ABSTRACT

KORTUM, AMANDA NOELE. Comparative Analysis of Tripartite Motif Containing 9 Protein Expression in Macrophages and Neutrophils. (Under the direction of Dr. Jeffrey Yoder).

The persistence of an immune response can contribute to the development of disease states, increasing the extent of tissue damage or perpetuating the disease condition. Chronic inflammation is often characterized by high numbers of macrophages and neutrophils (phagocytes) that release ROS, cytotoxic factors, and cytokines, which can further amplify inflammation. Phagocytes have been shown to contribute to disease in both natural and laboratory-induced models of chronic inflammation in veterinary species. Understanding the cellular mechanisms that mediate phagocyte function may reveal how these cells could be therapeutically targeted during persistent inflammation.

The E3 ubiquitin ligase TRIM9 is highly expressed in the brain where it mediates axon migration and synaptic vesicle release. While current literature on TRIM9 function in the brain is limited to human cell lines and mouse models, a recent study demonstrated TRIM9 is critical to normal macrophage cell shape and motility in zebrafish. In this study, I aimed to establish a novel mammalian model for the study of TRIM9 in phagocytes. Through the comparative analysis of the TRIM9 expression in macrophages and neutrophils from human, horse, pig, dog, and mouse, I identify TRIM9 isoforms exhibit tissue specificity that are observed across species. Furthermore, based on similar tissue expression of TRIM9 in humans and horses, I propose the use of an equine model for future studies on TRIM9 function in phagocytes.
Comparative Analysis of Tripartite Motif Containing 9 Protein Expression in Macrophages and Neutrophils.

by
Amanda Noele Kortum

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
2019

APPROVED BY:

______________________________  ______________________________
Dr. Jeffrey Yoder             Dr. Samuel Jones
Committee Chair

______________________________  ______________________________
Dr. Barbara Sherry            Dr. David Muddiman
DEDICATION

To my Great-Grandma Georgie,

who started my education early in life

with rag-bag-sag-nag and my ‘arithmetic’ lessons

and

My Grandma TheaLou,

who never hesitated putting dinner on the table two hours late

when a little girl butchering chickens asked “What’s this?”
BIOGRAPHY

Amanda grew up in the rural town of Ekalaka, Montana. This was 100-miles-from-a-McDonald’s, population 300, kind of rural. She spent much of her childhood on her grandparents’ ranch. From watching a caesarian section on a pregnant cow in her Grandpa’s barn to having ducks, rabbits, guinea pigs, dogs, cats, goats, and ferrets as pets, Amanda’s passion for animals blossomed early. It wasn’t until high school that a combined interest in biology, and specifically biological research, drew Amanda away from her original career goal of psychiatry. As the only female in a small Science Research class, Amanda learned the scientific method, developing her own hypotheses, creating experiments and analyzing the results to make conclusions, all while putting up with the antics of her esteemed colleagues, Reed (now Dr. Lambert), Eric (newspaper editor), and Nathan (almost Dr. Carroll).

During college at the University of Montana Western, Amanda pursued scientific research, working in the microbiology lab of her undergraduate mentor, Dr. Mike Morrow. Through four years of research on *Candida albicans*, the pathogenic yeast, Amanda found her true passion in life, infectious disease.

After graduating with a degree in Biology, Amanda went on to put her degree to work, building up her resume before applying to veterinary school. A year spent as a Scientific Researcher I, at the University of Washington School of Medicine only reassured her that pursuit of dual DVM and PhD degrees was the correct path for her. Through the culture shock of a big city and the concrete of Seattle, WA, Amanda chose to attend North Carolina State University College of Veterinary Medicine after many stressful interviews. It was also, coincidently, the choice of her future DVM/PhD/soulmate and partner-in-crime, Hannah Reynolds.
Amanda joined Dr. Jeff Yoder’s Comparative Immunology Lab due to a combined interest in the cat vaccines and the glowing zebrafish Jeff promised, but largely due to Jeff’s passion and excitement for his research. It was only after joining the lab that Amanda realized comparative immunology was the bridge that connected her goal of helping animals through veterinary medicine to her passion for research, scientist-minded brain, and perverse love of infectious disease.

Since beginning the DVM/PhD program, Amanda has had the privilege of learning veterinary medicine with 100+ now DVMs, she has seen graduate student colleagues become doctors and doctor doctors, and she met and married her husband, Josh. As a fellow in the USDA APHIS NBAF Scientist Training Program, Amanda will fulfill her longtime goal of working for the USDA Foreign Animal Disease and Diagnostic Laboratories at Plum Island, NY upon completion of DVM/PhD program. After the completion of the National Bio and Agro-Defense Facility, Amanda will move to Manhattan, KS to finish her service commitment to the USDA.
ACKNOWLEDGMENTS

My journey to this point has been filled with so many supportive and loving individuals; the list could be longer than my dissertation. First and foremost, I need to thank my parents for their unrequited belief in me, my dreams, and the journey it has taken me to get where I am today. My upbringing has prepared me for many of the challenges I have faced and have waiting for me in my future, and for that, I am eternally grateful. Likewise, I have numerous other family members that have offered their unconditional support to me in the last thirty years. I am so thankful for my grandfather and uncle, my brother and his wife, my sister Becky and her family-all people that have flown/driven me places and sent words and gifts of encouragement.

My husband, my patient, kind, creative, intelligent husband, Josh- you have seen me at my utter worst and I hope, what I consider my very best. You don’t count as people, because when I am all peopled out, and need to be alone, you are the only one I want with me. You are everything I could have ever wanted in a life partner and your selflessness, encouragement, and love have fueled these last few long years.

Countless friends deserve thanks: my childhood besties, Jen, Cecile, and Blake, my college pal, Trish, my friend forever in education with me, Hannah. You all build me up, in your own ways and are always there for me. Hannah, obviously I couldn’t have gotten married without you, but I also could not have survived these last 7 years without you. From vet school, Alex Davis (and Syd) are the friends I didn’t know I wanted, and you always know how to make me laugh. To the DVM/PhD crew, I truly believe one of the main reasons any of us have finished is that we are all in this together. The other reason is Sam Jones. Obviously. To Emily especially, you are one of the most positive, brilliant people I know and your inappropriate use of ‘that’s cute’ has made me smile many times over the years.
My labmates deserve a large round of acknowledgement. From putting up with disruptive chitchat to answering questions and only rolling your eyes 50% of the time, Dustin- I’m still cranky you finished before me. Jess, you are a role model in everything you do, vet school, research, motherhood, and being an overall amazing, kind person. Deb, Ashley, Hayley, and Ivan answered every question patiently, while Drake, Joy, Thomas, Emma, and Kathryn put up with obnoxious requests. Shannon Chiera, Alix Berglund, Caroline Johnson, and the Sheets and Jones Labs offered their expertise, samples, and encouragement.

I have to tribute my love of biology and research to my high school teachers, Mrs. Frye and Mrs. Carroll. You fostered my curiosity and opened my eyes to the scientific method. My undergraduate mentor, Dr. Michael Morrow, you made me realize that I thought like a scientist, that I would always have questions, and a PhD would give me the tools to answer those questions. You also exposed me to microbiology and from that, stemmed my interest in infectious disease. Dr. Mike, you always supported my goals, through words of encouragement, letters of recommendation, and pushes to apply for opportunities that opened doors, none of which ended when I left UMW.

Lastly, I would like to thank my committee, Drs. Barb Sherry, Sam Jones, and Dave Muddiman. You are all experienced, skilled, and proven in your field and I am eternally grateful for not only your scientific expertise, but your guidance towards my development as a scientist and my future career. To Dr. Jeff Yoder, I have nothing but appreciation for your support. I was never pressured to answer a scientific question that didn’t interest me, I always felt I could approach you with strange questions or goofy suggestions, even the BFP+ bacteria, and you allowed me to take my own path through my PhD training to gain experiences that would put me closer to my dream career.
## TABLE OF CONTENTS

LIST OF TABLES .......................................................................................................................... viii
LIST OF FIGURES ......................................................................................................................... ix

INTRODUCTION .......................................................................................................................... 1

CHAPTER 1: LITERATURE REVIEW ......................................................................................... 3
  Innate Immune Response .............................................................................................................. 3
  Phagocytes in Disease —
    Comparative Inflammatory Conditions ................................................................................. 6
  Activation of the NFκB Pathway and Type I Interferon Induction ............................................ 11
    NFκB Activation ......................................................................................................................... 11
    Induction of Type I Interferon ................................................................................................... 13
  Ubiquitin Pathway ....................................................................................................................... 15
  Proteasomal Degradation ............................................................................................................ 17
  TRIM9 ......................................................................................................................................... 19
  TRIM9 Function in Neurodevelopment ..................................................................................... 21
  TRIM9 in the Immune Response ................................................................................................. 24
  TRIM9 Associated Proteins in the Immune System .................................................................... 29
  TRIM Family Proteins in the Immune Response ......................................................................... 32
  Models for Studying TRIM9 ....................................................................................................... 35
  References ................................................................................................................................ 38

CHAPTER 2: COMPARATIVE ANALYSIS OF TRIM9 EXPRESSION IN PHAGOCYTES .......... 50
  Introduction ................................................................................................................................. 50
  Materials and Methods .............................................................................................................. 51
  Results ...................................................................................................................................... 57
  Discussion ................................................................................................................................. 78
  References ................................................................................................................................ 89

CHAPTER 3: CONCLUSIONS AND FUTURE DIRECTIONS .................................................... 92
  References ................................................................................................................................ 95

APPENDICES ............................................................................................................................... 96
APPENDIX A ................................................................................................................................. 97
APPENDIX B ................................................................................................................................. 111
LIST OF TABLES

Table 2.1  PCR Primers and Parameters ................................................................. 64

Table 2.2  TRIM9 Transcript Variants and Isoforms..................................................... 65

Table 2.3  TRIM9 antibody isoform specificity in the literature ................................. 66
LIST OF FIGURES

Figure 1.1 NFκB pathway activation and Type I Interferon induction: Dual roles for TRIM9 in the immune response .............................................................................. 37

Figure 2.1 TRIM9 is highly conserved at the protein level across five mammalian species .......... 67

Figure 2.2 TRIM9 is detected in the cerebral cortex of five mammalian species using a commercial antibody ................................................................................... 68

Figure 2.3 TRIM9 was not detected in LPS-stimulated J774.2 cells, a mouse macrophage-like cell line ......................................................................................... 69

Figure 2.4 TRIM9 is expressed in human and horse monocyte-derived macrophages .......... 70

Figure 2.5 An anti-TRIM9 antibody detects proteins in human, horse, pig, and dog neutrophils ........................................................................................................ 71

Figure 2.6 A comparative representation of all western blot results obtained using the anti-TRIM9 antibody from Proteintech ............................................................................. 72

Figure 2.7 Two additional TRIM9 antibodies detect proteins in human and horse phagocytes ............................................................................................................ 73

Figure 2.8 A comparative representation of protein detection in human neutrophils using three different anti-TRIM9 antibodies .................................................................. 74

Figure 2.9 The Origene Monoclonal Anti-TRIM9 antibody binds the FN3 domain of TRIM9 ................................................................................................................. 75

Figure 2.10 TRIM9 transcript variants encode three reported isoforms ...................................... 76

Figure 2.11 Multiple partial TRIM9 transcripts isolated from human neutrophils indicate many unidentified splice variants of TRIM9 may exist ............................................. 77
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2BAR</td>
<td>A2B adenosine receptor</td>
</tr>
<tr>
<td>Asap</td>
<td>Anomalies in sensory axonal patterning</td>
</tr>
<tr>
<td>β-TrCP</td>
<td>Beta-transducin repeat containing protein</td>
</tr>
<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>CC</td>
<td>Coiled Coil</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COS</td>
<td>C-terminal Subgroup One Signature</td>
</tr>
<tr>
<td>DAMPs</td>
<td>damage associated molecular patterns</td>
</tr>
<tr>
<td>DCC</td>
<td>Deleted in Colon Cancer</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Media</td>
</tr>
<tr>
<td>DUBs</td>
<td>deubiquitylases</td>
</tr>
<tr>
<td>F-actin</td>
<td>filamentous actin</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FN3</td>
<td>Fibernectin Type 3 Repeat</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IBD</td>
<td>irritable bowel disorder</td>
</tr>
<tr>
<td>IFA</td>
<td>immunofluorescence assay</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinases</td>
</tr>
<tr>
<td>IP</td>
<td>immunoprecipitation</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>IRF3</td>
<td>Interferon regulatory factor 3</td>
</tr>
<tr>
<td>IRI</td>
<td>ischemic reperfusion injuries</td>
</tr>
<tr>
<td>ISGs</td>
<td>IFN-stimulated genes</td>
</tr>
<tr>
<td>ISREs</td>
<td>interferon stimulated response elements</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitor of κB</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Burtani</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MADD2</td>
<td>muscle arm development defective 2</td>
</tr>
<tr>
<td>MAVS</td>
<td>mitochondrial antiviral signaling protein</td>
</tr>
<tr>
<td>MCAO</td>
<td>Medial cerebral artery occlusion</td>
</tr>
<tr>
<td>MDMs</td>
<td>monocyte-derived macrophages</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MTOC</td>
<td>microtubule organizing center</td>
</tr>
<tr>
<td>NEMO</td>
<td>NFκB Essential Modifier</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NIK</td>
<td>NFκB-inducing kinase</td>
</tr>
<tr>
<td>NLRs</td>
<td>nod-like receptors</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>p65</td>
<td>RelA</td>
</tr>
<tr>
<td>PAC</td>
<td>Parallel adapter capture</td>
</tr>
<tr>
<td>PAMPs</td>
<td>pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBMCs</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PRRs</td>
<td>pattern recognition receptors</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>RACE</td>
<td>Rapid Amplification of cDNA Ends</td>
</tr>
<tr>
<td>RBCC</td>
<td>RING-Bbox-Coiled Coil domain pattern</td>
</tr>
<tr>
<td>RING</td>
<td>Really Interesting New Gene</td>
</tr>
<tr>
<td>RIPA</td>
<td>radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RLRs</td>
<td>RIG-I-like receptors</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>SMART</td>
<td>Simple Modular Architecture Research Tool</td>
</tr>
<tr>
<td>SPRING</td>
<td>SNAP25-interacting RING finger protein</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>STING</td>
<td>stimulator of IFN genes</td>
</tr>
<tr>
<td>TBK1</td>
<td>TANK Binding Kinase 1</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRIF</td>
<td>TLR/IL-1R domain-containing adaptor protein inducing IFNβ</td>
</tr>
<tr>
<td>TRIM</td>
<td>Tripartite Motif Containing</td>
</tr>
<tr>
<td>TRIM9L</td>
<td>TRIM9 Long</td>
</tr>
<tr>
<td>TRIM9S</td>
<td>TRIM9 Short</td>
</tr>
<tr>
<td>TRIM9xL</td>
<td>TRIM9 Extra Long</td>
</tr>
<tr>
<td>VASP</td>
<td>vasodilator-stimulated phosphoprotein</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
</tr>
</tbody>
</table>
INTRODUCTION

When an organism experiences infection or injury, the body’s first response is activation of the innate immune system, resulting in inflammation. Inflammation is characterized by the recruitment of immune cells to the site of invading pathogens or tissue damage. Phagocytes, such as macrophages and neutrophils, are the first cells to be recruited and act quickly, targeting microbes through the release of cytotoxic factors and reactive oxygen species (ROS) and clearing infected, dead, or apoptotic cells through phagocytosis. As first responders, phagocytes also release cytokines, which recruit additional immune cells to the site of inflammation. Unfortunately, phagocytic functions that are essential to combat harmful pathogens such as viruses and bacteria can also contribute to the development of disease states, amplifying the extent of tissue damage or perpetuating the disease condition.

Therapeutics that target the anti-inflammatory pathway to treat autoimmune diseases or other chronic inflammatory conditions vary in availability and success and have a wide range of undesirable side effects. For example, certain medications can result in immune suppression, leaving the host vulnerable to infection or cancer. Consequently, the need for novel therapies that specifically target innate immune cells is crucial.

While there are numerous studies on the negative impact phagocytes can have in multiple disease states, there is still much to learn about the cellular and molecular mechanisms of phagocyte function during chronic inflammatory conditions. A more thorough understanding of the mechanisms and molecules involved in innate immune cell activation, recruitment, and function at the site of inflammation is critical in the development of more targeted therapeutic approaches that mediate harmful immune responses.
Tripartite Motif Containing 9 (TRIM9) is an E3 ubiquitin ligase that belongs to a large protein family known for their role in ubiquitylation of a multitude of protein substrates. First characterized in the brain, TRIM9 has been shown to be critical in controlled axonal migration as well as in neuron development\textsuperscript{1-3}. While TRIM9 has been implicated in the regulation of the NFκB pathway and Type I Interferon signaling, data on TRIM9’s function in the immune system is lacking, particularly in phagocytes\textsuperscript{4-6}. Recently, it was shown that TRIM9 is critical to macrophage morphology and chemotaxis in zebrafish\textsuperscript{7}. Little information is known on the expression of TRIM9 outside of human and mouse models and even less is known about the role TRIM9 plays in mammalian phagocytes. Through a comparative analysis of the TRIM9 expression in macrophages and neutrophils from human, horse, pig, dog, and mouse, this study aimed to establish a novel mammalian model for the future study of TRIM9 function in innate immune cells.
CHAPTER 1: LITERATURE REVIEW

Innate Immune Response

The immune system is responsible for the maintenance of health in living organisms. From a rudimentary system found in bacteria, to the complex, memory-forming system in mammals, it is vital in the defense against pathogens and plays an important role in maintaining homeostasis. In vertebrate species, the immune system is divided into two branches, the innate immune system and the adaptive immune system. The innate immune system consists of basic barrier defenses such as the skin as well as immune cells that provide a rapid, nonspecific response to invading pathogens. The adaptive response is slower to respond, but provides targeted protection against disease-causing organisms or threats to homeostasis and can result in the formation of immunological memory.

A state of inflammation is not solely dependent on the presence of traditional immune cells of the innate (e.g. neutrophils and macrophages) and adaptive (e.g. T and B lymphocytes) immune systems. Non-immune cells such as keratinocytes, fibroblasts, and epithelial cells can contribute to the immune response through sensing a threat and sending out danger signals\textsuperscript{8,9}. The detection of danger can occur through pattern recognition receptors (PRRs) present on non-immune cells as well as immune cells, which then activate complex signaling cascades, such as the nuclear factor kappa B (NF\kappa B) and interferon (IFN) pathways. Induction of the NF\kappa B pathway results in the production of pro-inflammatory mediators such as interleukins and tumor necrosis factor alpha (TNF\alpha), while Type I IFN signaling requires the activation of interferon regulatory factor 3 (IRF3) to produce interferons\textsuperscript{10}. The activation of both of these pathways ultimately leads to the recruitment of traditional innate immune cells, such as macrophages and neutrophils, to the site of inflammation.
Macrophages and neutrophils have the ability to engulf foreign material in a process
called phagocytosis and as such, are collectively referred to as professional phagocytes.
Neutrophils often patrol the vasculature and only migrate into the tissues in times of need, while
macrophages can be found in tissues or migrate from the periphery. Macrophages found in the
tissue are long-lived and referred to as tissue-resident macrophages\textsuperscript{11}. In response to a threat,
macrophage precursors, called monocytes, exit the vasculature, migrate to the tissues, and
differentiate into macrophages\textsuperscript{11}. The process in which these cells arrive to the damaged area is
sequential, with neutrophils arriving within minutes to an hour, followed by inflammatory
monocyte-derived macrophages (MDMs)\textsuperscript{9}. After clearance of the pathogen, a second population
of MDMs arrive, secreting factors that aid in wound healing and tissue repair. If the threat is not
resolved, cells from the adaptive response will also respond.

During an active immune response, neutrophils and macrophages migrate to the site of
inflammation in response to chemoattractants in a process called chemotaxis. These
chemoattractants are produced by local cells within the affected tissue such as keratinocytes,
fibroblasts, epithelial cells, dendritic cells, and resident macrophages. Well known
chemoattractants include leukotrienes, cytokines (IL-8), chemokines (RANTES, CXCL8, CCL2),
and complement peptides (C5a), which then create a concentration gradient that
phagocytes use to navigate to the site of inflammation\textsuperscript{12,13}. Chemotaxis is a step-wise, organized
process, involving the recruited cells, cells that make up the vasculature such as endothelium,
and the expression of cell surface receptors that mediate the interactions of these cells. For
example, neutrophils within the periphery express cell adhesion molecules such as L-selectins
which allow for loose connections to be made with E-selectin, P-selectin, and PSGL-1 molecules
on the surface of vascular endothelium\textsuperscript{13,14}. These interactions bring the recruited cells to a slow
rolling process. Conformational changes in integrin adhesion molecules such as LFA-1 and MAC-1 on neutrophils allow high-affinity ligand binding to occur with endothelial molecules that include ICAMs and VCAM-1\textsuperscript{13}. Following rolling arrest, the recruited cells cross the vasculature wall in a paracellular or transcellular manner in a process called extravasation\textsuperscript{13}. Once in the tissue, the cells migrate through the tissue across the chemoattractant gradient, in a process that requires structural changes within the cell, in order to form cellular extensions called pseudopodia\textsuperscript{13}. When they reach the site of inflammation, phagocytes can begin the process of combating pathogens as well as clearing debris, dead, and dying cells. They also further amplify the inflammatory response through the production of more cytokines. For example, neutrophils and macrophages recruit and activate more innate immune cells as well as those of the adaptive system through the production of leukotriene B\textsubscript{4}, CXCL8, IL-1, IL-6, IL-12, and TNF\textsuperscript{14,15}.

Neutrophils can act directly on pathogens through release of cytotoxic mediators such as proteases, antimicrobial peptides, ROS, nitric oxide (NO), and granules containing hypochlorous acid\textsuperscript{14}. The release of these mediators is termed degranulation and occurs through the exocytosis of membrane-bound secretory granules\textsuperscript{16}. There are 4 types of granules and the order in which they are released is tightly regulated. For example, secretory vesicles are the most readily released and contain albumin\textsuperscript{16,17}. Secondary and tertiary granules, containing Matrix Metalloproteinase 9 (MMP), are released next and lastly, primary granules sometimes called azurophilic granules are released\textsuperscript{16,17}. Exocytosis of primary granules occurs in response to the strongest stimuli, which typically occurs when cells arrive at the source of inflammation since these granules contain the most toxic mediators (elastase, defensins, lysozyme, myeloperoxidase)\textsuperscript{16,17}. 
Macrophages, and to a lesser extent neutrophils, indirectly combat invading pathogens through the phagocytosis of opsonized pathogens and infected cells, at which point they use their internal machinery to kill and digest the microbes. This process is dependent on phagolysosomal acidification. Other mechanisms of killing include the production of enzymes, ROS, NO, and antimicrobial peptides\(^\text{14}\). Macrophages can also contribute indirectly to the adaptive response by activating T-cells\(^\text{11}\).

The tactics phagocytes utilize to directly and indirectly fight invaders require cellular restructuring and complex cytoskeletal changes that require a cascade of cellular proteins and signals. Cytoskeleton rearrangement is critical in several phagocyte basic functions from extravasation and migration through the tissues to phagocytosis and degranulation. For example, the cellular polarization required during chemotaxis includes the formation of actin-rich pseudopodia at the leading edge of the migrating cell, while microtubule organizing center (MTOC) reorientation is important during phagocytosis\(^\text{13,18}\). Another instance where cytoskeletal rearrangement is critical is in neutrophil degranulation, as the first step of exocytosis is recruitment of the granules from the cytoplasm to the plasma membrane\(^\text{16}\). This requires rearrangement of the cytoskeletal microtubules and is followed by vesicle tethering, docking, and finally, fusion to the plasma membrane.

**Phagocytes in Disease – Comparative Inflammatory Conditions**

The release of cytotoxic factors, ROS, and cytokines by phagocytes are critical to combating invading pathogens. However, dysregulation of any of these functions can pose a threat to homeostasis as phagocytic functions that are essential to the inflammatory response can also contribute to the development of disease states, amplifying the extent of tissue damage or perpetuating the disease condition. There are many conditions in which innate immune cells
exacerbate the disease process or directly cause destructive inflammatory states. The following examples will highlight some of these conditions, provide relevant naturally occurring models, and collectively, demonstrate the critical need for tight regulation of innate immune cell function.

In patients with cardiovascular disease, phagocytes are recruited to the site of the lesion through chemoattractant signals released by epithelial and endothelial cells to help restore homeostasis\textsuperscript{19,20}. Macrophages and monocytes travel to atherosclerotic lesions to clear lipid blockage\textsuperscript{20,21}. The death of lipid laden macrophages can enhance lesion development by contributing to an unstable necrotic core, promoting plaque rupture which can lead to thrombosis, a major cause of myocardial infarction (MI)\textsuperscript{22–25}. After a MI, or another ischemic event, such as kidney injury, ischemic colitis, or organ transplant, injured tissues release chemotactic mediators during reperfusion that recruit innate immune cells to the injured site. Activated neutrophils, and possibly macrophages, are major contributors to ischemic reperfusion injuries (IRI) through superoxide, cytokine, and protease production\textsuperscript{26–31}. Cells of monocytic lineage, found in high numbers at these sites, have been shown to impair infarct healing and delay recovery of ventricular function after acute MI, although their role in IRI pathology is still unclear\textsuperscript{32–35}. Horses are highly susceptible to ischemic bowel injury and in the resultant IRI or endotoxemia, neutrophils play key roles in the pathogenesis of the disease\textsuperscript{36}. Furthermore, the merits of using experimental models of canine MI have long been known and in a study that directly compared mouse and dog MI models, researchers found that mice exhibit a more rapid and transient inflammatory response and differences in macrophage accumulation in the lesion when compared to large mammalian species\textsuperscript{37}. 
Damaging inflammatory states can also occur in response to infectious disease. In an attempt to prevent pathogenic bacterial colonization, neutrophils are prevalent in early periodontal disease; however, the same neutrophil proteases that kill microbes can damage extracellular matrix, exacerbating the disease state. In extreme cases such as septic shock, the accumulation and overactivation of neutrophils leads to the increased release of cytotoxic mediators that can have devastating systemic effects.

