ABSTRACT

CONLEY, HALEIGH ELIZABETH. A Comparative Investigation of MARCKS as a Therapeutic Target for Dysregulated Neutrophils. (Under the direction of Dr. Mary Sheats).

Neutrophils are the most abundant leukocyte circulating in the blood. These innate immune cells are termed the “first responders” of the immune system for their efficient recruitment to sites of infection and inflammation. Once at the site of infection, neutrophils deploy a variety of functions to destroy pathogens or resolve injury. In a normal state, neutrophils retreat when other immune cells arrive on the scene. However, neutrophils can become dysregulated and cause tissue damage in several disease states.

Once alerted to infection and inflammation, neutrophils migrate through the endothelial wall and into tissues via specialized cell surface receptors called β_2-integrins. B_2-integrins have been, and continue to be, desirable therapeutic targets to regulate neutrophil-mediated diseases. In Chapter 1 we review the tools, resources, and experiments available to assess β_2-integrins. In Chapter 2 we consider FcγRs and β_2-integrins, as these two cell surface receptors often cooperate in a complex mechanism of neutrophil activation and function. Therefore, we assess the nuance of using ICAM-1/Fc for studying β_2-integrins and present evidence that ICAM-1/Fc interacts with FcγRs and β_2-integrins.

Based on the unremarkable clinical success of targeting β_2-integrins for regulating neutrophil-mediated inflammation, there is a desire to look for other proteins that regulate neutrophils. One potential target is the MARCKS protein. Previous research in our lab determined MARCKS plays an essential role in several neutrophil inflammatory functions, including the finding that MARCKS function is essential for β_2-integrin-dependent respiratory burst but not β_2-integrin independent respiratory burst in equine neutrophils. This led us to investigate MARCKS role in neutrophil β_2-integrin activation and signaling in Chapter 3.

We and others have investigated MARCKS as a novel therapeutic target to regulate inflammation. In Chapter 4, we present data evaluating MARCKS in bovine neutrophil inflammatory responses to *Salmonella*. Some of the results of this work are similar to our previous findings, such as MARCKS inhibition attenuated STm-induced neutrophil adhesion and migration. However, we also provide the first report of enhanced neutrophil function with inhibition of MARCKS, specifically the finding that MANS peptide treatment enhanced *Salmonella* phagocytosis and respiratory burst.

The severe form of equine asthma syndrome (termed sEAS) is marked by severe neutrophilic lung inflammation. Previous *in vivo* studies demonstrated that MARCKS inhibitor peptides reduced neutrophilic inflammation in murine models of LPS-induced acute lung injury and ozone-induced airway neutrophilia and inflammation. In Chapter 5 we investigated MARCKS as a novel therapeutic target in equine neutrophilic asthma. Our data demonstrate that MARCKS levels are upregulated in horses with sEAS. We also demonstrate that MARCKS inhibition with MANS peptide attenuates inflammatory functions of peripheral blood neutrophils and alveolar macrophages stimulated with zymosan *ex vivo*.

Neutrophil extracellular trap (NET) formation is a more recently discovered neutrophil response to inflammation and infection. It has gained significant attention during the COVID-19 pandemic because patients with severe infection and ARDS exhibit NET formation in the lungs and elevated blood plasma markers of NETs. Like other neutrophil functional responses, some stimuli induce β_2-integrin-dependent NETs. In Chapter 6 we demonstrate that MANS peptide treatment attenuates NET formation induced by PMA.

Neutrophil-mediated diseases continue to impact both animals and humans alike. Our findings described here identify a role for MARCKS β_2-integrin activation and signaling,

supporting MARCKS as a potential therapeutic target for neutrophil-mediated diseases across species. We share these findings to address gaps in knowledge regarding MARCKS role in innate immunity and disease, neutrophil functions and β_2-integrin activation, and to further inform development of therapeutic strategies to diminish the harmful effects of neutrophil dysregulation in multiple disease states.

A Comparative Investigation of MARCKS as a Therapeutic Target for Dysregulated Neutrophils

by
Haleigh Elizabeth Conley

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Comparative Biomedical Sciences

Raleigh, North Carolina
March 10, 2022

APPROVED BY:

_____	_____
Dr. Mary Sheats Committee Chair	Dr. Samuel Jones
_____	_____
Dr. Jeffrey Yoder	Dr. Philip Sannes

DEDICATION

I dedicate this work to my husband, Coleman Conley, and my parents, Larry and Valerie Cole.

My accomplishments are the result of your unwavering support and sacrifices.

BIOGRAPHY

Haleigh Elizabeth Cole Conley is the oldest child of Larry and Valerie Cole. Haleigh grew up in Lebanon, Virginia with her younger brother Landon. Haleigh has always been serious about academics and loved science so much that she took more science classes than the average student at her high school. For her undergraduate education, Haleigh attended Emory & Henry College, a small liberal arts school not far from home. Although small, the college provided the perfect environment for Haleigh to excel in academics. Because of her original interest in dentistry and love of science, Haleigh pursued a bachelor's degree in chemistry with a minor of biology. Her love of science opened many other doors, including winning a summer fellowship to complete a research project at Wake Forest University and completing an honors research thesis with her advisor, Dr. Michael Lane. It was during her time at Emory & Henry that Haleigh's passion for research grew and was nurtured by the inspiring faculty in the chemistry and biology departments. Drs. Melissa Taverner, Christy Fleet, Michael Lane, and James Duchamp will forever be remembered as compassionate and supportive mentors. It was also during her time at Emory & Henry that Haleigh met her future husband, Cole. After completing her undergraduate degree, Haleigh worked as a research technician in the Moody Lab at the Duke Human Vaccine Institute. During her time at Duke, Haleigh discovered her true love immunology research, which was cultivated by her mentors Drs. Tony Moody and David Easterhoff. In August 2018, she began her Ph.D. and knew immediately that she had found the next right step for her. Completing her Ph.D. during the COVID-19 pandemic brought about several challenges; however, Haleigh embraced each opportunity to grow and learn under the outstanding mentorship of Dr. Katie Sheats.

ACKNOWLEDGEMENTS

I consider myself the luckiest graduate student to have Dr. Katie Sheats as my mentor over the past four years. She has supported and encouraged me throughout life's many challenges during my PhD. I am extremely grateful for her compassionate guidance as I've learned how to become an independent researcher and writer. Along with Dr. Sheats, I would like to thank Drs. Sam Jones, Jeff Yoder, and Phil Sannes for serving on my Ph.D. committee. I appreciate the time you invested to give me feedback and insight to make me a better scientist.

To the Sheats and Jones lab, thank you for your endless humor, support, and encouragement. Kaori, Rosemary, Jenna, Allison, Bethanie, and Alix, I'll forever remember this time together. It has been a pleasure to work with such brilliant people. To Chalise, I could not have survived summer 2021 without your help.

To my husband, Cole. Thank you for your endless support. I know I can always count on you to edit my work or make a late-night run to the lab. I could not have made it without you cheering me on and lifting me up when things were hard. Sharing an office with you during the pandemic was the only way I would have been able to finish my preliminary exam in high spirits. You made my dream possible, and I am forever grateful.

To my family: Mom, Dad, Grandma, Landon, Maitlyn, Steve, Larkin, Clif, Lauren, Debbie, Nanny, thank you for supporting me through my endless years of education. Your encouragement helped me shoot for the stars. To my Forest Hills family, thank you for becoming our family and always supporting me throughout this graduate school journey. To my WND friends, thank you for your endless laughter, encouragement, and for listening to the woes of graduate school. Kate Pennington and Sarah Bellamy, I couldn't have made it through 2020 without your encouragement, and I will remember it for the rest of my life.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 5

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 1

Targeting neutrophil beta2-integrins: A review of relevant resources, tools, and methods

Haleigh E. Conley[1,2] and M. Katie Sheats[1,2]

[1]Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, United States

[2]Comparative Medicine Institute, North Carolina State University, Raleigh, North Carolina, United States

Abstract

Neutrophils are important innate immune cells that respond during inflammation and infection. These migratory cells utilize β_2-integrin cell surface receptors to move out of the vasculature into inflamed tissues and to perform various anti-inflammatory responses. Although critical for fighting off infection, neutrophil responses can also become dysregulated and contribute to disease pathophysiology. Neutrophil-targeting therapies have focused on β_2-integrins as potential targets but so far have failed in clinical trials. As the field continues to move forward, a better understanding of β_2-integrin function and signaling will aid the design of future therapeutics. This review details relevant ligands, tools for assessment, experimental methods, and *in vivo* models utilized to examine neutrophil β_2-integrins more closely.

1. Introduction

Neutrophils are the predominant circulating leukocyte in the blood and are considered the first responders of the immune system. Neutrophils defend the host against invading pathogens via effector functions such as respiratory burst, phagocytosis, and NETosis. To accomplish these tasks, neutrophils must travel out of the vasculature and into the injured or infected tissue through a process known as transmigration. Beta2-integrins are specialized cell surface receptors that play a key role in a neutrophil's ability to transmigrate. The mechanisms of beta2-integrin function and signaling have been researched and reviewed extensively (Abram and Lowell, 2009; Bouti et al., 2021). While much has been learned, therapeutic efforts to target beta2-integrins to mitigate neutrophil-mediated host injury or disease have failed. Because neutrophils play a role in the pathophysiology of diseases ranging from acute lung injury and sepsis to rheumatoid arthritis and organ transplant rejection, the methods used to study neutrophil beta2-integrins, reviewed here, remain of interest to a wide array of health researchers.

2. Beta2-integrins and neutrophils

2.1 Beta2-integrin activation and signaling

Expressed exclusively on leukocytes, beta2 (β_2)-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,b,c,d) (Table 1) (Blythe et al., 2021; Schymeinsky et al., 2007). The two most prominent integrins on neutrophils are LFA-1 ($\alpha_L\beta_2$) and Mac-1 ($\alpha_M\beta_2$). Within circulating, quiescent neutrophils, these adhesion molecules are primarily contained within the cytoplasm, the secondary and tertiary granules, and the secretory vesicles. The few β_2-integrins that are present on the surface of resting neutrophils are maintained in an inactive or 'bent' conformation, termed the "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 unregulated activation could lead to damaging effects for the host. It is only when neutrophils encounter an activation signal, such as the binding of a chemoattractant (e.g., leukotriene B4 (LTB_4), N-Formylmethionine-leucyl-phenylalanine (fMLP)) to a G-protein coupled receptor (GPCR), that β_2-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" activation (Montresor et al., 2012). In contrast to "inside-out," "outside-in" activation occurs when the β_2-integrin extracellular domain interacts directly with extracellular matrix proteins or other cell surface ligands (ICAM-1, fibrinogen, etc.) and initiates its own signaling (Abram and Lowell, 2009; Williams and Solomkin, 1999). This triggers the phosphorylation of ITAM-bearing transmembrane adapters DAP-12 and Fc gamma

receptors which go on to activate Syk and initiate a signaling cascade for cytoskeletal reorganization (Jakus et al., 2007). These effects on the cytoskeleton are important for the role of β_2-integrins in neutrophil adhesion strengthening, cell spreading, and crawling (Schmidt et al., 2013). Despite being described, and studied *in vitro*, as distinct pathways, inside-out and outside-in activation are designed to work in concert *in vivo*, with signaling from one pathway reinforcing the other.

2.2 *Neutrophils in disease*

Neutrophils are essential as the "first responders" of the immune system following infection or injury and without them patients are at increased risk. Patients lacking functional β_2-integrins due to Leukocyte Adhesion Deficiency (LAD) experience frequent, life-threatening infections. However, neutrophils also cause damage to host tissue in numerous chronic inflammatory conditions and acute inflammatory events, making neutrophil-targeting therapies highly desirable. For example, patients with severe SARS-CoV-2 experience an influx of neutrophils into the lungs, resulting in alveolar damage and the development of acute respiratory distress syndrome (Borges et al., 2020; Yang et al., 2021). Numerous diseases and disorders involve neutrophils and specifically β_2 integrins (Table 2). Because of the essential role integrins play in neutrophil inflammatory functions, they are attractive therapeutic targets (Mitroulis et al., 2015; Zimmerman and Blanco, 2008). However, clinical trials have fallen short to identify efficacious β_2-integrin targeting therapies (Raab-Westphal et al., 2017), and additional research is needed to identify new methods of targeting integrins. Within the scientific literature, there have been many approaches to studying β_2-integrins. This review article will provide an overview of methods used for studying β_2-integrin function, activation, and signaling in neutrophils. Hopefully, with use of resources, tools and methods presented here, and continued

development of new approaches, researchers will discover a more comprehensive understanding of β_2-integrins that will lead to successful therapeutic targets to benefit patients with neutrophil-mediated disease.

3. Cell Types and Tools for Evaluating Integrins

3.1 Primary Cells

Neutrophils are hematopoietic cells that are terminally differentiated from myeloblasts. They are the most predominant circulating leukocyte in the blood, and their typical life span in circulation ranges between less than 24 hours to 5.4 days (Pillay et al., 2010; Tak et al., 2013). Most primary neutrophil research uses cells collected from either humans or mice. Over the years, several methods have been utilized to isolate neutrophils from human peripheral blood. Most protocols include an erythrocyte sedimentation step and a density centrifugation step. Erythrocyte sedimentation is typically done using dextran (varying concentrations 1-6%) or HetaSep (Wright et al., 2017). For density centrifugation, Ficoll and Percoll are the most common options utilized. The order of these steps often varies depending on the research group. Despite their common usage, there is concern that neutrophils isolated by Dextran and Ficoll are prematurely activated by the presence of monocytes (Quach and Ferrante, 2017). To avoid this background stimulation, some use a one-step high density Ficoll (1.114 g/mL) without erythrocyte sedimentation, or substitute a discontinuous gradient for Ficoll (Kuhns et al., 2016; Quach and Ferrante, 2017). Red blood cell lysis usually follows isolation of neutrophils; however, when the experimental method does not require removal of red blood cells, avoiding lysis may be one strategy to prevent unwanted neutrophil activation. Flow cytometry is an example of an experimental method that does not necessarily require removal of red blood cells.

These isolation methods routinely yield normal density neutrophils (NDNs) but fail to isolate low density neutrophils (LDNs) that exist in individuals with inflammatory conditions. To isolate LDNs, negative selection by magnetic beads of both the PBMC and granulocyte layers is necessary (Villanueva et al., 2012; Wright et al., 2017). While application of magnetic microbeads facilitates isolation of pure cell populations, this method increases the cost of isolation and may still require lysis to remove contaminating RBCs (Schweizer et al., 2021).

Primary human neutrophils are easy to obtain from willing donors, and a major benefit of using primary human cells is the ability to obtain samples from humans with diseases of interest. Neutrophils from LAD-I patients have been an invaluable resource for researchers interested in β_2-integrin-dependent and independent neutrophil functions and cell signaling downstream of β_2-integrins (Diacovo et al., 1996; Kuijpers et al., 1997). However, the risk to the patient versus the benefit of health discovery research must also be a consideration when obtaining samples from patients. For this reason, volume and cell number are likely to be limited when samples are obtained from diseased patients. While sampling from human populations can be extremely convenient, there are logistical barriers, such as IRB approvals and personnel, that can make human research more challenging. This has been especially true during the COVID-19 pandemic when IRB protocols changed to reduce risk for participants and researchers, resulting in reduced access to human participants.

Primary neutrophils from mice are commonly isolated from bone marrow or the peripheral blood. Similar to humans, density centrifugation with either Percoll or Histopaque discontinuous gradients are utilized (Alder et al., 2018; Wong et al., 2015; Xu et al., 2015). Due to the wide availability of species-specific resources, murine neutrophils can also be isolated

from bulk populations using magnetic microbeads or by fluorescence activated cell sorting (FACS) (Helou et al., 2019; Rivadeneyra et al., 2018).

Murine neutrophils are widely used due to the availability of models that duplicate neutrophil function during health and disease. However, they do not perfectly model humans regarding neutrophils. In humans, the predominantly circulating cell type in the blood is neutrophils (50-70% neutrophils, 30-50% lymphocytes), whereas mice have an abundance of lymphocytes (10-25% neutrophils, 75-90% lymphocytes) (Mestas and Hughes, 2004). Neutrophils are a heterogenous population consisting of normal density neutrophils (NDNs), low density neutrophils (LDNs), immature neutrophils, mature neutrophils, and neutrophils with immunosuppressive capabilities (Silvestre-Roig et al., 2019). Although mice do express these subpopulations of neutrophils, they do not always mimic the presentation of that in humans during certain disease states. During acute trauma and inflammation, murine neutrophils do not express the same cell surface markers as human neutrophils. Human autoimmune disease may result in increased circulation of proinflammatory LDNs, but murine models of autoimmune disease do not display heterologous populations of neutrophils (Silvestre-Roig et al., 2019). These phenotypic differences are also observed in murine models of cancer that also result in functional differences (Eruslanov et al., 2017; Silvestre-Roig et al., 2019). Human neutrophils also have significant transcriptional and epigenetic diversity. Females especially, have elevated gene expression levels related to immune responses that correspond with increased occurrences of autoimmune disease (Ecker et al., 2017). Further, murine models cannot model the impact of ethnic diversity on neutrophils, despite recent advances in high-diversity mouse populations (Atallah-Yunes et al., 2019; M. C. Saul et al., 2019).

3.2 Cell Lines

Although primary cells are highly desirable for understanding neutrophil β_2-integrins, primary cells are not always accessible or suitable for certain experiments. For example, although reported (Gupta et al., 2018; Liu et al., 2019), it is extremely difficult to manipulate RNA and protein expression in primary neutrophils, so cell lines are beneficial for researchers aiming to investigate roles of individual proteins through knockdown or overexpression studies. Although neutrophil-like cell lines have limitations and drawbacks, they can be an easier approach for studying integrin function and signaling via induced mutations rather than developing new transgenic mouse lines for each mutant desired.

The HL60 cell line is a human promyeoloblast cell line that can be differentiated into neutrophil-like cells utilizing dimethylsulfoxide (DMSO) or retinoic acid (Hickstein et al., 1987). Both methods of differentiation result in mature neutrophil-like cells; however, compared to DMSO, differentiation with retinoic acid resulted in dampened cellular responses to fMLP and increased random cellular migration (Sham et al., 1995). The two methods also result in different expression levels of Scar1 and WASP proteins (Launay et al., 2003). Functionally, DMSO-differentiated HL60 neutrophil-like cells are mostly similar to primary neutrophils but do express some differences (Table 3) (Hauert et al., 2002; Hickstein et al., 1987; Patcha et al., 2004). Despite these shortcomings, research using mutated HL60s has contributed to our understanding of LFA-1 in migration (Weber et al., 1997). This cell line has also be used to model host-pathogen interactions (Dumont et al., 2013).

Although not as commonly used as HL60s, the human myeloid cell line K562 has been utilized over the past 15 years in research focusing on granulocytes and β_2 integrins. Xue et al. used the K562 cell line to elucidate the mechanism of how kindlin-3 defects impact integrin function. Through these studies, it was demonstrated that kindlin-3 is required for β_2-integrin-

mediated adhesion and cell spreading (Xue et al., 2013). Another group of researchers used K562 cells as a means to express constructs of α_M fused to mCFP and β_2 fused to mYFP to assess the separation of Mac-1 cytoplasmic tail separation during integrin activation via FRET analysis (Lefort et al., 2009). With this technique, the authors were able to determine that integrin-ligand binding, or integrin-crosslinking, induces Mac-1 cytoplasmic tail separation to trigger outside-in signaling pathways. This finding may explain the lack of benefit seen in clinical trials with inhibitors designed to mimic integrin ligands (Dove, 2000; Harlan and Winn, 2002). K562 cells have been further used to evaluate potential small peptide inhibitors and monoclonal antibodies directed against β_2-integrins (Hafeez Faridi et al., 2013). Another benefit of K562 cells is that they only express the transfected β_2-integrin, allowing for studies examining just Mac-1 or just LFA-1 if desired. They also respond similarly to common stimuli of primary neutrophils, including Mn^{2+} (Celik et al., 2013; Lefort et al., 2009).

HoxB8 cells are immortalized murine hematopoietic progenitors that are differentiated to neutrophil-like cells using GM-CSF treatment. These cells perform many neutrophil and integrin-mediated functional responses similar to primary murine neutrophils, with a few discrepancies (Table 3) (Chu et al., 2019; S. Saul et al., 2019; M. E. Wilson et al., 2020). The usage of these cells has increased over the past several years, primarily because HoxB8 cells can be generated from transgenic mice and used to examine specific molecular contributions to β_2-integrins (Bromberger et al., 2021). Recently, investigators used HoxB8 neutrophil-like cells to show that Rap1 and Riam binding to talin is critical for β_2-integrin function (Bromberger et al., 2021). In addition to traditional methods of transfection and siRNA, CRISPR/Cas9 technology can be utilized to produce HoxB8 cells deficient in a specific integrin protein, such as talin (Bromberger et al., 2021). Bromberger and colleagues also used CRISPR/Cas9 to induce a

human β_2-integrin ortholog in murine HoxB8 neutrophil-like cells (humanized HoxB8 neutrophils).

3.3 Tools

Anti-integrin and fluorescently labeled antibodies

Antibodies are a common tool used to investigate β_2-integrins, and anti-integrin antibodies are a staple in this field of research (Berton et al., 1994; Diacovo et al., 1996; Dumont et al., 2013; Graham et al., 1993; Penberthy et al., 1995). These antibodies are advantageous for common lab use due to their ease of application and relative low cost. They are routinely used in three different ways: function blocking, integrin crosslinking, or fluorescent labeling. As a tool to block function, anti-integrin antibodies led to the discovery that Mac-1 is responsible for neutrophil firm adhesion (Diacovo et al., 1996). However, antibody binding of β_2-integrins can also cause activation, as demonstrated by antibody stimulation of neutrophils in the absence of a ligand (e.g., ICAM-1) and subsequent β_2-integrin outside-in activation and signaling (Lefort et al., 2009). This dual nature requires careful experimental planning to prevent unintentional crosslinking and/or Fc receptor engagement when used for inhibitory applications (Jakus et al., 2004). To avoid these unintentional interactions, researchers can use F(ab) and F(ab)'$_2$ fragments derived from monoclonal antibodies. These fragments are portions of antibodies where the Fc fragments are cleaved off to prevent non-specific binding of Fc receptors to antibody. Both types of fragments are helpful as blocking antibodies, and F(ab)'$_2$ provide additional capabilities for precipitating proteins of interest.

The use of fluorescently labeled antibodies has also been an invaluable tool for researchers. Using specific antibodies, surface expression levels of the integrins, including the high affinity conformation of CD11b, can be distinguished when determining how integrin

activation is upregulated (Feng et al., 2011). This type of labeling allowed for the discovery that neutrophils from patients with antiphospholipid syndrome (APS) have an upregulation of activated CD11b, which contributes to the increased neutrophil adhesiveness (Sule et al., 2020). Fluorescently labeled antibodies can also be used *in vivo*. Wilson et al. administered PE-anti-Ly6G intravenously to evaluate neutrophil infiltration induced by *P. aeruginosa* in talin-1 or kindlin-3 knockout mice (Wilson et al., 2017). As researchers continue to seek novel protein targets to regulate β_2-integrins, fluorescently labeled antibodies combined with flow cytometry may be a first step to understanding the impact inhibitors may have on β_2-integrin expression and activation.

Divalent Cations

Divalent cations (e.g., Mn^{2+}, Ca^{2+}, Mg^{2+}) are required for many biological processes, including the binding of integrins to their ligands. Both manganese (Mn^{2+}) and magnesium (Mg^{2+}) act through binding the metal ion dependent adhesion site (MIDAS) domain. Mn^{2+} binding to the MIDAS domain induces outside-in β_2 integrin activation by forcing integrins to assume a high affinity conformation that enhances ligand binding (Weber et al., 1997). Because of this nature, Mn^{2+} can also be applied as a rescue strategy when examining integrin defects caused by mutation or chemical inhibition. Xu et al. determined that Mn^{2+} treatment could not rescue *Mylk*$^{-/-}$ murine neutrophil binding effects, indicating a critical role for MYLK in outside-in β_2-integrin activation (Xu et al., 2008).

Like manganese, calcium and magnesium are required for biological processes. Therefore, it is common for researchers to include calcium and magnesium supplementation in media, and manipulation of cation presence has led to a better understanding of integrin regulation. In fact, calcium chelation is known to decrease integrin expression and is often used

as a positive control for inhibition in experiments (Celik et al., 2013; Diamond and Springer, 1993). Divalent cation stimulation of neutrophils with manganese or higher concentrations of magnesium induce the high affinity conformation of β_2-integrins without activating the neutrophil itself or increasing β_2-integrin surface expression (Spillmann et al., 2002). Through the manipulation of cation concentrations, this study demonstrated that β_2-integrins must be in their active/high affinity states to mediate adhesion (Spillmann et al., 2002). A complete understanding of how divalent cations impact neutrophil function and integrin activation is also useful for interpreting clinical information following certain treatments. For example, magnesium sulfate treatment for preterm birth impairs neonatal innate immune cell recruitment and β_2-integrin-dependent neutrophil responses (Mehta and Petrova, 2006).

3.4 Common Ligands

Recombinant ICAM-1

B_2 integrins bind to intracellular adhesion molecules (e.g., ICAM-1) expressed on the surface of endothelial cells to transmigrate from the vasculature into inflamed tissues. This binding interaction induces outside-in activation and signaling of neutrophil β_2-integrins. One of the most utilized ligands for understanding β_2-integrin activation and signaling is ICAM-1 because it is a powerful tool for modeling physiologically relevant neutrophil interactions. ICAM-1 is capable of stimulating neutrophil activation, adhesion, serving as a ligand in shear flow assays, and even induce clustering of neutrophil β_2-integrins (Celik et al., 2013; Lefort et al., 2009; Volmering et al., 2016; Xu et al., 2008). Although the usage of recombinant ICAM-1 is extremely common, there have been several discrepancies in the literature surrounding nomenclature and usage of this ligand. Recombinant ICAM-1/Fc is often used interchangeably for recombinant ICAM-1. Based on our own observations (unpublished findings) and those cited

in the literature, the Fc domain of ICAM-1/Fc is likely engaging Fc rectors on neutrophils and causing an inside-out activation cascade (Jakus et al., 2004; Jones et al., 1998). Therefore, we suggest that special attention must be given to the type of ICAM-1 utilized in studies to decipher inside-out or outside-in neutrophil activation.

Fibrinogen

Fibrinogen is a glycoprotein found in blood that is enzymatically converted to fibrin to promote clotting after damage occurs to vasculature or tissues. Neutrophil β_2-integrins bind fibrinogen at sites of inflammation; therefore, it is used *in vitro* to determine integrin dependent responses (Chu et al., 2019; Lowell et al., 1996; McMillan et al., 2013; Szczur et al., 2009; Volmering et al., 2016; Zen et al., 2011). Lowell et al. determined that Src family kinases were important for β_2 and β_3 integrin signaling by evaluating $hck^{-/-}$ $fgr^{-/-}$ double mutant murine neutrophils on fibrinogen (Lowell et al., 1996). The double mutant neutrophils failed to spread on fibrinogen, but PMA stimulation was able to overcome the defect, indicating that Src kinases act upstream of PKC during integrin-mediated signaling. Fibrinogen initiates outside-in integrin signaling in both β_2 and β_3 integrins (Cox et al., 1994; McMillan et al., 2013); therefore, experiments utilizing this ligand for β_2-integrins must rule out effects caused by β_3-integrin engagement as well.

PolyRGD

Beta-integrins bind extracellular matrix proteins (e.g., fibrinogen, fibronectin, collagen, and von Willebrand factor) via their RGD (arginine-glycine-aspartic acid – RGD) site (Ruoslahti and Pierschbacher, 1987). PolyRGD is a synthetic tripeptide used to engage integrins, and it is known for producing a robust CD18-dependent respiratory burst (Mócsai et al., 2002; Zhang et al., 2006). Other investigations found that Fc receptor knockout mice had decreased respiratory

burst in response to polyRGD (Mócsai et al., 2006), suggesting that inside-out activation via Fc receptors may also play a role in neutrophil responses to polyRGD. Because RGD binding sites exist on all β-integrins, polyRGD stimulates β_1, β_2, and β_3-integrins expressed on neutrophils (Bertram et al., 2012; Kim et al., 2014; Rossaint et al., 2014). Therefore, the PolyRGD is not the best tool for isolating β_2 integrin activation and signaling.

iC3b

Complement C3 fragment iC3b is a component of the complement system formed when complement factor I cleaves C3b. B_2-integrins bind iC3b and are recognized as complement receptor 3 (CR3) (Ueda et al., 1994). In a physiological context, iC3b is an opsonin to support β_2-integrin-mediated phagocytosis of pathogens (Li et al., 2011). Xue et al. used iC3b to stimulate K562 Kindlin-3 knockdown cells and determined that kindlin-3 is required for iC3b-mediated outside-in β_2-integrin signaling (Xue et al., 2013). IC3b can also be used as a coating for neutrophil adhesion or in shear flow experiments (Xue et al., 2013). Assays using iC3b as a tool are most relevant for *in vitro* modeling of diseases that may have iC3b-containing immune complex deposition contributing to neutrophil aggregate formation, such as Systemic Lupus Erythematosus (SLE) (Rosetti and Mayadas, 2016).

3.5 Assays

Flow cytometry

Flow cytometry is a high throughput technology that analyzes single cells from bulk populations. The technology detects and measures physical and chemical characteristics based on cell size and fluorescence. Neutrophils can easily be distinguished using flow cytometry based on their size and high granularity determined by a high side scatter profile when evaluating both side and forward scatter measurements. The usefulness of flow cytometry is widely known

across many fields of research, and leukocyte researchers have also harnessed this powerful tool to assess β_2-integrins. Using fluorescently labeled antibodies, researchers can assess expression, avidity, and affinity of β_2-integrins to learn more about how a protein or inhibitor impacts expression or to determine whether certain disease cause changes in β_2-integrin expression. Flow cytometry can also be used to measure neutrophil binding to ligands, such as ICAM-1, in the presence of pharmacological inhibitors or when isolated from transgenic mice (Lau et al., 2005; Newton and Hogg, 1998). Flow cytometry analysis of integrin expression is a relatively easy, but powerful assay to complement other experiments. One caution is that neutrophil populations may exhibit autofluorescence, including autofluorescence attributed to contaminating eosinophils (Dorward et al., 2013; Monsel et al., 2014; Naegele et al., 2012). Further, non-specific binding can also be a concern with flow cytometry due to neutrophil Fc receptors cross reacting with labeled monoclonal antibodies if Fc receptors were not first blocked. Non-specific staining can also occur if excessive concentrations of labeled antibody are used, yielding the importance of concentration optimization (Andersen et al., 2016). A significant benefit of flow cytometry is that multiple aspects of the neutrophil can be evaluated at once, such as integrin expression and cell viability. Imaging flow cytometry is a newer methodology used in neutrophil research. Specifically, this technology can be used to measure fluorescence and morphology of neutrophils during functions, such as phagocytosis (Smirnov et al., 2020). Because of its higher power in cellular analyses, this technique provides a breadth of information about cells. However, the large data files can create challenges for data management and analysis (Han et al., 2016).

Static Adhesion

Static adhesion is a common assay that has been used to assess neutrophil for over twenty years. Fluorescently labeled (e.g. calcein AM) neutrophils are added to ligand covered plates for

a certain amount of time followed by subsequent washing and fluorescence readings (Eckert et al., 2010; Sheats et al., 2014). This assay can evaluate the adhesion of neutrophil and neutrophil-like cells on most ligands, including human umbilical vein endothelial cell (HUVEC) monolayers (Shimizu et al., 1995; Takahashi et al., 2002). Unfortunately, static adhesion assays are subject to technical variability due to inversion procedure to "dump" cells. Further, static adhesion assays cannot fully recapitulate the physiological environment that occurs during shear flow adhesion. For example, neutrophil migration and adhesion under static adhesion requires vinculin; however, vinculin was not required for integrin-mediated migration and adhesion when neutrophils were examined under shear flow (Z. S. Wilson et al., 2020). Despite these differences, static adhesion assays do offer a high-throughput means to examine β_2 integrin-mediated firm adhesion (Sheats et al., 2014; Willeke et al., 2003).

FRET

Förster Resonance Energy Transfer (FRET) (also referred to as Fluorescence Resonance Energy Transfer) is a method that shows energy transfer between two light sensitive molecules based on distance (Shrestha et al., 2015). With this newer technology, two proteins of interest can be labeled to quantitatively measure the interactions between the proteins. FRET can detect neutrophil β_2-integrin conformational changes in the extracellular domain and the cytoplasmic tail when the alpha and beta chains are labeled separately. Lefort et al. used this method to determine how inside-out activation of Mac-1 results in integrin headpiece extension from the bent conformation. Cytoplasmic domain FRET in K562 cells demonstrated Mac-1 binding to ICAM-1 results in the separation of integrin cytoplasmic tails (Lefort et al., 2009). The sensitivity of this method has made it easier to determine protein interactions within living cells, including how integrins respond to neutrophil stimulation.

Integrin-crosslinking

Integrin-crosslinking is a technique where anti-integrin antibodies (e.g., anti-CD18 mAb) are coated on a plate and used as the stimulus for β_2-integrin activation and signaling (Mócsai et al., 2002). This approach has historically been used to induce outside-in signaling of integrins. However, Jakus and colleagues determined that there was cooperative interaction between FcγRIIa and integrins during integrin-crosslinking with anti-CD18 mAb due to the presence of Fc domains on mAbs. In this study, they demonstrated that anti-CD18 mAb crosslinking resulted neutrophil respiratory burst. When anti-CD18 $F(ab')_2$ was used instead, respiratory burst no longer occurred despite the occurrence of significant neutrophil adhesion. This finding ultimately led to the understanding that more than direct integrin activation is required for full neutrophil activation resulting in respiratory burst (Jakus et al., 2004). This technique provides many benefits to understanding the cooperative signaling of integrins and FcγRs, but researchers should use F(ab) or $F(ab')_2$ fragments when trying to stimulate outside-in β_2-integrin activation using this method.

Flow chamber assays

Neutrophils are migratory cells where dynamic motion is a critical part of their function. Many *in vitro* neutrophil assays are unable to capture the dynamic process of neutrophil diapedesis. Flow chamber experiments can determine neutrophil crawling velocity, arrest, polarization, migration patterns, and diapedesis using microscopy. This technique offers a multitude of options in ligands, cell type (whole blood, primary or differentiated neutrophil-like), chemoattractants, function-blocking antibodies, and immunofluorescence microscopy (Zhang et al., 2006). Flow chambers can also be coated with desired ligands and perfused with whole blood via tubing directly attached to murine carotid arteries. With this approach, Zarbock and

colleagues demonstrated that E-selectin engagement is required for LFA-1 dependent rolling on ICAM-1 (Zarbock et al., 2007).

In addition to the ligands utilized in shear flow assays, neutrophil interactions with cell monolayers can also be evaluated. Sule and colleagues demonstrated that neutrophils from patients with antiphospholipid syndrome display increased adhesion to HUVECs due to upregulated β_2-integrin activation (Sule et al., 2020). This system can also be designed to model organ specific neutrophil interactions, such as blood-brain barrier inflammation. Gorina and colleagues showed that neutrophils use β_2-integrins to crawl on ICAM-1 prior to diapedesis isolated primary mouse brain microvascular endothelial cells (Gorina et al., 2014). Therefore, shear flow assays are advantageous because they offer a multitude of options to model healthy and diseased states.

Immunoblotting and Co-immunoprecipitation

As the field continues to push toward effective drugs for targeting β_2-integrins, we must consider other proteins that may serve as therapeutic targets. Many of the assays already discussed can be adapted with any type of inhibitory peptide or antibody to probe the involvement of specific proteins in β_2-integrin activation and function. However, immunoblotting continues to be a desirable method for determining specific cell signaling patterns. A significant portion of our understanding of integrin signaling came from the use of immunoblotting experiments. Through immunoblotting, key signaling molecules downstream of integrin activation, such as Syk, have been identified (Mócsai et al., 2002). Specifically, Lefort et al. demonstrated that Mac-1 outside-in activation with ICAM-1 activates only the Akt apoptosis regulatory pathway and not the p38 MAPK pathway (Lefort et al., 2009). With immunoblotting,

researchers can determine the signaling mechanism that underlies a given functional response (Silva et al., 2020).

Co-immunoprecipitation (Co-IP) assays are used to identify the protein-protein interactions occurring within cells by indirectly capturing proteins that are bound to specific target proteins (Kaboord and Perr, 2008). The unknown proteins are then evaluated using traditional immunoblotting techniques. Co-IP has been useful for determining binding partners of β_2-integrins in both neutrophils and lymphocytes (Evans et al., 2011; Xu et al., 2008). Co-IP can also be used to determine CD18 binding partners on the surface of neutrophils (Burnett et al., 2017). Through Co-IP experiments, Willeke demonstrated that Syk binds to CD18 in fibrinogen stimulated neutrophils, expanding the understanding of Syk's role in β_2-integrin activation and signaling (Willeke et al., 2003).

3.5 In Vivo Experiments

We have primarily discussed *in vitro* experiments to evaluate neutrophil β_2-integrins; however, the use of mouse models has also made a significant impact on the field. The *in vitro* experiments utilizing primary cells or cell lines are great for building a fundamental understanding of β_2-integrins; however, findings *in vitro* do not always represent reality *in vivo*. Table 4 summarizes a selection of *in vivo* models where β_2-integrin dependence may vary depending on the stimulus or the organ in question. These differences have are also noted when comparing neutrophil migration within a 3D collagen matrix versus placed on top of a collagen matrix (Lämmermann and Germain, 2014).

Intravital microscopy

Intravital microscopy in the mouse cremaster muscle is a well-established method for examining neutrophil interactions in the blood vessels. Leukocyte recruitment can be visualized

in a variety of scenarios, including chemokine stimulation, fluorescently labeled leukocytes or eGFP mice, and/or the application of pharmacological inhibitors (Kranig et al., 2020; Phillipson et al., 2006; Zarbock et al., 2007). Phillipson and colleagues used this technique to decipher the different molecular mechanisms for LFA-1 and Mac-1 during MIP-2 induced neutrophil recruitment (Phillipson et al., 2006). Further, it was also used to determine that E-selectin induced slow rolling of neutrophils is LFA-1 dependent and not Mac-1 (Zarbock et al., 2007). As technology advances, researchers have expanded the field in intravital microscopy. Park and colleagues developed an intravital lung imaging system to examine neutrophil recruitment during sepsis-induced acute lung injury (ALI). In this study, they determined that decreased pulmonary microcirculation is due to obstructions of clustered neutrophils. Further, these neutrophils had high levels of surface Mac-1 expression, and the application of a Mac-1 inhibitor decreased sequestration in the pulmonary microvasculature (Park et al., 2019). Because of the breadth of options for examining neutrophils using intravital microscopy, this technique is an excellent next step for researchers wanting to translate *in vitro* findings into organ-specific *in vivo* scenarios (Margraf et al., 2019).

4. Conclusions

Over the years, many tools and assays have been developed to assess β_2-integrins in neutrophils and neutrophil-like cells. There is a great variety of options available to researchers with diverse questions regarding the biological function of β_2-integrins. The combination of these cell types, tools, and assays offers a powerful resource to further understand β_2-integrins and inform the next generation of neutrophil targeting therapies.

ABBREVIATIONS

CR3 – complement receptor 3

dHL60 – differentiated HL60 cells

DMSO - dimethylsulfoxide

fMLP – N-Formylmethionine-leucyl-phenylalanine

GM-CSF – granulocyte macrophage colony stimulating factor

GPCR – G-protein coupled receptor

ICAM-1 – intracellular adhesion molecule 1

IL-8R – interleukin 8 receptor

LDN – low density neutrophil

LFA-1 – lymphocyte function-associated antigen-1, $\alpha_L\beta_2$, CD11a/CD18

LTB_4 – leukotriene B4

Mac-1 – macrophage-1 antigen, $\alpha_M\beta_2$, CD11b/CD18

NDN – normal density neutrophil

PMA – phorbol 12-myristate 13-acetate

ROS – reactive oxygen species

TABLES

Table 1. Nomenclature for beta2-integrins expressed on neutrophils

B_2-Integrin	**Heterodimer**	**Other Names**
CD11a/CD18	$\alpha_L\beta_2$	LFA-1
CD11b/CD18	$\alpha_M\beta_2$	Mac-1, Complement receptor 3 (CR3)
CD11c/CD18	$\alpha_X\beta_2$	P150,95, Complement receptor 4 (CR4)
CD11d/CD18	$\alpha_D\beta_2$	

Table 2. Diseases and disorders where neutrophil beta2-integrins have been identified as key players in disease pathophysiology.

Disease or Disorder	Category	References
Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV)	Autoimmune disease	(Matsumoto et al., 2021)
Aspergillus fumigatus	Fungal infections	(Silva et al., 2020; Teschner et al., 2019)
Atrial fibrillation	Cardiovascular disease	(Friedrichs et al., 2014)
Blood-brain barrier inflammation	Acute illness	(Gorina et al., 2014; Marchetti and Engelhardt, 2020)
Candida albicans	Fungal infections	(Li et al., 2011)
COPD	Chronic disease	(Blidberg et al., 2013; Overbeek et al., 2011; Woolhouse et al., 2005)
Interstitial lung disease (ILD)	Chronic inflammation, autoimmune disease	(Zou et al., 2016)

Table 2 (continued).

Ischemia-Reperfusion injury	Acute injury/Sterile inflammation	(Chen and Nuñez, 2010; Dehnadi et al., 2017; Edwards and Bix, 2019; Volmering et al., 2016; Yago et al., 2015)
Leukocyte adhesion deficiency (LAD)	Genetic disorder	(Kuijpers et al., 1997; Moser et al., 2009; Wen et al., 2021)
Myocardial Infarction	Cardiovascular disease/Sterile inflammation	(Chen and Nuñez, 2010; Daseke II et al., 2021; Meisel et al., 1998; Volmering et al., 2016)
Rheumatoid arthritis	Autoimmune disease	(Khawaja et al., 2019)
SARS-CoV-2	Infectious disease	(Narasaraju et al., 2020; Simons et al., 2021)
Sepsis	Acute illness	(Yuki and Hou, 2020)
Sepsis-induced acute lung injury	Acute injury	(Park et al., 2019; Xu et al., 2008)
Solid organ transplant rejection	Transplant rejection	(Shimizu et al., 2008)

Table 2 (continued).

Systemic lupus erythematosus (SLE)	Autoimmune disease	(Fagerholm et al., 2013; Khawaja et al., 2019; Rosetti and Mayadas, 2016; Zhou et al., 2013)
Thrombosis	Cardiovascular disease	(Wang et al., 2017)
Transfusion-related acute lung injury (TRALI)	Acute injury	(Berthold et al., 2015)
Trauma/Vascular injury	Acute injury	(Hahm et al., 2013; Henrich et al., 2011)
Wiskott Aldrich syndrome	Genetic disorder	(Candotti, 2018; Zhang et al., 2006)

Table 3. Most common cell lines in neutrophil beta2-integrin research⁋

Cell Line	Requires Differentiation	Endogenous β_2-Integrin Expression	Limitations/Drawbacks
HL60	Yes – DMSO or retinoic acid (referred to as dHL60s)	$\alpha_L\beta_2$, differentiation required for $\alpha_M\beta_2$	• DMSO dHL60s have different IL-8R, signaling proteins, and α-actinin compared to humans • Lower $\alpha_M\beta_2$ and dampened upregulation to fMLP and LTB_4 compared to humans
K562	No	No	• Requires stable transfection for $\alpha_M\beta_2$ expression
HoxB8	Yes – GM-CSF	Yes - $\alpha_L\beta_2$ and $\alpha_M\beta_2$	• Additional cytokines needed to achieve "mature' neutrophilic cells • Lower levels of ROS production compared to murine neutrophils due to decrease $gp91^{phox}$ expression • Lower chemotactic responses • Not suitable for degranulation experiments

(⁋ see article text for references relevant for Table 2)

Table 4. Neutrophil infiltration dependence on beta2-integrins across *in vivo* murine models

Disease state	B_2-integrin dependent?	References
Alzheimer's disease	Yes	(Zenaro et al., 2015)
Atrial fibrosis	Yes	(Friedrichs et al., 2014)
HMGB1-induced peritonitis	Mac-1: Yes LFA-1: No	(Orlova et al., 2007)
Influenza	No	(Tak et al., 2018)
LTB_4-induced intestinal transepithelial migration	Yes	(Azcutia et al., 2021)
Pneumonia *S. pneumoniae* *P. aeruginosa* *E. coli* LPS	 No Yes Yes	 (Mizgerd et al., 1999) (Mizgerd et al., 1999; Wilson et al., 2017) (Mizgerd et al., 1999)
Pulmonary aspergillosis	No	(Teschner et al., 2019)
SLE-induced glomerular disease	Mac-1: No LFA-1: Yes	(Kevil et al., 2004)
Thioglycollate peritonitis	Mac-1: No LFA-1: Yes	(Coxon et al., 1996; Mizgerd et al., 1997; Orlova et al., 2007)

REFERENCES

Abram, C.L., Lowell, C.A., 2009. The Ins and Outs of Leukocyte Integrin Signaling. Annu Rev Immunol 339–362. https://doi.org/10.1146/annurev.immunol.021908.132554

Alder, M.N., Mallela, J., Opoka, A.M., Lahni, P., Hildeman, D.A., Wong, H.R., 2018. Olfactomedin 4 marks a subset of neutrophils in mice. Innate Immun. 0, 1–12. https://doi.org/10.1177/1753425918817611

Andersen, M.N., Al-Karradi, S.N.H., Kragstrup, T.W., Hokland, M., 2016. Elimination of erroneous results in flow cytometry caused by antibody binding to Fc receptors on human monocytes and macrophages. Cytom. Part A 89, 1001–1009. https://doi.org/10.1002/cyto.a.22995

Atallah-Yunes, S.A., Ready, A., Newburger, P.E., 2019. Benign Ethnic Neutropenia. Blood September. https://doi.org/10.1016/j.blre.2019.06.003.Benign

Azcutia, V., Kelm, M., Luissint, A., Boerner, K., Flemming, S., Quiros, M., Newton, G., Nusrat, A., Luscinskas, F.W., Parkos, C.A., 2021. Neutrophil expressed CD47 regulates CD11b/CD18-dependent neutrophil transepithelial migration in the intestine in vivo. Mucosal Immunol 14, 331–341. https://doi.org/10.1038/s41385-020-0316-4.Neutrophil

Berthold, T., Glaubitz, M., Muschter, S., GroB, S., Palankar, R., Reil, A., Helm, C.A., Bakchoul, T., Schwertz, H., Bux, J., Greinacher, A., Delcea, M., 2015. Human neutrophil antigen-3a antibodies induce neutrophil stiffening and conformational activation of CD11b without shedding of L-selectin. Transfusion 55, 2939–48. https://doi.org/10.1111/trf.13299

Berton, G., Fumagalli, L., Laudanna, C., Sorio, C., 1994. β2 Integrin-dependent Protein Tyrosine Phosphorylation and Activation of the FGR Protein Tyrosine Kinase in Human Neutrophils. J. Cell Biol. 126, 1111–1121.

