule out that the horses in this study were not experiencing endotoxemia as the correct time point for measurable endotoxin load may have been missed due to endotoxin concentrations being measured at a single time

point. In addition, endotoxin is rapidly cleared from the circulating blood volume, potentially making it difficult to detect when blood is collected from a peripheral site at a single time point (Fessler et al. 1989). Also, the exact concentration of endotoxin required to activate circulating neutrophils *in vivo* and cause delayed *ex vivo* apoptosis is unknown. However, additional mechanisms other than endotoxemia could have contributed to the *ex vivo* delayed neutrophil apoptosis observed herein.

Caspase-3, -8, and -9 activities were reduced in neutrophils isolated following the induction of colitis and cultured for 12 h. Similarly, humans diagnosed with sepsis have reduced neutrophil capsase-3 and -9 activities that contribute to delayed *ex vivo* neutrophil apoptosis (Paunel-Görgülü et al. 2009, Taneja et al.2004). The reduction in both caspase-8 (extrinsic initiator caspase) and caspase-9 (intrinsic initiator caspase) indicates that both the extrinsic and intrinsic pathways of neutrophil apoptosis are affected in neutrophils isolated from horses experiencing oligofructose-induced colitis. We have found a similar reduction in caspase-3, -8, and -9 activities in neutrophils isolated from horses following the induction of intestinal ischemia and reperfusion (Chapter 4). In contrast, our data show that *in vitro* LPS-delayed equine neutrophil apoptosis primarily affects the intrinsic pathway of apoptosis, ultimately resulting in reduced caspase-9 activity (Anderson et al. unpublished data). Taken together, our results suggest that additional unknown factors other than endotoxin exposure are contributing to delayed equine neutrophil apoptosis *in vivo*. Additional investigation is necessary to further elucidate the mechanism of delayed neutrophil apoptosis in horses experiencing a systemic inflammatory response due to colitis.

Interestingly, *in vitro* LPS treatment failed to delay apoptosis in neutrophils isolated following the induction of colitis. One reasonable explanation, which requires further

investigation, is that neutrophils were exposed to LPS *in vivo* and then developed tolerance, which abrogated the effects of LPS *in vitro*. Tolerance by immune cells is thought to prevent an overzealous, detrimental immune response and leads to the development of a compensatory anti-inflammatory response syndrome or CARS (Bone 1996, Cavaillon and Adib-Conquy 2006, Kopanakis et al. 2013, Medvedev et al. 2006). Much less is known about LPS tolerance in neutrophil biology compared to other immune cells, such as macrophages and monocytes. Additionally, the concept of tolerance to endotoxin is well-accepted by equine researchers, however the mechanisms behind this phenomenon have not been thoroughly investigated (Allen et al. 1996).

The induction of LPS tolerance in neutrophils is associated with reduced TLR4 surface expression and results in diminished neutrophil function, but maintenance of a pro-inflammatory phenotype (Parker et al. 2005). Similar to our results, neutrophil function in response to LPS stimulation *in vitro* was dampened in neutrophils isolated from septic humans (McCall et al. 1993). In contrast, another study demonstrated that activated neutrophils isolated from the lungs of horses with recurrent airway obstruction express high levels of TLR4 and retain the ability to respond to LPS *ex vivo* (Aharonson-Raz et al. 2012). It is possible that there is a difference in neutrophil tolerance to LPS when neutrophils migrate into tissues compared to those in circulation.

Endotoxin tolerance can also provide protection against non-endotoxin inflammatory disease processes, such as thermal or ischemia and reperfusion injury (Colleti et al. 1994, He et al. 1992). In these scenarios, inflammatory cytokine concentrations, such as TNF-α, and physical manifestations of inflammation are reduced with pre-LPS exposure, but the underlying mechanisms are unknown at the cellular and molecular level (Colleti et al. 1994, He et al. 1992).

Intracellular signaling during inflammatory processes is complex, but also redundant allowing for cross-activation of many cell processes. It is likely that multiple factors play a role in inflammation-delayed neutrophil apoptosis. Taken together, neutrophil function is altered by pre-treatment with LPS. To the authors' knowledge, this is the first study in any species demonstrating a tolerogenic response to *in vitro* LPS-delayed neutrophil apoptosis.

It is possible that the delayed *ex vivo* neutrophil apoptosis observed in the horses with colitis in this study was due to increased neutrophil concentration and density *in vivo,* as these factors have been shown to delay apoptosis *in vitro* (Hannah et al. 1998, Attalah et al. 2012). Indeed, all of the horses in this study had a significant increase in circulating neutrophil concentration following the induction of colitis. To the authors' knowledge, this mechanism of delayed apoptosis has not been explored using an *in vivo* inflammation model. An inflammation model introduces many confounding variables that make it less than ideal to study this mechanism of delayed neutrophil apoptosis *in vivo*; however, it more closely mimics a true clinical scenario. In live animals, concentration-dependent delay in neutrophil apoptosis may be due to dysfunction of neutrophil adhesion genes as is observed in profound neutrophilia that occurs in bovine leukocyte adhesion deficiency disease (Nagahata et al. 2004).

In conclusion, *ex vivo* neutrophil apoptosis was delayed following the induction of colitis and SIRS in horses. The role of delayed neutrophil apoptosis in the development of SIRS deserves further study in the horse as a potential therapeutic target, similar to what is already being performed in human medicine. The modulation of neutrophil apoptosis could prove to reduce inflammation and promote the resolution of inflammation all at once, mitigating the effects of an overzealous and life-threatening inflammatory response.

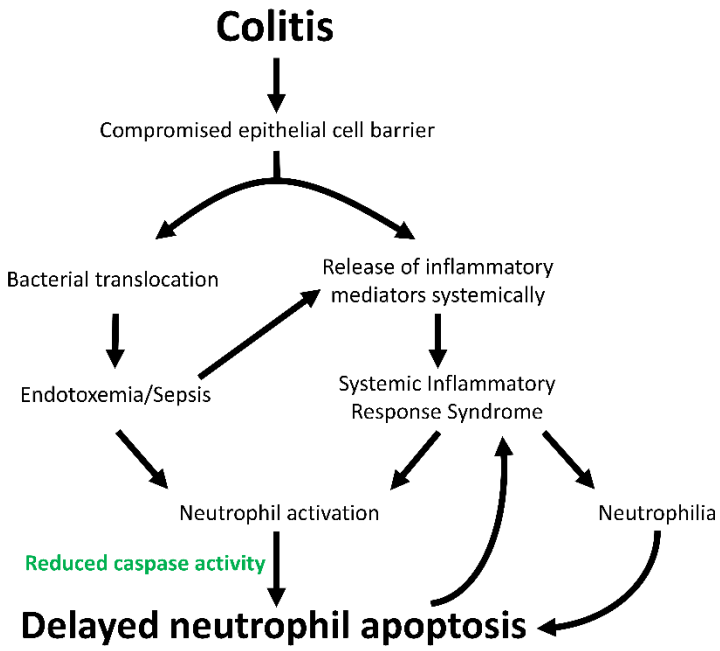


Figure 5.7 Potential mechanism for delayed *ex vivo* equine neutrophil apoptosis following the induction of colitis.

5.6 *Contineo* to Chapter 6

After determining that neutrophil apoptosis was delayed *ex vivo* following the induction of inflammation secondary to gastrointestinal insult, we were interested in exploring mechanisms to modulate neutrophil apoptosis. To our knowledge, the effect of co-incubation of apoptotic neutrophils with freshly isolated neutrophils has never been evaluated for its contribution on acceleration of neutrophil apoptosis *in vitro*. Therefore, a starting point was to determine if there were survival or non-survival signals that occur among neutrophil in the presence of apoptotic or aged neutrophils *in vitro* by assessing the occurrence of neutrophil apoptosis with co-incubation with 24 h-aged neutrophils. A similar phenomenon has been demonstrated for concentration-delayed neutrophil apoptosis where culture of freshly isolated neutrophils in supernatants from highly concentrated aged neutrophils prolonged lifespan due to a "community effect" (Atallah et al. 2012).

In addition, we were interested in evaluating the effect of apoptotic neutrophils on neutrophil lifespan with concurrent LPS treatment or neutrophils isolated from horses experiencing systemic inflammation. The administration of apoptotic neutrophils to mice experiencing endotoxic shock reduced inflammation and improved survival due to an as yet to be defined mechanism (Ren et al. 2008). The effect of administering apoptotic neutrophils on *ex vivo* neutrophil survival was not specifically evaluated in that study, but it is possible that the administration of apoptotic neutrophils could cause increased apoptosis of circulating neutrophils, accounting for the measured anti-inflammatory effects.

CHAPTER 6: LPS- OR SYSTEMIC INFLAMMATION-DELAYED EQUINE NEUTROPHIL APOPTOSIS IS NOT AFFECTED BY CO-INCUBATION WITH APOPTOTIC NEUTROPHILS

6.1 Abstract

Delayed neutrophil apoptosis may contribute to the development of a systemic inflammatory response syndrome (SIRS) in horses. Modulation of neutrophil apoptosis may prove to be an effective treatment, but requires investigation. We hypothesized that incubation of freshly isolated equine neutrophils with 24 h-aged autologous neutrophils would increase the occurrence of neutrophil apoptosis *in vitro* and rescue the delay in neutrophil apoptosis induced by LPS and inflammation. Neutrophils were isolated and placed into culture for 24 h at a low concentration ($5x10^5$ cells/ml) to induce apoptosis. Freshly isolated neutrophils from healthy horses were added to the 24 h-aged neutrophils with or without 1 μg/ml lipopolysaccharide (LPS). In another experiment, freshly isolated neutrophils from horses with oligofructose-induced colitis were added to the 24 h-aged neutrophils. The occurrence of apoptosis was assessed with Annexin V and propidium iodide staining and flow cytometric quantification after 24 h in culture. Neutrophil apoptosis was delayed in LPS-treated neutrophils and neutrophils isolated from horses after the induction of colitis (LPS: $P = 0.0003$, colitis: $P = 0.03$), but there was no effect of co-incubation with 24 h-aged neutrophils on the occurrence of apoptosis. Based on these results, apoptotic neutrophils do not appear to accelerate the occurrence of neutrophil apoptosis *in vitro*.

6.2 Introduction

Though not specifically studied in the horse, delayed neutrophil apoptosis contributes to collateral damage to tissues and delays macrophage-driven resolution of apoptosis, thereby

propagating an inflammatory response (Wright et al. 2010, Lucas et al. 2003). We have demonstrated that neutrophil apoptosis is delayed *ex vivo* in two separate intestinal inflammation models (Anderson et al. unpublished data). Horses subjected to these models developed clinical signs consistent with systemic inflammatory response syndrome (SIRS), including elevated heart rate, elevated neutrophil counts, and elevated body temperature.

Typically, horses that develop SIRS are treated with non-specifically acting non-steroidal anti-inflammatory drugs (NSAIDs) that may reduce inflammation, but may also delay the resolution of inflammation. Specifically, prostaglandin (PG) D_2 and PGJ_2, products of the arachidonic acid pathway that are inhibited by NSAID administration, have been shown to promote neutrophil apoptosis through inhibition of nuclear-factor kappa B (NF-κB, Ward et al. 2004). Therefore, administering NSAIDs during an inflammatory response may contribute to a prolonged neutrophil lifespan, delaying the resolution of inflammation.

Designing therapeutics that modulate neutrophil apoptosis may serve as an effective method to control an inflammatory process (Savill et al. 2002). Data from mice and human studies show that administration of apoptotic neutrophils into *in vivo* inflammation models modulates an inflammatory response (Ren et al. 2008, Morrison et al. 2013). Specifically, Ren et al. (2008) demonstrated that administering apoptotic neutrophils up to 24 h after inducing endotoxic shock in mice reduced circulating pro-inflammatory cytokines, neutrophil infiltration in target organs, and serum LPS concentrations. Thus far, similar *in vitro* or *in vivo* studies have not been performed in the horse.

