ABSTRACT

ROMANET, JESSICA LEIGH. A Role for TMEM150A in Cytokine Regulation. (Under the direction of Dr. Jeffrey Yoder).

Phosphatidylinositol 4-phosphate (PI(4)P) is a key limiting factor in phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$) production which plays critical roles in multiple cellular processes including the targeted recruitment of TIR domain containing adaptor protein (TIRAP) in the Toll-like Receptor 4 (TLR4) signal transduction pathway. A crucial kinase responsible for PI(4)P creation is phosphatidylinositol 4-kinase type III alpha (PI4KIIIα), which is known as staurosporine and temperature sensitive 4 (Stt4) in yeast, and is partially regulated by suppressor of four kinase protein 1 (Sfk1). The suggested mammalian homologue of Sfk1 is TMEM150A, a member of the damage-regulated autophagy modulator (DRAM) protein family. The DRAM family consists of five proteins with six transmembrane domains. As the name implies, three of the family members have been implicated in the mediation of autophagy. Although autophagy is most well-known for cellular homeostatic degradation of long-lived organelles and cell survival processes that occur during times of starvation, autophagy has clearly been linked to immune-related functions. In fact, it was demonstrated that DRAM1 can influence autophagy downstream of TLR4/MyD88 signaling in a pro-survival response to infection. Because of TMEM150A's connection to the TLR4 pathway by way of the DRAM family and as a potential regulator of PI4KIIIα, we hypothesized that there might be a direct connection between TMEM150A and the TLR4 cascade. Herein we provide the first evidence that the DRAM family member, TMEM150A, plays a key role in the regulation of cytokine production downstream of TLR4. These observations suggest that TMEM150A plays an important part in immune

function, particularly in regulating cytokine response, and, that it, and the pathway it affects, may provide novel targets for controlling common inflammatory diseases.

A Role for TMEM150A in Cytokine Regulation

by
Jessica Leigh Romanet

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

2017

APPROVED BY:

_____	_____
Dr. Jeffrey Yoder Committee Chair	Dr. Samuel Jones
_____	_____
Dr. Kenneth Adler	Dr. Nanette Nascone-Yoder

DEDICATION

To Zach, for teaching me to live an inspired life.

To my parents, for their love and support.

To my husband, for his tireless faith and belief in me.

And to Grayson and Asher, for reminding me of the power of true, unconditional love.

BIOGRAPHY

Jessica Leigh Romanet was born and raised in Southern New Jersey by her fun-loving and bright parents, Carol and Skip. Raised by two teachers, she always loved school and so it made sense to her, and probably her alone, why she chose to leave a successful and well-paid pharmaceutical sales career to pursue a veterinary medical degree. And then prolong her education by applying to the DVM/PhD combined program. But, it was during her time working for Pfizer while studying body system physiology and different drug mechanisms that made her realize just how much she loved science. The veterinary curriculum at North Carolina State College of Veterinary Medicine reinforced this and the idea that a veterinary clinician scientist plays a special role in advancing healthcare for both animals and humans compelled Jess to pursue research training. Graduate training has significantly enhanced Jess's ability to think critically and she is thankful for such an amazing and priceless education.

During the next year, Jess will be re-entering the veterinary school curriculum and is thrilled to be participating in the LEAD Peer Mentoring program. She is passionate and inspired about deepening her relationships with her peers. She hopes to help others realize their own amazing potential which will allow them to thrive even during the tumultuous and sometimes isolating graduate and DVM years. Jess truly believes it is relationships that create a wonderful and happy life.

Although Jess's southern Jersey roots come out each football season as she cheers for and is repeatedly disappointed by the NFL's Philadelphia Eagles, she has developed a deep love of the Triangle, especially her own town of Apex. Jess is married to Ryan, an extremely

supportive and hardworking Iraq and Afghan war veteran. Interestingly, Jess thought it was a good idea to birth two children during her graduate school years. (Actually, she didn't think it was a good idea, but it happened anyway). Jess wholeheartedly adores being a mom of two precious and mischievous sons, Grayson and Asher. This remains true even after putting a $3^{1/2}$ year-old back in his bed for the twentieth time in one night while trying to complete a dissertation and fantasizing about booking a hotel room. She humbly hopes to be a role-model for her boys of what strength, perseverance and passion can accomplish.

ACKNOWLEDGMENTS

I am extremely grateful to have worked under the guidance of Dr. Jeff Yoder. Jeff is a remarkable mentor who has been patient, very supportive, available and ever-encouraging. I would also like to thank my committee members, Dr. Nanette Nascone-Yoder, Dr. Ken Adler for their helpful insight and advice. Dr. Sam Jones also deserves a special thank you for his efforts to support and nurture the DVM/PhD program and its candidates. You are appreciated.

The members of the Yoder Lab have been a joy to work with, except for maybe Dustin, who I could always count on to say, "excuse you" when I sneezed. Kidding aside, Amanda, Dustin, Ashley, Deb, Hayley and Ivan always made the lab a wonderful place to be. Rest in peace, Mr. Fish. Additionally, I would be remiss if I didn't thank Shannon Chiera for answering a bunch of my questions over the years with patience and kindness. Thank you also goes to Steve Nagar for very helpful immunofluorescent staining advice! Plus, I would like to thank Katherine Cupo and Gretchen Scheffe for trusting me to mentor them and faithfully working to help troubleshoot our experiments.

I would not have made it past second year without the support of great friends. Emily and Becky, thank you for all the laughs, encouragement and camaraderie. I am grateful for the kindness of my friend Denisse, who has sacrificed her own time and freedom to help take care of my baby boy, Ash, while I am at school. Sabrina, you are a true joy. Thank you. Casey and Erin, you are like family. Aunt Ardis, thank you for always thinking of my boys and me.

Finally, I am overwhelmed by the support, patience, love and kindness from my parents, Carol and Skip, and my husband, Ryan. I would be nowhere without you. Gray and Ash, thank you for bringing me so much joy, each and every day. And, thank you to my very special guardian angel, Zachary Lou.

TABLE OF CONTENTS

LIST OF TABLES……....ix

LIST OF FIGURES……...…x

LIST OF ABBREVIATIONS……...xi

INTRODUCTION..……...……1

CHAPTER 1: LITERATURE REVIEW……...2

AUTOPHAGY……...……2

AUTOPHAGY (BASIC)……...2

AUTOPHAGY & INNATE IMMUNITY TOLL-LIKE RECEPTOR 4 (TLR4) SIGNALING.....4

DAMAGE-REGULATED AUTOPHAGY MODULATOR (DRAM) PROTEIN FAMILY……....7

DRAM1……8

DRAM2……...13

DRAM3……...14

TMEM150C……....16

TMEM150A……....16

PI4KIIIα: A PRIMER……..19

RATIONALE.....20

REFERENCES……24

CHAPTER 2: A ROLE FOR TMEM150A IN CYTOKINE REGULATION……28

INTRODUCTION……...29

RESULTS……31

DISCUSSION……..…51

EXPERIMENTAL PROCEDURES……54

REFERENCES……59

CHAPTER 3: SUMMARY AND FUTURE DIRECTIONS……..64

REFERENCES……71

LIST OF TABLES

CHAPTER 1

TABLE 1.1 Phases and associated proteins of the autophagic pathway……..21

CHAPTER 2

TABLE 2.1 Knockdown of TMEM150A in human lung epithelial cells causes changes in the transcription factor activity profile……46

LIST OF FIGURES

CHAPTER 1

FIGURE 1.1 The TLR4 signal transduction pathway induces cytokine production after activation by LPS……22

CHAPTER 2

FIGURE 2.1 Sequence conservation of TMEM150A.……37

FIGURE 2.2 Activation of TLR4, in the absence of TMEM150A, leads to increased CXCL8 production …….39

FIGURE 2.3 TMEM150a silencing affects cytokine regulation in human mucoepidermoid pulmonary carcinoma cell line (H292)……....41

FIGURE 2.4 Transcription Factor (TF) Activation Profiling Method…….45

FIGURE 2.5 Endogenous TMEM150A is localized in the nucleus and in a punctate pattern throughout the cytoplasm in H292 cells……..48

CHAPTER 3

FIGURE 3.1 Proposed model of how TMEM150A may influence cytokine response……68

LIST OF ABBREVIATIONS

AMPK	adenosine 5'-monophosphate-activated protein kinase
AP1	Activator protein 1 (JUN/FOS)
AP2	Activator protein 2
APL	acute promyelocytic leukemia
AR	Androgen receptor
ATF2	activating transcription factor 2
ATG	autophagy related gene
ATRA	all-trans retinoic acid
BCL-2	B-Cell CLL/Lymphoma 2
BCL-XL	B-Cell CLL/Lymphoma XL
BECN1	Beclin 1
Brn-3	POU domain, class 4, transcription factor 1
C/EBP	CCAAT/enhancer binding protein (C/EBP),alpha
CAR	nuclear receptor subfamily 1, group I, member 3
CBF	CCAAT/enhancer binding protein (C/EBP), zeta
CCL5	CCL5/Rantes
CD14	CD14 molecule
CDP	cut-like homeobox 1; CCAAT displacement protein
CREB	cAMP responsive element binding protein 1
CXCL8/IL-8	Interleukin 8
DRAM	Damage-regulated autophagy modulator
DRAM-1	FLJ11259
DRAM-2	TMEM77
E. coli	*Escherichia coli*
E2F-1	E2F transcription factor 1
EGR	Early growth response
ER	Estrogen receptor
Ets	v-ets erythroblastosis virus E26 oncogene homolog 1
FAST-1(FOXH1)	Forkhead box H1

GAS/ISRE	IFN-stimulated response element combing GAS and ISRE element
GATA	GATA transcription factor
GFP-LC3	green fluorescent protein tagged microtubule-associated protein light chain 3B (MAP1LC3B)
GMCSF	granulocyte macrophage colony stimulating factor
GR/PR	Glucocorticoid receptor/progesterone receptor
H292	National Cancer Institute H292 Lung mucoepidermoid pulmonary carcinoma cell line
HEK	Human Embryonic Kidney Cells
HEK TLR4	HEK cells expressing TLR4/MD2/CD14
HIF	Hypoxia inducible factor
HMrSV5	human peritoneal mesothelial cell line
HNF4	Hepatocyte nuclear factor 4
HRP	horseradish peroxidase
IFNg	interferon gamma
IL10	interleukin 10
IL12A	interleukin 12 a, one part of the IL12 heterodimer that dimerizes with IL12B to create interleukin 12
Il12B	interleukin 12 b, one part of the IL12 heterodimer that dimerizes with IL12B to create interleukin 12
IL6	interleukin 6
IL7	interleukin 7
IRF	Interferon regulatory factor
LPS	lipopolysaccharide
MAP1LC3B	microtubule-associated protein light chain 3B
MCL-1	Induced Myeloid Leukemia Cell Differentiation Protein
MD2	Lymphocyte Antigen 96
MEF2	Myocyte enhancer factor 2
Mm	*Mycobacterium marinum*
Mtb	*Mycobacterium tuberculosis*
mTORC1	mechanistic target of rapamycin complex 1
Myb	v-myb myeloblastosis viral oncogene homolog

Myc-Max	v-myc myelocytomatosis viral oncogene homolog (avian)
MyD88	myeloid differentiation factor
NCBI	National Center for Biotechnology Information
NF-1	Nuclear factor 1
NF-E2	Nuclear factor (erythroid-derived 2)
NFAT	Nuclear factor of activated T-cells
NFkB	nuclear factor kappa enhancer-binding protein
ng/ml	nanograms per milliliter
NIH 3T3	National Institutes of Health 3T3 mouse fibroblast cells
Oct4	POU class 5 homeobox 1
p53	Tumor protein p53
p73	tumor protein 73
PAMP	pathogen-associated molecular pattern
Pax-5	Paired box 5
PBMCs	peripheral blood mononuclear cells
Pbx1	Pre-B cell leukemia transcription factor-1
PI	Phosphoinositide
PI(4,5)P2	Phosphatidylinositol 4,5-bisphosphate aka PIP2
PI3KC3	phosphoinositide 3-kinase catalytic subunit type III
PI4KIIIα	phosphatidylinositol kinase III alpha
PI(4)P	phosphatidylinositol 4-phosphate
Pit	Pituitary specific transcription factor 1
PPAR	Peroxisome proliferator-activated receptor
PRR	pathogen recognition receptor
PXR	Steroid and xenobiotic receptor
qPCR	quantitative reverse transcriptase real-time polymerase chain reaction
RIP	receptor-interacting protein
SATB1	Special AT-rich sequence binding protein 1
Sfk1	suppressor of four kinase protein 1
SMAD (MADH)	SMAD family

Sp1	SP1 transcription factor
SRF	Serum response factor
Stat1	Signal transducer and activator of transcription 1
Stat3	Signal transducer and activator of transcription 3
Stat4	Signal transducer and activator of transcription 4
Stat5	Signal transducer and activator of transcription 5
Stat6	Signal transducer and activator of transcription 6
Stt4	staurosporine and temperature sensitive 4
TCF/LEF	Runt-related transcription factor 2
TFIID	TATA box binding protein
THP-1	acute monocytic leukemia cell line
TIR	Toll-interleukin receptor
TIRAP	TIR domain containing adaptor protein
TLR	Toll-like receptor
TMEM150A	DRAM5
TMEM150B	TMEM224/DRAM3
TMEM150C	DRAM4/TTN3
TNF	tumor necrosis factor aka tumor necrosis factor alpha
TR	Thyroid hormone receptor
TRIF	toll-interleukin 1 receptor domain-containing adaptor-inducing interferon-β
U937	histiocytic lymphoma cell line
ULK1	uncoordinated-51 like autophagy activating kinase 1
VPS	vacuolar protein sorting
YY1	YY1 transcription factor

Introduction

Immune signaling and a healthy immune response must include an inflammatory cascade to recognize, defend against, destroy and remove an insulting source, while mitigating the damaging effects against the host's tissues from a prolonged and exacerbated immune attack (Whicher, 1990). In this process, cytokines, which can be released by numerous cell types, play an important role in recruiting inflammatory cells to sites of infection or injury. Although cytokine production is essential to immunity, unchecked, overzealous or prolonged cytokine upregulation is implicated in numerous, costly diseases that include sepsis, cancer, inflammatory bowel disorder, psoriasis and arthritis (Baliwag et al., 2015; Dinarello, 2000; Hennessy et al., 2010; Tartour and Fridman, 1998). Identifying novel, targeted therapeutics to treat these conditions takes a nuanced understanding of the complexity of the pathways that trigger or dampen cytokine production. The mechanisms modulating cytokines are vast and diverse enough to encompass toll-like receptor signaling, autophagy and phosphatidylinositols. This work attempts to add to our understanding of the complex pathways of innate immune regulation by providing evidence that TMEM150A, a largely undefined but highly evolutionarily conserved protein, loosely linked to autophagy, phosphatidylinositols, and now to toll-like receptor signaling, plays a role in cytokine regulation.

Chapter 1: Literature Review

Autophagy introduction (Basic)

Autophagy ("self-eating") is a multifaceted, evolutionarily conserved, cellular homeostasis mechanism that is implicated in an array of cellular functions (Cadwell, 2016; Harris, 2011; Yin et al., 2016). Although autophagy is most well-known for cellular homeostatic degradation of long-lived organelles and cell survival processes that occur during times of starvation, autophagy has also been clearly linked to immune-related functions (Cadwell, 2016; Criollo et al., 2012; Harris, 2011; Li et al., 2011; Meijer and van der Vaart, 2014; Yin et al., 2016).

Autophagy occurs at a basal rate in a non-selective manner to facilitate disposal of damaged or surplus organelles (Cadwell, 2016; Johansen and Lamark, 2011; Yin et al., 2016) but is modulated for proper cellular responses to stress, starvation and infectious pathogens (Cadwell, 2016; Harris, 2011; Johansen and Lamark, 2011; Yin et al., 2016). It makes sense that multiple autophagic activation pathways have been reported since autophagy is implicated in a myriad of cellular responses (Cadwell, 2016; Crighton et al., 2007a; Harris, 2011; Johansen and Lamark, 2011; Meijer and van der Vaart, 2014; Xu et al., 2007; Yin et al., 2016) but the simplest and most studied activation pathway includes extracellular nutrient sensing regulation by adenosine 5'-monophosphate-activated protein kinase (AMPK) inhibition of mechanistic target of rapamycin complex 1 (mTORC1). In short, mTORC1 is inhibited when cells are starved and autophagy is induced by autophagy related gene 1 (ATG1)/uncoordinated-51 like autophagy activating kinase 1 (ULK1) (Cadwell, 2016;

Delgado and Deretic, 2009; Harris, 2011; Yin et al., 2016). Autophagosome biogenesis remains largely similar regardless of the instigating pathway and includes initiation, nucleation, expansion and maturation (Cadwell, 2016; Cordani et al., 2017). An outline of the steps and core proteins is categorized by Table 1.1 (Choi, 2013). In brief, ATG1/ULK1, phosphoinositide 3-kinase catalytic subunit type III (PI3KC3) complex (beclin1, vacuolar protein sorting 34 (VPS34), VPS15 and ATG14L work together to initiate nucleation of a phagosome (Cadwell, 2016). After initiation of autophagy, there is vesicle elongation leading to maturation of an autophagosome that in turn fuses with a lysosome to become an autolysosome finally leading to vesicle breakdown and degradation. The major mammalian autophagic proteins include ULK1, ATG3, ATG4b, ATG5, BECN1, ATG7, MAP1LC3B, ATG9A, ATG10, ATG12, ATG14, and ATG16, all which have evolutionarily conserved yeast homologues (Harris, 2011).

Autophagy can act in multiple ways to clear sources of the following: infection (xenophagy), defective mitochondria (mitophagy), protein aggregates (aggrephagy) as well as recycle cytoplasmic components (macroautophagy) to enhance cell survival or provide energy in times of stress or starvation (Cadwell, 2016; Harris, 2011; Yin et al., 2016). Since the topic of this thesis is related to innate immune signaling, the sections that follow focus on autophagy as it is related to innate immunity, specifically in terms of toll-like receptor 4 (TLR4) signaling.

Autophagy & Innate Immunity Toll-Like Receptor 4 (TLR4) Signaling

The lipopolysaccharide (LPS) component of gram-negative bacterial cell walls is a potent, innate immune stimulant and serves as a pro-inflammatory pathogen-associated molecular pattern (PAMP) and the ligand for the pathogen recognition receptor (PRR) TLR4 (Lu et al., 2008; Takeda and Akira, 2001; Tan and Kagan, 2014). A simple illustration of the canonical LPS-TLR4 signaling pathway is shown in Figure 1.1. The best described, downstream result of the TLR4 signal transduction pathway is activation of the transcription factors, nuclear factor kappa enhancer-binding protein B (NF-kB) and activator protein-1 (AP-1). NF-kB and AP-1 activation lead to the production of immune signaling peptides called cytokines (Lu et al., 2008). Cytokines, which can be released by numerous cell types, recruit inflammatory cells to sites of infection or injury and must be well regulated to prevent unnecessary and injurious tissue damage (Aguilar-Valles et al., 2015; Baliwag et al., 2015; Whicher and Evans, 1990; Zhang and An, 2007).

