Thesis for doctoral degree (Ph.D.) 2019

ELUCIDATION OF THE CELL SIGNALING PATHWAYS MEDIATING INNATE IMMUNITY AND HOST-PATHOGEN INTERACTIONS

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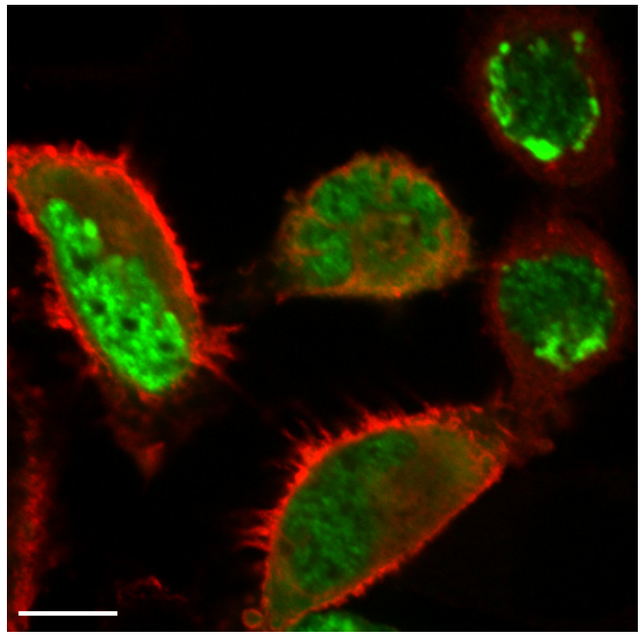

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Cover image: “CD38 drives TFEB nuclear translocation” by Neel R. Nabar

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Elucidation of the Cell Signaling Pathways Mediating Innate Immunity and Host-Pathogen Interactions

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To my loving family, whose unwavering support has been pivotal to my accomplishments

ABSTRACT

The ability to generate a robust immune response is integral to organismal homeostasis. Cells of the innate immune system are considered the first responders of immunity, and are therefore responsible for sensing both pathogens and endogenous danger signals and initiating a protective inflammatory response. To appropriately sense pathogens and danger signals, cells have developed intricate mechanisms for transducing signals from the extracellular environment into the cell. The integration of these signals is complex, resulting from crosstalk between many signaling pathways, but is critical to generating a coordinated biological response. In additional to the specialized mechanisms of innate immune cells to respond to antigens, these cells (like most) have evolved a complex set of adaptive mechanisms that maintain homeostasis during cell stress. Activation of innate immunity via pathogen invasion or the presence of danger signals can be considered an especially intense form of cell stress, thereby implicating these homeostatic pathways as components of the innate immune response.

The work presented in this thesis relates to the molecular mechanisms by which cells of the innate immune system integrate signals from the microenvironment to produce a coordinated biological response. The aim was to elucidate the mechanisms by which innate macrophages transduce extracellular signals to activate important effector pathways, and to describe crosstalk between cell signaling pathways that mediate adaptive responses to cell stress. Finally, we looked to extend our understanding to pathophysiological settings, and investigated the mechanisms by which pathogens that cause cell stress generate an aberrant inflammatory response. In doing so, we described novel components of these signaling pathways, which may be exploited in designing novel therapeutics.

In **paper I,** $G\alpha_{i2}$ was identified as a critical signaling molecule in macrophage phenotype determination, functioning to transduce signals from the microenvironment to fine tune macrophage propensity towards an M1 inflammatory or M2 anti-inflammatory phenotype. In **paper II,** the immune receptor CD38 was shown activate the master transcriptional regulation of the autophagic/lysosome machinery, TFEB. We further identified the large kinase LRRK2 as essential in signal transduction downstream of CD38. **In paper III,** we described adaptive crosstalk between TFEB, an essential component of the cell stress response, and the typically proliferative WNT signaling pathway. Finally, in **paper IV** we describe how the SARS-Coronavirus open reading frame-3a causes multimodal necrotic death by activating multiple cell stress and innate immune pathways, resulting in aberrant inflammation.

In summary, the work presented in this thesis extends our current understanding of the molecular mechanisms mediating the integration of signals in innate immune cells. We have identified several novel signaling mechanisms, which could lay the foundation for the development of targeted therapeutics.

LIST OF SCIENTIFIC PAPERS

I. Vural A*, Nabar NR*[#], Hwang IY, Sohn S, Park C, Karlsson MCI, Blumer JB, Kehrl JH[#]. **$G\alpha_{i2}$ signaling regulates inflammasome priming and cytokine production by biasing macrophage phenotype determination.** *J Immunol, 2018, 202(5): 1510-1520.*

II. Nabar NR[#], Shi CS, Hwang IY, Karlsson MCI, Kehrl JH[#]. **Identification of a functional CD38-LRRK2-TFEB signaling pathway in immune cells.** *In Manuscript.*

III. Xiao X, Nabar NR[#], Shi CS, Yue Y, Zhao W, Wang M, Kehrl JH[#]. **Transcription Factor EB limits Wnt/β-catenin signaling by directly binding β-catenin and promoting its degradation.** *In Revision.*

IV. Yue Y, Nabar NR[#], Shi CS, Kamenyeva O, Xiao X, Hwang IY, Wang M, Kehrl JH[#]. **SARS-Coronavirus Open Reading Frame-3a drives multimodal necrotic cell death.** *Cell Death Dis, 2018, 9(9): 904.*

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PUBLICATIONS NOT INCLUDED IN THE THESIS

I. Nabar NR, Shi CS, Kehrl JH. **Autophagy accompanies inflammasome activation moderating inflammation by eliminating active inflammasomes.** *In Hayat MA, Autophagy: Cancer, Other Patholgies, Inflammation, Immunity, Infection, and Aging, 2017, Vol 12 pp 343-357.*

II. Nabar NR, Shi CS, Kehrl JH. **Signaling by the Toll-like Receptors induces autophagy through modification of Beclin 1: Molecular Mechanism.** *In Hayat MA, Immunology: Immunotoxicology, Immunopathology, and Immunotherapy, 2017, Vol 1 pp 75-84.*

III. Nabar NR[#], Kehrl JH[#]. **The Transcription Factor EB links cellular stress to the immune response.** *Yale J Biol Med, 2017, 90(2): 301-315.*

IV. Harris J, Lang T, Thomas JPW, Sukkar MB, Nabar NR, Kehrl JH. **Autophagy and Inflammasomes.** *Mol Immunol, 2017, 86: 10-15.*

V. Nabar NR[#], Kehrl JH[#]. **Inflammasome inhibition links IRGM to innate immunity.** *Mol Cell, 2019 73(3): 391-392.*

VI. Shi CS, Nabar NR[#], Huang NN, Kehrl JH[#]. **SARS-CoV ORF8b triggers intracellular stress pathways and activates NRLP3 inflammasomes.** *Submitted.*

Co-corresponding authors

CONTENTS

LIST OF ABBREVIATIONS

ADPR	ADP-ribose
AGS	Activator of G-protein signaling
AIM2	Absent in melanoma 2
ALR	Aim2-like receptor
AMPK	AMP-activated protein kinase
ADCC	Antibody dependent cellular cytotoxicity
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BCR	B-cell receptor
BMDM	Bone marrow derived macrophage
cADPR	Cyclic ADP-ribose
CAMKKβ	Ca^{2+}/calmodulin-dependent protein kinase kinase-beta
CARD	Caspase activation and recruitment domain
CD	Cluster of differentiation
CICR	Calcium induced calcium release
CLR	C-type lectin receptor
COR	C-terminal of ROC
CoV	Coronavirus
DAMP	Damage-associated molecular pattern
DCs	Dendritic cells
DSS	Dextran sulfate sodium
ER	Endoplasmic reticulum
ELISA	Enzyme-linked immunosorbent assay
FZD	Frizzled
GAP	GTPase accelerating protein
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
HIV	Human immunodeficiency virus
ICAM1	Intracellular adhesion molecule 1

IFN	Interferon
IRF	Interferon regulatory factor
IMMs	Inflammatory monocyte-macrophages
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRIS	Inflammatory immune reconstitution syndrome
KI	Knock-in
KO	Knockout
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LRRK2	Leucine-rich repeat kinase 2
MAPK	Mitogen activated protein kinase
MRSA	Methicillin-resistant *Staphylococcus aureus*
MiT	Microphthalmia
MITF	Microphthalmia associated transcription factor
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
MyD88	Myeloid differentiation primary response 88
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NAADP	Nicotinic acid adenine dinucleotide phosphate
NFAT	Nuclear factor of activated T-cells
NLR	Nod-like receptor
NLRC4	NLR family CARD domain containing protein 4
NLRP3	NACHT, LRR, and PYD domains contain protein 3
NOD	Nucleotide-binding oligomerization domain
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PCD	Programmed cell death
PD	Parkinson’s disease
PKC	Protein kinase C

PRR	Pattern recognition receptor
PTX	Pertussis toxin
PYD	Pyrin domain
RGS	Regulator of G-protein signaling
RIPK	Receptor interacting protein kinase
RLR	RIG1-like receptor
ROC	Ras-of-complex
ROS	Reactive oxygen species
RyR	Ryanodine receptor
SARS	Severe acute respiratory syndrome
SARS 3a	SARS-CoV open reading frame-3a
TAM	Tumor associated macrophage
TCFs	T cell factor/lymphoid-enhancer binding factor proteins
TCR	T-cell receptor
TFE3	Transcription Factor E3
TFEB	Transcription factor EB
TFEC	Transcription Factor EC
TIR	Toll/IL-1 receptor
TLEs	Transducin-Like-Enhancer of split proteins
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNP	2, 4, 6,-Trinitrophenol
TPC	Two pore channel
TRIF	TIR domain containing adaptor-inducing IFN-β
VCAM1	Vascular adhesion molecular 1
WNT	Wingless/Integrated

1 INTRODUCTION

Mammalian species are constantly interacting with microbes, forming relationships that can be symbiotic or pathogenic [1]. The immune system, which is a complex network of cells, tissues, and molecules that functions to prevent and eradicate infections, has evolved to cope with both symbiotic and pathogenic microbes. The immune system can broadly be divided into two arms; innate immunity mediates initial protection against invading pathogens, while adaptive immunity develops more slowly and mounts a more effective defense against infections. Innate immunity is characterized by hallmark pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). Recognition of exogenous pathogenic or endogenous danger signals results in immediate activation of innate immune cells, initiating an inflammatory cascade that allows priming of innate immune effector cells and instructs the adaptive arm of immunity to generate pathogen specific protection [2]. Adaptive immunity, which develops over the course of days to weeks, entails the clonal expansion of cells that have undergone molecular rearrangement of DNA to express receptors specific for the invading pathogen. These cells then provide enhanced protection from the pathogen, and facilitate the production of long-lived cells with memory of the offending pathogen allowing rapid generation of an immune response if subsequently exposed [3].

The work presented in this thesis focuses primarily on cells of the innate immune system and elucidates some of the molecules and cell signaling pathways that are essential in integrating environmental signals into a coordinated biological response. Integration of these signals is complex and often requires crosstalk between multiple signaling pathways. This investigation is focused at the molecular level, revealing signal transduction mechanisms governing macrophage polarization, the upregulation of autophagy, cellular adaptation following cell stress, and pathogenic inflammation after severe acute respiratory syndrome (SARS) coronavirus (CoV) infection. Investigation of the molecular mechanisms underlying a process provides the advantage that it characterizes novel targets for the potential development of therapeutics.

1.1 THE IMMUNE SYSTEM

The immune system consists of a complex collection of cells, tissues, and molecules that mediates resistance to and clearance of infections. The immune system is broken down into the innate and adaptive arms, which mediate the initial non-specific response and the delayed specific response respectively [4]. Innate immunity consists first of physical barriers, such as the skin and mucosa, that function to keep invading pathogens (e.g. bacteria, viruses, and fungi) from entering the body. If pathogens breach this barrier, they are met by sentinel cells of the innate immune system that recognize antigens on pathogens and initiate the appropriate response. These cells include macrophages, dendritic cells (DCs), and neutrophils, whose key role is to identify pathogens via their PRRs and secrete cytokines and chemokines that

function to activate and recruit effector cells [5]. The initial secretion of chemokines and cytokines is an essential step in the amplification of the immune response, and macrophages play a key role in orchestrating the immune response via these processes. Macrophages and neutrophils additionally contribute to the innate immune response by directly engulfing and degrading pathogens via phagocytosis, an important mechanism for the eradication of pathogens [6]. DCs, on the other hand, are professional antigen presenting cells (APCs) that capture pathogens and present their antigens to lymphocytes (B-cells and T-cells) of adaptive immunity. While macrophages are also considered professional APCs, it is the DCs that typically migrate to the draining lymph node following antigen recognition and capture, where they facilitate generation of the adaptive response [7]. In this way, DCs act as a key bridge between innate and adaptive immunity. The adaptive response is generated over a period of days to weeks, as antigens presented by DCs results in the selection and activation of lymphocytes capable of recognizing antigens from the invading pathogen. Clonal expansion of the selected lymphocytes results in an improved ability to eradicate the invading pathogen. Finally, memory lymphocytes are generated during the adaptive immune response. These are typically senescent cells that lie dormant after eradication of the pathogen during primary challenge, but are rapidly activated upon exposure of the organism to the same pathogen, providing immunity [3].

A functional immune system is critical to human health and disease, which is underscored by the myriad of diseases resulting from both impaired or enhanced immune responses [8]. Immunodeficiencies can be classified based on the type of cell they affect, including phagocytes of innate immunity, T-cells of adaptive immunity mediating cellular immunity, or B-cells of adaptive immunity mediating humoral immunity. Primary immunodeficiencies of phagocytes, such as chronic granulomatous disease, presents symptomatically as recurrent bacterial infections and is associated with increased risk for life threating infections from common bacteria [9]. Children with severe combined immunodeficiency, which results in defects in both T-cell mediated cellular immunity and B-cell mediated humoral immunity, rarely live past the age of two if untreated [10]. While defects in immunity result in the obvious increased risk of life threating infections, aberrant inflammation similarly has negative effects on health and disease. The classical example of pathogenic aberrant inflammation is the clinical syndrome referred to as a cytokine storm. Dengue fever, Ebola virus, and several CoVs can result in an uncontrolled inflammatory response characterized by elevated cytokine levels; this results in increased vascular permeability, hemorrhage, organ failure, and can lead to death [11]. Immune reconstitution inflammatory syndrome (IRIS) subsequent to HIV infection is another example; the hallmark of IRIS is the paradoxical worsening of infection related symptoms following recovery of immune function due to treatment of HIV [12]. IRIS is believed to be mediated by hyperactivation of immune pathways [13].

One important feature of the immune system is the ability to differentiate between self and non-self, which in the context of the immune response to foreign pathogens is critical to avoid autoimmune disease [14]. However, the immune system has also been implicated in the

pathogenesis of non-infectious diseases [15]. For example, chronic exposure to sterile irritants such as asbestos or silica can lead to fibrosis of the lungs due to aberrant alveolar macrophage activation [16]. In atherosclerosis, macrophage mediated inflammation upon recognition of cholesterol crystals propagates disease pathogenesis [17]. In Alzheimer's disease, microglia produce pro-inflammatory cytokines in response to the hallmark amyloid plaques, promoting neurotoxicity [18]. The immune system has also been implicated in the development of tumors. In physiological setting, the immune system identifies and destroys cancerous and pre-cancerous cells [19], while in the pathophysiological setting the tumor microenvironment is immunosuppressive and contributes to tumor development [20]. The mechanisms of immune involvement in sterile inflammation are not as well understood as pathogen driven inflammation, but the ability of the innate immune system to recognizes endogenous DAMPs is believed to be important.

