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A MODEL OF
GLUCOCORTICOID-MEDIATED DELAY IN APOPTOSIS

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Doctoral degree in Animal Science

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BOVINE BLOOD NEUTROPHILS AT PARTURITION: A MODEL OF GLUCOCORTICOID-MEDIATED DELAY IN APOPTOSIS

By

Sally Ann Madsen-Bouterse

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Animal Science

2005

ABSTRACT

BOVINE BLOOD NEUTROPHILS AT PARTURITION: A MODEL OF GLUCOCORTICOID-MEDIATED DELAY IN APOPTOSIS

By

Sally Ann Madsen-Bouterse

Neutrophils play a pivotal role in the first line of host cellular immune defense against bacteria but can also cause significant tissue pathology if their activities are not tightly regulated through rapid apoptosis. In order to regulate apoptosis, neutrophils are equipped with a variety of receptors that respond to inflammatory and hormonal stimuli in their surrounding environment. Parturition presents a complex set of such stimuli to circulating neutrophils. In dairy cows, neutrophils respond to the parturient blood environment by altering gene expression and corresponding immune defense activities at a time when cows experience heightened severity of inflammatory diseases, and thus have been anecdotally linked. The goal of the current dissertation research was to study the physiological status of neutrophils at parturition in an effort to elucidate their functional changes along with mechanisms of these parturition-induced changes. The main objective of the first series of experiments was to profile neutrophil gene expression during the peripartum period using cDNA microarray analysis. A large cluster of altered genes related to apoptosis regulation was identified and provided an expression signature suggesting that parturition delayed the normally rapid program of cell death in circulating neutrophils. Because steroid hormones are integral to parturition and can regulate gene expression, the next objective was to determine if the steroid component of parturient serum influenced neutrophil spontaneous apoptosis. Neutrophils treated *in vitro* with a glucocorticoid receptor antagonist (RU486) removed the apoptosis-delaying capacity of

parturient serum (PS) and direct glucocorticoid treatment. In addition, charcoal removal of steroids from PS abolished its apoptosis delaying capacity, which could be restored when charcoal-treated serum was reconstituted with glucocorticoid concentrations found in PS. This was not the case when 17β-estradiol or progesterone was added-back to charcoal-treated serum. In the final objective, direct effects of glucocorticoid on maintenance of mitochondrial membrane stability and abundance of A1 and Bak mRNA and protein were assessed. A1 and Bak were selected for study because their mRNA abundance was changed during parturition (Objective 1) and available literature indicates these Bcl-2 family members are key in human and mouse models of neutrophil apoptosis regulation. In support of delayed apoptosis, mRNA and protein abundance of neutrophil A1 was increased following both *in vivo* and *in vitro* glucocorticoid treatment. Bak protein abundance was decreased after *in vivo* glucocorticoid treatment and mRNA abundance was decreased in cells treated *in vitro*. However, *in vitro* treated neutrophils did not display alterations in Bak protein abundance. *In vitro* changes in A1 and Bak occurred concurrently with increased stability of neutrophil mitochondrial membranes and decreased activity of caspase-9 in glucocorticoid treated cells. Thus, Bcl-2 family members A1 and Bak appear to be targets of glucocorticoid-induced delays in neutrophil spontaneous apoptosis and possibly influence changes at the level of mitochondrial membrane stability. These results establish glucocorticoid as one parturient serum factor capable of delaying the genetic program of spontaneous cell death in circulating bovine neutrophils. Further studies are needed to determine if a parturient delay in neutrophil apoptosis contributes to the heightened severity of inflammatory diseases typically observed during this stage of a cow's production cycle.

To Chad – for keeping a smile in my heart.

ACKNOWLEDGEMENTS

The work presented in this dissertation received funding support from USDA-IFAFS grant #2001-52100-11211, USDA-NRI grant # 2001-35204-10798, as well as Michigan Agricultural Experiment Station Projects MICL01836 (umbrella) and MICL02035 (NC1010). Personal financial support (graduate assistantship) was supplied by the Department of Animal Science and the Michigan Agricultural Experiment Station.

There are a number of people who have made my time at Michigan State a successful and enjoyable experience. First, I would like to thank everyone that has come and gone from the Immunogenetics Laboratory for their support and help with sample collections. Thanks to Bob Kreft and the crew at the MSU Dairy Teaching and Research facility for their assistance; be it cows calving or daily care of steers, this work would not have been completed without you. Thanks also to Rob Ashley and the Kellogg Biological Station Dairy for additional sample collections. Thanks to the Center for Animal Functional Genomics for their support, especially Sue Sipkovsky for her guidance with running microarrays.

I would like to thank my guidance committee members: Dr. Jeanne Burton (major professor), Dr. Paul Coussens, Dr. Guilherme Rosa, Dr. George Smith, and Dr. Lorraine Sordillo. Your comments, ideas, support, and encouragement have helped me to understand the importance of looking at the bigger picture even when studying something at the most basic level.

Several members of the Immunogenetics Laboratory are deserving of a special thank you. To Dr. Patty Weber – you have seen me through two graduate degrees and have offered never-ending support. Life in the lab would not have been the same without

you. To Janelle Durrett Tirrell and Kelly Buckham – thank you for the endless hours at the farm and neutrophil isolations back in the lab. Beyond the lab, you are two of the greatest friends I gained during my years at MSU and hope that as we continue down the paths of our lives we never lose contact.

Jeannie, where do I begin... Thank you for the opportunity to work with you and the great people you surround yourself with. You have guided me farther down the path to becoming not only a scientist but a thinker and I will be everlastingly thankful for the great ideas and your endless energy and passion you shared with me over the years. I look forward to learning and collaborating with you as a colleague as I begin the next stage of my career.

Finally, I would like to thank my friends and family. To my friends Dave and Christy for chats about school, chats about life, Spartan basketball games, happy hours, and always a place to crash. Thanks for making graduate school more than just research. Thank you to my parents, Helge and Marilyn, for teaching me that hard work pays off and to always see something through to the end not matter how hard it is. To my brother Toby, sister Mary, and my new family Keith, Toni, and Marcus for always being interested in what I was doing and your support and encouragement. Last but not least, thank you to my husband Chad. You were there when I started this journey and have stuck with me through the highs and the lows. I would never have gotten this far without you and am excited to start the next stage of our lives together.

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APPENDIX ONE

LIST OF ABBREVIATIONS

ACD	Acid Citrate Dextrose
AP-1	Activator Protein-1
Apaf-1	Apoptotic protease activating factor 1
ATP	Adenosine Triphosphate
BH	Bcl-2 Homology Domain (BH1, BH2, BH3, BH4)
BLASTn	Basic Local Alignment Search Tool - nucleotide
BOTL	Bovine Total Leukocyte
bp	Base Pairs
CD	Cluster of Differentiation (e.g CD14, CD18, CD62L)
cDNA	Complementary DNA
Cort	Cortisol
CTS	Charcoal-Treated Serum
DDRT-PCR	Differential Display Reverse Transcription Polymerase Chain Reaction
Dex	Dexamethasone
DISC	Death Inducing Signaling Complex
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleoside Triphosphate
DTT	Dithiothreitol
ECM	Extracellular Matrix
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene Glycol-bis(2-aminoethylether)-tetraacetic Acid

EIA	Enzyme Immuno Assay
ER	Estrogen Receptor
EST	Expressed Sequence Tag
Estr	Estrogen
E-value	Expectation Value
FasL	Fas Ligand
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
g	Gram
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
h	Hour
H. influenzae	*Haemophilus influenzae*
IAP	Inhibitor of Apoptosis Proteins
ICAM-1	Intracellular Adhesion Molecule-1
IFN-γ	Interferon-gamma
IGF-1	Insulin-like Growth Factor-1
IgG	Immunoglobulin G
IL	Interleukin (e.g. IL-1, IL-1β, IL-8)
KCl	Potassium Chloride

LOESS	Local-Weighted Regression and Smoothing Scatterplots
LPS	Lipopolysaccharide
M	Molarity
$MgCl_2$	Magnesium Chloride
MHC	Major Histocompatibility Complex
min	Minute
ml	Milliliter
MMP-2	Matrix Metalloproteinase-2
MMP-8	Matrix Metalloproteinase-8
MMP-9	Matrix Metalloproteinase-9
mRNA	Messenger Ribonucleic Acid
NaCl	Sodium Chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate, reduced form
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor kappa B
°C	Degrees Celsius
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
PR	Progesterone Receptor
Prog	Progesterone
PS	Parturient Serum
Q-RT-PCR	Quantitative real-time Reverse Transcription Polymerase Chain Reaction

RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPL-19	Ribosomal Protein L-19
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RU486	Mifepristone
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
S. aureus	*Staphylococcus aureus*
S. pneumoniae	*Streptococcus pneumoniae*
sFasL	Soluble Fas Ligand
tBID	Truncated Bid
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline containing 0.1% Tween
TGF-β	Transforming Growth Facto Beta
TIMP-2	Tissue Inhibitor of Metalloproteinase 2
TIMP-3	Tissue Inhibitor of Metalloproteinase 3
TNF-α	Tumor Necrosis Factor-Alpha
Tris-HCl	Tris-Hydrochloric Acid
US	United States of America
μg	Microgram

INTRODUCTION

Parturient dairy cows are well known for their heightened susceptibility to intramammary infections that cause clinical mastitis as well as severity of this disease immediately postpartum (Frost and Brooker, 1986; Shuster et al., 1996; Erskine et al., 2002). Losses due to mastitis cost the US dairy industry at least $2 billion annually, with estimates ranging from $35 to $239 per cow per year for most dairy enterprises (Bartlett et al., 1991; Hoblet et al., 1991; DeGraves and Fetrow, 1993). Mastitis is also of societal concern due to its effects on animal welfare and because antibiotic treatment for the disease leads to significant drug contamination of milk designated for human consumption (Erskine, 1996).

Neutrophils are granulocytic leukocytes that play a pivotal role in host defense against bacterial infections, especially opportunistic coliform bacteria that cause severe mastitis during times of immunosuppression, such as in newly calved cows (Burton and Erskine, 2003; Burvenich et al., 2003). Neutrophils develop in the bone marrow from myeloid-lineage progenitor cells over approximately 14 days. Once matured, these granulocytes are released into the circulation where they utilize surface adhesion molecules to marginate along blood vessel endothelia to survey peripheral tissues for signs of infection and inflammation (Kansas, 1996; Weber et al., 2004). During an inflammatory response, neutrophils use additional adhesion and chemokine receptors to migrate through the endothelium to the infection focus. Neutrophils then phagocytose and ultimately kill invading bacteria via oxidative and enzymatic mechanisms (Ward and Lentsch, 1999). These cells are also influential in up-regulating inflammatory and

adaptive immune responses through an ability to stimulate and secrete a wide variety of cytokines and chemokines resulting in recruitment of additional neutrophils, and ultimately lymphocytes, to the site of infection (Cassatella, 1999). However, neutrophils can cause significant tissue pathology if their pro-inflammatory activities are not tightly regulated. Neutrophil removal via rapid apoptosis is one such method of regulation.

During homeostasis, neutrophils have a short half-life of 6 to 10 hours in blood (Cartwright et al., 1964; Dancey et al., 1976) with ~85% of the cells at some stage of apoptosis (Shidham and Swami, 2000). This is in stark contrast to other leukocytes that can live for days, weeks, and even months. Neutrophil apoptosis is delayed for up to 48 hours during an inflammatory event to augment the number of pro-inflammatory cells available to clear invading pathogens (Whyte et al., 1999). In scenarios of both homeostasis and inflammation, neutrophils must ultimately die by apoptosis so they do not die by necrosis and risk the release of their potent enzymes and oxygen metabolites that would seriously damage surrounding healthy tissue.

Experimental animal models of inflammatory disease have shown that delayed apoptosis in neutrophils coincides with disease pathogenesis, magnified inflammatory tissue damage, and poor prognosis in affected animals (Turlej et al., 2001; Saba et al., 2002; Garlichs et al., 2004). Delayed apoptosis is essential for neutrophil accumulation at sites of bacterial infection and inflammation, but it is also a central concept for the maintenance of inflammation in many disease states (Dibbert et al., 1999; Haslett, 1999). Thus, apoptosis regulation in neutrophils may be a key control point in the rapid resolution of inflammation, and has been proposed as a worthy target for novel drug development (Haslett et al., 1994; Ward et al., 1999). In cattle, the powerful enzymes

and bactericidal substances in cytoplasmic granules of neutrophils are highly cytotoxic (Bainton, 1975). Pro-inflammatory tissue degrading enzymes are necessary for neutrophil migration but also can severely damage mammary tissue (Capuco et al., 1986). Neutrophils release these enzymes into the extracellular space as the cells degranulate during adhesion to extracellular matrix proteins and phagocytosis of pathogens (Xu and Hakansson, 2002). Recent studies suggest that rapid apoptosis of neutrophils may be a normal and effective protective mechanism to counteract their potential tissue degrading activities and thus prevent excess inflammatory damage during bovine mastitis (Sladek et al., 2000a,b; 2001; Boutet et al., 2004). If true, then it is reasonable to speculate that delayed apoptosis of neutrophils may contribute to the pathogenesis of severe mastitis in affected cows.

Neutrophil apoptosis regulation has been understudied in the context of bovine parturition and mastitis. On the other hand, numerous mastitis researchers have studied up-regulation of the inflammatory response and reported that migratory and bactericidal activities of blood neutrophils become suppressed in dairy cows around parturition (reviewed by Kehrli and Harp, 2001; Burton and Erskine, 2003). For example, observed decreases in expression of the surface adhesion molecules L-selectin and CD18 (Lee and Kehrli, 1998; Preisler et al., 2000; Weber et al., 2001) may lead to hampered endothelial margination and migration of the cells into infected mammary quarters. Neutrophils from parturient cows also exhibit lowered oxidative burst activity and generate fewer reactive oxygen species (ROS) during phagocytosis, which are proposed to result in an inability of the cells to effectively kill bacteria they have engulfed (Kehrli et al., 1989; Cai et al., 1994; Vangroenweghe et al., 2005). Some of these phenomena correlate with

the massive fluctuations in blood parturient steroids (cortisol, 17β-estradiol, and progesterone) that are necessary for parturition to occur (reviewed in Burton and Erskine, 2003; Burvenich et al., 2003; Burton et al., 2005). In turn, the observed delays in neutrophil migration and inhibited bactericidal functions have been used to explain heightened susceptibility to clinical mastitis in affected cows.

In spite of the numerous documented observations of decreased migration and ROS generating capacities, little is known about the molecular or physiological basis of how and why neutrophil activities change during bovine parturition. In addition, it is unclear how this relates to the severity of mastitis in newly calved cows. Human neutrophils have been shown to respond to multiple environmental stimuli, such as bacteria and cytokines, with altered expression of numerous gene clusters (Lloyd and Oppenheim, 1992; Itoh et al., 1998; Newberger et al., 2000). Our research group has begun to demonstrate that some of the altered phenotypes observed in neutrophils from parturient dairy cows may result from changes in gene expression. One such change is decreased mRNA and protein abundance of L-selectin (Weber et al., 2001), the key adhesion molecule needed for blood neutrophils to marginate along blood vessel endothelia. We demonstrated that increased cortisol levels at parturition most likely causes decreased L-selectin expression in circulating neutrophils (Weber et al., 2004). This correlates with neutrophilia and delayed neutrophil recruitment into infected mammary quarters (Burton and Kehrli, 1995). We have also shown that expression of a key apoptosis-associated death receptor, Fas, is decreased in circulating neutrophils from parturient dairy cows and that the fluctuating concentrations of serum cortisol correlate with Fas expression during the periparturient period (Chang et al., 2004). By using a

broader experimental approach to study gene expression, we further demonstrated that numerous genes involved in basic cellular functions, such as oxidative metabolism in the mitochondria and protein synthesis, have altered mRNA abundance in neutrophils of parturient dairy cows (Madsen et al., 2002). Although we did not directly identify the causative factors, significant statistical correlations between gene expression profiles and profiles of serum 17β-estradiol and progesterone suggested possible relationships between fluctuations in parturient steroids and neutrophil mRNA abundance changes (Madsen et al., 2002). Thus, neutrophils appear to be sensitive to the parturient blood environment and respond to it by altering gene expression. However, there is still much to learn about how neutrophils respond to the physiology of parturition, and why. With this information appropriate targets for development of effective mastitis prevention and treatments may be elucidated.

In the present study, the overall hypothesis was that circulating neutrophils respond to parturition with coordinated changes in their gene expression and phenotypic status which results from the cell's sensitivity to fluctuating concentrations of steroid hormones at parturition. A functional genomics approach was utilized to examine the physiological status of neutrophils from parturient dairy cows. The main objective of the first series of experiments was to profile changes in neutrophil gene expression during the peripartum period using cDNA microarrays and quantitative real time RT-PCR. This was followed by *in silico* approaches to cluster identified, differentially expressed genes by functional ontology in order to obtain a preliminary view of what the cells' physiological status might be. In addition, blood serum from parturient dairy cows was utilized *in vitro* to treat neutrophils from healthy steers and obtain preliminary evidence

that the factor(s) responsible for the physiological status of the cells at parturition is present in blood. This work is summarized in **Chapter Two** and is published (Madsen et al., 2004).

Based on observations from the work in **Chapter Two**, the next series of experiments were designed to determine the roles, if any, of three major parturient steroids (glucocorticoid, 17β-estradiol, and progesterone) and their cognate receptors in the altered physiological status of neutrophils around parturition. These steroid hormones were selected because of their pronounced fluctuations in blood at calving and their direct and profound effects on neutrophil L-selectin and Fas gene expression observed in our previous studies (Weber et al., 2001, 2004; Chang et al., 2004) and in other cell systems (Carson-Jurica et al., 1990; Beato et al., 1995). This work is summarized in **Chapter Three** and results are published (Burton et al., 2005).

Based on results presented in **Chapters Two** and **Three**, a final series of experiments were designed to test glucocorticoid effects on two key apoptosis regulating genes. The Bcl-2 family members A1/Bfl-1 (anti-apoptotic) and Bak (pro-apoptotic) are known to regulate mitochondria-mediated spontaneous apoptosis in human and rodent neutrophils. The work presented in **Chapter Four** demonstrates for the first time that these genes are targets for glucocorticoid regulation of spontaneous apoptosis in bovine blood neutrophils. Results of this research are currently being summarized into a manuscript to be submitted for peer-review (Madsen-Bouterse et al., in preparation).

CHAPTER ONE

A Review of Literature

I. INNATE IMMUNITY

An animal's ability to protect itself from invading pathogens, and potentially disease, begins with innate immunity. A system that is in place from the time of birth, innate (or nonspecific) immunity consists of three major defense categories: anatomic and physiologic barriers, humoral factors, and cellular factors (reviewed by Minchinton et al., 2004). The skin and mucous membranes provide the simplest level of innate immunity by inhibiting pathogens from entering the body while physiological barriers, such as high or low pH, may kill or inhibit pathogen growth (Kuby, 1997). Humoral components of innate immunity include complement proteins (e.g. C3b, C5a), collectins (e.g. mannose binding proteins), and acute phase proteins (e.g. C-reactive protein). These factors are integral in both sensing invading pathogens as well as facilitating their removal (Beutler, 2004; Minchinton et al., 2004). The cellular component of innate immunity consists of phagocytic leukocytes from the myeloid lineage, primarily neutrophils and macrophages. These leukocytes are integral to the immune response because of their ability to phagocytose and kill invading pathogens, thus removing them from the body (Beutler, 2004; Liu and Pope, 2004). If the innate immune response is dysfunctional, or an infection overcomes the phagocytes' abilities to clear pathogenic factors, adaptive (or specific) immunity will become activated over subsequent days and weeks. In a healthy individual, innate immunity is supplemented by this highly specific and targeted defense against invading pathogens resulting in an optimal immune response.

II. THE NEUTROPHIL: AN EFFECTOR CELL OF INNATE IMMUNITY

Neutrophils are granulocytic leukocytes that differentiate in the bone marrow from a pool of pluripotent stem cells into myeloblasts and promyelocytes via the process of granulopoeisis (Edwards, 1994). Development from pluripotent stem cells into mature, segmented neutrophils takes approximately 14 days, 7.5 days of which are spent in proliferation stages (myeloblast, promyelocyte, and myelocyte) and the remaining 6.5 days during which the cells mature through metamyelocyte, band cell, and segmented neutrophil stages (Bainton et al., 1971). Mature neutrophils are released into the blood circulatory system from the bone marrow fully equipped with a battery of defense proteins stored in their numerous granules and prepared to play an immediate role in innate immunity against invading pathogens. This is achieved through the cells five main functions which include surveillance, recruitment, receptor-mediated phagocytosis, respiratory burst, and fusion of phagolysosomes where cytoplasmic granules containing potent lytic enzymes (i.e. lysosomes) fuse with the phagosome and degranulate to aid in enzymatic killing of ingested pathogens. The following summary of information about these functions as well as mechanisms to control neutrophil death is provided so the critical roles of neutrophils in innate immunity and subsequent resolution of inflammation may be better appreciated.

A. Neutrophil Surveillance and Recruitment

Upon release from the bone marrow, neutrophils have a half-life of approximately 6 to 10 hours in the circulation (determined by the disappearance of neutrophils labeled *in vivo* with radioactive diisopropylfluorophosphate or tritiated thymidine in Cartwright et

al., 1964 and Dancey et al., 1976). Mature neutrophils can be identified in the blood by their large, multi-lobed, segmented nucleus in which chromatin is coarsely clumped (Gennaro et al., 1978; Edwards, 1994). Other characteristics of circulating neutrophils include numerous cytoplasmic granules, tubular mitochondria, small amounts of Golgi and endoplasmic reticulum, and some ribosomes. Cytoplasmic granules are particularly important to neutrophil bactericidal functions, thus many of the granular contents are preformed during development in bone marrow and stored for rapid action during innate immune responses.

Surveillance and recruitment into tissue occur primarily at the level of blood vessel endothelium and are the first two functions a mature neutrophil must perform. Circulating neutrophils are constantly surveying the body for infections and damage in peripheral tissues via margination along blood vessel endothelia. If an infection or inflammation is present, neutrophils are recruited by the endothelium and migrate through it into the affected peripheral tissue through the mechanisms of diapedesis and chemotaxis. Collectively, margination and migration are referred to as neutrophil trafficking. Neutrophil trafficking is mediated by a variety of adhesion molecules, termed selectins and integrins, which are located on the cell's surface. Under normal conditions, neutrophils attach lightly to blood vessel endothelial cells during margination via their surface L-selectin adhesion molecules (Liu and Pope, 2004). The shear force of blood flow facilitates the rolling of L-selectin tethered neutrophils along the vessel wall (Jutila, 1992; Kansas, 1996), and on other neutrophils already arrested on activated endothelia (Bargatze et al., 1994). Neutrophil rolling slows the movement of the cells enough to detect and respond to local increases in the chemokine, interleukin-8 (IL-8),

and pro-inflammatory cytokines (IL-1β, TNF-α, and IFN-γ) that help signal the location of invading pathogens and activate neutrophils for migration and immediate immune response.

When rolling neutrophils detect infection in underlying tissues, surface expression and activation of their granule stores of β_2-integrin adhesion molecules [such as LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18)] are increased (Beutler, 2004). Enhanced β_2-integrin expression is necessary to arrest rolling neutrophils on the activated endothelium. Up-regulation and engagement of these adhesion molecules with their ICAM ligands on the endothelium also causes granular release of gelatinase (primarily MMP-9) from the neutrophils, enabling diapedesis through the vessel wall and tissue extracellular matrix (ECM) (Jutila, 1992; Xu and Hakansson, 2002; Liu and Pope, 2004). The released enzymes also help shut down expression of L-selectin via proteolytic cleavage of the molecule at a membrane proximal site (Hafezi-Moghadam and Ley, 1999). Together, these processes halt the rolling phenotype of neutrophils and promote the arrested phenotype with subsequent migration into tissue (Jutila, 1992; Soler-Rodriguez et al., 2000). Therefore, proper trafficking of neutrophils requires regulated expression of the cells' L-selectin, β_2-integrins (Crockett-Torabi et al., 1995), and gelatinase enzymes (Xu and Hakansson, 2002).

Once neutrophils cross blood vessel endothelia, their directed migration through the tissue ECM to the site of infection is facilitated by a process called chemotaxis (reviewed by Wu, 2005). Migrating neutrophils utilize a variety of cell surface complement and cytokine receptors to follow concentration gradients of these molecules released at the infection focus (Beutler, 2004). The continuous exposure of neutrophils to

pro-inflammatory stimuli such as IL-8, G-CSF, TNF-α, and LPS during migration helps prime the cells for extended survival, degranulation, and phagocytosis and killing of the invading pathogen (Mitchell et al., 2003). Thus, effective margination and migration result in neutrophils that are properly placed and activated for rapid pathogen clearance in infected tissue.

B. Neutrophil Phagocytosis and Pathogen Clearance

Once in the infection focus, migrated neutrophils begin to clear pathogens through the endocytic processing pathway beginning with phagocytosis (Liu and Pope, 2004). This receptor-mediated event utilizes a variety of cell surface receptors located within the plasma membrane (e.g. CD14, toll-like receptors 2 and 4, Fc receptors, and complement receptors including CD18) to bind various bacterial cell wall components (endotoxin/LPS) and (or) host immune proteins (antibody and complement) that have opsonized the pathogen for enhanced phagocytosis by neutrophils (reviewed by Witko-Sarsat et al., 2000; Beutler, 2004). Receptor ligation initiates formation of pseudopods that encircle the bound pathogen for internalization (i.e. phagocytosis) into the neutrophil. This newly formed vesicle is known as a phagosome. In addition, the receptors transduce signals to the cell interior that set off respiratory burst activity critical to the endocytic processing pathway.

Neutrophil binding to the pathogen for phagocytosis signals a massive respiratory burst that generates a variety of highly reactive oxygen species (ROS). ROS-mediated oxidative degradation of the pathogens begins once the phagosome is closed to the outside of the cell (Dahlgren and Karlsson, 1999; Segal, 2005). ROS are produced by

shuttling electrons across the phagosome membrane's NADPH oxidase system resulting in the reduction of oxygen to superoxide anions, which is sometimes further converted to hydrogen peroxide. Various cytoplasmic granules, referred to collectively as lysosomes, fuse with the incoming phagosome during degranulation and release enzymes and anti-bacterial peptides into the phagolysosome to assist ROS in causing lipid peroxidation and oxidative damage to proteins, RNA, and DNA of the phagocytosed pathogen (Crockett-Torabi et al., 1995; Ward and Lentsch, 1999; Segal, 2005). Contents of the various granules include myeloperoxidase and proteolytic enzymes (in azurophil granules); lactoferrin, gelatinases, and lysozyme (in specific granules) (Smith, 1994, Segal, 2005), and bactenecins that are specific to the large granules in bovine neutrophils and have potent non-oxidative microbicidal activity (Zanetti et al., 1990). Myeloperoxidase aids in the oxidative damaging of pathogens. The other agents listed heighten damage already initiated by ROS and participate in complete digestion of the pathogen. For example, proteolytic enzymes aid in the digestion of bacterial structural proteins while lactoferrin sequesters numerous minerals and thus deprives the invading pathogen of essential nutrients. Lysozyme destroys the bacterial envelope protein matrix and gelatinase helps degrade components of the tissue ECM to prevent colonization by the pathogen as well as allow additional leukocytes to enter the infected region (Smith, 1994, Segal, 2005). In all, the functions of phagocytosis, respiratory burst, degranulation, and generation of phagolysosomes are highly connected and necessary to mediate successful killing and clearance of invading pathogens by neutrophils.

C. Neutrophil Death by Apoptosis

Rapid apoptosis of neutrophils during the resolution of inflammation is equally important as the pro-inflammatory activities of these cells in innate immunity and effective clearance of invading pathogens. Apoptosis, or programmed cell death, is essential for eliminating cells that are in excess or potentially dangerous to the host (Kerr et al., 1972). It is characterized morphologically by membrane blebbing, cell shrinkage, and condensation of the cytosol and nucleus with ultimate breakdown of chromosomal DNA. Apoptosis is an energy-requiring program of genetically regulated cell death that leads to rapid clearance of dying cells by the body's phagocytic networks (macrophages and fibroblasts), without inducing inflammatory responses (Squier et al., 1995; Whyte et al., 1999). This is in stark contrast to necrotic cell death, which does not require energy, causes membrane rupture with release of cytoplasmic contents and inflammation, and leads to pathological consequences for nearby healthy cells in what is called a "bystander effect" (Mahidhara and Billiar, 2000).

Apoptosis arises from two main signaling pathways in most cells, one mediated through intrinsic signals elaborated by mitochondria and the other through physiological factors extrinsic to the cell (Dragovich et al., 1998). Neutrophils are no exception. Upon release from bone marrow, mature neutrophils are primed for initiation of apoptosis through both intrinsic and extrinsic pathways (Liles et al., 1996; Sendo et al., 1996; Santos-Beneit and Mollinedo, 2000). In fact, approximately 85% of blood neutrophils are at some stage of apoptosis while in the circulation (Shidham and Swami, 2000). The intrinsic pathway can be initiated without any external influence and is controlled primarily by apoptosis regulatory proteins of the Bcl-2 family and ROS produced by

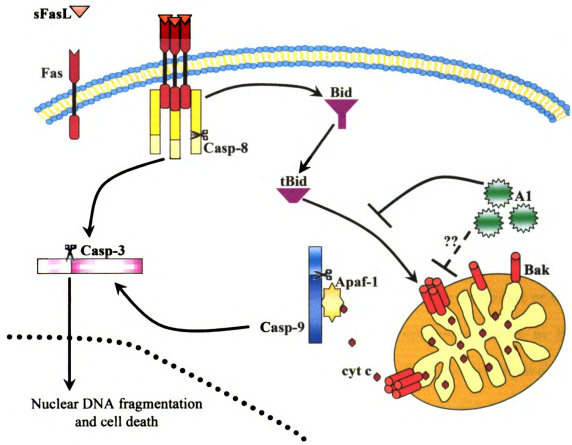
mitochondria during normal and accelerated cellular metabolism (Maianski et al., 2004a). This is in contrast to the extrinsic pathway, which requires extracellular signaling through membrane-bound death receptors such as Fas. Constant intrinsic and extrinsic signaling for apoptosis is critical to neutrophil homeostasis and ensures health of the organism by preventing serious oxidative and enzymatic damage to surrounding healthy tissue from activated and senescent cells (Homburg and Roos, 1996; Haslett, 1999).

i. Spontaneous neutrophil apoptosis – the intrinsic pathway to cell death

Spontaneous neutrophil apoptosis is initiated upon release of cytochrome c into the cytosol from the intermembrane space of mitochondria (**Figure 1.1**). In the cytosol, cytochrome c associates with apoptotic protease activating factor-1 (Apaf-1), ATP, and procaspase-9 (an initiator caspase) to form apoptosomes. This results in procaspase-9 cleavage and activation. Once activated, caspase-9 cleaves procaspase-3 (an executioner caspase). Activated caspase-3 affects downstream apoptotic events, including activation of a caspase-dependent deoxyribonuclease, that culminate in breakdown of the cell's cytoskeleton and nuclear demise (Nunez et al., 1998; Roy and Cardone, 2002). The initiation of this pathway is highly dependent on cytochrome c release into the cytosol. Whether or not cytochrome c is released from mitochondria is primarily under the control of a family of proteins referred to collectively as the Bcl-2 family.

In higher eukaryotes, the Bcl-2 family consists of approximately 30 homologous members that either induce or delay apoptosis (Borner, 2003). Not all homologs are present in every cell type, but neutrophils express both pro- and anti-apoptotic Bcl-2 family members. These proteins are grouped based on the presence of 4 homology domains termed BH1, BH2, BH3, and BH4. Pro-apoptotic family members typically

Figure 1.1 Neutrophils undergo apoptosis in response to intrinsic (mitochondrial-based pathway) as well as extrinsic (Fas) signals. Binding of Fas ligand (shown here in the soluble form, sFasL) leads to oligomerization of three membrane bound Fas molecules. The intracellular death domain of Fas recruits additional proteins resulting in the formation of a death inducing signaling complex (DISC) that includes procaspase-8. Activation of caspase-8 by cleavage of the pro-form in turn activates caspase-3, leading to fragmentation of genomic DNA and cell death. Inside the cell, the balance of pro-apoptotic (e.g. Bak) to anti-apoptotic Bcl-2 family proteins (e.g. A1) helps determine the rate of spontaneous apoptosis. Bak acts to disrupt the integrity of mitochondrial membranes, causing release of cytochrome c into the cytosol. Cytochrome c is a potent activator of caspase-9, which also leads to cell death by activating caspase-3. This pathway can be further amplified through caspase-8 induced activation of BH3-only proteins (e.g. Bid) that stimulate additional pore formation by Bak in the mitochondrial membrane. Potent anti-apoptotic proteins (e.g. A1) inhibit these pro-apoptotic activities by interacting with Bak, Bid, or both proteins. This results in stabilization of mitochondrial membranes and prevention of cytochrome c release into the cytoplasm.



possess BH1, BH2, and BH3 domains as well as a carboxy-terminal transmembrane domain that anchors the proteins in intracellular membranes (Reed, 1998; Borner, 2003). Two such proteins that are detectable in mature neutrophils are Bak (Bazzoni et al., 1999; Santos-Beneit and Mollinedo, 2000; Moulding et al., 2001) and Bax (Ohta et al., 1995; Moulding et al., 2001). Additional pro-apoptotic Bcl-2 family members found in neutrophils are the BH3-only proteins Bid, Bad, and Bik (Moulding et al., 2001). As per their name, this group of proteins is categorized by the presence of a singular BH domain, BH3. Mature neutrophils also express anti-apoptotic Bcl-2 family members. These include A1/Bfl-1 (referred to as A1 in the remainder of the text), Mcl-1 (Chuang et al., 1998; Moulding et al., 2001), Bcl-X_L (Weinmann et al., 1999), and Bcl-w (Santos-Beneit and Mollinedo, 2000), but not the prototypic family member Bcl-2 (Chuang et al., 1998; Santos-Beneit and Mollinedo, 2000; Moulding et al., 2001). Anti-apoptotic proteins contain all four BH domains but of those present in neutrophils only Bcl-X_L, Bcl-W, and Mcl-1 have a membrane-anchoring domain (Reed, 1998).

