ABSTRACT

SHEATS, MARY KATHERINE. Targeting Myristoylated Alanine Rich C Kinase Substrate (MARCKS) Protein to Inhibit Neutrophil Migration. (Under the direction of Samuel L Jones).

Inflammation is necessary for the maintenance of health and life and occurs most commonly in response to injury or infection. White blood cells involved in the inflammatory response, such as neutrophils, are essential both to host defense from invading microorganisms and wound healing and repair. However, inflammation that is recurrent or fails to resolve can severely damage host tissue; causing both acute and chronic diseases in animals and humans. Despite their inherent benefit, neutrophils are often involved in the pathophysiology of inflammatory diseases. In order to cause tissue damage, neutrophils must migrate through the blood vessel wall and into the interstitial tissue through coordinated signaling between chemoattractants in their external environment and reorganization of their actin cytoskeleton. The molecular regulators that coordinate this signaling are potential targets for pharmaceuticals designed to modulate neutrophil responses such as adhesion and migration. We believe that the MARCKS protein is one such potential target.

The Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) protein is a ubiquitously expressed protein in mammalian cells that is known to bind both the cell membrane and the actin cytoskeleton under the regulation of protein kinase C (PKC) phosphorylation and Ca^{++}/calmodulin. MARCKS is essential to several events requiring dynamic reorganization of the actin cytoskeleton including: myoblast adhesion and spreading, fibroblast migration, endothelial cell migration, mucin secretion in human bronchial epithelial cells, neurosecretion, maintenance of dendritic processes in neuron-like cells and regulation of dynamic adhesion in highly motile melanoma cells. Previous work
from our laboratory used a specific MARCKS inhibitor peptide known as MANS (identical to the N-terminal 24 amino acids of MARCKS) to show that MARCKS function was essential for chemoattractant-induced neutrophil migration and adhesion, two other cellular events that require dynamic actin reorganization. The work presented here expands on these findings, further clarifying MARCKS role in β2-integrin dependent functions and signaling in neutrophils. In Chapter II we investigate PKC-mediated MARCKS phosphorylation in human neutrophils; determining that MARCKS phosphorylation is mediated by δ-PKC. Furthermore, we provide evidence that δ-PKC mediated MARCKS phosphorylation is necessary for fMLF, LTB₄ and IL-8 stimulated neutrophil chemotaxis, as well as fMLF-mediated neutrophil adhesion. This is the first work to correlate the phosphorylation status of MARCKS to functional responses of neutrophils. We continue our investigation of MARCKS role in neutrophil migration, adhesion and respiratory burst in Chapter III, this time conducting a study using equine neutrophils. While this work is described as a comparative study to work previously completed using human and canine neutrophils, it goes beyond simply repeating the same experiments in a different species. The project presented in Chapter III provides important new evidence that further links MARCKS to regulation of neutrophil β₂-integrins, and it adds new information to the timing of MARCKS inhibition provided by the MANS peptide, which is an important consideration in terms of potential clinical utility. Finally, Chapter IV focuses on clarifying MARCKS role in neutrophil β₂-integrin “inside-out” and “outside-in” activation. In this chapter we set out to determine the effect of MARCKS inhibition on β₂-integrin function, affinity conformation, cluster formation and signaling. While some of this work is still ongoing, at this time we believe our
evidence supports the assertion that MARCKS function is essential to β2-integrin “outside-in” activation in neutrophils.

For several decades now we have understood the “yin and yang” of the neutrophil inflammatory response and are reminded of a line from Stan Lee’s *Spiderman* (1962, *Amazing Fantasy #15*); “With great power there must also come great responsibility.” Despite all of the safety measures that Nature so wisely put in place to help limit the destructive power of the neutrophil, their responses sometimes still go awry. Neutrophil mediated injury negatively impacts literally millions of people around the globe each year, causing injury and even death. Some of the diseases caused or exacerbated by neutrophils include acute lung injury (ALI), acute respiratory distress syndrome (ARDS), rheumatoid arthritis, ulcerative colitis, cystic fibrosis, chronic bronchitis, myocardial infarction, gut ischemia reperfusion injury and sepsis. Unfortunately, the clinically available therapeutic options to combat this wide array of neutrophil mediated diseases remain limited and largely supportive, and new medical choices are desperately needed. In order to aid in the development of new anti-inflammatory therapies, it is incumbent upon scientists to lead the way by identifying viable drug targets. As history has shown, the best new ideas often come from a better understanding. We have already shown that the MARCKS protein plays a key role in neutrophil responses. We believe that a better understanding of MARCKS function in neutrophils could have direct impact on the future of therapeutic strategies for targeting neutrophil dysregulation.
Targeting Myristoylated Alanine Rich C Kinase Substrate (MARCKS) Protein to Inhibit Neutrophil Migration

by
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DEDICATION

I dedicate this work to my two sons, Alexander McNeill Sheats (5 years old) and Emmett James Sheats (2 years old). Loving you both puts everything else in perspective.
BIOGRAPHY

On December 8, 1977, Nancy Jo Alexander Peed and Charles Onis Peed had their first child. She was named “Mary” for her mother’s sister Mary and “Katherine” for her father’s sister Anna Katherine. She was called “Katie”.

Katie grew up in Winston-Salem, NC with younger twin sisters Martha Nance and Jessie Elizabeth. They were visited in the summers and on holidays by their older half-sister Laurie Iola Monday. Unfortunately there are few details available about Katie’s demeanor as a child because her mother, by her own admission, has no memories; and Katie’s recollection of being a perfect angel should certainly be suspect. Nancy (Katie’s mother) did remember all of her children as being very nurturing; often caring for injured birds and bunnies (who were incidentally only in need of assistance because of the family cats and dog, respectively).

What Katie’s sisters remember is that Katie was a born leader (their word was “bossy”) and was very intelligent from the beginning (their word was “know-it-all”).

Despite some tensions that may have existed between Katie and her siblings, Katie’s childhood was a happy one and she was afforded an excellent education through the participation in the Highly Academically Gifted (HAG) program of the Forsyth County Public School System. Throughout her grade school education Katie encountered many wonderful, kind, motivated and innovative teachers who she still thinks about today. Whether it was Katie’s natural proclivity toward school in general, or the fact that school was a positive experience as a result of excellent teachers, she excelled in her studies and thoroughly enjoyed the time that they required.
In addition to the time she spent on schoolwork, Katie also spent a significant amount of her
time, truly the majority of it, “at the barn”. The barn was “Irish Oaks Stables” (located in
Pfafftown, NC) and Katie started taking lessons there in the disciple of Hunter Jumper when
she was 8 years old. It is difficult to determine where the idea for horseback riding came
from initially, neither of Katie’s parents had previous exposure to horses. In all likelihood the
idea came from Katie’s older sister Laurie, who once took a few lessons to pass the time
during a summer visit. Regardless of why it started, it stuck; and it wasn’t long before Katie
was at the barn every day. Even though access to horseback riding was clearly a privilege,
and something Katie did for fun, it also taught her that excellence required a great deal of
discipline, dedication and focus. It also taught her respect and responsibility, because
children at the barn were tasked with the care of their own horses. Daily grooming and stall
cleaning were requirements of the lease agreement for the ponies Katie rode over the years
including, Flossie Bobbsey, Shady Lady, Springer, and others. Most importantly, what Katie
learned from horseback riding was a love of horses, and that has made all the difference.
Katie continued riding horses throughout elementary, middle and high school and had to
work hard to find the balance between recreation and studies, because she truly enjoyed both.
In high school she discovered a particular interest in the biological sciences as a result of
taking Advanced Placement (AP) Biology. After graduating from high school in 1996, Katie
attended the University of North Carolina at Chapel Hill where she majored in Biology. After
two years of study, she realized that she ultimately wanted to attend veterinary school; so she
transferred to North Carolina State University (NCSU) to take advantage of their degree in
Animal Science. It was at NCSU that Katie, at the age of 21, met her future husband, Christopher McNeill Sheats.

In order to prepare for application to veterinary school Katie worked summers with Dr. George Hofmann (a small animal veterinarian) as a veterinary assistant. She also volunteered on her day off for Dr. Vikki Newell, an equine veterinarian. These experiences were both instructional in terms of applied veterinary medicine and invaluable in terms of highlighting the differences between lifestyle in different veterinary disciplines. Despite her enjoyment of horses from a personal perspective, Katie decided to pursue small animal medicine as a profession.

Although Katie’s first application to the North Carolina State University College of Veterinary Medicine was declined, she applied again and was successful on the second attempt. Katie started veterinary school in 2001 as a member of the Class of 2005. Veterinary school was everything Katie expected it to be; intellectually engaging, fascinating and challenging. Her excitement and interest is well characterized by one of her former professors Dr. Sam Jones, who fondly recalls her as “The one with all the questions.” Even though veterinary school was all that Katie anticipated, it ultimately did not lead to the career in small animal practice that she had anticipated.

To explain this divergence in her plan, Katie points to her thorough enjoyment of equine medicine on clinical rotations during the fourth and final year of veterinary school. Although Katie had a nice time on her small animal medicine rotation, she realized that working with horses was what truly motivated her. As a result of this shift in career goals, Katie spent her first year out of veterinary school as a rotating equine intern at the NCSU Veterinary
Teaching Hospital and then planned to enter private practice in Raleigh, NC. It was at this point that a different opportunity presented itself. A residency position became available in the NCSU Equine Medicine service and Katie leaped at the opportunity. Over the ensuing three years (2006 – 2009) Katie worked hard to become an excellent equine internist, developing her skills and knowledge in clinical diagnosis, patient directed therapy, disease pathophysiology, evidence based medicine and client communications. As one of the requirements of her residency program, Katie completed an original research project under the direction of a veterinary radiologist named Dr. Anthony Pease. Through this experience she discovered a real interest in answering important scientific question through research. Therefore, upon the completion of her equine medicine residency (2009), she entered a PhD program in Comparative Biomedical Science in the neutrophil biology laboratory of Dr. Sam Jones (NCSU). Also in 2009 (April 26th), Katie had her and Chris’s first child, a son they named Alexander McNeill Sheats.

From 2009 to the present day, Katie has worked happily to complete her PhD. In addition to her studies and research, Katie also spent her time as a graduate student serving on several university service committees and as vice president and then president of the Graduate Student Association. Along the way (May 11, 2012), she and Chris had their second son, Emmett James Sheats. Katie, Chris, Alex and Emmett currently live in Holly Springs, North Carolina on a small hobby farm with two cats (Hallie and Jillie), two aged horses (Star and Feature), three chickens (the numbers keep dropping) and their newest addition, a rescued pitbull they named “Asa”.
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Over the past 10 years I have been lucky to have Dr. Samuel Jones as my professor, trainer, counselor, critic, and mentor. He has supported and encouraged me at multiple career stages and I could not be more grateful to him for sharing his knowledge, perspective, wit and wisdom. Along with Dr. Jones, I would like to thank Drs. Kenneth Adler, Jeffrey Yoder and Jason Haugh for serving on my PhD committee. I recognize the demands placed on each of them in their busy careers and truly appreciate the feedback and insight that each of them provided. I also want to specifically acknowledge the valuable advice provided by Dr. Claire Doerschuck (UNC-CH) who is serving as a mentor on my K01.

To my fellow Jones lab graduate students, past and present, I offer my gratitude for their friendship and for making our office a wonderful place to work. Dr. Laura Ott was the senior graduate student in the neutrophil biology lab when I joined. She was an invaluable resource in terms of research training and academic knowledge and her energy and enthusiasm made each day brighter. Dr. Eui Jae Sung was “second in command” and an absolute “jack of all trades.” I honestly don’t know how we managed to keep the lab in working order since his graduation and departure. His diligence and remarkable work ethic inspired all of us to “Think harder” and “Think again”. (Unfortunately, we still don’t know how to fix any of our own equipment). Dr. Teresa Buchheit, who is finishing her Master’s just as I am finishing my PhD, has been both encouraging and kind; and has always been happy to let me share anecdotes about my little boys, for which I am grateful. Ms. Emily Medlin, a DVM/PhD student in the neutrophil biology lab, is an excellent listener for matters both professional and personal and she gives advice just as well as she receives it (even though she thinks she is the
one who always needs it). To Ms. Rebecca (“Becky”) Till, the newest graduate student in the Jones lab, I offer my thanks for sharing her spirit and vitality. To both Emily and Becky, I look forward to continuing our “philosophical Fridays”. I would also like to thank Ms. Katie Bell for her organizational and management skills; she is yet another person whose absence from the lab was sorely felt, but I am so excited to learn that her next adventure will be veterinary school in Michigan!

For their personal and professional support, I would like to thank Dr. Tracy Hill, Dr. Callie Fogle, Dr. Jonathan Fogle, Dr. Meghali Nigot and Dr. Kristen Messenger. I am also grateful to everyone who has served as a blood donor for my research over the past five years; the work truly couldn’t have been done without you.

In terms of funding and financial support, I would like to thank the North Carolina State University Center for Comparative Medicine Training Program (CCMTRTP) for my T32 funding as well as a pilot grant award. I would also like to thank the Office of the Director (NIH) for my K01 funding.

Finally I would like to thank my family. I am so appreciative to my parents for their high expectations balanced by their unwavering love. I am grateful to my sisters for staying, or coming back to, North Carolina. I am grateful for my husband’s family, who love me as one of their own. And finally, I want to say thank you to my husband; for learning the title of my research publication, “Use of Ultrasound to Evaluate Outcome Following Colic Surgery for Equine Large Colon Volvulus”, for not making me talk about work at home, for feeding my horses, for planning vacations around conferences and most importantly, for our boys.
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CHAPTER 1

Evolving ideas on sepsis and the role neutrophils play: an equine perspective

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Abstract

Sepsis is recognized as the host’s systemic inflammatory response to infection. This definition is fairly well accepted across species, by medical practitioners and biomedical researchers alike. Neutrophils, which are the most abundant leukocyte in circulation, play an essential role in defending the host during local tissue infection. Unfortunately, neutrophils play an equally important role in often catastrophic host injury during sepsis, making them important potential targets for sepsis treatment and prevention. In this review I will discuss current updates on opinions and concepts regarding the pathophysiology of sepsis and the systemic inflammatory response syndrome (SIRS). I will also review what we know about the neutrophil’s role in equine and human sepsis, based on evidence from species-specific research and clinical medicine. Future identification of clinically relevant sepsis therapies depends on a more thorough understanding of disease pathogenesis across species, which will only come from research studies that utilize a defined classification system of at-risk patients.

Introduction – The Impact of Sepsis

Sepsis, which is defined as the syndrome of dysregulated inflammation that occurs as a result of infection, claims millions of human lives worldwide each year. In North America alone, more than 750,000 people develop sepsis annually (Dellinger et al., 2004). While there is some positive news that the sepsis mortality rate in humans appears to be declining (27% in 2007 down from 39% in 2000), it is still unacceptably high (Dellinger and Vincent, 2005). The positive impact of this decline in mortality is further negated by the continued rise in the
rates of sepsis diagnosis, which reportedly doubled between 2000 and 2008 (Hall et al., 2013), making it the 11th leading cause of death in the United States in 2010 (Murphy et al., 2013). From a national health perspective the impact of sepsis is undeniable, as the Healthcare Cost and Utilization Project (HCUP) identified sepsis as the single most expensive condition treated in hospitals in 2011, with a total aggregated cost of $20.3 billion (Torio and Andrews, 2006). As a result of its impact, sepsis continues to be the focus of intense research in which the current understanding of pathophysiology is being re-evaluated, the relevance of various in vitro and in vivo models is being challenged (Deitch, 1998, Buras et al., 2005, Marshall et al., 2005a) and the importance of species specific differences (Rittirsch et al., 2007) is being recognized. It is certainly the hope of clinicians and basic scientists alike that this re-examination will lead to new approaches and new targets for the treatment and even prevention of this devastating condition.

As is almost always the case for disease impact on veterinary species, what we know about the cost and incidence of sepsis in equine patients is not nearly as well characterized as its impact on humans. While the development of a systemic inflammatory response is commonly recognized as a consequence of numerous infection- and/or endotoxin-associated conditions that affect horses, in equine medicine to date, there is no available epidemiologic data on the incidence and mortality associated with sepsis in horses (MacKay, 2000, Roy, 2004, Moore and Vandenplas, 2014). However, rates of sepsis mortality have been reported based on data obtained from individual veterinary hospital populations. In neonatal foals with sepsis, reported survival rates vary from 10% - 72%, with significant variability in sample population and sepsis definition between studies (Koterba et al., 1984, Hoffman et al., 1992,
Gayle et al., 1998, Peek et al., 2004). In general, lower survival rates are reported in studies that include positive blood-culture in the definition of sepsis. In terms of financial cost, one prospective study reported that the mean cost of hospitalization and treatment for foals that survived sepsis was $2842.00 (Peek et al., 2004), but based on severity of illness and duration of hospitalization, the individual patient costs can certainly be much higher. In contrast to the relatively large numbers of studies that have examined the impact of sepsis on survival in hospitalized foals, studies on sepsis mortality in adult horses, even in individual hospital populations, are few and far between. Furthermore, the frequent use of clinical ‘endotoxemia’ instead of SIRS further muddies interpretation of the data (Senior et al., 2011). Additional studies are needed to further clarify the impact of sepsis on survival in both adult horses and foals.

In 2006 the American College of Veterinary Emergency & Critical Care (ACVECC) made the first attempt to generate a consensus opinion of veterinary sepsis, and issued a survey on the diagnosis and treatment of sepsis in dogs, cats and horses to members of the ACVECC, Veterinary Emergency & Critical Care Society (VECCS), American College of Veterinary Internal Medicine (ACVIM), and Student Veterinary Emergency & Critical Care Society (SVECCS). Based on the responses to this survey (101 total surveys were completed) the estimated incidence of sepsis was 1-5% in feline patients and 6-10% in canine patients. In cats and dogs the median estimates of sepsis survival were 10-25% and 25-50%, respectively. The survey failed to generate any data in regards to equine sepsis due to a lack of response from equine practitioners (Otto, 2007b). While this lack of response is disappointing, it should in no way be misconstrued as a lack of interest in sepsis on the part
of equine practitioners and researchers. To the contrary, the scientific- and lay-literature is continually updated with new ideas and studies on equine sepsis pathophysiology, diagnosis and treatment; unfortunately these studies often consist of small patient populations and use inconsistent inclusion criteria or outdated terminology. Clearly, the time has come for those of us involved in equine practice and equine health research to unify our efforts. Areas that need to be addressed include the adoption of accurate definitions, a classification method for “at-risk” patients based on type and location of infection as well as possible genetic predispositions and identification of “markers” of sepsis that will help classify an individual patient’s immune response. This type of patient classification system would help to minimize the patient heterogeneity that has proven so detrimental to human clinical trials. With many of these individual pieces already underway, it is time we start fitting the puzzle pieces together.

**How do we define sepsis?**

*The Current Consensus*

In 1991, Roger C. Bone chaired a “Consensus Conference” of the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM); which was tasked with the “goal of agreeing on a set of definitions that could be applied to patients with sepsis and its sequelae” (Bone et al., 1992). It was expected that the definitions developed would be broad enough to improve early bedside detection of sepsis in order to allow for early therapeutic intervention. It was also anticipated that consensus on sepsis definitions would help to standardize research protocols, which would lead to “improved dissemination and
application of information derived from clinical studies” (Bone et al., 1992). The result was the development of a broad series of definitions (Table 1) (Bone et al., 1992) that are to this day, currently used in practice and have served as the basis for numerous human clinical trial inclusion criteria over the past two decades (Levy et al., 2003).

Importantly, as a result of this conference the term “systemic inflammatory response syndrome” was put forward as a criterion of the sepsis definition. As defined by Bone et al., SIRS is considered to be present when human patients have one or more of the following:

1) Body temperature > 38°C or < 36°C
2) Heart rate > 90 beats/minute
3) Hyperventilation (respiratory rate > 20 breaths/minute or PaCO2 < 32 mmHg)
4) White blood cell count > 12,000 cells/ul or < 4,000 cells/ul or > 10% band forms

The definition of SIRS, along with the definitions of “sepsis”, “severe sepsis” and “septic shock” as proposed by Bone et al., has also gained general acceptance within veterinary medicine, and are cited in numerous equine specific reviews on sepsis (Moore and Vandenplas, 2014, Palmer, 2014).

Although no census definitions are available, examples of published criteria used to diagnose SIRS in adult horses is 2 or more of the following (Epstein et al., 2011):

1) Body temperature > 101.5°F
2) Heart rate > 60 beats/minute
3) Hyperventilation (respiratory rate > 30 breaths/minute)
4) White blood cell count > 12,500 cells/ul or < 4,500 cells/ul and 10% band neutrophils
Criteria for the identification of SIRS in equine neonates have also been published by multiple investigators. One proposed definition is 2 or more of the following (Corley et al., 2005):

1) Body temperature > 39.2°C or <37.2°C
2) Heart rate > 120 beats/minute
3) Hyperventilation (respiratory rate > 30 breaths/minute)
4) White blood cell count > 12,900 cells/ul or < 4,00 cells/ul, or > 10% band neutrophils,

along with evidence of sepsis, cerebral ischemia or hypoxia, or trauma. Using these criteria, researchers determined that SIRS was present in nearly 30% of adult horses with colic (Epstein et al., 2011), and more than 40% of critically ill neonatal foals (Corley et al., 2005), who presented to a teaching hospital for evaluation and treatment. While the criteria used by these researchers are a reasonable equine adaptation of the ACCP/SCCM definitions, there has been no proposed consensus of equine specific definitions of SIRS or sepsis. As a result, the definitions and criteria used vary among individuals conducting equine specific research.

In 2001, a second International Sepsis Definitions Conference convened to respond to growing criticisms from experts in the field that the 1991 ACCP/SCCM definitions did not reflect an up to date understanding of the pathophysiology of human sepsis and its associated syndromes, and were contributing to erroneous and flawed clinical trial data (Bone, 1996a, Bone, 1996b, Bone, 1996c, Vincent, 1997, Abraham, 1999). Critics were primarily dissatisfied with the inclusion of the SIRS criteria in the definition of sepsis, arguing that the criteria lack specificity, and are too sensitive to be of any use in clinical diagnosis or clinical
trials (Vincent, 1997). This argument was supported by data showing that most intensive care unit (ICU) patients and many general ward patients met the SIRS criteria (Pittet et al., 1995, Rangel-Frausto et al., 1995, Salvo et al., 1995, Bossink et al., 1998). Further criticism claimed that a SIRS diagnosis provided no information regarding the underlying disease process and was simply a list of clinical signs routinely seen in patients having a potentially appropriate response to a physiologic insult. Another important criticism for the sepsis definition was the fact that actual infection could often only identified in 50% (or less) of patients who ‘appeared’ septic (Brun-Buisson et al., 1995, Rangel-Frausto et al., 1995, Deitch, 1998).

Given the aggravated list of complaints leveled against the 1991 ACCP/SCCM sepsis definitions, it is somewhat surprising that very few changes to the original definitions were made by the second Sepsis Definitions Conference (Table 2). The participants of the 2001 conference did concur that the SIRS criteria were too sensitive and lacked needed specificity. As a result, they created an expanded list of possible signs of systemic inflammation in response to infection (Table 3), with the caution that practitioners looking to establish a diagnosis of sepsis should only include those diagnostic criteria that cannot be easily explained by other causes. The 2001 Consensus concluded that more meaningful changes to the definition of sepsis and its associated conditions based on biomarkers was premature; and that future changes to these definitions would rely on an increased understanding of, and readily available means to diagnose, the immunological and biochemical processes involved in sepsis (Levy et al., 2003).
Sepsis and SIRS: the debate continues

It is important to note that the Consensus Statement from the ACCP/SCCM defined sepsis as SIRS plus infection; inexorably linking sepsis to a term used to describe a systemic activation of the innate immune response, regardless of cause. Indeed, SIRS is recognized in patients with numerous conditions including bacterial infections, viral infections, hemorrhage, trauma, thermal injury, or sterile inflammation (i.e. acute pancreatitis) (Bone et al., 1992). As one particular critic points out, an individual who experiences tachycardia and tachypnea as a result of exercise fits the current definition for systemic inflammatory response syndrome (SIRS) (Vincent et al., 2013). In equine patients SIRS has been identified in adult horses with gastrointestinal disease (i.e. colitis, enteritis, strangulating obstructions), pleuropneumonia, clostridial myositis and septic endometritis (Purvis and Kirby, 1994, MacKay, 2000, Roy, 2004, Epstein et al., 2011, Belknap and Black, 2012), as well as in foals with viral infection, bacteremia, pneumonia, septic arthritis and perinatal asphyxia syndrome (Perkins et al., 1999, Moore and Vandenplas, 2014, Palmer, 2014).

At the time that the SIRS definition was introduced, the focus from both a diagnostic and therapeutic potential was on the importance of the host response to infection; and rapid, readily available diagnostic criteria for sepsis were desperately needed to enroll patients in clinical trials for promising new therapeutics. Since that time over 40 approaches to target host signaling and response pathways involved in sepsis have failed to improve, or even lowered, human sepsis survival (Artenstein et al., 2013). Among the list of potential explanations for the failure of treatments that had appeared so promising in animal models of sepsis (i.e. inappropriate animal models, diverse etiologies of sepsis in the same study, lack
of control for comorbidities, flawed outcome measures) is the use of SIRS as entry criteria for human clinical trials (Abraham, 1999, Marshall, 2003, Marshall et al., 2005b, Rittirsch et al., 2007). In a recent guest editorial, veterinarian Cynthia Otto aptly summarizes the criticisms of human sepsis clinical trials. She points out that the use of SIRS criteria for enrollment may have resulted in a patient population with such diverse disease severity and etiology that the impact of therapeutics that may have otherwise proven beneficial to a specific subset of septic patients was undetectable in such a heterogeneous patient population. As we pursue investigations in equine-specific sepsis research, it is my sincere hope that we learn from the lessons of our predecessors, and strive to conduct meaningful and thoughtful investigations that will lead to advances in the diagnosis, monitoring and treatment of our septic patients.

**PIRO**

On tool that has been used to categorize patients with disease is a staging or scoring system. The concept of a clinically useful sepsis staging system was introduced by the 2001 International Sepsis Definitions Conference. The consensus stated that such a system would “stratif[y] patients by both their risk of an adverse outcome and their potential to respond to therapy” (Levy et al., 2003). The classification scheme proposed for sepsis – called PIRO – addressed 4 major areas to stage sepsis in a manner similar to the TNM (Tumor, Lymph Nodes, Metastasis) staging system (Mirsadraee et al., 2012) used for oncology patients. The first component, Predisposition, addresses the growing understanding that the same insult (i.e. infection) can cause a more severe response or worse outcome in some individuals compared to others. In human medicine the variables of Predisposition include genetics,
gender, age and nutritional status. Clinically, this category is also recognized in veterinary patients. A recent publication by Nemzek et al. demonstrated that the white blood cells of Rottweilers and Doberman Pinschers produced a marked inflammatory cytokine response compared with that of mixed breed dogs (Nemzek et al., 2007). The authors correlate their findings with the predisposition of these breeds for severe infection with canine parvovirus. The concept of Predisposition is also relevant to equine patients, whose white blood cells demonstrate extreme sensitivity to lipopolysaccharide (LPS), a structural component of the outer cell wall of gram-negative bacteria, when compared with other species (Moore and Vandenplas, 2014). Furthermore, in terms of cytokine production, LPS activation of equine monocytes is markedly pro-inflammatory when compared with other species (Figueiredo et al., 2009). These differences, along with others, likely make horses uniquely predisposed to experience detrimental inflammation during clinical conditions that lead to LPS exposure.

The second component of the PIRO staging system addresses Insult (i.e. infection). This category conveys the importance infection type and location, which is known to have an important impact on outcome in both humans (Martin et al., 2003) and horses. As evidence of this fact, a recent publication by DeClue and colleagues demonstrated that equine blood stimulated with components of Gram-negative and Gram-positive organisms produced differing cytokine profiles (Declue et al., 2012). Several other recent studies on equine sepsis in both foals and adults have moved beyond the traditional focus of Gram-negative infection/endotexemia’ to examine the impact of Gram-positive and mixed infections on clinicopathologic features of disease and outcomes in the development of equine systemic inflammatory response syndrome (Marsh and Palmer, 2001, Corley et al., 2007, Wereszka et
al., 2007, Russell et al., 2008, Johns et al., 2009). As we move forward with equine specific sepsis research, working to generate evidence-based medicine for the efficacy of various therapies for the treatment of sepsis, it will be particularly important to classify our patients by documented or suspected infection type, and discard the tired concept that all systemically inflamed horses are ‘endotoxemic’.

The third component of the PIRO sepsis staging system is the Response to sepsis. The physical and biochemical parameters that routinely describe septic human and veterinary patients are documented and well known, and incorporated into the currently accepted definitions of sepsis and its associated syndromes (Bone et al., 1992, Levy et al., 2003). Unfortunately, these parameters applied clinically (i.e. SIRS diagnosis) are often unable to help clinician’s gauge the severity of, or the prognosis for, an individual patient’s illness; and they lack sensitivity when it comes to inclusion criteria for clinical trials. It has been proposed that biomarkers of sepsis, in conjunction with other criteria, would be useful for diagnosing, guiding treatment and predicting outcomes for patients with sepsis (Sutherland et al., 2011, Faix, 2013, Ratzinger et al., 2013, Sankar and Webster, 2013, Vassiliou et al., 2014). As of 2013, there were a reported 178 biomarkers described in the scientific literature (Sankar and Webster, 2013), although few have made it to routine use in clinical practice. Biomarkers of inflammation have also been investigated recently in horses, particularly in septic foals (Pusterla et al., 2006, Gold et al., 2007, Paltrinieri et al., 2008) and adult horses with colic (Forbes et al., 2011, Silva et al., 2013). Although more research is certainly needed, it appears as though biomarkers in horses, as in people, will add a great deal to our ability to understand, diagnose and even potentially treat septic horses.
The final component of the PIRO staging system is Organ failure. Evidence from both human and veterinary medicine indicates that evidence of organ dysfunction is an important determinant of prognosis in patients with sepsis (Vincent et al., 1996, Marshall, 1999, Roy, 2004, Sheats et al., 2014), but the organ system affected, as well as molecular mechanisms of injury, can be very different depending on the species. At this time, the utility of organ failure as a component of staging sepsis in veterinary species is likely to be limited to determination of prognosis; as the high cost, as well as practical application (i.e. mechanical ventilation of adult horse), of ICU-level supportive care can be prohibitive in veterinary patients.