1.1.1 Innate Immunity

The innate immune system is an evolutionary conserved arm of host defense and is widely considered the first line against infection. While innate immunity includes physical barriers that prevent pathogen entry into the host, it also plays a critical role in initiating and propagating the inflammatory response following breaches of these barriers by pathogens. One defining feature of innate immunity is the use of non-specific PRRs that are germline encoded, which is in stark opposition to the highly specific receptors of adaptive immunity generated by lymphocyte clonal expansion from an infinitely diverse pool of receptors generated by gene rearrangement [2]. PRRs recognize conserved microbial patterns called PAMPs and endogenous danger signals called DAMPs.

Innate immune cells have distinct classes of PRRs, which are from different families based on protein homology and localize to different subcellular structures. The Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) are membrane bound; they are therefore found either on the cell surface associated with the plasma membrane or in endosomal compartments. Innate immune cells also have several cytosolic PRRs, including those of the NOD-like receptor (NLR) family, the RIG1-like receptor (RLR) family, and AIM2-like receptor (ALR) family [21, 22]. Of these, TLR signaling is the most well characterized and results in the activation of the critical inflammatory transcription factors, NF-κB, interferon-regulatory factors (IRFs), and AP-1. Protein expression downstream of these transcription factors is essential for orchestrating the inflammatory response and the subsequent eradication of microbes. All hematopoietic cells of the innate immune system express PRRs, and the functions of the major innate immune cell types are discussed herein.

1.1.1.1 Phagocytes: Neutrophils and Monocyte/Macrophages

Neutrophils and cells of the monocyte-macrophage lineage are typically the first responders to pathogenic insults, as they are quickly recruited to sites of infection where they both initiate the inflammatory response by recognizing pathogens and ingest microbes for intracellular killing [4]. Neutrophils are the most abundant leukocytes in the blood, and

production of neutrophils from the bone marrow results in a rapid rise in neutrophil levels during acute infection [23]. Neutrophils are particularly effective at trans-endothelial migration, and are classically considered to be short lived, as they die shortly after extravasation into tissues. Recent advances have extended the role of neutrophils, suggesting they play a role in chronic inflammation and instructing adaptive immunity, but these potential functions of neutrophils are less well defined and require continued study [24].

Monocytes/macrophages are less abundant than neutrophils but are more critical in orchestrating the immune response as they survive in tissues for long periods of time. Typically, monocytes in the blood are recruited to extravascular sites of infection, where they differentiate into macrophages [4]. Macrophages are particularly important in the immune response, as they not only mediate the initial propagation of inflammation, but are also involved in the suppression of inflammation following pathogen eradication in order to facilitate a return to homeostasis [25]. It is now appreciated that several macrophages subsets exist, and that macrophages have heterogeneous and flexible phenotypes allowing both pro- and anti-inflammatory roles. Classically activated macrophages (termed M1 macrophages) are pro-inflammatory and typically produce high levels of inflammatory cytokines, including IL-1β, IL-6, IL-12, and TNF-α. Macrophages can be polarized towards an M1 phenotype *in vitro* by stimulation with the bacterial wall component lipopolysaccharide (LPS) [26]. Alternatively activated (M2) macrophages are anti-inflammatory in nature and promote tissue repair. M2 macrophages produce high levels of anti-inflammatory cytokines, including IL-10 and TGF-β, and are polarized towards the M2 phenotype by IL-4 and IL-13 [27]. Both M1 and M2 polarized macrophages are essential to the propagation of inflammation and the physiological resolution of inflammation respectively, though the mechanisms and environmental signals biasing macrophage phenotype are still poorly understood.

1.1.1.2 Dendritic cells (DCs)

DCs are stellate shaped cells that function at the interface of innate and adaptive immunity [28]. They process antigens after capture and present them to T-cells to facilitate the adaptive response. They also produce cytokines and chemokines that recruit and activate lymphocytes of adaptive immunity. DCs have two major avenues for antigen presentation; they typically present cytoplasmic antigens to CD8+ cytotoxic T-cells via the MHC Class I complex, and extracellular antigens to CD4+ helper T-cells via MHC Class II [4]. The MHC Class I complex is present ubiquitously on cells throughout the body, while antigen presentation via MHC II is hallmark of professional APCs [29]. DCs have the unique capacity for cross-presentation, which is the ability to present extracellular antigens to cytotoxic CD8+ T-cells via MHC Class I [30]. This process is highly dependent on the cellular process of autophagy, which is a ubiquitous cell-autonomous homeostatic pathway [31]. DCs are a major mechanism of cross-talk between innate and adaptive immunity and are required for an integrated immune response.

1.1.1.3 NK Cells

Natural Killer (NK) cells are important lymphocytes in the response to intracellular (viral) infections and cancer. Many intracellular viruses and tumors have evolved mechanisms to evade immune recognition, including the downregulation of the typically expressed MHC Class I molecule. NK cells provide a backup mechanism to eradicate pathogens/tumors employing MHC I downregulation to evade immune detection, as they recognize and kill cells that fail to expression MHC Class I [32]. They kill infected cells by a variety of mechanisms, including induction of the programmed cell death pathway apoptosis, direct cell lysis via granzymes and perforin, and via production of interferon (IFN)-γ which upregulates the antiviral response and macrophage killing [32].

1.1.2 Inflammation

Inflammation is the primary physiological response to immunologic stimuli and is characterized by the delivery of leukocytes and plasma proteins to the site of pathogenic insult. Symptomatically, inflammation is described by heat, pain, redness, and swelling, and can vary in severity from a mild adaptive process to an aberrant severe life-threatening symptomology. Inflammation was initially described as a response to pathogenic invasion (PAMPs), but it is now appreciated that a myriad of stimuli including irritants and persistent endogenous danger signals (DAMPs) also initiate the inflammatory process [2]. Pathogenic insults typically occur in tissues, while most immune response proteins and cells are present in the blood. During inflammation, inflammatory exudate consisting of innate immune cells and blood proteins are delivered to the extravascular site of infection (or injury) via postcapillary venules [33]. Inflammation is critical in maintaining organism homeostasis as it promotes both pathogen eradication and wound/tissue repair, but excess inflammation is pathological and can result in autoinflammatory disease [33].

Propagation of the inflammatory response results from a well-orchestrated cascade that enhances the initial signal. The cytokines interleukin-1 beta (IL-1β) and tumor-necrosis factor alpha (TNF-α) are critical early pro-inflammatory cytokines that play central roles in initiating systemic inflammation [34]. The IL-1 protein family consists of eleven members, including IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), and IL-18, all of which have been well studied. Cells of innate immunity, including blood monocytes, tissue macrophages, and DCs, are the primary produces of IL-1β in humans. It is synthesized as an inactive precursor (pro-IL-1β), which is a substrate for caspase-1 mediated cleavage upon inflammasome activation. After production of cleaved IL-1β via caspase-1 cleavage, IL-1β undergoes noncanonical secretion to promulgate the inflammatory response in both an autocrine and paracrine manner. [35]. Cellular responses to IL-1β are mediated by the IL-1 receptor (IL-1R), which has a variety of systemic effects in the context of inflammation. IL-1β increases the mesenchymal expression of intracellular adhesion molecule 1 (ICAM-1) and endothelial expression of vascular adhesion molecule 1 (VCAM-1), allowing more efficient delivery of immunocompetent cells and immune proteins from the blood to the extravascular site of pathogenic insult [4]. IL-1β also increases the levels of blood neutrophils and several

important immunomodulatory proteins, including acute phase proteins and several cytokines (including IL-6) [36-38]. While IL-1β is important in eradication of infection following physiological inflammation, excess IL-1β can cause pathophysiological inflammation. Increased blood IL-1β levels presents clinically as fever, lowered pain threshold, vasodilation, and hypotension via IL-1R dependent increases in nitric oxide and prostaglandin E2. [39, 40]. Due to the pleiotropic effects and clinical manifestations of excess IL-1β, IL-1β neutralization has now become a mainstay treatment for autoinflammatory diseases.

In addition to delivering cells and proteins to the extravascular tissue or immediate protection against pathogens, inflammation also instructs adaptive immunity to generate a robust, long term response. This is mediated primarily by DCs, which migrate to the draining lymph node upon antigen capture to present antigens to lymphocytes to activate the adaptive response.

1.1.3 Adaptive Immunity

Adaptive immunity is mediated by B- and T-cells that express pathogen specific receptors and mediate long lasting immunity [3]. As cells of adaptive immunity are not the focus of this thesis, adaptive immunity will only be broadly described for completeness. During development, both B-cells and T-cells undergo somatic recombination of the genes encoding their receptors, the B-cell receptor (BCR) and T-cell receptor (TCR) respectively. Somatic recombination during development results in an infinitely diverse set of receptors, which are screened for reactivity against self via a complex set of mechanisms [41]. Only cells expressing functional receptors that do not bind self-antigens with high affinity are allowed to persist, minimizing the risk of B and T cell autoimmunity. Upon pathogenic invasion, DCs capture antigens and migrate to the germinal centers of lymph nodes, where they present antigens to B- and T-cells. Lymphocytes with receptors capable of recognizing the antigen are activated, where they expand (clonal selection) and eventually mediate immunity against the pathogen eliciting the response.

1.1.3.1 T-cells

T-cells are broken down into two major subsets, CD4+ helper and CD8+ cytotoxic T-cells [42]. CD4+ T-cells recognize antigens presented on MHC Class II molecules, while CD8+ T-cells recognize antigens presented on MHC Class I. T-cell differentiation occurs in the thymus, and CD4+/CD8+ lineage determination occurs in part through mechanisms related to the ability of a precursor T-cell's TCR to bind MHC Class I or MHC Class II. Upon CD4+ helper T-cell activation, they proliferate and result in the secretion of various cytokines. They help activate effector CD8+ cytotoxic T-cells, which can directly kill infected cells via induction of apoptosis or direct cell lysis by granzymes and perforin [43]. CD4+ T-cells can also help activate B-cells, which produce antibodies upon activation. The specific mechanisms through which CD4+ helper T-cells differentially activate CD8+ effector T-cells and B-cells are complex and out of the scope of this thesis [44].

1.1.3.2 B-cells

Naïve B-cells express one of two classes of membrane bound antibodies, IgD or IgM. They are able to recognize antigens through this receptor (which is termed the BCR), and antigen binding initiates signaling through the BCR resulting in the activation and maturation of the naïve B-cell into an antibody secreting plasma cell that mediates humoral immunity [45]. Antibody responses to antigens can be T-cell independent or T-cell dependent, depending on whether CD4+ helper T-cell co-stimulation is required for response to the antigen. Upon prolonged stimulation with an antigen, B-cells produce antibodies with different heavy chain classes due to isotype switching, with specific isotypes having specialized functions to combat specific types of microbes. Antibodies directed against an antigen on the surface of a microbe can bind the microbe in the circulation, which has several functional consequences mediating immunity. Antibody binding can neutralize the ability of the microbe to infect host cells, blocking the infection from taking hold. Antibody binding can also coat microbes, a process termed opsonization. This provides a signal promoting their phagocytosis by neutrophils and macrophages. Antibody binding can also promote killing by NK cells, a process termed antibody dependent cellular cytotoxicity (ADCC). Finally, antibody binding can promote initiation of the complement cascade, which further opsonizes microbes for phagocytosis and provides a positive feedback signal for the development of humoral immunity [46].

1.2 RECEPTORS AND SIGNALING PATHWAYS MEDIATING INNATE IMMUNITY

The work in this thesis focuses on the several signaling pathways that play important roles in macrophage biology and innate immunity, including signaling via TLRs (specifically TLR4), inflammasomes, G-protein coupled receptors, the calcium generating ectoenzyme CD38, and the WNT signaling pathway. These pathways all play an essential role in transducing signals from the extracellular environment into the cell and activate downstream pathways which functionally modulate the cell response. While most of these signaling pathways are conserved across cell types, cell specific variations exist for some of these pathways. Here, I present a brief introduction on each pathway, and discuss the known mechanisms through which these pathways signal with an emphasis on its role in macrophage biology.

1.2.1 Toll-like Receptors (TLRs)

1.2.1.1 TLR

The TLR family consists of 10 members in humans (TLR1-10) and 12 in mice (TLR1-9, TLR11-13). They are typically expressed on cells of the innate immune system, though they can also be found on cells of adaptive immunity. TLRs are membrane bound PRRs expressed either on the cell surface associated with the plasma membrane or in endocytic compartments [47, 48]. TLR4, which is the physiological receptor for LPS, is the most well studied TLR and localizes to the plasma membrane. Structurally, it is a type I transmembrane protein with

extracellular N-terminal LRR domain involved in PAMP/DAMP sensing and an intracellular Toll/IL-1 receptor (TIR) domain important for initiating signaling. Upon ligand engagement, TLR4 dimerizes with the two extracellular LRR domains sandwiching the ligand, while the intracellular TIR domain activates downstream signaling via homotypic interactions [47]. Several adaptor proteins exist that are critical in transducing signals downstream of TLR4, the most important of which are myeloid differentiation factor 88 (MyD88) and TIR domain-containing adaptor-inducing interferon-β (TRIF). Myd88- and TRIF- dependent signaling thus constitute the two major arms of the response downstream of TLR4 [49].

1.2.1.2 Myd88-dependent signaling and TRIF-dependent signaling

Engagement of TLR4 by LPS results in rapid recruitment of MyD88, which plays a scaffolding role in the formation of a large complex required for activation of downstream effectors. Both IRAK1 and IRAK4 are recruited to the complex and activated, allowing the binding of the RING-domain containing E3 ubiquitin ligase TRAF6 and subsequent activation of the kinase TAK1. TAK1 then directly activates the MyD88 downstream effectors, the NFκB pathway and MAPK signaling [50]. NFκB is a transcription factor responsible for the upregulation of many pro-inflammatory cytokines including TNF-α and IL-6, both of which are secreted from macrophages following LPS stimulation [51]. NFκB in macrophages is also essential in the upregulation of pro-IL-1β, which is not secreted unless a separate signal results in activation of the inflammasome [52, 53]. Initiation of the MAPK signaling cascade results in activation of the downstream transcription factors ERK1/2, JNK1/2, and p38 [54], which provide crosstalk aiding the upregulation of proinflammatory cytokines. While the MyD88-dependent rapidly activates transcription of pro-inflammatory cytokines, TRIF-dependent signaling is essential for the upregulation of type 1 IFN genes [55]. TRIF-dependent signaling results in the downstream activation of the kinases TBK1 and IKKi, which together phosphorylate and activate IRF3. IRF3 transcriptionally upregulates several type I IFN genes that are important in the response to LPS.

1.2.2 Inflammasomes

Inflammasomes are critical mediators of the early inflammatory response, as they generate IL-1β upon activation [56]. Inflammasomes consist of three key components: a sensor molecule, the adaptor protein ASC, and caspase-1. ASC contains two notable conserved structural domains; it has an N-terminal caspase activation and recruitment domain (CARD) and a C-terminal pyrin (PYD) domain [57]. Inflammasomes are typically named for their sensor molecule, which is a cytosolic PRR capable of recognizing specific PAMPs or DAMPs. The most well studied inflammasomes include the NLRP3, AIM2, and NLRC4 inflammasomes which recognize cellular stress, cytosolic DNA, and bacterial flagellin respectively. Activation of the sensor molecule results in its oligomerization and the subsequent recruitment of ASC, which forms a large protein speck due to homotypic PYD-PYD mediated multimerization. Caspase-1 is then recruited to the ASC speck via its CARD domain, promoting its autocatalytic activation. Active caspase-1 cleaves pro-IL-1β and pro-IL-18, generating their biologically active counterparts [58].