Utilizing a variety of *in vitro* culture systems consisting primarily of cell lines, it has been determined that Bcl-2 family members interact to form homo- and hetero-oligomers via their BH3 domains. In favor of apoptosis, BH1 and BH2 domains form a deep groove that accepts the BH3 domain of a neighboring protein to create Bak or Bax homo-oligomers (Ruffolo and Shore, 2003). This oligomerization results in the formation of pores in mitochondrial outer membranes through which cytochrome c is released (Degenhardt et al., 2002). Anti-apoptotic family members such as Mcl-1 and Bcl-X_L hetero-dimerize with Bak to limit pore formation (Willis et al., 2005). Reports of heterodimerization between A1 and Bak are inconsistent with one group demonstrating

interaction between these two family members with no subsequent inhibition of apoptosis (Holmgreen et al., 1999) while another group observed no significant binding between A1 and Bak (Willis et al., 2005). Instead, the link between A1 and Bak may lie in the BH3-only protein Bid. Truncated Bid (tBid), the protein's active pro-apoptotic form, interacts with Bak inducing its homo-oligomerization (Wang et al., 2001). However, tBid does not remain a permanent part of the pore formed by Bak in the mitochondrial membrane (Wei et al., 2000). To prevent this pore formation, A1 heterodimerizes with Bid in such a way that it can still be truncated but can no longer interact with Bak (Werner et al., 2002). By contrast, pro-apoptotic Bik and Bad bind anti-apoptotic Bcl-X_L inhibiting its hetero-dimerization with Bak, thereby allowing Bak-induced apoptosis to continue (Reed, 1998). Because of the ability of these proteins to form hetero- and homo-dimers with one another to inhibit or enhance their function, the relative abundance of anti-apoptotic to pro-apoptotic Bcl-2 family members within the cell is integral in determining apoptotic status (Dragovich et al., 1998).

Based on studies that have examined expression profiles of various Bcl-2 family members, mature neutrophils appear to be primed for rapid spontaneous apoptosis upon their release from bone marrow (Moulding et al., 2001). HL-60 cells (a human bone marrow cell line that can be induced to differentiate into myeloid-lineage cells, including neutrophils) express Bcl-2 during the proliferative phases of neutrophil development but this protein is inhibited during the differentiation process. Instead, A1 and Mcl-1 are increased in expression during neutrophil terminal differentiation (Chuang et al., 1998). Expression of Bak, Bax, and Bad are maintained or slightly increased during this same period (Santos-Beneit and Mollinedo, 2000). After several days of *in vitro* differentiation,

the expression pattern of these Bcl-2 family members is very similar between neutrophilic HL-60 cells and mature neutrophils that developed *in vivo* with the ratio of pro- to anti-apoptotic Bcl-2 family members in favor of apoptosis. This ratio may be shifted by increases in the potently anti-apoptotic A1 during an inflammatory response to delay apoptosis (see *iii. Delaying neutrophil apoptosis*). The critical role of A1 to regulation of neutrophil apoptosis was further demonstrated through the generation of A1 knockout mice. These animals exhibit accelerated neutrophil apoptosis (Hamaski et al., 1998) and a diminished acute inflammatory response (Orlofsky et al., 2002). In contrast, Bak knockouts do not exhibit any blatant neutrophil abnormalities but Bak/Bax double knockouts have multiple developmental defects and increased numbers of circulating neutrophils (Lindsten et al., 2000). Thus, A1, Bak, and Bax are major players in the regulation of spontaneous neutrophil apoptosis.

ii. Death-receptor induced apoptosis – extrinsic signals initiating cell death

Not only are mature neutrophils primed for spontaneous apoptosis through the mitochondrial pathway, they also express molecules that are critical to extrinsic pathways of cell death (Maianski et al., 2004a). Neutrophils receive these additional apoptosis signals primarily via the Fas/Fas ligand (FasL) system (**Figure 1.1**; Liles et al., 1996). Fas is a membrane-bound receptor while FasL is found in soluble and membrane bound forms, both of which are expressed by neutrophils (Santos-Beneit and Mollinedo, 2000). The extrinsic pathway of apoptosis is triggered upon binding of FasL to the extracellular portion of Fas (Dragovich et al., 1998). This results in Fas trimerization, bringing together in the cell's cytosol the highly conserved protein-protein binding death domains found in Fas' cytoplasmic tail (Nagata, 1999). This is followed by death domain

recruitment of a variety of adaptor proteins and ultimately procaspase-8. This newly formed death inducing signaling complex (DISC) induces apoptosis signaling beginning with cleavage of procaspase-8 (Algeciras-Schimnich et al., 2002). Similar to caspase-9 of the spontaneous apoptosis pathway, active caspase-8 magnifies and accelerates the death signal from Fas by cleaving procaspase-3. Activated caspase-3 affects downstream apoptotic events that cause rapid nuclear, cytoplasmic, and membrane collapse (Nunez et al., 1998; Roy and Cardone, 2002). Even though the Fas/FasL system on its own is highly effective in inducing neutrophil apoptosis, it also amplifies the spontaneous pathway of apoptosis through caspase-8 cleavage of Bid. Once truncated, tBid helps facilitate mitochondrial membrane destabilizing activities of Bak. Such activation of tBid occurs following FasL activation of Fas (Li et al., 1998) and in a ligand-independent manor initiated by ROS activation of Fas (Scheel-Toellner et al., 2004). Acting alone or together, the intrinsic and extrinsic pathways of apoptosis ensure that circulating neutrophils die rapidly if they are not recruited into sites of tissue infection or inflammation.

iii. Delaying neutrophil apoptosis

During homeostasis, it is important for neutrophils to undergo rapid apoptosis and be cleared by the body's phagocytic networks, thus preventing serious oxidative and enzymatic damage to healthy tissue from activated or senescent cells (Homburg and Roos, 1996; Haslett, 1999). However, it is also critical that neutrophil apoptosis be delayed during an innate immune response to infection. Pro-inflammatory factors that aid in neutrophil migration and priming of the cells for pathogen clearance, also delay neutrophil apoptosis. These factors include the cytokines G-CSF, GM-CSF, IL-8, TNF-α,

and IFN-γ, as well as a lipopolysaccharide (LPS, a major constituent of gram-negative bacteria) (reviewed by Simon, 2003; Maianski et al., 2004a). Several of these pro-inflammatory molecules regulate apoptosis by changing the relative proportions and (or) subcellular locations of expressed Bcl-2 family members in favor of neutrophil survival. For example, A1 mRNA abundance increased 1.5 to 4-fold in neutrophils following exposure to G-CSF, GM-CSF, TNF-α, and IFN-γ (Chuang et al., 1998; Moulding et al., 2001). LPS exposure increased blood neutrophil A1 expression at both mRNA and protein levels by 1.5 to 4-fold (Chuang et al, 1998; Moulding et al., 2001; Kotani et al., 2003). Lung neutrophils from LPS treated mice demonstrated 100-fold increases of A1 mRNA by gene array analysis (Kupfner et al., 2001). Protein abundance was increased 1.5 to 2-fold in these same cells, and was controlled by LPS activation of the NF-κB signaling pathway. The inducibility of A1 by multiple pro-inflammatory mediators in conjunction with its extremely short mRNA half-life (~3 hours; Moulding et al., 2001) makes expression of this Bcl-2 homologue a critical control point in the life-death decision of neutrophils.

Despite the importance of A1 expression in regulation of neutrophil life span, changes also have been observed in expression levels of pro-apoptotic family members during inflammatory scenarios that delay neutrophil apoptosis. For example, decreases in Bax protein levels occur following GM-CSF (Weinmann et al., 1999) and TNF-α (Moulding et al., 2001) treatment of human neutrophils. By contrast, Bak abundance remained relatively unchanged after exposure to GM-CSF, LPS (Moulding et al., 2001), TNF-α, and IFN-γ (Bazzoni et al., 1999). Interestingly, treatment of freshly isolated neutrophils with conditioned media from IL-8 treated neutrophils decreases Bak

(Grutkoski et al., 2002). The addition of neutralizing antibodies against IL-8, G-CSF, GM-CSF, and TNF-α did not block the observed decrease in Bak expression in freshly isolated neutrophils treated with the conditioned medium. Thus, the factors responsible for this effect have yet to be elucidated (Grutkoski et al., 2002). In yet another experimental scenario, it was not protein abundance changes but subcellular location of a Bcl-2 family protein that impacted neutrophil apoptotic status. Maianski and colleagues (2002) demonstrated that Bax protein levels did not change during delayed neutrophil apoptosis mediated by G-CSF. Instead, Bax translocation from the cytosol to mitochondrial membranes of neutrophils was drastically reduced. Bak and Bax proteins are extremely stable (half-life greater than 22 hours; Moulding et al., 2001). Accordingly, changes in protein abundance for those family members are often difficult to detect. However, rapid relocation of Bak and (or) Bax within the cell seems an efficient way to quickly alter rate of spontaneous apoptosis such as in the case of G-CSF stimulation.

Other factors not traditionally related to pro-inflammatory activities of cytokines also have been shown to delay neutrophil spontaneous apoptosis. For example, insulin-like growth factor 1 (IGF-1) delays apoptosis without increasing secretion of cytokines such as IL-8 and TNF-α that could contribute to its effect (Kooijman et al., 2002). The mechanism of IGF-1 delayed neutrophil apoptosis has yet to be described. Also, despite their well-known anti-inflammatory properties, glucocorticoids are potently anti-apoptotic for neutrophils (Cox, 1995; Liles et al., 1995; Meagher et al., 1996; Nittoh et al., 1998; Chang et al., 2004; Burton et al., 2005). The glucocorticoid-induced delay in neutrophil apoptosis requires new gene expression and protein synthesis (Cox and Austin, 1997). In one study, glucocorticoid directly and profoundly inhibited Fas mRNA

abundance and, with it, caspase-8 activation and cell death following sFasL stimulation (Chang et al., 2004). However, specific molecular information about how glucocorticoids mediate a delay in spontaneous apoptosis of neutrophils is currently unavailable.

Under non-inflammatory and non-stress situations, neutrophils rapidly lose their ability to perform the functions of trafficking, phagocytosis, and pathogen killing as they die by apoptosis. For example, as neutrophils age over 12 to 20 hours in the circulation during homeostasis, surface levels of L-selectin and margination capacity of the cells are decreased. This occurs in association with an increased susceptibility to apoptosis (Matsuba et al., 1997), and increasing deficiencies in the cell's ability to perform chemotaxis, phagocytosis, and degranulation (Whyte et al., 1993; Narayanan et al., 1997; Tanji-Matsuba et al., 1998). Thus, apoptotic neutrophils are poor defenders against invading pathogens and must be cleared by the body's phagocytic network to prevent their death by secondary necrosis, which could lead to systemic inflammation and death. However, neutrophils that have had their cell death program delayed by pro-inflammatory mediators during an innate immune response must also undergo timely apoptosis in the affected tissue. This has been shown to be a key factor in the resolution of inflammation and is likely due to the removal of apoptosis delaying factors (LPS, bacteria, cytokines, and chemokines) as the neutrophils successfully clear infecting pathogens (Sendo et al., 1996; Haslett, 1999). Whether the extracellular milieux signals for a delay or re-instatement of neutrophil apoptosis, these leukocytes respond to their environment with remarkable plasticity of their apoptotic programs by temporarily altering their genetic program of cell death.

III. PARTURITION: A CHANGING BLOOD ENVIRONMENT THAT ALTERS NEUTROPHIL PHENOTYPE

Parturition is a complex event that includes numerous physiological changes and must occur approximately every thirteen months for dairy cows to continue to produce milk at levels that benefit producers. Transition from the pregnant to non-pregnant state with a concurrent transition from non-lactating to a lactating state in high producing cows could come at a price. This may include significantly reduced dry matter intake, negative energy balance, loss of body weight, increased disease susceptibility, and associated pronounced fluctuations in blood concentrations of metabolic and endocrine factors, as well as pro-inflammatory mediators (Sordillo et al., 1995; Goff and Horst, 1997; Ingvartsen and Andersen, 2000). For example, plasma levels of leptin (Nikolic et al., 2003), insulin, and glucose (Holtenius et al., 2004) are all decreased in the immediate postpartum period. Greater differences between prepartum and postpartum levels of these metabolites associate with higher incidences of postpartum mastitis, which is an infection-induced inflammation of the mammary gland (Holtenius et al., 2004). Plasma calcium concentration decreases due to initiation of lactation also occur at parturition. If calcium homeostasis is not restored, milk fever (or severe periparturient hypocalcemia) can arise and sometimes result in death of the animal (Horst et al., 2005). Following parturition, plasma IGF-1 levels are decreased (Meikle et al., 2004) and this has been anecdotally associated with an apparent increase in apoptosis of blood neutrophils approximately one week postpartum (Van Oostveldt et al., 2001). By contrast, nonesterified fatty acids are increased during the periparturient period (Nikolic et al., 2003; Holtenius et al., 2004). This is particularly evident in over-conditioned animals

that have more depressed immune functions in the postpartum period relative to normal-conditioned cows (Rukkwamsuk et al., 1999). In humans, increases in prostaglandins act in conjunction with cytokines, such as IL-8, to recruit neutrophils to the cervix to aid in cervical ripening needed for parturition (Kelly, 1996). Increases in these and other pro-inflammatory cytokines have led to the hypothesis that human parturition actually induces a maternal inflammatory response (Molloy et al., 2004). Bovine parturition may also lead to a heightened pro-inflammatory condition. TNF-α, a potent pro-inflammatory cytokine that is critical to immune activation, but may also contribute to acute pathology, was increased during bovine parturition. This change was detected in mononuclear cells isolated from both blood and mammary lymph nodes during parturition (Sordillo et al., 1995), as well as in periparturient cow blood serum from 7 days prepartum through postpartum day 1 (Burton and Weber, unpublished observations). Thus, a heightened pro-inflammatory condition may contribute to the increased severity of mastitis and other inflammatory conditions noted immediately postpartum in cattle (Shuster et al., 1996; Goff and Horst, 1997; Burvenich et al., 2003).

Steroid hormones, including cortisol, estradiol, and progesterone, also fluctuate dramatically at parturition in dairy cows. Serum progesterone levels drop precipitously while cortisol and estradiol both peak at parturition, with all three hormones returning to low concentrations by approximately the second day postpartum (Smith et al., 1973; Weber et al., 2001). At term pregnancy, these highly coordinated fluctuations in blood steroids are initiated when the growing fetus responds to the increasingly small uterine environment with a classical stress response beginning with activation of its hypothalamic-pituitary-adrenal axis and ending with high levels of cortisol in the

maternal blood stream. This fetal cortisol response is instrumental in mediating placental changes that lead to conversion of progesterone into estradiol, and ultimately parturition. However, it may also affect neutrophils. Thus, the fetus appears to orchestrate changes in the cow's immune system and reproductive tract, as well as in the placenta, which enables inflammation and parturition (Senger, 1997; Smith et al., 2002). Paradoxically, the fluctuations in circulating steroids also have been associated temporally with compromised immune status and health of the cow and her neonatal calf (reviewed by Burton et al., 2005; Vangroenweghe et al., 2005). Key unanswered questions in the biology of bovine parturition include (1) what are the precise effects of parturition on the cow's immune system and (2) how do the effects of parturition influence cow health. The current dissertation work was designed to begin to address the first question.

A. Parturition Alters Neutrophil Bactericidal Functions in Temporal Correlation with Increased Susceptibility to Severe Inflammatory Disease

Parturient dairy cows are well known for their heightened susceptibility to severe clinical mastitis immediately postpartum (Frost and Brooker, 1986; Shuster et al., 1996; Erskine et al., 2002). Clinical mastitis results in altered mammary function, decreased milk production, and altered milk composition that costs the dairy industry over $2 billion annually (DeGraves and Fetrow, 1993). Herd estimates for mastitis range from $35 to $239 per cow per milking year, even in well managed herds (Bartlett et al., 1991; Hoblet et al., 1991). The etiology of severe clinical mastitis in the immediate postpartum period stems from intramammary infection by opportunistic environmental Gram-negative coliforms and Gram-positive streptococci (Hogan et al., 1989). These pathogens

take advantage of the fact that mammary glands are newly engorged with milk and the coincident immune system malfunctions that occur in response to parturition (Burton and Erskine, 2003; Vangroenweghe et al., 2005).

In studying the immunocompetence of periparturient dairy cows as a means to understand the heightened occurrence of severe clinical mastitis, many researchers have focused their investigations on neutrophils. These leukocytes are essential effector cells in innate immune defense against mastitis-causing bacteria and undergo functional changes from approximately 3 weeks prepartum to 3 weeks postpartum (reviewed by Mallard et al., 1998; Vangroenweghe et al., 2005). Of particular note to immune defense against mastitis, blood neutrophils of parturient cows demonstrate decreased respiratory burst activity (measured *in vitro*) and this phenotype statistically associates with increased occurrence of clinical mastitis (Kehrli et al., 1989; Mehrzad et al., 2001). Neutrophil recruitment into the mammary gland is delayed immediately postpartum in correlation with appearance of severe coliform mastitis (Hill et al., 1979; Shuster et al., 1996; Kehrli and Harp, 2001). Thus, correlative evidence supports a hypothesis that increased occurrence of severe coliform mastitis early postpartum is a function of delayed neutrophil migration into infected mammary quarters and decreased ability to kill pathogens once they do arrive in the tissue.

Some researchers have focused on particular aspects of neutrophil biology during the parturient period to try and discern the contribution of these leukocytes to clinical mastitis susceptibility early postpartum. For example, Guidry et al. (1976) found increases in circulating immature (band) neutrophils in blood of parturient cows, suggesting that neutrophils with undeveloped bactericidal functions are released

prematurely from the bone marrow under the influence of parturient blood factors. Their hypothesis was that band neutrophilia occurs in response to the high levels of blood cortisol (i.e. glucocorticoid) at parturition. This theory has been partly substantiated by experiments where glucocorticoids administered to cattle promoted gene expression changes in bone marrow cells indicative of neutrophil development and increased release of band and segmented neutrophils from this storage pool of the cells (Weber et al., 2004; Burton et al., 2005). Glucocorticoid administration to humans also results in band neutrophilia (Hetherington and Quie, 1985; Steele et al., 1987) and supports the first part of Guidry and colleagues' theory. Glucocorticoid effects on band neutrophil bactericidal functions are equivalent or even superior when compared to mature segmented neutrophils (Hetherington and Quie, 1985; Steele et al., 1987). *In vivo* glucocorticoid challenge increased neutrophil phagocytosis and killing of several pathogens including *E. coli, S. aureus, S. pneumoniae,* and *H. influenzae* (Steele et al., 1987) as well increased myleperoxidase release following *in vitro* PMA stimulation (Hetherington and Quie, 1985). Thus, it is difficult to accept Guidry's hypothesis about under-developed bactericidal functions in band neutrophils released prematurely from the bone marrow during glucocorticoid challenge.

Other groups have focused on neutrophil migration capacity during the periparturient period. The migration capacity of blood neutrophils, measured *in vitro* as random movement of the cells under agarose, was significantly reduced at parturition compared to prepartum (Nagahata et al., 1988; Kehrli et al., 1989; Detilleux et al., 1994). Reduced migration may contribute to the pronounced increase in circulating numbers of mature neutrophils that are consistently documented at parturition (Preisler et al., 2000;

Weber et al., 2001; Madsen et al., 2004), which results in part from significantly reduced L-selectin and β2-integrin adhesion molecule expression on the surface of blood neutrophils in response to the surge in cortisol (Burton et al., 1995; Lee and Kehrli, 1998; Kimura et al., 1999; Weber et al., 2001, 2004). These studies indicate that blood neutrophils, and possibly bone marrow neutrophils, respond to parturient physiology by altering the expression of their surface adhesion molecules. The migratory action of chemotaxis, detected as directed movement of neutrophils under agarose, was also significantly reduced when neutrophils were collected during the first week postpartum versus several weeks prepartum (Nagahata et al., 1988). In another study, decreases in chemotaxis of parturient cow blood neutrophils were associated with occurrences of clinical mastitis, retained placenta, and metritis (Cai et al., 1994). Both of these studies utilized serum-free media to limit *in vivo* versus *in vitro* environmental differences. Thus, alterations in neutrophil trafficking patterns in favor of reduced migration into infected mammary glands may contribute to increased risk of severe clinical mastitis early postpartum.

Functions of neutrophils known to be critical in bacterial uptake and killing by the cells have been examined *in vitro* and are markedly affected by bovine parturition. Guidry et al. (1976) showed that the total phagocytic capacity of neutrophils was increased during the period between 12 days prepartum to 2 days after parturition. This was due in part to the increased numbers of circulating neutrophils found at this time as well as increased average phagocytic capacity of each cell. However, from postpartum days 2 to 10 an overall decrease in neutrophil phagocytic ability was detected. Other researchers have demonstrated that while overall bacterial ingestion was increased around

parturition, other aspects of phagocytic activity such as number of bacteria phagocytosed and killed were decreased in parturient cows (Kehrli and Goff, 1989; Kehrli et al., 1989; Cai et al., 1994). Superoxide anion production, which is critical for pathogen damage following the respiratory burst in phagocytosing neutrophils, was significantly reduced during parturition (Kehrli and Goff, 1989; Detilleux et al., 1994). This was particularly evident in neutrophils from cows exhibiting clinical mastitis, metritis, and retained placenta (Cai et al., 1994). Myeloperoxidase activity, another important factor in pathogen damage within the phagolysosome, was also deficient in neutrophils from periparturient cows (Kehrli and Goff, 1989; Kehrli et al., 1989; Cai et al., 1994; Detilleux et al., 1994; Kimura et al., 1999), as was overall oxidative capacity of these phagocytes (Kehrli and Goff, 1989; Kehrli et al., 1989; Mehrzad et al., 2001). In summary, evidence supports alterations in neutrophil trafficking and killing functions induced by parturition. Not only does this show that neutrophils are highly sensitive to the physiology of parturition, results of past research may help explain the increased susceptibility to clinical mastitis in early postpartum dairy cows.

B. Changes in Serum Steroids Correlate with Altered Neutrophil Functions in Parturient Dairy Cows

Steroid hormone fluctuations of parturition have been hypothesized to instigate the immune dysfunctions and disease susceptibility of parturient dairy cows. For example, elevated blood glucocorticoid concentrations, such as the cortisol spike of parturition or following treatment of animals with dexamethasone, induce release of bone marrow neutrophils (Guidry et al., 1976; Hetherington and Quie, 1985) and cause down-

regulation of L-selectin expression on the surface of blood neutrophils (Burton et al., 1995; Lee and Kehrli, 1998; Nakagawa et al., 1999; Weber et al., 2001, 2004) and pronounced neutrophilia (Preisler et al., 2000; Weber et al., 2001). These studies, combined with observations from other investigators that glucocorticoids reduce other pro-inflammatory activities of neutrophils (Guidry et al., 1976; Detilleux et al., 1994), support arguments that the cortisol spike at parturition influences mastitis susceptibility in parturient dairy cows (Burton and Erskine, 2003).

Progesterone and estradiol effects on additional neutrophil functions have been evaluated using serum-free culture systems. While progesterone enhanced chemotaxis and migration of human neutrophils, these functions were reduced by estradiol following treatment *in vitro* (Miyagi et al., 1992). Neutrophils from progesterone treated steers also exhibited enhanced migration of neutrophils under agarose (Roth et al., 1982). A study of cow neutrophils collected during various stages of the estrous cycle demonstrated increased migration, measured *ex vivo*, when *in vivo* serum concentrations of progesterone or estradiol were high (Roth et al., 1983). Neutrophil myeloperoxidase activity, measured in response to *in vitro* phagocytosis, also was found to be decreased when serum progesterone concentrations were high (Roth et al., 1982 and 1983). By contrast, *in vitro* treatment with estradiol or progesterone did not alter bovine neutrophil oxidative burst activity (Winters et al., 2003). Thus, the fluctuations in steroid hormones may contribute to some, but not all of the altered phenotypes observed during the periparturient period.

C. Steroid Hormones Act via Intracellular Receptors

Clearly, bovine neutrophil sensitivity to the changing steroid hormone levels of parturition has begun to be documented. The most common mechanism of steroid action is via binding to homologous steroid receptors in or on cells (Beato et al., 1995). Human neutrophils possess estrogen receptors (Ito et al., 1995; Stefano et al., 2000) while progesterone receptors have been detected in mouse neutrophils (Tibbetts et al., 1999). Bovine neutrophils also contain estrogen receptors (Lamote et al., 2006), but have yet to demonstrate progesterone receptor expression (Winters et al., 2003). Glucocorticoid receptors are readily detected in bovine neutrophils and both hormone-binding capacity and mRNA abundance are highest immediately before the spike in serum cortisol at parturition (Preisler et al., 2000; Weber et al., 2001). In addition, decreases in L-selectin and Fas expression in neutrophils at parturition are glucocorticoid receptor mediated and occur at the level of gene transcription (Weber et al, 2004; Burton et al., manuscript in preparation). Transcriptional regulation is a common result of steroid receptor activation and may occur through several mechanisms. Because of known glucocorticoid-mediated transcriptional effects in bovine neutrophils, this system will be described in detail as an example of steroid receptor activation.

Unligated glucocorticoid receptors are located in the cytoplasm of most cells, including neutrophils (Chang et al., 2004). Being lipophilic, glucocorticoids easily cross the plasma membrane of target cells without the need for cell surface receptors. Once in the cytoplasm, the hormone binds to the glucocorticoid receptor's ligand-binding domain, which leads to receptor activation. Glucocorticoid binding causes a conformational change in the receptor that enables release of its associated heat shock proteins that

normally keep these receptors in the cell cytoplasm by blocking nuclear localization signal sequences (Bamberger et al., 1996). Hormone binding-induced conformational changes expose receptor dimerization sites and nuclear localization signals in the activated receptor molecules (Tsai and O'Malley, 1994). As a result, hormone-activated glucocorticoid receptors form homodimers and translocate into the nucleus to regulate gene transcription. Glucocorticoid receptors so activated can modulate transcription by binding hormone response elements (GRE) in target gene regulatory DNA through their DNA binding domain. This domain consists of two zinc-fingers that interact with the major groove of the DNA double helix (Tsai and O'Malley, 1994). Glucocorticoid receptors also interact with other nuclear factors to remodel nucleosomes and gain access to GREs (Hayashi et al., 2004). Examples include the nucleosome-remodeling proteins SWI/SNF and Ada-Gcn5, which alter histone-DNA interactions using energy from ATP hydrolysis allowing the glucocorticoid receptors' DNA binding domain to interact with GRE (McEwan, 2000). In these ways, hormone-activated glucocorticoid receptors become appropriately positioned on the genome to facilitate recruitment of basal transcriptional machinery and other proteins to target gene promoters that enable the modulation of target gene expression (Tsai and O'Malley, 1994).

GREs are most often located upstream of the transcription start site in glucocorticoid responsive genes. Gene transcription can be modulated at GRE through several mechanisms (reviewed by Jenkins et al., 2001; Smoak and Cidlowski, 2004), the simplest being glucocorticoid receptor binding to GRE and recruitment to the TATA box of the basal transcription machinery (Reichardt et al., 2000). This usually results in transactivation (up-regulation) of the target glucocorticoid responsive gene. More

complicated mechanisms of activation or repression can occur through glucocorticoid receptor binding of composite elements in target gene promoters, which normally bind other transcription factors in the absence of glucocorticoid receptors. For example, binding of a GRE/Ap-1 composite element can both activate and repress proliferin gene expression (Diamond et al., 1990; Miner and Yamamoto, 1992) where as GRE/NF-κB composite element binding results in inhibition of TNF-α, IL-1, and ICAM-1 gene expression (Scheinman et al., 1995; Liden et al., 2000). Additional repression can occur through binding of glucocorticoid receptors to transcription factors already bound to DNA (Wissink et al., 1997; Hayashi et al., 2004; Smoak and Cidlowski, 2004). This blocks the basal transcriptional machinery from binding the positive transcriptional activators. In addition, activated glucocorticoid receptors can bind negative GREs that are in close proximity to other response elements, inhibiting the binding of their cognate transcription factors (Drouin et al., 1989; Dostert and Heinzel, 2004). Negative GREs also may be located within the introns of genes and, as a result, act to disrupt transcription by interfering with the activity/movement of RNA polymerase. Thus, when glucocorticoids bind their receptors in the cell's cytoplasm, they have the ability to modulate transcription either alone or in conjunction with other co-activators in order to coordinate the cell's response to glucocorticoid stimulation.

IV. NEUTROPHILS RESPOND TO THEIR ENVIRONMENT WITH ALTERED mRNA ABUNDANCE

Despite being terminally differentiated cells with relatively low biosynthetic capacity, mature blood neutrophils express at least 750 mRNAs during their short life

span under normal conditions (Itoh et al., 1998). These genes encode for a variety of DNA-binding proteins, cytokines, MHC proteins and receptors, and cell surface membrane proteins. During an innate immune response, however, expression profiles for a large number of genes are altered in blood neutrophils by cytokine stimulation (Cowling and Birnboim, 2000) and exposure to various pathogenic and non-pathogenic bacteria (Newberger et al., 2000). A study with dexamethasone, a synthetic glucocorticoid, has demonstrated that neutrophil mRNA abundance of IL-1 receptor types I and II is increased following hormone administration into dairy cattle (Yu et al., 1997). Bovine neutrophils are also responsive to the parturient blood environment, and limited evidence indicates that fluctuations in steroid hormones may play key roles (Guidry et al., 1976; Nakagawa et al., 1999). For example, our laboratory has shown that parturient decreases in neutrophil L-selectin expression most likely result from glucocorticoid receptor mediated repression of L-selectin gene expression during the surge in serum cortisol (Weber et al., 2001, 2004). Using a broader technique to detect differential gene expression, called DDRT-PCR, we also showed that bovine parturition had pronounced effects on mRNA abundance of 14 neutrophil genes (Madsen, 2001), two of which are key in the regulation of energy metabolism in mitochondria and translation of mRNA into protein in the cytosol (Madsen et al., 2002). Changes in mRNA abundance for these neutrophil genes were statistically correlated with changing progesterone and estradiol concentrations in the serum of the cows (Madsen et al., 2002). In addition, expression of the Fas death receptor was decreased in neutrophils from parturient cows (Chang et al., 2004), an observation that the authors linked to the spike in cortisol by showing that neutrophil Fas expression is regulated transcriptionally via

glucocorticoid receptor activation (Burton et al., manuscript in preparation). Thus, it is feasible that glucocorticoids (such as cortisol) and the other steroids of bovine parturition have significant effects on expression of multiple genes in blood neutrophils. If true, deciphering gene expression profiles in neutrophils of parturient cows could help elucidate pathways to severe mastitis susceptibility in the early postpartum period and, with them, clues for the prevention or treatment of this costly disease. Accordingly, the overall hypothesis of the current dissertation research was that circulating neutrophils respond to parturition with coordinated changes in their gene expression and phenotypic status which results from the cell's sensitivity to the fluctuating concentrations of steroid hormones at parturition. The following three objectives were put forth to test this hypothesis:

Objective 1: Identify abundance changes in neutrophil expressed genes during the peripartum period;

Objective 2: Determine the roles, if any, of the major parturient steroids (glucocorticoid, 17β-estradiol, and progesterone) and their cognate receptors in altered apoptotic status of neutrophils (inferred from results of Objective 1);

Objective 3: Evaluate the direct effects of glucocorticoid on abundance of neutrophil apoptosis regulating genes and phenotypic status (inferred through the work of Objectives 1 and 2).

Results of this dissertation research, presented in Chapters Two, Three, and Four, are novel because they begin to elucidate the molecular basis for altered neutrophil physiology during bovine parturition. Despite numerous documented bactericidal dysfunctions of neutrophils from parturient cows, which are typical of apoptotic cells (Whyte et al., 1993; Narayanan et al., 1997; Matsuba et al., 1997; Tanji-Matsuba et al., 1998), results of the current research identifies alterations in the genetic program resulting in delayed neutrophil apoptosis during bovine parturition, and begins to address the role of glucocorticoids in regulating the neutrophil apoptosis program. Importantly, delayed neutrophil apoptosis is a central concept in the maintenance of inflammation and is correlated with clinical outcome in many infection-based inflammatory diseases (Haslett, 1999). While it may augment the number of neutrophils available to the mammary gland to protect against invading pathogens, it may also contribute to the severity of mastitis if not properly regulated (Boutet et al., 2004). Thus, increased understanding of how parturient glucocorticoids regulate neutrophil apoptosis may shed critical new light on a key issue of animal health and well being that continues to plague the dairy industry, namely susceptibility to exaggerated mammary inflammation and severe clinical mastitis in early postpartum cows.

CHAPTER TWO

Madsen SA, Chang LC, Hickey MC, Rosa GJM, Coussens PM, Burton, JL. 2004. Microarray analysis of gene expression in blood neutrophils of parturient cows. Physiol Genomics 16(2):212-21.