The determination of neutrophil lifespan is complex and is significantly affected by a neutrophil's environment (Fox et al. 2010). However, to the authors' knowledge, it is unknown whether apoptotic neutrophils can influence the lifespan of non-apoptotic neutrophils *in vitro*.

We hypothesized that co-incubation of 24 h-aged equine neutrophils that have begun the process of apoptosis would increase the occurrence of apoptosis in freshly isolated equine neutrophils. Additionally, we hypothesized that 24 h-aged neutrophils would rescue LPS- and inflammation-delayed neutrophil apoptosis.

6.3 Materials and Methods

6.3.1 Animals, model of systemic inflammation, and sample collection

For *in vitro* experiments, blood was collected from healthy horses used for teaching purposes at the Western College of Veterinary Medicine (n=12). Six healthy research horses were used for creating a model of systemic inflammation using an overdose of oligofructose (OGF) to induce colitis. Briefly, horses were sedated with xylazine (0.5 mg/kg IV) and administered OGF (10 g/kg bwt, OraftiP95®, Bene, Pemuco, Chile) dissolved in water via nasogastric tube intubation. Colitis developed over the next 12-15 hours. Blood was sampled for *ex vivo* experiments immediately before and 18 h after challenge with oligofructose (OGF). A physical examination was performed 1 h prior to and 18 h following OGF overdose. Throughout the study period, horses were offered hay and water *ad libitum.* All procedures and experimental protocols were approved by the University of Saskatchewan Committee on Animal Care and Supply and the University of Saskatchewan Animal Research Ethics Board.

6.3.2 Neutrophil isolation

Neutrophils were isolated using a previously reported protocol with minor modification (Cook et al. 2009). Blood was collected for neutrophil isolation aseptically from a jugular vein into vacuum tubes containing EDTA (Vacutainer™, BD, Mississauga, ON, Canada). Isolation was performed at room temperature (20-22°C) immediately after blood collection. The blood was allowed to separate into plasma and red cell fractions for 30-45 min at room temperature.

Plasma was layered onto 10 ml Ficoll-Paque™ Plus (GE Healthcare, Mississauga, ON, Canada) and centrifuged for 30 min at 400 x *g*. The subsequent pellet, containing erythrocytes and granulocytes, was retained after aspirating the supernatant and washed with Hank's Buffered Salt Solution (pH 7.4, HBSS) without phenol, magnesium, or calcium for 10 min at 200 x *g*. Erythrocytes were lysed with 2 ml sterile hypotonic distilled water for 25 s and restored to normotonicity with an equal volume hypertonic (2x) HBSS. Cells were washed 3 times with HBSS at 200 x *g* for 10 min, suspended in culture medium (Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 50 μg/ml streptomycin, and 25 mM Hepes, Lonza BioWhittaker®, VWR, Edmonton, AB, Canada), assessed for viability using Trypan blue staining, and counted with a hemocytometer. Cell purity was determined on cytology preparations made with a cytocentrifuge (Shandon Cytospin 4, Thermo Scientific, Waltham, MA, USA) at 1000 rpm for 4 min with medium acceleration and stained with a modified Giemsa stain (Hemacolor® EMD chemical, Gibbstown, NJ, USA).

6.3.3 Neutrophil culture

For all experiments, neutrophils were suspended in culture medium in plastic multi-well cell culture plates. To assess the effect of co-incubation of apoptotic neutrophils on the occurrence of freshly isolated neutrophils *in vitro*, neutrophils were first cultured at a concentration of 5×10^5 cells/ml for 24 h to allow cells to become apoptotic (24-h autologous neutrophils). Then freshly isolated neutrophils were cultured at a concentration of 2×10^6 cells/ml with no treatment, treatment with 1 μg/ml LPS (*Escherichia coli* 055:B5, Sigma-Aldrich Canada, Oakville, ON, Canada), co-incubation with 24 h-aged autologous neutrophils (1:8 ratio of aged:fresh

neutrophils), or co-incubation with 24 h-aged autologous neutrophils and 1 μg/ml LPS for 24 h at 37°C in 5% CO_2.

For *ex vivo* experiments, neutrophils were isolated prior to OGF overdose and cultured at a concentration of 5 $x10^5$ cells/ml for 24 h to allow cells to become apoptotic (24-h autologous neutrophils) or a standard concentration $2x10^6$ cells/ml to assess baseline occurrence of *ex vivo* apoptosis. Neutrophils were isolated 18 h after OGF overdose, placed into culture at a concentration of 2 x 10^6 cells/ml, and left untreated or co-incubated with 24 h-aged autologous neutrophils for 24 h at 37°C in 5% CO_2. Prior to performing apoptosis assays, neutrophils were taken out of culture, pelleted, and washed twice with HBSS at 400 x *g* for 8 min.

6.3.4 Determination of apoptosis

Neutrophils were stained with CF™488-conjugated Annexin V (AV) and propidium iodide (PI) per manufacturer's instructions (CF™488A-Annexin V and PI Apoptosis Assay Kit, Biotium, Hayward, CA, USA). Staining controls included unstained untreated cells, untreated cells stained with AV only, and untreated stained with PI only. A flow cytometer (CyFlow®, Partec, Swedesboro, NJ, USA) was used to count negatively stained (live) cells, AV positively stained (apoptotic) cells, or AV and PI positively stained (dead) cells. Flow cytometry data were analyzed with commercial software (FlowMax Software © Version 2.6, Quantum Analysis GmbH, Münster, Germany). The gated population was determined with unstained cells based on size (forward scatter) and granularity (side scatter). Quadrants were determined using untreated cells stained with only AV or PI. Data were acquired on at least 10,000 gated events.

6.3.5 Statistical analysis

Data were analyzed with a commercial software package (Graphpad Prism, Graphpad Software, Inc. La Jolla, CA, USA). For all comparisons, a *P*-value of < 0.05 was considered significant. All

data were analyzed using non-parametric tests. Differences among *in vitro* groups were assessed with a Kruskal-Wallis rank test with a Dunn's multiple comparisons test. The Wilcoxon matched-pairs signed rank test was used to compare occurrence of neutrophil apoptosis before and after the induction of colitis. The Mann-Whitney U test was used to compare untreated cells with cells co-incubated with 24-h autologous neutrophils in neutrophils isolated following the induction of colitis.

6.4 Results

6.4.1 Clinical data

See Chapter 5 for clinical data results.

6.4.2 Co-incubation of apoptotic equine neutrophils failed to rescue LPS- or systemic inflammation-delayed equine neutrophil apoptosis

LPS treatment delayed *in vitro* apoptosis ($P = 0.0003$, Figure 6.1); however, co-incubation with 24 h-aged neutrophils failed to rescue LPS-delayed neutrophil apoptosis (Figure 6.1). When colitis and SIRS were induced, *ex vivo* neutrophil apoptosis was delayed compared to baseline ($P = 0.03$, Figure 6.2); but, co-incubation with 24 h-aged neutrophils had no effect on the occurrence of *ex vivo* neutrophil apoptosis before or after the induction of colitis and SIRS (Figure 6.2). There was no significant difference in concentration of cultured neutrophils among treatment types for the *in vitro* or *ex vivo* experiments (Figure 6.3).

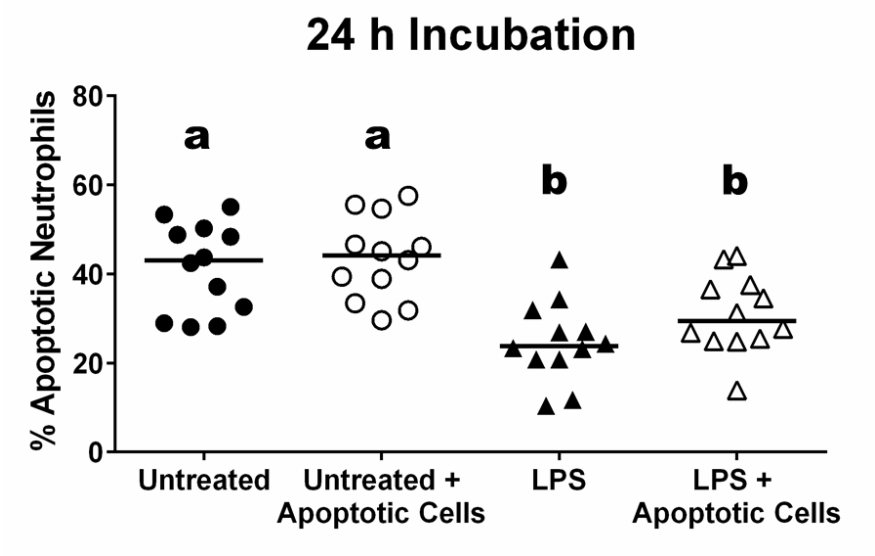


Figure 6.1 Co-incubation of freshly isolated neutrophils with apoptotic neutrophils failed to abolish LPS-delayed apoptosis after 24 h in culture. Data were analyzed with a Kruskal-Wallis rank test with a Dunn's multiple comparisons test. Bars represent median. Difference between letters indicates significant difference ($P < 0.01$).

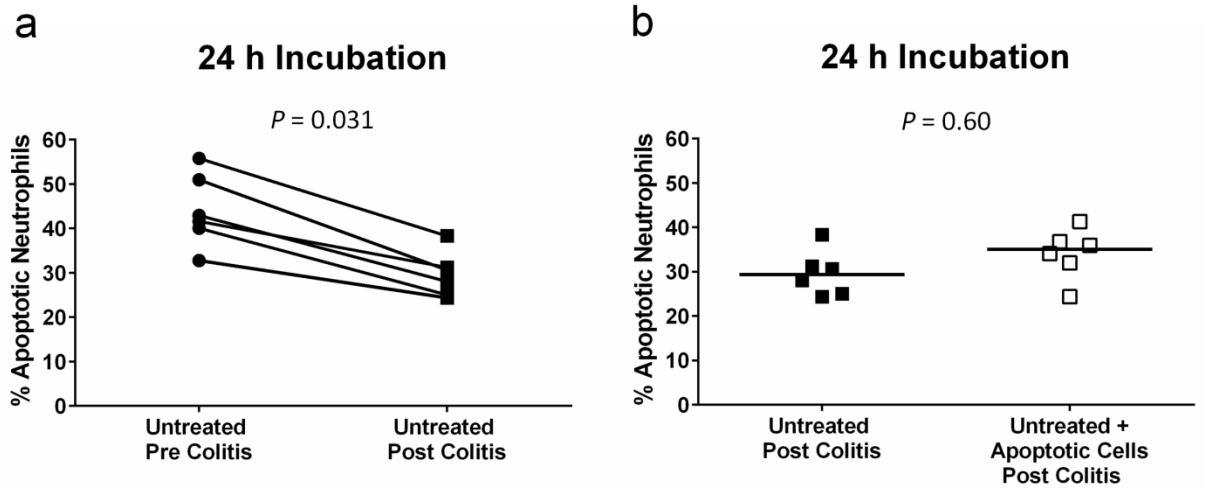


Figure 6.2 Co-incubation of freshly isolated neutrophils with apoptotic neutrophils fails to abolish inflammation-delayed apoptosis after 24 h in culture. Data in 6.2a were analyzed with a Wilcoxon matched-pairs signed rank test. Data in 6.2b were analyzed with a Mann-Whitney U test. Bars represent median.