Importantly, inflammasome activation is a two-step process. It first requires a priming signal (signal 1) which *in vitro* is typically provided by LPS. This signal activates NFκB and promotes the transcriptional upregulation of IL-1β. For the NLRP3 inflammasome, priming additionally induces the transcriptional upregulation of NLRP3 and licenses NLRP3 assembly by deubiquitination of NLRP3 [53]. Next, a sensor specific stimulus (signal two) is required for activation of the sensor molecule. In this way, inflammasome activation is tightly regulated, as aberrant inflammasome activity has serious pathologic consequences [59-61].

1.2.3 G-protein Signaling

1.2.3.1 G-protein coupled receptors (GCPRs)

G-protein coupled receptors are a large family of evolutionarily conserved proteins involved in transducing stimuli from the microenvironment into intracellular signals [62]. They mediate the majority of cellular responses to external stimuli, including light, odors, hormones, growth factors, and many immune signaling molecules [63]. GPCRs are of considerable interest to human health and disease, as the human genome encodes over 800 GPCRs and almost 35% of all FDA approved drugs target GPCRs. Nonetheless, 56% of non-olfactory GPCRs remain unexplored in clinical trials. Many of these unexplored GPCRs are known to have effects on the immune system, therefore GPCRs present an area with untapped therapeutic potential for immune-related diseases [64]. GPCRs transduce signals upon ligand binding by coupling to heterotrimeric G-proteins, which in their resting state consist of a GDP-bound Gα subunit in complex with a Gβγ heterodimer. GPCR activation induces GTP nucleotide exchange on Gα, causing its dissociation from the Gβγ heterodimer. Upon dissociation, both GTP-bound Gα and Gβγ heterodimers transduce signals via their corresponding downstream effector molecules. Signaling continues until the inherent GTPase activity of Gα subunits results in the hydrolysis of GTP to GDP, allowing re-association of GDP-bound Gα and Gβγ to terminate signaling. This cycle plays a critical role in cell signaling networks, allowing environmental signals to be transduced and integrated into a coordinated intracellular biological response [65].

1.2.3.2 $G\alpha_i$ and associated regulatory proteins

The functional versatility of GPCR signaling is mediated in part by the existence of several subtypes of G proteins. G-proteins are functionally defined by their Gα subunit, of which four families exist: $G\alpha_s$, $G\alpha_i$/ $G\alpha_o$, $G\alpha_q$/$G\alpha_{11}$, and $G\alpha_{12/13}$ [66]. The $G\alpha_i$ subunit is of particular importance in the immune system, as it is highly expressed across a variety of leukocytes [67]. Classically, the GTP-$G\alpha_i$ reduces intracellular cyclic-AMP levels by inhibiting certain adenylate cyclase isoforms [68]. In murine macrophages, which express the $G\alpha_{i2}$ and $G\alpha_{i3}$ isoforms, this is not believed to be a major mechanism of $G\alpha_i$ mediated signaling due to the lack of $G\alpha_i$ sensitive adenylate cyclase isoforms in these cells.

In addition to the traditional GPCR-G-protein-effector template, there are several G-protein regulatory proteins that exert their biological function by modulating G-protein signaling.

These regulatory proteins are typically classified into two groups, regulators of G-protein signaling (RGS) and activators of G-protein signaling (AGS) [69]. The RGS family contains more than 30 proteins and is defined by the presence of an RGS domain, which mediates their interaction with Gα subunits [67]. RGS proteins are negative regulators of G protein signaling, as they act as GTPase accelerating proteins (GAPs) thereby limiting the duration of Gα signaling [67, 70]. They enhance the intrinsic GTPase activity of Gα by stabilizing the GTPase transition state, which can accelerate the intrinsic Gα GTPase activity by 100-fold [71]. Accordingly, G-proteins modified to be insensitive to RGS function show higher basal levels of signaling [67]. As their name suggests, the AGS proteins function to activate G-proteins independent of receptor coupling [72]. AGS3 and AGS4, which enhance Gβγ signaling by directly binding GDP-bound Gα, are of particular interest due to their relatively high expression levels in macrophages [73, 74].

1.2.3.3 Role of $G\alpha_i$ in macrophages

The role of $G\alpha_i$ signaling in macrophages remains unclear, due to a variety of experimental approaches that have functionally implicated $G\alpha_i$ in macrophage biology without illuminating the molecular details. Studies on several $G\alpha_i$-coupled GPCRs in macrophages, including formyl-peptide receptor 2, the chemokine receptor CXCR3, and Chemeren receptor 23 have implicated these GPCRs in macrophage polarization, as multiple cognate ligands drive either M1 or M2 polarization in ligand specific manner [75-79]. However, experimental approaches studying $G\alpha_i$-coupled GPCRs are limiting in that it remains unclear which effects are due to $G\alpha_i$ signaling, Gβγ signaling, or non-canonical G-protein signaling. Other studies have implicated $G\alpha_i$ in signal transduction downstream of TLR4, but many of these studies relied on inhibition of $G\alpha_i$ by pertussis toxin (PTX) [80-82]. While treatment with PTX to inhibit $G\alpha_i$ is useful in many settings, it remains unclear if the effects of PTX truly phenocopy the loss of $G\alpha_i$. A few studies have used genetic deletion of $G\alpha_i$ in murine bone marrow derived macrophages (BMDMs) and show defects in phagocytosis and chemotaxis following loss of $G\alpha_i$ [83]. However, more studies are needed to fully understand the role of $G\alpha_i$ in macrophage biology.

1.2.4 CD38 and Calcium Signaling

CD38 is a member of the evolutionarily conserved ADP-ribosyl cyclase family of proteins, which are named for their ability to generated cyclic-ADP-ribose (cADPR) from nicotinamide adenine dinucleotide (NAD). The defining member of this family is the *Aplysia* ADP-ribosyl cyclase [84], and in addition to CD38, the human genome encodes for another family member named BST1 [85]. CD38 exists as a 46 kDa glycosylated type II membrane protein with a long extracellular C-terminal domain and a short 21 aa cytoplasmic tail [86]. CD38 is highly expressed in lymphoid and myeloid cells of the immune system, and has multiple enzymatic functions enabling the production of calcium mobilizing second messengers [87]. In addition to the earlier described ADP-ribosyl cyclase activity used to generate cADPR from NAD, CD38 can generate the second messenger ADP-ribose (ADPR) from NAD via its glycohydrolase activity [88, 89]. In acidic compartments, such as

endosomes and lysosomes, CD38 can utilize NADP to generate NAADP through a base exchange reaction [90, 91].

Notably, all second messengers generated by CD38 mobilize calcium from different sources. The major intracellular calcium stores include the endoplasmic reticulum and lysosomes, while calcium entry from the extracellular space can be mediated by receptors on the plasma membrane. ADPR binds TRPM2 on the plasma membrane to mobilize extracellular calcium [92], cADPR mobilizes ER calcium via Ryanodine Receptors (RyRs) [93], while NAADP targets two pore channels (TPCs) on the lysosome [94, 95]. Significant cross-talk has been shown to exist between these calcium stores. For example, NAADP directly targets the lysosomal two pore channels (TPCs), mobilizing lysosomal calcium. This then promotes amplification of the calcium signal by mobilizing calcium from other stores, a process termed calcium induced calcium release (CICR) [94, 96, 97]. In addition to initiating calcium signaling through production of second messengers, ligation of CD38 with antibodies can initiate distinct signaling pathways that do not require CD38 enzymatic activity. These pathways have been described in B-cells and have been shown to influence proliferation or death of several B cell subsets [98, 99].

Despite involvement in intracellular signaling pathways, the physiological function of CD38 remains unclear. In macrophages, CD38 is strongly upregulated after exposure to immune stimuli and has been suggested as a marker for M1 murine macrophage polarization [100, 101]. Studies have also implicated CD38 in promoting inflammation downstream of LPS and suggest CD38 plays a role in enabling phagocytosis by inducing calcium release after phagosome formation [102, 103]. Finally, CD38 has been implicated in autophagy. CD38 deficient cells have autophagic activation defects following stimulation with LPS [104], while *in vivo* studies in mouse models of coronary atherosclerosis similarly show autophagic defects in CD38 deficient mice [105, 106]. Finally, CD38 is very highly expressed in B cell tumors, especially in multiple myeloma. The monoclonal anti-CD38 antibody Daratumumab is already FDA approved for multiple myeloma, while the monoclonal Isatuximab is in phase III clinical trials [86, 107]. In addition to antibody-dependent cell-mediated cytotoxicty, these antibodies induce direct cytotoxicity via activation of intracellular pathways [108, 109]. Despite clinical use of these monoclonal antibodies (mAbs), the molecular mechanisms mediating signaling downstream of CD38 remain unclear.

1.2.5 WNT/β-catenin signaling

The final signaling pathway covered in this thesis is the Wingless/integrated (WNT) signaling pathway. WNT signaling has been primarily studied in the context of development and cancer, but recent advances show roles for WNT signaling in immune cells [110-112]. WNT signaling is a complex pathway activated by Wnts, which are the physiologic ligands of Frizzled (FZD) receptors. The most well studied WNT pathway is the WNT/β-catenin pathway, which results in downstream activation of T cell factor/lymphoid-enhancer binding factor (TCF/LEF) proteins (TCFs) [113]. In the WNT OFF state, TCFs bind Transducin-Like-Enhancer of split proteins (TLEs), where they act as transcriptional repressors on WNT

response elements. Upon activation of WNT signaling, TLEs in complex with TCFs are replaced by the transcriptional co-activator β-catenin, and TCF/β-catenin complexes promote transcription of WNT target genes [114-116].

β-catenin stabilization is the key step in Wnt/β-catenin signaling. In the absence of signaling, free β-catenin is quickly degraded by the Wnt destruction complex, which consists of the scaffold protein Axin1, the kinases CK1α/δ and GSK3α/β, the tumor suppressor protein APC, and β-catenin [117]. The kinases in this complex, CK1 and GSK3, mediate the constitutive phosphorylation of β-catenin inducing its ubiquitination by the E3 ligase β-TrCP and subsequent degradation [118-120]. Upon activation of WNT signaling, the destruction complex is localized to the cell membrane and inhibited, thereby allowing β-catenin levels to rise rapidly and accumulate both in the cytoplasm and nucleus [121]. Nuclear β-catenin then displaces TLEs to associate with TCFs, resulting in the transcriptional activation of Wnt target genes [115].

The WNT/β-catenin pathway is anabolic in nature, and has been implicated in several important processes including cell proliferation, cell migration, and cell fate determination [122]. The processes controlled by Wnt signaling are bioenergetic, requiring the consumption of energy [122]. Consistently, analysis of transcriptional changes downstream of WNT signaling suggest it plays a role in glutamine metabolism [123], which is important in cell growth and biosynthesis [124, 125]. In the immune response, upregulation of energetic pathways is important, but efficient utilization of energy towards initiating and sustaining inflammation precedes the need for cell growth and biosynthesis. There is limited data on the role of WNT/β-catenin signaling in macrophages, though a few studies exist. In alveolar macrophages, WNT/β-catenin has been implicated as a causative factor in excess fibrosis, which may be due to WNT/β-catenin mediated M2 macrophage polarization [126, 127].

1.3 HOMEOSTATIC CELLULAR FUNCTIONS INVOLVED IN INNATE IMMUNITY

In additional to the specialized mechanisms of innate immune cells to respond to immunogens, these cells also have a complex set of adaptive mechanisms to maintain cellular homeostasis. These mechanisms are shared among all cells and are especially prominent during conditions of cell stress. They function to either facilitate a return to homeostasis or delete severely dysregulated cells in order to maintain health of the organism [128]. These cell stress responses have both cell-autonomous and cell-extrinsic components, the latter of which contributes to systemic adaptations to stress conditions. Along this line of thinking, inflammation has been described as a part of a spectrum, with bona-fide systemic inflammation being the extreme end of a progressive spectrum that includes homeostasis, the physiological stress response, and finally inflammation [129]. Consistently, conditions that activate innate immunity including the presence of PAMPs or DAMPs can be considered intense forms of cell stress [130].

Two such conditions that are adaptive responses to disruption of cellular homeostasis are autophagy and programmed cell death. Autophagy is a critical mechanism for the clearance of cytoplasmic waste and is considered an adaptive mechanism as it recycles cellular nutrients during conditions of cell stress. It has also been implicated in the clearance of intracellular pathogens, which is important for defense against a variety of bacterial infections [131]. Programmed cell death (PCD) occurs when cellular homeostatic mechanisms are overcome. PCD can be anti-inflammatory in nature (apoptosis) or inflammatory (necroptosis, pyroptosis, etc) [132, 133]. In this section, I discuss the autophagy/lysosome system, its role as part of the stress response, and its contribution to innate immunity. I then move on to discuss the several PCD pathways, with emphasis on how they affect inflammation. The work in this thesis involves signaling controlling these pathways and their effects on innate immunity.

1.3.1 Autophagy/Lysosome System

Autophagy is a conserved cellular degradation pathway involved in the clearance of cytoplasmic waste. It delivers both cellular organelles and large protein complexes to the lysosome, enabling their destruction. From an evolutionary perspective, autophagy developed both as an adaptive mechanism mediating cellular recycling and a quality control mechanism for the clearance of harmful complexes [134]. Autophagy is orchestrated by complex mechanisms involving more than 30 proteins. Upon induction, a membrane sac termed the isolation membrane expands into a double membrane vesicle termed the autophagosome. Elongation of this membrane results in either the selective or nonselective envelopment of cytoplasmic constituents, which are then confined in the autophagosome lumen. The autophagosome eventually merges with a lysosome, resulting in a structure called the autophagolysosome which mediates the destruction of sequestered material via lysosomal proteases and the acidic pH of the compartment [135].

Though autophagy was initially recognized as a cellular response to nutrient starvation, it is now appreciated that autophagy is induced by many cell stress events. Immunologic stimuli are among those that induce autophagy, including stimulation with LPS. LPS stimulation activates TLR4, resulting in the targeting of Beclin-1 (a key component of the Class III PI(3)K autophagosome initiation complex) to the TLR adaptor proteins MyD88 and TRIF, which results in its TRAF6 mediated K63-linked ubiquitination and activation [136, 137]. Autophagy is also initiated following pathogenic invasion by many protozoa, bacteria, and viruses. Autophagy mediates immunity against intracellular pathogens by direct sequestration of pathogens, delivering them to the lysosome for destruction [131]. The importance of autophagy in the immune response is further highlighted by its function in antigen presentation [138]. While the complex mechanisms that regulating autophagy initiation are out of the scope of this thesis, it has recently been appreciated that autophagy is also transcriptionally controlled by Transcription Factor EB (TFEB).