Images in this dissertation are presented in color.

See **Appendix I** for updated BLASTn results of clones categorized as "Unknown" at the time of publication.

See **Appendix II** for Permission to Reprint

CHAPTER TWO

Microarray Analysis of Gene Expression in Blood Neutrophils of Parturient Cows

I. ABSTRACT

It is well documented that blood neutrophils from parturient dairy cows do not perform as well as neutrophils from non-parturient cows in laboratory assays of adhesion, migration, or phagocytosis-induced respiratory burst. However, little is known about the possible molecular basis for parturition-induced changes in neutrophils. cDNA microarray analysis was used in the current study to explore parturition-induced changes in gene expression profiles in bovine blood neutrophils. Total RNA from isolated blood neutrophils of 4 parturient Holstein cows was obtained before, during and after parturition, reverse transcribed into cDNA, and sequentially labeled with Cy3 or Cy5 dyes prior to paired hybridizations to 1056 member bovine total leukocyte (BOTL-3) microarrays in a loop design. Resulting gene expression data were LOESS normalized by array and analyzed using a mixed model approach. Results showed that expression profiles for 302 BOTL-3 genes were influenced by parturition. BLASTn analysis and preliminary clustering of affected genes by biological function indicated that the largest proportion (14%) of changed genes encode proteins critical to regulation of apoptosis. Independent confirmation of altered expression for 16 of these genes was achieved using Q-RT-PCR. A predominantly survival phenotype inferred from the microarray and Q-RT-PCR results was substantiated by monitoring apoptosis status of blood neutrophils from castrated male cattle cultured in the presence of sera from parturient cows. Thus, our combined gene expression and apoptosis phenotyping results suggest that bovine parturition may induce prolonged survival in normally short-lived blood neutrophils.

II. INTRODUCTION

Mammals undergo varying degrees of immunosuppression and disease susceptibility during late pregnancy and parturition (Krause et al., 1987; Kehrli and Harp, 2001; Osterlundh et al., 2001; Crouch et al., 1995). Leukocytes from dairy cows provide an excellent model for studying parturient immune suppression as these cells exhibit impaired inflammatory responses that associate with leukocytosis and increased susceptibility of the animals to opportunistic bacteria, such as Gram-negative coliforms that cause mastitis in infected mammary glands (Hill, 1981; Nagahata et al., 1988; Kehrli and Harp, 2001). Negative effects of parturition are readily detected in bovine blood neutrophils (Cai et al., 1994), which normally provide the main immunological defense against mastitis-causing bacteria (reviewed in Burton and Erskine, 2003). Specifically, neutrophil adhesion, migration, and phagocytosis-induced respiratory burst activities become depressed in some parturient cows to such an extent that intramammary bacteria get the upper hand in the host-pathogen battle (reviewed by Kehrli and Harp, 2001; Burton and Erskine, 2003). Recent studies have begun to elucidate potential molecular bases for certain parturition-induced neutrophil dysfunctions, showing that transcripts encoding key adhesion molecules, mitochondrial proteins, and ribosomal proteins are significantly decreased in neutrophils following the surge in blood steroids at parturition (Weber et al., 2001; Madsen et al., 2002). In the future, a broader examination of gene expression and corresponding phenotypic changes in neutrophils of periparturient cows may help researchers better understand and circumvent innate immune dysfunction and disease in female mammals.

Recently, we have developed bovine specific cDNA microarrays for studies on bovine immunobiology (Burton et al., 2001; Yao et al., 2001; Coussens and Nobis, 2002). Our preliminary microarray experiment used populations of bovine total leukocytes collected before and just after parturition to extend our previous findings (Weber et al., 2001; Madsen et al., 2002) that genes involved in leukocyte trafficking, phagocytic killing, maintenance of memory lymphocytes, energy metabolism, transcription, and translation are influenced by parturition (Burton et al., 2001). However, alterations in the proportions of circulating leukocyte sub-populations in blood samples used for that study precluded our ability to determine if the observed gene expression changes were real or simply due to changing leukocyte populations that contributed mRNA for the microarray analysis. Therefore, in the current study, we elected to use isolated populations of blood neutrophils for gene expression profiling during the periparturient period using our third generation bovine total leukocyte (BOTL-3) cDNA microarray. We were particularly interested in understanding gene expression changes in neutrophils that might explain the pronounced increase in circulating neutrophil counts that occur for approximately 2 days around parturition (Priesler et al., 2000; Weber et al., 2001). Our novel results suggest that patterns of expression for Bcl-2 family genes and Fas-related genes were changed by parturition in such a way as to suggest delayed apoptosis in the cells. Enhanced neutrophil survival was confirmed by follow-up phenotyping experiments and could partly explain parturition-induced neutrophilia.

III. MATERIALS AND METHODS

A. Animals and Sample Collection

Blood neutrophils utilized for all gene expression experiments of this study were obtained from periparturient Holstein cows (5 primiparous and 2 multiparous). Additional neutrophils were collected for phenotyping experiments from 3 young castrated male Holsteins (weighing between 225 and 325 kg). All animals were fed and housed according to standard operating procedures at the Dairy Teaching and Research facility and their use for the described experiments was approved by the All University Committee for Animal Use and Care, both of Michigan State University. Blood samples (30 ml) for neutrophil purifications intended for RNA isolations were collected by tail venipuncture into commercial ACD (acid citrate dextrose)-containing evacuated tubes (Vacutainer brand; BD Biosciences; San Jose, CA) from periparturient cows before (day –7), at (day 0), and after (days 0.25 and 1) parturition. An additional tube of blood (no anticoagulant) was taken from cows at each sampling for serum harvesting. Blood samples (60 ml) for neutrophil purifications intended for phenotyping assays were collected from male cattle through indwelling jugular cannulas into ACD-containing tubes. All blood samples were placed on ice as soon as they were collected and transported to the laboratory (~ 7 min drive) for immediate processing.

B. Sample Preparation

Upon arrival at the laboratory, 200 ul aliquots of whole blood from each ACD-anti-coagulated sample were reserved for monitoring total leukocyte counts (# cells/ml) by electronic counting (Beckman/Coulter Z1 Coulter Particle Counter and Zap-oglobin

Lytic Reagent; Beckman/Coulter; Miami, FL) and neutrophil differential counts [% $G1^+$ cells determined by immunostaining (clone MM20A from VMRD, Pullman, WA) and fluorescence activated flow cytometry (FACSCalibur with Cell Quest software; Becton Dickinson; San Jose, CA)], as in (Weber et al., 2001). Numbers of circulating neutrophils (# cells/ml whole blood) were then calculated as total leukocyte counts multiplied by % $G1^+$ cells. Remaining blood was used for neutrophil isolations ($\geq$ 93% purity) using Percoll density gradients (1.084 g/ml; Amersham Biosciences, Piscataway, NJ) followed by hypotonic lysis of red blood cells, as in (Weber et al., 2001). For mRNA abundance profiling, neutrophils were immediately lysed in TRIzol Reagent (Invitrogen, Carlsbad, CA) for 10 min at room temperature and stored in the same reagent at -80°C until use. For apoptosis phenotyping, the cells were suspended at a concentration of $1x10^7$/ml in culture media and treated as described below. Total time from blood collection to lysis in TRIzol or suspension for culture was < 3 h.

Total RNA was isolated from stored neutrophils according to the TRIzol manufacturer's instructions and concentration and purity determined using a DU-650 spectrophotometer (Beckman, Schaumburg, IL) and the 260 and 280 nm readings. RNA quality was checked in several randomly selected samples by 28S and 18S rRNA band visualization following gel electrophoresis and ethidium bromide staining (Weber et al., 2001).

Additional tubes of blood collected for serum harvesting were allowed to clot overnight (approximately 18 h) at 4°C. Samples were then centrifuged at 1000 x g for 30 min at 4°C. Following centrifugation, serum was transferred to microcentrifuge tubes in 1 ml aliquots and stored at -20°C until use in cell culture (see below).

C. cDNA Microarray Experiment

Neutrophil RNA from 4 of the primiparous Holstein cows was used for the microarray experiment. The general cDNA spotting design (including all control genes) used on the BOTL-3 microarrays is described in detail elsewhere [Coussens et al., 2002; National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO; http://www.ncbi.nlm.nih.gov/geo/) platform accession number GPL363], and included 3 spots for each of 1056 leukocyte genes present on the arrays. Expressed sequence tags (ESTs) of these spotted genes were obtained from three sources: 710 from our group's BOTL cDNA library (Yao et al., 2001), fourteen from previous differential display RT-PCR experiments (Madsen et al., 2002); and 332 from PCR-generated amplicons of targeted genes with known functions in immunobiology (Coussens and Nobis, 2002). These BOTL ESTs can be viewed on our website at http://nbfgc.msu.edu. Control genes spotted within and across the 48-patch microarrays (9 x 9 series of spots per patch) included 96 spots of synthetic Lambda Q cDNA (external control), 144 spots of GAPDH, 75 spots of β-actin, and 75 spots of RPL-19 (internal controls).

To preliminarily screen for parturition-induced neutrophil gene expression changes, RNA was pooled by day relative to parturition across the 4 test cows such that 20 μg per sample was available for cDNA synthesis and two dye coupling reactions. Accordingly, there was no biological replication (n = 1) in this screening assay. Assay replication per test gene was n = 6 (3 spots per gene by 2 dyes). The RNA pools were separated into two aliquots of 10 μg each for reverse transcription and dye labeling using a loop design (Kerr and Churchill, 2001). The experiment included 4 BOTL arrays with each sample labeled with Cy3 (green) and Cy5 (red) fluorescent dyes (Amersham

Pharmacia Biotech; Piscataway, NJ) and run in paired hybridizations on the arrays. Array #1 included a comparison between the day –7 and 0.25 samples, array #2 between the day 0.25 and 0 samples, array #3 between the day 0 and 1 samples, and array #4 between the day 1 and –7 samples. We employed this pairing strategy to directly compare the day –7 sample with the day 0.25 (array #1) and day 1 (array #4) samples because results from our previous studies suggested that the most pronounced differences in neutrophil gene expression may occur between day –7 and days 0.25 or 1 (Preisler et al., 2000; Weber et al., 2001; Madsen et al., 2002). Data from these microarray experiments can be found in GEO, Series accession number GSE544.

For cDNA synthesis, 10 μg of sample RNA was converted to cDNA (42°C for 60 min) using the Atlas Glass Fluorescent Labeling Kit (Clontech; Palo Alto, CA). Included in this reaction was 1.25 ng of Lambda Q synthetic mRNA which served as a cDNA synthesis and dye labeling control. Dye couplings and labeled cDNA purifications were performed according to the manufacturer's instructions. The cDNAs were incubated at 70°C for 5 min just prior to array hybridization using a GeneTAC Hybridization Station (Genomic Solutions; Ann Arbor, MI). Arrays were incubated 2 min at 75°C prior to probe addition. Following probe addition at 75°C, hybridization conditions included 3 h at 65°C, followed by 3 h at 55°C, and finally 12 h at 50°C. This was immediately followed by two medium stringency washes at 50°C, two high stringency washes at 42°C, and two washes with post wash buffer at 42°C (wash buffers and post wash buffer from Genomic Solutions). Finally, arrays were removed, rinsed in 2X SSC, and centrifuged in open 50-ml conical tubes (500 x g for 3 min at room temperature) to dry them. Array scanning was done using a GeneTAC LS IV (Genomic Solutions) and accompanying

software (version 3.01). Spot analyses were performed using GeneTAC Integrator Microarray Analysis Software, version 3.3.0 (Genomic Solutions). Total intensity values for each dye channel were stored as comma-separated value data files and exported into Excel spreadsheets for subsequent loading into SAS for data normalization and analysis (see below).

D. Statistical analysis of microarray data

Potential dye intensity biases in the microarray data sets were visualized using M versus A scatter plots constructed for each array, where log intensity ratios $M = \log(Cy3/Cy5) = \log Cy3 - \log Cy5$ were plotted against mean log-intensities $A = (\log Cy3 + \log Cy5)/2$ for each array spot, as described by Yang et al. (2002). Array-specific data normalization was then performed considering a robust local regression technique (Cleveland and Grosse, 1991) using the LOESS (locally-weighted regression and smoothing scatter plots) procedure of SAS (SAS Institute, 2000). The efficiency of LOESS normalization was assessed by monitoring *M-A* plots for data from each array before and after LOESS normalization. The normalized data were then back transformed prior to further statistical analyses using the formulas: $\log Cy3^{*} = A + \hat{M}/2$ and $\log Cy5^{*} = A - \hat{M}/2$, where $\log Cy3^{*}$ and $\log Cy5^{*}$ are the normalized log intensities. Here, $\hat{M}$ represents each of the normalized M values with $\hat{M}$ being the LOESS predicted value for each spot.

LOESS-adjusted log intensities were then analyzed statistically using a mixed model approach consisting of two steps (Wolfinger et al., 2001). The first step involved array-specific spatial variability normalization and the second step, gene-specific

analyses to test the effect of day relative to parturition on expression profiles for individual genes. The normalization model in the first step was as:

$$log(y_{ijklmn}) = \mu + T_i + D_j + A_l + P(A)_{lm} + \varepsilon_{ijklmn} ,$$

where y_{ijklmn} represents each observed fluorescent intensity signal; μ is an overall mean value; T_i is the main effect of time *i* (day relative to parturition); D_j is the main effect of dye *j*; A_l is the main effect of array *l*; $P(A)_{lm}$ is the effect of patch *m* within array *l*; and ε_{ijklmn} is a stochastic error (assumed to be normally distributed with mean 0 and variance σ^2). The second step of the statistical analysis consisted of gene-specific models for the estimated residuals ($\hat{\varepsilon}_{ijklmn}$) obtained from the normalization approach discussed above. These models were as:

$$\hat{\varepsilon}_{ijklmn} = \mu_k + T_{ki} + D_{kj} + A_{kl} + S(A)_{kln} + e_{ijklmn} ,$$

where all the effects have the same definitions as for the normalization model, except that now they are specific for each gene so they carry an additional index *k*. Moreover, the error terms e_{ijklmn} were assumed to have independent normal distributions with gene-specific variances σ_k^2. These analyses were computed using the MIXED procedure of SAS (SAS Institute, 2000). In the gene-specific analyses, the day relative to parturition effect was declared significant if $P < 0.01$. Overall effects of parturition on gene expression profiles were first visualized using GeneSpring software (Silicon Genetics, Redwood City, CA). To do this, day relative to parturition least squares means of genes for which a parturition effect was suggested in the primary statistical analysis were loaded into the software and expression levels on days 0, 0.25, and 1 plotted as ratios to expression levels on day –7 (our "normal" expression control). Resulting profiles were

then clustered using K-means clustering by general pattern of expression change across the four test days. In this way, clusters of genes that showed parturition-induced decreases were readily differentiated from genes that were induced by parturition. Next, the spotted cDNA sequences representing genes whose expression profiles were significantly influenced by day relative to parturition in this analysis were subjected to BLASTn analysis to reveal identities, and the biological functions of these genes determined through an extensive PubMed literature search. This information was used to form an ontological clustering of affected genes into broad functional categories, which was then used to determine which genes we would confirm as changed in expression due to parturition using an independent assay on individual RNA from neutrophils of three additional cows.

F. Confirmation of altered mRNA abundance profiles using quantitative real time RT-PCR

Confirmation of altered expression for several genes in the predominant ontology cluster that were identified as differentially expressed in our preliminary microarray screening experiment was pursued through the use of quantitative real time RT-PCR (Q-RT-PCR) in an Applied Biosystems 7000 DNA sequence detection system (Perkin Elmer Applied Biosystems; Foster City, CA). The 17 genes selected for real-time PCR validation were chosen based upon three criteria: (1) documented importance to apoptotic cell death in neutrophils or other immune cells; (2) *P*-value of effect of day relative to parturition in the microarray analysis; and (3) a mixture of up and down regulated genes. Individual RNAs from Percoll-purified blood neutrophils of two multiparous and one

primiparous periparturient Holstein cows (different from the 4 cows used for the microarray experiment) sampled on days -7, 0, 0.25, and 1 relative to parturition (on day 0) were converted into first-strand cDNA by combining 2 μg of the RNA with 10 mM oligo $(dT)_{12-18}$ primer and sterile water in a 10 μl volume that was incubated 5 min at 70°C followed by 5 min at 20°C. Master mix containing 4 μl of buffer (supplied by the RT manufacturer; final reagent concentrations of 50 mM Tris-HCL, pH 8.3, 75 mM KCl, and 3 mM $MgCl_2$), 200 units of Superscript II $RNaseH^-$ Reverse Transcriptase (Invitrogen Life Technologies), and a final concentration of 10 mM DTT and 0.5 mM dNTP were added to achieve a final reaction volume of 20 μl. Reverse transcription was allowed to proceed at 42°C for 60 min, heated to 70°C for 15 min, and cooled to 37°C prior to the addition of 2 units of DNase-Free RNaseH (Invitrogen Life Technologies). Incubation at 37°C was continued for 20 min in the presence of RNaseH to remove the original RNA template followed by enzyme inactivation via addition of 0.5 μl of 0.5M EDTA (pH 8.0). First-strand cDNAs were purified with Quick Clean resin (Clontech) followed by precipitation with sodium acetate and ethanol. Purified cDNAs were suspended in DNAse/RNAse free sterile water, quantified spectrophotometrically, diluted to a final concentration of 10 ng/μl, and stored at -20°C until use. Quantitative real time RT-PCR was performed using the SYBR Green PCR Master Mix (Perkin Elmer Applied Biosystems) and 17 gene-specific primer pairs (see Results section) designed using Primer Express Software (Perkin Elmer Applied Biosystems) and synthesized at a commercial facility (Qiagen-Operon, Inc.; Alameda, CA). Primers for β-actin were also made and this gene was included in all Q-RT-PCR analyses for the purpose of data normalization (Madsen et al., 2002). Results were recorded as relative gene expression

changes after normalizing for β-actin gene expression, computed using the $2^{-\Delta\Delta Ct}$ method described in detail by Livak and Schmittgen (2001). This method monitors relative gene expression changes across treatments (days relative to parturition in this case) based on differences in the PCR amplified target reaching a fixed threshold cycle (C_T) number at a set treatment (day –7) versus other treatments (days 0, 0.25, and 1). Thus, for our $2^{-\Delta\Delta Ct}$ analysis, the C_T for day –7 was the calibrator used to determine relative gene expression changes on all other days for each β-actin-normalized test gene. Statistical analysis of these data was performed by comparing day 0, 0.25, and 1 individually to day -7 using t-tests with pooled standard errors on a log ratio scale. Gene specific standard errors were estimated using independent analyses of variance (ANOVA), which included the effects of cow and time (0, 0.25 and 1 day). All the analyses were performed using the SAS System (SAS Institute, 2000).

G. Neutrophil apoptosis phenotyping

Based on results from our microarray screening and Q-RT-PCR experiments, we performed *ex vivo* apoptosis phenotyping on neutrophils from three Holstein steers. The periparturient blood environment was simulated in neutrophil cultures by adding heat inactivated (56°C for 30 min) blood sera collected at days -7, 0, 0.25, and 1 (relative to parturition) from the three cows used for neutrophil collections for the Q-RT-PCR experiment. Apoptosis phenotyping was assessed by two-color fluorescence-activated flow cytometric analysis of cultured neutrophils stained with Annexin V-FITC and propidium iodide (**PI**) (Weyts et al., 1998). Briefly, Percoll-isolated blood neutrophils from each of the three steers were reconstituted at 1 x 10^7 cells/ml in RPMI-1640

medium (Invitrogen) containing 0.25% penicillin-streptomycin (Invitrogen) of which 0.1 ml was seeded into wells of 96-well cell culture plates (Fisher Scientific, Pittsburgh, PA). Neutrophil cultures were supplemented with 20 or 40% of individual sera from three periparturient cows, such that the final culture volumes were 0.2 ml/well. Neutrophils were then incubated in moist 5% CO_2 air at 39°C (normal body temperature for cattle) for 24 h. After incubation, cells were centrifuged at 500 x g for 5 min at 4°C, washed twice with cold phosphate buffered saline, pH 7.2, and stained with FITC-conjugated AnnexinV and PI following the protocol contained in a commercial kit (Annexin V-FITC Apoptosis Detection Kit; BD Biosciences Pharmingen, San Diego, CA). Cells were then transferred to 5-ml polystyrene round bottom tubes (Becton Dickinson) and apoptosis data acquisition of 5000 cells per sample performed using a FACSCaliber flow cytometer and Cell Quest software (Becton Dickinson; San Jose, CA). Quadrants were set on resulting two-color flow cytometric density plots effectively separating Annexin V^-/PI^- non-apoptotic cells (lower left quadrant) from Annexin V^+/PI^- early apoptotic cells (lower right quadrant), AnnexinV^+/PI^+ late apoptotic cells (upper right quadrant), and Annexin V^-/PI^+ necrotic cells (upper left quadrant). For simplicity of data reporting, we selected cells in the lower left and right quadrants (i.e. % non-apoptotic and early apoptotic) for further statistical analyses and data presentation.

Statistical analysis was performed to test the fixed effect of day relative to parturition on the % Annexin V^-/PI^- and the % Annexin V^+/PI^- neutrophils using the MIXED procedure of SAS (SAS Institute, 2000). The statistical model also included random effects of steer (neutrophil donors) and cow (serum donors) as well as all two-

way interactions (steer x day, cow x day, and cow x steer). Significance of the effect of day relative to parturition was declared when $P < 0.05$.

IV. RESULTS

A. Characterization of neutrophils and RNA used

Blood samples collected from periparturient cows used in this study were subjected to electronic counting of total leukocytes and to G1 immunostaining and flow cytometric analysis of neutrophils. As expected (Preisler et al., 2000; Weber et al., 2001; Madsen et al., 2002), parturition caused leukocytosis (**Figure 2.1a**; $P = 0.012$) and neutrophilia (**Figure 2.1b**; $P < 0.001$) in the test cows that were particularly striking 6 h postpartum (day 0.25). Small aliquots of Percoll-isolated neutrophils from each blood sample were also subjected to G1 immunostaining and flow cytometric analysis to determine purity, which was always > 93% (not shown). The quantity of RNA averaged approximately 5 μg per sample and was considered of high quality based upon spectrophotometric and agarose gel electrophoretic analysis. However, the quantity of RNA we obtained from individual samples of purified neutrophils was relatively low, requiring that we pool them across cows within sample time to have enough RNA for the preliminary screening of gene expression changes using a loop design in the microarray experiment.

B. LOESS normalization of the microarray data

Representative *M-A* plots for fluorescence intensities of all spot data from array #3 (day 0 versus day 1), are shown before (**Figure 2.2a**) and after (**Figure 2.2b**) LOESS

normalization, which effectively adjusted spot intensities for the small Cy5 dye bias that was present at lower average intensities (**Figure 2.2a**). The LOESS-normalized data from each array were back-transformed and subjected to GeneSpring and statistical analyses to visualize and test the effect of day relative to parturition on gene-specific expression profile changes.

C. GeneSpring analysis of neutrophil gene expression profiles

Statistical analysis of the microarray data suggested that 302 neutrophil genes out of 1056 genes spotted on the BOTL microarray (i.e., ~30%) had expression profile changes ($P < 0.01$) induced by parturition. GeneSpring analysis of these genes revealed four main profiles based on the general type of expression changes over days –7, 0, 0.25, and 1. Clusters included genes that were highly induced on days 0, 0.25, and (or) 1 relative to expression on day -7, genes with moderately induced expression on days 0, 0.25, and (or) 1 relative to expression on day –7, genes whose expressions were inhibited on days 0, 0.25 and (or) 1 relative to expression on day –7, and genes with fluctuating expression changes indicating no particular pattern over time (data not shown). This analysis also demonstrated that expression of approximately one-third of the genes affected by parturition were up regulated on at least one test day while the remaining two-thirds were down regulated or fluctuated with no definitive pattern across test days.

D. In silico determination for function of genes affected by parturition

BLASTn searches of the GenBank and EST databases revealed that 36% of neutrophil genes affected by parturition were unknown (i.e., BLASTn hits were for

cloned genomic DNA and specific chromosomal regions with no genes identified). A combination of BLASTn analysis and exhaustive literature searching revealed clear identities for the remaining affected genes with expectation values (E-values) always < 10^{-4}. These could be grouped into 12 broad ontological clusters based on the best known function of their protein products. The clusters included apoptosis (14% of affected genes), leukocyte activation (7%), adhesion/trafficking (6%), signal transduction (5%), transcription/translation (5%), energy metabolism (5%), growth factors (4%), genes affecting cellular organelles (endoplasmic reticulum, cytoskeleton, ribosomes, and mitochondria; 4%), genes affecting steroid hormone receptors (2%), matrix metalloproteinases and their tissue inhibitors (2%), angiogenesis (1%), and one cluster we called "other" because the genes in it were seemingly unrelated and thus without an ontological theme (9%). We found it interesting that expression of 42 apoptosis-regulatory genes were putatively affected by parturition (**Table 2.1**), and selected 17 key genes from this ontological cluster for further independent validation by Q-RT-PCR.

E. Quantitative real time RT-PCR confirms expression changes in 16 out of 17 tested genes

As shown in **Table 2.2**, parturition-induced expression changes were confirmed by Q-RT-PCR for 16 out of 17 tested apoptosis regulatory genes ($0.0001 < P < 0.07$). β-actin, selected as the normalizing gene based on previous findings (Weber et al., 2001; Madsen et al., 2002), was not differentially expressed across sample days. Many of the genes confirmed to be affected by parturition influence spontaneous apoptosis in neutrophils (e.g., A1, Bag-1, Bak, Bax, Mcl-1) through actions on mitochondrial

membrane stability and release of mitochondrial cytochrome c. Several confirmed genes are also involved in the regulation of death signaling through plasma membrane death receptors (e.g., FADD, Daxx, FLASH, RIP). Other confirmed genes encode predominantly cytosolic proteins that influence neutrophil survival through effects on other apoptosis proteins (e.g., IAP), oxidative stress responses (thioredoxin-like 2), and expression of proinflammatory genes that also induce survival (e.g., IL-8). Expression profiles for 10 confirmed genes are shown in **Figure 2.3.**

F. Apoptosis phenotyping of neutrophils support microarray and Q-RT-PCR results

Because we confirmed that 16 key apoptosis regulatory genes of blood neutrophils were influenced in expression by parturition, we were curious to know how parturient blood affects the apoptosis phenotype of cultured neutrophils. We treated isolated neutrophils from donor steers with sera collected from the periparturient cows used for the gene expression experiments and assessed their apoptosis status flow cytometrically (**Figure 2.4).** Cultures treated with parturient sera (day 0) had higher % Annexin V^-/PI^- (non-apoptotic) neutrophils and lower % Annexin V^+/PI^- (early apoptotic) neutrophils at 12 and 24 h than cultures treated with sera from days -7, 0.25, or 1 ($P <$ 0.01). The main differences in both non-apoptotic and early apoptotic cells were between neutrophils cultured in day -7 sera versus day 0 sera. Identical results were observed when sera were used at 20% (**Figure 2.4**) and 40% (not shown) of the culture volume. Thus, the parturient blood environment induced temporary survival in otherwise normal blood neutrophils.

V. DISCUSSION

Neutrophils are mature, terminally differentiated leukocytes that normally survive for a short time in the circulation (6-12 h) before undergoing apoptosis and clearance from blood by the body's phagocytic cell network (Fanning et al., 1999). Thus, balance between production of new neutrophils in bone marrow and apoptosis in circulating cells determines blood neutrophil counts in healthy animals. In the current study, 42 neutrophil genes that encode apoptosis-regulatory proteins were found to be changed in expression during parturition (**Tables 2.1** and **2.2**). Noteworthy were expression profiles for key Bcl-2 family member genes (**Figure 2.3a-c**), Fas-signaling genes (**Figure 2.3d-g**), and the IL-8 gene (**Figure 2.3h**), all of which suggested a pro-survival gene expression pattern at parturition relative to 7 days prepartum. Subsequent apoptosis phenotyping of normal neutrophils treated with sera from periparturient cows substantiated this by demonstrating enhanced survival in the presence of serum collected at parturition (day 0) versus serum collected before (day-7) or after (days 0.25 and 1) parturition (**Figure 2.4**). Extended survival could partly explain the pronounced neutrophilia we observed in parturient cows (**Figure 1b**) because non-apoptotic neutrophils would not be cleared from circulation (Fanning et al., 1999).

The normally short life span of circulating neutrophils is thought to be due to their lack of Bcl-2 gene expression and relatively high level of Fas gene expression (Fanning et al., 1999). Bcl-2 is the prototypic anti-apoptosis protein in most cells that protects mitochondrial membranes from attack by pro-apoptotic Bcl-2 family members such as Bax and Bak (Reed, 1998). While lacking Bcl-2 expression, mature neutrophils do express other anti-apoptotic Bcl-2 family member genes, such as A1, Bag-1, and Mcl-1 in

addition to pro-apoptotic Bax and Bak (Orlofsky et al., 1999; Weinmann et al., 1999). A1 is the Bcl-2 homologue in mature neutrophils and its increased expression via NF-κB activation in cells treated with survival-inducing pro-inflammatory factors (e.g., LPS and G-CSF) rescues the cells from Bax/Bak-induced spontaneous apoptosis (Duriez et al., 2000; Kupfner et al., 2001). A1 protein works by protecting mitochondrial membranes from Bax/Bak-induced pore formation, preventing release of cytochrome c and subsequent activation of caspases 9 and 3 that effect DNA fragmentation and cell death (Orlofsky et al., 1999; Simon, 2001). Thus, expression ratios of A1 to Bax and Bak are the main determinants of whether neutrophils live or die by the mitochondrial cytochrome c release pathway (Weinmann et al., 1999; Kupfner et al., 2001). Our Q-RT-PCR experiment confirmed that parturition temporarily increased the A1:Bax and A1:Bak ratios in bovine blood neutrophils (**Figure 2.3a, b, c**) supporting the survival induction observed in normal neutrophils cultured in medium containing parturient serum (**Figure 2.4**).

While Bcl-2 family members are intracellular proteins, Fas (CD95/APO-1) is expressed on the plasma membranes of neutrophils (Liles and Klebanoff, 1995) and is a prototypic death receptor from the TNF receptor super family of molecules. Ligand-activated Fas induces aggressive apoptosis in neutrophils and other cells by recruiting adaptor proteins (e.g., FADD, Daxx, FLASH, and RIP) to its cytoplasmic death domain to form potent signaling complexes known as DISC (death-inducing signaling complex) (Scaffidi et al., 1998). DISC recruits and activates caspase 8, which then cleaves and activates down-stream caspases (e.g., 7 and 3) that inactivate normal survival signals (e.g., NF-κB) (Lin et al., 1999; Holler et al., 2000) and cleave cytoskeletal and DNA

repair proteins, leading to DNA fragmentation and irreversible cell death (reviewed by Nunez et al., 1998; Imai et al., 1999). Circulating neutrophils normally express higher levels of Fas than other leukocytes, easily explaining their sensitivity to apoptosis induction and short half-life in blood compared to lymphocytes, monocytes, and eosinophils (Fanning et al., 1999). Given that expression of genes encoding the 4 main DISC proteins were dramatically down-regulated in blood neutrophils of our study (**Figure 2.3d-g**), it would appear that the potent Fas-induction pathway of cell death may be disabled in the cells and support their temporary survival. If future studies prove that parturition interrupts formation of DISC at the death domains of Fas, expression changes in these proteins could become potential targets for drug development to manipulate neutrophil survival and inflammatory responses.

Given our results it appears that factor(s) in parturient blood influence expression of genes that change the apoptosis status of neutrophils. Though these factors have yet to be identified, biomedical literature has documented that glucocorticoids effectively induce survival in cultured human and rodent neutrophils (Cox, 1995; Nittoh et al., 1998). Glucocorticoids are dramatically increased in blood of cows at parturition (Preisler et al., 2000), including the cows used in the current study (data not shown), so this is one possibility that may explain the survival induction we observed. Given this, we are not certain how to reconcile the inhibited IAP (**Figure 2.3i**) and NF-κB p65 (**Figure 2.3j**) gene expression in neutrophils around parturition. The products of these genes are best known for their ability to confer survival by inhibiting caspases (IAP; reviewed by Doyle et al., 2002) and inducing pro-survival gene expression (NF-κB; see above and Kupfner et al., 2001) in most other leukocytes. While our BOTL microarrays

are valuable tools for studying gene expression in neutrophils, their limitation is that they do not contain all possible genes expressed by neutrophils, including apoptosis regulatory genes. Thus, other apoptosis genes not present on our microarrays that may override putative IAP and NF-κB survival systems may have been induced in the neutrophils in favor of extended survival. It is also possible that, in addition to acute survival induction via altered A1:Bax/Bak and DISC protein gene expression ratios, parturition induces a more chronic pro-death expression signature in genes such as IAP and NF-κB to ensure that the cells do eventually die by apoptosis. Supporting this possibility, most of the anti-apoptotic gene expression changes induced by parturition began to return to normal pro-apoptotic expression patterns observed 7 days prepartum by day 1 postpartum (**Figure 2.3**), when neutrophil numbers in blood were relatively normal (**Figure 2.1b**) and blood serum added to neutrophils *ex vivo* no longer supported prolonged survival (**Figure 2.4**). In fact, A1 expression was significantly repressed and Bax expression induced on day 1 post partum relative to day 7 prepartum, suggesting that this pathway was even more active after parturition than before parturition.

