

MECHANISMS AND CONSEQUENCES OF *STAPHYLOCOCCUS AUREUS* LEUKOCIDIN AB-MEDIATED  
ACTIVATION OF THE HOST NLRP3 INFLAMMASOME

Jason H. Melehani

A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in  
partial fulfillment of the requirements for the degree of Doctorate of Philosophy in the  
Department of Pharmacology in the School of Medicine

Chapel Hill  
2016

Approved by:

Robert A. Nicholas

Gary L. Johnson

Claire M. Doerschuk

Monte S. Willis

Joseph A. Duncan

© 2016  
Jason H. Melehani  
ALL RIGHTS RESERVED

## ABSTRACT

Jason H. Melehani: Mechanisms and consequences of *Staphylococcus aureus* Leukocidin AB-mediated activation of the host NLRP3 inflammasome  
(Under the direction of Joseph A. Duncan)

The NLRP3 inflammasome is a critical innate immune sensor implicated in the pathogenesis of dozens of infectious and non-infectious diseases. Activation of the NLRP3 inflammasome causes IL-1 $\beta$  and IL-18 secretion and necrotic cell death. *Staphylococcus aureus* is a common cause of infections in humans. *S. aureus* produces a family of pore-forming toxins that are cytotoxic to human immune cells. One recently discovered pore-forming toxin, Leukocidin AB, is the focus of studies herein.

Leukocidin AB is a human-specific, pore-forming toxin that binds CD11b to initiate pore formation. In order to characterize the mechanism of Leukocidin AB cytotoxicity and determine its significance, we evaluated the effects of Leukocidin AB on primary human monocytes and THP1 monocytic cells. Leukocidin AB was one of the most potent toxins in killing primary human monocytes. In THP1 cells, knockdown of NLRP3 or ASC by shRNA diminished Leukocidin AB-induced cytotoxicity and prevented secretion of IL-1 $\beta$  and IL-18. We also characterized the NLRP3 inflammasome in bacterial survival during phagocytosis. When *S. aureus* was phagocytosed by THP1 cells, LukAB triggered IL-1 $\beta$  secretion and cell death. shRNA-mediated depletion of NLRP3 or ASC suppressed IL-1 $\beta$  secretion but had no effect on Leukocidin AB-induced cell death. These data suggest that a separate mechanism is responsible for triggering

cell death when Leukocidin AB binds CD11b on the phagosome membrane instead of the plasma membrane.

We also initiated studies to characterize the role of kinases and phosphorylation in the response to Leukocidin AB. Using multiplex inhibitor bead chromatography and quantitative mass spectrometry, we identified eight kinases that rapidly decrease in activity during Leukocidin AB exposure. We demonstrated a role for Death-Associated Protein Kinase in the response to Leukocidin AB by showing that its inhibition suppressed Leukocidin AB-induced cytokine secretion and cytotoxicity. And finally, we used a novel transfection method for overexpressing mutant proteins in THP1 cells to show that the NLRP3 S198D/S201D mutant could spontaneously activate NLRP3 inflammasome signaling.

In total, these studies make significant contributions to the understanding of *S. aureus* pathogenesis and the regulation of innate immune NLRP3 inflammasome signaling in response to a human specific, *S. aureus* pore-forming toxin.

*To my dear family, friends, and mentors for their unwavering support*

## ACKNOWLEDGEMENTS

I would like to acknowledge and thank the directors of the UNC Medical Scientist Training Program past and present, the faculty of the Pharmacology Department with whom I interacted, the members of my thesis committee, the numerous faculty and staff at the University of North Carolina with whom I worked with, my collaborators at the University of North Carolina Chapel Hill and New York University, my colleagues in the Duncan Lab, my colleagues in the Medical Scientist Training Program and Pharmacology Graduate Program, the Infectious Disease Society of America for funding support, and my family and friends.

## TABLE OF CONTENTS

LIST OF TABLES.....	xiii
LIST OF FIGURES.....	xiv
LIST OF ABBREVIATIONS.....	xvi
CHAPTER 1: Discovery, activation and regulation of the NLRP3 inflammasome .....	1
1.1 A general overview of NLRP3 inflammasome form and function .....	1
1.2 Clinical relevance of NLRP3 inflammasome signaling .....	3
1.3 Interleukin-1 $\beta$ -driven monogenic hyperinflammatory fever disorders are caused by mutations in the NLRP3 inflammasome pathway.....	4
1.4 The NLRP3 inflammasome is a master innate immune sensor .....	7
1.5 Cautions in interpreting NLRP3 inflammasome research.....	8
1.5.1 Studies frequently overlook important outcomes of NLRP3 inflammasome activity.....	8
1.5.2 Dozens of activators and regulators complicate a comprehensive model for NLRP3 inflammasome activation .....	11
1.5.3 Commonly used Caspase 1 knockout mice are also Caspase 11 deficient.....	12
1.6 Priming the NLRP3 inflammasome for activation.....	13
1.6.1 Pattern-recognition receptor signaling upregulates transcription of NLRP3 inflammasome components.....	13
1.6.2 Negative feedback loops respond to prolonged priming to reign in NLRP3 inflammasome signaling.....	18
1.6.3 Kinase-mediated post-translational priming regulates NLRP3 inflammasome activity.....	22
1.6.4 Caspase 8 contributes to transcriptional and post-translational NLRP3 inflammasome priming.....	25

1.6.5 Multiple ubiquitination signals regulate NLRP3 inflammasome priming and protein degradation .....	28
1.6.6 Post-transcriptional regulation of NLRP3 inflammasome mRNA stability .....	32
1.6.7 NLRP3 inflammasome signaling without priming.....	33
1.7 Critical features of NLRP3 inflammasome activation .....	34
1.7.1 Potassium efflux is a universal requirement for NLRP3 inflammasome formation .....	35
1.7.2 Mitochondrial disruption triggers ROS production .....	37
1.7.3 Lysosome dysfunction liberates the cysteine protease Cathepsin B .....	43
1.7.4 Endoplasmic reticulum dysfunction disrupts protein folding .....	47
1.7.5 Calcium acts as a second messenger .....	49
1.7.6 Ubiquitination acts as a platform for regulator binding.....	58
1.7.7 Phosphorylation controls inflammasome oligomerization .....	62
1.7.7.1 Syk kinase couples C-type lectin domain family members to NLRP3 inflammasome signaling.....	62
1.7.7.2 RIPK1/RIPK3- and NLRP3-dependent necrosis overlap .....	65
1.7.7.3 Kinases have a wide ranging role in regulating the NLRP3 inflammasome ...	68
1.8 Canonical inflammasome activators: nigericin, ATP and monosodium urate .....	74
1.8.1 Nigericin is a potassium ionophore that activates the NLRP3 inflammasome .....	75
1.8.2 Extracellular ATP triggers NLRP3 activation by binding P2RX7 .....	85
1.8.3 Monosodium urate disrupts lysosomes to trigger the NLRP3 inflammasome .....	97
1.9 Consequences of NLRP3 inflammasome signaling in pulmonary diseases .....	105
1.9.1 The NLRP3 inflammasome is a sentinel in pulmonary infections.....	108
1.9.1.1 NLRP3 inflammasome activation helps clear respiratory viruses .....	108
1.9.1.2 The NLRP3 inflammasome is differentially required for clearing extracellular and intracellular bacterial infections.....	110
1.9.1.3 The NLRP3 inflammasome responds to acute and chronic fungal infections.....	113



1.9.2 Chronic obstructive pulmonary disease is influenced by long term NLRP3 inflammasome stimulation .....	115
1.9.3 NLRP3 may contribute to airway hyperreactivity in asthma, allergy and obesity.....	117
1.9.4 The NLRP3 inflammasome is critically activated in acute lung injury .....	122
1.9.5 The NLRP3 inflammasome drives fibrotic changes in pulmonary fibrosis .....	126
1.9.6 NLRP3 inflammasome signaling may predict risk of bronchopulmonary dysplasia in preterm infants .....	129
1.10 Concluding remarks .....	130
REFERENCES .....	131
CHAPTER 2: Inflammasome activation mediates tissue-specific pathogenesis in <i>Staphylococcus aureus</i> infection.....	
154	154
2.1 Introduction .....	154
2.2 <i>S. aureus</i> factors that activate inflammasomes.....	155
2.3 Inflammasomes that are activated by <i>S. aureus</i> .....	160
2.4 The role of inflammasomes in <i>S. aureus</i> infections.....	169
2.4.1 The NLRP3 inflammasome responds to hemolysins to control <i>S. aureus</i> dermal infections .....	169
2.4.2 <i>S. aureus</i> hijacks the NLRP3 inflammasome to exacerbate lung infection pathology .....	173
2.4.3 Microglia activate NLRP3 <i>in vitro</i> but depend on AIM2 for survival in <i>S. aureus</i> brain abscesses.....	177
2.4.4 IL-1 $\beta$ signaling is critical in soft tissue infections.....	179
2.5 How host inflammasomes can affect other immune processes .....	179
2.6 Integrating inflammasome studies to improve patient care.....	182
REFERENCES.....	184
CHAPTER 3: <i>Staphylococcus aureus</i> Leukocidin A/B (LukAB) kills human monocytes by co-opting host NLRP3 and ASC when extracellular, but not intracellular .....	
192	192
3.1 Introduction .....	192

3.2 Materials and Methods.....	195
3.2.1 Ethics statement .....	195
3.2.2 Mammalian cell lines .....	195
3.2.3 Purifying primary CD14+ human monocytes.....	196
3.2.4 Bacterial strains, culture conditions and generating mutants .....	196
3.2.5 Purifying toxins form <i>S. aureus</i> .....	197
3.2.6 Collecting culture filtrates.....	197
3.2.7 Transmission electron microscopy .....	198
3.2.8 Evaluating cell death by lactate dehydrogenase release .....	198
3.2.9 Evaluating cell death by propidium iodide staining.....	199
3.2.10 Measuring cytokine secretion by AlphaLISA.....	199
3.2.11 Measuring Caspase 1 activation with FLICA .....	200
3.2.12 Immunoblot analysis.....	200
3.2.13 Measuring THP1 surface CD11b levels .....	201
3.2.14 Evaluating infection assays by flow cytometry.....	201
3.3 Results.....	205
3.3.1 <i>S. aureus</i> kills human monocytes in a LukAB dependent manner .....	205
3.3.2 LukAB kills human monocytes by engaging its cellular receptor .....	209
3.3.3 LukAB-induced cell death displays necrotic features .....	213
3.3.4 LukAB induces activation of Caspase 1 in human monocytes.....	217
3.3.5 NLRP3 and ASC are necessary for LukAB-induced cytokine secretion and necrotic cell death .....	220
3.3.6 Caspase 1 is required for LukAB-induced cytokine secretion.....	223
3.3.7 LukAB promotes <i>S. aureus</i> escape from within human monocytes independent of NLRP3 or ASC, but dependent on CD11b .....	226
3.4 Discussion.....	230
REFERENCES .....	234

CHAPTER 4: Novel approaches to studying kinase-dependent regulation of the NLRP3 inflammasome .....	239
4.1 Introduction .....	239
4.2 Materials and Methods.....	242
4.2.1 Mammalian cell lines .....	242
4.2.2 Purifying LukAB from <i>S. aureus</i> .....	243
4.2.3 Evaluating cell death by propidium iodide staining.....	243
4.2.4 Measuring cytokine secretion by AlphaLISA.....	244
4.2.5 Measuring Caspase 1 activation with FLICA .....	244
4.2.6 Multiplex inhibitor bead chromatography .....	244
4.2.7 iTRAQ labeling.....	245
4.2.8 Mass spectrometry .....	245
4.2.9 Exploratory statistical analysis of MIB/MS results .....	246
4.2.10 DAPK inhibition experiments.....	246
4.2.11 Site directed mutagenesis of NLRP3 .....	246
4.2.12 Cloning of NLRP3 mutants into T7 expression vector .....	247
4.2.13 In vitro transcription of capped and poly-adenylated mRNA.....	247
4.2.14 Transfection of mRNA into THP1 cells.....	248
4.3 Results.....	248
4.3.1 LukAB causes rapid NLRP3 inflammasome activation and cell death .....	248
4.3.2 MIB/MS identifies 344 kinases expressed in THP1 cells.....	252
4.3.3 Eight kinases are modulated in response to LukAB intoxication.....	252
4.3.4 Inhibiting DAPK suppresses LukAB cytotoxicity and IL-1 $\beta$ and IL-18 secretion.....	255
4.3.5 mRNA transfection of NLRP3 S198D/201D into THP1 cells causes spontaneous IL-18 secretion not seen in S198D single mutant .....	256
4.4 Discussion.....	259
REFERENCES .....	264

CHAPTER 5: Concluding thoughts and future directions .....	267
APPENDIX A: Functional amyloid signaling via the inflammasome, necrosome and signalosome: new therapeutic targets in heart failure .....	271
A.1 Abstract.....	271
A.2 Introduction .....	272
A.3 Misfolded proteins in cardiac disease .....	272
A.4 Protein folding, preamyloid oligomers, and aggregation.....	275
A.5 Protein aggregates, proteotoxicity, and heart failure .....	278
A.6 How endogenous systems prevent protein misfolding.....	281
A.7 Pharmacological chaperone therapies .....	282
A.8 Amyloid proteins, heart failure, and activation of the inflammasome .....	295
A.9 Amyloid proteins, heart failure, and activation of “functional amyloid”: Necrosome .....	299
A.10 The COP9 signalosome enhances protein degradation to reduce the misfolded protein burden .....	300
A.11 Summary: The structural continuum between functional and pathological amyloids and the significance in heart failure .....	304
REFERENCES .....	308

## LIST OF TABLES

Table 1.1 – Kinases analyzed in NLRP3 inflammasome research .....	74
Table 3.1 – <i>Staphylococcus aureus</i> strains .....	203
Table A.1 – Clinical studies investigating tafamidis and siRNA TTR in transthyretin-associated (amyloid) cardiomyopathy .....	285
Table A.2 – Clinical studies investigating doxycycline in transthyretin-associated (amyloid) cardiomyopathy .....	291
Table A.3 – Clinical studies investigating ECGG in primary cardiac amyloidosis and Alzheimer’s disease .....	293
Table A.4 – Proteins forming amyloid as part of their functional role in cellular responses to the environment.....	307

## LIST OF FIGURES

Figure 1.1 – TLR signaling triggers NLRP3 inflammasome transcriptional priming.....	17
Figure 1.2 – Negative feedback loops limit NLRP3 inflammasome priming .....	21
Figure 1.3 – Kinases in non-transcriptional priming of the NLRP3 inflammasome .....	24
Figure 1.4 – Caspase 8 is critical for transcriptional and post-translational NLRP3 inflammasome priming .....	27
Figure 1.5 – Ubiquitination regulates NLRP3 protein levels and activity.....	31
Figure 1.6 – Accumulation of mitochondrial damage activates the NLRP3 inflammasome .....	42
Figure 1.7 – Lysosomal disruption contributes to NLRP3 inflammasome signaling.....	46
Figure 1.8 – TLR signaling may shift TAK1 between priming and NLRP3 inflammasome roles .....	103
Figure 2.1 – <i>S. aureus</i> pathogen-associated molecular patterns and virulence factors activate inflammasome priming and signaling .....	156
Figure 2.2 – NLRP3, NLRC5, NLRP7, AIM2 and other inflammasome-like signaling is activated in response to <i>S. aureus</i> .....	161
Figure 2.3 – NLRP3 inflammasome signaling plays unique rolls in skin and lung infections....	176
Figure 2.4 – Inflammasome signaling upregulates $\gamma\delta$ -T cells and a TH17 response .....	181
Figure 3.1 - <i>Staphylococcus aureus</i> LukAB induces cell death in human monocytic cells .....	207
Figure 3.2 – LukAB recognizes CD11b on human monocytes to potently induce cell death.....	211
Figure 3.3 – LukAB induces necrotic cell death and secretion of pro-inflammatory cytokines IL-1 $\beta$ and IL-18 .....	215
Figure 3.4 – LukAB is a potent activator of Caspase 1 .....	218
Figure 3.5 – LukAB activates the NLRP3 inflammasome leading to cell death and cytokine secretion.....	221
Figure 3.6 – NLRP3 and ASC contribute to <i>S. aureus</i> culture filtrate-mediated cytokine secretion and THP1 cell death.....	222
Figure 3.7 – Media supplementation with KCl inhibits LukAB-mediated FLICA-1 activation and cell death.....	223
Figure 3.8 – Genetic or pharmacologic disruption of Caspase 1 blocks LukAB-induced cytokine secretion but not cell death.....	224