1.3.2 Programmed Cell Death

PCD is a critical process for normal cell turnover, organismal development, and immune cell function. Canonically, PCD was believed only to occur through apoptosis, a caspase-dependent process resulting in non-inflammatory cell death. Inflammatory cell death is characterized by cellular leakage, cytoplasmic granulation, and organelle/cellular swelling (oncosis) and is termed necrosis. It was long assumed that necrotic cell death was accidental, due to the inability of cells to respond appropriately to cellular injury or stress. Accumulation of evidence over the years has challenged the view that necrosis only occurs accidently, leading to the understanding that regulated forms of necrosis exist [139-141]. It is now appreciated that there are multiple PCD pathways resulting in necrotic cell death, including necroptosis, pyroptosis, ferroptotis, MTP-mediated regulated necroptosis, parthanatosis, and NETosis/ETosis. All of these forms of PCD have different regulatory factors, execution factors, and physiologies [139-141]. Of these, necroptosis and pyroptosis are the best understood. As necroptotic and pyroptotic cell death result in the release of inflammation inducing DAMPs and are investigated later in this thesis, their basic mechanisms and signaling paradigms are discussed.

1.3.2.1 Apoptosis

Apoptosis, which was first described in a classic 1972 paper by Kerr, Wyllie, and Currie [142], is a morphologically distinct cell death characterized by cell shrinkage, an intact membrane that has undergone blebbing, cytoplasmic retention in apoptotic bodies, and nuclear DNA fragmentation [143]. The molecular machinery involved in apoptosis was first elucidated in C. elegans [144] and later worked out in mammals [145]. In short, members of the caspase family involved in apoptosis have been classified as initiator caspases (caspase-8, -9, and -10) or executioner caspases (caspase-3, -6, and -7). Both the extrinsic and intrinsic apoptosis pathways converge at activation of the executioner caspases, but upstream signaling differs. The extrinsic pathway is caspase-8 dependent and responds to FasR death signals, while the intrinsic pathway is caspase-9 dependent and responds to intracellular stresses (e.g. toxins, hypoxia, radiation) [143, 146]. Notably, apoptosis is considered a non-inflammatory form of cell death, as cell components remain neatly packaged inside apoptotic bodies precluding the release of DAMPs into the surrounding microenvironment [147]. Additional mechanisms exist to contain a potential inflammatory response, including “find me” and “eat me” signals on apoptotic bodies that recruit phagocytes facilitating their clearance [148]. Moreover, apoptotic cells and/or the phagocytes that clear them release anti-inflammatory cytokines such as IL-10 and TGF-β, ensuring minimal inflammatory responses in physiological conditions [147].

1.3.2.2 Necroptosis

Necroptosis refers to a form of programmed necrosis, which is an inflammatory cell death. The existence of programmed necrosis pathways was first suggested by Goodling’s group, who observed that TNF-α could induce both apoptosis and necrosis [149]. The authors used

two different cell lines sensitive to TNF-α mediated death, F17 (rat fibroblast line) and L-M (mouse fibroblast cell line), to examine the biochemical and morphological features associated with TNF-α induced death. The F17 cell line displayed apoptotic death, with low-molecular weight DNA and membrane blebbing seen by time-lapse microscopy (hallmarks of apoptosis). The L-M cell line, on the other hand, had no detectable low-molecular weight DNA release, and showed cellular swelling, cytoplasmic granulation, and eventual lysis by time lapse microscopy. This led the authors to suggest the potential formation of a "self-assembling membrane attack complex" a statement proven true more than 20 years later with the discovery of the necroptotic pathway [150].

The cellular signaling pathways mediating necroptosis have now been partially elucidated. Necroptosis is a RIPK1, RIPK3, and MLKL dependent pathway that is initiated by ligands of the death receptor family [151, 152]. Upstream activation requires RIPK1 kinase activity, which acts a molecular switch between proliferation/inflammation (RIPK1 complex 1 activity – NFkB activation) and necroptosis (RIPK1 complex 2b activity – programmed cell death). Initiation of necroptosis leads to trimerization of RIPK3, resulting in membrane recruitment of the effector protein MLKL which leads to formation of membrane holes and cell death [153]. Interestingly, basal levels of caspase-8 decrease RIPK1 kinase activity, indicating crosstalk between apoptotic and necroptotic PCD pathways. Necroptotic proteins are highly expressed in macrophages, which have been shown to undergo necroptosis in physiological conditions. Necroptosis is considered an inflammatory cell death pathway, as necrotic cell death results in the release of several DAMPs due to loss of membrane permeability [154]. There is also evidence suggesting that necroptotic death activates low levels of cytokine transcription in dying cells [155].

1.3.2.3 Pyroptosis

Pyroptosis is a highly inflammatory programmed cell death pathway that occurs following inflammasome activation. In short, activation of caspase-1 by inflammasome assembly results in cleavage of gasdermin D, allowing the N-terminal of gasdermin D to translocate to the plasma membrane and form pores [156]. The formation of membrane pores results in the release of several DAMPs, including ATP, DNA, ASC specks, the HMGB1 protein, and IL-1β, which strongly propagates inflammation [157].

1.4 LRRK2

Several non-receptor proteins were identified as novel signaling molecules downstream of the receptors investigated in this thesis, the Leucine-rich repeat kinase (LRRK2) is one of them. Thus, I provide essential background on the structure, biological function, and immunological functions of the LRRK2 protein.

1.4.1 LRRK2: Background

Leucine-rich repeat kinase 2 (LRRK2) is a large, 2527 amino acid protein with multiple functional and protein interaction domains. Autosomal dominant mutations in LRRK2 are the most common genetic cause of Parkinson's disease (PD) and result in late onset disease that is symptomatically indistinguishable from idiopathic PD [158]. LRRK2 mediated and idiopathic PD share common pathophysiologic pathways, including immune system involvement [159, 160]. In addition to PD, LRRK2 has been identified as a risk factor for Crohn's disease and leprosy [161, 162]. The common link between these diseases is systemic inflammation [163], further implicating LRRK2 in peripheral and innate immunity. Despite the vast body of research on neuronal LRRK2 (where it is lowly expressed), its physiological function remains unknown [164]. As LRRK2 is most strongly expressed in myeloid cells and B-lymphocytes [165], focus has turned to the role of LRRK2 in the immune system in hope of further elucidating its physiological and pathophysiological roles.

1.4.2 LRRK2: Structure and Function

LRRK2 is a large Roco family protein containing putative protein-protein interaction domains flanking a central catalytic region. Its core catalytic region consists of an N-terminal Ras-of-Complex (ROC) GTPase domain, a C-terminal serine/threonine kinase (MAPKKK-like, RIPK-like) domain, and a linker C-terminal of Roc (COR) domain. The N terminal of LRRK2 has an LRR (leucine rich repeat) domain and an ankyrin domain, while the C terminal contains WD40 repeats [166]. In line with its many protein interaction domains, a recent computational review of the literature shows more than 200 interaction partners for LRRK2 [167]. Given that multiple LRRK2 domains also have enzymatic activity, its structure suggests a multifaceted involvement in cell signaling and molecular scaffolding, implicating it as a potential regulatory hub with the ability to integrate and modulate multiple signaling pathways.

LRRK2 exists primarily as a cytosolic monomer, but in certain conditions forms dimers on the plasma membrane resulting in significant enhancement of kinase activity [168-170]. Recent advances have provided structural insight into the domain interactions mediating LRRK2 dimerization. Guaitoli et al. show that two monomers symmetrically interact in a head-to-tail orientation, with intramolecular interactions between the N-terminal ankyrin domain and C-terminal WD40 domain necessary for stabilization of intermolecular interactions by the central ROC-COR regions allowing dimer formation [171]. There is considerable evidence that LRRK2 undergoes autophosphorylation, with many of these events occurring in the ROC domain and regulating the ROC domain's GTPase activity [172, 173]. This observation raises the important point that LRRK2 catalytic domains likely regulate each other. Another study linked GDP/GTP binding state to dimerization in a LRRK2 homologue [174]. The study shows that a bacterial LRRK2 homologue is mainly dimeric in the unbound or GDP-bound state, but forms monomers upon GTP binding,

indicating a monomer-dimer cycle during GTP binding and hydrolysis [174]. Together, these findings suggest a complex mechanism by which LRRK2 kinase activity modulates its GTPase activity, which in turn regulates dimer formation and kinase activity (Figure 1). These important findings have significant implications in our attempt to better understand the pathogenic properties of disease causing LRRK2 mutants.

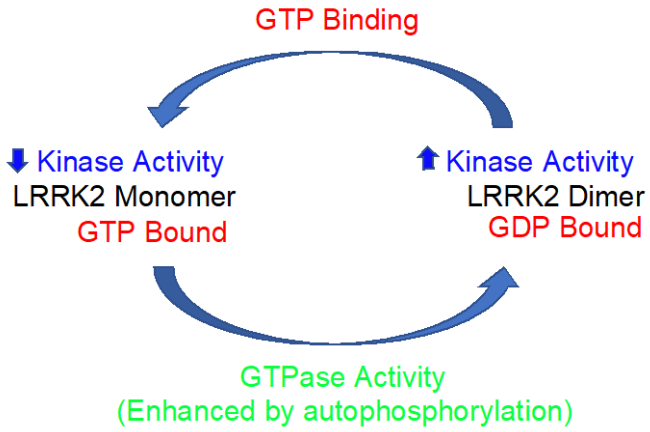

Figure 1. Schematic representation of hypothesized cross-regulation between LRRK2 kinase and GTPase activity.

The majority of PD causing LRRK2 mutants are in the core catalytic region [175]. The most common pathogenic mutation, G2019S, and the neighboring I2020T mutation are both in the kinase domain and increase kinase activity [176-178]. The increase in LRRK2 kinase activity appears to be sufficient for its pathogenic effects, as inhibition of kinase activity in these mutants slows LRRK2 mediated pathology [179]. The ROC domain of LRRK2 has high homology to Ras superfamily proteins and therefore both binds and hydrolyzes GTP [166]. The R1441 location in ROC domain is a hotspot for pathogenic mutations, as changes to C/G/H are all pathogenic [179]. It is unclear if mutations at this location increases kinase activity, but ROC domain autophosphorylation is clearly increased in these pathogenic mutants, as is similarly seen with increased kinase activity.

Finally, while many substrates for LRRK2 have been suggested, it is unclear which are physiological substrates due to many studies being done in overexpression models *in vitro* [180-183]. Recently, an unbiased screen found Rab1, Rab8a, Rab10 as bona-fide in vivo substrates of LRRK2 [184], and a second report corroborated that LRRK2 phosphorylates membrane bound Rabs [185, 186]. Moving forward, more research needs to be done to determine the pathophysiological consequences of Rab substrate hyperphosphorylation. Given the involvement of Rab proteins in membrane dynamics, it is possible that the pathogenic effects of LRRK2 are mediated by disruption of membrane associated pathways, including the autophagy/lysosome system.

1.4.3 LRRK2: Effects on the Autophagy/Lysosome Pathway

Although LRRK2 was linked to the endolysosomal system and autophagy almost 10 years ago [187], there is still no consensus on the effect of LRRK2 on these pathways. There exist many discordant reports within the field, with studies showing both positive and negative effects of LRRK2 on autophagy, a role for LRRK2 in different steps along the autophagic pathway, and discrepancies between the effects of LRRK2 kinase inhibition,

knockdown/knockout, and LRRK2 mutant expression (reviewed in [188, 189]). Here, I briefly summarize the existing data categorized by the biochemical approached used to study LRRK2: (1) LRRK2 G2019S mutant, (2) LRRK2 knockdown or KO, or (3) pharmacological LRRK2 kinase inhibition.

The pathogenic effects of LRRK2 G2019S are thought to be mediated primarily by an increase in kinase function – thus the G2019S mutant is often used to model LRRK2 kinase overactivity. Many studies on the LRRK2 G2019s mutation have shown it as a positive regulator of autophagy [187, 190-195]. Overexpression of LRRK2 G2019S in a neuronal cell line caused an increase in autophagosomes and neurite shortening [187]. Later studies overexpressing the LRRK2 kinase domain (WT and G2019S) corroborate this, showing increases in autophagosome number dependent on a CaMKK-β/AMPK and an NAADP dependent pathway [190]. Several other studies suggest LRRK2 G2019S as a positive regulator of autophagy; one reported enhanced autophagy due to LRRK2 G2019S phosphorylation of Thr^{56} on Bcl-2 [191], another showed increased in basal autophagy in LRRK2 G2019S patient fibroblasts [194], and yet another showed increased autophagosome formation in the cerebral cortex [195]. On the other hand, a study on LRRK2 G2019S patient derived stem cells suggested a decrease in autophagic flux despite increased autophagosome formation due to defects in autophagosome clearance [196]. This data is consistent with other reports showing defective lysosome degradation in LRRK2 G2019S cells [192, 193]. Studies from the Manzoni and coworkers suggest decreased autophagy/autophagic flux in G2019S cell lines and patient fibroblasts [197, 198]. Given the conflicting studies, there is a need for more reproducible data and better mechanistic insights on the effects of LRRK2 G2019S on autophagy.

Studies in LRRK2 KO models again show a complex picture. In LRRK2 KO mice, autophagic changes in kidney tissues were age dependent. Seven month old mice showed increased autophagy in the kidneys, while twenty month old mice showed the opposite [199]. An elegant study using LRRK2 knockdown macrophages provided some mechanistic insight into the role of LRRK2 in autophagy after LPS stimulation. LPS stimulation induced phosphorylation of LRRK2 at Ser^{935} and initiated the recruitment of LRRK2 to membranes; macrophages deficient in LRRK2 show less autophagosome formation and autophagic flux than controls [200]. Finally, a study using LRRK2 KO neuronal cells showed defects in autophagy dependent on endophilin A function, which LRRK2 directly phosphorylates [180, 201]. There have been a variety of studies using LRRK2 chemical inhibitors, with studies from Manzoni and coworkers showing an increase in autophagy after kinase inhibition [197, 202]. Studies using these chemical inhibitors are hard to interpret, as they effectively inhibit kinase activity but do not affect LRRK2 scaffolding or GTPase activity. Without chemical or biochemical tools to selectively inhibit other LRRK2 functions, studies using existing chemical inhibitors should be interpreted carefully.

Given the complexities and conflicting data, it is likely that we are missing a key mechanistic feature related to the effect of LRRK2 on autophagy. However, there do exist several valid

reasons that data on LRRK2 in autophagy appears discordant. First, there may be cell type differences stemming from variation in LRRK2 expression level from cell to cell and from variation in basal autophagy among cell types. Second, LRRK2 may affect multiple steps in the autophagy/lysosome pathway, with different phenotypes resulting from overexpression or loss of function. Finally, LRRK2 may differentially regulate autophagy dependent on the upstream stimulus, forming different complexes in response to different stimuli. In sum, while it is accepted in the scientific community that LRRK2 modulates the autophagy/lysosome pathway, detailed and reproducible studies on the effect of LRRK2 on each step of the autophagy/lysosome system are needed.

1.4.4 LRRK2: Role in the Immune System

Several lines of evidence exist suggesting an immune function for LRRK2. First, LRRK2 is most highly expressed in myeloid cells and B-cells [165]. In macrophages, its expression is upregulated by IFN-γ and viral particles [165, 203, 204], and LRRK2 is phosphorylated at Ser^{935} upon exposure to LPS [205]. Structurally, the LRR repeats on LRRK2 resemble those seen on PRRs (such as the TLRs and NLRs), and structural analysis shows that LRRK2's LRR domain resembles that of NLRP3 [206]. The LRRK2 kinase domain is a RIPK family member, and this family of proteins is intimately related to inflammation and cell death [151, 207, 208]. RIPK1 and RIPK3 act as molecular switches between inflammatory and necroptotic signaling [151]. RIP2 functions downstream of the PRRs NOD1/NOD2, and LRRK2 has been implicated in the NOD2/RIPK2 pathway as it directly interacts with these proteins. This interaction is required for the proper release of bacterial lysozyme in Paneth cells of the gut in response to commensal bacteria [209].