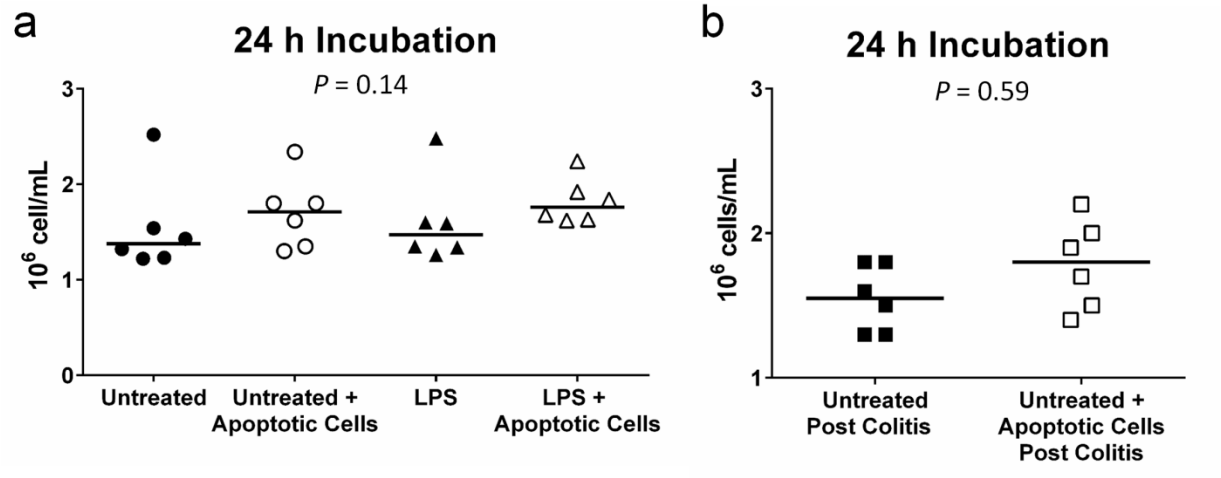


Figure 6.3 There was no difference in cell concentrations after 24 h incubation among treatments for *in vitro* experiments (a) or between treatments for *ex vivo* experiments (b) indicating that the 24-h aged neutrophils could not have contributed to concentration-delayed apoptosis. Data in figure 6.3a were analyzed with a Kruskal-Wallis rank test with a Dunn's multiple comparisons test and data in figure 6.3b were analyzed with a Mann-Whitney U test. Bars represent median.

6.5 Discussion

In contrast to our hypothesis, co-incubation of neutrophils, both LPS-activated and inactivated, with 24 h-aged neutrophils failed to have an effect on the occurrence of apoptosis *in vitro*. In addition, there was no difference in cell concentrations upon treatment with apoptotic neutrophils, indicating that an artificial increase in percentage of apoptotic cells was negligible.

To the authors' knowledge, this is the first report to evaluate the effect of co-incubation of aged neutrophils with freshly isolated neutrophils in order to determine the effect on the occurrence of neutrophil apoptosis *in vitro*. We chose to evaluate the effect of co-incubation of apoptotic neutrophils with fresh neutrophils because there is potentialp intercellular signaling that could occur amongst a population of neutrophils to control lifespan. Previous research has demonstrated that increased concentration or increased density of neutrophils significantly prolongs neutrophil lifespan (Attalah et al. 2012, Hannah et al. 1998). We have confirmed this finding with equine neutrophils (Anderson et al. unpublished data). For this reason, we chose to incubate our originally isolated neutrophils in this study at a low concentration and low density to rapidly promote apoptosis (data not shown). Attalah et al. 2012 demonstrated that human neutrophil apoptosis was delayed by increasing concentration *in vitro* via proteins S100A8 and S100A9, which were released into the supernatant to initiate intercellular signaling and the promotion of neutrophil survival. Therefore, we thought it possible that an opposite effect could occur whereby neutrophil apoptosis could be induced by co-incubating freshly isolated neutrophils with 24 h-aged neutrophils in their original media. However, that hypothesis was disproven by our results.

The use of apoptotic neutrophils to treat LPS-induced inflammation has shown promising results in mice (Ren et al. 2008). The mechanism underlying the effect of administering

apoptotic neutrophils in the face of LPS challenge is primarily due to modulation of monocyte cytokine secretion (Fadok et al. 1998, Ren et al. 2008). However, another mechanism by which apoptotic neutrophils modulate an inflammatory response may involve the neutralization of LPS (Ren et al. 2008). While apoptotic neutrophils have the capacity to bind LPS, they will lack the ability to de-granulate making this a potential method of neutralizing LPS *in vivo*. Co-incubation with apoptotic neutrophils has also been shown to reduce the pro-inflammatory functions of neutrophils, as measured by reactive oxygen species (ROS) production, in the face of LPS treatment (Esman et al. 2010).

From the results of this study, it is evident that apoptotic neutrophils do not induce apoptosis in normal neutrophils, LPS-activated freshly isolated neutrophils, or neutrophils isolated from horses experiencing systemic inflammation due to colitis suggesting that this mechanism is not important in modulating neutrophil lifespan. However, additional work needs to be performed to evaluate the effect of apoptotic equine neutrophils on macrophage function and LPS neutralization *in vitro* and *in vivo*.

CHAPTER 7: SUPPLEMENTAL DATA

7.1 Abstract

A horse from the sham celiotomy group for the third chapter of this thesis experienced severe acute aspiration pneumonia following general anesthesia. Lung sections obtained from the horse were stained for hematoxylin and eosin, revealing severe suppurative aspiration pneumonia characterized by a marked accumulation of neutrophils into the affected portion of the lung. Immunohistochemical staining was performed against the anti-apoptotic protein A1 and anti-calprotectin (MAC387) and revealed a marked expression of these proteins in neutrophils infiltrating the affected portions of the lung. This is the first report evaluating apoptotic protein expression in a severe neutrophilic inflammatory condition in the horse.

Accepted, in part, by the Journal of American Veterinary Medicine as a Pathology "What is Your Diagnosis?"

7.2 Case Details

An 8-year-old Quarter Horse gelding weighing 527-kg (1,161-lb) was placed under general anesthesia to undergo a research procedure via a ventral median celiotomy with approval by the institution's animal care and ethics committee. The horse was determined to be healthy by physical examination and complete blood count prior to surgery. The horse was fasted, but allowed access to water, over night before the induction of anesthesia and received no pre-operative treatments. Following general anesthesia where a ventral midline celiotomy was performed, the horse had a prolonged recovery from anesthesia (1 h and 40 min.) and required considerable encouragement to stand. Gastric contents were observed exiting from the nostrils during the early part of recovery.

Following recovery from anesthesia, the horse was moved to a stall for observation. Within 6 hours following recovery, the horse became febrile (102.1ºF), tachycardic (60 beats/minute), tachypneic (28 breaths per minute), the mucous membranes became injected, and he became markedly depressed. At 12 h post-operatively, his lung sounds became harsh on auscultation of the cranioventral thorax; and by 18 h post-operatively, harsh lung sounds could be heard throughout the entire thorax. A complete blood count performed 18 h post-operatively revealed a leukocytosis, neutrophilia with left shift, lymphopenia, and monocytosis.

The horse was euthanized for the purpose of research and a necropsy was performed. On gross examination, the cranial lobe and craniodorsal and caudodorsal parts of the caudal lobes of the right and left lung were firm, failed to collapse, discolored yellow and covered by an adherent layer of friable material (fibrin). There was marked congestion of the caudal dorsal surface of both lungs. On cut sections, feed material could be grossly observed within the large bronchi and marked separation of the interlobular septa (pulmonary edema) was present.

On histology, alveoli within affected areas were filled with large numbers of viable and degenerate neutrophils, fine fibrilar eosinophilic material (fibrin), protein rich edema fluid, and small amounts of hemorrhage (Figure 7.1A). Occasionally, alveoli were filled with cellular and nuclear debris (necrosis). Alveolar septa were moderately congested, and frequently necrotic. Interlobular septa were markedly expanded and filled with fibrin, edema and a similar inflammatory population to that seen in alveoli. Similar changes were present on the pleural surface of affected sections. Airways were largely clear of material and epithelium was intact. These changes are consistent with a severe acute multifocal to coalescing fibrinonecrotizing, suppurative pneumonia with marked pulmonary edema and fibrinous pleuritis.

Immunohistochemical staining was performed using primary antibodies against B-cell lymphoma (Bcl2)-related protein A1 (Santa Cruz, CA, USA), an anti-apoptotic proteins of the Bcl2 family, and anti-human macrophage antibody (MAC387, AbD Serotec®, Raleigh, NC, USA) that binds to calprotectin, a protein present in many inflammatory cells including macrophages and neutrophils. Affected lung sections had marked staining for both proteins in neutrophils (Figure 7.1B and 7.1C).

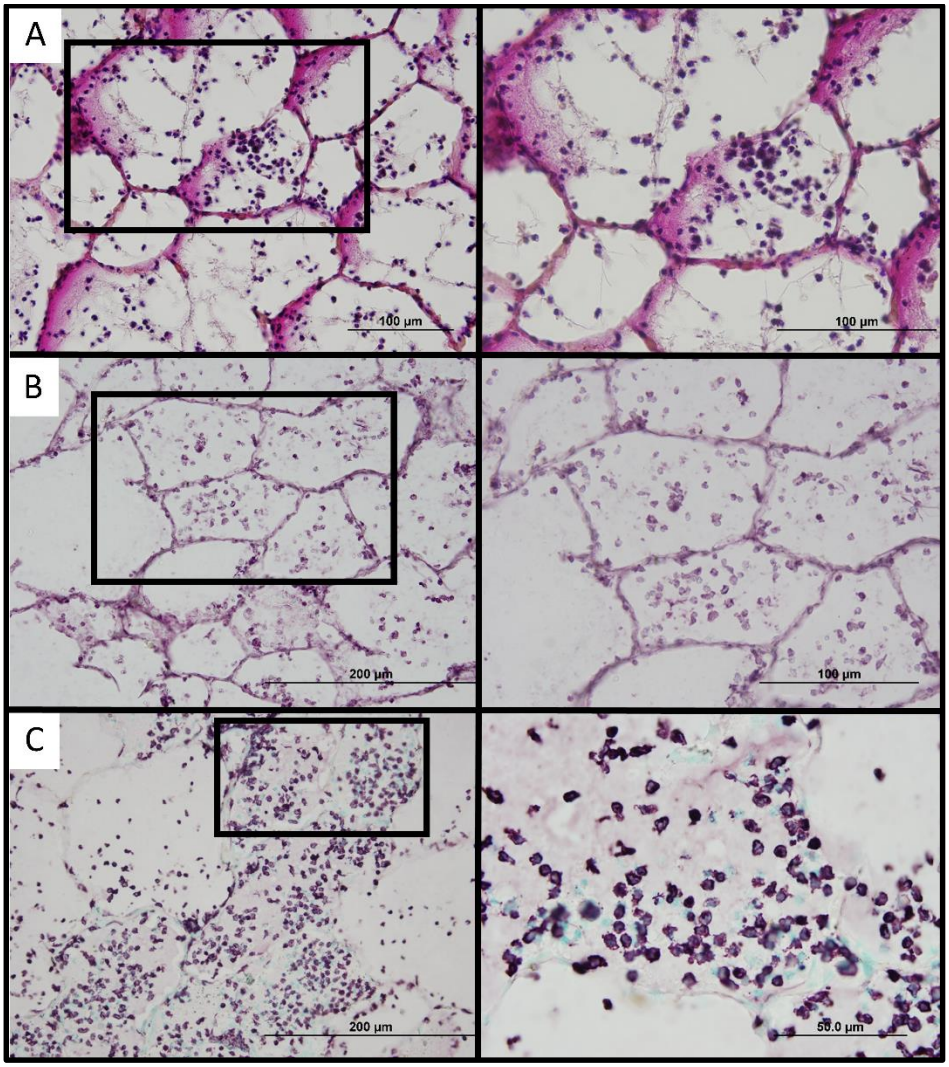


Figure 7.1 Photomicrographs of representative sections stained with hematoxylin and eosin (A) or with immunohistochemistry against the anti-apoptotic protein A1 (B) or anti-macrophage antibody MAC387 (C).