Bertram, A., Zhang, H., von Vietinghoff, S., de Pablo, C., Haller, H., Shushakova, N., Ley, K., 2012. Protein Kinase C-θ Is Required for Murine Neutrophil Recruitment and Adhesion Strengthening under Flow. J. Immunol. 188, 4043–4051. https://doi.org/10.4049/jimmunol.1101651

Blidberg, K., Palmberg, L., James, A., Billing, B., Henriksson, E., Lantz, A.S., Larsson, K., Dahlén, B., 2013. Adhesion molecules in subjects with COPD and healthy non-smokers: A cross sectional parallel group study. Respir. Res. 14, 1–10. https://doi.org/10.1186/1465-9921-14-47

Blythe, E.N., Weaver, L.C., Brown, A., Dekaban, G.A., 2021. β2 Integrin CD11d/CD18: From Expression to an Emerging Role in Staged Leukocyte Migration. Front. Immunol. 12, 1–16. https://doi.org/10.3389/fimmu.2021.775447

Borges, L., Pithon-Curi, T.C., Curi, R., Hatanaka, E., 2020. COVID-19 and Neutrophils: The Relationship between Hyperinflammation and Neutrophil Extracellular Traps. Mediators Inflamm. 2020, Article ID 8829674. https://doi.org/10.1155/2020/8829674

Bouti, P., Webbers, S.D.S., Fagerholm, S.C., Alon, R., Moser, M., Matlung, H.L., Kuijpers, T.W., 2021. β2 Integrin Signaling Cascade in Neutrophils: More Than a Single Function. Front. Immunol. 11, 1–20. https://doi.org/10.3389/fimmu.2020.619925

Bromberger, T., Klapproth, S., Rohwedder, I., Weber, J., Pick, R., Mittmann, L., Min-Weißenhorn, S.J., Reichel, C.A., Scheiermann, C., Sperandio, M., Moser, M., 2021. Binding of Rap1 and Riam to Talin1 Fine-Tune β2 Integrin Activity During Leukocyte Trafficking. Front. Immunol. 12, 1–10. https://doi.org/10.3389/fimmu.2021.702345

Burnett, A., Gomez, I., De Leon, D.D., Ariaans, M., Progias, P., Kammerer, R.A., Velasco, G., Marron, M., Hellewell, P., Ridger, V., 2017. Angiopoietin-1 enhances neutrophil

chemotaxis in vitro and migration in vivo through interaction with CD18 and release of CCL4. Sci. Rep. 7, 1–9. https://doi.org/10.1038/s41598-017-02216-y

Candotti, F., 2018. Clinical Manifestations and Pathophysiological Mechanisms of the Wiskott-Aldrich Syndrome. J. Clin. Immunol. 38, 13–27. https://doi.org/10.1007/s10875-017-0453-z

Celik, E., Faridi, M.H., Kumar, V., Deep, S., Moy, V.T., Gupta, V., 2013. Agonist leukadherin-1 increases CD11b/CD18-dependent adhesion via membrane tethers. Biophys. J. 105, 2517–2527. https://doi.org/10.1016/j.bpj.2013.10.020

Chen, G.Y., Nuñez, G., 2010. Sterile inflammation: Sensing and reacting to damage. Nat. Rev. Immunol. 10, 826–837. https://doi.org/10.1038/nri2873

Chu, J.Y., McCormick, B., Mazelyte, G., Michael, M., Vermeren, S., 2019. HoxB8 neutrophils replicate Fcγ receptor and integrin-induced neutrophil signaling and functions. J. Leukoc. Biol. 105, 93–100. https://doi.org/10.1002/JLB.1AB0618-232R

Cox, D., Aoki, T., Seki, J., Motoyama, Y., Yoshida, K., 1994. The pharmacology of the integrins. Med. Res. Rev. 14, 195–228. https://doi.org/10.1002/med.2610140203

Coxon, A., Rieu, P., Barkalow, F.J., Askari, S., Sharpe, A.H., Von Andrian, U.H., Arnaout, M.A., Mayadas, T.N., 1996. A novel role for the β2 integrin CD11b/CD18 in neutrophil apoptosis: A homeostatic mechanism in inflammation. Immunity 5, 653–666. https://doi.org/10.1016/S1074-7613(00)80278-2

Daseke II, M.J., Chalise, U., Becirovic-Agic, M., Salomon, J.D., Cook, L.M., Case, A.J., Lindsey, M.L., 2021. Neutrophil signaling during myocardial infarction wound repair. Cell. Signal. 77, 109816. https://doi.org/10.1016/j.cellsig.2020.109816

Dehnadi, A., Cosimi, A.B., Smith, R.N., Li, X., Alonso, J.L., Means, T.K., Arnaout, M.A., 2017. Prophylactic orthosteric inhibition of leukocyte integrin CD11b/CD18 prevents long-term

fibrotic kidney failure in cynomolgus monkeys. Nat. Commun. 8, 1–11. https://doi.org/10.1038/ncomms13899

Diacovo, T.G., Roth, S.J., Buccola, J.M., Bainton, D.F., Springer, T.A., 1996. Neutrophil Rolling, Arrest, and Transmigration Across Activated, Surface-Adherent Platelets Via Sequential Action of P-Selectin and the β2-Integrin CDllb/CDl8. Blood 88, 146–157.

Diamond, M.S., Springer, T.A., 1993. A Subpopulation of Mac-1 (CDllb/CD18) Molecules Mediates Neutrophil Adhesion to ICAM-1 and Fibfinogen. J. Cell Biol. 120, 545–556.

Dorward, D.A., Lucas, C.D., Alessandri, A.L., Marwick, J.A., Rossi, F., Dransfield, I., Haslett, C., Dhaliwal, K., Rossi, A.G., 2013. Technical Advance: Autofluorescence-based sorting: rapid and nonperturbing isolation of ultrapure neutrophils to determine cytokine production. J. Leukoc. Biol. 94, 193–202. https://doi.org/10.1189/jlb.0113040

Dove, A., 2000. CD18 trials disappoint again. Nat. Biotechnol. 18, 817–818. https://doi.org/10.1038/78410

Dumont, A.L., Yoong, P., Day, C.J., Iii, F.A., Mcdonald, W.H., Jennings, M.P., Torres, V.J., 2013. Staphylococcus aureus LukAB cytotoxin kills human neutrophils by targeting the CD11b subunit of the integrin Mac-1. PNAS 110, 10794–10799. https://doi.org/10.1073/pnas.1305121110

Ecker, S., Chen, L., Pancaldi, V., Bagger, F.O., Fernández, J.M., Carrillo de Santa Pau, E., Juan, D., Mann, A.L., Watt, S., Casale, F.P., Sidiropoulos, N., Rapin, N., Merkel, A., Stunnenberg, H.G., Stegle, O., Frontini, M., Downes, K., Kuijpers, T.W., Kuijpers, T.W., Rico, D., Valencia, A., Beck, S., Soranzo, N., Paul, D.S., Albers, C.A., Amstislavskiy, V., Ashford, S., Bomba, L., Bujold, D., Burden, F., Busche, S., Caron, M., Chen, S.H., Cheung, W.A., Clarke, L., Colgiu, I., Datta, A., Delaneau, O., Elding, H., Farrow, S., Garrido-

Martín, D., Ge, B., Guigo, R., Iotchkova, V., Kundu, K., Kwan, T., Lambourne, J.J., Lowy, E., Mead, D., Pourfarzad, F., Redensek, A., Rehnstrom, K., Rendon, A., Richardson, D., Risch, T., Rowlston, S., Shao, X., Simon, M.M., Sultan, M., Walter, K., Wilder, S.P., Yan, Y., Antonarakis, S.E., Bourque, G., Dermitzakis, E.T., Flicek, P., Lehrach, H., Martens, J.H.A., Yaspo, M.L., Ouwehand, W.H., 2017. Genome-wide analysis of differential transcriptional and epigenetic variability across human immune cell types. Genome Biol. 18, 1–17. https://doi.org/10.1186/s13059-017-1156-8

Eckert, R.E., Neuder, L.E., Park, J., Adler, K.B., Jones, S.L., 2010. Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) Protein Regulation of Human Neutrophil Migration. Am. J. Respir. Cell Mol. Biol. 42, 586–594. https://doi.org/10.1165/rcmb.2008-0394OC

Edwards, D.N., Bix, G.J., 2019. The Inflammatory Response After Ischemic Stroke: Targeting β2 and β1 Integrins. Front. Neurosci. 13, 540. https://doi.org/10.3389/fnins.2019.00540

Eruslanov, E.B., Singhal, S., Albelda, S.M., 2017. Mouse versus Human Neutrophils in Cancer: A Major Knowledge Gap. Trends in Cancer 3, 149–160. https://doi.org/10.1016/j.trecan.2016.12.006

Evans, R., Lellouch, A.C., Svensson, L., Mcdowall, A., Hogg, N., 2011. The integrin LFA-1 signals through ZAP-70 to regulate expression of high-affinity LFA-1 on T lymphocytes. Blood 117, 3331–3342. https://doi.org/10.1182/blood-2010-06

Fagerholm, S.C., Macpherson, M., James, M.J., Sevier-Guy, C., Lau, C.S., 2013. The CD11b-integrin (ITGAM) and systemic lupus erythematosus. Lupus 22, 657–663. https://doi.org/10.1177/0961203313491851

Feng, C., Zhang, L., Almulki, L., Faez, S., Whitford, M., Hafezi-Moghadam, A., Cross, A.S., 2011. Endogenous PMN sialidase activity exposes activation epitope on CD11b/CD18

which enhances its binding interaction with ICAM-1. J. Leukoc. Biol. 90, 313–321. https://doi.org/10.1189/jlb.1210708

Friedrichs, K., Adam, M., Remane, L., Mollenhauer, M., Rudolph, V., Rudolph, T.K., Andrié, R.P., Stö Ckigt, F., Schrickel, J.W., Ravekes, T., Deuschl, F., Nickenig, G., Willems, S., Baldus, S., Klinke, A., 2014. Induction of Atrial Fibrillation by Neutrophils Critically Depends on CD11b/CD18 Integrins. PLoS One 9. https://doi.org/10.1371/journal.pone.0089307

Gorina, R., Lyck, R., Vestweber, D., Engelhardt, B., 2014. β2 Integrin-Mediated Crawling on Endothelial ICAM-1 and ICAM-2 Is a Prerequisite for Transcellular Neutrophil Diapedesis across the Inflamed Blood-Brain Barrier. J. Immunol. 192, 324–337. https://doi.org/10.4049/jimmunol.1300858

Graham, I.L., Lefkowith, J.B., Anderson, D.C., Brown, E.J., Leikowith, J.B., Anderson, D.C., Browntln, E.J., 1993. Immune complex-stimulated neutrophil LTB4 production is dependent on β2 integrins. J. Cell Biol. 120, 1509–1517. https://doi.org/10.1083/jcb.120.6.1509

Gupta, S., Lee, C.M., Wang, J.F., Parodo, J., Jia, S.H., Hu, J., Marshall, J.C., 2018. Heat-shock protein-90 prolongs septic neutrophil survival by protecting c-Src kinase and caspase-8 from proteasomal degradation. J. Leukoc. Biol. 103, 933–944. https://doi.org/10.1002/JLB.4A0816-354R

Hafeez Faridi, M., Altintas, M.M., Gomez, C., Camilo Doque, J., Vazquez-Padron, R.I., Gupta, V., 2013. Small molecular agonists of integrin CD11b/CD18 do not induce global conformational changes and are significantly better than activating antibodies in reducing vascular injury. Biochim Biophys Acta 1830, 3696–3710.

https://doi.org/10.1016/j.bbagen.2013.02.018.Small

Hahm, E., Li, J., Kim, K., Huh, S., Rogelj, S., Cho, J., 2013. Extracellular protein disulfide isomerase regulates ligand-binding activity of αMβ2 integrin and neutrophil recruitment during vascular inflammation. Blood 121, 3789–800, S1-15. https://doi.org/10.1182/blood-2012-11-467985

Han, Y., Gu, Y., Zhang, A.C., Lo, Y.H., 2016. Review: Imaging technologies for flow cytometry. Lab Chip 16, 4639–4647. https://doi.org/10.1039/c6lc01063f

Harlan, J.M., Winn, R.K., 2002. Leukocyte-endothelial interactions: Clinical trials of anti-adhesion therapy. Crit. Care Med. 30. https://doi.org/10.1097/00003246-200205001-00007

Hauert, A.B., Martinelli, S., Marone, C., Niggli, V., 2002. Differentiated HL-60 cells are a valid model system for the analysis of human neutrophil migration and chemotaxis. Int. J. Biochem. Cell Biol. 34, 838–854. https://doi.org/10.1016/S1357-2725(02)00010-9

Helou, D.G., Braham, S., De Chaisemartin, L., Granger, V., Damien, M.-H., Pallardy, M., Kerdine-Römer, S., Chollet-Martin, S., 2019. Nrf2 downregulates zymosan-induced neutrophil activation and modulates migration. PLoS One 14, e0216465. https://doi.org/10.1371/journal.pone.0216465

Henrich, D., Zimmer, S., Seebach, C., Frank, J., Barker, J., Marzi, I., 2011. Trauma-activated polymorphonucleated leukocytes damage endothelial progenitor cells: Probable role of CD11b/CD18-CD54 interaction and release of reactive oxygen species. Shock 36, 216–222. https://doi.org/10.1097/SHK.0b013e3182236eba

Hickstein, D.D., Smith, A., Fisher, W., Beatty, P.G., Schwartz, B.R., Harlan, J.M., Root, R.K., Locksley, R.M., 1987. Expression of leukocyte adherence-related glycoproteins during differentiation of HL-60 promyelocytic leukemia cells. J. Immunol. 138, 513–9.

Jakus, Z., Berton, G., Ligeti, E., Lowell, C.A., Mócsai, A., 2004. Responses of Neutrophils to Anti-Integrin Antibodies Depends on Costimulation through Low Affinity FcγRs: Full Activation Requires Both Integrin and Nonintegrin Signals. J. Immunol. 173, 2068–2077. https://doi.org/10.4049/jimmunol.173.3.2068

Jakus, Z.N., Fodor, S., Abram, C.L., Lowell, C.A., Mócsai, A., 2007. Immunoreceptor-like signaling by β2 and β3 integrins. Trends Cell Biol. 17, 493–501. https://doi.org/10.1016/j.tcb.2007.09.001

Jones, S.L., Knaus, U.G., Bokoch, G.M., Brown, E.J., 1998. Two signaling mechanisms for activation of α(M)β2 avidity in polymorphonuclear neutrophils. J. Biol. Chem. 273, 10556–10566. https://doi.org/10.1074/jbc.273.17.10556

Kaboord, B., Perr, M., 2008. Isolation of Proteins and Protein Complexes by Immunoprecipitation, in: Posch, A. (Ed.), 2D PAGE: Sample Preparation and Fractionation. Humana Press, Totowa, NJ, pp. 349–364. https://doi.org/10.1007/978-1-60327-064-9_27

Kevil, C.G., Hicks, M.J., He, X., Zhang, J., Ballantyne, C.M., Raman, C., Schoeb, T.R., Bullard, D.C., 2004. Loss of LFA-1, but not Mac-1, protects MRL/MpJ-Faslpr mice from autoimmune disease. Am. J. Pathol. 165, 609–616. https://doi.org/10.1016/S0002-9440(10)63325-1

Khawaja, A.A., Pericleous, C., Ripoll, V.M., Porter, J.C., Giles, I.P., 2019. Autoimmune rheumatic disease IgG has differential effects upon neutrophil integrin activation that is modulated by the endothelium. Sci. Rep. 9. https://doi.org/10.1038/s41598-018-37852-5

Kim, H.Y., Skokos, E.A., Myer, D.J., Agaba, P., Gonzalez, A.L., 2014. Avβ3 Integrin Regulation of Respiratory Burst in Fibrinogen Adherent Human Neutrophils. Cell. Mol. Bioeng. 7, 231–242. https://doi.org/10.1007/s12195-014-0322-2

Kranig, S.A., Lajqi, T., Tschada, R., Braun, M., Kuss, N., Pöschl, J., Hudalla, H., 2020. Leukocyte infiltration of cremaster muscle in mice assessed by intravital microscopy. J. Vis. Exp. 2020, 1–10. https://doi.org/10.3791/60509

Kuhns, D.B., Long Priel, D.A., Chu, J., Zarember, K.A., 2016. Isolation and Functional Analysis of Human Neutrophils. Curr Protoc Immunol 111, 7.23.1-7.23.16. https://doi.org/10.1002/0471142735.im0723s111.Isolation

Kuijpers, T W, Verhoeven, A J, Roos, D, Kuijpers, Taco W, Van Lier, R.A.W., Hamann, D., De Boer, M., Yin Thung, L., Weening, R.S., Verhoeven, Arthur J, Roos, Dirk, 1997. Leukocyte adhesion deficiency type 1 (LAD-1)/variant. A novel immunodeficiency syndrome characterized by dysfunctional beta2 integrins. J. Clin. Invest 100, 1725–1733. https://doi.org/10.1172/JCI119697

Lämmermann, T., Germain, R.N., 2014. The multiple faces of leukocyte interstitial migration. Semin Immunpathol 36, 227–251. https://doi.org/10.1007/s00281-014-0418-8

Lau, D., Mollnau, H., Eiserich, J.P., Freeman, B.A., Daiber, A., Gehling, U.M., Brü, J., Rudolph, V., Mü, T., Heitzer, T., Meinertz, T., Baldus, S., 2005. Myeloperoxidase mediates neutrophil activation by association with CD11bCD18 integrins. PNAS 102, 431–436.

Launay, S., Brown, G., Machesky, L.M., 2003. Expression of WASP and Scar1/WAVE1 actin-associated proteins is differentially modulated during differentiation of HL-60 cells. Cell Motil. Cytoskeleton 54, 274–285. https://doi.org/10.1002/cm.10101

Lefort, C.T., Hyun, Y.-M., Schultz, J.B., Law, F.-Y., Waugh, R.E., Knauf, P.A., Kim, M., 2009. Conformational Changes in Integrin Mac-1 Outside-In Signal Transmission by. J. Immunol. 183, 6460–6468. https://doi.org/10.4049/jimmunol.0900983

Li, X., Utomo, A., Cullere, X., Choi, M.M., Milner, D.A., Venkatesh, D., Yun, S.H., Mayadas,

T.N., 2011. The β-glucan receptor dectin-1 activates the integrin Mac-1 in neutrophils via vav protein signaling to promote Candida albicans clearance. Cell Host Microbe 10, 603–615. https://doi.org/10.1016/j.chom.2011.10.009

Liu, Y.G., Teng, Y.S., Cheng, P., Kong, H., Lv, Y.P., Mao, F.Y., Wu, X.L., Hao, C.J., Chen, W., Yang, S.M., Zhang, J.Y., Peng, L.S., Wang, T.T., Han, B., Ma, Q., Zou, Q.M., Zhuang, Y., 2019. Abrogation of cathepsin C by Helicobacter pylori impairs neutrophil activation to promote gastric infection. FASEB J. 33, 5018–5033. https://doi.org/10.1096/fj.201802016RR

Lowell, C.A., Fumagalli, L., Berton, G., 1996. Deficiency of src family kinases p59/61hck and p58c-fgr results in defective adhesion-dependent neutrophil functions. J. Cell Biol. 133, 895–910. https://doi.org/10.1083/jcb.133.4.895

Marchetti, L., Engelhardt, B., 2020. Immune cell trafficking across the blood-brain barrier in the absence and presence of neuroinflammation. Vasc. Biol. 2, H1–H18. https://doi.org/10.1530/vb-19-0033

Margraf, A., Ley, K., Zarbock, A., 2019. Neutrophil Recruitment: From Model Systems to Tissue-Specific Patterns. Trends Immunol 40, 613–634. https://doi.org/10.1016/j.it.2019.04.010.Neutrophil

Matsumoto, K., Kurasawa, T., Yoshimoto, K., Suzuki, K., Takeuchi, T., 2021. Identification of neutrophil β2-integrin LFA-1 as a potential mechanistic biomarker in ANCA-associated vasculitis via microarray and validation analyses. Arthritis Res. Ther. 23, 1–12. https://doi.org/10.1186/s13075-021-02510-1

McMillan, S.J., Sharma, R.S., Mckenzie, E.J., Richards, H.E., Zhang, J., Prescott, A., Crocker, P.R., 2013. Siglec-E is a negative regulator of acute pulmonary neutrophil inflammation and

suppresses CD11b β2-integrin-dependent signaling. Blood 121, 2084–2094. https://doi.org/10.1182/blood

Mehta, R., Petrova, A., 2006. Intrapartum magnesium sulfate exposure attenuates neutrophil function in preterm neonates. Biol. Neonate 89, 99–103. https://doi.org/10.1159/000088560

Meisel, S.R., Shapiro, H., Radnay, J., Neuman, Y., Khaskia, A.R., Gruener, N., Pauzner, H., David, D., 1998. Increased expression of neutrophil and monocyte adhesion molecules LFA- 1 and Mac-1 and their ligand ICAM-1 and VLA-4 throughout the acute phase of myocardial infarction: Possible implications for leukocyte aggregation and microvascular plugging. J. Am. Coll. Cardiol. 31, 120–125. https://doi.org/10.1016/S0735-1097(97)00424-5

Mestas, J., Hughes, C.C.W., 2004. Of Mice and Not Men: Differences between Mouse and Human Immunology. J. Immunol. 172, 2731–2738. https://doi.org/10.4049/jimmunol.172.5.2731

Mitroulis, I., Alexaki, V.I., Kourtzelis, I., Ziogas, A., Hajishengallis, G., Chavakis, T., 2015. Leukocyte integrins: Role in leukocyte recruitment and as therapeutic targets in inflammatory disease. Pharmacol Ther 0, 123–135. https://doi.org/10.1016/j.pharmthera.2014.11.008

Mizgerd, B.J.P., Kubo, H., Kutkoski, G.J., Bhagwan, S.D., Scharffetter-kochanek, K., Beaudet, A.L., Doerschuk, C.M., 1997. Neutrophil Emigration in the Skin, Lungs, and Peritoneum: Different Requirements for CD11/CD18 Revealed by CD18-deficient Mice. J. Exp. Med 186, 1357–1364.

Mizgerd, J.P., Horwitz, B.H., Quillen, H.C., Scott, M.L., Doerschuk, C.M., 1999. Effects of CD18 deficiency on the emigration of murine neutrophils during pneumonia. J. Immunol.

163, 995–999.

Mócsai, A., Abram, C.L., Jakus, Z., Hu, Y., Lanier, L.L., Lowell, C.A., 2006. Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs. Nat. Immunol. 7, 1326–1333. https://doi.org/10.1038/ni1407

Mócsai, A., Zhou, M., Meng, F., Tybulewicz, V.L., Lowell, C.A., C, by F., Francisco, S., 2002. Syk is required for integrin signaling in neutrophils. Immunity 16, 547–558. https://doi.org/10.1016/S1074-7613(02)00303-5

Monsel, A., Lécart, S., Roquilly, A., Broquet, A., Jacqueline, C., Mirault, T., Troude, T., Fontaine-Aupart, M.P., Asehnoune, K., 2014. Analysis of autofluorescence in polymorphonuclear neutrophils: A new tool for early infection diagnosis. PLoS One 9, 1–10. https://doi.org/10.1371/journal.pone.0092564

Montresor, A., Toffali, L., Constantin, G., Laudanna, C., Ley, K., 2012. Chemokines and the signaling modules regulating integrin affinity. Front. Immunol. 3. https://doi.org/10.3389/fimmu.2012.00127

Moser, M., Bauer, M., Schmid, S., Ruppert, R., Schmidt, S., Sixt, M., Wang, H.V., Sperandio, M., Fässler, R., 2009. Kindlin-3 is required for β2 integrin-mediated leukocyte adhesion to endothelial cells. Nat. Med. 15, 300–305. https://doi.org/10.1038/nm.1921

Naegele, M., Tillack, K., Reinhardt, S., Schippling, S., Martin, R., Sospedra, M., 2012. Neutrophils in multiple sclerosis are characterized by a primed phenotype. J. Neuroimmunol. 242, 60–71. https://doi.org/10.1016/j.jneuroim.2011.11.009

Narasaraju, T., Tang, B.M., Herrmann, M., Muller, S., Chow, V.T.K., Radic, M., 2020. Neutrophilia and NETopathy as Key Pathologic Drivers of Progressive Lung Impairment in Patients With COVID-19. Front. Pharmacol. 11. https://doi.org/10.3389/fphar.2020.00870

Newton, R.A., Hogg, N., 1998. The Human S100 Protein MRP-14 Is a Novel Activator of the β2 Integrin Mac-1 on Neutrophils. J Immunol Ref. 160, 1427–1435.

Orlova, V. V, Choi, E.Y., Xie, C., Chavakis, E., Bierhaus, A., Ihanus, E., Ballantyne, C.M., Gahmberg, C.G., Bianchi, M.E., Nawroth, P.P., Chavakis, T., 2007. A novel pathway of HMGB1-mediated inflammatory cell recruitment that requires Mac-1-integrin. EMBO J. 26, 1129–1139. https://doi.org/10.1038/sj.emboj.7601552

Overbeek, S.A., Braber, S., Henricks, P.A.J., Kleinjan, M., Kamp, V.M., Georgiou, N.A., Garssen, J., Kraneveld, A.D., Folkerts, G., 2011. Cigarette smoke induces b 2-integrin-dependent neutrophil migration across human endothelium. Respir. Res. 12, 75. https://doi.org/10.1186/1465-9921-12-75

Park, I., Kim, M., Choe, K., Song, E., Seo, H., Hwang, Y., Ahn, J., Lee, S.H., Lee, J.H., Jo, Y.H., Kim, K., Koh, G.Y., Kim, P., 2019. Neutrophils disturb pulmonary microcirculation in sepsis-induced acute lung injury. Eur. Respir. J. 53. https://doi.org/10.1183/13993003.00786-2018

Patcha, V., Wigren, J., Winberg, M.E., Rasmusson, B., Li, J., S7rndahl, E., 2004. Differential inside-out activation of β2-integrins by leukotriene B4 and fMLP in human neutrophils. Exp. Cell Res. 300, 308–319. https://doi.org/10.1016/j.yexcr.2004.07.015

Penberthy, T., Jiang, Y., Luscinskas, F., Graves, D., 1995. MCP-1-stimulated monocytes preferentially utilize β2-integrins to migrate on laminin and fibronectin. Am. J. Physiol. 269, C60–C68.

Phillipson, M., Heit, B., Colarusso, P., Liu, L., Ballantyne, C.M., Kubes, P., 2006. Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade. J. Exp. Med. 203, 2569–2575.

https://doi.org/10.1084/jem.20060925

Pillay, J., Den Braber, I., Vrisekoop, N., Kwast, L.M., De Boer, R.J., Borghans, J.A.M., Tesselaar, K., Koenderman, L., 2010. In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. Blood 116, 625–627. https://doi.org/10.1182/blood-2010-01-259028

Quach, A., Ferrante, A., 2017. The Application of Dextran Sedimentation as an Initial Step in Neutrophil Purification Promotes Their Stimulation, due to the Presence of Monocytes. J. Immunol. Res. 2017, 10 pages. https://doi.org/10.1155/2017/1254792

Raab-Westphal, S., Marshall, J.F., Goodman, S.L., 2017. Integrins as therapeutic targets: Successes and cancers. Cancers (Basel). 9, 1–28. https://doi.org/10.3390/cancers9090110

Rivadeneyra, L., Charó, N., Kviatcovsky, D., de la Barrera, S., Gómez, R.M., Schattner, M., 2018. Role of neutrophils in CVB3 infection and viral myocarditis. J. Mol. Cell. Cardiol. 125, 149–161. https://doi.org/10.1016/j.yjmcc.2018.08.029

Rosetti, F., Mayadas, T.N., 2016. The many faces of Mac-1 in autoimmune disease. Immunol. Rev. 269, 175–193. https://doi.org/10.1111/imr.12373

Rossaint, J., Herter, J.M., Aken, H. Van, Napirei, M., Oring, Y.D.¨, Weber, C., Soehnlein, O., Zarbock, A., 2014. Synchronized integrin engagement and chemokine activation is crucial in neutrophil extracellular trap-mediated sterile inflammation. Blood 123, 2573–2584. https://doi.org/10.1182/blood-2013-07

Ruoslahti, E., Pierschbacher, M.D., 1987. New Perspectives in Cell Adhesion: RGD and Integrins. Science (80-.). 238, 491–497.

Saul, M.C., Philip, V.M., Reinholdt, L.G., Chesler, E.J., 2019. High-Diversity Mouse Populations for Complex Traits. Trends Genet. 35, 501–514.

https://doi.org/10.1016/j.tig.2019.04.003

Saul, S., Castelbou, C., Fickentscher, C., Demaurex, N., 2019. Signaling and functional competency of neutrophils derived from bone-marrow cells expressing the ER-HOXB8 oncoprotein. J. Leukoc. Biol. 106, 1101–1115. https://doi.org/10.1002/JLB.2A0818-314R

Schmidt, S., Moser, M., Sperandio, M., 2013. The molecular basis of leukocyte recruitment and its deficiencies. Mol. Immunol. 55, 49–58. https://doi.org/10.1016/j.molimm.2012.11.006

Schweizer, T.A., Mairpady Shambat, S., Vulin, C., Hoeller, S., Acevedo, C., Huemer, M., Gomez-Mejia, A., Chang, C., Baum, J., Hertegonne, S., Hitz, E., Scheier, T.C., Hofmaenner, D.A., Buehler, P.K., Moch, H., Schuepbach, R.A., Brugger, S.D., Zinkernagel, A.S., 2021. Blunted sFasL signalling exacerbates TNF-driven neutrophil necroptosis in critically ill COVID-19 patients. Clin. Transl. Immunol. 10, 1–12. https://doi.org/10.1002/cti2.1357

Schymeinsky, J., Mocsai, A., Walzog, B., 2007. Neutrophil activation via β2 integrins (CD11/CD18): Molecular mechanisms and clinical implications. Thromb. Haemost. 98, 262–273. https://doi.org/10.1160/TH07

Sham, R.L., Phatak, P.D., Belanger, K.A., Packman, C.H., 1995. Functional properties of HL60 cells matured with all-trans-retinoic acid and DMSO: Differences in response to interleukin-8 and fMLP. Leuk. Res. 19, 1–6. https://doi.org/10.1016/0145-2126(94)00063-G

Sheats, M.K., Pescosolido, K.C., Hefner, E.M., Sung, J., Adler, K.B., Jones, S.L., 2014. Myristoylated Alanine Rich C Kinase Substrate (MARCKS) is essential to β2-integrin dependent responses of equine neutrophils. Vet Immunol Immunopathol 160, 167–176. https://doi.org/10.1016/j.vetimm.2014.04.009

Shimizu, K., Libby, P., Shubiki, R., Sakuma, M., Wang, Y., Asano, K., Mitchell, R.N., Simon,

D.I., 2008. Leukocyte integrin Mac-1 promotes acute cardiac allograft rejection. Circulation 117, 1997–2008. https://doi.org/10.1161/CIRCULATIONAHA.107.724310

Shimizu, Y., Mobley, J.L., Finkelstein, L.D., Chan, A.S.H., 1995. A role for phosphatidylinositol 3-kinase in the regulation of β1 integrin activity by the CD2 antigen. J. Cell Biol. 131, 1867–1880. https://doi.org/10.1083/jcb.131.6.1867

Shrestha, D., Jenei, A., Nagy, P., Vereb, G., Szöllősi, J., 2015. Understanding FRET as a research tool for cellular studies. Int. J. Mol. Sci. 16, 6718–6756. https://doi.org/10.3390/ijms16046718

Silva, J.C., Rodrigues, N.C., Thompson-Souza, G.A., Muniz, V. de S., Neves, J.S., Figueiredo, R.T., 2020. Mac-1 triggers neutrophil DNA extracellular trap formation to Aspergillus fumigatus independently of PAD4 histone citrullination. J. Leukoc. Biol. 107, 69–83. https://doi.org/10.1002/JLB.4A0119-009RR

Silvestre-Roig, C., Fridlender, Z.G., Glogauer, M., Scapini, P., 2019. Neutrophil Diversity in Health and Disease. Trends Immunol. 40, 565–583. https://doi.org/10.1016/j.it.2019.04.012

Simons, P., Rinaldi, D.A., Bondu, V., Kell, A.M., Bradfute, S., Lidke, D.S., Buranda, T., 2021. Integrin activation is an essential component of SARS-CoV-2 infection. Sci. Rep. 11, 1–12. https://doi.org/10.1038/s41598-021-99893-7

Smirnov, A., Solga, M., Lannigan, J., Criss, A., 2020. Using Imaging Flow Cytometry to Quantify Neutrophil Phagocytosis, in: Neutrophil. Methods in Molecular Biology, Vol 2087.

Spillmann, C., Osorio, D., Waugh, R., 2002. Integrin activation by divalent ions affects neutrophil homotypic adhesion. Ann. Biomed. Eng. 30, 1002–1011. https://doi.org/10.1114/1.1511241

Sule, G., Kelley, W.J., Gockman, K., Yalavarthi, S., Vreede, A.P., Banka, A.L., Bockenstedt, P.L., Eniola-Adefeso, O., Knight, J.S., 2020. Increased Adhesive Potential of Antiphospholipid Syndrome Neutrophils Mediated by β2 Integrin Mac-1. Arthritis Rheumatol. 72, 114–124. https://doi.org/10.1002/art.41057

Szczur, K., Zheng, Y., Filippi, M.-D.D., 2009. The small Rho GTPase Cdc42 regulates neutrophil polarity via CD11b integrin signaling. Blood 114, 4527–4537. https://doi.org/10.1182/blood-2008-12-195164

Tak, T., Rygiel, T.P., Karnam, G., Bastian, O.W., Boon, L., Viveen, M., Coenjaerts, F.E., Meyaard, L., Koenderman, L., Pillay, J., 2018. Neutrophil-mediated Suppression of Influenza-induced Pathology Requires CD11b/CD18 (MAC-1). Am. J. Respir. Cell Mol. Biol. 58, 492–499. https://doi.org/10.1165/rcmb.2017-0021OC

Tak, T., Tesselaar, K., Pillay, J., Borghans, J.A.M., Koenderman, L., 2013. What's your age again? Determination of human neutrophil half-lives revisited. J. Leukoc. Biol. 94, 595–601. https://doi.org/10.1189/jlb.1112571

Takahashi, M., Nagao, T., Matsuzaki, K., Nishimura, T., Minamitani, H., 2002. Photodynamically induced endothelial cell injury and neutrophil-like HL-60 adhesion.pdf. J. Photoscience 9, 518–520.

Teschner, D., Cholaszczyńska, A., Ries, F., Beckert, H., Theobald, M., Grabbe, S., Radsak, M., Bros, M., 2019. CD11b regulates fungal outgrowth but not neutrophil recruitment in a mouse model of invasive pulmonary aspergillosis. Front. Immunol. 10. https://doi.org/10.3389/fimmu.2019.00123

Ueda, T., Rieu, P., Brayer, J., Amin Arnaout, M., 1994. Identification of the complement iC3b binding site in the β2 integrin CR3 (CD11b/CD18). Proc. Nati. Acad. Sci. USA 91, 10680–

10684.

Villanueva, E., Yalavarthi, S., Berthier, C.C., Hodgin, J.B., Khandpur, R., Lin, A.M., Rubin, C.J., Zhao, W., Olsen, S.H., Klinker, M., Shealy ||, D., Denny, M.F., Plumas, J., Chaperot, L., Kretzler, M., Bruce, A.T., Kaplan, M.J., 2012. Netting Neutrophils Induce Endothelial Damage, Infiltrate Tissues, and Expose Immunostimulatory Molecules in Systemic Lupus Erythematosus. J Immunol 187, 538–552. https://doi.org/10.4049/jimmunol.1100450

Volmering, S., Block, H., Boras, M., Lowell, C.A., Zarbock, A., 2016. The Neutrophil Btk Signalosome Regulates Integrin Activation during Sterile Inflammation. Immunity 44, 73–87. https://doi.org/10.1016/j.immuni.2015.11.011

Wang, Y., Gao, H., Shi, C., Erhardt, P.W., Pavlovsky, A., Soloviev, D.A., Bledzka, K., Ustinov, V., Zhu, L., Qin, J., Munday, A.D., Lopez, J., Plow, E., Simon, D.I., 2017. Leukocyte integrin Mac-1 regulates thrombosis via interaction with platelet GPIbα. Nat. Commun. 8. https://doi.org/10.1038/ncomms15559

Weber, C., Lu, F., Casasnovas, J.M., Springer, T.A., 1997. Role of alpha L beta 2 integrin avidity in transendothelial chemotaxis of mononuclear cells. J. Immunol. Methods 159, 3968–3975.

Wen, L., Marki, A., Roy, P., McArdle, S., Sun, H., Fan, Z., Gingras, A.R., Ginsberg, M.H., Ley, K., 2021. Kindlin-3 recruitment to the plasma membrane precedes high affinity β 2 integrin and neutrophil arrest from rolling. Blood 137, 29–38. https://doi.org/10.1182/blood.2019003446/1752695/blood.2019003446.pdf

Willeke, T., Schymeinsky, J., Prange, P., Zahler, S., Walzog, B., 2003. A role for Syk-kinase in the control of the binding cycle of the β2 integrins (CD11/CD18) in human polymorphonuclear neutrophils. J. Leukoc. Biol. 74, 260–269.

https://doi.org/10.1189/jlb.0102016

Williams, M.A., Solomkin, J.S., 1999. Integrin-mediated signaling in human neutrophil functioning. J. Leukoc. Biol. 65, 725–736. https://doi.org/10.1002/jlb.65.6.725

Wilson, M.E., Mccandless, E.E., Olszewski, M.A., Robinson, N.E., 2020. VA Ann Arbor Healthcare System. Res. Serv. https://doi.org/10.1016/j.tvjl.2020.105436

Wilson, Z.S., Ahn, L.B., Serratelli, W.S., Belley, M.D., Lomas-Neira, J., Sen, M., Lefort, C.T., 2017. Activated β2 integrins restrict neutrophil recruitment during murine acute pseudomonal pneumonia. Am. J. Respir. Cell Mol. Biol. 56, 620–627. https://doi.org/10.1165/rcmb.2016-0215OC

Wilson, Z.S., Witt, H., Hazlett, L., Harman, M., Neumann, B.M., Whitman, A., Patel, M., Ross, R.S., Franck, C., Reichner, J.S., Lefort, C.T., 2020. Context-Dependent Role of Vinculin in Neutrophil Adhesion, Motility and Trafficking. Sci. Rep. 10, 1–14. https://doi.org/10.1038/s41598-020-58882-y

Wong, S.L., Demers, M., Martinod, K., Gallant, M., Wang, Y., Goldfine, A.B., Kahn, C.R., Wagner, D.D., 2015. Diabetes primes neutrophils to undergo NETosis, which impairs wound healing. Nat. Med. 21, 815–819. https://doi.org/10.1038/nm.3887

Woolhouse, I.S., Bayley, D.L., Lalor, P., Adams, D.H., Stockley, R.A., 2005. Endothelial interactions of neutrophils under flow in chronic obstructive pulmonary disease. Eur. Respir. J. 25, 612–617. https://doi.org/10.1183/09031936.05.00086304

Wright, H.L., Makki, F.A., Moots, R.J., Edwards, S.W., 2017. Low-density granulocytes: functionally distinct, immature neutrophils in rheumatoid arthritis with altered properties and defective TNF signalling. J. Leukoc. Biol. 101, 599–611. https://doi.org/10.1189/jlb.5a0116-022r

Xu, J., Gao, X.-P., Ramchandran, R., Zhao, Y.-Y., Vogel, S.M., Malik, A.B., 2008. Nonmuscle myosin light-chain kinase mediates neutrophil transmigration in sepsis-induced lung inflammation by activating β2 integrins. Nat. Imm 9, 880–886. https://doi.org/10.1038/ni.1628

Xu, Z., Cai, J., Gao, J., White Ii, G.C., Chen, F., Ma, Y.-Q., 2015. Interaction of kindlin-3 and b 2-integrins differentially regulates neutrophil recruitment and NET release in mice. Blood 126, 373–377. https://doi.org/10.1182/blood-2015-03-636720

Xue, Z.-H., Feng, C., Liu, W.-L., Tan, S.-M., 2013. A Role of Kindlin-3 in Integrin aMβ2 Outside-In Signaling and the Syk-Vav1-Rac1/Cdc42 Signaling Axis. PLoS One 8, e56911. https://doi.org/10.1371/journal.pone.0056911

Yago, T., Petrich, B.G., Zhang, N., Liu, Z., Shao, B., Ginsberg, M.H., McEver, R.P., 2015. Blocking neutrophil integrin activation prevents ischemia-reperfusion injury. J. Exp. Med. 212, 1267–1281. https://doi.org/10.1084/jem.20142358

Yang, S.C., Tsai, Y.F., Pan, Y.L., Hwang, T.L., 2021. Understanding the role of neutrophils in acute respiratory distress syndrome. Biomed. J. 44, 439–446. https://doi.org/10.1016/j.bj.2020.09.001

Yuki, K., Hou, L., 2020. Role of β2 Integrins in Neutrophils and Sepsis. Infect. Immun. 88, e00031-20.

Zarbock, A., Lowell, C.A., Ley, K., 2007. Syk signaling is necessary for E-selectin-induced LFA-1-ICAM-1 association and rolling but not arrest. Immunity 26, 773–783. https://doi.org/10.1016/j.immuni.2007.04.011

Zen, K., Guo, Y.L., Li, L.M., Bian, Z., Zhang, C.Y., Liu, Y., 2011. Cleavage of the CD11b extracellular domain by the leukocyte serprocidins is critical for neutrophil detachment

during chemotaxis. Blood 117, 4885–4894. https://doi.org/10.1182/blood-2010-05-287722

Zenaro, E., Pietronigro, E., Bianca, V. Della, Piacentino, G., Marongiu, L., Budui, S., Turano, E., Rossi, B., Angiari, S., Dusi, S., Montresor, A., Carlucci, T., Nanì, S., Tosadori, G., Calciano, L., Catalucci, D., Berton, G., Bonetti, B., Constantin, G., 2015. Neutrophils promote Alzheimer's disease-like pathology and cognitive decline via LFA-1 integrin. Nat. Med. 21, 880–886. https://doi.org/10.1038/nm.3913

Zhang, H., Schaff, U.Y., Green, C.E., Chen, H., Sarantos, M.R., Hu, Y., Wara, D., Simon, S.I., Lowell, C.A., 2006. Impaired Integrin-Dependent Function in Wiskott-Aldrich Syndrome Protein-Deficient Murine and Human Neutrophils. Immunity 25, 285–295. https://doi.org/10.1016/j.immuni.2006.06.014

Zhou, Y., Wu, J., Kucik, D.F., White, N.B., Redden, D.T., Szalai, A.J., Bullard, D.C., Edberg, J.C., 2013. Multiple Lupus-associated ITGAM Variants Alter Mac-1 Functions on Neutrophils. Arthritis Rheum. 65, 2907–2916. https://doi.org/10.1002/art.38117

Zimmerman, T., Blanco, F., 2008. Inhibitors Targeting the LFA-1/ICAM-1 Cell-Adhesion Interaction: Design and Mechanism of Action. Curr. Pharm. Des. 14, 2128–2139. https://doi.org/10.2174/138161208785740225

Zou, J., Chen, J., Yan, Q., Guo, Q., Bao, C., 2016. Serum IL8 and mRNA level of CD11b in circulating neutrophils are increased in clinically amyopathic dermatomyositis with active interstitial lung disease. Clin. Rheumatol. 35, 117–125. https://doi.org/10.1007/s10067-015-3080-1

CHAPTER 2

Fc receptor engagement enhances neutrophil static adhesion to ICAM-1/Fc

Haleigh E. Conley[1,2], M. Katie Sheats[1,2]

[1]Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, 1060 William Moore Drive, Raleigh, NC 27607, United States

[2]Comparative Medicine Institute, College of Veterinary Medicine, North Carolina State University, 1060 William Moore Drive Raleigh, NC 27607, United Sates

Corresponding author: M. Katie Sheats, Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, 1060 William Moore Drive, Raleigh, NC 27607, United States. Tel: +1 919 513 7724; fax: +1 919 515 4237, mkpeed@ncsu.edu

Conflict of Interest Statement: The authors have declared that no competing interests exist.

ABSTRACT

Neutrophils express surface receptors known as β_2-integrins, which they use to migrate along vascular endothelial cells and into inflamed tissues. Endothelial cells express the surface protein intercellular adhesion molecule-1 (ICAM-1), which acts as a ligand for β_2-integrins. The binding of β_2-integrins to ICAM-1 is part of the "outside-in" signaling pathway for integrin activation. Acting in concert with "outside-in" signaling is "inside-out", which describes intracellular signaling events that activate β_2-integrins but are initiated by other cell surface receptors. Recent research has focused on deciphering the "inside-out" and "outside-in" signaling mechanisms of β_2-integrins. The ICAM-1/Fc chimeric protein is a common tool used to investigate this binding interaction *in vitro*. While most researchers utilize this protein to interrogate ICAM-1 mediated outside-in activation of β_2-integrins, the Fc portion of the protein could act as a ligand for neutrophil Fc receptors, contributing to inside-out activation of β_2-integrins. We hypothesized that neutrophil Fc receptors contribute to neutrophil adhesion to ICAM-1/Fc. We utilized primary human neutrophils and receptor blocking antibodies to compare static adhesion on ICAM-1 and ICAM-1/Fc coated plates. Our results show that Fc receptors contribute significantly to neutrophil adhesion to the ICAM-1/Fc protein. Therefore, we suggest investigators utilize neutrophil Fc receptor blocking prior to stimulation on ICAM-1/Fc, or use a pure ICAM-1 protein ligand, for *in vitro* investigation of β_2-integrin outside-in activation and signaling in primary human neutrophils.

Keywords: ICAM-1/Fc, ICAM-1, Beta2-integrin, adhesion, neutrophils

INTRODUCTION

B_2-integrins are expressed exclusively on leukocytes and play essential roles in cellular adhesion, spreading, crawling, migration, and inflammatory functions, such as respiratory burst. On neutrophils, these cell surface receptors interact with ICAM-1 expressed on endothelial cells to facilitate rolling, firm adhesion, and diapedesis into tissue at sites of infection and inflammation (Schmidt et al., 2013). B_2-integrins are activated and signal through two different but overlapping processes within cells. "Inside-out" activation occurs when β_2-integrins are activated by intracellular cell signaling cascades initiated by other neutrophil cell surface receptors such as Fc and G-protein coupled receptors, which are stimulated by ligands such as antibodies or chemoattractants. "Outside-in" activation occurs when β_2-integrins bind their extracellular ligands such as ICAM-1 and fibrinogen and initiate their own cell signaling cascade (Abram and Lowell, 2009). Because β_2-integrins play such a central role in neutrophil functions, they have been investigated as potential therapeutic targets for multiple inflammatory diseases; however, clinical trials for treatments designed to target β_2-integrins have not been successful (Raab-Westphal et al., 2017). It is possible that an improved understanding of β_2-integrin signaling will help to overcome the limitations of previous therapeutic strategies.

One tool used to assess β_2-integrin activation and signaling *in vitro* is the recombinant ICAM-1/Fc chimera protein (Nueda et al., 1995; Quek et al., 2010; Shulman et al., 2012; Wee et al., 2015; Zhou et al., 2013). Despite its common usage, to our knowledge, no studies have specifically evaluated whether the Fc portion of the protein acts as a ligand for neutrophil Fc receptors. Similar to β_2-integrins, neutrophil Fc receptors (FcγRII and FcγRIII) play important roles in neutrophil inflammatory responses, including adhesion. Stimulation of neutrophil Fc receptors with immune complexes cause a significant increase in surface expression of β_2-

integrins, which is one step of inside-out β_2-integrin activation and signaling (Jones et al., 1998). Given the known role of Fc receptors in inside-out β_2-integrin activation, we hypothesized that Fc receptors contribute to ICAM-1/Fc stimulated β_2-integrin dependent neutrophil adhesion.

MATERIALS AND METHODS

Isolation of human neutrophils

Human blood collection protocols were reviewed and approved by the Institutional Research Ethics Committee of NCSU (IRB approval #616). For all neutrophil experiments, 10 - 30 mL of whole blood was collected using heparinized syringes from healthy human volunteers that provided consent for participation. Neutrophils were isolated from whole blood using Ficoll-Paque Plus (GE Healthcare) density gradient centrifugation. Briefly, heparinized whole blood was mixed with 0.6% Dextran in a 15 mL polypropylene conical and allowed to settle at room temperature for 45-60 minutes. Up to 10 ml of leukocyte rich plasma was aspirated using a bulb syringe and layered on 5 mL of Fill in a separate 15 mL conical tube. Cells were then centrifuged at 1800 rpm for 20 minutes with the brake off. The supernatant was discarded and remaining blood cells within the cell pellet were removed by 60 seconds of hypotonic lysis. Isolated neutrophils were resuspended/washed in sterile HBSS without additives. Cell number and viability was quantified using trypan blue exclusion (1:1) and a manual hemocytometer count. Final suspension of cells was in $HBSS^{++}$ chemotaxis buffer [1x HBSS (Life Technologies), 1 mM Ca^{2+}, 1 mM Mg^{2+}, 5% fetal bovine serum (Gibco)] at the indicated concentration for each experiment. All experiments were completed within 4 to 6 hours of blood collection. Neutrophils from a sex-balanced cohort of individual human donors were used for all time points and treatment conditions for each experiment (i.e. “n” represents a separate human donor).