Figure 3.9 – LukAB produced by extracellular or phagocytized <i>S. aureus</i> kills human monocytes .....	228
Figure 4.1 – LukAB intoxication leads to rapid NLRP3 inflammasome activation, mitochondrial depolarization and cell death .....	250
Figure 4.2 – Mitochondrial depolarization is not blocked by extracellular potassium or shRNA-mediated depletion of NLRP3 or ASC.....	251
Figure 4.3 – Kinome analysis by MIB/MS identifies 344 kinases, 8 of which are significant changed at 10 separate time points .....	254
Figure 4.4 – DAPK inhibition blocks LukAB-mediated IL-1 $\beta$ and IL-18 secretion and cell death in THP1 cells .....	255
Figure 4.5 – GFP mRNA transfection efficiency is high and well tolerated in THP1 cells .....	258
Figure 4.6 – Phosphomimetic mutation of NLRP3 S198 and S201 leads to spontaneous NLRP3 inflammasome activity .....	259
Figure A.1 – Native, non-native, aggregates, and amyloid protein structures and the stressors that drive them.....	276
Figure A.2 – Protein aggregate and amyloid stimulate cellular dysfunction.....	280
Figure A.3 – The NLRP3 inflammasome is an amyloid-like fibrillar cytokine-processing platform, which senses amyloid and contributes to worsening heart failure .....	296
Figure A.4 – Cullin-RING ubiquitin ligase (CRL) activity is regulated via neddylation and deneddylation .....	303
Figure A.5 – Targeting pathological and functional aggregates in heart failure .....	305

## LIST OF ABBREVIATIONS

2-APB	2-aminoethoxydiphenyl borate
3-MA	3-methyladenine
ADAM10	A disintegrin and metalloprotease 10
ADCY	Adenylate cyclase
AIM2	Absent in melanoma 2
AL	Amyloid light-chain amyloidosis
ALI	Acute lung injury
ANOVA	Analysis of variance
AQP	Aquaporin
ASC	Apoptosis-associated speck-like protein
BAL	Bronchoalveolar lavage
BLM	Bleomycin
BMDC	Bone marrow dendritic cells
BMDM	Bone marrow-derived macrophages
BPD	Bronchopulmonary dysplasia
BSA	Bovine serum albumin
BTK	Bruton's tyrosine kinase
C5aR	Complement component 5a receptor 1
CA-MRSA	Community-acquired methicillin-resistant <i>S. aureus</i>
CaM	Ca <sup>2+</sup> /Calmodulin domain
cAMP	Cyclic AMP
CANTOS	Canakinumab anti-inflammatory thrombosis outcomes study
CaOx	Calcium oxalate
CAPS	Cryopyrin-associated periodic syndromes



CARD	Caspase activation and recruitment domain
CAS	Casamino acids
CASR	Calcium sensing receptor
CCCP	Cyanide <i>m</i> -chlorophenyl hydrazine
CCR	C-C motif receptor
CD11b	Cluster of differentiation 11b
CFR	Code of Federal Regulations
CHOP	C/EPB homologous protein
CINCA	Chronic infantile neurological cutaneous and articular syndrome
Cm	Chloramphenicol
COP9	Constitutive photomorphogenesis mutant 9
COPD	Chronic obstructive pulmonary disease
CR3	Complement receptor 3
CREB	Cyclic AMP-responsive element-binding protein
CRL	Cullin-RING-ligase
CS	Cyclic stretching
CSN	COP9 signalosome
CTSB	Cathepsin B
CXCR	CXC chemokine receptor
DAPK	Death-associated protein kinase
DPI	Diphenyliodonium
dsRNA	Double-stranded RNA
DUB	Deubiquitinating enzyme
EGCG	(-)-epigallocatechin-3-gallate
ER	Endoplasmic reticulum

FACS	Fluorescence-activated cell sorting
FADD	Fas-associated protein with death domain
FAP	Familial amyloidotic polyneuropathy
FBS	Fetal bovine serum
FCAS	Familial cold autoinflammatory syndrome
FEV1	Forced expiratory volume, 1 second
FLICA-1	FLICA-FMK Alexa Fluor 660
FMK	Fluoromethyl ketone
GFP	Green fluorescent protein
HALI	Hyperoxic acute lung injury
HBSS	Hank's balanced salt solution
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIES	Hyper IgE Syndrome
HIF1 $\alpha$	Hypoxia-inducible factor 1-alpha
HKSA	Heat-killed <i>S. aureus</i>
Hla	Alpha hemolysin
Hlb	Beta hemolysin
Hld	Delta hemolysin
HlgAB	Gamma hemolysin AB
HlgCB	Gamma hemolysin CB
HMGB1	High mobility group box 1 protein
HRP	Horseradish peroxidase
HSP	Heat shock protein
HV	High tidal volume

IAP	Inhibitor of apoptosis proteins
IFN	Interferon
IKBKE	Inhibitor of NF- $\kappa$ B kinase subunit epsilon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor-associated kinase
IRB	Institutional review board
IP3	Inositol 1,4,5-trisphosphate
LAC	Los Angeles County
LDH	Lactate dehydrogenase
LLMe	Leu-Leu-OMe
LLO	Listeriolysin
LOF	Loss of function
LRR	Leucine-rich repeat
LT	Lethal toxin
LTA	Lipoteichoic acid
LUBAC	Linear ubiquitin chain assembly complex
LukAB	Leukocidin AB
LukED	Leukocidin ED
LV	Low tidal volume
MAC	Membrane attack complex
Mac-1	Macrophage-1 antigen
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral signaling
MCU	Mitochondrial calcium uniporter

Mdm2	Mouse double minute 2 homolog
MDP	Muramyl dipeptide
MOI	Multiplicity of infection
MRSA	Methicillin-resistant <i>S. aureus</i>
MSU	Monosodium urate
mtDNA	Mitochondrial DNA
MVK	Mevalonate kinase
MWS	Muckle-Wells syndrome
NAC	N-acetylcysteine
NOX2	NADPH oxidase 2
Nec-1	Necrostatin-1
NHS	Normal human serum
NLRP3	NLR family, pyrin domain containing 3
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NSA	Necrosulfonamide
NTA	Nickel-nitrilotriacetic acid
OVA	Ovalbumin
p53	Tumor protein 53
PAK	p21-activated kinase
PAMP	Pathogen-associated molecular pattern
PARP	Poly(ADP-ribose) polymerase
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PDE	Phosphodiesterase

PFT	Pore-forming toxin
PGAM5	Phosphoglycerate mutase family member 5
PGN	Peptidoglycan
PI	Propidium iodide
PKR	RNA-dependent protein kinase
PLC	Phospholipase C
PML	Promyelocytic leukemia protein
PMN	Polymorphonuclear cell
Poly-Q19	Poly-glutamine (19 repeats)
Poly-Q83	Poly-glutamine (83 repeats)
PP2A	Protein phosphatase 2A
PRR	Pattern recognition receptor
PSM	Phenol-soluble modulins
PVL	Panton-Valentine leukocidin
PYD	Pyrin domain
RA	Rheumatoid arthritis
RHIMs	RIP homotypic interaction motifs
RIPK	Receptor interacting protein kinase
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SAA	Serum amyloid A
SDS	Sodium dodecyl sulfate
SERCA	Sarco/endoplasmic reticulum Ca <sup>2+</sup> ATPase
shRNA	Short hairpin RNA
siRNA	Small interfering RNA

SNAP	S-nitroso-N-acetylpenicillamine
SOCE	Store-operated Ca <sup>2+</sup> entry
STIM	Stromal interaction molecule
Syk	Spleen tyrosine kinase
TAB	TAK-1 binding protein
TBS	Tris-buffered saline
TRIM33	Tripartite motif 33
TRPM2	Transient receptor potential cation channel subfamily M
TRX	Thioredoxin
TSB	Tryptic soy broth
TTR	Transthyretin
TXNIP	Thioredoxin-interacting protein
TUDCA	Tauroursodeoxycholic acid
UNC	University of North Carolina
UPR	Unfolded protein response
UTR	Untranslated region
VILI	Ventilator-induced lung injury
XIAP	X-linked inhibitor of apoptosis protein
XOR	Xanthine oxidoreductase

## **Chapter 1. Discovery, activation and regulation of the NLRP3 inflammasome**

### **1.1 A general overview of NLRP3 inflammasome form and function**

The NLRP3 inflammasome is the most well characterized of a recently discovered family of sensors that activate Caspase 1 to initiate immune signaling. NLR proteins [1] (ex. Nucleotide-binding domain and leucine-rich repeat containing, pyrin-domain 3 – NLRP3) respond to pathogens and cellular stresses known as danger signals. Upon activation, NLR proteins interact with Caspase 1, either directly in those that contain a Caspase Activation and Recruitment Domain (CARD), such as in the NLRC family, or indirectly through pyrin-pyrin domain (PYD) interactions with Apoptosis-associated speck-like protein containing a CARD (ASC) [2]. Activation of Caspase 1 leads to proteolytic processing of critical inflammatory signals, including interleukin-1 $\beta$  (IL-1 $\beta$ ) and interleukin-18 (IL-18), to their mature form. Additionally, activation of inflammasomes, and NLRP3 in particular, can cause necrotic-type cell death. This cell death has been referred to as pyroptosis or pyronecrosis, largely depending on the requirement for Caspase 1. However, the use of these terms in the literature is sometimes inexact so they will be avoided here.

Activation of inflammasomes is also often times preceded by a “priming” step (or signal 1). Toll-like receptor (TLR) signaling primes inflammasome activation by: (1) upregulating NF- $\kappa$ B-dependent transcription of inflammasome components and pro-IL-1 $\beta$  and (2) initiating a series of post-translational modifications of inflammasome components and their regulators. In

some cells, usually those with high levels of expression of inflammasome components, such as THP1 cells, this step can be bypassed, though usually without IL-1 $\beta$  secretion.

Hundreds of unique agents activate the NLRP3 inflammasome. Because of the large number of different activating agents, a detailed comprehensive model of inflammasome activation has yet to emerge. Instead, the field is littered with disparate findings pertaining to different activators that are rarely confirmed system-wide. In this introduction I will summarize the current state (as of the end of 2015) of the NLRP3 inflammasome field. I will start by discussing the discovery of the NLRP3 inflammasome as the cause of heritable IL-1 $\beta$ -driven hyperinflammatory periodic fever disorders. Then, I will review the dominant hypotheses of how the NLRP3 inflammasome is regulated across different activators. Following that, I will discuss how each of these hypotheses fit in with each one another by reviewing the requirements for the three classical NLRP3 agonists: nigericin, extracellular ATP and monosodium urate (MSU). And lastly, I will highlight the role of NLRP3 in infectious and non-infectious pulmonary diseases, a personal and professional interest of mine, to demonstrate the wide ranging effects of the NLRP3 inflammasome. My dissertation research will follow. This will include:

- a comprehensive literature review of *S. aureus* and inflammasome interactions with a focus on the unique tissue specific roles of different inflammasomes
- primary research comparing the cytotoxicity of *S. aureus* exotoxins in killing human monocytes and identifying that Leukocidin AB (LukAB) activates the NLRP3 inflammasome
- primary research on the role of kinases broadly and DAPK specifically in regulating NLRP3 inflammasome activity in response to LukAB



- a review of novel therapeutic targets in heart failure, including specifics on a role for the NLRP3 inflammasome (this previously published paper will be included as an appendix)

At the end, I will conclude with my thoughts on significance of my work and the future of NLRP3 inflammasome research.

## **1.2 Clinical relevance of NLRP3 inflammasome signaling**

The NLRP3 inflammasome is increasingly being recognized as a source of pathology in a wide range of infectious and non-infectious diseases, and at the same time, critical for clearing many infections. *Staphylococcus aureus* is a highly drug-resistant and clinically important pathogen responsible for a growing number of skin and lung infections. Early research into the interaction between *S. aureus* and inflammasomes has revealed interesting insights into the pathogenesis of infections. In pneumonia, *S. aureus* pore-forming toxins (PFTs) such as alpha-hemolysin (Hla) activate the NLRP3 inflammasome leading to severe lung damage and death. In dermatitis, *S. aureus* PFTs and phenol-soluble modulins (PSMs) activate the NLRP3 inflammasome allowing the bacteria to infiltrate the keratinocyte barrier but also promote bacterial clearance by neutrophils. Additionally, the NLRP3 inflammasome is activated in human monocytes in response to PFTs that were only recently discovered because of their specificity for human cells over mice. As a deeper understanding of *S. aureus*-inflammasome interactions begins to emerge, the inflammasome signaling node may become an attractive target for clinical intervention to treat infections or reduce inflammation-associated tissue damage in conjunction with antibiotics.

### **1.3 Interleukin-1 $\beta$ -driven monogenic hyperinflammatory fever disorders are caused by mutations in the NLRP3 inflammasome pathway**

NLRP3 (also referred to in early literature as CIAS1, PYPAF1, NALP3 and Cryopyrin) was identified as a critical protein in inflammation when it was found to be the causal gene mutated in familial cold autoinflammatory syndrome (FCAS) and Muckle-Wells syndrome (MWS) [3]. Shortly thereafter, NLRP3 was also shown to be mutated in chronic infantile neurological cutaneous and articular (CINCA) syndrome (also known as neonatal-onset multisystem inflammatory disease [NOMID]) [4]. Mutations in NLRP3 have been confirmed in dozens of patients with these diseases [5], now collectively known as Cryopyrin-Associated Periodic Syndromes (CAPS) or Cryopyrinopathies.

FCAS is an autosomal-dominant systemic hyperinflammatory disease in which patients experience recurrent episodes of rash, arthralgia, fever and conjunctivitis after exposure to cold [6-8]. MWS is also an autosomal-dominant syndrome of periodic fevers, rash, arthralgia, sensorineural hearing loss, and sometimes amyloidosis [9,10]. CINCA syndrome is a severe early onset chronic inflammatory disease characterized by cutaneous symptoms, central nervous system involvement and arthropathy [11-13]. The mutations in NLRP3 that cause these three diseases are sometimes overlapping, suggesting that secondary modifying factors contribute to their distinct presentations. However, such modifying factors have yet to be identified.

Insight into the function of NLRP3 quickly followed discovery of CAPS causing mutations. A mammalian two-hybrid screen showed that ASC interacts with NLRP3 and co-expression of NLRP3 and ASC resulted in the formation of distinct cytoplasmic loci [14]. ASC

was also found to interact directly with pro-Caspase 1 [15] and, in conjunction with NLRP3, formed an IL-1 $\beta$  processing inflammasome [16].