Since its inception, the PIRO concept has been tested in human sepsis trials. Although some additions have recently been made, and additional suggestions exist for the future (i.e. biomarkers, a dynamic view of the patient daily clinical course) (Table 4), the PIRO scoring system has proven to be a reliable predictor of mortality in human patients with sepsis (Howell et al., 2011, Granja et al., 2013). It remains to be seen whether PIRO can, not only predict patient outcome, but also be used to impact it.

The thought of a staging system for sepsis in veterinary species is an interesting one. With our different species and breeds, we are well positioned to investigate the impacts of genetics on sepsis. In addition, our equine patients generally have a low rate of co-morbidities, (metabolic disease probably being the most common for adults, along with failure of passive transfer for foals) and this would likely simplify that aspect of patient stratification. But one challenge would be actual identification of patient sepsis based on infection. Currently, the blood culture status of our critically ill adult patients is not routinely investigated and
obtaining a positive blood culture result can be a challenge; but certainly more work in this area needs to be conducted to determine the true incidence of sepsis in equine patients with critical illness, and methods of microbial detection are improving. Certainly the most commonly expressed concern is that veterinary studies are generally conducted with small numbers of patients, and further patient stratification will likely reduce powers of statistical testing. But the alternative – putting patients that don’t belong together in the same clinical trial – is a recipe destined for failure, as our human counterparts can certainly attest. So as we pursue new avenues of sepsis research, let’s pause and make sure we’re on the right road.

Where does ‘endotoxemia’ fit in the definition of sepsis?

As pointed out in a recent article by Moore and Vandenplas (2014) (Moore and Vandenplas, 2014), a large contingent of equine clinicians use the term ‘endotoxemia’ to describe patients that have elevated heart and respiratory rates, dark or injected mucus membranes, prolonged capillary refill time and fever – all clinical criteria that are also used to diagnose sepsis (Table 3). Indeed, there are over 200 publications in the Pubmed database that are returned by the search terms ‘endotoxemia’ [and] ‘horse’. The precedent for the importance of endotoxin was set long before it was focused on in horses specifically. As early as the 1890s, researchers recognized that “toxins linked to the bacterial body substance” caused circulatory shock and death in laboratory animals (Rietschel and Cavaillon, 2003, Kumar and Sharma, 2010). As studies on the effects of purified endotoxin in horses began in the 1960s and 1970s, it was discovered that the dosage used to study LPS responses in other species was excessive for horses. Knowledge of the horse’s extremely sensitive immune response to
endotoxin, along with the strong clinical association between colic, mucosal permeability and Gram-negative bacteria, have led to general acceptance of the term ‘endotoxemia’ in describing horses exhibiting clinical signs of a systemic inflammatory response.

Interestingly, there is a parallel between clinical studies investigating equine endotoxemia and clinical trials investigating human sepsis. In multiple human studies that enrolled patients who met the clinical criteria for sepsis, only 50% or fewer were ultimately shown to have microbial infection as the cause of their clinical signs (Nathens and Marshall, 1996). Similarly, endotoxemia is routinely only identified in 25-41% of equine patients whose clinical signs are consistent with the ‘syndrome’ of endotoxemia (King and Gerring, 1988, Fessler et al., 1989, Steverink et al., 1994, Werners et al., 2005, Senior et al., 2011). While this parallel may simply be a reflection of the limitations of currently available detection methods, it should also serve as a caution against attributing a dysregulated host immune response (i.e. sepsis) to microorganisms or endotoxin, specifically.

The answer to the question, “Where does endotoxemia fit in the definition of equine sepsis?” is that it doesn’t. The clinical picture that equine clinicians associate with endotoxemia is defined by SIRS; and currently, the criteria for SIRS are how we define sepsis. While LPS-infusion is argued to be a readily available and reproducible “sepsis model”, it is highly likely, based on decades of failed human sepsis research, that it is not representative of clinical disease, even in the exquisitely LPS-sensitive equine species. We as veterinary scientists should heed the cautionary tail from our colleagues in human medicine, and remember that diagnostics derived from, and promising therapeutics identified by, single source models of sepsis may have limited translation to actual clinical patients.
You say endotoxemia, I say SIRS; let’s call the whole thing off.

There is no doubt that endotoxin is an important mediator of equine inflammation in a variety of clinical situations. Additionally, LPS-infusion models have yielded important discoveries for equine specific inflammatory cell signaling, and have helped define the clinical picture of equine systemic inflammation. However, our ability to illustrate the importance of endotoxin in specific case examples or in specific research settings does not mean we then have license to conduct studies in equine patients and attribute our findings to ‘endotoxemia’ (i.e. LPS in circulation) just because those patients fit the clinical description of being ‘endotoxemic’, or had gastrointestinal disturbance that may or may not have led to the absorption of endotoxin across the mucosal barrier. Interestingly, recent results in the equine laminitis literature have done a nice job illustrating that it is time to stop focusing on endotoxin as though it is the only cause of systemic inflammation in horses. It has long been recognized that post-operative colic patients were at an increased risk for the development of laminitis. Because these patients fit the clinical description of ‘endotoxemia’, and various levels of endotoxin could be isolated from the blood of horses with carbohydrate-overload (CHO) induced laminitis, many were convinced that gut-derived endotoxin was the cause of laminar injury in these post-operative patients. Consequently, researchers have made repeated attempts to use endotoxin administration to induce lamintis, but to no avail (Toth et al., 2009, Kwon et al., 2013). Most recently, Kwon et al. (2013) concluded that “factors other than endotoxin are responsible for the changes in laminar tissue gene expression that occur during the development of acute equine laminitis” (Kwon et al., 2013). This conclusion is an important step in recognizing that clinical conditions historically associated with endotoxemia do not
necessarily share a causal relationship with LPS, and that equine health researchers and clinicians should expand their focus from LPS alone to include other potential host and bacterial factors that might play a role in equine sepsis and its associated impacts (i.e. laminitis).

So what terminology should we use instead? A review of the recent equine literature clearly shows a trend toward the consensus definitions of SIRS and sepsis that have been proposed by our human colleagues (Moore and Vandenplas, 2014). But veterinarians should take note that significant debate on the use of SIRS in the field of human medicine continues. The criticisms of SIRS can be summarized by three major points (Vincent et al., 2013, Balk, 2014):

1. Too many patients fit the definition of SIRS for it to be useful in either a research setting, or clinically.

2. The definition doesn’t help differentiate patients having normal, physiologic host response from those whose response produces organ dysfunction.

3. SIRS is a diagnostic criterion of sepsis, but it remains difficult to determine the role of infection in this response, as SIRS can be seen with other, non-infectious conditions.

While the answer to these criticisms on the human side remains to be seen, it is interesting to note that in the survey of veterinarians conducted by the Society of Critical Care Medicine, the majority of veterinary respondents require 3 of 4 SIRS criteria to be present in order to diagnose sepsis in dogs and cats, compared to the human requirement for 2 out of 4 (Otto, 2007b).
Recent opinions on the most appropriate definition of human sepsis have made a point to emphasize that septic patients “look bad” due to some degree of organ dysfunction already being present (Vincent et al., 2013, Balk, 2014). In 2010, the International Sepsis Forum, held at the Merinoff Symposium, wrote, “sepsis is a life-threatening condition that arises when the body’s response to an infection injures its own tissues and organs” (Czura, 2011). When compared with the 1991 and 2001 Consensus definitions of sepsis, the distinction is clear. This newer definition conveys the concept that sepsis is not simply “the host response to infection”, it is a potentially life-threatening disorder that requires immediate treatment to avoid further patient deterioration and to optimize the likelihood of patient response to therapy. It remains to be seen whether the definition offered at the Merinoff Symposium will gain traction and the term ‘severe-sepsis’ will become obsolete.

Clearly, as this review has already discussed, there are problems with the ways in which the terms ‘endotoxemia’ and ‘SIRS’ have been used, by physicians, veterinarians and biomedical researchers alike. Instead of continuing to debate the use of these terms, let us instead turn our efforts to defining a reliable system of stratifying septic patients that will be applicable in terms of basic, translational and clinical research, as well as the practice of clinical medicine.

**Mechanism of Host Defense**

*Local Inflammation*

The four cardinal signs of inflammation (as described in 1st century AD) are ‘rubor et tumor cum calore et dolore’ (Rocha e Silva, 1978). This translates to redness and swelling with heat and pain. In today’s terms, we explain some of these ancient observations with the modern,
but no more accurate, terms of peripheral vasodilation (redness), vascular leakage (swelling) and fever (heat). We now understand that these concepts, recognized by the Ancient Greeks Galen and Celsus, are essential to local host defense from invading microorganisms. Within the environment plants and animals are continuous exposed to potentially invasive microorganisms, and as such are armed with defensive mechanisms. In mammals, the first line of defense is the physical barrier, including skin and mucus membranes, mucus layers that line the respiratory and gastrointestinal tracts, various enzymes and antimicrobial peptides. These barriers repel the vast majority of microorganisms, but when pathogens do “breach the wall,” vasodilation and vascular leakage, mediated by the local release of tumor necrosis factor-α (TNFα), interleukin (IL)-1β and histamine, promotes the recruitment of professional phagocytes (i.e. neutrophils) to the area (MacKay, 2000). Armed with bactericidal and proteolytic enzymes, as well as the ability to produce reactive oxygen intermediates, neutrophils are uniquely suited for killing pathogens. Additionally, local activation of the coagulation cascade slows the dissemination of the infectious organisms through the formation of microthrombi, which occlude small vessels. Without these basic, evolutionarily derived mechanisms of defense, the smallest injury to the host’s physical barrier (i.e. a cut) would have spelled certain doom. Unfortunately, when these immune mechanism intended for local host defense are activated systemically, it is the host, and not the microorganism, that suffers.
**Pathophysiology of Sepsis**

In response to microbial invasion, the host’s immune system mounts a response in order to eliminate the invading pathogen, heal the body’s defensive barriers and restore homeostasis. Sepsis occurs when the host response to infection becomes systemic, even if the actual infection isn’t. This systemic response has detrimental effects on tissues and organ systems remote from the site of initial injury (Iskander et al., 2013). The immune mechanisms and signaling cascades involved in the pathophysiology of sepsis are extremely complex. This review will provide an overview of these topics, with a focus on the role of neutrophil dysfunction in sepsis and organ damage. Equine relevant data will be included.

**Pathogen Recognition – a key component of host defense**

In vertebrates, there are two arms to the immune system: innate and adaptive immunity. The innate immune system was the first to evolve, and as such is present in all plants and animals, while adaptive immunity is found only in vertebrates. Both components of the vertebrate immune system have unique features that are vital for optimal host defense. The innate immune response is capable of instantaneous pathogen recognition and rapid mobilization of destructive cellular forces, but it is incapable of providing long-term immunity. Long-term immunity is the responsibility of the slower responding adaptive immune system. This review on sepsis focuses on components of the innate immune system.

*PAMPs, DAMPs and TLRs*

Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs, also “alarmins”) are key molecules in the immune system recognition of pathogens
and tissue injury, respectively. The list of PAMPs includes lipopolysaccharide (LPS), flagellin, peptidoglycan, lipoteichoic acid, double stranded viral DNA and unmethylated CpG motifs (Werners and Bryant, 2012). Molecules that act as DAMPs include hyaluronan, heparan sulfate, heat shock proteins, high mobility group protein B1 (HMGB1) and ATP (Chen and Nunez, 2010). While DAMPs have received recent attention as models of sterile inflammation, both are relevant to the discussion of sepsis pathophysiology. The hallmark of clinical sepsis is a harmful or damaging host response to infection. The PAMPs and DAMPs present in inflamed and/or infected tissues are recognized by signaling pattern recognition receptors (PRRs) on the surface of immune cells such as neutrophils, macrophages, mast cells and dendritic cells, as well as non-immune cells such as epithelial cells, endothelial cells, myocytes and fibroblasts (Rakoff-Nahoum et al., 2004, Matzinger, 2007, Tan et al., 2014). While it is generally accepted that PAMPs are involved in the initiation of the inflammatory response, it seems reasonable to suspect that tissue injury caused by invading pathogens and/or the host (i.e. neutrophil mediated damage) would generate DAMPs. DAMPs further activate the innate immune response, leading to additional inflammatory cytokine signaling, neutrophil recruitment and tissue damage. Considering the cross-reactivity of individual PRRs for PAMPs and DAMPs (Chen and Nunez, 2010, Szatmary, 2012, Tan et al., 2014), it is not difficult to imagine to vicious cycle of signal amplification that ultimately results in a global dysregulation of the immune response. Indeed, evidence shows that interaction of PAMPs and DAMPs with their receptors has been correlated with increased rates of fatality in an animal model of sepsis (Williams et al., 2003). Although at present the consequences of this amplification cycle in veterinary species is only inferred,
crossreactivity of equine PRRs with different DAMPs and PAMPs has been demonstrated (Benbarek et al., 1998, Declue et al., 2012).

There are numerous types of PRRs including: Toll-Like receptors (TLRs), leucine-rich repeat containing proteins (NLRs, previously called NOD-like receptors), C-type lectin receptors (CLRs) and RIG-I like receptors (RLRs) (Kawai and Akira, 2011, Szatmary, 2012). Only TLRs, with a focus on available equine specific data, will be addressed in the current review. As a key pattern recognition receptor, TLR signaling is an essential step in the activation of the innate immune response. TLRs are needed for the clearance of bacterial infection, but excessive TLR signaling can lead to systemic inflammation, which can have detrimental consequences to the host. Because of the relative importance of endotoxin in a number of equine diseases, Toll-Like receptor 4 (TLR4), which binds the lipid A component of LPS, has been the subject of much research and will be further discussed. Other members of the TLR family identified in horses (TLR 2, 3, 5, 8 and 9) are reviewed in detail elsewhere (Gornik et al., 2011, Werners and Bryant, 2012, Moore and Vandenplas, 2014).

In order to bind TLR4, LPS must first interact with LPS-binding protein (Zychlinsky et al., 2003, Schumann, 2011). Next, LPS monomers bind either soluble or membrane bound (myeloid cells) CD14. After CD14 binding, LPS then interacts with TLR4 and its co-receptor MD-2, which results in TLR/interleukin (IL)-1 receptor associated protein (TIRAP)-dependent recruitment of the MyD88 adaptor protein (Brikos and O’Neill, 2008, Szatmary, 2012). MyD88 activation causes activation of the transcription factor NF-κB, as well as phosphorylation of mitogen-activated protein kinases (MAPK). The most well recognized and frequently reported result of TLR4-LPS binding is NF-κB mediated upregulation of pro-
inflammatory mediators such as TNFα and IL-1β. However, LPS-mediated TLR4 activation on circulating monocytes in some species shows simultaneous upregulation of genes for IL-10 and IL-1Rα, showing that TLR4 signaling also activates anti-inflammatory mediators (Calvano et al., 2005). Importantly, this two-pronged activation of pro- and anti-inflammatory mediators serves to rapidly clear infection while limiting the overall level of immune system activation.

This reported simultaneous activation of pro- and anti-inflammatory mediators by TLR4 is somewhat different in the horse. TLR4 mediated production of IL-10 is regulated by the activation of two TLR4 adaptor molecules, TRIF and TRAM (Brikos and O'Neil, 2008, Siegemund and Sauer, 2012). In order for these adaptor molecules to become activated, TLR4 must be internalized in endosomes, where it then associates with TRAM-associated TRIF (Siegemund and Sauer, 2012). The time required for TLR4 internalization in equine cells is delayed, which may contribute to the delayed production of IL-10 following TLR4-LPS binding.

Based on differences in equine TLR4-LPS signaling, it would be fair to state that the equine monocyte response to LPS is more pro-inflammatory than that of other species. This is primarily due to the fact that LPS-activation of TLR4 in equine monocytes only signals by the MyD88 pathway, and not TRIF/IRF3 (Figueiredo et al., 2009). As a result of MyD88 signaling, LPS stimulation of equine monocytes elicits a rapid and short-lived induction of TNFα, a rapid and more sustained expression of IL-1β, and slower but sustained induction of IL-6. After a significant delay, IL-10 expression is also induced, although the level of induction is much lower than that seen with TLR3 agonists (i.e. double stranded viral DNA).
In general, MyD88 signaling is regarded as pro-inflammatory, while the TRIF pathway is considered anti-inflammatory. In other species, PI(3)Kδ regulates the switch in TLR4 signaling between MyD88 and TRIF, an event which does not seem to occur in equine monocytes. These attributes of TLR4 signaling specific for equine monocytes, taken together with their remarkable sensitivity to nanomolar concentrations of LPS, provide a scientific basis for the marked inflammatory response that horses experience in response to endotoxin exposure.

_SIRS, CARs and MARs (oh my)_

In the early days of human sepsis research, the focus was the systemic inflammatory response syndrome (SIRS), which was believed to be an overwhelming and unchecked spike in the host’s pro-inflammatory immune response (Iskander et al., 2013). Although SIRS can occur in response to trauma, anaphylaxis, ischemia, hemorrhage and sterile inflammation (i.e. pancreatitis), in the case of sepsis, the inciting event is documented or suspected infection. High levels of circulating pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα), interleukin-1 (IL-1) and interleukin-6 (IL-6), in both septic human patients and animals with experimentally induced sepsis, were evidence of SIRS in action. The belief that sepsis was a state of hyperinflammation led to numerous clinical trials investigating agents that would block, neutralize, remove or augment various pro-inflammatory mediators.

As more and more of these studies failed, the proposed hyperinflammatory pathogenesis of sepsis was re-examined. At that time, based on new evidence of immunosuppression in septic patients (Gomez et al., 2014), it was proposed that SIRS is only the initial phase of sepsis, and it is followed by a compensatory anti-inflammatory response syndrome (CARS), leading
to adverse events such as increased rate of nosocomial infections (Iskander et al., 2013). Hallmark characteristics of the CARS phase were cited as diminished expression of major histocompatibility complex class II molecules on circulating monocytes (mHLA-DR), massive apoptosis of circulating lymphocytes and elevated plasma levels of the anti-inflammatory cytokine IL-10 (Ward et al., 2008).

This notion of a multi-modal or two-phase inflammatory response to sepsis has sense been rejected. Most recently, definitive evidence of an integrated, highly mixed anti-inflammatory response syndrome (MARS) has been produced (Osuchowski et al., 2012, Iskander et al., 2013). While hindsight is 20/20, the evolution of understanding in SIRS, CARS and MARS is an excellent illustration of the fact that you can’t find what you’re not looking for. As research on sepsis in veterinary species continues, new tools such as multiplex cytokine assays and microarray analysis will undoubtedly assist in a comprehensive examination of both pro- and anti-inflammatory cytokines, furthering our knowledge of sepsis pathophysiology in our species of interest.

The cytokine features of equine sepsis and critical illness are currently the subject of intense study. At this time, much of the inflammatory cytokine data available is based on models of equine sepsis, such as intravenous LPS-infusion or oral administration of black walnut extract or carbohydrate overload. Based on these studies, we know that like other species, horses initially produce TNFα in response to LPS. Clinically speaking, high levels of circulating TNFα are also present in horses with colic, and have been linked to the horse’s systemic inflammatory response to endotoxin, and a higher rate of mortality in cases of equine colic (Morris et al., 1990, Morris et al., 1991). TNFα is known to induce the
expression of interleukin (IL)-1, and administration of these two cytokines together is enough to reproduce septic shock in animals (Tracey et al., 1986, Okusawa et al., 1988). In horses, IL-1β elevations have been documented in foals with naturally occurring sepsis and in adult horses following LPS-infusion (Castagnetti et al., 2012, Tadros and Frank, 2012). Another important cytokine during sepsis in humans and horses is IL-6, which is produced after cells are exposed to TNF and IL-1. While IL-6 is not considered to be a pro-inflammatory cytokine, neither is it considered anti-inflammatory. LPS-infusion in adult horses was shown to significantly increase circulating levels of IL-6 (Tadros and Frank, 2012). However, the data on IL-6 levels from foals with naturally occurring sepsis is a bit conflicted. In one study on naturally occurring neonatal sepsis, IL-6 levels were lower than healthy age matched controls (Burton et al., 2009). These authors provide evidence that healthy foals acquire a significant amount of IL-6 from the passive transfer of immunity, which may have bearing on their results. In addition, their study only included 15 septic foals, 2 of which were non-survivors. This low number of non-survivors prohibited comparison between these two groups. In other studies, IL-6 gene expression was significantly increased in non-surviving septic foals compared to foals that survived sepsis (Pusterla et al., 2006). In human sepsis trials, a persistently elevated level of IL-6 has been proposed to be a reliable indicator of disease severity and mortality (Gomez et al., 2014). It remains to be seen whether this cytokine, alone or in combination with others, will be a good prognostic indicator of sepsis in horse.

The most commonly investigated anti-inflammatory cytokine, in people and horses, is IL-10, although other anti-inflammatory mediators include: IL-4, IL-11, IL-13, transforming growth
factor β, soluble TNF receptors and IL-1 receptor antagonist (Roy, 2004, Grailer et al., 2014). Anti-inflammatory cytokines offer a counterbalance to the effects seen during SIRS, resulting in suppressed production of IL-1 and TNFα and chemokines and a decrease in expression of vascular adhesion molecules. An increased level of IL-10 in human patients with sepsis has been associated with poorer outcomes and an increased risk of MODS and death (Sherry et al., 1996, Giannoudis et al., 2000, Monneret et al., 2004). In equine neonatal sepsis, significantly elevated levels of IL-10 have been reported in non-survivors compared to survivors (Pusterla et al., 2006). However, another study was unable to detect elevation in circulating IL-10 in septic foals compared to healthy controls (Burton et al., 2009). Whether or not IL-10 levels correlate with outcome in adult equine patients with naturally occurring sepsis has yet to be investigated.

**The Neutrophil Response: a double-edged sword**

Neutrophils are essential for host defense against bacteria. This is illustrated by the fact that neutropenic patients or patients with leukocyte adhesion deficiency have an increased susceptibility to infection (Etzioni, 2010, Summers et al., 2010, Dotta et al., 2011, Kolaczkowska and Kubes, 2013, Schmidt et al., 2013). On the other hand, neutrophil effector functions (i.e. release of reactive oxygen intermediates, extracellular trap formation) can be very damaging to host tissue in a number of diseases including sepsis. Therefore, neutrophils are potential targets for combating sepsis associated organ damage. It remains hopeful that with an improved understanding of neutrophil functions, and molecular regulators of
neutrophil responses, that novel targets for therapeutic modulation of neutrophil dysfunction in sepsis will be identified.

*Neutrophils: an overview*

In 1882, Elie Metchnikoff observed recruitment of phagocytic cells to sites of injury in starfish embryos, and hypothesized that they (i.e. neutrophils) were involved in microbe digestion (Tauber and Chernyak, 1989). And so began more than a century of research, in which we have examined the role of neutrophils in host defense, host injury, microbial killing, resolution of inflammation, immunodeficiency and tissue healing. Research investigations on neutrophils continue to this day, and new facets of neutrophil functions, activation and signaling continue to be elucidated. The goal of this review is to cover broad topics of neutrophil physiology that are relevant to the role of neutrophils in the pathophysiology of sepsis, and the reader is referred elsewhere for a more detailed examination of neutrophil signaling.

There are several key features of neutrophils that make them particularly efficient microbial killers. First, neutrophils are the most abundant leukocyte in systemic circulation, with 2.5 – 7.5 x 10^9 cells/l circulating in healthy adult humans and horses. Importantly, these circulating neutrophils are maintained in a quiescent state until signals for recruitment and activation are received. This is an important safety mechanism that normally keeps the destructive power of neutrophils in check. To add to the large number of neutrophils in circulation, significant numbers can also be rapidly recruited from the reserve of neutrophils located in the bone marrow. This is particularly important, considering that the lifespan of neutrophils as terminally differentiated cells has been thought to be relatively short (i.e. half-life of 8 hours
in human circulation) (Malech and Gallin, 1987, Galli et al., 2011); although recent evidence has brought that belief under some dispute (Pillay et al., 2010). Second, under physiologic conditions, neutrophils can be found not only in circulation, but also as marginated pools in the spleen, liver and lung (Summers et al., 2010, Kolaczkowska and Kubes, 2013). Although the reason for the concentration of neutrophils in these organs remains uncertain, it has been postulated that organ-neutrophils are acting as sentinels, providing constant surveillance for the detection of, and defense against, microbial invasion and tissue damage (Kolaczkowska and Kubes, 2013). Third, in response to chemotactic stimuli neutrophils can migrate at extremely fast velocities (up to 12 um/min), meaning they have a tissue target response time as short as 3 hours after initial insult. An illustrative analogy for the neutrophil’s response time compared to the response time of other phagocytic white blood cells (i.e. monocytes) would be the comparison of a fighter jet (the neutrophil) to a tank (the monocyte), both destructive, but one definitely gets there faster. Finally, neutrophils are equipped with a remarkable contingent of surface receptors and adhesion molecules that facilitate neutrophil activation and endothelial transmigration, culminating in the arrival of neutrophils at remote sites of tissue injury or infection. All of these properties make neutrophils uniquely suited to the task of primary host defenders, and that is to say nothing for their actual mechanisms of killing (i.e. phagocytosis, respiratory burst) which are discussed in the sections below.

**Neutrophil recruitment**

Tissue damage due to either sterile injury or pathogenic invasion is recognized by immune cells such as macrophages and mast cells, as well as stromal cells (Arancibia et al., 2007, Zeytun et al., 2010, Sadik and Luster, 2012). Characteristic stimuli, including PAMPs and
DAMPs, activate these cells to release pro-inflammatory mediators such as IL-1β and TNFα, as well as chemoattractants for neutrophils (Sadik et al., 2011, Williams et al., 2011). The local release of these mediators causes activation of the neighboring vascular endothelium which leads to increased endothelial expression of P-selectin and then later, E-selectin and ICAM-1 (Bevilacqua et al., 1985, Gamble et al., 1985, Schleimer and Rutledge, 1986). Neutrophils passing the area of activated endothelium enter a reversible adhesion cascade that consists of six steps, which are mediated by specific ligand-receptor interactions and molecular mechanisms. The initial steps of the leukocyte adhesion cascade, capture and rolling, are mediated by induced endothelial expression of P- and E-selectin and neutrophil-expressed L-selectins and E-selectin ligand (ESL-1), P-selectin ligand (PSGL-1) and CD44, which are expressed constitutively (Mueller et al., 2010, Yago et al., 2010). The strength of these selectin mediated attachments only develops during the shear stress that neutrophils experience during laminar blood flow (Ley et al., 2007, Borregaard, 2010, Nauseef and Borregaard, 2014). Also important for the progression of neutrophil adhesion to the blood vessel wall is the presentation of endothelial-bound chemokines to their respective G-protein coupled receptors on the neutrophil cell surface. GPCR signaling leads to “inside-out” activation of neutrophil β2-integrins (Johnson et al., 2005). This activation of neutrophil β2-integrins Mac-1 (CD11b, αMβ2) and LFA-1 (CD11a, αLβ2) facilitates adhesion to their respective ligands on the endothelial surface, ICAM-1 and ICAM-2; which leads β2-integrin “outside-in” activation and subsequent slow rolling, arrest and finally adhesion strengthening (Ley et al., 2007). Following adhesion strengthening, neutrophils physically prepare for their transendothelial journey, taking on a characteristic polarized appearance with a leading edge
lamellipodium and a trailing edge uropod (Borregaard, 2010). Using β2-integrin dependent mechanisms, reviewed in detail elsewhere (Herter and Zarbock, 2013), neutrophils traverse the endothelium via transcellular or paracellular routes, cross the basement membrane, and home in on sites of bacterial invasion and/or tissue injury.

Neutrophil Chemotaxis

Circulating neutrophils, initially distant from the primary site of pathogen invasion, are recruited to the area of tissue infection by an uncontested gradient of endogenously produced host chemokines (i.e. IL-8 and LTB4), known as intermediate chemoattractants. As neutrophils cross the endothelium to enter the actual site of infection, they must ‘ignore’ the chemokine gradients of diffusely inflamed host tissue in order to ‘target’ invading microorganisms. End-stage chemoattractants derived from bacterial peptides and host complement factors, such as fMLP and C5a, signal neutrophils to converge at the point of bacterial invasion in order to phagocytose foreign material and release their arsenal of antimicrobial agents including oxidants, proteinases and cationic peptides (Nauseef, 2007, Bertram and Ley, 2011, Nauseef and Borregaard, 2014). Chemoattractant receptors play a vital role in translating the spatial cues of the chemoattractant gradient to the cellular machinery that drives neutrophil locomotion. Neutrophils “sense” these chemoattractant gradients by virtue of independent G-protein coupled chemoattractant receptors (GPCR) and “prioritize” intermediate and end-stage chemoattractant signals using the phosphatidylinositol-3-OH kinase (PI(3)K) and tensin homolog (PTEN) pathway and the p38 mitogen-activated protein kinase (MAPK) pathway, respectively (Heit et al., 2002, Heit et al., 2008a, Heit et al., 2008b). Without an intact “compass”, or damage to the machinery of
migration (as occurs in sepsis), neutrophils lose their sense of purpose and direction, fail to control infection, and become a liability to their host.