7.3 Discussion

Regurgitation leading to aspiration of stomach contents during anesthesia is exceedingly rare in horses due to the unique muscular distribution within their esophagus and strong cardiac sphincter, which prevents regurgitation of stomach contents, unlike carnivores (Carpenter and Hall 1981, Cunningham 2002, Davies et al. 2015, Taylor and Clarke 2007). However, it is common practice to induce a horse with an indwelling nasogastric tube for colic surgery to reduce the risk of aspiration from spontaneous gastric reflux that may occur from an overly distended stomach (Cunningham 2002, Taylor and Clarke 2007).

In humans and dogs, reflux of gastric contents during anesthetic events is not uncommon and aspiration of gastric acid may cause acute lung injury (Galato and Raptopoulos 1995, Many et al. 1986, Suratt and Parsons 2006, Warner et al. 1993). Acute lung injury (ALI) is defined clinically in veterinary patients by acute onset tachypnea and labored breathing, hypoxemia, evidence of pulmonary capillary leakage without increased pulmonary capillary pressure, and a known risk factor, such as SIRS, with or without evidence of diffuse pulmonary inflammation (Wilkins et al. 2007). Some of the criteria listed above were either not assessed or not present in this case; so based on clinical signs, it is difficult to state that the horse was experiencing ALI. However, to the authors' knowledge, ALI has never been reported in an adult horse in a clinical case. Additionally, based on the authors' research, it is possible that adult horses are highly resistant to developing clinically apparent ALI, as compared to other species and foals (Dunkel et al. 2007).

Regardless, it is clear that this horse suffered from severe aspiration pneumonia, which resulted in a marked neutrophilic inflammatory response in the lung and a systemic inflammatory response syndrome (SIRS). It has been shown that neutrophil apoptosis is delayed

upon activation with many substances (Fox et al. 2010). The increased expression of the anti-apoptotic protein A1 would suggest that the neutrophils infiltrating the lung in this case would be resistant to apoptosis. Similarly, in mice, installation of LPS into the lung resulted in increased expression of A1in lung neutrophils (Kupfner et al. 201). In the horse presented here, A1 expression was likely increased in neutrophils that had infiltrated into the lung due to neutrophil activation by the aspirated foreign material and gastric acid. The expression of anti-apoptotic proteins and other markers of apoptosis should be further evaluated in neutrophils taken from equine inflammatory models to further assess the contribution of delayed neutrophil apoptosis to inflammatory processes.

CHAPTER 8: DISCUSSION AND CONCLUSIONS

8.1 General Discussion

The goal of the research presented in this thesis was to investigate equine neutrophil lifespan in the face of inflammatory conditions. The majority of experiments were designed based on previously established protocols using human neutrophils, with additional experiments designed to investigate specific pathways of interest and the effect of inflammatory processes unique to the horse. We report for the first time that equine neutrophils experience delayed apoptosis in the face of various inflammatory scenarios *in vitro* and *in vivo*.

Neutrophils have short lifespans that may be prolonged upon activation to allow them to neutralize threats to the body (Fox et al. 2010). However, by prolonging their lifespan neutrophils potentiate an inflammatory process through release of their cytotoxic contents and production of reactive oxygen species (Jimenez et al. 1997). At the same time, macrophage-dependent resolution of inflammation is delayed when apoptotic neutrophils are unavailable for efferocytosis (Fadok et al. 1998, Huynh et al. 2002, Lucas et al. 2013). The mechanisms by which neutrophil lifespan is prolonged during inflammatory processes is still under investigation, and is relatively unknown in the horse.

One of the most commonly used substances to activate neutrophils *in vitro* is the bacterial endotoxin, LPS. In turn, LPS activation prolongs neutrophil lifespan *in vitro* by upregulating anti-apoptotic proteins of the Bcl2 protein family (Colotta et al. 1992, Jia et al. 2008, Milot et al. 2012, Sweeny et al. 1998, Sano et al. 2005, Yamamoto et al. 1993). However, an opposite effect of *in vitro* LPS treatment was recently demonstrated using equine neutrophils where neutrophils had increased occurrence of apoptosis with LPS treatment (Brazil et al. 2014). To assess the effect of LPS activation on equine neutrophil lifespan *in vitro*, experiments were designed to

measure the occurrence of neutrophil apoptosis *in vitro* using Annexin V and propidium iodide staining quantified with flow cytometry. Additionally, the mechanisms of apoptosis involved with LPS-treated neutrophils were explored.

As expected, LPS treatment delayed the occurrence of equine neutrophil apoptosis in a concentration-dependent manner. Though we were unable to demonstrate the involvement of specific Bcl2 proteins due to difficulties finding equine-compatible antibodies, we added novel insight into mechanisms that contribute to LPS-delayed neutrophil apoptosis by confirming TLR4 involvement and measuring reduced caspase-9 activities in equine neutrophils treated with LPS *in vitro* (Figure 3.8).

Neutrophil apoptosis is delayed in human patients suffering from sepsis, which may be attributed to endotoxemia (Jimenez et al. 1997). However, sepsis is also invariably associated with the development of SIRS, in which neutrophil apoptosis is delayed (Fanning et al. 1999, Jimenez et al. 1997, Shang et al. 2007). We used two equine models of intestinal inflammation that are associated with the development of endotoxemia and SIRS to evaluate neutrophil lifespan *in vivo*. Because apoptotic neutrophils are rapidly removed from the circulation, it is necessary to isolate neutrophils activated *in vivo* and culture them to assess the length of their lifespan. We failed to measure endotoxin in plasma from horses undergoing jejunal ischemia and reperfusion or oligofructose-induced colitis. However, it is possible that our method of detection plasma endotoxin was ineffective and circulating endotoxin was present. Regardless, horses with intestinal inflammation developed SIRS and *ex vivo* neutrophil apoptosis was delayed through reduced intrinsic and extrinsic caspase activity following the induction of intestinal inflammation.

The mechanism underlying delayed neutrophil apoptosis in these horses may be independent of endotoxemia, and may instead involve a cell concentration effect because data show that increased concentration of neutrophils delays apoptosis *in vitro.* We also demonstrate for the first time that the depletion of pulmonary intravascular macrophages, through administration of parenteral gadolinium chloride, rescues delayed *ex vivo* neutrophil apoptosis associated with intestinal ischemia and reperfusion injury. PIMs are a unique type of macrophage that, similar to Kupffer cells in the liver, are attached intimately to the endothelium of the alveolar capillaries and are exposed to the entire blood volume as it passes through the lung for oxygenation. Their proposed function is to sample the blood searching for pathogen- or danger-associated molecular patterns in order to mount an inflammatory response (Atwal and McDonell 2005). In addition, because of their attachment to the endothelium, it is possible that PIMs are involved in vascular inflammatory responses (Aharonson-Raz and Singh 2010). Their role in lung disease has been investigated in multiple species; however, their involvement in systemic inflammatory responses has not been studied.

The inflammatory (TNF-α and IL-1β) and anti-inflammatory (IL-10 and TGF-β) cytokine profile in PIM-depleted horses experiencing intestinal IR was significantly different than horses only experiencing IR, where PIM-depleted horses had a greater inflammatory response. These results could, in part, explain the difference measured in *ex vivo* neutrophil lifespan. Specifically, TNF-α is important in regulating neutrophil apoptosis (Fox et al. 2010, Murray et al. 1997, Salamone et al. 2001). Early or high concentration TNF-α exposure stimulates neutrophil apoptosis (Fox et al. 2010, Murray et al. 1997, Salamone et al. 2001). Indeed, Brazil et al. demonstrated enhanced apoptosis with early treatment (8 h) with equine recombinant TNF-α, but no effect after 20 h treatment (Brazil et al. 2014). However, late survival (after 12 h) is

promoted simultaneously through activation of the NF-κB pathway and subsequent up-regulation of the anti-apoptotic members of the Bcl-2 family, particularly A1 (Cross et al. 2008). Plasma TNF-α concentrations increased earlier in PIM-depleted horses, which would potentially induce apoptosis earlier in circulating neutrophils as evidenced in our experiment. Further experiments are necessary to evaluate the effect of TNF-α on equine neutrophil apoptosis.

Delayed neutrophil apoptosis is a self-propagating phenomenon. Based on the results from the *in vivo* models presented in this thesis, a potential scenario involves the exposure of neutrophils to a signal that delays their apoptosis (e.g. LPS), which, in conjunction with increased entry of new neutrophils into circulation, increases their concentration in the system, further prolonging their life through a currently undetermined mechanism. The prolongation of neutrophil lifespan then propagates an inflammatory response and contributes to the development of SIRS.

8.2 Future Directions

We still have insufficient understanding of neutrophil lifespan during inflammatory conditions in the horse (and other species) to achieve the ultimate goal of designing targeted therapeutics to modulate neutrophil apoptosis as a multimodal approach to treating inflammatory conditions. Many new questions were generated by the results presented in this thesis, which may be answered through further experiments.

A critical subsequent experiment must be performed to answer one of the main questions generated from the results of the *in vivo* models reported herein: Does endotoxemia cause delayed *ex vivo* neutrophil apoptosis? This experiment would evaluate the occurrence of equine neutrophil apoptosis before and after the administration of intravenous LPS. This model will remove confounding etiologies, such as local organ injury, from the development of a systemic

inflammatory response. At the same time, neutrophil tolerance may be assessed by performing a second *in vivo* LPS challenge or treating neutrophils isolated following LPS challenge with LPS *in vitro*. Similarly, the effect of concomitantly depleting PIMs using gadolinium chloride could potentially provide additional information on their role in determining neutrophil lifespan during systemic inflammation due to endotoxemia. Outcomes of these experiments need to focus on proteins involved in intracellular signaling of inflammation and apoptotic pathways, ideally using multiplex technology to assess multiple genes of interest over multiple time points.

8.3 Limitations of the Studies

Though we chose models that mimic clinical scenarios of endotoxemia, we failed to measure endotoxin in any horses at any time points. As previously discussed, it is possible that we missed the appropriate time point for measurement, so ideally endotoxin concentrations should have been measured over time. Also, it is possible that endotoxin is removed so rapidly from the blood that it is difficult to measure. Therefore, we would recommend using a non-terminal LPS infusion SIRS model to further evaluate the effects of LPS on *in vivo* neutrophil lifespan.

Additionally, more horses are required to lend sufficient power to the data presented in the intestinal ischemia and reperfusion model chapter. It is always a struggle to justify the sacrifice of animals to provide adequate power to a study; however, based on the interesting results with gadolinium chloride treatment, the sacrifice of additional animals is warranted to fully evaluate the effects of this treatment.

8.4 Concluding remarks

The study of delayed neutrophil apoptosis is a highly complex process that cannot be completed by simply exposing neutrophils to various cytokines, bacterial products, or conditions. *In vivo* neutrophil lifespan is most likely also affected by other immune cell signaling, particularly from

macrophages and monocytes. There is a distinct void in the literature on innate and adaptive immune cell signaling and its effect on neutrophil lifespan; therefore, this area requires additional study in all species.