It is now known that the engagement of TLR4 by LPS can induce autophagy. As reported by four different groups, TLR4 stimulation induces autophagy in RAW264.7 cells (a mouse macrophage cell line) causing green fluorescent protein (GFP)-tagged microtubule-associated protein light chain 3B (GFP-LC3) to go from a diffuse -- to a punctate pattern (a relatively reliable autophagy marker) indicating formation of autophagasomes (Delgado et al., 2008; Sanjuan et al., 2007; Shi and Kehrl, 2008; Xu et al., 2007). In addition, LPS stimulation results in the production of an abundance of the autophagy marker, LC-3II, in human (Li et al., 2011). Additionally, when key autophagy protein, Atg7, is knocked down (by siRNA), the downstream output of TLR4 activation,

cytokine production, is inhibited as demonstrated by reduced protein and transcript levels of CXCL8/IL-8 (Li et al., 2011). This last finding implicates autophagy as a positive regulator of cytokine production downstream of LPS-TLR4 signaling.

Although autophagy can contribute to both cell survival and cell death mechanisms, the LPS/TLR4 induced autophagy pathway is best characterized as promoting cell survival (Xu et al., 2007) through the enhancement of innate immune protection by stimulating additional TLR signaling and the translocation of NF-kB to the nucleus (Cadwell, 2016). Another example of the LPS/TLR4 autophagy cascade promoting cell survival is evidenced when the human peritoneal mesothelial cell line (HMrSV5) is challenged with *Escherichia coli (E. coli)* infection. Exposing HMrSV5 cells to LPS (1 μg/ml) triggers an increase in the autophagy markers BECN1, LC3II, and GFP-LC3 at 12 and 18 hours after exposure (Wang et al., 2013). When these same cells were infected with *E. coli* after LPS stimulation, the *E. coli* was more likely to be contained within autophagosomes and intracellular bactericidal activity increased (Wang et al., 2013). These host defense mechanisms were attenuated by siRNA mediated knockdown of BECN1 or by small molecule inhibition of autophagy (Wang et al., 2013). In this same study, both silencing and inhibition of TLR4 reduced LPS-triggered autophagy and bactericidal activity (Wang et al., 2013). Further, cell survival responses from TLR4 induced autophagy are evidenced by studies investigating autophagy and *Mycobacterium tuberculosis* infection. *M. tuberculosis* typically resides in autophagosomes and stunts maturation that would normally lead to degradation via the autolysosome (Xu et al., 2007). Autophagy inducing conditions, like starvation, exposure to rapamycin and stimulation by LPS lead to increased *M.*

tuberculosis in autophagic vacuoles and decreased mycobacterial survival (Xu et al., 2007). Similarly, knockdown of the autophagy inducer, damage-regulated autophagy modulator 1 (DRAM1), results in increased infection in a zebrafish-whole-organism model (Meijer and van der Vaart, 2014).

Further study is required to define the specifics of the LPS/TLR4 autophagy signaling pathway, but evidence suggests that there is more than one way to trigger autophagy via TLR4. TLR4 receptor activation leads to two separate arms of a signal transduction pathway, one that is myeloid differentiation factor (MyD88)-dependent and one that is MyD88-independent (FIGURE 1.1) (Lu et al., 2008; Takeda and Akira, 2001; Tan and Kagan, 2014). There is now evidence that autophagy can be induced by both MyD88-dependent and MyD88-independent signaling (Meijer and van der Vaart, 2014; Xu et al., 2007). The latter is dependent on Toll-interleukin 1 receptor domain-containing adaptor-inducing interferon-β (TRIF) (Takeda and Akira, 2015) and its activation by LPS induces autophagy downstream of receptor-interacting protein (RIP1) and p38 MAPK but not JNK (Xu et al., 2007). TRIF-dependent TLR4 signaling is known to induce late phase activation of NF-kB and MAPKs (Yamamoto et al., 2003) and it has been suggested that early TLR4 activation leads to acute phagocytosis of a pathogen or insulting source and late phase activation may result in autophagosome formation to clear that source (Delgado and Deretic, 2009; Xu et al., 2007).

Yet, autophagy can also be invoked by the MyD88-dependent pathway. TLR4 plays a major role in response to *mycobacterium* infection (Sánchez et al., 2010) and MyD88 mutation reduces the infection-dependent induction of the autophagy inducer DRAM1,

causing decreased LC3-GFP puncta in response to *Mycobacterium* infection in both human M2 macrophages and zebrafish embryos (Meijer and van der Vaart, 2014). These observations imply that LPS-induced autophagy signaling can result from either a TRIF-dependent or a MyD88-dependent pathway depending on the insulting source. Interestingly, NF-kB can stimulate BECN1 thereby activating autophagy (Criollo et al., 2012; Meijer and van der Vaart, 2014) but it has also been demonstrated that canonical NFkB nuclear translocation occurs only in the presence of an intact autophagic cascade (Criollo et al., 2012).

Temporal regulation of autophagy differs by instigating source. For instance, starvation-induced autophagy requires two hours (Kirkegaard et al., 2004) while LPS-induced autophagy is noted at 8 hours but maximal at 16 hours (Xu et al., 2007).

What is most clear from the literature is that autophagy is an evolutionary conserved biological process that plays an important role in the immune response to infectious disease, specifically in regard to TLR signaling. Notably, autophagy dysregulation can lead to inflammatory diseases like cancer, neurodegenerative disease and sepsis largely due to aberrant cytokine response: therefore, understanding the mechanisms that regulate autophagy is of high importance to human and animal health (Cadwell, 2016; Choi et al., 2013; Delgado and Deretic, 2009; Hayat, 2013; Saitoh and Akira, 2010) .

Damage-Regulated Autophagy Modulator (DRAM) Family

TMEM150a, the focus of this dissertation, is a gene related by homology to a family of proteins implicated in autophagy induction. This evolutionarily conserved family includes

five members in humans, *DRAM1*, *DRAM2* (aka *TMEM77*), *TMEM150B* (aka *TMEM224*, *TTN2* and *DRAM3*), *TMEM150C* (aka *TTN3* and/ *DRAM4)* and *TMEM150A* (aka *TM6P1*, *TTN1* and *DRAM5*) (O'Prey et al., 2009; Zhang et al., 2000). DRAM family members are highly conserved across vertebrate species and predicted to encode proteins with six-transmembrane domains with both the amino- and carboxyl-termini positioned on the cytoplasmic side of cellular membranes (Hong et al., 2016; Kerley-Hamilton et al., 2007; O'Prey et al., 2009). Members of the DRAM family are expressed across most tissue types but show differential expression patterns amongst themselves in mammalian tissues and human cancer cell lines (Crighton et al., 2007a; Hong et al., 2017; Kerley-Hamilton et al., 2007; Mrschtik et al., 2015; O'Prey et al., 2009; Zhang et al., 2000).

The family has been termed damage-regulated autophagy modulators (DRAM) by one group who has linked/DRAM1 and TMEM150B/DRAM3 to differential roles in autophagy (Crighton et al., 2007a; Mrschtik et al., 2015; O'Prey et al., 2009). However, the roles of the other DRAM family members remain to be clearly defined. The available literature describing each DRAM family member is summarized here.

DRAM1

DRAM1 is the best characterized and most studied member of the family. A screen for *TP53* target genes found that DRAM1 is induced by chemotherapeutic agents (DNA Damage) and contains both an upstream p53 response element (Kerley-Hamilton et al., 2007) and an intronic p53 response element (Crighton et al., 2006). Recombinant exogenous DRAM1 has been reported to localize to the plasma membrane as well as in a

punctate pattern throughout the cytoplasm in certain studies (Kerley-Hamilton et al., 2007) while specifically to the lysosome in other reports (Crighton et al., 2006; Park et al., 2009; van der Vaart et al., 2014; Yoon et al., 2012). There is also some conflict regarding how DRAM1 overexpression affects clonogenic survival under stressful conditions; Kerley-Hamilton et al reports that DRAM1 overexpression significantly increases clonogenic survival in three different cell types whereas Crighton et al maintains clonogenicity is unaffected in one of the same cell types (Crighton et al., 2006, 2007a; Kerley-Hamilton et al., 2007). Differences in DRAM1 constructs, overexpression levels, cellular stress or nutrient levels may be the source of this discrepancy. Meanwhile, there is evidence that DRAM1 contributes to cell death. Knockdown of DRAM1 with siRNA decreases apoptotic cell death after challenge with DNA damaging agents (Crighton et al., 2006). Thus, it has been suggested that DRAM1 is critical to p53-mediated programmed cell death but does not contribute to cell death alone (Crighton et al., 2006). The relationship between DRAM1 and p53 is supported by work showing that small molecule (3-nitropropionic acid; 3NP)--induced mitochondrial dysfunction upregulates DRAM1, mostly in a p53 dependent manner (Zhang et al., 2013).

Electron micrograph analysis shows that induction of DRAM1 creates an accumulation of double-membraned autophagic vesicles and changes the GFP-LC3 patterns from diffuse to punctate, indicating an increase in autophagic activity (Crighton et al., 2006). Likewise, GFP-LC3 patterns and LC3-II protein levels, after siRNA mediated knockdown of DRAM1 in a TetOn-p53 cell line, also support a role for DRAM1, downstream of p53, in autophagy modulation (Crighton et al., 2006).

In line with its relationship to p53, DRAM1 is downregulated in multiple oral tumor cell lines and in some squamous cancers, and has been proposed to be tumor suppressive (Crighton et al., 2006, 2007a).

However, p53 is not the only generator of DRAM1 expression. 3NP mitochondrial damage induced DRAM1, even in a p53 knockout cell line, but the DRAM1 levels did not match that of p53 competent cells (Zhang et al., 2013). In a separate report, p73, a p53-related protein, also involved in cell cycle arrest and apoptosis, was found to induce both DRAM1 expression as well as autophagy. Yet, in contrast to the relationship between p53 and DRAM1, DRAM1 is dispensible for p73-mediated induction of autophagy (Crighton et al., 2007b).

Additionally, p73 modulates the role DRAM1 plays in neutrophil differentiation. Neutrophil differentiation, in a cell line that has mutated, impaired p53, (NB4 acute promyelocytic leukemia (APL) cells), is both DRAM1 and autophagy dependent downstream of p73. Upon all-trans retinoic acid (ATRA)-induced neutrophil differentiation of APL cells, DRAM1 expression was induced by almost 12-fold and the same was not seen in NB4-ATRA resistant cells which do not differentiate. Reciprocally, knockdown of DRAM1 reduced neutrophil affiliated cell markers and there was reduced expression of core autophagy genes ATG5 and ATG7, suggesting decreased overall autophagy. Further, DRAM1 expression was modulated by p73 and p73 knockdown cells showed decreased neutrophil differentiation markers (Humbert et al., 2012).

More recently, DRAM1 was directly connected to the innate immune response mounted against mycobacterial infection in both human primary macrophages and in an *in*

vivo zebrafish model (Meijer and van der Vaart, 2014; van der Vaart et al., 2014). In these models, during mycobacterial infection, DRAM1-autophagy-induction is protective and downstream of the TLR4-MyD88-NF-kB pathway and independent of p53 (Meijer and van der Vaart, 2014; van der Vaart et al., 2014).

MyD88 null (*myd88*$^{-/-}$) zebrafish have an impaired immune response to *Mycobacterium marinum*, a natural fish pathogen and close relative to human *Mycobacterium tuberculosis) (van der Vaart et al., 2014).* Gene expression analysis of *myd88*$^{-/-}$ mutant zebrafish highlighted decreased *dram1* expression after infection. In contrast, MyD88 competent zebrafish (*myd88*$^{+/+}$) *dram1* expression increased over the course of the infection whereas, *p53* and key autophagy genes *atg5, lc3* and *p62* did not show this same change in expression (van der Vaart et al., 2014). Zebrafish *dram1* expression also increased over the course of infection in p53 mutant embryos demonstrating that DRAM1 induction was not p53 dependent in this case *(van der Vaart et al., 2014).*

Furthermore, TLR4-LPS stimulation also induced *dram1* expression but not in NF-kB inhibited zebrafish or *myd88*$^{-/-}$ embryos (van der Vaart et al., 2014). Similar results were obtained in human primary macrophage type1 (M1) and type 2 (M2) cells after infection with *M. tuberculosis*. DRAM1 was elevated four hours post infection, colocalized with *M. tuberculosis,* and NF-kB inhibition negated this result (van der Vaart et al., 2014). siRNA depletion of MyD88 showed decreased *DRAM1* expression in M2 cells but not M1 (Meijer and van der Vaart, 2014; van der Vaart et al., 2014).

Morpholino knockdown of *dram1* in zebrafish embryos infected with *M. marinum* had increased bacterial burdens, higher expression of *il1b*, and infected cells that were

overgrown or burst with bacteria in the cytoplasm rather than in phagosomes (van der Vaart et al., 2014). In concert, overexpression of Dram1 in zebrafish increased the quantity of GFP-LC3 and reduced *M. marinum* burden ((Meijer and van der Vaart, 2014; van der Vaart et al., 2014). The higher expression of the cytokine IL1b in this knockdown model is in contrast to the decreased levels of CXCL/IL8 cytokine in intestinal epithelial cells with ATG7 siRNA autophagy knockdown (Li et al., 2011). One important difference that should be understood is that IL1b samples were taken four days post infection for the *in vivo* model and sixteen hours post infection for the *in cellulo* model. In the zebrafish model, the increased *il1b* suggests a regulatory role for autophagy in cytokine production (van der Vaart et al., 2014). The take home message from these studies is that DRAM1 is host protective by being an inducer of mycobacterial autophagy.

The intricate details of how DRAM1 regulates autophagy are still being investigated. However, there is evidence using inhibitor assays showing that DRAM1 modulates autophagy by enhancing acidification and fusion of lysosomes with autophagosomes and also helping to manage autophagic flux by increasing turnover of autophagosomes (van der Vaart et al., 2014; Zhang et al., 2013).

In summary, DRAM1 induces autophagy and can be activated by multiple pathways including p53, p73 and TLR4. Autophagy modulation by DRAM1 occurs through lysosomal mediation and has been linked to tumor suppression, neutrophil differentiation and host defense to mycobacterial infection.

DRAM2/TMEM77

DRAM1 and DRAM2 (previously known as TMEM77) share 67% peptide sequence homology depending on the method utilized by the investigator (Park et al., 2009). Unlike DRAM1, DRAM2 expression is not induced by p53 or p73 (O'Prey et al., 2009) yet similar to DRAM1, both DRAM2 transcript and protein levels are reduced in ovarian tumors (Park et al., 2009).

Interestingly, there are conflicting reports about whether overexpression of DRAM2 induces autophagy or not (O'Prey et al., 2009; Yoon et al., 2012). This confusion is probably due to the different techniques used to measure autophagy by each group; only one group investigated knockdown of endogenous DRAM2 (instead of recombinant, overexpression of exogenous DRAM2) which did show a relationship with modulation of autophagy by inhibiting autophagosome formation, changing LC3 localization patterns and depleting LC3-II bands on Western Blot (Yoon et al., 2012).

Moreover, the combined expression of DRAM1 and DRAM2 synergistically activated autophagy in HEK293 cells and induced apoptotic-like nuclear blebbing (Park et al., 2009). Immunofluorescent labeling demonstrated that the majority of recombinant, exogenous DRAM2 colocalized in lysosomes with DRAM1 (O'Prey et al., 2009; Park et al., 2009). Similar to DRAM1, siRNA knockdown of DRAM2 enhanced cell viability after exposure to the chemotherapeutic, DNA damaging and p53-inducing agent--doxorubicin, possibly suggesting a role for DRAM2 in p53-induced cell death (Park et al., 2009).

All things considered, there is evidence that DRAM2 plays a role in autophagy, at least as it pertains to LC3B changes. Furthermore, DRAM1 and DRAM2 share downregulation in tumor tissues even though they have differential regulation.

TMEM150B/DRAM3

Similar to DRAM2 and in contrast to DRAM1, TMEM150B is not regulated by p53 and is not transcriptionally induced by the same chemotherapeutic drugs, inflammatory agents or starvation conditions. Based on this finding and the differential expression patterns of DRAM1 and TMEM150B across cell lines, coregulation of DRAM1 and TMEM150B is not evident (Mrschtik and Ryan, 2016; Mrschtik et al., 2015).

Recombinant, exogenous TMEM150B displays cytoplasmic punctate staining, colocalizing in similar ways as DRAM1 with endosomal, lysosomal and autolysosomal markers. In contrast to DRAM1 and DRAM2, TMEM150B appears to be enriched in the actin-rich focal adhesions of plasma membranes in cell culture; TMEM150B is not found in the endoplasmic reticulum, golgi apparatus or on phagophores (Mrschtik and Ryan, 2016; Mrschtik et al., 2015).

Stable expression of exogenous TMEM150B was attenuated over time indicating that overexpression of TMEM150B may affect cell health. However, a retroviral TMEM150B construct that induced lower levels of TMEM150B did not have this same effect. Interestingly, overexpression of TMEM150B in cells cultured in complete media showed increased LC3B puncta compared to control, but upon starvation conditions the difference was no longer significant (Mrschtik et al., 2015).

TMEM150B expression affects autophagic flux (autophagosome turnover) in normal cell growth conditions as evidenced by LC3BII accumulation after chloroquine (autophagosome turnover blocker) treatment. Reciprocally, CRISPR/Cas9 disruption of TMEM150B demonstrated a decrease in autophagic flux on multiple independent experiments. Interestingly, this was not always the case in every replicate suggesting that cellular nutrient status might be complicating the results. Unexpectedly, no differences were observed in localization or number of autophagy associated structures (phagophores, autophagosomes, autolysosomes) in TMEM150B CRISPR knock-out cells (Mrschtik and Ryan, 2016; Mrschtik et al., 2015).

Endogenous TMEM150B knock-out did not have an effect on cell viability, but TMEM150B overexpression under starved conditions increased clonogenic survival in an autophagy-independent (did not change upon chloroquine treatment) and apoptotic-independent (differences not seen in Annexin V-positive, PI-negative staining) manner hence leading the authors to predict that TMEM150B may be involved in necrotic cell death (Mrschtik and Ryan, 2016; Mrschtik et al., 2015).

In summation, TMEM150B is differentially regulated from DRAM1, localizes to multiple cellular compartments, including autolysosomes and the plasma membrane, has an impact on autophagic flux and promotes cell survival in an autophagy-independent manner after glucose starvation. Further investigation into the role of TMEM150B in cell survival and in autophagy regulation is required for a better functional understanding of this gene.

TMEM150C/DRAM4/TTN3

TMEM150C has not yet been implicated in autophagy modulation. TMEM150C has been implicated in conferring mechanosensitive currents in proprioceptive neurons and referred to as Tentonin 3 (TTN3) (Hong et al., 2016, 2017).