**Neutrophils dysfunction in sepsis**

*As in all things, timing matters*

An obvious theme in neutrophil-mediated defense and disease is that the properties that make neutrophils so effective at host defense can also, under certain circumstances, make them a threat to host health. Although neutrophils are widely associated with organ injury in sepsis, in both human and animal species, the actual data, depending on the study examined, can fail to give a clear picture, and indeed, may even appear contradictory. Before delving into the role neutrophils play in sepsis associated organ injury, it is important to point out that it is unlikely that one individual piece of evidence (i.e. one study) will paint a full picture of this complex syndrome. One variable that is often not included in studies designed to examine the role of neutrophils in sepsis is time. Yet sepsis is a process that lasts for days in most patients. In a recent study, Hoesel et al. (2005) addressed the issues of multiple organ failure, neutrophils and time, using a common mouse model of sepsis, cecal ligation and puncture (CLP) (Hoesel et al., 2005). Results of this study show that neutrophil depletion 12 hours after CLP significantly attenuates levels of bacteremia, organ dysfunction and cytokines, and ultimately improves survival. In contrast, neutrophil depletion before CLP resulted in increased bacteremia and organ dysfunction unchanged from controls. The important conclusion that the authors draw from these results is that neutrophils have both beneficial and harmful roles to play in sepsis, and their harmful affects seem to be associated with the
loss of their innate immune functions in the later phases of sepsis (i.e. 12 hours post-induction). Loss of immune function, along with other features of neutrophil dysfunction in sepsis, is discussed in further detail below.

**Abnormal numbers of blood neutrophils**

During sepsis, the number of neutrophils reserved in the bone marrow is decreased. Historically, this observation has been attributed to mobilization signals from cytokines, bacterial products and other inflammatory mediators (Reddy and Standiford, 2010). However, there is recent evidence of another player, the neutrophil chemoattractant CXCL12. During healthy, homeostatic states, CXCL12 is highly expressed by bone marrow stromal cells, effectively retaining neutrophils in the bone marrow (Christopher and Link, 2007); but during sepsis the expression of CXCL12 in the marrow is suppressed, while expression levels of CXCL12 in the blood and spleen do not change. The result is release of neutrophils stores from the bone marrow into circulation (Delano et al., 2011a). This may be one mechanism that contributes to the increase in immature neutrophil forms seen in the circulation of patients with SIRS and/or sepsis.

Increased numbers of immature neutrophils in circulation may explain some of the alterations seen in neutrophil function during sepsis. Compared to mature neutrophils, immature neutrophils have a relatively longer life span and are resistant to spontaneous apoptosis, they have higher basal levels of pro-inflammatory mediators compared to anti-inflammatory mediators (i.e. TNFα/IL-10 ratio) and are less efficient in conducting some immune functions (Drifte et al., 2013).
Neutrophil lifespan is directly affected by the rate of apoptosis, which is an important step in the resolution of inflammation. As a result of increased levels of circulating PAMPs and cytokines, neutrophil apoptosis is reduced in patients with SIRS, systemic infections, severe sepsis, and those at risk of MODS (Weiss and Evanson, 2003, Weiss et al., 2003). The anti-apoptotic effects of these soluble mediators are complimented by neutrophil binding to cytokine activated endothelium, which also extends the neutrophil lifespan (Ginis and Faller, 1997). It is believed that altered neutrophil lifespan contributes to increased numbers of activated neutrophils in circulation, which has been observed in horses with colic (Weiss and Evanson, 2003), and their subsequent damaging interactions with the endo- and epithelium.

**Altered rigidity and capillary bed sequestration**

It has been reported that neutrophil rigidity increases proportionally with sepsis severity (Skoutelis et al., 2000) and that this leads to an accumulation of neutrophils in the capillary beds of lung and liver sinusoids. Neutrophil rigidity induced in vitro by fMLF or TNFα is associated with the appearance of a submembrane ring of F-actin (Saito et al., 2002). Interestingly, in a rat model of bacterial pneumonia, neutrophils with submembrane F-actin were preferentially retained (over those neutrophils without F-actin) during passage through the lungs (Yoshida et al., 2006). It is unknown whether alterations in actin structure contribute to neutrophil accumulation within the laminae of horses with either BWE or CHO induced laminitis.

**Altered neutrophil migration**

The neutrophil’s response to inflammation is mediated in large part by chemokines and cytokines, which are ligands for neutrophil GPCRs. During sepsis the expression of these
neutrophil agonists can be aberrantly increased. This can effectively reduce neutrophil responsiveness in at least two ways. With a high rate of ligand binding, GRCPs are functionally desensitized due to a lack of gradient sensing ability. Additionally, GPCR-ligand binding leads to receptor internalization, which also effectively blunts GPCR-mediated neutrophil activation. These mechanisms are recognized clinically in sepsis patients, whose neutrophils have decreased surface expression of CXCR2 (also known as the IL-8 receptor) (Cummings et al., 1999).

In mice, sepsis has also been shown to upregulate the chemokine receptor CCR2 (Souto et al., 2011). The result of this upregulation, as shown by Souto et al., is an increase in random neutrophil migration, accumulation of neutrophils in remote organs and a failure of neutrophils to assimilate at the infection focus. In this study, severity of patient illness correlated positively with increasing neutrophil chemotaxis to CCR2 ligands and genetic or pharmacologic inhibition of CCR2 protected mice from CLP-induced mortality. The authors conclude that CCR2 may help drive the inappropriate infiltration of neutrophils into remote organs during sepsis. Interestingly, the altered expression patterns and functions of CXCR2 and CCR2 seen during sepsis are driven by TLR activation in neutrophils (Zhou et al., 2010, Souto et al., 2011). It is likely that both of these alterations in GPCR-mediated neutrophil function are contributing to dysfunctional migration of neutrophils during sepsis.

Evidence suggests that increased circulating levels of cytokines and chemokines may hinder normal neutrophil recruitment from the vasculature. Patients with sepsis frequently have elevated levels of IL-8, C5a and TNFα in their blood (Brown et al., 2006). Elevated levels of TNFα have also been documented in foals with presumed ‘septicemia’ (Morris and Moore,
1991). In vitro, IL-8 pretreatment led to reduced neutrophil migration across endothelial monolayers (Luscinskas et al., 1992); and in vitro exposure of neutrophils to C5a, at concentrations comparable to those in the blood of septic patients, completely paralyzed the migrational response (Ward, 2004, Ward et al., 2008). The effects of elevated TNFα in the blood of septic patients on neutrophils include decreased migration, inhibition of apoptosis, enhanced priming and production of reactive oxygen species and suppressed CXCR2 expression (Otsuka et al., 1990, Colotta et al., 1992, Ferrante, 1992). The sum of these effects is increasing numbers of neutrophils within the vasculature and decreasing numbers of neutrophils at the site of infection. The increased presence of primed neutrophils within the vasculature, which has been demonstrated in septic patients, may extend neutrophil-endothelial interactions and ultimately contribute to vascular and organ damage.

Altered effector functions

In order to combat pathogens, neutrophils produce inflammatory cytokines and chemokines, generate reactive oxygen species, phagocytose pathogens and release neutrophil extracellular traps (NETs) (Moraes et al., 2006, Fialkow et al., 2007, Kovach and Standiford, 2012). In neutrophils from septic patients, most (if not all) of these functions are altered, but the extent of these alterations, as well as whether they are enhanced or suppressed, is likely to vary depending on the sepsis model being studies, the timing or the investigation and the individual patient’s disease etiology and immune response.

In one recent study, Tang et al. used microarray analysis to profile the gene-expression of neutrophils collected from septic patients within 24 hours of hospital admission (Tang et al., 2007). These results document the down regulation of inflammatory response genes, immune
modulation genes and genes required for oxidant production. This is consistent with evidence of progressive impairment of oxidant production in murine neutrophils during *Pseudomonas spp.* sepsis (Delano et al., 2011b) and the impaired neutrophil phagocytic activity that has been correlated with poor outcomes in patients with sepsis (Danikas et al., 2008). One should not assume that the dampening of neutrophil effector functions at the gene expression level is the result of global immunosuppression. Indeed, peripheral blood leukocytes from sepsis patients have substantial induction of several antimicrobial genes (Kovach and Standiford, 2012). It has been suggested that this divergence in the regulation of host defense genes is supportive evidence for reprogramming of neutrophil effector functions during sepsis. Despite all the evidence for neutrophil dysfunction in sepsis, one effector function that appears to remain intact is phagocytosis. Indeed, neutrophils from septic patients show enhanced internalization and destruction of microorganisms. Unfortunately, intact phagocytic function is not helpful if neutrophils fail to arrive at the focus of infection. Indeed, several different animal studies have shown that a lack of neutrophil migration into infectious sites is associated with reduced survival (Alves-Filho et al., 2010). In addition to the blunting of neutrophil chemotaxis seen with excess levels of circulating chemotactic mediators, researchers also attribute this failure of migration to decreased rolling and adhesion of neutrophils to endothelium, observed during sepsis induction by CLP in mice (Benjamim et al., 2002). On the surface, it would seem difficult to resolve this observation with the evidence that neutrophil accumulation in tissue is contributing to sepsis-associated organ injury (i.e. MODS). Clearly, when it comes to altered neutrophil migration during sepsis, there are multiple mechanisms in play that differentially affect the neutrophils ability to
traverse endothelium, and the observed alterations in neutrophil recruitment and activation at the endothelial level is variable depending on the proximity of that endothelium to specific organs and sites of infection.

In order to destroy invading microorganisms, activated neutrophils produce reactive oxygen species (ROS), such as superoxide anion (O$_2^-$), through a process known as respiratory burst. In order to generate and release ROS, neutrophils must assemble a five-subunit enzyme complex known as NADPH at either the phagosomal or plasma membrane. Within the resting neutrophil, three of the subunits ($p40^{PHOX}$, $p47^{PHOX}$ and $p67^{PHOX}$) are consistently present within the cytosol, while the other two components ($p22^{PHOX}$ and gp91$^{PHOX}$) are located within the membranes of the secretory vesicles and specific granules. It is only when neutrophils are fully activated that the components assemble and ROS are released. This is an important protective mechanism for the host, as the release of ROS can damage not only microorganisms, but host tissue as well. This is true in septic patients, whose neutrophils have an enhanced ability to generate ROS compared to healthy people. In addition, the plasma from septic patients has been shown to cause ROS generation by human umbilical vein endothelial cells, which has been correlated with organ dysfunction and mortality. From human sepsis trials it was determined that decreasing neutrophil ROS generation by day 7 correlated positively with survival, whereas no significant decrease was associated with non-survival (Santos et al., 2012).

Neutrophil extracellular traps (NETs) are the most recently discovered tool in the neutrophil’s arsenal of host defense mechanisms (Brinkmann et al., 2004). NETs are formed when elastase, released from azurophlic granules, causes decondensation of nuclear
chromatic, which is then extruded (along with histones, myeloperoxidase, neutrophil elastase, cathepsin G and antimicrobial proteins) extracellularly. NETs are released from neutrophils in response to cytokines (i.e. IL-8), microbial components (i.e. LPS), phorbol esters (i.e. PMA) and activated platelets and endothelial cells. NETs enhance neutrophil bacterial killing by physically capturing bacteria and bringing them in to close proximity with antimicrobial proteins. Indeed, the effectiveness of NETs in killing a wide variety of Gram-positive and Gram-negative bacteria, as well as fungi (Candida albicans) and protozoa (Leishmania amazonensis) has been demonstrated both in vivo and under flow conditions in vitro (Seeley et al., 2012). While NETs are clearly beneficial for host defense, widespread release of NETs may have a role in organ injury during sepsis (Clark et al., 2007). In various studies, NETs have been shown to contribute to hepatocellular injury, damage endothelial cells, and instigate fibrin deposition and thrombus formation (Fuchs et al., 2010, Narasaraju et al., 2011). To my knowledge, NETosis of equine neutrophils has not yet been investigated, and the importance of NETs in equine sepsis pathophysiology remains to be determined.

*Neutrophil mediated cytokine production*

Neutrophils contribute to the production of pro-inflammatory cytokines during sepsis (Lloyd and Oppenheim, 1992). Lower levels of common pro-inflammatory cytokines, such as IL-1β, IL-6 and TNFα, may be benefit patients with sepsis (Tracey et al., 1987, Ohlsson et al., 1990, Riedemann et al., 2003). In animal models of sepsis (i.e. CLP), neutrophil depletion 12 hours after sepsis induction led to decreased levels of pro-inflammatory cytokines (as well as a reduction in the anti-inflammatory cytokine IL-10) and was associated with significantly improved rates of survival (Hoesel et al., 2005). However, in this same study, neutrophil
depletion prior to sepsis induction had no impact on cytokine levels compared to controls and significantly increased bacteremia and mortality. This result is significant, as it has implications for the potential usefulness of therapies designed to target neutrophils, in terms of when in the timing of sepsis they might benefit patient survival.

**Impact of TLR signaling**

In non-disease states, ligation of neutrophil TLRs elicits numerous responses including cytokine production, generation of reactive oxygen species, priming, receptor expression and phagocytosis (Hayashi et al., 2003). As is the theme with so many other aspects of the innate immune response, local TLR activation, namely TLR4, is absolutely essential for host defense against LPS-containing bacteria; but when activated systemically, TLR4 leads to septic shock and death (O'Brien et al., 1980). This impact of TLR4 on local and systemic immune responses was elucidated in studies with C3H/HeJ mice, initially described as being resistant to LPS-induced shock (Heppner and Weiss, 1965). It was later determined that they had a mutation of TLR4 (Coutinho et al., 1977). These same mice were later shown to have increased mortality when infected with Gram-negative bacteria (i.e. Salmonella sp., Neisseria sp. and E. coli) (O'Brien et al., 1980, Hagberg et al., 1984, Woods et al., 1988). This evidence demonstrates the importance of TLR4 in both host defense and sepsis pathophysiology.

So just how do alterations in neutrophil TLR contribute to LPS-mediated shock and increased sepsis mortality? It is suspected that one mechanism is persistent activation of TLR pathways in the early stages of sepsis, which causes increased expression of adhesion molecules (Zhou et al., 2005), excessive neutrophil expression of inflammatory cytokines
(O’Neill, 2006) and release of reactive oxygen species (Till et al., 1982, Zhou et al., 2010).

On the other hand, neutrophil exposure to TLR ligands (i.e. LPS) that is repeated or prolonged leads to a down regulation in TLR inflammatory signaling. Mechanisms of decreased neutrophil TLR signaling in sepsis include decreased expression of TLR2 and TLR4 (animal models and neutrophils from septic patients), induced expression of TLR signaling antagonists (i.e. IRAK-M) and paracrine inhibition by the anti-inflammatory cytokine IL-10 (Xiong and Medvedev, 2011, Kovach and Standiford, 2012). In summary, it appears as though TLR signaling that is enhanced in the early stages of sepsis becomes progressively unresponsive the longer sepsis continues.

**Neutrophil mediated organ damage**

After the successful elimination of bacteria, neutrophils undergo apoptosis, or programmed cell death (Haslett, 1992). But during sepsis, neutrophil apoptosis appears to be delayed (Jimenez et al., 1997). This may contribute the accumulation of neutrophils in the organs of patients with sepsis, an occurrence that is commonly documented in animal models of sepsis as well as during autopsies of human patients that died as a result of sepsis induced multiple organ failure (Brealey and Singer, 2000, Hoesel et al., 2005).

In humans with sepsis-mediated organ dysfunction, the lungs are the most common organ to undergo remote or secondary injury (Guo et al., 2002). This complication is referred to as acute lung injury (ALI) or acute respiratory distress syndrome (ARDS). Among the key players in the pathophysiology of ALI/ARDS, neutrophils are considered to have a crucial role in disease progression and outcome (Abraham et al., 2000, Abraham, 2003). During the initial exudative phase of ALI and ARDS, several pro-inflammatory mediators (i.e. IL-8)
initiate migration and pulmonary infiltration of neutrophils into the interstitial tissue where they cause injury and breakdown of the pulmonary parenchyma (Goodman et al., 2003, Matthay et al., 2003). In patients with ARDS, the concentration of IL-8, and the concentration of neutrophils, in the bronchoalveolar lavage (BAL) fluid correlates with ARDS severity and outcome (Parsons et al., 1985, Steinberg et al., 1994, Abraham, 2003), and severity of lung injury can be reduced by neutrophil depletion in mice (Neumann et al., 1999).

In both neonatal and adult horses, naturally occurring and experimentally induced systemic inflammation and/or sepsis has been associated with multiple organ dysfunction. Reports of organs injured include: lungs, heart, kidneys and laminae (Roy, 2004, Cotovio et al., 2008, Faleiros et al., 2008, Reddy et al., 2008, Stewart et al., 2009, Faleiros et al., 2011, Montgomery et al., 2014). Because of the significance of equine laminitis, a great deal of interest and research has been focused on determining the pathophysiology of sepsis-associated lamellar injury. Laminitis experts have compared sepsis mediated laminar injury in horses to sepsis induced organ failure in people based. This comparison is based on the observation that laminar changes during the prodromal and acute stages of induced laminitis in horses are similar to the changes observed in organs at risk of failure during human sepsis. These changes include an increase in tissue pro-inflammatory cytokines, leukocyte activation, endothelial activation and neutrophil tissue infiltration and accumulation (Belknap et al., 2009, Belknap and Black, 2012). Further, neutrophils are not only present in the laminae, they are contributing release of myeloperoxidase (BWE model) and likely contributing to epithelial injury (BWE- and CHO-model) (Riggs et al., 2007, Faleiros et al.,
2011, Leise et al., 2011). Are neutrophils a potential target for decreasing the risk of sepsis-associated laminitis in the horse? That question remains to be answered. The current standard of sepsis-associated laminitis prevention is cryotherapy, which experimentally, does inhibit the accumulation of neutrophils in the laminae, as well as the upregulation of pro-inflammatory cytokines (personal communication). In numerous animal studies, neutrophil depletion or targeted inhibition of neutrophil-endothelial adhesion (through the use of monoclonal antibodies), has prevented the development of sepsis-associated organ damage. This type of approach seems a reasonable next step in beginning to unravel the role of neutrophils in the pathophysiology of equine laminitis.

**Therapeutics: what’s working (early goal directed therapy), what’s not (everything else) and why.**

Of all of the potential therapies investigated for the treatment of human sepsis over the past 20 years, the only one that has made a positive impact on human survival is Early Goal Directed therapy (EGDT) (Dellinger et al., 2004, Trzeciak et al., 2008). EGDT is a systematic approach to resuscitation of the severely septic and septic shock patient. Its principles aim to optimize oxygen delivery to tissues through the rapid stabilization of cardiac pre-load, afterload and contractility (Rivers et al., 2001). Therapies utilized to achieve this goal include fluids, vasopressors or vasodilators (as indicated) and the option of intubation and mechanical ventilation. Routinely measured patient parameters include central venous pressure (CVP), mean arterial pressure (MAP), mixed venous oxygen saturation (SvO2) and urine output. The use of EGDT in equine medicine and critical care is gaining
traction (Boesch, 2013). While SvO2 is not routinely measured in equine patients at this time, other measures of tissue oxygenation include pH, base excess (BE), and lactate. In human medicine, “resuscitation end points” for EGDT have been clearly defined. Although similar end-points have not been defined for the horse, it is likely that agreement on such parameters would aid in equine critical care specialist with the application of these therapeutic principles.

As pointed out by Dr. Jon Palmer in a recent review on equine neonatal sepsis, there is a laundry list (over 40) of failed therapies for the treatment of human sepsis (Palmer, 2014). Why is it that therapies that seemed so promising in animal models of sepsis, have failed to translate benefit to human patients with sepsis, and in some cases have even increased mortality? The pontifications on the answer to this question are numerous. Among the most common are (Vincent et al., 2013, Balk, 2014):

1. Inappropriate clinical trial inclusion criteria (i.e. SIRS is too sensitive which leads to a lack of patient stratification)

2. Too much focus on blunting the pro-inflammatory response (i.e. lack of basic understanding of sepsis pathophysiology)

3. The use of inappropriate animal models of sepsis (i.e. models that are too simplistic and do not consider potential species differences)

What can we as veterinarians learn from this list of failures and what role might neutrophils play as future targets in sepsis therapy? I would argue that veterinary medicine has been given a great opportunity to make rapid progress in the understanding, diagnosis and treatment of sepsis. Fueled by the lessons from the past, we have a newfound appreciation for
the complexity of sepsis, the rigor that must be applied in order to make research truly translational, the importance of species-specific differences, and the potential tools of patient stratification for clinical trial design.

I would also argue that targeting neutrophils to combat injury to host tissue, in sepsis as well as other diseases is more of a reality each day. Historically speaking, the conundrum of neutrophil targeting has always been, “How do we strike a balance between eliminating the neutrophil’s contribution to host injury without sacrificing the neutrophil’s role in host defense?” Recently, there have been exciting developments that may begin to answer that question. In the February issue of Nature Nanotechnology, Wang and colleagues demonstrate the prevention of vascular inflammation and acute lung injury (in animal models of inflammation and sepsis, respectively) by nanoparticle targeting of neutrophils (Wang et al., 2014). The albumin derived nanoparticles, loaded with the spleen tyrosine kinase inhibitor piceatannol, were internalized preferentially by endothelial-adherent neutrophils via cell surface Fc receptors. Because Syk is required for β2-integrin “outside-in” signaling, these adherent neutrophils subsequently detached from the endothelium. Nanoparticles were not taken up by circulating neutrophils. This method of nanoparticle drug delivery is an important step in demonstrating the feasibility of inhibiting activated, pro-inflammatory neutrophils without inhibiting all neutrophils; and will no doubt lead to exciting impacts for the treatment of numerous inflammatory disorders such as rheumatoid arthritis, ulcerative colitis, myocardial infarction, and maybe even sepsis.
Future directions in sepsis

Collaboration is key

The pathophysiology of sepsis and its associated syndromes is extremely complex and important components not covered in this review include the role of endothelial damage, the acute phase response, coagulation and regulatory T cell responses. Given the complexity of this disease and the significant impact on our equine patients, more research is needed in order to: 1) better understand basics in pathophysiology, 2) justify or reject current treatment protocols and 3) discover new therapeutic modalities. In order to accomplish these goals, we as equine specialists need to establish reliable, consistent definitions of sepsis (and its associated syndromes) as well as a means for gathering and sharing real incidence and survival data. Without a Centers for Disease Control to service the veterinary community, it is incumbent upon us to develop strategies for conducting collaborative research. In 2006, ACVECC veterinarians helped create a framework for a web based sepsis registry, which had 141 reported members in 2007. Membership to the registry, funded by the Kindy French Foundation, is open to all veterinarians and veterinary technicians interested in sepsis (http://www.careanimalfoundation.org/). While the registry is currently specific for cats and dogs, it could readily be expanded to include other species.

From a veterinary standpoint, there is no doubt that future collaborations in sepsis research will benefit our equine patients, but with well-designed and accurately conducted studies, we also have the opportunity to make significant contributions to the understanding and treatment of human sepsis. Recent studies proposing canine sepsis models, and using equine sepsis models, have illustrated that point (Otto, 2007a, Sutherland et al., 2011). Where we go
from here is up to us. Will we remain isolated in our interests and efforts, and be destined to repeat past failures, or will we enter a new era of planning and collaboration that will change the face of sepsis research in veterinary species? I guess we will find out.
Table 1. ACCP/SCCM Consensus Conference Definitions (1991)

<table>
<thead>
<tr>
<th>ACCP/SCCM Consensus Conference Definitions</th>
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<tbody>
<tr>
<td>Sepsis – The clinical syndrome defined by the presence of both infection and a systemic inflammatory response.</td>
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<tr>
<td>Severe sepsis – Sepsis associated with organ dysfunction, hypoperfusion, or hypotension. Hypoperfusion and perfusion abnormalities may include, but are not limited to lactic acidosis, oliguria, or an acute alteration in mental status.</td>
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<tr>
<td>Septic shock – Sepsis-induced with hypotension despite adequate fluid resuscitation along with the presence of perfusion abnormalities that may include, but are not limited to lactic acidosis, oliguria, or an acute alteration in mental status. Patients who are receiving inotropic or vasopressor agents may not be hypotensive at the time that perfusion abnormalities are measured.</td>
</tr>
<tr>
<td>Multiple Organ Dysfunction Syndrome – Presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention.</td>
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*Bone et al., Chest, 1992.*

Table 2. SCCM/ESISM/ACCP/ATS/SIS Sepsis Definitions (2001)

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<th>SCCM/ESICM/ACCP/ATS/SIS Sepsis Definitions</th>
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<tr>
<td>Sepsis – The presence of infection, documented or strongly suspected, with a systemic inflammatory response.</td>
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<tr>
<td>Severe sepsis – Sepsis complicated by organ dysfunction.</td>
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<tr>
<td>Septic shock – Severe sepsis complicated by acute circulatory failure characterized by persistent arterial hypotension, despite adequate volume resuscitation, and unexplained by other causes.</td>
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<tr>
<td>Multiple Organ Dysfunction Syndrome – Presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention.</td>
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*Levy et al. Critical Care Medicine, 2003.*
Table 3. SCCM/ESICM/ACCP/ATS/SIS Diagnostic Criteria for Sepsis (2001)

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<tr>
<th>SCCM/ESICM/ACCP/ATS/SIS Expanded Diagnostic Criteria for sepsis</th>
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<tr>
<td><strong>Infection</strong> Documented or suspected <strong>and</strong> some of the following:</td>
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<td><strong>General parameters:</strong></td>
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<tr>
<td>Fever</td>
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<td>Hypothermia</td>
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<td>Tachycardia</td>
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<td>Tachypnea</td>
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<td>Altered mental status</td>
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<td>Significant edema or positive fluid balance</td>
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<td>Hyperglycemia (in the absence of diabetes)</td>
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<td><strong>Inflammatory parameters:</strong></td>
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<tr>
<td>Leukocytosis</td>
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<td>Leukopenia</td>
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<td>Normal white blood cell count with &gt; 10% immature forms</td>
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<td>Plasma C reactive protein &gt; 2 SD above normal value</td>
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<tr>
<td>Plasma procalcitonin &gt; 2 SD above the normal value</td>
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<tr>
<td><strong>Hemodynamic parameters</strong></td>
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<td>Arterial hypotension</td>
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<tr>
<td>Mixed venous oxygen saturation &gt; 70%</td>
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<td>Cardiac index &gt; 3.5 1min⁻¹m⁻²</td>
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<tr>
<td><strong>Organ dysfunction parameters</strong></td>
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<td>Arterial hypoxemia</td>
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<tr>
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<td>Creatinine increase</td>
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<td>Coagulation abnormalities</td>
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<td>Ileus</td>
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<td>Thrombocytopenia</td>
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<td>Hyperbilirubinemia</td>
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<td><strong>Tissue perfusion parameters</strong></td>
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<td>Hyperlactatemia</td>
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<td>Decreased capillary refill or mottling</td>
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Table 4. P I R O (Sepsis Patient Staging System)

<table>
<thead>
<tr>
<th>Available</th>
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<tr>
<td><strong>P</strong> Predisposition</td>
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<td>Assay of microbial products</td>
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<td>Detection of virulence factors</td>
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<td><strong>I</strong> Infection</td>
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<td>Susceptibility</td>
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<td><strong>Immune Response</strong></td>
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<td><strong>O</strong> Organ Dysfunction</td>
<td><strong>MODS</strong></td>
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CHAPTER 2

Protein kinase C-delta (δ-PKC) mediated MARCKS phosphorylation is required for human neutrophil migration and adhesion in vitro

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Abstract

Neutrophils are highly chemotactic innate immune cells that migrate from the blood into tissues in response to inflammatory signals. The primary function of neutrophils is to rapidly deliver their arsenal of reactive oxygen species and proteolytic enzymes to sites of bacterial invasion, defending the host from infection. Unfortunately, dysregulated release of these toxic substances can also be damaging to the host. This type of neutrophil mediated tissue injury is associated with both acute and chronic inflammatory disorders. In recent years, significant research efforts have focused on developing anti-inflammatory therapies that target molecular regulators of neutrophil functions. We have shown previously that MARCKS (Myristoylated Alanine Rich C-Kinase Substrate), a well-known PKC substrate protein, is a key regulator of neutrophil functions including migration, adhesion and respiratory burst, but further details regarding molecular mechanisms of MARCKS’ role in neutrophil functions is needed. In the current study we used a combination of biochemical analysis and in vitro migration and adhesion assays to investigate the regulation of, and the requirement for, MARCKS phosphorylation in neutrophil functions in vitro. We report that treatment of human neutrophils with the δ-PKC inhibitor rottlerin significantly attenuates fMLF induced MARCKS phosphorylation (IC50 = 5.709 μM), adhesion (IC50 = 8.4 μM) and migration (IC50 = 6.7 μM). Inhibition of α-, β- and ζ-PKC had no significant effect on MARCKS phosphorylation or neutrophil migration in response to fMLF stimulation. We conclude that δ-PKC mediated MARCKS phosphorylation is essential for human neutrophil migration and adhesion in vitro. These results implicate δ-PKC mediated MARCKS regulation as a key step in neutrophil inflammatory responses. Further studies are needed to
determine the role of δ-PKC mediated MARCKS phosphorylation in neutrophil functions \textit{in vivo}.