B_2-integrin inhibition and Fc Receptor Blocking

Neutrophils were pretreated at 37°C for 30 minutes with receptor blocking antibodies (**Table 1**) individually or in combination, prior to plating.

Adhesion assay

Individual wells of Immulon2HB flat bottom 96-well plates were coated with 10 μg/mL Recombinant ICAM-1/Fc (R&D Systems), 10 μg/mL Recombinant ICAM-1 (R&D Systems), 10 μg/mL Recombinant Human IgG1 Fc (R&D Systems), or 5% FBS (non-stimulated control). Isolated human neutrophils at 1 x 10^7/mL in HBSS were labeled with calcein AM at a concentration of 2 μg/mL and primed with human GM-CSF at 5 ng/mL for 30 minutes, protected from light. Cells were then centrifuged at 1000 rpm for 8 minutes and then resuspended in the chemotaxis buffer at 7.0x10^5 cells/mL. Indicated blocking antibodies and/or peptides were added to cells and incubated for 30 minutes at 37°C. Plates were washed once with 1X PBS prior to addition of cells. After the addition of cells, the plate was incubated for 10 minutes prior an initial fluorescence reading. Cells were gently dumped and washed with PBS, reading fluorescence at each wash step. The fluorescence values for each wash were divided by initial fluorescence and multiplied by 100 to calculate percent adhesion. The first wash that demonstrated less than 10% adhesion in the non-stimulated cells (plated on 5% FBS coating) was considered the result. Treatment groups were assessed in triplicate.

Statistical Analysis

Data are reported as mean ± SD of at least 3 independent experiments. All statistical tests were performed by ordinary one-way ANOVA using GraphPad Prism software (San Diego, CA). P values <0.05 were considered statistically significant. Data analyzed by ordinary one-way

ANOVA with Dunnett's multiple comparisons test. *Indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

RESULTS

Neutrophil adhesion was compared between individual and chimeric protein ligands Fc, ICAM-1, and ICAM-1/Fc in a 10-minute static plate-based neutrophil adhesion assay. Our results show a significant difference between neutrophil adhesion on ICAM-1/Fc compared to the individual Fc and ICAM-1 proteins, as well as to the 5% FBS (non-stimulated control) (**Figure 1**).

Given that ICAM-1/Fc demonstrated significantly greater neutrophil static adhesion than ICAM-1, we next evaluated whether Fc receptors were involved in ICAM-1/Fc induced neutrophil static adhesion. Neutrophils were pretreated with Fc-receptor blocking antibodies anti-CD32 monoclonal antibody and anti-CD16 $F(ab)'_2$. Our results show that antibody blocking of Fc receptors significantly attenuated ICAM-1/Fc induced neutrophil adhesion (**Figure 2**). Addition of anti-CD18 $F(ab)'_2$ to inhibit β_2-integrins further attenuated neutrophil adhesion, confirming the adhesion was β2-integrin dependent and supporting the specificity of Fc receptor blocking (**Figure 2**).

Next, we investigated which specific Fc receptors were involved in ICAM-1/Fc induced neutrophil static adhesion. Neutrophils were pretreated with Fc-receptor blocking antibodies anti-CD32 monoclonal antibody or anti-CD16 $F(ab)'_2$ and appropriate isotype controls. Inhibition of FcγRIII with the anti-CD16 $F(ab)'_2$ had no significant effect on neutrophil adhesion. Inhibition of FcγRII with the anti-CD32 mAb significantly attenuated ICAM-1/Fc induced adhesion (**Figure 3**).

DISCUSSION

Our finding that the ICAM-1/Fc chimeric protein stimulates greater leukocyte adhesion than ICAM-1 alone is consistent with previous reports. In a previous study of monocyte β_2-integrin interactions, ICAM-1/Fc provoked greater THP-1 adherence than the non-chimeric ICAM-1 protein (Quek et al., 2010). These authors suggested that Fc receptors could be contributing to enhanced adhesion; however, no further investigation was performed. Other investigators have addressed the concern of Fc enhanced adhesion by including the Fc ligand as a control in experiments using the chimeric ICAM-1/Fc (Shulman et al., 2012; Wee et al., 2015). Indeed, our own findings show that neutrophil adhesion to the Fc fragment was not significantly different from the 5% FBS unstimulated control (**Figure 1**), which might lead one to conclude that the Fc protein does not significantly affect neutrophil adhesion. However, the individual Fc fragment control does not account for the synergistic effect of Fc receptors and β_2-integrins on neutrophil adhesion, which is what our results demonstrate. This is consistent with a previous report demonstrating β_2-integrin and FcγRII/III cooperation in neutrophil activation during anti-integrin monoclonal antibody stimulation (Jakus et al., 2004). The current report shows that primary neutrophil adhesion to the chimeric ICAM-1/Fc protein involves both FcγR and β_2-integrin surface receptors, with FcγRII enhancing the β_2-integrin dependent adhesion. Despite these findings, we currently do not know how FcγRII enhances β_2-integrin dependent adhesion. Further research is needed to elucidate the cell signaling involved in crosstalk between Fc-receptors and β_2-integrins in neutrophils. However, based on the findings presented here, we suggest that investigators utilize neutrophil Fc receptor blocking prior to stimulation on ICAM-1/Fc, or use a pure ICAM-1 protein ligand, for *in vitro* investigation of β_2-integrin outside-in activation and signaling in primary human neutrophils.

TABLES

Table 1. **Receptor blocking antibodies**

Antibody	Receptor	Concentration	Corresponding Isotype Control
Anti-human CD18 F(ab)'$_2$ clone IB4 (Ancell)	CD18/β_2-integrin	30 µg/mL	Anti-human IgG2a F(ab)'$_2$ clone RPC5 (Ancell)
Anti-CD16 F(ab)'$_2$ clone 3G8 (Ancell)	CD16/FcγRIII	5 µg/mL	Mouse IgG1 F(ab)'$_2$ clone MPOC31C (Ancell)
Anti-CD32 mAb IV.3 (StemCell Technologies)	CD32/FcγRII	1 µg/mL	Mouse IgG2b, kappa, clone MPC-11 (StemCell Technologies)

FIGURES

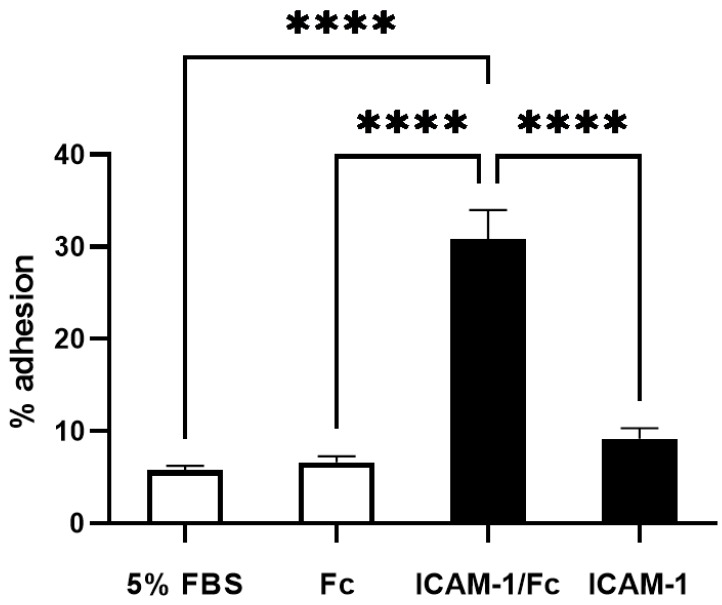


Figure 1. ICAM-1/Fc induces greater neutrophil adhesion than ICAM-1. Calcein-loaded neutrophils were plated on 5% FBS, Fc, ICAM-1/Fc, or ICAM-1 coated wells and allowed to adhere for 10 minutes prior to determining the percentage adhesion, as described in the materials and methods. Data represented as mean ± SD (n=3-7).

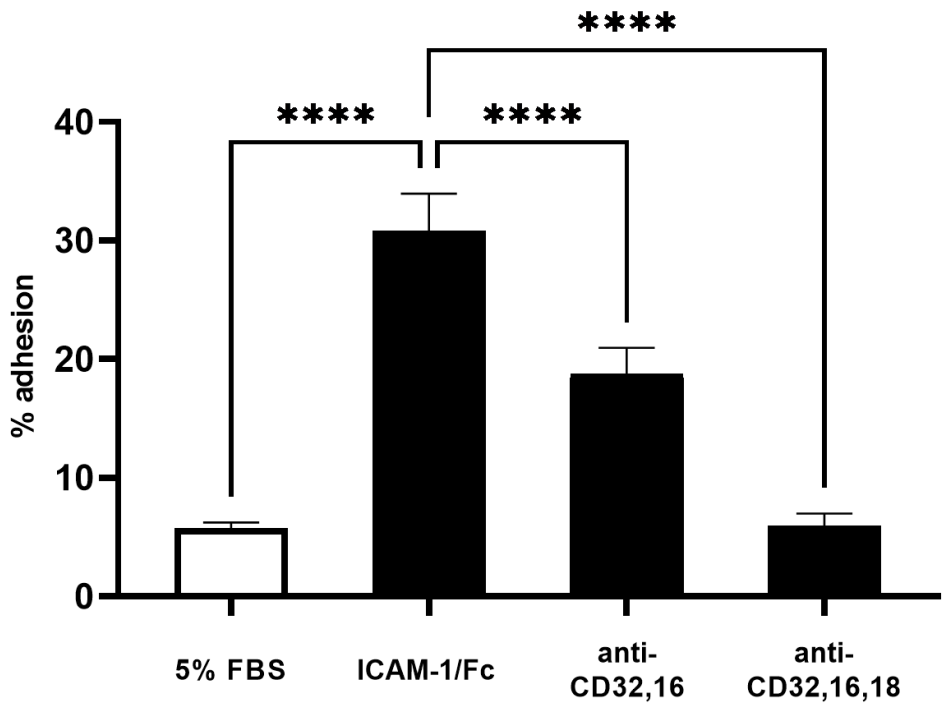


Figure 2. Fc receptor blocking attenuates neutrophil adhesion to ICAM-1/Fc. Calcein-loaded neutrophils were pre-treated for 30 minutes with anti-CD32 mAb, anti-CD16 F(ab)'2, and anti-CD18 F(ab)'2 prior to plating on ICAM-1/Fc coated wells. Data represented as mean ± SD (n=3-7).

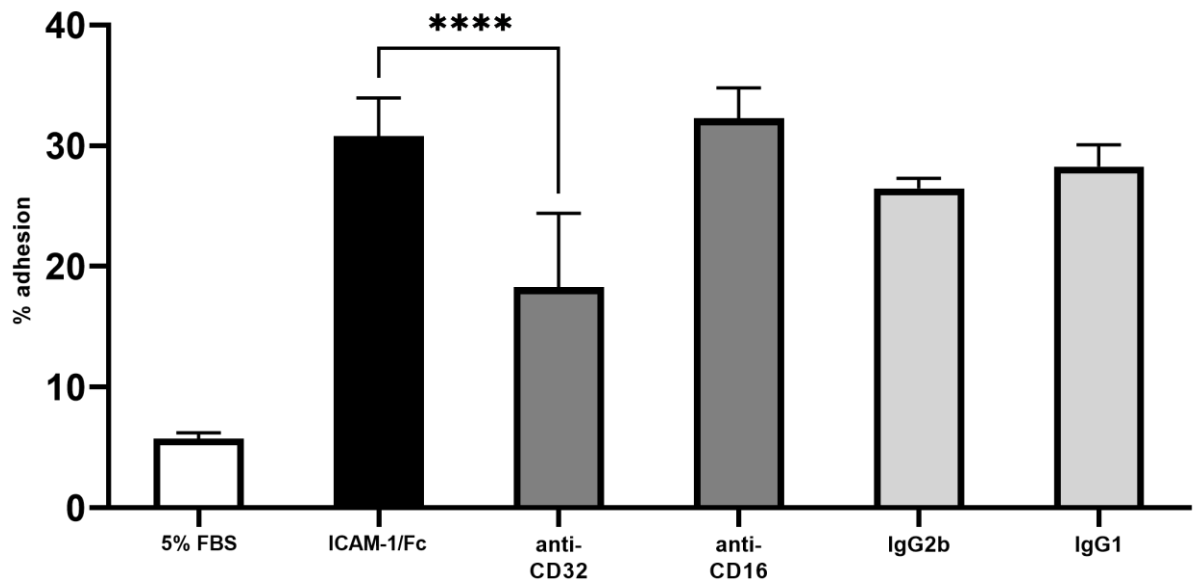


Figure 3. FcγRII (CD32) inhibition attenuates ICAM-1/Fc induced adhesion. Calcein-loaded neutrophils were pre-treated for 30 minutes with anti-CD32 mAb, anti-CD16 F(ab)'2, or appropriate isotype controls prior to plating on ICAM-1/Fc coated wells. Data represented as mean ± SD (n=3-7).

REFERENCES

Abram, C.L., Lowell, C.A., 2009. The Ins and Outs of Leukocyte Integrin Signaling. Annu Rev Immunol 339–362. https://doi.org/10.1146/annurev.immunol.021908.132554

Jakus, Z., Berton, G., Ligeti, E., Lowell, C.A., Mócsai, A., 2004. Responses of Neutrophils to Anti-Integrin Antibodies Depends on Costimulation through Low Affinity FcγRs: Full Activation Requires Both Integrin and Nonintegrin Signals. J. Immunol. 173, 2068–2077. https://doi.org/10.4049/jimmunol.173.3.2068

Jones, S.L., Knaus, U.G., Bokoch, G.M., Brown, E.J., 1998. Two signaling mechanisms for activation of α(M)β2 avidity in polymorphonuclear neutrophils. J. Biol. Chem. 273, 10556–10566. https://doi.org/10.1074/jbc.273.17.10556

Nueda, A., López-Rodríguez, C., Rubio, M.A., Sotillos, M., Postigo, A., DelPozo, M.A., Vega, M.A., Corbí, A.L., 1995. Hematopoietic cell-type-dependent regulation of leukocyte integrin functional activity: CD11b and CD11c expression inhibits LFA-1-Dependent aggregation of differentiated U937 cells. Cell. Immunol. https://doi.org/10.1006/cimm.1995.1157

Quek, B.Z., Lim, Y.C., Lin, J.H.R., Tan, T.E., Chan, J., Biswas, A., Schwarz, H., 2010. CD137 enhances monocyte-ICAM-1 interactions in an E-selectin-dependent manner under flow conditions. Mol. Immunol. 47, 1839–1847. https://doi.org/10.1016/j.molimm.2009.11.010

Raab-Westphal, S., Marshall, J.F., Goodman, S.L., 2017. Integrins as therapeutic targets:Successes and cancers. Cancers (Basel). 9, 1–28. https://doi.org/10.3390/cancers9090110

Schmidt, S., Moser, M., Sperandio, M., 2013. The molecular basis of leukocyte recruitment and its deficiencies. Mol. Immunol. 55, 49–58. https://doi.org/10.1016/j.molimm.2012.11.006

Shulman, Z., Cohen, S.J., Roediger, B., Kalchenko, V., Jain, R., Grabovsky, V., Klein, E., Shinder, V., Stoler-Barak, L., Feigelson, S.W., Meshel, T., Nurmi, S.M., Goldstein, I., Hartley, O., Gahmberg, C.G., Etzioni, A., Weninger, W., Ben-Baruch, A., Alon, R., 2012. Transendothelial migration of lymphocytes mediated by intraendothelial vesicle stores rather than by extracellular chemokine depots. Nat. Immunol. 13, 67–76. https://doi.org/10.1038/ni.2173

Wee, J.L., Schulze, K.E., Jones, E.L., Yeung, L., Cheng, Q., Pereira, C.F., Costin, A., Ramm, G., van Spriel, A.B., Hickey, M.J., Wright, M.D., 2015. Tetraspanin CD37 Regulates β 2 Integrin–Mediated Adhesion and Migration in Neutrophils . J. Immunol. 195, 5770–5779. https://doi.org/10.4049/jimmunol.1402414

Zhou, Y., Wu, J., Kucik, D.F., White, N.B., Redden, D.T., Szalai, A.J., Bullard, D.C., Edberg, J.C., 2013. Multiple Lupus-associated ITGAM Variants Alter Mac-1 Functions on Neutrophils. Arthritis Rheum. 65, 2907–2916. https://doi.org/10.1002/art.38117

ACKNOWLEDGEMENTS

NIH OD/ORIP K01OD015136 and NCSU College of Veterinary Medicine funded this research

CHAPTER 3

A Role for Myristoylated Alanine Rich C-Kinase Substrate (MARCKS) in Neutrophil Outside-In Beta2-Integrin Activation and Signaling

Haleigh E. Conley[1,2], Rebecca L. Till[1], Alix Berglund[1,2], Samuel L. Jones[1], M. Katie Sheats[1,2]

[1]Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, 1060 William Moore Drive, Raleigh, NC 27607, United States

[2]Comparative Medicine Institute, College of Veterinary Medicine, North Carolina State University, 1060 William Moore Drive Raleigh, NC 27607, United Sates

ABSTRACT

MARCKS (Myristoylated Alanine Rich C-Kinase Substrate) is an actin and PIP2 binding protein that plays an essential role in primary neutrophil migration and adhesion; however, the molecular details regarding MARCKS function in these processes remains unclear. Neutrophil adhesion and migration also require the cell surface receptors β_2-integrins. Activation of β_2-integrins consists of a well-described sequence of events including increased cell surface expression (upregulation), change in conformation (affinity), clustering (avidity), and signaling. In this study, we hypothesize that MARCKS protein function is essential for β_2 integrin activation and signaling. Isolated peripheral blood neutrophils were pretreated with specific inhibitor peptide MANS to inhibit MARCKS function or the scrambled control peptide RNS. Using flow cytometry to quantify surface expression of total and high affinity CD11b, our data demonstrate that MARCKS function is not essential for fMLP-stimulated inside-out activation of β_2-integrins. In a model of outside-in adhesion using ICAM-1 and Mn^{2+}, MARCKS inhibition significantly attenuated neutrophil static adhesion. MARCKS inhibition with MANS also diminished neutrophil cell spreading and β_2 integrin clustering, as determined by immunofluorescence microscopy. MANS peptide treatment also significantly attenuated β_2-integrin-dependent respiratory burst induced by insoluble immune complexes (IIC) but did not inhibit β_2-integrin independent respiratory burst induced by PMA. Using immunoblotting, we determined that MARCKS inhibition with MANS peptide has no effect on p38 MAPK activation in IIC-stimulated neutrophils. These results indicate MARCKS plays an essential role in outside-in β_2-integrin activation and signaling.

INTRODUCTION

Neutrophils play an essential role during the innate immune response. To transmigrate from the vasculature to sites of inflammation and/or microbial infection, neutrophils must be recruited from the bloodstream using specialized cell surface receptors, called beta2 (β_2)-integrins, that interact with adhesion molecules expressed on the surface of endothelial cells (e.g., intracellular adhesion molecule 1 (ICAM-1)). The details of neutrophil capture, rolling, endothelial adhesion, post-adhesion strengthening, crawling, and transmigration have largely been determined; however, there are still gaps in knowledge regarding the specific cell signaling mechanisms (Herter and Zarbock, 2013; Kolaczkowska and Kubes, 2013), especially regarding the involvement of β_2-integrins. It is clear that β_2-integrins play an important role in adhesion strengthening, crawling, and transmigration (Schmidt et al., 2013), all of which are required processes for normal biological functions and host defense mechanisms. This is evidenced by the severe consequences of lack of functional β_2-integrins, which severely weakens the host immune system, resulting in recurrent and/or chronic bacterial infections leading to significant morbidity and mortality in patients with leukocyte adhesion deficiency (LAD) (Kuijpers et al., 1997).

Although critical for host survival and injury resolution, neutrophils are considered a double-edged sword. When their destructive powers designed to combat pathogens become dysregulated, excessive neutrophil infiltration or neutrophil activation can cause significant damage to host tissues (Mittal et al., 2014). In fact, neutrophil derived tissue injury is known to play a role in numerous acute and chronic inflammatory diseases, including myocardial infarction (Meisel et al., 1998), stroke (Edwards and Bix, 2019), ischemia-reperfusion injury (Schofield et al., 2013; Yago et al., 2015), rheumatoid arthritis (Lin et al., 2019; Wright et al., 2017), severe SARS-CoV-2 (Middleton et al., 2020; Wang et al., 2020), and sepsis-associated

organ damage (i.e., acute lung injury) (Giacalone et al., 2020; Neumann et al., 1999). Several animal models have highlighted the potential and/or ability to target neutrophils by inhibiting β_2-integrins (Neumann et al., 1999; Wang et al., 2014); however, the use of monoclonal antibodies targeting β_2-integrins in clinical settings have not been successful (Harlan and Winn, 2002; Vicente-Manzanares and Sá Nchez-Madrid, 2018). Regardless, β_2-integrins remain a desirable target for modulating neutrophil responses (Zimmerman and Blanco, 2008). A more detailed understanding of how β_2-integrins are regulated may help identify novel targets for new and beneficial anti-inflammatory therapies.

B_2-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). Within circulating, inactive neutrophils, these adhesion molecules are primarily contained within the cytoplasm, secondary and tertiary granules, and secretory vesicles. The few β_2-integrins that are present on the surface of resting neutrophils exist in an inactive/low affinity state with a 'bent' conformation. 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 damaging effects on host tissue and vasculature. When neutrophils receive an activation signal through intracellular signaling pathways, β_2-integrin surface expression is increased, conformational changes take place ("affinity"), and increased mobility within the membrane leads to cluster formation ("avidity/valency"). This type of integrin stimulation is termed 'inside-out' activation and is driven by intracellular signals received by G protein coupled receptors (GPCRs) and Fc receptors, among others (Jones et al., 1998). B_2-integrins can also become activated when the extracellular domain interacts directly with extracellular matrix proteins or other cell surface

ligands (ICAM-1, fibrinogen, etc.) and initiates its own signaling, termed "outside-in" activation (Abram and Lowell, 2009; Herter and Zarbock, 2013; Schymeinsky et al., 2007; Williams and Solomkin, 1999). Details regarding the downstream signaling pathway from outside-in β_2-integrin activation are incomplete; however, multiple lines of evidence show that this type of activation is linked to neutrophil firm adhesion and cell spreading (Abram and Lowell, 2009).

Myristoylated Alanine Rich C Kinase Substrate (MARCKS) is a well-known protein kinase C (PKC) substrate and actin-binding protein. It is essential for several integrin-dependent cellular functions, such as adhesion, cell spreading, and migration in numerous cell types (Eckert et al., 2010; Ott et al., 2013; Sheats et al., 2014). In most resting cells, MARCKS protein is associated with the inner leaflet of the plasma membrane due to hydrophobic insertion of an N-terminal myristoyl-moiety into the lipid bilayer, and electrostatic interactions between the MARCKS effector domain (ED) and 3-4 phosphatidylinositol 4,5-bisphosphate (PIP2) (Kalwa and Michel, 2011; Wang et al., 2002, 2001). The ED of MARCKS is subject to reversible regulation by PKC phosphorylation or Ca^{++}/calmodulin binding. Either of these events displace MARCKS from the membrane to the cytosol and releases PIP2. Once dephosphorylated, MARCKS reassociates with the plasma membrane (Aderem, 1992; Arbuzova et al., 2002; Blackshear, 1993; Sundaram et al., 2004). This reversible association with the plasma membrane is a key aspect of normal MARCKS function and is known as the "myristoyl-electrostatic switch" (McLaughlin and Aderem, 1995; Seykora et al., 1996).

The interest in MARCKS role in integrin regulation began after the discovery that MARCKS homolog protein, MARCKSL1 (MARCKS-like 1, MacMARCKS), is an essential component of β_2-integrin activation in macrophages (Yue et al., 2000; Zhou and Li, 2000). Zhou et al. used a model of inside-out β_2-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 (Zhou and Li, 2000). Further, myoblasts plated on fibronectin to study outside-in β_1-integrin activation clearly linked $\alpha_5\beta_1$-mediated PKC activation to phospho-MARCKS dependent regulation of cell spreading and adhesion (Disatnik et al., 2004). Finally, previous work in our lab determined that MARCKS plays an essential role in β_2-integrin-dependent adhesion and migration in neutrophils when MARCKS function was inhibited with a peptide inhibitor (Eckert et al., 2010; Sheats et al., 2014). In the current study we examine the role of MARCKS in β_2-integrin dependent activation and signaling in human primary neutrophils using methods to target both "inside-out" and "outside-in" β_2-integrin activation pathways. Our results suggest that MARCKS plays an essential role in β_2-integrin outside-in activation and signaling in human neutrophils.

MATERIALS & METHODS

Isolation of human neutrophils

Human blood collection protocols were reviewed and approved by Institutional Research Ethics Committee of NCSU (IRB approval #616). For all neutrophil experiments, 10 - 30 ml of whole blood was collected using heparinized syringes from healthy human volunteers that provided informed consent for participation. Neutrophils were isolated from whole blood using Ficoll-Paque Plus (GE Healthcare) density gradient centrifugation. Briefly, heparinized whole blood was mixed with 0.6% Dextran in a 15 mL polypropylene conical and allowed to settle at room temperature for 45-60 minutes. Up to 10 ml 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 with the brake off. The supernatant was discarded and remaining blood cells within the cell pellet were removed by 60 seconds of hypotonic lysis.

Isolated neutrophils were resuspended/washed in sterile HBSS (Life Technologies) without additives. Cell number and viability was quantified using trypan blue exclusion (1:1) and a manual hemocytometer count. Final suspension of cells was in $HBSS^{++}$ chemotaxis buffer [1x HBSS, 1 mM Ca^{2+}, 1 mM Mg^{2+}, 5% fetal bovine serum (Gibco)] at the indicated concentration for each experiment. All experiments were completed within 4 to 6 hours of blood collection. Neutrophils from individual human donors were used for all time points and treatment conditions for each experiment (i.e., "n" represents a separate human donor).

Peptide Treatment

The MyristoylAted N-terminal Sequence (MANS) and Random Nucleotide Sequence (RNS) peptides were synthesized by Genemed Synthesis, Inc. The sequence of MANS is identical to the first 24 amino acids of the human MARCKS protein: myristic acid-GAQFSKTAAKGEAAAERPGEAAVA. The RNS peptide is a randomly scrambled control: myristic acid-GTPAPAAEGAGAEVKRASAEAKQAF. Peptide working solutions were resuspended in sterile PBS. Where indicated, pretreatment of cell suspensions with indicated peptide concentrations occurred at 37 °C for 30 minutes.

Fluorescence labeling of neutrophils

For adhesion experiments, isolated neutrophils (1×10^7/mL in HBSS) were incubated with the fluorescent dye calcein AM (Corning) at 2 μg/mL for 30 minutes at room temperature, protected from light. Cells were then centrifuged at 1200 rpm for 10 minutes and resuspended in $HBSS^{++}$ with 2% heat inactivated FBS (chemotaxis buffer) to the appropriate final experimental concentration.

Beta2-integrin inhibition

As a positive control β2-integrin inhibition, isolated human neutrophils were pretreated with 30 μg/mL anti-human CD18 $F(ab')_2$ (Ancell Corp) at 37 °C for 30 minutes. Anti-human IgG2a $F(ab')_2$ (Ancell Corp) was used as an isotype control.

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 fMLP (Sigma) or VC for 5 minutes, diluted with 1 volume ice cold PBS and placed on ice for 5 minutes, spun at 1200 rpm for 5 minutes, resuspended in sterile PBS with 5% FBS and labeled with PE-conjugated anti-CD11b antibody clone ICFR44 (Novus Biologicals), PE-conjugated anti-CD11b CBRM1/5 antibody (Biolegend), or PE-conjugated IgG1 and IgG2a isotype controls (Biolegend). Flow cytometry was performed using Calibur FACScan. Flow cytometry experiments were performed in the Flow Cytometry and Cell Sorting facility at North Carolina State University – College of Veterinary Medicine.

Static adhesion assay

Individual wells of Immulon2HB flat bottom 96-well plates were coated with 10 μg/mL Recombinant ICAM-1 (R&D Systems) or 5% FBS (for controls). Isolated human neutrophils at 1 x 10^7/mL in HBSS were labeled with calcein AM (Corning) at a concentration of 2 μg/mL and primed with human GM-CSF (EMD Millipore) at 5 ng/mL for 30 minutes, protected from light. Cells were then centrifuged at 1000 rpm for 8 minutes and then resuspended in the chemotaxis buffer at 7.0x10^5 cells/mL. Appropriate blocking antibodies and/or peptides were added to cells and incubated for 30 minutes at 37°C. The ICAM-1 coated plate was washed once with 1X PBS before cells were added to individual wells. Cells were added to wells and allowed to settle for

10 minutes at 37°C. 0.5 mM Mn^{2+} was added to individual wells. Plates were incubated for 10 minutes prior to initial fluorescence reading. Cells were gently dumped and washed with PBS, reading fluorescence (485 nm excitation, 535 nm emission) at each wash step. Fluorescence washing was divided by initial fluorescence and multiplied by 100 to calculate percent adhesion. The first was that demonstrated less than 10% adhesion in the non-stimulated cells (plated on 5% FBS coating) was considered the result. Treatment groups were tested in triplicate.

Immunofluorescence microscopy

Chambered coverslips were either coated with 5% FBS in PBS (unstimulated control) or 10 µg/mL ICAM-1/Fc (R&D Systems) overnight. Isolated primary neutrophils were primed with 5 ng/mL human GM-CSF for 30 minutes at room temperature protected from light. Cells were then centrifuged at 1000 rpm for 8 minutes and then resuspended in the chemotaxis buffer at $1.0x10^6$ cells/mL. Appropriate concentrations of peptides were added to cells and incubated for 30 minutes at 37°C. The ICAM-1/Fc (or ICAM-1) coated plate was washed once with 1X PBS before cells were added to individual wells. 130,000 cells were added to the ICAM-1/Fc (or ICAM-1) coated chamber coverslips for 10 minutes. For fMLP stimulated wells, cells were allowed to settle for 10 minutes prior to addition of 100 nM fMLP or VC for 5 minutes. Cells were then fixed to the coverslip, washed with PBS, blocked with rabbit serum, and labeled with AF549-conjugated anti-CD11b antibody clone M1/70 (Biolegend) before imaging with an Olympus IX83 Inverted Microscope.

Respiratory Burst

Isolated neutrophils were resuspended in chemotaxis buffer to a final concentration of $3.0x10^6$ cells/mL. Cells were then incubated with indicated treatments at 37°C for 30 minutes prior to each experiment and 100 µL of cells from indicated treatment groups were placed in individual

wells of 5% FBS or insoluble immune complex (IIC) coated Immulon2HB plates. Plates were coated overnight at 4°C with BSA (100 µg/mL). To generate IIC substrate, BSA (Sigma) coated wells were washed three times and recoated with anti-BSA antibody (50 µg/well) and incubated for 2 hours at 37°C. Prior to the addition of cells, all wells were washed three times with sterile PBS. For PMA-stimulated respiratory burst, cells were allowed to settle for 10 minutes prior to the addition of dihydrorhodamine-123 (DHR-123) (Sigma) (10 µM final concentration) and PMA (100 ng/mL final concentration) (Sigma). 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.

Cell Lysate Stimulation

Prior to the experiment, low density insoluble immune complex was prepared as follows. Wells of a 24 well tissue culture plate was coated with 100 µg/mL bovine serum albumin overnight at 4°C. The wells were washed three times with PBS before the addition of coating with 33.35 µg/mL rabbit anti-bovine albumin antibody (Sigma) for 2 hours at 37°C. The wells were washed three times with PBS prior to the addition of cells. Isolated neutrophils ($7x10^6$) were pretreated with PBS, 50 µM MANS, 50 µM RNS, 30 µM 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-D] pyrimidine (PP2) (Sigma), or 30 µg/mL anti-CD18 F(ab)'$_2$ prior to stimulation with immune complexes for 15 minutes at 37°C. An Eppendorf tube of cells remained unstimulated for a suspension control. Plates were centrifuged at 1200 rpms for 10 minutes then 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 and phosphatase inhibitors for 30 minutes shaking on ice. After lysis, cell supernatants were centrifuged for at 1300 rpm for 10 minutes at 4°C. Supernatants were collected and frozen at -80ºC until immunoblotting.

Immunoblotting

Frozen samples were thawed on ice and protein concentrations were measured using BCA Protein Assay Reagent (Pierce). 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 diH20] and boiled for 10 minutes. Equal amount of protein was analyzed in 4-12% SDS-PAGE. Resolved samples were transferred to Immobilon-P PVDF membrane (Millipore). Total protein was determined using No-Stain Protein Labeling Reagent (Invitrogen) followed by blocking 1 hour with 5% non-fat dry milk with Tween-20 (TBS/T; 136 μM NaCl, 20 μM Tris-base (pH: 7.6) and 0.1% Tween-20 v/v). Primary antibody (1:1000) was incubated overnight in 5% BSA TBS/T at 4°C. Membranes were washed with TBS/T and incubated with anti-rabbit horseradish peroxidase (HRP) secondary antibody (1:2000) (Cell Signaling Technology) in 5% BSA TBS/T for one hour, then washed three times for 5 minutes, developed using Bio-Rad Clarity Western ECL Substrate, and imaged using a Bio-Rad ChemiDoc. Western blot images were analyzed in Image Lab.

Statistical Analysis

Data are reported as mean ± SD. All statistical tests were performed using GraphPad Prism software (San Diego, CA) with the appropriate test details in the figure legend. P values <0.05 were considered statistically significant. *Indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

RESULTS

MARCKS protein is not essential for inside-out activation of neutrophil β_2-integrins

Neutrophils within the vasculature are non-adherent and express low numbers of surface β_2-integrins. When neutrophils are stimulated by chemoattractants, there is a significant increase in surface expression of β_2-integrins (CD18 and CD11b), termed "inside-out" activation. To determine whether MARCKS function is essential for inside-out activation of β_2-integrins, neutrophils pretreated with MANS or RNS were stimulated with fMLP, and CD11b surface expression was measured by flow cytometry. As shown in Figure 1A, fMLP stimulation induced a significant increase in total CD11b on the surface of neutrophils compared to control. MANS peptide treatment had no effect on fMLP-induced surface expression of CD11b. Calcium chelation with EDTA was used as a positive control for inhibition of CD11b upregulation.

In addition to increasing the number of surface expressed β_2-integrins, stimulation by chemoattractants also cause integrin conformation to shift from low to high affinity conformation, which facilitates ligand binding. Stimulation with fMLP resulted in a significant increase in the surface expression of high affinity CD11b compared to controls, as detected by CBRM1/5 antibody binding (Figure 1B). MANS peptide treatment had no effect on fMLP-induced β_2-integrin high affinity conformation surface expression. Calcium chelation with EDTA was used as a positive control for inhibition of high affinity conformation expression. Given that MARCKS inhibition has no impact on total CD11b upregulation or high affinity CD11b expression, this evidence demonstrates that MARCKS function is not essential for fMLP-induced inside-out activation of neutrophil β_2-integrins.

MARCKS protein is essential for outside-in β_2-integrin mediated neutrophil adhesion

When neutrophils become activated by chemoattractants through inside-out stimulation, the high affinity conformation encourages β_2-integrin binding to cellular adhesion molecules expressed on endothelial cells, such as intercellular adhesion molecule 1 (ICAM-1) (Fan et al., 2016; Spillmann et al., 2002). This binding results in "outside-in" activation and signaling. Outside-in activation and signaling controls firm adhesion, which is a necessary step for several neutrophil effector functions (Giagulli et al., 2006; Zarbock and Ley, 2009). Although inside-out and outside-in activation happen together *in vivo*, we utilized an *in vitro* model of outside-in adhesion to determine if MARCKS protein was essential for outside-in activation of β_2-integrins. Static neutrophil adhesion was induced on ICAM-1 coated wells and enhanced with the application of Mn^{2+} (Jones et al., 1998). This model induced significant static neutrophil adhesion (Figure 2). MARCKS inhibition with MANS peptide significantly attenuated ICAM-1/Mn^{2+} induced adhesion in a concentration-dependent manner. The addition of anti-CD18 F(ab)'$_2$ confirmed that ICAM-1/Mn^{2+} adhesion is β_2-integrin dependent. The RNS control peptide and isotype controls had no effect on neutrophil adhesion. These results indicate that MARCKS protein plays an essential role in outside-in β_2-integrin mediated adhesion.

MARCKS protein is essential for neutrophil cell spreading and β_2-integrin clustering

The finding that MARCKS protein is required for β_2-integrin mediated adhesion led us to investigate how MANS peptide was impacting neutrophil adhesion. To do this, β_2-integrin clustering and neutrophil spreading were investigated using immunofluorescence staining of CD11b of neutrophils stimulated on an ICAM-1/Fc surface. Neutrophils stimulated on ICAM-1/Fc exhibited cell spreading and punctate staining of clustered β_2-integrins (Figure 3B). MANS treatment significantly inhibited both cell spreading and β_2-integrin clustering (Figure 3C) to a

level that resembled the unstimulated control (Figure 3A). The addition of the inside-out stimulus fMLP did not restore the clustering and spreading defects caused by MANS peptide (Figure 3F). These results were also consistent in neutrophils stimulated with ICAM-1/Mn^{2+} (Supplemental Figure 1). These data demonstrate that MARCKS function is essential for neutrophil spreading and β_2-integrin integrin clustering.

MARCKS protein function is essential for β_2-integrin dependent neutrophil respiratory burst

The data presented thus far have demonstrated a role for MARCKS protein in the activation of β_2-integrins. Integrins not only are responsible for the physical interactions of neutrophils with ligands, but they are also responsible for transmitting intracellular signals to elicit appropriate cellular responses. We next evaluated MARCKS role in β_2-integrin signaling. Reactive oxygen species (ROS) are essential mediators of cellular signaling, so we first examined whether MARCKS function would be essential for respiratory burst. Neutrophil respiratory burst was stimulated with either low density insoluble immune complexes (IIC) or phorbol myristate acetate (PMA) and measured using DHR-123, which emits fluorescence once it is oxidized by reactive oxygen species.

As shown in Figure 4, both IIC and PMA stimulated robust and sustained neutrophil respiratory burst. MANS peptide treatment significantly inhibited IIC-induced respiratory burst (Figure 4A), while RNS peptide had no effect. IIC-induced respiratory burst was β_2-integrin dependent, as shown by significant inhibition with anti-CD18 F(ab)'$_2$ treatment and was PKC dependent, as indicated by staurosporine inhibition of respiratory burst (Figure 4A). MANS peptide treatment had no effect on PMA-induced respiratory burst of neutrophils. PMA-induced respiratory burst was independent of β_2-integrins, as indicated by lack of inhibition seen with

anti-CD18 F(ab)'$_2$ treatment, but was dependent on PKC, as indicated by staurosporine inhibition of respiratory burst (Figure 4B). These results demonstrate that MARCKS protein is essential for β_2-integrin dependent respiratory burst but not for β_2-integrin independent respiratory burst. These data also indicate that MARCKS protein functions upstream of assembly of the NADPH oxidase complex.

MARCKS protein is not essential for IIC-induced activation of p38 MAPK

The p38 MAPK is an important cell signaling protein known to play an essential role in neutrophil functions (Eckert et al., 2009; Kim and Haynes, 2013; Zu et al., 1998). Previous studies have identified a role for p38 MAPK in β_2-integrin-dependent neutrophil adhesion and ROS production (Detmers et al., 1998; Forsberg et al., 2001). Neutrophil stimulation with immune complexes induces activation (phosphorylation) of p38 MAPK, and p38 MAPK inhibition decreases neutrophil ROS production on IIC (Behnen et al., 2014). To determine whether MARCKS inhibition of IIC-induced respiratory burst d p38 MAPK activation, we evaluated the phosphorylation of p38 MAPK in MANS treated neutrophils stimulated by low density IIC. Isolated neutrophils were treated with either MANS, RNS, PP2, or anti-CD18 F(ab)'$_2$ prior to stimulation with IICs (Figure 5). The Src kinase inhibitor PP2 was included as a positive control for inhibition of p38 MAPK phosphorylation in IIC-stimulated neutrophils (Behnen et al., 2014). MANS and RNS peptides had no effect on p38 MAPK phosphorylation. Treatment with anti-CD18 F(ab)'$_2$ also did not inhibit p38 MAPK phosphorylation. These results indicate that inhibition of MARCKS with the MANS peptide does not alter IIC-induced p38 MAPK activation in neutrophils.

DISCUSSION

In the present study, we demonstrate a role for MARCKS protein in neutrophil β_2-integrin activation and signaling. MARCKS is an actin binding protein that is known to play a key role in neutrophil adhesion, migration, and respiratory burst. Based on previous work in our lab and others pointing toward a role for MARCKS in integrin-dependent functions (Disatnik et al., 2004; Sheats et al., 2014; Yue et al., 2000; Zhou and Li, 2000), we evaluated β_2-integrin activation and signaling in primary human neutrophils.

It is well documented that neutrophil activation through chemoattractant stimulation upregulates β_2-integrin surface expression (Jones et al., 1998; Montresor et al., 2012; Patcha et al., 2004). Therefore, we first evaluated whether MARCKS function was essential for upregulation of CD11b surface expression in neutrophils stimulated with the fMLP, otherwise known as inside-out activation. Our results demonstrate that neither MANS, nor the control RNS, peptide had any effect on fMLP-induced upregulation of CD11b (Figure 1A). This finding is in alignment with previous research showing that MARCKS inhibition with MANS did not affect CD18 surface expression induced by fMLP (Eckert et al., 2010). After upregulation of surface expression occurs, integrin conformation shifts from low affinity to high affinity to support the binding of β_2-integrins to ligand (Fan et al., 2016; Spillmann et al., 2002). We next evaluated whether MARCKS was required for fMLP-stimulated β_2-integrin affinity conformation change using an antibody specific for the high affinity conformation. N-formyl-methionyl-leucyl-phenylalanine (fMLP) stimulation induced a significant increase in surface expression of the high affinity conformation of CD11b that was not affected by MANS peptide treatment (Figure 1B). In a previous study, Eckert et al. showed that inhibition of MARCKS with the MANS peptide significantly attenuated fMLP-stimulated neutrophil migration and adhesion

(Eckert et al., 2010). These findings, combined with our data showing that MARCKS is not involved in inside-out β_2-integrin activation, led us to a closer investigation of MARCKS role in outside-in β_2-integrin activation.

Outside-in β_2-integrin activation occurs when neutrophil β_2-integrins interact with ligands, leading to cell signaling that promotes neutrophil firm adhesion (Abram and Lowell, 2009; Zarbock and Ley, 2009). Common neutrophil ligands are located on the endothelium or within the extracellular matrix. To model outside-in activation, recombinant ICAM-1 was utilized as a ligand to stimulate neutrophil adhesion. However, ICAM-1 alone is not a very strong adhesion stimulus for neutrophils *in vitro*. To overcome this, a second stimulus can be added to further promote adhesion (Abram and Lowell, 2009; Jakus et al., 2004). The addition of manganese is commonly used to induce the high affinity conformation of β_2-integrins *in vitro* (Weber et al., 1997) (Figure 2). Our results demonstrate that MARCKS inhibition with MANS peptide attenuates ICAM-1/Mn^{2+} induced neutrophil adhesion in a concentration dependent manner. ICAM-1/Mn^{2+} adhesion was also β_2-integrin dependent, as evident by significant attenuation in neutrophils treated with anti-CD18 F(ab)'$_2$. These findings demonstrate that MARCKS plays an essential role in outside-in β_2-integrin mediated adhesion.

The last step of activation is clustering of β_2-integrins on the surface, which increases the number of receptors interacting with ligand (valency) and increases the strength of adhesion (avidity) (Carman and Springer, 2003; Kim et al., 2004). Based on MANS peptide inhibition of β_2-integrin outside-in adhesion, we hypothesized that MARCKS plays a role in β_2-integrin clustering and neutrophil spreading. Immunofluorescence microscopy was used to evaluate neutrophil spreading and β_2-integrin clustering on ICAM-1/Fc. Neutrophils were stimulated on ICAM-1/Fc (Figure 3) or ICAM-1/Mn^{2+} (Supplemental Figure 1) coated chambered coverslips.

Both surfaces induced neutrophil spreading and β_2-integrin clustering, but ICAM-1/Fc induced a more pronounced response, likely due to engagement of neutrophil Fc receptors with the Fc portion of the ICAM-1/Fc protein. MARCKS inhibition with MANS peptide disrupted neutrophil spreading and β_2-integrin clustering on both surfaces (Figure 3C and Supplemental Figure 1E). These findings further inform our understanding of the effect of MANS peptide on neutrophil adhesion. Interestingly, the addition of fMLP as an inside-out stimulus did not restore the defects caused by MANS peptide. These results are consistent with previous studies that showed that MARCKS plays an essential role in cell spreading in several cell types (Disatnik et al., 2004; Li et al., 1996; Manenti et al., 1997; Monn Myat et al., 1997; Yue et al., 2000). Taken together, these data suggest that MARCKS plays an essential role in neutrophil β_2-integrin outside-in activation.

Respiratory burst is an important neutrophil effector function and window into neutrophil intracellular signaling. In the current study, we evaluated MARCKS role in both β_2-integrin dependent (IIC) and independent (PMA) respiratory burst. MANS peptide treatment significantly inhibited IIC-induced respiratory burst but not PMA-stimulated respiratory burst (Figure 4). From these findings, we 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 confirm 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.

Fc receptors and β_2-integrins cooperate in many neutrophil functional responses. Zhou and Brown demonstrated that β_2-integrin and FcγRIII cooperation generates a respiratory burst

that is unproductive when each receptor is stimulated separately. The respiratory burst response was also inhibited when either receptor was individually blocked (Zhou and Brown, 1994). Other studies have also demonstrated the requirement for β_2-integrins in neutrophil respiratory burst (Coxon et al., 1996; Jones et al., 2001; Moser et al., 2009), and our results further confirm that β_2-integrins are required for IIC-induced neutrophil respiratory burst (Figure 4A). This finding is in contrast with a different study showing that superoxide production by immune complex adherent neutrophils was not β_2-integrin dependent (Graham et al., 1993). However, superoxide production was only measured 20 minutes into the stimulation and the density of immune complex was not specified. In the current report, anti-CD18 F(ab)'$_2$ treated cells are significantly different than untreated cells at the 30-minute time point but not at 15 minutes (Figure 4A). It is possible that the limited time point analysis is the reason that the study by Graham *et al*. did not capture any differences attributed to β_2-integrin inhibition.

P38 MAPK is known to play an important role in inflammation and several neutrophil functions (Detmers et al., 1998; Eckert et al., 2009; Herlaar and Brown, 1999). P38 is also involved in neutrophil β_2-integrin mediated adhesion, as either p38 inhibition or Mac-1 receptor blocking antibodies significantly diminish IL-8 induced adhesion to ICAM-1 (Lomakina and Waugh, 2010). Interestingly, similar to our results with inhibition of MARCKS, inhibition of p38 MAPK did not attenuate PMA-stimulated neutrophil adhesion or ROS production (Detmers et al., 1998). Based on these similarities, we hypothesized that MARCKS could play a role in a pathway involving p38 MAPK and β_2-integrin dependent adhesion. Interestingly, we found that MANS peptide has no effect on p38 MAPK activation in IIC-stimulated primary neutrophils. This is in contrast to previous work that showed MANS treatment significantly decrease both LPS-induced cytokine production and p38 phosphorylation in murine macrophages (Lee et al.,

2015). However, p38 is activated by several different pathways, so the differences between our findings and Lee *et al.* may be explained by different cell types or mechanisms of stimulation (LPS vs IIC).