TMEM150C was identified by a screen that demonstrated its knockdown caused a decrease in the amplitude of slow adapting muscle efferents (SA) after mechanical stimuli (Hong et al., 2016, 2017). Recombinant, exogenous TMEM150C expression localized to the plasma membrane in HEK293T cells and also demonstrated significantly higher peak current amplitudes after mechanical activation compared to cells expressing exogenous GFP, TMEM150A and TMEM150B (Hong et al., 2016).

Results from *TMEM150C* siRNA knockdown in dorsal root ganglion (DRG) neurons and *TMEM150C* knockout mice suggest that TMEM150C is required for SA currents in DRG neurons (Hong et al., 2016). TMEM150C is found in myelinated neurons and proprioceptive sensory neurons and *TMEM150C* knockout mice have significantly noticeable motor coordination deficiencies, suggesting a role in proprioception and motor coordination (Hong et al., 2016).

TMEM150A/TM6P1/DRAM5

In 2000, *TMEM150A* (originally named *TM6P1*), was identified from a subtracted cDNA library screen of liver transcripts from fasted rats (Zhang et al., 2000). *TMEM150A* is predicted to encode an integral membrane protein with six transmembrane domains, three N-glycosylation sites, three casein kinase II phosphorylation sites, and seven N-myristoylation

sites. Two protein isoforms are predicted with translation likely initiating frequently from a second start codon that conforms to the Kozak consensus sequence for translation initiation (Kozak, 1991; Zhang et al., 2000). Initiation at the second start codon would eliminate the amino-terminal transmembrane domain, resulting in a five-transmembrane domain protein. Zhang et al report that a recombinant TMEM150A-GFP fusion protein localized to the perinuclear region, the Golgi apparatus and the plasma membrane (Zhang et al., 2000).

Northern blot analysis demonstrated that *TMEM150A* expression was significantly upregulated two-fold in the liver of fasted rats and nearly ubiquitously expressed across fourteen different rat tissues examined (Zhang et al., 2000). Highest transcript levels of *TMEM150A* were observed in placenta, liver, and kidney; lowest levels were observed in brain and thymus (Zhang et al., 2000).

Since this initial report by Zhang et al, only one report has been published regarding TMEM150A function. Chung, et al propose that TMEM150A is the mammalian homologue of yeast Sfk1 and involved with plasma membrane interactions of phosphatidylinositol kinase III alpha (PI4KIIIα) (Chung et al., 2015). (Phosphatidylinositols and their kinases are vital to a myriad of cellular processes including membrane trafficking, immunity and mRNA processing and are discussed in slightly more detail in the next section) (De Craene et al., 2017; Dornan et al., 2016; Kagan and Medzhitov, 2006).

Previous work has demonstrated that PI4KIIIα (Stt4 in yeast) localizes to the plasma membrane in a complex that includes two adaptor proteins, EFR3 and TTC7 (Ypp1 in yeast). However, the yeast regulator of Stt4 (PI4KIIIα in mammals), Sfk1, had not been identified in mammals (Chung et al., 2015). Yeast Sfk1 is a multipass transmembrane protein that

localizes to the plasma membrane and thus the investigators decided to evaluate if TMEM150a may be behaving in a manner similar to yeast Sfk1 (Chung et al., 2015).

Recombinant, carboxy-terminally tagged TMEM150A-GFP was localized predominantly in the plasma membrane of mammalian cells with a minor pool localized on intracellular structures; the carboxy-terminal tail was determined to be within the cytoplasm (Chung et al., 2015) confirming the membrane topography of TMEM150A predicted by (Chung et al., 2015; Zhang et al., 2000). Western blot analysis indicated that TMEM150A but not TMEM150B (DRAM3) or TMEM150C (DRAM4) coprecipitated with PI4KIIIα and that the carboxy-terminal cytoplasmic tail of TMEM150A is mediates this interaction (Chung et al., 2015).

PI4KIIIα was only targeted to the plasma membrane when overexpression of TMEM150A was coexpressed with EFR3 and TTC7 (Chung et al., 2015). Overexpression of TMEM150A alone as well as TMEM150A siRNA mediated knock down did not change localization patterns of PI4KIIIα, EFR3 or TTC7 (Chung et al., 2015). Yet, immunoprecipitation and immunofluorescent studies demonstrated that TMEM150A interacts directly with both PI4KIIIα and EFR3 but not TTC7, suggesting that the interaction of TMEM150A with PI4KIIIα and EFR3 is mutually exclusive from the interaction of PI4KIIIα, EFR3 and TTC7 (Chung et al., 2015).

Both TMEM150A overexpression and knockdown did not change the total levels of PI(4)P (the direct target of PI4KIIIα phosphorylation) or PI(4,5)P_2 (the upstream metabolite of PI(4)P) (Chung et al., 2015). However, in a model of PI(4,5)P_2 resynthesis after depletion

that requires PI(4)P generation by PI4KIIIα, TMEM150A overexpression accelerated $PI(4,5)P_2$ recovery. Conversely knock-down of TMEM150A decreased the resynthesis recovery rate (Chung et al., 2015).

Altogether, the data from Chung et al. substantiates that TMEM150a may be the mammalian homologue of yeast Sfk1 which plays a role in interactions with PI4KIIIα (Chung et al., 2015).

PI4KIIIα: A Primer

Phosphoinositides (PI) are lipids that are crucial to normal cellular function as their roles include a key part in membrane trafficking, organelle physiology and identity, cellular growth, and as second messengers in cell membrane signaling (Balla, 2013; Dornan et al., 2016). PI(4)P is most-well known as a biosynthetic precursor to phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$) (aka PIP_2) (D'Angelo et al., 2008; Dornan et al., 2016). $PI(4,5)P_2$ is fundamental to many cellular processes which includes the recruitment of TIR domain containing adaptor protein (TIRAP) to the TLR4 complex and hence crucial to proper and effective TLR4 activation (Kagan and Medzhitov, 2006; Tan and Kagan, 2014). Because of the fundamental roles that PI(4)P and $PI(4,5)P_2$ play in a multiplicity of cellular functions, the enzymes that regulate/phosphorylate them must be tightly controlled (D'Angelo et al., 2008; Dornan et al., 2016). The availability of PI(4)P is a limiting factor in the synthesis of $PI(4,5)P_2$ (D'Angelo et al., 2008). PI4KIIIα generates PI(4)P by phosphorylation of phosphatidylinositol at the 4-position and is thereby a critical enzyme upstream of PI(4)P and $PI(4,5)P_2$ (Dornan et al., 2016).

Rationale

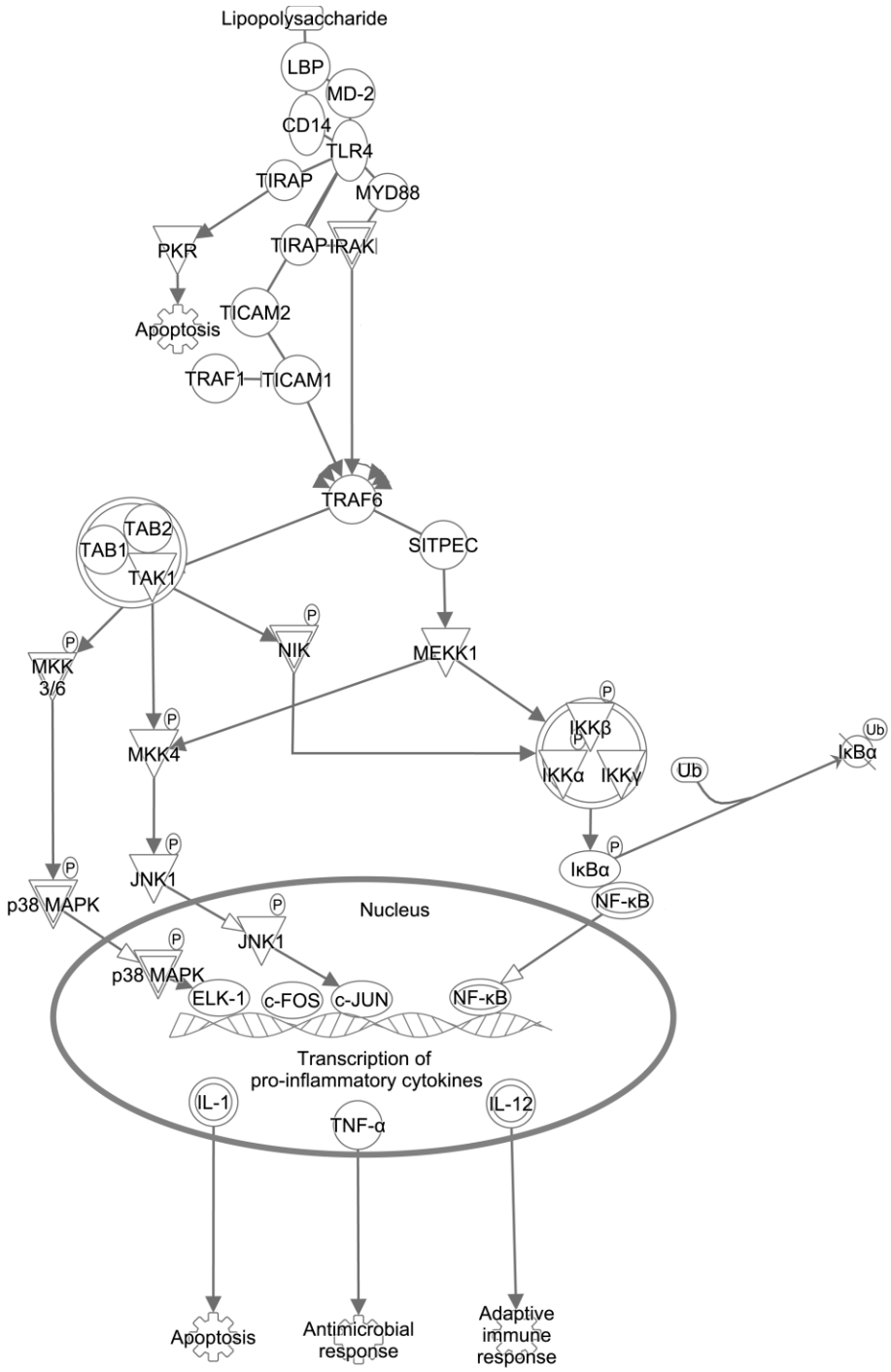
TMEM150A has been linked to the TLR4 pathway in two ways. First, by being related to DRAM1 which has a direct connection to the TLR4-mediated immune response to LPS. Second, because TMEM150A plays a role in recruitment of PI4KIIIα and thus could be an indirect component that limits $PI(4,5)P_2$ availability to enrich the TLR4 complex after LPS activation. Thus, we set out to investigate the role that TMEM150A might play in the TLR4 signal transduction pathway by monitoring the production and expression of cytokines normally induced by TLR4 activation.

Table 1.1. Phases and associated proteins of the autophagic pathway*

AUTOPHAGY STAGE	STRUCTURE	KEY ASSOCIATED PROTEINS
INITIATION	Phagophore Assembly Site	ULK1-ATG13-FIP200-ATG101 (aka mTOR substrate complex)
NUCLEATION	Phagophore	BECN1-ATG14-VPS15-VPS34 (Beclin 1 class PI3K complex)
EXPANSION	Autophagosome	ATG5-ATG12-ATG16L and LC3II
NUTRIENT RECYCLING	Autolysosome	LAMP2A

***Information adapted from Choi, 2013**

FIGURE 1.1 The TLR4 signal transduction pathway induces cytokine production after activation by LPS. In the most well-studied model, LPS is sensed by a complex formed by the combination of CD14, MD-2 and TLR4. After LPS binds TLR4, subsequent recruitment of adaptor proteins like those of MyD88 and TIRAP lead to NFkB nuclear translocation and the production of proinflammatory cytokines. Enrichment of PI(4,5)P2 in actin-rich membrane ruffles helps target and recruit TIRAP (not shown) (Tan and Kagan, 2014). Figure created by Ingenuity Pathway Analysis via Qiagen.


Lipopolysaccharide
LBP
MD-2
CD14
TLR4
TIRAP
MYD88
PKR
TIRAP
IRAK
Apoptosis
TICAM2
TRAF1
TICAM1
TRAF6
TAB2
TAB1
TAK1
SITPEC
MKK 3/6
NIK
MEKK1
MKK4
IKKβ
IKKα
IKKγ
Ub
IκBα
JNK1
IκBα
p38 MAPK
NF-κB
Nucleus
JNK1
p38 MAPK
ELK-1
c-FOS
c-JUN
NF-κB
Transcription of pro-inflammatory cytokines
IL-1
TNF-α
IL-12
Apoptosis
Antimicrobial response
Adaptive immune response

References

Aguilar-Valles, A., Inoue, W., Rummel, C., and Luheshi, G.N. (2015). Obesity, adipokines and neuroinflammation. Neuropharmacology *96*, 124–134.

Baliwag, J., Barnes, D.H., and Johnston, A. (2015). Cytokines in psoriasis. Cytokine *73*, 342–350.

Balla, T. (2013). Phosphoinositides: tiny lipids with giant impact on cell regulation. Physiol. Rev. *93*, 1019–1137.

Cadwell, K. (2016). Crosstalk between autophagy and inflammatory signalling pathways: balancing defence and homeostasis. Nat. Rev. Immunol. *16*, 661–675.

Choi, A.M.K., Ryter, S.W., and Levine, B. (2013). Autophagy in human health and disease. N. Engl. J. Med. *368*, 1845–1846.

Chung, J., Nakatsu, F., Baskin, J.M., and De Camilli, P. (2015). Plasticity of PI4KIIIα interactions at the plasma membrane. EMBO Rep. *16*, 312–320.

Cordani, M., Butera, G., Pacchiana, R., and Donadelli, M. (2017). Molecular interplay between mutant p53 proteins and autophagy in cancer cells. Biochim. Biophys. Acta *1867*, 19–28.

Crighton, D., Wilkinson, S., O'Prey, J., Syed, N., Smith, P., Harrison, P.R., Gasco, M., Garrone, O., Crook, T., and Ryan, K.M. (2006). DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. Cell *126*, 121–134.

Crighton, D., Wilkinson, S., and Ryan, K.M. (2007a). DRAM links autophagy to p53 and programmed cell death. Autophagy *3*, 72–74.

Crighton, D., O'Prey, J., Bell, H.S., and Ryan, K.M. (2007b). p73 regulates DRAM-independent autophagy that does not contribute to programmed cell death. Cell Death Differ. *14*, 1071–1079.

Criollo, A., Chereau, F., Malik, S.A., Niso-Santano, M., Mariño, G., Galluzzi, L., Maiuri, M.C., Baud, V., and Kroemer, G. (2012). Autophagy is required for the activation of NFκB. Cell Cycle *11*, 194–199.

D'Angelo, G., Vicinanza, M., Di Campli, A., and De Matteis, M.A. (2008). The multiple roles of PtdIns(4)P -- not just the precursor of PtdIns(4,5)P2. J. Cell Sci. *121*, 1955–1963.

De Craene, J.-O., Bertazzi, D.L., Bär, S., and Friant, S. (2017). Phosphoinositides, Major Actors in Membrane Trafficking and Lipid Signaling Pathways. Int. J. Mol. Sci. *18*.

Delgado, M.A., and Deretic, V. (2009). Toll-like receptors in control of immunological autophagy. Cell Death Differ. *16*, 976–983.

Delgado, M.A., Elmaoued, R.A., Davis, A.S., Kyei, G., and Deretic, V. (2008). Toll-like receptors control autophagy. EMBO J. *27*, 1110–1121.

Dinarello, C.A. (2000). Proinflammatory cytokines. Chest *118*, 503–508.

Dornan, G.L., McPhail, J.A., and Burke, J.E. (2016). Type III phosphatidylinositol 4 kinases: structure, function, regulation, signalling and involvement in disease. Biochem. Soc. Trans. *44*, 260–266.

Harris, J. (2011). Autophagy and cytokines. Cytokine *56*, 140–144.

Hayat, M.A. (2013). Autophagy: Cancer, Other Pathologies, Inflammation, Immunity, Infection, and Aging: Volume 3 - Role in Specific Diseases (Academic Press).

Hennessy, E.J., Parker, A.E., and O'Neill, L.A.J. (2010). Targeting Toll-like receptors: emerging therapeutics? Nat. Rev. Drug Discov. *9*, 293–307.

Hong, G.-S., Lee, B., Wee, J., Chun, H., Kim, H., Jung, J., Cha, J.Y., Riew, T.-R., Kim, G.H., Kim, I.-B., et al. (2016). Tentonin 3/TMEM150c Confers Distinct Mechanosensitive Currents in Dorsal-Root Ganglion Neurons with Proprioceptive Function. Neuron *91*, 708–710.

Hong, G.-S., Lee, B., and Oh, U. (2017). Evidence for Mechanosensitive Channel Activity of Tentonin 3/TMEM150C. Neuron *94*, 271–273.e2.

Humbert, M., Mueller, C., Fey, M.F., and Tschan, M.P. (2012). Inhibition of damage-regulated autophagy modulator-1 (DRAM-1) impairs neutrophil differentiation of NB4 APL cells. Leuk. Res. *36*, 1552–1556.

Johansen, T., and Lamark, T. (2011). Selective autophagy mediated by autophagic adapter proteins. Autophagy *7*, 279–296.

Kagan, J.C., and Medzhitov, R. (2006). Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. Cell *125*, 943–955.

Kerley-Hamilton, J.S., Pike, A.M., Hutchinson, J.A., Freemantle, S.J., and Spinella, M.J. (2007). The direct p53 target gene, FLJ11259/DRAM, is a member of a novel family of transmembrane proteins. Biochim. Biophys. Acta *1769*, 209–219.

Kirkegaard, K., Taylor, M.P., and Jackson, W.T. (2004). Cellular autophagy: surrender, avoidance and subversion by microorganisms. Nat. Rev. Microbiol. *2*, 301–314.

Kozak, M. (1991). Structural features in eukaryotic mRNAs that modulate the initiation of translation. J. Biol. Chem. *266*, 19867–19870.

Li, Y.-Y., Ishihara, S., Aziz, M.M., Oka, A., Kusunoki, R., Tada, Y., Yuki, T., Amano, Y., Ansary, M.U., and Kinoshita, Y. (2011). Autophagy is required for toll-like receptor-mediated interleukin-8 production in intestinal epithelial cells. Int. J. Mol. Med. *27*, 337–344.

Lu, Y.-C., Yeh, W.-C., and Ohashi, P.S. (2008). LPS/TLR4 signal transduction pathway. Cytokine *42*, 145–151.

Meijer, A.H., and van der Vaart, M. (2014). DRAM1 promotes the targeting of mycobacteria to selective autophagy. Autophagy *10*, 2389–2391.

Mrschtik, M., and Ryan, K.M. (2016). Another DRAM involved in autophagy and cell death. Autophagy *12*, 603–605.

Mrschtik, M., O'Prey, J., Lao, L.Y., Long, J.S., Beaumatin, F., Strachan, D., O'Prey, M., Skommer, J., and Ryan, K.M. (2015). DRAM-3 modulates autophagy and promotes cell survival in the absence of glucose. Cell Death Differ. *22*, 1714–1726.