A variety of studies have shown that LRRK2 deficiency generally increases susceptibility to intracellular bacterial infection, with focus on the role of LRRK2 in macrophages [165, 209, 210]. Mice deficient in LRRK2 showed increased susceptibility to *Salmonella Typhimurium* [210], while *in vitro* infection of LRRK2 deficient macrophages with *Salmonella* reveal defects in bacterial killing [165]. LRRK2 deficient mice also show increased susceptibility to *Listeria* infection [209]. Finally, LRRK2 mediated changes in autophagy are likely to have important consequences on pathogen clearance, as autophagic engulfment of foreign pathogens (called xenophagy) is an important mechanism in maintaining host homeostasis [131]. Overall, these studies suggest that LRRK2 is a proinflammatory protein, and that lack of LRRK2 increases susceptibility to foreign pathogens.

Consistent with the notion that LRRK2 is a positive regulator of inflammation, several studies suggest that LRRK2 deficiency rescues pathogenicity caused by aberrant host inflammation [211-216]. In a murine model of neuro-HIV, LRRK2 kinase inhibition and LRRK2 deficiency decreased pathological inflammation after phagocytosis of the HIV-protein Tat [211, 212]. Two additional studies in LRRK2 deficient microglia showed an attenuation of proinflammatory signals, including TNF-α and iNOS after LPS stimulation [213, 214]. Moreover, LRRK2 pathogenic mutants appear to be proinflammatory, with the R114G KI mice having increase inflammatory markers and LRRK2 G2019S myeloid cells

showing increased migration to cytokines [215, 217]. Finally, asymptomatic patients with the LRRK2 G2019S mutation show increases in peripheral inflammation, including increases in IL-1β, IL-8, and other proinflammatory markers [216].

Apart from myeloid cells, LRRK2 is highly expressed in DCs and B-cells. In DCs, LRRK2 was shown to regulate expression of the Na^+/K^+ ATPase and Na^+/Ca^{2+} pumps in the plasma membrane [218, 219]. This results in LRRK2 KO cells having increased calcium mobilization upon store operated calcium entry from the ER. In B cells, LRRK2 is expressed in B-2 cells but not B-1 cells [220]. A preliminary immunophenotyping of the B cell compartment in LRRK2 KO mice showed increased basal IgA levels and increased IgM after immunization with TNP-Ficoll [221, 222]. A more recent study implicated LRRK2 function in B cells to systemic erythematous lupus (SLE) [223]. LRRK2 was shown to promote autoantibody production in a pristine induced SLE model, and LRRK2 KO mice were protected against development of disease. Accordingly, LRRK2 KO mice showed decreased germinal center and plasma cell levels basally [223].

1.5 TRANSCRIPTION FACTOR EB (TFEB)

TFEB is the master transcriptional regulator of the autophagy/lysosome machinery and an integral component of the cell stress response. The autophagy/lysosome system and its role in cellular stress is investigated several times in this thesis, and TFEB is implicated downstream of a variety of signaling pathways. Thus, I provide necessary background on this essential transcription factor.

1.5.1 TFEB: Background

TFEB is an evolutionarily conserved transcription factor belonging to the microphthalmia (MiT) family of proteins, which include the closely related transcription factors MITF, TFE3, and TFEC. TFEB is the master transcriptional regulator of the autophagy and lysosome machinery [224], and shares some functional overlap with TFE3 which has also been shown to regulate the autophagy/lysosome machinery [225]. TFEB has been implicated in the pathogenesis of several cancers and pursued as a therapeutic target for the treatment of several diseases resulting from accumulation of toxic aggregates [226]. While previously thought to be a static housekeeping organelle, it is now appreciated that the lysosome is tightly regulated by both transcriptional activators and repressors [227-229]. TFEB was first described as a response to nutrient deprivation, as starvation promotes mTOR dependent TFEB nuclear translocation [230]. Once in the nucleus, TFEB binds a 10-base palindromic sequence (GTCACGTGAC) called CLEAR binding elements resulting in the transcriptional upregulation of TFEB response genes, including those of the autophagy/lysosome system [228]. Via these pathways, TFEB promotes the clearance of aggregated proteins, which has therapeutic implications [231-235]. In addition to its function on the autophagy/lysosome system, TFEB regulates endosome dynamics and lysosomal exocytosis [224]. TFEB also

promotes transcription of several immune genes and those that function as part of the integrated stress response [236, 237].

1.5.2 TFEB: Molecular Mechanisms of Activation

TFEB is regulated primarily by post translational modifications, as it contains many phosphorylation sites governing its protein-protein interactions and subcellular localization (reviewed in [224]) **(Figure 2)**. TFEB was first described as an adaptive response to nutrient starvation, which causes its nuclear translocation and the transcription of autophagy related genes to promote nutrient recycling [228]. The mammalian target of rapamycin complex 1 (mTORC1) is an essential nutrient sensor responsible for integrating environmental signals [238]. The mTORC1 complex contains the important kinase mTOR, which was quickly determined to regulate TFEB subcellular localization by directly phosphorylating TFEB Ser^{211} [239]. In nutrient replete conditions, mTOR-dependent phosphorylation of TFEB on Ser^{211} mediates its interaction with 14-3-3 proteins, resulting in its cytoplasmic sequestration and inactivation [240, 241]. Inhibition of mTOR in nutrient starved conditions conversely results in the dephosphorylation of Ser^{211}, freeing TFEB from interaction with 14-3-3 proteins and allowing its nuclear translocation [228]. More recently, it was shown that mTORC1 is also responsible promoting TFEB cytoplasmic sequestration by phosphorylating TFEB on Ser^{122} [242].

The discovery of mTOR independent TFEB activation pathways led to the hypothesis that additional mechanisms regulate TFEB Ser^{211} phosphorylation. Calcineurin, a heterodimer consisting of the calmodulin-binding catalytic subunit calcineurin A and a smaller Ca^{2+}-binding subunit calcineurin B, was subsequently identified as the phosphatase responsible for dephosphorylating Ser^{211}. Under certain conditions, including starvation, ER stress, and ROS exposure, calcineurin is activated by a Ca^{2+} signal allowing the dephosphorylation of Ser^{211} and subsequent nuclear translocation of TFEB [236, 243, 244]. The source of the Ca^{2+} signal responsible for calcineurin activation has been hotly debated, though it is now understood that Ca^{2+} mobilization from a variety of sources can activate TFEB. In the context of TFEB activation following nutrient starvation, lysosomal Ca^{2+} released through MCOLN1 has been implicated as the Ca^{2+} source. Ca^{2+} release via MCOLN1 was later found to be important for TFEB nuclear translocation following ROS exposure and Fc-mediated phagocytosis [243-245]. A more recent study definitively showed that TFEB activation also occurs upon calcium release from the ER and mitochondria following pharmacological stimulation with known TFEB activators, underscoring the importance of Ca^{2+} mobilization in TFEB activation [246].

There are a variety of mTOR independent pathways that regulate TFEB nuclear translocation, stability, and activation status. Ser^{142} is phosphorylated by ERK1/2 promoting its cytoplasmic sequestration [247]. Recently, GSK3β and Akt were shown to be important kinases controlling TFEB [248, 249]. GSK3β phosphorylates TFEB on Ser^{134} and Ser^{138}, sites that

are important in directing TFEB to the lysosome surface enabling mTOR phosphorylation [248]. Protein kinase C (PKC), which is upstream of GSK3β, is an important signaling molecule controlling activation of this pathway, and pharmacological manipulation of PKC has proven to be an effective method to stimulate TFEB activity [248]. The C-terminus of TFEB contains a serine rich region with 5 phosphorylation sites. Akt phosphorylates TFEB on its C-terminus at Ser^{467}, inhibiting its nuclear translocation [249]. Meanwhile, a previous study showed that PKCβ mediated C-terminal phosphorylation in osteoclasts resulted in the stabilization of TFEB and promoted its activity. Thus, more studies are needed to parse out the individual contributions of these C-terminal phosphorylation sites on TFEB activity. A summary of TFEB phosphorylation sites and the kinases involved are illustrated schematically (**Figure 2).**

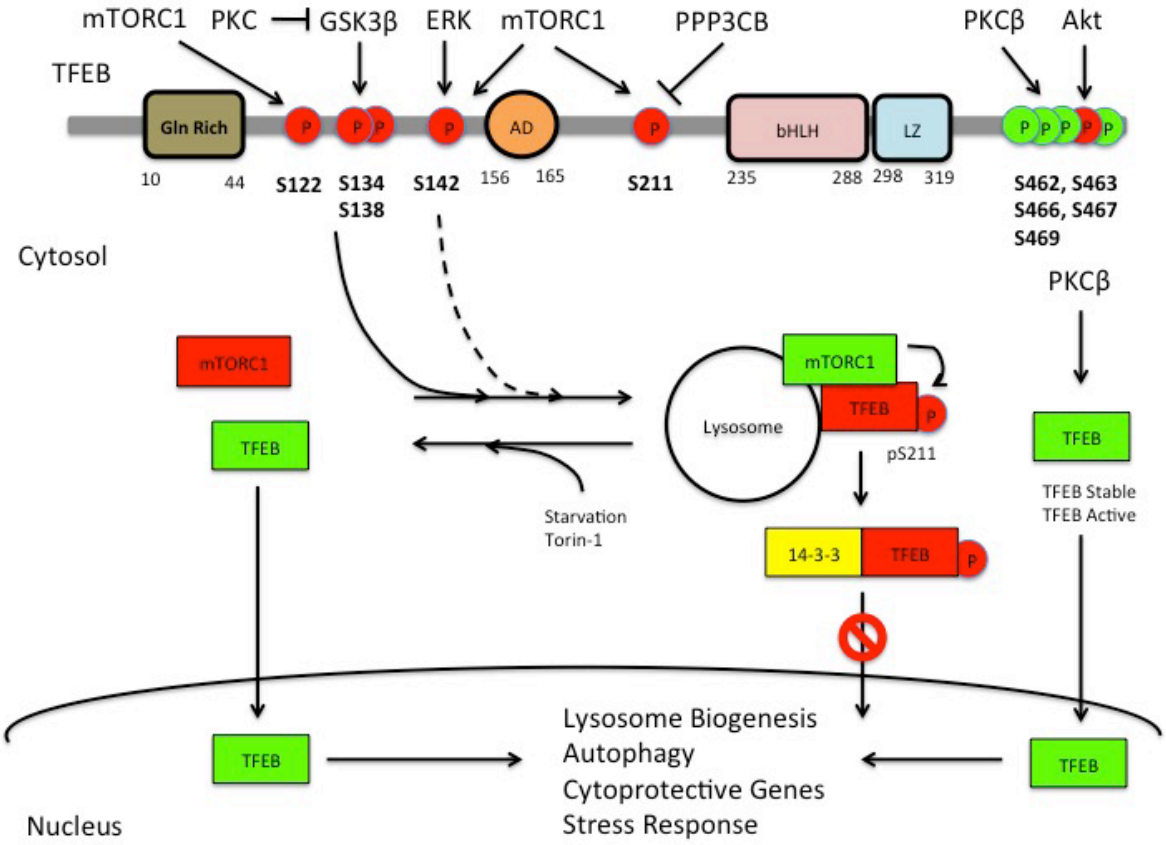

Figure 2. Schematic illustration of TFEB phosphorylation sites and implicated kinases. Red = inhibitory, green = activating. Figure originally published in Nabar and Kehrl, *Yale J Biol Med, 2017, 90(2): 301-315.*

1.5.3 TFEB: Role in the Cell Stress Response

While TFEB was first discovered as a response to starvation, it is now clear that TFEB responds to a variety of cellular stresses (reviewed in [250]). TFEB activation is believed to be an adaptive response to cell stress, as it results in the upregulation of transcriptional networks that maintain cellular homeostasis in the face of the environmental stress sensed.

TFEB is strongly activated in response to lysosomal stress, which physiologically occurs when the autophagy/lysosomal system is unable to maintain homeostasis resulting in an inability to properly degrade cellular waste [228, 241, 251]. In response, TFEB activates transcriptional networks that promote lysosomal biogenesis and autophagy to maintain protein homeostasis during lysosome stress conditions..

TFEB also responds to mitochondrial stress, as it activated after induction of mitophagy or treatment with mitochondrial membrane permeabilizers [244, 252]. In response, TFEB upregulates autophagy machinery which helps remove damaged mitochondria via mitophagy, and transcriptionally activates PGC-1α which promotes mitochondrial biogenesis [253]. Additionally, TFEB functions as an integral component in the unfolded protein response in conditions of ER stress [236]. In this scenario, TFEB activates ATF4 and CHOP, which promote cell survival. Activation of the autophagy/ lysosome pathway promotes clearance of unfolded proteins which accumulate after ER Stress, further helping maintain homeostasis. Finally, TFEB has also been implicated in genotoxic and oxidative stress. In conditions of genotoxic stress such as DNA damage, TFEB amplifies the p53 dependent response to coordinate cell cycle check points and cell death pathways [254]. In oxidative cell stress conditions, TFEB is activated in an mTORC1-independent fashion, which may have implications in cell growth during immune activation and cancer [255].

1.5.4 TFEB: Immune function

Considering the role of TFEB in the cell stress response, it is unsurprising that it is implicated in immune function. Pathogenic invasion itself can be considered a type of cell stress and is known to activate a variety of homeostatic cell stress pathways. However, evolutionary analysis of TFEB homologues suggest a larger role for TFEB in the immune response than expected. The *Caenorhabditis elegans* TFEB homologue HLH-30 transcriptionally controls 80% of immune genes in the worm [256]. HLH-30 is strongly activated in response to *Staphylococcus aureus*, and loss of HLH-30 greatly decreases worm tolerance to infection. Interestingly, *C. elegans* does not contain the critical immune transcription factor NFκB, while mammalian species do. It is likely that with the evolutionary emergence of NFκB, specialization of transcription factor functions resulted in HLH-30 homologues losing transcriptional control of certain immune genes while maintaining control of others. A recent study using TFEB, TFE3, and TFEB/TFE3 double KOs convincingly showed direct TFEB binding to immune gene promoters and an associated increase in immune related gene transcription. Specifically, they show that TFEB is upregulated in response to macrophage activation, and that macrophages lacking TFEB/TFE3 have decreased CSF2, IL-1β, IL-2, and CCL2 at the protein and transcript level. Finally, CHIP-seq experiments in this study showed that TFEB binds CLEAR sequences in many immune response genes, though they typically bind farther from the promoter than seen for lysosome/autophagy genes [237].

In addition to direct transcriptional activation of immune genes, many cellular processes controlled by TFEB are important in the immune response, including autophagy (for intracellular pathogen degradation), vesicle dynamics (for phagocytosis and antigen

presentation), and lysosomal exocytosis (for signal secretion). TFEB has been shown to be activated by a variety of immune receptors and aid in the clearance of several pathogens. For example, IFN-γ results in the calcineurin mediated nuclear translocation of TFEB, which is importance in the clearance of *Mycobacterium tuberculosis* [257]. Upon infection with *Staphylococcus aureus,* activation of an unknown GPCR leads to Gαq coupling and phospholipase C mediated calcium mobilization, promoting TFEB activation. A study from our group showed that AGS3 is an essential regulator of TFEB [73]. Macrophages from AGS3 KO mice are more susceptible to infection by intracellular bacteria, including *Mycobacterium Tuberculosis*, methicillin resistant *Staphylococcus aureus* (MRSA), and *Burkholderia cenocepacia*, underscoring the role of TFEB in intracellular infection.