In our results reported here, anti-CD18 F(ab)'$_2$ did not attenuate p38 activation. This is in contrast with previously published results showing that β_2-integrin blocking antibodies did inhibit p38 activation; however, there are key differences between these previous studies and our current study that are worth mentioning. In the current report, we utilized low density insoluble immune complexes to stimulate neutrophil activation, which is mediated by both FcγRs and β_2-integrins. The prior studies utilized chemoattractants (e.g., TNFα and IL-8) to stimulate inside-out β_2-integrin responses on a variety of ligands (Detmers et al., 1998; Forsberg et al., 2001; Jakus et al., 2004; Lomakina and Waugh, 2010). The differences in cell signaling pathways initiated by these different stimuli could explain differences in our findings. Additionally, p38 has also been implicated in inside-out β_2-integrin activation by studies that showed that inhibition of p38 MAPK decreased TNFα-induced upregulation of CD11b in neutrophils (Forsberg et al., 2001; Tandon et al., 2000). However, we determined in Figure 1 that MARCKS inhibition does not impact inside-out β_2-integrin activation stimulated by fMLP; therefore, MANS peptide treatment is unlikely to affect p38 activation induced by IICs if p38 MAPK activation is linked to inside-out β_2-integrin activation and signaling. Finally, our finding that MANS peptide does not decrease p38 MAPK activation provides further indirect support for MARCKS role in outside-in β_2-integrin activation, as opposed to FcγR signaling. Indeed, several reports have demonstrated that outside-in β_2-integrin activation does not result in p38 phosphorylation (Bouaouina et al., 2004; Lefort et al., 2009). Therefore, p38 activation in our IIC-stimulated neutrophils is likely the result of FcγR engagement, and it was not altered in

MANS treated neutrophils. This indicates that either MARCKS function is downstream of p38 phosphorylation or that MARCKS function is operating a separate parallel and essential pathway that leads to IIC-induced respiratory burst in neutrophils.

Based on the evidence presented, we suggest that MARCKS plays an essential role in outside-in β_2-integrin activation and signaling. There are several reasons for this conclusion: (1) MARCKS inhibition with MANS peptide does not affect inside-out upregulation of CD11b or shift from low to high affinity CD11b stimulated by fMLP. (2) MARCKS inhibition with MANS significantly attenuates neutrophil adhesion to ICAM-1 enhanced with Mn^{2+}. Prior research in equine neutrophils also demonstrated that MANS attenuates IIC-induced neutrophil adhesion (Sheats et al., 2014). (3) MARCKS inhibition causes defects in neutrophil cell spreading and β_2-integrin clustering on ICAM-1/Fc. The β_2-integrin Mac-1 is required for sustained neutrophil spreading on immune complexes, and Mac-1 deficient neutrophils display a rounded morphology and lack spreading on immune complexes, similar to our results with MANS treated cells on ICAM-1/Fc (Tang et al., 1997). Additionally, clustering of β_2-integrins is considered to be an essential part of post-adhesion strengthening and sustained adhesion to ligand (Maheshwari et al., 2000; Zuchtriegel et al., 2021). (4) MARCKS inhibition with MANS attenuates IIC-induced neutrophil respiratory burst. FcγR stimulation induces inside-out signaling of β_2-integrins (Jones et al., 1998); however, our data in Figure 4 demonstrates that the respiratory burst induced by IICs is at least partially β_2-integrin-dependent despite FcγR-induced inside-out activation. (5) MARCKS inhibition with MANS does not decrease p38 activation induced by IICs. Prior studies demonstrated that p38 was clearly linked to FcγRs. Crosslinking of FcγRII/III in wild type mice resulted in p38 phosphorylation. Further, p38 was not phosphorylated in $Fc\gamma R3^{-/-}$ murine neutrophils stimulated with anti-CD18 mAbs (Jakus et al., 2004).

In summary, these data point toward a role for MARCKS protein in the underlying adhesive interactions that take place during neutrophil functions. It is well documented that neutrophil respiratory burst in response to immune complexes and other various stimuli is adhesion dependent (Chilcoat et al., 2002; Detmers et al., 1998; Moser et al., 2009; Zarbock and Ley, 2009; Zhou and Brown, 1993). Using neutrophils from patients with LAD, Nathan and colleagues demonstrated cytokine-induced neutrophil respiratory burst requires adhesion. These results were further confirmed using an anti-CD18 blocking antibody (Nathan et al., 1989). Thus, the disruption of β_2-integrin clustering and lack of cell spreading in MANS peptide treated cells certainly explains how MARCKS inhibition attenuates respiratory burst, adhesion, and chemotaxis in neutrophils (Eckert et al., 2010; Sheats et al., 2014).

Prior to this study, several studies pointed toward a role for MARCKS in integrin-dependent cellular responses, but the molecular details for why the protein is so important were not described. This report helps to expand understanding of the role of MARCKS function in neutrophil β_2-integrin-dependent responses. Future studies will further elucidate the molecular mechanism of MARCKS involvement in β_2-integrin outside-in signaling and determine how these findings relate to MARCKS regulation of the actin cytoskeleton in cell types beyond neutrophils.

FIGURES

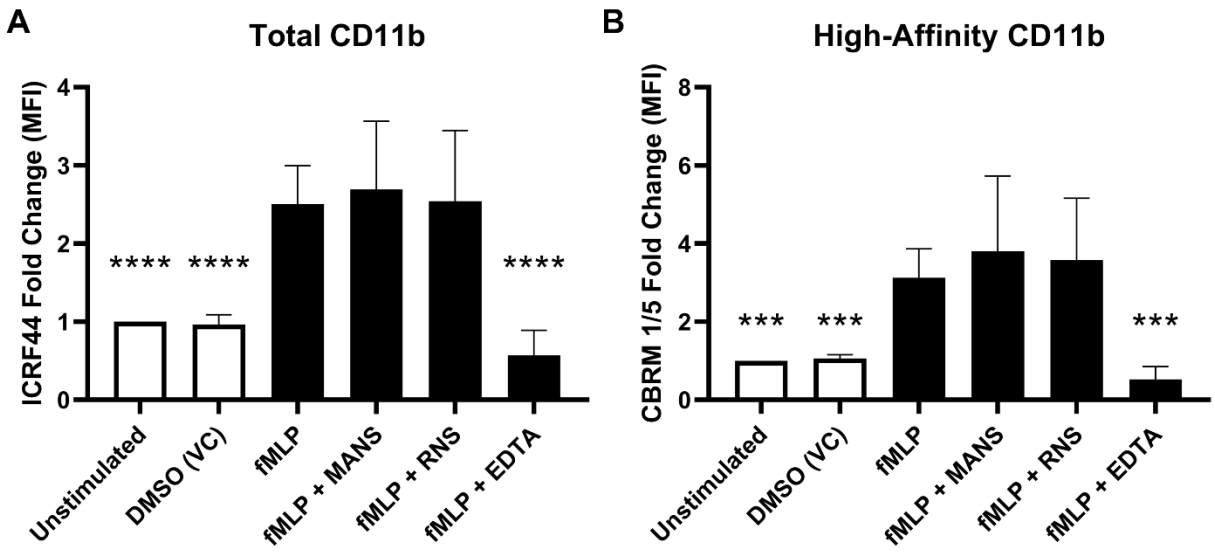


Figure 1. MARCKS function is not essential for fMLP-induced β_2-integrin affinity conformation change. Neutrophils were pretreated with 50 μM MANS, 50 μM RNS, 10 mM EDTA, or PBS for 30 minutes then stimulated with 100 nM fMLP or vehicle control for 5 minutes. Flow cytometry was used to measure total CD11b (ICRF44) (A) and high affinity CD11b (CBRM1/5) (B) as described. As a positive control for inhibition, pretreatment of neutrophils with EDTA significantly attenuated fMLP-induced surface expression of total CD11b (A) and high affinity CD11b (B). Data are represented as mean ± SD, n=4-9. One-way ANOVA with Dunnett's multiple comparisons test. *$p<0.05$ when compared to fMLP stimulation.

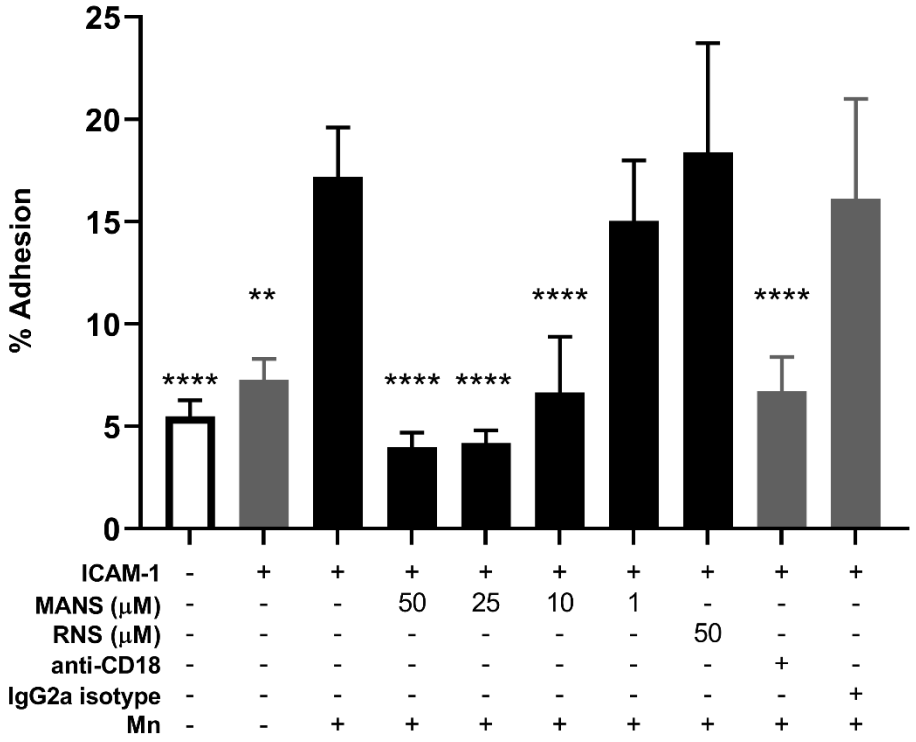


Figure 2. MARCKS function is essential for outside-in β_2-integrin-mediated neutrophil adhesion. Neutrophils were pretreated with indicated concentrations of MANS and RNS peptides, 30 μg/mL anti-CD18 F(ab)'$_2$, or 30 μg/mL IgG2a control F(ab)'$_2$ for 30 minutes prior to application to 96 well plate coated with 10 μg/mL ICAM-1. Cells were allowed to settle for 10 minutes before application of 0.5 mM Mn^{2+} for 10 minutes. Adhesion was quantified as described in the materials and methods section. MANS inhibition of MARCKS demonstrated concentration-dependent inhibition of ICAM-1/Mn^{2+}-induced adhesion. Data represented as mean ± SD, n=6. One-way ANOVA with Dunnett's multiple comparisons test. *$p<0.05$ when compared to ICAM-1 + Mn group.

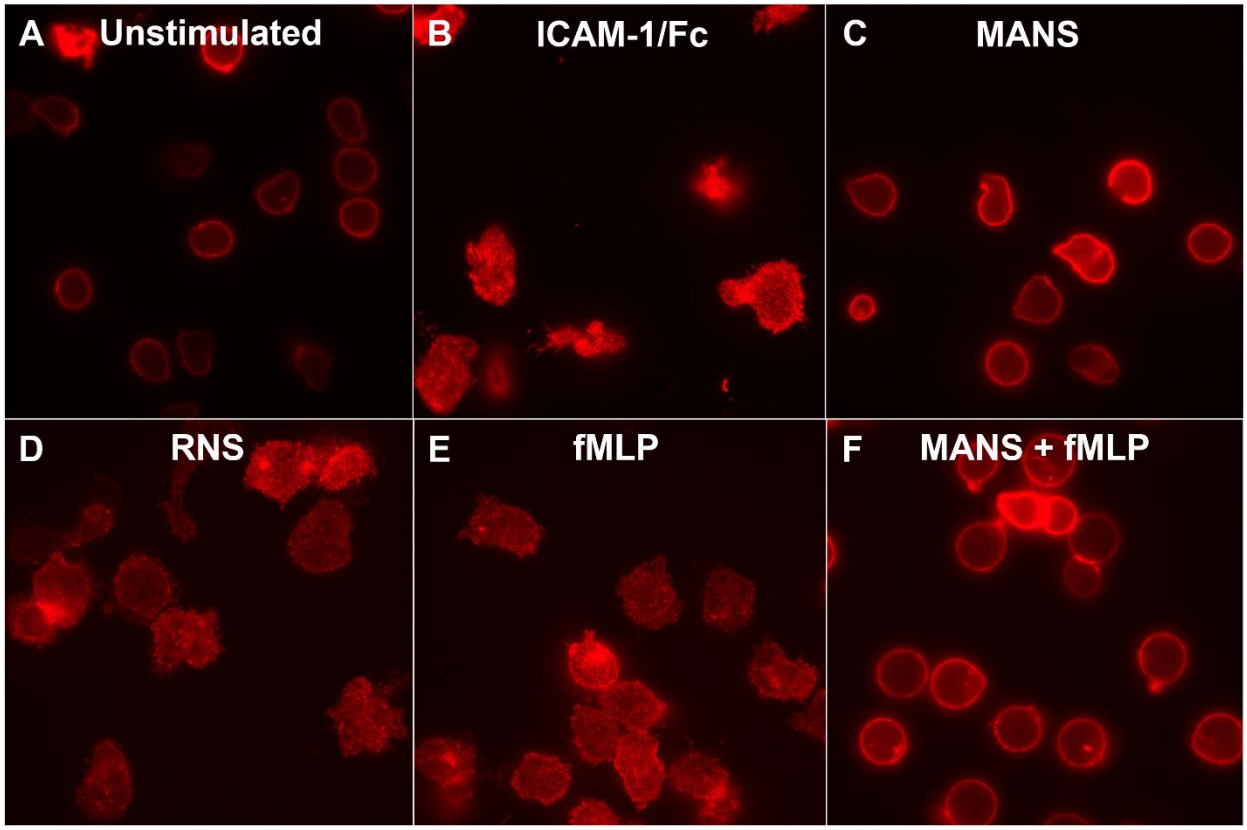


Figure 3. MARCKS function is essential for neutrophil spreading and β_2-integrin clustering on ICAM-1/Fc. fMLP does not restore defects. Immunofluorescence microscopy of CD11b: Neutrophils were pretreated with 50 µM MANS, 50 µM RNS, or PBS for 30 minutes prior to application to ICAM-1/Fc coated chambered coverslip. Cells were incubated for 10 minutes at 37°C. fMLP was applied 5 minutes into stimulation. Cells plated on ICAM-1/Fc surface exhibits both cell spreading and β_2-integrin clustering (B). MANS pretreatment (C) inhibited both cell spreading and β_2-integrin clustering. The control peptide RNS (D) had no effect on neutrophil spreading and integrin clustering. Application of fMLP to MANS (F) treated cells did not restore cell spreading and clustering defects caused by MANS peptide (C).

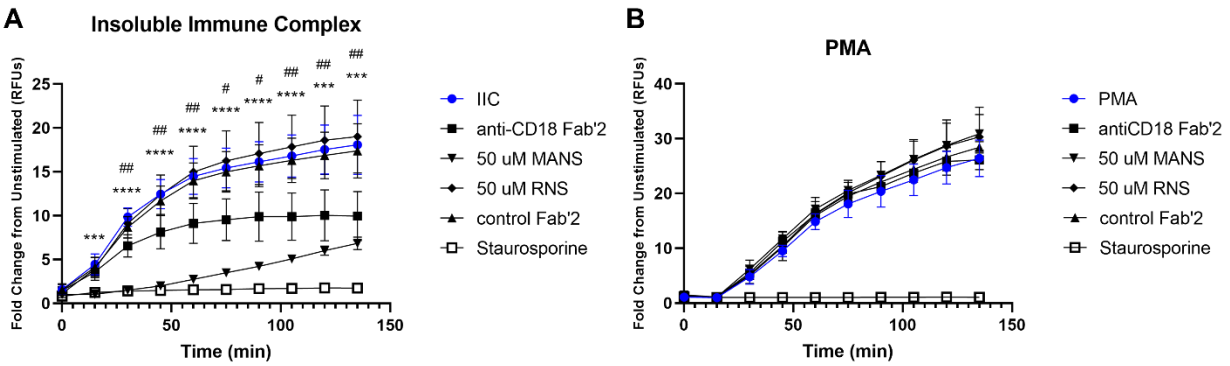


Figure 4. MARCKS function is essential for β2-integrin dependent neutrophil respiratory burst stimulated by insoluble immune complexes but not PMA-stimulated β2-integrin independent neutrophil respiratory burst. Neutrophils were pretreated for 30 minutes with 30 ug/mL anti-CD18 F(ab)'$_2$, 30 ug/mL IgG2a control F(ab)'$_2$, 50 µM MANS, 50 µM RNS, 100 nM staurosporine, or media alone (control). Respiratory burst was quantified by DHR-123. Cells were stimulated by insoluble immune complexes (IIC) (A) or PMA (B). Data are represented as mean ± SD, n=4. One-way ANOVA with Dunnett's multiple comparisons test. *$p<0.05$ MANS compared to IIC. #$p<0.05$ anti-CD18 F(ab)'$_2$ compared to IIC. Staurosporine $p<0.05$ for all time points beginning at 30 minutes.

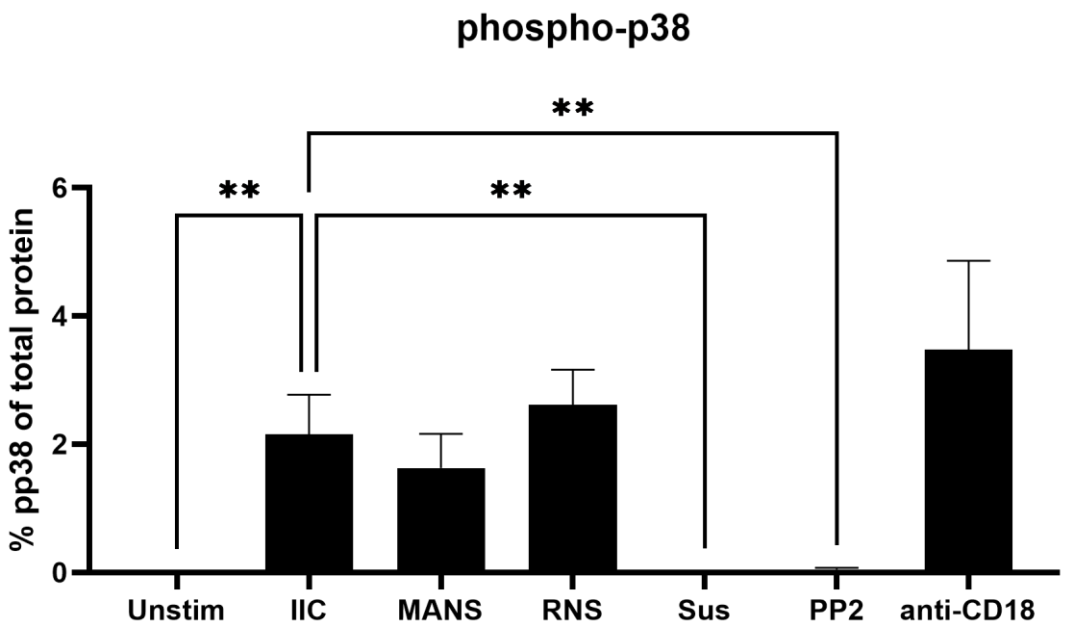
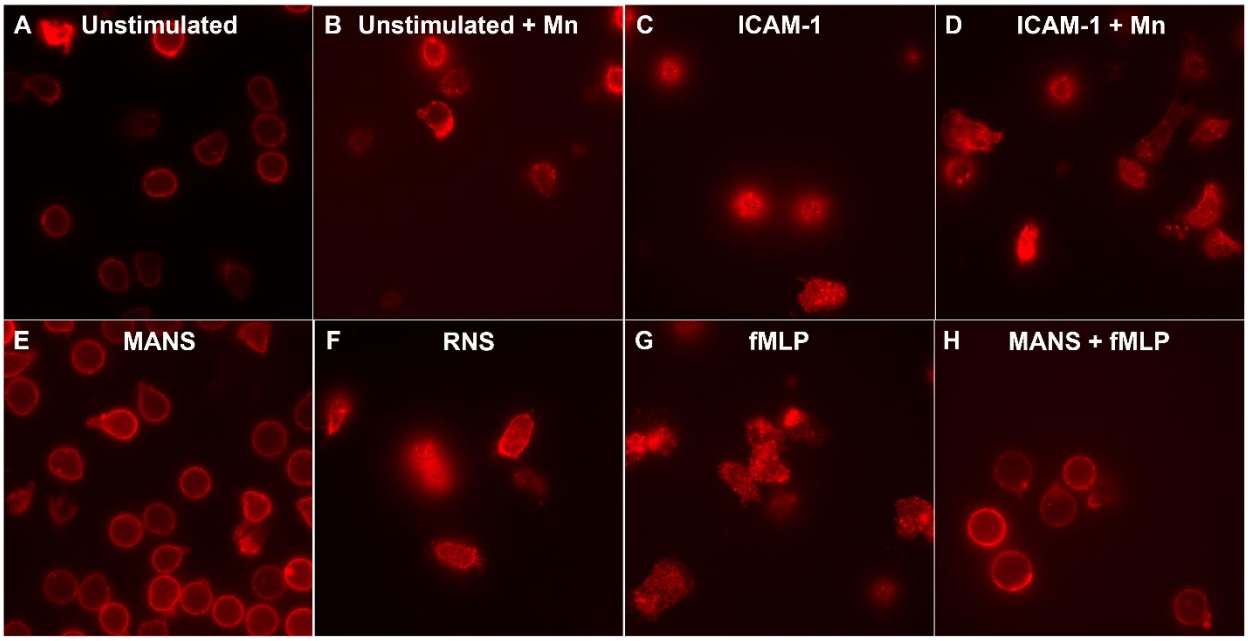


Figure 5. MARCKS function is not essential for IIC-induced p38 activation in neutrophils. Neutrophils ($7x10^6$) were pretreated with either PBS, 50 µM MANS, 50 µM RNS, 30 µM PP2 (positive control for inhibition), or 30 µg/mL anti-CD18 $F(ab)'_2$ then stimulated on low density immune complexes for 15 minutes or left in suspension (Sus). Phosphorylation of p38 was analyzed by Western blotting of whole-cell lysates. Equal loading was confirmed following transfer with Invitrogen No-Stain Protein Labeling Reagent. The percentage of phosphop38 was determined based on the total protein in the respective lane and the signal of the phosphop38 band. Blot shown is representative of two independent experiments.



Supplemental Figure 1. Stimulation of human neutrophils with ICAM-1/Mn^{2+}.

Immunofluorescence microscopy of CD11b: Neutrophils were pretreated with 50 µM MANS, 50 µM RNS, or PBS for 30 minutes prior to application to ICAM-1 coated chambered coverslip. Cells were incubated for 10 minutes at 37°C prior to application of Mn^{2+}. fMLP was applied 5 minutes after Mn^{2+}. Cells plated on ICAM-1 surface exhibits both cell spreading and β_2-integrin clustering (C). This response was enhanced with the application of Mn (D). MANS pretreatment (E) inhibited both cell spreading and β_2-integrin clustering. The control peptide RNS (F) had no effect on neutrophil spreading and integrin clustering. Application of fMLP to MANS (H) treated cells did not restore cell spreading and clustering defects caused by MANS peptide (E).

REFERENCES

Abram, C.L., Lowell, C.A., 2009. The Ins and Outs of Leukocyte Integrin Signaling. Annu Rev Immunol 339–362. https://doi.org/10.1146/annurev.immunol.021908.132554

Aderem, A., 1992. The Marcks brothers: A family of protein kinase C substrates. Cell 71, 713–716. https://doi.org/10.1016/0092-8674(92)90546-O

Arbuzova, A., Schmitz, A.A.P., Res, G.V., 2002. Cross-talk unfolded : MARCKS proteins. Biochem. J 362, 1–12.

Behnen, M., Leschczyk, C., Möller, S., Batel, T., Klinger, M., Solbach, W., Laskay, T., 2014. Immobilized Immune Complexes Induce Neutrophil Extracellular Trap Release by Human Neutrophil Granulocytes via FcγRIIIB and Mac-1. J. Immunol. 193, 1954–1965. https://doi.org/10.4049/jimmunol.1400478

Blackshear, P.J., 1993. The MARCKS Family of Cellular Protein Kinase C Substrates. J. Biol. Chem. 268, 1501–1504.

Bouaouina, M., Blouin, E., Halbwachs-Mecarelli, L., Lesavre, P., Rieu, P., 2004. TNF-Induced β 2 Integrin Activation Involves Src Kinases and a Redox-Regulated Activation of p38 MAPK . J. Immunol. 173, 1313–1320. https://doi.org/10.4049/jimmunol.173.2.1313

Carman, C. V., Springer, T.A., 2003. Integrin avidity regulation: Are changes in affinity and conformation underemphasized? Curr. Opin. Cell Biol. 15, 547–556. https://doi.org/10.1016/j.ceb.2003.08.003

Chilcoat, C.D., Rowlingson, K.A., Jones, S.L., 2002. The effects of cAMP modulation upon the adhesion and respiratory burst activity of immune complex-stimulated equine neutrophils. Vet. Immunol. Immunopathol. 88, 65–77. https://doi.org/10.1016/S0165-2427(02)00137-X

Coxon, A., Rieu, P., Barkalow, F.J., Askari, S., Sharpe, A.H., Von Andrian, U.H., Arnaout, M.A., Mayadas, T.N., 1996. A novel role for the β2 integrin CD11b/CD18 in neutrophil apoptosis: A homeostatic mechanism in inflammation. Immunity 5, 653–666. https://doi.org/10.1016/S1074-7613(00)80278-2

Detmers, P.A., Zhou, D., Polizzi, E., Thieringer, R., Hanlon, W.A., Vaidya, S., Bansal, V., 1998. Role of stress-activated mitogen-activated protein kinase (p38) in beta 2-integrin-dependent neutrophil adhesion and the adhesion-dependent oxidative burst. J. Immunol. 161, 1921–9.

Disatnik, M.-H.M.-H., Boutet, S.C., Pacio, W., Chan, A.Y., Ross, L.B., Lee, C.H., Rando, T.A., 2004. The bi-directional translocation of MARCKS between membrane and cytosol regulates integrin-mediated muscle cell spreading. J. Cell Sci. 117, 4469–79. https://doi.org/10.1242/jcs.01309

Eckert, R.E., Neuder, L.E., Park, J., Adler, K.B., Jones, S.L., 2010. Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) Protein Regulation of Human Neutrophil Migration. Am. J. Respir. Cell Mol. Biol. 42, 586–594. https://doi.org/10.1165/rcmb.2008-0394OC

Eckert, R.E., Sharief, Y., Jones, S.L., 2009. p38 mitogen-activated kinase (MAPK) is essential for equine neutrophil migration. Vet. Immunol. Immunopathol. 129, 181–191. https://doi.org/10.1016/j.vetimm.2008.11.007

Edwards, D.N., Bix, G.J., 2019. The Inflammatory Response After Ischemic Stroke: Targeting β2 and β1 Integrins. Front. Neurosci. 13, 540. https://doi.org/10.3389/fnins.2019.00540

Fan, Z., Mcardle, S., Marki, A., Mikulski, Z., Gutierrez, E., Engelhardt, B., Deutsch, U., Ginsberg, M., Groisman, A., Ley, K., 2016. Neutrophil recruitment limited by high-affinity bent β2 integrin binding ligand in cis. Nat. Commun. 7, 12658. https://doi.org/10.1038/ncomms12658

Forsberg, M., Löfgren, R., Zheng, L., Stendahl, O., 2001. Tumour necrosis factor-α potentiates CR3-induced respiratory burst by activating p38 MAP kinase in human neutrophils. Immunology 103, 465–472. https://doi.org/10.1046/j.1365-2567.2001.01270.x

Giacalone, V.D., Margaroli, C., Mall, M.A., Tirouvanziam, R., 2020. Neutrophil adaptations upon recruitment to the lung: New concepts and implications for homeostasis and disease. Int. J. Mol. Sci. 21, 1–21. https://doi.org/10.3390/ijms21030851

Giagulli, C., Ottoboni, L., Caveggion, E., Rossi, B., Lowell, C., Constantin, G., Laudanna, C., Berton, G., 2006. The Src family kinases Hck and Fgr are dispensable for inside-out, chemoattractant-induced signaling regulating β2 integrin affinity and valency in neutrophils, but are required for β2 integrin-mediated outside-in signaling involved in sustained adhesion. J. Immunol. 177, 604–11. https://doi.org/10.4049/JIMMUNOL.177.1.604

Graham, I.L., Lefkowith, J.B., Anderson, D.C., Brown, E.J., Leikowith, J.B., Anderson, D.C., Browntln, E.J., 1993. Immune complex-stimulated neutrophil LTB4 production is dependent on β2 integrins. J. Cell Biol. 120, 1509–1517. https://doi.org/10.1083/jcb.120.6.1509

Harlan, J.M., Winn, R.K., 2002. Leukocyte-endothelial interactions: Clinical trials of anti-adhesion therapy. Crit. Care Med. 30. https://doi.org/10.1097/00003246-200205001-00007

Herlaar, E., Brown, Z., 1999. p38 MAPK signalling cascades in inflammatory disease. Mol. Med. Today 5, 439–447. https://doi.org/10.1016/S1357-4310(99)01544-0

Herter, J., Zarbock, A., 2013. Integrin Regulation during Leukocyte Recruitment. J. Immunol. 190, 4451–4457. https://doi.org/10.4049/jimmunol.1203179

Jakus, Z., Berton, G., Ligeti, E., Lowell, C.A., Mócsai, A., 2004. Responses of Neutrophils to Anti-Integrin Antibodies Depends on Costimulation through Low Affinity FcγRs: Full

Activation Requires Both Integrin and Nonintegrin Signals. J. Immunol. 173, 2068–2077. https://doi.org/10.4049/jimmunol.173.3.2068

Jones, S.L., Knaus, U.G., Bokoch, G.M., Brown, E.J., 1998. Two signaling mechanisms for activation of α(M)β2 avidity in polymorphonuclear neutrophils. J. Biol. Chem. 273, 10556–10566. https://doi.org/10.1074/jbc.273.17.10556

Jones, S.L., Sharief, Y., Chilcoat, C.D., 2001. Signaling mechanism for equine neutrophil activation by immune complexes. Vet. Immunol. Immunopathol. 82, 87–100. https://doi.org/10.1016/S0165-2427(01)00350-6

Kalwa, H., Michel, T., 2011. The MARCKS Protein Plays a Critical Role in Phosphatidylinositol 4,5-Bisphosphate Metabolism and Directed Cell Movement in Vascular Endothelial Cells * □ S. J. Biol. Chem. 286, 2320–2330. https://doi.org/10.1074/jbc.M110.196022

Kim, D., Haynes, C.L., 2013. The Role of p38 MAPK in Neutrophil Functions: Single Cell Chemotaxis and Surface Marker Expression. Analyst 138. https://doi.org/10.1039/b000000x/Kim

Kim, M., Carman, C. V., Yang, W., Salas, A., Springer, T.A., 2004. The primacy of affinity over clustering in regulation of adhesiveness of the integrin α L β 2. J. Cell Biol. 167, 1241–1253. https://doi.org/10.1083/jcb.200404160

Kolaczkowska, E., Kubes, P., 2013. Neutrophil recruitment and function in health and inflammation. Nat. Rev. Immunol. 13, 159–175. https://doi.org/10.1038/nri3399

Kuijpers, T W, Verhoeven, A J, Roos, D, Kuijpers, Taco W, Van Lier, R.A.W., Hamann, D., De Boer, M., Yin Thung, L., Weening, R.S., Verhoeven, Arthur J, Roos, Dirk, 1997. Leukocyte adhesion deficiency type 1 (LAD-1)/variant. A novel immunodeficiency syndrome characterized by dysfunctional beta2 integrins. J. Clin. Invest 100, 1725–1733.

https://doi.org/10.1172/JCI119697

Lee, S.M., Suk, K., Lee, W.H., 2015. Myristoylated alanine-rich C kinase substrate (MARCKS) regulates the expression of proinflammatory cytokines in macrophages through activation of p38/JNK MAPK and NF-κB. Cell. Immunol. 296, 115–121. https://doi.org/10.1016/j.cellimm.2015.04.004

Lefort, C.T., Hyun, Y.-M., Schultz, J.B., Law, F.-Y., Waugh, R.E., Knauf, P.A., Kim, M., 2009. Conformational Changes in Integrin Mac-1 Outside-In Signal Transmission by. J. Immunol. 183, 6460–6468. https://doi.org/10.4049/jimmunol.0900983

Li, J., Zhu, Z., Bao, Z., 1996. Role of MacMARCKS in Integrin-dependent Macrophage Spreading and Tyrosine Phosphorylation of Paxillin. J. Biol. Chem. 271, 12985–12990.

Lin, J., He, Y., Wang, B., Xun, Z., Chen, S., Zeng, Z., Ou, Q., 2019. Blocking of YY1 reduce neutrophil infiltration by inhibiting IL-8 production via the PI3K-Akt-mTOR signaling pathway in rheumatoid arthritis. Clin. Exp. Immunol. 195, 226–236. https://doi.org/10.1111/cei.13218

Lomakina, E.B., Waugh, R.E., 2010. Signaling and dynamics of activation of LFA-1 and Mac-1 by immobilized IL-8. Cell. Mol. Bioeng. 3, 106–116. https://doi.org/10.1007/s12195-009-0099-x

Maheshwari, G., Brown, G., Lauffenburger, D.A., Wells, A., Griffith, L.G., 2000. Cell adhesion and motility depend on nanoscale RGD clustering. J. Cell Sci. 113, 1677–1686. https://doi.org/10.1242/jcs.113.10.1677

Manenti, S., Malecaze, F., Darbon, J.M., 1997. The major myristoylated PKC substrate (MARCKS) is involved in cell spreading, tyrosine phosphorylation of paxillin, and focal contact formation. FEBS Lett. 419, 95–98. https://doi.org/10.1016/S0014-5793(97)01438-5

McLaughlin, S., Aderem, A., 1995. The myristoyl-electrostatic switch: a modulator of reversible protein-membrane interactions. TIBS 20.

Meisel, S.R., Shapiro, H., Radnay, J., Neuman, Y., Khaskia, A.R., Gruener, N., Pauzner, H., David, D., 1998. Increased expression of neutrophil and monocyte adhesion molecules LFA- 1 and Mac-1 and their ligand ICAM-1 and VLA-4 throughout the acute phase of myocardial infarction: Possible implications for leukocyte aggregation and microvascular plugging. J. Am. Coll. Cardiol. 31, 120–125. https://doi.org/10.1016/S0735-1097(97)00424-5

Middleton, E., Cody, M., Rondina, M., 2020. Neutrophil Extracellular Traps (NETs) Contribute to Immunothrombosis in COVID-19 Acute Respiratory Distress Syndrome. Blood 136, 1169–1179. https://doi.org/10.1182/blood.2020007008/1747487/blood.2020007008.pdf

Mittal, M., Siddiqui, M.R., Tran, K., Reddy, S.P., Malik, A.B., 2014. Reactive oxygen species in inflammation and tissue injury. Antioxidants Redox Signal. 20, 1126–1167. https://doi.org/10.1089/ars.2012.5149

Monn Myat, M., Anderson, S., Allen, L.-A.H., Aderem, A., 1997. MARCKS regulates membrane ruffling and cell spreading. Curr. Biol. 7, 611–614.

Montresor, A., Toffali, L., Constantin, G., Laudanna, C., Ley, K., 2012. Chemokines and the signaling modules regulating integrin affinity. Front. Immunol. 3. https://doi.org/10.3389/fimmu.2012.00127

Moser, M., Bauer, M., Schmid, S., Ruppert, R., Schmidt, S., Sixt, M., Wang, H.V., Sperandio, M., Fässler, R., 2009. Kindlin-3 is required for β2 integrin-mediated leukocyte adhesion to endothelial cells. Nat. Med. 15, 300–305. https://doi.org/10.1038/nm.1921

Nathan, C., Srimal, S., Farber, C., Sanchez, E., Kabbash, L., Asch, A., Gailit, J., Wright, S.D., 1989. Cytokine-induced Respiratory Burst of Human Neutrophils: Dependence on Extracellular Matrix Proteins and CDU/CD18 Integrins. J. Cell Biol. 109, 1341–1349.

Neumann, B., Zantl, N., Veihelmann, A., Emmanuilidis, K., Pfeffer, K., Heidecke, C.-D., Holzmann, B., 1999. Mechanisms of acute inflammatory lung injury induced by abdominal sepsis, International Immunology.

Ott, L.E., Sung, E.J., Melvin, A.T., Sheats, M.K., Haugh, J.M., Adler, K.B., Jones, S.L., Sung, J., Melvin, A.T., Sheats, M.K., Haugh, J.M., Adler, K.B., Jones, S.L., 2013. Fibroblast Migration Is Regulated by Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) Protein. PLoS One 8, e66512. https://doi.org/10.1371/journal.pone.0066512

Patcha, V., Wigren, J., Winberg, M.E., Rasmusson, B., Li, J., S7rndahl, E., 2004. Differential inside-out activation of β2-integrins by leukotriene B4 and fMLP in human neutrophils. Exp. Cell Res. 300, 308–319. https://doi.org/10.1016/j.yexcr.2004.07.015

Schmidt, S., Moser, M., Sperandio, M., 2013. The molecular basis of leukocyte recruitment and its deficiencies. Mol. Immunol. 55, 49–58. https://doi.org/10.1016/j.molimm.2012.11.006

Schofield, Z.V., Woodruff, T.M., Halai, R., Wu, M.C.L., Cooper, M.A., 2013. Neutrophils - A key component of ischemia-reperfusion injury. Shock 40, 463–470. https://doi.org/10.1097/SHK.0000000000000044

Schymeinsky, J., Mocsai, A., Walzog, B., 2007. Neutrophil activation via β2 integrins (CD11/CD18): Molecular mechanisms and clinical implications. Thromb. Haemost. 98, 262–273. https://doi.org/10.1160/TH07

Seykora, J.T., Monn Myat, M., Allen, L.-A.H., Ravetch, J. V, Aderem, A., 1996. Molecular Determinants of the Myristoyl-electrostatic Switch of MARCKS. J. Biol. Chem. 271,

18797–18802.

Sheats, M.K., Pescosolido, K.C., Hefner, E.M., Sung, J., Adler, K.B., Jones, S.L., 2014. Myristoylated Alanine Rich C Kinase Substrate (MARCKS) is essential to β2-integrin dependent responses of equine neutrophils. Vet Immunol Immunopathol 160, 167–176. https://doi.org/10.1016/j.vetimm.2014.04.009

Spillmann, C., Osorio, D., Waugh, R., 2002. Integrin activation by divalent ions affects neutrophil homotypic adhesion. Ann. Biomed. Eng. 30, 1002–1011. https://doi.org/10.1114/1.1511241

Sundaram, M.;, Cook, H.W., Byers, D.M., 2004. The MARCKS family of phospholipid binding proteins: regulation of phospholipase D and other cellular components. Biochem. Cell Biol. 82, 191–200.

Tandon, R., Sha'afi, R.I., Thrall, R.S., 2000. Neutrophil β2-integrin upregulation is blocked by a p38 MAP kinase inhibitor. Biochem. Biophys. Res. Commun. 270, 858–862. https://doi.org/10.1006/bbrc.2000.2540

Tang, T., Rosenkranz, A., Assmann, K.J.M., Goodman, M.J., Gutierrez-Ramos, J.-C., Carroll, M.C., Cotran, R.S., Mayadas, T.N., 1997. A Role for Mac-1 (CDIIb/CD18) in Immune Complex-stimulated Neutrophil Function In Vivo: Mac-1 Deficiency Abrogates Sustained Fc Receptor-dependent Neutrophil Adhesion and Complement-dependent Proteinuria in Acute Glomerulonephritis. J. Exp. Med 186, 1853–1863.

Vicente-Manzanares, M., Sá Nchez-Madrid, F., 2018. Targeting the integrin interactome in human disease. Curr. Opin. Cell Biol. 55, 17–23. https://doi.org/10.1016/j.ceb.2018.05.010

Wang, J., Arbuzova, A., Hangyas-Mihalyne, G., McLaughlin, S., 2001. The Effector Domain of Myristoylated Alanine-rich C Kinase Substrate Binds Strongly to Phosphatidylinositol 4,5-

Bisphosphate. J. Biol. Chem. 276, 5012–5019. https://doi.org/10.1074/jbc.M008355200

Wang, J., Gambhir, A., Ngyi Hangyá S-Mihá Lyné, G., Murray, D., Golebiewska, U., Mclaughlin, S., 2002. Lateral Sequestration of Phosphatidylinositol 4,5-Bisphosphate by the Basic Effector Domain of Myristoylated Alanine-rich C Kinase Substrate Is Due to Nonspecific Electrostatic Interactions*. J. Biol. Chem. 277, 34401–34412. https://doi.org/10.1074/jbc.M203954200

Wang, J., Li, Q., Yin, Y., Zhang, Y., Cao, Y., Lin, X., Huang, L., Hoffmann, D., Lu, M., Qiu, Y., 2020. Excessive Neutrophils and Neutrophil Extracellular Traps in COVID-19. Front. Immunol. 11, 1–13. https://doi.org/10.3389/fimmu.2020.02063

Wang, Z., Li, J., Cho, J., Malik, A.B., 2014. Prevention of vascular inflammation by nanoparticle targeting of adherent neutrophils. Nat. Nanotechnol. 9, 204–210. https://doi.org/10.1038/nnano.2014.17

Weber, C., Lu, F., Casasnovas, J.M., Springer, T.A., 1997. Role of alpha L beta 2 integrin avidity in transendothelial chemotaxis of mononuclear cells. J. Immunol. Methods 159, 3968–3975.

Williams, M.A., Solomkin, J.S., 1999. Integrin-mediated signaling in human neutrophil functioning. J. Leukoc. Biol. 65, 725–736. https://doi.org/10.1002/jlb.65.6.725

Wright, H.L., Makki, F.A., Moots, R.J., Edwards, S.W., 2017. Low-density granulocytes: functionally distinct, immature neutrophils in rheumatoid arthritis with altered properties and defective TNF signalling. J. Leukoc. Biol. 101, 599–611. https://doi.org/10.1189/jlb.5a0116-022r

Yago, T., Petrich, B.G., Zhang, N., Liu, Z., Shao, B., Ginsberg, M.H., McEver, R.P., 2015. Blocking neutrophil integrin activation prevents ischemia-reperfusion injury. J. Exp. Med.

212, 1267–1281. https://doi.org/10.1084/jem.20142358

Yue, L., Lu, S., Garces, J., Jin, T., Li, J., 2000. Protein Kinase C-regulated Dynamitin-Macrophage-enriched Myristoylated Alanine-Rice C Kinase Substrate Interaction Is Involved in Macrophage Cell Spreading. J. Biol. Chem. 275, 23948–23956. https://doi.org/10.1074/jbc.M001845200

Zarbock, A., Ley, K., 2009. Neutrophil Adhesion and Activation under Flow. Microcirculation 16, 31–42. https://doi.org/10.1080/10739680802350104

Zhou, M.-J., Brown, E.J., 1994. CR3 (Mac-l, CgM 2, CDllb/CD18) and Fc /RIII Cooperate in Generation of a Neutrophil Respiratory Burst: Requirement for FcTRII and Tyrosine Phosphorylation. J. Cell Biol. 125, 1407.

Zhou, M., Brown, E.J., 1993. Leukocyte response integrin and integrin-associated protein act as a signal transduction unit in generation of a phagocyte respiratory burst. J. Exp. Med. 178, 1165–1174. https://doi.org/10.1084/jem.178.4.1165

Zhou, X., Li, J., 2000. Macrophage-enriched Myristoylated Alanine-rich C Kinase Substrate and Its Phosphorylation Is Required for the Phorbol Ester-stimulated Diffusion of 2 Integrin Molecules*. J. Biol. Chem. 275, 20217–20222. https://doi.org/10.1074/jbc.M909129199

Zimmerman, T., Blanco, F., 2008. Inhibitors Targeting the LFA-1/ICAM-1 Cell-Adhesion Interaction: Design and Mechanism of Action. Curr. Pharm. Des. 14, 2128–2139. https://doi.org/10.2174/138161208785740225

Zu, Y.L., Qi, J., Gilchrist, A., Fernandez, G.A., Vazquez-Abad, D., Kreutzer, D.L., Huang, C.K., Sha'afi, R.I., 1998. p38 mitogen-activated protein kinase activation is required for human neutrophil function triggered by TNF-alpha or FMLP stimulation. J. Immunol. 160, 1982–9.

Zuchtriegel, G., Uhl, B., Pick, R., Ramsauer, M., Dominik, J., Mittmann, L.A., Canis, M., Kanse, S., Sperandio, M., Krombach, F., Reichel, C.A., 2021. Vitronectin stabilizes intravascular adhesion of neutrophils by coordinating B2 integrin clustering. Haematologica 106, 2641–2653.

ACKNOWLEDGEMENTS

NIH OD/ORIP K01OD015136 and NCSU College of Veterinary Medicine funded this research

CHAPTER 4

MARCKS inhibition alters bovine neutrophil responses to *Salmonella* Typhimurium

Haleigh E. Conley[1,2], Chalise F. Brown[1], Trina L. Westerman[3], Johanna R. Elfenbein[3], M. Katie Sheats[1,2]

[1]Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, United States

[2]Comparative Medicine Institute, North Carolina State University, Raleigh, North Carolina, United States

[3]Department of Pathobiological Sciences, University of Wisconsin-Madison, Madison, Wisconsin, United States

Abstract

Salmonella enteritis infections are of great concern in both humans and livestock. Due to growing concerns over increasing antimicrobial resistance of non-typhoidal salmonellae (NTS), novel, non-anti-microbial host-directed targets are being explored as an alternative strategy for treating this pathogen. During enteric salmonellosis, the host experiences severe diarrhea characterized by severe neutrophilic enterocolitis. Normally, neutrophils serve as the first and most effective line of host defense against bacterial pathogens; however, *Salmonella* is a unique pathogen that can utilize the toxic mediators released by neutrophils to further colonize the intestine. Because neutrophils may cause the host more harm than good during *Salmonella* infection, neutrophils represent a potential therapeutic target. MARCKS is an actin-binding protein that plays an essential role in many neutrophil effector responses. We hypothesized that inhibition of MARCKS protein would alter bovine neutrophil responses to *Salmonella* Typhimurium (STm). Our data demonstrate that MARCKS inhibition attenuates STm-induced neutrophil adhesion and chemotaxis but enhances neutrophil phagocytosis and respiratory burst in response to STm. This is the first report implicating a role for MARCKS protein in neutrophil antibacterial responses.

Introduction

Non-typhoidal salmonellae (NTS) are the leading cause of human bacterial foodborne gastroenteritis. Annually, NTS cause more than 150 million illnesses worldwide and the highest number of food-borne disease related deaths in the United States (Healy and Bruce, 2020; Kirk et al., 2015; Sell and Dolan, 2018; Voetsch et al., 2004). Infection can result from contaminated food or water or direct contact with individuals or animals shedding the pathogen (Hoelzer et al., 2011). Despite efforts to decrease the number of infections, the incidence of NTS illnesses has

not changed over the past 20 years (Henao et al., 2015). In addition to increasing infections, The World Health Organization reports that the incidence of *Salmonella* antimicrobial resistance (AMR) is increasing. The high rate of disease, increasing antimicrobial resistance, and high degree of morbidity and mortality associated with multiple-drug resistant (MDR) NTS infection drive the need to develop new interventions to combat this important pathogen (Chiu et al., 2009; Coburn et al., 2007).

Bovine salmonellosis is a significant concern for both humans and cattle. The bovine host-adapted serotype Dublin is a significant cause of calf sepsis, pneumonia, diarrhea, encephalitis, and mortality, placing a significant economic burden on the dairy industry (Mohler et al., 2009). In addition, cattle of all ages are clinically affected by NTS, including serotype Typhimurium (STm) (Mohler et al., 2009). Infected cattle present significant contamination to human food supply, contributing to the increase in MDR *Salmonella* corresponding to human disease (Jackson et al., 2013; Rodriguez-Rivera et al., 2014; Valenzuela et al., 2017). Therefore, development of non-antimicrobial strategies to mitigate bovine *Salmonella* carriage and shedding is of great importance to protect food supply.