Finally, our results have posed an apparent anomaly. It is not intuitive why parturition would induce a temporary pro-survival gene expression pattern in blood neutrophils or how this would relate to heightened mastitis susceptibility in newly calved cows. One could postulate that neutrophils in survival mode would actually augment the supply of available neutrophil defense around parturition due to resulting neutrophilia. Why is it then that parturient dairy cows tend to lack efficient inflammatory responses in peripheral tissues making them susceptible to clinical mastitis (Burton and Erskine, 2003)? Alternatively, could altered expression profiles of additional genes related to

trafficking, migration, and energy metabolism suggested by our microarray experiment and other studies (Weber et al., 2001; Madsen et al., 2002) better explain mastitis at parturition? If survival induction is a by-product of altered inflammatory phenotypes resulting from changed expression in these other genes, it may occur simply to protect animals from the growing numbers of neutrophils that would cause harmful systemic inflammation if they died by necrosis or out-numbered the capacity of the phagocytic network to clear them from the circulation. These questions are important to answer because the balance between neutrophil survival and programmed cell death ultimately determines the outcome of all inflammation, including in the bovine mammary gland. Guided by results of this study, our future work will be aimed at addressing these questions.

Table 2.1 Forty-two apoptosis regulatory genes detected in blood neutrophils by cDNA microarray analysis as putatively altered in expression by parturition (listed in alphabetical order).

Gene Name	BOTL clone* (38) or PCR amplicon (5)	Most Commonly Reported Effect on Apoptosis
A1	PCR	anti-apoptotic
Bak	PCR	pro-apoptotic
BAFF	PCR	pro-apoptotic
Bcl-10	PCR	anti-apoptotic
Bag-1	PCR	anti-apoptotic
Bax-alpha	PCR	pro-apoptotic
BIK	PCR	anti-apoptotic
Calpain II	10_H08	pro-apoptotic
CD27-binding protein transcript variant 1 (SIVA)	11_F02	pro-apoptotic
CD3 gamma	PCR	pro-apoptotic
chemokine (C-C motif) receptor 9	PCR	anti-apoptotic
Cyclin A (Cyclin-A2)	PCR	pro-apoptotic
DAP	PCR	pro-apoptotic
Daxx	PCR	pro-apoptotic
Death effector domain containing protein (DEDD)	PCR	pro-apoptotic
Serine/threonine kinase, DRAK-1	PCR	pro-apoptotic
FADD	PCR	pro-apoptotic
FLASH	PCR	pro-apoptotic
Growth factor receptor tyrosine kinase (STK-1)	PCR	anti-apoptotic
I kappa B kinase alpha	PCR	pro-apoptotic
Immunity associated protein (Imap38)	4XC01R	pro-apoptotic
Interleukin-8 (IL-8)	PCR	anti-apoptotic

Table 2.1 continued...

Kruppel-like factor	6XG09R	anti-apoptotic
LIM domain only 4	3XD11R	anti-apoptotic
Mcl-1	PCR	anti-apoptotic
MIL6GP Membrane glycoprotein gp130	PCR	anti-apoptotic
MYC1 mRNA encoding the c-myc oncogene	PCR	pro-apoptotic
NF-kappaB binding subunit	3XF01R	anti-apoptotic
NF-kappaB p105 (DNA binding subunit)	PCR	anti-apoptotic
NF-kappaB p65 subunit	6XH02R	anti-apoptotic
Nuclear transcription factor HMG-2	1XC07R	pro-apoptotic
PEBP2aC runt domain encoding gene	13_F04	anti-apoptotic
Death domain containing protein, PIDD	13_G01	pro-apoptotic
Poly A binding protein, cytoplasmic 1	8_D10	pro-apoptotic
IAP (putative inhibitor of apoptosis)	9_H08	anti-apoptotic
RIP protein kinase	PCR	pro-apoptotic
Serine protease-like protein (Granzyme B)	PCR	pro-apoptotic
Similar to RAS suppressor protein 1	11_D11	anti-apoptotic
TANK	PCR	anti-apoptotic
Thioredoxin-like 2 (TXNL2)	4XF05R	anti-apoptotic
TIAF-1	PCR	anti-apoptotic
TRAF6	PCR	anti-apoptotic

*All BOTL clone numbers present in our database (http://www.nbfgc.msu.edu) are preceded by 'BOTL01000'.
(38) = Yao et al., 2001
(5) = Coussens and Nobis, 2002

Table 2.2 Quantitative real time RT-PCR confirmation of 16 of 17 apoptosis regulatory genes altered in expression by parturition (listed in alphabetical order).

Gene name	Primer sequences (5' to 3')	Size of amplicon (bp)	Tm (°C)	Change relative to day –7 (*P*-value)
A1	Forward: ACTGCCAGAACAATATTCAACCAA	78	76	Increase
	Reverse: GGTTACAATCCTGCCCCAGTT			(< 0.01)
Bag-1	Forward: AGGCCTTCCTAGCCGAGTGT	104	83	Decrease
	Reverse: CTCCGCCTCACCTCAGTCA			(< 0.05)
Bak	Forward: CAACAGCAFACAACGCCTATG	70	81	Decrease
	Reverse: CCAGTTGATCCGCTCTCAAAC			(< 0.01)
Bax	Forward: TCCCCGAGAGGTCTTTTTCC	71	79	Increase
	Reverse: TCCCCGAGAGGTCTTTTTCC			(< 0.01)
DAP5	Forward: CCGACTAAAAGAACCAGCCAAA	78	76	Decrease
	Reverse: GGTTAGGTCAAATGCAGTTACATCA			(< 0.01)
Daxx	Forward: CAGAGGGAGCTGGTCACCAA	70	83	Decrease
	Reverse: TGGCTCCAGTTGCTGATTCC			(< 0.01)
FADD	Forward: TGCCAGAACCACATTAGCAAGA	96	85	Decrease
	Reverse: CTCGGCGTTCAGCTCGTT			(< 0.05)
FLASH	Forward: CCATCCAGGAATTGTTTGGATTTA	95	75	Decrease
	Reverse: CCATATTCTGCCTGCAAATCATT			(< 0.01)
IAP	Forward: ACTGTTCTTCCATTGGTAAATCTGAA	92	73	Decrease
	Reverse: TGATCCCATGTTATACAAGCGTTT			(< 0.01)

Table 2.2 continued

IL-8	Forward: GGAAAAGTGGGTGCAGAAGGT	80	77	Increase
	Reverse: GGTGGTTTTTTCTTTTTCATGGA			(< 0.07)
IκB kinaseα	Forward: CGTCCCTTGTAGGATCCAGTCTAG	87	82	Decrease
	Reverse: CCACGCATGACAGAGGATGT			(> 0.50)
Mcl-1	Forward: TGTGGCCAAACACTTGAAGAGT	82	77	Decrease
	Reverse: CCTTACGAGAACATCTGTGATGCTT			(> 0.01)
NF-κB p65	Forward: GGAATTCCAGTACCTGCCAGATA	89	78	Decrease
	Reverse: TCATGATGCTCTTGAAGGTCTCA			(< 0.01)
RIP	Forward: GCCAAAATGAAATTGTAAAGAGAATG	90	77	Decrease
	Reverse: GCTGTTCTGTGGCTGAATTTGAC			(< 0.01)
TANK	Forward: TCCGGCATCTTAATTCACACTTA	135	73	Decrease
	Reverse: AATGATCTTGAGGCTAAGTTTGCAATC			(< 0.01)
Thioredoxin-like 2	Forward: TTCTCCTTTCAGTATAGGCAGCAA	85	80	Decrease
	Reverse: TGTGAAGGGCGAGCTTGTC			(< 0.01)
TRAF6	Forward: AGGGTCGCCTTGTAAGACAAGA	74	78	Decrease
	Reverse: TACATACATGCTCTGGGTTTCCA			(< 0.01)
β–actin	Forward: CGCCATGGATGATGATATTGC	66	84	No Change
	Reverse: AAGCGGCCTTGCACAT			(> 0.70)

Figure 2.1 Whole blood counts (number of cells/ml) of total leukocytes and neutrophils from cows sampled for the microarray and Q-RT-PCR experiments. Panel (**a**) shows leukocytosis (P = 0.012) at and 6 h after parturition (days 0 and 0.25) compared to prepartum (day –7) and 24 h postpartum (day 1). Leukocytosis was driven by neutrophilia (**b**; $P < 0.001$). *Daily means significantly different ($P < 0.05$) from the day -7 mean.

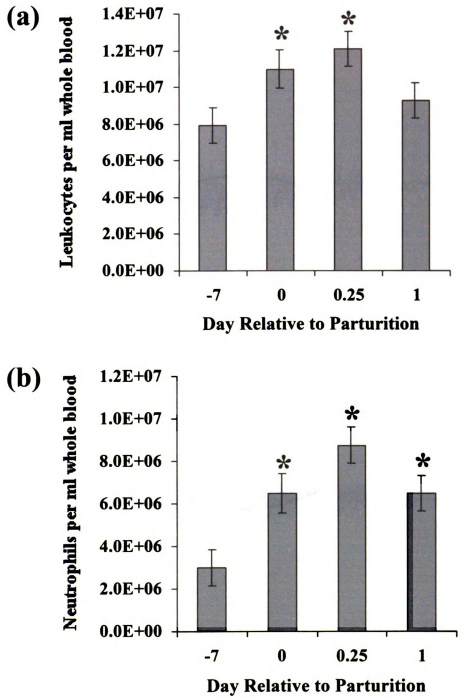


Figure 2.2 LOESS normalization effectively removed a slight Cy5 dye bias that was apparent for low intensity spots (negative control and some test spots) in the BOTL microarray data sets. Yellow dots are internal GAPDH control spots, pink dots are external lambda Q control spots, green dots are internal β-actin control spots, blue dots are internal RPL-19 control spots, and red dots are assay blanks (negative controls). Black dots represent the 1056 thrice-spotted leukocyte genes. Shown in panel **(a)** is the pre-normalization *M-A* plot for array #3, which was hybridized with RNA from day 0 (Cy3 labeled) and day 1 (Cy5 labeled) neutrophils. Shown in panel **(b)** is the post-LOESS normalization *M-A* plot for the same array shown in (a).

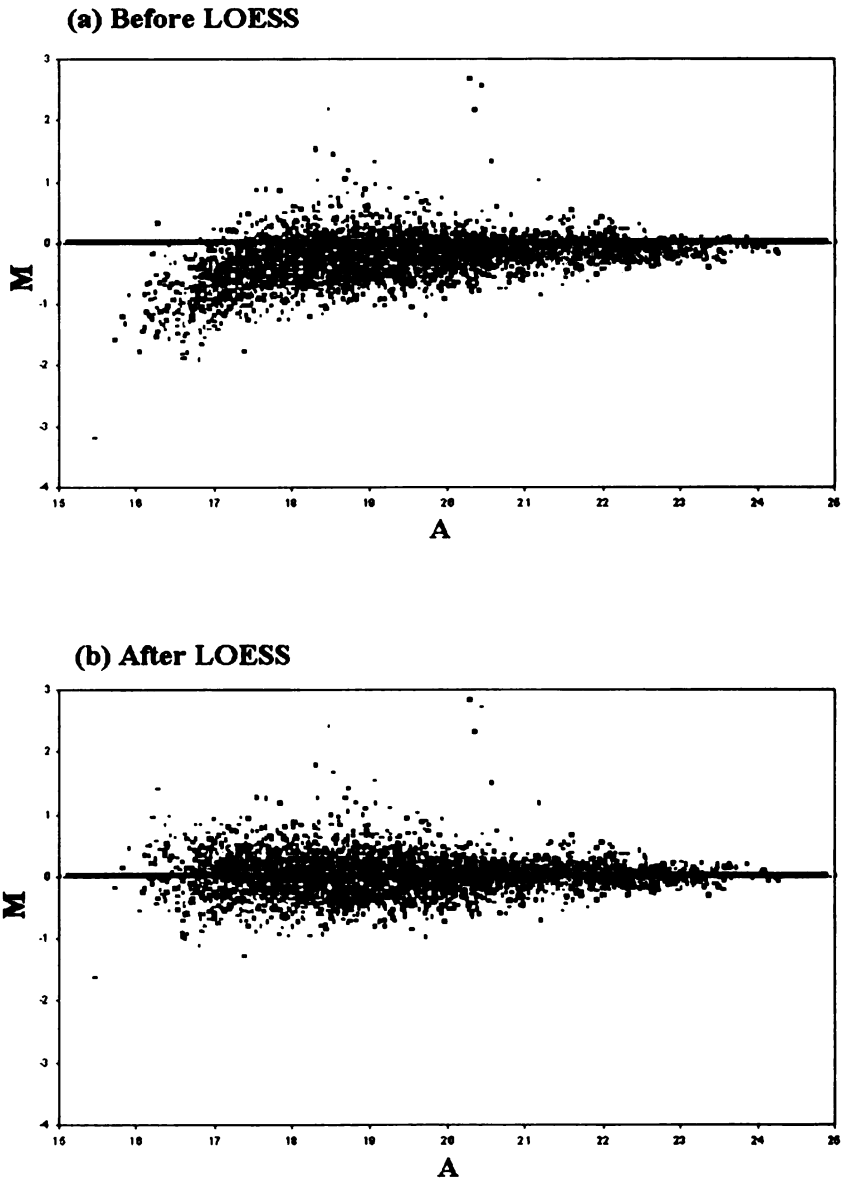


Figure 2.3 Mean expression changes (± SEM) of key neutrophil apoptosis regulatory genes during the peripartum period. Data were derived from Q-RT-PCR assay of RNA from three cows sampled on days –7, 0, 0.25 and 1 relative to parturition (on day 0) and expressed ratios of expression on day –7. Parturition increased A1 expression and decreased Bak expression, effectively increasing both A1:Bak and A1:Bax ratios (panels a-c). Parturition also decreased expression of genes encoding key Fas-signaling death proteins (panels d-g), and induced pronounced expression of the pro-survival chemokine, IL-8 (panel h). Parturition decreased expression of IAP and NF-κB p65 genes (panels i and j, respectively), which have anti-apoptotic roles in many cell types but are not well described in neutrophils.
** $P \leq 0.01$; * $P \leq 0.05$; ‡ $0.05 < P < 0.07$

Figure 2.3

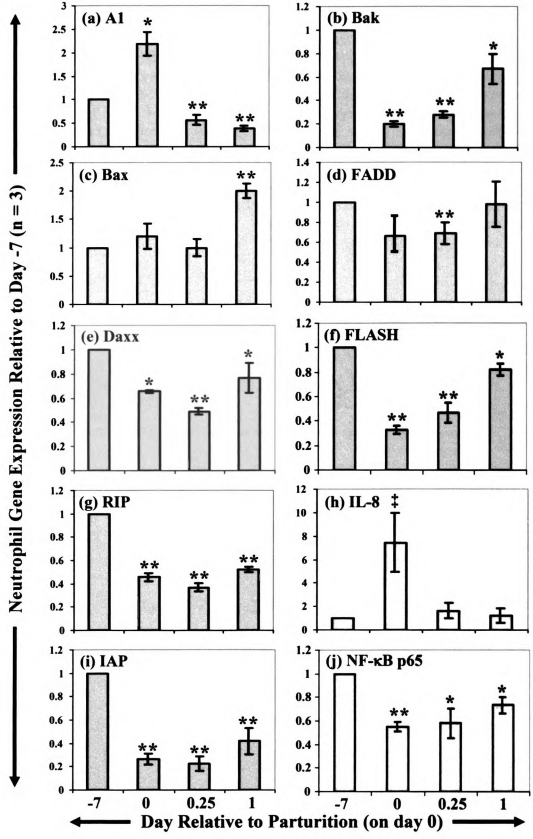
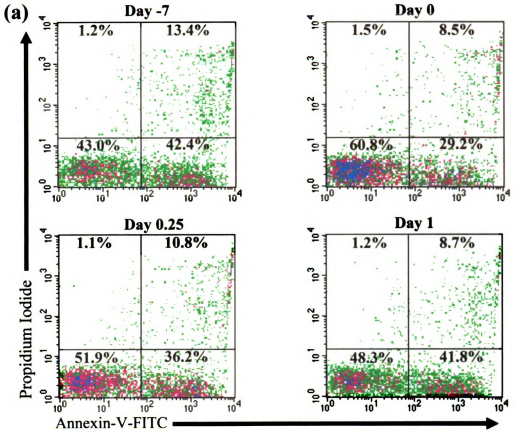

(a) A1
(b) Bak
(c) Bax
(d) FADD
(e) Daxx
(f) FLASH
(g) RIP
(h) IL-8
(i) IAP
(j) NF-κB p65
Neutrophil Gene Expression Relative to Day -7 (n = 3)
-7
0
0.25
1
Day Relative to Parturition (on day 0)

Figure 2.4 Blood serum from parturient cows prolong neutrophil survival *ex vivo*. Data in panel **(a)** are representative two-color flow cytometric density dot plots for neutrophils from one steer incubated for 24 h in periparturient sera from one cow collected on days -7 (upper left), day 0 (upper right), day 0.25 (lower left), and day 1 (lower right) relative to parturition (on day 0). Annexin-V-FITC fluorescence of the cells is on the X-axes and propidium iodide (PI) fluorescence of the cells is on the Y-axes. Color changes within each quadrant of these plots represent a 50% change in density on the $\log_{10}$ scale of the two-color stained cells. Percentage non-apoptotic neutrophils (i.e., %Annexin V^-/PI^- in lower left quadrants) was higher, and % early apoptotic neutrophils (i.e., %Annexin V^+/PI^- in lower right quadrants) was lower when cells were cultured in day 0 sera compared to sera from days –7, 0.25, or 1. In panel **(b)**, means (± SEM) for % non-apoptotic and % early apoptotic neutrophils cultured for 12 and 24 hours in the various periparturient sera are summarized across all steers and cows tested (*P* – values indicate significant effects of day relative to parturition).

Figure 2.4



(b)

Day Relative to Parturition	12 Hour Incubation		24 Hour Incubation	
	% AnnexinV$^-$/PI$^-$	% AnnexinV$^+$/PI$^-$	% AnnexinV$^-$/PI$^-$	% AnnexinV$^+$/PI$^-$
-7	56.8 ± 4.9	35.7 ± 4.9	35.1 ± 5.5	47.9 ± 3.2
0	69.9 ± 4.9	23.2 ± 4.9	46.6 ± 5.5	41.9 ± 3.2
0.25	62.5 ± 4.9	30.8 ± 4.9	39.0 ± 5.5	46.1 ± 3.2
1	62.2 ± 4.9	30.9 ± 4.9	37.3 ± 5.5	48.5 ± 3.2
***P*-value**	0.007	0.010	0.005	0.083

CHAPTER THREE

Several figures in the following chapter were published previously in:

Burton JL, Madsen SA, Chang LC, Weber PS, Buckham KR, van Dorp R, Hickey MC, Earley B. 2005. Gene expression signatures in neutrophils exposed to glucocorticoids: a new paradigm to help explain "neutrophil dysfunction" in parturient dairy cows. Vet Immunol Immunopathol 105(3-4):197-219.

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CHAPTER THREE

High Glucocorticoid Concentrations Found in Blood Serum of Parturient Cows Delays Neutrophil Apoptosis

I. ABSTRACT

Bovine blood neutrophils are documented as having bactericidal abnormalities typical of apoptotic cells at the time of parturition, which have often been indirectly correlated with the increased susceptibility to mastitis at this time. However, recent functional genomics data from our laboratory showed that neutrophils from parturient cows have a gene expression signature typical of healthy cells that are in a state of extended survival, including increased expression of anti-apoptotic A1 and decreased expression of pro-apoptotic Bak. Furthermore, we showed that soluble factor(s) present in blood serum from these cows dramatically extended the *ex vivo* survival of normal steer neutrophils for 24 to 48 h. A number of serum factors could have been responsible, including steroid hormones (cortisol, 17β-estradiol, and progesterone) that fluctuate dramatically at parturition. Based on evidence from biomedical studies, we hypothesized that glucocorticoid was a parturient serum factor responsible for extending neutrophil life span *ex vivo*. The objectives were to manipulate the concentrations of all three steroids in parturient serum, antagonize their homologous receptors in steer neutrophils, and administer glucocorticoid directly into steers to determine which steroid(s) may be responsible for delaying spontaneous neutrophil apoptosis. In the first experiment, blood neutrophils from healthy donor steers were treated *ex vivo* with intact parturient serum (PS) or charcoal-treated parturient serum (CTS, steroid hormones removed). As expected, the cells exhibited a dose dependent delay in apoptosis to increasing amounts of added PS,

a delay not observed when the cells were cultured in the presence of CTS. In a second set of experiments, treatment of isolated neutrophils directly with the glucocorticoid and progesterone receptor antagonist, RU486, negated the apoptosis delaying capacity of PS. Steer neutrophils were shown to express only glucocorticoid and estrogen receptors, thus RU486 was not acting through the progesterone receptor. Treatment of the cells with tamoxifen (an estrogen receptor antagonist) in combination with PS had no effect on cell survival. In addition, apoptosis was delayed when glucocorticoids were added to CTS at levels normally found in PS or to fetal bovine serum (FBS) supplemented neutrophils at 10^{-7} M concentrations (approximately equal to level in PS). Treatment with progesterone and 17β-estradiol at levels found in PS or 10^{-7} M did not delay apoptosis when added to CTS or FBS, respectively. In a final experiment, Western blot analysis was used to evaluate abundance of A1 and Bak proteins in neutrophil cytosols from glucocorticoid-treated steers. Administration of glucocorticoid caused the expected leukocytosis (neutrophil-based) within 9 h with a concurrent 2.4 fold increase in A1 and a 5.4 fold decrease in Bak protein abundance in isolated blood neutrophils. Combined, results of this study point to glucocorticoids as playing a significant role in the homeostasis of circulating neutrophils in parturient and steroid treated cattle, possibly by altering the cells' normal program of spontaneous apoptosis through A1 and Bak abundance changes and extending their life span approximately 3 to 4 fold.

II. INTRODUCTION

Blood neutrophil homeostasis is profoundly impacted by events surrounding bovine parturition. Increased susceptibility to diseases such as mastitis, metritis, and

retained placenta have all been linked to altered neutrophil functional capacity at parturition (reviewed by Burton and Erskine, 2003). Some neutrophil activities are altered in ways considered to be dysfunctional, yet others are maintained or even enhanced at parturition. In general, the impairments appear to involve decreased expression of adhesion molecules responsible for neutrophil margination on blood vessel endothelium (Lee and Kehrli, 1998; Weber et al., 2001), rate of cell migration into infected mammary quarters (Frost and Brooker, 1986; Hill et al., 1979), and oxidative killing of the invading pathogens (Kehrli and Goff, 1989; Cai et al., 1994). Paradoxically, the neutrophil count in blood increases (Weber et al., 2001) as does the cells' capacity for phagocytosis (Kehrli and Goff, 1989; Cai et al., 1994).

A recent study by our group has demonstrated that neutrophils from parturient cows exhibit gene expression patterns suggestive of delayed apoptosis (Chapter Two; Madsen et al., 2004) with increased inflammatory potential of the cells (Burton et al., 2005). Indeed, significant delays in spontaneous apoptosis and increases in phagocytic capacity were observed when blood neutrophils from healthy steers were cultured in the presence of blood serum from the parturient cows (Madsen et al., 2004 and our unpublished data). This surprising result was in contrast to the widely held belief that parturition leads to neutrophil dysfunctions, such as decreased cell adhesion and respiratory burst activity, typical of apoptotic cells (Mehrzad et al., 2001; VanOostveldt et al., 2001; Mehrzad et al., 2002).

Under normal conditions, neutrophils circulate in blood for approximately 12 h (Cartwright et al., 1964; Dancey et al., 1976) with close to 85% of these cells being at some stage of apoptosis (Shidham and Swami, 2000). This is in stark contrast to

monocytes and lymphocytes, which can live for days, weeks, or even months. Upon release from bone marrow, mature neutrophils are primed for spontaneous apoptosis as is evidenced by their high expression of pro-apoptotic Bak/Bax and low expression of anti-apoptotic Bcl-2 molecules, which are important to intrinsic apoptosis signaling initiated through mitochondria (Lin et al., 1996; Sendo et al., 1996). Additional extrinsic apoptosis signals occur when membrane-bound Fas molecules interact with Fas ligand (FasL) as they marginate on the vascular endothelium (Liles et al., 1996; Fanning et al., 1999). The constant intrinsic and extrinsic signaling for apoptosis in circulating and marginating neutrophils is critical to their homeostasis and the resolution of inflammation at sites of tissue infection. As neutrophils age in the circulation or infection foci, they become apoptotic and are cleared as intact cells by the body's phagocytic networks without inducing further inflammation (Savill et al., 1989; Haslett, 1999). This is an appropriate fate since apoptotic neutrophils are deficient in chemotaxis, phagocytosis, and degranulation, and are thus poor defenders against infections (Narayanan et al., 1997; Tanji-Matsuba et al., 1998). This is in contrast to non-apoptotic neutrophils, which are highly active in these important disease-fighting functions.

Blood neutrophils are exposed to a rapidly changing steroid hormone environment during parturition. The sharp increase in cortisol has already been linked to several altered functions of the cells. For example, observational studies have described relationships between changing corticosteroid levels and increased neutrophil phagocytic ability during parturition (Guidry et al., 1976), as well as altered neutrophil trafficking and neutrophilia due to decreased surface expression of L-selectin adhesion molecules (Lee and Kehrli, 1998; Weber et al., 2001, 2004). Although glucocorticoids, such as

cortisol, are potent anti-inflammatory agents, studies with human and rat blood neutrophils have demonstrated that this steroid also acts to delay spontaneous apoptosis in the cells (Cox, 1995; Liles et al., 1995; Meagher et al., 1996; Nittoh et al., 1998). Of the other major parturient steroids, 17β-estradiol and progesterone have little to no effect on neutrophil apoptosis in these species (Meagher et al., 1996; Nittoh et al., 1998).

In light of the past work demonstrating major effects of glucocorticoids on neutrophil apoptosis, the hypothesis of the current study was that cortisol in parturient serum was a factor responsible for extending neutrophil life span *ex vivo* (Chapter Two; Madsen et al., 2004). The objectives of this study were to manipulate the concentrations of all three steroids in parturient serum, antagonize their homologous receptors in steer neutrophils, and administer glucocorticoid directly into steers to determine which steroid(s) may be responsible for delaying spontaneous neutrophil apoptosis. Two experiments were performed using isolated neutrophils from young healthy Holstein steer donors. Apoptosis was assessed in these experiments after multiple *in vitro* treatments using flow cytometric assays that detect early and later stages of apoptosis. In the third experiment, glucocorticoid was administered to healthy steers to determine *in vivo* effects of the steroid on neutrophil abundance of the A1 and Bak proteins.

III. MATERIALS AND METHODS

A. Animals and blood sample collections

Neutrophils for all experiments were obtained from young male Holsteins that were castrated at roughly 1 month of age (steers). These steers weighed between 225 and 325 kg at the time of blood collections. Blood (120 ml or 240 ml depending on

experiment) for neutrophil isolations was collected from the steers through indwelling jugular catheters, as described in Weber et al. (2004). The samples were aliquoted into a series of four to eight 50-ml conical tubes (depending on the experiment), each preloaded with 4 ml of acid citrate dextrose (ACD) anticoagulant, and gently mixed. These blood samples were placed on ice and transported to the laboratory (~7 min drive) for immediate processing (see section B). Cow blood for serum harvesting was collected from primiparous parturient Holsteins on days -7, 0, 0.25, and 1 relative to parturition (on day 0). This blood (100 ml per animal) was collected by tail venipuncture using evacuated tubes with no anticoagulant and was allowed to clot overnight (~18 h) at 4°C. The coagulated samples were then centrifuged at 1000 x g for 30 min at 4°C, and the sera collected, pooled within sample time across animals, and stored at -20°C until use in various assays (see below). The steers and cows were fed and housed according to standard operating procedures at the MSU Dairy Teaching and Research Facility and their use for the described experiments was approved by MSU's All University Committee on Animal Care and Use (approval # 03/99-031-00 and # 07/04-104-00).

B. Isolation of blood neutrophils by Percoll density centrifugation

Upon arrival in the laboratory, the 50-ml conical tubes containing ACD anti-coagulated blood were centrifuged at 1000 X g for 20 min at 4°C to separate plasma and buffy coats from the red cell packs that contained neutrophils. Plasma, buffy coat, and approximately the upper two-thirds of the red cell pack were discarded and 16.5 ml of cold PBS per tube was added to the remaining red cell packs. Aliquots (6 ml each) of the diluted red cell packs were then transferred into new 50 ml tubes that were preloaded

with 12 ml of cold PBS. This cell suspension was then under layered with 12 ml of 1.084 g/ml Percoll (Sigma Chemical Company; St. Louis, MO). Neutrophils were pelleted through the Percoll by centrifugation at 400 X g for 40 min at 22°C. Supernatant, mononuclear cell layer, and Percoll were aspirated and discarded, leaving the red cell pellet that contained neutrophils. Lysis of erythrocytes in this cell pellet was performed as described in Weber et al. (2001), leaving neutrophils intact and enriched. Neutrophils were enumerated by electronic counting using a Z1 Coulter Particle Counter system (Beckman Coulter Particle Characterization, Miami, FL) prior to suspension of the cells to 5 x 10^6 neutrophils/ml in basic culture medium [RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 0.25% penicillin-streptomycin (Invitrogen) and in some experiments, 1% fetal bovine serum (FBS; Hyclone, Logan, UT)]. Purity and viability of the neutrophil preparations was immediately assessed flow cytometrically (Weber et al., 2001), using forward and side scattering properties and PI staining of the cells, and were found to be > 95% for all samples.

C. Preparation of blood serum for *in vitro* assays

Blood serum from the periparturient cows was thawed at room temperature and heat inactivated (to denature complement) at 56°C for 30 min (with gentle swirling every 10 min). Small volumes (1 ml) of the sera were aliquoted for steroid hormone assays (see below). Remaining day 0 (parturient) samples from all of the animals were pooled and split into two aliquots. One aliquot was filter sterilized prior to storage in 10-ml volumes at -20°C and will be referred to subsequently as parturient serum, or PS. The other aliquot was treated with dextran-coated activated charcoal (Sigma Chemical Co.,

St. Louis, MO) for 1 h at room temperature with rotation to remove steroid hormones. Centrifugation at 2060 x g for 30 min at 4°C was used to pellet the charcoal and remaining sera were filtered using 0.22 micron Millex-GS® syringe driven filter units (Millipore Corporation, Bedford, MA) to remove any residual charcoal. The final steroid-removed serum product (which will be referred to hereafter as charcoal-treated serum or CTS) was stored in 10-ml volumes at -20°C. Prior to storage, small aliquots of both PS and CTS were removed for assessment of steroid hormone concentrations.

Cortisol, 17β-estradiol, and progesterone concentrations in individual sera from all cows and in the PS, CTS, and a negative control serum (heat inactivated FBS) were assayed using Correlate-EIA Immunoassay kits (Assay Designs, Inc., Ann Arbor, MI). Each serum sample was assayed in duplicate.

D. Western blot analysis of steroid hormone receptors

The presence of glucocorticoid, estrogen, and progesterone receptors in steer neutrophils was assessed by Western blot analysis using whole cell extracts. Bovine endometrium extract (kindly provided by Dr. George Smith's laboratory) was used as a positive control sample. To obtain these extracts, neutrophils and endometrium tissue were suspended in 200 µl of cell disruption solution [0.34 M sucrose, 0.01 M Tris-HCl pH 6.8, 0.005 M EGTA, and Complete, Mini Protease Inhibitor Cocktail™ tablet (1 tablet per 10 ml disruption solution; Roche Applied Science; Indianapolis, IN)]. Sample buffer (50 µl; 5X buffer containing 0.25M Tris-HCl pH 6.8, 50% glycerol, 10% SDS) was added and samples were boiled for 10 min. Following centrifugation (10,000 x g for

10 min at room temperature) supernatants were transferred to new 1.5 ml microfuge tubes and stored until use at -20°C.

Total protein from the cell extracts (60 μg) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gels. Proteins were transferred overnight at 4°C to nitrocellulose membranes and blocked for 1 h at room temperature with 5% non-fat milk in TBST buffer (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20). After washing once with TBST buffer, membranes were incubated overnight at 4°C with anti-glucocorticoid receptor antibody (rabbit polyclonal IgG; cat# Pa1-511A; Affinity BioReagents, Golden, CO), anti-estrogen receptor antibody (mouse monoclonal IgG1; cat# MA1-310; Affinity BioReagents, Golden, CO), or anti-progesterone receptor antibody (mouse monoclonal IgG1; cat# MA1-410; Affinity BioReagents, Golden, CO) in TBST buffer containing 0.5% non-fat milk, washed again, and further labeled with detection antibody [(goat anti-rabbit IgG horseradish peroxidase conjugated; Pierce Biotechnology, Rockford, IL) or (goat anti-mouse IgG1 horseradish peroxidase conjugated; Bethyl Laboratories, Montgomery, TX)] added at room temperature for 1 h. Blots were developed using the SuperSignal West Pico chemiluminescent substrate system (Pierce Biotechnology), photographed, stripped, and re-probed with anti- β-actin antibody (mouse monoclonal IgG1; cat # AC-15: ab6276; Abcam Limited, Cambridge, MA) as a lane loading control (Weber et al., 2004).