TFEB has been shown to have cell specific functions in immune cells. In macrophages, in addition to being upregulated by LPS [237], TFEB is strongly activated in response to Fc-Receptor mediated phagocytosis [245]. Functionally, TFEB is involved in the production of ROS in the phagosome upon Fc-receptor mediated phagocytosis, and silencing TFEB results in bacterial killing defects [245]. Several studies have suggested a role for TFEB in macrophage polarization, showing that TFEB activation biases macrophages towards an M1 phenotype [258-260]. The first study showed that TFEB is downregulated in tumor associated macrophages (TAMs) by signals in the tumor microenvironment, causing polarization towards an M2 phenotype [260]. The next study showed that TFEB is required in reprogramming TAMs back to an M1 phenotype during treatment with chloroquine, as TFEB promotes the glycolytic metabolic switch critical to M1 polarization [258]. Finally, a third study showed that Lamptor1 KO myeloid cells results in hyperactivation of TFEB, and myeloid-specific Lamptor1 conditional KO mice are hypersensitive to LPS [259].

In dendritic cells, TFEB has been shown to play an important role in antigen presentation. Lysosomal signaling is important in cross presentation; high levels of lysosome degradation promote less cross presentation and MHC Class II signaling, while low lysosome degradation levels enhance cross presentation [30]. TFEB acts as a molecular switch in dendritic cells and can either inhibit or promote cross presentation in different conditions [261]. Finally, TFEB has been shown to regulate dendritic cell migration by modulating cytoskeletal organization after bacterial sensing [262]. Much less is known about the function of TFEB in lymphoid cells. It has been shown that TFEB is upregulated upon T-cell receptor (TCR) ligation in T-cells, and that TFEB is required for CD40L expression on T-cells [263]. The role of TFEB in B cells remains unclear, and merits rigorous investigation moving forward.

2 AIM

The aim of this thesis was to elucidate molecular mechanisms by which cells of innate immunity transduce and integrate extracellular signals to generate a coordinated biological response.

The specific aims were:

Paper I. To investigate the role of $G\alpha_i$ signaling in macrophage polarization and describe its effects on inflammasome activation and cytokine release.

Paper II. To investigate the signaling pathways through which the cell surface receptor CD38 and the large kinase LRRK2 regulate autophagy in macrophages.

Paper III. To investigate the mechanisms mediating cross-talk between the cell stress response transcription factor TFEB and the proliferative Wnt signaling pathway.

Paper IV. To investigate the cellular mechanisms by which the SARS-CoV ORF-3a potentiates aberrant inflammation.

3 RESULTS AND DISCUSSION

3.1 Gαi2 REGULATES INFLAMMASOME PRIMING AND CYTOKINE RELEASE BY BIASING MACROPHAGE POLARIZATION (PAPER I)

Macrophages can be broadly categorized as proinflammatory M1 or anti-inflammatory M2 macrophages, and signaling from the microenvironment plays an important role in influencing their phenotype [264]. $G\alpha_{i2}$ and $G\alpha_{i3}$ are highly expressed in murine macrophages [67], and $G\alpha_i$ signaling has previously been shown to regulate macrophage chemotaxis, phagocytosis, and activation [67, 83, 265]. Given that the aim of this study is to characterize pathways by which innate immune cells transduce cellular signals into biological responses, and that the role of G-protein signaling in macrophage polarization is unknown, we investigated whether $G\alpha_i$ biases macrophage phenotype determination [266].

To test this hypothesis, we first looked at IL-1β release following activation of various inflammasomes (NLRP3, AIM2, and NLRC4) in bone marrow derived macrophages (BMDMs) from several genetically modified mice. $G\alpha_{i2}$ deficient BMDMs showed significant reductions in IL-1β release for all inflammasomes assayed (Figure 3A). BMDMs lacking $G\alpha_{i3}$ showed no changes in IL-1β release in the same assays, indicating this phenotype is specific to the loss of $G\alpha_{i2}$. We then used BMDMs derived from RGS-insensitive *Gnai2*$^{G184S/G184S}$ knock-in (KI) mice to determine if excess $G\alpha_{i2}$ signaling caused reciprocal changes. Consistently, G184S KI mice had increased IL-1β release for all inflammasomes assayed (Figure 3B).

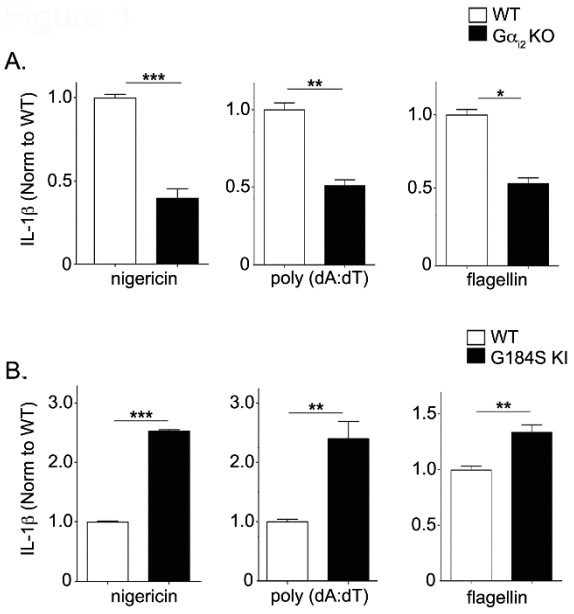

Figure 3. IL-1β release after activation of the indicated inflammasome in BMDMs generated from (A) WT and $G\alpha_{i2}$ deficient or (B) WT and G184S KI mice. First published in Vural et al., *J Immunol, 2019, ji1801145.*

We reasoned $G\alpha_{i2}$ was unlikely to be affecting inflammasome assembly, as consistent changes were noted across all three inflammasomes despite different assembly mechanisms. Thus, we turned our attention to determining whether $G\alpha_{i2}$ modulates the LPS priming phase or the IL-1β secretion phase. To do so, we mimicked signal 1 by stimulating with LPS and monitored TNF-α, IL-6, and IL-10 release by ELISA in $G\alpha_{i2}$ deficient, G184S KI, and control BMDMs. IL-1β secretion is mechanistically distinct from secretion of other cytokines, thus changes in IL-1β without corresponding cytokine changes during priming indicates involvement of an IL-1β specific secretion mechanism. Conversely, changes in IL-1β release with concomitant cytokine secretion changes during priming indicates involvement

of the LPS priming phase [58]. $G\alpha_{i2}$ deficient cells showed decreases in TNF-α and IL-6, while reciprocal changes were observed in G184S KI BMDMs (Figure 4A, B). These results suggest that the IL-1β release defect observed in $G\alpha_{i2}$ deficient BMDMs is due to defects during LPS priming. As pro- IL-1β transcriptional upregulation is controlled by the LPS priming phase, we immunoblotted for pro- IL-1β in $G\alpha_{i2}$ deficient and G184S BMDMs, consistently noting decreased and increased expression respectively compared to the WT control after LPS stimulation (Figure 4C, D).

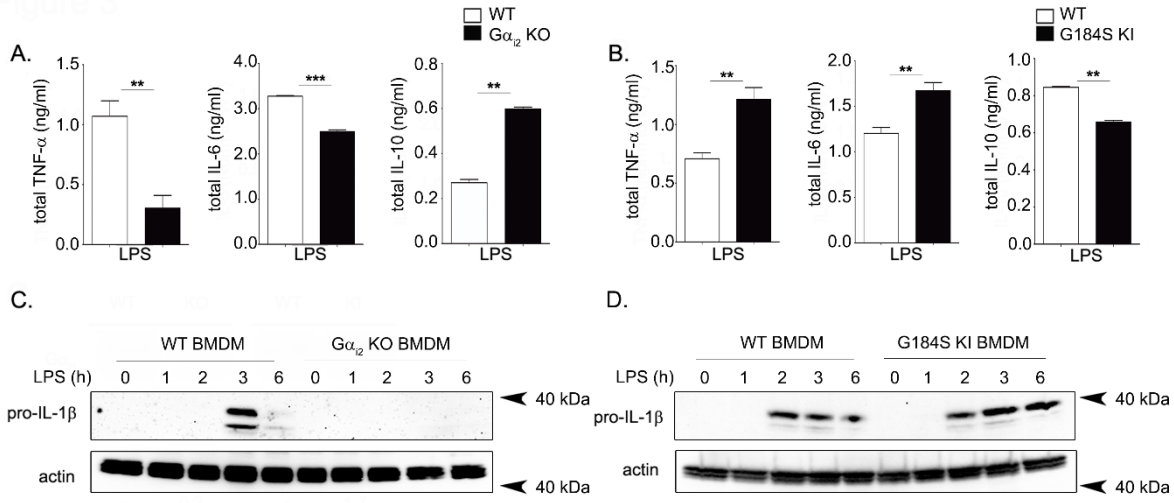

Figure 4. Supernatant TNF-α, IL-6, and IL-10 levels in LPS stimulated BMDMs from (A) WT and $G\alpha_{i2}$ deficient and (B) WT and G184S KI mice. Immunoblots for pro-IL-1β following LPS stimulation for the indicated time in BMDMs from (C) WT and $G\alpha_{i2}$ deficient and (D) WT and G184S KI mice. Adapted from figure first published in Vural et al., *J Immunol, 2019, ji1801145*

Inflammasome priming is partially regulated by macrophage polarization [267]. Additionally, we noted increased IL-10 (an anti-inflammatory cytokine) despite decreases in TNF-α and IL-6 in $G\alpha_{i2}$ deficient BMDMs, while G184S KI macrophages showed decreased IL-10 in the face of increased TNF-α and IL-6 secretion after LPS stimulation. Together, these observations prompted us to assay macrophage polarization by looking at important M1 genes (*iNOS*, *TNF-α*, and *IL-12p40*) after M1 macrophage polarization with LPS and important M2 genes (*Arg1*, *Fizz1*, *Ym1*) after IL-4 mediated M2 polarization by qRT-PCR. $G\alpha_{i2}$ deficient BMDMs showed decreased M1 gene expression after LPS mediated polarization and enhanced M2 gene expression following IL-4 mediated polarization, suggesting that loss of $G\alpha_{i2}$ biases macrophages towards the M2 phenotype (Figure 5A, B). Similarly, G184S KI macrophages show increased M1 genes after LPS polarization and decreased M2 genes upon IL-4 stimulation, suggesting that enhanced $G\alpha_{i2}$ signaling promotes the pro-inflammatory M1 phenotype (Fig 5C, D). We verified these results using a functional T-cell suppression assay, and as expected the M2 biased $G\alpha_{i2}$ deficient BMDMs more effectively suppressed T-cell proliferation than control cells, while the opposite was true for G184S KI macrophages.

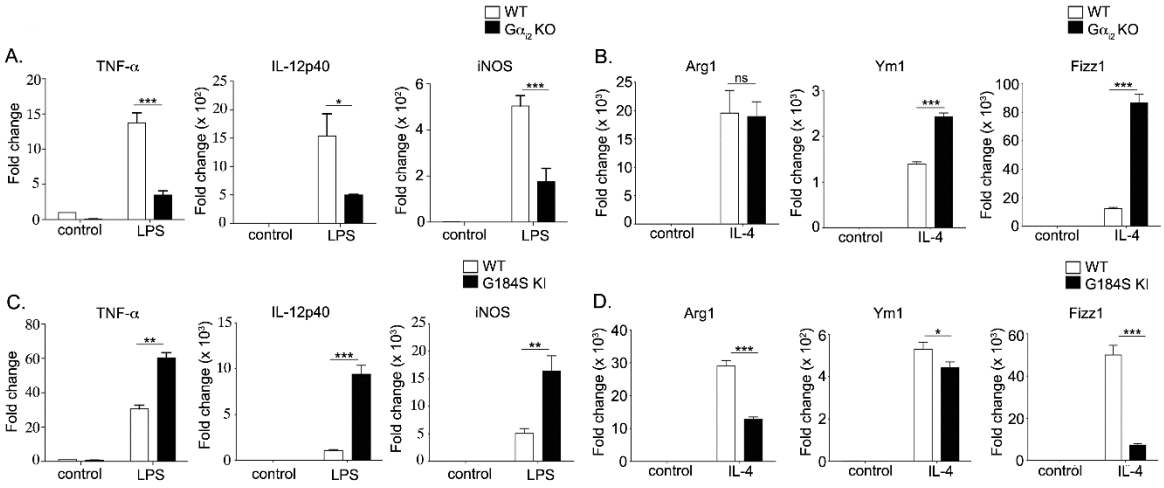

Figure 5. A) Expression of M1 genes in Gα_{i2} deficient BMDMs after treatment with LPS. B) Expression of M2 genes in Gα_{i2} deficient BMDMs after treatment with IL-4 C) Expression of M1 genes in G184S KI BMDMs after treatment with LPS D) Expression of M2 genes in G184S KI BMDMs after treatment with IL-4. Adapted from figure first published in Vural et al., *J Immunol, 2019, ji1801145*

Finally, we assessed the signaling pathways downstream of LPS and IL-4 in Gα_{i2} deficient cells to determine which signaling molecules may be involved. Gα_{i2} deficient BMDMs had defects in ERK1/2 and STAT3 signaling and enhanced Akt activation following LPS stimulation. After IL-4 stimulation, Gα_{i2} deficient BMDMs had enhanced STAT6 activation, which is important in M2 polarization. In sum, our study identified Gα_{i2} as a critical mediator of macrophage polarization. Excess Gα_{i2} signaling promotes an M1 pro-inflammatory phenotype, while a deficiency in Gα_{i2} promotes an M2 anti-inflammatory phenotype.

3.2 CD38 SIGNALS THROUGH LRRK2 TO ACTIVATE TFEB (PAPER II)

CD38 is an important cell surface receptor that is highly expressed on both B cells and macrophages [107]. In hepatocytes, it has been implicated as a positive regulator of autophagy through an NAADP-dependent pathway [104]. LRRK2, which is the most common genetic cause of PD, has also been implicated in autophagy and NAADP signaling [268]. In this paper we show that CD38 promotes the nuclear translocation of TFEB, the master transcriptional regulator of the autophagy and lysosome machinery, by a calcium signal that requires LRRK2. We then investigate the mechanisms through which this occurs in both macrophages and B cells. This paper again aims to elucidate signaling pathways from the cell surface that activate functions related to innate immunity, namely the transcriptional activation of the autophagy/lysosome system.

To first determine if CD38 activates TFEB, we stimulated B cells and macrophages with an α-CD38 monoclonal antibody (clone 90) and immunoblotted for TFEB after nuclear fractionation. Ligation of CD38 induced more than a two-fold increase in nuclear TFEB in both B-cells and macrophages. As CD38 generates several second messengers that mobilize calcium [89] and TFEB has previously been shown to be activated by calcium activated calcineurin mediated dephosphorylation [243], we determined whether CD38 mediated TFEB activation is calcium and calcineurin dependent. Immunostaining for endogenous TFEB in BMDMs showed that while CD38 ligation induces TFEB nuclear translocation, this is reversed both by calcium chelation and calcineurin inhibition (Figure 6A). Co-staining of CD38 1 hour after ligation with clone 90 shows a primarily intracellular pattern, indicating receptor internalization (Figure 6B).