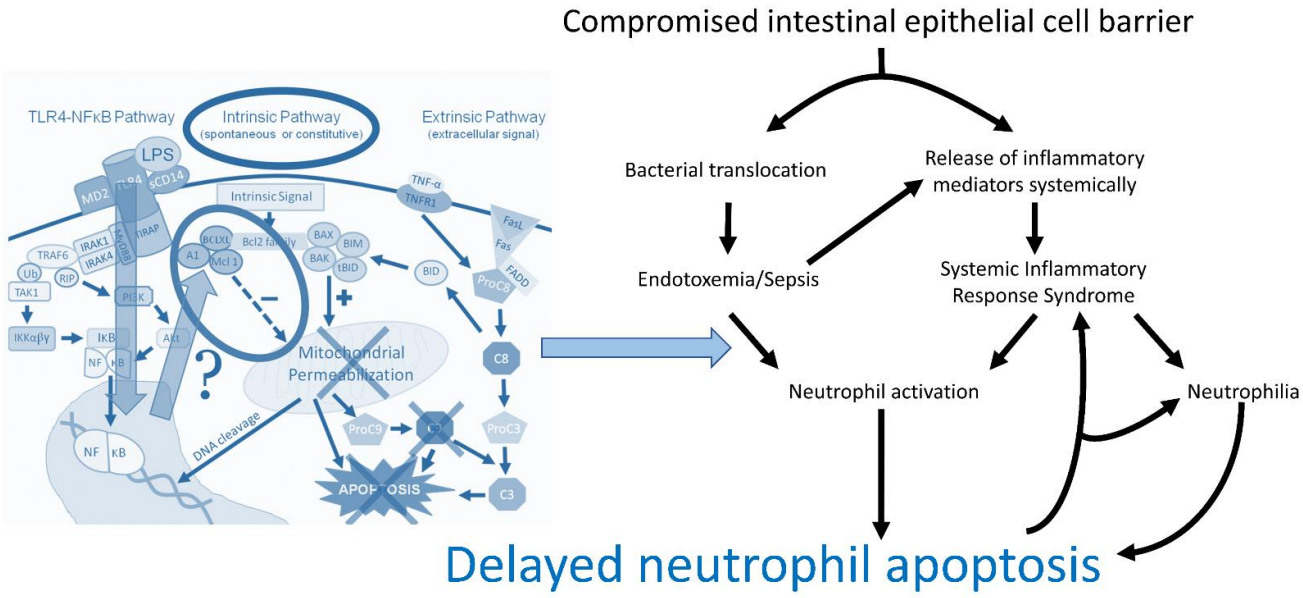
Because delayed neutrophil apoptosis is an important process for the body to maintain a large effector cell population to deal with threats to the body, redundant mechanisms likely exist. Within this thesis, we focused on very specific causes for delayed neutrophil apoptosis. However, the major conclusion one should garner from this body of work is that neutrophil apoptosis was delayed in inflammatory conditions originating from the gastrointestinal tract, and the delay in neutrophil apoptosis may be an important contributor to SIRS.

The contribution of delayed neutrophil apoptosis on the propagation and resolution of inflammation is still under investigation. Intuitively, promoting neutrophil apoptosis would seem to reduce inflammation, but at what cost to the effectiveness of the innate immune response? The ability to induce neutrophil apoptosis, whether locally or systemically, may prove to be an important therapeutic target in treating inflammatory diseases; however, the timing of promoting neutrophil apoptosis will be critical in developing effective treatment regimens.

This body of work contributes significantly to the literature on the equine innate immune system. Despite the horses' propensity to experience life-threatening inflammation, there is a distinct void in research into the equine innate immune system. Rather, researchers focus on treatment regimens that have been extrapolated from human medicine, where similar basic knowledge on the innate immune response may be lacking. Before effective treatments can be developed, understanding of the equine innate immune response must be more complete. Additionally, we propose that the horse could serve as a valuable model for studying inflammatory diseases in humans, specifically sepsis and endotoxemia.

8.5 Conclusions

- LPS treatment delays equine neutrophil apoptosis *in vitro* through reduced caspase-9 activity and TLR4 signaling.
- *Ex vivo* equine neutrophil apoptosis is delayed following the induction of intestinal inflammation and SIRS through reduced caspase activity.
- Pulmonary intravascular macrophages are involved in modulating systemic inflammatory cytokine concentrations that may affect neutrophil apoptosis.
- Equine neutrophil apoptosis is delayed by increased cell concentration *in vitro*.
- Equine neutrophils become refractory to the *in vitro* effect of LPS on their lifespan following the induction of SIRS *in vivo*.



8.1 Pathophysiology of delayed equine neutrophil apoptosis originating from intestinal inflammation.

LIST OF REFERENCES

Aharonson-Raz K, Singh B. Pulmonary intravascular macrophages and endotoxin-induced pulmonary pathophysiology in horses. Can J Vet Res. 74:45-49, 2010.

Aharonson-Raz K, Lohmann KL, Townsend HG, Marques F, Singh B. Pulmonary intravascular macrophages as proinflammatory cells in heaves, an asthma-like equine disease. Am J Physiol Lung Cell Mol Physiol 303:L189-198, 2012.

Akgul C, Moulding DA, Edwards SW. Molecular control of neutrophil apoptosis. FEBS Letters 487:318–322, 2001.

Allen GK, Campbell-Beggs C, Robinson JA, Johnson PJ, Green EM. Induction of early-phase endotoxin tolerance in horses. Equine Vet J 28:269-274, 1996.

Allenbach C, Zufferey C, Perez C, Launois P, Mueller C, Tacchini-Cottier F. Macrophages induce neutrophil apoptosis through membrane TNF, a process amplified by Leishmania major. J Immunol 176:6656-6664, 2006.

Almzaiel AJ, Billington R, Smerdon G, Moody AJ. Hyperbaric oxygen enhances neutrophil apoptosis and their clearance by monocyte-derived macrophages. Biochem Cell Biol 93:405-416, 2015.

Atallah M, Krispin A, Trahtemberg U, Ben-Hamron S, Grau A, Verbovetski I, Mevorach D. Constitutive Neutrophil Apoptosis: Regulation by Cell Concentration via S100 A8/9 and the MEK – ERK Pathway. PLoS ONE 7:e29333, 2012.

Atwal OS, McDonell W. In vivo interaction of pulmonary intravascular macrophages with activated platelets in microvessels of equine lung after multiple exposures to halothane, isoflurane, and thiamylal: a comparative ultrastructural and cytochemical study. Anat Rec A Discov Mol Cell Evol Biol 284:574-584, 2005.

Bae HB, Zmijewski JW, Deshane JS, Zhi D, Thompson LC, Peterson CB, Chaplin DD, Abraham E. Vitronectin inhibits neutrophil apoptosis through activation of integrin-associated signaling pathways. Am J Respir Cell Mol Biol. 46:790-796, 2012.

Barnes PJ: Glucocorticoids: current and future directions. Br J Pharmacol 163:29-43, 2011.

Bates JJ, Watson RW, Glynn CM, O'Neill AJ, Fitzpatrick JM, Buggy DJ. Aspirin preserves neutrophil apoptosis after cardiopulmonary bypass. Shock 21:495-499, 2004.

Belknap JK, Black SJ. Sepsis-related laminitis. Eq Vet J 44:738-740, 2012.

Belknap JK, Moore JN, Crouser EC. Sepsis-from human organ failure to laminar failure. Vet Immunol Immunopathol 129:155-157, 2009.

Benbarek H, Deby-Dupont G, Deby C, Serteyn D. Direct stimulation of the oxidative activity of isolated equine neutrophils by TNF-alpha and IL-1beta. Vet Immunol Immunopathol 121:101-106, 2008.

Bone RC. Sir Isaac Newton, sepsis, SIRS, and CARS. Crit Care Med 24:1125-1128, 1996.

Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, KNaus WA, Schein RM, Sibbald WJ. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. Chest 101:1644-1655, 1992.

Brain JD, Molina RM, DeCamp MM. Pulmonary intravascular macrophages: their contribution to the mononuclear phagocyte system in 13 species. Am J Physiol Lung Cell Mol Physiol 276:L146-L154, 1999.

Brazil TJ, Dixon PM, Haslett C, Murray J, McGorum BC. Constitutive apoptosis in equine peripheral blood neutrophils in vitro. Vet J 202:536-542, 2014.

Brockow K. Contrast media hypersensitivity--scope of the problem. Toxicology 209:189-192, 2005.

Brockow K, Ring J. Anaphylaxis to radiographic contrast media. Curr Opin Allergy Clin Immunol 11:326-331, 2011.

Brown SB, Bailey K, Savill J. Actin is cleaved during constitutive apoptosis. Biochem J 323:233-7, 1997.

Brown SB, Savill J. Phagocytosis triggers macrophage release of Fas ligand and induces apoptosis of bystander leukocytes. J Immunol 162:480-485, 1999.

Burrows GE. Dose-response of ponies to parenteral Escherichia coli endotoxin. Can J Comp Med 45:207-210, 1981.

Cavaillon JM, Adib-Conquy M. Bench-to-bedside review: endotoxin tolerance as a model of leukocyte reprogramming in sepsis. Crit Care 10:233, 2006.

Chen KW, Groß CJ, Vásquez Sotomayor F, Stacey KJ, Tschopp J, Sweet MJ, Schroder K. The neutrophil NLRC4 inflammasome selectively promotes IL-1β maturation without pyroptosis during acute *Salmonella* challenge. Cell Reports 8:570-582, 2014.

Carpenter I, Hall LW. Regurgitation in an anaesthetised horse. Vet Rec 108:209, 1981.

Chuang PI, Yee E, Karsan A, Winn RK, Harlan JM. A1 is a constitutive and inducible Bcl-2 homologue in mature human neutrophils. Biochem Biophys Res Commun 249:361-365, 1998.

Colletti LM, Remick DG, Campbell DA Jr. LPS pretreatment protects from hepatic ischemia/reperfusion. J Surg Res 57:337-343, 1994.

Colotta F, Polentarutti N, Sozzani S, Mantovani A. Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. Blood 80:2012-2020, 1992.

Cook VL, Jones Shults J, McDowell MR, Campbell NB, Davis JL, Marshall JF, Blikslager AT. Anti-inflammatory effects of intravenously administered lidocaine hydrochloride on ischemia-injured jejunum in horses. Am J Vet Res 70:1259-1268, 2009.

Cook VL, Neuder LE, Blikslager AT, Jones SL. The effect of lidocaine on in vitro adhesion and migration of equine neutrophils. Vet Immunol Immunopathol 129:137-142, 2009.

Cox G, Austin RC. Dexamethasone-induced suppression of apoptosis in human neutrophils requires continuous stimulation of new protein synthesis. J Leukoc Biol 61:224-230, 1997.

Cross A, Moots RJ, Edwards SW. The dual effects of TNF on neutrophil apoptosis are mediated via differential effects on expression of Mcl-1 and Bfl-1. Blood 111:878-884, 2008.

Cunningham JG. Movements of the gastrointestinal tract. In: Cunningham JG, ed. Textbook of Veterinary Physiology. 2nd ed. Philadelphia: Saunders Elsevier, 235, 2002.

Davies JA, Fransson BA, Davis AM, et al. Incidence of and risk factors for postoperative regurgitation and vomiting in dogs: 244 cases (2000-2012). J Am Vet Med Assoc 246:327-335, 2015.

De Lorenzo BH, Godoy LC, Novaes e Brito RR, Pagano RL, Amorim-Dias MA, Grosso DM, Lopes JD, Mariano M. Macrophage suppression following phagocytosis of apoptotic neutrophils is mediated by the S100A9 calcium-binding protein. Immunobiology 215:341-347, 2010.

Devitt A, Marshall LJ. The innate immune system and the clearance of apoptotic cells. J Leukoc Biol 90:447-457, 2011.

Drifte G, Dunn-Siegrist I, Tissières P, Pugin J. Innate immune functions of immature neutrophils in patients with sepsis and severe systemic inflammatory response syndrome. Crit Care Med 41:820-832, 2013.

Duffin R, Leitch AE, Fox S, Haslett C, Rossi AG. Targeting granulocyte apoptosis: mechanisms, models, and therapies. Immunol Rev 236:28-40, 2010.

Dunkel B, Dolente B, Boston RC. Acute lung injury/acute respiratory distress syndrome in 15 foals. Eq Vet J 37:435-440, 2005.

Edwards SW, Derouet M, Howse M, Moots RJ. Regulation of neutrophil apoptosis by Mcl-1. Biochem Soc Trans 32:489-492, 2004.