\textbf{Introduction}

As essential components of the innate immune system, neutrophils provide a first line of defense against bacterial invasion through rapid accumulation in tissue, release of proteolytic enzymes, production of reactive oxygen species, and phagocytosis of microbial pathogens. The toxic substances released by neutrophils invariably result in some degree of host tissue injury and inflammation, but this so called “collateral damage” is normally limited by elimination of inciting pathogens and subsequent return to homeostasis. However, neutrophil responses that are misdirected or dysregulated can result in acute and chronic forms of inflammation that are debilitating and even life-threatening. This type of neutrophil mediated host injury occurs in diseases such as rheumatoid arthritis, ulcerative colitis, acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) (Malech and Gallin, 1987).

Additional medications are desperately needed to treat these acute and chronic inflammatory conditions, and neutrophils, as the cause of damaging inflammation, are a viable target for therapeutic intervention (Segel et al., 2011). For this reason, significant research efforts have focused on discerning essential cell signaling components involved in various neutrophil functions (Gaestel et al., 2009).

Protein Kinase C (PKC) is one such molecular regulator known to play a significant role in neutrophil inflammatory signaling (Cohen, 2009). Human neutrophils express several PKCs including conventional isoforms α- and β, novel isoform δ and atypical isoform ζ. These PKC isoforms are implicated in various neutrophil functions including adhesion, migration,
Recently, δ-PKC has emerged as a key regulator of inflammation and promising target for anti-inflammatory therapy (Ramnath et al., 2010). δ-PKC is activated by numerous inflammatory signals including host derived cytokines (i.e. TNFα and IL-1) and bacterial components such as LPS (Kilpatrick et al., 2002, Page et al., 2003, Kilpatrick et al., 2010). Studies targeting δ-PKC by use of a dominant negative peptide showed this PKC isoform to be involved integrally in adherence and migration of human neutrophils *in vitro* (Mondrinos et al., 2014). Intra-tracheal administration of this same inhibitor peptide significantly reduced inflammatory cell infiltration and pulmonary edema following cecal ligation and puncture in rats (an animal model of ARDS) (Kilpatrick et al., 2011). However, the molecular basis for the beneficial effects seen with δ-PKC inhibition remains uncertain.

Myristoylated Alanine Rich C-Kinase Substrate (MARCKS) protein is another key regulator of neutrophil functions including migration, adhesion, degranulation and respiratory burst (Takashi et al., 2006, Eckert et al., 2009, Sheats et al., 2014). As a prominent protein kinase C substrate and known actin-binding protein, MARCKS has a demonstrated role in the regulation of cellular events requiring dynamic actin reorganization (Thelen et al., 1990, Thelen et al., 1991). In most resting cells, non-phosphorylated MARCKS is localized to the inner leaflet of the plasma membrane by hydrophobic insertion of the N-terminal myristoyl-moiety and electrostatic interactions of the basic, serine-rich effector domain (ED) (Arbuzova et al., 2002). Membrane bound MARCKS is believed to stabilize the cytoskeleton through actin cross-linking. Upon PKC phosphorylation of the ED, MARCKS is displaced to the cytosol and actin cross-linking is diminished, effectively relaxing the cellular cytoskeleton.
(Hartwig et al., 1992b, Nairn and Aderem, 1992). Within the cytosol MARCKS is dephosphorylated by specific phosphatases, re-establishing MARCKS ability to both cross-link actin and return to the plasma membrane (Aderem, 1992a, Aderem, 1992b, Blackshear, 1993).

To better understand the role of MARCKS phosphorylation in neutrophil functions, we sought to determine which PKC isoforms were regulating MARCKS in human neutrophils, and whether or not those same isoforms were required for neutrophil migration and adhesion. To that end, we investigated PKC isotypes α, β, δ and ζ using specific PKC isotype inhibitors Go6976, CG53353, rottlerin and ζ pseudosubstrate, respectively. Using subcellular fractionation, we demonstrate that of the four isotypes examined, only δ-PKC translocates from cytosol to membrane in neutrophils in response to stimulation (fMLF and PMA).

Because MARCKS phosphorylation is known to occur at the plasma membrane, this pattern of δ-PKC translocation is consistent with MARCKS regulation. We further report that the δ-PKC inhibitor rottlerin inhibits MARCKS phosphorylation in human neutrophils following fMLF stimulation in a concentration-dependent manner (IC$_{50} =$ 5.709 μM). Interestingly, rottlerin pre-treatment also inhibits fMLF mediated human neutrophil migration and adhesion in a concentration-dependent manner (IC$_{50}$ of 8.385 uM and 7.624 uM, respectively). We conclude that δ-PKC is the primary isotype regulating MARCKS phosphorylation in human neutrophils, and that δ-PKC mediated MARCKS phosphorylation is essential for neutrophil migration and adhesion. Previous research from our lab and others suggests that MARCKS and δ-PKC are both potential targets for future anti-inflammatory therapies. The results
presented here shed further light on the integral relationship between these two important signaling molecules in the neutrophil’s response to inflammation.

**Materials and Methods**

*Human Subjects*

The human neutrophils utilized for this study were isolated from the peripheral blood of healthy, adult volunteers using the protocol approved by the Institutional Research Ethics Committee of North Carolina State University (IRB approval #616). Prior to donation, all participants provided written informed consent using the approved consent form.

*Neutrophil isolation*

Human neutrophils were isolated from whole blood using Ficoll gradient centrifugation of dextran-sedimented leukocyte rich plasma. Briefly, approximately 6ml of plasma was layered on 5ml of sterile, endotoxin-free Ficoll-Paque solution and spun at 600g for 20 minutes. Neutrophils were used if they demonstrated greater than 98% viability, as determined by trypan blue exclusion. Red blood cells were removed by hypotonic lysis and remaining neutrophils were washed once with HBSS. Cells were re-suspended in HBSS with 20mM HEPES, 8.9 mM sodium bicarbonate, 1mM Ca\(^{2+}\) and 1mM Mg\(^{2+}\) prior to biochemical assays (HBSS++) and in HBSS with 1mM Ca\(^{2+}\), 1mM Mg\(^{2+}\) and 2% FCS (chemotaxis buffer) prior to adhesion and migration assays.

*Fluorescence labeling of neutrophils*

For migration and adhesion experiments, isolated neutrophils (1 x 10^7/ml in HBSS) were incubated with the fluorescent dye calcein am (Anaspec, Fremont, CA) at 2 ug/ml for 30
minutes at room temperature. Cells were then centrifuged at 1000 rpm for 8 min and resuspended in chemotaxis buffer to the appropriate final experimental concentration.

Antibodies and reagents

Ficoll-Paque Plus and Dextran T500 were from GE Healthcare (Sweden). Dimethyl sulfoxide (Me₂SO), f-Met-Leu-Phe (fMLF), phorbol 12-myristate 13-acetate (PMA), Triton-X 100, pepstatin, HEPES, staurosporine, Protein kinase Cζ pseudosubstrate and poly-l-lysine were from Sigma Chemical Co. (St. Louis, MO). Rottlerin, Go6976 and CG53353 were from Calbiochem (Billerica, MA). Powdered phosphate-buffered saline (PBS) and Hank’s balanced salt solution (HBSS) were from Life Technologies (Grand Island, NY). Ethylenediamine tetraacetate dihydrate (EDTA) was from Fisher Scientific (Atlanta, GA). Leukotriene B₄ (LTB₄) was from Cayman Chemical (Ann Arbor, MI). Interleukin 8 (IL-8) was from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Fetal calf serum (FCS) was obtained from Hyclone (Logan, UT). MARCKS, Phospho-MARCKS, PKC α, PKC δ, PKC ζ primary antibodies and anti-rabbit HRP-conjugated secondary antibody were obtained from Cell Signaling (Beverly, MA). PKC β was from Invitrogen (Frederic, MD). Diisopropylfluorophosphate (DFP) was from BD Biosciences (San Diego, CA).

Lysate preparation and western blot assay

Purified human neutrophils (2.5 x 10⁷ cells/ml) were suspended in HBSS++ and incubated with or without PKC isoform inhibitors for 15 to 30 min with varying concentrations in microcentrifuge tubes and incubated at 37°C. Cells were then stimulated with 100 nM fMLF, 50 ng/mL PMA or appropriate vehicle control (VC) for the indicated time periods during incubation at 37°C. Incubated cells were immediately transferred onto ice and lysed with ice
cold RIPA buffer [1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 5 mM sodium pyrophosphate and 50 mM sodium fluoride] containing protease inhibitors [1mM phenylmethanesulphonylfluoride (PMSF), 100μg/mL pepstatin, 1mM iodoacetamide, and 10 μg/mL aprotinin/leupeptin] for 30 minutes on ice. After lysis, cell solutions were spun at 13,400 g for 5 minutes. Supernatants were collected and protein concentrations were measured using BCA Protein Assay Reagent (Pierce, Rockford, IL). Cell lysate was mixed with 5X sample buffer [25% glycerol, 2% SDS, 60mM Tris-HCL (pH 6.8), 5% β-mercaptoethanol, 0.1% bromophenol blue in diH2O] and boiled for 5 minutes. Equal amount of protein was analyzed in 10% SDS-PAGE. Resolved samples were transferred to Immobilon-P PVDF membrane (Millipore, Billerica, MA) and blocked for 1 hour with 5% non-fat dry milk in tris buffered saline with Tween-20 (TBS/T; 136 μM NaCl, 20 μM Tris-base (pH: 7.6) and 0.1% Tween-20 v/v) prior to overnight incubation with the 1:1000 dilution of phospho MARCKS or MARCKS primary antibody in 5% BSA in TBS/T at 4°C. Membranes were washed in TBS/T and incubated with 1:4000 dilution of anti-rabbit horseradish peroxidase (HRP) secondary antibody (Cell Signaling Technology, Danvers, MA) in 5% non-fat dry milk in TBS/T for 1 hour with gentle agitation. Membranes were washed at room temperature in TBS/T three times for 5 mins and developed using enhanced chemiluminescence and radiograph film. Films were scanned and the density of the bands was measured with densitometric software (Scanalytics, Fairfax, VA).

Subcellular fractionation

Subcellular fractionation assay was performed as previously described (Eckert et al., 2009). Briefly, stimulated cells were lysed by sonication in lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM sodium pyrophosphate and 50 mM sodium fluoride)] containing protease inhibitors [1mM phenylmethanesulphonylfluoride (PMSF), 100μg/mL pepstatin, 1mM iodoacetamide, and 10 μg/mL aprotinin/leupeptin] for 30 minutes on ice. After lysis, cell solutions were spun at 13,400 g for 5 minutes. Supernatants were collected and protein concentrations were measured using BCA Protein Assay Reagent (Pierce, Rockford, IL). Cell lysate was mixed with 5X sample buffer [25% glycerol, 2% SDS, 60mM Tris-HCL (pH 6.8), 5% β-mercaptoethanol, 0.1% bromophenol blue in diH2O] and boiled for 5 minutes. Equal amount of protein was analyzed in 10% SDS-PAGE. Resolved samples were transferred to Immobilon-P PVDF membrane (Millipore, Billerica, MA) and blocked for 1 hour with 5% non-fat dry milk in tris buffered saline with Tween-20 (TBS/T; 136 μM NaCl, 20 μM Tris-base (pH: 7.6) and 0.1% Tween-20 v/v) prior to overnight incubation with the 1:1000 dilution of phospho MARCKS or MARCKS primary antibody in 5% BSA in TBS/T at 4°C. Membranes were washed in TBS/T and incubated with 1:4000 dilution of anti-rabbit horseradish peroxidase (HRP) secondary antibody (Cell Signaling Technology, Danvers, MA) in 5% non-fat dry milk in TBS/T for 1 hour with gentle agitation. Membranes were washed at room temperature in TBS/T three times for 5 mins and developed using enhanced chemiluminescence and radiograph film. Films were scanned and the density of the bands was measured with densitometric software (Scanalytics, Fairfax, VA).

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1 mM EDTA, 1 mM DTT). The lysate was centrifuged at 800g for 10 min to remove cell debris and nuclei. Supernatant was then centrifuged at 20,000g for 1 hour. The supernatant was collected as the cytosolic fraction and the remaining pellet was regarded as the membrane fraction and resuspended in lysis buffer containing 1% Triton X-10. Protein concentration was determined by Protein 660 (Pierce, Rockford, IL) and samples were diluted in 5x sample buffer and boiled for 5 minutes. Equal protein concentrations were loaded onto a 10% SDS-PAGE and western blots were performed for EGFP expression, as described above.

**Neutrophil chemotaxis assay**

Neutrophil chemotaxis assays were performed using the 8 micron pore size Neuro Probe ChemoTx® system as previously described (Eckert et al., 2009). Briefly, each upper well was loaded with 5 x 10^4 calcein-labeled neutrophils in 25 ul of chemotaxis buffer. Cells were allowed to migrate for 1 hour at 37°C toward lower wells containing chemoattractants (100 nM fMLF, 100 nM LTB4 or 100 ng/mL IL-8) in PBS or vehicle controls (DMSO and EtOH). Following the hour migration, non-migrated cells were removed from the top of the membrane with a cell scraper. Following membrane removal, fluorescence of the bottom wells was measured (485 nm excitation, 530 nm emission) using an fMax fluorescence plate reader (Molecular Devices). Percent migration was calculated by dividing the fluorescence of the experimental bottom wells by the fluorescence of bottom wells containing a known number of cells (5 x 10^4 cells). Treatment groups were tested in triplicate.
Neutrophil adhesion assay

To assess fMLF mediated adhesion, 1.2 x 10^5 calcein-labeled neutrophils (60 ul) from the various treatment groups were aliquoted into individual wells of Immulon2HB flat bottom 96-well plates (Thermo Fischer Scientific) previously coated with 5% FCS in sterile PBS, as previously described (Eckert et al., 2009). The plates were then placed in a 37°C incubator while the cells settled for 10 minutes. Following the addition of fMLF (100 nM final concentration) or vehicle control (DMSO) the plate was floated in the 37°C water bath for 3 minutes. Following incubation, neutrophil adhesion was assessed using an fMax fluorescence plate reader (Molecular Devices). After an initial fluorescence reading (485 nm excitation, 530 nm emission) the plates were gently dumped in a single inverted motion; the wells were filled with 150 ul sterile PBS and another fluorescence reading was obtained. This procedure was repeated for a total of three washes to remove non-adhered cells. Fluorescence after each washing was divided by the initial fluorescence to calculate percent adhesion. The first wash that demonstrated 10% adhesion of non-stimulated cells or less (second wash on average) was considered the final result. Treatment groups were tested in triplicate.

Statistical analysis

Films were scanned and the density of the bands was measured with densitometric software (SigmaScan 5). A student t-test was used to calculate the indicated p values. Migration and adhesion data are reported as mean ± SEM. Results were analyzed by one way repeated measures ANOVA (Holm-Sidak multiple comparison testing) assuming equal variance, with p < 0.05 considered statistically significant.
Results

δ-PKC translocates upon neutrophil stimulation.

Circulating neutrophils, initially distant from the primary site of pathogen invasion, are recruited to areas of inflammation and/or infection by a gradient of intermediate- (i.e. IL-8 and LTB4) and end-stage chemoattractants (i.e. fMLF) (Futosi et al., 2013). This signaling, which occurs through transmembrane G-protein coupled chemoattractant receptors (GPCR), activated various isoforms of protein kinase C (Bertram and Ley, 2011). Translocation of PKC from the cytosol to the membrane compartments upon cell stimulation is indicative of intracellular PKC activation (Mochly-Rosen et al., 1990, Kent et al., 1996). To determine PKC isoform activation in human neutrophils, we performed sub-cellular fractionation of cell lysates following stimulation with either 100 nM fMLF or 50 ng/mL PMA. In unstimulated human neutrophils, α-, δ- and ζ-PKC isoforms were primarily located within the cytosolic fraction, while β-PKC was partitioned to the membrane (Figure 1). Following stimulation, PKC was the only isoform that translocated from the cytosol to the membrane. Additionally, translocation of δ-PKC was extremely rapid in response to the physiologically relevant chemoattractant fMLF (Figure 1). Of the four PKC isoforms investigated, δ-PKC was the only isoform that appeared activated upon stimulation of human neutrophils with either 100 nM fMLF or 50 ng/ml PMA.

MARCKS is transiently phosphorylated upon fMLF stimulation of primary human neutrophils

MARCKS plays a key role in human neutrophil migration (Eckert et al., 2009) and its bi-directional movement between the plasma membrane and the cytosol is a well described
aspect of its function (Disatnik et al., 2004). Early studies by Thelen et al. show that the translocation of MARCKS between the plasma membrane and the cytosol in human neutrophils is dependent upon reversible PKC-phosphorylation of MARCKS ED (Thelen et al., 1991). Consistent with previous reports, we observed rapid phosphorylation of MARCKS following non-specific PKC activation with PMA (Figure 2A). However, with this receptor-independent form of activation, there was no evidence of subsequent MARCKS de-phosphorylation. This finding is consistent with the non-physiologic PKC activation seen with PMA, which is a synthetic analog of the second messenger diacylglycerol (DAG). With physiologically relevant activation using fMLF (100 nM) rapid MARCKS phosphorylation (30 seconds) was followed by return to baseline phosphorylation levels by 3 minutes (Figure 2B). A second phosphorylation peak was observed at 10 minutes. Thus, stimulation of human neutrophils with 100 nM fMLF caused a physiologically relevant pattern of MARCKS phosphorylation, which was both rapid and transient. Based on these results, we chose 30 seconds fMLF stimulation as the time point for maximal MARCKS phosphorylation for the remaining biochemical studies.

δ-PKC MARCKS phosphorylation is required for neutrophil chemotaxis

Next, we sought to determine the requirement for MARCKS phosphorylation in migrating human neutrophils. As shown in Figure 3A, pretreatment of human neutrophils with the pan-PKC inhibitor staurosporine attenuated fMLF mediated MARCKS phosphorylation in a concentration-dependent manner, with significant inhibition seen at concentrations of 1.0 uM and greater (Figure 3B). From this result, we calculated that the staurosporine IC_{50} for fMLF-induced MARCKS phosphorylation is 1.094 μM (Figure 3C). Staurosporine treatment
also significantly attenuated fMLF, LTB4 and IL-8 induced migration of human neutrophils in a concentration - dependent manner (Figures 3D and 3F). Staurosporine inhibition of neutrophil migration for both intermediate (LTB4 and IL-8) and end-stage (fMLF) chemoattractants was significant at concentrations of 100 nM and greater. The staurosporine IC$_{50}$ for fMLF-induced migration is 0.1027 uM (Figure 3E). Our findings indicate that the staurosporine IC$_{50}$ for inhibition of migration is 10-fold lower than the IC$_{50}$ calculated for the inhibition of MARCKS phosphorylation in human neutrophils. The lower inhibitory concentration for migration compared to MARCKS phosphorylation may potentially be explained by non-specific effects of pan-PKC inhibition on other signaling pathways and regulatory molecules in addition to MARCKS. It is interesting to note that in previous reports staurosporine failed to inhibit fMLF and IL-8 induced human neutrophil adhesion and migration in vitro (Laudanna et al., 1998), but these findings were reported as “data not shown”. Without knowing the concentrations of staurosporine examined, it is difficult to speculate on the reasons for the difference between this previous report and our findings. 

δ-PKC regulates MARCKS phosphorylation, migration and adhesion in human neutrophils

Treatment of human neutrophils with the pan-PKC inhibitor staurosporine inhibited both MARCKS phosphorylation and human neutrophil migration (Figure 3), verifying PKC-mediated MARCKS phosphorylation is an essential event for neutrophil chemotaxis. Neutrophils express several different PKC isotypes including the conventional α- and β-PKC, the novel δ-PKC, and the atypical ζ-PKC (Kent et al., 1996). In the next series of experiments we utilized specific PKC isoform inhibitors to identify the PKC isoform(s)
responsible for phosphorylating MARCKS in human neutrophils. Using Western blot analysis, we observed that treatment of human neutrophils with the δ-PKC inhibitor rottlerin significantly attenuated fMLF induced MARCKS phosphorylation at concentrations of 10 uM and greater (Figures 4a and 4B). Based on these results, we determined the rottlerin IC₅₀ for fMLF-induced MARCKS phosphorylation of human neutrophils is 5.709 μM (Figure 4C). Thus, δ-PKC appears to be required for fMLF-induced MARCKS phosphorylation in human neutrophils.

Next, we investigated the effect of δ-PKC inhibition on neutrophil migration. As with fMLF-induced MARCKS phosphorylation, rottlerin concentrations of 10 uM and greater significantly inhibited both fMLF- and LTB4-induced neutrophil chemotaxis (Figure 4D and 4F). Interestingly, significant inhibition of IL-8 mediated neutrophil chemotaxis was only achieved with rottlerin concentrations of 20 uM and greater, suggesting IL-8 mediated chemotaxis may be less dependent on δ-PKC and/or MARCKS (Figure 4F). The rottlerin IC₅₀ for fMLF-induced migration was found to be 8.385 uM (Figure 4E).

In order to emigrate from the vasculature into tissues, neutrophils must adhere to the endothelium (Schmidt et al., 2013). We next sought to determine whether δ-PKC mediated MARCKS phosphorylation was required for neutrophil adhesion. Consistent with our findings on MARCKS phosphorylation and migration, rottlerin concentrations of 10 uM and greater significantly inhibited fMLF-induced adhesion of human neutrophils (Figure 4G) with an IC₅₀ of 7.624 uM (Figure 4H). We conclude from this series of experiments that δ-PKC mediated MARCKS phosphorylation is required for human neutrophil migration and adhesion.
PKC isoforms α, β, and ζ do not regulate MARCKS phosphorylation in human neutrophils

As neutrophils express several isotypes of PKC, we next evaluated whether inhibition of α-, β- or ζ-PKC would affect fMLF-induced MARCKS phosphorylation in human neutrophils. Based on Western blot analysis of lysates from fMLF-stimulated human neutrophils, the specific PKC isotype inhibitors Go6976, CG53353 and PKC ζ pseudosubstrate had no effect on MARCKS phosphorylation. Indicating that δ-PKC is the only PKC-isotype responsible for fMLF-induced MARCKS phosphorylation in human neutrophils.

ζ-PKC plays a role in human neutrophil migration that is downstream or independent of MARCKS phosphorylation

Utilizing the same inhibitors as our phosphorylation study, we next investigated the requirement for α-, β- and ζ-PKC isotypes in LTB₄, IL-8 and fMLF mediated human neutrophil migration. Inhibitors of α- and β-PKC (Go6976 and CG53353, respectively) had no effect on LTB₄, IL-8 or fMLF mediated neutrophil chemotaxis (Figure 6A and 6B); but the ζ-PKC pseudosubstrate inhibitor did significantly inhibit IL-8 induced neutrophil migration at concentrations of 5 uM and greater (Figure 6C). These findings are consistent with previous reports implicating the atypical ζ-PKC, and not classical PKC isoforms, in the regulation of neutrophil migration and adhesion (Laudanna et al., 1998, Hirai and Chida, 2003, Xiao and Liu, 2013). Based on our findings, it appears that ζ-PKC plays a role in IL-8 mediated neutrophil migration that is either independent or downstream of δ-PKC mediated MARCKS phosphorylation.
Discussion

The goal of the current study was to determine the PKC isoform(s) responsible for MARCKS phosphorylation in human neutrophils, and to evaluate the requirement for MARCKS phosphorylation in neutrophil chemotaxis in vitro. Using a combination of biochemical analysis and in vitro migration and adhesion assays, we investigated four PKC isotypes known to be expressed in human neutrophils: alpha (α), beta (β), delta (δ) and zeta (ζ). We conclude that δ-PKC is the isotype responsible for MARCKS phosphorylation in fMLF-stimulated human neutrophils and that MARCKS phosphorylation is essential for in vitro neutrophil chemotaxis. These conclusions are supported by the following experimental results: 1. δ-PKC, but not other PKC isotypes, redistributed from the cytosol to the membrane following fMLF stimulation of isolated human neutrophils (Figure 1). Translocation is consistent with PKC activation. 2. Pretreatment of human neutrophils with the δ-PKC inhibitor rottlerin, but not other PKC isotype inhibitors (Figure 5), prevented fMLF-induced MARCKS phosphorylation in a concentration-dependent manner (Figure 4A). 3. The range of rottlerin concentrations that blocked MARCKS phosphorylation in fMLF stimulated neutrophils also significantly attenuated fMLF-induced migration (Figure 4D) and adhesion (Figure 4G). Taken together, these findings provide strong evidence that δ-PKC is essential for neutrophil migration and adhesion. Importantly, this work also sheds new light on how δ-PKC might be regulating these important neutrophil functions – through the phosphorylation of a key substrate, MARCKS. Additional studies are needed to determine whether δ-PKC mediated MARCKS phosphorylation is relevant to neutrophil functions in vivo.
The protein kinase C (PKC) family encompasses 10-15 isoforms (depending on species) that are activated by phospholipid second-messengers downstream from a variety of surface receptor interactions. PKC activation is associated with translocation of the enzyme from the cytosol to various membrane compartments (Mochly-Rosen et al., 1990), depending on cell type. Following activation, PKCs phosphorylate serine and threonine residues on a large number of proteins (Steinberg, 2008). The large family of PKC enzymes is divided into three subfamilies: conventional PKCs (cPKC), novel PKCs (nPKC) and atypical PKCs (aPKC). The first two subfamilies, cPKC and nPKC, have high sequence homology and are similarly activated by phorbol esters, as well as the lipid-derived second messenger diacylglycerol (DAG). In addition to DAG, cPKCs also require calcium for their activation (Bertram and Ley, 2011). Atypical PKC isoforms share less sequence homology and do not respond to DAG, calcium or phorbol esters; but are instead activated by other membrane lipid components such as phosphatidic acid (PA) or phosphatidylinositol trisphosphates (PIP₃) (Ono et al., 1988, Chou et al., 1998).

PKC isoforms expressed in human neutrophils include two conventional isoforms (α- and β-PKC), one novel isoform (δ-PKC) and one atypical isoform (ζ-PKC) (Smallwood and Malawista, 1992, Balasubramanian et al., 1998, Bertram and Ley, 2011). Several different studies have linked one or more of these PKC isoforms to a wide array of cellular processes including migration, apoptosis, adhesion and respiratory burst (Larsson, 2006b, Park et al., 2007, Carpenter and Alexander, 2008, Geraldes and King, 2010, Kilpatrick et al., 2010, Gray et al., 2013, Fogh et al., 2014). To date, the most convincing evidence supports a role for novel (δ-PKC) and atypical (ζ-PKC) PKC isoforms in neutrophil functions including
migration, adhesion and respiratory burst. In 1996, Kent et al. demonstrated that δ-PKC is activated in fMLF stimulated human neutrophils (Kent et al., 1996). This is in agreement with our results, which showed δ-PKC translocation from the cytosol to the membrane following stimulation of human neutrophils with 100 nM fMLF (Figure 1). The study by Kent et al. also showed translocation of β-PKC from cytosolic to membrane fractions following fMLF neutrophil stimulation (Kent et al., 1996). This finding is in contrast to the current study results (Figure 1). However, it is possible that this discrepancy could be explained by differences in buffer conditions. The HBSS++ used to suspend cells in this study contains 1 mM calcium. Added calcium in the extracellular environment can lead to increased intracellular calcium (Ohkura et al., 1998), and as a member of the conventional PKC subfamily, β-PKC is sensitive to calcium activation (Majumdar et al., 1991). We therefore suggest that added calcium in the cell suspension media may have resulted in early β-PKC activation and subsequent accumulation within the membrane fraction, even prior to fMLF stimulation. We also saw differences in our ζ-PKC translocation results compared to a previous report by Laudanna et al. (Laudanna et al., 1998). Based on our subcellular fractionation and Western blot analysis of isolated human neutrophils, ζ-PKC was primarily located in the cytosol and showed no evidence of subcellular redistribution with either fMLF or PMA stimulation. In contrast, Laudanna et al. report that ζ-PKC did translocate from the particulate fraction in resting neutrophils to the light membrane fraction of fMLF or PMA stimulated neutrophils (Laudanna et al., 1998). Because we used a different method of subcellular fractionation, it is possible that there was ζ-PKC translocation that we did not detect.
Further evidence for the involvement of δ-PKC in neutrophil inflammatory functions comes from studies that utilize δ-PKC gene deficient mice. Neutrophils from these mice show markedly reduced adhesion in vitro following fMLF or IL-8 stimulation, compared to wild type neutrophils (Chou et al., 2004)(Chou et al., 2004). In addition, neutrophil adhesion, migration, superoxide generation and degranulation are reduced in δ-PKC null mice in vivo (Chou et al., 2004). Additional in vivo studies have utilized a δ-PKC specific inhibitor peptide (TAT-PKCδ) to examine the role of this PKC isotype in neutrophil mediated lung injury. Intra-tracheal instillation of this dominant negative peptide significantly attenuates neutrophil infiltration, chemokine expression and pulmonary edema following cecal-ligation and puncture induced sepsis in rats (Kilpatrick et al., 2011). From these studies it is clear that δ-PKC is activated during sepsis-induced pulmonary inflammation (Kilpatrick et al., 2011), and that δ-PKC plays an important role in neutrophil recruitment to the inflamed lung (Mondrinos et al., 2014), but the molecular mechanisms of this regulation remain uncertain. Like δ-PKC, previous study results have also implicated MARCKS as an important regulator of various neutrophil functions. In 2006, Takashi et al. demonstrated that inhibition of MARCKS, using a MARCKS specific inhibitor peptide known as MANS, caused a decrease in PMA-mediated neutrophil degranulation in vitro (Takashi et al., 2006). MANS mediated MARCKS inhibition also abrogated ozone-(Damera et al., 2010) and elastase (Foster et al., 2010) induced airway neutrophilia in mice in vivo. Subsequent studies by our laboratory utilized the MANS peptide to show that MARCKS function is essential for in vitro neutrophil functions including migration, adhesion and respiratory burst (Eckert et al., 2009). Most recently, work by Nordone et al. (Li et al., 2013) showed that MANS treatment of LPS-
stimulated canine neutrophils in vitro significantly attenuates production of inflammatory cytokines including IL-8 and TNFα. Interestingly, the MANS peptide, which is thought to act through inhibition of the MARCKS amino-terminus, has no known effect on MARCKS phosphorylation. But based on studies in several different cell types, MARCKS phosphorylation is a key step in regulating a variety of cellular events including spreading (Myat et al., 1997, Yue et al., 2000, Disatnik et al., 2004), adhesion (Estrada-Bernal et al., 2009, Yamaguchi et al., 2009), motility (Rosen et al., 1990, Kalwa and Michel, 2011, Lee et al., 2012), neurosecretion (Tanimukai et al., 2002), membrane trafficking (Salli et al., 2003, Siskova et al., 2006, Park et al., 2007, Chappell et al., 2009) and tumorigenesis (Finlayson and Freeman, 2009, Chen and Rotenberg, 2010). Also, the PKC isotype responsible for MARCKS phosphorylation varies depending on cell type (Uberall et al., 1997, Song et al., 1999, Kim et al., 2000, Dulong et al., 2004, Park et al., 2007, Sugiya et al., 2009, Gadi et al., 2011). In this study we hypothesized that MARCKS phosphorylation in human neutrophils would be regulated by a specific PKC isotype. We further hypothesized that MARCKS phosphorylation would be required for neutrophil functions (i.e. migration and adhesion) in vitro. To our knowledge, this is the first study to concurrently investigate PKC activation and MARCKS phosphorylation in human neutrophil functions in vitro.