Not surprisingly, innate immune cells can contribute to the pathology observed in autoimmune disorders and allergy. For example, in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), it has been hypothesized that phagocytes contribute to autoantibody production. Developing as a common theme, neutrophils in both SLE and RA produce molecules that directly damage vascular endothelial cells or cartilage, respectively. In fact, synovial neutrophils from RA patients had higher levels of basal NADPH oxidase activity when compared to healthy controls and after stimulation, had higher ROS production than blood neutrophils, suggesting they were primed. Type IV allergies, also referred to as delayed-type hypersensitivities, are a T-cell mediated disease that result in large recruitment of macrophage and neutrophil populations. The clinical presentation of flea allergy, scabies, and demodecosis are all similar to those in humans with contact allergies to metal or certain drug allergies. In fact, recent work has demonstrated that canine macrophages can be polarized towards the alternatively activated, anti-inflammatory M2 phenotype in vitro, which will prove a valuable model for allergy studies.

Complex diseases and chronic disorders such as asthma and irritable bowel disorder (IBD) have been extensively researched, and while there is still much to learn about the roles phagocytes play in the pathological findings, utilization of naturally occurring disease in novel
animal models may offer insight. For example, while IBD in humans is still not fully understood and believed to be multi-factorial disorder, it is well established that macrophages recruited from the blood infiltrate the intestinal mucosa\textsuperscript{51}. Although inflammatory bowel conditions in dogs and horses have also proven difficult to characterize, horses with the IBD granulomatous enteritis resemble Crohn’s disease at the villus level and also have high numbers of macrophages in affected regions\textsuperscript{49,51}. Furthermore, neutrophils are the second most common clinicopathological finding in equine IBD and in human IBD, neutrophils accumulate in lesions where they display increased levels of ROS\textsuperscript{49,52}. These naturally occurring models of IBD are of value to the scientific community as genetically altered mice models of IBD have questionable physiological relevance and in fact, genetically modified mice that lack human IBD-associated genes (identified through genome wide association studies) do not develop IBD\textsuperscript{53}.

Asthma in humans is a heterogenous group of respiratory diseases that are believed to have both genetic and environmental contributions\textsuperscript{54}. Feline asthma and equine asthma are also collective terms used to describe a complex set of diseases characterized by inflammation of the lower airway\textsuperscript{49}. The disease in both species is influenced by environmental factors and associated with breed predispositions reminiscent of the genetic aspect seen in humans\textsuperscript{49}. Asthma in humans, horses, and cats all present with increased mucus production of the airways, increased airway reactivity, and airway remodeling\textsuperscript{49,55,56}. Furthermore, humans and horses suffering from asthma often have a chronic cough, while humans and cats experience air-trapping, difficulty exhaling against the obstruction of mucus and edema within the bronchi\textsuperscript{49}. Increased neutrophil levels are observed in the lungs of all three species with certain subtypes of asthma, and neutrophils in humans and horses have been shown to perpetuate the disease\textsuperscript{54,57–61}. Research on the role of neutrophils and macrophages in equine asthma provides compelling evidence that
innate immune cells are responsible for a certain degree of pathology during the disease course. For example, the most apparent pathological consequence of the characteristic infiltration of neutrophils in equine asthma is their ability to produce proteases and ROS that directly damage tissue, as well as cytokines that further recruit other immune cells. In a study of severe asthma (previously known as heaves/recurrent airway obstruction) by Lindberg et al., data suggest that leukotriene B\textsubscript{4} produced by neutrophils contributes to additional neutrophil infiltration into the lungs and further tissue damage\textsuperscript{62–64}. Comparatively, neutrophils from asthmatic humans have been shown to have enhanced functional capabilities such as increased migratory capacity and cytokine production (TNF\textalpha, GM-CSF, IFN\gamma)\textsuperscript{54}. In addition to the evident role neutrophils have in equine asthma, macrophages have been implicated in the disease process as well. Both macrophages and neutrophils produce damaging MMPs in severe asthma\textsuperscript{63,64}. While MMP8 expression correlates with neutrophil number and disease severity in bronchoalveolar lavage fluid (BALF), a study by Raulo et al. found that lung macrophages were the major sources of MMP8 in horses with severe asthma\textsuperscript{62,63}. Another study found a correlation between protein levels, gelatinolytic activity of MMP9, and neutrophil levels of BALF from horses with severe asthma and similarly, increased levels of MMP9 has been reported in the neutrophils of asthmatic humans\textsuperscript{54,64}.

A better understanding of the mechanisms mediating macrophage and neutrophil activation, migration, and function during an immune response can lead to the development of more efficient, targeted therapeutics to mediate phagocytes’ roles in damaging inflammatory conditions. The need for new treatments for exuberant inflammation is highlighted by the side effects that accompany one of the common medications for chronic inflammation, corticosteroids. The mode of action of these drugs, in combination with systemic delivery results
in targeting a broad range of cell types. Side effects can include hypokalemia, adrenal insufficiency, and immunosuppression which can leave the patient susceptible to infection\textsuperscript{65–67}. These drawbacks of currently available treatment options demonstrate a critical need for a more thorough understanding of the mechanisms by which innate immune cells are activated, recruited and participate in the immune response. Furthermore, the discrepancies between \textit{in vivo} mouse studies and the translational relevance to humans demonstrate a need for new models\textsuperscript{68}. Through utilization of naturally occurring disease models in large animals, the cellular and molecular mechanisms mediating innate immune cell migration may be discerned, contributing to the development of therapies that target this process during damaging inflammatory states.

\textbf{Activation of the NFκB Pathway and Type I Interferon Induction}

\textit{NFκB activation}

NFκB-mediated signaling is one of the most influential, complex pathways activated during an immune response. The complexity arises from a wide variety of inducers/activators of the pathway as well as the vast number of proteins and processes (such as protein-protein interactions and post-translational modifications) mediating the signaling cascades once activated. The substantial influence of NFκB is reflected by the thousands of genes it can activate or repress as a transcription factor.

In resting cells, NFκB exists either as mature proteins that are inhibited by a second protein or inactive precursor subunits in the cytoplasm. After loss of inhibition or activation of precursors, respectively, these subunits form dimers, translocate to the nucleus, and bind enhancer or promoter regions of NFκB-stimulated genes. Inhibited subunits, RelA (p65), RelB and c-Rel, are synthesized as mature proteins and inhibited by the Inhibitor of κB (IκB) proteins: IκBα, IκBβ, IκBε, BCL-3, IκBz, and IκBNS\textsuperscript{69}. p100 (IκBδ) and p105 (IκBy) are the inactive
precursors of NFκB and after C-terminal cleavage, their subunits, p52 and p50 respectively, are activated\textsuperscript{69}.

There are two pathways of NFκB activation, canonical and alternative, both of which result in the loss of NFκB inhibitors or activation of the precursor proteins (Figure 1). During canonical activation, IκB kinases (IKK) phosphorylate IκB inhibitory proteins which leads to their degradation and subsequent activation of NFκB subunits\textsuperscript{69}. Canonical IKK is a complex of proteins made up of NFκB Essential Modifier (NEMO), IKK\(\alpha\), and IKK\(\beta\). IKK\(\beta\) can phosphorylate IκB\(\alpha\) or IκB\(\beta\), which mark them for polyubiquitylation by the E3 ubiquitin ligase, Beta-transducin repeat containing protein (β-TrCP)\textsuperscript{70}. This ubiquitylation targets the IκB proteins for proteasomal degradation. During alternative activation, dimerized IKK\(\alpha\), independent of the canonical IKK complex, phosphorylates inactive NFκB precursor p100, allowing β-TrCP to ubiquitylated p100, leading to proteasomal processing that yields the active p52 subunit\textsuperscript{69–71}. For the sake of simplicity, this review will not cover the numerous proteins upstream of the IKK proteins that vary depending on the method of induction/receptor stimulated.

Receptors known to induce the canonical NFκB pathway include Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and nod-like receptors (NLR)\textsuperscript{72}. Inducers of the canonical NFκB pathway include pro-inflammatory cytokines as well as pathogen-associated molecular patterns (PAMPs) found on harmful organisms such as bacteria and viruses. Inducers of the canonical pathway potently activate immune cells such as lymphocytes, macrophages, neutrophils, and dendritic cells and result in the release of pro-inflammatory cytokines and chemokines\textsuperscript{69}. The alternative pathway is induced largely by TNF receptor family proteins and can also be activated by certain pathogens such as RNA viruses\textsuperscript{73,74}. Through alternative
activation, NFκB plays critical roles in processes such as lymphoid organ development, B-cell survival, and osteoclast differentiation\textsuperscript{69,74}. It is critical to reiterate that the ligand and receptor combination dictate which downstream proteins involved in the signaling cascade lead to IKK activity and ultimately, NFκB translocation into the nucleus. Furthermore, there are multiple mechanisms of regulation throughout the NFκB pathway, including post-translational modifications that can alter the interactions of the many signal transducing proteins, as well as positive and negative feedback loops.

\textit{Induction of Type I Interferon}

The IFN pathway is most notably involved in antiviral responses, but IFN production can be induced by a number of ligands, including PAMPs and damage associated molecular patterns (DAMPs) through PRRs. Members of the IFN superfamily are categorized into three subtypes. Type I Interferons include IFN\textgamma, IFN\textbeta, IFN\kappa, IFN\epsilon, and IFN\omega, while Type II consists of IFN\gamma\textsuperscript{75}. Type III Interferons are not well characterized, but contain proteins that are distantly related to the IL-10 cytokine family\textsuperscript{75}.

Many types of cells, both professional immune and non-immune cells, have the ability to produce IFN and after being produced, all IFNs signal through the well-studied Janus kinase(JAK)-signal transducer and activator of transcription(STAT) pathway\textsuperscript{76,77}. JAK-STAT signaling results in the activation of a wide variety of innate and adaptive immune responses, critical in combatting pathogens. The cell type, receptors expressed on it, and the inducing stimuli collectively dictate which IFN subtype will be produced. As mentioned above, many cell types have the ability to produce IFN, but often, specific IFNs are associated with particular cell types due to their ability to produce high amounts of a particular IFN. For example, IFN\beta is the
predominant form of IFN produced by macrophages, while IFNγ production is mainly associated with T-cells\textsuperscript{77}.

IFN signaling is an extremely complex process with many proteins involved, countless methods of regulation, and a wide variety of outcomes. Therefore, the focus of this section will be an introductory overview of the induction of Type I IFN production through IRF3 and the main protein players involved, rather than the process and outcomes of IFN signaling.

IRF3, a key regulator in the initiation of Type I IFN expression induced by viruses, acts as a transcription factor\textsuperscript{78–80}. The PRRs known to result in the activation of IRF3 include TLR3, TLR4, RLRs, NLRs, and cytosolic DNA sensors\textsuperscript{79,81}. In response to detection of PAMPs, there are two main signaling cascades through which IRF3 can be activated (Figure 1). The first occurs after activation of TANK Binding Kinase 1 (TBK1) through signaling of one of three adapter proteins: TLR/IL-1R domain-containing adaptor protein inducing IFNβ (TRIF), mitochondrial antiviral signaling protein (MAVS), and stimulator of IFN genes (STING)\textsuperscript{82}. Activated TBK1 facilitates the phosphorylation of IRF3 and upon phosphorylation, IRF3 translocates to the nucleus\textsuperscript{83}. In a TBK1-independent manner, the second pathway of IRF3 activation requires the phosphorylation of β-catenin by GSK3, which then interacts with IRF3, resulting in its translocation to the nucleus\textsuperscript{83}. Within the nucleus, IRF3 undergoes another phosphorylation event, dimerizes and then binds interferon stimulated response elements (ISREs) which can lead to the transcription of over 20 genes that encode for Type I interferons\textsuperscript{79,83,84}. Similarly, IRF7 is another transcription factor activated by TBK1 and plays a role in positive feedback, creating an amplification loop that further induces Type I IFN gene expression\textsuperscript{83}.

As alluded to through the sheer complexity of the activation of these pathways, the outcome of NFκB and IFN signaling can have widespread implications on host immunity.
Furthermore, while often categorized as two separate pathways, the activation, regulation, and protein mediators of NFκB and IFN signaling often overlap, adding to the intricacies of these critical mediators of the immune response. For example, TLR3 signaling leads to TRIF recruitment of a protein complex that includes TRAF3, TBK1, and the NFκB kinase, IKKε. This group of proteins then activate IRF3 to induce Type I IFN production. Conversely, there are several interesting examples that highlight the regulatory cross-over between these pathways. A study in mice demonstrated that when a critical upstream component of alternative NFκB signaling, NFκB-inducing kinase (NIK), is knocked out, macrophages produce elevated levels of Type I IFN in response to viral infection, suggesting components of the NFκB pathway negatively regulate Type I IFN responses. Similarly, in the cytoplasm of resting cells, IRF3 can interfere with IKKβ activity, serving as an example of the Type I IFN system inhibiting canonical NFκB activation. Lastly, IRF3-activating kinase TBK1 has been shown to mediate the phosphorylation and subsequent degradation of NIK, illustrating a protein that promotes Type I IFN response and negatively regulates non-canonical NFκB signaling (Figure 1).

**Ubiquitin Pathway**

Ubiquitylation is a reversible post-translational modification that enzymatically adds one or more ubiquitins onto a specific lysine of the target substrate. Ubiquitin is a highly conserved 76 amino acid protein and ubiquitylation of a protein can target it to one of many pathways. The type of ubiquitin linkage, the number of ubiquitin added, and the branching pattern of the ubiquitin dictates the fate of the substrate. There are three general classifications of ubiquitin modification: monoubiquitylation, multi-monoubiquitylation, and polyubiquitylation. Ubiquitin linkage occurs on a lysine residue of the substrate protein and then additional ubiquitin peptides can be added to the chain through linkages with one of seven lysine residues within ubiquitin.
(K6, K11, K27, K29, K33, K48, K63) or the starting methionine, M1. Polyubiquitylation that consists of ubiquitin in chains linked in the same manner is considered homotypic, while ubiquitin chains formed through multiple types of linkages are heterotypic. Heterotypic ubiquitylation is often referred to as mixed, branched, or hybrid ubiquitin chains. Due to obvious reasons of complexity, homotypic ubiquitylation is better understood than heterotypic ubiquitylation. Homotypic ubiquitin linkage to any of the above mentioned eight residues except K63 and M1 can result in targeting to the most well characterized ubiquitin pathway, proteasomal degradation (expanded upon in Proteasomal Degradation). K63 and M1 homotypic linkages allow for the formation of large protein complexes that are involved in DNA repair, NFκB activation, or protein synthesis. Additional pathways that substrate proteins can be targeted to, through both homotypic and heterotypic polyubiquitylation, include localization to certain cellular compartments such as the ER, nucleus, or cell surface, endocytosis, involvement in histone regulation, and mitophagy.

The process of ubiquitylation occurs when an E1 protein transfers ubiquitin onto the E2 protein, and an E3 ubiquitin ligase can then catalyze the transfer of ubiquitin from the E2 protein onto the substrate protein. As a reversible modification, removal of ubiquitin from a substrate protein occurs by deubiquitylases (DUBs). In mammals, only two E1 proteins have been identified, while E2 and E3 proteins are numerous. E3 proteins contain RING, HECT, UBOX, or RDR domains that possess enzymatic ubiquitin ligase activity. RING-containing proteins are the majority of E3 ligases, and many RING containing proteins have subunits that serve as scaffolds that tether the substrate and the E2-ubiquitin complex. At least 600 E3 ubiquitin ligases have been identified in the human genome and it is predicted these collectively have >9,000 substrate targets. To complicate matters further, the ubiquitin system is tightly regulated.
through various methods, one of which is through ubiquitylation itself. For example, E3 ligases can self-ubiquitylate in a cis-manner or alternatively, one ligase can ubiquitylate another\textsuperscript{88}. Ubiquitylation of an E3 ligase can target it for degradation or can modify protein-protein interactions. In addition to ubiquitylation, other types of post-translational modifications (phosphorylation as an example) either within a substrate or on the E3 ligase, as well as cellular conditions (such as oxygen concentration) can modify protein-protein interactions between an E3 ligase and its substrate protein\textsuperscript{70,91,92}.

**Proteasomal Degradation**

As an important method of regulation, it is not surprising that the ubiquitin pathway is critical in many aspects of the immune system. The most well-known consequence of ubiquitylation is the targeting of a substrate protein to the 26S proteasome. The following discussion will provide a basic description of the proteasome and how it functions as well as highlight well-studied instances where ubiquitin-mediated proteasomal degradation is essential in immune responses.

Found in both the nucleus and cytoplasm of cells, the 26S proteasome is a large molecule, 50-100X larger than extracellular proteases, and is dependent on ATP hydrolysis\textsuperscript{92}. There are two defined reasons a substrate protein would be ubiquitylated and targeted to the proteasome. First, this system has been shown to act as a method of quality control in the cell. Quality control E3 ligases have the ability to detect degenerate motifs, degrons, that are exclusively presented by misfolded proteins, and are undetectable in non-accessible regions of correctly folded proteins\textsuperscript{89}. Consequently, incorrectly folded proteins are degraded by the proteasome and in fact, 10-15% of all newly synthesized proteins are disposed of in this manner\textsuperscript{89}. Alternatively, ubiquitylation that targets correctly folded, functional proteins for
degradation is often a process critical in many signaling cascades. Thus, this method of protein disposal through ubiquitylation is a regulatory mechanism. Perhaps the best known example of ubiquitin-mediated proteasomal degradation in the immune response occurs when IκB, the inhibitory partner of NFκB, is phosphorylated by IKK. This targets IκB for ubiquitylation by β-TrCP and subsequent proteasomal degradation, which results in canonical NFκB activation (see NFκB Activation above)\(^70\).

The 26S proteasome’s active cleavage chamber contains six proteolytic sites: two sites cleave after hydrophobic residues, two sites cleave after acidic residues, and the final two cleave after basic residues\(^92\). The amino acid cleavage that the proteolytic sites utilize is unique to the proteasome, and therefore, blocking this process requires highly specific inhibitors such as lactacystin.

There are two main ways the 26S proteasome degrades proteins. The first, most frequent method begins with protein unfolding and then entry of the entire polypeptide inside the 20S chamber. The polypeptide is then cleaved continuously until peptides of only 3-25 amino acids remain, which are then released and quickly digested into single amino acids by endopeptidases and aminopeptidases\(^92\). This mode of proteasomal degradation differs from classical protease activity, which only cleaves peptides once and releases the two remaining fragments. The other infrequent, but still quite important method, is partial degradation of a protein, yielding an active component. This is the method the proteasome utilizes to produce the active p52 subunit of the alternative NFκB pathway (see NFκB Activation above)\(^92\).

Indeed, there are countless instances where proteasomal degradation results in the activation or inhibition of important mediators in immune signaling pathways. However, with the relatively new discovery of a specialized proteasome, the immunoproteasome, it is apparent that
the importance of proteasomal degradation extends past signaling pathways. Found abundantly in immune cells, such as antigen presenting cells, immunoproteasomes have novel proteolytic cleavage sites that allow for the production of larger peptides than the 26S proteasome\textsuperscript{92,93}. These larger peptides are then able to be processed and presented by the MHC Class I complexes\textsuperscript{92,94}. Although not fully understood, new roles for the immunoproteasome, such as during differentiation of non-immune cells, have been recently reported\textsuperscript{93}. While there is much to learn about these specialized structures, it is of note that immunoproteasome expression is inducible by cytokines such as INF\textgreek{g} and TNF\textsuperscript{93}.

**TRIM9**

The Tripartite Motif Containing (TRIM) family is a large family of proteins known for their pivotal role in the ubiquitin pathway. These proteins are highly conserved across species and contain the Tripartite Motif which includes an amino-terminal Really Interesting New Gene (RING) domain followed by one or two Bbox domains and a Coiled Coil (CC) domain\textsuperscript{95–97}. This Ring-Bbox-Coiled Coil domain pattern (RBCC) is then followed by a variable carboxyl-terminal that dictates which subfamily a TRIM protein belongs to and is also important in protein-protein interactions\textsuperscript{97–99}. TRIM proteins are E3 ubiquitin ligases, the third protein in the ubiquitin pathway which catalyze the transfer of ubiquitin onto substrate proteins\textsuperscript{100,101}. This ubiquitin ligase activity is dependent on the zinc-binding RING domain, while the Bbox-Coil Coiled domains seem to be critical in target specificity\textsuperscript{79,95,96,100,101}. The Bbox domains are unique to TRIM proteins and also contain zinc-binding motifs, although these differ from that of the RING domain\textsuperscript{96,97,101}. It is hypothesized that the Bbox domains may contribute to ubiquitin activity, while the Coiled Coil region is essential in homodimerization\textsuperscript{97,99,102}. 
As described above in the *Ubiquitin Pathways* and *Proteasomal Degradation* sections, ubiquitylated proteins are targeted to one of many pathways, the best characterized is that of proteasomal degradation. Other pathways include protein localization to certain cellular compartments such as the ER or nucleus\(^89,90\). TRIM proteins are involved in two main types of ubiquitylation-linkage, K48 and K63. K48 ubiquitylation results in the proteasomal degradation of the target protein, while K63 ubiquitylation is implicated in the NFκB pathway, DNA repair, and lysosomal targeting\(^72,73,103\).

TRIM9 contains the amino-terminal RBCC motif conserved in TRIM proteins, while the carboxyl-terminal domain places TRIM9 in the C-I subfamily of TRIMs\(^98\). The C-I subfamily also contains TRIM1, 18, 36, 46, and 67 and are characterized by the presence of a C-terminal Subgroup One Signature (COS) domain, a Fibronectin Type 3 Repeat (FN3) domain, and a PRY-SPRY domain\(^98\). The functions of each of the domains have yet to be fully characterized, but a few key roles have been demonstrated (see *TRIM9 Function in Neurodevelopment* and *TRIM9 in the Immune Response* sections below). In general, the COS domain binds microtubules and may be involved in cytoskeletal rearrangement, while the PRY-SPRY domain, sometimes referred to as B30.2, contains an immunoglobulin-like fold and has been shown to have antiviral activity in some TRIM proteins (see *TRIM Family Proteins in the Immune Response*)\(^98,104,105\).

TRIM9 is highly expressed in the brain and has been shown to play critical roles during the development and maturity of the neural network in both invertebrates and vertebrates\(^1,2,97\). The *TRIM9* gene is located on human chromosome 14 and the longest transcript encodes 10 exons and is 4.9 kilobases in length\(^97\). Although the first report of TRIM9 predicted 3 isoforms, only two of these isoforms have been studied in depth: TRIM9 long (TRIM9L) and TRIM9 short (TRIM9S)\(^97\). TRIM9L contains all the above mentioned domains and is translated from the
transcript that contains Exons 1-10. TRIM9S lacks the SPRY-PRY domain and the corresponding transcript contains Exons 1-7. Until recently, most studies were focused on TRIM9L, or failed to make a distinction, and therefore, much of what is known about TRIM9 function is presumably based on TRIM9L.

**TRIM9 Function in Neurodevelopment**

First identified in the brain, TRIM9 was originally thought to be specific to the central nervous system (CNS)\(^97\). The first report on TRIM9 function in vertebrates was in a rat model where the gene was initially named SNAP25-interacting RING finger protein (SPRING)\(^{106}\). In this study, researchers identify TRIM9 (SPRING) as a negative regulator of SNARE complex formation through its interactions with SNAP25\(^{106}\). Through competitive binding of SNAP25, dependent on the Bbox-CC domains, TRIM9 prevents Syntaxin1 and VAMP2 from interacting with SNAP25 to form the SNARE complex\(^{106}\). This interaction effectively suppresses synaptic vesicle exocytosis. Interestingly, several of the components of the SNARE complex, as well as TRIM9, have been shown to be downregulated in the brains of rabies-infected mice\(^{107}\). Similarly, TRIM9 expression is decreased in the brains of humans with Parkinson’s Disease and dementia with Lewy bodies present\(^{108}\). In that study, it was also demonstrated that TRIM9 has E3 ubiquitin ligase activity\(^{108}\).

A candidate for an invertebrate TRIM9 homolog was first identified in a forward genetic screen on *Caenorhabditis elegans* mutants that had fewer muscle arms, an extension of the plasma membrane that contains body muscles\(^{109}\). Muscle arm development defective 2 (MADD2) is the only TRIM that belongs to the TRIM C-I subfamily in invertebrates\(^{110,111}\). Reports vary on whether MADD2 is a true TRIM9 homolog or a homolog of another C-I TRIM protein (see *Models for Studying TRIM9* section below), but regardless of classification,
MADD2, as well as the *Drosophila melanogaster* homolog Anomalies in sensory axonal patterning (Asap), show many functional similarities to TRIM9\textsuperscript{110-113}. Initial studies done in invertebrate species identified many MADD2/Asap-interacting proteins and were then later corroborated by studies of TRIM9 in mammals. For example, through interactions between UNC6 and UNC40, MADD2 mediates ventral axon migration and branching and MADD2\textsuperscript{-/-} mutant *C. elegans* have defects in midline muscle orientation\textsuperscript{110,113}. UNC6 is a nematode homolog of mammalian signaling molecule netrin, while UNC40 is a homolog for mammalian Deleted in Colon Cancer (DCC), which is a netrin receptor\textsuperscript{114}. The comparative relevance of these findings is expanded upon below.

Since the initial studies in invertebrate models identified UNC40/DCC as a TRIM9 binding partner, much work has been done to elucidate the functional relevance of this interaction. Highly expressed in the CNS and critical to neural development, netrin is a soluble, laminin-like protein that mediates both the chemoattraction and repulsion of neurons. This dual guidance property is dependent on the receptor repertoire it acts on and includes the netrin family receptors: DCC, neogenin, UNC5, and the A2B adenosine receptor (A2BAR). The best characterized instance of netrin bifunctionality is through binding to DCC, which leads to homodimerization with another DCC receptor through cytoplasmic tail P3 domains, resulting in guidance-cue mediated axonal attraction\textsuperscript{114}. However, when both DCC and UNC5b are present, netrin induces repulsive effects on neuron migration\textsuperscript{114}. Winkle et al. demonstrated that the negative regulation TRIM9 exerts on SNARE complex formation occurs through DCC and is netrin dependent\textsuperscript{1,106}. In the cerebral cortex, TRIM9 binds and ubiquitylates the intracellular portion of the DCC receptor in a non-degradative manner and in conjunction with its interactions with SNAP25, prevents SNARE complex formation in the absence of netrin\textsuperscript{1,3,106}. These
interactions are dependent on functional RING and CC domains and result in controlled, basal levels of axonal branching in the absence of netrin\textsuperscript{1}. However, when netrin is introduced, TRIM9 releases SNAP25 which then assembles with other SNARE complex proteins\textsuperscript{1}. Concurrently, netrin induces DCC clustering to form multimers and through a reduction in TRIM9-mediated ubiquitylation of DCC, focal adhesion kinase (FAK) is able to bind the cytoplasmic domain of DCC\textsuperscript{3}. FAK then mediates vesicle fusion in a phosphorylation dependent manner, which results in local plasma membrane expansion\textsuperscript{1,3}. This series of events allows for controlled axon branch formation and in the absence of TRIM9, constitutive SNARE complex formation results in unconstrained axon branching, independent of netrin\textsuperscript{1}.