When NLRP3 with common disease-associated mutations (R260W, D303N, and E637G) was expressed in THP1 cells, these mutants constitutively associated with ASC and induced spontaneous IL-1 $\beta$  secretion, whereas wildtype NLRP3 did not [16,17]. Macrophages from MWS patients also spontaneously secreted active IL-1 $\beta$  [16]. Monocytes from patients with FCAS were cold sensitive and responded to incubation at 32°C by secreting IL-1 $\beta$  [18], mimicking the sensitivity of FCAS patients generally. Knockin mice expressing the D301N NLRP3 mutation (the ortholog of D303N in humans) exhibited widespread neutrophilia and high levels of serum inflammatory markers. They also exhibited skeletal abnormalities seen in CINCA patients including knee joint deformity, growth retardation and severe postnatal osteopenia resulting from increased osteoclastogenesis, accelerated bone resorption and loss of chondrocytes [19].

Early hints that IL-1 $\beta$  dysregulation may drive pathology in CAPS led to therapeutic trials of the recombinant human IL-1-receptor (IL-1R) antagonist anakinra (brand name: Kineret). Anakinra led to a dramatic, rapid, and sustained resolution of symptoms in patients with MWS [20] that has been reproduced in hundreds of patients [21], including patients with FCAS [22] and CINCA [23]. The clinical experience with anakinra has become the basis for clinical guidelines [24]. Clinical trials have also shown success with Riloncept, an IL-1 Trap that acts as a long-acting and potent inhibitor of IL-1 $\beta$ , in patients with FCAS and MWS [25,26]. A third IL-1 $\beta$  disrupting therapy, Canakinumab, a human anti-IL-1 $\beta$  monoclonal antibody, has also been used successfully [27].

Additional periodic fever syndromes have been identified with casual genes that relate to inflammasome signaling providing greater insight into the activity and regulation of the NLRP3 inflammasome-IL-1 $\beta$  signaling axis. Sterile Multifocal Osteomyelitis with Periostitis and Pustulosis (OMPP) [28,29] is caused by a deficiency in the IL-1R antagonist (IL1-RN) that leads to unopposed IL-1 $\beta$  signaling and life-threatening systemic inflammation [30]. Familial Mediterranean Fever (FMF) is an autosomal recessive disorder caused by mutations in the MEFV gene that encodes for Pyrin [31]. Pyrin negatively regulates the NLRP3 inflammasome by disrupting the interaction between NLRP3 and ASC, and thus, when mutated, leads to increased inflammasome activation [32]. Hyper-IgD and Periodic Fever Syndrome is caused by mutations in mevalonate kinase (MVK) [33,34] that disrupt autophagy, which is thought to negatively regulate the NLRP3 inflammasome and IL-1 $\beta$  secretion, leading to a hyperinflammatory state [35]. Pyogenic Sterile Arthritis, Pyoderma Gangrenosum, and Acne (also known as PAPA Syndrome - PAPAS) [36], is caused by mutation in the PSTPIP1 gene [37]. PSTPIP1 binds to Pyrin and disease-associated mutations exert a dominant-negative effect on Pyrin leading to increased IL-1 $\beta$  production in patients [38].

It is interesting to note that in all Mendelian disorders affecting the NLRP3 inflammasome pathology results from increased activation of this pathway, not decreased. This makes the NLRP3 inflammasome fairly unique among critical immune signaling pathways in that outright disease resulting from absent NLRP3 inflammasome signaling has not yet been identified. It is likely that low or absent function NLRP3 alleles do exist in the human population, but their susceptibility to infection may be so minimal so as to not be easily recognizable.

#### **1.4 The NLRP3 inflammasome is a master innate immune sensor**

Activation of the NLRP3 inflammasome typically proceeds in two stages. First, a “priming” step upregulates transcription and translation of inflammasome components and pro-IL-1 $\beta$  through the well-studied TLR-NF- $\kappa$ B signaling pathway. Additionally, priming prepares the NLRP3 inflammasome for activation through a series of post-translational modifications. Second, Caspase 1 catalytic activity is triggered through NLRP3 inflammasome oligomerization leading to processing of cytokines to their mature form for secretion and necrotic cell death. In the absence of priming, cells that constitutively express NLRP3 inflammasome components can trigger IL-18 secretion and cell death, without IL-1 $\beta$  secretion. However, the physiologic significance of this partial activation is still unclear.

Early functional studies of the NLRP3 inflammasome drew comparisons to the Toll-like receptor system, a family of pattern recognition receptors (PRRs) for sensing pathogen-associated molecular patterns (PAMPs). While other NLR proteins detect a narrow range of PAMPs (ex. NLRC4 binds flagellin and NLRP1 binds muramyl dipeptide - MDP), NLRP3 is activated by hundreds of pathogen-associated molecules, endogenous danger signals, and environmental and man-made pollutants. The only common feature of these NLRP3-activating molecules identified thus far is that their activity is inhibited by increasing the extracellular concentration of potassium. As such, the NLRP3 inflammasome would be more accurately placed in a category of its own – as a master innate immune sensor – to distinguish its high degree of promiscuity from the PRRs that sense a narrow range of molecules.

As a result of this promiscuity, the NLRP3 inflammasome research field is full of gaps. Before discussing the NLRP3 inflammasome in more detail, the reader will be warned in section 1.5 of these gaps and how they hamper generalizability of important data.

## **1.5 Cautions in interpreting NLRP3 inflammasome research**

Research on the NLRP3 inflammasome has been expanding rapidly with each passing year and with no signs of slowing down. A search of “NLRP3” on PubMed at the end of 2015 returned 2182 results, of which, nearly half, 1035 (47.4%), were published in 2014 or 2015. Because NLRP3 inflammasome research is still a nascent field, many new discoveries are disjointed and little effort is made to integrate new findings into a coherent and comprehensive model for NLRP3 inflammasome activity. This issue has been exacerbated by the large number of different activating stimuli. Below, as a brief aside, I will highlight some of the common faults in the field in order to prepare the reader for some of the unfortunate confusion and shortcomings that exist in the data.

### **1.5.1 Studies frequently overlook important outcomes of NLRP3 inflammasome activity**

The vast majority of research on the NLRP3 inflammasome focuses on two readouts of NLRP3 activity: cleavage of pro-Caspase 1 into its active form and secretion of mature IL-1 $\beta$ . As previously mentioned, activation of the NLRP3 inflammasome actually triggers Caspase 1 activation, IL-1 $\beta$  and IL-18 secretion, and necrotic cell death. These are also only the most well-known outcomes. Activation of Caspase 1 is also thought to result in processing and secretion of

IL-33 [39] and cleavage of possibly up to 41 other cellular proteins including glycolytic enzymes [40].

While activation of Caspase 1 and maturation of IL-1 $\beta$  are appropriate readouts for NLRP3 inflammasome activation, ignoring these other downstream events is certain to result in faulty assumptions and missed opportunities. For example, the secreted *Listeria monocytogenes* (Lm) p60 protein activated the NLRP3 inflammasome to trigger IL-1 $\beta$  and IL-18 secretion but not cell death. Diphenyliodonium (DPI) treatment to block production of reactive oxygen species (ROS) blocked secretion of IL-1 $\beta$  but not IL-18. Additionally, Caspase 11 deficient mice failed to secrete IL-1 $\beta$  in response to Lm p60 but IL-18 secretion was fully intact [41]. Of course, it would be unreasonable to evaluate forty different targets of Caspase 1 cleavage, but by more rigorously evaluating downstream results of NLRP3 inflammasome activity this one study demonstrated at least two unique aspects of Lm p60 that would have otherwise been unappreciated. First, that Lm p60-induced NLRP3-dependent IL-1 $\beta$  and IL-18 secretion are regulated by separate mechanisms, and second, that NLRP3 inflammasome activation in response to Lm p60 does not cause cell death.

Numerous studies also support a larger role for Caspase 1 beyond IL-1 $\beta$  signaling (reviewed [42]). Interestingly, despite early studies establishing a role for Caspase 1 in NLRP3-mediated cell death, many have since demonstrated NLRP3-dependent, Caspase 1-independent cell death, most notably the pore-forming toxins of *S. aureus* [43-45]. By just measuring IL-1 $\beta$  secretion, studies miss an opportunity to characterize the mechanism of NLRP3-mediated cell death in their system. My work, detailed in Chapter 3, also serves as an example showing that even when the NLRP3 inflammasome is activated, other pathways may contribute to the phenotype observed. *S. aureus* LukAB binding to CD11b on the phagosomal membrane

activated NLRP3 and killed THP1 cells. shRNA-mediated depletion of NLRP3 blocked LukAB-induced IL-1 $\beta$  secretion but did not diminish LukAB-induced cell death, suggesting that a NLRP3-independent cell death pathway is responsible [45].

Most importantly, though, when studies of the NLRP3 inflammasome focus exclusively on IL-1 $\beta$ , there exists a potential to do harm to patients. As mentioned previously, disruption of IL-1 $\beta$  signaling has successfully been used as a treatment for CAPS. However, evidence from mouse models suggests that both IL-18 and cytokine-independent processes play an important role in disease pathogenesis as well. Expression of NLRP3-activating mutations A352V or L353P (commonly associated with MWS and FCAS respectively) in mice resulted in neonatal or perinatal lethality. Breeding each mutation onto an IL-1R knockout background resulted in an incomplete phenotypic rescue. The A352V MWS mutation combined with IL-1R knockout mice still had a diminished growth rate and limited cutaneous inflammation when compared to the parental strain mice. The L353 FACS mutation combined with IL-1R knockout was more severe with eighty percent of mice dying by 35 days after birth [46]. These data raised the possibility of non-IL-1 $\beta$ -mediated effects in CAPS.

The MWS and FCAS mutations were also crossed onto an IL-18 receptor (IL-18R) knockout background in a subsequent study. FCAS IL-18R knockout mice had equivalent survival to FCAS IL-1R knockouts and MWS IL-18R knockout mice had vastly improved survival compared to MWS IL-1R knockouts. Knockout of Caspase 1 was completely protective against the FCAS mutation. However, knockout of both IL-1R and IL-18R does not fully protect against the lethality of the FCAS mutation. These data suggest that there are IL-1 $\beta$ - and IL-18-independent processes resulting from Caspase 1 activation that are important for disease pathogenesis in this mouse model [47]. Of course, the generalizability of discoveries in mice to



humans is not exact. While there have not been widespread reports of treatment failure with anti-IL-1 therapy in CAPS, these studies point to a theoretical shortcoming that must be investigated further.

### **1.5.2 Dozens of activators and regulators complicate a comprehensive model for NLRP3 inflammasome activation**

Studies of the NLRP3 inflammasome typically focus on identifying new activators or new regulators of NLRP3 inflammasome signaling. Mechanistic discoveries are being made across a wide range of cellular processes including organelle perturbation, calcium signaling, ubiquitinating enzymes and kinases. The breadth of these discoveries has created tremendous difficulty in relating these discoveries to one another. Additionally, because new regulators are often identified with only a small subset of activators, it is not clear that each regulatory element is critical across the range of all NLRP3 activating molecules.

Compounding this difficulty, studies usually only validate mechanistic discoveries across activators using a narrow range of assays, rather than the full extent used to prove the original requirement. For example, in a typical publication, one that shows a role for Xanthine oxidoreductase (XOR) in regulating the NLRP3 inflammasome in response to crystalline activators, only one experiment using an inhibitor of XOR in ATP- and nigericin-induced IL-1 $\beta$  secretion is shown to extend the regulatory role of XOR to those activators. This was presented as sufficient evidence in light of the experiments on crystalline activators to substantiate an argument for a general requirement for XOR in NLRP3 inflammasome activation [48].

Additionally, this makes it unlikely that future research will comprehensively address those claims because of the “first to publish” mentality.

In some cases, when a well-studied protein or molecule is found to regulate the NLRP3 inflammasome, the data provide no mechanistic hints except to rule out involvement of previously characterized pathways. One example of this is a study that showed the anti-inflammatory eicosanoid 15-deoxy-delta-12,14-PGJ2 (15d-PGJ2) inhibits inflammasomes, including the NLRP3 inflammasome, but not through its known actions on PPAR $\gamma$ , NRF2, or COX-1 [49]. While these studies are important starting points, they must be followed up on in order to avoid creating too many holes in the field.

### **1.5.3 Commonly used Caspase 1 knockout mice are also Caspase 11 deficient**

Caspase 1 activation is the most immediate result of NLRP3 inflammasome activation. The Caspase 1 knockout mouse strain commonly used in published studies in the field was produced using strain 129 embryonic stem cells. Strain 129 mice harbor a mutation in the Caspase 11 locus that attenuates Caspase 11 expression and segregates with Caspase 1 because of close proximity in the genome, approximately 1,500 base pairs. As such, the C57BL/6 Caspase 1 knockout mouse first published in Li et al in 1995 [50] was determined to be deficient in both Caspase 1 and Caspase 11 activity in 2011 [51]. Unfortunately, many studies that preceded this discovery, and even some that have followed it, have not been clarified to determine the role of Caspase 1 and Caspase 11 separately. Caspase 11 activating stimuli are now commonly referred to as non-canonical inflammasome stimuli, but the failure to recognize the loss of Caspase 11 activity in these mice makes it difficult to appreciate the unique roles of

these two Caspases. When possible, in the discussion below, these mice will be called “Caspase 1/Caspase 11 deficient” to highlight this important distinction.

## **1.6 Priming the NLRP3 inflammasome for activation**

Priming of the NLRP3 inflammasome is often required for the full complement of NLRP3 inflammasome activity. Classically, priming involves transcriptional upregulation of NLRP3 inflammasome components and pro-IL-1 $\beta$  through NF- $\kappa$ B signaling. It has more recently been expanded to include non-transcriptional events including post-translational modifications that enhance eventual NLRP3 inflammasome activity. In this section, I will review current data on priming of the NLRP3 inflammasome.

### **1.6.1 Pattern-recognition receptor signaling upregulates transcription of NLRP3 inflammasome components**

Toll-like receptor ligand binding activated NF- $\kappa$ B leading to transcriptional upregulation of pro-IL-1 $\beta$  and components of the NLRP3 inflammasome. Ligand binding to plasma membrane-based TLR2 or TLR4, or endosomal membrane-based TLR3 or TLR7, are all sufficient to induce priming of the NLRP3 inflammasome. TLR4 binding to LPS signaled through two adapters, MyD88 and TRIF. Macrophages deficient in MyD88 or TRIF responded normally to LPS priming but macrophages deficient in both MyD88 and TRIF did not, suggesting that these two downstream adapters can compensate for each other [52]. IRAK4, a kinase required for NF- $\kappa$ B activation downstream of MyD88, is also required for priming by

TLR ligands that signal exclusively through MyD88 [52]. Bay11-7082, an inhibitor of I $\kappa$ B- $\alpha$  phosphorylation and NF- $\kappa$ B signaling [53], and cycloheximide, an inhibitor of protein synthesis, both blocked transcriptional priming [52].

RNA from both Gram-positive and Gram-negative bacteria can prime NLRP3 inflammasome activity in bone marrow-derived macrophages (BMDM) and bone marrow dendritic cells (BMDC). This response was independent of established nucleic acid sensing endosomal TLRs, including TLR3, 7 and 9, and the TLR-adaptor TRIF. Bacterial RNA-based priming required UNC93B, an endoplasmic reticulum protein that has a known role in delivery of TLRs to the endosome, and MyD88. Knockout of UNC93B or TLR2/3/7/9 had no impact on LPS-induced priming but blocked R848, CpG or polyI:C induced priming, as would be expected for these ligands. These data suggest that there is a yet unidentified intracellular nucleic acid receptor involved in bacteria RNA-mediated NLRP3 inflammasome priming that signals through MyD88 [54]. Despite the Toll-like receptor system being very well studied, this study highlights the importance of thoroughly evaluating the role of previously worked out pathways in the context of NLRP3 inflammasome priming.