El Kebir D, József L, Pan W, Filep JG. Myeloperoxidase delays neutrophil apoptosis through CD11b/CD18 integrins and prolongs inflammation. Circ Res 103:352-359, 2008.
El Kebir D, Damlaj A, Filep JG. Toll-like receptor 9 signaling delays neutrophil apoptosis by increasing transcription of Mcl-1. PLoS ONE 9:e87006, 2014.

El Keibir D, Filep JG. Targeting neutrophil apoptosis for enhancing the resolution of inflammation. Cells 2:330-348, 2013.

Ellett JD, Atkinson C, Evans ZP, Amani Z, Balish E, Schmidt MG, van Rooijen N, Schnellmann RG, Chavin KD. Murine Kupffer cells are protective in total hepatic ischemia/reperfusion injury with bowel congestion through IL-10. J Immunol 184:5849-5858, 2010.

Epstein KL, Brainard BM, Gomez-Ibanez SE, Lopez MAF, Barton MH, Moore JN. Thrombelastography in horses with acute gastrointestinal disease. J Vet Intern Med 25:307–314, 2011.

Esmann L, Idel C, Sarkar A, Hellberg L, Behnen M, Möller S, van Zandbergen G, Klinger M, Köhl J, Bussmeyer U, Solbach W, Laskay T. Phagocytosis of apoptotic cells by neutrophil granulocytes: diminished proinflammatory neutrophil functions in the presence of apoptotic cells. J Immunol 184(1):391-400, 2010.

Fadeel B, Åhiln A, Henter JI, Orrenius S, Hampton MB. Involvement of caspases in neutrophil apoptosis: regulation by reactive oxygen species. Blood 92:4808-4818, 1998.

Fadok VA, Savill JS, Haslett C, Bratton DL, Doherty DE, Campbell PA, Henson PM. Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. J Immunol 149:4029-4035, 1992.

Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J Clin Invest 101:890-898, 1998.

Fanning NF, Kell MR, Shorten GD, Kirwan WO, Bouchier-Hayes D, Cotter TG, Redmond HP. Circulating granulocyte macrophage colony-stimulating factor in plasma of patients with the systemic inflammatory response syndrome delays neutrophil apoptosis through inhibition of spontaneous reactive oxygen species generation. Shock 11(3):167-174, 1999.

Fessler JF, Bottoms GD, Coppoc GL, Gimarc S, Latshaw HS, Noble JK. Plasma endotoxin concentrations in experimental and clinical equine subjects. Eq Vet J Suppl 7:24-28, 1989.

Fink SL, Cookson BT. Apoptosis, pyroptosis, and necrosis: mechanisitic description of dead and dying eukaryotic cells. Infection and Immunity 73:1907-1916, 2005.

Fox S, Leitch AE, Duffin R, Haslett C, Rossi AG. Neutrophil apoptosis: relevance to the innate immune response and inflammatory disease. J Innate Immun 2:216-227, 2010.

François S, El Benna J, Dang PM, Pedruzzi E, Gougerot-Pocidalo MA, Elbim C. Inhibition of neutrophil apoptosis by TLR agonists in whole blood: involvement of the phosphoinositide 3-

kinase/Akt and NF-kappa B signaling pathways, leading to increased level of Mcl-1, A2, and phophorylated Bad. J Immunol 174:3633-3642, 2005.

Frasch SC, Fernandez-Boyanapalli RF, Berry KZ, Leslie CC, Bonventre JV, Murphy RC, Henson PM, Bratton DL. Signaling via macrophage G2A enhances efferocytosis of dying neutrophils by augmentation of Rac activity. J Biol Chem 286:121088-121022, 2011.

Freitas M, Porto G, Lima JL, Fernandes E. Isolation and activation of human neutrophils in vitro. The importance of the anticoagulant used during blood collection. Clin Biochem 41:570-575, 2008.

Freitas M, Costa VM, Ribeiro D, Couto D, Porto G, Carvalho F, Fernandes E. Acetaminophen prevents oxidative burst and delays apoptosis in human neutrophils. Toxicol Lett 219(2):170-177, 2013.

Galato AD, Raptopoulos D. Gastro-esophageal reflux during anaesthesia in the dog: the effect of age, positioning, and type of surgical procedure. Vet Rec 137:513-516, 1995.

Guzma´n-de la Garza FJ, Ibarra-Herna´ndez JM, Cordero-Pe´rez P, Villegas-Quintero P, Villarreal-Ovalle CI, Torres-Gonza ´ lez L. Temporal relationship of serum markers and tissue damage during acute intestinal ischemia/reperfusion. Clinics 68:1034-1038, 2013.

Gautam S, Kirschnek S, Wiesmeier M, Vier J, Häcker G. Roscovitine-induced apoptosis in neutrophils and neutrophil progenitors is regulated by the Bcl-2-family members Bim, Puma, Noxa and Mcl-1. PLoS One 8(11):e79352, 2013.

Geering B, Simon HU. Pecularities of cell death mechanism in neutrophils. Cell Death Diff 18:1457-1469, 2011.

Gordy C, Pua H, Sempowski GD, He YW. Regulation of steady-state neutrophil homeostasis by macrophages. Blood 17:618-629, 2011.

Guo RF, Sun L, Gao H, Shi KX, Rittirsch D, Sarma VJ, Zetoune FS, Ward PA. In vivo regulation of neutrophil apoptosis by C5a during sepsis. J Leukoc Biol 80:1575-1583, 2006.

Hagar JA, Powell DA, Aachoui Y, Ernst RK, Miao EA. Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. Science 341:1250-1253, 2013.

Hallett JM, Leitch AE, Riley NA, Duffin R, Haslett C, Rossi AG. Novel pharmacological strategies for driving inflammatory cell apoptosis and enhancing the resolution of inflammation. Trends Pharmacol Sci 29:250-257, 2008.

Hannah S, Mecklenburgh K, Rahman I, Bellingan GJ, Greening A, Haslett C, Chilvers ER. Hypoxia prolongs neutrophil survival in vitro. FEBS Lett 372:233–237, 1995.

Hannah S, Nadra I, Dransfield I, Pryde JG, Rossi AG, Haslett C. Constitutive neutrophil apoptosis in culture is modulated by cell density independently of beta2 integrin-mediated adhesion. FEBS Lett 421:141-146, 1998.

Hasegawa T, Suzuki K, Sakamoto C, Ohta K, Nishiki S, Hino M, Tatsumi N, Kitagawa S. Expression of the inhibitor of apoptosis (IAP) family members in human neutrophils: up-regulation of cIAP2 by granulocyte colony-stimulating factor and overexpression of cIAP2 in chronic neutrophilic leukemia. Blood 101: 1164-1171, 2003.

Haslett C, Guthrie LA, Kopaniak MM, Henson PM. Modulation of multiple neutrophil functions by preparative method or trace concentrations of bacterial lipopolysaccharide. Am J Pathol 119:101-110, 1985.

He W, Fong Y, Marano MA, Gershenwald JE, Yurt RW, Moldawer LL, Lowry SF. Tolerance to endotoxin prevents mortality in infected thermal injury: association with attenuated cytokine responses. J Infect Dis 165:859-864, 1992.

Hofman P. Molecular regulation of neutrophil apoptosis and potential targets for therapeutic strategy against the inflammatory process. Curr Drug Targets Inflamm Allergy 3:1-9, 2004.

Hur J, Kang MK, Park JY, Lee SY, Bae YS, Lee SH, Park YM, Kwak JY. Pro-apoptotic effect of high concentrations of histamine on human neutrophils. Int Immunopharmacol 3:1491-1502, 2003.

Huynh ML, Fadok VA, Henson PM. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. J Clin Invest 109:41-50, 2002.

Jia SH, Li Y, Parodo J, Kapus A, Fan L, Rotstein OD, Marshall JC. Pre–B cell colony–enhancing factor inhibits neutrophil apoptosis in experimental inflammation and clinical sepsis. J Clin Invest 113:1318–1327, 2004.

Jia SH, Parodo J, Kapus A, Rotstein OD, Marshall JC. Dynamic regulation of neutrophil survival through tyrosine phosphorylation or dephosphorylation of caspase-8. J Biol Chem 283:5402-5413, 2008.

Jimenez MF, Watson RW, Parodo J, Evans D, Foster D, Steinberg M, Rotstein OD, Marshall JC. Dysregulated expression of neutrophil apoptosis in the systemic inflammatory response syndrome. Arch Surg 132:1263–1269, 1997.

Johnson L, Montgomery JB, Schneider JP, Townsend HGG, Ochs M, Singh B. Morphometric examination of the equine adult and foal lung. Anat Rec 297:1950-1962, 2014.

Kalck KA, Frank N, Elliott SB, Boston RC. Effects of low-dose oligofructose treatment administered via nasogastric intubation on induction of laminitis and associated alterations in glucose and insulin dynamics in horses. Am J Vet Res 70:624-632, 2009.

Kamada S, Kikkawa U, Tsujimoto Y, Hunter T. Nuclear translocation of caspase-3 is dependent on its proteolytic activation and recognition of a substrate-like protein(s). J Biol Chem 280:857-860, 2005.

Kaplan JM, Cook JA, Hake PW, O'Connor M, Burroughs TJ, Zingarelli B. 15-Deoxy-delta(12,14)-prostaglandin J(2) (15D-PGJ(2)), a peroxisome proliferator activated receptor gamma ligand, reduces tissue leukosequestration and mortality in endotoxic shock. Shock 24(1):59-65, 2005.

Kawamoto T, Ii M, Kitazaki T, Iizawa Y, Kimura H. TAK-242 selectively suppresses Toll-like receptor 4-signaling mediated by the intracellular domain. Eur J Pharmacol 584:40-48, 2008.

Kettritz R, Choi M, Salanova B, Wellner M, Rolle S, Luft FC: Fever-like temperatures affect neutrophil NF-B signaling, apoptosis, and ANCA-antigen expression. J Am Soc Nephrol 17:1345-1353, 2006.

Knepper-Nicolai B, Savill J, Brown SB. Constitutive apoptosis in human neutrophils requires synergy between calpains and the proteasome downstream of caspases. J Biol Chem 273:30530-30536, 1998.

Kobayashi SD, Braughton KR, Whitney AR, Voyich JM, Schwan TG, Musser JM, DeLeo FR. Bacterial pathogens modulate an apoptosis differentiation program in human neutrophils. PNAS 100:10948-10953, 2003.

Koedel U, Frankenberg T, Kirschnek S, Obermaier B, Häcker H, Paul R, Häcker G. Apoptosis is essential for neutrophil functional shutdown and determines tissue damage in experimental pneumococcal meningitis. PLoS Pathog 5:e10000461, 2009.

Kopanakis K, Tzepi IM, Pistiki A, Carrer DP, Netea MG, Georgitsi M, Lymperi M, Droggiti DI, Liakakos T, Machairas A, Giamarellos-Bourboulis EJ. Pre-treatment with low-dose endotoxin prolongs survival from experimental lethal endotoxic shock: Benefit for lethal peritonitis by Escherichia coli. Cytokine 62:382-388, 2013.

Kranke P, Bennett MH, Martyn-St James M, Schnabel A, Debus SE, Weibel S. Hyperbaric oxygen therapy for chronic wounds. Cochrane Database Syst Rev 6:CD004123, 2015.

Krista KM, White NA, Barrett JG, Furr MO, Buechner-Maxwell VA. Evaluation of neutrophil apoptosis in horses with abdominal disease. Am J Vet Res 74:999-1004, 2013.

Kun T, Jakubowski L. Influence of MRI contrast media on histamine release from mast cells. Pol J Radiol 77:19-24, 2012.

KupfnerJG, Arcaroli JJ, Yum H-K, Nadler SG, Yang K-Y, Abraham E. Role of NF-κB in endotoxemia-induced alterations of lung neutrophil apoptosis. J Immunol 167:7044-7051, 2001.

Lawrence T, Willoughby SA, Gilroy DW. Anti-inflammatory lipid mediators and insights into the resolution of inflammation. Nat Rev Immunol 2:787-795, 2002.