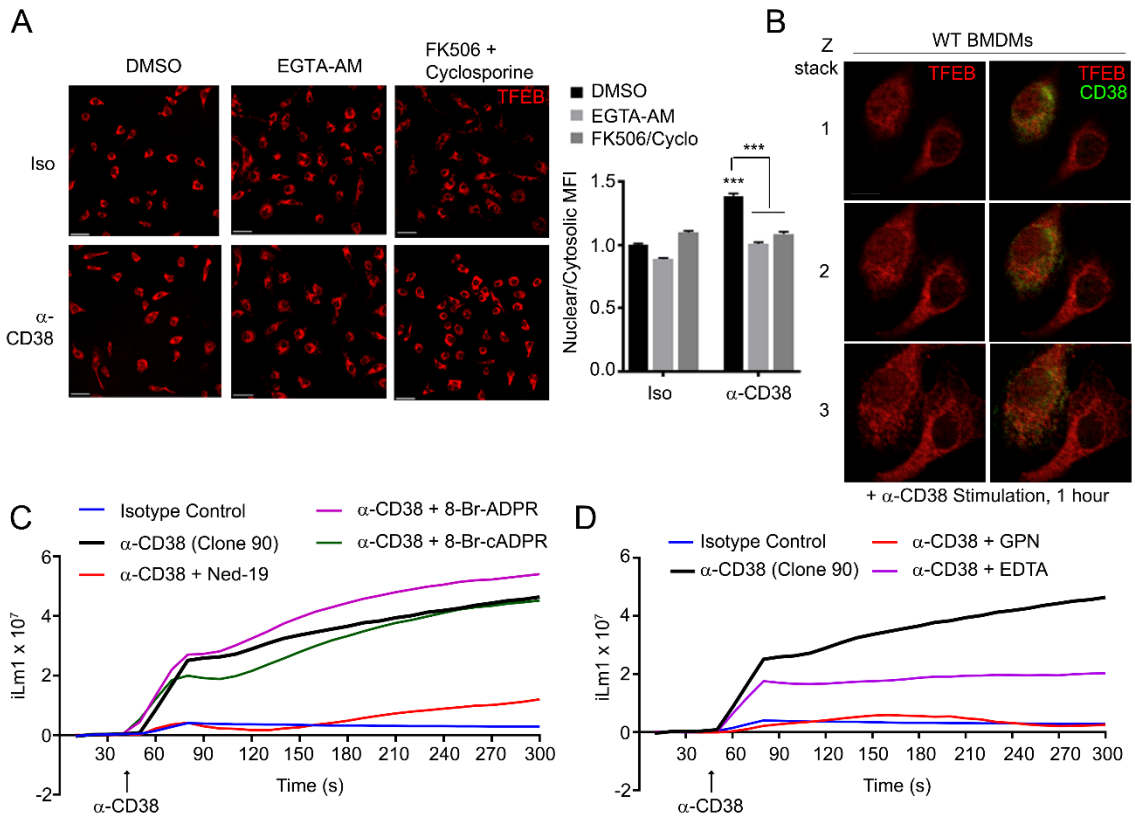

Figure 6. (A) Immunostaining of endogenous TFEB after clone 90 stimulation with or without pretreatment with EGTA-AM and calcineurin inhibitors. (B) Immunostaining to show CD38 localization after stimulation with clone 90. (C, D) Real time calcium assays measuring cytosolic calcium after clone 90 stimulation with or without indicated inhibitors.

To validate that ligation of CD38 by clone 90 results in calcium mobilization, we measured calcium in real time after stimulation of both B cells and macrophages with clone 90. The observed calcium response was biphasic, with an initial sharp peak followed by a slower ramp phase (Figure 6C, black line). We used various inhibitors to determine the second messengers and calcium stores involved in this calcium signal. We determined that the initial sharp peak required NAADP and lysosomal calcium, while the slow ramp phase required

extracellular calcium (Figure 6C, D). Our results additionally showed that ER calcium stores contributed to cytosolic calcium increase during the slow ramp phase, suggesting that NAADP/lysosomal calcium induce calcium release from other stores through CICR. Together, this data shows that CD38 ligation drives TFEB nuclear translocation through NAADP-dependent calcium signaling.

As LRRK2 has been previously implicated in NAADP-dependent calcium signaling, we tested whether CD38 mediated TFEB activation was dependent on LRRK2. In LRRK2 KO macrophages and B-cells, TFEB nuclear translocation was not observed following ligation of CD38 with clone 90. Using real time calcium assays, we verified that LRRK2 KO B-cells do not produce a calcium response following clone 90 ligation (Figure 7A) and note that pretreatment with a LRRK2 kinase inhibitor also ablates the calcium response seen after ligation of clone 90. To further verify the importance of LRRK2 kinase activity in mediating the calcium response downstream of CD38, we performed the experiment in LRRK2 G2019S KI B-cells, which express a kinase overactive version of LRRK2 that is the most common pathogenic variant. The LRRK2 G2019S cells showed an enhanced calcium response compared to the WT cells (Figure 7B). Finally, we note that CD38 and LRRK2 strongly interact by co-immunoprecipitation and colocalize as discrete puncta on the cell membrane (Figure 7C). Taken together, these results suggest that LRRK2 is required for signaling downstream of CD38 ligation by clone 90, and that CD38 and LRRK2 form a complex at the plasma membrane.

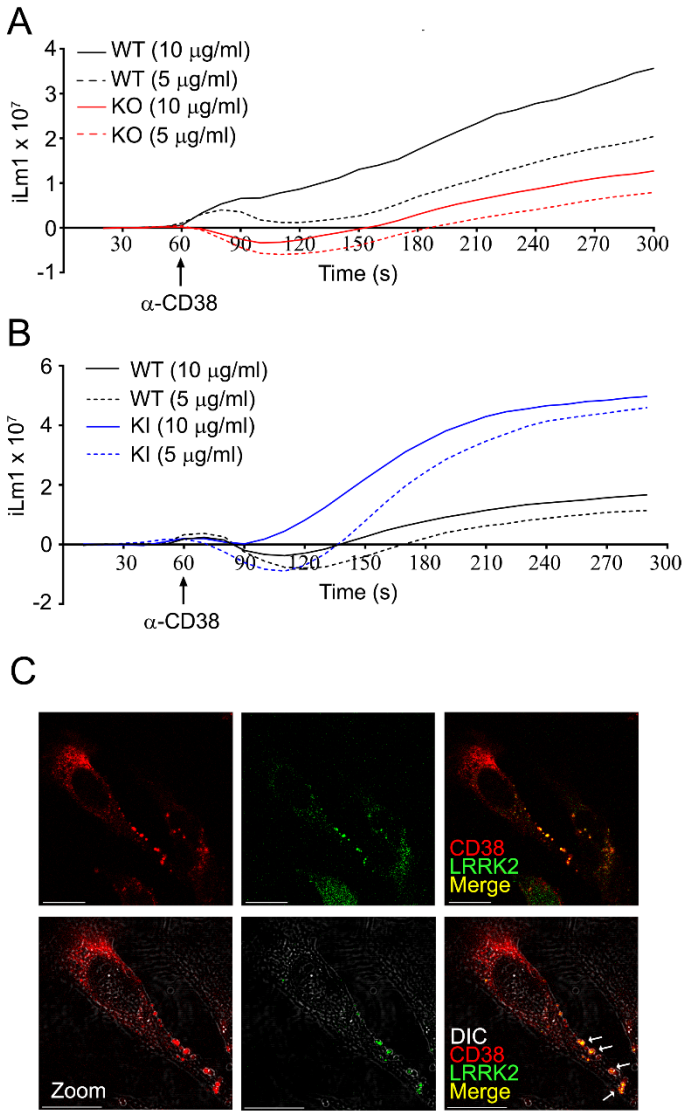

Figure 7. Real time monitoring of cytosolic calcium following ligation of clone 90 in WT and (A) LRRK2 KO and (B) LRRK2 G2019S KI B-cells. (C) Confocal imaging showing the colocalization of CD38 and LRRK2 on the plasma membrane.

After elucidating the relationship between CD38 and LRRK2, we shifted our focus to determine if LRRK2 KO cells have TFEB activation defects. We find that LRRK2 KO macrophages fail to upregulate TFEB in response to LPS as seen in WT controls. Additionally, LRRK2 KO B-cells fail to upregulate TFEB following stimulation with B-cell

activators. Finally, we show that overexpression of the kinase overactive LRRK2 G2019S results in the activation of TFEB, both by increasing its stability via phosphorylation on its C-terminus and by driving its nuclear translocation in an NAADP-TPC2-lyosomal calcium dependent manner. This expectedly results in the transcriptional upregulation of TFEB target genes with corresponding increases at the protein level. In sum, in this paper we characterize a novel CD38-LRRK2-TFEB signaling pathway active in multiple types of immune cells.

3.3 TFEB NEGATIVELY REGULATES WNT SIGNALING BY DIRECTLY BINDING β-CATENIN AND PROMOTING ITS DEGRADATION (PAPER III)

The WNT signaling pathway is anabolic in nature, as it promotes cell growth, cell differentiation, and cell division [110]. These events require significant energy expenditure at the cellular level, and accordingly bioenergetic pathways are activated downstream of WNT signaling [123]. TFEB, on the other hand, is an adaptive cellular response that promotes nutrient recycling and preserves homeostasis. It is activated by various forms of cell stress to facilitate cell survival and prevent metabolic collapse [226]. TFEB belongs to the MiT family of proteins, which includes the closely related transcription factor MITF [225]. MITF has been implicated in augmenting WNT/β-catenin signaling, and additional crosstalk has been identified as WNT signaling reciprocally stabilizes MITF [269]. Despite the link between MITF and WNT signaling, the physiological and biochemical crosstalk between TFEB and WNT remains unknown. Given that cells have intricate crosstalk mechanisms to ensure downregulation of anabolic pathways during periods of stress [270], we hypothesized that TFEB may negatively regulate the WNT/β-catenin signaling pathway due to their opposing functional roles.

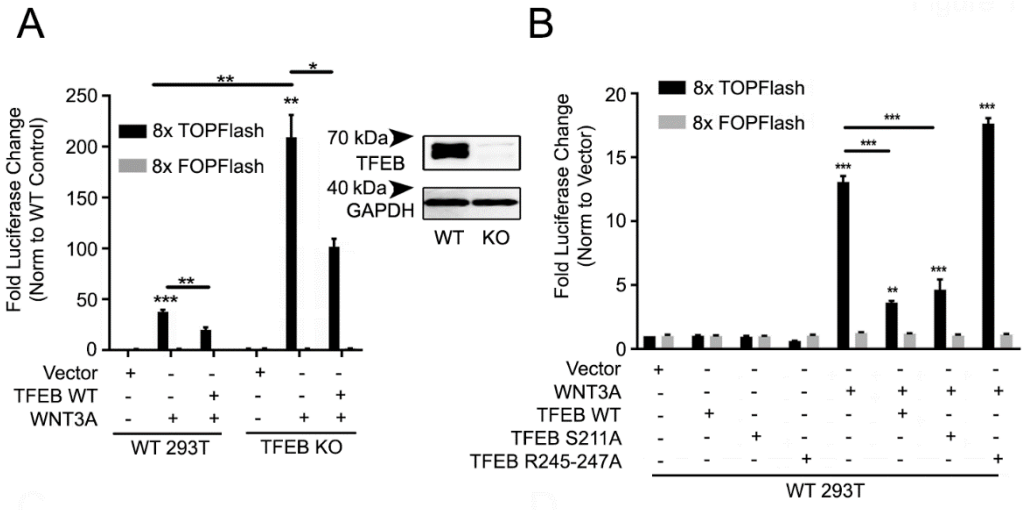

Figure 8. Normalized Super 8x TOPFlash & Super 8x FOPFlash activity in (A) WT and TFEB KO 2393T cells transfected with the indicated proteins, and (B) WT 293T cells transfected with TFEB mutants restricting subcellular localization.

To test our hypothesis, we used CRISPR/Cas9 to generate TFEB KO HEK 293T cells. We generated 3 clones, and critical experiments were repeated in all clones to ensure the observed phenomenon were specific to the knockout of TFEB. First, we activated the WNT/β-catenin pathway by transfection of WNT3A and monitored TCF/LEF activity in WT and TFEB KO cells. In the absence of WNT signaling, no difference was observed between WT and TFEB KO cells. However, upon transfection of WNT3A, WT cells showed a 40-fold induction of luciferase activity, while TFEB KO cells showed more than a 200-fold induction. Indicating that the observed increase was specific to the loss of TFEB, reconstitution of TFEB KO cells with overexpressed TFEB reduced TCF/LEF activity by more than half (Figure 8A). This trend was preserved across all 3 TFEB KO clones, strongly suggesting that TFEB negatively regulates the WNT/β-catenin pathway in the WNT ON state. To determine the subcellular localization where TFEB exerts its influence on the WNT/β-catenin pathway, we monitored TCF/LEF activity after transfection with a either a control vector, WT TFEB, nucleus restricted TFEB (S211A), or cytoplasm restricted TFEB (R245-247A). In the WNT ON state, WT and nuclear TFEB reduced TCF/LEF activity compared to the control, but the cytoplasm restricted TFEB mutant was unable to do so (Figure 8B). This observation suggests that TFEB exerts its effects on the WNT/β-catenin signaling pathway in the nucleus.

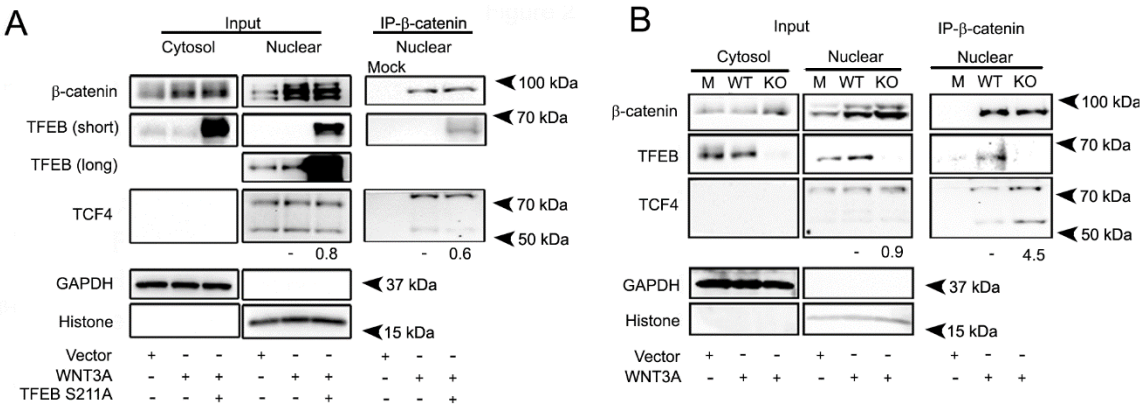

Figure 9. Immunoblots for the indicated proteins after immunoprecipitations of nuclear lysates from (A) WT 293T cells with our with TFEB S211A, and (B) WT or TFEB KO 293T cells.

As β-catenin is critical for the activation of TCF/LEF target genes, we investigated whether TFEB regulates WNT signaling by interacting with β-catenin. We found that TFEB robustly interacts with β-catenin, and that this interaction is enhanced upon activation of WNT signaling. We then went on to show that TFEB and β-catenin interact in the nucleus, and that overexpression of TFEB reduces the β-catenin/TCF interaction (Figure 9A). This suggests that TFEB may competitively interact with β-catenin, decreasing its binding with TCF/LEFs. We then repeated the same experiment using WT and TFEB KO cells. We observed a stronger β-catenin/TCF interaction in TFEB KO cells than WT controls (Figure 9B). Taken together, this data shows that TFEB inhibits the WNT/β-catenin pathway in part by competitively binding β-catenin in the nucleus and hindering its interaction with TCF.