E. *In vitro* culture experiments

Two main *in vitro* experiments were executed to determine if glucocorticoids in parturient serum contribute to delayed neutrophil apoptosis. In the first experiment, steer

neutrophil cultures were supplemented with increasing amounts of PS or CTS, added at 5, 10, 20, and 40% of the total volume of culture medium (no FBS included). The cultures were incubated at 39°C (normal body temperature for cattle) in 5% CO_2 for up to 24 h. Paired cultures of the neutrophils were also treated with RU486 (a glucocorticoid and progesterone receptor antagonist) or tamoxifen (an estrogen receptor antagonist) for 30 min prior to incubation with PS for up to 24 h. Hormone receptor antagonist treatments were used at 1000-fold excess to the cortisol or 17β-estradiol levels detected in PS. For experiment 2, CTS cultures were supplemented with cortisol, 17β-estradiol, and progesterone (all from Sigma) at levels found in PS. The synthetic glucocorticoid, dexamethasone (Schering Plough, Animal Health; Kenilworth, NJ), was also utilized as a glucocorticoid positive control treatment. Cultures supplemented with PS and CTS at optimal levels found in experiment 1 served as additional controls in this experiment. Additional cultures supplemented with 1% FBS and dexamethasone, 17β-estradiol, or progesterone at 10^{-7} M (the approximate level of cortisol in PS) were also prepared.

F. Apoptosis phenotyping

In the *in vitro* experiments described above, early apoptosis in the neutrophils was assessed at 0, 12, and 24 h of culture using dual annexin V-FITC/propidium iodide (PI) staining with fluorescence-activated flow cytometric analysis (Weyts et al., 1998; Zhang et al., 2001). Later stages of apoptosis, such as extensive DNA fragmentation, was assessed at 24 h of culture using PI staining of genomic DNA and fluorescence-activated flow cytometric analysis of hypodiploidy (Meagher et al., 1996).

Briefly, 0, 12, and 24 h neutrophils cultured with the various test sera were cold-shocked by placing the culture plates on ice for 5 min, and the cells transferred to 5-ml polysterene round bottom tubes (Becton Dickinson) at 5 x 10^5 cells/tube. These were assayed in duplicate for early apoptosis, using the protocol contained in a commercial kit (Annexin V-FITC Apoptosis Detection Kit; BD Biosciences Pharmingen, San Diego, CA), with data recorded as % non-apoptotic neutrophils (i.e., the percentage of cells negative for both annexin-V-FITC and PI in 2-color flow cytometric density dot plots). At 24 h, duplicate cultures of 5 x 10^5 of the variously treated neutrophils were also monitored flow cytometrically for late apoptosis by hypodiploidy analysis. These cells were washed twice in 500 µl of cold PBS and fixed for 20 min at 4°C using 200 µl of ice cold 70% ethanol. After gentle centrifugation (600 x g for 5 min at 4°C), the cell pellets were suspended in 200 µl of PBS that contained 1.3 mg/ml RNase A (Sigma) and 33 µg/ml PI (Sigma) for nuclear DNA staining, and incubated for 30 min at 4°C in the dark. Degree of apoptosis was detected flow cytometrically using one-color PI fluorescence histogram plots that displayed two peaks, one labeled as PI^{dim} (hypodiploid, or apoptotic cells) and the other as PI^{bright} (diploid, or non-apoptotic neutrophils).

G. Western blot analysis of A1 and Bak

In a third and final experiment, cytosolic abundance changes in A1 and Bak proteins in response to glucocorticoid administration *in vivo* was evaluated in blood neutrophils from three steers before and 9 h after injection of dexamethasone (0.1 mg/kg body weight; Weber et al., 2004). Neutrophil cytosolic fractions were prepared as described in Weber et al. (2004) and 40 µg of total cytosolic protein subjected to SDS-

PAGE using 15% gels (for A1 detection) or 12.5% gels (for Bak detection). Proteins were transferred to nitrocellulose membranes overnight at 4°C and blocked for 1 h at room temperature with SuperBlock™ Blocking Buffer in TBS (Pierce Biotechnology). After washing once with TBST buffer, membranes were probed with either anti-Bak antibody (rabbit polyclonal IgG, cat# H-211: sc7873, Santa Cruz Biotechnology, Inc) or anti-A1 antibody (rabbit polyclonal IgG, cat# FL-175: sc8351, Santa Cruz Biotechnology, Inc.) for 1 h at room temperature, washed again, and probed with detection antibody (goat anti-rabbit IgG horseradish peroxidase conjugated; Pierce Biotechnology) added at room temperature for 1 h. Blots were developed using the SuperSignal West Pico chemiluminescent substrate system (Pierce Biotechnology), photographed, stripped, and re-probed with anti- β-actin antibody (mouse monoclonal IgG_1; cat# AC-15: ab6276; Abcam Limited, Cambridge, MA) as a lane loading control (Weber et al., 2004). Resulting autoradiographs were analyzed with a scanning densitometer (GS-710 Calibrated Imaging Densitometer and Multi-Analyst Software; BioRad) and A1 and Bak protein abundance recorded as density ratios to β-actin.

H. Statistical analysis

Data are summarized in the results as least squares means ± SEM. Hormone data were statistically analyzed using the MIXED procedure of SAS (SAS Institute, 2000) where the model included a fixed effect of time relative to parturition and random effects of cow (n = 4) and cow x time interaction. Apoptosis phenotype data (% non-apoptotic or % hypodiploid neutrophils) were also analyzed using the MIXED procedure of SAS, this time the model included a fixed effect of treatment (PS, CTS, PS with receptor

antagonist, CTS with hormone added back, FBS with hormone, etc.) and random effects of steer (n = 2 to 4 depending on experiment) and steer x treatment interaction. Regression analysis was utilized to determine dose responsiveness of PS and CTS in the first *in vitro* experiment. Neutrophilia as well as A1 and Bak protein abundance changes due to *in vivo* dexamethasone treatment were analyzed by paired t-tests. Differences were considered significant at $P \leq 0.05$ in all experiments.

IV. RESULTS

A. Steroid hormone profiles in sera from parturient cows and presence of homologous hormone receptors in blood neutrophils of donor steers

Blood serum levels of cortisol, 17β-estradiol, and progesterone were measured to ensure that the sera to be used for all *in vitro* experiments had been collected from animals undergoing normal parturition. All hormones tested exhibited profiles similar to those observed in Weber et al. (2001) and no cow showed any signs of clinical disease. Mean serum cortisol concentration was significantly increased around parturition (day 0 and 0.25) and returned to pre-partum levels by day 1 post-partum (**Figure 3.1a**). Estradiol also was increased at the time of parturition (**Figure 3.1b**) whereas progesterone was very low at and after parturition relative to pre-partum concentrations (**Figure 3.1c**). Thus, the cow serum donors appeared to undergo normal parturition, as witnessed by their good health and normal fluctuations in serum concentrations of cortisol, estradiol, and progesterone.

In addition to measuring serum steroids, it was also important to determine if blood neutrophils from the donor steers (n = 2) expressed receptors for these hormones.

The representative blot in **Figure 3.1d** shows that steer neutrophils contain glucocorticoid receptor (GR) as well as low levels of estrogen receptor (ER). In contrast, the cells did not express either the A or B forms of the progesterone receptor (PR). Both GR and PR were present in endometrial cell extracts that served as a positive control. The unexpected lack of ER expression in this positive control tissue may have been due to the stage of the estrous cycle when the tissue was collected, which was performed at a slaughterhouse with no knowledge of the animal's stage of estrous. As no differences were detectable in the level of β-actin expression, it is not likely that the differences in PR expression observed between neutrophils and endometrium were due to differences in protein loading of the Western blot.

B. Charcoal treatment of parturient serum significantly reduced steroid hormone concentrations

Parturient cow blood serum (day 0) was pooled across the four donor animals and assayed in duplicate for cortisol, 17β-estradiol, and progesterone by EIA. Results indicated that the pooled parturient serum (PS) contained 29,000 pg/ml of cortisol, 1500 pg/ml of 17β-estradiol, and 900 pg/ml of progesterone (**Table 2.1**). Following charcoal treatment of the PS, resulting CTS contained considerably lower levels of these steroids, including 150 pg/ml of cortisol, 75 pg/ml of 17β-estradiol, and 110 pg/ml of progesterone. The negative control FBS contained low concentrations of cortisol (985 pg/ml), 17β-estradiol (869 pg/ml), and progesterone (370 pg/ml) relative to PS. Fetal bovine serum did not undergo charcoal treatment, as levels of all three hormones were less than that found in parturient serum (See **Table 3.1**). Therefore, charcoal treatment

successfully reduced steroid hormone levels in parturient cow blood serum to levels below those detected in serum collected seven days pre-partum.

C. Charcoal treatment of parturient serum removed its apoptosis delaying factor(s)

The effects of PS and CTS on blood neutrophil apoptosis were observed during *ex vivo* aging of the cells utilizing two independent flow cytometric assays. Representative flow cytometric data in **Figure 3.2** demonstrated that significantly more blood neutrophils were non-apoptotic at 12 h in culture (**Figure 3.2a**; $P < 0.001$) and had significantly less hypodiploidy at 24 h (**Figure 3.2b**; $P < 0.001$) when supplemented at 10% of the total culture volume with PS versus CTS. In addition, the delay in apoptosis induced by PS was significantly ($P < 0.05$) dose-dependent when PS was added at ranges from 5 to 40% of the total culture volume (**Figure 3.2 c** and **d**). No such dose-responsiveness was observed for the CTS (**Figures 3.2 c and d**). Thus, charcoal treatment of the parturient serum effectively removed the factor(s) responsible for its apoptosis delaying capacity.

D. The delay in neutrophil apoptosis afforded by culture in parturient serum was glucocorticoid receptor mediated

The GR and PR antagonist, RU486, and the ER antagonist, tamoxifen, were utilized to determine if the PS-induced delay in spontaneous neutrophil apoptosis was mediated through steroid hormone receptor activation. Neutrophils were treated with the antagonists for 10 min prior to an additional 12 to 24 h culture in 20% PS. The RU486 pre-treatment significantly inhibited the apoptosis delaying capacity of the PS (**Figure**

3.3). As PR was not detected in neutrophils from one of the donor steers used in this study (**Figure 3.1d**), it is unlikely that the observed inhibition of the PS-induced delay of neutrophil apoptosis was mediated through the progesterone receptor. Instead, RU486 likely blocked GR activation to inhibit this PS effect. Tamoxifen pretreatment did not affect the rate of neutrophil apoptosis (**Figure 3.3**). These experiments thus demonstrated that one factor in parturient serum capable of delaying neutrophil spontaneous apoptosis was the glucocorticoid, cortisol.

E. Glucocorticoid addition to charcoal treated parturient serum reconstituted its ability to delay neutrophil apoptosis

Glucocorticoid, 17β-estradiol, and progesterone were added individually to CTS at levels found in PS to determine if a single steroid hormone contributed to the neutrophil apoptosis delaying effect of PS. Serum supplementation was set at 20% of the total culture volume based on dose responsiveness to PS established in the first experiment (**Figure 3.2**). Reconstitution of CTS with either cortisol or dexamethasone (i.e., glucocorticoids) at the concentration of cortisol found in PS (~ 10^{-7} M) resulted in delayed neutrophil apoptosis and a reduction in hypodiploidy to near levels achieved with 20% PS (**Figures 3.4 a** and **b**). However, reconstitution of CTS with progesterone or estradiol at the concentrations found in PS had no effect on neutrophil apoptosis or hypodiploidy status. Additional neutrophil cultures were supplemented with 1% FBS and either dexamethasone, 17β-estradiol, or progesterone, each at a high dose of 10^{-7} M to validate that it was not merely the level of glucocorticoid added to CTS that delayed neutrophil apoptosis. Results showed that only dexamethasone significantly ($P < 0.001$)

delayed neutrophil apoptosis over 24 h in culture (**Figure 3.4c**). Thus, we are confident that the glucocorticoid component of parturient serum plays a significant role in survival induction in bovine blood neutrophils.

F. Neutrophil protein abundance of A1 and Bak was altered in glucocorticoid treated steers

Alterations in circulating leukocyte counts and neutrophil cytosolic A1 and Bak abundance in glucocorticoid (dexamethasone)-treated steers were assessed in a final experiment. Leukocyte counts in the whole blood were increased 9 h post-dexamethasone injection (**Figure 3.5a**). This increase was due to increased neutrophil numbers (Weber et al., 2004) and approached significance ($P = 0.067$). By 9 h post-dexamethasone administration, neutrophil cytosolic A1 protein abundance was increased ~ 2.4 fold relative to cells sampled prior to dexamethasone treatment ($P < 0.01$; **Figure 3.5b** and **c**), whereas a 5.4 fold decrease in Bak protein abundance was detected ($P < 0.01$; **Figure 3.5b** and **d**). Dexamethasone was shown previously to have no effect on β-actin protein abundance (Weber et al., 2004), an observation confirmed in the current study. Therefore, a glucocorticoid-induced abundance change in these key apoptosis regulatory proteins was detected and coincided with the increase in circulating leukocyte counts. The A1 and Bak protein changes observed were also consistent with changes in mRNA abundance for these genes in neutrophils obtained during the cortisol surge in parturient cows (Chapter Two; Madsen et al., 2004).

V. DISCUSSION

Bovine blood neutrophils exhibit a number of altered trafficking and anti-bactericidal functions at the time of parturition. In addition, and quite surprisingly, the cells also exhibit a pronounced pro-survival gene expression signature during parturition (Madsen et al., 2004; Burton et al., 2005). The goal of this study was to determine if steroid hormones in parturient cow serum play a role in delaying neutrophil apoptosis, and if steroid-induced changes in pro- and anti-apoptotic molecules of the Bcl-2 family may be involved. We determined for the first time that the high cortisol concentration in parturient serum was primarily responsible for delaying spontaneous apoptosis in steer blood neutrophils treated *in vitro*. Indeed, parturient levels of 17β-estradiol and progesterone had no effect on neutrophil apoptosis, perhaps due to the low and undetectable expression of ER and PR (respectively; **Figure 3.1**). In addition, the glucocorticoid effect on neutrophil apoptosis delay may be mediated through activation of GR, because it was steroid dose-dependent and inhibited by pretreatment of the cells with RU486 (**Figure 3.2** and **Figure 3.3**).

Direct effects of glucocorticoids on neutrophil spontaneous apoptosis were studied in a number of species in the mid 1990's. Our results agree with those studies, which demonstrated that glucocorticoids significantly delay spontaneous apoptosis of cultured human and rat neutrophils (Cox, 1995; Liles et al., 1995; Meagher et al., 1996; Nittoh et al., 1998). The absence of 17β-estradiol and progesterone effects on neutrophil apoptosis in our study coincides with previous work (Meagher et al., 1996; Nittoh et al., 1998). Several previous studies also demonstrated inhibition of the glucocorticoid effect on neutrophil apoptosis by RU486 (Cox, 1995; Meagher et al., 1996; Nittoh et al., 1998),

something clearly demonstrated using bovine neutrophils in the current study. What is novel about this study is the demonstration of effects of glucocorticoids in blood serum collected at a physiologically relevant time in the cow's production cycle, namely parturition, when neutrophilia predominates and cows are highly susceptible to exaggerated mammary inflammation in response to mastitis-causing pathogens (Kehrli and Harp, 2001; Burton and Erskine, 2003). Rapid apoptosis in neutrophils is required for timely resolution of inflammation during infectious insults (reviewed by Savill, 1997; Haslett, 1999; Simon, 2003), so it is possible that the 3-4 fold increase in neutrophil life span due to high circulating glucocorticoids contributes to the increased severity of mastitis in newly calved cows. Because manipulation of neutrophil apoptosis may be an easy and effective way to manage the inflammation of mastitis in peripartum cows, this possibility warrants testing in future studies.

In addition to our *in vitro* experiments described above, our previous studies indicated that bovine neutrophil expression of apoptosis regulatory genes was significantly altered in favor of cell survival in conjunction with the cortisol surge during parturition (Madsen et al., 2004; Burton et al., 2005). Increases in A1 mRNA and decreases in Bak mRNA at parturition significantly correlated with serum cortisol concentrations (A1: r-value = 0.90, $P = 0.002$; Bak: r-value = -0.82, $P = 0.01$; Burton et al., 2005), and as a result, we elected to evaluate protein abundance of A1 and Bak in glucocorticoid-treated steers to substantiate our previous gene expression observations. Dexamethasone treatment clearly increased neutrophil protein abundance of A1 while Bak protein abundance was decreased, thus demonstrating that glucocorticoids alter the program of spontaneous apoptosis in bovine blood neutrophils.

Normal blood neutrophils constitutively express Bak and A1 (Chuang et al., 1998; Bazzoni et al., 1999), but at differing ratios depending on the presence or absence of inducing and inhibiting factors in the vicinity of the cells (Moulding et al., 2001). Bak is a pro-apoptotic molecule that works at the mitochondria to induce pore formation for the release of cytochrome c and subsequent activation of caspase-9 (Degenhardt et al., 2002). In contrast, A1 is a potent anti-apoptotic molecule, the prominent Bcl-2 homologue in neutrophils, and inhibits cell death by blocking mitochondrial release of cytochrome c (Wang et al., 1999; Werner et al, 2002). High levels of A1 and low levels of Bak mRNA detected during the cortisol surge in our parturition study supports a hypothesis that glucocorticoids induce neutrophil survival via altered abundance of pro- and anti-apoptotic Bcl-2 family members (Madsen et al., 2004; Burton et al., 2005). Results from the current study substantiate this hypothesis by showing that glucocorticoids both delay neutrophil spontaneous apoptosis (**Figure 3.4**) and increase the ratio of cytosolic A1 to Bak following hormone administration (**Figure 3.5**). The observed changes in A1 and Bak proteins suggest that glucocorticoids may modulate neutrophil apoptosis by protecting mitochondria membrane integrity but this needs to be verified through appropriately designed experiments and its significance to animal health elucidated using parturient cows and other appropriate models of production-stressed cattle exposed to pathogens.

In light of our presented results, a question still remains as to what glucocorticoid-mediated extension in neutrophil life span and accompanying neutrophilia mean to dairy cows during the periparturient period. Reduced expression of neutrophil surface adhesion molecules substantially contributes to parturient neutrophilia (reviewed by

Burton and Erskine, 2003). This down-regulation has been demonstrated to be a result of high glucocorticoid concentrations at parturition (Weber et al., 2001) and is now known to be GR-mediated and occur at the gene transcription level in neutrophils (Weber et al., 2004; Burton and Weber, unpublished observations). However, our current results suggest that a glucocorticoid-induced delay in neutrophil apoptosis also may be a GR-mediated event that contributes to neutrophilia at parturition. Typically, parturient neutrophilia was thought to leave the mammary gland somewhat unprotected resulting in increased mastitis susceptibility. We offer an additional possibility that extended cell survival under the influence of cortisol may contribute to parturient neutrophilia and serve as a way to augment large numbers of potentially pro-inflammatory neutrophils in the circulation. In humans, massive recruitment of blood-derived neutrophils into the placenta, cervix, and uterus is essential for tissue remodeling processes that enable fetal membrane rupture, cervical dilation, fetal expulsion, and separation of the fetal and maternal membranes during parturition (Thomson et al., 1999; Winkler et al., 1999a, 1999b). It also has been shown that bovine placental tissue produces soluble factors at parturition that induce chemotactic responses and increased phagocytosis by bovine blood neutrophils (Hoedemaker et al., 1992a, 1992b). Thus, neutrophil lifespan extension by cortisol at parturition may be more related to the needs of the reproductive tract and placenta than to the well described dysfunctions that have been linked postpartum to mastitis susceptibility. The previously unexplained increases in neutrophil phagocytosis (Giudry et al., 1979; Cai et al., 1994) and depressed ROS production (Kehrli and Goff, 1989; Detilleux et al., 1994) at parturition also may be indicative of a need for longevity and heightened reproductive tissue remodeling capacity of the cells. If

this proves to be true, then parturition may occur at the temporary expense of neutrophil-mediated mammary defense against mastitis-causing bacteria, a possibility that is currently under study in our laboratory.

In summary, results from this study clearly supported our hypothesis and initial observations that the normal genetic program for spontaneous apoptosis is altered in such a way by glucocorticoids as to extend neutrophil life span. Current results further extend our previous work by demonstrating that the glucocorticoid component of parturient blood delays neutrophil apoptosis via homologous receptor activation and that glucocorticoid treatment significantly alters protein abundance ratios for A1 and Bak in favor of neutrophil survival. Extended survival may contribute to the neutrophilia of parturition and glucocorticoid treatment in cattle, possibly acting to increase the pool of circulating cells available for remodeling of tissues in need of such activity. Future studies that are beyond the scope of this dissertation will be necessary to address this novel hypothesis.

Figure 3.1 Serum concentrations of three steroid hormones of bovine parturition. In panel (a), concentrations of serum cortisol rise sharply at parturition and return to basal by day 1 of lactation. In panel (b), serum estradiol concentrations peak at parturition, and return to basal concentration by day 1 of lactation. In panel (c), serum progesterone concentrations are high before parturition but plummet at parturition. Data are presented as raw daily means (± SEM) with a, b, c relaying significant differences at $P < 0.05$. Western blot analysis of steroid hormone receptors is shown in panel (d) with E = endometrium (control tissue) and N = neutrophil. Panels (a), (b), and (c) n = 3; panel (d) representative blot of n = 2 steer's neutrophils.
[(a) is reprinted from Vet Immunol Immunopathol 105(3-4); Burton JL, Madsen SA, Chang LC, Weber PS, Buckham KR, van Dorp R, Hickey MC, Earley B; Gene expression signatures in neutrophils exposed to glucocorticoids: a new paradigm to help explain "neutrophil dysfunction" in parturient dairy cows; pp197-219; Copyright 2005 with permission from Elsevier.]

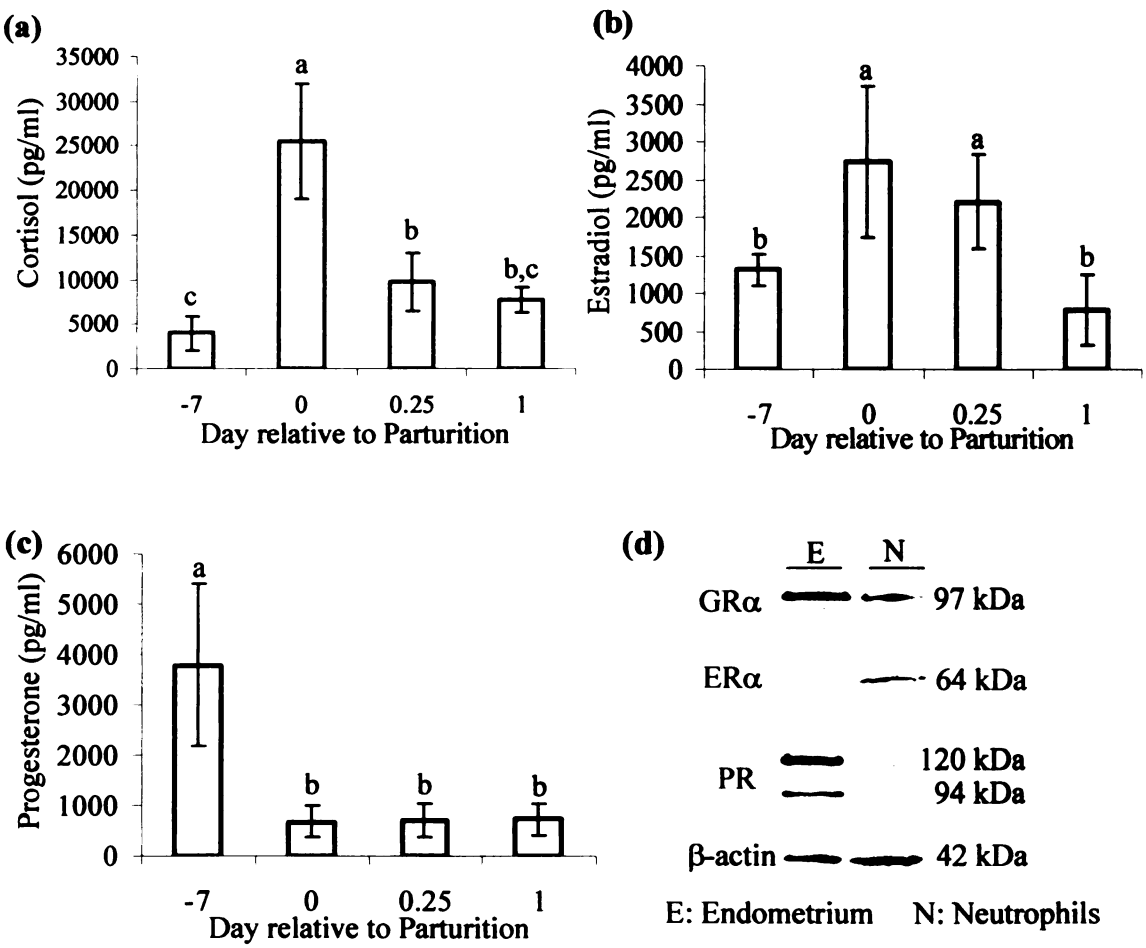


Table 3.1 Charcoal treatment of parturient cow blood serum significantly reduced steroid hormone concentrations to below those found in commercial fetal bovine serum.‡

	Parturient Serum	Charcoal Treated Parturient Serum	Fetal Bovine Serum
Cortisol	**29,000 pg/ml***	**150 pg/ml**	**985 pg/ml**
17β-Estradiol	**1500 pg/ml**	**75 pg/ml**	**869 pg/ml**
Progesterone	**900 pg/ml**	**110 pg/ml**	**370 pg/ml**

*** Equivalent to approximately 10^{-7}M**

‡Reprinted from Vet Immunol Immunopathol 105(3-4); Burton JL, Madsen SA, Chang LC, Weber PS, Buckham KR, van Dorp R, Hickey MC, Earley B; Gene expression signatures in neutrophils exposed to glucocorticoids: a new paradigm to help explain "neutrophil dysfunction" in parturient dairy cows; pp197-219; Copyright 2005 with permission from Elsevier.

Figure 3.2 Charcoal treatment of parturient serum removes its dose dependent apoptosis delaying effects on neutrophils. Panels (a) and (b) display representative flow cytometric plots from two independent apoptosis phenotyping assays. Panel (a): Two-color density dot plots were used to assess early apoptotic events in neutrophils following 12 hours of culture in parturient serum (PS, left plot) or charcoal treated parturient serum (CTS, right plot). Dots in the lower left quadrants represent neutrophils negative for both annexin V and propidium iodide staining, indicating non-apoptotic cells. Panel (b): Fluorescence histograms were utilized for DNA fragmentation detection (late apoptosis) in neutrophils at 24 hours of culture. Healthy neutrophils stained with propidium iodide following permeabilization and RNase A treatment contain mostly diploid DNA (PI^{BRIGHT} peak). Apoptotic cells contain hypodiploid, or fragmented, DNA as is evidenced by the left shift of the fluorescence peak (PI^{DIM} peak). Panels (c) and (d) summarize such data for neutrophils from steers (n = 2) assayed in duplicate. PS (gray bars) and CTS (white bars) were supplemented at increasing levels of the total culture volume. % Non-apoptotic neutrophils was significantly increased (panel c) and % hypodiploid neutrophils was significantly decreased (panel d) in the presence of increasing PS. No change was detected between varying levels of CTS but significant differences between PS and CTS began to occur with 10% serum in both assays.
a, b, c significantly different at $P < 0.05$ across treatment dose;
* significantly different at $P < 0.05$ between treatments.
[(a) and (b) reprinted from Vet Immunol Immunopathol 105(3-4); Burton JL, Madsen SA, Chang LC, Weber PS, Buckham KR, van Dorp R, Hickey MC, Earley B; Gene expression signatures in neutrophils exposed to glucocorticoids: a new paradigm to help explain "neutrophil dysfunction" in parturient dairy cows; pp197-219; Copyright 2005 with permission from Elsevier.]

Figure 3.2

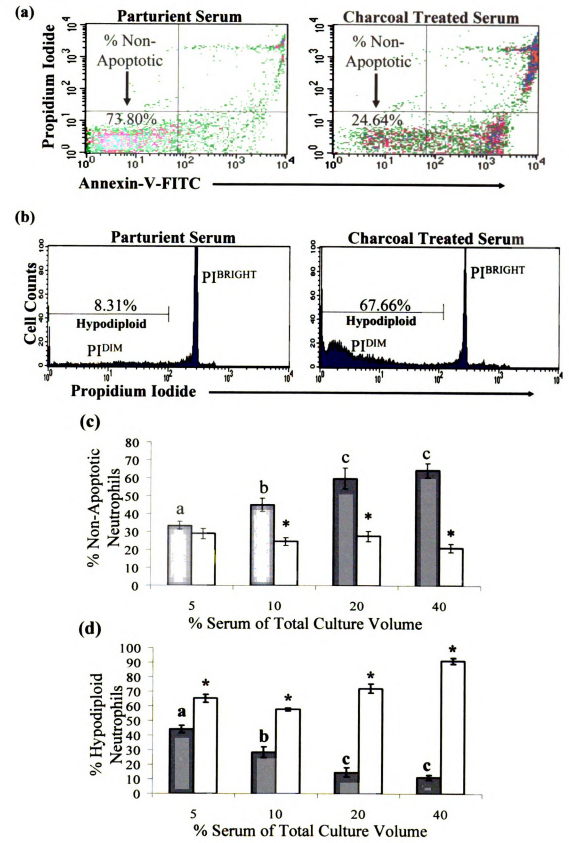

(a)
Parturient Serum
Charcoal Treated Serum
Propidium Iodide
% Non-Apoptotic
73.80%
24.64%
Annexin-V-FITC
(b)
Parturient Serum
Charcoal Treated Serum
Cell Counts
PI BRIGHT
PI DIM
8.31%
67.66%
Hypodiploid
Propidium Iodide
(c)
% Non-Apoptotic Neutrophils
% Serum of Total Culture Volume
(d)
% Hypodiploid Neutrophils
% Serum of Total Culture Volume

Figure 3.3 Glucocorticoid-delayed apoptosis in bovine neutrophils is mediated by the glucocorticoid receptor. Treatment of neutrophils with the glucocorticoid and progesterone receptor antagonist, RU486 (10^{-4} M concentration), prior to culture with PS (right hatched bars) reduced the apoptosis delaying effects of PS (gray bars) to the level found when cultures were supplemented with CTS (white bars). Panel (a) depicts % non-apoptotic neutrophils where as % hypodiploid neutrophils are found in panel (b). Treatment of the cells with the estrogen receptor antagonist, tamoxifen (10^{-6} M concentration; checkered bars), did not alter the level of apoptosis. (Steer n = 2)
a,b,c Bars are significantly different at $P < 0.05$.
[Reprinted from Vet Immunol Immunopathol 105(3-4); Burton JL, Madsen SA, Chang LC, Weber PS, Buckham KR, van Dorp R, Hickey MC, Earley B; Gene expression signatures in neutrophils exposed to glucocorticoids: a new paradigm to help explain "neutrophil dysfunction" in parturient dairy cows; pp197-219; Copyright 2005 with permission from Elsevier.]

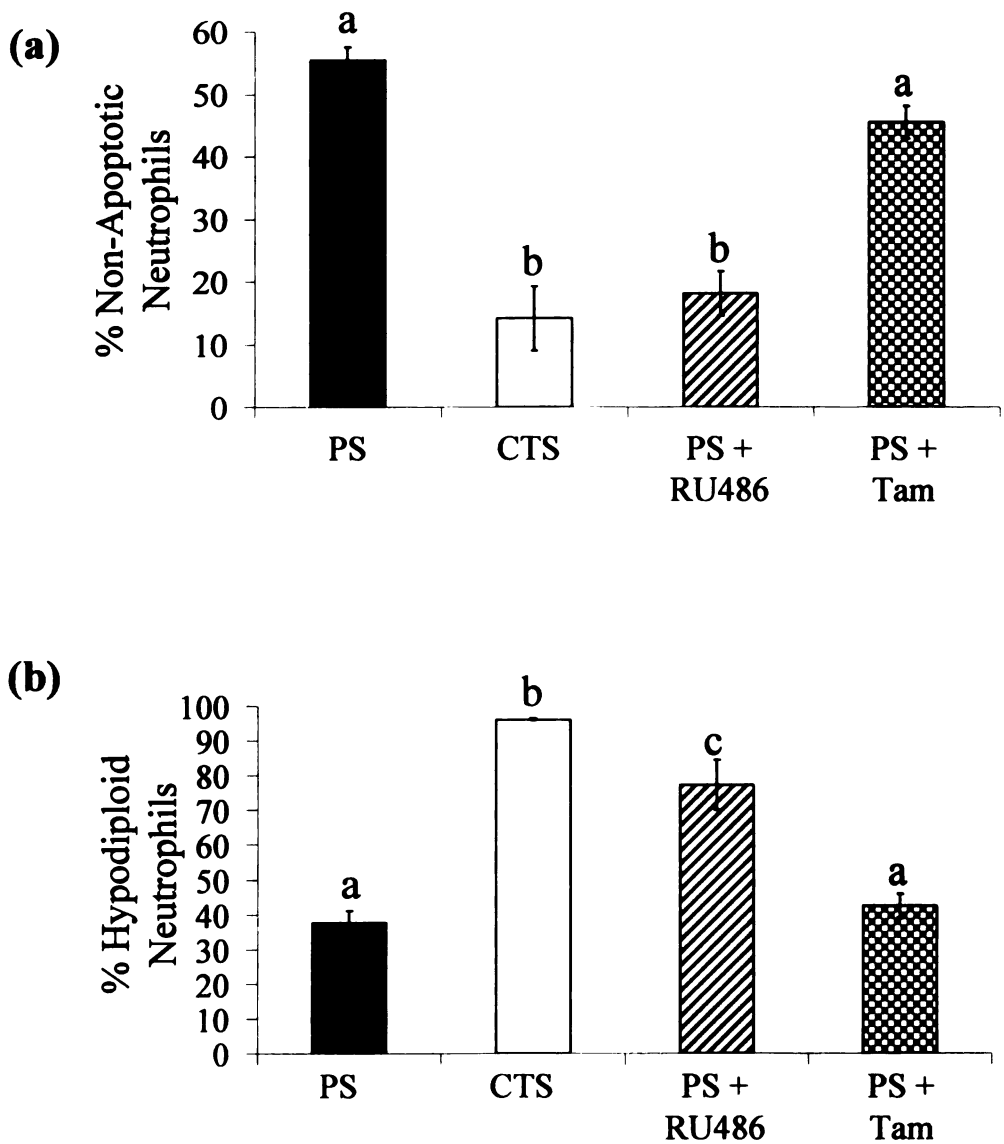


Figure 3.4 Glucocorticoid supplementation of steroid-stripped parturient serum (CTS) reconstituted its ability to delay neutrophil apoptosis to levels near those observed with intact parturient serum (PS). Panel (a) displays % non-apoptotic neutrophils following 12 hours of culture whereas panel (b) depicts % hypodiploid neutrophils at 24 hours in culture. Addition of dexamethasone or cortisol to CTS resulted in suppression of neutrophil apoptosis similar to that observed in neutrophils treated with PS. Treatments: PS (gray bars), CTS (white bars), CTS + glucocorticoid (dexamethasone = hatched bars; cortisol = horizontal striped bars), CTS + 17β-estradiol (checkered bars), and CTS + progesterone (vertical striped bars). Hormone additions to the CTS were at levels found in parturient blood serum (see Table 1). Neutrophil apoptosis was also assessed in cultures supplemented with 1% FBS ± hormones at 10^{-7}M. Panel (c) demonstrates that addition of dexamethasone (hatched bar) but not 17β-estradiol (checkered bar) or progesterone (vertical striped bar) to 1% FBS containing culture medium (gray bar) resulted in a profound apoptosis delay at 24 hours in culture. (Steer n = 2)
a, b Bars significantly different at $P < 0.05$.
[(a) and (b) reprinted from Vet Immunol Immunopathol 105(3-4); Burton JL, Madsen SA, Chang LC, Weber PS, Buckham KR, van Dorp R, Hickey MC, Earley B; Gene expression signatures in neutrophils exposed to glucocorticoids: a new paradigm to help explain "neutrophil dysfunction" in parturient dairy cows; pp197-219; Copyright 2005 with permission from Elsevier.]