TRIM9 also mediates cortical axon development through ubiquitylation of vasodilator-stimulated phosphoprotein (VASP), a protein that assembles filamentous actin (F-actin) bundles in the growth cone\textsuperscript{2,115}. At the end of axons, growth cones contain filamentous filopodial protrusions which are made of F-actin and possess the ability to respond to growth cues, such as those given by netrin. The filopodia of growth cones depend on VASP function in order to respond to netrin\textsuperscript{116}. Menon et al. demonstrated that when TRIM9 ubiquitylates VASP, localization of VASP at the tips of filopodia is lost and deubiquitylation is required to restore netrin-induced stability and axon turning\textsuperscript{2}. These results suggest that TRIM9 acts as a negative regulator of filopodia stability and growth\textsuperscript{2}. These findings translate to the hippocampus as well, and in fact, TRIM9 deletion results in impaired learning and memory, further demonstrating the important role TRIM9 plays in neuron morphogenesis\textsuperscript{117}.

Most recently, a role for TRIM9 in CNS cancer has been elucidated. Liu and colleagues found that TRIM9 expression is decreased in glioblastoma and through interactions of M KK6,
TRIM9S creates a positive feedback loop for p38 signaling that suppresses glioblastoma progression\textsuperscript{118}.

**TRIM9 in the Immune Response**

Many of the TRIM family proteins have well established roles in the immune system and with the growing interest in the clear role the ubiquitin pathway plays in immunity, a number of studies have aimed to identify functions for TRIM9 in the immune response. The result of this work is highlighted below.

Initial microarray data comparing expression in human monocytes throughout differentiation into macrophages and then polarized M1 or M2 phenotypes revealed *TRIM9* expression was detectable\textsuperscript{119}. While *TRIM9* was not differentially expressed across macrophages in varying states of differentiation, a later publication reported significant increases of *TRIM9* in response to immune stimuli\textsuperscript{41,119}. Type I IFN stimulation did not significantly alter *TRIM9* levels in human MDMs, while Type II IFN decreased *TRIM9* mildly\textsuperscript{41}. Interestingly, *TRIM9* expression was significantly upregulated by immune complexes and out of a total 72 TRIMs tested, only one other gene, *TRIM54*, was significantly upregulated in response to the immune-complex induced Fc\(\gamma\)R stimulation\textsuperscript{41}. *TRIM54* shared similar patterns of expression throughout the experiment, with low levels in resting MDMs and downregulation in response to both types of IFN stimulation\textsuperscript{41}. TRIM9, belonging to the TRIM C-I subset, and TRIM54, belonging to the C-II subset, share similar domain architecture with possession of a COS domain\textsuperscript{98}. This domain is only found in 8 other TRIMs (belonging to the TRIM subsets C-I through C-III), and is thought to be involved in microtubule binding\textsuperscript{98}. Authors note that Fc\(\gamma\)R stimulation of macrophages can activate signaling pathways other than IFNs, in addition to playing a role in the negative regulation of IFN induced signaling\textsuperscript{41}. It is interesting to speculate that the COS domains of these
two TRIMs could be playing key roles in FcγR-stimulated macrophages. For example, MTOC reorientation occurs quickly, exclusively during FcγR-mediated phagocytosis\textsuperscript{18}. The findings from Carthagena et al. support a role for TRIM9 in innate immune cells and one that may be independent of the signaling pathways discussed below.

Conversely to data from humans, Rajsbaum et al. reported TRIM9 was undetectable in mouse macrophages and dendritic cells, during both resting and stimulated conditions\textsuperscript{120}. Interestingly, TRIM9 was detected in both resting and stimulated IL-10\textsuperscript{+} Tregs as well as in stimulated Th2 cells\textsuperscript{120}. The TRIM9 expression pattern across these cell types was mirrored by 3 other TRIMs (1, 18, and 46), all members of the TRIM C-I subset family\textsuperscript{120}.

In 2013, a study by Versteeg et al. investigated the TRIM family and using full length mRNAs from GenBank and Spliceminer, found that 90\% of all TRIMs have more than one splice variant\textsuperscript{121}. Interestingly, 52\% of these variants encode proteins that lack at least one major domain and 63\% of those lack the tripartite motif\textsuperscript{121}. The authors hypothesized that splice variants negatively regulate full-length proteins\textsuperscript{121}. Recombinant expression of TRIM proteins in the presence of a proteasomal inhibitor revealed that 24 TRIM proteins, including TRIM9, showed a >100\% increased level of expression\textsuperscript{121}. These findings suggest that many of the TRIM proteins may self-ubiquitylate, targeting them for proteasomal degradation. Through a series of experiments using reporter plasmids, TRIM9L was capable of enhancing constitutive activity as well as inducing IFNβ and ISRE promoter activity alone\textsuperscript{121}. This work is supported by Qin et al., that reported moderate antiviral activity when TRIM9 is overexpressed in HEK cells\textsuperscript{6}. The reporter experiments also revealed enhanced IFNβ and ISRE promoter activity by 90\% of TRIM proteins containing a COS domain tested\textsuperscript{121}. These findings add further support to Carthagena et al.’s earlier theory that the COS domain may play a role in innate immune function\textsuperscript{41,121}. Viral
infection in HeLa cells expressing recombinant TRIM9 induced changes in the localization of the recombinant protein from microtubule-associated to a dispersed cytoplasmic distribution. Lastly, this report also investigated TRIM family expression in lung-derived A549 cells and monocyte-like THP-1 cells in response to stimuli. Transcript amplification primers for this work were located in Exons 1 and 2 of TRIM9, which likely resulted in the amplification of multiple TRIM9 transcript variants. Viral infection led to increased TRIM9 expression in A549 cells but interestingly, did not change TRIM9 expression in THP-1 cells. However, TRIM9 was downregulated when THP-1 cells were treated with IFNβ. Furthermore, multiple TRIM genes in this study exhibited opposite expression patterns across cell types in response to infection, suggesting cell-type specific regulatory roles for TRIM proteins in immune response.

Shi and colleagues made the first report of TRIM9 involvement in the NFκB pathway. β-TrCP plays an important role in both the canonical and alternative activation of the NFκB pathway through ubiquitylation of IκBα and p100, respectively. As a result of both types of activation, NFκB translocates to the nucleus and activates many critical genes in the immune response. This study demonstrated that TRIM9 negatively regulates NFκB activation and subsequent cytokine production such as IL-6. This finding is supported by similar studies on the TRIM9 homolog found in the Hong Kong Oyster (Crassostrea hongkongensis). Their concluding model hypothesizes that in a manner dependent on TRIM9 phosphorylation, TRIM9L interacts with β-TrCP, preventing it from ubiquitylating its target proteins (Figure 1). This work supports earlier findings from Versteeg et al., that reported NFκB was not induced by TRIM9 overexpression in reporter assays.

Perhaps the most informative study to reveal TRIM9’s complex role in the regulation of immune signaling was done by Qin et al. in 2016. Researchers investigated the ability of
TRIM9S to positively regulate IFN production\textsuperscript{6}. Protein and mRNA analysis demonstrated both TRIM9L and TRIM9S are expressed in peripheral blood mononuclear cells (PBMCs), comparable to liver levels, although at lower levels than the lung or spleen\textsuperscript{6}. The authors then cloned both isoforms from human PBMCs and evaluated the effect of their overexpression in HEK293T cells during vesicular stomatitis virus (VSV) infection\textsuperscript{6}. Overexpression of TRIM9S was associated with lower levels of VSV infection and increased $IFN\beta$, although in the absence of infection, $IFN\beta$ was undetectable\textsuperscript{6}. Through immunoprecipitation assays combined with knockdown or overexpression of TRIM9S, researchers demonstrated that TRIM9S binds TBK1 in a RING-domain dependent manner and also binds GSK3\textbeta, independent of the RING domain\textsuperscript{6}. Ultimately, this study revealed that ubiquitylated TRIM9S, but not TRIM9L, mediates the interactions between TBK1 and GSK3\textbeta to enhance TBK1 phosphorylation and subsequent IRF3 activation\textsuperscript{6}. Furthermore, neither TBK1 nor GSK3\textbeta were target substrates of TRIM9, but rather oligomerized TRIM9 undergoes K63-linked self-ubiquitylation, and recruits GSK3\textbeta to initiate activation of TBK1 (Figure 1)\textsuperscript{6}. Self-ubiquitylation of TRIM9 is supported by earlier findings by Versteeg et al\textsuperscript{121}. Lastly, the ability of both TRIM9 isoforms to repress NF\kappaB activation was investigated and findings demonstrated that while only TRIM9S activates ISREs in a ligase dependent manner, both isoforms can repress NF\kappaB activity in a manner dependent on phosphorylation of TRIM9 at S76 and S80\textsuperscript{6}. While these residues are located in the RING domain, ubiquitin ligase activity was not a requirement for NF\kappaB repression\textsuperscript{6}.

The first in vivo demonstration of negative regulation of the NF\kappaB pathway by TRIM9 was in a mouse model of ischemic stroke\textsuperscript{5}. Using a medial cerebral artery occlusion (MCAO) model, RNAseq data revealed TRIM9 was upregulated in mice after MCAO and protein levels reflected these findings\textsuperscript{5}. Three isoforms were observed in this study, but data was a reflection of
TRIM9L. Using TRIM9−/− mice, investigators show TRIM9 deficiency leads to more severe brain injury, increased production of pro-inflammatory mediators such as IL-6, TNFα, IL-1β, and CCL2, and higher infiltrating immune cells such as CD45highGR1+CD11b+ granulocytes, inflammatory monocytes, and CD45highCD11b−CD3e+ T cells. In vivo lentiviral transduction of TRIM9 resolved the severity of injury to the level of TRIM9+/+ mice. Due to an age-associated risk for stroke, experimenters looked at differences in ischemia response between young and old mice. Interestingly, after MCAO, TRIM9 expression in the brain is lower in old mice compared to younger individuals.

TRIM9 demonstrates variable endogenous expression across cell types which is mirrored by differential expression in response to varying immune stimuli. Indeed, the data presented in the studies described above, as well as data from our lab utilizing the zebrafish model and human cell lines, suggest that constitutive TRIM9 expression is dependent on cell type and inducibility of expression varies on the immune stimulation. After detecting trim9 expression in zebrafish innate immune cells, our lab investigated the hypothesis that TRIM9 could act as a mediator of immune cell migration, similar to its role in neurons. Through experiments utilizing a dominant negative transgenic zebrafish model, TRIM9 function was disrupted and the chemotactic ability of macrophages measured in vivo. Resting macrophages lacking Trim9 ubiquitin ligase activity exhibited abnormal morphology, migrated limited distances, and in response to TLR2 and TLR3 stimuli, chemotaxed at significantly slower velocities. This is the first report of TRIM9 influencing the migration of cells outside of the nervous system as well as a novel description of TRIM9 function in zebrafish.
TRIM9 Associated Proteins in the Immune System

Roles for TRIM9 have been reported in signaling pathways, cytoskeletal interactions, exocytosis, and cellular migration/chemotaxis. The extensive amount of literature on TRIM9 covered in the previous sections provides some insight into potential TRIM9 interacting proteins within innate immune cells.

In neurons, TRIM9 interacts with the cytoplasmic tail of DCC, a receptor that mediates axonal migration and branching in response to netrin. Interestingly, current literature reports little to no expression of DCC in immune cells, although a number of studies have investigated netrin signaling in the immune system. Ly et al. first demonstrated netrin-mediated inhibition of leukocyte migration in 2005\textsuperscript{124}. Years later, netrin was shown to be upregulated by hypoxia and positively regulated by the NFκB pathway\textsuperscript{42,125}. Since the Ly et al. publication, several studies have implicated netrin, netrin receptors, or both molecules as influential in regulating immune cell migration\textsuperscript{122,124–132}.

Within the vertebrate netrin receptor family, DCC is 50% homologous at the amino acid level to neogenin and the secondary structures of these molecules, especially the extracellular portions, are highly similar\textsuperscript{133}. Neogenin, which is more prevalent outside of the CNS than DCC, has been shown to be crucial in the development of other organ systems during embryogenesis, such as angiogenesis, myogenesis, and mammary cap gland formation\textsuperscript{133}. Other netrin receptor family members include UNC5b and A2BAR and all netrin receptors have been implicated in the dual guidance of netrin within the nervous system. Neogenin expression is increased in stimulated T-cells and T-cell chemotaxis is influenced by neogenin\textsuperscript{126}. Furthermore, neogenin expression is induced in neutrophils during IRI, and mice lacking neogenin ( NEO\textsuperscript{−/−}) exhibit lower numbers of immune-responsive neutrophils as well as decreased pro-inflammatory...
cytokine production\textsuperscript{132}. Similar studies using models of acute peritonitis and acute pulmonary injury in mice corroborate these findings\textsuperscript{130,131}.

Conversely, netrin signaling through UNC5b inhibits the recruitment of MDMs during immune stimulation\textsuperscript{124,128}. This was corroborated in a study that utilized primary peritoneal macrophages from mice and measured their response to multiple chemoattractants\textsuperscript{42}. Interestingly, this study demonstrated that netrin itself inhibited the typical cytoskeletal rearrangements seen during macrophage stimulation\textsuperscript{42}. Similarly, A2BAR expression has been shown in neutrophils and is hypothesized to be required for netrin-controlled inhibition of neutrophil migration\textsuperscript{125}.

While these experiments provide essential information about netrin signaling in immunological processes, a comprehensive understanding of these mechanisms is lacking. For example, several studies that show migratory inhibition is dependent on netrin signaling through UNC5b failed to look for concurrent neogenin expression\textsuperscript{42,124,128}. Likewise, studies that demonstrate that increased neogenin expression is associated with pro-inflammatory conditions \textit{in vivo} did not investigate netrin expression levels in these diseased states\textsuperscript{130–132}. Additionally, only one of these studies tested whether exposure to netrin enhances netrin receptor expression on immune cells\textsuperscript{126}.

A role for TRIM9 in netrin signaling in leukocyte migration has yet to be demonstrated and the only netrin receptor TRIM9 is known to interact with, DCC, is undetectable in immune cells. However, the failure to detect DCC in immune cells may be a reflection of low basal levels and the inability to identify induction conditions. In fact, published studies investigating tissue-wide DCC expression changes in response to immune stimuli are lacking, and as mentioned above, there is limited literature on the influence of netrin on netrin receptor expression. DCC,
along with neogenin and UNC5b, are dependence receptors and as such, cells that express these receptors rely on netrin binding and in its absence, these cells undergo caspase-mediated apoptosis\textsuperscript{134}. Whether failure to detect DCC in immune cells is due to a true absence of receptor expression or the apoptosis of DCC\textsuperscript{+} cells in netrin-deficient conditions has yet to be studied. Interestingly, although DCC expression outside of the CNS is debated, morpholino knockdown of DCC in zebrafish embryos leads to defects in lymphangiogenesis\textsuperscript{135}.

Of the TRIM family, TRIM9 is most similar to TRIM67, sharing 67\% sequence similarity. While TRIM67 has not been characterized in the immune system in depth, it has been shown to induce reporter activity of IFN\(\beta\) and ISRE promoters, similar to TRIM9, which suggests it is directly involved in activating these immune pathways\textsuperscript{121}. Recent work has shown that TRIM9 and TRIM67 heterodimerize in neurons\textsuperscript{112}. Further, TRIM67 competitively binds VASP to prevent TRIM9 binding and subsequent ubiquitylation of VASP\textsuperscript{136}. It would be interesting to investigate a role for TRIM9-TRIM67 interactions in the regulation of the immune pathways that have been previously reported for TRIM9.

In axons, VASP mediates F-actin remodeling in response to netrin in a phosphorylation-dependent process\textsuperscript{2,137}. Through ubiquitylation, TRIM9 disrupts VASP localization at the tips of filopodia, acting as a negative regulator of filopodia stability and growth\textsuperscript{2}. As phosphorylation of VASP decreases when filopodia density increases, it is hypothesized that the ubiquitylation and phosphorylation of VASP work in tandem to alter VASP localization and subsequently, its function in filopodia formation\textsuperscript{2}. Interestingly, VASP is also involved in the chemotaxis of leukocytes. Two initial studies in neutrophils identified VASP as a regulator of CXCR2-mediated chemotaxis and polarity, as well as \(\beta2\) integrin expression\textsuperscript{137,138}. More recently, VASP
has been shown to mediate the movement of activated T-cells during inflammation and negatively regulates the chemotaxis of monocytes and neutrophils after injury\textsuperscript{139,140}.

In the nervous system, TRIM9 acts as a negative regulator of SNARE complex formation through its interactions with SNAP25, preventing its interaction with Syntaxin1 and VAMP2\textsuperscript{1,106}. This interaction effectively suppresses synaptic vesicle exocytosis. Many of the molecular processes required for synaptic vesicle exocytosis are shared by neutrophils during granule release. In fact, SNAP25, Syntaxin1, and VAMP2 expression have been detected in neutrophils\textsuperscript{16,17,141,142}. Specifically, SNAP25 and VAMP2 associate with tertiary granules and secretory vesicles, while VAMP2 is also directly involved in the exocytosis of secondary granules\textsuperscript{16,141}. It is interesting to speculate that if TRIM9 negatively regulates exocytosis of neutrophil degranulation through interactions with SNAP25 as it does in neurons, then the role it would play in mediating inappropriate inflammation would be quite important. Intriguingly, p38 has also been shown to play a role in neutrophil degranulation and just recently, a role for TRIM9S in p38 signaling was demonstrated by Liu and colleagues\textsuperscript{118}.

**TRIM Family Proteins in the Immune Response**

Proteins of the TRIM family are involved in a wide variety of cellular processes, from DNA repair and cell cycle regulation to apoptosis, epigenetic silencing, and differentiation. The number of TRIM proteins expanded rapidly with the evolution of vertebrates, with 6 TRIM genes in *Drosophila*, approximately 30 in non-jawed vertebrates, and more than 70 known TRIM proteins in humans\textsuperscript{143}. This expansion across species suggests that the evolutionarily older TRIMs have conserved functions while many of the newer TRIMs have demonstrated roles in the immune system, from cytokine signaling to direct antiviral activity\textsuperscript{143}. Furthermore,
disruptions to TRIM-mediated pathways or mutations in TRIM proteins themselves have been implicated in a number of inflammatory states and autoimmunity.

A number of studies have performed TRIM family-wide screens to investigate the role these proteins play in many immune signaling pathways. Among these screens, a multitude of TRIM proteins that positively or negatively regulate the NFκB and IFN pathways were identified. Similarly, the differential expression of TRIM genes in response to immune stimuli has been studied many times using a variety of experimental methods and cell types. The following examples of these findings are meant to highlight some of the better understood roles of TRIM proteins in immunity and are not an exhaustive list.

Over 20 TRIM proteins have demonstrated anti-retroviral activity and as such, it should not be surprising that the expression of many TRIM genes are induced in response to IFN. However, not all antiviral TRIMs exert antiviral properties through regulation of the IFN pathway. For example, in many TRIMs, the SPRY domain mediates the interaction with the virus, while ubiquitin ligase activity is required for antiviral activity. TRIM22 does this by directly ubiquitylating an encephalomyocarditis virus protease that is crucial in viral processing, which results in proteasomal degradation. TRIM28 acts as a transcriptional silencer, through histone H3 trimethylation, on endogenous retroviruses in murine stem cells. One of the best characterized TRIMs in antiviral immunity, TRIM5α has direct antiviral activity against retroviruses. Through the formation of multimers via RBCC interactions, TRIM5 proteins form a hexagonal structure that effectively acts as a net. This structure ‘traps’ the virus by its capsid, prevents uncoating of the viral genome, and targets it for autophagosomal degradation. In another role within the immune system, TRIM5α mediates the activation of TAK1 which then activates the NFκB pathway.
Several other TRIMs regulate the NFκB pathway and have been well characterized. Similar to TRIM9, TRIM27 inhibits the degradation of a NFκB inhibitor, IκB, which represses NFκB activation\textsuperscript{79}. In a different approach, TRIM19 sequesters active NFκB in nuclear bodies, preventing it from binding gene promoters\textsuperscript{79}. TRIM21 plays an unique role in immunity through its involvement in opsonization. The SPRY domain of TRIM21 has the ability to bind the constant region of IgG and upon binding, targets IgG bound to the opsonized protein for proteasomal degradation through RING-dependent ubiquitylation\textsuperscript{143}. Conflicting reports have published roles for TRIM21 in both the NFκB and IFN pathways. For example, one study found that TRIM21 ubiquitylates IRF3, targeting it for degradation, and consequently negatively regulates the Type I IFN system while a separate study found that TRIM21 acts as a positive regulator by interfering with an interaction between IRF3 and the IRF3 inhibitor, PIN1\textsuperscript{79}. 

One member of the TRIM family that is of particular interest is TRIM33. Similar to the findings Tokarz et al. published on the importance of TRIM9 in macrophage mobility, Demy et al. revealed that TRIM33 was critical in the migration of innate immune cells towards an area of infection in zebrafish\textsuperscript{7,147}. The translational relevance of this work was supported by \textit{ex vivo} studies in murine MDMs\textsuperscript{147}. This study revealed that macrophage movement in an amoeboid-fashion, but not mesenchymal migration, was dependent on TRIM33\textsuperscript{147}. TRIM33 is also known for its role in regulating the TNFβ pathway where it ubiquitylates Smad4\textsuperscript{101}. TRIMs have also been implicated in immunological diseases and inflammatory conditions. For example, TRIM21 is an autoantigen in SLE and Sjogren’s syndrome and mutations in TRIM20 are associated with familial Mediterranean fever\textsuperscript{79,148}. 
Models for Studying TRIM9

The C-I subtype TRIM protein, MADD2/Asap, has been well studied in the invertebrate models, *C. elegans* and *D. melanogaster*, respectively. However, the classification of invertebrate TRIM9 homologs has not been straightforward. Identification of MADD2 as a TRIM homolog was initially based on similarities of domain architecture which placed it in the TRIM family subgroup C-I, the only member of that subgroup in *C. elegans*. After identifying MADD2, Alexander and colleagues then characterized it as a homolog of MID1/TRIM18 based on functional similarities. Another study published later that year acknowledged that sequence homology indicated MADD2 could be a homolog for either TRIM9, TRIM 18, or TRIM67, but expression patterns were similar to TRIM9 expression observed in the CNS of mammals. Two studies then published in 2011 concurrently reclassified MADD2/Asap as a TRIM9 homolog based on phylogenetic analysis and the protein’s role in netrin signaling. Interestingly, a more recent publication questioned this classification based on phenotypic differences seen between *TRIM9* mice and *MADD2* C. elegans, and hypothesized that MADD2 was a true homolog for TRIM67 instead. However, their results demonstrated TRIM67 deletion in mice also does not phenocopy MADD2 knockout in *C. elegans*, leaving the classification of this invertebrate TRIM homolog unclear.

Preliminary identification of the *TRIM9* gene was in the human genome and expression was investigated in the mouse. The first functional studies on TRIM9 were done in rats, where the TRIM9 homolog was initially called SPRING. Since then, mouse and human TRIM9 have been investigated through *ex vivo* studies of brain tissue, *in vitro* neural cell culture lines, and transgenic work using common cancer cell lines such as HeLa and HEK293 cells. A *TRIM9* mouse was generated and has been used to investigate TRIM9 function and identify binding
partners in multiple studies\textsuperscript{1,5,112}. Reports of TRIM9 as mentioned above (see \textit{TRIM9 in the Immune Response} section) have offered inconsistent accounts of expression in immune cells. Two studies in human MDMs and two studies in the monocyte-like cell line THP-1 identified \textit{TRIM9}, with varying differential response to immune stimuli\textsuperscript{41,119,121,145}. Another study identified \textit{TRIM9} and cloned it from PBMCs for functional assays\textsuperscript{6}. Conversely, \textit{TRIM9} was undetectable in resting and induced MDMs, but observed in certain subsets of T-cells in mice\textsuperscript{120}.

The role of TRIM9 in disease has been briefly studied and is limited to three investigations. TRIM9 shows differential expression in human brains of individuals with Parkinson’s disease and dementia with Lewy Bodies as well as in mice infected with rabies\textsuperscript{107,108}. A more recent study identified anti-TRIM9 autoantibodies in two human patients that do not seem to have pathogenic effects on neuronal viability or development, but may be useful biomarkers in the future for paraneoplastic cerebellar degeneration\textsuperscript{150}. To our knowledge, our lab published the first report on TRIM9 function utilizing a zebrafish model, which was also a novel report on TRIM’s role in immune cell migration\textsuperscript{7}. Other studies using non-mammalian vertebrate models have not been reported.
Figure 1. NFκB pathway activation and Type I Interferon induction: Dual roles for TRIM9 in the immune response. Through the recognition of PAMPs, PRRs start the signaling cascades that lead to the induction of Type I IFN production and NFκB activation. Once activated, TBK1 phosphorylates IRF3 and phosphorylated IRF3 translocates to the nucleus to act as a transcription factor for Type I IFN genes. Activation of the canonical and alternative NFκB pathways lead to the ubiquitylation of inhibitory or inactive proteins. Proteasomal processing of these proteins result in the activation of NFκB, which then enters the nucleus and initiates the transcription of pro-inflammatory genes. This figure was created using BioRender.com.
References


96. Reddy, B. & Etkin, L. A novel zinc finder coiled-coil domain in a family of nuclear


110. Alexander, M. *et al.* MADD-2, a homolog of the opitz syndrome protein MID1, regulates


122. Liu, Y. *et al.* The first molluscan TRIM9 is involved in the negative regulation of NF-κB activity in the Hong Kong oyster, Crassostrea hongkongensis. *Fish Shellfish Immunol.* **56**, 46


150. Do, L. D. *et al.* TRIM9 and TRIM67 Are New Targets in Paraneoplastic Cerebellar
CHAPTER 2: COMPARATIVE ANALYSIS OF TRIM9 EXPRESSION IN PHAGOCYTES

Introduction

Inflammation is the immune system’s response to infection or injury and is characterized by the recruitment of immune cells to the site of invading pathogens or tissue damage. The persistence of an immune response is often characterized by high numbers of macrophages and neutrophils that release reactive oxygen species (ROS), cytotoxic factors, and cytokines. Phagocytes can contribute to the development of disease states, increasing the extent of tissue damage or perpetuating the disease condition. Characterizing the molecular pathways that regulate phagocytic cell function and subsequently contribute to persistent, damaging inflammatory states is necessary for the development of targeted therapeutics to mediate these conditions.