Using rottlerin to inhibit δ-PKC activation, we observed concentration-dependent inhibition of MARCKS phosphorylation (Figure 4A), migration (Figure 4D) and adhesion (Figure 4G) in fMLF stimulated human neutrophils with markedly similar IC₅₀ concentrations of 5.074 uM (Figure 4C), 8.385 uM (Figure 4E) and 7.624 uM (Figure 4H), respectively. Inhibitors of α-, β- and ζ-PKC had no effect on MARCKS phosphorylation (Figure 5A-C). Furthermore,
we demonstrate that LTB₄ and IL-8 induced migration are also dependent on δ-PKC mediated MARCKS phosphorylation (Figure 4F). It is worth noting, however, that significant inhibition of IL-8 induced migration requires a higher rottlerin concentration (i.e. 20 uM) than inhibition of migration induced by fMLF or LTB₄ (i.e. 10 uM). This difference in inhibitory effect may reflect variability in the importance of certain PKC isotypes depending on the signal of activation.

One perceived drawback of inhibitor studies, like the one reported here, is the potential for “off-target” effects of inhibitors. In the current report we utilized rottlerin as an inhibitor of δ-PKC. Previous reports identify rottlerin as a specific δ-PKC inhibitor with an IC₅₀ of 3.0 to 6.0 uM. Rottlerin also reportedly inhibits conventional (IC₅₀ for α- and β-PKC = 30-42 μM), as well as atypical (IC₅₀ for ζ-PKC = 80-100 μM) PKC isoforms (Gschwendt et al., 1994, Villalba et al., 1999). Based on these reports, rottlerin mediated δ-PKC inhibition is 5 to 10 times more potent than conventional PKC inhibition and almost 30 times more potent than atypical PKC inhibition. But critics of rottlerin claim that this inhibitor causes non-specific δ-PKC inhibition due to mitochondrial uncoupling and reduced levels of cellular ATP (Soltoff, 2007). However, a recent study using dermal fibroblasts shows that rottlerin-induced up-regulation of mitochondrial uncoupling agents is limited. Moreover, this study showed agreement in specificity among three methods of δ-PKC inhibition (i.e.δ-PKC siRNA knock-down, δ-PKC inhibitory peptide and rottlerin treatment) based on RNA and microarray analysis (Wermuth et al., 2011). While we feel there is ample evidence to support the use of rottlerin as a specific δ-PKC inhibitor (Hendey et al., 2002, Pabla et al., 2011, Yanase et al.,
2012), we plan to further investigate MARCKS phosphorylation in neutrophils with more therapeutically relevant methods of δ-PKC inhibition in vivo.

Another key finding of the current study was the observation that ζ-PKC inhibition had no effect on MARCKS phosphorylation in human neutrophils (Figure 5C), but did significantly inhibit IL-8 mediated chemotaxis (Figure 6C). We also observed a downward trend of fMLF and LTB4 induced migration with ζ-PKC inhibition, but this was not significant. Our findings are in agreement with previous reports that ζ-PKC is a key regulator of motility in hematopoietic cells (Xiao and Liu, 2013). Several studies have shown that treatment of isolated human neutrophils with ζ-PKC pseudosubstrate inhibitor peptide significantly attenuates IL-8 induced adhesion (Laudanna et al., 1998). Specifically, results from Chakrabarti et al. point to a roll for ζ-PKC in the regulation of the β2-integrin CD11b (also known as MAC-1) affinity and avidity (Chakrabarti and Patel, 2008). We take great interest in the finding that ζ-PKC attenuates IL-8 mediated neutrophil chemotaxis in vitro with no apparent affect on MARCKS phosphorylation and plan to perform follow up investigations.

As presented here, our study results show that δ-PKC activation is essential for MARCKS phosphorylation in human neutrophils, and that inhibition of MARCKS phosphorylation correlates with a decrease in neutrophil migration and adhesion. Our results also show that ζ-PKC, which had no observed effect on MARCKS phosphorylation, plays a key role in neutrophil migration, at least toward the intermediate chemoattractant IL-8. Based on the known physiology of the signaling components involved, we propose the following narrative to explain our observations. In neutrophils, δ-PKC (a novel PKC isotype) is activated downstream of GPCR signaling by the secondary messenger DAG (Dewald et al., 1988).
Activation of δ-PKC involves translocation to the plasma membrane (Kent et al., 1996), where the substrate MARCKS is localized in resting cells (Thelen et al., 1991). δ-PKC phosphorylation of MARCKS ED disrupts the electrostatic interaction with the plasma membrane and displaces MARCKS to the cytosol (Yang and Glaser, 1996, Ohmori et al., 2000, Disatnik et al., 2004). This PKC-mediated regulation of MARCKS has at least two significant effects on the cell. 1. MARCKS phosphorylation decreases actin cross-linking and allows for dynamic actin reorganization (Aderem, 1992b, Hartwig et al., 1992a, Nairn and Aderem, 1992, Myat et al., 1997, Laux et al., 2000, Arbuzova et al., 2002, Salli et al., 2003, Disatnik et al., 2004, Larsson, 2006a, Yamaguchi et al., 2009). 2. MARCKS phosphorylation displaces it from the membrane and effectively liberates pools of phosphoinositides (i.e. PIP$_2$ and PIP$_3$) to be involved in additional cell signaling (Glaser et al., 1996, Laux et al., 2000, Wang et al., 2001, McLaughlin et al., 2002, Yamaguchi et al., 2009, Gadi et al., 2011, Kalwa and Michel, 2011). We propose that an increase in unsequestered PIP$_3$, as occurs with MARCKS phosphorylation, may facilitate the activation of ζ-PKC (Nakanishi et al., 1993), potentially placing the role of this atypical PKC isotype downstream of δ-PKC mediated MARCKS phosphorylation.

**Conclusions**

We conclude that novel isotype δ-PKC is responsible for MARCKS phosphorylation in human neutrophils following fMLF stimulation and that MARCKS phosphorylation is essential for neutrophil migration and adhesion. Additionally, we find that ζ-PKC, which is an essential signaling component in IL-8 induced neutrophil migration, may be activated downstream of δ-PKC mediated MARCKS phosphorylation. Ultimately, our goal is to better...
understand the complex molecular interactions regulating neutrophil migration and adhesion in an effort to identify potential targets for new anti-inflammatory therapies. We submit that the current report contributes valuable information regarding two key components of neutrophil inflammatory functions, δ-PKC and MARCKS. To our knowledge, this study is the first report suggesting these two proteins may be integrally linked in the regulation of neutrophil migration. We plan to validate the importance of these in vitro findings with additional studies in vivo.
Figure 1. δ-PKC translocates from cytosol to membrane upon neutrophil stimulation. (performed by EJ Sung)

Isolated human neutrophils were stimulated with fMLF (100 nM), PMA (50 ng/mL) or VC (DMSO) at 37°C for the indicated times. Prepared lysates were subsequently divided into membrane and cytosolic fractions using ultracentrifugation. Western blot analysis was performed to detect α-, β-, δ- and ζ-PKC in the two fractions. Data are representative of three separate experiments using different neutrophil donors.
Figure 2. MARCKS is rapidly phosphorylated upon stimulation of human neutrophils. (performed by EJ Sung)

Isolated human neutrophils were stimulated with (A) 100 nM fMLF, (B) 50 ng/mL PMA or (C) VC (DMSO) at 37°C for times ranging from 30 – 1800 seconds. Western blot analysis of lysates was performed to detect phosphorylated MARCKS (Phospho MARCKS) and total MARCKS protein (MARCKS). Data are representative of three separate experiments using different neutrophil donors.
Figure 3. PKC-mediated MARCKS phosphorylation is required for human neutrophil chemotaxis. (performed by MK Sheats and EJ Sung)

(A-C) Isolated human neutrophils were pretreated at 37°C for 30 minutes with indicated concentrations of the pan-PKC inhibitor staurosporine or VC (Me₂SO) and then stimulated with 100 nM fMLF. (A) Representative dose response experiment (30 second fMLF stimulation). Western blot analysis of equal protein amounts from prepared lysates was performed to detect phosphorylated MARCKS (Phospho MARCKS) and total MARCKS protein (MARCKS). (B) Densitometry of phospho MARCKS and total MARCKS bands was determined and “% MARCKS phosphorylation” was calculated by dividing the phospho MARCKS band by the total MARCKS band. Data are presented as the mean ± SE. (*) indicates significant (p<0.05) difference in % MARCKS phosphorylation compared to VC f(+). (C) IC₅₀ value (1.094 μM) was calculated by plotting percent inhibition of MARCKS phosphorylation against log₁₀ staurosporine concentration (uM). (D-F) Chemotaxis of calcein-labeled human neutrophils pretreated with indicated concentrations of staurosporine was measured using an 8 micron ChemoTx plate (Neuro Probe Inc.) (D) % neutrophil migration toward 100 nM fMLF or VC (Me₂SO) was calculated by dividing fluorescence of migrated cells by fluorescence of total cells. Data are presented as the mean ± SE. (*) indicates significant (p<0.05) difference in % migration compared to VC f(+). (E) IC₅₀ value (0.1027 μM) was calculated by plotting percent inhibition of neutrophil migration against log₁₀ staurosporine concentration (uM). (F) % neutrophil migration toward 100 nM LTB₄, 100 ng/mL IL-8 or VC (EtOH)) was calculated by dividing fluorescence of migrated cells by fluorescence of total cells. Data are presented as the mean ± SE. * indicates significant
(p<0.05) difference in % migration of LTB4 vs. VC ("0 uM" staurosporine), † indicates significant (p<0.05) difference in % migration of IL-8 vs. VC ("0 uM" staurosporine). (A-F)

All data are representative of at least three separate experiments using different neutrophil donors.
A. Staurosporine

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Phospho MARCKS
Total MARCKS

B. % MARCKS Phosphorylation

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Inhibitor Concentration

C. IC₅₀

% Inhibition

Staurosporine Concentration (μM)

(performed by EJ Sung)
(performed by MK Sheats)
F.

![Graph showing the effect of Staurosporine concentration on migration as a function of time.](image)

- LTB4 100 nM
- IL8 100 ng/mL
- Unstimulated

(permformed by MK Sheats)
Figure 4. δ-PKC regulates MARCKS phosphorylation, migration and adhesion in human neutrophils. (performed by MK Sheats and EJ Sung)

(A-C) Isolated human neutrophils were pretreated at 37°C for 30 minutes with indicated concentrations of the δ-PKC inhibitor rottlerin or VC (Me₂SO) and then stimulated with 100 nM fMLF. (A) Representative dose response experiment (30 second fMLF stimulation). Western blot analysis of equal protein amounts from prepared lysates was performed to detect phosphorylated MARCKS (Phospho MARCKS) and total MARCKS protein (MARCKS). (B) Densitometry of phospho MARCKS and total MARCKS bands was determined and “% MARCKS phosphorylation” was calculated by dividing the phospho MARCKS band by the total MARCKS band. Data are presented as the mean ± SE. (*) indicates significant (p<0.05) difference in % MARCKS phosphorylation compared to VC f(+). (C) IC₅₀ value (5.709 μM) was calculated by plotting percent inhibition of MARCKS phosphorylation against log₁₀ staurosporine concentration (μM).

(D-F) Chemotaxis of calcein-labeled human neutrophils pretreated with indicated concentrations of rottlerin was measured using an 8 micron ChemoTx plate (Neuro Probe Inc.) (D) % neutrophil migration toward 100 nM fMLF or VC (Me₂SO) was calculated by dividing fluorescence of migrated cells by fluorescence of total cells. Data are presented as the mean ± SE. (*) indicates significant (p<0.05) difference in % migration compared to VC f(+). (E) IC₅₀ value (8.385 μM) was calculated by plotting percent inhibition of neutrophil migration against log₁₀ rottlerin concentration (μM). (F) % neutrophil migration toward 100 nM LTB₄, 100 ng/mL IL-8 or VC (EtOH)) was calculated by dividing fluorescence of migrated cells by fluorescence of total cells. Data are presented as the mean ± SE. (*) indicates significant
(p<0.05) difference in % migration compared to VC (“0 uM” rottlerin). (G-H) Adhesion of calcein-labeled human neutrophils pretreated with indicated concentrations of rottlerin using an Immulon 2HB plate coated with 5% FCS. (G) Neutrophil adhesion was stimulated with 100 nM fMLF for 3 minutes at 37°C. Fluorescence of wells after 2 washes was divided by the initial fluorescence to calculate % adhesion. Data are presented as the mean ± SE. (*) indicates significant (p<0.05) difference in % adhesion compared to VC f (+). (H) IC_{50} value (7.624 μM) was calculated by plotting percent inhibition of neutrophil adhesion against log_{10} rottlerin concentration (μM). (A-H) All data are representative of at least three separate experiments using different neutrophil donors.
A. Rotterin
100 nM IMLF

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B. % MARCKS Phosphorylation

C. % Inhibition

(performed by EJ Sung)
(performed by MK Sheats)
(performed by MK Sheats)
Figure 5. PKC isoforms alpha, beta, and zeta do not regulate MARCKS phosphorylation in human neutrophils. (performed by EJ Sung)

(A-C) Isolated human neutrophils were pretreated at 37°C with indicated concentrations of (A) α-, (B) β- and (C) ζ-PKC inhibitors or vehicle control (Me2SO) and then stimulated with 100 nM fMLF. (A-C) Representative dose response experiments with Go6976 (A), CG53353 (B), PKC ζ pseudosubstrate (C) (30 second fMLF stimulation). Western blot analysis of equal protein amounts from prepared lysates was performed to detect phosphorylated MARCKS (Phospho MARCKS) and total MARCKS protein (MARCKS). Data are representative of at least three separate experiments using different neutrophil donors.
Figure 6. ζ-PKC plays a role in IL-8 mediated chemotaxis of human neutrophils. (performed by MK Sheats)

(A-C) Chemotaxis of calcein-labeled human neutrophils pretreated with indicated concentrations of (A) α-, (B) β- and (C) ζ-PKC inhibitors or vehicle control (Me₂SO) was measured using an 8 micron ChemoTx plate (Neuro Probe Inc.) % neutrophil migration toward 100 nM fMLF, 100 nM LTB₄, 100 ng/mL IL-8 or VC was calculated by dividing fluorescence of migrated cells by fluorescence of total cells and is presented as the mean ± SE. † indicates significant (p<0.05) difference in % migration of IL-8 vs. VC (“0 uM” staurosporine). Data are representative of at least three separate experiments using different neutrophil donors.
(performed by MK Sheats)
(performed by MK Sheats)
References


CHAPTER 3

Myristoylated Alanine Rich C Kinase Substrate (MARCKS) is essential to β2-integrin dependent responses of equine neutrophils

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Abstract

Neutrophil infiltration is a prominent feature in a number of pathologic conditions affecting horses including recurrent airway obstruction, ischemia-reperfusion injury, and laminitis. Cell signaling components involved in neutrophil migration represent targets for novel anti-inflammatory therapies. In order to migrate into tissue, neutrophils must respond to chemoattractant signals in their external environment through activation of adhesion receptors (i.e. integrins) and reorganization of the actin cytoskeleton. Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS), a highly conserved actin-binding protein, has a well demonstrated role in cytoskeletal dependent cellular functions (i.e. adhesion, spreading, and migration), but the details of MARCKS involvement in these processes remain vague. We hypothesized that MARCKS serves as a link between the actin cytoskeleton and integrin function in neutrophils. Using a MARCKS-specific inhibitor peptide known as MANS on equine neutrophils in vitro, we demonstrate that inhibition of MARCKS function significantly attenuates β2-integrin-dependent neutrophil functions including migration, adhesion, and immune complex-mediated respiratory burst. The MANS peptide did not, however, inhibit the β2-integrin-independent PMA mediated respiratory burst. These results attest to the essential role of MARCKS function in regulating neutrophil responses, and strongly implicate MARCKS as a potential regulator of β2-integrins in neutrophils.
1. Introduction

Although they are essential for normal host defense, neutrophils feature prominently in the pathophysiology of a number of important equine diseases, including laminitis, ischemia-reperfusion injury and recurrent airway obstruction (Moore et al., 1995, Gerard et al., 1999, Little et al., 2005, Marinkovic et al., 2007, de la Rebiere de Puyade, G. and Serteyn, 2011). Mechanisms of neutrophil-mediated tissue injury include release of proteolytic enzymes and production of reactive oxygen species (Wong et al., 2012). Despite ample research efforts directed toward understanding neutrophil recruitment, activation, and mechanisms of injury, clinically applicable treatments for neutrophil-mediated diseases remain limited.

Neutrophils in systemic circulation are recruited to sites of tissue infection or inflammation by host- or bacterial-derived chemoattractants; first adhering to the luminal surface of post-capillary venules, then moving across the endothelium, and finally crawling through the extracellular matrix to their final destination (Colditz, 1985, Baggioolini, 1998). To accomplish this arduous journey, neutrophils must recognize, interpret, and physically migrate along a chemokine gradient through a process known as chemotaxis. Neutrophil chemotaxis is a complex process that requires coordinated participation of essential cell surface receptors, a hierarchy of secondary cell signaling molecules and dynamic restructuring of the actin cytoskeleton (Dillon et al., 1988, Foxman et al., 1997, Cicchetti et al., 2002). Thus, inhibition of cellular regulators of neutrophil chemotaxis could be utilized to prevent or minimize unwanted neutrophil accumulation in tissues, and are therefore potential targets for novel anti-inflammatory therapies.
In order to move from the vasculature to sites of tissue inflammation, neutrophils must adhere to, and crawl along, inflamed endothelium via an integrin-dependent process (Kurtel et al., 1992). Integrins are transmembrane receptors that consist of non-covalently bound heterodimers of α and β chains. While neutrophils express several integrin heterodimers from the β1, β2 and β3 families, intraluminal adhesion and migration are dependent on activation of β2-integrins specifically (Schmidt et al., 2013). There are three key steps to activation of β2-integrins in neutrophils. 1) Increased surface expression of β2-integrins is achieved when secretory vesicles, which contain high numbers of preformed β2-integrins on their membranes, fuse with the neutrophil plasma membrane during exocytosis. 2) Intermediate and high-affinity conformations of β2-integrins are induced by chemoattractant binding to G-protein coupled receptors (“inside-out” signaling) or by direct integrin-ligand binding (“outside-in” signaling). 3) Increased binding avidity occurs when integrins are released from their cytoskeletal constraints and are able to diffuse throughout the cell membrane, resulting in formation of clusters (Nishida et al., 2006, Schymeinsky et al., 2007).

While many of the signaling details regulating integrin affinity and avidity remain unclear, PKC-mediated release of cytoskeletal constraints is known to play a key role in β2-integrin activation (Springer, 1990, Hynes, 1992, Clark and Brugge, 1995, Rosales and Juliano, 1995, Zhou and Li, 2000, Larsson, 2006). As a prominent PKC substrate and actin-binding protein, the MARCKS protein (Myristoylated Alanine Rich C-Kinase Substrate) has been proposed as a key link between PKC, actin, and integrin molecules (Aderem, 1992a, Hartwig et al., 1992a, Blackshear, 1993, Arbuzova et al., 2002). Indeed, previous research from our laboratory has demonstrated that inhibition of MARCKS function attenuates the β2-integrin-
dependent processes of migration and adhesion in human neutrophils in vitro (Eckert et al., 2009). In the current study, our goal was to further investigate the potential link between β2-integrin-dependent neutrophil functions and MARCKS. To this end, we measured the β2-integrin-dependent neutrophil functions of migration, adhesion and respiratory burst in vitro, with and without MARCKS inhibition. These data were compared to results from similar experiments conducted with or without β2-integrin-specific inhibition. Equine neutrophils were utilized in order to gain comparative species data to complement our previous study, to expand on our previous results with a human-relevant animal model, and to conduct research relevant to veterinary species, as well as humans.

To block MARCKS function, we utilized the MARCKS-specific inhibitor peptide known as “MANS” (myristoylated n-terminal sequence) as previously described (Singer et al., 2004, Takashi et al., 2006, Eckert et al., 2009, Li et al., 2013). RNS (random n-terminal sequence), which is a scrambled version of the same 24 amino acids as MANS, was used as a control. To block β2-integrin function we inhibited the integrin β chain (CD18) with the F(ab1)2 portion of an anti-CD18 antibody (αCD18). Interestingly, these results show that inhibition of β2-integrin (using αCD18) or MARCKS (using 50 uM MANS) attenuates equine neutrophil migration, adhesion and respiratory burst to a similar degree. Our findings also demonstrate that MARCKS is essential for β2-integrin-dependent neutrophil functions, but is not essential for β2-integrin-independent functions (i.e. PMA-mediated respiratory burst) in equine neutrophils. Taken together, these results strongly suggest that MARCKS function is essential to β2-integrin-dependent processes in neutrophils. Studies are currently underway to determine which aspects of integrin activation and/or signaling are dependent on MARCKS
function. Our findings support the assertion that specific inhibitors of MARCKS deserve further study as potential therapies for neutrophil mediated tissue injury.

2. Materials & Methods

2.1. Donors and neutrophil isolation

Animal use protocols were reviewed and approved by the institutional IACUC review board. For all neutrophil experiments, 30 to 60 mL of whole blood was collected using heparinized syringes from the jugular vein of adult horses from the teaching animal unit herd at the College of Veterinary Medicine, North Carolina State University. All donors were fed and housed under the same conditions and were receiving no medical treatment at the time of blood collection. Neutrophils were isolated from whole blood using Ficoll-PaqueTM Plus (GE Healthcare, Sweden) density gradient centrifugation (Nauseef, 2007). Briefly, heparinized whole blood was aliquoted into 15 ml polypropylene conical tubes (Sarstedt) and allowed to settle at room temperature for 45-60 min. Up to 12 mls of leukocyte rich plasma was aspirated using a bulb syringe and layered on 5 ml of Ficoll in a separate 15 ml conical tube. Cells were then centrifuged at 1800 rpm for 20 minutes. The supernatant was discarded and remaining red blood cells within the cell pellet were removed by 60 seconds of hypotonic lysis. Isolated neutrophils (> 96% by Wright’s Geimsa staining) were resuspended/washed in sterile HBSS (Cellgro, Inc.) without additives. Cell number and viability was quantified using trypan blue dye exclusion (1:1) and a manual hemocytometer count. Viability was routinely > 99%. Final suspension of cells was in HBSS++ chemotaxis buffer [1x HBSS, 1mM Ca2+, 1mM Mg2+, 5% fetal bovine serum (Gemini bio-product)] at the indicated concentration for each experiment. With this isolation protocol, all experiments
were completed within 4-6 hours of blood collection. Neutrophils from individual donor horses were used for all time points and treatment conditions for each experiment (i.e. “n” represents a separate horse donor).

2.2. MARCKS inhibition using a cell permeant peptide

2.2.1. Peptide pretreatment

MANS and RNS peptides, as previously described, were synthesized by Genemed Synthesis, Inc. (San Francisco, CA, USA) (Singer et al., 2004). The sequence of MANS is identical to the first 24 amino acids of the human MARCKS protein: myristic acid-GAQFSKTAAKGEEAAERPGEAAVA. The RNS peptide is a randomly scrambled control: myristic acid-GTAPAAEGAGAEVKRASAEAKQAF. Where indicated, pretreatment of cell suspensions with indicated peptide concentrations occurred at 37°C for 30 min. Neutrophils were confirmed viable after peptide treatment by trypan blue dye exclusion.

2.2.2. Effect of peptide without pretreatment

In addition to experiments in which neutrophils were pretreated with MANS and RNS, we also investigated the inhibitory effect of the MANS peptide without pretreatment. For these experiments, MANS and RNS (50 uM) were added to the cell suspension immediately prior to performing the assay.

2.3. Fluorescence labeling of neutrophils

For migration and adhesion experiments, isolated neutrophils (1 x 10^7/ml in HBSS) were incubated with the fluorescent dye calcein am (Anaspec, Fremont, CA) at 2 ug/ml for 30 minutes at room temperature. Cells were then centrifuged at 1000 rpm for 8 min and
resuspended in HBSS++ (chemotaxis buffer) to the appropriate final experimental concentration.

2.4. β2-integrin inhibition

As a positive control for β2-integrin inhibition in both adhesion and respiratory burst assays, isolated equine neutrophils were pretreated with indicated concentrations of α-human CD18 F(ab1)2 (Ancell Corp, Bayport, MN) at 37°C for 30 min. Anti-human IgG1 F(ab1)2 (Ancell Corp, Bayport, MN) was used as an isotype control. The F(ab1)2 fragment, as opposed to whole antibody, was utilized to avoid unintended neutrophil Fcγ receptor crosslinking and activation.

2.5. Western blotting

PMA (ACROS organics, Belgium), platelet-activating factor (PAF) (Cayman Chemical, Ann Arbor, MI) and leukotriene B4 (LTB4) (Sigma, St. Louis, MO) stimulated equine neutrophils (2.5 X 10^7 cells/ml) were lysed with RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 5 mM sodium pyrophosphate and 50 mM sodium fluoride) containing EDTA-free protease and phosphatase inhibitor tablets (Roche, Germany). Lysates were incubated on ice with agitation for 20 minutes and then cleared by centrifugation for 10 minutes at 9,000 x g at 4°C. Protein concentrations were determined by BCA assay (Pierce, Rockford, IL). Samples were then diluted in 5X Sample Buffer containing 2-ME and boiled for 5 minutes prior to storing at -20°C and subsequent analysis by 10% SDS-PAGE (Invitrogen). Resolved samples were transferred to Immobilon-P PVDF membrane (Millipore), blocked in 5% non-fat dry milk (Labscientific, Livingston, NJ) in sterile dulbecco’s powdered phosphate-buffered saline (PBS) (GIBCO, Gland Island, NY).
for 1 hour prior to overnight incubation with phospho-MARCKS or total MARCKS primary antibodies (Santa Cruz) at 4°C. Following 2 hour incubation with the appropriate HRP-conjugated secondary antibody (Santa Cruz) the PVDF membrane was developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) and radiograph film.

2.6. Migration & Adhesion assays

2.6.1. Neutrophil migration

The 3 micron pore size Neuro Probe ChemoTx® system consisting of a 96-well microtiter plate was used to assess neutrophil migration (Frevert et al., 1998). Each upper well was loaded with 4 x 10^4 calcein-labeled neutrophils in 20 ul of the appropriate media. Treatment groups were tested in triplicate. HBSS++ was used for chemotaxis; while HBSS++ with chemoattractant (concentration equal to the bottom wells) was used to measure chemokinesis. Cells were allowed to migrate for 1 hour at 37° C toward lower wells containing HBSS++, HBSS++ with chemoattractant (10 nM LTB4 or 10 nM PAF), or HBSS++ with VC (EtOH). Following the hour migration, non-migrated cells were removed from the top of the membrane with a cell scraper. Following centrifugation at 1000 rpm for 1 minute, fluorescence of the bottom wells was measured (485 nm excitation, 530 nm emission) using an fMax fluorescence plate reader (Molecular Devices). Percent migration was calculated by dividing the fluorescence of the experimental bottom wells by the fluorescence of bottom wells containing a known number of cells (4 x 104 cells).
2.6.2. Neutrophil adhesion

1. To assess PMA and LTB4 mediated adhesion, 1x10^5 calcein-labeled neutrophils (100 ul) from the various treatment groups were allowed to settle for 10 minutes in individual wells of Immulon2HB flat bottom 96-well plates (Thermo Fisher Scientific) coated with 5% FCS. The addition of 10 ng/ml PMA per well (final concentration) or vehicle control (DMSO), or 10 nM LTB4 per well (final concentration) or vehicle control (EtOH) was followed by indicated experimental incubation times at 37°C. Treatment groups were tested in triplicate.