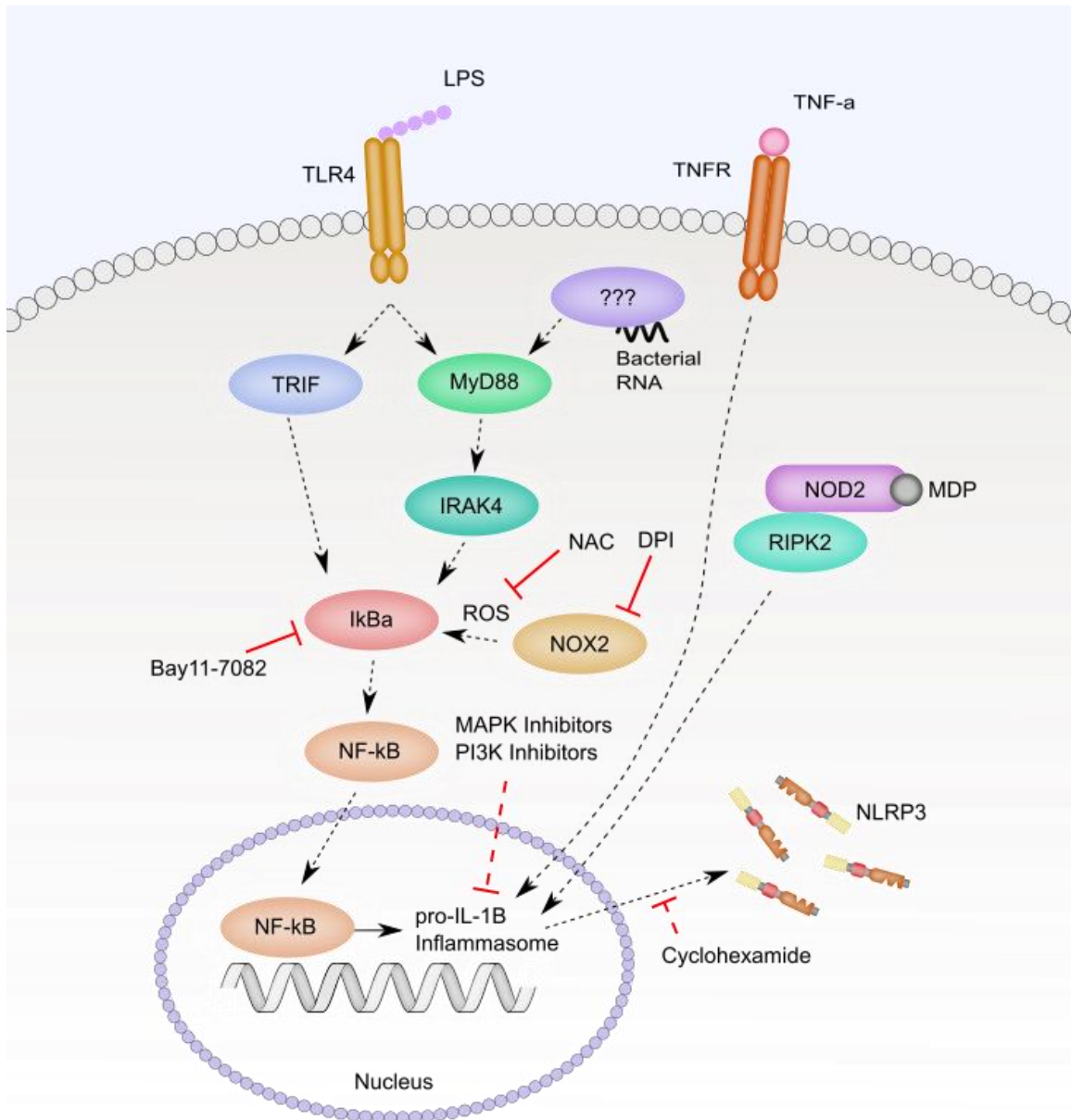
NOD2, the intracellular receptor for muramyl dipeptide, a component of peptidoglycan, also promoted transcriptional priming of the NLRP3 inflammasome through a receptor interacting protein kinase 2 (RIPK2)-dependent mechanism [52]. TNFR1 or TNFR2 binding to TNF- $\alpha$  was also sufficient for transcriptional priming of the NLRP3 inflammasome as loss of both these receptors blocked priming by TNF- $\alpha$  [52,55]. Likewise, exposure to IL-1 $\alpha$  or IL-1 $\beta$  induced a lower levels of transcription as compared to TNF- $\alpha$ , but was sufficient to prime the NLRP3 inflammasome for ATP-induced Caspase 1 activation [55].

Interestingly, whereas murine macrophages required priming for NLRP3 inflammasome activation, transcriptional priming was not required for AIM2 or NLRC4 activation in response to poly(dA:dT) and Flagellin, respectively. Cycloheximide had no effect on AIM2 or NLRC4 activation but significantly reduced NLRP3-dependent Caspase 1 activation in response to LPS and nigericin. The AIM2 inflammasome is an ASC- and Caspase 1-containing inflammasome, suggesting that neither of these components are critically regulated by transcriptional priming. Transcriptional priming was necessary for transcription and translation of NLRP3, as constitutive expression of NLRP3 from an exogenous promoter was able to overcome the priming requirement [56]. In a second study, while constitutive expression of NLRP3 in BMDM was able to overcome the requirement for priming, LPS-induced priming still enhanced Caspase 1 activation further, suggesting that other factors may also be induced or modified by priming [57].

DPI, an inhibitor of Flavin-containing cofactors that blocks NOX2-dependent ROS production, and N-acetylcysteine (NAC), an ROS scavenger, both blocked induction of NLRP3 and IL-1 $\beta$  mRNA. Treatment of macrophages with DPI or NAC prior to LPS exposure suppressed IL-1 $\beta$  secretion but had no effect on IL-1 $\beta$  secretion when DPI was added after priming of the NLRP3 inflammasome was complete [56,58]. DPI treatment blocked phosphorylation of I $\kappa$ B- $\alpha$  and prevented downstream NF- $\kappa$ B activation [58]. Constitutive expression of NLRP3 from an exogenous promoter overcame sensitivity to DPI, further supporting the notion that DPI was blocking transcriptional priming [56]. It is important to note that in this series of experiments, DPI and NAC both also affected LPS-induced TNF- $\alpha$  secretion [56], consistent with a role for ROS in NF- $\kappa$ B-dependent transcriptional priming.

In addition to activation of NF- $\kappa$ B, TLR signaling activates MAPK signaling pathways leading to nuclear translocation of cyclic AMP (cAMP)-responsive element-binding protein

(CREB) and activator protein 1 (AP1) [59]. Inhibitors of MAPK signaling, including the MEK1 inhibitor PD98059 and the JNK1/2 inhibitor SP600125, but not the p38 inhibitor SB203580, blocked the LPS-induced increases in NLRP3 expression. The JAK2 inhibitor AG490 and the PI3-kinase inhibitors LY294002 and wortmannin also inhibited LPS-induced upregulation of NLRP3 protein [58]. This collection of inhibitor experiments suggests that multiple signaling paths downstream from TLRs all coordinate to promote induction of NLRP3 mRNA during LPS priming.



**Figure 1.1. TLR signaling triggers NLRP3 inflammasome transcriptional priming.** This figure summarizes the discussion in section 1.6.1. LPS binding to TLR4 triggered activation of both TRIF and MyD88 which compensated for each other to transduce signaling through IκB-α to activate NF-κB. Signaling through MyD88 also required IRAK4. Treatment of cells with the NOX2 inhibitor DPI, the ROS scavenger NAC, or the IκB-α inhibitor Bay11-7082 all blocked

transcriptional priming. MAPK and PI3K inhibitors blocked transcription of NLRP3 from the endogenous locus. Use of cyclohexamide demonstrated a requirement for transcriptional priming by blocking all protein translation. Solid lines represent direct interactions and dotted lines represent indirect interactions. Only relationships investigated in the context of NLRP3 inflammasome priming are including, not all previously known TLR signaling components. Other pattern recognition receptors, such as NOD2 binding to MDP, and immune receptors such as TNFR binding to TNF- $\alpha$  can trigger transcriptional priming. A requirement for a new bacterial RNA sensor was also identified, demonstrating the importance of comprehensively evaluating these well-studied pathways in the context of NLRP3 inflammasome signaling. Solid lines represent direct interactions and dotted lines represent indirect interactions.

### **1.6.2 Negative feedback loops respond to prolonged priming to reign in NLRP3 inflammasome signaling**

Activation of the NLRP3 inflammasome and secretion of inflammatory mediators dramatically changes tissue structure and physiology. Just as the activation of the NLRP3 inflammasome is critical for defense against many pathogens, it must be carefully regulated to avoid detrimental effects of inflammation. In this section, I will focus on mechanisms that translate prolonged TLR signaling into decreases in NLRP3 inflammasome activity.

NLRP3 inflammasome transcriptional priming seems to integrate signals into time-sensitive negative feedback loops. Murine macrophages stimulated with LPS for 12 hours secreted significantly less IL-18 in response to ATP and nigericin as compared to murine macrophages stimulated with LPS for 4 hours. In an inducible nitric oxide synthase (iNOS)

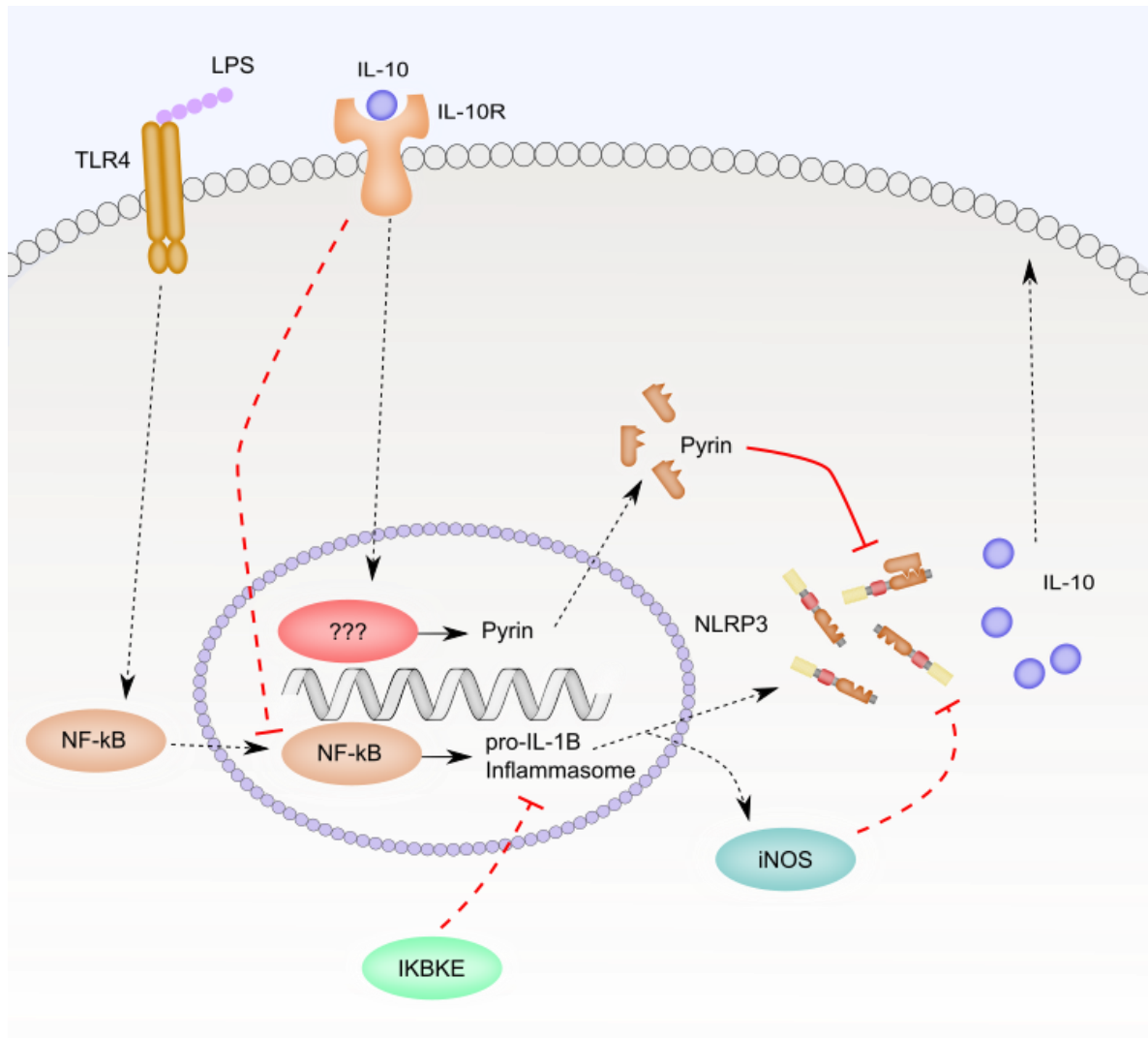


knockout strain, IL-18 secretion was nearly equivalent in both four and twelve hour-long LPS incubations. Long-term (twelve hour) stimulation of macrophages with LPS induced type I interferon (IFN) production leading to upregulation of iNOS and nitric oxide (NO) synthesis. Endogenous NO and the NO donor S-nitroso-N-acetylpenicillamine (SNAP) inhibited the NLRP3 inflammasome. S-nitrosylation of NLRP3 and Caspase 1 was detected in long-term LPS-treated cells and was proposed, but not confirmed, to be responsible for iNOS-dependent suppression of NLRP3 inflammasome activity [60]. A second study investigating the immunopathology of tuberculosis showed that IFN- $\gamma$ , a type II IFN, suppressed IL-1 $\beta$  secretion in an iNOS-dependent manner. They also observed s-nitrosylation of NLRP3 and Caspase 1 and demonstrated that ascorbate, a reducing agent, reversed the modification and release inhibition of IL-1 $\beta$  secretion in a dose dependent manner [61].

Long term stimulation of macrophages with LPS also required inhibitor of NF- $\kappa$ B kinase subunit epsilon (IKBKE) for negative feedback control of NLRP3 inflammasome priming. Loss of IKBKE enhanced LPS-induced NLRP3 and IL-1 $\beta$  mRNA transcription in both a four-hour and twenty four-hour LPS exposure. Ultimately, this led to IKBKE deficient cells secreting more IL-1 $\beta$  relative to wildtype controls. Similar results were seen in adipose tissues [62]. IKBKE is also involved in activating production of type I IFNs in response to viral infections, suggesting the possibility that IKBKE could upregulate iNOS as well. However, the exact mechanism by which IKBKE decreased NLRP3 and IL-1 $\beta$  mRNA remains unexplored.

A study on hemorrhagic shock highlights how multiple different signals can be integrated to balance NLRP3 inflammasome priming. Mice subjected to hemorrhagic shock then given LPS intratracheally experienced an increase in NLRP3 expression, Caspase 1 activation and IL-1 $\beta$  secretion in the lung. Pro-IL-1 $\beta$  mRNA levels were equivalent in sham operation mice treated

with LPS as in hemorrhagic shock mice, but IL-1 $\beta$  secretion was much higher in hemorrhagic shock, leading to the suggestion that hemorrhagic shock induces a NLRP3 inflammasome modifying factor. Hemorrhagic shock suppressed IL-10 secretion and these mice had lower levels of Pypin, a negative regulator of the NLRP3 inflammasome. Blocking IL-10 with an antibody in sham treated mice decreased Pypin expression and increased IL-1 $\beta$  secretion, while administering IL-10 exogenously to hemorrhagic shock treated mice increased Pypin expression and decreased IL-1 $\beta$  secretion. Knockdown of Pypin in mouse lung vascular endothelial cells (MLVECs) led to an increase in IL-1 $\beta$  secretion that could not be blocked by administration of exogenous IL-10 [63]. The ability of IL-10 to suppress NLRP3 inflammasome activity during chronic LPS stimulation was confirmed in a subsequent study of LPS and ATP-treated BMDMs. In this setting, IL-10 decreased expression of NLRP3 leading to decreased Caspase 1 activation and IL-1 $\beta$  secretion in response to LPS and ATP [64].