Tripartite Motif Containing 9 (TRIM9) is an E3 ubiquitin ligase highly expressed in the brain where it plays critical roles in neuronal development\(^1\)\(^-\)\(^3\). Importantly, recently published studies support a role for TRIM9 in the immune response, but this work was performed almost exclusively in murine neural tissue or mammalian cell lines of non-immune lineage\(^4\)\(^-\)\(^6\). Additionally, \textit{in vitro} cell culture experiments heavily relied on the overexpression of TRIM9 through plasmid transfection, while knockdown or knockout of TRIM9 was performed in cells that may have little endogenous TRIM9 expression or may lack the proper co-expression of other immune genes that TRIM9 acts with or upon. The single study that studied TRIM9 in innate immune cells was published from our lab using a zebrafish model\(^7\). In that report, TRIM9 function was disrupted in macrophages using a dominant negative approach which resulted in decreased motility in response to immune stimuli and abnormal morphology \textit{in vivo}\(^7\). These results suggested that TRIM9 is critical to macrophage function. While the zebrafish model is
beneficial for in vivo experiments, low numbers of innate immune cells make ex vivo studies difficult and comparative relevance is essential. There is a need for a mammalian model in which higher numbers of innate immune cells can be isolated from a single animal.

Preliminary data from our lab as well as the literature has demonstrated TRIM9 is expressed in mammalian innate immune cells, albeit at low levels\textsuperscript{5,8–11}. The study of immune cells such as phagocytes can be challenging: neutrophils are short-lived cells, while in vitro differentiation of monocyte-derived macrophages (MDMs) results in high rates of cell death. Thus, there is a limitation on the number and viability of harvestable phagocytes from small research animals such as the mouse. Through a comparative study on TRIM9 expression in mammalian innate immune cells, I aimed to establish a new animal model that could be employed in future studies on TRIM9 function in immune cells.

Materials & Methods

Protein sequence comparison

The human TRIM9 protein sequence (NP_055978) was obtained from GenBank and used as a query in a protein BLAST (BLASTp) analysis to identify and determine sequence homology in putative equine, canine, and porcine protein homologs. Murine TRIM9 isoform 1 has already been reported: NP_444397.2. Protein sequences were aligned using T-Coffee and Boxshade\textsuperscript{12} (v3.21). SMART analysis was used to predict protein domains\textsuperscript{13}.

GST fusion protein expression

The pGEX-6P-1 parental plasmid and pGEX-6P-1-TRIM9 fusion plasmids were a kind gift from the Stephanie Gupton lab (UNC, Chapel Hill, NC). These vectors contained full isoforms TRIM9L (NP_055978) and TRIM9S (NP_443210) and the following TRIM9 domain or domain combinations: Bbox-Coiled Coil-Cos, Bbox-Coiled Coil, SPRY, and FN3. Cultures
were started in Terrific Broth (24 g/L yeast extract, 20 g/L Tryptone, 4% glycerol, 100 mL/L 1X Phosphate buffered saline[PBS]) containing 0.2% glucose and ampicillin. After overnight incubation at 225 RPM, 37 °C, 12.5 mL of this culture was used to inoculate 250 mL of Luria-Burtani (LB) broth (5 g/L yeast extract, 10 g/L tryptone, 10 g/L sodium chloride) containing ampicillin. This culture was incubated at 37 °C, 225 RPM until it reached an OD600 of 1.0 (~3 hours). The bacteria were induced with 10 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and then incubated overnight at 25 °C at 240 RPM. The next day, cultures were centrifuged at 5,000 RPM, at 4 °C for 10 minutes, the supernatant was collected, and aliquoted for storage at -80 °C for future use.

**J774.2 cell culture**

A macrophage-like mouse cell line, J774.2, derived from BALB/c mice, was purchased from Sigma-Aldrich (85011428). Cells were grown and maintained in complete Dulbecco’s Modified Eagle Media (DMEM with 4.5 g/L glucose and sodium pyruvate without L-glutamine (Corning; 15013) supplemented with 10% heat inactivated fetal bovine serum (FBS; Corning; 35-011-CV), 1X penicillin/streptomycin (Corning, 30-002-Cl), and 2 mM GlutaMAX (Gibco; 35050061) at 37 °C, 5% CO₂. As needed, cells were collected with a cell scraper, centrifuged at 1,500 RPM for three minutes, resuspended in the above media and passaged. Cells were plated at 350,000 cells/well in complete DMEM and incubated for 48 hours. PBS (GenClone; 25-507) or lipopolysaccharide (LPS *Escherichia coli* 055:B5; Sigma; L2880) was added to the wells at 0.1 or 1 μg/mL. At the indicated times, media was aspirated, PBS was added, and cells were scraped and collected. Samples were centrifuged at 1,400 RPM for 3 minutes and the supernatant was removed. Cell pellets from each well were resuspended in 100 μL of radioimmunoprecipitation assay (RIPA) lysis buffer (150 mm NaCl, 5 mM EDTA, pH 8, 50 mM Tris, pH 8, 1% Triton-X
100, 0.1% sodium dodecyl sulfate[SDS]) containing Halt protease inhibitor cocktail (ThermoFisher; 78430).

**Tissue collection**

Brain tissue was generously donated by the following groups at NCSU: CVM Diagnostic Laboratories – Dr. Debra Tokarz (horse, dog), Clinical Immunology – Laura Edwards (pig), Molecular Education, Technology and Research Innovation Center – Dr. David Muddiman (human), and the Ghashghaei lab – Dr. Caroline Johnson (mouse). Tissue (30-150mg) was homogenized using a pellet pistol in 600 μL of RIPA lysis buffer containing Halt protease inhibitor cocktail.

**Blood collection and phagocyte isolation**

Venous blood was collected from anonymous human volunteers, NCSU CVM research animals (Jones lab; horses), purchased from the NCSU Clinical Laboratories (dog), or donated by the Piedrahita lab (pig) using heparinized syringes. Human blood mixed 1:5 with 6% dextran (Alfa Aesar; J63789), horse blood with no additional additives, pig blood mixed 1:1 with 6% dextran, and dog blood mixed 1:1 with 3% dextran were incubated for one hour at room temperature to allow erythrocytes to settle. Leukocyte rich plasma was then harvested and layered onto Ficoll® Paque PLUS (GE Healthcare;17-1440-02) in 15 mL conical tubes in a 2:1 ratio using a bulb pipet. Tubes were then centrifuged at 550Xg for 20 minutes at room temperature with no brake upon deceleration.

**Human and horse monocyte isolation and differentiation into macrophages**

Peripheral blood mononuclear cells (PBMCs) were collected and mixed 1:5 with ice cold 1X PBS. They were centrifuged at 400Xg for 5 minutes and pellets were washed two additional times with ice cold 1X PBS. Total PBMC population was counted using the Cellometer Vision
CBA cell imaging system (Nexcelom) and then plated in 60 mm plates in complete RPMI 1640 medium (GenClone; 25-506) containing 10% FBS and 1X penicillin/streptomycin. After 24 hours of incubation at 37 °C, 5% CO₂, nonadherent cells were washed away with warm complete RPMI media and plated cells continued to incubate in complete media supplemented with recombinant human GM-CSF (25 ng/mL; Gibco; PHC2015). Human cells were incubated for 7 days total, with fresh media and GM-CSF being replaced every 2-3 days. Horse cells incubated for 48 additional hours after nonadherent cells were washed away in complete RPMI media with no additional factors added. They were then counted using a Cellometer imaging system and viability was measured. MDMs were lysed in 150μL RIPA buffer containing Halt protease inhibitor cocktail per 2 X 10⁶ cells. To establish that differentiation conditions were appropriate, a board certified pathologist viewed Romanowsky stained slides prepared by cytospin.

**Neutrophil isolation**

Neutrophil pellets were gently resuspended in 3 mL of eBioscience 1X RBC Lysis buffer (Invitrogen, 00-4333-57). Ten mL of 1X PBS was added immediately afterwards and cells were centrifuged at 400Xg for 4 minutes at room temperature. This step was repeated once more if necessary. Neutrophils were then counted using a Cellometer imaging system and viability was measured. Neutrophils were lysed in 100 μL RIPA buffer containing Halt protease inhibitor cocktail per 2.5 X 10⁷ cells. Cytospin and staining was done to ensure species-specific protocols yielded highly pure cell populations.

**Cell lysis for protein isolation**

Samples of J774.2 cells, homogenized brain, MDMs, and neutrophils in RIPA buffer containing protease inhibitors were incubated on ice, rocking at 60 RPM for 15 minutes. After incubation, samples were sonicated five times at ten second intervals using the cuphorn
Ultrasonic Processor (Misonix) on setting 5. Samples were then incubated on ice, rocking at 60 RPM for an additional 10 minutes and were centrifuged at 14,000Xg for 15 minutes at 4 °C. Supernatant was collected and aliquoted for storage at -80 °C. Protein lysates were quantified using the Pierce™ BCA Protein Assay Kit (23227).

**Western blot**

Lysates were prepared with 5X SDS Loading buffer (250mM Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 500mM DTT, 0.1% bromophenol blue) and boiled for 10 minutes. They were then loaded on Mini PROTEAN stain-free 10% TGX gels (BioRad; 4561034) in 1X Tris/Glycine/SDS Running buffer (BioRad; 1610732) and ran for 60 to 75 minutes at 100V. Ten μL of PageRuler Plus Prestained Ladder (ThermoFisher; 26619) were also loaded. Gels were transferred using the TransBlot Turbo Transfer Pack, Mini format, onto 0.2 μM PVDF (BioRad; 1704156) set to 2.5 A, 25 V. Blots were immediately washed with 1X TBST (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 0.1% Tween-20) and then stained with Ponceau S (Sigma; 7170) to ensure complete transfer. Blots were washed with deionized water until stain was removed and then incubated in 5% blocking buffer (non-fat dry milk, 1X TBST) overnight, rocking at 4 °C. Membranes were washed three times rocking at room temperature for five minutes and then incubated with primary antibodies. Antibodies were diluted in 5% blocking buffer in the following ratios: rabbit anti-TRIM9 polyclonal 1:1000 (Proteintech; 10786-1-AP), mouse anti-TRIM9 monoclonal 1:2000 (Origene; TA800044), rabbit anti-TRIM9 polyclonal 1:2000 (Gupton lab), anti-GST 1:1000, mouse anti-β-actin monoclonal 1:800 (Santa Cruz; 47778 ), or goat anti-GAPDH polyclonal conjugated to horseradish peroxidase (HRP) 1:5000 (Santa Cruz; 20357). After an overnight incubation, rocking at 4 °C, blots were washed three times in 1X TBST rocking at room temperature for five minutes and then incubated with secondary antibodies in
5% blocking buffer: 1:5000 anti-rabbit IgG-HRP (Promega; W401B) for Proteintech and Gupton anti-TRIM9 and 1:2500 anti-mouse IgG-HRP (Promega; W4012) for Origene anti-TRIM9, anti-GST, and anti-β-actin. Membranes were washed three times as above and then visualized using Clarity™ Western ECL Substrate (BioRad; 1705061). Chemiluminescence was imaged using the BioRad ChemiDoc™ MP Imaging System and ImageLab6.0.1 software. Blots were incubated at room temperature for 10 minutes with Restore™ Western Blot Stripping Buffer (ThermoScientific; 21059) or at 50 °C for 45 minutes in Tris HCl, pH 6.8, 0.5 M, 2% SDS, and 0.8% BME when reprobing for loading controls.

**RNA isolation, Rapid Amplification of cDNA Ends (RACE), and RT-PCR**

For neutrophil RNA isolation, 3 X 10⁷ cells were lysed in one mL of TRIZol™ reagent (Invitrogen; 15596026) and samples were stored at -80 °C or immediately processed. Chloroform (0.2 mL) was added and samples incubated at room temperature for three minutes and then centrifuged at 12,000Xg for 15 minutes at 4 °C. The aqueous upper phase was collected and 0.5 mL of isopropanol was added. After a 10 minute incubation, samples were centrifuged for 10 minutes at 12,000Xg, 4 °C. Pellets were resuspended in 1 mL of 75% ethanol, vortexed quickly and then centrifuged for 5 minutes at 7,500Xg, 4 °C. RNA pellets air dried for 10 minutes at room temperature, were resuspended in 30 μL of molecular grade water, and incubated at 55 °C for 10 minutes. RNA was stored at -80 °C. Total RNA (1 µg) was used to prepare cDNA using the Superscript™ IV VILO™ Master Mix with ezDNase™ Enzyme (Invitrogen; 11766050) or RACE ready cDNA using the GeneRacer™ Kit with SuperScript™ III (Invitrogen; L150201). cDNA was diluted 1:10 with molecular grade water and used as a template for nested or hemi-nested PCR. The type of cDNA used, primer sets, enzymes, and cycling parameters are listed in Table 1.
Results

TRIM9 protein sequence is highly conserved across human, mouse, horse, dog, and pig

BLASTp analysis revealed predicted sequences that encode human TRIM9 isoform 1 in the horse (XP_023480921), dog (XP_013971371), and pig (XP_013848850). All three protein sequences were predicted from automated computational analyses of genomic sequence. An alignment of these protein sequences to human TRIM9 isoform 1 and mouse Trim9 isoform a revealed high homology across all five species (Figure 1).

A commercial anti-TRIM9 antibody detects TRIM9 in brain protein lysate from multiple species

Based on the high homology of TRIM9 sequences across the species of interest, it was reasoned that it was likely that a polyclonal antibody (Proteintech) would detect TRIM9 in multiple species. Proteintech anti-TRIM9 was raised in rabbit against the 350 N-terminal amino acids of human TRIM9. This region of TRIM9 contains the highly conserved Tripartite motif found in both well-studied isoforms, TRIM9 Short (TRIM9S) and TRIM9 Long (TRIM9L; Figure 2a).

To confirm antibody sensitivity across species, brain lysates were analyzed by western blot. Proteins were detected in cerebral cortex samples from all species investigated at ~100 kDa and 73 kDa (Figure 2b). Longer exposures revealed a smaller band at 61 kDa in the human and horse samples (not shown on representative blot; Figure 6). Interestingly, a faint band in the pig sample was observed near the top of the blot that would represent a higher molecular weight protein than any reported isoform of TRIM9. These findings were supported by similar results obtained from brainstem protein lysate from mouse, horse, and dog (blots not pictured; Figure 6).

TRIM9 is undetectable in a mouse macrophage-like cell line
Limitations in obtaining high numbers of phagocytes from individual mice led to an initial approach of investigating TRIM9 expression in mammalian macrophages through the use of a macrophage-like cell line of mouse origin. When resting J774.2 cells were analyzed by western blot for TRIM9 expression using the Proteintech antibody, no detectable levels of TRIM9 were visible. Hypothesizing that TRIM9 expression may only be induced in response to immune stimuli, a timecourse of LPS stimulation using two different concentrations was performed and analyzed by western blot (Figure 3). Stimulation of J774.2 cells failed to result in visibly detectable levels of TRIM9 as compared to the mouse brain lysate controls. Detection of β-actin demonstrates protein loading and protein transfer to the membranes was adequate. These findings indicated that J774.2 cells may not be an ideal model for studying TRIM9 in phagocytes and it was then hypothesized that primary phagocytic cells from large animal species could be feasibly collected and were biologically more relevant than mammalian phagocyte-like cell lines. *Proteintech anti-TRIM9 detects proteins of many molecular weights in MDMs and neutrophils from multiple species*

Human and horse MDMs were analyzed by western blot using an anti-TRIM9 antibody (Proteintech). Proteins were detected in both samples at 63 and 14 kDa (Figure 4). Multiple bands in the 37-46 kDa range are also seen but band intensity varied between species. A prominent band at 93 kDa in human MDMs is also observed. TRIM9 expression in pig and dog MDMs was investigated, but limited cell numbers led to low total protein yield and western blot loading controls demonstrated undetectable levels of protein (data not shown).

Representative images of western blots of multi-species neutrophils using anti-TRIM9 antibody (Proteintech) show proteins in each species that migrated at much lower molecular weights than expected for TRIM9L and TRIM9S (Figure 5). Of note, is the presence of strong
bands at ~26 kD and 15 kDa in human, horse, and dog neutrophils. While less prominent bands are observed at those locations in neutrophil lysate from pigs, results from another animal confirmed the presence of faint bands at these regions (data not shown). Other species’ differences include the presence of a faint band at 100 kDa in horse, a prominent 40 kDa protein in pig, and a faint band at 58 kDa in the canine sample.

A compilation of all western blot data using the Proteintech anti-TRIM9 antibody is shown in Figure 6. Each band depicted is the representation of protein that was observed in MDMs and neutrophils of two or more different animals within a species (≥2 biological replicates). For brain TRIM9 expression, bands represent proteins that were observed in at least 2 technical replicates (≥2 separate blots). Exceptions to these stipulations include observations from samples in which cells were harvested from only one animal of that species (canine neutrophils). This composite analysis reveals other cell-type specific and species-specific patterns of protein expression not depicted or apparent in representative blots shown in Figures 4 and 5. For example, the anti-TRIM9 antibody from Proteintech detects a 40 kDa protein in both MDMs and neutrophils of several species that is absent in all brain tissue tested. Additionally, human MDMs express a ~100 kDa protein that is also observed in the cerebrum. The presence of a protein detected migrating at ~74 kDa is seen in the cerebrum of three species as well as the mouse brainstem. Interestingly, products that migrated at >100 kDa were observed in the brain of some species.

Two additional anti-TRIM9 antibodies detect bands in human and horse phagocytes

The comparative analysis of TRIM9 expression using the anti-TRIM9 Proteintech antibody provides new and intriguing data that suggests that TRIM9 may have uncharacterized isoforms that are unique to phagocytes. To investigate this hypothesis further, human and horse
phagocytes were used for the next set of experiments. If the bands observed in protein lysate from human, horse, pig, and dog phagocytes represent true isoforms or products derived from TRIM9, then these findings would likely be corroborated with the use of other anti-TRIM9 antibodies.

A rabbit polyclonal antibody raised against mouse TRIM9 Bbox domains was a kind gift from Dr. Stephanie Gupton at the University of North Carolina at Chapel Hill. A second antibody, a mouse monoclonal antibody raised against a recombinant protein consisting of the carboxyl-terminal 385 amino acids of human TRIM9 (Origene) was also chosen with the aim of increasing the protein specificity (Figure 7a). To test species reactivity and specificity of each anti-TRIM9 antibody, human and horse cerebral cortex lysates were analyzed through western blotting (Figure 7b). The Origene TRIM9 antibody detected several proteins in each species. Interestingly, with the exception of a band at ~73 kDa in horse brain, all proteins are below the predicted molecular weight of TRIM9S (61 kDa).

Through western blotting, the anti-TRIM9 Origene antibody revealed two prominent bands at 60 and 35 kDa in human MDM protein lysate (Figure 7c). Inadequate loading of horse MDMs led to the failure of detectable protein by the Origene anti-TRIM9 or anti-GAPDH antibodies. The Gupton anti-TRIM antibody reveals eight bands in both human and horse samples that range from 21 kDa to >250 kDa. The pattern of these bands is similar in each species, although several proteins in the human sample are ~5 kDa smaller than their horse counterpart.

A comparative analysis of TRIM9 expression in neutrophils reveal that the Origene antibody detected proteins that migrated at 28 and 16 kDa in human neutrophil samples (Figure 7d). The equine sample also shows a band at 15 kDa using this antibody. Multiple bands were
seen in both species’ neutrophils using the Gupton antibody. Of note is a distinct protein signal shared between both species located at approximately 51 kDa. The four proteins detected in horse neutrophils are also represented at the same molecular weights in MDMs.

A compilation of all human neutrophil western blot data is shown in Figure 8 and compares the results obtained using the Proteintech polyclonal, Gupton polyclonal, and the Origene monoclonal anti-TRIM9 antibodies. Unless indicated, each band represents a protein observed in two separate western blots that was within 3 kDa of the other. Consistently, across 5 humans, bands are detected using the Origene antibody at 15-17 kDa, corroborating earlier findings using the Proteintech antibody. Similarly, the Origene antibody detected a 24-28 kDa protein in three different humans, two of which also contained a protein of that size detected by the Proteintech antibody.

These results collectively add support to the likelihood that the proteins detected in mammalian phagocytes are products of TRIM9 transcripts. The next focus of this study was to investigate if these proteins represent true TRIM9 isoforms or products of degraded TRIM9 protein. Preliminary transcriptomic studies failed to detect TRIM9 transcript variants that differ from the canonical sequence (data not shown). By determining which TRIM9 domains are present in the proteins observed, a more targeted approach to identifying mRNA transcripts through PCR could be utilized.

*Monoclonal anti-TRIM9 antibody detects TRIM9 FN3 domain*

In order to discern the TRIM9 epitope location of the monoclonal anti-TRIM9 antibody by Origene, a series of TRIM9 expression constructs were used to generate GST-fusion proteins containing two full TRIM9 isoforms (TRIM9L and TRIM9S) and various domain combinations of TRIM9 (Figure 9a; *left*). Western blot analysis performed on the fusion proteins using an anti-
GST antibody revealed that all constructs were being expressed and migrated at the predicted rate based on molecular weight (Figure 9a). The anti-TRIM9 antibody (Origene) recognized protein in the GST-FN3 sample at 36 kDa, corresponding to the combined predicted molecular weight of GST (26 kDa) and the FN3 domain (9.15 kDa; Figure 9b). The Origene antibody also recognized proteins in the samples containing GST-TRIM9S and GST-TRIM9L. The polyclonal anti-TRIM9 antibody (Proteintech) and the anti-TRIM9-Bbox domain antibody (Gupton lab) were used to verify these findings. Both GST-TRIM9 isoforms are strongly detected by the Proteintech and Gupton antibodies (Figure 9c and d). The Proteintech antibody detected a distinct band in lane 5, which contains a fusion protein consisting of the first 350 amino-terminal residues of TRIM9. The Gupton antibody strongly detects two additional TRIM9-domain-fusion proteins in lanes 4 and 5. These findings confirm the regions of immunogenicity within TRIM9 for the Proteintech and Gupton antibodies. For both polyclonal antibodies, faint bands are visible in all lanes at the predicted molecular weight for the corresponding fusion proteins. These may represent contaminating anti-GST antibodies that were produced in conjunction with anti-TRIM9 antibodies since both immunogens were GST-fusion proteins. The presence of a faint band in lane 1, the GST control, for both antibodies and the absence of this band in the Origene blot supports this reasoning. Figure 9f reflects the findings of this experiment and demonstrates the updated immunoreactive portions of TRIM9 for all antibodies used in this study.

**Partial TRIM9 transcripts amplified from human neutrophils**

To corroborate protein data suggesting that novel FN3 domain-containing TRIM9 isoforms are expressed, mRNA transcript analysis through PCR in human neutrophils was performed. Neutrophils offer a few technical advantages over macrophages (abundance, harvested in mature form) and furthermore, *TRIM9* expression has been detected in T-cells and
both mRNA and protein expression has been observed in MDMs \(^8,9,14\). An investigation of TRIM9 in neutrophils has yet to be published and the characterization of *TRIM9* expression in human neutrophils would provide novel information supporting a role for TRIM9 function in innate immune cells.