2. For insoluble immune complex (IIC)-mediated adhesion, experimental and blank wells of Immulon2HB plates were each coated with 200 ul of 100 ug/mL BSA (Sigma) and incubated overnight at 4°C (Jones et al., 1998). The next day, wells were emptied and washed 3 times with sterile PBS, followed by the addition of either 5 ug/well (low density) or 20 ug/well (high density) rabbit-anti-BSA antibody (Sigma) in 200 uL sterile PBS. Plates were incubated at 37°C for 2 hours; then immediately prior to the assay the wells were emptied and washed three times with sterile PBS. 1x10^5 calcein-labeled neutrophils (100 ul) from the various treatment groups were then added to individual wells and incubated at 37°C for 30 min.

3. Following indicated incubation times, neutrophil adhesion was assessed using an fMax fluorescence plate reader (Molecular Devices). After an initial fluorescence reading (485 nm excitation, 530 nm emission) the plates were gently dumped and washed with 150 ul sterile PBS and a second fluorescence reading was obtained. This procedure was repeated for a total of three to four washes to remove non-adhered cells. Fluorescence after each washing was divided by the initial fluorescence to calculate percent adhesion. The first wash that
demonstrated less than 10% adhesion of non-stimulated cells (third wash on average) was considered the final result.

2.7. Neutrophil ROS generation

To conduct respiratory burst experiments, isolated neutrophils were resuspended in HBSS++ with 2% FCS to a final concentration of 3.0 x 10^6/ml. Cells were then incubated with indicated treatments at 37°C for 30 minutes prior to each experiment. 3.0 x 10^5 cells (100 ul) from various treatment groups were placed in individual wells of 5% FBS or IIC coated Immulon HB2 plates. For PMA-stimulated respiratory burst, cells were allowed to settle for 10 minutes prior to the addition of dihydorhodamine-123 (DHR-123) (Sigma) (10 uM final concentration) and PMA (100 ng/ml final concentration) (Hurley et al., 2006). In the case of IIC-mediated respiratory burst, DHR-123 was added immediately following addition of cells to the well. An fMax fluorescence plate reader (Molecular Devices) was used to measure initial fluorescence (485 nm excitation, 530 nm emission) followed by a fluorescence reading every 15 minutes for 120 minutes. Results are reported as nm fluorescence.

2.8. Statistical analysis

Data are reported as mean ± SEM. Data were analyzed by one way repeated measures ANOVA (Holm-Sidak multiple comparison testing) assuming equal variance, with p < 0.05 considered statistically significant.

3. Results

3.1. MARCKS amino-terminus is highly conserved

Myristoylated Alanine-Rich C Kinase Substrate (MARCKS) is both highly conserved and ubiquitously expressed in mammalian species (Li et al., 2013). A specific inhibitor of
MARCKS, the MANS peptide, is identical to the first 24 amino acids of human MARCKS (NCBI RefSeq: NP_002347.5). In order to establish the suitability of MANS as an inhibitor of equine MARCKS, we investigated available equine reference sequences. Because there was no identifiable protein or nucleotide sequence for equine MARCKS present in GenBank, we employed the human MARCKS mRNA sequence (NCBI RefSeq: NM_002356.5) as a query to search (BLASTn) the equine genome database. This search identified a highly homologous region on chromosome 10 (EquCab2.0 scaffold_12, whole genome shotgun sequence, RefSeq: NW_001867364.1) with an E value of 0.0. Although the entire sequence homology was >80%, a significant portion of the coding region was missing from the reference genome; therefore a full length protein sequence could not be predicted. However, an alignment between the N-terminus of the two sequences shows that the first 34 amino acids of the equine MARCKS protein are nearly identical to human MARCKS (Fig. 1A).

As a prominent protein kinase C (PKC) substrate, MARCKS is rapidly phosphorylated following neutrophil stimulation (Thelen et al., 1991). Treatment of isolated equine neutrophils with PMA resulted in rapid MARCKS phosphorylation, as detected by western blot (Fig. 1B). Equine neutrophils stimulated with known chemoattractants, leukotriene B4 (LTB4) and platelet activating factor (PAF) showed maximal MARCKS phosphorylation at 30 seconds with a return to baseline by 3 minutes (Fig 1C). This pattern of transient phosphorylation followed by de-phosphorylation is a key feature of MARCKS’ reversible, phosphorylation-dependent association with the plasma membrane, and is thought to be an essential mechanism for MARCKS regulation of the actin cytoskeleton (Disatnik et al., 2004). Based on identifiable nucleotide sequence homology, cross-reactivity with human
MARCKS antibody and a pattern of phosphorylation consistent with reports in other species, we concluded that the MARCKS protein present in equine neutrophils is structurally and functionally homologous to human MARCKS, and therefore that the MANS peptide should be an effective MARCKS inhibitor in equine neutrophils.

3.2. MANS peptide pretreatment inhibits equine neutrophil migration

Cell permeant peptides MANS (myristoylated N-terminal sequence) and RNS (random N-terminal sequence) were used to investigate the requirement for MARCKS function in equine neutrophil migration in vitro. In isolated equine neutrophils pretreated with varying concentrations of MANS (1 – 50 uM), a dose dependent decrease in chemotaxis toward LTB4, was observed, starting at 10 uM. Directed migration of equine neutrophils pretreated with 50 uM MANS peptide was markedly attenuated, with only 14% migration toward 10 nM LTB4 compared to the maximal migration of 76% seen in neutrophils pretreated with PBS (“0” uM MANS) (Figure 2A). Pretreatment of cells with up to 50 uM RNS had no effect on LTB4 mediated neutrophil chemotaxis. The IC50 of MANS, 29.3 uM, was determined by plotting the % reduction in migration of MANS vs. vehicle control treated cells, against MANS concentration (data not shown).

We next investigated MANS effect on both random (chemokinesis) and directed (chemotaxis) neutrophil migration using uniform and polarized gradients of LTB4 and PAF. PBS treated neutrophils responded to both cytokines (LTB4 and PAF) with significant chemotaxis (74% and 50%, respectively) and chemokinesis (45% and 42%, respectively) compared to “background migration” of cells not exposed to chemoattractant (VC). Consistent with the effects seen on chemotaxis, only 16% and 13% of neutrophils pretreated
with 50 uM MANS migrated to the lower well in polarized gradients of LTB4 and PAF, respectively. Pretreatment with 50 uM MANS also inhibited equine neutrophil chemokinesis, as only 13% of LTB4-stimulated cells and 12% of PAF-stimulated migrated across the membrane (Figure 2B). Pretreatment of equine neutrophils with 50 uM RNS had no significant effect on chemotaxis or chemokinesis compared to PBS treated control. Because MANS peptide inhibited both directed (chemotaxis) and non-directed (chemokinesis) migration, we were able to establish that MARCKS function is not limited to pathways involving the neutrophil’s ability to “sense” direction. The next aspect of neutrophil migration that we chose to investigate was adhesion.

3.3. MANS peptide pretreatment inhibits PMA- and IIC-induced adhesion

In order to migrate along activated endothelium, neutrophils must first adhere to the endothelial surface through β2-integrin (CD11a/CD18 and CD11b/CD18) dependent ligand interactions. Therefore, we next investigated whether MANS was interfering with neutrophil migration via inhibition of β2-integrin dependent adhesion. Stable neutrophil adhesion was induced with two different neutrophil activators, phorbol 12-myristate 13-acetate (PMA) or insoluble immune complexes (IIC). PMA is a synthetic mimic of diacylglycerol and a direct activator of novel and conventional PKCs, whereas IIC ligates and activates Fcγ receptors. High- and low-densities of IIC were used to investigate varying degrees of IIC-mediated neutrophil activation. Both methods induce stable neutrophil adhesion through β2-integrin activation. Treatment with PMA stimulated approximately 50% adhesion of neutrophils that received pretreatment with PBS only (MANS and RNS “0 uM”), while unstimulated cells (VC) showed less than 3% “background” adhesion. Neutrophil groups that were pretreated
with 10, 25 and 50 uM MANS had 26%, 12% and 7% adherent cells following 30 minutes PMA treatment, respectively; which was significantly less than cells treated with VC (DMSO)(Figure 3A). Similar results were observed with IIC-mediated adhesion. Of the PBS treated (MANS and RNS “0 uM”) neutrophils added to wells coated with either high (20 ug/well) or low (5 ug/well) density IIC, approximately 47% and 38% adhered, respectively. On high density IIC coated wells, only 8% of 50 uM MANS pretreated neutrophils adhered, a significant reduction compared to PBS treated cells. On low density IIC coated wells, 25 uM and 50 uM MANS treatment significantly inhibited neutrophil adhesion (3% and 1%, respectively) compared to PBS treated cells (Figure 3B).

We next examined the effect of 50 uM MANS pretreatment, along with specific inhibition of β2-integrins, on PMA- and IIC-mediated equine neutrophil adhesion. As shown in Figure 3C, less than 1% of neutrophils treated with 50 uM MANS remained adherent following PMA or IIC-activation, significantly less than the nearly 50% adhesion observed with PBS (MANS “0 uM”) treated cells. A similar attenuation of adhesion was seen with specific β2-integrin inhibition using either 10 ug/mL (data not shown) or 30 ug/mL αCD18 F(ab1)2 pretreatment (Figure 3C).

3.4. MANS inhibits neutrophil adhesion and migration without pretreatment

The clinical benefit of minimizing neutrophil mediated tissue injury has been demonstrated in animal models of sepsis, ischemia-reperfusion injury, acute and chronic airway disease and arthritis (Kurtel et al., 1992, Fujishima and Aikawa, 1995, Moore et al., 1995, Kraan et al., 2000, Cazzola et al., 2012, Uriarte et al., 2013, Berger et al., 2014). However, most patients that would benefit from therapies targeting neutrophil migration would not
realistically have an opportunity for “pretreatment” prior to neutrophil activation. With this in mind, the next set of experiments investigated MANS effect on neutrophil migration and adhesion without pretreatment. Using 3 micron pore sized chemotaxis plates we measured directed migration of calcein-labeled equine neutrophils toward LTB4 at 15, 30 and 60 minutes. At these respective time points, 46%, 80% and 95% of PBS treated neutrophils migrated to the lower wells. These percentages were not significantly different in RNS treated cells. However, 50 uM MANS peptide, even without pretreatment, significantly inhibited LTB4 induced migration at all three time points when compared to treatment with RNS (Figure 4A). We next investigated whether MANS peptide, without pretreatment, would inhibit the rapid and transient adhesion induced by chemoattractant activation. The percentage of PBS treated equine neutrophils adherent at 75, 150 and 300 seconds following LTB4 stimulation was 25%, 24% and 22%, respectively. Even without pretreatment, MANS inhibition proved to be extremely rapid, with a significant inhibition of LTB4-induced adhesion seen at all three time points. There was no difference in percent adhesion with RNS treatment. Interestingly, the percent adhesion of MANS treated cells was also significantly less than the average unstimulated adhesion of approximately 10% seen in unstimulated (VC) neutrophils (Figure 4B).

3.5. MANS inhibits β2-integrin dependent respiratory burst

Our findings to this point clearly demonstrate similar effects of MARCKS inhibition and β2-integrin inhibition on neutrophil migration and adhesion. Therefore, we chose to further investigate the apparent link between MARCKS and β2-integrins by determining the effect of MANS on generation of reactive oxygen species (i.e. H2O2) stimulated in a β2-integrin
dependent (IIC) or independent (PMA) manner. With both PMA and IIC stimulation, DHR-123 detected a robust and sustained production of intracellular H2O2 in equine neutrophils for over 2 hours (Figure 5A and 5B). Inhibition of β2-integrin with αCD18 F(ab1)2 caused an average 70% reduction in the IIC-mediated respiratory burst compared to untreated cells (Figure 5A), while PMA-mediated respiratory burst was unaffected (Figure 5B). Similar to αCD18 F(ab1)2, pretreatment of equine neutrophils with MANS peptide significantly attenuated IIC-mediated respiratory burst (approximately 80% inhibition compared to UTC), but had no effect on PMA-mediated respiratory burst. This result demonstrates that MANS treatment does not interfere with the assembly of the NADPH complex or generation of ROS, but with activation and/or signaling upstream of PKC activation in a manner consistent with inhibition of β2-integrins.

4. Discussion
The current study was conducted to investigate the potential link between β2-integrins and the actin binding protein MARCKS, in equine neutrophils. Consistent with our previous findings in human neutrophils (Eckert et al., 2009), these results confirm that inhibition of MARCKS with the MANS peptide attenuates β2-integrin dependent migration (Figure 2) and adhesion (Figure 3) of equine neutrophils in vitro. Using LTB4 to stimulate migration and adhesion, we further demonstrate that MANS is an effective inhibitor of these neutrophil functions in vitro, even without pretreatment. Finally, we report that MARCKS function is necessary for IIC-mediated respiratory burst, but is not required for the β2-integrin independent process of PMA-mediated respiratory burst. This novel finding suggests that MANS mediated MARCKS inhibition disrupts β2-integrin signaling upstream of PKC.
activation. This significant discovery opens the door to exciting hypotheses regarding the role of MARCKS in integrin-dependent pathways.

Our ultimate goal with MARCKS inhibition is the development of therapeutics that will prevent or minimize neutrophil mediated tissue injury in acute and/or chronic inflammatory diseases. Most patients that would benefit from this type of treatment already have increased levels of endogenous inflammatory cytokines causing various degrees of neutrophil activation. In these clinical situations, a drug requiring pretreatment would have very limited applications. For this reason, we investigated chemotaxis and adhesion of equine neutrophils exposed simultaneously to MANS peptide and chemoattractant. Our results demonstrate that even without pretreatment, the MANS peptide rapidly inhibits equine neutrophil migration and adhesion in response to LTB4 (Figure 4A and 4B). To more closely simulate conditions in vivo, future in vitro studies will determine if functions of previously activated neutrophils (i.e. stable adhesion, chemotaxis) can be disrupted by MANS treatment.

The current report adds significant information to our previous understanding of MARCKS function in neutrophils. In this study we observed that MANS peptide treatment of equine neutrophils significantly inhibits IIC-mediated respiratory burst, but fails to inhibit PMA-mediated respiratory burst. As shown by pretreatment of neutrophils with an αCD18 antibody F(ab’)² (Figure 5), the result seen with MANS treatment is consistent with those of β2-integrin inhibition. Insoluble IC activation of oxidative burst is triggered by FcγRIII-receptors, but this process also requires the cytoskeletal dependent activation of β2-integrins (Jones et al., 1998, Williams and Solomkin, 1999, Jones et al., 2001). PMA, which activates respiratory burst at the level of novel and/or conventional PKCs, is independent of β2-integrin
and the actin cytoskeleton (Lehmeyer et al., 1979). Inhibition of both IIC- and PMA-mediated respiratory burst by the pan-PKC inhibitor staurosporine confirmed that both methods of stimulation rely on a PKC dependent pathway (data not shown). Because our findings demonstrate that MANS treated neutrophils are able to undergo respiratory burst when stimulated by PMA, but not by IIC, we conclude that at least one aspect of MARCKS function is essential in β2-integrin dependent processes in neutrophils between the level of β2-integrin receptors and PKC activation.

Beta2- integrins, composed of two non-covalently bound α- (CD11a, CD11b and CD11c) and β- (CD18) subunits, are trans-membrane adhesion proteins essential to neutrophil functions including adhesion, migration, degranulation, respiratory burst and phagocytosis. Prior to neutrophil activation, β2-integrins are expressed in an inactive or “bent” conformation in relatively small numbers on the cell surface, and movement within the membrane is restricted by cytoskeletal constraints. Upon neutrophil stimulation, β2-integrins undergo several changes: 1) their surface expression is increased by exocytosis of secretory vesicles, as well as tertiary and specific granules; 2) their conformation changes from low- to intermediate- or high-affinity in order to engage specific ligands and matrix proteins; and 3) release from the actin cytoskeleton leads to increased lateral mobilization and cluster formation, termed “avidity”. Neutrophil β2-integrins are unique in terms of receptors because they can originate (“outside-in”) or be the target of (“inside-out”) activation signals. During “inside-out” signaling integrins are the downstream target of intracellular signaling initiated by cell surface receptors that recognize mediators of neutrophil activation (i.e. LTB4, PAF). During “outside-in” signaling, β2-integrins engage specific ligands and initiate signaling that
regulates processes from cytoskeletal rearrangement and degranulation, to cytokine production and apoptosis (Nishida et al., 2006, Ley et al., 2007, Luo et al., 2007, Schymeinsky et al., 2007). However, neither of these processes occurs independently, and the complexity of this network is why so many questions regarding the regulation and function of neutrophil β2-integrins remain unanswered. The results reported here indicate that MARCKS function is essential to β2-integrin dependent equine neutrophil functions. What remains unclear is whether that function is relevant to “outside-in” or “inside-out” β2-integrin signaling/activation, or both.

Previous reports linking MARCKS to β1-integrin dependent processes in myoblasts and oligodendrocytes clearly demonstrate that MARCKS is an essential regulator of the actin cytoskeleton downstream of integrin-mediated PKC activation (Disatnik et al., 2004, Siskova et al., 2006). While our results show that inhibition of MARCKS function with the MANS peptide disrupts β2-integrin dependent functions of adhesion, migration and respiratory burst in neutrophils, the mechanism of MANS inhibition has yet to be determined. Eckert et al. (2009) previously showed that the MANS peptide does not affect spreading or polarization of human neutrophils following PMA or fMLF stimulation, respectively, and had minimal effect on the fMLF induced rise in F-actin. Additionally, these authors showed no effect of MANS on up-regulation of CD18 following either PMA or fMLF stimulation of human neutrophils in vitro (Eckert et al., 2009). We are currently conducting studies to investigate both MARCKS role in, and the effect of MANS on, additional aspects of β2-integrin activation and signaling in neutrophils.
5. Conclusions

Viable therapeutic options are needed to treat neutrophil mediated tissue damage in human and veterinary species alike. In horses, neutrophils play a key role in the pathophysiology of significant diseases including laminitis, recurrent airway obstruction and ischemia-reperfusion injury. Research identifying key cellular regulators of neutrophil functions will assist in the development of novel anti-inflammatory therapies. Based on the promising results of the current study, as well as key in vivo findings by our colleagues (Singer et al., 2004, Takashi et al., 2006, Li et al., 2013), we hope to move forward with investigation of MANS or other MARCKS inhibitors as potential anti-inflammatory therapy for recurrent airway obstruction in the horse.

Conflict of Interest Statement

KBA holds 150,000 founders shares of a start-up biotech company, BioMarck, and serves as a scientific consultant and member of the scientific advisory board without monetary compensation. KBA receives over $100,000 yearly in research grants from National Institutes of Health (NIH) and U.S. Environmental Protection Agency. KBA is Editor-in-Chief of the American Journal of Respiratory Cell and Molecular Biology and receives a stipend of <$100,000 per year from the American Thoracic Society for this. The remaining authors have declared that no competing interests exist.

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Figure 1. MARCKS protein is expressed in equine neutrophils. (A) Box-shade demonstrating homology of amino-terminus of human MARCKS sequence (NCBI RefSeq: NP_002347.5) with predicted amino-terminus of equine MARCKS. The equine MARCKS sequence was derived from alignment of human MARCKS mRNA (NCBI RefSeq: NW_001867364.1) with EquCab2.0 scaffold_12 whole genome shotgun sequence. Western blots of MARCKS phosphorylation in equine neutrophils stimulated with PMA (B) or cytokines LTB₄ and PAF (C). Human mAbs for total MARCKS and phospho-MARCKS consistently detected a single protein dimer at a molecular weight of 50 kDa.
Figure 2. MANS peptide inhibits equine neutrophil migration toward multiple chemoattractants. MANS peptide demonstrated a dose dependent inhibition (A) of LTB4 stimulated equine neutrophil migration in vitro. (B) Compared to RNS 50 uM treatment, MANS 50 uM significantly inhibited both chemoattractant stimulated (gradient and non-gradient) and non-stimulated (VC) migration. Data presented as mean ± sem, * p < 0.05 MANS vs. RNS, n = 6.
Figure 3. MANS peptide inhibits β2-integrin dependent equine neutrophil adhesion.

MANS peptide, but not RNS, demonstrated a dose dependent inhibition of PMA- (A) and IC-mediated (B) equine neutrophil adhesion in vitro. (C) Both MANS 50 μM and αCD18 F(ab')₂ (30 μg/mL shown) significantly inhibited PMA – and IIC – mediated equine neutrophil adhesion. Data presented as mean ± sem, *p < 0.05 vs. PBS (“MANS 0 μM”), n = 6.
Figure 4. MANS peptide inhibits migration and adhesion without pretreatment. Addition of peptide concurrent with LTB₄ stimulation demonstrated that MANS 50 uM rapidly inhibits equine neutrophil adhesion (A) and migration (B). Data presented as mean ± sem, * p < 0.05 vs. LTB₄ + PBS, n = 6.
Figure 5. MANS peptide inhibits β2-integrin dependent respiratory burst. MANS peptide (50 uM) significantly inhibited IC-stimulated respiratory burst (A), but had no significant effect on respiratory burst stimulated by PMA (B). As demonstrated by inhibition of β2-integrin with αCD18 F(ab’)2 (30 ug/mL), IC-stimulated respiratory burst is β2-integrin dependent, while PMA-stimulated respiratory burst is β2-integrin independent. Respiratory burst was measured by addition of DHR-123. Data presented as mean ± sem, * p < 0.05 MANS vs. Control, † p < 0.05 αCD18 F(ab’)2 vs. Control, n = 6.
References


CHAPTER 4

Myristoylated Alanine Rich C Kinase Substrate (MARCKS) is involved in “outside-in” β2-integrin activation in neutrophils

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Abstract

Beta₂-integrins present a fascinating therapeutic target for drugs designed to modulate neutrophil response and/or function, but many molecular details regarding neutrophil β₂-integrin regulation and signaling remain unclear. Previous work in our lab determined that the actin binding protein MARCKS (myristoylated alanine rich C kinase substrate) plays an essential role in the β₂ integrin-dependent neutrophil functions of migration and adhesion. In this previous report we utilized a 24 amino acid MARCKS specific inhibitor peptide known as MANS to study MARCKS function. In the current report, we sought to further investigate the effects of MARCKS inhibition (with MANS) on key aspects of β₂-integrin activation and signaling in human neutrophils. As with other β₂-integrin dependent functions (i.e. migration and adhesion) we found that MANS treatment caused significant inhibition of immune complex-mediated respiratory burst (β₂-integrin dependent), but not PMA-mediated respiratory burst (β₂-integrin independent). Interestingly, we saw no effect of MARCKS inhibition on increased β₂-integrin expression (CD18 and CD11b) or affinity (CD11b), as measured by flow cytometry, following fMLF-stimulation of neutrophils. This result suggests that β₂-integrin “inside-out” activation by GPCR signaling was intact in MANS treated cells. This conclusion was further supported with results of biochemical analysis in which we monitored Syk phosphorylation as a marker of β₂-integrin activation. These experiments show normal Syk phosphorylation in immune complex and TNFα-stimulated neutrophils, even with MANS treatment. However, MANS treatment did inhibit Syk phosphorylation in unstimulated neutrophils plated on fibrinogen. MANS treatment also inhibited Mn²⁺ induced neutrophil adhesion to substrates of IIC or 5% FBS. From these
preliminary results, we hypothesize that MANS inhibition of MARCKS function is disrupting β2-integrin “outside-in” signaling, potentially through effects on avidity. This hypothesis requires further evaluation.

**Introduction**

The members of the β2 integrin family play an essential role in neutrophil recruitment and effector functions during the innate immune response. In order to transmigrate from the vasculature to sites of microbial tissue invasion neutrophils must be recruited from the rapid flow of blood by several types of receptors located on both neutrophil and endothelial cell surfaces. Although this process of neutrophil capture, rolling, endothelial adhesion, post-adhesion strengthening, crawling and finally transmigration has been extensively researched and reviewed in detail (Herter and Zarbock, 2013, Kolaczkowska and Kubes, 2013), the complexity of this series of events means that there are still molecular details that remain uncertain. What is certain about neutrophil transmigration is that β2-integrins play an especially important role in adhesion strengthening, crawling and transmigration, depending on the site of tissue emigration (Schmidt et al., 2013). This is important from the standpoint of both normal host defense and disease pathophysiology. In terms of defense, a lack of functional β2-integrin weakens the host immune system so severely that patients suffer recurrent and/or chronic bacterial infections, leading to significant morbidity and even mortality as seen in people with leukocyte adhesion deficiency (LAD) (Springer, 1990). But for every ‘yin’ there is a ‘yang’, and the destructive power of neutrophils that is so beneficial in fighting off microbial invaders can be extremely detrimental if tissue infiltration is excessive or activation is dysregulated (Tong et al., 2013). Indeed, neutrophil derived tissue
injury is known to play a role in numerous acute and chronic inflammatory diseases
including myocardial infarction, gut ischemia-reperfusion injury, ulcerative colitis,
rheumatoid arthritis and sepsis associated organ damage (i.e. acute lung injury, acute kidney
injury). In several different animal models of neutrophil mediated inflammatory disease,
researchers have inhibited neutrophils or their migration to demonstrate the potential benefit
of β2-integrin inhibition (Neumann et al., 1999, Wang et al., 2014). Unfortunately, to date,
inhibition of β2-integrins in the clinical setting, with the use of monoclonal antibodies, has
not yielded the results that researchers anticipated; but β2-integrins remain a desirable target
for modulating neutrophil responses. A more detailed understanding of the molecular details
regulating neutrophil β2-integrins may lead to as of yet undiscovered targets for new and
beneficial anti-inflammatory therapies.
Expressed exclusively on leukocytes, β2-integrins are transmembrane heterodimers that
consist of a common β-subunit (CD18), which is non-covalently associated with one of the
four known α-subunits (CD11a, CD11b, CD11c, and CD11d) (Schymeinsky et al., 2007).
Both subunits possess a large extracellular domain, a transmembrane domain and a short
cytoplasmic tail. Within circulating, quiescent neutrophils these adhesion molecules are
primarily contained within the cytoplasm; present in increasing numbers within neutrophil
secondary and tertiary granules and secretory vesicles. The few β2-integrins that are present
on the surface of resting neutrophils are maintained in an inactive or ‘bent’ conformation,
otherwise known as a “low-affinity” state. This combination of low surface expression and
inactive conformation are control measures to help prevent non-specific neutrophil binding
and activation, as this could have untoward effects on host tissue and vasculature (as
previously mentioned). It is only once neutrophils encounter an “activation signal” that β2-integrin surface expression is increased, conformational changes take place (affinity) and increased mobility within the membrane leads to cluster formation (avidity/valency). This method of activation in which integrin affinity and avidity is altered by intracellular signals that affect change at the integrin cytoplasmic tail is known as “inside-out” control. On the opposite end (literally), “outside-in” activation occurs when the β2-integrin extracellular domain interacts directly with extracellular matrix proteins or other cell surface ligands and initiates its own signaling (Williams and Solomkin, 1999, Laudanna and Alon, 2006, Nishida et al., 2006, Schymeinsky et al., 2007, Abram and Lowell, 2009, Montresor et al., 2012, Herter and Zarbock, 2013). While research efforts have only recently begun to better define β2-integrin outside-in signaling, this form of activation has been clearly linked adhesion strengthening and cell spreading.

In the current report, we examine the role of MARCKS (Myristoylated Alanine Rich C Kinase Substrate) in inside-out and outside-in activation of β2-integrins in human neutrophils. As a well-known PKC substrate and actin-binding protein, MARCKS plays a key role in integrin-dependent processes such as adhesion, cell spreading and migration in numerous cell types. In most resting cells, this 32 kDa protein is associated with the inner leaflet of the plasma membrane as a result of hydrophobic insertion of an N-terminal myristoyl-moiety and electrostatic interaction between the acidic phospholipids of the plasma membrane and a basically charged region in the middle of the protein, known as the effector domain (ED). The ED of MARCKS is subject to regulation by PKC phosphorylation or Ca++/calmodulin binding, and either of these changes to the ED displaces MARCKS from the
membrane to the cytosol. Importantly, these changes to MARCKS ED are reversible.

Following ED de-phosphorylation by cytosolic phosphatases or decreased Ca^{++}/calmodulin binding, MARCKS can re-associate with the plasma membrane (Aderem, 1992b, Hartwig et al., 1992, Blackshear, 1993, Arbuzova et al., 2002, Sundaram et al., 2004). This “on-off” association with the plasma membrane, which has been described as the “myristoyl-electrostatic switch”, is a key aspect of normal MARCKS function. Because membrane bound MARCKS ED preferentially associates with 3-4 molecules of PIP_{2} (Sundaram et al., 2004, Kalwa and Michel, 2011), it has been hypothesized that MARCKS dissociation from, and re-association with, the plasma membrane serves to regulate PIP_{2} availability in specific sub-regions of the cell’s inner leaflet. This function would indeed give MARCKS a very important job, as PIP_{2} availability, both as a substrate and as a protein binding site (i.e. those with PH-domains), has major effects on both cell signaling and function.

Another reported function of the MARCKS ED is actin binding. In 1992, Hartwig et al. presented evidence that MARCKS protein can bind to and cross-link actin in vitro, and that this effect is disrupted when the ED is either phosphorylated by PKC or bound by Ca^{++}/calmodulin (Hartwig et al., 1992). Further evidence showed that peptides analgous to MARCKS ED were able to induce actin polymerization and bundle existing actin filaments. This body of research led to a general hypothesis that MARCKS acts as a “cross-bridge”, translating extracellular signals into changes in actin organization by integrating PKC and Ca^{++}/calmodulin signaling with reversible, MARCKS-mediated, actin crosslinking (Arbuzova et al., 2002). Subsequent studies linked MARCKS to actin-dependent process including: membrane ruffling in fibroblasts, lamellipodia formation in neuron-like cells and
altered stress-fiber formation in cultured endothelial cells (Myat et al., 1997, Yamaguchi et al., 2009, Kalwa and Michel, 2011). While most MARCKS researchers agree with the characterization of MARCKS as a “link” between environmental signals and actin-dependent cellular responses, the physiologic relevance of MARCKS physical interaction with the actin network is still under debate (Arbuzova et al., 2002).