O'Prey, J., Skommer, J., Wilkinson, S., and Ryan, K.M. (2009). Analysis of DRAM-related proteins reveals evolutionarily conserved and divergent roles in the control of autophagy. Cell Cycle *8*, 2260–2265.

Park, S.-M., Kim, K., Lee, E.-J., Kim, B.-K., Lee, T.J., Seo, T., Jang, I.-S., Lee, S.-H., Kim, S., Lee, J.-H., et al. (2009). Reduced expression of DRAM2/TMEM77 in tumor cells interferes with cell death. Biochem. Biophys. Res. Commun. *390*, 1340–1344.

Saitoh, T., and Akira, S. (2010). Regulation of innate immune responses by autophagy-related proteins. J. Cell Biol. *189*, 925–935.

Sánchez, D., Rojas, M., Hernández, I., Radzioch, D., García, L.F., and Barrera, L.F. (2010). Role of TLR2- and TLR4-mediated signaling in Mycobacterium tuberculosis-induced macrophage death. Cell. Immunol. *260*, 128–136.

Sanjuan, M.A., Dillon, C.P., Tait, S.W.G., Moshiach, S., Dorsey, F., Connell, S., Komatsu, M., Tanaka, K., Cleveland, J.L., Withoff, S., et al. (2007). Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. Nature *450*, 1253–1257.

Shi, C.-S., and Kehrl, J.H. (2008). MyD88 and Trif target Beclin 1 to trigger autophagy in macrophages. J. Biol. Chem. *283*, 33175–33182.

Takeda, K., and Akira, S. (2001). Regulation of innate immune responses by Toll-like receptors. Jpn. J. Infect. Dis. *54*, 209–219.

Takeda, K., and Akira, S. (2015). Toll-like receptors. Curr. Protoc. Immunol. *109*, 14.12.1–10.

Tan, Y., and Kagan, J.C. (2014). A cross-disciplinary perspective on the innate immune responses to bacterial lipopolysaccharide. Mol. Cell *54*, 212–223.

Tartour, E., and Fridman, W.H. (1998). Cytokines and cancer. Int. Rev. Immunol. *16*, 683–704.

van der Vaart, M., Korbee, C.J., Lamers, G.E.M., Tengeler, A.C., Hosseini, R., Haks, M.C., Ottenhoff, T.H.M., Spaink, H.P., and Meijer, A.H. (2014). The DNA damage-regulated autophagy modulator DRAM1 links mycobacterial recognition via TLR-MYD88 to autophagic defense [corrected]. Cell Host Microbe *15*, 753–767.

Wang, J., Feng, X., Zeng, Y., Fan, J., Wu, J., Li, Z., Liu, X., Huang, R., Huang, F., Yu, X., et al. (2013). Lipopolysaccharide (LPS)-induced autophagy is involved in the restriction of Escherichia coli in peritoneal mesothelial cells. BMC Microbiol. *13*, 255.

Whicher, J.T., and Evans, S.W. (1990). Cytokines in disease. Clin. Chem. *36*, 1269–1281.

Xu, Y., Jagannath, C., Liu, X.-D., Sharafkhaneh, A., Kolodziejska, K.E., and Eissa, N.T. (2007). Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. Immunity *27*, 135–144.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., et al. (2003). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science *301*, 640–643.

Yin, Z., Pascual, C., and Klionsky, D.J. (2016). Autophagy: machinery and regulation. Microb. Cell Fact. *3*, 588–596.

Yoon, J.-H., Her, S., Kim, M., Jang, I.-S., and Park, J. (2012). The expression of damage-regulated autophagy modulator 2 (DRAM2) contributes to autophagy induction. Mol. Biol. Rep. *39*, 1087–1093.

Zhang, J.-M., and An, J. (2007). Cytokines, inflammation, and pain. Int. Anesthesiol. Clin. *45*, 27–37.

Zhang, J., D'Ercole, A.J., and Underwood, L.E. (2000). Identification of a new gene (rat TM6P1) encoding a fasting-inducible, integral membrane protein with six transmembrane domains. Biochim. Biophys. Acta *1492*, 280–284.

Zhang, X.-D., Qi, L., Wu, J.-C., and Qin, Z.-H. (2013). DRAM1 regulates autophagy flux through lysosomes. PLoS One *8*, e63245.

Chapter 2: A Role for TMEM150A in Cytokine Regulation

Introduction

Phosphoinositides (PI) are lipids that are crucial to normal cellular function as their roles include a key part in membrane trafficking, organelle physiology and identity, cellular growth, and as second messengers in cell membrane signaling (Balla, 2013; Dornan et al., 2016). An important PI is phosphatidylinositol 4-phosphate (PI(4)P); PI(4)P is enriched in the golgi apparatus and is important to membrane trafficking between the golgi and the endoplasmic reticulum, has been implicated in regulation of phagolysosome maturation but is most well known as a biosynthetic precursor to phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$) (aka PIP_2) (D'Angelo et al., 2008; Dornan et al., 2016). $PI(4,5)P_2$ is critical in cell membrane dynamics that include phagocytosis, and cytoskeleton-membrane attachments (Czech, 2000; Raucher et al., 2000), in membrane trafficking like endocytosis and also in cell signaling (Czech, 2000). For example, $PI(4,5)P_2$ is essential for the proper activation of innate immune sensor, Toll-like Receptor 4 (TLR4) signal transduction because of its role in the mediation of recruitment of TIR domain containing adaptor protein (TIRAP) (Kagan and Medzhitov, 2006; Tan and Kagan, 2014). Because of the fundamental roles that PI(4)P and $PI(4,5)P_2$ play in a multiplicity of cellular functions, the enzymes that regulate/phosphorylate them must be tightly controlled (D'Angelo et al., 2008; Dornan et al., 2016). Phosphatidylinositol 4-kinase type III alpha (PI4KIIIα), also known as staurosporine and temperature sensitive 4 (Stt4) in yeast, generates PI(4)P by phosphorylation of

phosphatidylinositol at the 4-position (Dornan et al., 2016). In yeast, Stt4 is partially regulated by suppressor of four kinase protein 1 (Sfk1) (Dornan et al., 2016). It has recently been suggested that TMEM150A is the mammalian homologue of yeast Sfk1, because it plays a role in interactions with PI4KIIIα in mammalian cells (Chung et al., 2015).

TMEM150A, is related by homology to an evolutionarily conserved family of proteins termed damage-regulated autophagy modulators (DRAM) (O'Prey et al., 2009). The DRAM protein family consists of five proteins that are predicted to encode proteins with six-transmembrane domains with both the amino- and carboxy-termini positioned on the cytoplasmic side of cellular membranes (Hong et al., 2016; Kerley-Hamilton et al., 2007; O'Prey et al., 2009). DRAM1 is most well known as being a target gene of tumor suppressor p53 and an important factor in autophagy modulation (Crighton et al., 2006, 2007). Two other DRAM family members, DRAM2 and TMEM150B, are also implicated in the mediation of autophagy (Mrschtik et al., 2015; Yoon et al., 2012).

Autophagy ("self-eating") is a multifaceted, evolutionarily conserved, cellular homeostasis mechanism that is implicated in an array of cellular functions (Cadwell, 2016; Harris, 2011; Yin et al., 2016). Although autophagy is most well-known for cellular homeostatic degradation of long-lived organelles and cell survival processes that occur during times of starvation, autophagy has also been clearly linked to immune-related functions (Cadwell, 2016; Criollo et al., 2012; Harris, 2011; Li et al., 2011; Meijer and van der Vaart, 2014; Yin et al., 2016). In fact, it is now known that the engagement of Toll-like receptor 4 (TLR4) by the lipopolysaccharide (LPS) component of gram negative bacteria can induce autophagy (Delgado and Deretic, 2009; Delgado et al., 2008; Xu et al., 2007, 2008).

Recently it was demonstrated that DRAM1 can also influence autophagy downstream of TLR4/MyD88 signaling in a prosurvival response to infection (Meijer and van der Vaart, 2014; van der Vaart et al., 2014). To date, the other DRAM protein family members have not been implicated in immune-related functions.

We hypothesized that there might be a direct connection between TMEM150A and the TLR4 signalling pathway because of the following observations:

- The DRAM family of proteins has been implicated in autophagy, a functional output of the TLR4 pathway
- TMEM150A has been suggested to play a role in modifying the interactions of PI4KIIIα and is thus an upstream regulator of $PI(4,5)P_2$ which is well established to be a known player in the TLR4 pathway

This hypothesis is supported by the observation that TMEM150A is downregulated in human monocyte (peripheral blood mononuclear cells (PBMCs), THP-1 monocytes, U937 monocytes) and macrophage-like (THP-1 macrophages, U937 macrophages) cell lines after LPS exposure (Heffelfinger, thesis data not published) as well as in seven different gene expression analyses profiled on NCBI's Gene Omnibus (Edgar et al., 2002). Thus, we investigated the role that TMEM150A might play in the TLR4 signal transduction pathway by monitoring the production and expression of cytokines normally induced by TLR4 activation. Herein we provide the first evidence that the DRAM family member, TMEM150A, plays a key role in the regulation of cytokine production downstream of TLR4.

Results

In silico analysis of TMEM150A highlights a 6-transmembrane protein with strong evolutionary conservation

TMEM150A is linked by homology and synteny to the DRAM protein family (Figure 2.1C) (Saitou and Nei, 1987; Tamura et al., 2013). Similar to the other DRAM proteins, TMEM150A is a highly conserved gene encoding a Type III transmembrane protein that possesses six transmembrane domains (Figure 2.1A). Figure 2.1B illustrates the protein sequence alignments for human, mouse and zebrafish TMEM150A (Sievers and Higgins, 2014). The observed high homology indicates that there has been high selective pressure on TMEM150A to remain conserved over 450 million years of evolution. Three DRAM proteins have been linked to autophagy regulation and DRAM1 is directly linked to the immune-related arm of autophagy function downstream of TLR4. Yet, TMEM150A's role in immune function remains undefined. An *in silico* search investigating the relationship between *TMEM150A* expression and TLR4 provides evidence that LPS, the TLR4 agonist, can downregulate *TMEM150A* (not shown).

Activation of TLR4, in the absence of TMEM150A, leads to increased CXCL8 production

In order to investigate a possible link between LPS-induced immune modulation and TMEM150A function, we used human embryonic kidney cells, (HEK293), that stably express TLR4 and two cofactors necessary for LPS response—MD2, and CD14 ($HEK^{TLR4/MD2/CD14}$ cells, hereafter referred to as HEK^{TLR4}). This cell line is used to

independently study LPS-induced activation of TLR4 because other TLRs, including TLR2, are either not present or nonfunctional (Takeuchi et al., 1999). By combining siRNA techniques to knockdown TMEM150A with HEK^{TLR4} cells (Figure 2.2A) and challenging them with different concentrations of LPS ranging from 0 to 1000 ng/ml for 18 hours, we could assess how loss of TMEM150A affects TLR4 induced release of CXCL8 (Figure 2.2B). Interestingly, TMEM150A-deficient cells secreted significantly more CXCL8 protein across LPS concentrations (Figure 2.2B). Although this increase in CXCL8 secretion may reflect an increase in the release of CXCL8, it might also reflect increases in cytokine transcript and protein production.

In order to investigate if TMEM150A knockdown affects transcription of *CXCL8,* quantitative real-time PCR (qPCR) was employed with TMEM150A knockdown in HEK^{TLR4} cells. Changes in *CXCL8* transcript levels were observed in TMEM150A knockdown samples (Figure 2.2C). These data implicate TMEM150A in regulating *CXCL8* production at the transcript level. Importantly, ELISA-based experiments using HEK293 cells that do not stably express TLR4 failed to detect any CXCL8 production after LPS exposure (not shown). In sum, in the shortage of TMEM150A, activation of TLR4 in this model leads to significantly increased amounts of the chemokine CXCL8 when compared to control cells. Coupled with the transcriptional response data, and the lack of other functional TLR receptors in this cell type, this demonstrates that TMEM150A plays a specific role in the the production of cytokines via the TLR4 signaling pathway.

Knockdown of TMEM150A in human lung epithelial cells causes increased protein and transcript levels of multiple cytokines

In order to determine if the changes in cytokine levels after knockdown of TMEM150A were limited to HEK^{TLR4} cells and/or limited to CXCL8 cytokine production, we evaluated if knockdown of TMEM150A would have a more global response and affect additional cytokines in a lung carcinoma epithelial cell line, NCI-H292s (H292). Since H292 cells originate from barrier-type epithelium at the interface of the interstitium and vasculature as well as the outside air and inhalants, H292s are more endogenously equipped to respond to immune stimuli in comparison to HEK cells. Because the epithelium of the lung is an area of trafficking between vital nutrients and microbes, and is thus in constant contact with potential pathogens, H292 lung epithelial cells act as a model for this important barrier where cytokine production and control are critical and are known to secrete cytokines like CXCL8, CCL5(Rantes), and IL6 (Kato and Schleimer, 2007). Additional cytokine transcripts, including IL7, IL10, IL12B, IFNg and TNF have been reported in airway epithelial cells (Adachi et al., 1997; Kato and Schleimer, 2007; Lam et al., 2011; Lee et al., 2010; Matsukura et al., 1996; Oyanagi et al., 2017). Therefore, we employed similar protocol for siRNA knockdown of TMEM150A and LPS exposure using H292 cells and assessed for a more universal cytokine response (Adachi et al., 1997; Lam et al., 2011; Matsukura et al., 1996). We quantified secretion of CXCL8, IL-6 and Rantes after LPS stimulation by ELISA (Figure 2.3A) and demonstrated that IL-6 and Rantes protein production was significantly higher after LPS exposure in cells lacking TMEM150A compared to untransfected control, vehicle control, and scrambled siRNA control cells. CXCL8 showed the same trend in H292 cells,

but with a more variable response. To address an array of additional cytokine protein levels we employed milliplex map technology. Figure 2.3B shows the average cytokine fold change for IL7, IL10, IL12B (which dimerizes with IL12a to produce IL12), IFNg and TNF and recapitulates the trend observed by ELISA for CXCL8, IL6 and Rantes. Increased levels of these cytokines were observed with the knockdown of TMEM150A and no LPS exposure, and exposure to LPS further increased cytokine levels. In contrast, granulocyte macrophage colony stimulating factor (GMCSF) production increased only after exposure to 100 and 300 ng/ml of LPS.

To investigate if these cytokine aberrations were the result of changes in transcript levels we employed a multiarray qPCR strategy (Figure 2.3C). At the transcript level, *Rantes, IL6, CXCL8* and *TNFa* showed similar expression profiles and reflected protein level results. TMEM150A-deficient H292 cells had an increase in transcripts of these cytokines compared to controls. No and low (30 ng/ml) levels of LPS stimulation elicited the highest transcript fold changes versus 100ng/ml LPS. *IL12a* transcripts did not mirror *IL12b* protein secretion changes. *IL12a* transcript levels of both TMEM150A-deficient cells and siRNA scrambled control cells were similar (Figure 2.3C). Overall, reduction of TMEM150A leads to an increase in both transcript and protein levels of multiple cytokines suggesting that TMEM150A plays a role upstream of cytokine transcription.

Knockdown of TMEM150A in human lung epithelial cells causes changes in the transcription factor activation profile

In order to determine if TMEM150A regulates transcription factor activity, we investigated how knockdown of TMEM150A affected the activity of NFkB and AP-1, two key transcription factors in the TLR4 pathway (Kaisho and Akira, 2001; Lu et al., 2008; Takeda and Akira, 2015; Takeuchi et al., 1999). Unexpectedly, in both HEK^{TLR4} cells and H292 cells, NFkB and AP1 activation, quantified by luciferase-based reporter assays, did not show significant differences compared to controls (data not shown). We then expanded the investigation of how TMEM150A may regulate transcription to include 48 different transcription factors using a transcription factor activation profiling array (Figure 2.4). Since it takes 24 hours for the siRNA to produce an observable reduction in TMEM150A, we evaluated transcription factor activation profiles at 24 and 32 hours post siRNA transfection. For this assay, a difference of a two-fold change between samples is considered significant (Signosis) and is shown on Table 2.2. At 24 hours post transfection, nine transcription factors showed significant activation in TMEM150A-impaired samples compared to control whereas fourteen showed decreased activation (Table 2.2). At 32 hours, nine transcription factors displayed greater than a two-fold change in activation with eleven transcription factors negatively affected regarding activation. P53, Glucocorticoid receptor/Progesterone receptor (GR/PR), and Myocyte enhancer factor 2 (MEF2) are more activated at both time points whereas Hypoxia inducible factor (HIF) is deactivated versus control at both time points. In contrast, Peroxisome proliferator-activated receptor (PPAR), SP1 transcription

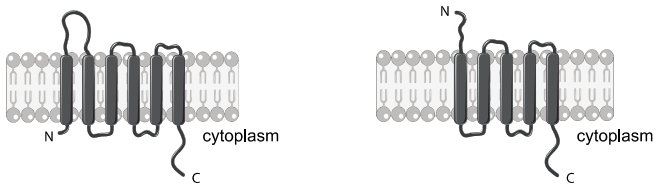
factor (SP1) and Signal transducer and activator of transcription 3 (STAT3) show greater activation at 24 hours yet decreased activation at 32 hours post transfection.

TMEM150A localizes to both the nucleus and punctate staining in the cytoplasm in epithelial cells

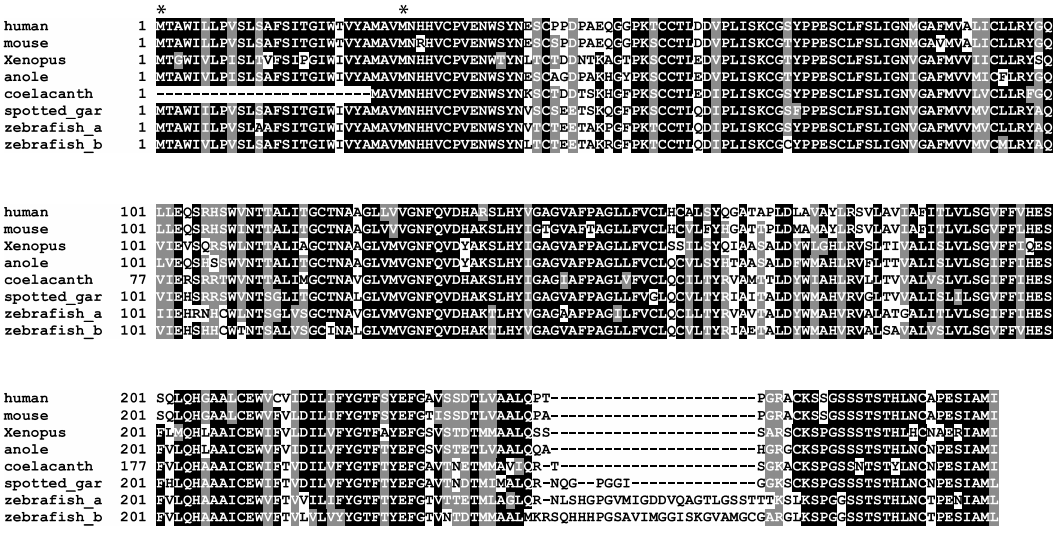
Knowing that knockdown of TMEM150A causes changes in transcription factor activation, interacts with PI4KIIIα and because to date only recombinant, GFP- or HA-tagged TMEM150A has been evaluated in transfected cells, we investigated the subcellular localization of endogenous TMEM150A within epithelial cells. In order to identify the cellular location of endogenous TMEM150A, we employed a TMEM150A-specific antibody (Figure 2.2A) for indirect immunofluorescence. We observed that TMEM150A localizes within and around the nucleus, with notable vacuoles and in a punctate pattern in the cytoplasm (Figure 2.5). This staining pattern was common among H292 lung epithelial cells, HEK^{TLR4} cells, and NIH3T3 mouse fibroblasts (not shown). This observation is in contrast to the localization of recombinant, exogenous TMEM150A (Chung et al., 2015; Zhang et al., 2000).