The two well characterized TRIM9 isoforms, TRIM9L and TRIM9S are encoded by two GenBank reviewed transcript variants (Table 2; Figure 10). There is an additional *TRIM9* transcript that is computationally predicted to be protein-coding and is supported by the literature (See Discussion)\(^1,6\). Using the known transcript variant sequences and a combination of PCR strategies, I sought to identify novel *TRIM9* transcripts (Table 1). Through these experiments, five partial *TRIM9* transcripts were identified from two humans (Figure 11). For all transcripts, predicted start and stop codons were mapped based on the assumption that no frameshift or nonsense mutations exist upstream and downstream of the partial sequence.
### Table 1. PCR Primers and Parameters

<table>
<thead>
<tr>
<th>Transcript Identified (Fig. 11)</th>
<th>Type of PCR</th>
<th>Enzyme</th>
<th>Primer set</th>
<th>Primary Primer Set</th>
<th>Sequence (5’-3’)</th>
<th>Annealing temp(°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcript A</td>
<td>Nested</td>
<td>KAPA2G Robust</td>
<td>Primary</td>
<td>T9-midEx1-For</td>
<td>CATGTGCGAACAGTGTCG</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T9-Ex10-Rev</td>
<td>TAGGCTATGTATGCTCTGCTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Secondary</td>
<td>T9-Ex2-For</td>
<td>GCAACATGTTCCAGCACG</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T9-Ex9-Rev</td>
<td>CTGGTCAGGTGACGC</td>
<td></td>
</tr>
<tr>
<td>Transcript B</td>
<td>Hemi-nested</td>
<td>KAPA2G Robust</td>
<td>Primary</td>
<td>T9-midEx1-For</td>
<td>CATGTGCGAACAGTGTCG</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T9-Ex10-Rev</td>
<td>TAGGCTATGTATGCTCTGCTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Secondary</td>
<td>T9-midEx1-For</td>
<td>CATGTGCGAACAGTGTCG</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T9-Ex9-Rev</td>
<td>CTGGTCAGGTGACGC</td>
<td></td>
</tr>
<tr>
<td>Transcript B</td>
<td>RACE</td>
<td>KAPA HiFi</td>
<td>Primary</td>
<td>GSPE3-For</td>
<td>CACCTGACAGACACGAGC</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Generater 3’ Primer</td>
<td></td>
<td>TGGAGAAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GAGTTTCTGGGAGCTACGG</td>
<td>GCTGTCACGATACTGACGTAGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Secondary</td>
<td>GSPE2-8junc-For</td>
<td>GTGGACGTCTGGTACAG</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Generater 3’ Nested Primer</td>
<td></td>
<td>CACGTACGTTACGGCATGACA</td>
<td></td>
</tr>
<tr>
<td>Transcript C</td>
<td>Nested</td>
<td>KAPA2G Robust</td>
<td>Primary</td>
<td>T9-midEx1-For</td>
<td>CATGTGCGAACAGTGTCG</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T9-Ex10-Rev</td>
<td>TAGGCTATGTATGCTCTGCTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Secondary</td>
<td>T9-Ex2-For</td>
<td>GCAACATGTTCCAGCACG</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T9-Ex9-Rev</td>
<td>CTGGTCAGGTGACGC</td>
<td></td>
</tr>
<tr>
<td>Transcript D</td>
<td>Nested</td>
<td>KAPA HiFi</td>
<td>Primary</td>
<td>T9-midEx1-For</td>
<td>CATGTGCGAACAGTGTCG</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T9-Ex10-Rev</td>
<td>TAGGCTATGTATGCTCTGCTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Secondary</td>
<td>T9-Ex2-For</td>
<td>GCAACATGTTCCAGCACG</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T9-Ex9-Rev</td>
<td>CTGGTCAGGTGACGC</td>
<td></td>
</tr>
<tr>
<td>Transcript E</td>
<td>Nested</td>
<td>KAPA HiFi</td>
<td>Primary</td>
<td>T9-midEx1-For</td>
<td>CATGTGCGAACAGTGTCG</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T9-Ex10-Rev</td>
<td>TAGGCTATGTATGCTCTGCTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Secondary</td>
<td>T9-Ex2-For</td>
<td>GCAACATGTTCCAGCACG</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T9-Ex9-Rev</td>
<td>CTGGTCAGGTGACGC</td>
<td></td>
</tr>
<tr>
<td>Transcript E</td>
<td>RACE</td>
<td>KAPA HiFi</td>
<td>Primary</td>
<td>GSPE3-For</td>
<td>CAGCTGCTCAGCCCGCTGCAAC</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Generater 3’ Primer</td>
<td></td>
<td>TGGAGAAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GAGTTTCTGGGAGCTACGG</td>
<td>GCTGTCACGATACTGACGTAGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Secondary</td>
<td>GSPE5-8junc-For</td>
<td>GTGGACGTCTGGTACAG</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Generater 3’ Nested Primer</td>
<td></td>
<td>CACGTACGTTACGGCATGACA</td>
<td></td>
</tr>
<tr>
<td>GenBank Transcript Name</td>
<td>GenBank Transcript Accession Number</td>
<td>Exons</td>
<td>GenBank Isoform Name</td>
<td>GenBank/ uniprot Protein Accession Number</td>
<td>Isoform Designation</td>
<td>Length (amino acids)</td>
</tr>
<tr>
<td>------------------------</td>
<td>------------------------------------</td>
<td>-------</td>
<td>----------------------</td>
<td>-------------------------------------------</td>
<td>---------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>TRIM9 transcript variant 1</td>
<td>NM_015163</td>
<td>10</td>
<td>E3 ubiquitin-protein ligase TRIM9 isoform 1</td>
<td>NP_055978/Q9C026</td>
<td>TRIM9 Long/Canonical TRIM9</td>
<td>710</td>
</tr>
<tr>
<td>TRIM9 transcript variant 2</td>
<td>NM_052978</td>
<td>7</td>
<td>E3 ubiquitin-protein ligase TRIM9 isoform 2</td>
<td>NP_443210/Q9C026-5</td>
<td>TRIM9 Short</td>
<td>550</td>
</tr>
<tr>
<td>TRIM9 transcript variant X1</td>
<td>NM_011536389</td>
<td>12</td>
<td>E3 ubiquitin-protein ligase TRIM9 isoform X1</td>
<td>Q9C026-4</td>
<td>TRIM9aL</td>
<td>802</td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trin9 transcript variant 1</td>
<td>NM_053167</td>
<td>15</td>
<td>E3 ubiquitin-protein ligase TRIM9 isoform a</td>
<td>NP_444597</td>
<td>Isoform a</td>
<td>788</td>
</tr>
<tr>
<td>Trin9 transcript variant 2</td>
<td>NM_00110202</td>
<td>12</td>
<td>E3 ubiquitin-protein ligase TRIM9 isoform b</td>
<td>NP_001103672/ Q8C7M3-3</td>
<td>Isoform b</td>
<td>714</td>
</tr>
<tr>
<td>Trin9 transcript variant 3</td>
<td>NM_00110203</td>
<td>9</td>
<td>E3 ubiquitin-protein ligase TRIM9 isoform c</td>
<td>NP_001103673</td>
<td>Isoform c</td>
<td>547</td>
</tr>
</tbody>
</table>
Table 3. TRIM9 antibody isoform specificity in the literature\textsuperscript{1,2,4–6,15,16}

<table>
<thead>
<tr>
<th>Study</th>
<th>Antibody source</th>
<th>TRIM9 Immunogen*</th>
<th>Specificity</th>
<th>Molecular weight of proteins</th>
<th>Tissue Source/Species of protein lysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current study</td>
<td>Proteintech</td>
<td>1-350aa</td>
<td>Polyclonal</td>
<td>100, 73, 60</td>
<td>Brain (human, horse, pig, dog, mouse)</td>
</tr>
<tr>
<td></td>
<td>Origene</td>
<td>284-669aa</td>
<td>Monoclonal</td>
<td>50, 25, 18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gupton</td>
<td>mouse Bbox domain</td>
<td>Polyclonal</td>
<td>97, 70, 60, 47, 31, 60</td>
<td></td>
</tr>
<tr>
<td>Zeng et al.</td>
<td>Gupton</td>
<td>Mouse Bbox domain</td>
<td>Polyclonal</td>
<td>&gt;75, 75, &lt;75</td>
<td>Brain (mouse)</td>
</tr>
<tr>
<td>Winkle et al.,</td>
<td>Gupton</td>
<td>Mouse Bbox domain</td>
<td>Polyclonal</td>
<td>&gt;75, 75, &lt;75</td>
<td>Brain (mouse)</td>
</tr>
<tr>
<td>Menon et al.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Qin et al.</td>
<td>Proteintech</td>
<td>1-350aa</td>
<td>Polyclonal</td>
<td>&gt;75, &lt;75</td>
<td>recombinant expression (human); Cell lines(human); PBMCs(human)</td>
</tr>
<tr>
<td></td>
<td>Origene</td>
<td>284-669aa</td>
<td>Monoclonal</td>
<td>&lt;75</td>
<td></td>
</tr>
<tr>
<td>Shi et al.**</td>
<td>UR</td>
<td>1-261aa</td>
<td>Polyclonal</td>
<td>80-85 &amp; 72</td>
<td>neural cell line (human) neurons (rat)</td>
</tr>
<tr>
<td></td>
<td>Abnova</td>
<td>1-109aa</td>
<td>Monoclonal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tanji et al.</td>
<td>Proteintech</td>
<td>1-350aa</td>
<td>Polyclonal</td>
<td>105, 80, 55</td>
<td>Brain (human); Brain (mouse);</td>
</tr>
<tr>
<td></td>
<td>Rabbit serum</td>
<td>C-terminus</td>
<td>Polyclonal</td>
<td>80, 55</td>
<td>recombinant (human)</td>
</tr>
<tr>
<td>Li et al.</td>
<td>UR</td>
<td>Rat 138-151aa</td>
<td>Polyclonal</td>
<td>80, 60</td>
<td>Brain (rat)</td>
</tr>
</tbody>
</table>

*Unless specified, immunogen was of human origin

**Study did not specify which antibodies were used in each experiment

UR: unreported
Figure 1. TRIM9 is highly conserved at the protein level across five mammalian species.
Protein sequences of Human TRIM9 isoform 1 (TRIM9 Long; NP_055978), mouse TRIM9 isoform a (NP_444397), and BLASTp predicted TRIM9 sequences in dog (XP_013971371), horse (XP_023480921), and pig (XP_013848850.1) were aligned using T-coffee. Identical residues are shaded black and structurally similar residues are shaded gray. Sequences that correspond to the Bbox domains are marked above them with blue and the FN3 domain is boxed in red.
Figure 2. **TRIM9 is detected in the cerebral cortex of five mammalian species using a commercial antibody.** (A) The protein domains of two TRIM9 isoforms, TRIM9 Short and TRIM9 Long, are depicted. An amino-terminal RING domain, two BBox domains, and a Coiled Coil domain make up the characteristic TRIM motif and are present in both isoforms. The carboxyl-terminus contains a COS domain and FN3 domain, while TRIM9 Long also contains a PRY/SPRY domain. The commercial polyclonal anti-TRIM9 antibody from Proteintech used in the following experiments was raised against the amino-terminal 350 amino acids of human TRIM9 in rabbit. The indicated domains correspond to the immunogenic portion. (B) TRIM9 protein expression in human, horse, pig, dog and mouse cerebral cortex was evaluated by western blot and the antibody indicated in (A). Expression of GAPDH or Beta actin served as loading controls.
Figure 3. TRIM9 was not detected in LPS-stimulated J774.2 cells, a mouse macrophage-like cell line. TRIM9 expression in LPS-stimulated J774.2 cells was analyzed by western blotting. Cells were treated with a vehicle control or stimulated with 0.1 μg or 1.0 μg of LPS and harvested at four different time points. Whole mouse brain was included as a positive control. Twenty micrograms of total protein lysate were loaded and β-actin was detected as a loading control.
Figure 4. TRIM9 is expressed in human and horse monocyte-derived macrophages. This is a representative image of western blots analyzing TRIM9 expression in human (n=2) and horse (n=6) monocyte-derived macrophages. Ten micrograms of protein lysate were loaded onto SDS-PAGE gels and blots were incubated with polyclonal anti-TRIM9 antibody (Proteintech). Detection of GAPDH served as a loading control.
Figure 5. An anti-TRIM9 antibody detects proteins in human, horse, pig, and dog neutrophils. This is a representative image of western blot analysis performed using a polyclonal antibody to detect TRIM9 (Proteintech) in neutrophils from multiple species. Neutrophil lysates from human (20 μg; n=2), horse (20 μg; n=7), pig (90 μg; n=2), and dog (undetermined conc; n=1) were analyzed. GAPDH and β-actin were detected as a loading control.
Figure 6. A comparative representation of all western blot results obtained using the anti-TRIM9 antibody from Proteintech. Each band depicted is the representation of at least two biological samples (observed in the phagocytes of ≥2 humans or animals) or two technical replicates (observed in brain lysate of ≥2 separate blots) within 6 kDa of each other. Exceptions: dog neutrophils with one biological sample analyzed once; pig cerebrum analyzed once.
Figure 7. Two additional TRIM9 antibodies detect proteins in human and horse phagocytes. Western blot analysis of TRIM9 expression was performed using two different antibodies. (A) The polyclonal rabbit antibody was raised against the Bbox domains of human TRIM9 and was a generous gift from Dr. Stephanie Gupton (UNC-CH). The monoclonal Origene antibody was raised in mouse against human TRIM9 amino acids 284 through 669. Protein lysate from human and horse cerebral cortex (B), MDMs (C), and neutrophils (D) was analyzed and representative images are shown. GAPDH expression was evaluated as a loading control.
Figure 8. A comparative representation of protein detection in human neutrophils using three different anti-TRIM9 antibodies. Each band depicted is the representation of at least two technical replicates that produced bands within 3 kDa of each other, with the exception of individuals indicated (*).
Figure 9. The Origene Monoclonal Anti-TRIM9 antibody binds the FN3 domain of TRIM9. (A) GST fusion proteins containing TRIM9 Long, TRIM9 Short, or various domain combinations were expressed for later use in western blot analysis. The construct designation refers to the indicated lanes in B-D. The final predicted molecular weight of these constructs is the sum of the molecular weight of GST (26 kDa) and the predicted molecular weight of the TRIM9 variant (left). Construct expression was verified using an anti-GST antibody and western blot analysis (right). (B-D) GST-TRIM9 variants were analyzed through western blotting using three different TRIM9 antibodies: Origene (B), Proteintech (C), and the Gupton antibody (D). Due to the high level of protein detection, stripping these blots was ineffective, therefore, these blots are the product of separate SDS-PAGE gels. (F) Based on (B), the epitope of the monoclonal Origene anti-TRIM9 antibody was determined to be located in the FN3 domain of TRIM9.
Figure 10. *TRIM9* transcript variants encode three reported isoforms. (A) SMART analysis was used to predict protein domains of human TRIM9\textsuperscript{13}. The indicated protein domains are encoded by the corresponding exons depicted below them in B-D. (B) *TRIM9* transcript variant 1 (NM_015163) is considered the canonical sequence and encodes for TRIM9 Long (710aa). (C) *TRIM9* transcript variant 2 (NM_052978) encodes for TRIM9 Short (550aa). (D) *TRIM9* transcript variant XM_011536389 encodes for isoform 4 (Q9C026-4), an 810 residue protein. Exons are shown in dark blue solid rectangles. Introns are depicted by dashed lines and are not to scale. Retained introns are shown as light blue rectangles. The start codon is indicated by a vertical green line and the stop codon is shown in red.
Figure 11. Multiple partial TRIM9 transcripts isolated from human neutrophils indicate many unidentified splice variants of TRIM9 may exist. Partial TRIM9 transcripts A-E were identified through RACE PCR strategies and images depict predicted full length forms if no other alterations in the transcript exist. Exons are shown in dark blue solid rectangles. Introns are depicted by dashed lines and are not to scale. Retained introns are not shown. The location of the primer set used to identify each partial transcript are depicted by arrows above each transcript. The predicted start and stop codons are indicated by vertical green and red lines. Transcript B was isolated in two separate cases from two different individuals. Transcript E was identified twice from the same human. A BLASTx analysis showed transcript C is 98% similar through 100% of the query to predicted TRIM9 transcript variant 16 (XM_017020950).
Discussion

Absence of TRIM9 in mouse macrophages

The failure to detect protein in resting and stimulated macrophage-like cells from mice using an anti-TRIM9 antibody suggests that mice may not be an adequate model for studying TRIM9 in phagocytes, which is supported by the literature\textsuperscript{10}. Furthermore, as an immortalized cell line, J774.2 cells may not exhibit the same TRIM9 expression pattern as endogenous macrophages. The lack of signal for TRIM9 in these cells is likely not due to insufficient protein loading as ten micrograms of total protein was sufficient to detect TRIM9 in human and horse MDMs using the same Proteintech antibody. However, it should be noted that the exposure time for the mouse western blots was limited by the detection of high concentrations of TRIM9 in the brain which produced an overwhelming signal after a short exposure.

Discrepancies in reported TRIM9 isoform sizes

The analysis of TRIM9 expression in tissues outside of the central nervous system (CNS) is limited and largely, the presence of isoforms that differ from the canonical TRIM9L have been neglected. In this study, three TRIM9 isoforms are detected in the cerebral cortex of human and two isoforms were detected in the mouse. These results are validated by the manufacturer’s (Proteintech) report on species specificity to human, mouse, and rat. The same sized proteins are observed in the brain of horse, pig, and dog, demonstrating the Proteintech anti-TRIM9 antibody exhibits cross-reactivity with these species (Figure 2b). However, a discrepancy in the antibody manufacturer's predicted molecular weight (90 and 61 kDa) and the proteins observed in these experiments (100 and 73 kDa) raised concerns which spurred an extensive literature review to compare findings of this study with others (Table 3).
GenBank reports twelve isoforms of human TRIM9, two of which have been reviewed (Table 2) and 10 that are predicted. In depth functional studies have been done on TRIM9L and to a lesser extent, TRIM9S\textsuperscript{1,3–5,15–17}. A third isoform (Q9C026-4; GenBank Isoform X1 XP_011534691), containing 802 residues, is predicted to have a molecular weight higher than TRIM9L and is designated TRIM9xL in our study (Table 2).

The predicted molecular weight of these isoforms suggests that the two proteins detected in brain samples using our western blotting conditions are TRIM9xL and TRIM9L. Due to differences in western blotting materials and methods (anti-TRIM9 antibodies, gel gradients, SDS-PAGE running parameters) and the cell type being studied, there is no apparent consensus in the molecular weights of TRIM9 isoforms when analyzed by western blotting. The following studies, summarized in Table 3, show proteins with varying molecular weights detected by anti-TRIM9 antibodies.

Shortly after the first reported genomic analysis of human TRIM9 predicted three protein-coding transcript variants, two proteins were detected in rat brain using an antibody against a region of TRIM9 conserved in all three isoforms\textsuperscript{16,18}. After recombinant expression of a 710 amino acid isoform and detection of a 80 kDa protein, the authors conclude the smaller band observed at 61 kDa represent a degradation product\textsuperscript{16}.

The anti-TRIM9 antibody by Proteintech has been used in two additional studies\textsuperscript{5,15}. This antibody detects three distinct proteins in human cerebral cortex (Table 3)\textsuperscript{15,present study}. Tanji et al. validated the presence of the two lower proteins in mouse brain using a second antibody source and make no further mention of the 105 kDa protein\textsuperscript{15}. More recently, TRIM9S and TRIM9L-encoding transcripts were cloned from PBMCs\textsuperscript{5}. Upon recombinant expression and western blotting, the Proteintech anti-TRIM9 antibody detected both isoforms, migrating just
above and below the 75 kDa ladder mark\textsuperscript{5}. Two proteins migrating at the same rate as recombinant isoforms were also detected in primary PBMCs and a human monocytic leukemia cell line, THP-1\textsuperscript{5}. Detection of other sized proteins was not mentioned and the blots were cropped around the two proteins of interest. The same study also demonstrated that the monoclonal anti-TRIM9 from Origene exclusively detects TRIM9\textsuperscript{5}.

The Gupton polyclonal antibody raised against mouse TRIM9 Bbox domains clearly detects three proteins expressed in mouse brain that correspond to TRIM9 isoform a, b, and c (Table 2)\textsuperscript{1,2,6}. The middle protein, isoform b, migrates at 75 kDa, but the molecular weights of isoforms a and c are unable to be determined due to an absence of additional weight markers in these studies. Zeng et al. used this antibody to study isoform b in a mouse model of ischemic stroke\textsuperscript{6}. Curiously, when researchers used this antibody with human neural progenitor derived neurons, only one isoform is distinct, although the blot may have been cropped to depict this\textsuperscript{6}.

The reported variation of two or three isoforms expressed in the brain are likely a reflection of the area of brain and species investigated, which is supported by our data (Table 3; Figure 6). For example, three isoforms were identified in horse and human cerebrum, while only two were observed in the mouse. Conversely, mouse brainstem expressed three isoforms and horse exhibited two isoforms. The data in this study also validates the presence of a third TRIM9 isoform, TRIM9xL, in the brain. It was concluded that the Proteintech antibody detects three isoforms of TRIM9 in human brain that migrate at 100, 73, and 60 kDa, which correspond to TRIM9xL, TRIM9L and TRIM9S. Furthermore, the Proteintech anti-TRIM9 antibody exhibits cross-reactivity to horse, dog, and pig homologs of these isoforms.

*Interpretation of phagocyte western blots*
After establishing that the Proteintech anti-TRIM9 antibody is cross-reactive in our species of interest, TRIM9 expression in phagocytes was investigated. While the detection of TRIM9 by the Proteintech anti-TRIM9 antibody in MDMs and neutrophils from multiple species was hypothesized, distinct bands that represent proteins smaller than TRIM9S were surprising. In human MDMs, TRIM9xL and TRIM9S were detected and it is likely that horse MDMs also express TRIM9S (Figure 4). The ~74 kDa protein seen in the cerebrum of three species as well as the mouse brainstem was not observed in innate immune cells, suggesting TRIM9L could be brain specific. Collective MDM results also show multiple proteins ranging from 15-45 kDa in both species (Figure 6). Similarly, neutrophils from all species display proteins at ~40, 27, and 15 kDa. The presence of these bands in multiple species, and their absence from the brain, suggests specific recognition of the immunogen by the antibody rather than non-specific binding. Through the use of additional TRIM9 antibodies, I aimed to confirm these findings.

Our literature review on TRIM9 antibody isoform specificity revealed a polyclonal antibody (Gupton) that detects all three isoforms in mouse (Table 3)\textsuperscript{1,2,6}. Due to the high level of conservation in the immunogenic region of TRIM9, the Bbox domains (Figure 1 and 7a), it was predicted this antibody would cross react to human and horse TRIM9. Another antibody of interest from our literature review was a monoclonal antibody by Origene that a recent publication had deemed TRIM9S specific (Table 3). Through recombinantly expressing FLAG-tagged TRIM9L and TRIM9S, anti-FLAG immunoprecipitation (IP) and western blotting, researchers demonstrated that the Proteintech antibody detects both isoforms, while the Origene anti-TRIM9 exclusively detects TRIM9S\textsuperscript{5,15}. The immunogen of this antibody is located in the carboxyl-terminus of human TRIM9 which offered a unique region of detection in our study and as a monoclonal antibody, theoretically offers higher specificity (Figure 7a).
Confirming species cross-reactivity in brain tissue of human and horse proved more difficult than anticipated. The Origene antibody detected bands which are smaller than the predicted molecular weight of TRIM9S in both species (Figure 7b). Horse brain showed a faint band that may represent TRIM9L, which disputes current reports on the TRIM9S specificity of this antibody. However, data on the Origene antibody is lacking, as the use of this antibody in CNS tissue has not been reported. In fact, the study by Qin et al. is the sole publication listed by the manufacturer and the validation of antigen specificity from the manufacturer is limited to recombinant expression of TRIM9S in HEK cells\(^5\). On the other hand, the Gupton antibody detected proteins that likely represent all three TRIM9 isoforms in both species (Figure 7b).

TRIM9S was detected by the Origene antibody in human MDMs (Figure 7c). Equine samples were uninterpretable due to poor protein loading. In the MDMs of both species, the Gupton antibody detected several distinct proteins of varying sizes, none of which corresponded to known TRIM9 isoforms. Neutrophil data show a shared 15 kDa band detected by Origene in both species, but of particular interest is the strong band at 27 kDa detected by both antibodies in humans, despite poor protein loading (Figure 7d). Curiously, the 40 kDa protein seen in both cell types across species with the Proteintech antibody (Figure 6) was not replicated in these experiments.

A composite of all human neutrophil data collected using the three anti-TRIM9 antibodies is shown in Figure 9. These data represent bands observed in at least two separate western blots, the exceptions being all samples utilizing the Gupton antibody and the Origene investigation on human 10. The failure of the Origene antibody to consistently detect proteins above 26 kDa may be a reflection of its monoclonal nature and the potential for its target epitope site to be blocked by post-translational modifications such as ubiquitylation. Collectively, these
results demonstrate through the use of multiple antibodies with western blotting that TRIM9 is expressed in human and horse phagocytes.

**Biological relevance of small FN3 domain-containing proteins**

Experiments with TRIM9 GST-fusion proteins and the monoclonal Origene antibody allowed us to conclude that neutrophils, and to a lesser extent macrophages, express small FN3-containing proteins or protein products. To state that this is novel information is in general impossible because most studies only publish the ‘region of interest’ on the blot. A growing awareness of how misleading this can be has come with transcriptomic studies demonstrating that splice variants are more common than previously thought. In fact, a number of papers have been published lately criticizing this standard practice of ‘cropping’ and have even gone so far as to call it scientific misconduct\(^\text{19,20}\). Nonetheless, it must not be assumed that these are true TRIM9 isoforms, and alternative hypotheses must be considered. The remainder of this discussion will review three possibilities that are supported in part by data in this study:

I. Antibody cross-reactivity with another structurally similar protein

II. Degradation or purposeful cleavage of TRIM9

III. Novel isoforms of TRIM9

I. Antibody cross-reactivity with another structurally similar protein

The protein products detected below the molecular weight of TRIM9S (60 kDa) could be the result of non-specific binding of an unknown protein. However, the consistency across multiple species and throughout samples within a species, the specificity within particular cell types, and the reproducibility with more than one antibody suggests non-specific binding is unlikely. If we conclude these bands represent true protein products, the next possibility to consider is whether these proteins represent TRIM9 or another structurally similar protein that
could exhibit cross-reactivity to multiple antibodies. To discern this, I aimed to identify the domain of TRIM9 which contained the epitope for the only monoclonal anti-TRIM9 antibody (Origene) used in this study. Through expression of GST fusion proteins, I identified the FN3 domain as the region of TRIM9 recognized by the Origene antibody. It stands to reason that the proteins detected by this antibody in neutrophils, as well as the other samples, contain this domain. Cross reactivity to another structurally similar protein would require that protein to exhibit high homology to the FN3 domain of TRIM9. A BLASTp analysis using the TRIM9 FN3 domain as a query reported the most similar sequence in a non-TRIM9 protein was the FN3 domain of TRIM67. These proteins are 77% similar across 97% of the FN3 domain. Epitopes are typically 5-8 amino acids so it is possible that TRIM67 (NP_001004342) could be recognized by this antibody. There are two reviewed TRIM67 isoforms, which encode proteins with predicted molecular weights of 78 and 83 kDa, suggesting that if the Origene antibody is cross reacting with the FN3 domain of TRIM67, it is detecting a novel isoform or degradation product. The investigation into the computationally predicted isoforms of TRIM67 warrants further attention.