While questions remain about direct interactions between MARCKS and actin, the associations between MARCKS and cellular events that require dynamic actin reorganization are well described. MARCKS function has been examined in various cell types by a variety of methods including inhibitor peptides, transgenic expression of mutant proteins, RNAi knock-down and knock-out mice. To date, in vitro research has demonstrated a role for MARCKS protein in exo-, endo- and phagocytosis, embryogenesis, growth-cone formation, myoblast spreading and adhesion, mast cell and neutrophil degranulation, tumor cell motility, and directed migration of vascular endothelial cells, fibroblast and neutrophils (Sundaram et al., 2004, Takashi et al., 2006, Park et al., 2007, Estrada-Bernal et al., 2009, Chen and Rotenberg, 2010, Foster et al., 2010, Ott et al., 2011, Green et al., 2012, Chen et al., 2013, Ott et al., 2013, Sheats et al., 2014). In vivo research shows that MARCKS knockdown negatively affects the developing cerebral cortex and post-natal survival in mice and embryogenesis in zebrafish, that the level of MARCKS protein expression in various types of cancer is associated with rates of metastasis, and that intratracheal instillation of a MARCKS inhibitor peptide (MANS) abrogates mucin-hypersecretion and pulmonary leukocyte infiltration in a mouse model of airway inflammation. Given the obvious importance MARCKS in such a broad array of cellular and biological functions, it seems remarkable that
a cohesive understanding of the function of this ubiquitously expressed protein remains
untenable; but the diversity of cell types studies, along with the often seemingly
contradictory roles of MARCKS in adhesion and cell motility, have left those of us looking
for a unifying theory on MARCKS, wanting.

One theme of MARCKS function, to emerge as of late, is integrin regulation. Interest in
MARCKS in integrin dependent cell processes seems to have stemmed from evidence
identifying a MARCKS homolog protein, MARCKSL1 (MARCKS-like 1, MacMARCKS),
as an essential component of β2-integrin activation in macrophages (Yue et al., 2000, Zhou
and Li, 2000). Specifically, studies by Zhou et al. (2000) used a model of “inside-out” β2-
integrin activation (i.e. PMA stimulation) to demonstrate that MARCKSL1 phosphorylation
is required for releasing the cytoskeletal constraints on integrin molecules during PKC-
mediated integrin activation. More recently, studies by Disatnik et al., using myoblasts plated
on fibronectin to study “outside-in” β1-integrin activation, clearly linked α5β1-mediated PKC
activation to phospho-MARCKS dependent regulation of cell spreading and adhesion
(Disatnik et al., 2004). Finally, previous work in our lab used a MARCKS inhibitor protein,
known as MANS, to determine that MARCKS plays as essential role in β2 integrin-
dependent adhesion and migration in human neutrophils (Eckert et al., 2009). In the current
report we further examine MARCKS role in β2-integrin dependent function and signaling in
human primary neutrophils, using methods to specifically target both “inside-out” and
“outside-in” β2-integrin activation. The work presented, while preliminary, offers some
exciting “hints” regarding MARCKS role in β2-integrin regulation in human neutrophils.
Materials and Methods

Human Subjects

The human neutrophils utilized for this study were isolated from the peripheral blood of healthy, adult volunteers using the protocol approved by the Institutional Research Ethics Committee of North Carolina State University (IRB approval #616). Prior to donation, all participants provided written informed consent using the approved consent form.

Neutrophil isolation

Human neutrophils were isolated from whole blood using Ficoll gradient centrifugation of dextran-sedimented leukocyte rich plasma, as previously described. Briefly, approximately 6ml of plasma was layered on 5ml of sterile, endotoxin-free Ficoll-Paque solution and spun at 600g for 20 minutes. Neutrophils were used if they demonstrated greater than 98% viability, as determined by exclusion of trypan blue dye incorporation. Red blood cells were removed by hypotonic lysis and remaining neutrophils were washed once with HBSS. Cells were re-suspended in HBSS with 20 mM HEPES, 8.9 mM sodium bicarbonate, 1mM Ca++ and 1mM Mg++ prior to flow cytometry and biochemical assays (HBSS++) and in HBSS with 1mM Ca++, 1mM Mg++ and 2% FCS (chemotaxis buffer) prior to adhesion assays.

Fluorescence labeling of neutrophils

For adhesion experiments, isolated neutrophils (1 x 10⁷/ml in HBSS) were incubated with the fluorescent dye calcein am (Anaspec, Fremont, CA) at 2 ug/ml for 30 minutes at room temperature. Cells were then centrifuged at 1000 rpm for 8 min and resuspended in chemotaxis buffer to the appropriate final experimental concentration.
Peptide pretreatment

MANS and RNS peptides were synthesized by Genemed Synthesis, Inc. (San Francisco, CA, USA) (Singer et al., 2004). The sequence of MANS is identical to the first 24 amino acids of the human MARCKS protein: myristic acid-GAQFSKTAAKGEAAERPGEAAVA. The RNS peptide is a randomly scrambled control: myristic acid - GTAPAAEGAGAEVKRASAEAKQAF. Previous work by our laboratory has demonstrated the essential role of the MARCKS amino-terminus in both human neutrophil and NIH-3T3 fibroblast migration using the MANS peptide (Eckert et al., 2009, Ott et al., 2013). Where indicated, pretreatment of cell suspensions with indicated peptide concentrations occurred at 37°C for 30 min. Neutrophil viability (routinely ≥ 98%) was not affected by peptide treatment, as assessed by trypan blue dye exclusion.

β2-integrin inhibition

As a positive control for β2-integrin inhibition in both adhesion and respiratory burst assays, isolated equine neutrophils were pretreated with indicated concentrations of α-human CD18 F(ab’)2 (Ancell Corp, Bayport, MN) at 37°C for 30 min. Anti-human IgG1 F(ab’)2 (Ancell Corp, Bayport, MN) was used as an isotype control. The F(ab’)2 fragment, as opposed to whole antibody, was utilized to avoid unintended neutrophil Fcγ receptor crosslinking and activation.

Antibodies and reagents

Ficoll-Paque Plus and Dextran T500 were from GE Healthcare (Sweden). Bovine serum albumin (BSA), rabbit-anti-BSA antibody, dimethyl sulfoxide (Me₂SO), f-Met-Leu-Phe (fMLF), phorbol 12-myristate 13-acetate (PMA), pepstatin, HEPES and staurosporine were
from Sigma Chemical Co. (St. Louis, MO). Rottlerin, chelerythrine chloride and calphostin C were from Calbiochem (Billerica, MA). Diisopropylfluorophosphate (DFP) was from BD Biosciences (San Diego, CA). Powdered phosphate-buffered saline (PBS) and Hank's balanced salt solution (HBSS) were from Life Technologies (Grand Island, NY). Ethylenediamine tetraacetate dihydrate (EDTA) was from Fisher Scientific (Atlanta, GA). Fetal etal calf serum (FCS) was obtained from Hyclone (Logan, UT). Phospho-Syk, total Syk and β-actin primary antibodies and anti-rabbit HRP-conjugated secondary antibody were from Cell Signaling (Beverly, MA). Phycoerythrin (PE)-conjugated anti-MHC Class I (W6/32) and PE-conjugated anti-CD18 (IB4) antibodies were from Santa Cruz Biotechnologies (Dallas, TX). For detection of total CD11b a PE-conjugated anti-CD11b antibody (ICFR44) was obtained from Novus Biologicals (Littleton, CO) and PE-conjugated CBRM1/5 antibody (Biolegend, San Diego, CA) was used to detect the high affinity conformation of CD11b. PE-conjugated IgG1 and IgG2a isotype control antibodies were also from Biolegend (San Diego, CA).

**Neutrophil ROS generation**

To conduct respiratory burst experiments, isolated neutrophils were resuspended in chemotaxis buffer to a final concentration of $3.0 \times 10^6$/ml. Cells were then incubated with indicated treatments at 37°C for 30 minutes prior to each experiment and 100 uL of cells from indicated treatment groups were placed in individual wells of 5% FBS or IIC coated Immulon HB2 plates. (Plates were coated as previously described for adhesion.) For PMA-stimulated respiratory burst, cells were allowed to settle for 10 minutes prior to the addition of dihydrorhodamine-123 (DHR-123) (Sigma) (10 uM final concentration) and PMA (100
ng/ml final concentration). In the case of IIC-mediated respiratory burst, DHR-123 was added immediately following addition of cells to the well. An fMax fluorescence plate reader (Molecular Devices) was used to measure initial fluorescence (485 nm excitation, 530 nm emission) followed by a fluorescence reading every 15 minutes for 120 minutes. Results are reported as nm fluorescence. Treatment groups were tested in triplicate.

**Neutrophil adhesion**

**IIC stimulation.** For insoluble immune complex (IIC)-mediated adhesion, experimental and blank wells of Immulon2HB plates were each coated with 200 ul of 100 ug/mL BSA (Sigma) and incubated overnight at 4°C (Jones et al., 1998). The next day, wells were emptied and washed 3 times with sterile PBS, followed by the addition of 20 ug/well rabbit-anti-BSA antibody (Sigma) in 200 uL sterile PBS. (BSA coated control wells did not receive anti-BSA antibody). Plates were incubated at 37°C for 2 hours; then immediately prior to the assay the wells were emptied and washed three times with sterile PBS. 1.0x10⁵ calcein-labeled neutrophils (100 ul) from indicated treatment groups were added to individual wells and incubated at 37°C for 30 min. Treatment groups were tested in triplicate.

**Mn²⁺ stimulation.** To assess Mn²⁺-stimulated adhesion, 1.0x10⁵ calcein-labeled neutrophils (100 ul) from indicated treatment groups were allowed to settle for 10 minutes in individual wells of Immulon2HB flat bottom 96-well plates (Thermo Fischer Scientific) coated with 5% FCS in PBS. The addition of Mn²⁺ (0.5 mM final concentration) or PBS was followed by 10 minutes incubation at 37°C. Treatment groups were tested in triplicate.

**Quantification.** Following indicated incubation times, neutrophil adhesion was assessed using an fMax fluorescence plate reader (Molecular Devices). After an initial fluorescence
reading (485 nm excitation, 530 nm emission) the plates were gently dumped in a single inverted motion; the wells were filled with 150 ul sterile PBS and a second fluorescence reading was obtained. This procedure was repeated for a total of two to three washes to remove non-adhered cells. Fluorescence after each washing was divided by the initial fluorescence and multiplied by 100 to calculate percent adhesion. The first wash that demonstrated less than 10% adhesion of non-stimulated cells was considered the final result.

Flow cytometry
To avoid unintended stimulation of isolated neutrophils, hypotonic lysis of red blood cells was not performed prior to flow cytometry. Cells were pretreated as specified, stimulated with 100 nM fMLF or VC for 5 minutes, diluted with 1 volume ice cold PBS and placed on ice for 5 minutes. Cells were then spun at 1200 rpm for 5 minutes, resuspended in sterile PBS with 5% FCS and labeled with the specified primary antibodies. Flow cytometry was performed using Calibur FACScan, as previously described.

Lysate preparation and western blot assay
Prior to the experiment, 12-well tissue culture plates were coated overnight at 4°C with BSA (100 ug/mL) or fibrinogen (250 ug/mL). To generate insoluble immune complex (IIC) substrate, BSA coated wells were washed three times and recoated with anti-BSA antibody (50 ug/well) and incubated for 2 hours at 37°C. Prior to the addition of cells, all wells were washed three times with sterile PBS. Next, purified human neutrophils (5 × 10⁶ cells/ml) were suspended in HBSS++ and pretreated for 30 minutes (37°C) with MANS, RNS, anti-CD18 F(ab’)2 or media alone. 500 uL of cell suspensions were then added to individual wells of a 12-well tissue culture plate. For TNFα stimulation, final concentration was 100 ng/mL.
Plates were incubated at 37°C (5% CO2) for 30 minutes and then spun at 400 rpms for 90 seconds. The supernatant was removed by gentle aspiration and cells on the plate were lysed with 90 uL ice cold RIPA buffer [1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 5 mM sodium pyrophosphate and 50 mM sodium fluoride] containing protease inhibitors [1mM phenylmethanesulphonylfluoride (PMSF), 100μg/mL pepstatin, 1mM iodoacetamide, and 10 μg/mL aprotinin/leupeptin] for 30 minutes on ice, with agitation. After lysis, cell solutions were transferred to ependorff tubes, sonicated for 2-3 seconds and spun at 13,400 g for 10 minutes. Supernatants were collected and protein concentrations were measured using BCA Protein Assay Reagent (Pierce, Rockford, IL). Samples were stored at -80°C until analyzed. Lysate samples containing equal amounts of protein were prepared with 4X LDS sample buffer (Novex, NuPAGE®) and 10X reducing agent (Novex, NuPAGE®), heated to 70°C for 10 minutes, and analyzed by SDS-PAGE. Resolved samples were transferred to Immobilon-P PVDF membrane (Millipore, Billerica, MA) and blocked for 1 hour with 5% non-fat dry milk in tris buffered saline with Tween-20 (TBS/T; 136 μM NaCl, 20 μM Tris-base (pH: 7.6) and 0.1% Tween-20 v/v) prior to overnight incubation (4°C) with a 1:1000 dilution of phospho-Syk, total Syk or β-actin primary antibody in 5% BSA in TBS/T. Membranes were washed in TBS/T and incubated with 1:3000 dilution of anti-rabbit horseradish peroxidase (HRP) secondary antibody (Cell Signaling Technology, Danvers, MA) in 5% non-fat dry milk in TBS/T for 1 hour with gentle agitation. Membranes were washed at room temperature in TBS/T three times for 5 minutes and developed using enhanced chemiluminescence and radiograph film.
Statistical analysis

Data are reported as mean ± SEM. Data were analyzed by one way repeated measures ANOVA (Holm-Sidak multiple comparison testing) assuming equal variance, with $p < 0.05$ considered statistically significant.

Results

**MARCKS function is essential for $\beta_2$-integrin dependent respiratory burst**

The members of the $\beta_2$ integrin family play an essential role in neutrophil recruitment from the vasculature to sites of tissue infection and inflammation. Previous findings by our laboratory identified an essential role for MARCKS protein in $\beta_2$-integrin dependent processes including adhesion and migration. Based on these results, we hypothesized that MARCKS would have an essential role in other $\beta_2$-integrin dependent neutrophil processes, such as respiratory burst. Respiratory burst of isolated human neutrophils was stimulated via $\beta_2$-integrin dependent and independent methods, using insoluble immune complexes (IIC) and phorbo-myristate acetate, respectively. Intracellular respiratory burst was measured by the $\text{H}_2\text{O}_2$ mediated conversion of non-fluorescent dihydrorhodamine (DHR)-123 to the fluorescent molecule, rhodamine. Following baseline readings, fluorescence was measured every 15 minutes for 2 hours.

As shown in Figure 1, both PMA and insoluble immune-complexes stimulated robust, and sustained, respiratory burst. PMA-induced respiratory burst was independent of $\beta_2$-integrin CD18, as indicated by lack of inhibition seen with $\alpha\text{CD18 F(ab')}_2$, but was dependent on protein kinase C, as indicated by staurosporine inhibition of respiratory burst (Figure 1A). Neither MANS or RNS peptide treatment had an effect on PMA-stimulated respiratory burst
of human neutrophils. IIC-induced respiratory burst was dependent on β2-integrin CD18, as indicated by significant inhibition seen with αCD18 F(ab’)_2, as well as PKC. Importantly, IIC-stimulated respiratory burst was also significantly inhibited by MANS peptide, while the RNS peptide had no effect. From this result we are able to conclude that MANS treatment does not hinder the assembly and function of the NADPH oxidase complex. Furthermore, because the pan-PKC inhibitor staurosporine blocked both types of respiratory burst, we know that PKC is required for neutrophils to undergo successful respiratory burst. Therefore, we suggest that at least one aspect of MARCKS function, which is inhibited by the MANS peptide, must be essential for IIC-stimulated respiratory burst between the level of FcγR-signaling and PKC activation.

“Inside-out” activation of neutrophil β2-integrins is not affected by MARCKS inhibition

Neutrophils within the vasculature are non-adherent, expressing low numbers of surface β2-integrins. Upon stimulation with 100 nM fMLF the number of β2-integrins on the cell surface (CD18 and CD11b) increase significantly. As shown in Figures 2A and 2D, 100 nM fMLF caused a 2- to 3-fold increase in CD18 and CD11b, respectively, as measured by flow cytometry. The measured increase in CD18 and CD11b surface expression was not affected by treatment with MANS or RNS peptide (Figures 2A and 2D). This is consistent with previous results published by Eckert et al. (2009). Calcium chelation with EDTA (10 mM) was used as a positive control for inhibition.

In addition to increasing the number of surface β2-integrins, 100 nM fMLF also causes a significant increase in the affinity of β2-integrins (CD11b) on the surface of neutrophils. Upon stimulation of isolated human neutrophils with 100 nM fMLF there was an average
2.5-fold increase in CBRM1/5 antibody binding. This antibody recognizes the exposed binding epitope of CD11b. The measured increase in CBRM1/5 binding indicates a significant increase in “high-affinity” Mac-1 on the surface of fMLF stimulated human neutrophils. The fMLF-induced increase in β2-integrin affinity was not affected by treatment with MANS or RNS peptide (Figure 2F). Calcium chelation with EDTA (10 mM) was used as a positive control for inhibition.

“Outside-in” activation of neutrophil β2-integrins may require MARCKS protein

a. Mn++ fails to rescue adhesion of MANS treated neutrophils

Prior to neutrophil stimulation, integrins are maintained in an inactive or ‘bent’ conformation, which effectively masks the N-terminal ligand binding site known as the I-domain. In order for β2-integrin to engage cellular- or substrate-ligands, the molecule undergoes a conformational change from a bent to extended form, which unmasksthe binding site at the I-domain. In this extended form the “affinity” of monovalent protein/ligand interactions is increased. Neutrophil stimulation with fMLF, as used in the preceeding experiments, induces the extended or “high-affinity” form of β2-integrins through “inside-out” signaling. Exposure of neutrophils to manganese cations (Mn++) has also been shown to cause an increase in β2-integrin affinity. Because Mn++ treatment acts directly on the extracellular domain of the integrins, and does not rely on an intracellular signaling cascade, it can be used to differentiate between mediators that are essential to “inside-out” versus “outside-in” β2-integrin activation. In the following series of adhesion experiments, we utilized MnCl2 treatment to further investigate a role for MARCKS in β2-integrin activation.
As shown in Figure 3A, sustained adhesion of isolated human neutrophils to insoluble immune complex (IIC) substrate is dependent on β2-integrins and is effectively blocked by anti-CD18 (IB4) and anti-CD11b (ICRF44) antibodies. While the addition of Mn++ is able to “rescue” adhesion of neutrophils treated with β2-integrin blocking antibodies, it was unable to recover adhesion of MANS treated cells to IIC (Figure 3A). As previously demonstrated by Jones et al., IIC stimulated “inside-out” activation of β2-integrins is dependent on PI3K. This is shown in Figure 3B by wortmannin inhibition of neutrophil adhesion to IIC. Because Mn++ activates integrins from the “outside-in”, treatment with 0.5 mM MnCl₂ is able to partially rescue adhesion of wortmannin treated cells to IIC substrate. In this separate set of experiments, Mn++ again had no effect on increasing adhesion of MANS treated cells (Figure 3B). Finally, we investigated the role of various PKC subclasses in Mn++ stimulated adhesion of isolated human neutrophils. Calphostin C, an inhibitor of classical PKC isoforms, had no effect on Mn++ stimulated neutrophil adhesion. Rottlerin, a novel PKC inhibitor, only partially abrogated Mn++ induced neutrophil adhesion. Chelerythrine chloride, which inhibits atypical PKCs, completely blocked Mn++ mediated adhesion of neutrophils. The inhibitory effect of chelerythrine chloride were similar to results seen with MANS peptide treatment (Figure 3C), suggesting that both MARCKS and an atypical isoform of PKC are necessary for “outside-in” β2-integrin activation in human neutrophils. RNS peptide treatment had no effect on Mn++ adhesion in any of the experimental conditions.

b. MANS treatment inhibits integrin-mediated Syk phosphorylation in neutrophils

Neutrophil β2-integrins play a vital role as signaling molecules, mediating numerous cellular processes including adhesion, migration, phagocytosis and respiratory burst; and the
signaling pathways subject to integrin regulation are the focus of ongoing research.

Activation of the non-receptor spleen tyrosine kinase (Syk) by immunoreceptor tyrosine activating motif (ITAM), downstream of Src-family kinases, has been identified as an initial signaling event triggered by β2-integrin “outside-in” activation (Dib, 2000). Syk is also activated by other neutrophil cell surface receptors that utilize ITAM signaling, including FcRs and selectins (Futosi et al., 2013). Using biochemical analysis, we investigated the effect of MARCKS inhibition (with the MANS peptide) on Syk activation in adherent neutrophils. Previous experiments in our lab established that MARCKS inhibition with the MANS peptide abolishes human neutrophil adhesion to insoluble immune complexes; but the molecular details of MANS interference with neutrophil adhesion remain uncertain. FcRγ crosslinking by IgG is known to lead to Syk activation in neutrophils. As expected, we observed strong Syk activation in human neutrophils plated on IIC. The level of Syk activation, as measured by a phospho-Syk specific antibody, did not appear affected by neutrophil pretreatment with either MANS or RNS peptides. Neutrophils were pretreated with αCD18 F(ab’)2 (30 ug/mL) in order to demonstrate the level of Syk phosphorylation mediated by FcγR-IgG binding, as opposed to β2-integrin mediated Syk activation. From this experiment we conclude that MARCKS inhibition does not interfere with FcγR signaling to the level of Syk activation.

Next we investigated Syk activation in neutrophils adherent to fibrinogen (250 ug/mL), with and without TNFα stimulation. TNFα stimulation is known to lead to Syk activation in both hematopoietic and non-hematopoietic cells (Takada et al., 2007). As shown in Figure 4B, Syk phosphorylation was detected in TNFα-stimulated neutrophils. We did see a slight
decrease in Syk phosphorylation in neutrophils pretreated with αCD18 F(ab’)². Treatment with MANS or RNS peptides appeared to have no effect on TNF-receptor mediated Syk activation. Consistent with previous reports, we also observed Syk activation, albeit relatively weak, in unstimulated neutrophils plated on fibrinogen (Figure 4B) (Willeke et al., 2003b). Interestingly, in this preliminary experiment, MANS peptide pretreatment completely inhibited Syk activation in this model of “outside-in” integrin signaling. We saw no effect of CD18 blocking F(ab’)² or RNS peptide treatment on Syk phosphorylation in fibrinogen plated neutrophils. From these results we conclude that MANS peptide treatment has no effect on Fcγ- or TNF-receptor signaling to the level of Syk phosphorylation. We did observe that inhibition of MARCKS with the MANS peptide inhibited Syk activation in this static adhesion model of “outside-in” integrin activation, suggesting that MARCKS may play a role in “outside-in” integrin signaling. This hypothesis will require further investigation.

**MARCKS may play a role in neutrophil adhesion beyond β²-integrin function**

While β²-integrins are essential for neutrophils to sustain adhesion, the initial adhesion (< 10 minutes) of neutrophils to a substrate of IIC is not dependent on β²-integrin expression, number or affinity (Graham et al., 1993, Jones et al., 1998). Our previous experiments clearly show that the MANS peptide inhibits adhesion of neutrophils to IIC, but the 30 minutes time point used for assessment of adhesion in these prior studies was only indicative of sustained adhesion. In this final experiment, we investigate the effects of MANS treatment on neutrophil adhesion to IIC at three different time points: 7, 12 and 30 minutes. Consistent with findings by Jones et al., our results show that initial adhesion (7 minutes) of neutrophils to IIC was only partially inhibited by CD18 function blocking F(ab’)² (IB4) and that maximal
inhibition with IB4 treatment was obtained at 30 minutes (Figure 5). In contrast, MANS peptide treatment completely blocked neutrophil adhesion to IIC at all time points (Figure 5) while the RNS peptide had no effect. This result suggests that MANS mediated MARCKS inhibition is affecting function or signaling that is essential to initial neutrophil adhesion to IIC, which may or may not be related to regulation of β2-integrins.

Discussion

Endothelial cells are activated during inflammation by inflammatory cytokines, causing them to express adhesion molecules and present chemokines and chemoattractants on their luminal surface. These activated endothelial cells make passing neutrophils aware of proximate tissue trauma or infection and signal them to begin the process of transmigration. The series of steps involved in neutrophil extravasation from blood into tissues includes tethering, rolling, adhesion, crawling and transmigration. The molecular regulators of this process are numerous and have been reviewed in detail (Ley et al., 2007), but of particular significance is the role of β2-integrins. Beta2-integrins LFA-1 (CD11a/CD18 or αLβ2) and MAC-1 (CD11b/CD18 or αMβ2) are essential for neutrophil adhesion and intraluminal crawling, respectively (Schenkel et al., 2004, Phillipson et al., 2006). Without successful endothelial adhesion and transmigration, neutrophils are unable to enter sites of tissue infection or inflammation, where the release of their arsenal of matrix degrading enzymes and reactive oxygen species takes place (Nathan et al., 1989, Nathan, 2002, Tong et al., 2013). Indeed, without functional β2-integrins (as occurs with leukocyte adhesion deficiency or LAD) the host experiences recurrent or chronic infections which result in significant morbidity and even mortality. Unfortunately, conditions also exist in which neutrophil responses that are
excessive or dysregulated are actually damaging to the host, rather than beneficial.

Neutrophil-mediated tissue injury has been implicated in a number of diseases including acute lung injury, sepsis, rheumatoid arthritis, ischemia-reperfusion injury, myocardial infarction, ulcerative colitis and chronic bronchitis (Segel et al., 2011, Wong et al., 2012, Schmidt et al., 2013, Grailer et al., 2014, Mondrinos et al., 2014, Wang et al., 2014). In fact, inhibition of β2-integrin has proven beneficial for protecting hosts from neutrophil mediated tissue injury in several animal models of both acute and chronic inflammation (Nathan, 2002). Clearly, the drawback of generalized β2-integrin inhibition is immunosuppression of the host’s normal innate immune response (Wang et al., 2014), and this has hindered the use of β2-integrins as a therapeutic target. But with a more thorough understanding of β2-integrin signaling and function at the molecular level, and recent advances in cell-specific delivery of therapeutics (Wang et al., 2014), we believe there is significant potential for the discovery of novel and clinically beneficial targets for new anti-inflammatory therapies that would benefit patients with neutrophil mediated disease.

We believe one such potential target is the actin binding MARCKS (Myristoylated Alanine Rich C Kinase Substrate). As a ubiquitously expressed protein, MARCKS role in integrin dependent cellular processes including adhesion, cell spreading, and membrane directed vesicular transport and migration has been investigated in numerous cell types (see Introduction). Of particular interest, recent studies of cells with motile behavior (i.e. melanoma, nerve growth cones) have identified a PKC-MARCKS dependent pathway in which un-phosphorylated, membrane bound MARCKS co-localizes with, and stabilizes, integrin-mediated dynamic adhesions (Gatlin et al., 2006, Estrada-Bernal et al., 2009).
Further, these studies demonstrated that an alteration in PKC-dependent MARCKS translocation between the membrane and the cytosol (i.e. MARCKS silencing or overexpression) significantly disrupts migratory responses of these cells to their relevant stimuli. An important role for MARCKS in the inflammatory response of neutrophils was first suggested by Thelen et al. (1990), who demonstrated that neutrophil priming agents TNFα and LPS induced marked synthesis of MARCKS protein, which is rapidly phosphorylated by neutrophil chemotactic agonists (Thelen et al., 1990). Previous data from our lab provided further evidence for the importance of MARCKS function in neutrophils by demonstrating that peptide specific inhibition of MARCKS (with MANS) significantly reduced β2-integrin mediated neutrophil migration in response to fMLF, LTB4 and IL-8, as well as PMA- and immune complex-mediated neutrophil adhesion (Ecker et al., 2009). The purpose of the current study was to further investigate the effect of MARCKS inhibition on in vitro β2-integrin activation and signaling in human neutrophils.

One mechanism used to study β2-integrins in neutrophils is to measure functional responses that require β2-integrin activation, such as respiratory burst. Based on our previous results, it was not surprising that we observed inhibition of β2-integrin dependent respiratory burst in neutrophils treated with the MARCKS inhibitor peptide, MANS (Figure 1B). It is noteworthy, however, that β2-integrin independent respiratory burst, stimulated by PMA, was not affected by MARCKS inhibition (Figure 1A). This result shows that MANS inhibition of respiratory burst is not due to the cells inability to assemble a functional NADPH complex.