FIGURE 2.1. Sequence conservation of TMEM150A. (A) Alternative predicted protein structures of TMEM150A. Protein encoded by the initial start codon is on the left. Protein encoded by the second start codon is on the right. **(B) TMEM150A is highly conserved across species.** TMEM150A protein sequences from multiple vertebrate species were aligned by Clustal Omega (Sievers and Higgins, 2014). Methionines reflective of alternative start codons are indicated by asterisks. Identical residues are shaded black and structurally similar residues are shaded gray. **(C) Phylogenetic comparison of vertebrate TMEM150A protein sequences.** Sequences of human TMEM150B, TMEM150C, DRAM1 and DRAM2 are included for comparison. Tree was constructed with the Neighbor-Joining method (Saitou and Nei, 1987) and 2000 bootstrap replicates using MEGA6 (Tamura et al., 2013). Species include human, mouse (*Mus musculus*), Carolina anole (*Anolis carolinensi*), *Xenopus tropicalis*, West Indian Ocean coelacanth (*Latimeria chalumnae*), spotted gar (*Lepisosteus oculatus*) and zebrafish (*Danio rerio*). Sequence Genbank identification numbers are included in panel C.

A.


N
cytoplasm
C
N
cytoplasm
C

B.


human
mouse
Xenopus
anole
coelacanth
spotted_gar
zebrafish_a
zebrafish_b

C.

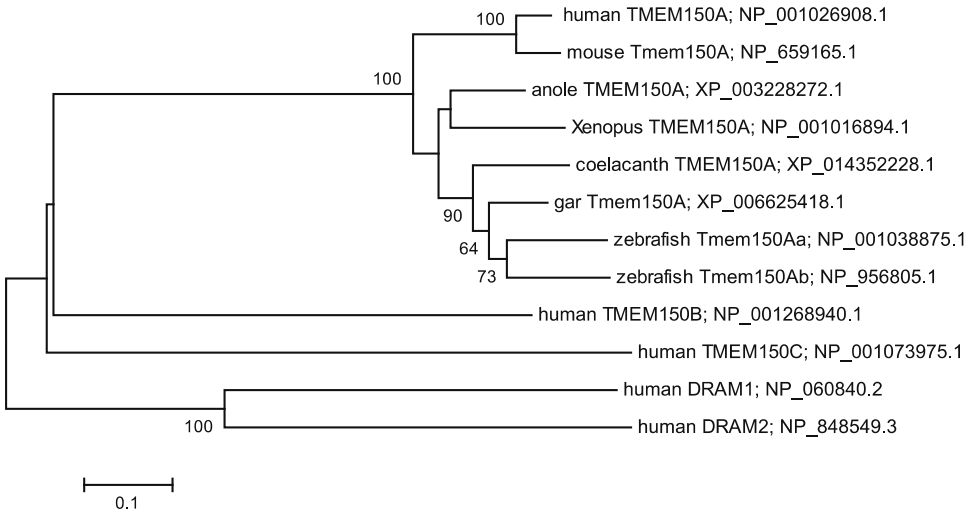

100
human TMEM150A; NP_001026908.1
mouse Tmem150A; NP_659165.1
100
anole TMEM150A; XP_003228272.1
Xenopus TMEM150A; NP_001016894.1
coelacanth TMEM150A; XP_014352228.1
90
gar Tmem150A; XP_006625418.1
64
zebrafish Tmem150Aa; NP_001038875.1
73
zebrafish Tmem150Ab; NP_956805.1
human TMEM150B; NP_001268940.1
human TMEM150C; NP_001073975.1
human DRAM1; NP_060840.2
100
human DRAM2; NP_848549.3
0.1

FIGURE 2.2. Activation of TLR4, in the absence of TMEM150A, leads to increased CXCL8 production (**A) Endogenous TMEM150A protein levels are knocked down by TMEM150A siRNA.** HEK^{TLR4} cells were not transfected or transfected with scrambled siRNA or TMEM150A siRNA. Knockdown of protein was evaluated by Western blot with anti-TMEM150a antibody. **(B) Silencing of endogenous TMEM150a increases CXCL8 protein production after TLR4 ligand stimulation.** HEK^{TLR4} cells were not transfected or transfected with TMEM150A siRNA or controls and allowed to rest for 30 hours. Media was replaced and cells stimulated for 18 hours with 0, 30, 100, 300, or 1000 ng/ml of LPS. Supernatant was then collected and CXCL8 protein levels quantified by Milliplex technology. Fold change was calculated based on protein levels in non-transfected, 0 ng/ml LPS treated cell supernatant. Results were confirmed by ELISA. ELISA graph is representative of three biological replicates. **(C) Silencing of endogenous TMEM150A results in an increase in *CXCL8* transcripts.** HEK^{TLR4} cells were treated as described above but 18 hours after LPS exposure, RNA was extracted and *CXCL8* transcript levels measured by qPCR. Relative *CXCL8* levels were normalized to levels of *β actin* and fold-change calculated based on non-transfected 0ng/ml LPS samples using the $2^{-\Delta\Delta Ct}$ method (LIVAK and SCHMITTGEN 2001).

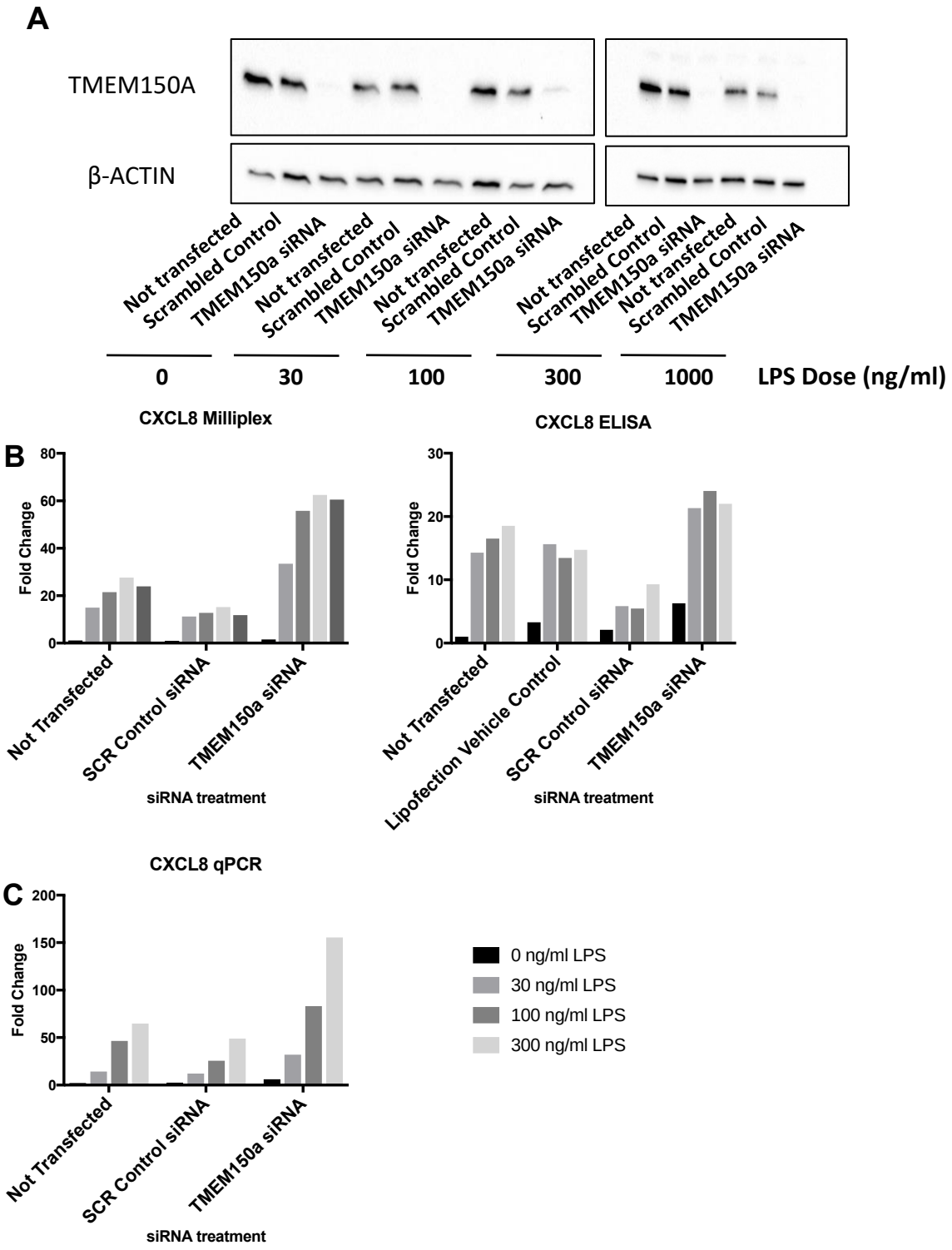
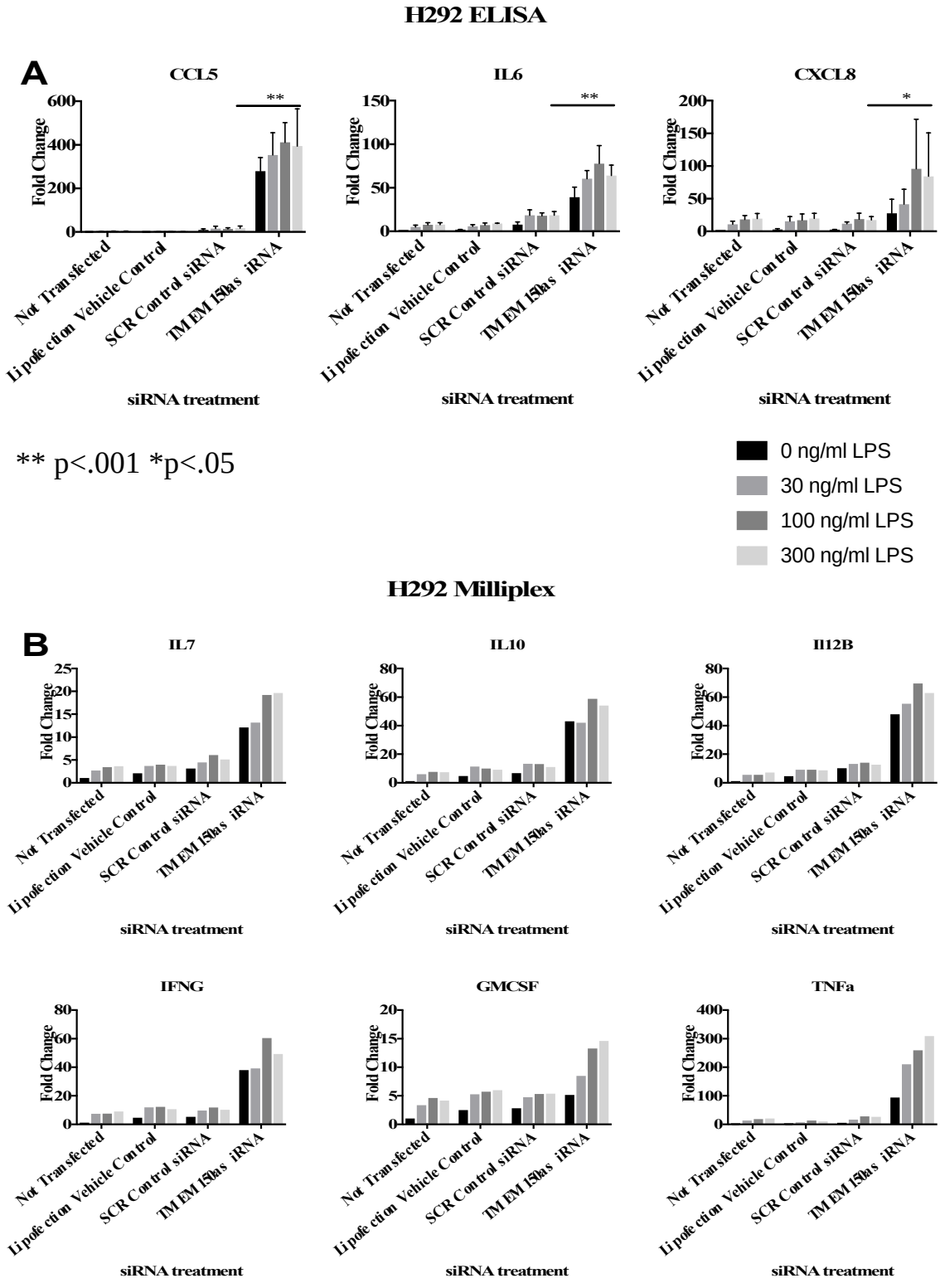
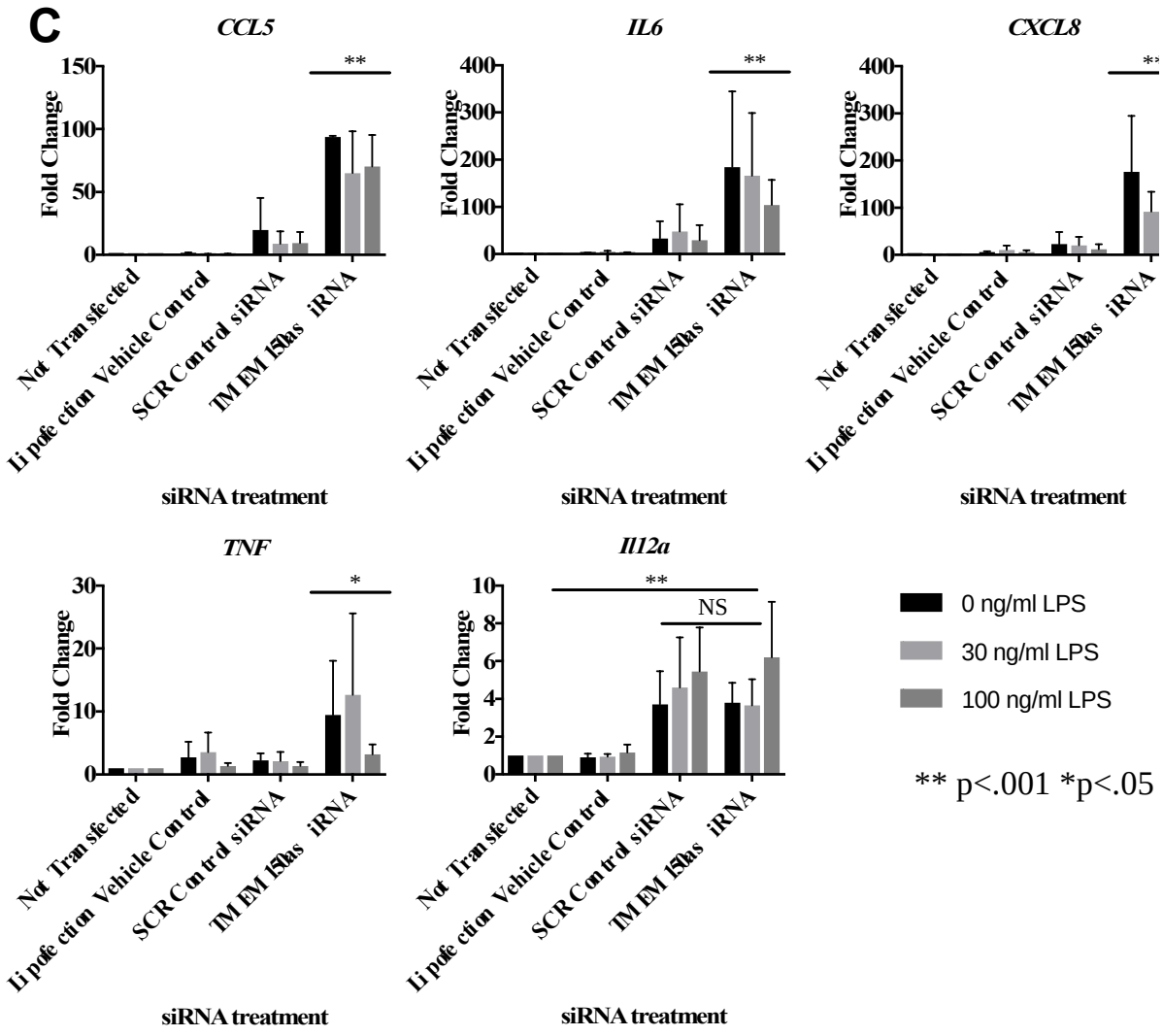

A
TMEM150A
β-ACTIN
Not transfected
Scrambled Control
TMEM150a siRNA
0
30
100
300
1000
LPS Dose (ng/ml)
B
CXCL8 Milliplex
Fold Change
Not Transfected
SCR Control siRNA
TMEM150a siRNA
siRNA treatment
CXCL8 ELISA
Lipofection Vehicle Control
C
CXCL8 qPCR
0 ng/ml LPS
30 ng/ml LPS
100 ng/ml LPS
300 ng/ml LPS

FIGURE 2.3. TMEM150a silencing affects cytokine regulation in human mucoepidermoid pulmonary carcinoma cell line (H292) (A) TMEM150a knockdown results in increased protein production of multiple cytokines. H292 cells were not transfected or transfected with TMEM150a siRNA or controls and allowed to rest. Media was replaced and cells stimulated with either 0, 30, 100, or 300 ng/ml of LPS. Supernatant was collected after 18 hours of LPS stimulation (48 hours post siRNA transfection), and cells were counted. Cytokine protein production was quantified by ELISA and normalized to cell counts. Fold change was calculated using not transfected 0 ng/ml LPS cell supernatant as baseline. ELISA graphs are the average of three biological replicates. Error bars represent standard error of the mean (SEM). Statistical analyses were performed by two-factor ANOVA testing significance and interaction of siRNA and LPS. TMEM150a siRNA treated samples, independent of LPS exposure, produced significantly more cytokines than controls (**P<0.001 *P<.05). **(B)** H292 cells were treated as described above and supernatant was quantified by Milliplex map technology for multiple cytokines. Values represent the average of two biological replicates. **(C) Cytokine transcript levels are affected by TMEM150a knockdown.** H292 cells were treated as described above and RNA extracted 18 hours after LPS stimulation (48 hours post siRNA transfection). Transcript levels were normalized to *GAPDH* and fold change was calculated using the $2^{-\Delta\Delta Ct}$ method. Figures are the average of three biological replicates. Error bars reflect SEM. Statistical analyses were performed by two-way ANOVA. With the exception of *IL12a*, TMEM150a siRNA treated samples displayed significantly higher cytokine transcript levels than all controls (**P<0.001

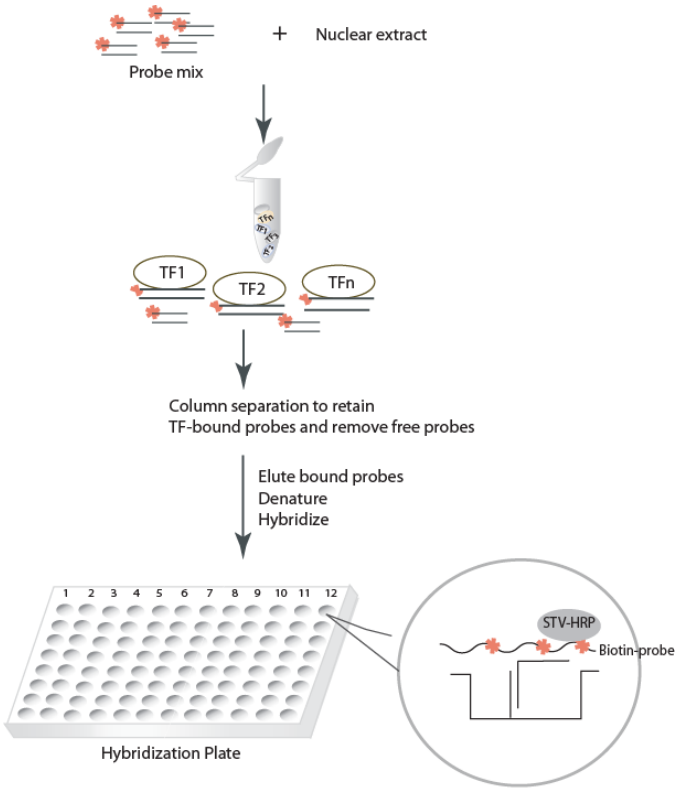
FIGURE 2.3 (Continued)
*P<.05). *IL12a* transcript levels were increased by both TMEM150A siRNA and the scrambled (SCR) control siRNA.