II. Degradation or purposeful cleavage of TRIM9

Consistent recognition of proteins of unexpected sizes, specifically smaller than TRIM9S, by multiple antibodies would also occur if they are degradation products of TRIM9. The use of protease inhibitors during the preparation of lysate samples as well as their absence in brain samples make it unlikely this degradation is occurring during sample processing. Furthermore, when neutrophils were boiled with SDS immediately after isolation and analyzed by western blot, the Origene antibody detected the expression of three proteins at 40, 26, and 15 kDa (data not shown). Another possibility is that TRIM9 is being targeted for degradation as a normal cellular process and in fact, this hypothesis could be supported by several findings reported in the
literature. TRIM5 auto-ubiquitylates itself, inducing proteasomal degradation, and results in high TRIM5 protein turnover\textsuperscript{21}. Fletcher et al. hypothesize this is a form of negative regulation that allows TRIM5 to be produced and poised for an acute viral introduction, while constant degradation prevents the inappropriate activation of innate immune pathways such as NFκB\textsuperscript{21}. This type of self-regulation through ubiquitin activity is suspected of many TRIM proteins\textsuperscript{22,23}. Three studies demonstrate that TRIM9 has this capability\textsuperscript{5,10,15}. In fact, the appearance of proteins well above the predicted molecular weight of TRIM9xL, such as those observed in mouse brain and horse neutrophils are likely ubiquitylated TRIM9 (Figure 6). Ubiquitylation can add up to 20 ubiquitin proteins onto a target and with a molecular weight of 8.6 kDa, this can result in an increase in molecular weight up to 200 kDa\textsuperscript{24}. Ubiquitylation of TRIM9 could target it to the proteasome, and in fact, when TRIM9 was recombinantly expressed in the presence of a proteasomal inhibitor, increased levels of TRIM9 were observed\textsuperscript{10,15}.

The observation of western blot bands below the predicted 60 kDa of TRIM9S is not exclusive to this study. In a brain western blot by Tanji et al., all three known TRIM9 isoforms as well as an additional faint band migrating below 50 kDa were observed with the Proteintech antibody\textsuperscript{15}. Additionally, recombinantly expressed TRIM9S was accompanied by three distinct bands below 55 kDa upon western blotting. Li et al. make note of smaller protein products in both rat brain preparations and recombinant samples and suggest these are likely degradation products since their presence varies from preparation to preparation\textsuperscript{16}. Lastly, a paper in Nature Communications provided all extended blots in the supplemental findings, which reveal many proteins above and below their 80-85 kDa protein of interest although make no mention of other TRIM9 isoforms\textsuperscript{4}. These studies highlight the frequency at which unexpected western blot signals are dismissed as artifacts or degradation products as a result of sample processing. The
presence of additional proteins, particularly those as small as observed in the neutrophils of the present study, would easily be overlooked if authors were interested in TRIM9L which migrates around 70 kDa. Targeted degradation of TRIM9 may be a process amplified in innate immune cells as compared to neurons. It is of note that the smaller proteins observed in phagocytes were consistently detected by antibodies that recognize separate regions of TRIM9. This suggests that if the small proteins are TRIM9 degradation products, they are produced through specific targeted cleavage that produces fragments of TRIM9 that retain the different regions immunogenicity for each antibody.

III. Novel isoforms of TRIM9

Five partial transcripts of TRIM9 were identified that, if encoded, represent novel splice variants (Figure 11). Of note, Transcript A is the only partial mRNA recovered that would encode a protein containing portions of the FN3 domain. This protein has a predicted molecular weight of 41-45 kDa. Transcript B was isolated from two different humans using two different PCR strategies and predicted to encode a 33 kDa protein. Interestingly, a BLASTx analysis revealed that transcript C is 98% similar through 100% of the query to predicted TRIM9 transcript variant 16 (XM_017020950). While the biological relevance of these transcripts is questionable at present, there is an increasing amount of published data demonstrating a severe underappreciation for alternatively spliced transcripts. In fact, a recent publication suggests that as few as 10% of all human genes only encode a single transcript²⁰. There is a growing body of work that suggests that different isoforms of a gene have adopted distinct functions in a tissue-specific manner and more often than not, behave like completely distinct proteins, with the majority of isoforms sharing less than 50% of their interactions¹⁷. In other words, not only is it likely that TRIM9 has unreported splice variants, but also that they encode biologically relevant
proteins that function independently of the reference protein, TRIM9L, in a tissue-specific manner. In fact, this study found that out of 10% of all human protein-coding genes, 64% of isoforms identified differed from the ‘reference’ isoform\(^\text{17}\). While the total abundance of reference transcripts were higher than the alternative transcripts in certain tissues, there were some instances where alternative transcripts were the predominant product\(^\text{17}\). A relevant example was the detection of an alternative isoform of TRIM26 in testis, placenta, and brain, while the reference isoform was undetectable in all five tissues investigated\(^\text{17}\). Authors suggest that these findings provide evidence that the concept of ‘reference’ isoforms is arbitrary\(^\text{17}\).

The functional differences in TRIM9 isoforms have recently gained appreciation and new roles for TRIM9S have been identified. Interestingly, one role is as a regulator of the immune response. While both TRIM9S and TRIM9L have the capability to negatively regulate the NFκB pathway, TRIM9S exclusively positively regulates the Type I IFN pathway\(^\text{4,5}\). Another study on signaling pathways demonstrated that TRIM9S, but not TRIM9L, promotes the activation of p38, effectively suppressing glioblastoma progression\(^\text{25}\). While these studies demonstrate a promising change in the way protein isoforms are regarded, there are still instances where researchers incorrectly assume functional equivalence across isoforms. For example, in a study of TRIM9 response during ischemic stroke, researchers measured expression through the detection of isoform b on western blots\(^\text{6}\). Later, in rescue experiments in TRIM9\(^{-/-}\) mice, adenovirus vectors were used for in vivo expression of TRIM9. However, the vector contained a transcript that encodes for isoform c, which was reflected in the western blot validation of protein expression\(^\text{6}\). Lastly, in situ hybridization experiments utilized a RNA probe that detects a murine transcript which encodes for isoform a\(^\text{6}\). The discrepancy between studying the endogenous expression of transcript variant 1, protein expression of isoform b, and using isoform
c for *in vivo* rescue experiments was not addressed. Furthermore, differences in isoform function may indeed explain the failure of TRIM9 knockout mice to phenocopy dominant negative models in *C. elegans*¹,²⁶.

The idea of different isoforms performing very different functions in the TRIM family is not novel. Several functional isoforms of TRIM5 are the products of differential splicing and similarly to TRIM9, the longest form contains a SPRY domain while smaller isoforms may not²⁷. Interestingly, a comparison of TRIM5 between humans and rhesus monkeys show that the SPRY domain confers anti-HIV-1 and SIV-1 properties²⁷. However, in isoforms that lack this domain, those properties are diminished. Furthermore, it has been demonstrated that interactions between TRIM5α, the longest isoform, and a second isoform TRIM5ι, decrease TRIM5’s activity against HIV-1²⁸.

I conclude that TRIM9S is expressed in both human and horse MDMs. TRIM9xL and TRIM9L may also be expressed in innate immune cells, but this expression was unable to be validated by a second antibody, and therefore, the presence of these isoforms cannot be concluded with confidence. For example, Gupton antibody detects a TRIM9L-sized protein in human neutrophils, but these findings were not technically replicated, nor seen with other antibodies. Smaller proteins exist in the phagocytes of both species that are detected with multiple anti-TRIM9 antibodies. Of note, multiple anti-TRIM9 antibodies detect 15 kDa proteins in the neutrophils of both horses and human, 27 kDa proteins in human neutrophils, and 48, 36, and 21 kDa proteins in human MDMs. Further work to characterize the origin of these proteins is needed and may offer insight into the role of TRIM9 isoforms in innate immune cells.
References


20. Yan, R. *et al.* Probably less than one-tenth of the genes produce only the wild type protein without at least one additional protein isoform in some human cancer cell lines. *Oncotarget* **8**, 82714–82727 (2017).


CHAPTER 3: CONCLUSIONS AND FUTURE DIRECTIONS

Macrophages and neutrophils are often contributors to damaging inflammatory states. A more thorough understanding of the molecules involved in their activation, recruitment, and migration to the site of inflammation can contribute to the development of more targeted approaches to therapies aimed at mediating harmful immune responses. In this investigation, I aimed to establish a mammalian model for the study of TRIM9, a protein that has recently been implicated in NFκB and Type I Interferon (IFN) signaling, as well as macrophage chemotaxis.\(^1-4\)

In the course of this study, TRIM9 homologs were identified in the horse, pig, and dog and protein expression was observed in the brain of all species investigated. Three TRIM9 isoforms, TRIM9S, TRIM9L, and TRIM9xL are expressed in the brain of humans and horses. Through a comparative analysis of TRIM9 expression in innate immune cells, TRIM9S expression was identified in human and horse MDMs. Furthermore, macrophages and neutrophils of both species express small FN3 domain-containing proteins that multiple anti-TRIM9 antibodies recognize. While a positive identification of these proteins has yet to be made, it is likely these are novel TRIM9 isoforms or degradation products of already characterized TRIM9 isoforms.

The role TRIM9 plays in the immune response of phagocytes is likely isoform-specific and largely influenced by TRIM9’s level of expression. The low level of expression of the well-characterized TRIM9 isoforms in phagocytes, combined with the appearance of what may be degradation products of TRIM9 could suggest that endogenous protein turnover of TRIM9 is quite high and leads us to wonder what biological significance, if any, TRIM9 has in phagocytes. On the other hand, the possibility that the small protein products recognized by anti-TRIM9
antibodies are indeed unreported TRIM9 isoforms that exhibit phagocyte specificity offers the exciting potential of discovering novel TRIM9 functions in the immune response.

The present findings could lead to several lines of investigation. Of particular interest is the identification of the small proteins recognized by anti-TRIM9 antibodies in phagocytes. Discerning whether these are true TRIM9 isoforms could be performed through a more thorough identification of novel splice variants. Using PCR strategies, five partial novel transcript variants of TRIM9 were identified. Confirming the full sequences of these splice variants, recombinant expression, and western blotting techniques could corroborate these findings. However, if the proteins observed at low molecular weights are degradation products of TRIM9, alternative strategies are needed.

Based on these findings, it is hypothesized that if TRIM9 is being degraded in the MDM and neutrophil samples analyzed, the degradation is likely a result of normal cellular processes, rather than sample handling. A common pathway of degradation is that of the ubiquitin proteasomal pathway. It is not unlikely that TRIM9 exhibits self-ubiquitylation in phagocytes and as such, is degraded. In fact, it has been shown that TRIM9 possesses self-ubiquitylation capabilities\(^2,5^-7\). However, demonstrating this process will be met with a series of challenges. For example, identification of E3 ubiquitin ligase substrates can be difficult due to the low abundance of substrate proteins\(^8\). In this case, TRIM9, already expressed at low levels in phagocytes, would act as both the substrate protein and the ubiquitin ligase. In addition, the challenge of actively identifying the limited and weak transient interactions between enzyme and substrate make subsequent identification of ubiquitylated substrate proteins difficult, especially if those proteins are now targeted for proteasomal degradation\(^8\). Promising new approaches to identify substrate-E3 ligase interactions may overcome some of the difficulties in identifying
substrates of the ubiquitylation pathways. Parallel adapter capture (PAC) proteomics consists of mass spectrometry of immunoprecipitated E3 ligase-complex samples before and after treatment with a proteasomal inhibitor\(^8\). This approach could be helpful in determining if TRIM9 is self-ubiquitylating and undergoing proteasomal degradation as a mode of regulation in phagocytes.

Our findings also suggest that TRIM9 isoforms have cell-type specificity and while Zeng et al. found no phenotypic differences in Iba1\(^+\) microglia between TRIM9\(^{-/-}\) mice and wildtype mice, it would be valuable to identify TRIM9 in brain-specific immune cells\(^3\). By determining if isoform expression of TRIM9 differs in cells such as microglia from their peripheral counterparts, we could discern whether isoform expression is influenced by cell lineage, or alternatively, the microenvironment of the cell. For example, if brain-specific factors regulate the expression of TRIM9, the presence of all three TRIM9 isoforms would be expected in microglia. However, if isoform expression is truly cell-type specific, microglia would likely exhibit TRIM9S expression in addition to the unique small proteins identified in the MDMs in the present study.

In summary, the results of the present study support a role for TRIM9S in regulating the immune response in macrophages. These findings are corroborated by studies that demonstrate TRIM9S-specific roles in the regulation of IFN Type I induction and p38 activation\(^1,2,9\). Furthermore, this study provides the groundwork for establishing a large animal model to study TRIM9 in phagocytes. Based on similar findings across all tissues observed, I propose the use of an equine model for future studies.
References


APPENDICES
APPENDIX A

The attached published paper was a collaborative effort by many individuals. Of note, I performed the RACE and cloning experiments to identify Zebrafish PIGRL transcripts as well as the RT-PCR analysis of *pigrl* and PIGRL transcript levels from zebrafish tissues. Bioinformatics and analysis were performed by Jeffrey Yoder with additional support in design and analysis by Ivan Rodriguez-Nunez, Jibing Yang, Juong Shim, Donna Runft, Marci O’Driscoll, Robert Haire, John Cannon, Poem Turner, Ronda Litman, Carol Kim, Melody Neely, and Gary Litman.
Differential expression and ligand binding indicate alternative functions for zebrafish polymeric immunoglobulin receptor (pIgR) and a family of pIgR-like (PIGRL) proteins

Amanda N. Kortum · Ivan Rodriguez-Nunez · Jibing Yang · Juyoung Shim · Donna Runft · Marc L. O’Driscoll · Robert N. Haire · John P. Cannon · Poem M. Turner · Ronda T. Litman · Carol H. Kim · Melody N. Neely · Gary W. Litman · Jeffrey A. Yoder

Received: 5 December 2013 / Accepted: 10 January 2014 / Published online: 28 January 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract The polymeric immunoglobulin (Ig) receptor (pIgR) is an integral transmembrane glycoprotein that plays an important role in the mammalian immune response by transporting soluble polymeric Igs across mucosal epithelial cells. Single pIgR genes, which are expressed in lymphoid organs including mucosal tissues, have been identified in several teleost species. A single pIgR gene has been identified on zebrafish chromosome 2 along with a large multi-gene family consisting of 29 pIgR-like (PIGRL) genes. Full-length transcripts from ten different PIGRL genes that encode secreted and putative inhibitory membrane-bound receptors have been characterized. Although PIGRL and pIgR transcripts are detected in immune tissues, only PIGRL transcripts can be detected in lymphoid and myeloid cells. In contrast to pIgR, which binds Igs, certain PIGRL proteins bind phospholipids. PIGRL transcript levels are increased after infection with Streptococcus iniae, suggesting a role for PIGRL genes during bacterial challenge. Transcript levels of PIGRL genes are decreased after infection with Snakehead rhabdovirus, suggesting that viral infection may suppress PIGRL function.

Keywords Teleost · Innate immunity · Lipid binding · Genome evolution

Electronic supplementary material The online version of this article (doi:10.1007/s00251-014-0759-4) contains supplementary material, which is available to authorized users.

A. N. Kortum · J. Rodriguez-Nunez · J. Yang · P. M. Turner · J. A. Yoder (✉)
Department of Molecular and Biomedical Sciences and Center for Comparative Medicine and Translational Research, North Carolina State University, 1060 William Moore Drive, Raleigh, NC 27695, USA
E-mail: Jeff_Yoder@ncsu.edu

J. Shim · C. H. Kim
Department of Molecular and Biomedical Sciences, University of Maine, Orono, ME 04469, USA

D. Runft · M. N. Neely
Department of Immunology and Microbiology, Wayne State School of Medicine, Detroit, MI 48201, USA

M. L. O’Driscoll · R. N. Haire · J. P. Cannon · R. T. Litman · G. W. Litman
Department of Pediatrics, University of South Florida Morsani College of Medicine, 140 7th Avenue South, St. Petersburg, FL 33701, USA

G. W. Litman
Department of Molecular Genetics, All Children’s Hospital Johns Hopkins Medicine, 501 6th Avenue South, St. Petersburg, FL 33701, USA

Present Address:
J. Yang
Patronary and Critical Care Medicine, University of Michigan, Ann Arbor, MI, USA

G. W. Litman
H. Lee Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, Tampa, FL 33612, USA
Introduction

The polymeric immunoglobulin (Ig) receptor (pIgR) is an integral transmembrane glycoprotein that plays an important role in the mammalian immune response by transporting soluble polymeric immunoglobulins (pIg; dimeric IgA and in some mammals, pentameric IgM) across mucosal epithelial cells, such as those lining the gastrointestinal tract. Polymeric Ig is expressed by plasma cells present in the lamina propria underlying the intestinal epithelium and is bound by pIgR on the basolateral surface of epithelial cells. pIgR transports pIgA through the epithelial cell by transcytosis. Once on the apical surface, pIgR undergoes a cleavage event in which the secretory component is released from cells either as a free form or bound to pIgA as the secretory IgA complex (SlgA) that liberates it from the plasma membrane (Asano and Komiyama 2011; Kaeztel 2005). The transport of pIg by pIgR to the intestinal lumen is essential for protecting the host from invading pathogens and maintaining homeostasis (Johansen et al. 1999).

The mammalian pIgR is encoded by a single copy gene (PIGR) that encodes five extracellular Ig domains (D1–D5), a cleavage site, a transmembrane domain, and a cytoplasmic tail (Asano and Komiyama 2011; Kaeztel 2005). An alternatively spliced PIGR transcript lacking the D2 and D3 domains has been reported in rabbit and cow (Detichler and Mostov 1986; Kulseth et al. 1995). pIgR from chicken and Xenopus possesses four Ig domains; the D2 domain found in mammals is missing in these species (Braunton et al. 2007; Wieland et al. 2004). Full-length transcripts encoding a pIgR homolog, which possesses two Ig domains, have been identified in multiple fish species; however, only partial transcripts of this gene have been reported in zebrafish (Danio rerio) (Feng et al. 2009; Hamuro et al. 2007; Montgomery et al. 2011; Rombout et al. 2008; Tadiso et al. 2011; Zhang et al. 2010). Teleost (bony fish) pIgR is expressed by lymphoid organs including mucosal tissues (intestine, skin, and gills) (Feng et al. 2009; Hamuro et al. 2007; Rombout et al. 2008; Tadiso et al. 2011). Teleost pIgR has been shown to bind both IgM and IgG1 (Feng et al. 2009; Hamuro et al. 2007; Zhang et al. 2010).

Single pIgR-like (PIGRI) transcripts that have been identified in Atlantic salmon (Salmo salar) and common carp (Cyprinus carpio) share sequence and structural similarities with teleost pIgR, modular domain immune-type receptors (MD1Rs) from clearstone skate (Raja eglanteria), and the mammalian CD300/TREM family of receptors (Cannon et al. 2006; Ribeiro et al. 2011; Tadiso et al. 2011). The carp PIGRI protein was shown to be abundantly expressed in macrophages and secreted upon immune stimulation (referred to as a soluble immune-type receptor; SITR, in Ribeiro et al. 2011). Data mining of the zebrafish genome database previously revealed the presence of multiple PIGRI sequences on chromosome 2 (Cannon et al. 2006; Ribeiro et al. 2011; Tadiso et al. 2011). We herein report the characterization of this multi-gene family that includes the single pIgR gene and 29 PIGRI genes; characterize full-length transcripts from the zebrafish pIgR gene and ten PIGRI genes; demonstrate that certain PIGRI proteins bind phospholipids; examine the expression of pIgR and PIGRI genes in adult tissues, during embryonic development and from leukocyte lineages; and quantify changes in pIgR and PIGRI transcript levels after bacterial and viral infection.

Methods

Animals

Zebrafish were purchased from EldkWill WildLife Resources (Ruakun, FL). All experiments involving live zebrafish were performed in accordance with relevant institutional and national guidelines and regulations and were approved by the Institutional Animal Care and Use Committees of North Carolina State University, University of Maine or Wayne State University.

Bioinformatics

Genomic sequences encoding zebrafish pIgR and the majority of candidate PIGRI Ig domains were identified on chromosome 2 (Zv9 scaffold 234, GenBank NW_001878710.3; Zv9 scaffold 235, GenBank NW_001878708.3; and Zv9 scaffold 3509 GenBank NW_003362635.1) using BLAST searches using zebrafish MIR1 as query. Additional Ig domains were identified by individually translating segments of each scaffold in silico in all six reading frames and submitting the output to SMART analyses (Letunic et al. 2012). Ig domains from two predicted PIGRI genes encode a frame shift and/or premature stop codon and have been classified as pseudogenes. Protein sequences were aligned by Clustal W (Larkin et al. 2007). Phylogenetic trees were constructed from pairwise Poisson correction distances with 2,000 bootstrap replications by MEGA5 software (Tamura et al. 2011). Protein sequence domains were identified with SMART software (Letunic et al. 2012). Variability plots were generated with the Protein Variability Server (Garcia-Borronat et al. 2008).

Sequences analyzed include pIgR from common carp (C. carpio, Cyca; GenBank ADB97624), fugu (Takifugu rubripes, Toru; GenBank BAF56575), orange-spotted grouper (Epinephelus coioides, Epo; GenBank ACV91878), Atlantic salmon (S. salar, Sasa; GenBank ACX44838), rainbow trout (Oncorhynchus mykiss, Onmy; GenBank ADB81776), haman (Homo sapiens, Homo; GenBank NP_002635), mouse (Mus musculus, Mus; GenBank NP_035212), chicken (Gallus gallus, Goga; GenBank NP_001038109), and Xenopus laevis (Xela; GenBank ABK62772).
Zebrafish pig and PIGRL transcripts and genes

Rapid amplification of cDNA ends (RACE) and reverse transcriptase–polymerase chain reaction (RT-PCR) strategies were employed to obtain full-length pigr and PIGRL cDNAs. RACE-ready cDNA was prepared from RNA pooled from zebrafish kidney and spleen using the GeneRacer Kit™, Superscript™ III Reverse Transcriptase (Invitrogen), and Titanium Taq DNA polymerase (Clontech). The resulting amplicons were ligated into pGEM-T Easy (Promega) and sequenced. Experimental details are included in Electronic Supplementary Methods and Supplementary Table S1. The genomic organization of pigr and PIGRL genes was deduced by comparing cDNA sequences to zebrafish Zv9 genomic reference sequences: chromosome 2 scaffolds 234 and 235 and unplaced scaffold 3509.

Lipid binding assays

Recombinant soluble proteins of zebrafish pigr and PIGRL D1 and D2 ectodomains fused to a human IgG Fc domain were generated by cloning various ectodomains (amplified from pooled hematopoietic tissue cDNA) into the pcDNA3hslgG1Fc-Avi fusion vector as described (Cannon et al. 2011, 2012; Haire et al. 2012). Chimeric proteins were expressed and secreted by HEK293T cells (Haire et al. 2012). Cleared cell culture supernatant harvests were concentrated 10- to 100-fold and the Fc fusion proteins were characterized by Western analyses and quantified using the Easy-Titer Human IgG Assay kit (Thermo Scientific). The mouse CLM7-hFc protein was previously reported to bind lipids (Cannon et al. 2012; Haire et al. 2012).

Solid-phase enzyme-linked immunosorbent assays (ELISAs) were conducted as described previously (Cannon et al. 2012; Haire et al. 2012). Bacterial sources and the method for phospholipid extraction have been described (Haire et al. 2012). Either 0.5 μg purified cardiolipin or 50 μl of MBT/ethanol bacterial extract was used to coat plates. Negative control wells were treated in parallel with solvent (100% methanol). Binding efficiency was determined after color development as absorbance at 450 nm. Values were corrected by subtracting the value from negative control wells. Corrected ELISA values less than 0.10 were scored as zero; 0.10–0.15 as +1; 0.15–0.20 as +2; 0.20–0.25 as +3; 0.25–0.30 as +4; and >0.30 was scored as +5.

Reverse transcriptase–polymerase chain reaction

Tissues were harvested from 20 adult zebrafish. Embryos were collected by natural mating, maintained at 28 °C (Westerfield 2007), and ten embryos from each of the following time points were collected: 0, 6, 12, 24, 36, 48, and 72 h post fertilization (hpf) and 6 days post fertilization (dpf). Myeloid and lymphoid cell lineages were purified from the kidneys of ten adult zebrafish as described (Traver et al. 2003; Traver 2004). In brief, kidneys from adult zebrafish were dissected and homogenized with a 40-μm nylon-mesh filter in ice-cold PBS+5 % FBS. Propidium iodide was added to a concentration of 1 μg/ml. Myeloid and lymphoid cells were isolated from this single-cell suspension by sorting based on propidium iodide exclusion, forward scatter and side scatter with a BD FACS Aria II SORP flow cytometer (Beckton Dickinson). Cell populations were sorted twice to optimize cell purity. Total RNA was purified from zebrafish tissues, embryos, and cells using TRIzol reagent (Life Technologies). Five micrograms RNA from each tissue, 2.0 μg RNA from each embryonic time point, or 0.5 μg RNA from each leukocyte population were reverse transcribed using Superscript™ III Reverse Transcriptase (Life Technologies) and oligo dT primers. Tissue and embryonic cDNA was diluted 1:5 and leukocyte cDNA was diluted 1:2.5 in H2O for PCR with Titanium Taq DNA polymerase (Clontech) using the gene family specific primers and cycling parameters specified in Electronic Supplementary Table S2. The resulting amplicons were cloned into pGEM-T Easy and sequenced to confirm their identity.

Streptococcus iniae infections

Adult zebrafish were anesthetized with 0.016 % tricaine and either injected intramuscularly with 10 μl 1×10⁷ CFU/ml S. iniae or mock injected with media (THY + P) as described (Neddy et al. 2002). We have shown previously that this dose is lethal to over 90% of the fish within 4 days (Lowe et al. 2007). Zebrafish were maintained at 28 °C and euthanized for dissection at 2, 4, 8, 12, and 24 h post inoculation (hpi). Tissues from ten zebrafish per treatment and time point were pooled for RNA extraction.

Snakehead rabdovirus infections

Adult zebrafish were anesthetized as above and either injected intraperitoneally with 10⁷ TCID₅₀ (Tissue Culture Infectious Dose) of snakehead rhabdovirus (SHRV) virl or mock injected with PBS as described (Phelan et al. 2005; Pressey et al. 2005). Mortalities exceeded 40 % under these conditions. Zebrafish were maintained at 28 °C and euthanized for dissection at 2, 4, 8, 12, and 24 hpi. Tissues from ten zebrafish per treatment and time point were pooled for RNA extraction.