Furthermore, treatment with staurosporine, a pan-PKC inhibitor, confirmed that both IIC- and PMA-stimulated neutrophil respiratory burst relied on signaling by PKC. Because
MANS inhibited IIC-mediated respiratory burst, but was unable to inhibit PMA-mediated respiratory burst, we conclude that MARCKS role in respiratory burst regulation must be upstream of PKC. This is somewhat surprising since MARCKS is a known PKC substrate and would therefore be expected to be downstream of PKC activation. To further confuse the narrative, previous results from our laboratory have demonstrated that PMA-induced adhesion of human neutrophils is inhibited by MANS peptide. As PMA is a synthetic analog of DAG, it essentially activates the cell at the level of PKC. Because MANS can block PMA-mediated adhesion, this would seem to place MARCKS function in β2-integrin dependent adhesion downstream of PKC. There are at least two potential explanations for this apparent contradiction: 1) the signaling pathway for respiratory burst initiated by immune complexes, which are a physiologic stimulant, is different from the respiratory burst induced by the non-physiologic stimulant PMA, or 2) MARCKS could be downstream of a PKC isoenzyme specifically activated by ligation of immune complexes, but upstream of a different PKC isoenzyme involved in a respiratory burst signaling pathway common for both IC and PMA-stimulation. A third potential explanation is that MARCKS is involved in a feedback loop of PKC signaling in which PKC is initially activated in discrete, subcellular locations where it phosphorylates MARCKS, MARCKS then releases the plasma membrane and translocates to the cytosol, which causes changes in cytoskeletal organization and PIP2 signaling that further activate PKC, continuing the phosphorylation of MARCKS. With conflicting evidence for the role of multiple isoforms of PKC in various neutrophil functions (Merritt et al., 1997, Pongracz and Lord, 1998), this is clearly an area that requires further study.
In order to study β2-integrin activation, we stimulated respiratory burst of isolated human neutrophils with a substrate of adhered rabbit anti-BSA antibodies. To verify that this form of respiratory burst was β2-integrin dependent, neutrophils were pretreated with 30 ug/mL αCD18 F(ab’)² for 30 minutes at 37°C. While αCD18 F(ab’)² treatment did significantly attenuate IC-mediated respiratory burst in our study (Figure 1B), it was not completely inhibited. This finding is potentially explained by previous reports that differentiate between the roles of FcγRII and FcγRIII in the immune complex stimulated respiratory burst of neutrophils (Zhou and Brown, 1994, Fossati et al., 2002). As these studies demonstrate, immune complex mediated respiratory burst shows different requirements for FcγRII and FcγRIII receptors depending on whether the immune complexes are soluble or insoluble (Fossati et al., 2002). Insoluble immune complex-mediated respiratory burst requires the cytoskeletal dependent cooperation of β2-integrins (CD11b has been specifically investigated) and FcγRIII, in addition to intact FcγRII signaling (Zhou and Brown, 1994, Fossati et al., 2002). In contrast, soluble immune complexes are able to stimulate FcγRII-mediated respiratory burst (Fossati et al., 2002), which is independent of both FcγRIII and β2-integrin ligation (Zhou and Brown, 1994). It is possible that soluble immune complexes were present in our assay and this led to the activation of intracellular H₂O₂ production through FcγRII. It is therefore potentially noteworthy that MANS peptide treatment had a more pronounced effect on immune complex mediated respiratory burst than did αCD18 F(ab’)². This observation potentially lends itself to the hypothesis that MARCKS plays a role in neutrophil signaling beyond the regulation of β2-integrin dependent functions. On the other hand, it is also possible that treatment with 30 ug/mL αCD18F(ab’)² was simply insufficient
to completely block insoluble immune complex stimulation of FcγRIII and some amount of β2-integrin dependent respiratory burst, completely blocked by 50 uM MANS peptide, was able to proceed. Higher concentrations of αCD18 F(ab’)2 pretreatment did not seem necessary based on a review of the previous literature, but follow up studies using higher treatment concentrations would be one approach to resolving this question.

To continue our study of MARCKS role in β2-integrin activation we next utilized flow cytometry to study the induction of increased β2-integrin surface expression and affinities, which are initial steps in β2-integrin “inside-out” activation. As covered in the Introduction, “inside-out” activation describes the process whereby intracellular signals: 1) induce changes at the β2-integrin cytoplasmic tail that ‘extends’ the conformation of the extracellular domain and exposes the N-terminal ligand-binding site (affinity) and 2) result in cytoskeletal relaxation such that β2-integrin mobility within the plasma membrane facilitates assembly of integrin clusters and an increased number of protein-ligand interactions (avidity/valency).

Exposure of neutrophils to soluble chemoattractants, such as fMLF, also increases the number of β2-integrins on the cell surface (Hughes et al., 1992, Berger et al., 2014). We chose fMLF stimulation as our model of GPCR mediated “inside-out” activation because previous studies by Eckert et al. demonstrated that MARCKS inhibition with the MANS peptide significantly attenuated fMLF induced migration and adhesion of human neutrophils in vitro (Eckert et al., 2009). As shown in Figure 3, we saw no significant effect of MANS or RNS peptide treatment on upregulation of total CD18 (IB4) or total CD11b (ICFR44) following 100 nM fMFL stimulation of human neutrophils. Neither did we see an effect of MANS or RNS peptide treatment on increased binding of antibody CBRM1/5, which is used
to detect the high affinity conformation of CD11b. In fact, there was even a slight trend towards an increase in CBRM1/5 binding in MANS treated cells, but with an “n” of 3, this result was not significant. These results were consistent, whether or not neutrophils were paraformaldehyde fixed.

Based on these findings, we conclude that MARCKS function is not required for fMLF induced upregulation of β₂-integrin CD11bCD18 (also known as Mac-1 or α₅β₂), or “inside-out” β₂-integrin activation to the level of conformation change. Our findings in regards to MANS effect on β₂-integrin upregulation are consistent with those already published (Eckert et al., 2009). What clues in regards to MARCKS function can be gleaned from this result? As previous investigations have shown, upregulation of surface β₂-integrins upon neutrophil activation primarily results from fusion of neutrophil secretory vesicles (which have large stores of β₂-integrins incorporated into their vesicle membrane) with the cell’s plasma membrane (Borregaard et al., 1995, Borregaard and Cowland, 1997, Lacy, 2006, Lacy and Eitzen, 2008). This transformation of resting neutrophils to β₂-integrin presenting cells is a key event in neutrophil-endothelial adhesion, allowing neutrophils to transition from tethering and rolling to arrest, adhesion strengthening and crawling (Herter and Zarbock, 2013). Mobilization of secretory vesicles therefore happens very rapidly in response to both neutrophil selectin binding and exposure to inflammatory mediators. Fitting with their role in this rapid response, neutrophil secretory vesicles are mobilized by nanomolar concentrations of inflammatory mediators that have little effect on exocytosis of neutrophil granules (Sengelov et al., 1993, Borregaard et al., 1995). The signal for mobilization is regulated, at least in part, by changes in cytosolic Ca²⁺. As shown in early studies by Sengelov et al.
(1993), rising levels of cytosolic Ca\(^{++}\) are tightly coupled to the hierarchical release of neutrophil secretory vesicles, gelatinase granules, specific granules, and azurophil granules (in that order); and elimination of Ca\(^{++}\) from the cell media effectively prevents the mobilization of secretory vesicles (Sengelov et al., 1993) (as we saw with EDTA treatment of fMFL stimulated neutrophils). Unfortunately, the protein targets involved in Ca\(^{++}\) mediated mobilization of secretory vesicles remain unclear (Lacy, 2006), but it would appear from our findings that MARCKS function is not required for exocytosis of neutrophil secretory vesicles. How then, do we resolve this result with previous evidence that MARCKS function is required for exocytosis of mucin granules from human bronchial epithelial cells (Singer et al., 2004, Li et al., 2013), as well as neutrophil degranulation as evidenced by release of myeloperoxidase (Takashi et al., 2006)?

An important distinction between the process of secretory vesicle exocytosis and degranulation, relevant to the discussion of possible MARCKS functions, is the role of the actin cytoskeleton. As reviewed by Porat-Shliom et al. (2014) (Porat-Shliom et al., 2013), there is seemingly conflicting evidence for the role of the actin cytoskeleton in regulated exocytosis. In some cases, it would seem that actin acts as a barrier to exocytosis that must be removed in order for small, swiftly mobilized vesicles to access the plasma membrane; while in other situations/cell types, it appears that the actin cytoskeleton plays a more active role in directing the movement and docking of large, cargo-laden granules (i.e. mucin containing granules, myeloperoxidase containing primary granules) with the cell’s plasma membrane (Porat-Shliom et al., 2013). In terms of actin regulation, it may be that the initial, rapid (< 30 sec) dissociation of MARCKS from the plasma membrane is all that is required to disrupt the
cortical actin network and allow secretory vesicle fusion in neutrophils. It would make sense then that this process is unaffected by MANS, as treatment with this peptide actually promotes MARCKS dislocation from the membrane to the cytosol even in unstimulated cells (Eckert et al., 2009). What MANS may interfere with though, through competitive inhibition of MARCKS N-terminus, is the association of MARCKS protein with membrane specific binding sites, including those on granule membranes (Singer et al., 2004). This would prohibit the actin binding protein from physically linking granules, such as those containing mucin, to active cytoskeletal regulation. MARCKS role in vesicle exocytosis and degranulation in numerous cell types is an ongoing area of active research.

Of final consideration in terms of “inside-out” β2-integrin activation, we must point out that difference in activation signals exist, depending upon the type of stimulation. For example, immune complex induced signaling that leads to increased affinity of CD11b is dependent on both PI3K and the actin cytoskeleton; while fMLF induced activation signaling is not dependent on PI3K or actin (Jones et al., 1998). With these facts in mind, it will be necessary to investigate other stimulators of β2-integrin dependent neutrophil functions before offering final conclusions on MARCKS role, or lack there of, in “inside-out” β2-integrin activation.

The signaling steps that occur as a result of β2-integrin-ligand binding in neutrophils induces rearrangement of the actin cytoskeleton that is required for firm cell adherence and spreading, as well as phagocytosis and respiratory burst (Abram and Lowell, 2009). This is referred to as “outside-in” activation. Binding of integrins to ligand in response to soluble stimuli is dependent on the presence of Mg^{++} cation (Lundgren-Akerlund et al., 1992), but binding can also be induced by treatment of neutrophils with MnCl$_2$ (which dissociates to
Mn\(^{++}\) in aqueous solution). The effect of these cations (not Ca\(^{++}\)) on β2-integrin affinity is exerted through binding at a divalent metal binding motif called the “metal ion-dependent adhesion site” (MIDAS) present on the extracellular integrin I-domain. This binding induces a conformational change that exposes a ligand binding epitope (Michishita et al., 1993, Rabb et al., 1993, Oxvig et al., 1999, Wagner and Roth, 2000). Because Mn\(^{++}\) only activates neutrophils extracellularly, it is commonly used by investigators to study “outside-in” integrin signaling (Berton et al., 1994, Berton et al., 2005). Mn\(^{++}\) activation of β2-integrin affinity and clustering is sufficient to induce neutrophil spreading and adhesion to fibrinogen (Berton et al., 2005, Chakrabarti and Patel, 2008) even in the absence of other stimuli. Impaired immune complex adhesion of neutrophils treated with β2-integrin blocking antibodies can be “rescued” by the addition of 0.5 mM MnCl\(_2\), which acts at the extracellular level to induce the high affinity conformation and clustering of β2-integrins, along with subsequent ligand engagement (Jones et al., 1998, Williams and Solomkin, 1999, Chakrabarti and Patel, 2008). This was verified by our current study, which showed that Mn\(^{++}\) treatment not only recovered immune complex mediated adhesion of IB4 and CBRM1/5 treated neutrophils, it enhanced the adhesion of neutrophils in control groups (Control, IgG1 and RNS). Mn\(^{++}\) was also able to partially rescue immune complex adhesion of wortmannin treated neutrophils, which fail to adhere due to inhibition of PI3-kinase (Jones et al., 1998). Interestingly, Mn\(^{++}\) failed to restore IIC-mediated adhesion of neutrophils treated with the MANS peptide. This result is particularly significant because it informs us that even when β2-integrins are forced into a high affinity conformation (by Mn\(^{++}\)), MANS peptide prevents adhesion.
To gain further insight on MARCKS role in “outside-in” signaling from β₂-integrins, we stimulated neutrophil adhesion to a substrate of 5% FCS with Mn²⁺ treatment only, in the presence of MANS or RNS peptides, as well as calphostin C, rottlerin and chelerythrine chloride, inhibitors of classical, novel and atypical PKCs, respectively. PKC isotypes δ and ζ have previously been implicated in neutrophil adhesion and migration (Kent et al., 1996) (Sheats and Sung, unpublished data – see Chapter 2), but because these studies utilized static adhesion assays initiated by GPCR signaling (i.e. fMLF, LTB₄, IL8), it remains unclear whether regulation by these PKC isoforms is located upstream or downstream of neutrophil β₂-integrin activation (Bertram and Ley, 2011). In order to isolate β₂-integrin activation, we treated neutrophils with 0.5 mM Mn²⁺ and measured adhesion to 5% FCS. Not surprisingly, we found that MANS peptide treatment completely abrogated Mn²⁺ induced adhesion of human neutrophils. In agreement with a previous report (Chakrabarti and Patel, 2008), we also found that calphostin C mediated inhibition of classical and novel PKCs had no effect on Mn²⁺ induced neutrophil adhesion, while inhibition of atypical PKCs with chelerythrine chloride did significantly inhibit Mn²⁺ stimulated neutrophil adhesion. The results of this study provide evidence of similar roles for an atypical PKC isotype and MARCKS in β₂-integrin “outside-in” signaling/function. In light of this finding, it is tempting to propose that MARCKS, which is a substrate of PKC, could be regulated by an atypical PKC isotype in human neutrophils. As compelling as this conclusion would be, we have previously demonstrated that the novel PKC isotype δ, and not the atypical PKC isotype ζ, is responsible for fMLF-stimulated MARCKS phosphorylation in human neutrophils (Sheats and Sung, unpublished data – see Chapter 2). Interestingly, results from the current study show that
inhibition of δ-PKC (rottlerin) did attenuate Mn\textsuperscript{++} induced neutrophil adhesion. This is in contrast to the findings by Chakrabarti et al. (2008) who report no effect of novel PKC inhibition on MnCl\textsubscript{2} induced neutrophil adhesion, but their study did not investigate the effects of rottlerin. From this preliminary investigation, we conclude that δ-PKC, ζ-PKC and MARCKS are likely to be involved in β\textsubscript{2}-integrin “outside-in” signaling. Follow up studies will look closely at the effects of specific δ-PKC, ζ-PKC and MARCKS peptide inhibitors on proximal signaling events in β\textsubscript{2}-integrin “outside-in” signaling including activation of Src-family kinases Hck and Fgr, the spleen tyrosine kinase Syk and the downstream target of Syk activation, Pyk2.

The most well described proximal signaling events of “outside-in” β\textsubscript{2}-integrin activation in neutrophils are activation of the non-receptor tyrosine kinases of the Src-family (Hck and Fgr), as well as the spleen tyrosine kinase, Syk. Initial studies implicated a role for these cellular proteins by demonstrating robust tyrosine phosphorylation that was essential for adhesion induced cellular responses (Fuortes et al., 1993, Yan and Berton, 1998, Willeke et al., 2000, Willeke et al., 2003b, Laudanna and Alon, 2006, Schymeinsky et al., 2007). Also significant, genetic deficiencies of these kinases in mice led to physiologic defects in neutrophil functions (i.e. respiratory burst, degranulation, firm adhesion) during β\textsubscript{2}-integrin mediated cellular activation (Mocsai et al., 1999, Mocsai et al., 2002, Mocsai et al., 2010, Lowell, 2011). Syk phosphorylation and activation upon β\textsubscript{2}-integrin mediated adhesion of neutrophils (Mocsai et al., 2002, Willeke et al., 2003a) is believed to be mediated by an immunoreceptor tyrosine based activation (ITAM) motif, which is the mechanism of Syk phosphorylation mediated by other receptor types such as lymphocyte antigen receptors (B-
and T-cells) and immunoglobulin Fc-receptors (Schymeinsky et al., 2007). When we investigated neutrophil activation by insoluble immune complexes, we determined that MARCKS inhibition (with the MANS peptide) had no effect on FcγR-mediated Syk phosphorylation. This finding supports the assertion that MANS inhibition of IC-mediated neutrophil adhesion and respiratory burst is mediated through β2-integrin-, rather than FcγR-, dependent signaling.

In order to isolate β2-integrin mediated, “outside-in” Syk activation, we next plated neutrophils on fibrinogen without additional stimulation. This method of Syk activation was verified in a previous study by Willeke et al., who showed that adhesion of human neutrophils to immobilized fibrinogen resulted in Syk phosphorylation and co-precipitation with CD18 (Willeke et al., 2003b). Interestingly, unlike results seen with IC- and TNFα-stimulation, Syk activation initiated by neutrophil exposure to immobilized fibrinogen was completely inhibited by MANS peptide. However, our results also indicate that fibrinogen is not a β2-integrin specific substrate, because pretreatment of neutrophils with αCD18 F(ab’)_2 did not inhibit Syk phosphorylation in neutrophils plated on immobilized fibrinogen. While fibrinogen is generally regarded as a β2-integrin specific substrate for neutrophils, there are reports of β3 and β1 (both of which are present in neutrophils) fibrinogen binding as well (Werr et al., 1998, Werr et al., 2000, Siskova et al., 2006, Jakus et al., 2007, Takada et al., 2007). While these interactions are generally dismissed as inconsequential, it is incumbent upon us to consider that MARCKS function may be required not only for β2-integrin “outside-in” signaling, but β3- and/or β1-signaling as well. Future studies will employ the use of β1 and β3 specific blocking antibodies, in addition to β2, to determine which integrin
receptors are involved in activation of Syk subsequent to neutrophil adhesion to fibrinogen. Because MANS peptide treatment completely blocked Syk phosphorylation in neutrophils exposed to fibrinogen, these future studies will offer key insights on the role of MARCKS in neutrophil integrin signaling.

With preliminary evidence suggesting a role for MARCKS protein in non-β2-integrin dependent Syk phosphorylation, we wanted to further investigate the effect of MARCKS inhibition on other β2-integrin independent neutrophil functions. To that end, we examined the effect of MANS treatment on the two phases of neutrophil adhesion to immune complex coated surfaces. As shown by Graham et al. (1993), initial adhesion (< 10 minutes) of human neutrophils to IIC does not require β2-integrins, while sustained immune complex adhesion of neutrophils is β2-integrin dependent (Graham et al., 1993). In our experiment, αCD18 F(ab’)2 did partially inhibit the “initial” phase of neutrophil adhesion to immune complex; however, the level of inhibition was not nearly as marked as that seen with MANS peptide treatment. Indeed, there appeared to be no difference in adhesion of MANS treated cells at any of the three time points and MANS treated cells displayed even less adhesion than that of unstimulated cells (control) exposed to a substrate of BSA. In both fMLF-mediated adhesion of human neutrophils (Eckert et al., 2009) and LTB4-mediated adhesion of equine neutrophils (Sheats et al., 2014), MANS peptide has demonstrated significant inhibition of rapid (<3 minutes) adhesion even without pretreatment. Although MANS peptide treatment has no demonstrated effect on fMLF or PMA mediated MARCKS phosphorylation (Park et al., 2007, Eckert et al., 2009), it is interesting that MANS immediate inhibition of neutrophil migration and adhesion mirrors kinetics of MARCKS phosphorylation, which is extremely
rapid (~30 seconds) following neutrophil stimulation (Thelen et al., 1991, Eckert et al., 2009)(Sheats and Sung, unpublished data). Following initial phosphorylation, which displaces MARCKS from the plasma membrane to the cytosol, cytosolic phosphatases dephosphorylate MARCKS (~3 minutes), allowing MARCKS to reassociate with the plasma membrane. Previous studies by Disatnik et al. clearly demonstrate the importance of MARCKS reversible association with the plasma membrane to the series of cytoskeletal changes that determine the initial stages of cell adhesion as well as the later stages of cell spreading (Disatnik et al., 2004). More recently, MARCKS PKC-dependent cycling between the membrane and the cytosol has also been linked to the regulation of integrin dependent dynamic adhesions in highly motile cell types (Gatlin et al., 2006, Estrada-Bernal et al., 2009). While the MANS peptide has no known effect on MARCKS phosphorylation in neutrophils, it has been shown to displace MARCKS from the plasma membrane to the cytosol. It has been hypothesized that the MANS peptide, identical to the N-terminal 24 amino acids of MARCKS, could interfere with MARCKS membrane association (Singer et al., 2004, Eckert et al., 2009). Additional studies will be required to determine if MANS-mediated disruption of MARCKS association with the plasma membrane could offer an explanation for MANS inhibition of β2-integrin dependent and independent phases of IIC-mediated neutrophil adhesion.

One aspect of neutrophil β2-integrin activation we have yet to investigate, in terms of MARCKS function, is the cytoskeletal dependent process of increased lateral mobilization and cluster formation, referred to as avidity. An increase in avidity enhances the overall binding strength of the cell in at least two ways: 1. integrin mobilization increases the
potential number of integrin-ligand interaction and 2. mobility leads to the formation of clusters of integrins in the membrane, which initiate important “outside-in” signaling that directs numerous cellular functions (Abram and Lowell, 2009). As previously mentioned, avidity in neutrophils, and leukocytes in general, is a cytoskeletal dependent process. In resting leukocytes, $\beta_2$-integrins are located diffusely across the membrane and impeded from lateral movement by cytoskeletal constraints (Kucik et al., 1996, Stewart and Hogg, 1996, Jin and Li, 2002). Activation of PKC, either as a component of receptor signaling, or directly through phorbol esters, causes relaxation of the cytoskeleton and increases integrin diffusion within the membrane (Zhou and Li, 2000, Zhou et al., 2001). Increased lateral mobility of integrins can also be induced by leukocyte treatment with low concentrations of the depolymerizing agent cytochalasin D, providing evidence for the role cytoskeletal involvement (Kucik et al., 1996). As an abundant PKC substrate that also binds actin as well as the plasma membrane, MARCKS seems highly qualified to regulate PKC-dependent cytoskeletal constraints on integrin molecules. Indeed, direct evidence indicates that PKC phosphorylation of the MARCKS homolog protein, MARCKSL1 (expressed primarily in macrophages) is required for releasing cytoskeletal constraint on integrin molecules in macrophages (Zhou and Li, 2000). MARCKSL1 is also functionally relevant for macrophages, as siRNA induced downregulation significantly inhibited chemotaxis of the macrophage cell line RAW264.7 cells (Chun et al., 2009). Given the high level of similarity between MARCKS and MARCKSL1 (Aderem, 1992a), in sequence, structure and binding partners, an investigation of MARCKS role in the lateral mobility and cluster formation of $\beta_2$-integrins in neutrophils is clearly indicated. Attempts to examine the formation of integrin
surface clusters in MANS treated cells were made with the use of immunofluorescent-antibody staining and confocal microscopy, but the washing of coated coverslips removed all of the MANS treated cells (which fail to adhere) and prohibited any conclusion (unpublished data). In future studies, we plan to utilize imaging flow-cytometry to compare induced β2-integrin cluster formation, with and without MARCKS inhibition.

Conclusions

Over the past two decades we have been presented with evidence that β2-integrins are not only targets of intracellular signals that direct neutrophil functions, but are themselves, essential regulators of neutrophil adhesion, migration, phagocytosis, respiratory burst and apoptosis. This bi-directional nature of integrins as both “effecters” and “affecters” of cell function is often divided into “inside-out” and “outside-in” signaling, respectively. While conceptually it is reasonable to draw the distinction between these two methods of activation, practically and technically speaking it is actually quite difficult to examine these processes in isolation. Differentiating between these two mechanisms of integrin activation is further complicated by the presence of alternate and/or parallel signaling pathways (depending on the method of cell activation), as well as the fact that many molecules (i.e. PKCs, PI3K) appear to be involved in both “inside-out” and “outside-in” signaling. This circular pattern of complex and dynamic signaling is not surprising, considering that cells like neutrophils have to rapidly sense and respond to extracellular signals with both accuracy and speed. Despite these challenges, key molecular events in the regulation of integrin signaling and activation continue to be elucidated. In this report, we provide evidence that MARCKS protein is involved in “outside-in” β2-integrin activation in neutrophils. Studies that are currently
underway will hopefully determine in what capacity, MARCKS is affecting β2-integrin function and/or signaling in neutrophils.
Figure 1. MARCKS function is essential for β2-integrin dependent respiratory burst. Isolated human neutrophils were pretreated for 30 minutes with 30 µg/mL αCD18 F(ab’)2 and IgG1 control F(ab’)2 antibodies, MANS (50 µM), RNS (50 µM), staurosporine (100 nM) or media alone (control). Respiratory burst of isolated human neutrophils was quantified by measuring conversion of DHR-123 (10 µM final concentration) to the fluorescent product rhodamine. This conversion is catalyzed by intracellular H2O2 production in neutrophils. Cells were stimulated by IIC (A) or PMA (B), to measure β2-integrin dependent and β2-integrin independent respiratory burst, respectively. Data are presented as mean ± sem, * p < 0.001 MANS vs. IIC or PMA, † p < 0.001 αCD18 F(ab’)2 vs. IIC or PMA, n = 6.
Figure 2. MANS treatment does not affect upregulation or increased affinity of Mac-1 following fMLF stimulation. Isolated human neutrophils were pretreated with MANS (50 uM), RNS (50 uM), EDTA (10 mM) or PBS for 30 minutes and then stimulated at 37°C with 100 nM fMLF or vehicle control (5 minutes). Stimulation was stopped by the addition of 1 volume ice cold PBS and incubation on ice for 5 minutes. Cells were then resuspended and incubated with PE-conjugated antibodies specific for total CD18 (IB4), total CD11b (ICRF44) and high affinity CD11b (CBRM1/5), and flow cytometry was performed as described in Materials and Methods. (A and B) Stimulation of human neutrophils with 100 nM fMLF increased surface expression of CD18 (as measured by IB4 binding) but had no effect on the control receptor MHC class 1 (W6/32). (C) A representative histogram showing the effects of indicated treatments on total CD11b surface expression following fMLF stimulation. (D) 100 nM fMLF stimulation of human neutrophils significantly increased surface expression of CD11b (as measured by ICRF44 binding). (E) A representative histogram showing the effects of indicated treatments on high affinity CD11b surface expression following fMLF stimulation. (F) 100 nM fMLF stimulation of human neutrophils significantly increased surface expression of high affinity CD11b (as measured by CBRM1/5 binding). (A, B, D and F) Data are presented as mean ± standard error. * p < 0.05 vs. VC.
Figure 3. Mn++ does not rescue adhesion of MANS treated neutrophils. (A and B) Calcein labeled human neutrophils were pretreated for 30 minutes with 30 ug/mL of αCD18 F(ab’)2 (IB4), anti-CD11b (CBRM1/5) and control (IgG1) antibodies, MANS (50 uM), RNS (50 uM), wortmannin or media alone (control) before being added to wells coated with 20 ug IIC or 100 ug/mL BSA alone (UTC). Following 30 minutes incubation (37°C, 5% CO2) fluorescence was measured initially and then again after each of three washes, using an fMax plate reader (485 excitation, 530 emission). Percent adhesion (adhesion index, AI %) was determined by dividing final fluorescence by initial fluorescence x 100. Each condition was repeated in triplicate. (C) Calcein labeled human neutrophils were pretreated with calphostin C (1uM), rottlerin (20 uM), chelerythrine chloride (20 uM), MANS (50 uM), RNS (50 uM) or vehicle control, as described in Materials and Methods. Cells were then added to wells coated with 5% FBS and allowed to settle for 10 minutes (37°C, 5% CO2) prior to the addition of 0.5 mM Mn++ or PBS. Following 10 minutes incubation (37°C, 5% CO2) fluorescence was measured initially and then again after each of three washes, using an fMax plate reader (485 excitation, 530 emission). Percent adhesion (adhesion index, AI %) was determined by dividing final fluorescence by initial fluorescence x 100. Each condition was repeated in triplicate. Where applicable, data are presented as mean ± standard error.
Figure 4. MANS treatment inhibits “outside-in” Syk activation in neutrophils. Isolated human neutrophils were pretreated with 50 uM MANS (M), 50 uM RNS (R), 30 ug/mL αCD18 F(ab’)2 (IB4) or PBS (C) for 30 minutes. (A) Following pretreatment, 500 uL of cells (5x10^6 cells/mL) were added to 2 cm diameter coated wells. Plates were incubated for 30 minutes (37°C, 5% CO2) and neutrophil lysates were analyzed by western blot as described in Materials and Methods. (A) Western blot of phospho-Syk (Tyr 525/526) and total Syk in neutrophils plated on BSA and IIC. IIC stimulated adhesion induced Syk phosphorylation that does not appear to be affected by peptide treatment (MANS or RNS) or CD18 blocking. Syk phosphorylation was not detected in neutrophils plated on BSA. (B) Western blot of phospho-Syk (Tyr 525/526) and total Syk in neutrophils plated on fibrinogen (250 ug/mL) alone or also stimulated with TNFα (100 ng/mL). In this initial experiment, Syk phosphorylation in human neutrophils plated on fibrinogen is inhibited in MANS treated neutrophils. MANS does not appear to affect Syk phosphorylation in TNFα-stimulated neutrophils.
Figure 5. MANS inhibition of neutrophil adhesion to IIC is not β₂-integrin specific. Calcein labeled human neutrophils were pretreated for 30 minutes with 30 ug/mL anti-CD18 and IgG1 control F(ab’)2 antibodies, MANS (50 uM), RNS (50 uM) or media alone (control) before being added to wells coated with 20 ug IIC or 100 ug/mL BSA alone (untreated). Following 7, 12 or 30 minutes incubation (37°C, 5% CO₂), fluorescence was measured initially and then again after each of three washes, using an fMax plate reader (485 excitation, 530 emission). Percent adhesion (adhesion index, AI %) was determined by dividing final fluorescence by initial fluorescence x 100. Each condition was repeated in triplicate.
References


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