During the nuclear immunoprecipitations, we noted that β-catenin levels were consistently increased in TFEB KO cells compared to WT cells. Thus, we determined if TFEB regulates the stability of β-catenin. We found that in the WNT ON state, TFEB KO cells have significantly higher levels of β-catenin than WT controls. We also found that overexpression of TFEB significantly decreases β-catenin levels in the WNT ON but not the WNT OFF state. Using a series of sequential immunoprecipitations, we then confirmed that TFEB interacts with free β-catenin but not β-catenin as part of the WNT destruction complex. Importantly, this finding mechanistically explains why TFEB negatively regulates WNT/ β-catenin signaling only in the WNT ON state. Finally, we determined that overexpression of β-catenin also reciprocally decreases levels of TFEB, suggesting the TFEB/β-catenin may be targeted for degradation.

In summary, we identified TFEB as a novel repressor of the WNT/β-catenin signaling pathway. We determined that TFEB inhibits WNT/β-catenin signaling by two mechanisms. First, it competitively binds β-catenin in the nucleus, inhibiting its ability to bind TCF/LEF and activate WNT target genes. Second, TFEB directly binds and promotes the degradation of free β-catenin, decreasing its overall levels. Notably, β-catenin reciprocally promotes the degradation of TFEB, suggesting that these two pathways reciprocally regulate one another.

3.4 SARS-CORONAVIRUS OPEN READING FRAME-3A DRIVES MULTIMODAL NECROTIC DEATH BY INSERTING INTO MEMBRANES

The SARS-CoV causes illness with a 10% mortality rate; it presents as flu-like symptoms but can rapidly progress to respiratory failure [271, 272]. Lung tissue from fatal cases exhibits severe inflammation, showing diffuse alveolar damage and infiltration of inflammatory monocyte-macrophages (IMMs) [273, 274]. It is now understood that the lung damage is not simply caused by the virus itself, but rather by both cytotoxic effects of the virus and an aberrant host inflammatory response to viral components [275]. A recent study in mouse models highlighted the importance of IMMs in SARS pathogenesis, as depletion of these cells rescues infected mice from fatal challenge [275]. Though the importance of IMMs in SARS pathogenesis is now appreciated, the mechanisms mediating their aberrant inflammatory state remain poorly understood. The SARS-CoV codes eight accessory proteins, of which open reading frame-3a (SARS 3a) is of interest as deletion of ORF-3a rescues mice from lethal challenge in murine models [276]. SARS 3a has been shown to oligomerize based on the formation of disulfide bonds at its Cys^{133} residue, enabling it to insert into membranes and function as a potassium ion channel [277]. In this paper, we investigated downstream consequences of SARS 3a membrane insertion.

Based on structural similarities between the necroptosis effector protein MLKL and SARS 3a, namely the ability to oligomerize and insert into membranes, we investigated whether RIPK3 could target SARS 3a to drive necrotic death.

Overexpression of SARS 3a caused very little necrotic death alone, but induced significant necrotic death as assayed by protease release and ATP depletion when co-expressed with RIPK3 (Figure 10A). Notably, SARS 3a induced cell death was not inhibited by addition of a pan-caspase inhibitor, further suggesting necrotic death. Time lapse confocal imaging confirmed that expression of SARS 3a in the presence of RIPK3 induces rapid cell death, and we next found that SARS 3a and RIPK3 interact when co-expressed. Next, we determined if RIPK3-SARS 3a mediated cell death was dependent on SARS 3a oligomerization and RIP3K's kinase activity. Co-transfection of RIP3K and the SARS 3a C133A oligomerization deficient mutant reduced cell death compared to WT SARS 3a, indicating oligomerization of SARS 3a plays a role in cell death. However, the RIP3K kinase dead mutant induced cell death to similar levels as WT RIP3K when expressed with SARS 3a, indicating RIP3K's kinase activity is dispensable for SARS 3a induced necrotic death.

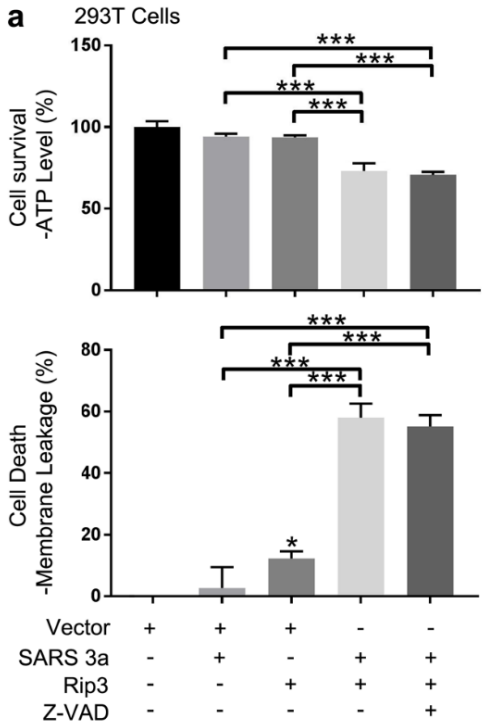

Figure 10. (A) ATP depletion (top) and membrane leakage (bottom) in cells expressing SARS-3a with or without RIP3. Adapted from figure first published in Yue et al, *Cell Death Dis*, 2018: 904

Having shown that SARS 3a oligomerization is important for cell death, we checked whether RIPK3 enhances SARS 3a oligomerization. Notably, both WT RIPK3 and kinase dead RIPK3 enhanced SARS 3a oligomerization, which is consistent with previous findings. We validated the oligomer band, as both reduction of lysates with DTT and heating of cross-linked samples to 100 ^{o}C abrogated oligomer detection (Figure 11A). Finally, we validated that SARS 3a similarly causes cell death in a human lung cell line expressing endogenous RIP3 (A459 treated with the hypomethylating agent decitabine) [278]. These results underscore the pathophysiological relevance of SARS 3a induced necrosis.

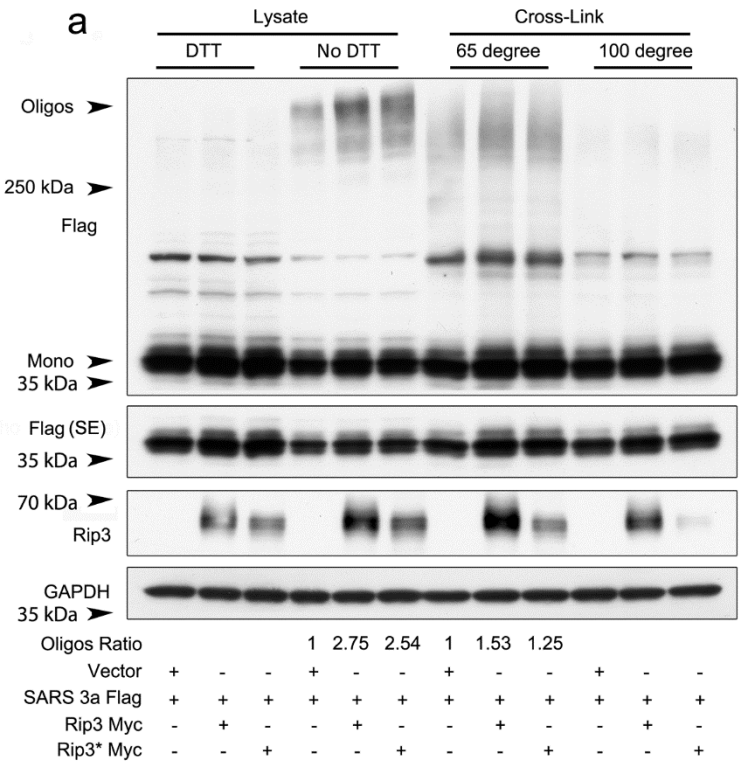

Figure 11. (A) Immunoblotting showing the oligomerization of SARS-3a is driven by RIP3K. Adapted from figure first published in Yue et al, *Cell Death Dis*, 2018: 904

Next, we investigated the pathophysiological consequences of SARS 3a insertion into membranes other than the plasma membrane. Co-transfection of SARS 3a and RIP3K results in clear lysosomal co-localization, which results in lysosomal damage as assayed by galectin-3 puncta formation [279]. Consistently, SARS 3a expressing cells showed defective lysosomal degradation capacity, as well as cytosolic cleavage of Bid consistent with release of lysosomal cathepsins into the cytosol. Unsurprisingly, TFEB nuclear localization was seen accompanying lysosomal damage, causing upregulation of TFEB target genes. Finally, as SARS 3a is known to function as a potassium channel and the NLRP3 inflammasome is activated by potassium efflux, we tested whether SARS 3a triggers the NLRP3 inflammasome. Transfection of SARS 3a into the human macrophage cell line Thp-1 results in significant caspase-1 cleavage, suggesting activation of pyroptotic pathways in inflammasome competent cells. In summary, we identified that SARS 3a induces necrotic cell death by direct insertion into the plasma membrane, which is promoted by RIPK3 mediated oligomerization. We find that in inflammasome competent cells, SARS 3a drives IL-1β release and pyroptotic death. These mechanisms may contribute to the aberrant systemic inflammation seen patients following SARS-CoV infection.

3.5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The aim of this thesis was to elucidate molecular mechanisms by which cells of the innate immune system transduce and integrate extracellular signals to generate a coordinate biological response. This thesis provides insights into multiple signaling pathways effecting macrophage function and uncovers crosstalk mechanisms by which cells adapt to stressful stimuli. Specifically, the major findings of this thesis are:

- The identification of $G\alpha_{i2}$ as a critical regulator of macrophage polarization, with sigificant effects on important processes including cytokine release, inflammasome priming, and T cell supression.
- The discovery and description of a novel CD38-LRRK2-TFEB signaling pathway that is present in both B-cells and macrophages.
- The identification of TFEB as a novel repressor of the WNT/β-catenin signaling pathway and detailed elucidation of the molecular mechanisms by which TFEB inhibits the WNT signaling pathway.
- The identification that SARS ORF-3a causes necrotic cell death via RIPK3 mediated oligomerization and activates the NLRP3 inflammasome in immune cells, which may contribute to the aberrant inflammatin seen in SARS-CoV infected patients.

Paper I identifies $G\alpha_{i2}$ as a critical regulator of macrophage polarization, showing that excess $G\alpha_{i2}$ signaling biases macrophages towards a proinflammatory M1 phenotype while defective $G\alpha_{i2}$ signaling biases macrophages towards the alternatively activated M2 phenotype. There are several lines of investigation worth exploring in following up this study. One particularly interesting observation was that while $G\alpha_{i2}$ deficient macrophages release significantly less

IL-1β than WT macrophages upon inflammasome activation, short-term inhibition of $G\alpha_{i2}$ with PTX (overnight) did not recapitulate this phenotype. This observation suggests that signaling downstream of TLR4 during LPS priming is not responsible for the macrophage polarization phenotype that manifests as decreased IL-1β release, because short-term PTX treatment also inhibits nucleotide exchange during LPS stimulation. It is unlikely that nucleotide exchange independent effects of $G\alpha_{i2}$ are responsible for these changes, given the reciprocal phenotypes of $G\alpha_{i2}$ deficient and G184S KI BMDMs. We observed that long term inhibition of $G\alpha_{i2}$ with PTX (7 days) during development was able to recapitulate the macrophage polarization phenotype seen in $G\alpha_{i2}$ deficient macrophages. The important question that remains unanswered is whether prolonged $G\alpha_{i2}$ inhibition itself can reprogram macrophages towards the M2 phenotype, or whether $G\alpha_{i2}$ inhibition during differentiation is critical to mediating reprogramming. The former scenario has major therapeutic implications, as long-term targeting of macrophage specific $G\alpha_{i2}$-coupled GPCRs could lay the foundation for the treatment of several diseases via macrophage reprogramming [266].

Paper II identifies a novel CD38-LRRK2-TFEB signaling pathway in B cells and macrophages. This is the first paper to identify LRRK2 as part of the signaling cascade downstream of CD38, as the mechanisms mediating CD38 signaling are largely unknown. The work in this paper has several clinical implications, as monoclonal antibodies against CD38 are already FDA approved for multiple myeloma and LRRK2 inhibitors are currently in clinical trials for the treatment of PD. With regards to the implications of this study for the development of cancer therapeutics, it has already been reported that Daratumumab induces apoptosis following cross-linking in a variety of hematologic cell lines. Our study shows that inhibition of LRRK2 during Daratumumab cross-linking enhances cell death in the Ramos Burkitt's lymphoma cell line, warranting follow up on the role of LRRK2 inhibitors as adjuvant therapy for Daratumumab. The monoclonal antibody Isatuximab currently in phase III clinical trials has been shown to initiate lysosomal cell death characterized by cytoplasmic cathepsins. As we also show that CD38 controls the activation of TFEB, it will be interesting to see if TFEB has any role in the observed lysosomal cell death following Isatuximab treatment. With regards to LRRK2 inhibitors in clinical trials, this study underscores that LRRK2 has significant immune function. Clinicians administering LRRK2 inhibitors must remain vigilant to the fact that LRRK2 inhibitors could increase patient risk for serious infections by inhibiting LRRK2's immune function.

Paper III identifies TFEB is a negative regulator of the WNT/β-catenin signaling pathway and elucidates some of the molecular mechanisms mediating TFEB repression of WNT. We find that TFEB directly binds β-catenin in the nucleus, and that this interaction may hinder β-catenin/TCF binding required for the activation of WNT target genes. We also find that TFEB promotes the degradation of β-catenin, potentially through direct binding of free β-catenin and subsequent degradation of the complex. It remains unclear what role, if any, TFEB transcriptional targets have in TFEB mediated inhibition of WNT signaling. It may be illuminating to perform these studies with TFEB constructs that are unable to activate transcription. Furthermore, it may be useful to extend the scope of these studies to

physiological contexts. The drawback of this study is that WNT signaling was activated by transfection of WNT3A, which results in very high levels of expression. Similar studies using recombinant WNT ligands may be more relevant to the physiological context. Furthermore, TFEB overexpression was frequently used in this study to activate TFEB. It may be useful to determine if TFEB still strongly affects WNT signaling when activated by physiological inducers of TFEB activity, such as starvation or initiation of cell stress pathways.

Paper IV implicates the SARS-CoV ORF-3a protein in necrotic cell death. SARS 3a mediated necrotic death occurs after SARS 3a oligomerization and membrane insertion, which is enhanced by RIPK3. We also show that oligomerized SARS 3a can insert into lysosomal membranes, causing lysosomal damage and triggering cell stress. Lastly, we show that SARS 3a can activate the NLRP3 inflammasome, potentially activating pyroptotic death in inflammasome competent cells. While it is already known that deletion of SARS 3a rescues murine models from lethal challenge, it would be interesting to determine if abrogation of SARS 3a oligomerization by genetic mutation similarly rescues mice from lethal challenge. If oligomerization of SARS 3a is in fact a key mechanism for promoting aberrant inflammation, targeted therapies blocking this can be developed. Furthermore, the IMMs that have been implicated as important in SARS pathogenesis presumably express both high levels of RIP3K and of NLRP3. Blocking these two pathways that initiate the inflammatory cascade may help prevent the aberrant inflammatory response associated with SARS-CoV infection.

In conclusion, this work expands our knowledge of the cell signaling pathways important in innate immunity. The results underscore the complexity of cell signaling networks, and open up new avenues worthy of future study.

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