Reverse transcriptase–quantitative polymerase chain reaction

TaqMan primer/probe sets designed to amplify and detect zebrafish pigr, pigrl1.4, pigrl2.3, pigrl3.10, and pigrl4.2 transcripts were purchased from Applied Biosystems. Reverse transcription was completed as described above. Quantitative PCR were executed on a MyIQ Real-time PCR detection system with
IQ5 Optical system software (Bio-Rad). Reactions were completed in triplicate and average relative transcript levels calculated by normalizing to transcript levels of the ubiquitin translation elongation factor 1 alpha 1, like 1 (softalll) gene by the ΔΔCt method (Livak and Schmittgen 2001).

Results and discussion

Zebrafish polymeric immunoglobulin receptor, plgR.

The zebrafish plgR gene maps to chromosome 2 and a partial, 5’ truncated cDNA sequence (GenBank EF539183) has been reported (Feng et al. 2009; Rombout et al. 2008; Tadiso et al. 2011). 5’ RACE and RT-PCR were employed to amplify two full-length allelic variants of zebrafish plgR that differ by three residues (GenBank KF932324 and KF932325). Alignment of plgRs from multiple fish species at the peptide level indicates a high degree of similarity in both Ig domains (Fig. 1a). Prior sequence comparisons of teleost plgR to mammalian plgR led to the conclusion that the first and second Ig domains of teleost plgR correspond to D1 and D5 of mammalian plgR (Feng et al. 2009; Hamuro et al. 2007; Rombout et al. 2008). Comparison of individual Ig domains from plgR of human, mouse, chicken, Xenopus, zebrafish, and multiple teleost species (Fig. 1b and c) confirms that the second Ig domain from teleost plgRs is most similar to D5 from tetrapod plgR. However, the analyses presented here with an expanded gene set, indicate that the first Ig domains of teleost plgR and tetrapod plgR are phylogenetically distinct. It also was reported that the first Ig domain of teleost plgR shares more similarity to D5 of mammalian plgR than does the second Ig domain of teleost plgR (Tadiso et al. 2011). Although the first and second Ig domains of the teleost plgR have been referred to as D1 and D5 (suggesting similarity to mammalian D1 and D5), the domains are designated as D1 and D2 in this report.

It has been shown that a conserved sequence in complementarity determining region 1 (CDR1) in the D1 domain of the tetrapod plgR is required for non-covalent Ig binding (Kaetzel 2003; Roe et al. 1999); however, a conserved equivalent of this sequence is lacking in teleost plgR (Feng et al. 2009). The sequence differences between tetrapod and teleost plgRs could reflect co-evolution of the receptor function with different classes of Igs. Work in other systems has demonstrated that mammalian plgR binds IgA and IgM whereas teleost plgR binds IgM and IgZ/IgT (Feng et al. 2009; Hamuro et al. 2007; Zhang et al. 2010).

Zebrafish plgR-like Ig domains and genes

The zebrafish plgR-like (PIGRIL) sequences on chromosome 2 were identified initially through BLAST searches of the zebrafish genome with Ig domains from modular domain immune-type receptors from skate, a salmon PIGRl, and a carp PIGRl/STIR (Cannon et al. 2006; Ribeiro et al. 2011; Tadiso et al. 2011). All identifiable plgR and PIGRIL sequences in the current version of the zebrafish genome (Zv9) can be identified in three scaffolds: 234, 235, and 3509. The zebrafish plgR gene, 13 PIGRIL genes (including one pseudogene), and the defender against cell death 1 (dadc1) gene are linked tightly on scaffold 234 (Fig. 2a). An additional 18 PIGRIL genes (including one pseudogene), one “orphan” Ig domain, and a leucine rich repeat containing 24 gene (lrc24 or lrc24-like) map to scaffolds 235 and 3509 (Fig. 2b and c). No overlap can be identified between scaffold 234 and either scaffold 235 or scaffold 3509, suggesting that additional PIGRIL may be present in the zebrafish genome. The high level of identity between parts of scaffold 235 (pigrl3.8, pigrl3.9, pigrl4.1, pigrl4.2, and lrc24) and scaffold 3509 (pigrl3.11, pigrl3.12, pigrl4.3, pigrl4.4, and lrc24l, respectively), coupled with the high degree of allelic polymorphism in immune-type genes in zebrafish make it likely that these scaffolds represent two different haplotypes for a single loci (Electronic Supplementary Fig. S1).

Phylogenetic comparisons indicate that all D1 and D2 domains predicted to be encoded by PIGRIL genes (Electronic Supplementary Fig. S2) group in two major distributions (indicated in Fig. 3). Whereas, the D2 domains of the PIGRILs are highly conserved between individual loci, the D1 domains distribute into four major groups named 1 through 4. As multiple genes are present in each group, genes are differentiated by an additional number (e.g., the gene symbol for PIGRIL group 2, gene 1 is = pigrl2.1). The relationship between D2 domains does not consistently mirror the relationships between D1 domains, e.g., the D2 domains of Pigrl1.4 and Pigrl2.1 group together, but the D1 domains from these genes are distinctly different (Fig. 3). Recombination and exon swapping between innate immune receptor genes has been described previously (Litman et al. 2001).

A number of observations can be made from a phylogenetic analysis comparing Ig domains from teleost plgRs, zebrafish PIGRILs, a salmon PIGRl, a carp PIGRl/STIR, a putative PIGRl from medaka (Oryzias latipes), and representatives of zebrafish novel immunoglobulin-like transcript (NIET; Ster et al. 2005), diverse immunoglobulin domain-containing protein (DICP; Haire et al. 2012), and novel immune-type receptor (NITR; Yoder 2009) proteins (Electronic Supplementary Fig. S3y (1) PIGRIL proteins are distinct from NITR and DICPs, (2) zebrafish NILTs are more related closely to plgR than they are to NITRs and DICPs, but are distinct from PIGRILs, (3) the previously reported carp STIR (Ribeiro et al. 2011) can be classified as a PIGRIL, (4) the D2 domain of the salmon PIGRIL is more related to the D2 of teleost plgRs than it is to the D2 of zebrafish PIGRILs, and (5) the current medaka genome reveals eight Ig domains that likely represent a single plgR as well as three PIGRIL genes (discussed below).
The PIGR1 gene cluster is absent in fugu, stickleback, and tilapia but present in medaka.

As a PIGR1 transcript has been identified from salmon (Tadiso et al. 2011), we mined the salmon genome in order to determine the gene organization at the PIGR locus. From the salmon shotgun sequence database, contigs could be identified that encode DAD1, PIGR, PIGR1, or LRR24L, but none of these contigs overlap precluding any conclusion about the PIGR/PIGR1 loci in this species. Although, a carp PIGR/SITR transcript has also been identified, a carp genome sequence database is not yet available.

The genome sequence databases of other teleosts were searched in an effort to identify the genomic organization of PIGR1/PIGR1 loci in other species. In zebrasfish, the pigr gene and a number of PIGR1 genes are adjacent to dadi1 on scaffold 234; whereas a different set of PIGR1 genes are adjacent to lre24 on scaffold 235 (Fig. 2a–e). In fugu pufferfish (T. rubripes), stickleback (Gasterosteus aculeatus), and tilapia (Oreochromis niloticus), the PIG1 gene is flanked by the DAD1L and LRR24L genes. No genomic evidence is seen for PIGR1 genes at this locus in these species (Fig. 2d–f).

A BLASTN search of the current medaka reference genome using zebrafish Dadi1, Lre24, and pigr as queries identified a region of unannotated 190 (GenBank NW_004088020.1) that
encodes eight Ig domains between the DADIL and LRRCC4L genes (Fig. 2g). Although transcripts that correspond to these Ig domains have not been identified, gene prediction software predicts that the two Ig domains closest to LRRCC4L are encoded by a single transcript (GenBank XM_004079122) and the remaining six Ig domains are encoded by a second transcript (GenBank XM_004079121). The former transcript encodes a membrane-bound receptor with high similarity to plgR from other fish (61% identical to zebrafish plgR, E-value = 1e-152) as well as D1 and D2 domains that are phylogenetically similar to plgR from other teleosts (Electronic Supplementary Fig. S3). The additional six Ig domains encoded by the latter transcript are more similar to plgR than to PIGRL; phylogenetic analyses indicate that these Ig domains are present in a D1-D2-D1-D2-D1-D2 configuration and are likely derived from tandem D1-D2 duplication events. Although transcripts encoding these medaka sequences are not available, the organization of the zebrafish PIGRL genes suggests that the eight homologous medaka Ig domains represent a cluster of four genes with a D1-D2 configuration, consisting of a single plgR gene and three PIGRL genes. As mentioned above, the salmon PIGRL D1 domain is most similar to zebrafish PIGRL D1 domains whereas the D2 domain from the same protein is most similar to teleost plgR D2 domains.

Although the DAD1-PIGR-LRRCC4 gene organization appears to be well conserved in multiple teleost lineages, it may
be that only certain species encode PIGRL genes. Teleosts include four major lineages: otophysi (cyprinids [zebrafish and carp] and siluriformes [catfish]), euteleostomorphs (salmonids [salmon], tetraodontiformes [fugu]), perciformes [tilapia] and ovalentariae [medaka]), osteoglossomorpha (arapaima) and elopomorphs (turtles, ladyfishes, and eel) (Betancur et al. 2013; Broughton et al. 2013). The identification of definitive PIGRL sequences from zebrafish and carp indicates that these genes are present within the otophysi lineage of teleostei. However, the presence of PIGRL sequences in some euteleostomorphs (salmon and medaka) but not others (fugu and tilapia) and the phylogenetic differences between the zebrafish, salmon, and medaka PIGRL sequences (Electronic Supplementary Fig. S3) confounds efforts to model the history of the PIGRL gene cluster. The PIGRL gene clusters in zebrafish and medaka may reflect recent and independent gene expansions or a more ancient gene family that has been lost in multiple teleost lineages. The complete sequencing of additional teleost genomes will help understand the origins of these genes.

The DAD1-PIGR-LRRC24 locus is not conserved in mammals.

In the human genome, PIGRL, LRRC24, and DAD1 map to three different chromosomes (chromosomes 1, 8, and 14, respectively), demonstrating a lack of conserved synteny between the zebrafish and human PIGR loci. Although LRRC24 encodes an Ig domain of the C2 type, no other genes encoding Ig domains can be identified in the vicinity of this human gene. In contrast, the human, mouse, and Xenopus tropicalis DAD1 gene is adjacent to the TCRα constant domain locus (TRAC), whereas chicken DAD1 is adjacent to the TCRβ locus (Electronic Supplementary Fig. S4). Although, the zebrafish TCRα constant domain (trac) and the pig/PIGR gene cluster both map to chromosome 2, they are predicted to be separated by approximately 15 Mb. The human PIGR gene is flanked by BIM3 (Bcl2 apoptotic inhibitory molecule 3, aka FcuR, and TOSO) and FCAMR (FcRµR and CD351); this three-gene cluster (Electronic Supplementary Fig. S4) is conserved in numerous mammalian species (Mumukumu et al. 2012; Sakamoto et al. 2001; Shibuya et al. 2000, Shimizu et al. 2001). Both of these mammalian receptors possess a
single Ig domain that binds IgM (FAIM3) or both IgA and IgM (FCAMR). Although the PIGR gene has been identified in mammals, birds, and amphibians, this cluster of Ig receptors (FAIM3, PIGR, and FCAMR) may be specific to mammals as BAIM3 and FCAMR have not been identified adjacent to PIGR in chicken, zebra finch, turkey, or Xenopus.

Conserved features of PIGRL Ig domains

An alignment of the zebrafish PIGRL D1 and D2 domains reveals residues and extended regions that are conserved and variable (Fig. 4). Both D1 and D2 domains encode residues that are conserved with V domains (G<sup>16</sup>, C<sup>25</sup>, W<sup>41</sup>, L<sup>60</sup>, G<sup>100</sup>, Y<sup>112</sup>, and C<sup>109</sup>) (Barclay et al. 1997; Barclay 2003; Williams and Barclay 1988). The D1 domains of zebrafish plgR and PIGRLs share an IPCXV motif at the C<sup>16</sup> position, and a FTV motif amino terminal to L<sup>112</sup> (Figs. 1 and 4). A comparison of the zebrafish PIGRL D1 and D2 domains reveals three main regions of hypervariability (HV1–HV3), which likely influence ligand recognition and binding (Fig. 4).

The D1 domains of the zebrafish plgR, PIGRL1, PIGRL3, and PIGRL4 proteins as well as the D2 domain of plgR and
Fig. 5. PIGRL transcripts, exons, and predicted proteins. a Transcript (cDNA) numbers are listed on the left, adjacent to the gene that best matches the transcript. Exons are represented by rectangles. The position of start codons and stop codons are indicated by green and red vertical lines, respectively. D1 domain exons are color-coded as in Figs. 2 and 3. Other exons are color-coded to indicate nearly identical sequences. b Schematic representations of zebrafish pigr and PIGRL sequences from salmon and carp (Ribeiro et al. 2011; Tadiso et al. 2011). c Schematic representation of PIGRL proteins deduced from transcripts. Positioning of D1, D2, transmembrane (TM), cytoplasmic tyrosines (T), immunoreceptor tyrosine-based inhibition motif (ITIM), and ITIM-like (iITIM) sequences are indicated. Transcripts that best match the gene shown are listed below the protein structure and the amino acid (aa) variation between the proteins encoded by these transcripts indicated. Peptide sequences corresponding to cytoplasmic domains encoded by three PIGRL genes are listed. Variable residues encoded by polymorphic transcripts are in parentheses. Full-length protein sequences are available in Electronic Supplementary Fig. S5. Sequences have been deposited in GenBank with accession numbers KP932324–KP932349.

Nearly all PIGRL D2 domains possess two additional cysteines (C308 and C387/C385). These cysteines also are present in the D1 and D5 domains of tetrapod pigr and the D1 and D2 domains of zebrafish pigr in which their spacing is highly conserved at seven residues (CX-C). This spacing is conserved in zebrafish PIGRL3 and PIGRL4 D1 domains and in all PIGRL D2 domains. In contrast, this spacing is nine residues (CXa CX) in the D1 domain of zebrafish PIGRL1 proteins. Structural models of the salmon PIGRL predict that these additional cysteines form intrachain disulfides (Tadiso et al. 2011).

PIGRL transcripts encode secreted and inhibitory forms. RACE strategies were used to clone 24 different full-length PIGRL2, PIGRL3, and PIGRL4 transcripts (GenBank KP932326–KP932349), which likely represent polymorphic variations of ten different genes (Fig. 5 and Electronic Supplementary Fig. S5). Only partial PIGRL1 transcripts were recovered (see below, GenBank KP932330–KP932357). Using these full-length transcripts and the reference genome, the exon organization of multiple PIGRL genes could be defined or predicted. Twenty-one of the PIGRL transcripts encode a single D1 domain and a single D2 domain. The PIGRL3.10 gene and its three transcripts have a D1-D2-D2 exon organization that maintains the reading frame, allowing for both D2 domains to be translated (Fig. 5 and Electronic Supplementary Fig. S5). One distinguishing difference between the organization of the zebrafish pigr and PIGRL genes is that the pigr D2 domain is encoded by two exons whereas the D2 domain of all PIGRL genes is encoded by a single exon (Fig. 5 and Tadiso et al. 2011).
Transcripts recovered from pigr12.4, pigr12.5, and pigr13.5 encode type I transmembrane proteins that possess cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) or ITIM-like (Itim) sequences (Barrow and Trowsdale 2006; Billadeau and Leibson 2002), consistent with inhibitory function upon ligand recognition (Fig. 5d). It is possible that activating forms may be encoded by PIGRL genes for which transcripts have not yet been identified. The remaining PIGRL transcripts encode secreted proteins similar to the carp PIGRL/STIR with no identifiable signaling motifs. The role of secreted PIGRL proteins remains to be resolved; however, the carp PIGRL/STIR is secreted from macrophages after infection with a protozoan parasite (Ribeiro et al. 2011).

BLASTp searches of the human reference protein database using full-length Pigr12.4, Pigr13.5, and Pigr14.2 protein sequences as queries identified pgr as the human protein with the most similarity to PIGRLs (Electronic Supplementary Table S3). Additional proteins that share similarity to these PIGRL sequences include members of the CD300, CD300-like, TREM-like, and natural cytotoxicity receptors (Nkp44); however, this similarity is restricted to the Ig domains. This relationship between PIGRL and human innate immune receptor families has been reported for the salmon and carp PIGRLs (Ribeiro et al. 2011; Tedino et al. 2011).
Flavobacteria classes (Haire et al. 2012). Only the PIGR1-D1-hFc fusion proteins were found to exhibit lipid binding (Fig. 6). The recombinant proteins Pigr1.1-D1-hFc and Pigr1.7-D1-hFc bound lipid extracts from all classes of bacteria. The plgR-D1-hFc protein did not bind lipids in this assay as would be predicted based on other teleost plgRs binding IgM and IgG (Feng et al. 2009; Hamuro et al. 2007; Zhang et al. 2010). Lipid binding could not be detected for: Pigr1.2-6-D1-hFc, Pigr3.2-D1-hFc, Pigr3.2-D2-hFc, and Pigr4.1-D2-hFc. The ligands for PIGR1.2, PIGR3.2, and PIGR4.4 proteins remain to be identified. The ability of PIGR1.1 D1 domains to bind lipids is shared with Ig domains from the zebrasfish diverse immunoglobulin domain-containing protein family of innate immune receptors that also share sequence and structural similarities with the CD300 and TREM families (Haire et al. 2012).

Expression analyses of zebrafish pigr and PIGRL genes

PIGR transcripts have been detected using RT-PCR in multiple lymphoid organs of fugu, carp, and grouper including the kidney, spleen, gut, skin, and gills (Feng et al. 2009; Rombout et al. 2008) and in the ovary or gonad of fugu and grouper (Feng et al. 2009; Hamuro et al. 2007). Similar expression profiles are observed for pigr in zebrafish (Fig. 7a). The observations that pigr is expressed in the ovary and during embryogenesis suggests that it may function in some aspect of development, possibly for transport of maternal antibodies (Wang et al. 2012).

Four sets of degenerate primers were used to evaluate the expression of zebrasfish PIGR1.1, PIGR1.2, PIGR1.3, and PIGR1.4 genes (Fig. 7a). Transcripts from all PIGR1 groups can be detected from liver, kidney, spleen, intestine, gill, and skin as well as ovary; however, PIGR1 transcripts are not abundant during embryonic development.

In order to evaluate pigr and PIGRL expression in zebrafish hematopoietic lineages, RT-PCR analyses were conducted using lymphoid and myeloid cells sorted from zebrafish kidneys (Trauer 2004; Yoder et al. 2010). Although pigr transcripts were not detected in either leukocyte lineage, PIGRL gene expression was detected in both myeloid and lymphoid cells (Fig. 7b). The absence of pigr transcripts from zebrafish leukocytes is consistent with a role in transporting antibodies across mucosal epithelium. Expression of pigr in epidermal cells of the skin and intestine of fugu has been reported (Hamuro et al. 2007). PIGR1.1 transcripts were detected primarily in the lymphoid lineage, whereas PIGR1.2 transcripts were detected primarily from the myeloid lineage. This is consistent with the report that a carp PIGR1 (aka SITR), which groups with the zebrafish PIGR1.2 sequences (Electronic Supplementary Fig. S3), is expressed primarily on
myeloid cells (Ribeiro et al. 2011). In contrast, PIGRL3 and PIGRL4 transcripts were expressed at comparable levels in myeloid and lymphoid cell lineages. The observation that the PIGRL genes are expressed in leukocytes, whereas pigr transcripts are not detected in these cell types, suggests that the PIGRLs are functionally distinct from pigr in zebrafish.

As PIGR and PIGRL transcript levels were shown to be increased after copepod infection in salmon (Tadiso et al. 2011) we employed RT-qPCR to investigate if transcript levels of zebrafish pigr, pigrl1.4, pigrl2.3, pigrl3.10, and pigrl4.2 were altered after bacterial infection (Fig. 8a–c). After infection with the fish pathogen S. iniae (Lowe et al. 2007; Neely et al. 2002) transcript levels of all five genes were altered. In the intestine of infected zebrafish, pigrl1.4 and pigrl3.10 had the largest increases in transcript level which occurred at 4 hpi (17-fold and 25-fold increases, respectively). In the spleen, pigrl4.2 (12-fold increase) and pigrl2.3 (8-fold increase) had the largest increases in transcript level which occurred at 12 and 24 hpi, respectively. No dramatic increase in pigr or PIGRL transcript levels was observed in the kidney over the 24 h of infection. The most dramatic change in pigr transcript levels occurred at 4 hpi in the intestine (3-fold increase) and spleen (5-fold increase). Thus, the highest increase in PIGRL expression during bacterial infection was observed in the intestine which is a major site for mucosal immunity.

An effort was made to determine if transcript levels of pigr, pigrl2.3, pigrl3.10, and pigrl4.2 changed during infection with the viral fish pathogen Snakehead rhabdovirus (Phei et al. 2005). In contrast to changes in transcript level after bacterial infection, the most dramatic changes after viral infection were decreased transcript levels (Fig. 5d–f). Transcript levels of pigrl3.10 in the intestine decreased by 16-fold from 2 hpi and transcript levels of pigrl2.3 in the kidney decreased by 19-fold from 4 hpi. The most dramatic change in pigr transcript levels (6-fold decrease) was observed 2 hpi in the spleen. The viral-induced down-regulation of PIGRL transcripts is similar to the response of CD300a/c in human dendritic cells (DCs): TR7 and TR9 activation on DCs leads to down-regulation of CD300a/c transcripts (Ju et al. 2008). Collectively, these observations suggest that SHRV may suppress the zebrafish immune response by down-regulating pigr and PIGRL gene expression.

Summary

Teleost pigR (1) is expressed in epithelial cells, (2) binds soluble immunoglobulins, and (3) is well conserved across multiple species. In contrast, zebrafish PIGRL proteins (1) are expressed in leukocytes, (2) bind lipids, and (3) are not well conserved between fish species. The zebrafish PIGRL gene cluster encodes both membrane-bound receptors with potential inhibitory function and secreted proteins. Some PIGRL proteins bind bacterially-derived phospholipid extracts. Following bacterial infection the transcript levels of multiple PIGRL genes increase, suggesting a role in immunity. In contrast, after a viral infection the transcript levels of multiple PIGRL genes decrease possibly as a result of viral induced immune suppression. These features of the PIGRL family are reminiscent of the CD300 and TREM families of mammalian innate immune receptors.

Acknowledgments We thank John Whitesides and Pati McDermott (Duke Human Vaccine Institute) for assistance with cell sorting; John Rawls (Duke University), Karen Guillerman, and Erik Miltge (University of Oregon), and Ed Noga (North Carolina State University) for bacterial and Barb Pryor for editorial assistance. The zebrafish PIGRL gene nomenclature has been approved by the Zebrafish Nomenclature Committee. The authors are supported by the National Institutes of Health (R01 AD057559 to GWL and JAY and R01 AI23357 to GWL).

References


Yoder JA (2009) Form, function, and phylogeneses of NTRs in fish. Dev Comp Immunol 33:135–144


APPENDIX B

The attached manuscript has recently been submitted to the Journal of Veterinary Internal Medicine. I contributed to the experimental design and data interpretation, performed the indirect immunofluorescence assays (IFA), trained Emma in IFA techniques, and aided in the composition and revising of the manuscript. Emma Stafford primarily designed and performed the experiments with support in design and analysis by Jeffrey Yoder, and Natasha Olby. Aude Castel, Lauren Green, Jeanie Lau, Peter Early, Karen Munana, and Christopher Mariani contributed in sample collection and manuscript revision.
ABSTRACT

Background: Presumed autoimmune diseases affecting the canine central nervous system (CNS) are common. In people, antibodies against neuronal cell surface antigens have been identified that are associated with a wide variety of neurological syndromes. The prevalence of autoantibodies that target neuronal cell surface proteins has not been reported in dogs with neurologic disorders.

Hypothesis/Objectives: Autoantibodies to neuronal cell surface antigens can be found in the cerebrospinal fluid (CSF) of dogs with inflammatory CNS disease. The aim of this study was to determine the prevalence of six neuronal cell surface autoantibodies in CSF of dogs diagnosed with inflammatory and non-inflammatory CNS disease.

Animals: Client owned dogs with CNS disease and a complete diagnostic evaluation including magnetic resonance imaging (MRI) and CSF analysis were included. One healthy research dog was included as a negative control.

Methods: CSF was tested for six antigenic targets (NMDAR1, GABA_A1/2, AMPAR1/2, DPPX, CASPR, LGI1) using a commercially available indirect immunofluorescence assay (IFA) test kit.

Results: Of the 32 dogs with neurological disease in this study, 19 were diagnosed with inflammatory disease and 13 with non-inflammatory disease. Anti-NMDAR1 antibodies were detected in three dogs (n=3/32; 9.38%). Two of these dogs were diagnosed with meningoencephalitis of unknown etiology (MUE) and the third had normal neurodiagnostics and was treated for suspected MUE. All 3 dogs responded to prednisone therapy.
21 Conclusion/Clinical Importance: Further evaluation of the prevalence of CSF antibodies to neuronal cell surface antigens is warranted. Defining antigenic targets associated with canine encephalitis might allow diagnostic categorization of MUE antemortem.
Canine NMDA receptor encephalitis

INTRODUCTION

Meningoencephalomyelitis is one of the most common causes of neurological disease in dogs, with small breed, young adult dogs particularly at risk. While some cases are caused by CNS infections, the overwhelming majority of cases do not have an identifiable underlying infection and are considered immune-mediated. There are several different recognized syndromes, including a primarily cerebellar syndrome (idiopathic cerebellitis or generalized tremor syndrome), eosinophilic meningoencephalitis, and encephalitides defined as necrotizing or granulomatous histopathologically. Due to the difficulty in defining these latter conditions antemortem, the term ‘meningoencephalomyelitis of unknown etiology’ (MUE) has been coined. This umbrella term includes meningoencephalomyelitis diagnosed by a combination of MRI and CSF findings and negative infectious disease testing. Extensive efforts to identify an underlying infectious cause for these idiopathic inflammatory conditions have failed and they are managed by immunosuppression.