**Figure 1.2. Negative feedback loops limit NLRP3 inflammasome priming.** This figure summarizes the discussion from section 1.6.2. Long term stimulation of cells with LPS leads to production of iNOS which regulates NLRP3 inflammasome signaling through production of nitric oxide and s-nitrosylation of NLRP3. IKKBE also negatively regulates NLRP3 inflammasome signaling in long term LPS exposures. Solid lines represent direct interactions and dotted lines represent indirect interactions. Only relationships investigated in the context of NLRP3 inflammasome priming are including.

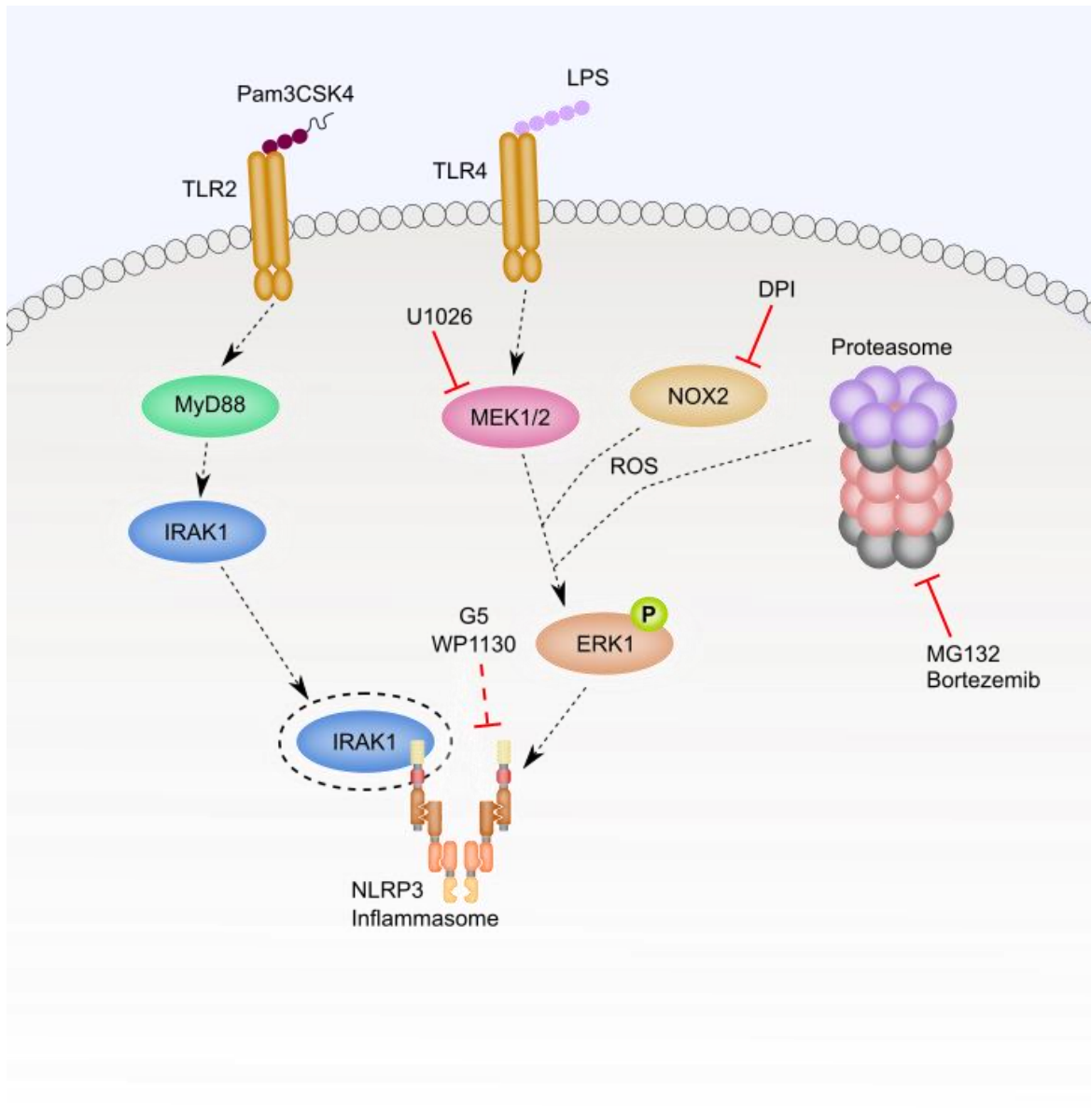
### **1.6.3 Kinase-mediated post-translational priming regulates NLRP3 inflammasome activity**

Careful time course analysis of shorter than typical priming exposure followed by ATP treatment demonstrated unique outcomes that depended on the TLR signal adapter used. Specifically, priming with Pam3CSK4, a TLR2 ligand that signals through MyD88, resulted in biphasic activation of Caspase 1, with levels of active Caspase 1 higher at 10 minutes and 180 minutes of Pam3CSK4 stimulation and lower following 30 minutes and 60 minutes. Priming with poly I:C, a TLR3 ligand that signals through TRIF, induced maximal Caspase 1 activation following 30 and 60 minutes of poly I:C stimulation and almost none seen at 10 minutes and 180 minutes. In contrast, when priming with LPS, a TLR4 agonist that signals through MyD88 and TRIF, robust Caspase 1 activation was observed at all four time points. Interestingly, knockout of MyD88 or TRIF led to LPS-induced priming that mirrored Pam3CSK4 or poly I:C, depending on the signaling adaptor present. Cells treated with just ATP did not activate Caspase 1 [65].

These priming adaptations are more rapid than one would expect for transcriptional changes. Actinomycin D, an inhibitor of RNA chain elongation, was used to test for a requirement for transcription directly. Actinomycin D treatment had no effect on Pam3CSK4-, poly I:C- or LPS-induced priming of Caspase 1 activation at any time point but blocked upregulation of NLRP3 and pro-IL-1 $\beta$ . IRAK1 and IRAK4 were critically required for NLRP3 inflammasome priming during a 10-minute exposure of Pam3CSK4 or LPS. Loss of IRAK1 or IRAK4 resulted in decreased ASC oligomer formation during simultaneous treatment with Pam3CSK4 and ATP. NLRP3 and IRAK1 co-immunoprecipitated together in macrophages and the interaction appeared to be enhanced by Pam3CSK4 treatment [65]. A subsequent study confirmed these findings, showing that simultaneous exposure to LPS and ATP induced IRAK1

and NLRP3 inflammasome co-localization leading to IRAK1-, NLRP3-, and Caspase 1-dependent IL-18 secretion and cell death [66].

NOX2- and proteasome-dependent phosphorylation of ERK was also required for post-translational priming of the NLRP3 inflammasome in human monocytes. Co-stimulation with LPS and ATP triggered Caspase 1 activation and IL-18 secretion from human monocytes in as little as 5 minutes whereas ATP treatment alone was insufficient. LPS induced phosphorylation of ERK, but ATP did not. Treatment of monocytes with U0126, an inhibitor of MEK1/2 that prevents phosphorylation of ERK, blocked IL-18 secretion in response to rapid LPS and ATP exposure. siRNA specifically targeting ERK1, but not ERK2, confirmed this finding. ERK was phosphorylated in response to LPS and treatment with MG132 or bortezomib, both inhibitors of the proteasome, blocked ERK phosphorylation, Caspase 1 activation and IL-18 secretion. Treatment of cells with DPI to block ROS production also suppressed ERK1/2 phosphorylation and IL-18 secretion. The deubiquitinating (DUB) enzyme inhibitors G5 and WP1130 blocked IL-18 secretion but had no effect on ERK1/2 phosphorylation, suggesting that they acted downstream or separately from this pathway. Wortmannin, a PI3K inhibitor, SB203580, a p38 MAPK inhibitor, and SP600125, a JNK inhibitor, had no effect on IL-18 secretion. Loss of NLRP3 or Caspase 1 also had no effect on ERK phosphorylation in response to LPS [67]. The requirement for proteasome activity strongly suggests that priming of the NLRP3 inflammasome initiated destruction of some factor that blocked ERK phosphorylation, though the identity of the proteasome target is still unknown.



**Figure 1.3. Kinases in non-transcriptional priming of the NLRP3 inflammasome.** This figure summarizes the discussion in section 1.6.3. Rapid priming by Pam3CSK4 binding to TLR 2 triggered MyD88-dependent activation of IRAK1. IRAK1 co-immunoprecipitated with the NLRP3 inflammasome and this association was enhanced by Pam3CSK4 priming. IRAK1 promoted ASC oligomerization. LPS binding to TLR4 promoted activation of MEK1/2 and phosphorylation of ERK1 leading to rapid inflammasome priming. Inhibition of MEK1/2 with

U1026, NOX2 with DPI or the proteasome with MG132 or bortezomib, suppressed ERK phosphorylation and IL-18 secretion. The DUB inhibitors G5 and WP1130 had no effect on ERK phosphorylation, but blocked IL-18 secretion. Solid lines represent direct interactions and dotted lines represent indirect interactions. Only relationships investigated in the context of NLRP3 inflammasome priming are including.

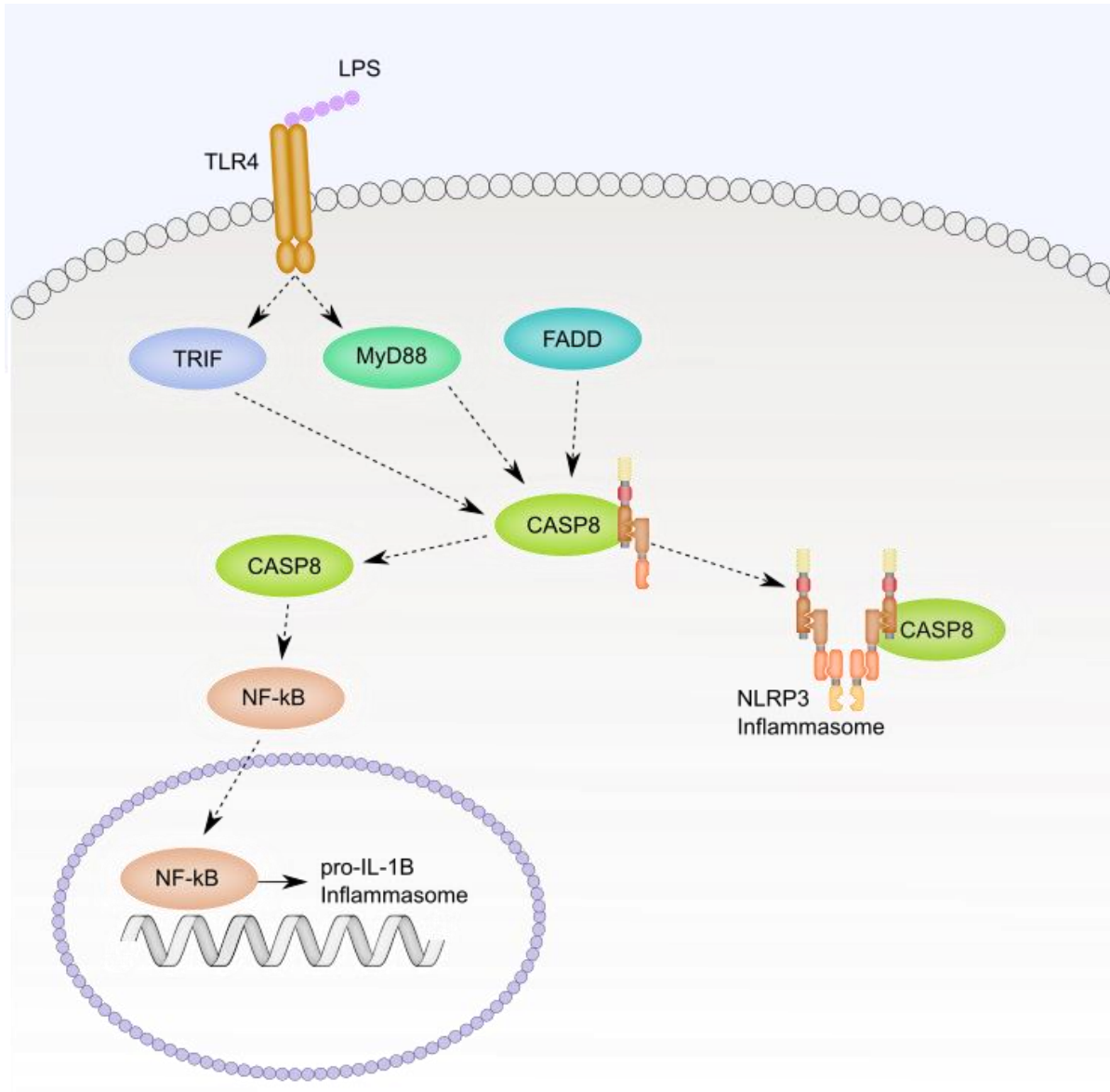
#### **1.6.4 Caspase 8 contributes to transcriptional and post-translational NLRP3 inflammasome priming**

Caspase 8 played a bifunctional roll in priming the NLRP3 inflammasome through both transcriptional and post-translational means. Previous research has noted that deletion of Fas-associated protein with death domain (FADD), the activator of Caspase 8, leads to defects in myeloid cell differentiation [68]. Differentiation can be normalized with additional deletion of RIPK3, allowing for the study of FADD and Caspase 8 in BMDMs. LPS-primed and ATP or nigericin-stimulated BMDMs required TLR4, TRIF, MyD88 and FADD for Caspase 8 maturation. Deletion of Caspase 8 resulted in decreased ATP- and nigericin-induced Caspase 1 activation, IL-1 $\beta$  secretion and cell death, suggesting that Caspase 8 regulates NLRP3 inflammasome activity [69,70]. Deletion of FADD significantly decreased LPS induction of NLRP3 and pro-IL-1 $\beta$  mRNA. Similar results were observed with Caspase 8 deletion as well [69].

Interestingly, Caspase 8 maturation also required NLRP3 and ASC, but not Caspase 1 or Caspase 11, suggesting a possible interaction between Caspase 8 and the core of the NLRP3 inflammasome. Confocal immunofluorescence showed significant co-localization of Caspase 1

and Caspase 8 in LPS and ATP-stimulated macrophages. Pharmacologic inhibition of Caspase 8 with Ac-IETD-fmk was equally as effective in suppressing Caspase 1 activation in response to LPS and ATP when used both as a pretreatment and following LPS priming [69]. Activation of the NLRP3 inflammasome as measured by dsRNA also required Caspase 8 but, contrary to the previous study, could not be blocked by another Caspase inhibitor, the pan Caspase inhibitor OMe-zVAD-fmk [71].





**Figure 1.4. Caspase 8 is critical for transcriptional and post-translational NLRP3 inflammasome priming.** This figure summarizes the discussion in section 1.6.4. Caspase 8 maturation in response to LPS stimulation required TLR4, TRIF, MyD88, FADD, NLRP3 and ASC. Caspase 8 co-localized with Caspase 1 in BMDM stimulated with LPS plus ATP. Loss of Caspase 8 impacted both production of NLRP3 and IL-1 $\beta$  mRNA and Caspase 1 activation, suggesting a dual role in NLRP3 inflammasome priming. Solid lines represent direct interactions

and dotted lines represent indirect interactions. Only relationships investigated in the context of NLRP3 inflammasome priming are including.