Figure 3.4

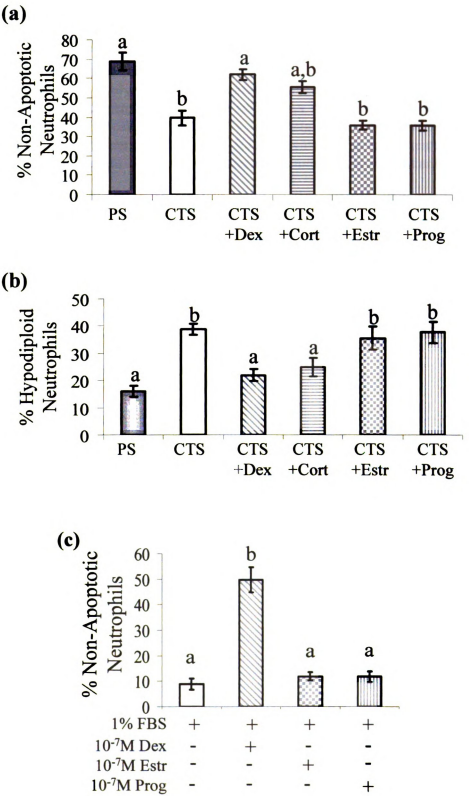
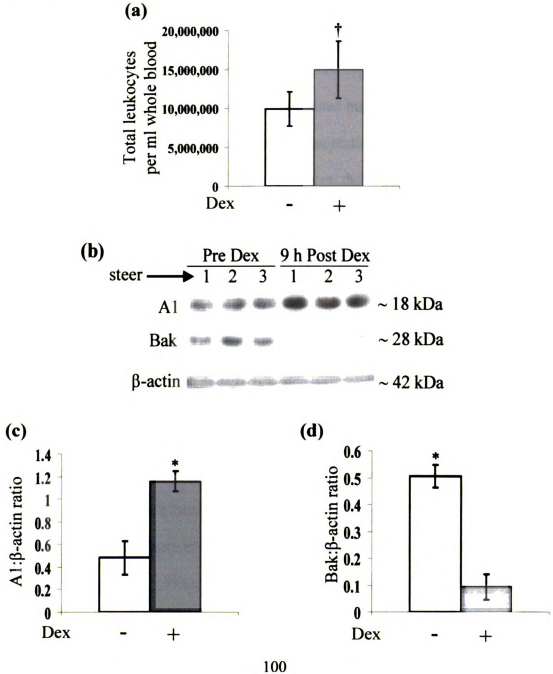

(a)
% Non-Apoptotic Neutrophils
80
70
60
50
40
30
20
10
0
a
b
a
a,b
b
b
PS
CTS
CTS +Dex
CTS +Cort
CTS +Estr
CTS +Prog
(b)
% Hypodiploid Neutrophils
50
40
30
20
10
0
a
b
a
a
b
b
PS
CTS
CTS +Dex
CTS +Cort
CTS +Estr
CTS +Prog
(c)
% Non-Apoptotic Neutrophils
60
50
40
30
20
10
0
a
b
a
a
1% FBS + + + +
10-7M Dex - + - -
10-7M Estr - - + -
10-7M Prog - - - +

Figure 3.5 Dexamethasone administration into steers altered the abundance of A1 and Bak proteins in circulating neutrophils. Total leukocyte numbers in whole blood were increased ($P = 0.067$) in steers after dexamethasone treatment (panel a) due to increased numbers of neutrophils (Weber et al., 2004). The Western blots in panel (b) shows that A1 protein was increased whereas Bak protein was decreased in neutrophils 9 h following dexamethasone administration. β-actin did not change (steer n = 3). Autoradiographs were analyzed with a scanning densitometer and A1 and Bak protein abundance recorded as density ratios to β-actin. As shown in panel (c), A1 protein exhibited a 2.4-fold increase in neutrophils following dexamethasone administration. Bak, on the other hand, demonstrated a 5.4-fold decrease in the same neutrophils (panel d). Differences in protein abundance between treatments was significant at $P < 0.05$ (designated by *).



CHAPTER FOUR

Glucocorticoid Modulation of Bcl-2 Family Members A1 and Bak during Delayed Spontaneous Apoptosis of Bovine Blood Neutrophils

I. ABSTRACT

Neutrophils play a pivotal role as the first line of host cellular immune defense against bacteria but can also cause significant tissue pathology if their bactericidal activities and life span are not tightly regulated. Glucocorticoids are known to delay spontaneous apoptosis in neutrophils from humans, rodents, and cattle but mechanisms involved are unknown. In the current study it was hypothesized that glucocorticoids directly alter neutrophil apoptotic status via maintained mitochondrial membrane stability under a scenario of increased abundance of anti-apoptotic A1 and decreased abundance of pro-apoptotic Bak, two Bcl-2 family members that are important in regulating neutrophil spontaneous apoptosis. To address this hypothesis, bovine blood neutrophils were aged *ex vivo* and treated with dexamethasone, a potent glucocorticoid, with and without glucocorticoid receptor antagonism via RU486 pretreatment. Optimal doses of glucocorticoid had pronounced protective effects on mitochondrial membrane stability and were associated with steroid-induced reductions in caspase-9 activation and delayed apoptosis. These dexamethasone effects were reversed by RU486. Glucocorticoid treatment also altered abundance of A1 and Bak mRNA in favor of delayed apoptosis. Effects on Bak mRNA were reversed by RU486 pretreatment while A1 mRNA was unexpectedly increased by glucocorticoid receptor antagonism. Glucocorticoid-mediated increases in A1 mRNA were reflected in A1 protein changes, but not Bak which is a relatively stable protein. Thus, glucocorticoid modulation of neutrophil spontaneous

apoptosis putatively occurs at the level of mitochondrial membrane stability and may involve altered abundance of Bcl-2 family members A1 and Bak.

II. INTRODUCTION

Neutrophils are granulocytic leukocytes integral in the first line of immune defense against invading bacteria. Equally important to their bactericidal activities is the tight regulation of their death, which occurs rapidly under normal physiological scenarios by a process of spontaneous (mitochondrial-induced) apoptosis and prevents unnecessary inflammatory tissue damage. Accordingly, neutrophils have a short half-life of approximately 6 to 10 h in blood (Cartwright et al., 1964; Dancey et al., 1976) and 24 to 48 h in infected tissues (Homburg and Roos, 1996). As neutrophils age in the circulation during homeostasis, they display apoptotic features and are cleared in the spleen and liver as intact cells by the body's phagocytic networks without inducing systemic inflammation (Savill et al., 1989; Haslett, 1999). The clearance of apoptotic neutrophils in infected tissue is also necessary to limit release of cytotoxic oxygen free radicals and proteolytic enzymes that would lead to further tissue damage. In fact, timely apoptosis of neutrophils is a key event in the resolution of inflammation during tissue infection and trauma (Savill and Haslett, 1995; Haslett, 1999). Rapid clearance of apoptotic tissue neutrophils by neighboring macrophages is an appropriate fate for the cells, which become deficient in chemotaxis, phagocytosis, and degranulation as they age and are thus poor defenders against infectious pathogens (Narayanan et al., 1997; Tanji-Matsuba et al., 1998). This is in contrast to non-apoptotic neutrophils, which are highly active in

these important disease-fighting functions but also capable of causing significant inflammatory tissue damage (Boutet et al., 2004; Garlichs et al., 2004).

Neutrophil apoptosis is regulated by both intrinsic and extrinsic signaling pathways. Whereas extrinsic signals are initiated by death receptor ligation at the cell's surface, intrinsic signaling is controlled at the level of mitochondria by several Bcl-2 family members (Simon, 2003). Bak and Bax are two pro-apoptotic family members known to be expressed in neutrophils (Santos-Beneit and Mollinedo, 2000, Moulding et al., 2001). In contrast to most other cell types that express Bcl-2 as the prototypical anti-apoptotic protein, mature neutrophils utilize family members A1 and Mcl-1 as their main survival inducing Bcl-2 family members. Mcl-1 has been shown to be up-regulated in neutrophils during cytokine-induced apoptosis delays (Moulding et al., 2001). Knockout of Mcl-1 results in peri-implantation embryonic lethality (Rinkenberger et al., 2000) and Mcl-1 down-regulation in Jurkat cells induces apoptosis (Nencioni et al., 2005) but does not affect HeLa cell apoptosis. Effects of Mcl-1 loss on neutrophil apoptosis have yet to be elucidated. On the other hand, the importance of A1, Bak, and Bax in neutrophil spontaneous apoptosis has begun to be revealed through the utilization of knockout mice (reviewed by Simon, 2003). Abolishment of A1 function results in accelerated neutrophil apoptosis (Hamaski et al., 1998) while double knockouts for Bak and Bax exhibited increased blood neutrophil numbers (Lindsten et al., 2000). Thus, regulation of A1, Bak, and (or) Bax appear to be critical control points in the regulation of neutrophil spontaneous apoptosis.

In most cell systems it is the ratio of anti-apoptotic to pro-apoptotic Bcl-2 family members that is critical in the determination of apoptotic status (Dragovich et al., 1998).

When pro-apoptotic members such as Bak are in excess, this signifies a state of apoptosis. In contrast, high levels of anti-apoptotic members, including A1, results in heterodimerization with pro-apoptotic members (Holmgreen et al., 1999; Werner et al., 2002) limiting their death-initiating actions and thereby delaying apoptosis. This life/death balance is in constant flux in neutrophils and is highly dependent on the cytokine and steroid milieux of their surrounding blood or tissue environment.

During neutrophil homeostasis, Bak and Bax facilitate apoptosis by forming homo-oligomers to create pores in mitochondria outer membranes (Degenhardt et al., 2002). These pores allow for the release of cytochrome c from the intermembrane space into the cytosol where it comes together with apoptotic protease activating factor-1 (Apaf-1), ATP, and procaspase-9 to form apoptosomes. This results in procaspase-9 cleavage and activation, which in turn activates caspase-3. Caspase-3 affects downstream apoptotic events that culminate in plasma membrane instability and nuclear demise (Nunez et al., 1998; Roy and Cardone, 2002). This spontaneous apoptosis pathway can be altered at multiple points as well as amplified during death receptor ligation through a BH3-only Bcl-2 family member called Bid (Figure 1.1; Wei et al., 2000; Maianski et al., 2004b). Ultimately, it is changes in the Bak/Bax death-initiation events at the mitochondria that are often seen in neutrophils during cytokine-induced cell survival.

During an inflammatory response, neutrophil apoptosis is delayed for 24 to 48 h. Pro-inflammatory factors known to delay neutrophil apoptosis include the cytokines G-CSF, GM-CSF, IL-8, TNF-α, and IFN-γ, as well as LPS (reviewed by Simon, 2003; Maianski et al., 2004a). Several of these factors also change the relative proportions and

(or) subcellular locations of expressed Bcl-2 family members that, in general, favor neutrophil survival. For example, neutrophil A1 mRNA increased 1.5 to 4-fold following exposure to G-CSF, GM-CSF, TNF-α, and IFN-γ (Chuang et al., 1998; Moulding et al., 2001). Exposure to LPS increased blood neutrophil A1 mRNA and protein levels 1.5 to 4-fold (Chuang et al, 1998; Moulding et al., 2001; Kotani et al., 2003). A 100-fold increases in A1 mRNA and 1.5 to 2-fold increases in A1 protein was observed in lung neutrophils of LPS treated mice (Kupfner et al., 2001). Decreases in Bax protein levels also were observed following GM-CSF (Weinmann et al., 1999) and TNF-α (Moulding et al., 2001) stimulation of neutrophils whereas Bak abundance remained relatively unchanged. That said, IL-8 treatment of isolated neutrophils conditions medium in such a way as to decrease Bak expression in freshly isolated cells (Grutkoski et al., 2002). In some experiments, it was not protein abundance changes but subcellular location of Bcl-2 family members that impacted neutrophil apoptotic status. For example, Bax protein levels did not change following G-CSF treatment of neutrophils but Bax translocation from the cytosol to mitochondrial membranes was drastically reduced (Maianski et al., 2002). In total, more inflammatory mediators appeared to target A1 than Bak and Bax in such studies.

It is perhaps not surprising that many more inflammatory factors target A1 expression over Bak expression in delaying neutrophil apoptosis because anti-apoptotic Bcl-2 family members are generally very labile (A1 and Mcl-1 mRNA half-life ~3 h, Mcl-1 protein half-life ~6 h) relative to pro-apoptotic family members (Bak and Bax protein half-life >22 h; Moulding et al., 2001). In addition, A1 is inducible through NF-κB signaling (Wang et al., 1999; Kupfner et al., 2001), which is readily activated in

neutrophils during exposure to pro-inflammatory cytokines and LPS (Chen et al., 2001). Thus, it may be more efficient for neutrophils to alter relative ratios of pro- to anti-apoptotic Bcl-2 family members and consequently apoptotic status by altering A1 expression. When neutrophils have served their purpose of pathogen clearance during the acute inflammatory event, the A1:Bak and A1:Bax ratios revert back to levels that favor apoptosis and the mitochondrial death pathway is re-initiated.

Despite their well-known anti-inflammatory properties, glucocorticoids have been identified as potently anti-apoptotic in neutrophils (Chapter Three; Cox, 1995; Liles et al., 1995; Meagher et al., 1996; Nittoh et al., 1998; Chang et al., 2004; Burton et al., 2005). Although it has been demonstrated that glucocorticoid-induced delay in neutrophil apoptosis requires new gene expression and protein synthesis (Cox and Austin, 1997), specific molecular information about how glucocorticoids achieve this delay in spontaneous cell death is unavailable. We have demonstrated in two *in vivo* models of glucocorticoid challenge, bovine parturition (Chapter Two) and administration of dexamethasone (Chapter Three) that neutrophil expression of A1 is increased and Bak decreased in conjunction with neutrophilia. Thus, we hypothesized in the current study that glucocorticoids directly alter neutrophil apoptotic status via maintained mitochondrial membrane stability with concurrent changes in A1 and Bak abundance.

III. MATERIALS AND METHODS

A. Animals and blood neutrophil isolations

Bovine neutrophils for this study were obtained as needed from five young male Holstein donors that were castrated at 1 month of age (steers). The steers were 3 to 6

months of age during the blood collection period. All animals were fed and housed according to standard operating procedures at the Dairy Teaching and Research Facility and their use for the described experiments was approved by the All University Committee on Animal Care and Use (approval # 07/04-104-00), both organizations of Michigan State University.

Blood was drawn by jugular venipuncture into ACD anti-coagulant and neutrophils were isolated according to our published Percoll density gradient centrifugation protocol (Weber et al., 2004). Total neutrophils were enumerated by electronic counting using a Z1 Coulter Particle Counter system (Beckman Coulter Particle Characterization, Miami, FL). Purity of all neutrophil preparations was always greater than 95% as assessed flow cytometrically (FACSCaliber flow cytometer and CellQuest software; Becton Dickinson, San Jose, CA) using our published G1 immunostaining protocol (Weber et al., 2001). In this assay, neutrophils are distinguished from other cell types by their high granularity and intense G1 staining on side scatter versus fluorescence density dot plots. The primary antibody used was clone MM20A (VMRD, Pullman, WA) and the secondary antibody was goat anti-mouse IgG_1 (Code # M32004; Caltag Laboratories, Burlingame, CA).

B. Culture of bovine blood neutrophils

Isolated neutrophils were cultured in basic medium consisting of RPMI 1640 (Invitrogen, Carlsbad, CA), 1.0% fetal bovine serum (low endotoxin FBS; Hyclone, Logan, UT), and 25 Units/ml penicillin plus 25 μg/ml streptomycin (Invitrogen Life Technologies, Carlsbad, CA). Cultures were incubated at 39°C (normal body

temperature for cattle) in humidified 5% CO_2 for up to 24 h with or without added glucocorticoid. The glucocorticoid used was dexamethasone (Azium; Schering Plough, Animal Health, Kenilworth, NJ) because it binds with high specificity and affinity to glucocorticoid receptors (Miller et al., 1994). Hormone action was antagonized in relevant experiments by neutrophil pretreatment (30 min) with mifepristone (RU486; Sigma Chemical Co., St. Louis. MO). Concentrations of dexamethasone and RU486 used are described in the various experiments below.

C. Apoptosis dose response to glucocorticoid receptor agonism and antagonism

Neutrophil apoptosis was assessed by dual annexin V-FITC/propidium iodide staining with fluorescence-activated flow cytometric analysis (Madsen et al. 2004). To determine glucocorticoid dose responsiveness of the delay in apoptosis, duplicate cultures of 5 x 10^5 neutrophils were treated with increasing molar concentrations of dexamethasone (0, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M) in 96-well culture plates. At each assay time point (0, 6, 12, and 24 h), culture plates were placed on ice for 5 min to ensure maximum neutrophil retrieval from the wells. Neutrophils were transferred to 5-ml polysterene round bottom tubes (Becton Dickinson) and assayed for apoptosis using the protocol contained in a commercial kit (Annexin V-FITC Apoptosis Detection Kit; BD Biosciences Pharmingen, San Diego, CA). Resulting flow cytometric data were recorded as % non-apoptotic (i.e., viable) neutrophils, which were the percentage of cells that were negative for both annexin V-FITC and propidium iodide staining in 2-color density dot plots (Madsen et al., 2004). Apoptosis was also assessed in neutrophils pre-treated for 30 min with the glucocorticoid receptor antagonist, RU486, at 10-, 100-, or 1000-fold

amounts relative to the optimal dose of dexamethasone. The most favorable doses of dexamethasone and RU486 were used in all subsequent experiments.

D. Assessment of neutrophil apoptotic status by mitochondrial membrane staining

Mitochondria membrane integrity was assessed in fresh cells and following treatment of the cells with 0 or 10^{-7} M of dexamethasone for 12 h using MitoTracker® Green FM staining (Invitrogen-Molecular Probes, Carlsbad, CA). Neutrophils, 2.5 x 10^5 cells per treatment, were incubated for 30 min in a 5% CO_2 incubator at 39°C with 100 nM MitoTracker® Green FM (Maianski et al., 2002). Cells were centrifuged at 500 x g, suspended in basic medium, and cytocentrifuged onto glass microscope slides (Shandon Cytocentrifuge; Thermo Shandon Cytospin 4, Pittsburgh, PA) prior to microscopic evaluation with a Leica DM IL Microscope fitted with a Leica DFC480 Digital Camera system (Leica Microsystems Inc., Bannockburn, IL).

E. Assessment of neutrophil apoptotic status by caspase-9 activity

Neutrophil caspase-9 activity was assessed after dexamethasone treatment (0 or 10^{-7} M) ± RU486 (10^{-6} M) for 0, 0.75, 1.5, 3, 6, and 9 h using a commercially available assay (APOPCYTO™ Caspase-9 Colorimetric Assay Kit; MBL International Corporation, Woburn, MA). Neutrophils incubated for 3 h with and without 100 ng/ml of soluble Fas ligand (recombinant human sFasL; Axxora Life Sciences, San Diego, CA) served as the positive control in this assay. At each time point, 1 x 10^7 neutrophils were transferred from 12-well culture plates to microfuge tubes and pelleted by centrifugation

(500 x g) for 5 min at 4°C. Supernatants were discarded and the cell pellets stored at -80°C until the time of assay.

A follow-up experiment was conducted to assess whether dexamethasone decreased caspase-9 activity levels to levels observed with a known caspase-9 inhibitor and to begin evaluating whether caspase-8 activation contributed to caspase-9 activation during spontaneous apoptosis. Neutrophils from a single steer were treated *in vitro* with the following: dexamethasone (10^{-7} M), caspase-9 inhibitor (50 μM z-LEHD-fmk; Calbiochem, La Jolla, CA), and caspase-8 inhibitor (50 μM z-IETC-fmk; Calbiochem, La Jolla, CA) with and without dexamethasone. During the final 3 h of incubation, sFasL was added to neutrophils treated with and without caspase-8 inhibitor ± dexamethasone. After 9 h of total culture time, neutrophil cell pellets were collected and stored as described above.

Assessment of caspase-9 activity was performed in duplicate according to the manufacturer's protocol. Briefly, cell pellets were thawed on ice for 15 min, suspended in 115 μl ice cold Cell Lysis Buffer, and incubated on ice for 10 min. Samples were centrifuged at 10,000 x g for 5 min at 4°C to pellet debris and supernatants (cell lysates) were transferred to new tubes. The following were then added to the wells of a flat-bottom 96-well plate: 50 μl/well of the various cell lysates, 50 μl/well of 2X reaction buffer containing 0.01 M DTT, and 5 μl/well of 0.01 M LEHD-*p*-nitroanilide (*p*NA) substrate. Plates were incubated at 37°C for 19.5 h after which the absorbance of *p*NA freed by caspase-9 activity was measured at 405 nm (Benchmark Plate Reader and Microplate Manager III Analysis Software; BioRad Laboratories, Hercules, CA). Specific activity was calculated using a standard curve for absorbance of *p*NA (supplied

with the kit) and total cell lysate protein added to each well was determined by Lowry assay. Caspase-9 activity was normalized against total protein added per well.

F. RNA isolation and quantitative real-time RT-PCR analysis of A1 and Bak mRNA

Analysis of A1 and Bak mRNA abundance was assessed in neutrophils cultured with and without dexamethasone (10^{-7} M) ± RU486 (10^{-6} M) for 0, 1, 2, and 4 h. Neutrophils were lysed in TRIzol Reagent (Invitrogen) at a concentration of 1 x 10^7 cells per milliliter TRIzol for 10 min at room temperature and stored in the same reagent at -80°C. RNA was isolated according to the manufacturer's instruction and treated with RQ1 RNase-Free DNase (Promega, Madison, WI) as described by the manufacturer. Total RNA concentration and purity were determined with a ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). cDNA was synthesized from 2 μg of total RNA using Superscript II RNaseH$^-$ Reverse Transcriptase (Invitrogen Life Technologies).

Quantitative real time RT-PCR was performed using the SYBR Green PCR Master Mix system for real time fluorescence detection in a PE7700 thermal cycler (Perkin Elmer Applied Biosystems; Foster City, CA) and gene-specific primers for A1 and Bak (Madsen et al, 2004). A1 and Bak PCR amplicons used for the development of standard curves in these assays were created with the following primers:

A1 (284 bp): forward primer 5'CCAGGCAGAAGATGACAG3'

reverse primer 5'GGTTACAATCCTGCCCCAGTT3'

Bak (306 bp): forward primer 5'AGGAGCAGGTAGCCCAGGAC3'

reverse primer 5'CCAGTTGATCCGCTCTCAAAC3'

The PCR products were amplified in a reaction mixture containing 1X PCR buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM forward primer, 0.2 μM reverse primer, 35 ng cDNA template (from bovine leukocytes), and 1 U per reaction Taq DNA polymerase (Invitrogen), brought to a final volume of 25 μl with sterile Milli-Q water. Reaction conditions used to generate A1 and Bak amplicons were: denature at 95°C for 5 min followed by 35 cycles of: 95°C for 30 seconds (denature), 52°C for 30 seconds (anneal), and 72°C for 30 seconds (extend), and a final extension at 72°C for 10 min. The resulting single band amplicons were gel purified, ligated into the pGEM-T Easy vector (Promega) and the recombinant plasmids were transformed into JM109 competent *E. coli* cells (Promega). Positive clones containing the A1 and Bak cDNA inserts were selected by blue/white colony screening and confirmed by DNA sequence analysis. Plasmids from white colonies were isolated using the Mini-prep Plasmid DNA Isolation kit (Promega; Madison, WI). A1 and Bak were then amplified from their respective plasmids with the primers described above and gel purified prior to dilution for their use as templates in the Q-RT-PCR standard curves. Seven concentrations of the A1-284bp amplicon (10^{-12} to 10^{-18} g/μl), five concentrations of the Bak-306bp amplicon (10^{-12} to 10^{-16} g/μl), or 20 ng of neutrophil cDNA were added as templates to Q-RT-PCR reaction mixtures that contained Q-RT-PCR primers (A1 or Bak from Madsen et al., 2004; see Chapter Two) and SYBR Green PCR Master Mix. A1 and Bak mRNA abundance in treated neutrophil samples was calculated using the equation for the linear standard curves for A1-284bp and Bak-306bp, which were plotted as the number of PCR cycles to threshold (Ct) versus starting amplicon concentration (in femtograms, fg). All reactions, including a negative control (no cDNA template) and were run in triplicate.

G. Western blot analysis of A1 and Bak

To assess protein abundance changes of A1 and Bak, neutrophils were cultured for 9 h in the presence or absence of dexamethasone (10^{-7} M) ± RU486 (10^{-6} M). Neutrophil cytosolic fractions were prepared as described (Weber et al., 2004). Pellets obtained from the 100,000 x g centrifugation step were suspended in sample buffer (200 µl; 1X buffer containing 0.05 M Tris-HCl pH 6.8, 10% glycerol, 2% SDS) and boiled for 10 min. Following centrifugation (10,000 x g for 10 min at room temperature) supernatants were transferred to new 1.5 ml microfuge tubes and stored until use at -20°C. This preparation resulted in the neutrophil organelle fraction. To obtain whole cell extracts, neutrophil pellets were suspended in 200 µl of cell disruption solution [0.34 M sucrose, 0.01 M Tris-HCl pH 6.8, 0.005 M EGTA, and Complete, Mini Protease Inhibitor Cocktail™ tablet (1 tablet per 10 ml disruption solution; Roche Applied Science; Indianapolis, IN)]. Sample buffer (50 µl; 5X buffer containing 0.25M Tris-HCl pH 6.8, 50% glycerol, 10% SDS) was added and samples were boiled for 10 min. Following centrifugation (10,000 x g for 10 min at room temperature) supernatants were transferred to new 1.5 ml microfuge tubes and stored until use at -20°C. Cytosolic, organelle, or whole cell extracts (40 µg total protein per lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% gels for A1 detection and 12.5% gels for Bak detection. Proteins were transferred overnight at 4°C to nitrocellulose membranes and blocked for 2 h at room temperature with BupH Tris buffered saline (Pierce Biotechnology, Inc.; Rockford, IL) containing 0.1% Tween 20 (Sigma; St. Louis, MO) and 5% non-fat milk. After washing with TBST buffer (BupH Tris buffered saline containing 0.1% Tween 20), immunodetection was performed with

either anti-Bak (H-211: sc7873, Santa Cruz Biotechnology, Inc.) or anti-A1 (FL-175: sc8351, Santa Cruz Biotechnology, Inc.) polyclonal antibodies for 1 h at room temperature (cytosolic fractions) or overnight at 4°C (whole cell and organelle fractions), the blots were washed again, and detection antibody (goat anti-rabbit IgG horseradish peroxidase conjugated; Pierce) was added at room temperature for 1 h. Blots were developed using the SuperSignal West Pico chemiluminescent substrate system (Pierce), photographed, stripped, and re-probed with anti- β-actin antibody (AC-15: ab6276, Abcam Limited) as a lane loading control (Weber et al., 2004).

H. Statistical analysis

Data are summarized in the results as least squares means ± SEM. When necessary for statistical analysis, some data were log transformed for better normal distribution approximation. Statistical analysis was performed using the MIXED procedure of SAS (SAS Institute, 2000) and a model that included the fixed effect of experimental group (no treatment, dexamethasone, RU486, dexamethasone plus RU486) and random effects of steer (n = 3 to 5) and steer x treatment interaction. Regression analysis was utilized in the dexamethasone and RU486 dose response experiments to determine optimal concentrations of each for use in all subsequent experiments. When the steer x treatment interaction was significant, such as with the mRNA abundance data, the effect of one factor (e.g. RU486) were examined within each level of the other factor (e.g. dexamethasone 0 M vs 10^{-7} M) using the SLICE function within the MIXED procedure. Significant differences between treatments were declared when $P \leq 0.05$.

Data from the caspase inhibition study were obtained from a single animal and will not be statistically analyzed until this work been repeated in multiple animals.

IV. RESULTS

A. Dexamethasone caused a dose-dependent delay in spontaneous neutrophil apoptosis that was inhibited by all doses of RU486 tested

Before examining direct effects of dexamethasone on mitochondrial membrane stability, caspase-9 activity, and A1 and Bak expression, it was necessary to confirm the dose of steroid at which spontaneous neutrophil apoptosis would be optimally delayed. Neutrophil cultures containing 10^{-9}, 10^{-8}, 10^{-7}, or 10^{-6} M dexamethasone all had significantly more non-apoptotic cells at 24 h relative to cultures with no dexamethasone (0 M, **Figure 4.1a**). The dose response was quadratic in nature with the dose of dexamethasone that optimally inhibited neutrophil apoptosis at 24 h calculated to be between 10^{-8} and 10^{-7} M. The dose of 10^{-7} M was selected for use in all subsequent experiments because this is the approximate concentration of cortisol found in parturient cow blood serum (Chapter Three). Pretreatment of neutrophils with 10^{-6}, 10^{-5}, or 10^{-4} M of RU486, a glucocorticoid and progesterone receptor antagonist, removed the apoptosis delaying effect of 10^{-7} M dexamethasone. **Figure 4.1b** demonstrates that as little as a 10-fold excess of RU486 abrogated the dexamethasone effect without itself inducing apoptosis. In Chapter Three, we demonstrated that steer neutrophils express glucocorticoid receptors while progesterone receptors were undetectable. Thus, the dexamethasone-induced delay in neutrophil spontaneous apoptosis in this study was

mediated via glucocorticoid receptor activation and inhibited by RU486 antagonism of the receptor.

B. Dexamethasone prevented mitochondrial membrane instability in neutrophils aged *ex vivo*

The integrity of neutrophil mitochondrial membranes was examined in this study with the aid of MitoTracker® Green FM staining. Freshly isolated neutrophils demonstrated a bright green staining of their tubular mitochondria (**Figure 4.2a** top panel) and did not stain with annexin V-FITC or propidium iodide (**Figure 4.2b** top panel). After 12 h of *ex vivo* aging without added dexamethasone, most neutrophils showed a diffuse cytosolic staining pattern indicative of mitochondrial membrane demise, and this correlated with advanced apoptosis in the cells (**Figure 4.2a, b** middle panels). In contrast, neutrophils aged for 12 h in the presence of 10^{-7} M dexamethasone showed bright green staining, similar to that observed in freshly isolated cells, indicating that the organelles' membranes stayed intact and correlating well with delayed apoptosis (**Figure 4.2a, b** bottom panels). Thus the optimal dose of dexamethasone that delayed spontaneous apoptosis in neutrophils likely did so by delaying mitochondrial membrane decay.

C. Dexamethasone delayed activation of caspase-9 in neutrophils aged *ex vivo*

Maintenance of mitochondria outer membranes occurred in conjunction with decreased caspase-9 activation in dexamethasone treated neutrophils (**Figure 4.3a**). Significantly less caspase-9 activity was observed after 6 and 9 h in dexamethasone

treated neutrophils relative to untreated cells. Pre-incubation of the neutrophils with RU486 removed the inhibiting effect of dexamethasone on caspase-9 activation (**Figure 4.3b**). Preliminary results (n = 1) indicate that dexamethasone-mediated decreases in caspase-9 activity were similar to decreases observed in neutrophils treated with LEHD-fmk, a known caspase-9 inhibitor (**Figure 4.4a**). Inhibition of caspase-8 with IETC-fmk, a known caspase-8 inhibitor, also lowered the activity of caspase-9 under scenarios of spontaneous apoptosis as well as sFasL induced apoptosis (**Figure 4.4b**). Dexamethasone further decreased caspase-8 activity in the spontaneous apoptosis scenario, but was not as effective in delaying sFasL induced apoptosis. Further analysis is necessary to fully understand the role of caspase-8 in caspase-9 activation during spontaneous apoptosis. Nevertheless, the mitochondria morphology and caspase-9 activity data from aging neutrophils in this study indicate that the actions of glucocorticoids on neutrophil apoptosis are at least partially targeted at preservation of mitochondrial membrane integrity.