In people, the description of antibodies to neuronal cell-surface or synaptic proteins has revolutionized the understanding of neurologic disorders in recent years. Syndromes previously defined as ‘idiopathic’ have been characterized as autoimmune by identification of antibodies to a variety of self proteins. The most famous example of this, due in part to a book and the film it inspired, titled ‘Brain on Fire’, is N-methyl-D-aspartate receptor encephalitis (NMDARE). This can present as a psychiatric disorder, resulting in mismanagement of patients, but is now diagnosable through the detection of antibodies in the CSF. Patients who receive early and aggressive immunotherapy have an excellent prognosis.
Canine NMDA receptor encephalitis

48 with over 80% of patients returning to their baseline functioning. The prevalence of these
49 autoimmune disease diagnoses in people has increased dramatically since commercial antibody
50 testing has become available with NMDARE becoming the most common non-infectious
51 encephalitis in people.17,18

52
53 In veterinary medicine, antibodies against glial fibrillary acidic protein (GFAP), an astrocytic
54 protein, have been identified in the CSF of dogs with granulomatous meningoencephalomyelitis
55 (GME) and necrotizing meningoencephalitis (NME)19,20. In addition, anti-NMDAR autoantibodies
56 were identified in the CSF of a captive polar bear after he died following a generalized seizure
57 and elevated levels of anti-voltage-gated potassium channel complex (VGKC), specifically
58 anti-leucine-rich glioma-inactivated protein 1 (LGI1) antibodies have been identified in serum of
59 cats with limbic epilepsy22, raising the question of whether antigenic targets identified in
60 human encephalitis could be associated with encephalitis in other mammals. We hypothesized
61 that increased levels of antibodies to neuronal surface proteins would be present in the CSF of
62 dogs with inflammatory, non-infectious CNS disease. The objective of this study was to
63 determine the prevalence of six autoantibodies associated with human autoimmune
64 encephalitis (AE) in CSF of dogs with inflammatory and non-inflammatory CNS disease.

65
66 METHODS

67 Bioinformatics- Protein sequence comparison. A BLASTp analysis using the following human
68 proteins as a query in the NCBI database was performed to identify canine homologs:
69 glutamate receptor ionotropic, NMDA1 receptor (NMDAR1/GRIN1), α-amino-3-hydroxy-5-
Canine NMDA receptor encephalitis

methyl-4-isoxazolepropionic acid receptors 1 and 2 (AMPAR1/R2), γ-aminobutyric acid type B
type receptors 1 and 2 (GABA_A1/2), LGI1, contactin-associated protein receptor (CASPR2), and
dipeptidyl aminopeptidase-like protein (DPPX) \(^2\); see Table 1 for accession numbers. Human
and canine NMDAR1 sequences were aligned using T-Coffee \(^2\).

74

Cerebrospinal Fluid Samples. CSF samples were selected from the Neurology Service CSF bank
at the ______________. These samples were obtained from dogs undergoing diagnostic
evaluation for CNS disorders. Samples were obtained from the cerebellomedullary or lumbar
cistern and submitted for routine analysis (measurement of protein concentration, red and
white blood cell counts, and cytology). Residual CSF was then stored at -80 °C within 24 hours
of sampling as per the immunofluorescence assay manufacturer’s directions. Samples were
selected from dogs with neurological signs localizing to the brain or spinal cord that also had a
brain or spinal cord MRI and CSF analysis. Clinical details of all dogs were extracted from their
medical records and the final diagnosis and treatment were recorded. All dogs diagnosed with
MUE also had negative infectious disease testing which included negative PCR in blood and CSF
for canine distemper virus, West Nile virus, Borrelia burgdorferi, Neospora hughesi and
Caninum, Toxoplasma gondii, Anaplasma phagocytophilum, Ehrlichia canis and Rickettsia spp.*.

Additional serology was performed in certain cases at the discretion of the clinician. CSF banked
from a healthy, neurologically normal beagle dog taking part in a separate research study
served as the negative control. All research samples were acquired in accordance with

* Canine Neurological Panel, Real-time PCR Research and Diagnostics Core Facility, University of
Ca, Davis, Ca.
Canine NMDA receptor encephalitis

Guidelines and approval of the Institutional Animal Care and Use Committee (protocol number: 17-053-0).

Immunofluorescence assay. Autoimmune Encephalitis Mosaic 6 testing kit (EUROIMMUN, Lübeck, Germany, FA 112d-1003-6), is an indirect immunofluorescence assay (IFA) used to screen for autoantibodies against six targets associated with human autoimmune encephalitic diseases. It tests for antibodies against NMDAR1, AMPAR1, AMPAR2, GABA\(_A\)R1, and GABA\(_A\)R2 receptors, along with other protein targets: CASPR2, LGI1, and DPPX. To minimize background signal in samples from dogs with severe inflammatory conditions, CSF samples with a protein concentration greater than 50 mg/dL were diluted to a final protein concentration of 50 mg/dL using 1X phosphate buffered saline (PBS) containing 0.2% Tween-20 (PBST). CSF samples were applied to the kit’s proprietary ‘biochip’ according to the manufacturer’s instructions. An anti-human NMDAR1 antibody, supplied by the manufacturer, was used as a positive control. A sample from a healthy beagle dog was included in each technical replicate as a negative control. After incubation, samples were washed with 1X PBST and secondary antibodies were applied per manufacturer’s instructions. Secondary antibodies provided by the manufacturer included a fluorescein-labeled goat anti-dog IgG (EUROIMMUN, Lübeck, Germany, AF 102-0115). A) applied to all canine samples while a fluorescein-labeled goat anti-human antibody (EUROIMMUN, Lübeck, Germany, FA 112d-1003-6) was used for the positive control. Biochips were incubated and washed with PBST. Fluorescent and differential interference contrast (DIC) images were acquired with a Leica DM5000B fluorescent microscope at 20X. Each reaction field was captured in four quadrants using the same imaging technique. Images were reviewed
Canine NMDA receptor encephalitis

for presence of immunofluorescent cells. Manufacturer’s instructions note that not all cells are
transfected with the antigen, thus presence of immunofluorescence is not expected of all cells
within a field. The characteristics of cellular immunofluorescence differ between the antigens
and a full description of expected staining patterns was provided by the manufacturer and used
to determine the presence of positive staining. Samples from dogs that had positive results
were retested to ensure the results were repeatable.

RESULTS

Conservation of protein sequences. The NMDA receptor is made of three protein subunits,
NMDAR1, NMDAR2, and NMDAR3. The epitope of NMDA receptor autoantibodies in people is
located on the NMDAR1 subunit specifically, at amino acids 368B and 369G. A canine
NMDAR1 homolog was identified as 99% similar to the longest human NMDAR1 isoform (Table
1). Alignment of the canine and human NMDAR1 protein sequences revealed that the critical
368N and 369G immunogenic epitope is conserved in dogs (Figure 1). For AMPAR1/2,
GABA_{\alpha}R1/2, LGI1, CASPR2, and DPPX, high similarities (94-99%) were observed between the
human and putative canine homologs (Table 1).

Animals

Thirty-two dogs with CNS disease and one healthy beagle dog were included in the study. Full
details of each dog’s presenting signs, diagnostic findings and treatment are provided in
Supplementary data Table 1. The study included 4 Maltese, 4 Golden Retrievers, 3 Poodles
(Toy/Standard/Miniature), 3 Mixed Breeds, 2 French Bulldogs, 2 Dachshunds, 2 Labrador
Canine NMDA receptor encephalitis

Retrievers, and one each of the following breeds or breed mixes: Beagle, Cavalier King Charles Spaniel, Peekapoo, Yorkiepoo, Goldendoodle, Labradoodle, Boston Terrier, Bernese Mountain Dog, Scottish Terrier, Japanese chin, American Staffordshire Terrier, Schnauzer. Of these dogs, 13 were female (all spayed) and 19 were male (all neutered) and they ranged from 8 months to 11 years in age with a mean age of 6.2 years (SD: 3.1). Nineteen dogs were diagnosed with non-infectious inflammatory disease of the CNS; 1 with idiopathic cerebellitis, 1 with steroid responsive meningitis arteritis (SRMA) and 17 with an antemortem diagnosis of MUE. Two of these 17 dogs underwent necropsy with histopathological diagnoses of GME (n=2). One dog, a 1-year-old FS French bulldog presented with multifocal CNS signs but neurodiagnostics were normal. This dog responded to 1 mg/kg prednisone daily as well as continued treatment of previously diagnosed tooth root abscesses with antibiotics. Eleven dogs were diagnosed with non-inflammatory CNS conditions. Three of these dogs were diagnosed with idiopathic epilepsy, 1 with unknown epilepsy, 4 with brain neoplasia (1 gliomatosis cerebri, 1 anaplastic oligodendrogloma, and 2 presumptive meningiomas based on imaging characteristics), 2 with thoracolumbar intervertebral disc extrusions and 1 with degenerative myelopathy. The final dog had a differential diagnosis list of MUE versus round cell neoplasia versus gliomatosis cerebri based on MRI appearance and CSF analysis, but was euthanized with no necropsy so a final diagnosis was not established.

Immunofluorescent Assay Results: IFA results demonstrated a high level of immunofluorescence (IF) for the positive NMDAR1 control as expected, while a lack of IF was observed for all antibodies using the healthy canine sample (Figure 2). Of the 6 antibodies, the only positive IF was seen to NMDAR1 in three dogs (Figure 3) while the five other antibodies
Canine NMDA receptor encephalitis

were negative in all dogs. The three positive NMDAR1 dogs included a 6-year old FS miniature poodle diagnosed with MUE, a 1-year old MC standard poodle diagnosed with MUE, and a 1-year old FS French bulldog diagnosed with a tooth root abscess (dogs 4, 10, and 17 respectively in Supplementary data Table 1). All three dogs presented with multifocal CNS signs (Table 2).

Dog 4 had both inflammatory CSF and inflammatory lesions on the brain MRI, with negative infectious disease testing and responded well to immunosuppression with prednisone 2mg/kg/day tapering course (Westward Pharmaceuticals, Eatontown, NJ) and cytosine arabinoside 300mg/m² SQ, 3 weeks for 8 doses (Zydus, Hopewell Township NJ; Fresenius Kabi, Bad Homburg, Germany). At the time of publishing, the dog was alive and neurologically normal 22 months post-diagnosis and remains on 0.33mg/kg of prednisone once a day. Reduction of prednisone below this dose resulted in neurologic deterioration. Dog 10 had a normal MRI but inflammatory CSF with negative infectious disease testing and responded well to immunosuppression with a tapered course of prednisone 2mg/kg/day (HIKMA Pharmaceuticals, Amman, Jordan). The dog is currently normal and not on any treatment. Dog 17 presented with a signalment and signs of multifocal CNS dysfunction typical of dogs with MUE but with a complex history. Nine days prior to presentation at our institution, the dog presented to the primary veterinarian with a 3-day history of coughing and snorting. Thoracic radiographs and a urinalysis were performed and were unremarkable. The dog was treated with marbofloxacin 37.5mg PO q24h and carprofen 25mg PO q24h. The next day the dog’s breathing appeared to deteriorate, and it started to circle, wander and bark at random objects as well as stumble unexpectedly. The dog was anesthetized by the family veterinarian who performed brachycephalic airway syndrome surgery. The veterinarian also extracted three abscessed
maxillary teeth and noted purulent discharge. Treatment with marbofloxacin and carprofen was
continued and metronidazole was added for the next two days for diarrhea that developed
after surgery. During this time the dog developed circling to the right and was referred to
_______. On presentation the dog had a serosanguinous discharge from the left nostril,
ptyalism, and frequent snorting with normal lung sounds and eupnea. On neurological
examination, the dog was dull and intermittently disoriented, with a gait characterized by wide
circles to the right and a right head tilt with postural reaction deficits in the right thoracic and
pelvic limbs. The dog’s cranial nerve examination was unremarkable with the exception of the
head tilt. The clinical signs were localized to the right forebrain and brainstem. No
abnormalities were identified in the brain on MRI, but the left maxilla was abscessed with
involvement of the left nasal cavity. There was no evidence of extension of this pathology into
the cranial cavity. Cerebrospinal fluid sampled from the cerebellomedullary cistern was within
normal limits. Infectious disease testing was negative, and a bile acid tolerance test was within
normal limits. Given the presenting signs, breed and age, the dog was treated with a 6-week
tapering course of prednisone (Westward Pharmaceuticals, Eatontown, NJ) starting at
1mg/kg/day while also treating the tooth root abscesses with clindamycin 150mg po q12h for
21 days (Ohm Laboratories, New Brunswick, NJ) and minocycline 100mg po q12h for 21 days
(Aurobino, Dayton, NJ). Clinical signs initially resolved but recurred as the prednisone dose was
tapered. The dose was increased back to 1mg/kg/day and then tapered again over a 3-month
period and signs resolved.
Canine NMDA receptor encephalitis

Discussion

In this preliminary study, we screened CSF from 32 dogs diagnosed with a variety of neurologic disorders using a commercially available IFA kit that detects antibodies to 6 different neuronal cell surface or synaptic targets associated with human AE. Cerebrospinal fluid samples from 3 dogs were positive by IF indicating antibodies against NMDAR were present, while all other antibody targets were negative in all dogs. Two of the 3 dogs that were positive for anti-NMDAR antibodies were diagnosed with MUE and responded to immunosuppression. A specific diagnosis was not established in the third dog but this dog responded to a combination of antibiotics and prednisone therapy. Our results suggest that autoantibodies against NMDAR deserve further investigation for their role in neurologic signs in dogs with encephalitis.

Autoimmune encephalitis is an increasingly important diagnosis in people since the first compelling description of the presence of antibodies against CNS neuronal surface antigens in six patients with limbic epilepsy in 2005. Antibodies that target neuronal cell surface proteins are considered a distinct entity from antibodies against intracellular neuronal antigens, such as anti-Hu antibodies. Antibodies against intracellular neuronal antigens have been recognized for much longer, are always associated with cancer, and typically associated with neurologic dysfunction resulting from irreversible T-cell cytotoxicity. Since the 2005 publication, over 20 different neuronal cell surface antigenic targets have been identified to be associated with a number of neurologic conditions. These include such diverse signs as limbic epilepsy, neuropsychiatric disorders, memory loss, cerebellar ataxia, chronic pain, and nystagmus. The most common antigenic targets include NMDAR, a complex of 2 proteins closely associated...
Canine NMDA receptor encephalitis

with the VGKC named LGI1 and CASPR2, VGCC, the metabotropic glutamate receptor 1 and 5 (mGluR1, mGluR5) as well as AMPA and GABA<sub>B</sub> (See supplementary data Table 2 for details).

While cancer is not always present, several of these autoantibodies are known to be associated with certain cancers, such as the NMDAR antibody and ovarian teratoma. Autoantibodies are hypothesized to cause direct neurotoxicity through downregulation of the target antigen.

Although complement mediated damage is also possible, it is likely minimal and many other mechanisms are potentially involved<sup>30,31</sup>. Importantly, these conditions are not always associated with MRI changes, as in 2 of the canine cases we describe with NMDAR antibodies<sup>15</sup>.

Due to the difficulty of accessing CSF antibody testing in a timely fashion, and the importance of early intervention with immunomodulatory drugs and appropriate screening for neoplasia, clinical guidelines for recognition of human AE have been developed. These guidelines allow treatment to be initiated prior to detection of antibodies<sup>12</sup>. However, a recent publication highlighted the wide spectrum of presenting clinical signs, noting that there was a clinical suspicion of encephalitis in only 16 of 50 patients that were ultimately diagnosed with AE. Of these 16 patients, an infectious cause was suspected in 9<sup>29</sup>. Thus, AE was considered as a differential at time of presentation and initial work up in only 7/50 (14%) of patients ultimately diagnosed with the condition<sup>29</sup>. This highlights the importance of considering autoimmune mechanisms for CNS disorders currently believed to be ‘idiopathic’.

The expanding knowledge of neuronal surface antigen targets as a cause of AE in people, the recent publication on the presence of anti-NMDAR antibodies in a polar bear<sup>21</sup>, and the
frequency with which MUE is diagnosed in dogs led us to investigate whether homologous autoantibodies against some of the more common human targets were present in dogs. We designed the study to screen CSF from dogs with a wide variety of neurological conditions including conditions such as epilepsy and intervertebral disc extrusion as well as encephalitis to determine firstly whether these autoantibodies were present in dogs with neurological disease, and secondly, with which diseases they were associated. Within this population of 32 dogs with neurologic disease and one healthy control, antibodies were not identified against AMPA1/2 or GABA<sub>B</sub>1/2 receptors or CASPR2, LG11, and DPPX in any dog. Three dogs had CSF that tested positive for antibodies against NMDAR1, the most common antigenic target associated with AE in people<sup>50</sup>. Notably, dogs in this study that were diagnosed with brain neoplasia, idiopathic or unknown epilepsy, intervertebral disc extrusion and degenerative myelopathy did not have CSF antibodies to this target.

NMDA receptor encephalitis is well described in people and classically affects young women and children causing a ‘rapid’ onset (defined as less than 3 months) of ‘flu-like’ symptoms, that progress to abnormal behavior or cognitive dysfunction, speech abnormalities, seizures, movement disorders (including dyskinesias and rigidity), decreased level of consciousness and autonomic dysfunction. A fatal catatonic state can occur but appropriate treatment with immunosuppression results in an favorable outcome<sup>11,12</sup>. A diagnosis of NMDARE can be made in patients with rapid onset of four of the above six categories of neurological signs, and abnormal EEG or CSF with pleocytosis or oligoclonal bands and reasonable exclusion of other disorders. It is notable that the syndrome is associated with teratomas in nearly 40% of
Canine NMDA receptor encephalitis

patients and MRI findings are normal in 66% of patients\textsuperscript{15,18}. Also of note is a genetic predisposition to the syndrome associated with a particular HLA-1 allele\textsuperscript{32}.

The 3 dogs with positive CSF for anti-NMDAR1 antibodies underwent extensive diagnostic workups. The first dog had a history and multifocal neurological signs typical of encephalitis as well as both multifocal MRI changes and CSF pleocytosis. The second dog had severe cerebellar signs, consistent with the atypical presentation reported in people more recently\textsuperscript{29}, as well as having CSF pleocytosis. In both dogs, infectious causes were excluded and their response to immunosuppression supports an autoimmune etiology although a causal relationship with the anti-NMDAR antibodies cannot be established from these early observations. The third case was more anomalous; as a one-year-old FS French bulldog presenting with progressive, severe, multifocal CNS signs, the top differential diagnosis at presentation was MUE. However, the dog's history was complicated by the presence of multiple abscessed teeth treated with broad spectrum antibiotics and recent airway surgery for brachycephalic airway syndrome. The normal appearance of the brain MRI and CSF analysis were puzzling given the severity of the neurological signs. In light of the signs of CNS dysfunction and the presence of resolving dental abscesses, treatment with antibiotics and prednisone at a dose rate of 1mg/kg/day was initiated. The dog's clinical signs responded to this treatment but relapsed as prednisone was tapered and then resolved with a longer course of prednisone. While the dog's clinical diagnosis remains mysterious, the presence of CSF anti-NMDAR1 antibodies suggests a possible autoimmune etiology, potentially triggered by the bacterial infection.
Canine NMDA receptor encephalitis

This pilot study had several limitations. Although high homology of all protein targets between species allowed for confidence in results, the commercially available kit used antigen targets designed for humans specifically. This project was developed as a pilot study to screen CSF banked from dogs with CNS dysfunction due to a variety of causes for the presence of CSF antibodies against neuronal surface targets. While the panel of antigenic targets was negative in dogs with epilepsy and degenerative conditions, the number of dogs with these conditions was limited and a much larger study of the prevalence of these antibodies is warranted in light of our results. Finally, the retrospective nature of this study means that dogs with positive CSF titers were not followed prospectively and serum titers were not measured.

We conclude that anti-NMDAR1 antibodies can be found in the CSF of dogs with encephalitis. While their pathologic role is unclear, this work suggests that neuronal surface protein antibodies might be a cause of AE and a much wider search for autoantibodies is warranted. Indeed, it is possible that dogs with idiopathic syndromes such as idiopathic cerebellitis (generalized tremor syndrome) or fly biting might have antibodies to an identifiable antigenic target in their CSF. Identification of these autoantibodies may allow better antemortem categorization of MUE as specific types of AE and improve our ability to provide a prognosis. A much larger prospective study evaluating a wide range of neuronal cell surface or synaptic antigenic targets is warranted.
REFERENCES


Canine NMDA receptor encephalitis


Canine NMDA receptor encephalitis


Canine NMDA receptor encephalitis

### Table 1. Sequence homology between dog and human protein targets of each antibody.

<table>
<thead>
<tr>
<th>Human Protein</th>
<th>Symbol</th>
<th>GenBank ID</th>
<th>Top Canine Blastp hlt (Canis lupus familiaris)</th>
<th>Top Blastp protein ID</th>
<th>Query cover (blastp)</th>
<th>Homology (blastp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>glutamate receptor ionotropic, NMDA 1 isoform GluN1-1a precursor</td>
<td>NMDAR1/GRIN1</td>
<td>NP_015566</td>
<td>glutamate receptor ionotropic, NMDA 1 precursor</td>
<td>NP_001008717</td>
<td>95%</td>
<td>99%</td>
</tr>
<tr>
<td>gamma-aminobutyric acid type B receptor subunit 1 isoform a precursor</td>
<td>GABAR1</td>
<td>NP_001461</td>
<td>gamma-aminobutyric acid type B receptor subunit 1</td>
<td>XP_022270239</td>
<td>92%</td>
<td>98%</td>
</tr>
<tr>
<td>gamma-aminobutyric acid type B receptor subunit 2 precursor</td>
<td>GABAR2</td>
<td>NP_005440</td>
<td>gamma-aminobutyric acid type B receptor subunit 2</td>
<td>XP_538749.2</td>
<td>100%</td>
<td>99%</td>
</tr>
<tr>
<td>glutamate receptor 1 isoform 1 precursor</td>
<td>AMPA1</td>
<td>NP_000818</td>
<td>glutamate receptor 1 isoform X2</td>
<td>XP_853398.1</td>
<td>100%</td>
<td>99%</td>
</tr>
<tr>
<td>glutamate receptor 2 isoform 1 precursor</td>
<td>AMPA2</td>
<td>NP_000817</td>
<td>glutamate receptor 2 isoform X1</td>
<td>XP_013975091.1</td>
<td>92%</td>
<td>99%</td>
</tr>
<tr>
<td>dipeptidyl aminopeptidase-like protein 6 isoform 3</td>
<td>DPPX</td>
<td>NP_001034</td>
<td>dipeptidyl aminopeptidase-like protein 6 isoform X4</td>
<td>XP_005629758.1</td>
<td>98%</td>
<td>94%</td>
</tr>
<tr>
<td>leucine-rich glioma-inactivated protein 1 isoform 2 precursor</td>
<td>LG1</td>
<td>NP_001295</td>
<td>leucine-rich glioma-inactivated protein 1 isoform X3</td>
<td>XP_013964506.1</td>
<td>100%</td>
<td>99%</td>
</tr>
<tr>
<td>contactin-associated protein-like 2 precursor</td>
<td>CASPR2</td>
<td>NP_054860</td>
<td>contactin-associated protein-like 2 isoform X1</td>
<td>XP_003432128.1</td>
<td>100%</td>
<td>95%</td>
</tr>
</tbody>
</table>
Table 2. Details of diagnostic findings in dogs with CSF anti-NMDAR1 antibodies.

<table>
<thead>
<tr>
<th>Dog</th>
<th>Signalement</th>
<th>Clinical signs</th>
<th>CSF</th>
<th>MRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>6y FS miniature poodle</td>
<td>Torticollis, non-ambulatory tetraparesis</td>
<td>WBC: 1567 cells/μL, RBC: 1675 cells/μL, Protein: 674 mg/dL, Mixed pleocytosis</td>
<td>Ill-defined bilateral T2 hyperintensity within thalamus, subthalamus, tegmentum and medulla</td>
</tr>
<tr>
<td>10</td>
<td>1y MC standard poodle</td>
<td>Non-ambulatory tetraparesis with hypermetria and intention tremors</td>
<td>WBC: 43 cells/μL, RBC: 23 cells/μL, Protein: 52.6mg/dL</td>
<td>Lymphocytic pleocytosis, Normal MRI</td>
</tr>
<tr>
<td>17</td>
<td>1y FS French bulldog</td>
<td>Circling to right, right head tilt, behavioral changes, right hemiparesis</td>
<td>WBC: 2 cells/μL, RBC: 48 cells/μL, Protein: 12.2mg/dL</td>
<td>Cytologically unremarkable CSF, Normal CNS structures on MRI, Tooth root abscesses in left maxilla extending into nasal cavity via a fistula.</td>
</tr>
</tbody>
</table>

CSF: cerebrospinal fluid sampled from the cerebellomedullary cistern
MRI: magnetic resonance imaging
Y: year
FS: female spayed
Canine NMDA receptor encephalitis

406 MC: male castrated

407 WBC: white blood cells

408 RBC: red blood cells

409 CSF samples were taken from the cerebellomedullary cistern and normal ranges of WBC: 0-5 cells/µL, protein: 0-25 mg/dL.
FIGURE LEGENDS

Figure 1. Amino acid sequence for NMDAR1 in people (*Homo sapiens; Hosa) and dogs (*Canis lupus familiaris; Calu) starting at amino acid 361. Critical amino acid positions 368 and 369 (*) show conservation of amino acids responsible for immunogenicity in human NMDARE.

Figure 2. Immunofluorescent assay results for the presence of CSF NMDAR1. A: Human positive control; B: negative results in a healthy dog; C: negative results in dog 3.

Figure 3: Immunofluorescent assay results for the presence of CSF NMDAR1 in 3 dogs with positive results. A: dog 4; B: dog 17 and C: dog 10.
Figure 1. Amino acid sequence for NMDAR1 in people (Homo sapiens; Hsa) and dogs (Canis lupus familiaris; Can) starting at amino acid 361. Critical amino acid positions 368 and 369 (*) show conservation of amino acids responsible for immunogenicity in human NMDARE.

79x77mm (300 x 300 DPI)
Figure 2. Immunofluorescent assay results for the presence of CSF NMDAR1. A: Human positive control; B: negative results in a healthy dog; C: negative results in dog 3.
Figure 3: Immunofluorescent assay results for the presence of CSF NMDAR1 in 3 dogs with positive results. A: dog 4; B: dog 17 and C: dog 10.