Invasion of the intestinal epithelium is an essential starting point to *Salmonella* infection. *Salmonella* uses the type-III secretion system-1 (TTSS-1) to secrete effector proteins directly into the epithelial cell cytosol, manipulate the actin cytoskeleton, and direct invasion. This process begins when effector proteins nucleate actin at the invasion site to induce plasma membrane ruffling (Patel and Galán, 2005; Srikanth et al., 2011). The plasma membrane reorganizes and closes the phagosome around *Salmonella* (Bakowski et al., 2007). After internalization, the expression of TTSS-1 is reduced, and the actin cytoskeleton returns to homeostasis (Fu and Galán, 1999). Epithelial invasion also activates the inflammatory response

through epithelial cell release of inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α, and IL-18) (Broz et al., 2012; Hobbie et al., 1997; Jung et al., 1995). These cytokines induce neutrophil transmigration across the epithelium causing neutrophilic enteritis, which is a hallmark of enteric salmonellosis and key to *Salmonella's* survival in the intestine (Loetscher et al., 2012; Zhang et al., 2003).

NTS are unique because they benefit from the neutrophilic inflammatory response that is designed to kill most bacteria. When stimulated, bovine neutrophils release toxic products that *Salmonella* is relatively resistant to, which gives the pathogen a competitive advantage over resident microflora (Diaz-Ochoa et al., 2016; Gallois et al., 2001; Stecher et al., 2007; Winter et al., 2010). The success of NTS during intestinal inflammation suggests that a host-directed therapeutic strategy designed to reduce inflammation will reduce NTS success in the intestine and ultimately diminish the host *Salmonella* burden without contributing to the development of antimicrobial resistance.

Protein kinase-C (PKC) enzymes are essential players in regulating neutrophil-mediated inflammation (Bertram et al., 2011; Larsson, 2006; Sheats et al., 2015). One way that PKC enzymes regulate inflammation is through a reversible phosphorylation of Myristoylated Alanine Rich C-kinase Substrate (MARCKS). MARCKS is an actin binding protein that is ubiquitously expressed in mammalian cells and regulates events requiring dynamic actin reorganization, such as adhesion, phagocytosis, and cell migration (Allen and Aderem, 1995; Arbuzova et al., 2002; Nairn and Aderem, 1992). MARCKS alters these processes through a cycle of reversible binding of phosphatidylinositol 4,5-bisphosphate (PIP2) and actin. When dephosphorylated, MARCKS is anchored to the plasma membrane through hydrophobic insertion of the N-terminal myristoyl-moiety into the plasma membrane and through electrostatic interaction of the polybasic effector

domain (ED) with the negatively charged phospholipid PIP2 (Arbuzova et al., 2002; Wang et al., 2001). Dephosphorylated MARCKS concentrates PIP2 within the membrane and facilitates actin polymerization and crosslinking (Arbuzova et al., 2002; Hartwig et al., 1992). Phosphorylated MARCKS, on the other hand, is displaced to the cytosol, which increases PIP2 availability at the plasma membrane and decreases MARCKS-actin binding (Disatnik et al., 2004; Hartwig et al., 1992).

In addition to cellular actin regulation, MARCKS also has a clear role in inflammatory responses. MARCKS inhibition with N-terminus or phosphorylation blocking peptides inhibits immune cell functions including adhesion, migration, ROS production, and pro-inflammatory cytokine production (C.-H. Chen et al., 2014; Disatnik et al., 2004; Eckert et al., 2010; Estrada-Bernal et al., 2009; Green et al., 2012; Monn Myat et al., 1997; Ott et al., 2013; Sheats et al., 2014, 2015). Given that MARCKS plays a key role in neutrophil-mediated immune responses, we investigated whether MARCKS inhibition would be a strategy to alter bovine neutrophil inflammatory responses to *Salmonella*.

MATERIALS & METHODS

Reagents

RPMI Medium 1640 (Gibco), Recombinant Bovine GM-CSF (Kingfisher Biotech), 1.077 g/mL Ficoll (Cytvia), Calcein AM (Corning), DHR-123 (Sigma), Diphenyleneiodonium chloride (DPI) (Sigma), HBSS (Corning), UltraPure 0.5M EDTA (Invitrogen), Interleukin-8 (Sigma), Dead Cell Apoptosis kit (Invitrogen), Difco LB Broth, Miller (BD), Difco LB Agar, Miller (BD).

Peptide Treatment

The MyristoylAted N-terminal Sequence (MANS) and Random Nucleotide Sequence (RNS) peptides were synthesized by Genemed Synthesis, Inc. The sequence of MANS is identical to the

first 24 amino acids of the human MARCKS protein: myristic acid-GAQFSKTAAKGEAAAERPGEAAVA. The RNS peptide is a randomly scrambled control: myristic acid-GTPAPAAEGAGAEVKRASAEAKQAF. The Effector Domain (ED) and Control Peptide (CP) peptides were synthesized by Sigma Aldrich. The sequence of ED: KKKKKRFSFKKSFKLSGFSFKKNKKGRKKRRQRRRPQ. The sequence of CP: CEIEEHAWNTVEMFSSFPGTQLYNDAGRKKRRQRRRPQ. Peptide working solutions were resuspended in sterile PBS. Where indicated, pretreatment of cell suspensions with indicated peptide concentrations occurred at 37°C for 30 minutes.

Bovine Neutrophil Isolation

Jugular or tail vein blood was collected with 1.5 mg/mL EDTA loaded syringes and 25 mL or less placed in a 50 mL conical. An equal amount of PBS was added to each conical and carefully mixed by inversion. 30 mL of diluted blood was then layered on top of 15 mL Ficoll (1.077 g/mL). The gradient was centrifuged at 1,100 x g for 30 minutes at 10ºC with the brake off. The supernatant was removed via aspiration. Hypotonic lysis was done by adding 20 mL cold 0.2% NaCl to the RBC/granulocyte pellet. The tube was mixed by gentle inversion and allowed to lyse for 30-40 seconds. Then 20 mL cold 1.6% NaCl was added, and tubes were inverted to mix. The tubes were then centrifuged at 100 x g for 8 minutes at 4ºC with the brake on. The supernatants were gently removed via aspiration. The lysis and centrifugation step were repeated for a second time. Cells were resuspended in HBSS prior to counting via Trypan blue and allowed to rest for 1 hour. Because of the prevalence of parasites in dairy cows, we also performed a differential count for each individual isolation. Cytology slides were prepared and stained with Wright Giemsa prior to counting. Cell counts for experiments were normalized to the neutrophil percentage based on the differential staining.

Pooled Whole Serum Preparation

Whole blood was collected via tail vein from 10 healthy lactating dairy cows in the NCSU CVM TAU Diary herd and placed in anti-coagulant free serum tubes. Tubes were allowed to settle for 1 hour prior to centrifugation at 2,000 x g for 10 minutes at 4ºC. Serum was isolated and pooled prior to the preparation of frozen aliquots. Pooled serum was used as indicated for experiments.

Bacterial Strains and Growth Conditions

All bacterial strains were derivatives of *Salmonella* Typhimurium (STm) ATCC 14028.s. HA420 wild type strain was used for all experiments except phagocytosis. pTurboGFP was used for phagocytosis. Bacteria were grown on Luria Bertani (LB) agar or in LB broth at 37°C with agitation (225 rpm). Media was supplemented with the following antibiotics as appropriate: nalidixic acid (50 mg/L) and carbenicillin (100 mg/L). For all neutrophil stimulations, bacteria were grown to late exponential phase by diluting overnight cultures 1:100 into LB broth and incubating at 37°C with agitation for 3.5 hours (Westerman et al., 2021). Bacterial cultures were washed in phosphate buffered saline (PBS) and cell density was estimated by optical density (600 nm). Bacteria were diluted in PBS or indicated buffer and maintained on ice until use. Where indicated, bacteria were opsonized as detailed in individual experiments. Diluted bacteria were plated to establish the number of viable colony forming units.

Neutrophil Adhesion

Immulon2HB clear 96 well plates were coated overnight with 5% HI FBS in HBSS and washed 1x with PBS prior to experiment. Isolated neutrophils were resuspended to $10x10^6$ cells/mL in HBSS, primed with 10 ng/mL GM-CSF, and loaded with 2 µg/mL Calcein AM for 30 minutes at room temperature protected from light. The cells were then centrifuged at 100 x g for 8 minutes, supernatant removed by aspiration, then resuspended in HBSS supplemented with 1 mM $CaCl_2$,

1 mM $MgCl_2$, and 2% HI FBS at $1x10^6$ cells/mL. Neutrophils were then pretreated in Eppendorf tubes with MARCKS inhibitor peptides as indicated for 30 minutes at 37ºC with 5% CO_2. 100,000 cells with appropriate treatments were plated in the wells and allowed to settle for 10 minutes at room temperature, protected from light. PBS or late exponential-phase STm (MOI 50:1) was added to the cells. The plate was then incubated for 1 hour at 37ºC with 5% CO_2. After the incubation, the fluorescence was measured using a microplate reader at 485 nm excitation 525 nm emission. Cells were dumped and washed with PBS, reading fluorescence at each wash step. The fluorescence values for each wash were divided by initial fluorescence and multiplied by 100 to calculate percent adhesion. The first wash that demonstrated less than 10% adhesion in the non-stimulated cells (plated on 5% FBS coating) was considered the result. Treatment groups were assessed in triplicate.

Chemotaxis

Isolated primary neutrophils were rested for 1.5 hours at room temperature in HBSS. Neutrophils were primed with 10 ng/mL bovine GM-CSF and loaded with calcein at 2 µg/mL in HBSS at $1x10^7$ cells/mL for 30 minutes at room temperature protected from light. Cells were centrifuged at 100 x g for 8 minutes then resuspended in HBSS supplemented with 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 2% HI FBS (chemotaxis buffer) at $2.2x10^6$ cells/mL and pretreated with indicated concentrations of inhibitory peptides or controls for 30 minutes at 37ºC with 5% CO_2. Chemoattractants (30 µL) were added in triplicate to the bottom wells of a standard PCTE NeuroProbe ChemoTx plate (5 µm pore size, well capacity 30 µL, 3.2 mm cell site diameter). For this assay, STm was normalized to the determined OD_{600} for an MOI for 50:1, centrifuged, then resuspended in chemotaxis buffer and stored cold until ChemoTx plate was setup. Cells (50,000 cells in 25 µL) were added to the 100% migration wells in the bottom and to the top of

the membrane for all groups. The ChemoTx plate was incubated for 60 minutes at 37ºC with 5% CO_2. The non-migrated cells were then removed from the top of the membrane using a cell scraper and 10 µL 0.5 M EDTA was added to the top of the membrane for 10 minutes at room temperature protected for light. The excess EDTA was then removed using a cell scraper and the ChemoTx plate was centrifuged for 5 minutes at 1,000 x rpm. The membrane was removed, and the fluorescence of the bottom wells was measured (485 nm excitation, 525 nm emission) using a Biotek microplate reader. Percent migration was calculated by dividing the fluorescence of the experimental bottom wells by the fluorescence of bottom wells containing the known cell number (100% migration group). Treatment groups were tested in triplicate (Sheats et al., 2015).

Phagocytosis – Flow Cytometry

Neutrophils were primed with 10 ng/mL bovine GM-CSF in HBSS at $1x10^7$ cells/mL for 30 minutes at room temperature protected from light. Cells were centrifuged at 100 x g for 8 minutes then resuspended in RPMI supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 2% HI FBS at $1.67x10^6$ cells/mL and pretreated with indicated concentrations of MARCKS inhibitor peptides or PBS for 30 minutes at 37ºC with 5% CO_2. 450,000 cells were aliquoted (300 µL) into BD Falcon polystyrene round bottom tubes and allowed to settle for 10 minutes at room temperature. Immediately prior to experiment, GFP-STm were opsonized with RPMI + 10% pooled bovine serum for 30 minutes at 37ºC then used to induce phagocytosis by stimulating cells with a 25:1 MOI for the indicated amount of time. At the end of incubation, 40% PFA was added to the tube to reach a final concentration of 2% and the tube was placed on ice for 10 minutes prior to dilution with 1 volume of PBS (Westerman et al., 2018). Tubes were stored at 4ºC until ready to prep for acquisition. Tubes were centrifuged at 1,100 x g for 10 minutes, supernatants gently dumped, resuspended in 200 µL 0.125% Trypan blue in PBS, and transferred

to a polypropylene tube then immediately acquired on a BD LSRII cytometer (Jin et al., 2018). The neutrophil population was gated by a forward scatter versus side scatter plot to discriminate from cell debris. Singlet cells were included by gating based on side scatter-area versus side scatter-width and forward scatter-area versus forward scatter-width. Neutrophils not exposed to STm were used as a negative control for GFP-negative cells. Data from at least 7,000 events gated on singlet neutrophils were collected. FCS files were analyzed by FlowJo. We calculated the percentage of GFP positive neutrophils using histograms. The fluorescence intensity was divided into two peaks: the GFP-negative cells that did not have phagocytosed bacteria (left peak) and phagocytosing GFP-positive cells (right peak) (Boero et al., 2021). Flow cytometry experiments were performed in the Flow Cytometry and Cell Sorting facility at North Carolina State University – College of Veterinary Medicine.

Phagocytosis - Microscopy

The neutrophil and GFP-STm co-culture was prepared as described for flow cytometry and incubated for 75 minutes. Cells were transferred to Eppendorf tubes, centrifuged at 160 x g for 5 minutes, supernatant discarded, and pellet resuspended in 200 µL PBS. Cells were fixed for 10 minutes on ice with PFA (2% final concentration), then stained with 2 µg/mL AF954-conjugated wheat germ agglutinin (WGA) (Invitrogen) at room temperature for 10 minutes. Cell suspensions were diluted with an additional 200 µL PBS, and 200 µL were centrifuged onto glass slides using a cytospin. Slides were air dried and cover glass was mounted with DAPI Flouromount (Southern Biotech) before imaging with an Olympus IX83 inverted microscope.

Respiratory Burst

Clear-bottom black 96 well plates were coated overnight with 5% HI FBS in HBSS and washed 1x with PBS prior to experiment. Isolated primary neutrophils were resuspended to $1.0x10^7$

cells/mL in HBSS and primed with 10 ng/mL GM-CSF for 30 minutes at room temperature protected from light. Cells were centrifuged at 100 x g for 8 minutes and resuspended in RPMI supplemented with 10% autologous serum, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. 150,000 cells were added to Eppendorf tubes containing indicated concentrations of MANS peptide or controls and incubated for 30 minutes at 37ºC with 5% CO_2. Cells were added to plate, allowed to settle for 10 minutes, and 10 µM DHR-123 was added to each well. PBS or STm (MOI 50:1) was added to the plate and fluorescence immediately read every 15 minutes for 2 hours at 485 nm excitation, 525 nm emission for 2 hours. All groups were run in triplicate. A control consisting of DHR-123 added to media alone was used for normalization prior to determining fold change from unstimulated control.

Propidium Iodide Staining

Isolated primary neutrophils were rested for 1 hour at room temperature in HBSS. Neutrophils were primed with 10 ng/mL bovine GM-CSF in HBSS at $1x10^7$ cells/mL for 30 minutes at room temperature protected from light. Cells were centrifuged at 100 x g for 8 minutes then resuspended in RPMI supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 2% HI FBS at $1.0x10^6$ cells/mL. 100 µL of cells were added to Eppendorf tubes containing indicated concentrations of MARCKS targeting peptides or PBS. Cells were incubated for 90 minutes at 37ºC with 5% CO_2, immediately place on ice, and 1 volume of cold PBS was added. Cells were centrifuged at 300 x g for 5 minutes at 4°C. Samples were stained with 1 µL of 100 µg/mL propidium iodide following the manufacturer's recommendations (Invitrogen Dead Cell Apoptosis kit) and immediately acquired on a BD LSRII cytometer. The neutrophil population was gated by a forward scatter versus side scatter plot to discriminate from cell debris. Singlet cells were included by gating based on side scatter-area versus side scatter-width and forward scatter-area

versus forward scatter-width. Data from at least 10,000 events gated on singlet neutrophils were collected. Flow cytometry experiments were performed in the Flow Cytometry and Cell Sorting facility at North Carolina State University – College of Veterinary Medicine.

Statistical Analyses

All results were analyzed using GraphPad Prism software version 9 using repeated measures one-way ANOVA unless otherwise noted. Data are reported as mean ± SD with sample size indicated on each figure. P values <0.05 were considered statistically significant. *Indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when compared to STm stimulation.

RESULTS

Effect of MARCKS inhibitor peptides on primary neutrophil viability

In this study, we investigated two different peptide inhibitors of MARCKS: MANS and ED peptides. MANS peptide is a function blocking peptide identical to the first 24 amino acids of MARCKS. Reported mechanisms of action for MANS peptide include displacing MARCKS from the plasma membrane to the cytosol, altering cytoskeletal dynamics, and decreasing MARCKS phosphorylation (C.-H. Chen et al., 2014; Eckert et al., 2010; Li et al., 2001). ED peptide is a 37 amino acid peptide that mimics the effector domain of MARCKS. Reported mechanisms of action for ED include decreasing MARCKS phosphorylation, direct binding to lipopolysaccharide (LPS), and acting as a nuclear localization signal and regulator of nuclear membrane PIP2, with downstream effects on gene expression (Manček-Keber et al., 2012; Rohrbach et al., 2017, 2015). MANS and ED peptides each have a corresponding control peptide, RNS and CP, respectively. Cell viability of peptide-treated cells was evaluated using propidium iodide staining. Isolated primary neutrophils were treated with relevant in vitro concentrations of MANS, RNS, ED, and CP peptides. Neutrophil viability at 90 minutes was not

significantly affected by any of the tested concentrations of MANS peptide (Figure 1). Neutrophil viability at 90 minutes was significantly affected by all tested concentrations of ED peptide (Figure 1). Further, ED peptide induced neutrophil respiratory burst without the presence of any other stimulus (Supplemental Figure 1). Given these results, we chose to focus on the MANS and RNS peptides in our subsequent investigations of the effects of MARCKS inhibition on STm-induced neutrophil responses.

Effect of MARCKS inhibition on STm-induced neutrophil adhesion

Neutrophil adhesion to the vasculature and to the basolateral membrane of the intestine is critical for neutrophil extravasation (Fournier and Parkos, 2012; Schmidt et al., 2013). Previous work in our lab demonstrated an essential role for MARCKS in equine neutrophil adhesion (Sheats et al., 2014). Therefore, we hypothesized that MARCKS inhibition would decrease STm-induced adhesion of primary bovine neutrophils. Static neutrophil adhesion was induced using STm for 1 hour. Pretreatment with MANS peptide for 30 minutes prior to stimulation significantly attenuated adhesion in a concentration dependent manner (Figure 2). The control peptide RNS had no effect on neutrophil adhesion. This evidence demonstrates an essential role for MARCKS protein in STm-induced neutrophil adhesion.

Effect of MARCKS inhibition on STm-induced neutrophil migration

The histopathological hallmark of *Salmonella* enterocolitis is a massive influx of neutrophils into the intestine. Previous research demonstrated a clear role for MARCKS in neutrophil migration *in vitro* and *in vivo* (Damera et al., 2010; Sheats et al., 2014), so we next evaluated the effect of MARCKS inhibition on *Salmonella*-induced neutrophil migration. Neutrophil migration can be modeled using specialized ChemoTx plates where fluorescently-labeled neutrophils are plated on top of a permeable membrane and stimulated by the presence of

chemoattractants (e.g., IL-8, fMLP, LTB_4) or bacteria in wells below the membrane (Sheats et al., 2015, 2014). Migrated neutrophils are then detected using fluorescence. Previous reports using this model for bacteria-induced migrations were limited (Adusumilli et al., 2005); therefore, two different approaches for stimulating STm-induced neutrophil migration were utilized. For the first approach, STm alone was used to stimulate neutrophil migration. For the second approach, a low concentration of IL-8 (10 ng/mL) was added to the prepared STm, knowing that IL-8 should enhance neutrophil migration.

STm alone induced significant neutrophil migration (~55%) compared to the unstimulated control (~25%), and MANS peptide treatment significantly attenuated migration in a concentration-dependent manner (Figure 3A). IL-8 significantly enhanced STm-induced neutrophil migration (~85% migration), and MANS peptide significantly attenuated migration in a concentration-dependent manner. RNS control peptide had no effect on neutrophil migration. This evidence demonstrates an essential role for MARCKS protein in STm-induced neutrophil migration *in vitro*.

Effect of MARCKS inhibition on STm-induced neutrophil respiratory burst

Neutrophil reactive oxygen species (ROS) production, also termed respiratory burst, is a critical host response to pathogens. Previous research in our lab demonstrated that MARCKS inhibition with MANS peptide significantly inhibited immune complex-mediated ROS production (Sheats et al., 2014); therefore, we hypothesized that MARCKS inhibition would attenuate *Salmonella*-induced neutrophil respiratory burst. DHR-123 was used to measure reactive oxygen species production in STm-stimulated neutrophils for 2 hours. Peak respiratory burst occurred around 90 minutes (not shown), and the effect of MANS peptide treatment on respiratory burst at specific time points and as total production (Figure 4A-D). MANS peptide

treatment had no significant effect on STm-induced respiratory burst at 60-, 75-, and 90-minutes post stimulation. However, peptide treatment with lower concentrations of MANS (25 μM and 10 μM) significantly enhanced total respiratory burst, as measured by area under the curve compared to STm alone (Figure 4D). The control peptide RNS had no effect on respiratory burst. These data suggest that MARCKS inhibition with low concentrations of MANS peptide enhances STm-stimulated neutrophil respiratory burst.

Effect of MARCKS inhibition on neutrophil phagocytosis of *Salmonella*

Phagocytosis also plays a key role in the immune response to bacterial infections. Based on the respiratory burst data, we hypothesized that the increased neutrophil respiratory burst may be due to an increase in neutrophil phagocytosis of STm. To determine the effect of MARCKS inhibition on STm phagocytosis, neutrophils were incubated with pooled serum-opsonized GFP expressing *Salmonella* wild type strain for 15, 75, and 90 minutes. The fluorescence of extracellular GFP-STm was quenched with Trypan blue before detection of phagocytosis using flow cytometry. Our results demonstrate that MANS peptide treatment increased the number of GFP+ neutrophils at 75- and 90-minutes post-stimulation, indicating an increase in phagocytosis (Figure 5A-B). Furthermore, MANS peptide treatment also resulted in an increase in GFP+ MFI at 75- and 90-minutes post-stimulation (Figure 5D-E), showing that there was an increase in the number of ingested bacteria (Schreiner et al., 2011). The control peptide RNS had no effect on neutrophil phagocytosis of GFP-STm. Phagocytosis was confirmed using immunofluorescence microscopy (Figure 5C&F). These results demonstrate that MARCKS inhibition with MANS peptide enhances neutrophil phagocytosis of opsonized-STm.

DISCUSSION

MARCKS protein is known to play an important role in neutrophil effector functions. This investigation is the first to examine whether the inhibition of MARCKS function affects neutrophil responses to bacteria. Two MARCKS inhibitor peptides, MANS and ED, were used to inhibit MARCKS function in neutrophils. MANS is a function blocking peptide that is known to displace MARCKS from the plasma membrane to the cytosol (Eckert et al., 2010). MANS peptide treatment has also been shown to decrease MARCKS phosphorylation, although the mechanism is unknown (C.-H. Chen et al., 2014; Lee et al., 2015). In addition to previous studies demonstrating MANS peptide inhibition of neutrophil migration, adhesion, and respiratory burst, the peptide also regulates LPS-induced inflammatory responses of macrophages (Eckert et al., 2010; Lee et al., 2015; Sheats et al., 2014). The ED peptide and other peptides that mimic the MARCKS effector domain inhibit MARCKS phosphorylation across multiple cell types, presumably through competitive inhibition with PKC-mediated MARCKS phosphorylation (Elzagallaai et al., 2000; Rohrbach et al., 2017). Prior studies with ED peptide or effector domain mimetic peptides have demonstrated success in suppressing cancer cell migration and metastasis *in vitro* and *in vivo* (C. H. Chen et al., 2014). Other studies have highlighted the potential use of ED peptides in targeted delivery to cells for cell-specific killing mechanisms (Eustace et al., 2020; Tamura, 2017).

The cytotoxicity of both peptides on neutrophils was determined, and the results demonstrate that ED peptide had significant cytotoxic effects on neutrophil viability (Figure 1). ED peptide treatment also induced respiratory burst of neutrophils without the addition of other stimuli (Supplemental Figure 1). ED peptide induced cytotoxicity has been reported previously; however, there were no previous reports of this peptide being evaluated in primary neutrophils.

In a previous study, ED peptide demonstrated significant cytotoxic effects on several lung cell lines at a concentration lower than evaluated in our study (Rohrbach et al., 2017). Another study demonstrated that ED peptide selectively induced cytotoxic effects on a glioblastoma cell line in a manner unique from apoptosis (Eustace et al., 2020). Our data also demonstrate cytotoxic effects of ED peptide on primary neutrophils that are not indicative of apoptosis (Supplemental Figure 2). Taken together, these findings indicate that ED peptide likely has off-target effects beyond inhibition of MARCKS that are contributing to significant neutrophil cell death, precluding our ability to utilize this peptide as a tool to investigate the role of MARCKS in STm-induced neutrophil responses. MANS peptide had no effect on neutrophil viability, which is consistent with previous findings that MANS peptide is not cytotoxic to normal human bronchial epithelial cells (C.-H. Chen et al., 2014). Therefore, we present data evaluating only MANS peptide inhibition of MARCKS protein. The effects of ED peptide in selected neutrophil functional assays are reported as supplemental data (Supplemental Figure 3).

Adhesion is one of the first neutrophil functions in response to infection and inflammation. Therefore, we sought to determine whether MARCKS protein plays a significant role in STm-induced neutrophil adhesion. There are limited reports of whole bacteria-induced neutrophil adhesion (Darveau et al., 1995; Drevets, 1997); however, STm induced significant static adhesion after 1 hour of stimulation. Our results demonstrate that MANS significantly attenuates neutrophil adhesion induced by STm, which is consistent with results of neutrophil adhesion induced by other stimuli (Eckert et al., 2010; Sheats et al., 2014).

Previous reports have also identified MARCKS as a key player in neutrophil migration. *Salmonella*-induced migration was evaluated using two different experimental approaches. The first was STm alone and the second included STm + IL-8. This approach was taken due to

limited evidence of bacteria alone stimulating migration in the ChemoTx plates. Further, IL-8 plays a prominent role in inducing neutrophil migration into the intestinal epithelium during *Salmonella* infection (Lee et al., 2000). Both models induced significant and robust migration that was significantly attenuated with MANS peptide treatment. These findings are also consistent with previous reports identifying a role for MARCKS in *in vitro* neutrophil chemotaxis (Sheats et al., 2015, 2014).

Salmonella is known for utilizing reactive oxygen species for survival, and the bacterium is known to induce respiratory burst in human neutrophils (Hiyoshi et al., 2018; Westerman et al., 2018). We sought to determine whether MARCKS protein played a role in STm-induced respiratory burst. Previous findings demonstrated that STm-induced neutrophil respiratory burst required whole serum (Westerman et al., 2018). We utilized autologous serum in the respiratory burst experiments to eliminate autofluorescence issues observed with purchased whole bovine serum and to obtain maximal respiratory burst. Our results demonstrate marked variability in respiratory burst in our study population, which is likely due to the varying age, gestation stage, and prior exposure to *Salmonella*. We also observed relatively low levels of respiratory burst activity compared with our previous reports in STm-stimulated human neutrophils (Westerman et al., 2018). Despite this, our AUC data show that treatment of primary bovine neutrophils with low concentrations of MANS peptide enhanced STm-stimulated neutrophil respiratory burst. This is a novel finding, given that previous research demonstrated that MARCKS inhibition (with the MANS peptide) either attenuated immune-complex induced respiratory burst, or had no effect on PMA-induced respiratory burst (Sheats et al., 2014).

Neutrophils are designed to kill bacteria through phagocytosis and subsequent release of ROS into the phagolysosome. We next sought to determine whether MARCKS played a role in

neutrophil phagocytosis of a GFP-expressing wild type STm. Similar to the respiratory burst data, our results demonstrate that inhibition of MARCKS with the MANS peptide enhances neutrophil phagocytosis of STm. The finding that MARCKS inhibition enhanced neutrophil respiratory burst and phagocytosis of STm is novel. This is the first report of enhancement of neutrophil effector functions with MANS-mediated MARCKS inhibition. In previous studies using MARCKS$^{-/-}$ and WT fetal liver derived murine macrophages, Carballo et al. found no difference or a slight decrease in MARCKS$^{-/-}$ macrophages, depending on the stimulus (Carballo et al., 1999). The differences between our result and theirs could be due to differences in stimulus, differences between a function blocking peptide versus protein knockout, or the extremely high phagocytic index in their study that could preclude their ability to detect enhancement. Enhanced neutrophil phagocytosis and ROS production with MARCKS inhibition could suggest that MARCKS has a negative regulatory role in these functions or cooperates with a different negative regulator protein. There are a few known negative regulators of phagocytosis, including the Src kinase Fgr and SHIP (Cox et al., 2001; Gresham et al., 2000). Further studies are needed to determine how MARCKS regulates *Salmonella*-induced neutrophil phagocytosis and respiratory burst.

On a molecular level, it is possible to speculate how MARCKS inhibition may enhance neutrophil phagocytosis. During early phagocytosis, PIP2 is synthesized, accumulates at the phagocytic cup, and may control actin assembly at the phagosome. Phospholipase C (PLC) degrades PIP2, producing the lipid messenger diacylglycerol (DAG). Further cytoskeletal changes occur due to either the decrease in PIP2 or increase in DAG. At the same time, phosphatidylinositol 3-kinase (PI3K) converts to PIP2 to PIP3, triggering closure of the phagosome (May and Machesky, 2001). While at the plasma membrane, MARCKS is known to

sequester 3-4 PIP2 molecules. Therefore, when MARCKS inhibition with MANS displaces MARCKS protein from the plasma membrane to the cytosol, there is an increase in PIP2 at the plasma membrane. Given that PIP2 plays such an important role in phagocytosis, it is possible that the increase in PIP2 availability is the reason for enhanced phagocytosis during MARCKS inhibition.

Neutrophils utilize both β_2-integrins (also known as complement receptor 3 – CR3) and Fc receptors to mediate the engulfment of pathogens. In the current study, STm were serum-opsonized prior to stimulation, which likely initiated a phagocytosis mediated by both CR3 and Fc receptors (Ishibashi and Arai, 1996; van Bruggen et al., 2007). However, we did not use any blocking antibodies to determine if either of the receptors had a dominant effect. In addition to Fc receptor and CR3 recognition of *Salmonella*, toll-like receptors 2, 4, and 5 are also important in the recognition and internalization of *Salmonella* (Arpaia et al., 2011; Blander and Medzhitov, 2004; Feuillet et al., 2006; van Bruggen et al., 2007). TLR2/4 stimulation is also known to activate the β_2-integrin Mac-1 (Torres-Gomez et al., 2020). Thus, it is apparent that neutrophil recognition of *Salmonella* results in the activation of β_2-integrins either directly or indirectly through Fc receptors or toll-like receptor signaling. Our findings that MARCKS inhibition attenuated both neutrophil adhesion and migration is consistent with our previously published results. Both adhesion and migration are known to be β_2-integrin-dependent, and we have previously ascribed a role for MARCKS in neutrophil β_2-integrin-dependent neutrophil functions (Sheats et al., 2014). LPS stimulation induces β_2-integrin-dependent cell spreading (Schmidt et al., 2001), reinforcing the likelihood that *Salmonella* induces β_2-integrin-dependent adhesion. Although our current investigation did not utilize specific β_2-integrin inhibitors, we hypothesize that diverging effects of MARCKS inhibition on STm-induced neutrophil responses could be

explained by the difference β_2-integrin-dependent (adhesion and migration) and independent (phagocytosis and respiratory burst) neutrophil effector functions induced by *Salmonella*.

Formation of the Nicotinamide adenine dinucleotide phosphate-oxidase/Nox2 (NADPH) oxidase system and subsequent respiratory burst is intimately associated with neutrophil phagocytosis. Assembly of the 5 subunits of the phagocyte oxidase complex (gp91phox, p22phox, p40phox, p47phox, and p67phox) can be detected as early as 30 seconds after the onset of phagocytosis (Allen et al., 1999; DeLeo et al., 1999; Karimi et al., 2014). In light of this connection between these mechanisms, it seems plausible that the slight enhancement of ROS production that we observed is simply a predictable sequela to the enhanced phagocytosis caused by inhibition of MARCKS with the MANS peptide. At this time, it is unknown whether the enhanced phagocytosis and respiratory burst results in increased killing of *Salmonella*. Experiments to determine whether MANS peptide treatment enhances the killing of STm are ongoing.

Our results show that MARCKS protein function plays a significant role in multiple neutrophil effector responses to *Salmonella* Typhimurium *in vitro* but is dispensable for others. Future investigations will determine whether this difference is due to differences in β_2-integrin dependent versus independent neutrophil responses to *Salmonella*. From a novel target standpoint, our results regarding MARCKS inhibition as a way to limit neutrophil-mediated host damage are promising. MARCKS inhibition may offer a strategy to limit excess neutrophil recruitment to sites of infection or inflammation (inhibition of adhesion and migration), while still preserving neutrophil host defense mechanisms (phagocytosis and respiratory burst). In this study we utilized the MANS peptide was used to inhibit MARCKS function in neutrophil responses to *Salmonella*. Although MANS peptide worked well in our *in vitro* evaluation, the

peptide is quite small and would not withstand the harsh protease-rich environment of the intestine. Therefore, alternative delivery methods or compound modifications would be needed in order to further investigate MARCKS-targeting as a therapeutic strategy *in vivo*.

FIGURES

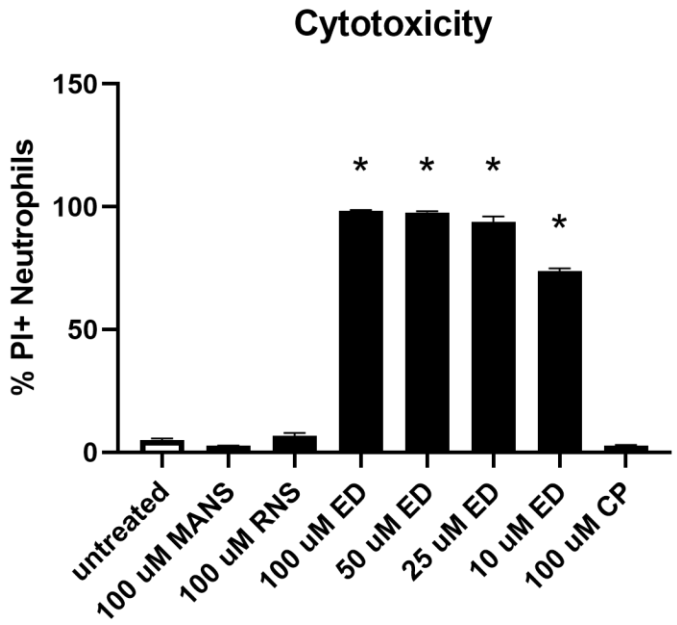


Figure 1. Treatment of primary bovine neutrophils with ED peptide compromises cell viability. Neutrophils were treated with indicated concentrations of peptide or PBS (untreated) for 90 minutes, stained by propidium iodide, then analyzed by flow cytometry. Data represented as mean ± SD (n=2). p*<0.05 when compared to untreated cells.

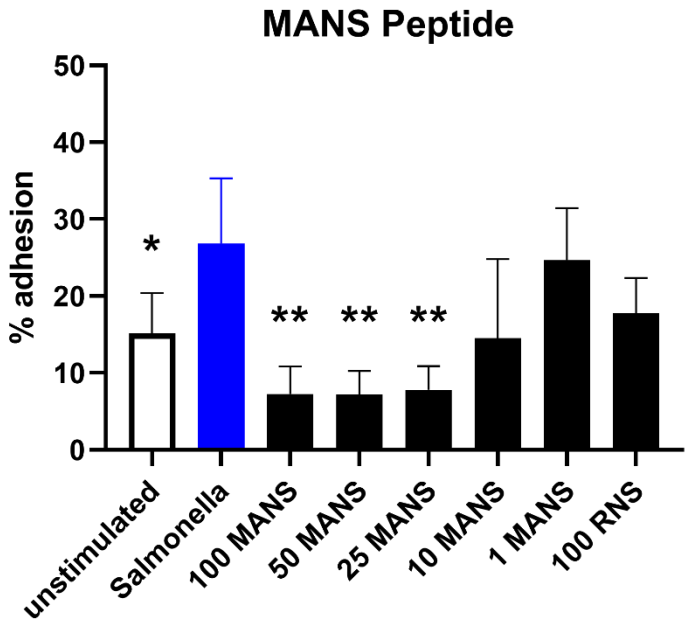


Figure 2. Treatment of primary bovine neutrophils with MANS peptide attenuates STm-induced neutrophil adhesion. Calcein-loaded neutrophils were stimulated with STm at MOI 50:1 for 1 hour prior to serial washing and fluorescence measurements as detailed in the methods section. Data represented as mean ± SD (n=7). p*<0.05 vs STm.

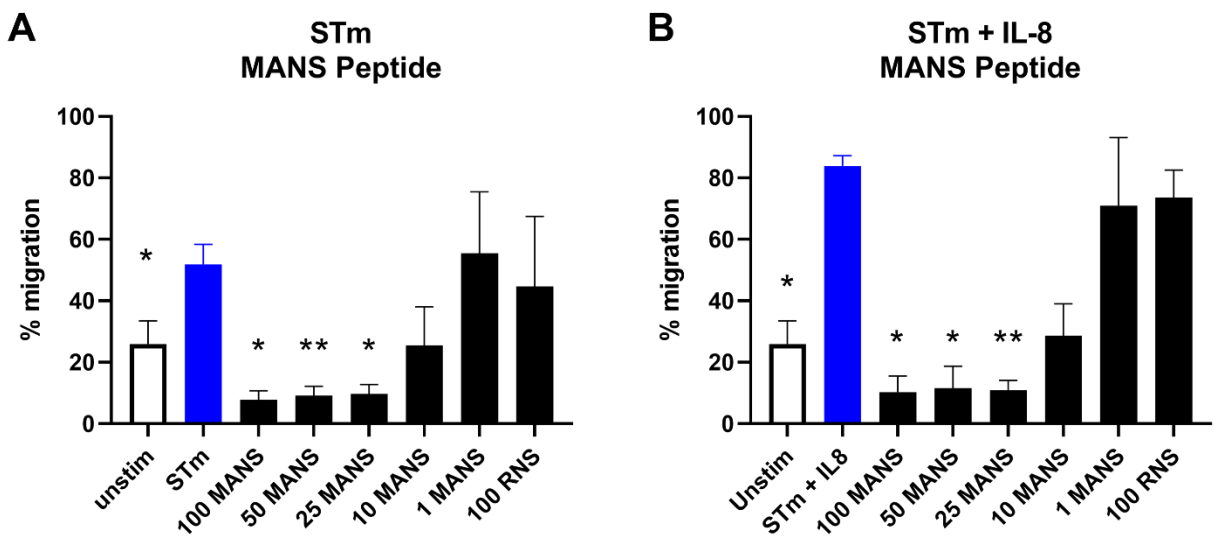


Figure 3. Treatment of primary bovine neutrophils with MANS attenuates STm- and STm+IL8-induced neutrophil migration. Calcein-loaded neutrophils were pretreated with indicated concentrations of peptide and placed on top of the membrane in a ChemoTx plate and either STm alone (MOI 50:1) (A) or STm (50:1) + 10 ng/mL IL-8 (B) were to the bottom wells to induce chemotaxis. The chemotaxis plate was incubated for 1 hour at 37ºC and 5% CO_2. The cells on top of the membrane were removed and the fluorescence of migrated cells were determined. Data represented as mean ± SD (n=3). p*<0.05 vs STm.

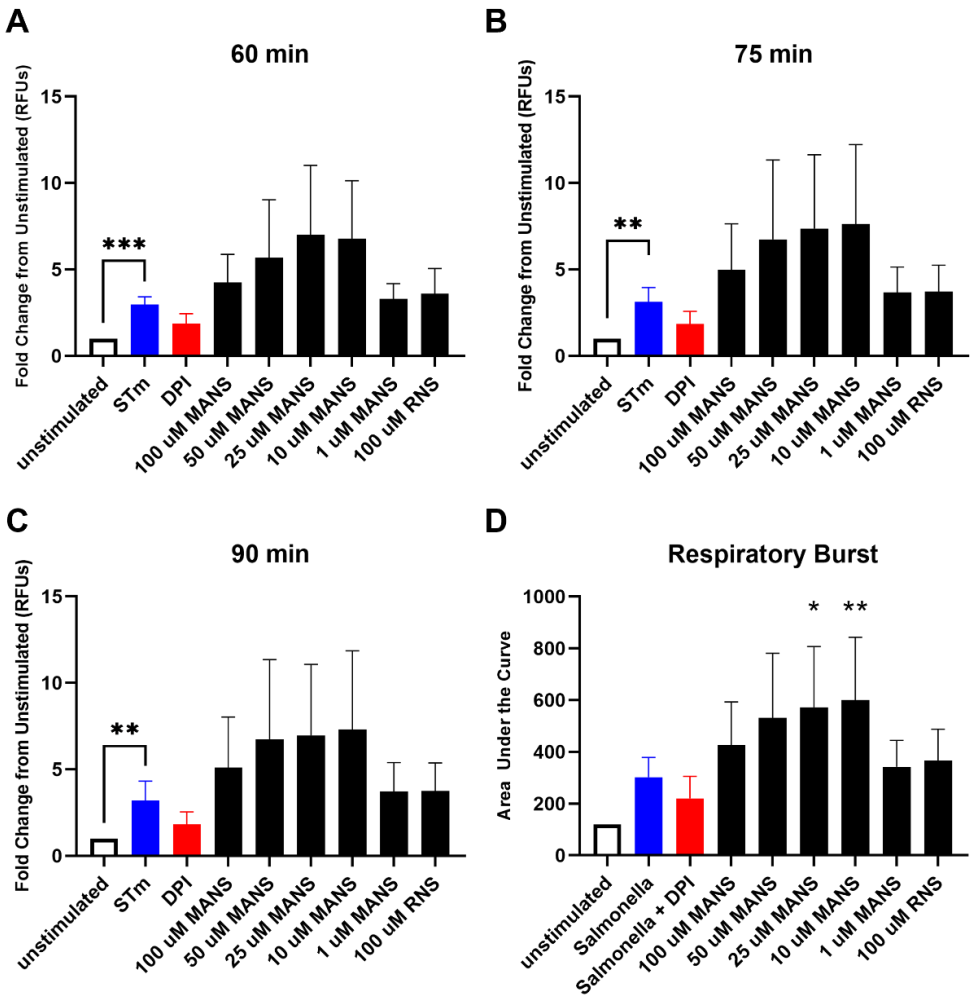


Figure 4. Treatment of primary bovine neutrophils with low concentrations of MANS peptide enhances STm-induced neutrophil respiratory burst. Neutrophils were pretreated with indicated concentrations of MANS peptide for 30 minutes prior to plating in 96 well plate. DPI NADPH oxidase inhibitor was used as a positive control for inhibition. DHR-123 was added to wells, and cells were stimulated with STm (MOI 50:1). Fluorescence was measured for 2 hours and fold change from the unstimulated control was calculated for 60 minutes (A), 75 minutes (B), and 90 minutes (C) into stimulation. Area under the curve was also analyzed by ordinary one-way ANOVA with Dunnett's multiple comparisons test (D). Data represented as mean ± SD (n=7). p*<0.05 vs STm.

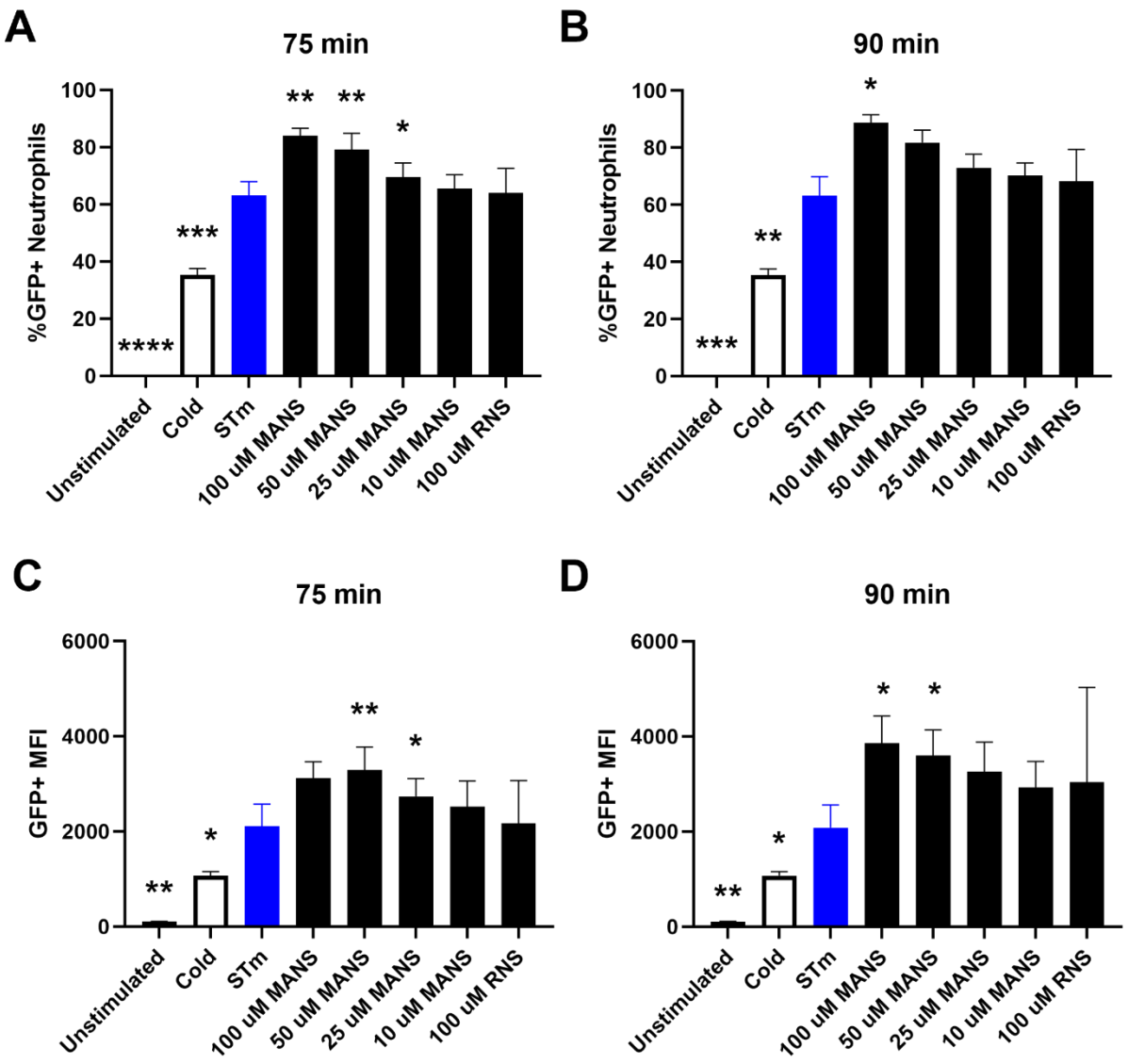


Figure 5. Treatment of primary bovine neutrophils with MANS peptide enhances neutrophil phagocytosis of GFP-STm. Peptide-treated bovine peripheral neutrophils were incubated with opsonized GFP-STm (25:1) for indicated times at 37°C with 5% CO_2. A cold control containing cells and GFP-STm was included as a negative control for phagocytosis. Percentage of GFP-positive neutrophils was determined using histogram gating (A-B), and mean fluorescence intensity (MFI) was also determined using FlowJo analysis (C-D). Data represented as mean ± SD (n=5). p*<0.05 vs STm.