D. Dexamethasone directly affects neutrophil abundance of A1 and Bak

To evaluate dexamethasone effects on A1 and Bak mRNA abundance, neutrophils were incubated up to 4 h in the presence or absence of dexamethasone ± RU486 pretreatment and their RNA analyzed for A1 and Bak mRNA abundance by Q-RT-PCR. Representative standard curves for the calculation of neutrophil A1 and Bak mRNA abundance are depicted in **Figure 4.5a** and **c** ($R^2 > 0.98$). Using these standard curves, absolute quantification of mRNA was calculated for each neutrophil treatment and culture time. A modest 1.4-fold increase in A1 mRNA abundance was observed at 2 h in

neutrophils cultured in 10^{-7} M dexamethasone versus cells cultured in 0 M dexamethasone (**Figure 4.5b**). Surprisingly, A1 mRNA abundance was also significantly increased in all cultures containing RU486. Dexamethasone decreased Bak mRNA abundance by 1.7-fold after 4 h of culture and addition of RU486 abrogated this glucocorticoid effect (**Figure 4.5d**).

Finally, Western blot analysis was utilized to evaluate direct effects of dexamethasone on neutrophil A1 and Bak protein abundance. Both proteins were readily detectable in neutrophil whole cell extract and cytosolic fraction whereas only Bak was present in the organelle fraction (**Figure 4.6a**). Moderate increases in neutrophil A1 were observed in whole cell extracts after 9 h of dexamethasone treatment when compared to untreated cells (**Figure 4.6b**). Addition of RU486 appeared to further increase A1, which was in agreement with changes in A1 mRNA abundance described above. Glucocorticoid-induced increases in A1 were more prominent in the cytosolic fractions (**Figure 4.6c**) but effects of RU486 were unclear due to animal related variation (data not shown). In contrast to our mRNA data, protein abundance of Bak appeared to be relatively unchanged in whole cell (**Figure 4.6b**) and cytosolic fractions (**Figure 4.6c**). Western blots generated from the organelle fraction demonstrated minimal changes in Bak abundance due to dexamethasone treatment (**Figure 4.6d**), but responses were variable from animal to animal. As a result, clear increases or decreases were not observed and further investigation is required to determine *in vitro* dexamethasone effects on neutrophil Bak protein abundance.

V. DISCUSSION

Glucocorticoid-induced delays in spontaneous apoptosis have been documented in human (Cox, 1995; Liles et al., 1995), rat (Nittoh et al., 1998), carp (Weyts et al., 1998), and more recently in bovine neutrophils (Chapter Three; Chang et al., 2004; Burton et al., 2005). The current study shows for the first time that glucocorticoids delay spontaneous neutrophil apoptosis by targeting the cells' mitochondrial membrane stability system. Key findings supporting this observation were visualization of mitochondria membrane integrity with MitoTracker Green FM staining (**Figure 4.2**) and decreased caspase-9 activation (**Figure 4.3**) in neutrophils aged *ex vivo* and treated with dexamethasone versus those not receiving the steroid. These results extend the growing list of how glucocorticoids delay neutrophil apoptosis, which has previously included neutrophil apoptosis initiated by reactive oxygen species (Ruiz et al., 2002) and soluble Fas ligand (Chang et al., 2004).

Having observed that glucocorticoids affect neutrophil mitochondrial membrane stability during the delay in spontaneous apoptosis, it was of interest to determine if changes in A1 and Bak expression also occurred, as was suggested in previous data collected from neutrophils of parturient dairy cows and dexamethasone-treated steers (see Chapters Two and Three). In the current study, neutrophils treated with dexamethasone and aged *ex vivo* had obvious increases in A1 protein abundance (**Figure 4.6b, c**), which may have resulted from the modest but significant increases in A1 mRNA abundance (**Figure 4.5b**). Interestingly, A1 was further increased in the presence of RU486. Although RU486 impairs glucocorticoid receptor nuclear localization, the receptor still undergoes some level of activation (reviewed by Agarwal, 1996). For example, if

binding of RU486 resulted in release of heat shock and Src proteins from the cytoplasmic glucocorticoid receptor complex, these proteins would now be free in the cytoplasm to elicit non-genomic effects that could affect A1 abundance. Src tyrosine kinases are involved in NF-κB activation (Kang et al., 2005) and NF-κB is a known transcriptional inducer of A1 (Kupfner et al. 2001). Released heat shock proteins may bind cytoplasmic mRNAs to direct processes including subsequent translation or degradation (Henics et al., 1999). Thus, A1 abundance may be under the control of indirect or non-genomic effects following RU486 treatment. In contrast, Bak mRNA abundance was substantially reduced by dexamethasone treatment and this glucocorticoid effect was inhibited by RU486 pretreatment of the cells indicating that this Bcl-2 family member may be regulated by genomic effects of glucocorticoid receptor activation (**Figure 4.5d**). However, corresponding decreases in Bak protein abundance were not detected in this *in vitro* glucocorticoid exposure experiment (**Figure 4.6d**). Clear decreases in Bak protein abundance were observed following *in vivo* glucocorticoid challenge. Thus, the *in vitro* observations may have resulted from the minimal culture system utilized (i.e. Bak down-regulation may require additional factors related to protein turnover) as well as the relatively short culture time of 9 h (Bak protein half-life >22 h; Moulding et al., 2001).

Previous work has demonstrated that changes in A1 occur when neutrophils are exposed to a variety of pro-inflammatory factors. These include G-CSF (Chuang et al., 1998), GM-CSF, TNF-α, IFN-γ (Moulding et al., 2001), and LPS (Chuang et al, 1998; Kupfner et al., 2001; Kotani et al., 2003). Our results extend these findings to include glucocorticoids as a modulator of A1 abundance in neutrophils. Also, while dexamethasone directly altered Bak mRNA in the current study, Bak abundance was not

affected following GM-CSF, TNF-α, IFN-γ, and LPS treatments (Moulding et al., 2001). Some research suggests that Bak protein is present only in mitochondria fractions (Wei et al., 2000; Mikhailov et al., 2003). However, our results show the presence of Bak protein in bovine neutrophil cytosolic preparations, which is in agreement with the intracellular location of this protein described by Griffiths et al. (2001). Under normal pro-apoptotic scenarios, Bak forms homo-oligomers in the outer mitochondrial membrane and creates pores that allow release of cytochrome c (Degenhardt et al., 2002). While we did not measure cytochrome c release from mitochondria in the current study, our up-stream measurement of enhanced mitochondrial membrane integrity and down-stream measurements of inhibited caspase-9 activation and delayed spontaneous apoptosis in the dexamethasone treated neutrophils indicate that the steroid-induced increase in A1 mRNA and protein abundance may be biologically meaningful. We also did not directly test the roles of Bak or A1 in the dexamethasone-induced delays in mitochondrial membrane demise and apoptosis but, based on the data we did collect, we provide here several hypotheses as to how glucocorticoids may delay spontaneous apoptosis in bovine neutrophils.

The first hypothesis is that dexamethasone-induced decreases in Bak abundance alone were responsible for delaying neutrophil apoptosis. Although we did not observe protein abundance changes in *in vitro* treated neutrophils, recall from Chapter Three that dexamethasone treatment *in vivo* did result in a pronounced decrease in neutrophil Bak abundance. Decreases in Bak mRNA observed following glucocorticoid treatment may impact Bak protein synthesis in neutrophils newly released from the bone marrow, while the lack of detection of decreased Bak protein following *in vitro* treatment with

glucocorticoid may result from slow turn-over of Bak protein (half-life greater than 22 h; Moulding et al., 2001). However, another possibility is that A1 abundance changes are relatively more important to the apoptotic status of neutrophils than changes in Bak, as was demonstrated in A1 and Bak knockout mice (Hamaski et al., 1998; Lindsten et al., 2000). Thus, a second hypothesis worthy of testing is that increased A1 under the influence of glucocorticoid creates competition with Bak for the formation of A1/Bak heterodimers thereby limiting mitochondrial membrane pore formation by reducing the number of Bak/Bak homodimers. Reports of A1 and Bak interaction are not consistent. Holmgreen et al. (1999) described co-immunoprecipitation of these two proteins that appeared to be associated with a highly conserved glycine in the BH1 domain of A1. More recently, it was found that heterodimerization of Bak with Mcl-1 or Bcl-X_L delayed apoptosis while no significant interaction between Bak and A1 was observed (Willis et al., 2005). Thus, it would be difficult to accept this second hypothesis without the generation of additional supporting data.

A final hypothesis regarding the connection between A1, Bak, and glucocorticoid induced delay in neutrophil spontaneous apoptosis involves the BH3-only Bcl-2 family member Bid. Bid is cleaved into its active form, tBid, in the cells' cytosol following activation of caspase-8 by death receptor (e.g., Fas) ligation or activation by ROS (Scheel-Toellner et al., 2004) at the plasma membrane (Figure 1.1). The tBid, in turn, has been shown to induce cytochrome c release from mitochondria (Li et al., 1998). The tBid apparently binds Bak to induce an open conformation in Bak that allows for enhanced Bak/Bak homo-oligomerization (Ruffolo and Shore, 2003). This interaction is necessary for subsequent cytochrome c release through Bak pores but tBid is not a

permanent part of this complex (Wei et al., 2000). It also has been demonstrated that A1 associates with both Bid and tBid. Although A1 does not inhibit the processing of Bid to tBid, it does inhibit tBid activation of Bak (Werner et al., 2002). Therefore, the increases in A1 expression we observed during glucocorticoid treatment of neutrophils may have delayed the cells' apoptosis by decreasing interactions between tBid and Bak. While Bid cleavage is most often a result of cell surface death receptor activation via ligand binding (Droin and Green, 2004; Sprick and Walczak, 2004), its cleavage in neutrophils undergoing spontaneous or ROS induced apoptosis has also been observed (Maianski et al., 2004b; Scheel-Toellner et al., 2004). Our observation that caspase-9 activation was decreased by caspase-8 inhibition to the same level as observed for glucocorticoid treatment (n = 1; **Figure 4.4b**) begins to address this final hypothesis but additional studies are necessary to substantiate this result. If confirmed, and viewed in conjunction with our observations on A1 abundance, it could indicate that glucocorticoid control of A1 is a key regulatory point in the life-death decision of neutrophils as well as a link between the spontaneous and death receptor induced apoptosis pathways (Chang et al., 2004). It is perhaps not coincidental, and indeed, may be critical to apoptosis regulation, that glucocorticoids also inhibit expression of ROS generating genes (Burton et al., 2005), ROS production (Dandona et al., 1999), and Fas expression in treated neutrophils (Chang et al., 2004).

In summary, our observations suggest that glucocorticoid-induced changes in A1 abundance may be part of the mechanism by which this steroid preserves mitochondrial membrane integrity and delays spontaneous apoptosis in bovine blood neutrophils. In the case of Bak, it may be necessary for glucocorticoids to influence or act in conjunction

with other factors that would be present in a whole animal system to transform the *in vitro* mRNA abundance changes we observed to changes in Bak protein abundance. Whether changes in A1 and Bak alone or in conjunction with glucocorticoid-mediated decreases in death receptor signaling were responsible for observed stability of mitochondria membranes and inhibited caspase-9 activation in glucocorticoid treated neutrophils has yet to be determined. In either scenario, results of this study solidify the role of mitochondria and Bcl-2 family members A1 and Bak in the glucocorticoid regulation of neutrophil life span, indicating the pronounced impact of this organelle in apoptosis regulation of mature neutrophils despite its relatively limited abundance in these cells.

Figure 4.1 Glucocorticoid delayed neutrophil apoptosis is dose responsive and inhibited by RU486. (a) A significantly higher % of non-apoptotic neutrophils were detected flow cytometrically as negative for Annexin V-FITC/propidium iodide staining after 24 h of *ex vivo* aging in the presence of dexamethasone. The equation represents the quadratic regression observed among the variously treated neutrophil samples. Utilizing this equation, the optimal dose of dexamethasone was calculated to be between 10^{-8} and 10^{-7} M. (b) Treatment with a 10-fold excess of the glucocorticoid receptor antagonist, RU486 (10^{-6} M), prior to addition of 10^{-7} M dexamethasone removed the apoptosis delaying effect of the glucocorticoid on bovine neutrophils. (Steer n = 5) * $P \leq 0.05$ relative to all other treatments.

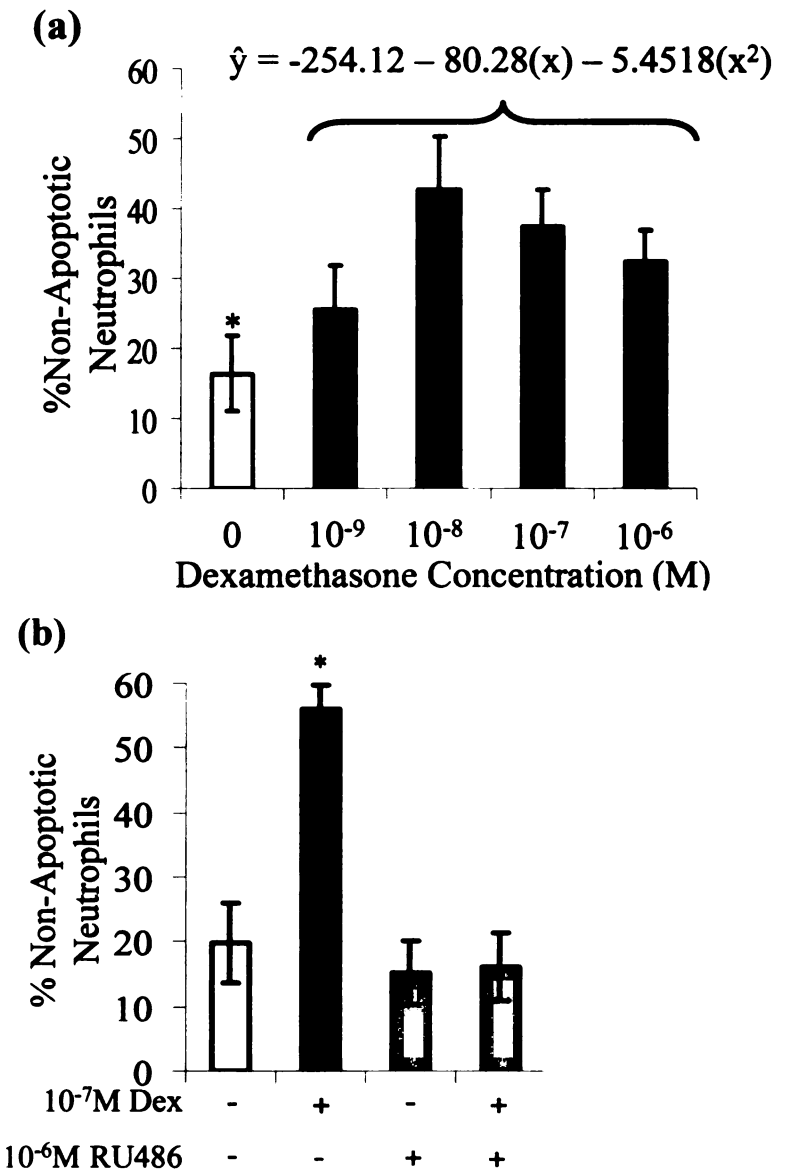


Figure 4.2 Glucocorticoid treatment of neutrophils during *ex vivo* aging delayed their spontaneous apoptosis in conjunction with maintenance of mitochondrial membrane stability. Freshly isolated neutrophils (a - top panel) contain tubular mitochondria, detected by bright green staining with Mitotracker® Green FM, and no annexin V-FITC binding (b – top panel). Neutrophils cultured in basic medium for 12 h displayed diffuse staining (indicating decay of mitochondrial membranes; a – middle panel) which coincided with a high level of annexin V-FITC binding (74.05% of neutrophils; b – middle panel). Inclusion of dexamethasone for the 12 h incubation resulted in maintained stability of mitochondrial membranes (observed as long, tubular organelles; a – bottom panel) and decreased annexin V-FITC binding (26.43% of neutrophils; b – bottom panel). Representative of neutrophils from n = 3 steers.

Figure 4.2

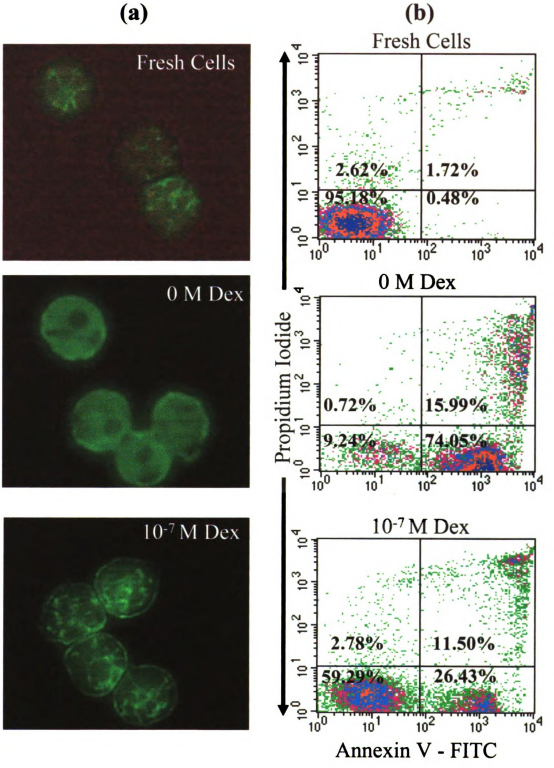

(a)
(b)
Fresh Cells
0 M Dex
10-7 M Dex
Fresh Cells
2.62%
1.72%
95.18%
0.48%
0 M Dex
0.72%
15.99%
9.24%
74.05%
10-7 M Dex
2.78%
11.50%
59.29%
26.43%
Propidium Iodide
Annexin V - FITC

Figure 4.3 Glucocorticoid treatment delayed the activation of caspase-9 in neutrophils aged *ex vivo* over 9 h. (a) Dexamethasone (10^{-7} M) significantly inhibited caspase-9 activation at 6 and 9 h of treatment. (b) Caspase-9 activation in dexamethasone treated neutrophils aged for 9 h *ex vivo* was not significantly different from that observed in freshly isolated neutrophils. No dexamethasone and inclusion of RU486 resulted in high caspase-9 activity that was similar to that observed in positive control cells (neutrophils treated with sFasL). Cells from n = 4 steers. $^{*}P \leq 0.05$ relative to no dexamethasone.

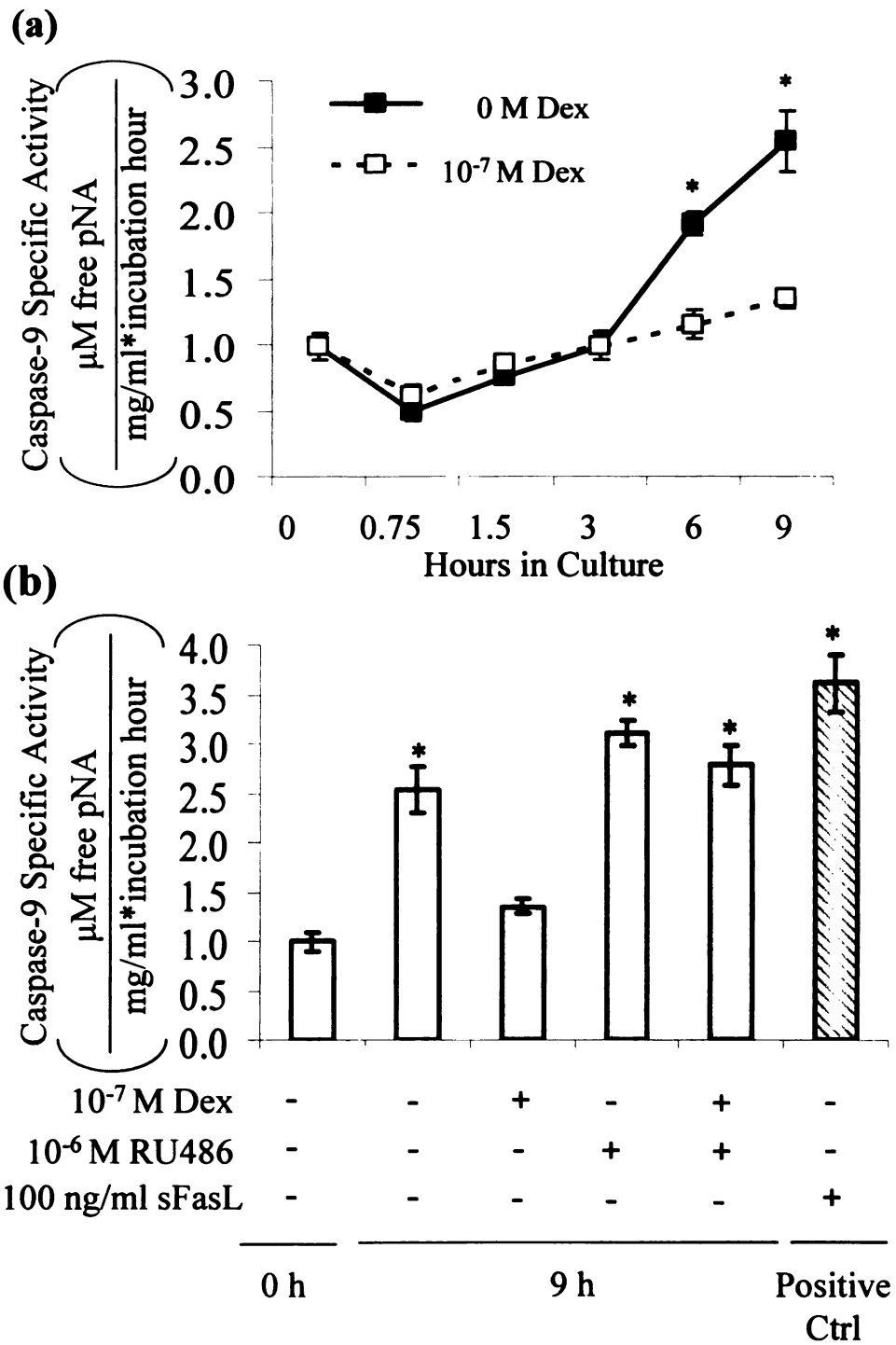


Figure 4.4 Glucocorticoid-mediated decreases in neutrophil caspase-9 activity after 9 h in culture were similar to that observed with known caspase inhibitors in neutrophils from a single steer. (a) Dexamethasone (10^{-7} M) and the caspase-9 inhibitor, LEHD-fmk, substantially decreased caspase-9 activation at 9 h of treatment. (b) Neutrophil caspase-9 activation was also decreased by a known caspase-8 inhibitor, IETD-fmk, both in the absence and presence of dexamethasone. Caspase-9 activation by sFasL is mediated by activation of caspase-8 but any effect of dexamethasone on this system is currently unclear. As the data presented are from n = 1 assayed in duplicated, repeating this experiment with additional steers is necessary to substantiate this scenario.

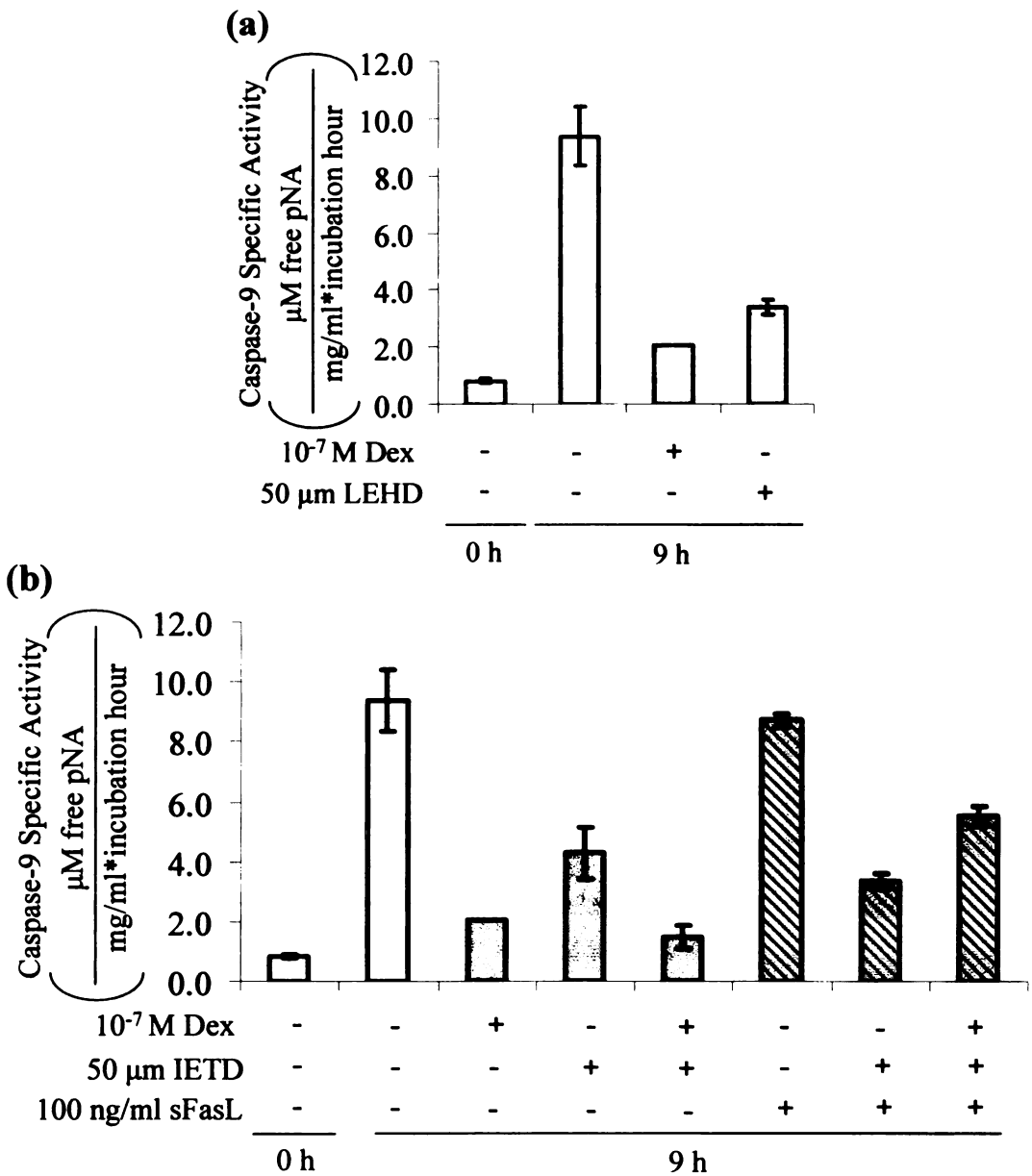


Figure 4.5 Abundance of A1 and Bak mRNA was altered by dexamethasone treatment at 2 and 4 h of *ex vivo* aging, respectively. (a) A1 standard curve made with the A1-286bp amplicon. (b) A1 mRNA abundance was significantly increased following 2 h of dexamethasone treatment. Addition of RU486 for 2 h also resulted in a significant increase in A1 mRNA. (c) Bak standard curve made with the Bak-306bp amplicon. (d) Bak mRNA abundance was significantly decreased after 4 h of exposure to dexamethasone. RU486 eliminated this effect. Panels (b) and (d) neutrophils from n = 4 steers. † $P = 0.07$, * $P \leq 0.05$ relative to no dexamethasone.

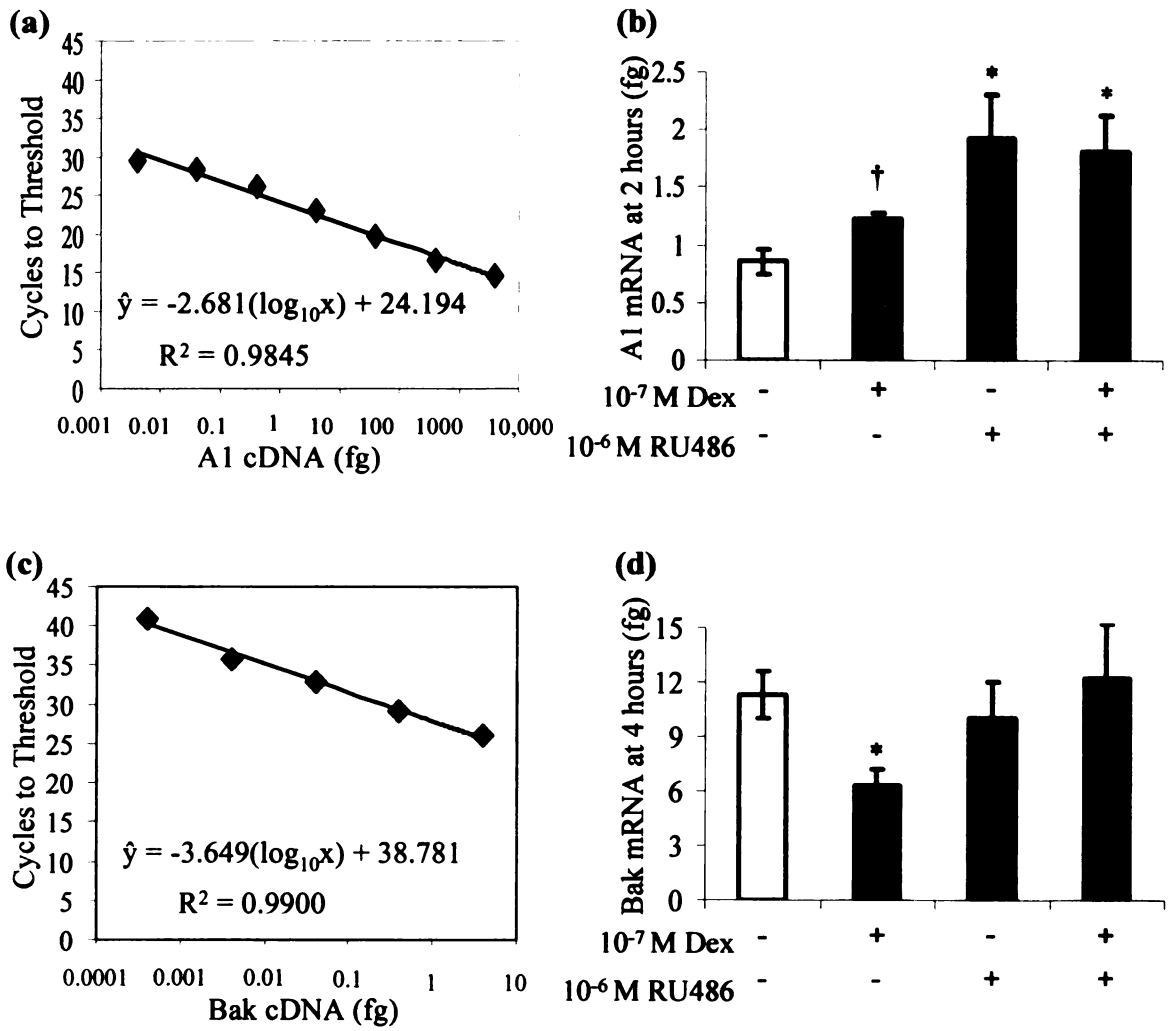
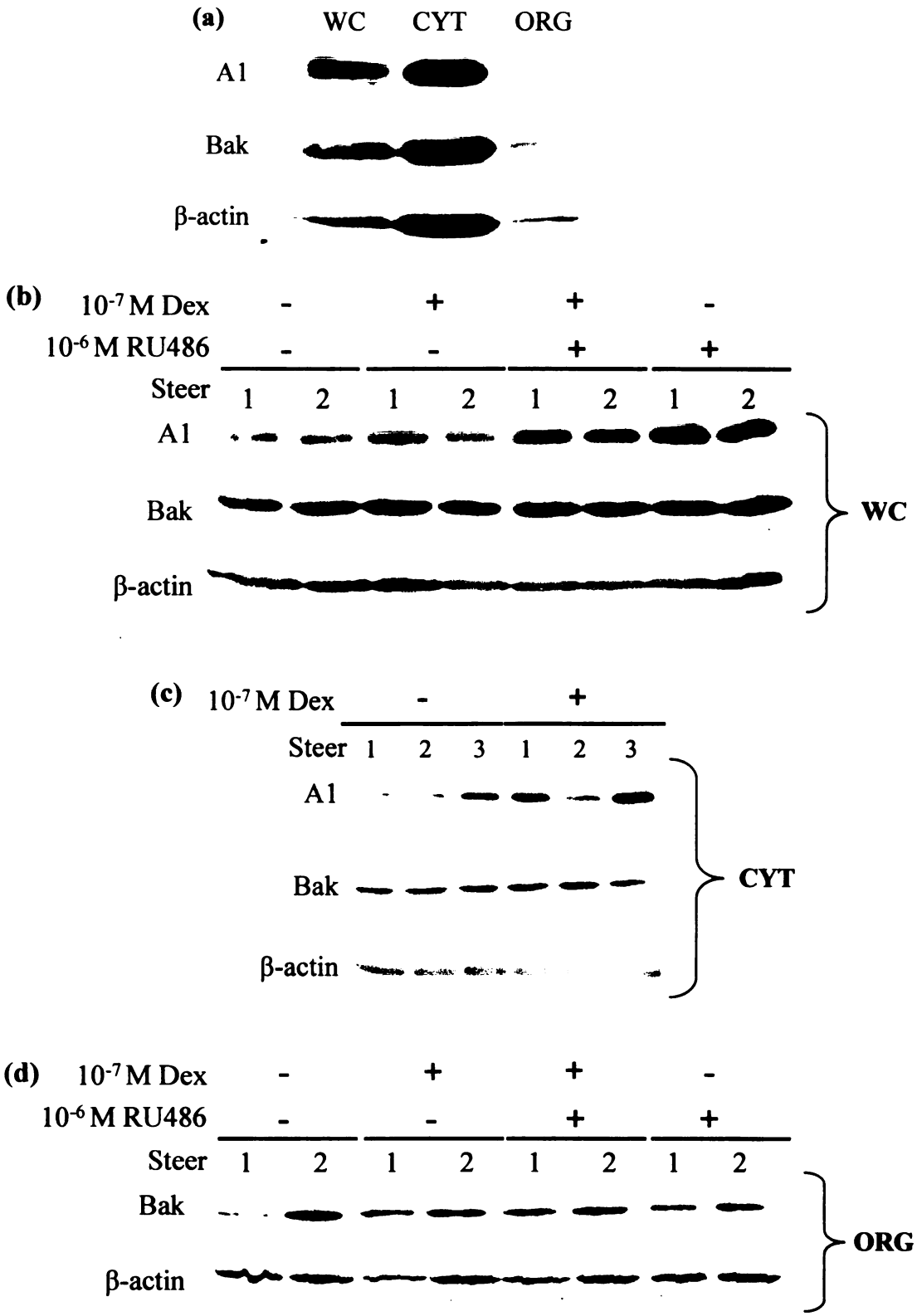


Figure 4.6 Neutrophil A1 protein abundance was altered by dexamethasone treatment over 9 h of *ex vivo* aging while Bak appeared unchanged. A representative blot of A1 and Bak distribution in the three protein preparations (whole cell extract = WC; cytosolic fraction = CYT; organelle fraction = ORG) is found in panel (a). A1 was detectable in only WC and CYT where as Bak was present in all three fractions. (b) Abundance of A1 appears to be slightly increased in WC from neutrophils (from n = 2 steers) treated with dexamethasone as well as RU486 and the combination of the two. This is in agreement with A1 mRNA abundance data (Figure 4.5b). Bak appeared to be unchanged in WC preparations. (c) Increases in neutrophil A1 were more readily detectable in CYT preparations of dexamethasone treated cells from n = 3 steers. As was observed in WC, there were no apparent changes in Bak abundance in CYT. (d) Changes in Bak abundance were highly variable from animal to animal in ORG (n = 2), thus no clear decreases or increases were observed due to dexamethasone treatment. β-actin served as a loading control on all membranes.