H292 ELISA
A
CCL5
IL6
CXCL8
Fold Change
siRNA treatment
Not Transfected
Lipofection Vehicle Control
SCR Control siRNA
TMEM150as iRNA
** p<.001 *p<.05
0 ng/ml LPS
30 ng/ml LPS
100 ng/ml LPS
300 ng/ml LPS
H292 Milliplex
B
IL7
IL10
Il12B
IFNG
GMCSF
TNFa

H292 qPCR


C
CCL5
IL6
CXCL8
TNF
Il12a
Fold Change
siRNA treatment
Not Transfected
Lipofection Vehicle Control
SCR Control siRNA
TM EM 150as iRNA
**
*
NS
0 ng/ml LPS
30 ng/ml LPS
100 ng/ml LPS
** p<.001 *p<.05

A



B

	1	2	3	4	5	6	7	8	9	10	11	12
A	AP1	CDP	GATA	NF-1	Pit	Stat3	AP1	CDP	GATA	NF-1	Pit	Stat3
B	AP2	CREB	GR/PR	NFAT	PPAR	Stat4	AP2	CREB	GR/PR	NFAT	PPAR	Stat4
C	AR	E2F-1	HIF	NF-E2	PXR	Stat5	AR	E2F-1	HIF	NF-E2	PXR	Stat5
D	ATF2	EGR	HNF4	NFkB	SMAD	Stat6	ATF2	EGR	HNF4	NFkB	SMAD	Stat6
E	Brn-3	ER	IRF	OCT4	Sp1	TCF/LEF	Brn-3	ER	IRF	OCT4	Sp1	TCF/LEF
F	C/EBP	Ets	MEF2	p53	SRF	TFIID	C/EBP	Ets	MEF2	p53	SRF	TFIID
G	CAR	FAST-1	Myb	Pax-5	SATB1	TR	CAR	FAST-1	Myb	Pax-5	SATB1	TR
H	CBF	GAS/ISRE	Myc-Max	Pbx1	Stat1	YY1	CBF	GAS/ISRE	Myc-Max	Pbx1	Stat1	YY1

Figure 2.4. Transcription Factor (TF) Activation Profiling Method (reprinted from manufacturer's manual) (Signosis, CA). **(A)** In brief, nuclear extracts are incubated with biotin labeled probes (which correspond to TF DNA binding sites) to create TF-DNA/Probe complexes. Unbound probes are removed and bound complexes are eluted and then assessed using a hybridization plate pre-coated with complimentary sequences. Strepavidin-HRP binds biotin with high affinity and relative light units (RLUs) are measured on a luminometer. **(B)** 48 TFs assessed comparing TMEM150a siRNA and scrambled siRNA control nuclear extract samples. Image reflects layout in 96-well plate.

TABLE 2.1. Knockdown of TMEM150A in human lung epithelial cells causes changes in the transcription factor activity profile

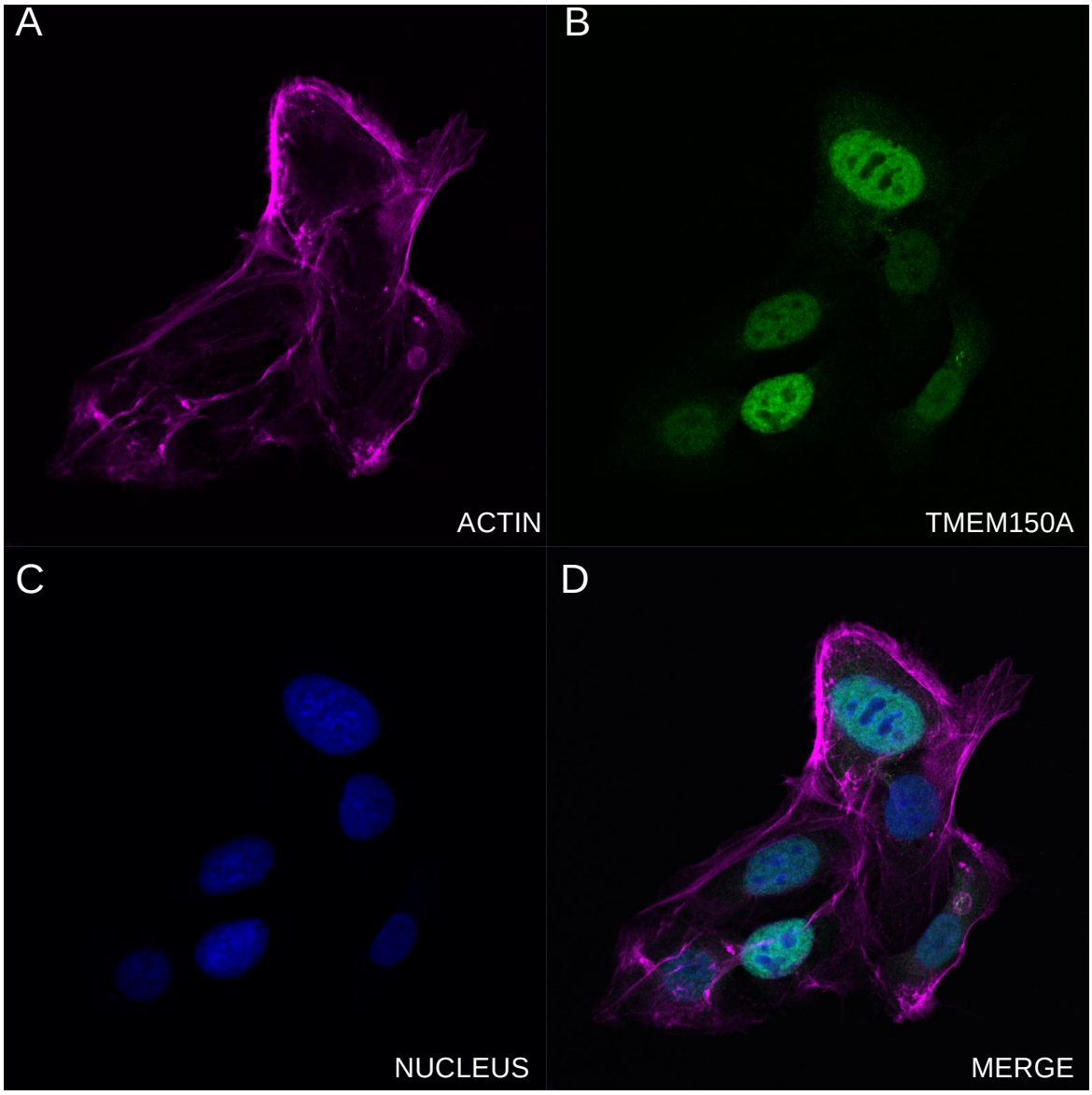
Transcription Factor Symbol	TF Description*	24 h Fold Change**	32 h Fold Change**
AP1	Activator protein 1 (JUN/FOS)	-1.11	1.58
AP2	Activator protein 2	1.40	**-2.98**
AR	Androgen receptor	1.22	1.73
ATF2	activating transcription factor 2	-1.55	1.15
Brn-3	POU domain, class 4, transcription factor 1	**-2.15**	1.83
C/EBP	CCAAT/enhancer binding protein (C/EBP),alpha	**-3.21**	**2.75**
CAR	nuclear receptor subfamily 1, group I, member 3	**2.14**	-1.82
CBF	CCAAT/enhancer binding protein (C/EBP), zeta	**3.81**	**2.69**
CDP	cut-like homeobox 1; CCAAT displacement protein	-1.37	**4.09**
CREB	cAMP responsive element binding protein 1	1.69	**3.18**
E2F-1	E2F transcription factor 1	-1.98	-1.18
EGR	Early growth response	-1.37	1.04
ER	Estrogen receptor	-1.16	**-2.86**
Ets	v-ets erythroblastosis virus E26 oncogene homolog 1	**-11.36**	-1.15
FAST-1(FOXH1)	Forkhead box H1	1.36	-1.24
GAS/ISRE	IFN-stimulated response element combing GAS and ISRE element	**-3.67**	**4.71**
GATA	GATA transcription factor	**2.00**	-1.08
GR/PR	Glucocorticoid receptor/progesterone receptor	**5.86**	**18.17**
HIF	Hypoxia inducible factor	**-32.48**	-1.60
HNF4	Hepatocyte nuclear factor 4	1.28	**-2.05**
IRF	Interferon regulatory factor	**-6.79**	**-2.78**
MEF2	Myocyte enhancer factor 2	**7.22**	**10.58**
Myb	v-myb myeloblastosis viral oncogene homolog	**-5.24**	-1.21
Myc-Max	v-myc myelocytomatosis viral oncogene homolog (avian)	**4.21**	1.51
NF-1	Nuclear factor 1	1.01	**-3.23**
NFAT	Nuclear factor of activated T-cells	**-4.13**	1.27
NF-E2	Nuclear factor (erythroid-derived 2)	1.83	-9.11
NFkB	nuclear factor of kappa light polypeptide gene	1.26	-1.88
OCT4	POU class 5 homeobox 1	1.38	-1.18
p53	Tumor protein p53	**4.64**	**44.88**
Pax-5	Paired box 5	-1.28	1.59
Pbx1	Pre-B cell leukemia transcription factor-1	**-3.63**	**-2.43**
Pit	Pituitary specific transcription factor 1	-1.67	1.77
PPAR	Peroxisome proliferator-activated receptor	**2.12**	**-25.36**

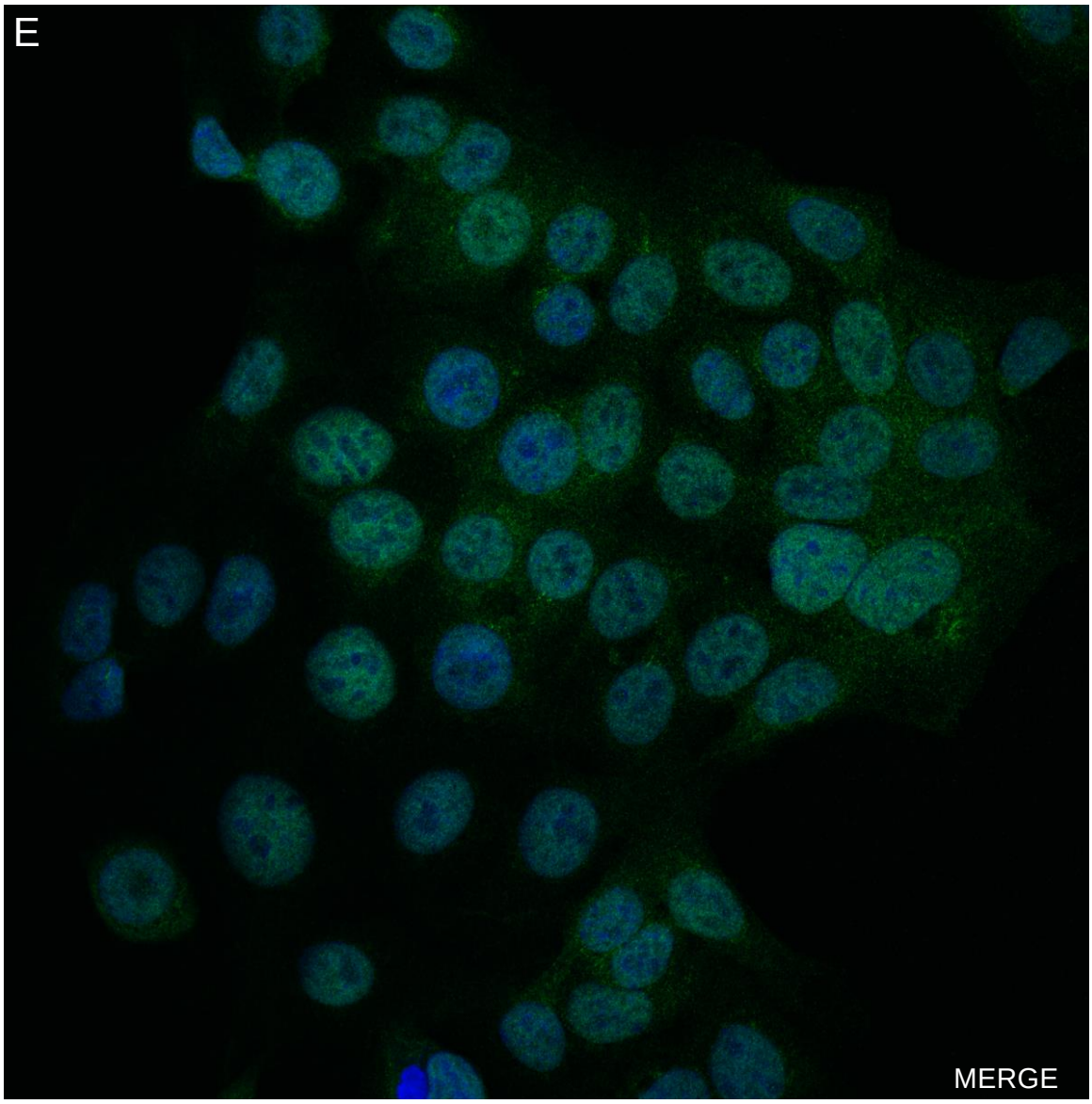
TABLE 2.1. (Continued)

Transcription Factor Symbol	TF Description*	24 h Fold Change**	32 h Fold Change**
PXR	Steroid and xenobiotic receptor	**-6.31**	1.00
SMAD (MADH)	SMAD family	**-2.09**	**-3.00**
Sp1	SP1 transcription factor	**23.05**	**-29.84**
SRF	Serum response factor	**-2.05**	-1.26
SATB1	Special AT-rich sequence binding protein 1	-1.30	-1.55
Stat1	Signal transducer and activator of transcription 1	1.40	-1.22
Stat3	Signal transducer and activator of transcription 3	**11.68**	**2.00**
Stat4	Signal transducer and activator of transcription 4	1.30	**-8.28**
Stat5	Signal transducer and activator of transcription 5	1.17	**4.50**
Stat6	Signal transducer and activator of transcription 6	**-2.07**	-1.11
TCF/LEF	Runt-related transcription factor 2	1.79	-1.61
TFIID	TATA box binding protein	-1.90	1.73
TR	Thyroid hormone receptor	**-2.35**	-1.30
YY1	YY1 transcription factor	**-2.16**	-1.05

*Transcription factor symbol and description as provided in manual (Signosis)
**The data are expressed as ratio of RLU values of TMEM150A siRNA treated samples over scrambled control siRNA treated samples. Time points are indicated. A ratio > +/-2.0 indicates a significant change in promoter activity in TMEM150A siRNA treated samples compared to control. A value >2 indicates up-regulatory effect. A value of < -2 indicates a down-regulatory effect.

FIGURE 2.5 Endogenous TMEM150A is localized in the nucleus and in a punctate pattern throughout the cytoplasm in H292 cells. H292 cells were stained with **(A)** phalloidin to label actin (pink), **(B)** with an anti-TMEM150A antibody (green), and with **(C)** DAPI to identify the nucleus (blue). **(D)** Merge. **(E)** 20X image reflecting merge of anti-TMEM150A antibody (green) merged with DAPI (nucleus-Blue). Fluorescence images were obtained using a LSM 880 confocal laser scanning microscope


A
ACTIN
B
TMEM150A
C
NUCLEUS
D
MERGE


E
MERGE

Discussion

TMEM150A is evolutionarily conserved across vertebrate species with minimal amino acid changes over hundreds of millions of years, thus suggesting that TMEM150A plays an important and vital role in vertebrate survival. Yet, heretofore, TMEM150A function in immunity is undefined. Prior research substantiates a role for TMEM150A as part of a highly evolutionarily conserved complex that helps control the targeting of PI4KIIIα to the cell plasma membrane (Chung et al., 2015). PI4KIIIα generates PI(4)P, a limiting factor and key precursor of $PI(4,5)P_2$ (Dornan et al., 2016). $PI(4,5)P_2$ enhances the TLR4 response to LPS ultimately leading to the production of large amounts of cytokines (Kagan and Medzhitov, 2006; Płóciennikowska et al., 2016). Cytokine production is critical for eradication of pathogens yet tight cytokine control is absolutely necessary to prevent excessive tissue damage and the induction of inflammatory diseases (Charo and Ransohoff, 2006). Here we show evidence that DRAM family member, TMEM150A, plays a role in regulation of multiple cytokines.

The data collected from HEK^{TLR4} cells suggests that TMEM150A modulates the TLR4 signal transduction pathway. This is based on two major observations after siRNA mediated knockdown of TMEM150A: (1) in an LPS challenge, a dose dependent response in CXCL8 was noted in HEK^{TLR4} cells which specifically isolate the TLR4 pathway, and (2) HEK cells that did not stably express the TLR4 pathway showed no effect on CXCL8 production. Knockdown of TMEM150A in H292s, a better representative model of immune barrier cells, produced cytokine changes that illuminated role of TMEM150A in regulating multiple cytokines. The total cytokine response was not necessarily LPS and TLR4-

activation dependent however since this pathway is not isolated in this cell type. H292 cells are more well equipped to mount endogenous responses to cellular stress in comparison to HEK^{TLR4} cells so it is possible and likely that other cellular stress pathways were activated over the eighteen-hour incubation. Hence, reduction of TMEM150A, may be implicated in regulating cytokine production outside of the TLR4 pathway, and play a more global regulatory role.