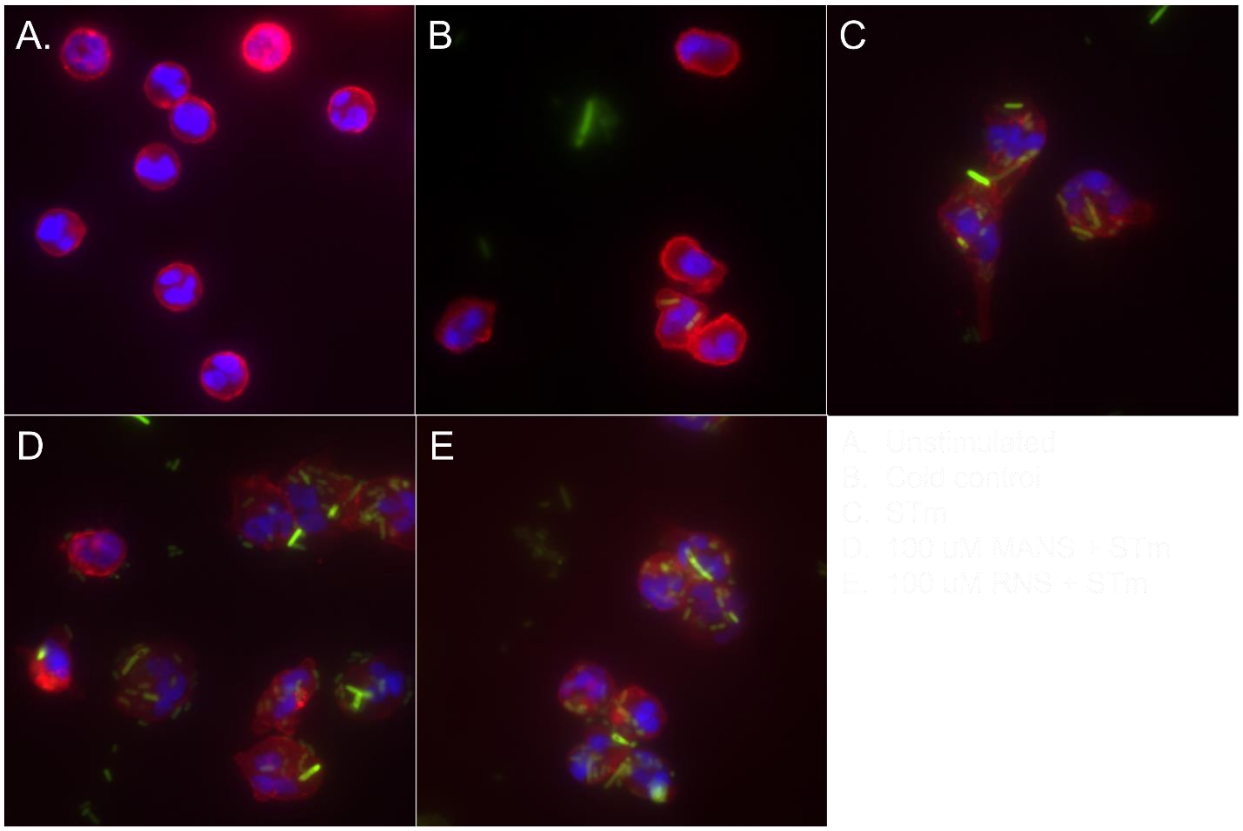
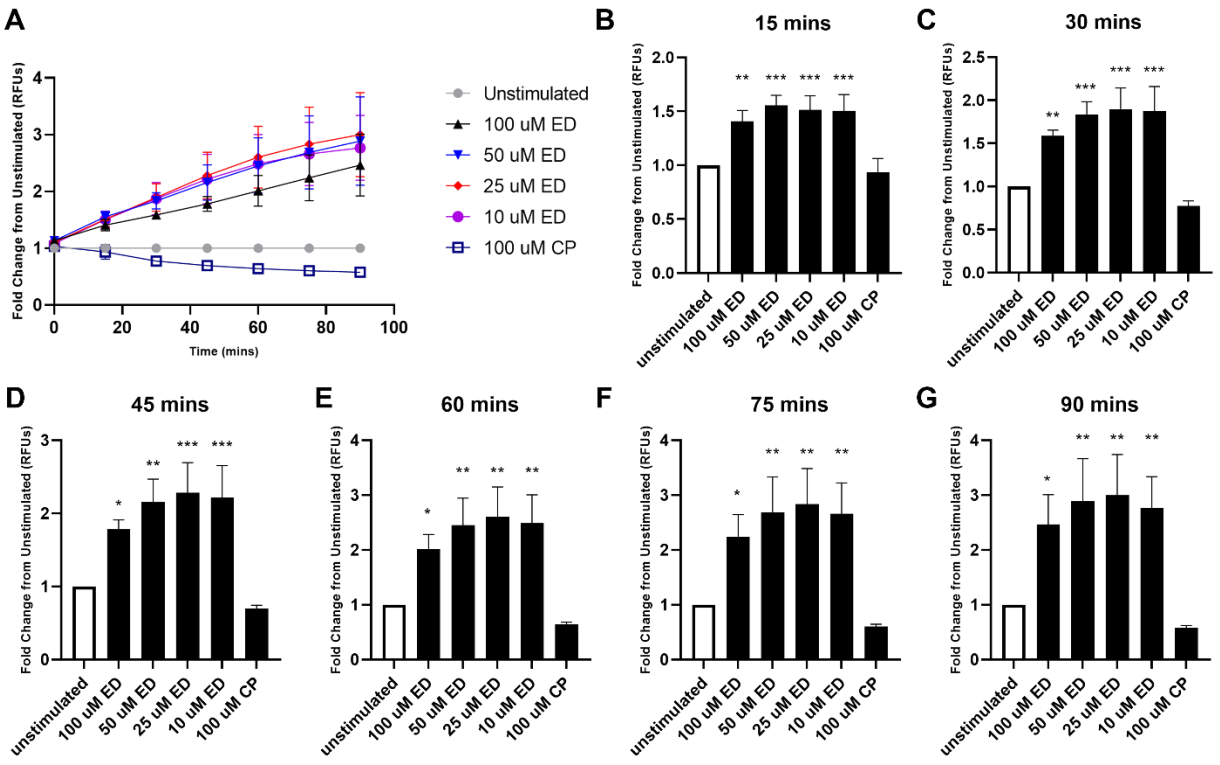
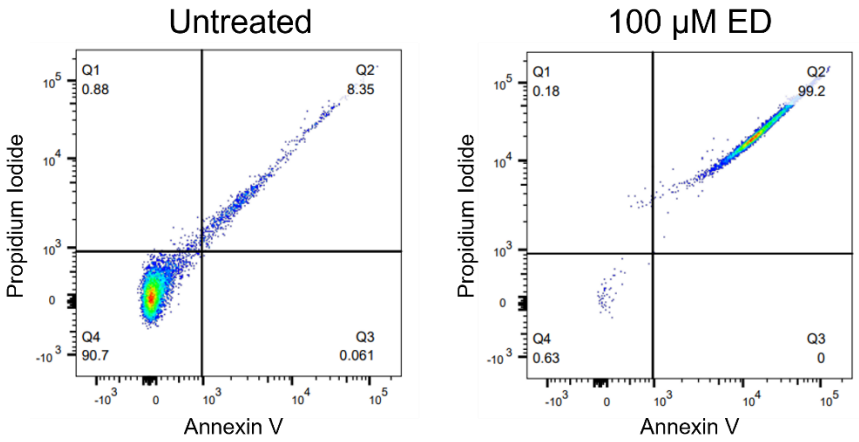


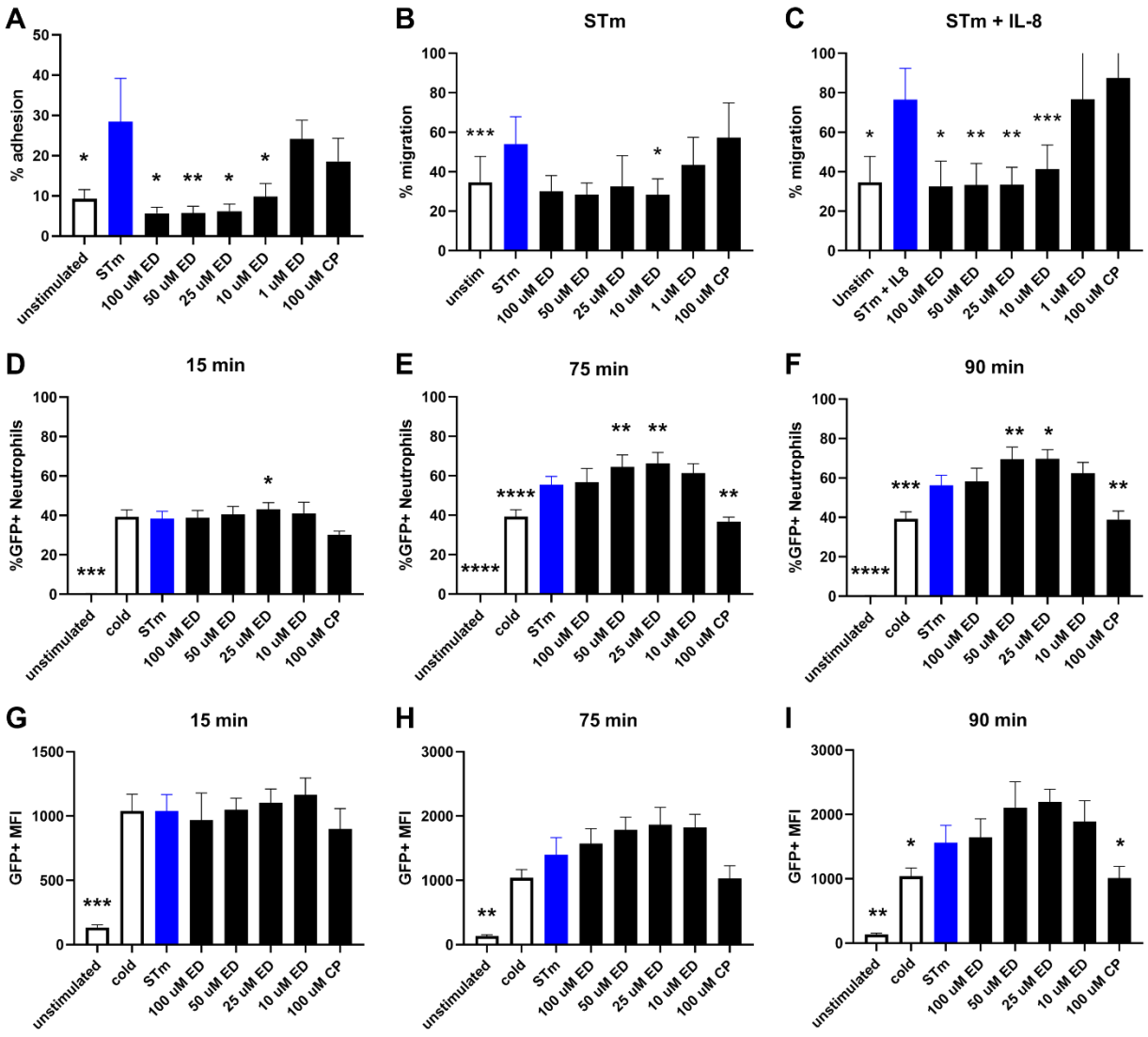
Figure 6. Neutrophil phagocytosis of GFP-STm. Peptide-treated bovine peripheral neutrophils were incubated with opsonized GFP-STm (25:1) for 75 minutes at 37°C with 5% CO_2 and then stained with wheat germ agglutinin (WGA) (red) and DAPI (blue). (A) unstimulated, (B) neutrophils + GFP-STm kept on ice (cold control), (C) STm stimulation, (D) 100 µM MANS, and (E) 100 µM RNS. Representative image of three independent experiments.



Supplemental Figure 1. ED peptide induces respiratory burst in neutrophils. Isolated neutrophils were primed for 30 minutes with GM-CSF then treated with indicated concentrations of peptide for 30 minutes. Cells were plated in 96 well plate, DHR added, and fluorescence was read every 15 minutes for 90 minutes. Data represented as mean ± SD (n=3). Ordinary one-way ANOVA with Dunnett's multiple comparisons test. *p<0.05 vs unstimulated.



Supplemental Figure 2. ED peptide induces cell cytotoxicity that is unique from apoptosis. Neutrophils were treated with indicated concentrations of peptide or PBS (untreated) for 90 minutes, stained by propidium iodide and Annexin V, then analyzed by flow cytometry. Representative image of three independent experiments.



Supplemental Figure 3. Effects of ED peptide on Salmonella-induced neutrophil inflammatory functions. (A) STm-induced adhesion of ED peptide treated neutrophils (n=6), (B) Neutrophil chemotaxis induced by STm (n=6), (C) Neutrophil chemotaxis induced by STm + 10 ng/mL IL-8. Phagocytosis of GFP-STm (25:1) by ED peptide treated neutrophils determined by %GFP+ neutrophils (D-F) and GFP+ MFI (G-I) (n=5).

REFERENCES

Adusumilli, S., Mve-Obiang, A., Sparer, T., Meyers, W., Hayman, J., Small, P.L.C., 2005. Mycobacterium ulcerans toxic macrolide, mycolactone modulates the host immune response and cellular location of M. ulcerans in vitro and in vivo. Cell. Microbiol. 7, 1295–1304. https://doi.org/10.1111/j.1462-5822.2005.00557.x

Allen, L.A.H., Aderem, A., 1995. A role for MARCKS, the α isozyme of protein kinase c and myosin I in zymosan phagocytosis by macrophages. J. Exp. Med. 182, 829–840. https://doi.org/10.1084/jem.182.3.829

Allen, L.A.H., DeLeo, F.R., Gallois, A., Toyoshima, S., Suzuki, K., Nauseef, W.M., 1999. Transient association of the nicotinamide adenine dinucleotide phosphate oxidase subunits p47phox and p67phox with phagosomes in neutrophils from patients with X-linked chronic granulomatous disease. Blood 93, 3521–3530. https://doi.org/10.1182/blood.v93.10.3521.410k21_3521_3530

Arbuzova, A., Schmitz, A.A.P., Res, G.V., 2002. Cross-talk unfolded : MARCKS proteins. Biochem. J 362, 1–12.

Arpaia, N., Godec, J., Lau, L., Sivick, K.E., McLaughlin, L.M., Jones, M.B., Dracheva, T., Peterson, S.N., Monack, D.M., Barton, G.M., 2011. TLR signaling is required for salmonella typhimurium virulence. Cell 144, 675–688. https://doi.org/10.1016/j.cell.2011.01.031

Bakowski, M.A., Cirulis, J.T., Brown, N.F., Finlay, B.B., Brumell, J.H., 2007. SopD acts cooperatively with SopB during Salmonella enterica serovar Typhimurium invasion. Cell. Microbiol. 9, 2839–2855. https://doi.org/10.1111/j.1462-5822.2007.01000.x

Bertram, A., Ley, K., Hirszfeld, Ó.L., 2011. Protein Kinase C Isoforms in Neutrophil Adhesion

and Activation. Arch. Immunol. Ther. Exp 59, 79–87. https://doi.org/10.1007/s00005-011-0112-7

Blander, J.M., Medzhitov, R., 2004. Regulation of Phagosome Maturation by Signals from Toll-Like Receptors. Science (80-.). 304, 1014–1018. https://doi.org/10.1126/science.1096158

Boero, E., Brinkman, I., Juliet, T., van Yperen, E., van Strijp, J.A.G., Rooijakkers, S.H.M., van Kessel, K.P.M., 2021. Use of Flow Cytometry to Evaluate Phagocytosis of Staphylococcus aureus by Human Neutrophils. Front. Immunol. 12, 1–15. https://doi.org/10.3389/fimmu.2021.635825

Broz, P., Ohlson, M.B., Monack, D.M., 2012. Innate immune response to Salmonella typhimurium, a model enteric pathogen. Gut Microbes 3, 62–70. https://doi.org/10.4161/gmic.19141

Carballo, E., Pitterle, D.M., Stumpo, D.J., Sperling, R.T., Blackshear, P.J., 1999. Phagocytic and macropinocytic activity in MARCKS-deficient macrophages and fibroblasts. Am J Physiol 277, C163-173.

Chen, C.-H., Thai, P., Yoneda, K., Adler, K.B., Yang, P.-C., Wu, R., 2014. A peptide that inhibits function of Myristoylated Alanine-Rich C Kinase Substrate (MARCKS) reduces lung cancer metastasis. Oncogene 33, 3696–706. https://doi.org/10.1038/onc.2013.336

Chen, C.H., Statt, S., Chiu, C.L., Thai, P., Arif, M., Adler, K.B., Wu, R., 2014. Targeting myristoylated alanine-rich C kinase substrate phosphorylation site domain in lung cancer: Mechanisms and therapeutic implications. Am. J. Respir. Crit. Care Med. 190, 1127–1138. https://doi.org/10.1164/rccm.201408-1505OC

Chiu, H.C., Kulp, S.K., Soni, S., Wang, D., Gunn, J.S., Schlesinger, L.S., Chen, C.S., 2009. Eradication of intracellular Salmonella enterica serovar typhimurium with a small-molecule,

host cell-directed agent. Antimicrob. Agents Chemother. 53, 5236–5244. https://doi.org/10.1128/AAC.00555-09

Coburn, B., Grassl, G.A., Finlay, B.B., 2007. Salmonella, the host and disease: A brief review. Immunol. Cell Biol. 85, 112–118. https://doi.org/10.1038/sj.icb.7100007

Cox, D., Dale, B.M., Kashiwada, M., Helgason, C.D., Greenberg, S., 2001. A regulatory role for Src homology 2 domain-containing inositol 5′-phosphatase (SHIP) in phagocytosis mediated by Fcγ receptors and complement receptor 3 (αMβ2; CD11b/CD18). J. Exp. Med. 193, 61–71. https://doi.org/10.1084/jem.193.1.61

Damera, G., Jester, W.F., Jiang, M., Zhao, H., Fogle, H.W., Mittelman, M., Haczku, A., Murphy, E., Parikh, I., Panettieri, R.A., 2010. Inhibition of myristoylated alanine-rich C kinase substrate (MARCKS) protein inhibits ozone-induced airway neutrophilia and inflammation. Exp Lung Res 36, 75–84. https://doi.org/10.3109/01902140903131200

Darveau, R.P., Cunningham, M.D., Bailey, T., Seachord, C., Ratcliffe, K., Bainbridge, B., Dietsch, M., Page, R.C., Aruffo, A., 1995. Ability of Bacteria Associated with Chronic Inflammatory Disease To Stimulate E-Selectin Expression and Promote Neutrophil Adhesion. Infect. Immun. 63, 1311–1317.

DeLeo, F.R., Allen, L.A., Apicella, M., Nauseef, W.M., 1999. NADPH oxidase activation and assembly during phagocytosis. J. Immunol. 163, 6732–40.

Diaz-Ochoa, V.E., Lam, D., Lee, C.S., Klaus, S., Behnsen, J., Liu, J.Z., Chim, N., Nuccio, S.P., Rathi, S.G., Mastroianni, J.R., Edwards, R.A., Jacobo, C.M., Cerasi, M., Battistoni, A., Ouellette, A.J., Goulding, C.W., Chazin, W.J., Skaar, E.P., Raffatellu, M., 2016. Salmonella Mitigates Oxidative Stress and Thrives in the Inflamed Gut by Evading Calprotectin-Mediated Manganese Sequestration. Cell Host Microbe 19, 814–825.

https://doi.org/10.1016/j.chom.2016.05.005

Disatnik, M.-H.M.-H., Boutet, S.C., Pacio, W., Chan, A.Y., Ross, L.B., Lee, C.H., Rando, T.A., 2004. The bi-directional translocation of MARCKS between membrane and cytosol regulates integrin-mediated muscle cell spreading. J. Cell Sci. 117, 4469–79. https://doi.org/10.1242/jcs.01309

Drevets, D.A., 1997. Listeria monocytogenes infection of cultured endothelial cells stimulates neutrophil adhesion and adhesion molecule expression. J. Immunol. 158, 5305–5313.

Eckert, R.E., Neuder, L.E., Park, J., Adler, K.B., Jones, S.L., 2010. Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) Protein Regulation of Human Neutrophil Migration. Am. J. Respir. Cell Mol. Biol. 42, 586–594. https://doi.org/10.1165/rcmb.2008-0394OC

Elzagallaai, A., Rosé, S.D., Trifaró, J.M., 2000. Platelet secretion induced by phorbol esters stimulation is mediated through phosphorylation of MARCKS: A MARCKS-derived peptide blocks MARCKS phosphorylation and serotonin release without affecting pleckstrin phosphorylation. Blood 95, 894–902. https://doi.org/10.1182/blood.v95.3.894.003k15_894_902

Estrada-Bernal, A., Gatlin, J.C., Sunpaweravong, S., Pfenninger, K.H., 2009. Dynamic adhesions and MARCKS in melanoma cells. J. Cell Sci. 122, 2300–2310. https://doi.org/10.1242/jcs.047860

Eustace, N.J., Anderson, J.C., Warram, J.M., Widden, H.N., Pedersen, R.T., Alrefai, H., Patel, Z., Hicks, P.H., Placzek, W.J., Gillespie, G.Y., Hjelmeland, A.B., Willey, C.D., 2020. A cell-penetrating MARCKS mimetic selectively triggers cytolytic death in glioblastoma. Oncogene 39, 6961–6974. https://doi.org/10.1038/s41388-020-01511-9.A

Feuillet, V., Medjane, S., Mondor, I., Demaria, O., Pagni, P.P., Galán, J.E., Flavell, R.A.,

Alexopoulou, L., 2006. Involvement of toll-like receptor 5 in the recognition of flagellated bacteria. Proc. Natl. Acad. Sci. U. S. A. 103, 12487–12492. https://doi.org/10.1073/pnas.0605200103

Fournier, B.M., Parkos, C.A., 2012. The role of neutrophils during intestinal inflammation. Mucosal Immunol. 5, 354–366. https://doi.org/10.1038/mi.2012.24

Fu, Y., Galán, J.E., 1999. A Salmonella protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. Nature 401, 293–297. https://doi.org/10.1038/45829

Gallois, A., Klein, J.R., Allen, L.-A.H., Jones, B.D., Nauseef, W.M., 2001. Salmonella Pathogenicity Island 2-Encoded Type III Secretion System Mediates Exclusion of NADPH Oxidase Assembly from the Phagosomal Membrane. J. Immunol. 166, 5741–5748. https://doi.org/10.4049/jimmunol.166.9.5741

Green, T.D., Park, J., Yin, Q., Fang, S., Crews, A.L., Jones, S.L., Adler, K.B., 2012. Directed migration of mouse macrophages in vitro involves myristoylated alanine-rich C-kinase substrate (MARCKS) protein. J. Leukoc. Biol. 92, 633–639. https://doi.org/10.1189/jlb.1211604

Gresham, H.D., Dale, B.M., Potter, J.W., Chang, P.W., Vines, C.M., Lowell, C.A., Lagenaur, C.F., Willman, C.L., 2000. Negative regulation of phagocytosis in murine macrophages by the Src kinase family member, Fgr. J. Exp. Med. 191, 515–528. https://doi.org/10.1084/jem.191.3.515

Hartwig, J.H., Thelen, M., Rosen, A., Janmey, P.A., Nairn, A.C., Aderem, A., 1992. MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. Nature 356, 618–622.

Healy, J.M., Bruce, B.B., 2020. Salmonellosis (Nontyphoidal) [WWW Document]. CDC Yellow

B. Chapter 4 Travel. Infect. Dis. URL https://wwwnc.cdc.gov/travel/yellowbook/2020/travel-related-infectious-diseases/salmonellosis-nontyphoidal#:~:text=Nontyphoidal salmonellae are one of,57%2C000 deaths globally each year.

Henao, O.L., Jones, T.F., Vugia, D.J., Griffin, P.M., 2015. Foodborne diseases active surveillance network—2 decades of achievements, 1996–2015. Emerg. Infect. Dis. 21, 1529–1536. https://doi.org/10.3201/eid2109.150581

Hiyoshi, H., Wangdi, T., Lock, G., Saechao, C., Raffatellu, M., Cobb, B.A., Bäumler, A.J., 2018. Mechanisms to Evade the Phagocyte Respiratory Burst Arose by Convergent Evolution in Typhoidal Salmonella Serovars. Cell Rep. 22, 1787–1797. https://doi.org/10.1016/j.celrep.2018.01.016

Hobbie, S., Chen, L., Davis, R., Galan, J., 1997. Involvement of mitogen-activated protein kinase pathways in the nuclear responses and cytokine production induced by Salmonella typhimurium in cultured intestinal epithelial cells. J. Immunol. 159, 5550–5559.

Hoelzer, K., Switt, A.I.M., Wiedmann, M., 2011. Animal contact as a source of human non-typhoidal salmonellosis. Vet. Res. 42, 34. https://doi.org/10.1186/1297-9716-42-34

Ishibashi, Y., Arai, T., 1996. A possible mechanism for host-specific pathogenesis of Salmonella serovars. Microb. Pathog. 21, 435–446. https://doi.org/10.1006/mpat.1996.0074

Jackson, B.R., Griffin, P.M., Cole, D., Walsh, K.A., Chai, S.J., 2013. Outbreak-associated salmonella enterica serotypes and food commodities. Emerg. Infect. Dis. 19, 1239–1244.

Jin, Q., Jiang, L., Chen, Q., Li, X., Xu, Y., Sun, X., Zhao, Z., Wei, L., 2018. Rapid flow cytometry-based assay for the evaluation of γδ T cell-mediated cytotoxicity. Mol. Med. Rep. 17, 3555–3562. https://doi.org/10.3892/mmr.2017.8281

Jung, H.C., Eckmann, L., Yang, S.-K., Panja, A., Fierer, J., Morzycka-Wroblewska, E., 1995. A Distinct Array of Proinflammatory Cytokines is Expressed in Human Colon Epithelial Cells in Response to Bacterial Invasion. J. Clin. Invest. 95, 55–65. https://doi.org/10.1016/j.saa.2011.09.017

Karimi, G., Houée Levin, C., Dagher, M.C., Baciou, L., Bizouarn, T., 2014. Assembly of phagocyte NADPH oxidase: A concerted binding process? Biochim. Biophys. Acta - Gen. Subj. 1840, 3277–3283. https://doi.org/10.1016/j.bbagen.2014.07.022

Kirk, M.D., Pires, S.M., Black, R.E., Caipo, M., Crump, J.A., Devleesschauwer, B., Döpfer, D., Fazil, A., Fischer-Walker, C.L., Hald, T., Hall, A.J., Keddy, K.H., Lake, R.J., Lanata, C.F., Torgerson, P.R., Havelaar, A.H., Angulo, F.J., 2015. World Health Organization Estimates of the Global and Regional Disease Burden of 22 Foodborne Bacterial, Protozoal, and Viral Diseases, 2010: A Data Synthesis. PLoS Med. 12, 1–21. https://doi.org/10.1371/journal.pmed.1001921

Larsson, C., 2006. Protein kinase C and the regulation of the actin cytoskeleton. Cell. Signal. 18, 276–284. https://doi.org/10.1016/j.cellsig.2005.07.010

Lee, C.A., Silva, M., Siber, A.M., Kelly, A.J., Galyov, E., McCormick, B.A., 2000. A secreted Salmonella protein induces a proinflammatory response in epithelial cells, which promotes neutrophil migration. Proc. Natl. Acad. Sci. U. S. A. 97, 12283–12288. https://doi.org/10.1073/pnas.97.22.12283

Lee, S.M., Suk, K., Lee, W.H., 2015. Myristoylated alanine-rich C kinase substrate (MARCKS) regulates the expression of proinflammatory cytokines in macrophages through activation of p38/JNK MAPK and NF-κB. Cell. Immunol. 296, 115–121. https://doi.org/10.1016/j.cellimm.2015.04.004

Li, Y., Martin, L.D., Spizz, G., Adler, K.B., 2001. MARCKS Protein Is a Key Molecule Regulating Mucin Secretion by Human Airway Epithelial Cells in Vitro. J. Biol. Chem. 276, 40982–40990. https://doi.org/10.1074/jbc.M105614200

Loetscher, Y., Wieser, A., Lengefeld, J., Kaiser, P., Schubert, S., Heikenwalder, M., Hardt, W.D., Stecher, B., 2012. Salmonella transiently reside in luminal neutrophils in the inflamed gut. PLoS One 7. https://doi.org/10.1371/journal.pone.0034812

Manček-Keber, M., Benčina, M., Japelj, B., Panter, G., Andrä, J., Brandenburg, K., Triantafilou, M., Triantafilou, K., Jerala, R., 2012. MARCKS as a Negative Regulator of Lipopolysaccharide Signaling. J. Immunol. 188, 3893–3902. https://doi.org/10.4049/jimmunol.1003605

May, R.C., Machesky, L.M., 2001. Phagocytosis and the actin cytoskeleton. J. Cell Sci. 114, 1061–1077. https://doi.org/10.1242/jcs.114.6.1061

Mohler, V.L., Izzo, M.M., House, J.K., 2009. Salmonella in Calves. Vet. Clin. North Am. - Food Anim. Pract. 25, 37–54. https://doi.org/10.1016/j.cvfa.2008.10.009

Monn Myat, M., Anderson, S., Allen, L.-A.H., Aderem, A., 1997. MARCKS regulates membrane ruffling and cell spreading. Curr. Biol. 7, 611–614.

Nairn, A.C., Aderem, A., 1992. Calmodulin and protein kinase C cross-talk: the MARCKS protein is an actin filament and plasma membrane cross-linking protien regulated by protein kinase C phosphorylation and calmodulin. Ciba Found. Symp. 164, 145–54; discussion 154-61. https://doi.org/10.1038/356618a0

Ott, L.E., Sung, E.J., Melvin, A.T., Sheats, M.K., Haugh, J.M., Adler, K.B., Jones, S.L., Sung, J., Melvin, A.T., Sheats, M.K., Haugh, J.M., Adler, K.B., Jones, S.L., 2013. Fibroblast Migration Is Regulated by Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS)

Protein. PLoS One 8, e66512. https://doi.org/10.1371/journal.pone.0066512

Patel, J.C., Galán, J.E., 2005. Manipulation of the host actin cytoskeleton by Salmonella - All in the name of entry. Curr. Opin. Microbiol. 8, 10–15. https://doi.org/10.1016/j.mib.2004.09.001

Rodriguez-Rivera, L.D., Wright, E.M., Siler, J.D., Elton, M., Cummings, K.J., Warnick, L.D., Wiedmann, M., 2014. Subtype Analysis of Salmonella Isolated from Subclinically Infected Dairy Cattle and Dairy Farm Environments Reveals the Presence of Both Human- and Bovine-Associated Subtypes. Vet Microbiol 170, 307–316. https://doi.org/10.1016/j.vetmic.2014.02.013.Subtype

Rohrbach, T.D., Jones, R.B., Hicks, P.H., Weaver, A.N., Cooper, T.S., Eustace, N.J., Yang, E.S., Jarboe, J.S., Anderson, J.C., Willey, C.D., 2017. MARCKS phosphorylation is modulated by a peptide mimetic of MARCKS effector domain leading to increased radiation sensitivity in lung cancer cell lines. Oncol. Lett. 13, 1216–1222. https://doi.org/10.3892/ol.2016.5550

Rohrbach, T.D., Shah, N., Jackson, W.P., Feeney, E. V., Scanlon, S., Gish, R., Khodadadi, R., Hyde, S.O., Hicks, P.H., Anderson, J.C., Jarboe, J.S., Willey, C.D., 2015. The effector domain of MARCKS Is a nuclear localization signal that regulates cellular PIP2 levels and nuclear PIP2 localization. PLoS One 10, 1–14. https://doi.org/10.1371/journal.pone.0140870

Schmidt, A., Caron, E., Hall, A., 2001. Lipopolysaccharide-Induced Activation of β2-Integrin Function in Macrophages Requires Irak Kinase Activity, p38 Mitogen- Activated Protein Kinase, and the Rap1 GTPase. Mol. Cell. Biol. 21, 438–448. https://doi.org/10.1128/mcb.21.2.438-448.2001

Schmidt, S., Moser, M., Sperandio, M., 2013. The molecular basis of leukocyte recruitment and

its deficiencies. Mol. Immunol. 55, 49–58. https://doi.org/10.1016/j.molimm.2012.11.006

Schreiner, L., Huber-Lang, M., Weiss, M.E., Hohmann, H., Schmolz, M., Schneider, E.M., 2011. Phagocytosis and digestion of pH-sensitive fluorescent dye (Eos-FP) transfected E. coli in whole blood assays from patients with severe sepsis and septic shock. J. Cell Commun. Signal. 5, 135–144. https://doi.org/10.1007/s12079-010-0112-0

Sell, J., Dolan, B., 2018. Common Gastrointestinal Infections. Prim. Care Clin. Off. Pract. 45, 519–532.

Sheats, M.K., Pescosolido, K.C., Hefner, E.M., Sung, J., Adler, K.B., Jones, S.L., 2014. Myristoylated Alanine Rich C Kinase Substrate (MARCKS) is essential to β2-integrin dependent responses of equine neutrophils. Vet Immunol Immunopathol 160, 167–176. https://doi.org/10.1016/j.vetimm.2014.04.009

Sheats, M.K., Sung, E.J., Adler, K.B., Jones, S.L., 2015. In vitro neutrophil migration requires protein kinase c-delta (δ-PKC) mediated MARCKS (Myristoylated Alanine Rich C-Kinase Substrate) phosphorylation. Inflammation 38, 1126–1141. https://doi.org/10.1007/s10753-014-0078-9

Srikanth, C. V., Mercado-Lubo, R., Hallstrom, K., McCormick, B.A., 2011. Salmonella effector proteins and host-cell responses. Cell. Mol. Life Sci. 68, 3687–3697. https://doi.org/10.1007/s00018-011-0841-0

Stecher, B., Robbiani, R., Walker, A.W., Westendorf, A.M., Barthel, M., Kremer, M., Chaffron, S., Macpherson, A.J., Buer, J., Parkhill, J., Dougan, G., Von Mering, C., Hardt, W.D., 2007. Salmonella enterica serovar typhimurium exploits inflammation to compete with the intestinal microbiota. PLoS Biol. 5, 2177–2189. https://doi.org/10.1371/journal.pbio.0050244

Tamura, R., 2017. 1. Study on the effector domain of MARCKS protein as a cell penetrating peptide. 2. Development of photoactivatable prodrugs of doxazolidine.

Valenzuela, J.R., Sethi, A.K., Aulik, N.A., Poulsen, K.P., 2017. Antimicrobial resistance patterns of bovine Salmonella enterica isolates submitted to the Wisconsin Veterinary Diagnostic Laboratory: 2006–2015. J. Dairy Sci. 100, 1319–1330. https://doi.org/10.3168/jds.2016-11419

van Bruggen, R., Zweers, D., Diepen, A. Van, Dissel, J.T. Van, Roos, D., Verhoeven, A.J., Kuijpers, T.W., 2007. Complement Receptor 3 and Toll-Like Receptor 4 Act Sequentially in Uptake and Intracellular Killing of Unopsonized Salmonella enterica Serovar Typhimurium by Human Neutrophils □. Infect. Immun. 75, 2655–2660. https://doi.org/10.1128/IAI.01111-06

Voetsch, A.C., Van Gilder, T.J., Angulo, F.J., Farley, M.M., Shallow, S., Marcus, R., Cieslak, P.R., Deneen, V.C., Tauxe, R. V., 2004. FoodNet estimate of the burden of illness caused by nontyphoidal Salmonella infections in the United States. Clin. Infect. Dis. 38, 127–134. https://doi.org/10.1086/381578

Wang, J., Arbuzova, A., Hangyas-Mihalyne, G., McLaughlin, S., 2001. The Effector Domain of Myristoylated Alanine-rich C Kinase Substrate Binds Strongly to Phosphatidylinositol 4,5-Bisphosphate. J. Biol. Chem. 276, 5012–5019. https://doi.org/10.1074/jbc.M008355200

Westerman, T.L., Bogomolnaya, L., Andrews-Polymenis, H.L., Sheats, M.K., Elfenbein, J.R., 2018. The Salmonella type-3 secretion system-1 and flagellar motility influence the neutrophil respiratory burst. PLoS One 13, e0203698. https://doi.org/10.1371/journal.pone.0203698

Westerman, T.L., Sheats, M.K., Elfenbein, J.R., 2021. Sulfate import in salmonella typhimurium

impacts bacterial aggregation and the respiratory burst in human neutrophils. Infect. Immun. 89, 1–15. https://doi.org/10.1128/IAI.00701-20

Winter, S.E., Thiennimitr, P., Winter, M.G., Butler, B.P., Huseby, D.L., Crawford, R.W., Russell, J.M., Bevins, C.L., Adams, L.G., Tsolis, R.M., Roth, J.R., Bäumler, A.J., 2010. Gut inflammation provides a respiratory electron acceptor for Salmonella. Nature 467, 426–429. https://doi.org/10.1038/nature09415

Zhang, S., Adams, L.G., Nunes, J., Khare, S., Tsolis, R.M., Bäumler, A.J., 2003. Secreted effector proteins of Salmonella enterica serotype typhimurium elicit host-specific chemokine profiles in animal models of typhoid fever and enterocolitis. Infect. Immun. 71, 4795–4803. https://doi.org/10.1128/IAI.71.8.4795-4803.2003

ACKNOWLEDGEMENTS

This study was funded by the USDA (MKS and JRE). CFB received funding from the VSP Scholarship Program and FFAR Veterinary Fellowship. The authors would like to thank Mike Veach (CVM TAU Dairy), Dr. Rosemary Bayless, Dr. Bethanie Cooper, and Abby Kepley for their assistance with bovine blood collection. Special thanks to Grayson Walker and Mitzu Suyemoto for technical assistance.

CHAPTER 5

Evaluation of MARCKS protein as a therapeutic target in a naturally occurring equine model of asthma

Haleigh E. Conley[*1,2], Kaori Uchiumi Davis[*1,2], Jean-Pierre Lavoie[3], and Mary Katherine Sheats[1,2] (*co-first authors)

[1]Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, 1060 William Moore Dr., Raleigh, NC 27607, United States

[2]Comparative Medicine Institute, North Carolina State University, 1060 William Moore Dr., Raleigh, NC 27607, United States

[3]Département des Sciences Cliniques, Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, QC J2S 2M2, Canada

ABSTRACT

Introduction: Asthma is a chronic inflammatory airway disease that affects millions of people worldwide. Aside from cats, horses are the only other species to develop spontaneous asthma, positioning them as a relevant and translational model for human asthma. Many studies have implicated MARCKS protein, an important regulator of inflammatory cells, as a potential therapeutic target for asthma. Using horses as a natural model of asthma, we investigated MARCKS protein levels in horses with no, mild/moderate or severe airway inflammation. Further, we investigated whether MARCKS inhibition decreased inflammatory responses in zymosan-stimulated alveolar macrophages and peripheral neutrophils. **Methods:** BALF from pastured teaching horses was evaluated for cytology, asthma classification, and MARCKS protein analysis. Isolated alveolar macrophages and peripheral neutrophils were stimulated with zymosan to evaluate MARCKS inhibition in respiratory burst and cytokine secretion. **Results:** We found an upregulation of MARCKS protein in BALF in mild/moderate and severe asthmatic horses when compared to normal horses. MARCKS inhibition attenuated zymosan-stimulated respiratory burst in both alveolar macrophages and peripheral neutrophils but had no effect on release of TNFα, IL-1β, and IL-8. **Conclusion:** This study is the first to describe changes in MARCKS protein expression in BAL cells from horses with asthma and demonstrate MARCKS inhibition of zymosan-stimulated alveolar macrophage and neutrophil respiratory burst.

INTRODUCTION

Asthma is a chronic inflammatory airway disease that manifests with cough, shortness of breath, chest tightness and wheeze caused by bronchorestriction, airway hyper-responsiveness, excess mucus, and airway remodeling. There are several phenotypes of asthma, including early versus late onset, allergic versus non-allergic, steroid resistant versus steroid sensitive, and asthma of variable disease severity (Hamilton and Lehman, 2020). Animal modeling in asthma has been difficult to achieve because rodents do not naturally develop asthma and fail to translate well to human asthma (Davis and Sheats, 2020); however, horses have recently gained recognition as a relevant and translational asthma model. Aside from cats, horses are the only other animal species that spontaneously develops asthma, referred to as equine asthma syndrome (EAS) (Leclere et al., 2011). The most common form of EAS is mild/moderate (mEAS) where 60% or more horses are afflicted. However, 10-20% of adult horses in temperate climates suffer from severe EAS (sEAS) (Davis and Sheats, 2019).

EAS and human asthma both result from similar environmental exposures and/or genetic predisposition (Ewart and Robinson, 2007), especially asthma occurring in farmers (Sheats et al., 2019). Airborne organic dust exposure is the most common environmental trigger in agriculture workers and horses with asthma. Organic dust consists of a mixture of many potential antigens, including particulate matter, endotoxins or lipopolysaccharide (LPS), peptidoglycans, noxious gases, and β-D-glucans (Ivester et al., 2014; Poole and Romberger, 2012). Following inhalation, the airborne organic dust is recognized by the respiratory epithelial cells and alveolar macrophages, causing these cells to release proinflammatory mediators and neutrophil chemoattractants in addition to undergoing respiratory burst.

Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) has been identified as an essential regulator of inflammatory cells and a promising target for asthma treatment. MARCKS is a 32 kDa substrate for Protein Kinase C (PKC) that is expressed ubiquitously in eukaryotic cells. In various types of cells at rest, including macrophages (Rosen et al., 1990), MARCKS localizes to the inner leaflet of the plasma membrane, crosslinking actin filaments. MARCKS binds the plasma membrane via its highly basic serine-rich effector domain (ED), which interacts electrostatically through with lipids, and the myristoylated N-terminus, which inserts hydrophobically into the membrane core (Arbuzova et al., 2002). While localized to the plasma membrane, MARCKS ED domain binds to and sequesters up to four molecules of phosphatidylinositol 4,5-bisphosphate (PIP2). MARCKS-PIP2 binding is disrupted when cells receive activation signals that induce PKC-mediated phosphorylation or calcium/calmodulin binding of the ED domain. MARCKS is then displaced to the cytosol, resulting in actin reorganization and release of PIP2 molecules (Arbuzova et al., 2002; Sundaram et al., 2004; Ziemba and Falke, 2018). Due to its critical role in actin reorganization and PIP2 molecule sequestration, MARCKS has been recognized to play pivotal roles in diverse biological processes including cell motility, phagocytosis, membrane trafficking and secretion (Arbuzova et al., 2002; Green et al., 2011; Seykora et al., 1996). MARCKS is also a key regulator of neutrophil functions including migration, adhesion, degranulation, cytokine secretion and respiratory burst (Eckert et al., 2010; Li et al., 2013; Sheats et al., 2014; Takashi et al., 2006). A MARCKS-specific inhibitor peptide known as MANS (Myristoylated N-terminal Sequence) peptide is identical to the first 24 amino acids of the N-terminal region of MARCKS and has been reported to attenuate equine neutrophil migration, adhesion and respiratory burst (Sheats et al., 2014), as well as production of LPS-induced cytokines in canine neutrophils (Li et al., 2013).

Early studies on MARCKS showed that levels of this protein were significantly increased in neutrophils following LPS and TNFα stimulation, coinciding with increased MARCKS phosphorylation (Thelen et al., 1990). In a recent study using a murine model of steroid-resistant asthma, Wang and colleagues showed that suppressing MARCKS phosphorylation attenuated asthma symptoms and severe neutrophilic inflammation (Wang et al., 2019). Given the important role that MARCKS plays the inflammatory functions of neutrophils and macrophages and the animal model evidence supporting MARCKS as a potential target for asthma, we investigated MARCKS as a therapeutic target to combat cellular inflammatory responses in severe asthma.

MATERIALS AND METHODS

Main study population – North Carolina State University (NCSU)

All procedures performed for the purposes of this study were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC 16-0740O and 19-779). Horses included in this study were university owned adult teaching horses that lived on the pasture or client-owned horses. All horses lived in the southeastern United States. The horses were 6 to 24 years of age, and of mixed breed and gender (Table 1). The horses received no medications during the study. All horses received a physical exam and a BAL. Horses sampled for the protein ELISA also received a clinical score. The clinical score was determined based on the previously described scoring system, which considers respiratory rate, frequency of cough, nostril flare, abdominal lift and nasal discharge (Davis and Sheats, 2019).

Subpopulation – Equine Respiratory Tissue Bank (ERTB)

BAL cell lysates from an additional 24 horses with EAS were obtained from the Equine Respiratory Tissue Biobank (ERTB) (JP Lavoie). These samples were used to conduct a separate secondary investigation of MARCKS levels in the BAL cell lysates of horses with documented

clinical signs and pulmonary function abnormalities. The horses of the subpopulation were 9 to 24 years of age and of mixed breeds and gender (Table 2). Each horse received a physical examination, lung function test including Delta PpL, pulmonary resistance and elastance, and a BAL, except for three horses where lung function test was not performed. Delta PpL was computed as the difference between airway pressure and pleural pressure. Pleural pressure was measured indirectly as esophageal pressure using an esophageal balloon catheter. Pulmonary resistance and elastance were calculated from the airflow data that was measured using pneumotachograph mounted on the face mask and connected to a differential pressure transducer.

Bronchoalveolar lavage and sample processing

BALF samples were collected as described previously (Davis and Sheats, 2019). Briefly, after intravenous premedication with sedative (0.005-0.01mg/kg detomidine and 0.02-0.04 mg/kg butorphanol), a local anesthetic gel (lidocaine) was applied at the nostril. The BAL was then performed with a 3-meter cuffed Bivona® tube, with two 150-250mL boluses of sterile isotonic saline solution. Recovered BALF samples were pooled and placed on ice for protein lysate collection, or kept at room temperature for ex vivo experiments, and processed within an hour of collection. One Protease inhibitor (cOmplete™ ULTRA, Roche) tablet was added per 50mL BAL samples processed for cell lysate.

Cytological analysis

Cells (100 µL) were collected on object slides by cytospin centrifugation with 50 µL 30% BSA and stained by Wrights-Giemsa. Differential count was performed using a light microscope by counting 300 cells/slide for ELISA samples and 500 cells/slide for ex vivo experiment samples.

Disease classifications

Horses were classified as normal, mild/moderate inflammation, or severe inflammation according to their physical exam and BAL cytology (Table 3). Horses with respiratory signs at rest were considered severe. Fractional numbers were rounded to the nearest whole number.

Lysate preparation

The BALF was filtered through sterile gauze to remove mucus and was spun at 1500 rpm for 10 minutes. The mixed BAL cells were resuspended in Hank's balanced salt solution (HBSS). The cells were lysed with ice cold RIPA buffer [1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 5 mM sodium pyrophosphate and 50mM sodium fluoride] containing protease inhibitors (cOmplete™ ULTRA tablets mini, Roche) and phosphatase inhibitors (Pierce™ Phosphatase Inhibitor Mini Tablets, Thermo Fisher) for 30 min on ice. After lysis, cell solutions were spun at 13,000 rpm for 10 min at 4 °C. Supernatants were collected stored at -80 °C.

Immunoblotting

Protein concentrations in cell lysates were measured using BCA Protein Assay Reagent (Pierce). Cell lysate was mixed with NuPAGE LDS Sample Buffer (4X) (Thermo Fisher) and Sample Reducing Agent (10X) (Thermo Fisher) and boiled for 10 minutes. Equal amount of protein was analyzed in 4-12% SDS-PAGE with MES running buffer. Resolved samples were transferred to Immobilon-P PVDF membrane (Millipore) and blocked for 1 hours with 5% non-fat dry milk or 5% BSA (for Phospho-MARCKS (Cell Signaling Technology)) 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 primary antibody in TBS/T at 4°C. Membranes were washed with TBS/T and incubated with 1:2000 dilution of anti-rabbit horseradish peroxidase (HRP) secondary antibody (Cell Signaling Technology) in 5% non-fat dry milk in TBS/T for one hour, washed three times

for 5 minutes each, developed using Bio-Rad Clarity Western ECL Substrate, and imaged using a Bio-Rad ChemiDoc. Western blot images were analyzed in Image Lab.

ELISA

Analysis of MARCKS was carried out using equine-specific MARCKS ELISA kit (MyBioSource) per the manufacturer's protocol. MARCKS ELISA results were normalized to the total protein concentrations determined using either BCA Protein Assay Reagent (Pierce), or nanodrop (used due to limited quantity of lysate from the ERTB). Equine-specific TNFα (R&D systems), IL-1β (R&D systems), and IL-8 (Genorise) ELISAs were carried out per the manufacturer's protocol. For the TNFα ELISA, cell culture supernatants were diluted 1:100 prior to analysis.