### **1.6.5 Multiple ubiquitination signals regulate NLRP3 inflammasome priming and protein degradation**

TLR receptor signaling also primes the NLRP3 inflammasome through ubiquitination and degradation of the NLRP3 inflammasome and its regulators. It was noted that NLRP3 protein levels increased in response to LPS priming in U937 human monocytic cells even in the presence of cycloheximide, a protein translation inhibitor. Blocking protein degradation with the proteasome inhibitor MG132, but not the lysosomal inhibitor leupeptin, prevented steady-state degradation of NLRP3 in untreated cells, leading to increased NLRP3 protein levels. These data suggest that the ubiquitin-proteasome targeting of NLRP3 is regulated by TLR priming to control the half-life of NLRP3.

FBXL2 catalyzed NLRP3 ubiquitination *in vitro*, and interacted with NLRP3 when overexpressed in cells. LPS treatment decreased the interaction between FBXL2 and NLRP3 and also decreased ubiquitination of NLRP3. Additionally, depletion of FBXL2 by siRNA in human macrophages led to increased levels of NLRP3. Mutation of NLRP3 lysine 689 to arginine blocked ubiquitination, suggesting that K689 was the ubiquitin acceptor site [72].

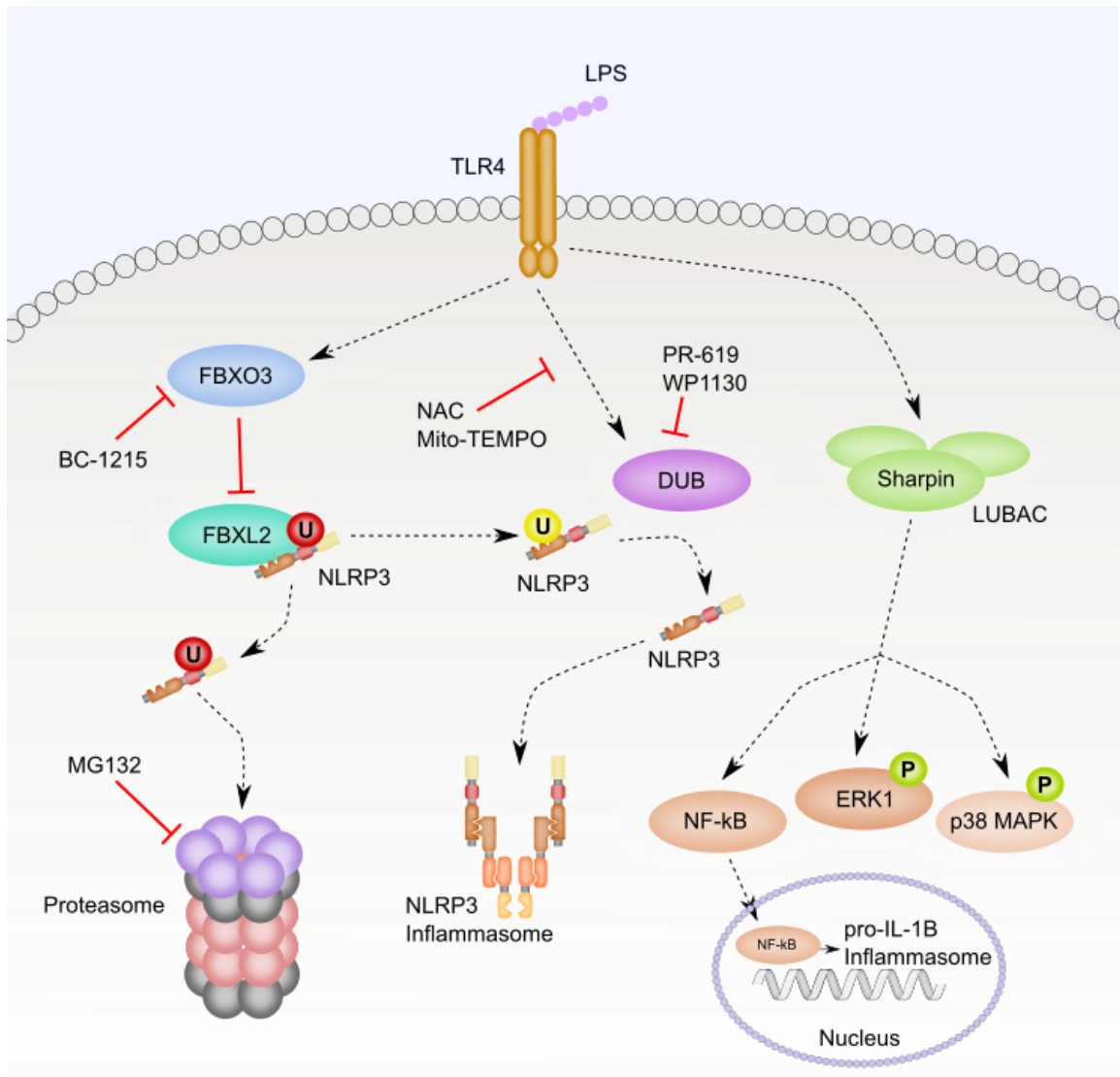
FBXO3 is an E3 ligase subunit that regulates activity of FBXL2 when FBXL2 is phosphorylated GSK $\beta$  [73]. The FBXO3 inhibitor BC-1215 increased FBXL2 levels and decreased NLRP3 levels. Importantly, this FBXO3 inhibitor opposed LPS-induced

accumulations in NLRP3 but had no effect on mutant K689R NLRP3, suggesting that FBXO3 was responsible for degrading FBXL2 leading to increased NLRP3 protein levels [72].

A loss-of-function (LOF) polymorphism was identified in FBXO3 (V221I) that has an allele frequency of 6.2% in European Caucasians. Peripheral blood mononuclear cells (PBMCs) from patients carrying the LOF allele produced less IL-1 $\beta$  when treated with LPS. Mice injected with a lentivirus encoding for wildtype FBXO3, but not FBXO3 V221I, had increased cellular infiltration and lung damage in response to *Pseudomonas aeruginosa* pneumonia, suggesting increased activity from the NLRP3 inflammasome [73]. The discovery of a naturally occurring allele that would result in decreased NLRP3 inflammasome activity is particularly interesting. Mendelian disorders of NLRP3 are the result of activating mutations, but now with a reasonably abundant allele causing decreased NLRP3 activity, it would be interesting to compare patient outcomes in NLRP3-driven processes such as *S. aureus* pneumonia or acute lung injury. However, this would not be a perfect correlation with NLRP3 activity as FBXO3 and FBXL2 also regulate TRAF [73].

Deubiquitination of NLRP3 is also required for LPS-mediated priming. The general DUB enzyme inhibitors PR-619 and WP1130 blocked deubiquitination of NLRP3 in response to LPS. Similarly, treatment with ROS scavenging NAC or mitochondrial ROS specific Mito-TEMPO inhibited LPS-induced deubiquitination of NLRP3. ROS scavenging blocked LPS plus ATP-induced Caspase 1 activation, but not Caspase 1 activation resulting from ATP treatment alone, suggesting a relationship between TLR signaling, ROS production and deubiquitination-mediated priming. This deubiquitination did not result in accumulation of NLRP3, suggesting that ubiquitin is acting to suppress NLRP3 activity in this case and not acting to target NLRP3 for degradation [74].

The linear ubiquitin chain assembly complex (LUBAC) plays a role in priming of the NLRP3 inflammasome. Knockout of Sharpin, one of the three components of the LUBAC - along with HOIP and HOIL-1, in BMDMs leads to defective pro-IL-1 $\beta$  synthesis in response to LPS stimulation. Loss of Sharpin decreases LPS-induced NF- $\kappa$ B activation, phosphorylation of ERK and phosphorylation of p38 MAPK [75]. The exact point of intersection between the LUBAC and LPS-induced priming is unclear, but the broad effects on these downstream activators suggests it is relatively early in the signaling pathway.



**Figure 1.5. Ubiquitination regulates NLRP3 protein levels and activity.** This figure summarizes the discussion in section 1.6.5. LPS priming triggered numerous changes through ubiquitination-deubiquitination. First, LPS activated FBXO3 to prevent FBXL2 mediated ubiquitination and proteasomal degradation of NLRP3. Treatment with the FBXO3 inhibitor BC-1215 opposed LPS-induced activation of FBXO3. Treatment with the proteasome inhibitor MG132 increased NLRP3 protein levels. LPS also activated deubiquitinating enzymes that regulated NLRP3 activity rather than abundance. Treatment with NAC and Mito-TEMPO to

suppress ROS blocked deubiquitination of NLRP3. Additionally, the deubiquitinating enzyme inhibitors PR-619 and WP1130 blocked LPS-mediated priming of NLRP3 by preventing deubiquitination of NLRP3. Lastly, LPS treatment required Sharpin for full NF- $\kappa$ B signaling, ERK phosphorylation and p38 MAPK phosphorylation. “P” and “U” indicate the post-translational modifications phosphorylation and ubiquitination, respectively. The color of the “P” and “U” reflects the effect of the post-translational modification: green is activating, yellow is inhibiting, and red is targeted for degradation. Solid lines represent direct interactions and dotted lines represent indirect interactions.

#### **1.6.6 Post-transcriptional regulation of NLRP3 inflammasome mRNA stability**

While TLR-induced priming upregulated transcription, other mechanisms exist to facilitate tight control of NLRP3 inflammasome mRNA. The myeloid-specific microRNA miR-223 was a critical regulator of NLRP3 inflammasome expression. miR-223 interacted with the 3' untranslated region (UTR) of NLRP3 transcripts to target them for degradation. Overexpression of miR-223 led to decreased NLRP3 protein levels and sequestration of miR-223, by expression of GFP with a 3' UTR containing multiple miR-223 binding sites, led to increased NLRP3 protein levels. The consequence of these changes in NLRP3 protein levels carry through to differences in IL-1 $\beta$  secretion as well. miR223 is not induced during priming, however. It has been suggested to act as a threshold regulator of transcriptional priming – requiring a strong enough activating signal to overcome endogenous miR-223-mediated repression [76,77].

Interestingly, Epstein-Barr virus (EBV) produced a microRNA that binds to the same site as miR-223. EBV miR-BART15 decreased NLRP3 protein levels leading to decreased IL-1 $\beta$

secretion. When EBV-infected B cells and THP1 cells were cultured in the same media divided by a 3- $\mu$ m pore-containing filter, miR-BART15 was secreted from infected B cells via exosomes that were ultimately taken up by the THP1 cells leading to decreased NLRP3 protein levels and inhibition of IL-1 $\beta$  secretion [77].

### **1.6.7 NLRP3 inflammasome signaling without priming**

THP1 cells are able to activate the NLRP3 inflammasome independent of priming. This is thought to be the result of sufficiently high expression of NLRP3, ASC and pro-Caspase 1, allowing THP1 cells to respond to NLRP3-activating stimuli without priming. In this case, NLRP3 inflammasome activation led to secretion of IL-18 and necrotic cell death without IL-1 $\beta$  secretion. THP1 cells are a human leukemic cell line established from a patient with monocytic leukemia [78], so it is unclear how relevant this response is to physiologically normal cells. However, in experiments presented in Chapter 3, I have found that primary human CD14+ monocytes responded to LukAB stimulation with production of IL-18 and necrotic cell death, even in the absence of priming, similar to THP1 cells.

There are likely to be differences in the wide range of phagocytic cell phenotypes, including not just dendritic cells vs monocytes and macrophages, but alveolar macrophages of the lung versus Kupffer cells of the liver and Langerhans dendritic cells versus plasmacytoid dendritic cells, for example. The Immunological Genome Project [79], an effort to catalog gene expression profiles across a wide range of immune cells using microarrays, suggested that tissue-specific phenotypes may influence NLRP3 inflammasome activity. Expression of NLRP3 was highest in Langerhans dendritic cells in the skin but was nearly 10-fold lower in spleen

plasmacytoid dendritic cells. Classical monocytes and bone marrow neutrophils expressed an intermediate level of NLRP3. IL-1 $\beta$  transcripts follow the same pattern [79]. Research into tissue-specific cell type differences may reveal important distinctions in NLRP3 inflammasome priming which may be important in establishing the best models to study tissue-specific infections.

If priming-independent NLRP3 inflammasome activation is physiologic, then it presents the possibility of temporally regulating NLRP3-dependent signaling events. Exposure to most infectious pathogens likely results in both priming and NLRP3 inflammasome activation, but because transcriptional upregulation is a slower process than post-translational proteolytic processing, IL-18 signaling may initiate an immediate response that is only later followed by secretion of IL-1 $\beta$ .

### **1.7 Critical features of NLRP3 inflammasome activation**

As mentioned previously, because of the large number of different activating molecules, it has been difficult for the field to converge on a core set of features required for NLRP3 inflammasome activation. Currently though, there are a few overarching hypotheses outlining the necessary cellular changes that promote NLRP3 inflammasome activation. The most well established and universal requirement in NLRP3 inflammasome activation is a requirement for potassium efflux. Following that, a substantial amount of evidence points to a role for mitochondrial disruption and reactive oxygen species production for most, but not all NLRP3 agonists. Additional key features include lysosomal disruption, endoplasmic reticulum disruption, calcium signaling, ubiquitination, and phosphorylation.



Different activators rely upon these processes to different extents to activate the NLRP3 inflammasome and this might explain why such a wide range of unique molecules activate NLRP3 as compared to other inflammasomes. Additionally, multiple processes are at probably at play at the same time, but the details of connections between them have not yet been uncovered. While the convergence point of all these changes is NLRP3 inflammasome activation, there is no satisfying explanation for how so many different pathways can result in formation of this complex.

Below, I will briefly describe each of the previously mentioned critical signaling pathways to NLRP3 inflammasome activation and the evidence supporting them. I will focus on the most significant studies and highlight the activating molecules used and the endogenous regulators that have been implicated. During this discussion it will become obvious to the reader (and at the same time be entirely disappointing) of how little is known about the requirement for certain regulators across a wide range of activators. Following this discussion, in section 1.8, I will focus on the three most popular canonical activators of NLRP3 inflammasome signaling, ATP, nigericin and MSU, and attempt to outline how each of the features discussed in section 1.7 come together to mediate the response to each of these three activators. Unfortunately, because of space and time limitations, I will not be able to touch on all endogenous regulators or all NLRP3 agonists.

### **1.7.1 Potassium efflux is a universal requirement for NLRP3 inflammasome formation**

Potassium efflux appears to be the only absolute requirement for activation of the NLRP3 inflammasome, subsequent cytokine processing and cell death [80]. In mouse macrophages and

human monocytes, NLRP3-dependent Caspase 1 activation and IL-1 $\beta$  secretion in response to R837, MSU, PGN, nigericin, ATP, and *E. coli* was blocked by addition of KCl to the media [81]. Since this early discovery, inhibition of NLRP3 inflammasome activation by increasing extracellular potassium has been demonstrated with nearly every activator including the particulates alum [82], silica and asbestos [83]; malarial hemozoin [84]; *S. aureus* pore-forming toxins [43,45]; and more. In fact, this author is unaware of and was unable to find any NLRP3 agonists that were not suppressed by extracellular potassium.

Unfortunately, little is known about why potassium efflux is critical in NLRP3 inflammasome activation. The simplest, and perhaps best explanation is that oligomerization of ASC is sensitive to decreases in potassium concentration. When THP1 cells were lysed then incubated at 30°C for 60 minutes, spontaneous ASC oligomerization occurred leading to Caspase 1 activation and pro-IL-1 $\beta$  processing [85]. Furthermore, increasing the concentration of potassium in these lysates blocked spontaneous ASC oligomerization *in vitro* [86].