This model is in line with its relationship to both PI4KIIIα and DRAM family members that modulate autophagy, which are connected to, but not isolated from, the TLR4 immune response. Notably, NFkB, the canonical transcription factor responsible for cytokine production, was not significantly activated after knockdown of TMEM150A at the time points investigated. Therefore, follow up analysis is warranted at earlier time points. However, we did observe a number of other transcription factors altered by knockdown of TMEM150A. The transcription factors most affected by a reduction in TMEM150A, such as P53, PPAR, STAT3, and HIF have been implicated in lysosomal stress and autophagic response (Pietrocola et al., 2013; You et al., 2015). For example, STAT3 fine tunes autophagy by regulating transcription of certain autophagy inhibitor genes that sequester BECN1 (BCL-2, BCL-X_L, MCL-1) (Pietrocola et al., 2013) and PPARγ plays a role in the prosurvival autophagic response (Pellerito et al., 2014). Further, PXR, ETS-1 and Myb have also been suggested to play roles in autophagy mediation (Wang et al., 2010; Yuan et al., 2015; Zhang et al., 2017).

The nuclear and cytoplasmic localization pattern of TMEM150A illustrated in Figure 5 is not unexpected although previous studies localized recombinant, exogenous

TMEM150A to the plasma membrane. It is possible that overexpression of TMEM150A may affect its cellular localization (Snapp, 2005). We predict that our method of using antibody labeling for localizing endogenous TMEM150A is more reflective of its natural position within a cell. In fact, a human interactome report (Bioplex Network), that utilized high-throughput affinity-purification mass spectrometry, identified TMEM150A as co-localizing with nuclear proteins (Huttlin et al., 2015).

Taken together, TMEM150A may interact with PI4KIIIα in and around the nucleus to regulate or sequester its activity and thereby act as an upstream regulator of $PI(4,5)P_2$ dampening the TLR4-cytokine response to infection. Another possibility is that the interaction of TMEM150a and PI4KIIIα could modify cytoskeleton arrangements, as it has been reported that Il-6 and CXCL8 mRNA stability is affected by cytoskeleton distortion in airway epithelial cells (van den Berg et al., 2006). Alternatively, TMEM150A's interactions with PI4KIIIα could affect autophagic flux and thereby cytokine production. There is evidence that PI(4)P, the direct product of PI4KIIIα activity, is central to late phagolysosomal maturation (Jeschke et al., 2015) and thereby significant in moderating autophagic flux. Deeper investigation into the interactions between TMEM150A and PI4KIIIα, with a focus on how they affect autophagic flux, may be the next step in understanding this somewhat universal inflammatory upregulation in TMEM150A deficient cells.

Experimental Procedures

Cell culture

HEK cells stably expressing TLR4/MD2/CD14 (HEK^{TLR4}) were provided by Dr. Shila Nordone, and cultured in DMEM media 4.5g/L glucose (Corning Cellgro) and supplemented with 10% fetal bovine serum (Corning Cellgro). NCI-H292 cells (ATCC CRL-1848) were cultured in DMEM/F12 50/50 media (Corning cellgro) supplemented with 10% fetal bovine serum (Corning cellgro). Cells were incubated at 37 º C at 5% CO2.

Western Blot

Cell protein extraction was done by concentrated radioimmunoprecipitation assay (RIPA) lysis buffer (0.2% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 2% NP-40, 10mM sodium pyrophosphate and 100mM sodium fluoride) containing Halt™ Protease Inhibitor Cocktail (78430, ThermoFisher). Cells were agitated on ice for 15 minutes, sonicated briefly, and agitated for another 10 minutes before centrifugation at 14,000 times g for 15 minutes at 4ºC. Supernatant was collected and stored at -80 º C until use. Protein concentrations in lysates were determined by bicinchoninic acid (BCA) Assay (Pierce, Thermo) Equal concentrations of protein were loaded onto precast 4-20% or 10% Mini-PROTEAN TGX Stain-free Gels (BioRad, 17000436) for electrophoresis. After electrophoresis, gels were activated for one minute with UV per manufacturer's instructions and the protein was transferred using the BioRad Transblot turbo system (Transblot Turbo Transfer system & Transblot Turbo Mini PVDF packs, 170-4156). After transfer, membranes were blocked with 5% skim milk for 1 hour at room temperature. TMEM150A Primary

antibody (Novus Biologicals, NBP1-81885) incubation was done at 4 º C overnight at 1:750. □-actin primary antibody (cell signal) incubations were done for 1 hour at room temperature at 1:5000. Membranes were washed with tris-buffered-saline with 0.1% Tween (TBST) and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies in 5% skim milk for 1 hour at room temperature. Membranes were washed and developed using BioRad Clarity ECL (1705060) and visualized with the BioRad ChemiDoc MP system. Image normalization and quantification was completed with BioRad Image Lab software.

siRNA transfection

TMEM150A was knocked down using siRNA. TMEM150A siRNA and control siRNA was obtained from Origene Technologies (catalog #s SR315062 & SR30004). siRNA was transfected into cultured cells using Lipofectamine RNAiMax (Life Tech 13778) according to manufacturer's protocol. For H292 cells were transfected with 5 pmol of siRNA concurrent with plating the cells in 24-well plates. HEKTLR4 cells were transfected with 5 pmol siRNA after the cells were adherent (about 18 hr) post plating. SiRNA transfection efficacy was validated by western blot and qPCR as previously described.

ELISA/Milliplex

H292 or HEKTLR4 cells were plated and transfected with siRNA,or with transfection reagent only (Lipofectamine RNAiMax) or were not transfected at all. One day post transfection, media was changed and cells were stimulated with 0, 30, 100, or 300 ng/ml lipopolysaccharide (LPS, *Escherichia coli* 055:B5 SIGMA L2880) for 18 hours. At 18 hours

cell supernatant was collected, centrifuged at 4 ºC for 5 minutes, aliquoted and immediately stored in the -80º C. Adherent cells were washed with PBS and trypsinized. Cells were collected and counted using a Nexcelom Vision cellometer. Supernatant was evaluated with ELISAs to measure protein secretion of CXCL8 (IL 8), Rantes (CCL5), and IL6 (R&D systems Quantikine catalog numbers D8000C, DRN00B, D6050) . The readout was analyzed by 4-parameter logistic regression and output was normalized to cell counts.

For Milliplex analysis, cells and supernatant were treated as described above and protein secretion was measured by a Milliplex Map Human Cytokine Immunoassay (Millipore catalog # HSCYMAG60SPMX13). Biomarker profiling was performed in the Duke Regional Biocontainment Laboratory (RBL) Immunology Unit (Durham, NC) under the direction of Dr. Gregory D. Sempowski.

RNA isolation and Quantitative real-time PCR

Cells were homogenized using a QIA shredder and RNA was isolated using an RNeasy Mini Kit per manufacturer's instructions (Qiagen). RNA quantity and quality was verified with both NanoDrop and an Agilent 2100 Bioanalyzer. RNA samples with integrity numbers (RIN) less than 8.0 were excluded from analyses. cDNA was synthesized using equal quantities of RNA and the Maxima First Strand cDNA Synthesis Kit (Thermo K1671). Quantitative real-time PCR was performed using Applied Biosystems Taqman Universal PCR Master Mix II and Taqman Gene Expression array (4413266) for 18s rRNA (Hs99999901_s1), GAPDH (Hs02758991_g1), IL10(Hs00961622_m1), IL12A (Hs01073447_m1), IL6 (Hs00985639_m1), CXCL8 (Hs00174103_m1), CCL5

(Hs00982282_m1), TNF (Hs01113624_g1). Fold changes in transcript levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Transcription Factor Array

Used the supplied in tandem with the TF Activation Profiling Plate Array I (FA-1001). H292 cells were transfected as previously described with TMEM150A siRNA or scrambled control siRNA from Origene. 24 and 32 hours post transfection nuclear extraction was performed using a Nuclear Extraction kit (SK-0001) as described by the manufacturer (Signosis). Nuclear extract was assessed for 48 different transcription factors noted in Table 2.2. TF Activation Array protocol was followed as recommended by manufacturer (FA-1001, Signosis). In brief, 9ug of nuclear extracts were incubated with biotin labeled probes (which correspond to TF DNA binding sites) to create TF-DNA/Probe complexes. Unbound probes were removed and bound complexes were eluted and then assessed using a hybridization plate pre-coated with complimentary sequences. Biotin labeled probes were detected with Streptavidin-HRP and then chemiluminescent relative light units (RLUs) were measured on a Perkin Elmer Victor 3 luminometer. Figure 2.4 A reprinted from the manual illustrates the method. Figure 2.4B reflects layout in 96-well plate.

Cellular Imaging

Cells were fixed with 4% PFA for 15 min, washed three times with PBS. Cells were permeabilized with 0.3% Triton X-100 and subsequently blocked with 5% goat serum/PBS for 30 min. TMEM150A antibody (Novus Biologicals) was diluted in 1% goat serum/PBS

and cells were incubated overnight at 4ºC. After washing three times with PBS, the coverslips were incubated for one hour with the secondary antibody (goat-anti rabbit conjugated to Alexa 488, A32731, Thermofisher) and phalloidin Alexa 568 stain (A12380, ThermoFisher). Coverslips were fixed glass slides using ProLong Gold Antifade Mountant with DAPI (P36931, ThermoFisher). Fluorescence images were obtained using a Zeiss LSM 880 confocal laser scanning microscope.

References

Adachi, M., Matsukura, S., Tokunaga, H., and Kokubu, F. (1997). Expression of cytokines on human bronchial epithelial cells induced by influenza virus A. Int. Arch. Allergy Immunol. *113*, 307–311.

Balla, T. (2013). Phosphoinositides: tiny lipids with giant impact on cell regulation. Physiol. Rev. *93*, 1019–1137.

van den Berg, A., Freitas, J., Keles, F., Snoek, M., van Marle, J., Jansen, H.M., and Lutter, R. (2006). Cytoskeletal architecture differentially controls post-transcriptional processing of IL-6 and IL-8 mRNA in airway epithelial-like cells. Exp. Cell Res. *312*, 1496–1506.

Cadwell, K. (2016). Crosstalk between autophagy and inflammatory signalling pathways: balancing defence and homeostasis. Nat. Rev. Immunol. *16*, 661–675.

Charo, I.F., and Ransohoff, R.M. (2006). The many roles of chemokines and chemokine receptors in inflammation. N. Engl. J. Med. *354*, 610–621.

Chung, J., Nakatsu, F., Baskin, J.M., and De Camilli, P. (2015). Plasticity of PI4KIIIα interactions at the plasma membrane. EMBO Rep. *16*, 312–320.

Crighton, D., Wilkinson, S., O'Prey, J., Syed, N., Smith, P., Harrison, P.R., Gasco, M., Garrone, O., Crook, T., and Ryan, K.M. (2006). DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. Cell *126*, 121–134.

Crighton, D., O'Prey, J., Bell, H.S., and Ryan, K.M. (2007). p73 regulates DRAM-independent autophagy that does not contribute to programmed cell death. Cell Death Differ. *14*, 1071–1079.

Criollo, A., Chereau, F., Malik, S.A., Niso-Santano, M., Mariño, G., Galluzzi, L., Maiuri, M.C., Baud, V., and Kroemer, G. (2012). Autophagy is required for the activation of NFκB. Cell Cycle *11*, 194–199.

Czech, M.P. (2000). PIP2 and PIP3: complex roles at the cell surface. Cell *100*, 603–606.

D'Angelo, G., Vicinanza, M., Di Campli, A., and De Matteis, M.A. (2008). The multiple roles of PtdIns(4)P -- not just the precursor of PtdIns(4,5)P2. J. Cell Sci. *121*, 1955–1963.

Delgado, M.A., and Deretic, V. (2009). Toll-like receptors in control of immunological autophagy. Cell Death Differ. *16*, 976–983.

Delgado, M.A., Elmaoued, R.A., Davis, A.S., Kyei, G., and Deretic, V. (2008). Toll-like receptors control autophagy. EMBO J. *27*, 1110–1121.

Dornan, G.L., McPhail, J.A., and Burke, J.E. (2016). Type III phosphatidylinositol 4 kinases: structure, function, regulation, signalling and involvement in disease. Biochem. Soc. Trans. *44*, 260–266.

Edgar, R., Domrachev, M., and Lash, A.E. (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. *30*, 207–210.

Harris, J. (2011). Autophagy and cytokines. Cytokine *56*, 140–144.

Hong, G.-S., Lee, B., Wee, J., Chun, H., Kim, H., Jung, J., Cha, J.Y., Riew, T.-R., Kim, G.H., Kim, I.-B., et al. (2016). Tentonin 3/TMEM150c Confers Distinct Mechanosensitive Currents in Dorsal-Root Ganglion Neurons with Proprioceptive Function. Neuron *91*, 708–710.

Huttlin, E.L., Ting, L., Bruckner, R.J., Gebreab, F., Gygi, M.P., Szpyt, J., Tam, S., Zarraga, G., Colby, G., Baltier, K., et al. (2015). The BioPlex Network: A Systematic Exploration of the Human Interactome. Cell *162*, 425–440.

Jeschke, A., Zehethofer, N., Lindner, B., Krupp, J., Schwudke, D., Haneburger, I., Jovic, M., Backer, J.M., Balla, T., Hilbi, H., et al. (2015). Phosphatidylinositol 4-phosphate and phosphatidylinositol 3-phosphate regulate phagolysosome biogenesis. Proc. Natl. Acad. Sci. U. S. A. *112*, 4636–4641.

Kagan, J.C., and Medzhitov, R. (2006). Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. Cell *125*, 943–955.

Kaisho, T., and Akira, S. (2001). Toll-like receptors and their signaling mechanism in innate immunity. Acta Odontol. Scand. *59*, 124–130.

Kato, A., and Schleimer, R.P. (2007). Beyond inflammation: airway epithelial cells are at the interface of innate and adaptive immunity. Curr. Opin. Immunol. *19*, 711–720.

Kerley-Hamilton, J.S., Pike, A.M., Hutchinson, J.A., Freemantle, S.J., and Spinella, M.J. (2007). The direct p53 target gene, FLJ11259/DRAM, is a member of a novel family of transmembrane proteins. Biochim. Biophys. Acta *1769*, 209–219.

Lam, W.Y., Yeung, A.C.M., and Chan, P.K.S. (2011). Apoptosis, cytokine and chemokine induction by non-structural 1 (NS1) proteins encoded by different influenza subtypes. Virol. J. *8*, 554.

Lee, K.J., Moon, J.Y., Choi, H.K., Kim, H.O., Hur, G.Y., Jung, K.H., Lee, S.Y., Kim, J.H., Shin, C., Shim, J.J., et al. (2010). Immune regulatory effects of simvastatin on regulatory T cell-mediated tumour immune tolerance. Clin. Exp. Immunol. *161*, 298–305.

Li, Y.-Y., Ishihara, S., Aziz, M.M., Oka, A., Kusunoki, R., Tada, Y., Yuki, T., Amano, Y.,

Ansary, M.U., and Kinoshita, Y. (2011). Autophagy is required for toll-like receptor-mediated interleukin-8 production in intestinal epithelial cells. Int. J. Mol. Med. *27*, 337–344.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402–408.

Lu, Y.-C., Yeh, W.-C., and Ohashi, P.S. (2008). LPS/TLR4 signal transduction pathway. Cytokine *42*, 145–151.

Matsukura, S., Kokubu, F., Noda, H., Tokunaga, H., and Adachi, M. (1996). Expression of IL-6, IL-8, and RANTES on human bronchial epithelial cells, NCI-H292, induced by influenza virus A. J. Allergy Clin. Immunol. *98*, 1080–1087.

Meijer, A.H., and van der Vaart, M. (2014). DRAM1 promotes the targeting of mycobacteria to selective autophagy. Autophagy *10*, 2389–2391.

Mrschtik, M., O'Prey, J., Lao, L.Y., Long, J.S., Beaumatin, F., Strachan, D., O'Prey, M., Skommer, J., and Ryan, K.M. (2015). DRAM-3 modulates autophagy and promotes cell survival in the absence of glucose. Cell Death Differ. *22*, 1714–1726.

O'Prey, J., Skommer, J., Wilkinson, S., and Ryan, K.M. (2009). Analysis of DRAM-related proteins reveals evolutionarily conserved and divergent roles in the control of autophagy. Cell Cycle *8*, 2260–2265.

Oyanagi, T., Takizawa, T., Aizawa, A., Solongo, O., Yagi, H., Nishida, Y., Koyama, H., Saitoh, A., and Arakawa, H. (2017). Suppression of MUC5AC expression in human bronchial epithelial cells by interferon-γ. Allergol. Int. *66*, 75–82.

Pellerito, O., Notaro, A., Sabella, S., De Blasio, A., Vento, R., Calvaruso, G., and Giuliano, M. (2014). WIN induces apoptotic cell death in human colon cancer cells through a block of autophagic flux dependent on PPARγ down-regulation. Apoptosis *19*, 1029–1042.

Pietrocola, F., Izzo, V., Niso-Santano, M., Vacchelli, E., Galluzzi, L., Maiuri, M.C., and Kroemer, G. (2013). Regulation of autophagy by stress-responsive transcription factors. Semin. Cancer Biol. *23*, 310–322.

Płóciennikowska, A., Hromada-Judycka, A., Dembińska, J., Roszczenko, P., Ciesielska, A., and Kwiatkowska, K. (2016). Contribution of CD14 and TLR4 to changes of the PI(4,5)P2 level in LPS-stimulated cells. J. Leukoc. Biol. *100*, 1363–1373.

Raucher, D., Stauffer, T., Chen, W., Shen, K., Guo, S., York, J.D., Sheetz, M.P., and Meyer, T. (2000). Phosphatidylinositol 4,5-Bisphosphate Functions as a Second Messenger that Regulates Cytoskeleton–Plasma Membrane Adhesion. Cell *100*, 221–228.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for

reconstructing phylogenetic trees. Mol. Biol. Evol. *4*, 406–425.

Sievers, F., and Higgins, D.G. (2014). Clustal Omega, accurate alignment of very large numbers of sequences. Methods Mol. Biol. *1079*, 105–116.

Snapp, E. (2005). Design and use of fluorescent fusion proteins in cell biology. Curr. Protoc. Cell Biol. *Chapter 21*, Unit 21.4.

Takeda, K., and Akira, S. (2015). Toll-like receptors. Curr. Protoc. Immunol. *109*, 14.12.1–10.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., and Akira, S. (1999). Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity *11*, 443–451.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. *30*, 2725–2729.