Alveolar macrophage isolation

The BALF was filtered through sterile gauze to remove mucus and was spun at 1500 rpm for 10 minutes. The mixed BAL cells were resuspended in Hank's balanced salt solution (HBSS), and following total cell count and differential count determination, centrifuged at 1500 rpm for 10 minutes. Based on the calculated total alveolar macrophage count, mixed BAL cells were resuspended in RPMI 1640 medium without phenol red (Thermo Fisher) supplemented with 10% heat inactivated fetal calf serum (Gibco). Alveolar macrophages were then seeded into plates at equivalent densities (between 700,000 and $1.0x10^6$ cells depending on BALF macrophage %) and incubated for an hour to allow alveolar macrophages to adhere to the bottom of the well. The wells were then washed three times to remove nonadherent cells, and the remaining alveolar macrophages were incubated at 37 °C with 5% CO_2 for at least 20 hours.

Zymosan stimulation of alveolar macrophages

Alveolar macrophages were seeded in 24 well plates based on total alveolar macrophage count and incubated in air at 37 °C with 5% CO_2 for an hour to allow alveolar macrophages to adhere to the bottom of the well. The wells were then washed three times so that nonadherent cells would be removed, and the remaining alveolar macrophages were incubated at 37 °C with 5% CO_2 for at least 20 hours. The media was changed to RPMI 1640 medium without phenol red (Thermo Fisher) supplemented with 10% equine whole serum and the cells were then primed with 1 ng/mL equine GM-CSF (Kingfisher Biotech). Following 30-minute incubation at 37 °C with 5% CO_2, cells were stimulated with 100 μg/mL zymosan for 6 hours. For MARCKS inhibition, immediately after priming, cells were treated with 10, 25, 50 or 100 μM MANS peptide, 100 μM control RNS peptide or vehicle (PBS) for 30 minutes at 37 °C followed by zymosan for the pretreatment groups. For post-treatment groups, appropriate concentrations of MANS, RNS, or control were added 1 hour following zymosan stimulation. After 6-hour incubation at 37 °C with 5% CO_2, the plate was centrifuged 300 x G for 5 minutes, supernatants collected into Eppendorf tubes, then centrifuged again at 1300 x rpm for 3 minutes to pellet remaining cellular components. Cell-free supernatants were collected and frozen at -80°C until ELISA analysis.

Peripheral blood neutrophil isolation

Peripheral blood neutrophils were isolated from equine whole blood using density gradient centrifugation method. Heparinized whole blood was collected from donor horses via jugular venipuncture. The collected whole blood was kept at room temperature in sterile conical tubes to allow erythrocytes to settle at the bottom. The supernatant (leukocyte-rich plasma) was collected,

placed onto Ficoll-Paque Plus (GE Healthcare) layer. Following centrifugation, erythrocyte contamination within the neutrophil pellet was removed by 1-minute hypotonic lysis.

Respiratory burst

Production of reactive oxygen species (ROS) was determined by luminol-enhanced chemiluminescence. Alveolar macrophages were seeded into 96-well plate at a density of 100 x 10^3 cells per well and incubated at 37 °C with 5% CO_2 for an hour to allow alveolar macrophages to adhere to the bottom of the well. The wells were then washed three times so that nonadherent cells would be removed, and the remaining alveolar macrophages were incubated at 37 °C with 5% CO_2 for at least 20 hours. The media was changed to RPMI 1640 medium without phenol red (Thermo Fisher) supplemented with 10% equine whole serum and the cells were then primed with 1 ng/mL equine GM-CSF (Kingfisher Biotech). For the MARCKS inhibition experiment, immediately after priming, the cells were treated with 10, 25, 50 or 100 μM MANS peptide, 100 μM control RNS peptide or vehicle for 30 minutes at 37 °C. The cells were then stimulated with 100 μg/mL zymosan (Millipore Sigma) or vehicle. 1mM luminol was added to each well, and luminescence was measured every 15 minutes for 5 hours using a Synergy HTX Multi-Mode Microplate Reader (Biotek). Peripheral blood neutrophils were also stimulated with 100 μg/mL zymosan or vehicle as described previously (Sheats et al., 2014) and detected using luminescence.

Statistical Analyses

All analyses were performed using GraphPad Prism (GraphPad Software). Data were tested for normality by Shapiro-Wilk test. One-way ANOVA with Tukey's post-hoc test was used to analyze normally distributed data. Nonparametric Kruskal-Wallis ANOVA with Dunn's post-hoc test was used to analyzed non-normally distributed data. Chi-square test was performed

to determine whether there was a significant difference in the sex distribution among groups. Correlation between the normalized MARCKS and MARCKSL1 levels and the BALF cytology results was determined by Pearson's correlation coefficient analysis. For all analyses, $p<0.05$ was considered statistically significant.

RESULTS

MARCKS upregulation in BAL cell lysates from horses with mild/moderate and severe inflammation

To investigate whether there were alterations in MARCKS protein in horses with inflammation, nineteen pastured horses were sampled. Results of physical examination as well as BALF differential cell counts from the NCSU population are provided in Table 1. Of those, one horse had respiratory signs at rest despite <20% BALF neutrophils and was therefore included in the severe inflammation group. Of the remaining eighteen horses, four horses had normal BALF cytology, ten had mild/moderate inflammation, and four had severe inflammation. The results from the ERTB population are provided in Table 2. Out of the 24 horses, seven horses were considered normal, ten were mild/moderate, and seven were severe. The ERTB population was also tested for pulmonary function. Delta PpL and pulmonary resistance were significantly different between the normal, mild/moderate, and severe groups (Supplemental Figure 1).

BAL cell lysates from both the NCSU and the ERTB populations were analyzed by ELISA to determine if MARCKS protein was upregulated in the lungs of horses with EAS. MARCKS equine specific ELISA showed a significant difference in normalized MARCKS levels in BAL cell lysates from the NCSU population horses with mild/moderate inflammation and severe inflammation compared to normal horses (Figure 1A). A significant difference was also found in the normalized MARCKS levels in the ERTB population lysates from horses with

no inflammation vs. severe inflammation (Figure 1B). In the NCSU population, there was no significant correlation between the normalized MARCKS levels and percentage of specific cell types in the BALF (Supplemental Figure 2A-E). Similarly, no significant correlation was found in between normalized MARCKS levels and absolute counts of any specific cell types in the BALF (Supplemental Figure 2F-J). For the ERTB samples, there were significant positive correlations between normalized MARCKS levels and % BALF neutrophils and a trend towards positive correlation between MARCKS levels and absolute neutrophil counts (Supplemental Figure 3A, F). Significant negative correlation was found in MARCKS levels and % BALF macrophages (Supplemental Figure 3D).

Given that levels of MARCKS were increased in mild/moderate and severe EAS groups, we investigated whether phospho-MARCKS and total MARCKS protein were increased in BAL cells from horses with EAS. Total MARCKS expression was increased in BAL samples from horses with EAS (Figure 2), supporting our ELISA data. However, phospho-MARCKS did not appear to be increased in our samples.

The effects of MARCKS inhibition on zymosan-stimulated equine alveolar macrophages and neutrophils

Airway neutrophilia is marked in horses with heaves, and these neutrophils express mRNA for cytokines including tumor necrosis factor alpha (TNFα) and interleukin-8 (IL-8) (Ainsworth et al., 2006, 2003; Giguère et al., 2002; Horohov et al., 2005). Previous studies have shown that inhibition of MARCKS with the MANS peptide is able to inhibit *in vitro* mRNA and protein expression of the pro-inflammatory mediators IL-8 and TNFα by canine neutrophils (Li et al., 2013) and IL-6 and TNFα by murine peritoneal macrophages (Lee et al., 2015). Zymosan is a cell well preparation of Saccharomyces cerevisiae, which is primarily composed of β1,3-

glucan core linked to chitin and β1,6-glucans. Beta-glucans are one of the main environmental triggers of EAS (Ivester et al., 2018) and are also common triggers for farmers with asthma (Poole and Romberger, 2012; Sheats et al., 2019). Therefore, the effect of MANS-mediated MARCKS inhibition on *in vitro* cytokine secretion from alveolar macrophages stimulated by zymosan was evaluated. Rested alveolar macrophages were primed with equine GM-CSF, treated with MANS peptide for 30 minutes prior to stimulation with 100 ug/mL zymosan for 6 hours. The supernatants were harvested and analyzed for TNFα and IL-8. Zymosan induced significant cytokine production of both TNF α and IL-8 in alveolar macrophages. However, MANS peptide treatment had no effect on the production of either cytokine (Figure 3). Staurosporine-mediated PKC inhibition significantly decreased TNFα production (Figure 3A). Although insignificant, MANS peptide treatment trended to decrease IL-8 production (Figure 3B).

Reactive oxygen species production (ROS) by innate immune cells, such as neutrophils and macrophages, contributes to the progression of inflammation in the lung. Excessive ROS production results in oxidative stress, resulting in tissue damage and encourage disease progression (Bullone and Lavoie, 2017; Mittal et al., 2014; Paola Rosanna and Salvatore, 2012). Given that ROS production is such an important driver of lung inflammation, the effect of MARCKS inhibition on ROS production was evaluated in zymosan-stimulated alveolar macrophages using luminescence. Zymosan elicits a robust respiratory burst response in alveolar macrophages within 30 minutes of stimulation (Figure 4). At peak stimulation, MANS peptide treatment attenuated zymosan-stimulated ROS production in a concentration dependent manner. In severe EAS, neutrophil infiltration induces lung inflammation. The respiratory burst response of peripheral blood neutrophils stimulated by zymosan was determined. Similar to alveolar

macrophages, zymosan stimulated significant ROS production by neutrophils (Figure 5). MANS peptide treatment significantly attenuated respiratory burst in a concentration dependent manner. The control peptide RNS had no effect. Taken together, these data demonstrate that MARCKS inhibition with MANS peptide attenuates zymosan-induced ROS production in both alveolar macrophages and peripheral blood neutrophils.

DISCUSSION

The goal of this study was to evaluate MARCKS protein as a potential target for asthma treatment. Using horses as a spontaneously occurring disease model, we first determined whether levels of MARCKS and phospho-MARCKS are altered in BAL isolated airway cells from horses with mild/moderate and/or severe EAS. We found that the levels of MARCKS protein was significantly increased in the BAL cell lysates from horses with inflammatory BALF cytology results compared to those from horses with no lower airway inflammation. The significance of MARCKS protein in inflammation is well characterized. To this date, many studies have associated MARCKS with neutrophil and macrophage function (Carballo et al., 1999; Green et al., 2012; Lee et al., 2015; Sheats et al., 2015, 2014). The increase in MARCKS protein levels seen in the BAL cell lysates from horses with lower airway inflammation is in agreement with these previous studies and are also consistent with the previous reports that describe significant activation of airway neutrophils and alveolar macrophages in horses with EAS (Joubert et al., 2011; Vargas et al., 2017). Supporting our ELISA results, we were able to show an increase in total MARCKS in BAL cell lysates from horses with mild/moderate and severe EAS. The increase in the MARCKS protein levels in the BAL cell lysates from horses with lower airway inflammation is consistent with a previous study that showed increased MARCKS levels in TNFα- and LPS-stimulated human neutrophils (Thelen et al., 1990).

A critical regulator of MARCKS function is phosphorylation by PKC (Bubb et al., 1999; Seykora et al., 1996), which results in the release of actin and PIP2. MARCKS plays an essential role in many actin- and PIP2-dependent events such as migration (Eckert et al., 2010; Sheats et al., 2015; Ziemba and Falke, 2018), adhesion (Sheats et al., 2014), cytokine secretion (Damera et al., 2010; Haddock et al., 2014) and respiratory burst (Sheats et al., 2014), all of which are important inflammatory cellular functions. A previous study demonstrated increased MARCKS phosphorylation in the lung homogenates of mice using a murine model of steroid-resistant asthma (Wang et al., 2019). Based on the previous studies, it can be hypothesized that MARCKS phosphorylation is increased in the BAL cells from horses with EAS. However, we were unable to show an upregulation of phospho-MARCKS in BAL cell lysates from horses with EAS. The samples used for western blotting were from the mostly asymptomatic NCSU population; therefore, future studies using symptomatic horses will be warranted to properly assess MARCKS phosphorylation during severe asthma.

The MARCKS levels were further analyzed to evaluate correlations between the protein levels and the BALF cytology results. The overall BALF cellularity increases in horses with EAS (Barton and Gehlen, 2016), although differential cell count does not reflect the change in the overall cellularity. Also, % macrophages in the BALF is known to decrease in horses with EAS as % neutrophils increase (Couëtil et al., 2016). Therefore, the correlations between protein levels and absolute cell counts in addition to %BALF cell types were analyzed. Given the airway neutrophilia in horses with EAS and increase in the MARCKS in neutrophils, we hypothesized that MARCKS levels would be positively correlated with % BALF neutrophils and absolute neutrophil count in the BALF. When correlations between MARCKS levels and BALF cytology results were evaluated, the results were variable between the two populations. While there was a

significant positive correlation between normalized MARCKS levels and %BALF neutrophils in macrophages in the ERTB population, no significant correlations were found in the normalized MARCKS levels and %BALF cell types in the NCSU population. There were no significant correlations found in the normalized MARCKS levels and absolute counts of any specific cell types in either of the populations. The lack of consistent patterns of correlation between MARCKS and the percentage and the absolute count of specific cell types may be explained by the global expression of MARCKS. The difference in the degree of cell activation may also contribute to the variable correlation results of other cell types including neutrophils. This is a possibility considering that EAS affected horses in the ERTB population were symptomatic while the EAS affected horses in the NCSU population were mostly asymptomatic. Future studies evaluating the correlation between MARCKS protein levels, and the cell activity markers will likely help evaluate this possibility.

Horses with mEAS, by definition, do not show respiratory signs at rest. Therefore, BALF cytology is considered a useful method to detect lower airway inflammation in horses especially in horses with mEAS. BAL is a noninvasive procedure that requires minimal equipment. However, BALF cytology has limitations as it does not always correlate with lung biopsy findings (Naylor et al., 1992). For the investigation of MARCKS protein levels, the horses that participated were grouped as normal, mEAS or sEAS based on the BALF cytology. The horses in the subpopulation received pulmonary function testing to aid in the disease classification, although this testing was not available for the horses in the main population. Performing further testing, such as evaluation for clinical signs upon exercise, airway hyperreactivity or lung biopsy, would have added useful information regarding the disease status of the horses included in this study and provide better information for translating this research to human asthma.

Following exposure to organic dust, alveolar macrophages become activated and release proinflammatory mediators including IL-6, CXCL1 and CXCL2 (Poole et al., 2012), as well as reactive oxygen species (Bullone and Lavoie, 2017). In response to these signals, large numbers of neutrophils are recruited to the airways. Indeed, as neutrophils are removed from the airways via phagocytosis and apoptosis, the severity of clinical signs of horses with EAS diminishes (Brazil et al., 2005). In a mouse model of neutrophilic airway inflammation, Damera et al. reported that MARCKS inhibition significantly attenuates neutrophilic infiltration and significantly reduced BALF levels of CXCL1, IL-6 and TNFα (Damera et al., 2010). Taken together, the responses of alveolar macrophages and neutrophils are contributing factors of the symptoms and tissue damage in the airway of EAS affected horses, and thus targeting the inflammatory functions of neutrophils and macrophages is a viable strategy for the management of severe neutrophilic asthma.

To investigate our hypothesis that MARCKS plays an essential role in the secretion of proinflammatory cytokines and ROS production, alveolar macrophages and peripheral blood neutrophils were stimulated with zymosan. Alveolar macrophages were treated with MANS peptide 30 minutes before stimulation with zymosan. Significant cytokine production of TNFα and IL-8 by zymosan stimulation was observed, but no significant differences mediated by MANS peptide were observed in cells stimulated for 6 hours. Although insignificant, MANS treatment trended to decrease IL-8 production by alveolar macrophages. In future studies, extending the incubation time may provide more information about cytokine production. Similar to previous studies, MANS peptide treatment attenuated reactive oxygen species production in both alveolar macrophages and peripheral blood neutrophils (Sheats et al., 2014). Our data

suggest that MARCKS inhibition may decrease the overall inflammation in the lungs by lowering ROS production, ultimately reducing oxidative stress-mediated tissue damage.

Only alveolar macrophages and peripheral blood neutrophils were included in our ex vivo sEAS model. While alveolar macrophages and neutrophils are key cell types in the initiation and perpetuation of the lower airway inflammation in response to airborne triggers of severe asthma, there are other cell types that significantly contribute to the pathophysiology, including airway epithelial cells. As the lung's first line of defense against airborne irritants, the airway epithelial cells secrete a variety of proinflammatory cytokines and chemokines that modulate host immune response (Message and Johnston, 2004; Takizawa, 1998). In the future, we plan to conduct studies using cultured respiratory epithelial cells to determine the role of MARCKS in the inflammatory responses of airway epithelial cells.

TABLES

Table 1. Population data from NCSU herd.

Horse ID	Breed	Age	Sex	Respiratory rate (/min)	Clinical score	Nucleated Cell Count /ul	Neutrophil %	Lymphocyte %	Macrophage %	Mast Cell %	Eosinophil %	BALF cytology classification
1	Thoroughbred	13	Mare	20	1	310	4.7	49.7	42.3	2.3	1	Normal
2	Thoroughbred	10	Mare	28	1	240	6.3	56.3	37.0.3	0.3	0	Normal
3	Quarterhorse	20	Mare	20	3	315	2.7	38.7	57.3	1.3	0	Normal
4	Paint	19	Mare	16	1	588	3	44.7	50.7	1.3	0.3	Normal
5	Warmblood Cross	13	Mare	12	1	343	1.7	48	47.3	2.3	0.7	Normal
6	Quarterhorse	6	Mare	16	0	118	1.7	40	54	3.6	0.7	mEAS
7	British Riding Pony	14	Gelding	32	2	235	9	37	49	5	0	mEAS
8	Quarterhorse/Thoroughbred Cross	9	Mare	12	0	235	7	31	60.3	1.7	0	mEAS
9	Quarterhorse	16	Mare	18	1	410	5	32.3	59.3	3.4	0	mEAS
10	Tennessee Walking Horse	9	Gelding	16	2	375	15	16	66	2	1	mEAS
11	Quarterhorse	18	Mare	20	1	145	2.7	45.3	47	5	0	mEAS
12	Paint	10	Mare	24	0	443	10.8	42	45	1.7	0.5	mEAS
13	Quarterhorse	21	Mare	16	0	285	17.3	47.7	34.3	0.7	0	mEAS
14	Quarterhorse	24	Mare	24	1	445	12	48.3	28.7	8	3	mEAS
15	Quarterhorse/Thoroughbred Cross	12	Gelding	12	0	70	9.3	45	39.7	6	0	mEAS
16	Quarterhorse/Thoroughbred Cross	13	Gelding	12	0	136	2	30.3	58.4	9	0.3	mEAS
17	Quarterhorse/Thoroughbred Cross	7	Gelding	12	0	346	2.3	40	52.4	5.3	0	mEAS
18	Thoroughbred	18	Mare	12	1	293	2.3	40.3	51.7	5	0	mEAS
19	Paint	19	Mare	16	1	513	12	44	42.7	1.3	0	mEAS
20	Throughbred	8	Gelding	12	0	380	16	50	33	1	0	mEAS
21	Throughbred	13	Mare	28	2	113	4	48	44	4	0	mEAS
22	Arabian Cross	19	Mare	16	2	300	3	59.3	33	4.7	0	mEAS
23	Pony	11	Mare	32	3	378	22	42	31.7	1.3	3	sEAS
24	Quarterhorse	19	Mare	18	1	280	27.8	36	33.6	2.3	0.3	sEAS
25	Quarterhorse	21	Mare	16	0	410	22.3	43.7	31.3	2.3	0.3	sEAS
26	Quarterhorse	18	Mare	12	1	235	30	26.3	41	2.7	0	sEAS
27	Allen						12.3	35	49.3	3	0.3	sEAS*
28	Treasure						53.7	5	39	0.7	1.7	sEAS

*Indicates horse grouped as sEAS due to respiratory signs at rest.

Table 2. Population data from ERTB herd.

Horse ID	Breed	Age	Sex	Delta PpL	Resistance	Elastance	Neutrophil %	Macrophage %	Mast Cell %	Lymphocyte %	Eosinophil %	Symptomatic?	BALF cytology classification
1	Standardbred	13	Mare	No lung function performed			2.75	45.75	0	51.5	0	No	Normal
2	Thoroughbred Cross	20	Mare	5	0.2	0.4	2	37.75	1	59.75	0	No	Normal
3	Arabian	18	Gelding	9.15	0.873	1.096	1.75	53.5	0	41.5	3.25	No	Normal
4	Standardbred	9	Mare	7.36	0.61	0.78	3.75	50	0.25	46	0	No	Normal
5	Morgan	19	Gelding	5.813	0.432	0.672	3	47.5	0.25	46	3.25	No	Normal
6	Standardbred	19	Mare	5.63	0.438	0.309	1	50.5	0	48.5	0	No	Normal
7	Quarte Horse	20	Mare	No lung function test performed			1.25	48.25	0	50.5	0	No	Normal
8	Quarter Horse	14	Gelding	56.955	2.732	7.073	8.75	49.75	0	41.5	0	Yes	mEAS
9	Standardbred	15	Mare	58.766	4.317	6.059	14.25	22	0.5	58	0	Yes	mEAS
10	Belgian	22	Mare	28.37	1.49	0.71	15.75	37	0.25	46.5	0.05	Yes	mEAS
11	Canadian	15	Mare	52.885	3.027	4.492	14.75	14.75	0.25	70.5	0	Yes	mEAS
12	Arabian	15	Mare	No Lung Function test performed			10	66.25	0.25	38.5	0	Yes	mEAS
13	Arabian X Quarter Horse	16	Mare	31.547	1.936	1.995	10.75	25.25	0.25	63	0.75	Yes	mEAS
14	Quarter Horse	13	Mare	29.19	1.63	2.2	14.5	27.5	0.75	57.5	0	Yes	mEAS
15	Standardbred	19	Mare	64.63	4.1	7.56	16	29	0.75	54.5	0	Yes	mEAS
16	Quarter Horse	11	Gelding	30.27	3.08	2.61	7.75	30.25	1.25	60.75	0	Yes	mEAS
17	Quarter Horse	20	Mare	89.449	2.775	16.166	12.5	19.25	2	66.25	0	Yes	mEAS
18	Standardbred	24	Mare	23.370	2.088	2.166	29	18	1	52	0	Yes	sEAS
19	Cross	20	Gelding	29.288	2.37	1.813	63.5	20	0	16.5	0	Yes	sEAS
20	Paint Horse	12	Gelding	58.527	4.100	3.600	36.25	23.75	1	38	0	Yes	sEAS
21	Quarter Horse Cross	20	Mare	38.312	2.105	3.115	29.5	19.75	0.5	50.25	0	Yes	sEAS
22	Canadian	9	Mare	69.875	3.829	6.087	27.75	29.5	0.5	42.25	0	Yes	sEAS
23	Paint Horse	16	Mare	74.47	3.64	12.58	34.75	22.5	0.25	42.5	0	Yes	sEAS
24	Quarter Horse	15	Gelding	35	1.99	0.51	81.5	9	0.5	9	0	Yes	sEAS

Table 3. Equine asthma disease classifications based on BAL cytology

Disease Classification	% Neutrophils	% Mast Cells	% Eosinophils
Normal	≤ 6%	≤ 2%	≤ 1%
Mild/Moderate	≥ 7% but ≤ 20%	(and/or) ≥ 3%	(and/or) > 1%
Severe	≥ 20%		

FIGURES

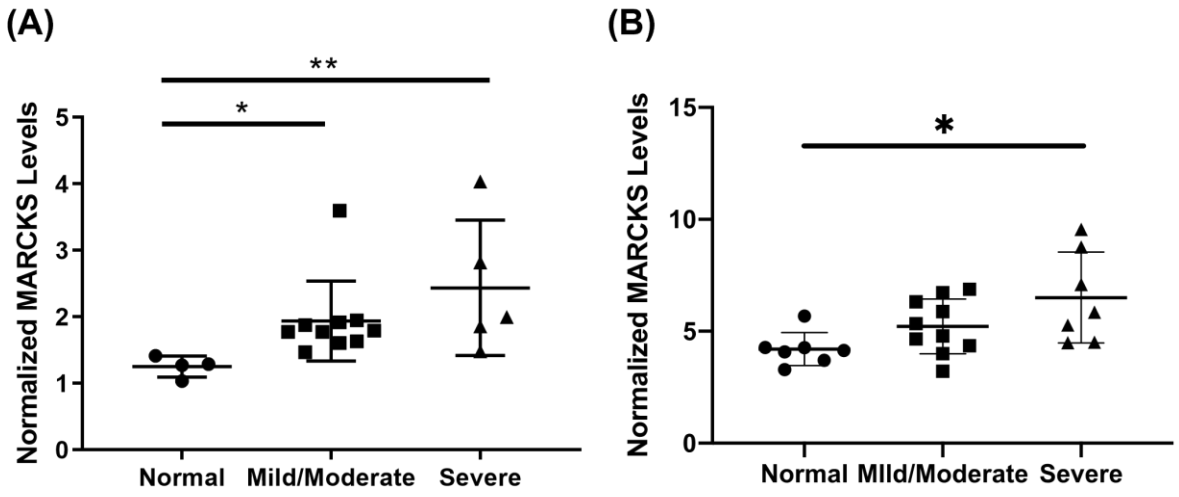


Figure 1. MARCKS Levels in Horse Populations. MARCKS levels in BAL cell lysates from the NCSU population (n=18) (A) and the ERTB population (n=24) (B). The samples were tested for normality. The NCSU population was analyzed using nonparametric one-way ANOVA with Dunn's multiple comparisons test. The ERTB population was analyzed using ordinary one-way ANOVA with Tukey's multiple comparisons test. *$p<0.05$, **$p<0.005$.

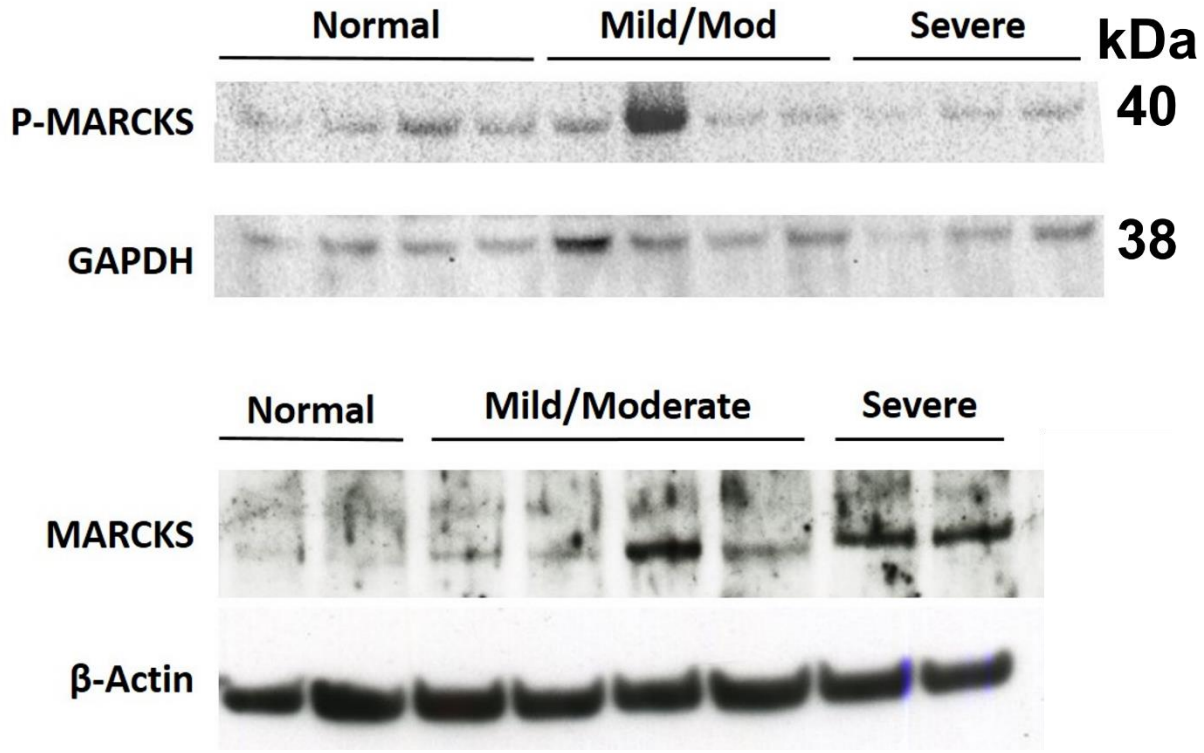


Figure 2. Immunoblot of phospho- and total MARCKS in equine BAL cell lysates. BAL cell lysates from normal horses and horses with mild/moderate and severe disease were immunoblotted for phospho-MARCKS and total MARCKS.

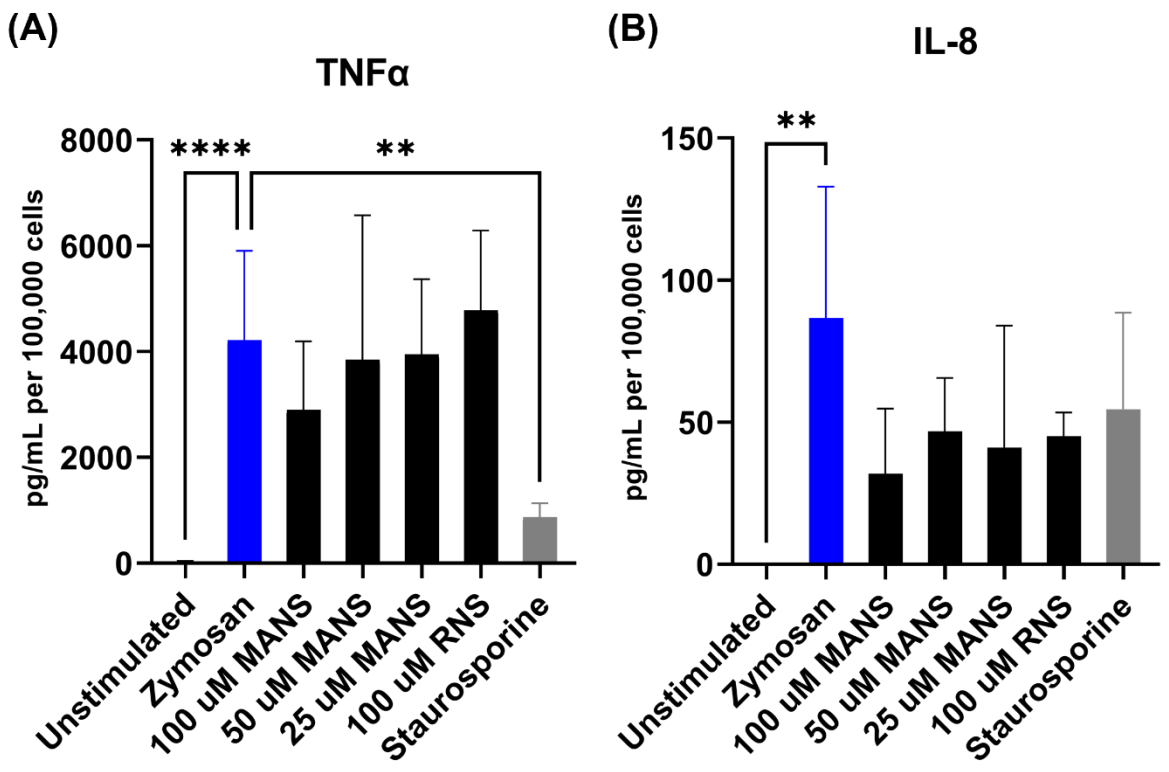


Figure 3. Effects of MANS peptide treatment on alveolar macrophage cytokine secretion. Isolated alveolar macrophages were treated with indicated concentrations of MANS peptide either 30 minutes before or after stimulation with 100 ug/mL zymosan. Supernatants were collected for ELISA analysis of equine specific (A) TNFα and (B) IL-8. Data represented as mean ± SD, (n=7-9) for (A), n=3 for (B). Data analyzed by one-way ANOVA with Dunnett's multiple comparisons test.

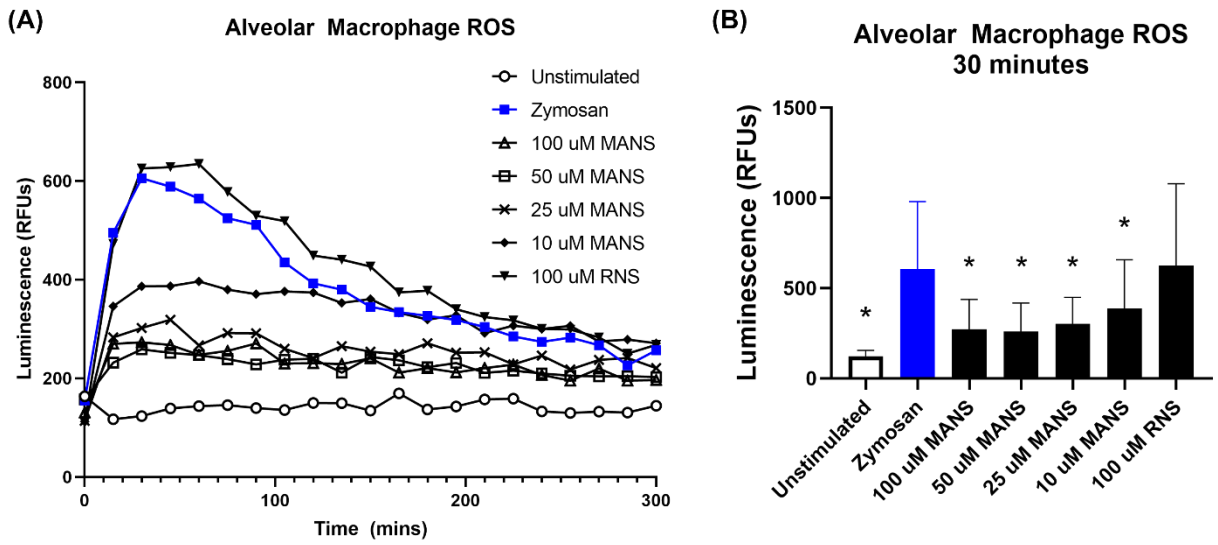


Figure 4. ROS production by zymosan-stimulated equine alveolar macrophages pretreated with MARCKS inhibiting MANS peptide. (A) Stimulation with 100 ug/mL zymosan caused robust ROS production by alveolar macrophages. (B) Pretreatment with MANS peptide significantly attenuated ROS production by zymosan-stimulated alveolar macrophages at 30 minutes. Data analyzed by repeated measures one-way ANOVA with Dunnett's multiple comparisons test, n=8. *$p<0.05$ when compared to zymosan stimulation.

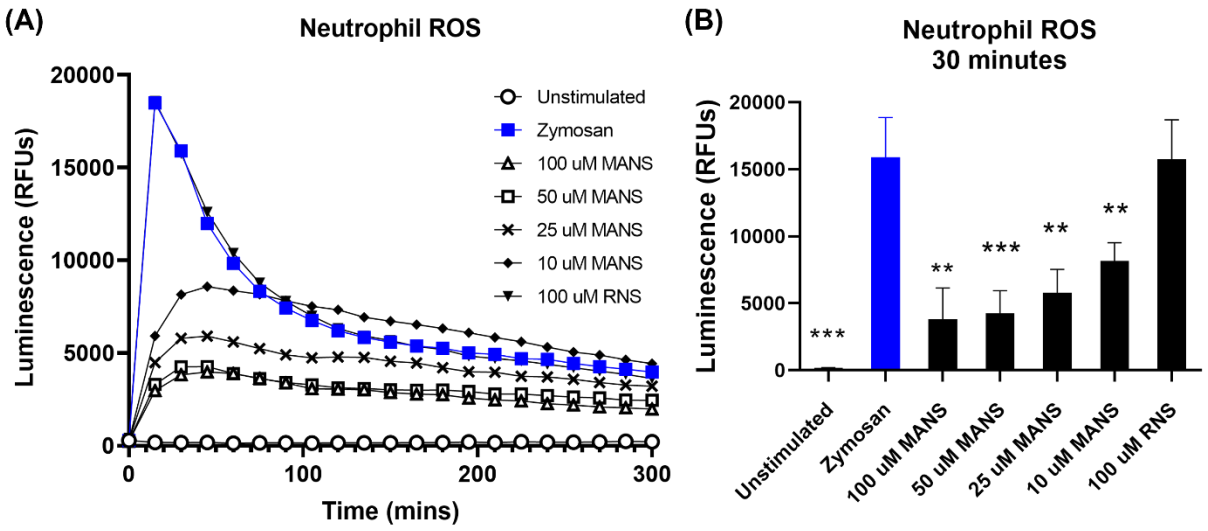
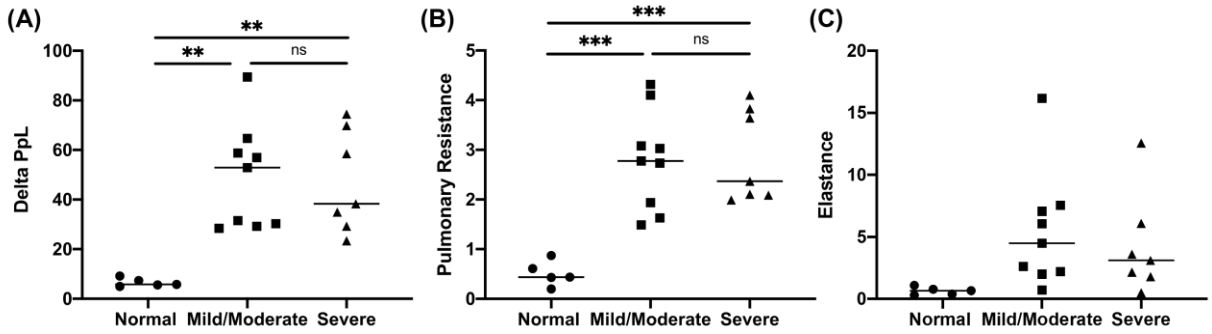
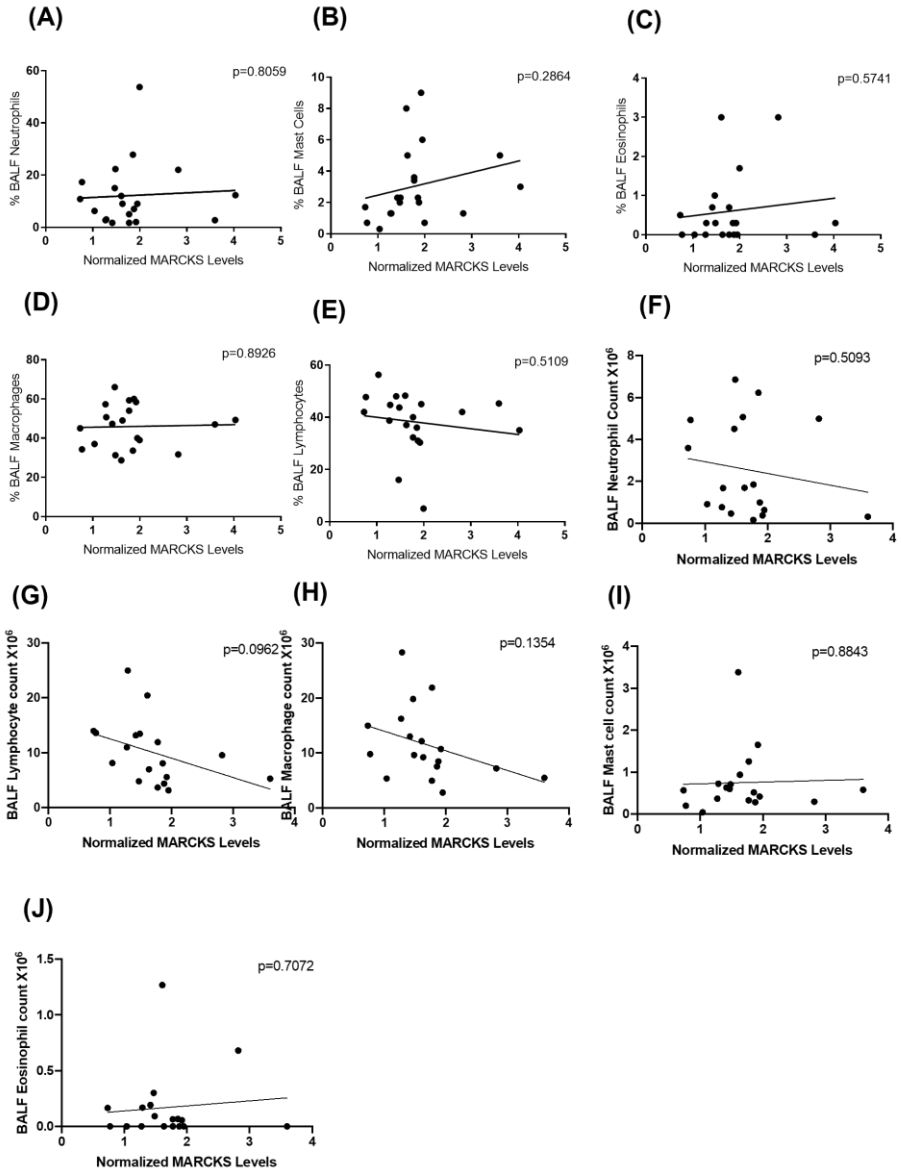


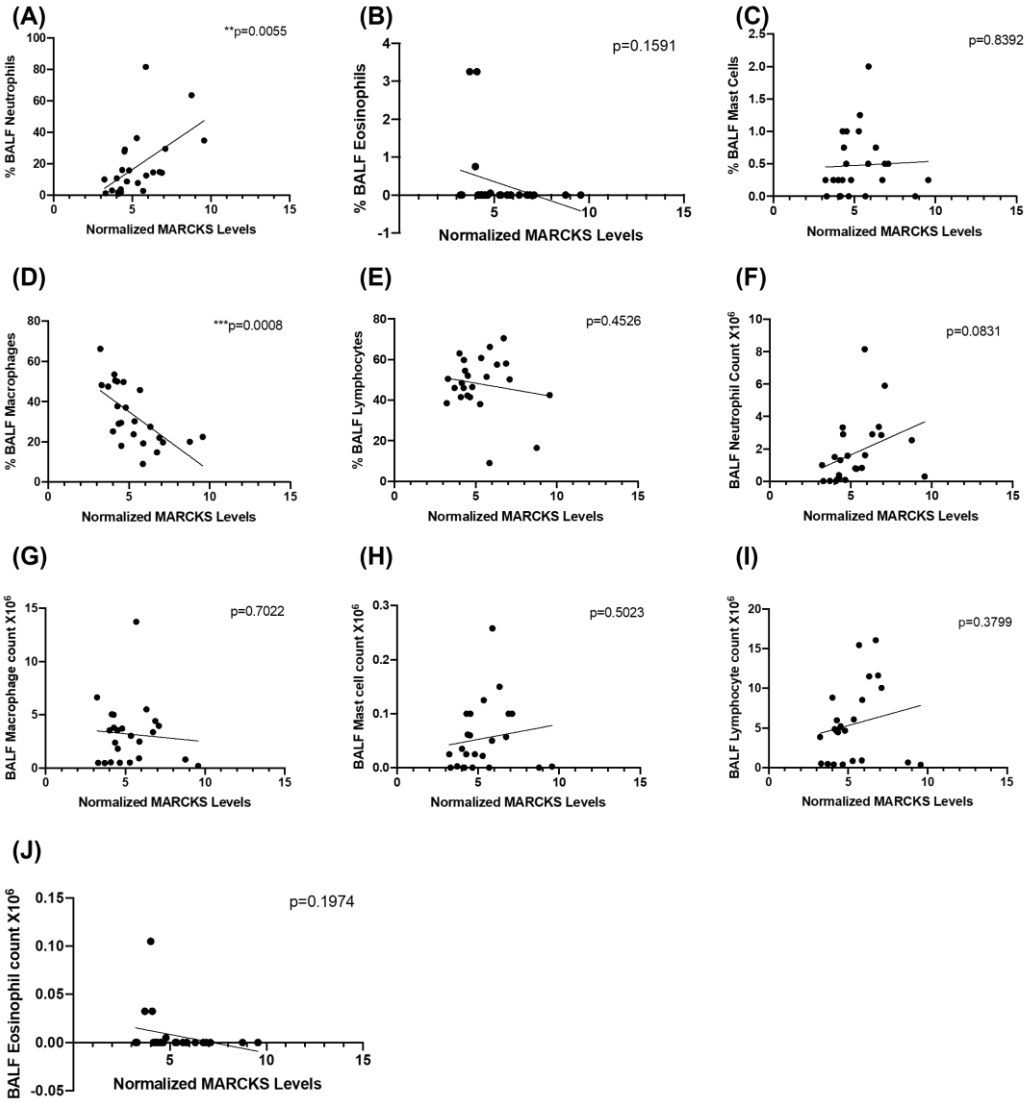
Figure 5. ROS production by zymosan-stimulated equine peripheral blood neutrophil pretreated with MARCKS inhibiting MANS peptide. (A) Stimulation with 100 ug/mL zymosan caused robust ROS production by alveolar macrophages. (B) Pretreatment with MANS peptide significantly attenuated ROS production by zymosan-stimulated alveolar macrophages at 30 minutes. Data analyzed by repeated measures one-way ANOVA with Dunnett's multiple comparisons test, n=6. *$p<0.05$ when compared to zymosan stimulation



Supplemental Figure 1. Pulmonary function test results of horses from ERTB subpopulation. (A) Delta PpL, (B) pulmonary resistance, and (C) Elastase were determined in the ERTB horse population. One-way ANOVA with Tukey's multiple comparisons test (n=21). **p<0.005, ***p=0.001.



Supplemental Figure 2. Correlations between normalized MARCKS levels and BALF cells in NCSU population. Correlation between normalized MARCKS levels and BALF % neutrophils (A), % mast cells (B), % eosinophils (C), % macrophages (D), and % lymphocytes (E). Correlation between normalized MARCKS levels and absolute BALF counts of neutrophils (F), lymphocytes (G), macrophages (H), mast cells (I), and eosinophils (J). No significant correlation was found in between normalized MARCKS levels and the BALF cytology results. p values were calculated with Pearson's correlation coefficient analysis. n=18



Supplemental Figure 3 Correlations between normalized MARCKS levels and BALF cells in ERTB population. Correlation between normalized MARCKS levels and BALF % neutrophils (A), % mast cells (B), % eosinophils (C), % macrophages (D), and % lymphocytes (E). Correlation between normalized MARCKS levels and absolute BALF counts of neutrophils (F), macrophages (G), mast cells (H), lymphocytes (I) and eosinophils (J). Significant positive and negative correlations are found between % BALF neutrophils and macrophages and normalized MARCKS levels, respectively. p values were calculated with Pearson's correlation coefficient analysis. n=24.

REFERENCES

Ainsworth, D., Grünig, G., Matychak, M., Al., E., 2003. Recurrent airway obstruction (RAO) in horses in characterized by IFN-gamma and IL-8 production in bronchoalveolar lavage cells. Vet Immunol Immunopathol 96, 83–91.

Ainsworth, D., Wagner, B., Franchini, M., Grunig, G., Erb, H., Tan, J., 2006. Time-dependent alterations in gene expression of interleukin-8 in the bronchial epithelium of horses with IFN-gamma and IL-8 production in bronchoalveolar lavage cells. Am J Vet Res 67, 669–677.

Arbuzova, A., Schmitz, A.A.P., Res, G.V., 2002. Cross-talk unfolded : MARCKS proteins. Biochem. J 362, 1–12.

Barton, A., Gehlen, H., 2016. Pulmonary Remodeling in Equine Asthma: What Do We Know about Mediators of Inflammation in the Horse? Mediators Inflamm. 2016.