However, despite all the NLRP3 agonists that are blocked by potassium efflux inhibition, potassium efflux is not a defining feature of the NLRP3 inflammasome. Treatment of murine macrophages with lethal toxin (LT) from *Bacillus anthracis* activated the NLRP1 inflammasome and was responsive to potassium efflux inhibition [81]. Activation of the NLRC4 inflammasome by *Pseudomonas aeruginosa* can be blocked by increased extracellular potassium as well [87]. Alternatively, potassium efflux is not required for *Salmonella typhimurium*-induced activation of IPAF1 inflammasome signaling [81] or dsDNA-induced activation of AIM2 inflammasome signaling [80]. So in addition to potassium efflux, other signals must help differentiate NLRP3 activating stimuli from NLRP1- and NLRC4-activating stimuli. There is likely more to the story

of potassium efflux than its effect on ASC as the AIM2 and IPAF1 inflammasomes contain ASC but are not potassium-responsive.

### **1.7.2 Mitochondrial disruption triggers ROS production**

Mitochondria intersect with NLRP3 inflammasome signaling in numerous different ways. First, the earliest discovered and most direct, is that mitochondrial production of ROS led to NLRP3 inflammasome activation. Both NAC, an ROS scavenger, and DPI, an inhibitor of NOX2-dependent ROS production, inhibited NLRP3-dependent Caspase 1 activation and IL-1 $\beta$  secretion in response to numerous different activators [81,88-91]. Loss of NLRP3 does not impact ROS production, supporting the notion that ROS production is upstream of NLRP3 inflammasome activation [92]. However, not all NLRP3 agonists depend on ROS. Both nigericin and gramicidin acted independently of ROS [80], as did linezolid [93].

Thioredoxin-interacting protein (TXNIP) is an ROS-sensor that regulates the NLRP3 inflammasome. TXNIP natively interacts with thioredoxin (TRX). When ROS are produced in the cell, TXNIP and TRX dissociate and TXNIP interacts with the NLRP3 inflammasome. This interaction is essential for inflammasome formation as loss of TXNIP led to dramatically decreased MSU-induced IL-1 $\beta$  secretion [94].

ROS production has also been tied to calcium signaling leading to NLRP3 inflammasome activation. ROS production triggered opening of the calcium channel transient receptor cation channel subfamily M member 2 (TRPM2). This occurred because ROS activated poly(ADP-ribose) polymerase (PARP) and TRPM2 is a ADP-ribose gated channel [95]. Alum and silica triggered calcium fluxes that were blocked by knockout of TRPM2, treatment with DPI to block

ROS production, or treatment with DPQ, an inhibitor of PARP. All three of these interventions led to a decreased alum-, silica- and MSU-mediated IL-1 $\beta$  secretion [96].

Accumulation of damaged mitochondria, and failure to appropriately clear them, may lead to increased ROS production. BMDCs from RIPK2 knockout mice were defective in autophagy of mitochondria. Influenza infection of RIPK2-deficient BMDCs led to hyperactivation of Caspase 1 and decreased LC3-II levels, a marker of autophagy, as compared to BMDCs from the parental mouse strain. Deficiency in RIPK2 caused accumulation of damaged mitochondria and extremely elevated levels of ROS production. This is because RIPK2 phosphorylated ULK1 to induce autophagy. Loss of ULK1 mimicked the accumulation of damaged mitochondria and Caspase 1 hyperactive seen upon influenza infection in RIPK2 deficient cells. The autophagy defect resulting from RIPK2 deficiency can be rescued by rapamycin treatment to induced autophagy downstream of RIPK2 leading to more subdued Caspase 1 activation levels and increased LC3-II [97]. The  $\gamma$ -aminobutyric acid A receptor-associated protein (Gabarap) also played a role in clearing damaged mitochondria. Gabarap deficiency led to accumulation of damaged mitochondria and ROS production during LPS plus ATP treatment of macrophages. Ultimately, Gabarap deficiency led to increased Caspase 1 activation and IL-1 $\beta$  and IL-18 secretion [98].

Mitochondrial homeostasis requires a delicate balance of mitochondrial fission and fusion, mechanisms by which new mitochondria are created and old mitochondria are recycled, respectively. As would be expected based off the studies implicating autophagy discussed above, both mitochondrial fission and fusion are important in NLRP3 inflammasome activation.

The protonophore carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) can chemically induce mitochondrial fission and inhibited ATP and nigericin-induced NLRP3 inflammasome formation. Knockdown of dynamin-related protein 1 (DRP1) caused elongation of mitochondrial as a result of defective fission. In turn, DRP1 deficiency caused increased Caspase 1 activation and IL-1 $\beta$  secretion in BMDMs treated with LPS plus ATP or just ATP alone. Loss of DRP1 led to enhanced phosphorylation of ERK, and inhibition of MEK with U1026 blocked IL-1 $\beta$  secretion in DRP1-deficient cells [99]. Although the authors do not propose a mechanism by which ERK might be regulating NLRP3 inflammasome activity, it appears from their data that it might be through priming, as pro-IL-1 $\beta$  production seems to be decreased in U1026 treated cells. This would be consistent with a previously discussed report that found a roll for ERK1 in NLRP3 inflammasome priming [67]. If that is the case, it appears DRP1-mediated fission is important for both priming and activation of the inflammasome.

On the other end, mitofusin 2, a mediator of mitochondrial fusion, interacted with NLRP3 upon loss of mitochondrial membrane polarization after infection with influenza virus. Knockdown of mitofusin 2 reduced IL-1 $\beta$  secretion following infection without significantly affecting pro-IL-1 $\beta$  production [100].

The mitochondria might serve as a scaffold for inflammasome assembly and regulation in order to tightly couple mitochondrial homeostasis and NLRP3 inflammasome activation. The mitochondria are an important site of negative regulators of NLRP3 too. During NLRP3 inflammasome activation, both small heterodimer partner (SHP) and NLRP3 are recruited to mitochondria where SHP interacted with NLRP3 to block association of NLRP3 and ASC. As such, SHP blocked Caspase 1 activation and IL-1 $\beta$  secretion in cells stimulated with ATP,

nigericin or dsRNA. Loss of SHP further disrupted mitochondrial integrity during ATP stimulation and ultimately led to mtDNA translocating to the cytosol [101].

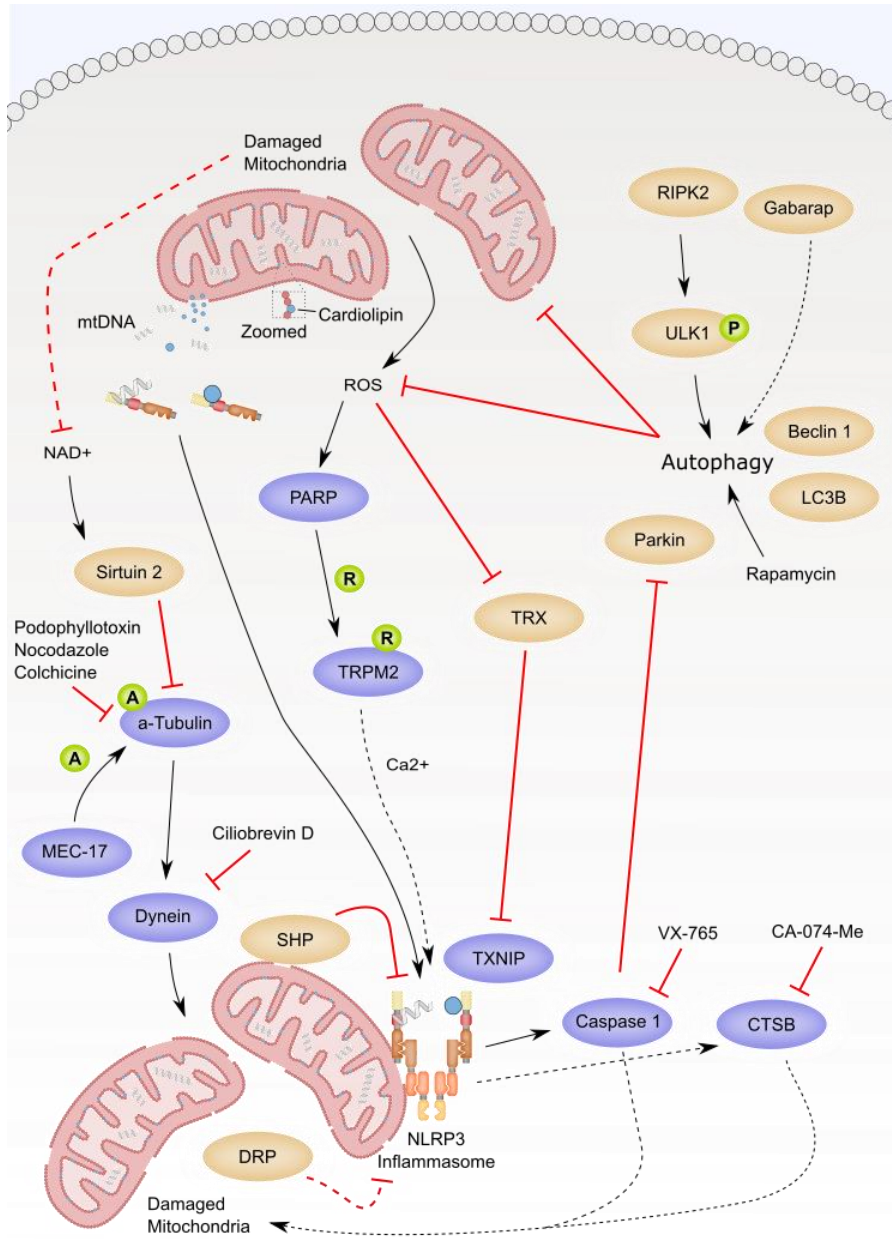
Perturbation of mitochondrial homeostasis by NLRP3 agonists led to decreased intracellular  $\text{NAD}^+$ . Addition of  $\text{NAD}^+$  to cells blocked nigericin-, ATP-, silica- and MSU-induced NLRP3 inflammasome activation. Decreased  $\text{NAD}^+$  concentrations led to inactivation of the  $\text{NAD}^+$ -dependent  $\alpha$ -tubulin deacetylase sirtuin 2 and accumulation of acetylated  $\alpha$ -tubulin. shRNA-mediated depletion of MEC-17, an acetyltransferase for  $\alpha$ -tubulin, decreased IL-1 $\beta$  secretion, suggesting acetylated  $\alpha$ -tubulin accumulation was critical for NLRP3 inflammasome activation. Acetylated  $\alpha$ -tubulin caused dynein-dependent rearrangement of mitochondria and endoplasmic reticulum so that they approximated each other. Disruption of dynein activity with ciliobrevin-D or microtubule dynamics with colchicine, nocodazole or podophyllotoxin all decreased IL-1 $\beta$  secretion from a broad range of agonists. These features were all unique to NLRP3 agonists, as flagellin and dsDNA-induced IL-1 $\beta$  secretion was unaffected [102]. This study, because of its use of a wide range of different agonists and its comprehensive analysis, in particular, is one of the best pieces of evidence demonstrating the scaffolding role of mitochondria in spatially regulating NLRP3 inflammasome activation.

This juxtaposition of NLRP3 and mitochondria might be arranged because the mitochondria is the source of two different ligands that triggered NLRP3 inflammasome activation. Mitochondrial cardiolipin, a specialized lipid found in the mitochondrial inner membrane, bound directly to NLRP3. Cardiolipin-containing liposomes were sufficient to activate NLRP3 inflammasome formation. Knockdown of cardiolipin synthesis with siRNA targeting cardiolipin synthase decreased linezolid-, silica- and ATP-induced IL-1 $\beta$  secretion. The authors propose that cardiolipin is at the intersection of ROS-dependent and ROS-independent

signaling pathways, because silica and ATP required ROS and linezolid did not, that converge to damage mitochondria and activate the NLRP3 inflammasome [93].

The other NLRP3 ligand identified is oxidized mitochondrial DNA (mtDNA). Mitochondrial ROS production led to oxidation of mtDNA. ATP-induced mitochondrial dysfunction caused release of oxidized mtDNA into the cytosol. Oxidized mtDNA co-localized with the activated NLRP3 inflammasome when expressed and activated in 293 cells. Transfection of oxidized mtDNA directly is sufficient to induce IL-1 $\beta$  secretion from LPS-primed BMDMs [103]. Oxidized mtDNA is thought to translocate from damaged mitochondria into the cytosol. Depletion of autophagic proteins LC3B and Beclin 1 led to accumulation of damaged mitochondria and increased cytosolic mtDNA in response to ATP treatment. Disruption of autophagy increased Caspase 1 activation and IL-1 $\beta$  secretion. Interestingly, culturing cells in low levels of ethidium bromide blocks replication of mitochondrial DNA, allowing one to culture cells free of mitochondrial DNA after several passages. Loss of mitochondrial DNA severely limited LPS plus ATP-induced Caspase 1 activation [104].

Damage to the mitochondria is also a consequence of NLRP3 inflammasome activation. Expression of NLRP3 Y570C, a CAPS-associated mutation, in THP1 cells caused a decrease in mitochondrial membrane polarization. Pretreatment of cells with the Cathepsin B (CTSB) inhibitor CA-074-Me completely blocked the decrease [105]. The Caspase 1 inhibitor VX-765 and deletion of Caspase 1 and Caspase 11 can block ATP and CAPS mutation-induced damage to mitochondria and ROS production, suggesting these features may also be important downstream of inflammasome activation. Interestingly, Parkin, a critical protein in promoting mitophagy, was cleaved by Caspase 1 leading to inhibition of mitophagy [106]. These data suggest a possible positive feedback cycle to amplify NLRP3 inflammasome signaling.



**Figure 1.6. Accumulation of mitochondrial damage activates the NLRP3 inflammasome.**

This figure summarizes the discussion in section 1.7.2. All pathways were combined for the purpose of comparison and brevity. Concurrence of different pathways has not been established.

Tan circles indicate proteins that oppose NLRP3 inflammasome activation. Purple circles indicate proteins that promote NLRP3 inflammasome activation. Solid lines represent direct interactions and dotted lines represent indirect interactions. My hypothesis is that accumulated



damage to mitochondria is central in these NLRP3 inflammasome activating pathways. Low NAD<sup>+</sup> concentration decreased activity of Sirtuin 2, an  $\alpha$ -Tubulin deacetylase. Accumulation of acetylated  $\alpha$ -Tubulin resulted in dynein-dependent microtubules rearrangement of mitochondria, binding of NLRP3 and activation of the NLRP3 inflammasome. Loss of MEC-17, an  $\alpha$ -Tubulin acetyltransferase, prevented microtubule and dynein-dependent rearrangement. Oxidized mtDNA and cardiolipin both bound directly to NLRP3 and promoted NLRP3 inflammasome activation. ROS production activated PARP and led to production of ADP-ribose. ADP-ribose opened the Ca<sup>2+</sup> channel TRPM2 which was required for NLRP3 inflammasome activation. ROS production blocked the association of TRX and TXNIP and allowed TXNIP to interact with NLRP3 promoting NLRP3 inflammasome formation. Functional autophagy opposed NLRP3 inflammasome activation. RIPK2 phosphorylated ULK1, a requirement for autophagy. Rapamycin treatment bypassed loss of RIPK2 to promote autophagy. Loss of Gabarap prevented autophagy through an unknown mechanism. Loss of DRP resulted in defective mitochondrial fission and NLRP3 inflammasome activation. SHP associated with the mitochondria and inhibited NLRP3 inflammasome formation. Activation of the NLRP3 inflammasome resulted in Caspase 1- and CTSB-dependent mitochondrial ROS production and damage.