Figure 4.6


(a)
WC
CYT
ORG
A1
Bak
β-actin
(b)
10-7 M Dex
10-6 M RU486
Steer
1 2 1 2 1 2 1 2
A1
Bak
β-actin
WC
(c)
10-7 M Dex
Steer 1 2 3 1 2 3
A1
Bak
β-actin
CYT
(d)
10-7 M Dex
10-6 M RU486
Steer
1 2 1 2 1 2 1 2
Bak
β-actin
ORG

CHAPTER FIVE

General Discussion

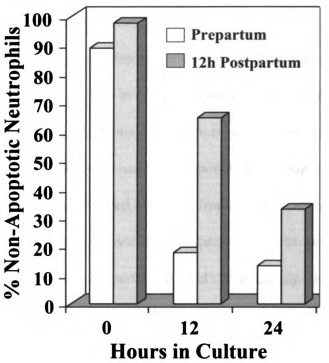
The parturient dairy cow undergoes profound physiological changes (physical, metabolic, nutritional, and hormonal) relative to her pre-partum condition. Animal health, especially of the mammary glands, is often compromised during this time in association with some of these physiological changes. Numerous mastitis researchers have focused their investigations on blood neutrophils of parturient cows due to the critical role these leukocytes play in innate immunity and host defense against intramammary infections that lead to clinical mastitis (Frost and Brooker, 1986; Shuster et al., 1996; Erskine et al., 2002). Collectively, previous studies have demonstrated that key neutrophil bactericidal activities are impaired in parturient dairy cows. For example, neutrophils demonstrate a decreased ability to migrate *in vitro* (Nagahata et al., 1988; Kehrli et al., 1989; Detilleux et al., 1994), reduced ability to produce ROS when stimulated *in vitro* (Kehrli and Goff, 1989; Detilleux et al., 1994), and possess lowered myeloperoxidase activity and oxidative burst capacity compared to neutrophils from cattle not undergoing parturition (Kehrli and Goff; 1989; Kehrli et al., 1989; Detilleux et al., 1994). Despite this knowledge, little is known about the molecular or physiological basis for the observed neutrophil dysfunctions at parturition. Thus, effective treatment and prevention of disease has been elusive.

The experiments described in Chapters 2-4 describe several novel findings and may help elucidate some molecular basis for neutrophil physiology in parturient dairy cows. The first noteworthy finding was that neutrophils from parturient dairy cows

possess a gene expression signature indicative of delayed apoptosis. This is in spite of the numerous documented neutrophil dysfunctions which are more typical of apoptotic cells. The significance of these findings was further substantiated by *ex vivo* aging experiments where neutrophils were incubated in the presence of blood sera from parturient dairy cows prior to apoptosis phenotyping. This phenotype has yet to be absolutely confirmed *in vivo* using neutrophils from multiple parturient dairy cows, but our preliminary results from two cows do support the anti-apoptosis gene expression signature we observed (**Figure 5.1**). In addition, we found that a number of the apoptosis gene expression profiles in neutrophils significantly correlated with changes in blood cortisol around parturition (Burton et al., 2005). In light of this, and because of the well-documented effects of glucocorticoids on gene expression in other cell systems (Carson-Jurica et al., 1990; Beato et al., 1995), we hypothesized that the high glucocorticoid concentration found in blood serum at parturition was partly responsible for delaying neutrophil apoptosis

Being lipophilic, steroid hormones easily cross the plasma membrane of target cells without the need for surface receptors and bind to high affinity receptors that are present in the cell's cytoplasm (Bamberger et al., 1996). Hormone binding induces conformational changes in the steroid receptors, converting them into potent transcription factors that act in the nucleus on transcription of hormone-responsive genes (Carson-Jurica et al., 1990). Steroid receptor action on hormone responsive genes is often direct and mediated by receptor binding to DNA motifs in the regulatory regions of the genes. Using a bioinformatics approach, putative GRE half-sites have been detected upstream of transcription start sites in human A1 and Bak genomic DNA (Madsen-Bouterse, Halgren,

Figure 5.1 Delayed neutrophil apoptosis of parturient cow neutrophils versus mid-lactation cow neutrophils. Blood neutrophils were isolated on post partum d 0.5 from one animal and control neutrophils from a pregnant cow in mid-lactation and aged *ex vivo* over 24 h. Apoptosis was assessed using 2-color flow cytometric analysis with non-apoptotic neutrophils detected as Annexin V negative and propidium iodide negative. Substantially more non-apoptotic neutrophils were detected in the sample from the parturient cow compared to very few non-apoptotic neutrophils at 12 and 24 h from the mid-lactation cow. This experiment will be repeated with additional animals in the near future.



and Burton; preliminary observations). However, future studies will be necessary to confirm the presence and activity of these sites in the bovine A1 and Bak genes. In addition to direct effects, hormone activated steroid receptors can also repress or induce target gene expression indirectly by interfering with or activating other inducing or repressing transcription factors that then act on target gene transcription (Beato et al., 1995). Whether direct or indirect, the actions of steroids on target gene expression are typically rapid and pronounced.

Before examining direct effects of glucocorticoids on the expression of candidate apoptosis genes identified in Chapter Two (Madsen et al., 2004), it was important to demonstrate that fluctuations in serum concentrations of this steroid hormone were associated with a delay in neutrophil spontaneous apoptosis. We did this using a cell culture system where isolated blood neutrophils from normal healthy steers were aged *ex vivo* in the presence of various periparturient sera prior to apoptosis assessment. Neutrophils cultured in parturient and immediately postpartum sera demonstrated significantly less apoptosis at 24 and 48 h of culture relative to cells cultured in sera collected seven days prior to parturition. These results were also associated with increased cortisol concentrations in the periparturient sera used.

A search of the literature revealed numerous studies in which glucocorticoid added *in vitro* to cultured neutrophils from humans, rodents, and fish delayed their spontaneous apoptosis for 24 to 48 h (Cox, 1995; Nittoh et al., 1998; Weyts et al., 1998). However, results of the present dissertation are the first to show glucocorticoid effects on apoptosis mRNA abundance during physiologically relevant scenarios (parturition and steroid therapy) and in cattle (Chapters Two and Three; Madsen et al., 2004; Burton et

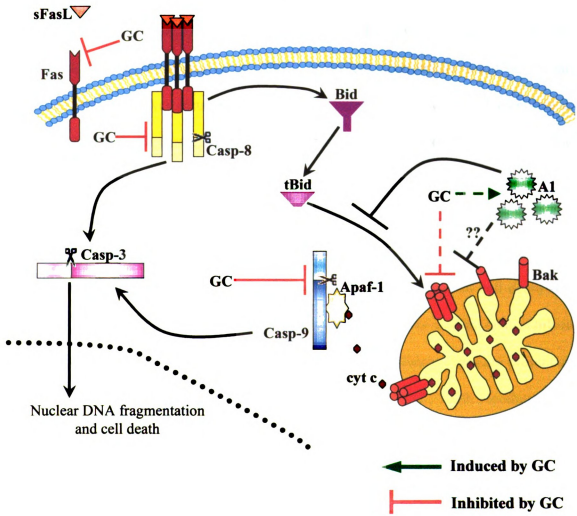
al., 2005). In addition, results of the described studies are the first to demonstrate that glucocorticoids delay neutrophil spontaneous apoptosis by targeting the cell's mitochondrial membrane stability system (Chapter Four). Neutrophil abundance of A1, a key anti-apoptotic Bcl-2 family member identified as increased at parturition and during glucocorticoid treatment, was consistent with maintenance of mitochondrial membrane stability and decreased caspase-9 activation in the steroid-exposed cells. Changes in pro-apoptotic Bak, depressed at parturition and during glucocorticoid treatment, also supports the notion that glucocorticoids act on the mitochondrial pathway to delay spontaneous apoptosis. However, the current study has not addressed the mechanism behind glucocorticoid regulation of A1 and Bak. Further research is necessary to determine whether mRNA and protein abundance changes were due to glucocorticoid effects on transcription, mRNA stability, translation, or protein stability.

While changes in A1 and Bak abundance coincided with maintained mitochondrial membrane stability and decreased caspase-9 activity, research presented in this dissertation is primarily observational. Future experiments are essential to evaluate the direct roles of A1 and Bak in the regulation of spontaneous apoptosis by glucocorticoids. Such experiments might evaluate interactions between A1 and Bak through co-immunoprecipitation and Western blot analysis or intracellular staining for these two proteins to determine if they co-localize to the same region of the cell in the presence and absence of glucocorticoids. Additional intracellular staining experiments could be used to determine interactions of A1 and (or) Bak with the mitochondria. In lieu of these potential experiments, the observed changes in A1 and Bak abundance suggest

that glucocorticoids target these two Bcl-2 family members to induce a delay in neutrophil spontaneous apoptosis.

In other studies, our group has demonstrated that Fas mRNA is decreased in correlation with the glucocorticoid surge at parturition (Chang et al., 2004). Direct glucocorticoid treatment of neutrophils *in vitro* also resulted in decreased Fas mRNA and protein abundance, causing a significant delay in soluble Fas ligand-induced activation of caspase-8 and apoptotic cell death (Chang et al., 2004; Burton et al., manuscript in preparation). The observed decreases in Fas mRNA appear to result from decreases transcription (Burton and Chang, manuscript in preparation). In addition, glucocorticoids appear to target the death receptor pathway of neutrophil apoptosis as was apparent from the large number of Fas-related genes altered in expression around parturition in our microarray experiment (Chapter Two; Madsen et al., 2004) and our preliminary caspase inhibition studies (Chapter Four). Collectively these studies demonstrate that elevated glucocorticoids *in vivo* and glucocorticoid treatment *in vitro* targets neutrophil apoptosis through both the intrinsic (mitochondria mediated) and extrinsic (death receptor mediated) pathways of programmed cell death (**Figure 5.2**).

Figure 5.2 Glucocorticoids (GC) modulate neutrophil apoptosis pathways initiated at the mitochondria as well as through death receptor ligation. Pro-apoptotic Bcl-2 family members such as Bak oligomerize to create pores in outer mitochondrial membranes resulting in release of cytochrome c. Apaf-1, procaspase-9, and cytochrome c form a complex called the apoptosome which cleaves procaspase-9. Once activated, caspase-9 can cleave caspase-3 which will in turn activate enzymes involved in nuclear DNA fragmentation and ultimately cell death. Death receptors, such as Fas, trimerize following sFasL binding which leads to cleavage and activation of procaspase-8. Like caspase-9, caspase-8 cleaves and activates caspase-3. Caspase-8 also cleaves Bid, resulting in a truncated Bid that induces Bak pore formation thus amplifying the intrinsic pathway of apoptosis. Results of described studies indicate that glucocorticoids increase A1 and decrease Bak abundance. These observations were concurrent with maintenance of mitochondrial membrane stability and decreased caspase-9 activation. The mechanism of by which glucocorticoids-mediate these changes will be the focus of future work. Other studies from our laboratory indicated that glucocorticoids directly decrease Fas expression as well as caspase-8 activity (Chang et al., 2004). Thus, glucocorticoids are shown in this figure to inhibit neutrophil apoptosis by down regulating activity of both the mitochondrial and Fas pathways of programmed cell death.



Though the research presented in this dissertation clearly demonstrates that glucocorticoids act at the level of the mitochondria in bovine neutrophils to delay apoptosis, questions still remain as to why apoptosis is delayed in this manner at parturition when the cells also exhibit multiple bactericidal dysfunctions. Based on available literature, we have formulated several hypotheses that may be tested with future experiments to answer this question. A first hypothesis is that neutrophil apoptosis is delayed at parturition to increase circulating numbers of these cells. In support of this possibility, high blood cortisol concentrations that are important to the processes of parturition in cattle are strongly associated with a pronounced neutrophilia at this time (Priesler et al., 2000, Weber et al., 2001; Madsen et al., 2004). Although often described as a result of decreased expression of adhesion molecules on the surface of neutrophils (Lee and Kehrli, 1998; Weber et al., 2001), parturient neutrophilia could be partially accounted for by delayed neutrophil apoptosis. However, this does not reconcile the documented neutrophil dysfunctions during parturition or what benefit increasing blood neutrophil numbers may provide.

Our second hypothesis focuses on the known depression in adhesion molecule expression on circulating neutrophils of parturient cows. We postulate that neutrophils during this time must have a requirement for delaying their program of cell death since they are not able to gain access to peripheral tissues. Once in the tissues, neutrophils are normally cleared by macrophages as they die by apoptosis. Macrophages are present and active in peripheral tissues but not in the circulation (Savill et al., 1989; Homburg et al., 1996). At parturition, when neutrophils lack the adhesion molecules necessary to migrate into peripheral tissues, possibly contributing to mastitis susceptibility at this time (Burton

and Kehrli, 1995; Lee and Kehrli, 1998; Weber et al., 2001), there are no macrophages present in the blood to clear apoptotic neutrophils leading to increased risk of their death by secondary necrosis (reviewed by Savill and Haslett, 1995). Necrotic neutrophils release enzymes, proteinases, and ROS normally contained within their granules. Because circulating neutrophil numbers increase so dramatically during parturition, a potentially harmful inflammatory scenario exists for the vascular system that may require a delay in neutrophil apoptosis until the cells regain expression of their surface adhesion molecules (~ 2 days post partum; Weber et al., 2001) and entry into peripheral tissues. If apoptosis is not delayed during this time, a vicious cycle of systemic inflammation and potentially irreversible blood vessel damage may occur. Thus, it may be critical to animal survival that neutrophil migration capacity and rate of apoptosis are co-regulated by glucocorticoids during parturition.

On the other hand, it is also possible that a parturient delay in neutrophil apoptosis could result in increased severity of clinical mastitis immediately postpartum. In experimental animal models of inflammatory disease, delayed neutrophil apoptosis coincides with disease pathogenesis, magnified inflammatory tissue damage, and poor prognosis in affected animals (Turlej et al., 2001; Saba et al., 2002; Garlichs et al., 2004). In dairy cattle, it has been suggested that rapid apoptosis of neutrophils may be a normal and effective protective mechanism to prevent excess tissue damage during episodes of mastitis (Sladek et al., 2000a,b; 2001; Boutet et al., 2004). If the neutrophil apoptosis delay of parturition extends into the postpartum period, it is possible that extended release of granule contents during resumed neutrophil migration into infected mammary quarters and phagocytosis of pathogens therein could severely damage mammary parenchymal

tissue and disrupt the blood-mammary barrier (Capuco et al., 1986). If true, the parturient delay in neutrophil apoptosis may contribute to the dramatic increase in mastitis severity of newly calved cows through overactive local and systemic inflammatory events. This third hypothesis is currently being tested in our laboratory.

A fourth hypothesis as to why neutrophil apoptosis may be delayed at parturition stems from some of the other data we obtained during our microarray experiment (Chapter Two; Madsen et al., 2004). In addition to the apoptosis regulatory genes, expression of a cluster of genes that regulate extracellular matrix degradation was altered in circulating neutrophils at parturition, with a gene expression signature indicating heightened tissue remodeling capacity of the cells. This cluster included genes for two key neutrophil matrix metalloproteinases (MMP-8 and MMP-9), as well as their natural tissue inhibitors (TIMP-2 and TIMP-3) and the pro-collagen synthesis, anti-inflammatory cytokine TGF-β. Transcripts for both MMPs were increased in abundance at and just after parturition, while the TIMPs and TGF- β were decreased at these times (Burton et al., 2005). These neutrophil mRNA abundance profiles were significantly correlated with serum cortisol profiles from the same animals. In addition, MMP-9 protein abundance was increased in neutrophils from dexamethasone treated steers (**Figure 5.3**) and MMP-9 activity is increased in blood serum from parturient dairy cows (**Figure 5.4**). Thus, glucocorticoids may contribute to altered neutrophil functions and inflammatory capacity during parturition, priming the cells for increased tissue degradation activities that are longer lived than normal.

Figure 5.3 Glucocorticoid treatment increases neutrophil cytosolic MMP-9 protein abundance. Blood neutrophils were collected from three steers before and 9 h after dexamethasone administration (see Chapter Three – Materials and Methods). Immunodetection was performed with a mouse monoclonal anti-MMP-9 (cat # 550942; BD Pharmingen, San Diego, CA) and detection antibody (goat anti-mouse IgG1 horseradish peroxidase conjugated; Bethyl Laboratories, Montgomery, TX). β-actin immunodetection (performed as described in Chapter Three) was used for normalization. Normalized data was analyzed by paired T-test. * $P = 0.02$

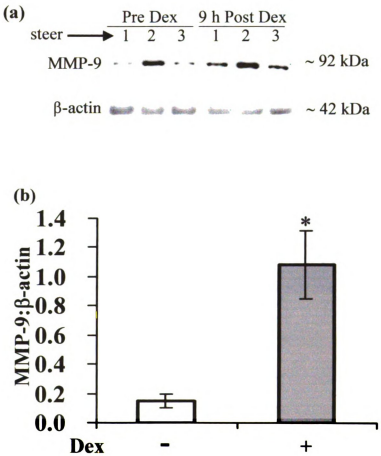
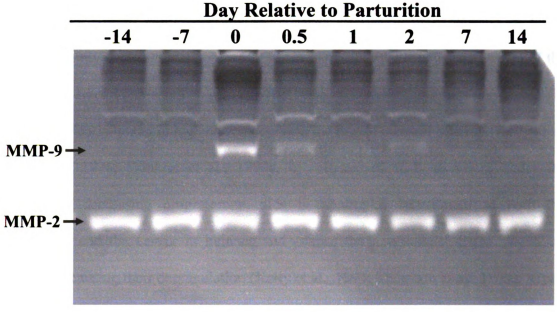


Figure 5.4 Parturition increases MMP-9 activity in blood serum. Blood sera collected on multiple days during the periparturient period were subjected to gelatin zymography to assess MMP-9 activity. The representative zymogram containing samples from a single cow demonstrated that the greatest activity of MMP-9 was observed at the time of parturition (day 0). MMP-2 activity was present at what appears to be equal levels at all time points. Our laboratory is currently pursuing studies to determine if increases in neutrophil MMP-9 abundance result in increased neutrophil MMP-9 activity as well as contribute to the observed activity increases detected in parturient serum.



In light of our preliminary results on the neutrophil MMP/TIMP gene expression system, we have hypothesized that delayed neutrophil apoptosis paired with increased tissue degradation activity must be critical to the processes of labor, parturition, and placental removal in cattle (Burton et al., 2005). This hypothesis is substantiated by available biomedical literature describing key roles of neutrophils in human parturition. For example, large numbers of neutrophils are detected in the myometrium at parturition relative to pre-partum, putatively due to increases in E-selectin expression in blood vessels of the myometrium that apparently aid in recruiting neutrophils to this area of the reproductive tract (Thomson et al., 1999). Also, neutrophil numbers in the cervix increase substantially and in high correlation with the rate of cervical dilation (Kelly et al, 1994; Kelly, 1996; Winkler et al., 1999a, b). Increased IL-8 secretion, under the influence of decreasing progesterone, aids in neutrophil recruitment to both the myometrium and the cervix in humans and primes the neutrophils' tissue degrading activities by causing their degranulation (Kelly et al., 1994; Thomson et al., 1999). Upon histochemical analysis, it appeared as if neutrophils in the human reproductive tract at parturition had fewer specific granules, which contain these MMPs (Junqueira et al., 1980). Thus, increasing IL-8 appears to facilitate increased neutrophil recruitment and degranulation with MMP release into the lower uterine tract and cervix, phenomena that are readily demonstrated immunohistochemically (Winkler et al., 1999a). In cattle, it has been shown that factor(s) produced by placental tissue (including cotyledon) at parturition induce chemotactic responses and increased phagocytosis by bovine neutrophils (Hoedemaker et al., 1992a, 1992b) with the chemotactic response inhibited by anti-IL-8 antibody (Kimura et al., 2002). So, it is possible that the role of neutrophils

in the reproductive tract at parturition is similar in humans and cattle. If true, the cortisol-induced delay in neutrophil apoptosis in combination with its associated decrease in adhesion molecule expression, increased expression of MMP-8 and MMP-9, and decreased expression of MMP inhibitors (TIMP-2, TIMP-3, and TGF-β) suggest that the parturient surge in glucocorticoid may be a way of augmenting supplies of longer lived neutrophils that are focused on and primed for rapid extracellular matrix degradation in the reproductive tract and placenta. These possibilities also are currently under investigation in our laboratory.

With the above hypotheses put forth to explain why apoptosis may be delayed in neutrophils from parturient dairy cows, it is still unclear what this phenotype means for increased mastitis susceptibility and severity at the time of calving. In our microarray experiment, most of the apoptosis related genes returned to pre-partum levels by post partum day 1 (Chapter Two, Madsen et al., 2004; Burton et al., 2005). There is still the question of what happens to neutrophil bactericidal behavior during and after the delay in apoptosis. As neutrophils resume their apoptotic program, do they regain expression of surface adhesion molecules to aid in migration from blood into tissues where macrophages can phagocytose the dying cells before they become necrotic? If this scenario is reality, do the corresponding increases in MMP-8 and -9 expression and neutrophil degranulation lead to excessive extracellular matrix degradation in infected peripheral tissues? Or, do the neutrophils lose these functions quickly and regain some of their lost bactericidal capacities once the delay in apoptosis is terminated? If so, do the cells begin to clear pathogens as well as is typically observed in neutrophils from healthy non-parturient animals? If the latter were true, then why is there an increased

susceptibility to disease? Could this be the fault of cells other than neutrophils? On the other hand, genes encoding protein products known to regulate key neutrophil inflammatory and immune functions continue to be decreased on day 1 postpartum and beyond (Weber et al., 2001; Chang et al., 2004; Burton et al., 2005). Several ontological gene clusters were identified in our microarray experiment that relate to traditional bactericidal functions of neutrophils including leukocyte activation, cell adhesion and migration, as well as energy metabolism (Madsen et al., 2002; Madsen et al., 2004). Validation of mRNA and protein abundance decreases for several of these genes may help describe some of the dysfunctions commonly observed in neutrophils from parturient dairy cows (Burton et al., 2005). Altered genes included L-selectin and CD18 (critical to cell adhesion and migration), IL-8 receptor β and FcRN (a cell-surface receptor involved in chemokinesis and antibody-mediated phagocytosis, respectively), as well as mitochondrial cytochrome b and PSST (important in the regulation of ROS generation). Although theses genes may serve as promising candidates for previously described neutrophil dysfunction, it has yet to be determined what factor(s) are responsible for their changed gene expression, if these genes have roles in the described neutrophil behaviors, and if these changes have any relation to disease susceptibility during the parturient period. Thus, it may be necessary to examine neutrophils at molecular and behavioral levels on multiple days postpartum. Continuing studies that link the physiology of parturition with molecular and behavioral changes in neutrophils will only increase the opportunity for researchers to obtain a clear understanding of increased mastitis susceptibility and severity in parturient dairy cows, and thus to develop novel preventatives and therapies for this common and costly disease.

APPENDIX ONE

Figure A.1 Altered expression of 302 genes from **Chapter Two** clustered into 11 clear ontological groups. This clustering was performed in Summer 2002 when the manuscript describing the microarray experiment was initially prepared. At that time the 36% of genes in the "Unknown" group consisted of BOTL clones that did not have a significant BLASTn result and those that exhibited high homology to cloned genomic DNA or specific chromosomal regions.

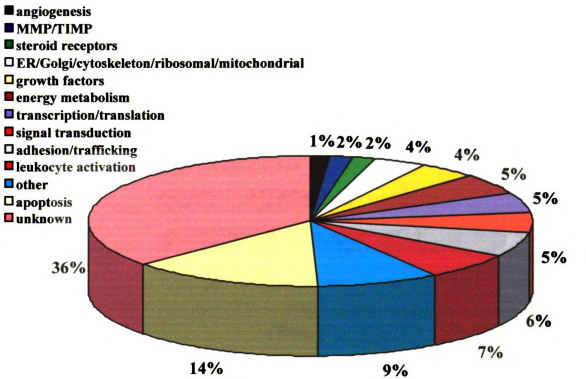


Figure A.2 BOTL clones clustering to the "Unknown" group (**Figure A.1**) were re-analyzed using "BLASTn vs. GenBank" in the "Search Libraries" tool on the MSU Center for Animal Functional Genomics website (http://www.cafg.msu.edu). Eight of the formerly unknown clones now demonstrate significant homology to several known genes (**See Table A.1**), as well as to predicted bovine genes. These predictions were made using a bioinformatics approach and are based on sequence similarities in the bovine genome to known genes in other species utilizing NCBI reference sequences (http://www.ncbi.nlm.nih.gov/RefSEq/). Even now, a few of the original BOTL clones did not have significant homology to any sequences in GenBank whereas the largest number of unknown clones still had significant homology only to cloned genomic DNA or specific chromosomal regions (listed as "Still Unknown").

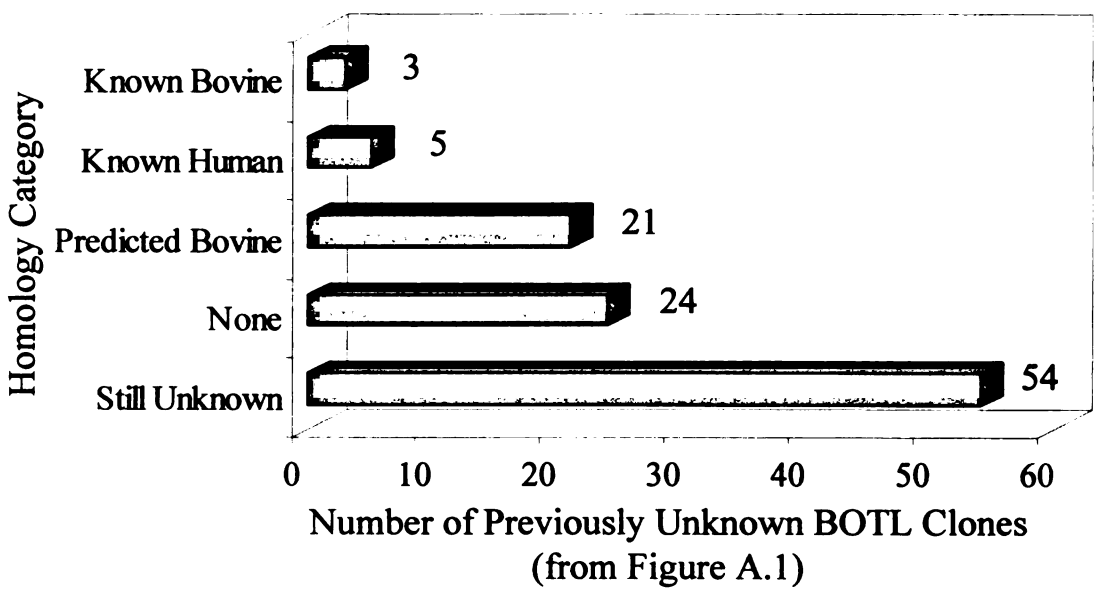


Table A.1 BOTL clones previously grouped in the "Unknown" ontology cluster (Figure A.1) that displayed significant homology to known bovine and human proteins and the ontological cluster to which they may be grouped.

Clone*	BLAST Result	Ontological Cluster	Reference
1XE05R	Bos taurus cell division cycle 10 (CDC10) E-value = 0**	Cytoskeleton	Kartmann and Roth, 2001 Liauw et al., 2002
3XE04R	Bos taurus semaphoring 4A E-value = 0	Leukocyte activation	Takegahara et al., 2005
4XA05R	Bos taurus voltage-dependant anion channel 2 (VDAC2) E-value = 10^{-17}	Apoptosis	Sampson et al., 1997 Cheng et al., 2003
3XH02R	Homo sapiens zinc finger, DHHC domain containing 7 E-value = 10^{-53}	Transcription	Putilina et al., 1999
6XA10R	Homo sapiens zinc finger protein 106 homolog E-value = 10^{-55}	Transcription	Grasberger and Bell, 2005
8_E06	Homo sapiens CCR4-NOT transcription complex subunit 3 E-value = 10^{-174}	Transcription/ Translation	Collart, 2003
9_D11	Homo sapiens CASK interaction protein 2 E-Value = 10^{-20}	Signal transduction	Tabuchi et al., 2002
11_H03	Homo sapiens bridging integrator 1 E-value = 10^{-12}	Apoptosis	Elliott et al., 2000 DuHadaway et al., 2001

*All BOTL clone numbers present in our database (http://www.cafg.msu.edu) are preceded by 'BOTL010000'.

**E-value is a parameter that describes the number of hits one can "expect" to see just by chance when searching a database of a particular size. The closer it is to "0" the more "significant" the match is. (http://www.ncbi.nlm.nih.gov/blast/blast_FAQs.shtml#Expect)

APPENDIX TWO

MICHIGAN STATE
UNIVERSITY

September 13, 2005

Ms. Penny Ripka
Publications Office
American Physiological Society
9650 Rockville Pike
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Dear Ms. Ripka:

I am writing to you in regards to the article:

"Microarray analysis of gene expression in blood neutrophils of parturient cows."

by Madsen et al. that was published in 2004 in *Physiological Genomics* volume 16, pages 212-221. I am a doctoral candidate at Michigan State University and am scheduled to defend my dissertation during Fall semester 2005. I request permission to include this manuscript as Chapter Two of my dissertation.

Thank you for your prompt consideration of this manner!

Sincerely,

Sally A. Madsen-Bouterse

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Publ Mgr & Exec Editor

MICHIGAN STATE
UNIVERSITY

September 13, 2005

Ms. Catherine Nielsen
Copyright Manager
Health Science Rights Department
Elsevier Inc
625 Walnut Street
Philadelphia PA 19106

Dear Ms. Nielsen:

I am writing to you in regards to the article:

"Gene expression signatures in neutrophils exposed to glucocorticoids: A new paradigm to help explain "neutrophil dysfunction" in parturient dairy cows."

by Burton, Madsen, Chang, et al. that was published in 2005 in *Veterinary Immunology and Immunopathology* volume 105, numbers 3-4, pages 197-219. I am a doctoral candidate at Michigan State University and am scheduled to defend my dissertation during Fall semester 2005. I request permission to include Figures 2a, 5, and 6 as well as portions of Table 1 and all of Table 2 in Chapter Three of my dissertation. These data were integral in my dissertation research to demonstrate that glucocorticoids are a key component of parturient cow blood serum in delaying neutrophil apoptosis.

Thank you for your prompt consideration of this manner!

Sincerely,

Sally A. Madsen-Bouterse

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Publication: Figures 2a, 5, 6 and Tables 1 and 2 from VETERINARY IMMUNOLOGY & IMMUNOPATHOLOGY, 105(3-4):197-219, Burton, Madsen, Chang et al: "Gene expression signatures...," copyright 2005 Elsevier B.V.

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