Tan, Y., and Kagan, J.C. (2014). A cross-disciplinary perspective on the innate immune responses to bacterial lipopolysaccharide. Mol. Cell *54*, 212–223.

van der Vaart, M., Korbee, C.J., Lamers, G.E.M., Tengeler, A.C., Hosseini, R., Haks, M.C., Ottenhoff, T.H.M., Spaink, H.P., and Meijer, A.H. (2014). The DNA damage-regulated autophagy modulator DRAM1 links mycobacterial recognition via TLR-MYD88 to autophagic defense [corrected]. Cell Host Microbe *15*, 753–767.

Wang, K., Damjanov, I., and Wan, Y.-J.Y. (2010). The protective role of pregnane X receptor in lipopolysaccharide/D-galactosamine-induced acute liver injury. Lab. Invest. *90*, 257–265.

Xu, Y., Jagannath, C., Liu, X.-D., Sharafkhaneh, A., Kolodziejska, K.E., and Eissa, N.T. (2007). Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. Immunity *27*, 135–144.

Xu, Y., Liu, X.-D., Gong, X., and Eissa, N.T. (2008). Signaling pathway of autophagy associated with innate immunity. Autophagy *4*, 110–112.

Yin, Z., Pascual, C., and Klionsky, D.J. (2016). Autophagy: machinery and regulation. Microb. Cell Fact. *3*, 588–596.

Yoon, J.-H., Her, S., Kim, M., Jang, I.-S., and Park, J. (2012). The expression of damage-regulated autophagy modulator 2 (DRAM2) contributes to autophagy induction. Mol. Biol. Rep. *39*, 1087–1093.

You, L., Wang, Z., Li, H., Shou, J., Jing, Z., Xie, J., Sui, X., Pan, H., and Han, W. (2015).

The role of STAT3 in autophagy. Autophagy *11*, 729–739.

Yuan, J., Zhang, Y., Sheng, Y., Fu, X., Cheng, H., and Zhou, R. (2015). MYBL2 guides autophagy suppressor VDAC2 in the developing ovary to inhibit autophagy through a complex of VDAC2-BECN1-BCL2L1 in mammals. Autophagy *11*, 1081–1098.

Zhang, J., D'Ercole, A.J., and Underwood, L.E. (2000). Identification of a new gene (rat TM6P1) encoding a fasting-inducible, integral membrane protein with six transmembrane domains. Biochim. Biophys. Acta *1492*, 280–284.

Zhang, X., Wu, D., Aldarouish, M., Yin, X., Li, C., and Wang, C. (2017). ETS-1: A potential target of glycolysis for metabolic therapy by regulating glucose metabolism in pancreatic cancer. Int. J. Oncol. *50*, 232–240.

Chapter 3: Summary and Future Directions

The purpose of this work was to investigate a role for TMEM150A in innate immune function. We hypothesized that TMEM150A may play a role in the TLR4 signal transduction cascade and thereby affect cytokine response. This hypothesis was based on the following three items: previous research that provides both evidence that TMEM150A is the yeast homologue of Sfk1 and possible regulator of PI4KIIIα (Chung et al., 2015); the highly conserved nature of TMEM150A and its relationship to the DRAM family of proteins linked to autophagy modulation (O'Prey et al., 2009); and finally the observation *in silico* and in pilot studies in monocyte/macrophage-like cell lines that demonstrated an overall reduction in *TMEM150A* transcripts following LPS stimulation (Edgar et al., 2002, Heffelfinger, unpublished data).

To test this hypothesis, cytokine protein and transcript levels were quantified downstream of LPS stimulation in two cell culture models, one which isolated the TLR4 pathway and one which reflected a more endogenous, airway epithelium-type response. The results from these studies suggest for the first time, that TMEM150A, a largely undefined, novel protein and member of the DRAM family, plays a role in cytokine regulation. Because overactive, unchecked cytokine response is implicated in numerous, costly diseases that include sepsis, cancer, inflammatory bowel disorder, psoriasis and arthritis (Baliwag et al., 2015; Dinarello, 2000; Hennessy et al., 2010; Tartour and Fridman, 1998), the impact of this new finding could be profound. Identifying novel, targeted therapeutics to treat these conditions takes a nuanced understanding of the complexity of the pathways that trigger or

dampen cytokine production. Although, this work merely introduces TMEM150A as a cytokine regulator, we have moved one step forward in our understanding. Of course, there are still fundamental questions about the role TMEM150A in immune response that need to be investigated.

First, investigating transcription factor activity along an earlier time course after siRNA knockdown of TMEM150A may help unravel more nuance as well as verify that we didn't simply miss NFkB or AP1 activity changes. Preliminary studies in our lab however have not yet led us to believe that NFkB is significantly changed versus control. Additionally, it would be interesting and insightful to fully explore autophagy markers by Western Blot like LC3II after TMEM150a knockdown illuminating a more direct connection between TMEM150A and autophagy regulation.

The next few steps in clarifying how TMEM150A contributes to cytokine regulation might include a closer look into the interactions between TMEM150A and PI4KIIIα with a focus on how they affect autophagic flux. For instance, we found that in epithelial cells, TMEM150A localized to the nucleus and in a punctate pattern in the cytoplasm. Since PI4KIIIα has also been reported to localize to the nucleus (D'Angelo et al., 2008), immunofluorescent microscopy could possibly reveal if TMEM150A and PI4KIIIα colocalize without the use of recombinant overexpression plasmids. Further, it would be interesting to investigate how siRNA mediated knockdown of either protein affected the localization patterns of the other, as well as the patterning of the autophagy marker, LC3B.

Another question makes sense to ask is if the inhibition of PI4KIIIα rescues the cytokine production phenotype caused by knockdown of TMEM150A. PI4KIIIα is sensitive

to wortmannin (Clayton et al., 2013; D'Angelo et al., 2008) and could be easily inhibited. Unfortunately, this method would also inhibit phosphoinositide 3-kinase (PI3K), a key component of autophagosome formation and thereby does not isolate PI4KIIIα (Yin et al., 2016). On a similar train of thought, reduction in TMEM150A caused a change in the activity of several transcription factors that have been linked to autophagy regulation. We would be able to test if small molecule inhibition of these transcription factors rescues the cytokine phenotype produced from knockdown of TMEM150A and subsequently narrow the pathway. Further, gaining additional information regarding the protein-protein interactions and binding partners of TMEM150A would aid in fine-tuning the cellular pathways it plays a role in modifying.

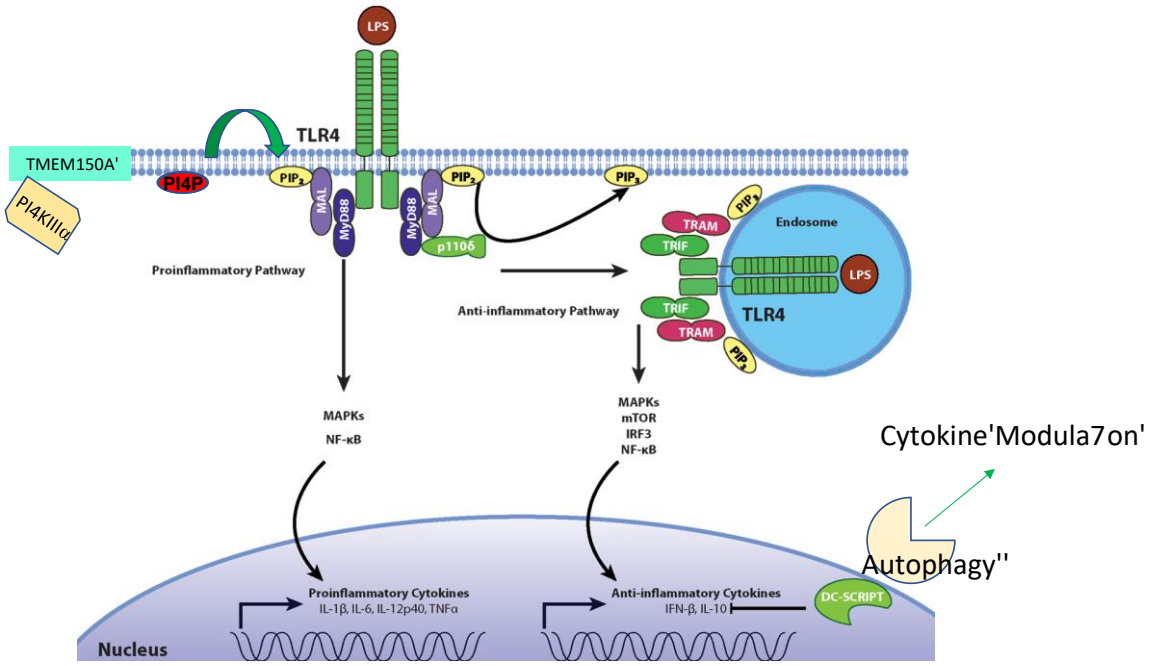
Importantly, transitioning to a whole organism *in vivo* system will be key to translating our findings. Studies using morpholino mediated knockdown of TMEM150A in zebrafish embryos suggested TMEM150A is essential for proper development (not shown). In pilot studies in the same model, cytokine transcript level changes were detected that mirrored the cell culture results presented here, as well as an increased susceptibility to infection (not shown). The intricacies of working with an *in vivo* model leave some question to the validity of the results however. Cytokine transcript level changes, for example, were not uniformly consistent, therefore further fine-tuning of the methodology needs to be done to determine if these findings can be replicated.

In sum, although much more investigation is warranted, all together, the results presented suggest a functional role for TMEM150A in cytokine regulation. The next step would be to tease out what underlying TMEM150A is affecting. We hypothesize that

cytokine regulation may involve subtleties in the interactions of PI4KIIIα with TMEM150A that in turn has an effect on both the TLR4 pathway and autophagic flux. Figure 3.1 illustrates a proposed model for how TMEM150A might be playing a role in cytokine regulation. TMEM150A, during normal conditions, sequesters PI4KIIIα, which in turn dampens the pool of PI(4)P which subsequently decreases both PI(4,5)P2 available to enrich TLR4 activation as well as the autophagy-driven cytokine response thus leading to a reduction in overall cytokine transcription. Whereas under stressful conditions like gram-negative bacterial infection or LPS exposure, TMEM150A transcript levels are reduced, leaving more PI4KIIIα available to phosphorylate PI and create PI(4)P. The increased availability of PI(4)P leads to downstream events already discussed that upregulate cytokine transcription. Further investigation into the dynamics of TMEM150A will help elucidate the validity of this proposed model.

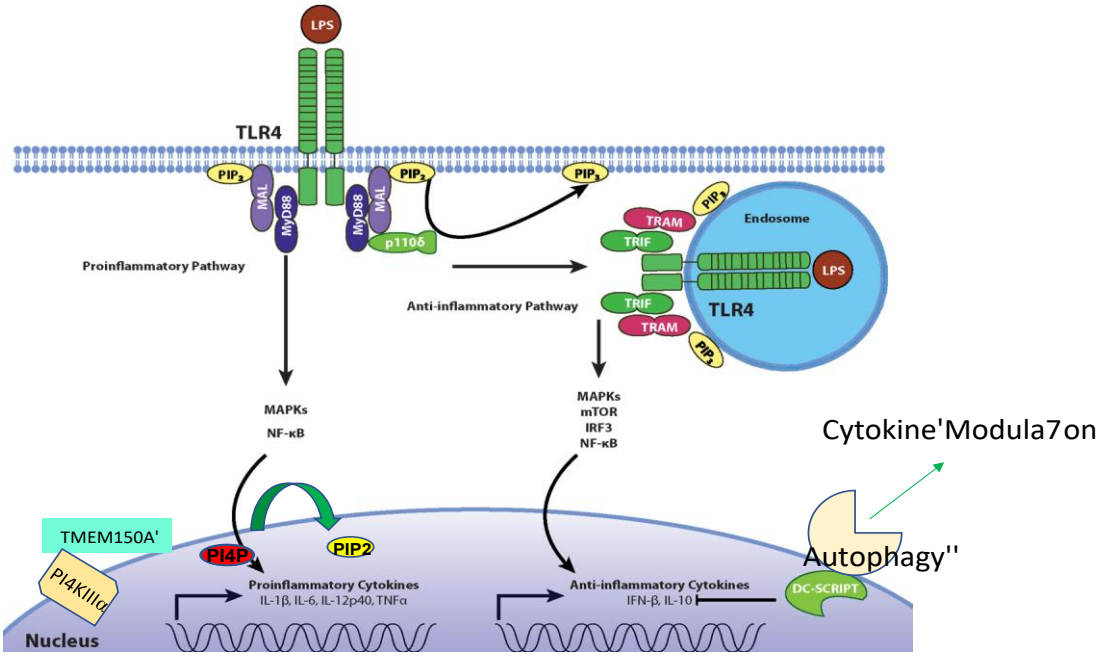
Figure 3.1 Hypothesis regarding how TMEM150A may interact with PI4KIIIα and thereby influence cytokine response downstream **(A)** Illustration of how TMEM150A might interact with PI4KIIIα at the plasma membrane as predicted by Chung et al. The interaction of TMEM150A and PI4KIIIα may have an effect on PI(4,5)P2 (labeled as PIP2 in the diagram) synthesis and subsequently affect the TLR4 pathway. **(B)** Illustration of how TMEM150A might interact with PI4KIIIα at the nuclear membrane and thereby affect nuclear pools of PI(4,5)P2. **(C)** TMEM150A interacts with PI4KIIIα and limits PI4KIIIα kinase activity thereby regulating the amount of PI(4)P available for PI(4,5)P2 synthesis and the amount of PI(4)P available for mediation of autophagolysosomal maturation. In turn, the TLR4 signal transduction and the autophagolysosomal pathway that results in cytokine production is dampened. **(D)** Proposed cytokine changes in the event that TMEM150A transcription is reduced after LPS exposure. We hypothesize that less TMEM150A sequestering PI4KIIIα it may allow for a greater pool of PI(4)P available for PI(4,5)P2 synthesis, and thereby enhance the TLR4 cascade by enrichment and recruitment of adaptor molecule, TIRAP (not shown). Additionally, the availability of PI(4)P may also enhance autophagy signaling. These two events could lead to increased cytokine production in the reduction of TMEM150A. (Figure is adapted with changes by permission from LI, J. 2013. Frontiers in Immunology, https://doi.org/10.3389/fimmu.2013.00347).

A



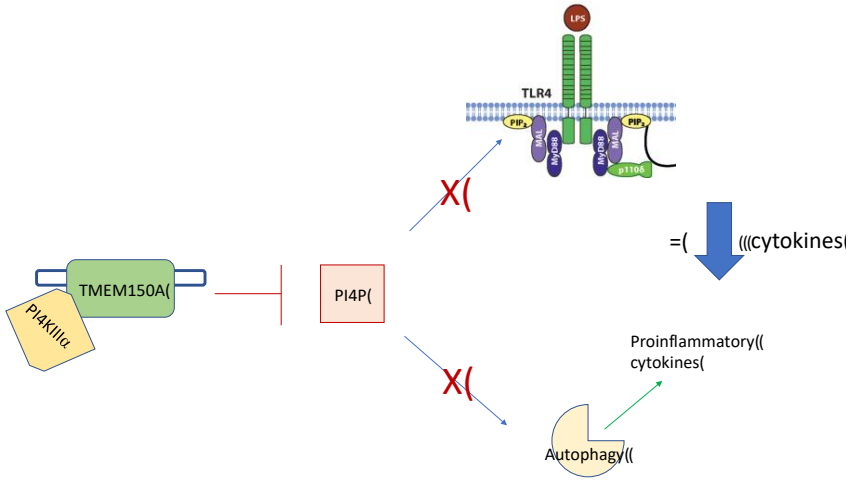
Adapted'from'LI,'J.'2013.'Fron7ers'in'Immunology''

B



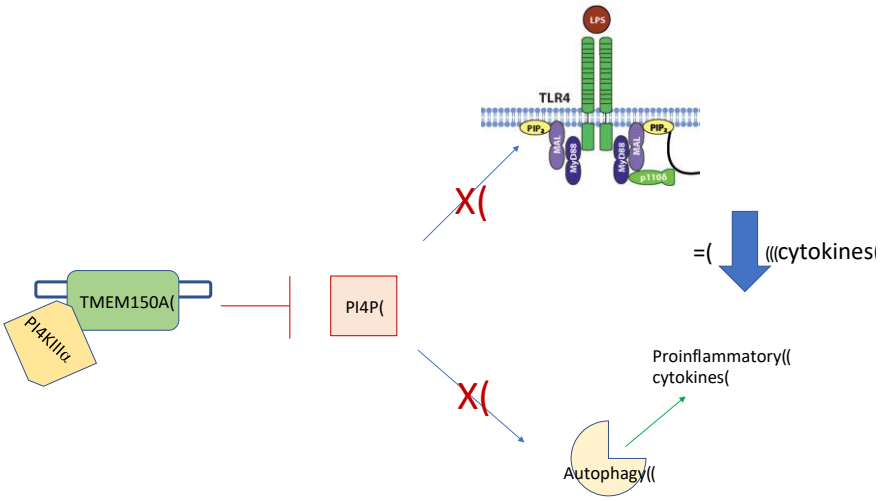
Adapted'from'LI,'J.'2013.'Fron7ers'in'Immunology''

C



Adapted'from'LI,'J.'2013.'Fron7ers'in'Immunology''

D



Adapted'from'LI,'J.'2013.'Fron7ers'in'Immunology''

References

Baliwag, J., Barnes, D.H., and Johnston, A. (2015). Cytokines in psoriasis. Cytokine *73*, 342–350.

Chung, J., Nakatsu, F., Baskin, J.M., and De Camilli, P. (2015). Plasticity of PI4KIIIα interactions at the plasma membrane. EMBO Rep. *16*, 312–320.

Clayton, E.L., Minogue, S., and Waugh, M.G. (2013). Mammalian phosphatidylinositol 4-kinases as modulators of membrane trafficking and lipid signaling networks. Prog. Lipid Res. *52*, 294–304.

D'Angelo, G., Vicinanza, M., Di Campli, A., and De Matteis, M.A. (2008). The multiple roles of PtdIns(4)P -- not just the precursor of PtdIns(4,5)P2. J. Cell Sci. *121*, 1955–1963.

Dinarello, C.A. (2000). Proinflammatory cytokines. Chest *118*, 503–508.

Edgar, R., Domrachev, M., and Lash, A.E. (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. *30*, 207–210.

Hennessy, E.J., Parker, A.E., and O'Neill, L.A.J. (2010). Targeting Toll-like receptors: emerging therapeutics? Nat. Rev. Drug Discov. *9*, 293–307.

Li, J., Lee, D.S.W., and Madrenas, J. (2013). Evolving Bacterial Envelopes and Plasticity of TLR2-Dependent Responses: Basic Research and Translational Opportunities. Front. Immunol. *4*, 347.

O'Prey, J., Skommer, J., Wilkinson, S., and Ryan, K.M. (2009). Analysis of DRAM-related proteins reveals evolutionarily conserved and divergent roles in the control of autophagy. Cell Cycle *8*, 2260–2265.

Tartour, E., and Fridman, W.H. (1998). Cytokines and cancer. Int. Rev. Immunol. *16*, 683–704.

Yin, Z., Pascual, C., and Klionsky, D.J. (2016). Autophagy: machinery and regulation. Microb. Cell Fact. *3*, 588–596.