Lawrence DW, King SB, Frazier WA, Koenig JM. Decreased CD47 expression during spontaneous apoptosis targets neutrophils for phagocytosis by monocyte-derived macrophages. Early Hum Dev 85:659-663, 2009.

Leblebicioglu B, Walters J. Alkaline conditions accelerate polymorphonuclear leukocyte apoptosis in vitro. Infect Immun 67:2019-2021, 1999.
Lee A, Whyte MK, Haslett C. Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. J Leukoc Biol 54:283-288, 1993.

Lee A, Whyte MKB, Haslett C. Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. J Leukoc Biol 54:283-288, 1993.

Lefebvre JS, Lévesque T, Picard S, Paré G, Gravel A, Flamand L, Borgeat P. Extra domain A of fibronectin primes leukotriene biosynthesis and stimulates neutrophil migration through activation of Toll-like receptor 4. Arthritis Rheum 63:1527-1533, 2011.

Leitch AE, Riley NA, Sheldrake TA, Festa M, Fox S, Duffin R, Haslett C, Rossi AG. The cyclin-dependent kinase inhibitor R-roscovitine down-regulates Mcl-1 to override pro-inflammatory signalling and drive neutrophil apoptosis. Eur J Immunol 40:1127-1138, 2010.
Leuenroth SJ, Grutkoski PS, Ayala A, Simms HH. Suppression of PMN apoptosis by hypoxia is dependent on Mcl-1 and MAPK activity. Surgery 128:171–177, 2000.

Liles WC, Dale DC, Klebanoff SJ. Glucocorticoids inhibit apoptosis of human neutrophils. Blood 86:3181-3188, 1995.

Little D, Tomlinson JE, Blikslager AT. Post operative neutrophilic inflammation in equine small intestine after manipulation and ischaemia. Equine Vet J 37:329-335, 2005.

Liu D, Geng Z, Zhu W, Wang H, Chen Y, Liang J. 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 ameliorates endotoxin-induced acute lung injury in rats. Chin Med J (Engl) 127(5):815-820, 2014.

Loison F, Zhu H, Karatepe K, Kasorn A, Liu P, Ye K, Zhou J, Cao S, Gong H, Jenne DE, Renold-O'Donnell E, Xu Y, Luo HR. Proteinase 3-dependent caspase-3 cleavage modulates neutrophil death and inflammation. J Clin Invest 124: 4445-4458, 2014.

Lucas M, Stuart LM, Savill J, Lacy-Hulbert A. Apoptotic cells and innate immune stimuli combine to regulate macrophage cytokine secretion. J Immunol 171:2610-2615, 2003.

Maianski NA, Geissler J, Srinivasula SM, Alnemri ES, Roos D, Kuijpers TW. Functional characterization of mitochondria in neutrophils: a role restricted to apoptosis. Cell Death Diff 11:143-153, 2004.

Manny J, Manny N, Lelcuk S, et al. Pulmonary and systemic consequences of localized acid aspiration. Surg Gynecol Obstet 162:259-267, 1986.

Marwick JA, Dorward DA, Lucas CD, Jones KO, Sheldrake TA, Fox S, Ward C, Murray J, Birttan M, Hirani N, Duffin R, Dransfield I, Haslett C, Rossi AG. Oxygen levels determine the ability of glucocorticoids to influence neutrophil survival in inflammatory environments. J Leukoc Biol 94:1285-1292, 2013.

McCall CE, Grosso-Wilmoth LM, LaRue K, Guzman RN, Cousart SL. Tolerance to endotoxin-induced expression of interleukin-1β gene in blood neutrophils of humans with sepsis syndrome. J Clin Invest 91:853-861, 1993.

McCracken JM, Allen LH. Regulation of human neutrophil apoptosis and lifespan in health and disease. J Cell Death 7:15-23, 2014.

Mecklenburgh KI, Walmsley SR, Cowburn AS, Wiesener M, Reed BJ, Upton PD, Deighton J, Greening AP, Chilvers ER. Involvement of a ferroprotein sensor in hypoxia-mediated inhibition of neutrophil apoptosis. Blood 100:3008-3016, 2002.

Medvedev AE, Sabroe I, Hasday JD, Vogel SN. Tolerance to microbial TLR ligands: molecular mechanisms and relevance to disease. J Endotoxin Res 12:133-150, 2006.

Meszaros AJ, Reichner JS, Albina JE. Macrophage-induced neutrophil apoptosis. J Immunol 165:435-441, 2000.

Miles K, Clarke DJ, Lu W, Sibinska Z, Beaumont PE, Davidson DJ, Barr TA, Campopiano DJ, Gray M. Dying and necrotic neutrophils are anti-inflammatory secondary to the release of alpha-defensins. J Immunol 183:2122–2132, 2009.

Milot E, Fotouhi-Ardakani N, Filep JG. Myeloid nuclear differentiation antigen, neutrophil apoptosis and sepsis. Front Immunol 3:397, 2012.

Mizgerd JP, Molina RM, Stearns RC, Brain JD, Warner AE. Gadolinium induces macrophage apoptosis. J Leukoc Biol 59:189-195, 1996.

Molloy EJ, O'Neill AJ, Grantham JJ, Sheridan-Pereira M, Fitzpatrick JM, Webb DW, Watson RWG. Sex-specific alterations in neutrophil apoptosis: the role of estradiol and progesterone. Blood 102:2653-2659, 2003.

Molloy EJ, O'Neill, Grantham JJ, Sheridan-Pereira M, Fitzpatrick JM, Webb DW, Watson RWG. Granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor have differential effects on neonatal and adult neutrophil survival and function. Pediatr Res 57:806-812, 2005.

Montgomery JB, Hamblin B, Suri SS, Johnson LE, New D, Johnston J, Kelly J, Wilson DG, Singh B. Remote lung injury after experimental intestinal ischemia-reperfusion in horses.

Histol Histopathol 29:361-375, 2014.

Moore JN, Vandenplas ML. Is it the systemic inflammatory response syndrome or endotoxemia in horses with colic? Vet Clin Equine 30:337-351, 2014.

Moore JN, White NA, Berg JN, Trim CM, Garner HE. Endotoxemia following experimental intestinal strangulation obstruction in ponies. Can J Comp Med 45:330-332, 1981.

Morrison CA, Moran A, Patel S, Vidaurre Mdel P, Carrick MM, Tweardy DJ. Increased apoptosis of peripheral blood neutrophils is associated with reduced incidence of infection in trauma patients with hemorrhagic shock. J Infect 66:87-94, 2013.

Moulding DA, Akgul C, Derouet M, White MR, Edwards SW. BCL-2 family expression in human neutrophils during delayed and accelerated apoptosis. J Leukoc Biol 70:783-792, 2001.

Murray J, Barbara JAJ, Dunkley SA, Lopez AF, Van Ostade X, Condliffe AM, Dransfield I, Haslett C, Chilvers ER. Regulation of neutrophil apoptosis by tumor necrosis factor-α: requirement for TNFR55 and TNFR75 for induction of apoptosis in vitro. Blood 90:2772-2783, 1997.

Nagahata H, Higuchi H, Teraoka H, Takahashi K, Inanami O, Kuwabara M. Decreased apoptosis of β_2-integrin-deficient bovine neutrophils. Immunol Cell Biol 82:32-37, 2004.

Negrotto S, Malaver E, Alvarez ME, Pacienza N, D'Atri LP, Pozner RG, Gómez RM, Schattner M. Aspirin and salicylate suppress polymorphonuclear apoptosis delay mediated by proinflammatory stimuli. J Pharmacol Exp Ther 319(2):972-979, 2006.

Niedzwiedz A, Jaworski Z, Tykalowski B, Smialek M. Neutrophil and macrophage apoptosis in bronchoalveolar lavage fluid from healthy horses and horses with recurrent airway obstruction (RAO). BMC Vet Res 10:29, 2014.

Nourian AR, Baldwin GI, van Eps AW, Pollitt CC. Equine laminitis: ultrastructural lesions detected 24-30 hours after induction with oligofructose. Equine Vet J 39:360-364, 2007.

O'Brien MA, Kirby R. Apoptosis: A review of pro-apoptotic and anti-apoptotic pathways and dysregulation in disease. J Vet Emerg Crit Care 18:572-585, 2008.

Ortiz-Muñoz G, Mallavia B, Bins A, Headley M, Krummel MF, Looney MR. Aspirin-triggered 15-epi-lipoxin A4 regulates neutrophil-platelet aggregation and attenuates acute lung injury in mice. Blood. 2014 124:2625-2634, 2014.

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

Parker LC, Jones EC, Prince LR, Dower SK, Whyte MK, Sabroe I. Endotoxin tolerance induces selective alterations in neutrophil function. J Leukoc Biol 78:1301-1305, 2005.

Paunel-Görgülü A, Zörnig M, Lögters T, Altrichter J, Rabenhorst U, Cinatl J, Windolf J, Scholz M. Mcl-1-mediated impairment of the intrinsic apoptosis pathway in circulating neutrophils from critically ill patients can be overcome by Fas stimulation. J Immunol 183:6198-6206, 2009.

Perianayagam MC, Balakrishnan VS, Pereira BJ, Jaber BL. C5a delays apoptosis of human neutrophils via an extracellular signal-regulated kinase and Bad-mediated signalling pathway. Eur J Clin Invest 34(1):50-56, 2004.

Ren Y, Xie Y, Jiang G, Fan J, Yeung J, Li W, Tam PKH, Savill J. Apoptotic cells protect mice against lipopolysaccharide-induced shock. J Immunol 180:4978–4985, 2008.

Riedemann NC, Guo RF, Neff TA, Laudes IJ, Keller KA, Sarma VJ, Markiewski MM, Mastellos D, Strey CW, Pierson CL, Lambris JD, Zetoune FS, Ward PA Increased C5a receptor expression in sepsis. J Clin Invest 110:101–108, 2002.

Rossi AG, Sawatzky DA, Walker A, Ward C, Sheldrake TA, Riley NA, Caldicott A, Martinez-Losa M, Walker TR, Duffin R, Gray M, Crescenzi E, Martin MC, Brady HJ, Savill JS, Dransfield I, Haslett C. Cyclin-dependent kinase inhibitors enhance the resolution of inflammation by promoting inflammatory cell apoptosis. Nat Med 12:1056-1064, 2006.

Rowe SJ, Allen L, Ridger VC, Hellewell PG, Whyte M. Caspase-1-deficient mice have delayed neutrophil apoptosis and a prolonged inflammatory response to lipopolysaccharide-induced acute lung injury. J Immunol 169:6401-6407, 2002.

Sabroe I, Jones EC, Usher LR, Whyte MK, Dower SK. Toll-like receptor (TLR)2 and TLR4 in human peripheral blood granulocytes: a critical role for monocytes in leukocyte lipopolysaccharide responses. J Immunol 168:4701-10, 2002.

Sabroe I, Dower SK, Whyte MK. The role of Toll-like receptors in the regulation of neutrophil migration, activation, and apoptosis. Clin Infect Dis 41:S421-6, 2005.

Saffar AS, Dragon S, Ezzati P, Shan L, Gounni AS. Phosphatidylinositol 3-kinase and p38 mitogen-activated protein kinase regulate induction of Mcl-1 and survival in glucocorticoid-treated human neutrophils. J Allergy Clin Immunol 121:492-498, 2008.

Sakamoto C, Suzuki K, Hato F, Akahori M, Hasegawa T, Hino M, Kitagawa S. Antiapoptotic effect of granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and cyclic AMP on human neutrophils: protein synthesis-dependent and protein synthesis-independent mechanisms and the role of the Janus kinase-STAT pathway. Int J Hematol 77:60-70, 2003.

Salamone G, Giordano M, Trevani AS, Gamberale R, Vermeulen M, Schettinni J, Geffner JR. Promotion of neutrophil apoptosis by TNF-α. J Immunol 166:3476-3483, 2001.