Brazil, T.J., Dagleish, M.P., McGorum, B.C., Dixon, P.M., Haslett, C., Chilvers, E.R., 2005. Kinetics of pulmonary neutrophil recruitment and clearance in a natural and spontaneously resolving model of airway inflammation. Clin. Exp. Allergy 35, 854–865. https://doi.org/10.1111/j.1365-2222.2005.02231.x

Bubb, M.R., Lenox, R.H., Edison, A.S., 1999. Phosphorylation-dependent Conformational Changes Induce a Switch in the Actin-binding Function of MARCKS*. J. Biol. Chem. 274, 36472–36478.

Bullone, M., Lavoie, J., 2017. The Contribution of Oxidative Stress and Inflamm-Aging in Human and Equine Asthma. Int J Mol Sci 18.

Carballo, E., Pitterle, D.M., Stumpo, D.J., Sperling, R.T., Blackshear, P.J., 1999. Phagocytic and macropinocytic activity in MARCKS-deficient macrophages and fibroblasts. Am J Physiol

277, C163-173.

Couëtil, L., Cardwell, J., Gerber, V., Lavoie, J., Léguillette, R., EA, R., 2016. Inflammatory Airway Disease of Horses--Revised Consensus Statement. J Vet Intern Med 30, 503–515.

Damera, G., Jester, W.F., Jiang, M., Zhao, H., Fogle, H.W., Mittelman, M., Haczku, A., Murphy, E., Parikh, I., Panettieri, R.A., 2010. Inhibition of myristoylated alanine-rich C kinase substrate (MARCKS) protein inhibits ozone-induced airway neutrophilia and inflammation. Exp Lung Res 36, 75–84. https://doi.org/10.3109/01902140903131200

Davis, K.U., Sheats, M.K., 2020. The Role of Neutrophils in the Pathophysiology of Asthma in Humans and Horses. Inflammation. https://doi.org/10.1007/s10753-020-01362-2

Davis, K.U., Sheats, M.K., 2019. Bronchoalveolar Lavage Cytology Characteristics and Seasonal Changes in a Herd of Pastured Teaching Horses. Front Vet Sci 6, 74. https://doi.org/10.3389/fvets.2019.00074

Eckert, R.E., Neuder, L.E., Park, J., Adler, K.B., Jones, S.L., 2010. Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) Protein Regulation of Human Neutrophil Migration. Am. J. Respir. Cell Mol. Biol. 42, 586–594. https://doi.org/10.1165/rcmb.2008-0394OC

Ewart, S.L., Robinson, N.E., 2007. Genes and respiratory disease: a first step on a long journey. Equine Vet J 39, 270–4.

Giguère, S., Viel, L., Lee, E., MacKay, R., Hernandez, J., Franchini, M., 2002. Cytokine induction in pulmonary airways of horses with heaves and effect of therapy with inhaled fluticasone propionate. Vet Immunol Immunopathol 85, 147–158.

Green, T.D., Crews, A.L., Park, J., Fang, S., Adler, K.B., 2011. Regulation of mucin secretion and inflammation in asthma; A role for MARCKS protein? Biochim Biophys Acta 1810, 1110–1113. https://doi.org/10.1016/j.bbagen.2011.01.009

Green, T.D., Park, J., Yin, Q., Fang, S., Crews, A.L., Jones, S.L., Adler, K.B., 2012. Directed migration of mouse macrophages in vitro involves myristoylated alanine-rich C-kinase substrate (MARCKS) protein. J. Leukoc. Biol. 92, 633–639. https://doi.org/10.1189/jlb.1211604

Haddock, B.J., Zhu, Y., Doyle, S.P., Abdullah, L.H., Davis, C.W., 2014. Role of MARCKS in regulated secretion from mast cells and airway goblet cells. Am J Physiol Lung Cell Mol Physiol 306, 925–936. https://doi.org/10.1152/ajplung.00213.2013

Hamilton, D., Lehman, H., 2020. Asthma Phenotypes as a Guide for Current and Biologic Therapies. Clin. Rev. Allergy Immunol. 59, 160–174.

Horohov, D., Beadle, R., Mouch, S., Pourciau, S., 2005. Temporal regulation of cytokine mRNA expression in equine recurrent airway obstruction. Vet Immunol Immunopathol 108, 237–245.

Ivester, K., Couëtil, L., Moore, G., 2018. An observational study of environmental exposures, airway cytology, and performance in racing thoroughbreds. J Vet Intern Med 32, 1754–1762.

Ivester, K., Couëtil, L., Moore, G., Zimmerman, N., 2014. Environmental exposures and airway inflammation in young thoroughbred horeses. J Vet Intern Med 28, 918–924.

Joubert, P., Cordeau, M., Lavoie, J., 2011. Cytokine mRNA expression of pulmonary macrophages varies with challenge but not with disease state in horses with heaves or in controls. Vet Immunol Immunopathol 142, 236–242.

Leclere, M., Lavoie-Lamoureux, A., Lavoie, J., 2011. Heaves, an asthma-like disease of horses. Respirology 16, 1027–1046.

Lee, S.M., Suk, K., Lee, W.H., 2015. Myristoylated alanine-rich C kinase substrate (MARCKS)

regulates the expression of proinflammatory cytokines in macrophages through activation of p38/JNK MAPK and NF-κB. Cell. Immunol. 296, 115–121. https://doi.org/10.1016/j.cellimm.2015.04.004

Li, J., D'Annibale-Tolhurst, M., Adler, K., Al, E., 2013. A myristoylated alanine-rich C kinase substrate-related peptide suppresses cytokine mRNA and protein expression in LPS-activated canine neutrophils. Am J Respir Cell Mol Biol 48, 314–321.

Message, S.D., Johnston, S.L., 2004. Host defense function of the airway epithelium in health and disease: clinical background. J. Leukoc. Biol. 75, 5–17. https://doi.org/10.1189/jlb.0703315

Mittal, M., Siddiqui, M.R., Tran, K., Reddy, S.P., Malik, A.B., 2014. Reactive oxygen species in inflammation and tissue injury. Antioxidants Redox Signal. 20, 1126–1167. https://doi.org/10.1089/ars.2012.5149

Naylor, J., Clark, E., Clayton, H., 1992. Chronic obstructive pulmonary disease: Usefulness of clinical signs, bronchoalveolar lavage, and lung biopsy as diagnostic and prognostic aids. Can Vet J 33, 591–598.

Paola Rosanna, D., Salvatore, C., 2012. Reactive Oxygen Species, Inflammation, and Lung Diseases. Curr. Pharm. Des. 18, 3889–3900. https://doi.org/10.2174/138161212802083716

Poole, J., Gleason, A., Bauer, C., Al., E., 2012. CD11c(+)/CD11b(+) cells are critical for organic dust-elicited murine lung inflammation. Am J Respir Cell Mol Biol 47, 652–659.

Poole, J., Romberger, D., 2012. Immunological and inflammatory responses to organic dust in agriculture. Curr Opin Allergy Clin Immunol 12, 126–132.

Rosen, A., Keenan, K.F., Thelen, M., Nairn, A.C., Aderem, A., 1990. Activation of Protein Kinase C Results in the Displacement of its Myristoylated, Alanine-rich Substrate from

Punctate Structures in Macrophage Filopodia. J. Exp. Med. 172, 1211–1215.

Seykora, J.T., Monn Myat, M., Allen, L.-A.H., Ravetch, J. V, Aderem, A., 1996. Molecular Determinants of the Myristoyl-electrostatic Switch of MARCKS. J. Biol. Chem. 271, 18797–18802.

Sheats, M.K., Davis, K.U., Poole, J.A., 2019. Comparative Review of Asthma in Farmers and Horses. Curr. Allergy Asthma Rep. 19. https://doi.org/10.1007/s11882-019-0882-2

Sheats, M.K., Pescosolido, K.C., Hefner, E.M., Sung, J., Adler, K.B., Jones, S.L., 2014. Myristoylated Alanine Rich C Kinase Substrate (MARCKS) is essential to β2-integrin dependent responses of equine neutrophils. Vet Immunol Immunopathol 160, 167–176. https://doi.org/10.1016/j.vetimm.2014.04.009

Sheats, M.K., Sung, E.J., Adler, K.B., Jones, S.L., 2015. In vitro neutrophil migration requires protein kinase c-delta (δ-PKC) mediated MARCKS (Myristoylated Alanine Rich C-Kinase Substrate) phosphorylation. Inflammation 38, 1126–1141. https://doi.org/10.1007/s10753-014-0078-9

Sundaram, M.;, Cook, H.W., Byers, D.M., 2004. The MARCKS family of phospholipid binding proteins: regulation of phospholipase D and other cellular components. Biochem. Cell Biol. 82, 191–200.

Takashi, S., Park, J., Fang, S., Koyama, S., Parikh, I., Adler, K.B., 2006. A Peptide Against the N-Terminus of Myristoylated Alanine-Rich C Kinase Substrate Inhibits Degranulation of Human Leukocytes In Vitro. Am J Respir Cell Mol Biol 34, 647–652. https://doi.org/10.1165/rcmb.2006-0030RC

Takizawa, H., 1998. Airway epithelial cells as regulators of airway inflammation. Int J Mol Med 1, 367–378.

Thelen, M., Rosen, A., Nairn, A.C., Aderem, A., 1990. Tumor necrosis factor a modifies agonist-dependent responses in human neutrophils by inducing the synthesis and myristoylation of a specific protein kinase C substrate. Proc. Nati. Acad. Sci. USA 87, 5603–5607.

Vargas, A., Boivin, R., Cano, P., Murcia, Y., Bazin, I., Lavoie, J., 2017. Neutrophil extracellular traps are downregulated by glucocorticosteroids in lungs in an equine model of asthma. Respir. Res. 18, 207.

Wang, C.-N., Lin, Y.-C., Chang, B.-C., Chen, C.-H., Wu, R., Lee, C.-C., 2019. Targeting the phosphorylation site of myristoylated alanine-rich C kinase substrate alleviates symptoms in a murine model of steroid-resistant asthma Background and Purpose: Myristoylated alanine-rich C kinase substrate. Br J Pharmacol 176, 1122–1134. https://doi.org/10.1111/bph.14596

Ziemba, B.P., Falke, J.J., 2018. A PKC-MARCKS-PI3K regulatory module links Ca 2+ and PIP 3 signals at the leading edge of polarized macrophages. PLoS One 13, e0196678. https://doi.org/10.1371/journal.pone.0196678

CHAPTER 6

Investigating MARCKS role in neutrophil extracellular trap (NET) formation in primary human neutrophils

Haleigh E. Conley[1,2]

[1]Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, 27607, United States

[2]Comparative Medicine Institute, North Carolina State University, Raleigh, NC, 27607, United States

Abstract

Neutrophil extracellular trap (NET) formation is the most recently discovered neutrophil effector function deployed during infection and inflammation. NETs consist of extruded DNA that is decorated with antimicrobial proteins, such as neutrophil elastase, myeloperoxidase, and citrullinated histone 3. Although the mechanisms of NET release are yet to be fully elucidated, this process, like other neutrophil effector functions, may utilize β_2-integrins. Previous research in our lab demonstrated that MARCKS protein plays an essential role in β_2-integrin dependent neutrophil effector functions; therefore, we hypothesized that MARCKS protein would also be essential for NET formation. Neutrophils were treated with two different MARCKS inhibitor peptides and stimulated with PMA to induce NETs. We measured NETs using 3 different assays: SYTOX green detection of DNA, neutrophil elastase ELISA, and fluorescence microscopy. Our results demonstrate that MARCKS inhibition decreased the release of NET-DNA and obtained microscopy evidence that MARCKS inhibition diminished NET formation. MARCKS inhibition did not significantly affect the release of neutrophil elastase. These preliminary findings indicate that MARCKS may have an essential role in NET formation, but further studies are needed to determine the extent and the mechanism of its involvement.

Introduction

Neutrophils are considered the first responders of the immune system. When an infection occurs, tissue resident cells recognize the infection and produce chemoattractants, such as IL-8, for neutrophil recruitment. These signals form a gradient to the bloodstream, where circulating neutrophils will respond to the signal via G-protein coupled receptors (GPCRs). Neutrophil recognition through GPCRs trigger inside-out activation of neutrophil β_2-integrins, resulting in the engagement of ligands, such as ICAM-1, on the vascular endothelium. Neutrophils then

crawl and move between endothelial cells, traveling to the infected tissue. Upon arrival at the site of infection, neutrophils unleash an arsenal of inflammatory functions to eliminate the pathogen or source of inflammation. One potential function is neutrophil extracellular trap (NET) formation, where the neutrophil attempts to capture the pathogen for destruction.

NETs have been observed in response to various stimuli: inflammatory mediators (Brinkmann et al., 2004; Mitroulis et al., 2011), bacteria (Alyami et al., 2019; Brinkmann et al., 2004; Fuchs et al., 2007), fungi (Byrd et al., 2013), parasites (Muñoz-Caro et al., 2015), and viruses, including SARS-CoV-2 (Jenne et al., 2013; Middleton et al., 2020; Narasaraju et al., 2011; Saitoh et al., 2012; Thierry and Roch, 2020; Veras et al., 2020; Wardini et al., 2010). The process is characterized by the release of extracellular DNA that is decorated with antimicrobial proteins, such as citrullinated histone 3 (citH3), myeloperoxidase (MPO), and neutrophil elastase (NE) (Brinkmann et al., 2004). Two distinct pathways of NET formation have been identified; however, they are still incompletely understood. NADPH oxidase 2 (Nox)-dependent NET formation is induced by PMA, LPS, and microbes, while Nox-independent NETs are triggered by calcium ionophores and involve PAD4-mediated histone citrullination (Khan and Palaniyar, 2017). In order for NETs to form, neutrophils must be able to adhere to the endothelium, extracellular matrix, or tissues (Kamoshida et al., 2018). NET formation also involves the neutrophil cell surface receptors β_2-integrins (Byrd et al., 2013; Raftery et al., 2014; Rossaint et al., 2014). Our lab has previously shown that the actin-binding protein MARCKS plays an essential role in many β_2-integrin dependent neutrophil functional responses, including adhesion, migration, and respiratory burst. We presented evidence in Chapter 3 that MARCKS protein has a demonstrated role in β_2-integrin activation and signaling. The combination of this evidence led us to hypothesize that MARCKS protein would also have an essential role in NET formation.

Therefore, this short pilot study was designed to evaluate the role of MARCKS protein in NETs. Peptide inhibitors of MARCKS (MANS and BIO-11006) were used to evaluate MARCKS function in NET formation. MANS peptide inhibits MARCKS function by displacing MARCKS from the plasma membrane to the cytosol (Eckert et al., 2010). BIO-11006 is a smaller analog of MANS and has the same MARCKS-inhibitory effects. BIO-11006 reduces neutrophil-mediated lung inflammation in mice (Damera et al., 2010; Yin et al., 2016) and has completed phase 2 clinical trials for several lung inflammatory conditions (www.biomarck.com/science/). Our results indicate that MARCKS may play an important role in NET formation.

MATERIALS & METHODS

Materials

Isolation of human neutrophils

Human blood collection protocols were reviewed and approved by the Institutional Research Ethics Committee of NCSU (IRB approval #616). For all neutrophil experiments, 10 - 30 mL of whole blood was collected using heparinized syringes from healthy human volunteers that provided consent for participation. Neutrophils were isolated from whole blood using Ficoll-Paque Plus (GE Healthcare) density gradient centrifugation. Briefly, heparinized whole blood was mixed with 0.6% Dextran in a 15 mL polypropylene conical and allowed to settle at room temperature for 45-60 minutes. Up to 10 ml of leukocyte rich plasma was aspirated using a bulb syringe and layered on 5 mL of Fill in a separate 15 mL conical tube. Cells were then centrifuged at 1800 rpm for 20 minutes with the brake off. The supernatant was discarded and remaining blood cells within the cell pellet were removed by 60 seconds of hypotonic lysis. Isolated neutrophils were resuspended/washed in sterile HBSS without additives. Cell number and viability was quantified using trypan blue exclusion (1:1) and a manual hemocytometer count.

Final suspension of cells was in HBSS++ chemotaxis buffer [1x HBSS, 1 mM Ca2+, 1 mM Mg2+, 5% HI fetal bovine serum] at the indicated concentration for each experiment. All experiments were completed within 4 to 6 hours of blood collection. Neutrophils from a sex-balanced cohort of individual human donors were used for all time points and treatment conditions for each experiment (i.e. "n" represents a separate human donor).

Peptide Treatment

MANS and RNS peptides were synthesized by Genemed Synthesis, Inc. The sequence of MANS is identical to the first 24 amino acids of the human MARCKS protein: myristic acid-GAQFSKTAAKGEAAAERPGEAAVA. The RNS peptide is a randomly scrambled control: myristic acid-GTPAPAAEGAGAEVKRASAEAKQAF. BIO-11006 acetate is an analog of the MANS peptide that was designed by Biomarck Pharmaceuticals and synthesized by MedChemExpress. Dr. Ken Adler at North Carolina State University gifted us this peptide. Where indicated, pretreatment of cell suspensions with indicated peptide concentrations occurred at 37 ℃ for 30 minutes.

SYTOX Green Assay

Isolated human neutrophils were resuspended in RPMI at a concentration of $1.0x10^6$ cells/mL and were pretreated with indicated concentrations of peptide for 30 minutes at 37 ºC. 100,000 cells were plated and allowed to settle for 10 minutes then stimulated with 50 nM PMA or PBS at 37ºC or 4 hours. SYTOX green dye (5 µM) was then added to the cells for 10 minutes prior to detection at 485 nm excitation, 523 nm emission using a BioTek plate reader.

Microscopy

Isolated human neutrophils were resuspended in RPMI with 2% FBS at a concentration of $1x10^6$ cells per/mL. Neutrophils were then pretreated with either 100 µM MANS, 100 µM RNS, or

PBS prior to being plated in wells of a chambered coverslip. Cells were then stimulated with 50 nM PMA for 4 hours at 37ºC. After stimulation, neutrophils were fixed with 4% PFA for 15 minutes at room temperature. Cells were then permeabilized with 0.1% Triton X-100 for 15 minutes at room temperature. Wells were blocked with 5% BSA in PBS for 1 hour prior. Cells were then stained with anti-neutrophil elastase antibody (1:500) and anti-histone H3 antibody (1:500) in 5% BS for 1 hour. Cells were then stained with secondary antibody for 1 hour, washed with PBS, and then mounted with DAPI Fluorimount (Southern Biotech).

Neutrophil Elastase Assay

Isolated human neutrophils were pretreated with indicated concentrations of peptide for 30 minutes at 37ºC. $1x10^6$ cells were added to a 24 well plate, allowed to settle for 10 minutes, then stimulated with 50 nM PMA for 4 hours at 37ºC. The plate was then centrifuged prior to removing the supernatant by aspiration to remove free elastase. The wells were then washed twice with warm RPMI, removing the buffer each time. 500 µL S7 nuclease (15 U/mL) was then added to the wells and incubated for 15 minutes at 37ºC to disrupt the NETs. 10 µL EDTA (500 mM) was then added to stop the reaction. The cell suspension was centrifuged, and the supernatants were aliquoted and frozen for analysis. Neutrophil elastase levels were measured using the R&D Systems Human Neutrophil Elastase ELISA per manufacturer's recommendations. Samples were diluted 1:10 prior to analysis using the provided reagent diluent.

Statistics

Data are reported as mean ± SD of independent experiments as listed. All statistical tests were performed using GraphPad Prism software (San Diego, CA) with the appropriate test details in

the figure legend. P values <0.05 were considered statistically significant. *indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

RESULTS

The effect of MARCKS inhibition on NET DNA release

Neutrophil extracellular traps (NETs) are characterized by the extrusion of extracellular DNA, which can be measured using the SYTOX green DNA binding dye. To determine whether MARCKS inhibition decreased the release of DNA from NETS, neutrophils were with phorbol myristate acetate (PMA). PMA is a commonly used stimulant for NET formation *in vitro* and is known to directly activate PKC (Keshari et al., 2013). Pretreatment with MANS peptide (Figure 1A) decreased extracellular DNA induced by PMA stimulation in a concentration dependent manner. Similarly, BIO-11006 decreased NET DNA, although not following a concentration dependent trend (Figure 1B). PMA is known to induce NET formation in a β_2-integrin-dependent manner that is also Nox-dependent. Therefore, anti-CD18 F(ab)'$_2$ and DPI NADPH oxidase inhibitors were included as positive controls for inhibition (Figure 1).

MANS peptide, but not BIO-11006 peptide, interferes with detection of DNA via SYTOX green.

Based on the observations that MANS peptide, but not BIO-11006, decreased NET DNA in a concentration dependent manner, we decided to investigate whether the peptides interfered with the detection of DNA. To do this, a standard curve of DNA was determined and incubated DNA with either 100 µM or 1 µM peptide in a similar manner to experiments containing cells. The results demonstrate that MANS peptide (Figure 2A) significantly interfered with the detection of DNA using SYTOX green but BIO-11006 did not (Figure 2B).

The effect of MARCKS inhibition on neutrophil elastase release

Elastase is contained in the primary/azurophilic granules of neutrophils and is considered one of the most powerful and toxic weapons utilized by neutrophils during infection (Brinkmann et al., 2004). Elastase release is commonly associated with NETs and used as a marker for NET formation *in vitro* and the plasma of patients with inflammatory disorders (Aldabbous et al., 2016). Supernatants from MANS or BIO-11006 pretreated neutrophils stimulated with PMA were evaluated for the presence of NE using an ELISA assay. PMA induced a significant increase in neutrophil elastase after 4-hour stimulation with PMA; however, neither MARCKS targeting inhibitor peptide had a significant effect on elastase levels (Figure 3). MANS peptide did trend to reduce the levels of neutrophil elastase (Figure 3A).

The effect of MARCKS inhibition on PMA-induced NET formation

Although NET formation is commonly measured using single assays, such as SYTOX green or NE detection, microscopy is the best tool for evaluating the presence of NETs because many NET components can be visualized together. Microscopy was used to evaluate how MANS peptide treatment impacted NETs stimulated with PMA. In Figure 4 panels A-C, the unstimulated cells show a rounded morphology with DNA and neutrophil elastase both remaining contained within the cell without significant colocalization. Once stimulated with PMA, there are extrusions from the cells that contain both DNA and neutrophil elastase, indicating NET formation (panels D-F). MANS treatment abrogated the extrusion of NETs, as shown in panels G-I, but RNS control peptide treatment did not hinder the formation of NETs following PMA stimulation (panels J-L). Therefore, our data demonstrate that MARCKS inhibition with the MANS peptide diminishes PMA-stimulated NET formation.

DISCUSSION

Neutrophil extracellular trap (NET) formation has been observed in many inflammatory conditions, including severe SARS-CoV-2 infection. NET formation is just one of many neutrophil inflammatory functions, many of which require functional MARCKS protein. Therefore, to investigate MARCKS role in NET formation, PMA was used to stimulate NET formation in primary human neutrophils, a highly characterized model for *in vitro* analyses. There are many ways to quantify NET formation, including microscopy and high-throughput plate-based assays of SYTOX green and ELISAs. This study utilized a combination of these approaches to assess NET formation.

The first assay looked at the extracellular DNA using SYTOX green (Figure 1). The results demonstrate that PMA stimulates a robust release of DNA inhibited by the NADPH oxidase inhibitor DPI. MARCKS inhibitor peptides decreased the fluorescence and the amount of DNA detected. However, it was then determined that the MANS peptide inhibitor for MARCKS interferes with the detection of DNA using SYTOX green (Figure 2), which likely explains the observed concentration dependence for MANS peptide but not BIO-11006 in Figure 1.

PMA has been shown to stimulate NETs in a β_2-integrin-dependent manner (Raftery et al., 2014); therefore, the addition of anti-CD18 F(ab)'$_2$ antibody served as a positive control the DNA release experiments. Our data aligns with others that have also demonstrated this effect (Raftery et al., 2014). Given that previous studies highlighted MARCKS role in other β_2-integrin-dependent neutrophil functions (Sheats et al., 2014) and that we have demonstrated a role in β_2-integrin activation (Chapter 3), we hypothesize that MARCKS does play an essential role in some types of NET formation. MARCKS role in NETs induced by other stimuli, such as

calcium ionophores, have not yet been evaluated; therefore, future experiments should consider both Nox-dependent and Nox-independent stimuli. There are various physiological stimuli that form β_2-integrin-dependent NETs (Raftery et al., 2014; Silva et al., 2020), so there is a strong likelihood that MARCKS-targeting peptides would inhibit NETs induced in those conditions.

Neutrophil elastase is an enzyme released from the azurophilic granules and cleaves histones during NET formation (Papayannopoulos et al., 2010). We did not observe any significant effect of MARCKS inhibition on neutrophil elastase release (Figure 3), despite MANS treatment trending to decrease NE release (Figure 3A). Examining MARCKS inhibition on NE production is likely not the best predictor of reducing overall NET burden; inhibition of NE during NET formation did not decrease NET-induced cytotoxicity of A549 cells (Saffarzadeh et al., 2012). Previous studies demonstrated MARCKS inhibition with MANS peptide decreased the release of neutrophil myeloperoxidase (MPO) from azurophilic granules at 1, 2, and 3 hours post PMA stimulation (50 nM) (Takashi et al., 2006). Although both NE and MPO are release from azurophilic granules, they display different surface charge and size, indicating they may not be released similarly. In the current study, NE release was evaluated 4 hours post PMA stimulation, so it is possible that different effects would be seen with earlier time points similar to the MPO experiments.

Although DNA and NE were examined individually, there is hesitation in the field to use assays detecting single components as a definitive indicator of NET formation. Therefore, microscopy is always warranted and combination assays, such as DNA-NE and MPO-DNA ELISAs are more trustworthy compliments to the microscopy since those components are commonly located in tandem in the physical NET. To determine if MARCKS inhibition impacted the formation of NETs, immunofluorescence microscopy of DNA, NE, and

citrullinated histone 3 (citH3) was evaluated. PMA induced NETs that stained for DNA and NE, but citH3 was not detected. This finding was not particularly surprising given that PMA is Nox-dependent stimulus of NETosis, which does not induce significant citrullination of histone 3 like that of calcium ionophores (Khan and Palaniyar, 2017). This preliminary microscopy experiment confirmed that MARCKS inhibition with MANS peptide decreased NET formation induced by PMA. We do not have microscopy data with the BIO-11006 peptide; however, the evidence presented with this peptide is particularly interesting given that BIO-11006 has been in phase 2 clinical trials for ARDS treatment. ARDS can be induced in various trauma or diseases, including SARS-CoV-2. NET formation is pronounced in severe COVID-19 patients, and it is possible that BIO-11006 could provide therapeutic benefit against NETs and ARDS in these patients (Middleton et al., 2020).

CONCLUSION

Our preliminary findings demonstrate a putative role for MARCKS in NET formation. Future studies should include physiologically relevant stimuli and evaluate NETs using combinatorial assays to best assess the effects of MARCKS inhibition.

FIGURES

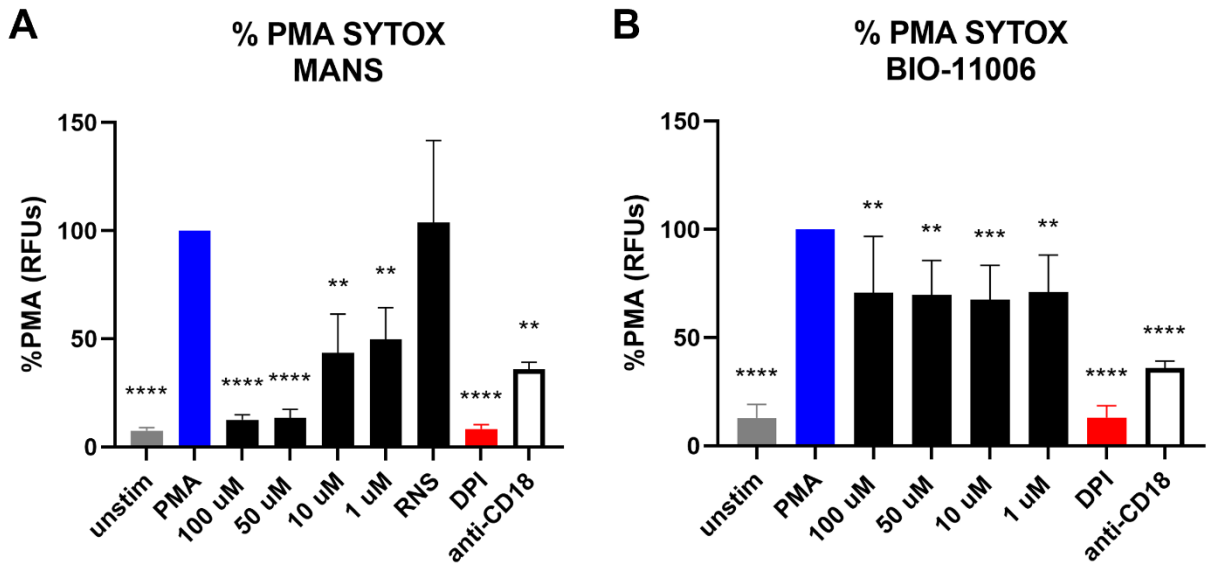


Figure 1. MANS and BIO-11006 peptides attenuate PMA-induced NET-DNA release. Isolated neutrophils pretreated with MARCKS inhibitor peptides, DPI, or anti-CD18 F(ab)'$_2$ were stimulated with 50 nM PMA for 4 hours. Extracellular DNA was measured using SYTOX green. Data are presented as mean ± SD (n=2-7). (*) indicates significant difference in RFUs when compared to PMA. One-way ANOVA with Holm Sidak's multiple comparisons test.

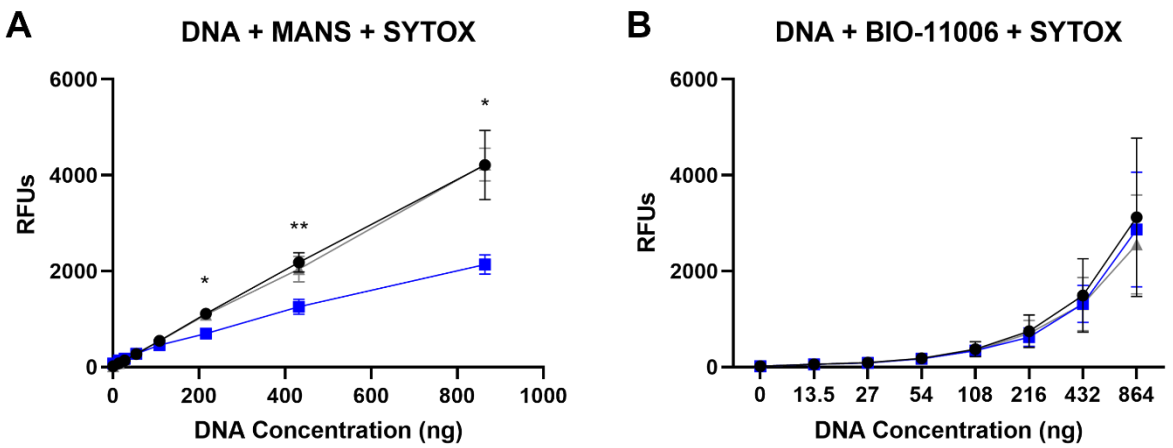


Figure 2. MANS peptide interferes with detection of DNA via SYTOX green.

A standard curve of DNA (●) was generated and incubated with either 100 µM MANS (▲) or 1 µM MANS (■) for 4 hours followed by detection of DNA using SYTOX green. Data are presented as mean ± SD (n=3). (*) indicates significant difference in RFUs when compared to the standard curve. Repeated measures one-way ANOVA with Holm-Sidak's multiple comparisons test.

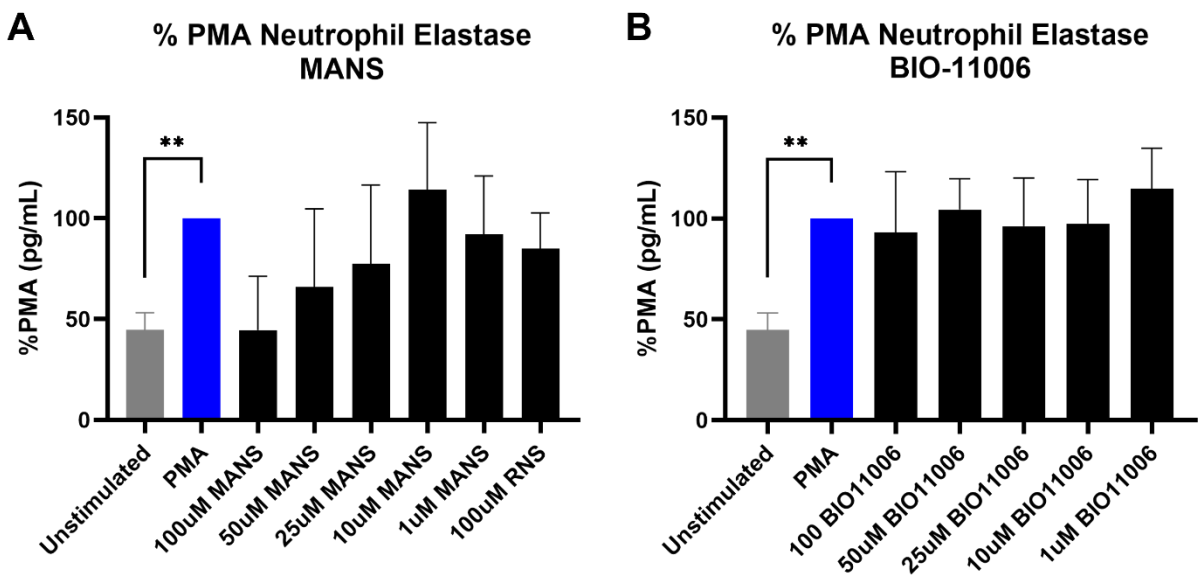
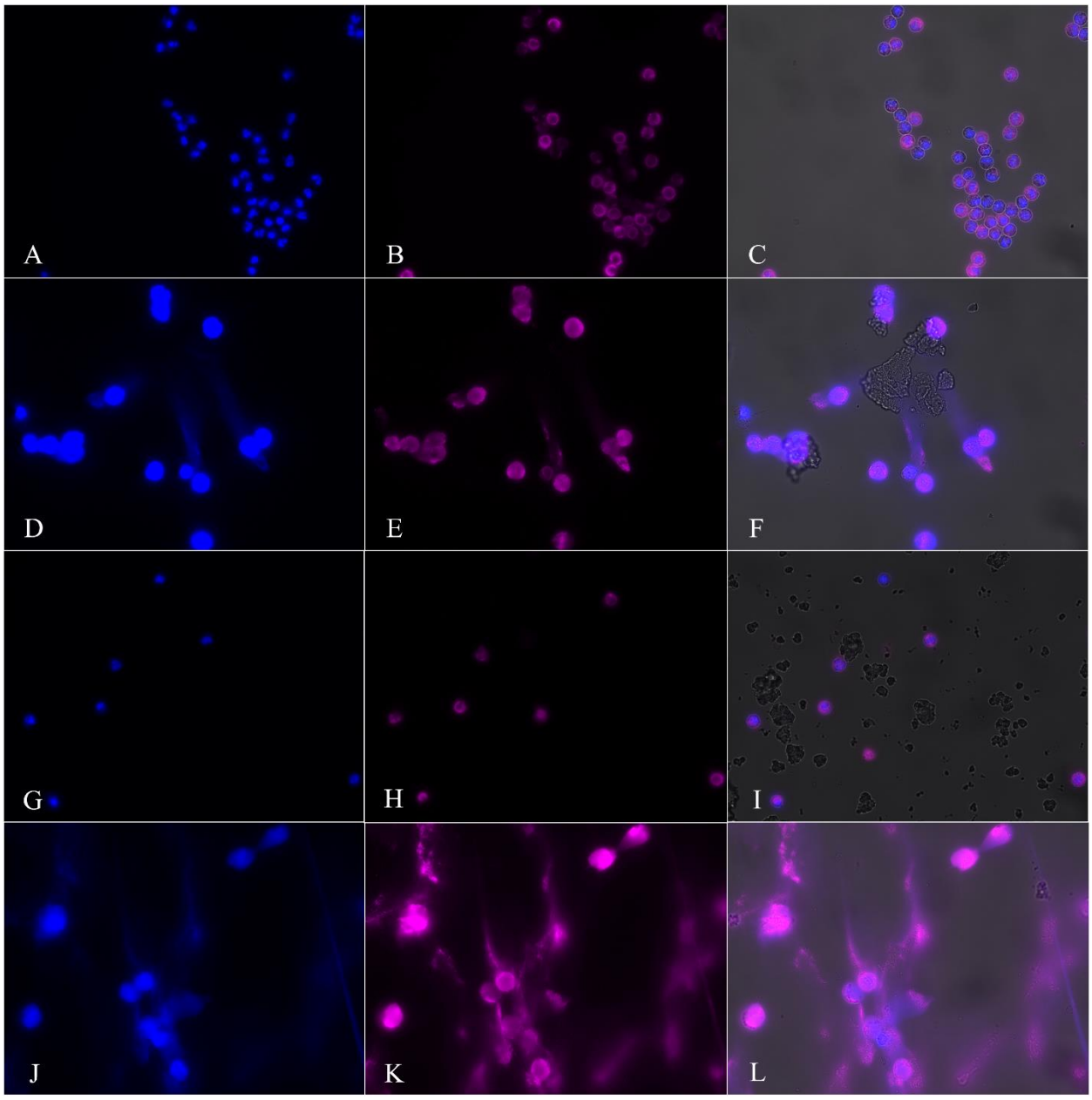


Figure 3. MANS peptide trends to decrease PMA-induced elastase release.

Isolated neutrophils pretreated with MARCKS inhibitor peptides were stimulated with 50 nM PMA for 4 hours. Supernatants were assayed by ELISA for neutrophil elastase. Data are represented as mean ± SD (n=4). (*) indicates significant when compared to PMA. One-way ANOVA with Holm Sidak's multiple comparisons test.

Figure 4. MANS peptide decreases PMA-induced NET formation.

Isolated human neutrophils were pretreated with indicated concentrations of peptide for 30 minutes at 37 ºC. Cells were then added to a chambered microscope coverslip and stimulated with 50 nM PMA for 4 hours. Cells were then fixed and stained prior to imaging using a Keyence BZ-X810. DAPI (blue) (left column), neutrophil elastase (magenta) (middle column), and overlayed with a brightfield image (right column). (A-C) unstimulated, (D-F) 50 nM PMA, (G-I) MANS, and (J-L) RNS. (n=1).


A
B
C
D
E
F
G
H
I
J
K
L

REFERENCES

Aldabbous, L., Abdul-Salam, V., Mckinnon, T., Duluc, L., Pepke-Zaba, J., Southwood, M., Ainscough, A.J., Hadinnapola, C., Wilkins, M.R., Toshner, M., Wojciak-Stothard, B., 2016. Neutrophil extracellular traps promote angiogenesis. Arterioscler. Thromb. Vasc. Biol. 36, 2078–2087. https://doi.org/10.1161/ATVBAHA.116.307634

Alyami, H.M., Finoti, L.S., Teixeira, H.S., Aljefri, A., Kinane, D.F., Benakanakere, M.R., 2019. Role of NOD1/NOD2 receptors in Fusobacterium nucleatum mediated NETosis. Microb. Pathog. 131, 53–64. https://doi.org/10.1016/j.micpath.2019.03.036

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., Zychlinsky, A., 2004. Neutrophil Extracellular Traps Kill Bacteria. Science (80-.). 303, 1532–1535.

Byrd, A.S., O'brien, X.M., Johnson, C.M., Lavigne, L.M., Reichner, J.S., 2013. An extracellular matrix-based mechanism of rapid neutrophil extracellular trap formation in response to C. albicans. J. Immhol 190, 4136–4148. https://doi.org/10.4049/jimmunol.1202671

Damera, G., Jester, W.F., Jiang, M., Zhao, H., Fogle, H.W., Mittelman, M., Haczku, A., Murphy, E., Parikh, I., Panettieri, R.A., 2010. Inhibition of myristoylated alanine-rich C kinase substrate (MARCKS) protein inhibits ozone-induced airway neutrophilia and inflammation. Exp Lung Res 36, 75–84. https://doi.org/10.3109/01902140903131200

Eckert, R.E., Neuder, L.E., Park, J., Adler, K.B., Jones, S.L., 2010. Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) Protein Regulation of Human Neutrophil Migration. Am. J. Respir. Cell Mol. Biol. 42, 586–594. https://doi.org/10.1165/rcmb.2008-0394OC

Fuchs, T.A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., Weinrauch, Y., Brinkmann, V., Zychlinsky, A., 2007. Novel cell death program leads to neutrophil

extracellular traps. J. Cell Biol. 176, 231–241. https://doi.org/10.1083/jcb.200606027

Jenne, C.N., Wong, C.H., Zemp, F.J., McDonald, B., Rahman, M.M., Forsyth, P.A., McFadden, G., Kubes, P., Snyder, J., 2013. Neutrophils Recruited to Sites of Infection Protect from Virus Challenge by Releasing Neutrophil Extracellular Traps. Cell Host Microbe 13, 169–180. https://doi.org/10.1016/j.chom.2013.01.005

Kamoshida, G., Kikuchi-Ueda, T., Nishida, S., Tansho-Nagakawa, S., Ubagai, T., Ono, Y., 2018. Pathogenic bacterium Acinetobacter baumannii inhibits the formation of neutrophil extracellular traps by suppressing neutrophil adhesion. Front. Immunol. 9. https://doi.org/10.3389/fimmu.2018.00178

Keshari, R.S., Verma, A., Barthwal, M.K., Dikshit, M., 2013. Reactive oxygen species-induced activation of ERK and p38 MAPK mediates PMA-induced NETs release from human neutrophils. J. Cell. Biochem. 114, 532–540. https://doi.org/10.1002/jcb.24391

Khan, M.A., Palaniyar, N., 2017. Transcriptional firing helps to drive NETosis. Sci. Rep. 7, doi: 10.1038. https://doi.org/10.1038/srep41749

Middleton, E., Cody, M., Rondina, M., 2020. Neutrophil Extracellular Traps (NETs) Contribute to Immunothrombosis in COVID-19 Acute Respiratory Distress Syndrome. Blood 136, 1169–1179. https://doi.org/10.1182/blood.2020007008/1747487/blood.2020007008.pdf

Mitroulis, I., Kambas, K., Chrysanthopoulou, A., Skendros, P., Apostolidou, E., Kourtzelis, I., Drosos, G.I., Boumpas, D.T., Ritis, K., 2011. Neutrophil Extracellular Trap Formation Is Associated with IL-1b and Autophagy-Related Signaling in Gout. PLoS One 6, e29318. https://doi.org/10.1371/journal.pone.0029318

Muñoz-Caro, T., Mena Huertas, S.J., Conejeros, I., Alarcón, P., Hidalgo, M.A., Burgos, R.A., Hermosilla, C., Taubert, A., 2015. Eimeria bovis-triggered neutrophil extracellular trap

formation is CD11b-, ERK 1/2-, p38 MAP kinase- and SOCE-dependent. Vet. Res. 46. https://doi.org/10.1186/s13567-015-0155-6

Narasaraju, T., Yang, E., Perumal Samy, R., Hian Ng, H., Peng Poh, W., Liew, A.-A., Chee Phoon, M., van Rooijen, N., Chow, V.T., 2011. Excessive Neutrophils and Neutrophil Extracellular Traps Contribute to Acute Lung Injury of Influenza Pneumonitis. Am. J. Pathol. 179, 199–210. https://doi.org/10.1016/j.ajpath.2011.03.013

Papayannopoulos, V., Metzler, K.D., Hakkim, A., Zychlinsky, A., 2010. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J. Cell Biol. 191, 677–691. https://doi.org/10.1083/jcb.201006052

Raftery, M.J., Lalwani, P., Krautkrämer, E., Peters, T., Scharffetter-Kochanek, K., Krüger, R., Hofmann, J., Seeger, K., Krüger, D.H., Schönrich, G., 2014. β2 integrin mediates hantavirus-induced release of neutrophil extracellular traps. J. Exp. Med. 211, 1485–1497. https://doi.org/10.1084/jem.20131092

Rossaint, J., Herter, J.M., Aken, H. Van, Napirei, M., Oring, Y.D.¨, Weber, C., Soehnlein, O., Zarbock, A., 2014. Synchronized integrin engagement and chemokine activation is crucial in neutrophil extracellular trap-mediated sterile inflammation. Blood 123, 2573–2584. https://doi.org/10.1182/blood-2013-07

Saffarzadeh, M., Juenemann, C., Queisser, M.A., Lochnit, G., Barreto, G., 2012. Neutrophil Extracellular Traps Directly Induce Epithelial and Endothelial Cell Death: A Predominant Role of Histones. PLoS One 7, 32366. https://doi.org/10.1371/journal.pone.0032366

Saitoh, T., Komano, J., Saitoh, Y., Misawa, T., Takahama, M., Kozaki, T., Uehata, T., Iwasaki, H., Omori, H., Yamaoka, S., Yamamoto, N., Akira, S., 2012. Neutrophil Extracellular Traps Mediate a Host Defense Response to Human Immunodeficiency Virus-1. Cell Host Microbe

12, 109–116. https://doi.org/10.1016/j.chom.2012.05.015

Sheats, M.K., Pescosolido, K.C., Hefner, E.M., Sung, J., Adler, K.B., Jones, S.L., 2014. Myristoylated Alanine Rich C Kinase Substrate (MARCKS) is essential to β2-integrin dependent responses of equine neutrophils. Vet Immunol Immunopathol 160, 167–176. https://doi.org/10.1016/j.vetimm.2014.04.009

Silva, J.C., Rodrigues, N.C., Thompson-Souza, G.A., Muniz, V. de S., Neves, J.S., Figueiredo, R.T., 2020. Mac-1 triggers neutrophil DNA extracellular trap formation to Aspergillus fumigatus independently of PAD4 histone citrullination. J. Leukoc. Biol. 107, 69–83. https://doi.org/10.1002/JLB.4A0119-009RR

Takashi, S., Park, J., Fang, S., Koyama, S., Parikh, I., Adler, K.B., 2006. A Peptide Against the N-Terminus of Myristoylated Alanine-Rich C Kinase Substrate Inhibits Degranulation of Human Leukocytes In Vitro. Am J Respir Cell Mol Biol 34, 647–652. https://doi.org/10.1165/rcmb.2006-0030RC

Thierry, A.R., Roch, B., 2020. Neutrophil Extracellular Traps and By-Products Play a Key Role in COVID-19: Pathogenesis, Risk Factors, and Therapy. J. Clin. Med. 9, 2942. https://doi.org/10.3390/jcm9092942

Veras, F.P., Pontelli, M., Silva, C., Toller-Kawahisa, J., de Lima, M., Nascimento, D., Schneider, A., Caetite, D., Rosales, R., Colon, D., Martins, R., Castro, I., Almeida, G., Lopes, M.I., Benatti, M., Bonjorno, L., Giannini, M., Luppino-Assad, R., Almeida, S., Vilar, F., Santana, R., Bollela, V., Martins, M., Miranda, C., Borges, M., Pazin-Filho, A., Cunha, L., Zamboni, D., Dal-Pizzol, F., Leiria, L., Siyuan, L., Batah, S., Fabro, A., Mauad, T., Dolhnikoff, M., Duarte-Neto, A., Saldiva, P., Cunha, T., Alves-Filho, J.C., Arruda, E., Louzada-Junior, P., Oliveira, R., Cunha, F., 2020. SARS-CoV-2 triggered neutrophil extracellular traps (NETs)

mediate COVID-19 pathology. J. Exp. Med. 217. https://doi.org/10.1101/2020.06.08.20125823

Wardini, A.B., Guimarã es-Costa, A.B., C Nascimento, M.T., Nadaes, N.R., M Danelli, M.G., Mazur, C., Benjamim, C.F., Saraiva, E.M., Pinto-da-Silva, L.H., Lucia Pinto-da-Silva, H., 2010. Characterization of neutrophil extracellular traps in cats naturally infected with feline leukemia virus. J. Gen. Virol. 91, 259–264. https://doi.org/10.1099/vir.0.014613-0

Yin, Q., Fang, S., Park, J., Crews, A.L., Parikh, I., Adler, K.B., 2016. An Inhaled Inhibitor of Myristoylated Alanine-Rich C Kinase Substrate Reverses LPS-Induced Acute Lung Injury in Mice. Am J Respir Cell Mol Biol 55, 617–622. https://doi.org/10.1165/rcmb.2016-0236RC