Salamone GV, Petracca Y, Fuxman Bass JI, Rumbo M, Nahmod KA, Gabelloni ML, Vermeulen ME, Matteo MJ, Geffner JR, Trevani AS. Flagellin delays spontaneous human neutrophil apoptosis. Lab Invest 90:1049-1059, 2010.

Saldanha-Gama RF, Moraes JA, Mariano-Oliveira A, Coelho AL, Walsh EM, Marcinkiewicz C, Barja-Fidalgo C. Alpha(9)beta(1) integrin engagement inhibits neutrophil spontaneous apoptosis: involvement of Bcl-2 family members. Biochim Biophys Acta 1803:848-857, 2010.

Sano J, Oguma K, Kano R, Yazawa M, Tsujimoto H, Hasegawa A. High expression of Bcl-xL in delayed apoptosis of canine neutrophils induced by lipopolysaccharide. Res Vet Sci 78:183-187, 2005.

Savill JS, Henson PM, Haslett C. Phagocytosis of aged human neutrophils by macrophages is mediated by a novel "charge-sensitive" recognition mechanism. J Clin Invest 84:1518-1527, 1989.

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 by macrophages. J Clin Invest 83: 865-875, 1989.

Savill JS, Fadok VA. Corpse clearance defines the meaning of cell death. Nature 407:784-788, 2000.

Savill J, Dransfield I, Gregory C, Haslett C. A blast from the past: clearance of apoptotic cells regulates immune responses. Nat Rev Immunol 2:965-975, 2002.

Schneberger D, Aharonson-Raz K, Singh B. Pulmonary intravascular macrophages and lung health: what are we missing? Am J Physiol Cell Mol Physiol 302:L498-503, 2012.

Schneberger D, Caldwell S, Suri SS, Singh B. Expression of toll-like receptor 9 in horse lungs. Anat Rec (Hoboken) 292:1068-1077, 2009.

Scheel-Toellner D, Wang K, Craddock R, Webb PR, McGettrick HM, Assi LK, Parkes N, Clough LE, Gulbins E, Salmon M, Lord JM. Reactive oxygen species limit neutrophil lifespan by activating receptor signaling. Blood 104:2557-2564, 2004.

Senior JM, Proudman CJ, Luwer M, Carter SD: Plasma endotoxin in horses presented to an equine referral hospital: correlation to selected clinical parameters and outcomes. Equine Vet J 43:585-591, 2011.

Serhan CN, Savill J. Resolution of inflammation: the beginning programs the end. Nat Immunol 6:1191-1197, 2005.

Shang D, Qi QH, Wang BZ, Chen HL, Bi W, Guan FL. Role of polymorphonuclear neutrophil apoptosis and expression of Fas and caspase-3 in the systemic inflammatory response syndrome. Zhongguo Wei Zhong Bing Ji Jiu Yi Xue 19:11-13, 2007.

Silva MT. When two is better than one: macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. J Leukoc Biol 87:93-106, 2010.

Silva MT. Macrophage phagocytosis of neutrophils at inflammatory/infectious foci: a cooperative mechanism in the control of infection and infectious inflammation. J Leukoc Biol 89:675-683, 2011.

Stark MA, Huo Y, Burcin TL, Morris MA, Olson TS, Ley K. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. Immunity 22:285-294, 2005.

Steverink PJ, Salden HJ, Sturk A, Klein WR, van der Velden MA, Németh F. Laboratory and clinical evaluation of a chromogenic endotoxin assay for horses with acute intestinal disorders. Vet Q 16:117-121, 1994.

Stone SF, Cotterell C, Isbister GK, Holdgate A, Brown SG. Elevated serum cytokines during human anaphylaxis: Identification of potential mediators of acute allergic reactions. J Allergy Clin Immunol 124:786-792, 2009.

Sun Z, Lei H, Zhang Z. Pre-B cell colony enhancing factor (PBEF), a cytokine with multiple physiological functions. Cytokine Growth Factor Rev 24:433-442, 2013.

Suratt BT, Parsons PE: Mechanisms of acute lung injury/acute respiratory distress syndrome. Clin Chest Med 27:579-589, 2006.

Suri SS, Janardhan KS, Parbhakar O, Caldwell S, Appleyard G, Singh B. Expression of toll-like receptor 4 and 2 in horse lung. Vet Res 37:541-551, 2006.

Sweeney JF, Nguyen PK, Omann GM, Hinshaw DB. Lipopolysaccharide protects polymorphonuclear leukocytes from apoptosis via tyrosine phosphorylation-dependent signal transduction pathway. J Surg Res 74:64-70, 1998.

Tadros EM, Frank N, Gomez De Witte F, Boston R. Effects of intravenous lipopolysaccharide infusion on glucose and insulin dynamics in horses with equine metabolic syndrome. Am J Vet Res 74:1020–1029, 2013.

Tak T, Tesselaar K, Pillay J, Borghans JA, Koenderman L. What's your age again? Determination of human neutrophil half-lives revisited. J Leukoc Biol 94(4):595-601, 2013.

Taneja R, Parodo J, Jia SH, Kapus A, Rotstein OD, Marshall JC. Delayed neutrophil apoptosis in sepsis is associated with maintenance of mitochondrial transmembrane potential and reduced caspase-9 activity. Crit Care Med 32:1460-1469, 2004.

Tatsuta T, Cheng J, Mountz JD. Intracellular IL-1β is an inhibitor of Fas-mediated apoptosis. J Immunol 157:3949-3957, 1996.

Taylor PM, Clarke KW. Anaesthesia in special situations. In: Taylor PM, Clarke KW , eds. Handbook of Equine Anaesthesia. 2nd ed. Philadelphia: Saunders Elsevier 187, 2007.

Tennenberg SD, Zemlan FP, Solomkin JS. Characterization of N-formyl-methionyl-leucyl-phenylalanine receptors on human neutrophils. Effects of isolation and temperature on receptor expression and functional activity. J Immunol 141:3937-3944, 1988.

Turlej RK, Fiévez L, Sandersen CF, Dogné S, Kirschvink N, Lekeux P, Bureau F. Enhanced survival of lung granulocytes in an animal model of asthma: evidence for a role of GM-CSF activated STAT5 signalling pathway. Thorax 56:696–702, 2001.

Unnewehr H, Rittirsch D, Sarma JV, Zetoune F, Flierl MA, Perl M, Denk S, Weiss M, Schneider ME, Monk PN, Neff T, Mihlan M, Barth H, Gebhard F, Ward PA, Huber-Lang M. Changes and regulation of the C5a receptor on neutrophils during septic shock in humans. J Immunol 190:4215-4225, 2013.

van Raam BJ, Drewniak A, Groenewold V, van den Berg TK, Kuijpers TW. Granulocyte colony-stimulating factor delays neutrophil apoptosis by inhibition of calpains upstream of caspase-3. Nature 112:2046-2054, 2008.

van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J Immunol Methods 174:83-93, 1994.

Vogler M. BCL2A1: the underdog in the BCL2 family. Cell Death Differ 19:67-74, 2012.

Walmsley SR, Print C, Farahi N, Peyssonnaux C, Johnson RS, Cramer T, Sobolewski A, Condliffe AM, Cowburn AS, Johnson N, Chilvers ER. Hypoxia-induced neutrophil survival is mediated by HIF-1alpha-dependent NF-kappaB activity. J Exp Med 201:105-115, 2005.

Wang J, Huang W, Wang C, Liu R. Dynamic process of phagocytosis and forms of macrophage cell death induced by ingestion of apoptotic neutrophils. Sci China Life Sci Sep 12, 2014.

Ward C, Walker A, Dransfield I, Haslett C, Rossi AG. Regulation of granulocyte apoptosis by NF-kappaB. Biochem Soc Trans 32:465-467, 2004.

Wardle DJ, Burgon J, Sabroe I, Bingle CD, Whyte MK, Renshaw SA. Effective caspase inhibition blocks neutrophil apoptosis and reveals Mcl-1 as both a regulator and a target of neutrophil caspase activation. PLoS ONE 6:e15768, 2011.
Watson RWG, Rotstein OD, Jimenez M, Parodo J, Marshall JC. Neutrophil apoptosis is modulated by endothelial transmigration and adhesion molecule engagement. Blood 89:4175-4181, 1987.

Warner MA, Warner ME, Weber JG. Clinical significance of pulmonary aspiration during the perioperative period. Anesthesiology 78:56 – 62, 1993.

Watson F, Robinson JJ, Edwards SW. Neutrophil function in whole blood and after purification: changes in receptor expression, oxidase activity, and responsiveness to cytokines. Biosci Rep 12:123-133, 1992.

Watson RWG, Rotstein OD, Nathens AB, Parodo J, Marshall JC. Neutrophil apoptosis is modulated by endothelial transmigration and adhesion molecule engagement. J of Immunol 158:945-953, 1997.

Watson RWG, Rotstein OD, Parodo J, Bitar R, Marshall JC. The IL-1β-converting enzyme (caspase-1) inhibits apoptosis of inflammatory neutrophils through activation of IL-1β. J Immunol 161:957-962, 1998.

Wauters J, Martens A, Pille F, Dumoulin M, Gasthuys F, Sys S, Meyer E. Viability and cell death of synovial fluid neutrophils as diagnostic biomarkers in equine infectious joint disease: a pilot study. Res Vet Sci 92(1):132-137, 2012.

Weinmann P, Scharffetter-Kochanek K, Forlow SB, Peters T, Walzog B. A role for apoptosis in the control of neutrophil apoptosis in the circulation: insights from CD18-deficient mice. Blood 101:739-746, 2003.

Werners AH, Bull S, Fink-Gremmels J. Endotoxaemia: a review with implications for the horse. Eq Vet J 37:371-383, 2005.

West MA, Koons A, Crandall M, Skinner R, Worley M, Shapiro MB. Whole blood leukocyte mitogen activated protein kinases activation differentiates intensive care unit patients with systemic inflammatory response syndrome and sepsis. J Trauma 62:805-811, 2007.

Whyte MK, Meagher LC, MacDermot J, Haslett C. Impairment of function in aging neutrophils is associated with apoptosis. J Immunol 50:5124-34, 1993.
Xu R, Lin F, Bao C, Huang H, Ji C, Wang S, Jin L, Sun L, Li K, Zhang Z, Wang FS. Complement 5a receptor-mediated neutrophil dysfunction is associated with a poor outcome in sepsis. Cell Mol Immunol Mar 2, 2015. doi: 10.1038/cmi.2014.136.

Wilkins PA, Otto CM, Baumgardner, JE, et al. Acute lung injury and acute respiratory distress syndrome in veterinary medicine: consensus definitions: the Dorothy Russell Havemeyer working group on ALI and ARDS in veterinary medicine. J Vet Emerg Crit Care 17:333-339, 2007.

Wright HL, Moots RJ, Bucknall RC, Edwards SW. Neutrophil function in inflammation and inflammatory disease. Rheumatology 49:1618-1631, 2010.

Xu X, Liu Y, Wang L, He J, Zhang H, Chen X, Li Y, Yang J, Tao J. Gambogic acid induces apoptosis by regulating the expression of Bax and Bcl-2 and enhancing caspase-3 activity in human malignant melanoma A375 cells. Int J Dermatol 48:186-192, 2009.

Yamamoto C, Yoshida S, Taniguchi H, Qin MH, Miyamoto H, Mizuguchi Y. Lipopolysaccharide and granulocyte colony-stimulating factor delay neutrophil apoptosis and ingestion by guinea pig macrophages. Infect Immun 61:1972-1979, 1993.

Zielińska-przyjemska M, Główka FK, Klaczyńska J. Modulatory effect of chiral nonsteroidal anti-inflammatory drugs on apoptosis of human neutrophils. Chirality 20(2):159-165, 2008.