### **1.7.3 Lysosome dysfunction liberates the cysteine protease Cathepsin B**

Early studies of NLRP3 inflammasome activation reported a critical requirement for CTSB activation. Expression of NLRP3 Y570C, a CAPS-associated mutation, in THP1 cells caused lysosomal leakage as measured by staining with the lysosome-accumulating dye acridine orange. Inhibition of CTSB with CA-074-Me eliminated lysosomal leakage in the NLRP3 mutant [105], a very unusual discovery given that CTSB is a protease that is activated inside

lysosomes normally. Inhibition of CTSB also blocked NLRP3 induced cell death [105], suggesting that CTSB leakage and activation might be driving necrosis. The requirement for CTSB in CAPS-associated mutant-mediated cell death has been confirmed in a subsequent study [107]. CTSB is also required for pathogen and particulate matter-associated NLRP3-induced cell death [107-109].

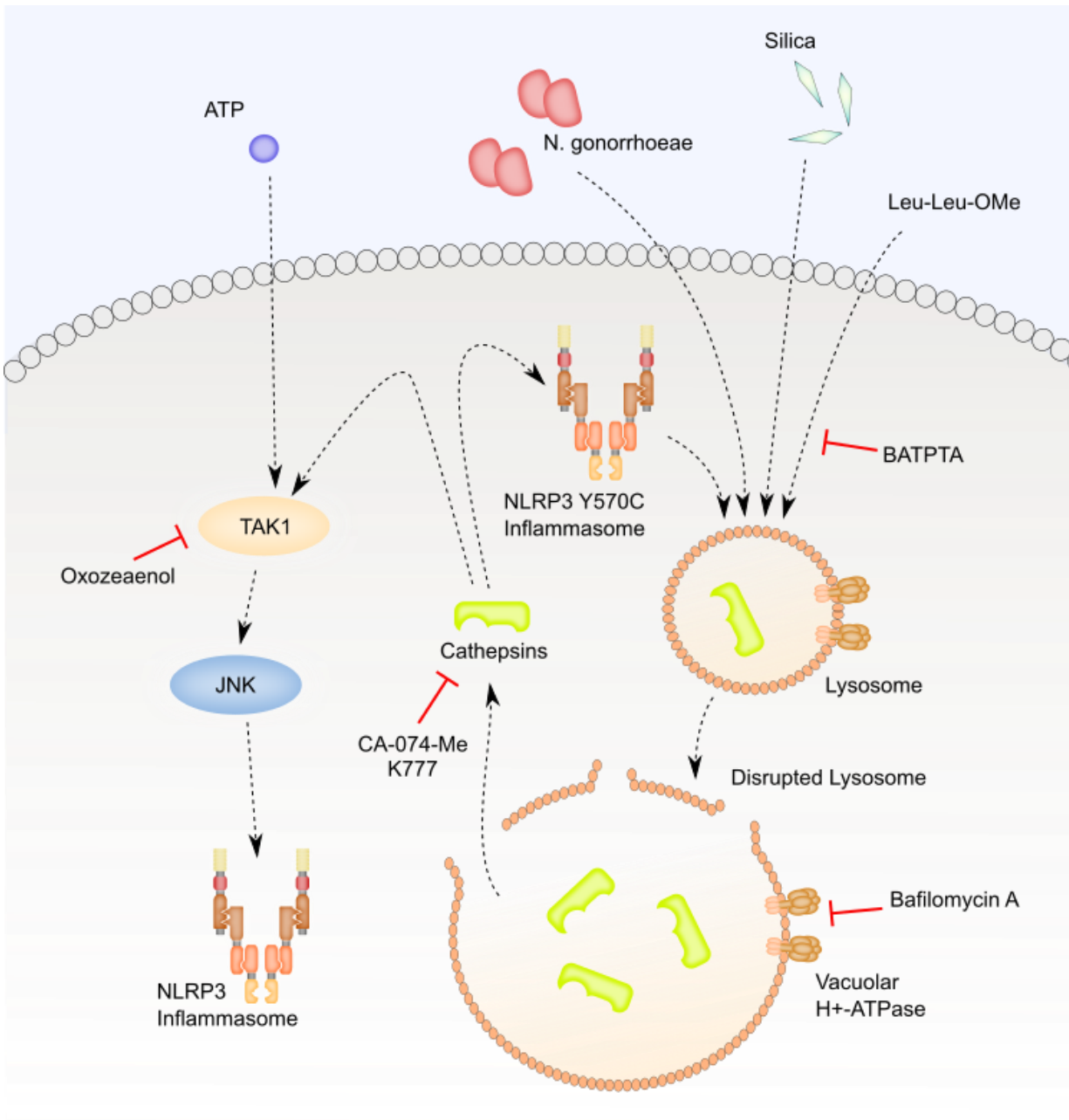
Bafilomycin A is an inhibitor of the vacuolar H<sup>+</sup>-ATPase in lysosomes that prevents acidification of lysosomes. Bafilomycin treatment of macrophages blocked both acidification of lysosomes, as measured with the lysomotropic pH sensitive dye lysosensor green, and suppressed lysosomal proteolytic function. This inhibition led to a decrease in silica-mediated IL-1 $\beta$  release but had no effect on ATP treatment. In this setting, activation of CTSB preceded NLRP3 activation, as mature CTSB was still detected in NLRP3 knockout cells [108]. During *Neisseria gonorrhoeae* infection of macrophages, the same effect was seen: inhibition of CTSB, but not Cathepsin L, blocked NLRP3-dependent IL-1 $\beta$  secretion and LDH release, a marker for cell death [109]. The requirement for CTSB in CAPS-associated mutants and in the activation of wildtype NLRP3 suggests that CTSB plays an amplifying role by acting on both ends of the NLRP3 inflammasome pathway.

The commonly used CTSB inhibitor CA-074-Me inhibits cathepsins family proteins more broadly at concentrations commonly used in NLRP3 inflammasome studies [110]. This broad inhibition of cathepsins turns out to be the real requirement for blocking NLRP3 inflammasome activation. Genetic disruption of individual cathepsins B, C, L, S, or X had no effect on silica-induced IL-1 $\beta$  secretion. Only by deleting all five cathepsins could an effect be seen [110]. Interestingly, a newly described broad cathepsin inhibitor, K777 [111], potently

blocked IL-1 $\beta$  secretion even in the absence of these five cathepsins [110], suggesting that there are still more cathepsins at play or that the inhibitor had additional off-target effects.

Once cathepsins are activated and released into the cytosol, their mechanism of action leading to NLRP3 activation is not well understood. One suggestion is that lysosome rupture leads to a cascade of kinase signaling that activates the NLRP3 inflammasome. Treatment of THP1 cells with Leu-Leu-OMe (LLME), a direct lysosome disrupting peptide, activated TAK1 (MAP3K7) leading to phosphorylation of JNK. Inhibiting cathepsins with CA-074-Me or bafilomycin A blocked phosphorylation of JNK. The TAK1 inhibitor, 5-Z-oxozeaenol, or siRNA-mediated knockdown of TAK1 blocked LLME-induced JNK phosphorylation. These inhibitory effects decreased activation of Caspase 1 in response to LLMe and MSU [112]. Interestingly, 5-Z-oxozeaenol prevented ATP-induced Caspase 1 activation, even though ATP has not previously been shown to activate cathepsins [112,113]. This finding suggests that TAK1 may be a common transducer of signaling to activate the NLRP3 inflammasome by both lysosome-dependent and -independent mechanisms. TAK1 and JNK phosphorylation in response to LLME was also blocked by chelation of intracellular calcium using BAPTA-AM [112], but the exact role of calcium was unclear.

Although the ties between cathepsin leak and NLRP3 inflammasome signaling are still not well established, activation of such a broad range of cathepsins is likely to have a profound effect on cells. Given the fact that Ca-074-Me blocked downstream TAK1 phosphorylation in response to LLMe, it would be interesting to examine the effects of cathepsins on protein phosphatases, and whether inhibition of protein phosphatases is sufficient to bypass inhibition of cathepsin activation.



**Figure 1.7 Lysosomal disruption contributes to NLRP3 inflammasome signaling.** This figure summarizes the discussion from section 1.7.3. Numerous NLRP3 inflammasome activating agents disrupted lysosomes and caused release of cathepsins into the cytosol. Inhibition of Vacuolar H<sup>+</sup>-ATPase with Bafilomycin A or of cathepsins with CA-074-Me and K777 prevented NLRP3 inflammasome activation. In a study investigating LLMe, Cathepsin

release triggered TAK1-JNK signaling leading to NLRP3 inflammasome activation. Solid lines represent direct interactions and dotted lines represent indirect interactions.

#### **1.7.4 Endoplasmic reticulum dysfunction disrupts protein folding**

The endoplasmic reticulum (ER) has also been proposed as central to NLRP3 inflammasome activation. Induction of ER stress directly using brefeldin A (BFA), which reversibly blocks protein transport from the ER to the Golgi, thapsigargin, which blocks ER SERCA pumps and depletes ER calcium stores, or tunicamycin, which blocks GlcNAc-1-phosphate transferase and prevents glycoprotein synthesis, led to IL-1 $\beta$  secretion in primary human macrophages. NLRP3 deficient cells or cells incubated in high potassium media did not secrete IL-1 $\beta$  in response to these ER stress triggers. Disruption of the unfolded protein response (UPR) by depleting IRE1 had no effect on BFA-induced IL-1 $\beta$  secretion, depletion of PERK had no effect on tunicamycin-induced IL-1 $\beta$  secretion and deletion of ATF6a had no effect on thapsigargin- or tunicamycin-induced IL-1 $\beta$  secretion [114]. These data suggest that ER stress-mediated NLRP3 inflammasome activation in this setting is independent of the well-studied UPR pathway. However, the incomplete analysis of treatments and effectors made it likely something could be missed, as we will see below.

Alternate mechanisms of NLRP3 activation might, however, triggered the UPR to activate the NLRP3 inflammasome. Silver nanoparticles of 15 nanometers (AgNP15) induced ER stress in THP1 monocytes and ultimately NLRP3 inflammasome-dependent Caspase 1 activation, IL-1 $\beta$  secretion and cell death. During AgNP15 treatment, ATF6, an ER stress sensor,

was rapidly degraded. Blocking ATF6 degradation with the Site-2 protease inhibitor 1,10-phenanthroline completely blocked AgNP15-induced NLRP3 inflammasome activation [115].

A few studies have found links between ER stress and mitochondrial disruption. ER stress-induced by thapsigargin caused IRE1 activation leading to upregulation of TXNIP by depleting the TXNIP mRNA regulator miR-17. As seen before with mitochondria, TXNIP is involved in activation of the NLRP3 inflammasome, and as such, thapsigargin-induced TXNIP upregulation led to NLRP3-dependent IL-1 $\beta$  secretion in THP1 cells [116,117]. Inhibition of IRE1 with STF-083010 was sufficient to block thapsigargin-induced IL-1 $\beta$  secretion, but not ATP-induced IL-1 $\beta$  secretion [117].

In BMDMs infected with *Brucella abortus* strain RB51, NLRP3 was activated leading to Caspase 1-dependent IL-1 $\beta$  secretion. Treatment of cells with the chemical chaperone tauroursodeoxycholic acid (TUDCA) to facilitate protein folding and alleviate ER stress or 4 $\mu$ 8c, an inhibitor of IRE1, blocked RB51-induced IL-1 $\beta$  secretion but had no effect on LPS plus ATP-induced IL-1 $\beta$  secretion. IRE1 upregulated TXNIP expression, as seen in previous studies. NLRP3 caused mitochondrial stress independent of ASC by activating Caspase 2-dependent cleavage and activation of Bid. Knockdown of Bid in BMDM prevented RB51-induced mitochondrial disruption, Caspase 1 activation and IL-1 $\beta$  secretion [118].

However, not all inflammasome activators trigger ER stress. Notably, MSU and R837, a NLRP3 inflammasome-activating anti-viral compound, did not induce CHOP activation, a marker for ER stress [114]. Therefore, ER stress-induced NLRP3 activation may be an uncommon requirement for only a unique set of activating molecules.

The ER has also been proposed to be a critical source for calcium signaling leading to NLRP3 inflammasome activation. That will be discussed in the next section.

### **1.7.5 Calcium acts a second messenger**

Another less prominent but still common regulatory schema controlling NLRP3 inflammasome activity is the mobilization of calcium ions ( $\text{Ca}^{2+}$ ). Evidence suggesting a role for  $\text{Ca}^{2+}$  began to emerge around the same time the NLRP3 inflammasome was discovered.

IL-1 $\beta$  does not have a signal sequence and is not secreted through the classical ER-Golgi route. Drugs that blocked intracellular transport of IL-6, TNF- $\alpha$  and other secreted proteins had no effect on IL-1 $\beta$  secretion [119]. BAPTA-AM, an intracellular calcium chelator, inhibited ATP-induced IL-1 $\beta$  processing and secretion. To test the opposite condition, 100 $\mu\text{M}$  ATP (sub-P2RX7 activating) and 1 $\mu\text{M}$  thapsigargin were used to mobilize calcium. Cells treated with thapsigargin or thapsigargin and ATP secreted significantly less IL-1 $\beta$  in response to both ATP and nigericin. Thapsigargin is an inhibitor of the sarco/endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase (SERCA), the channel that opposes  $\text{Ca}^{2+}$  leak from the ER. By blocking SERCA, thapsigargin increased intracellular  $\text{Ca}^{2+}$  concentration. Increased intracellular  $\text{Ca}^{2+}$  concentration was not sufficient to induced IL-1 $\beta$  secretion and premature depletion of  $\text{Ca}^{2+}$  from the ER blocked IL-1 $\beta$  secretion. Coordinated  $\text{Ca}^{2+}$  ER flux and cytoplasmic potassium depletion were together required for effective IL-1 $\beta$  secretion [120].

$\text{Ca}^{2+}$ -dependent NLRP3 inflammasome activation extends beyond classical NLRP3 agonists. Cholesterol-dependent cytolysins, exotoxins secreted by many Gram-positive bacteria that bind cholesterol to form pores in eukaryotic cell membranes, activated the NLRP3

inflammasome in a  $\text{Ca}^{2+}$ -dependent manner as BAPTA-AM treatment suppressed IL-1 $\beta$  secretion. This study also confirmed that BAPTA-AM treatment can block ATP- and nigericin-induced IL-1 $\beta$  secretion [121].

Rhinovirus ion channel protein 2B (P2B) triggered the NLRP3 inflammasome in a  $\text{Ca}^{2+}$ -dependent manner. Expression of P2B in bronchial cells caused an increase in cytosolic  $\text{Ca}^{2+}$  and thapsigargin pretreatment reduced the P2B-dependent increase in  $\text{Ca}^{2+}$  suggesting that they both pulled from the same source – the ER. Further supporting that notion, P2B and NLRP3 co-localized at the Golgi and disruption of the Golgi with the fungal metabolite brefeldin A (BFA) caused a redistribution of P2B and blocked NLRP3 inflammasome activation by rhinovirus. However, BFA did not have the same effect on ATP-induced IL-1 $\beta$  secretion, suggesting that this mechanism may be unique to Golgi targeted proteins. Lastly, as was done in previous studies, BAPTA-AM treatment of rhinovirus infected cells caused a significant decrease in IL-1 $\beta$  secretion, further implicating  $\text{Ca}^{2+}$  in this process [122].

Similarly, encephalomyocarditis virus (EMCV) viroporin 2B activates the NLRP3 inflammasome and localized to the Golgi where it caused an increase in intracellular  $\text{Ca}^{2+}$  concentration. EMCV-induced IL-1 $\beta$  secretion was blocked by BAPTA-AM treatment, but not suppression of mitochondrial ROS or inhibition of Cathepsin B with Ca-074 Me [123]. This may be a common theme in picornaviruses as multiple others, including coxsackievirus, polio virus, hepatitis A virus, enterovirus, and foot-and-mouth disease virus, all produce similar 2B proteins that localize to the Golgi or ER or effect Golgi and ER integrity [124].

One study on keratinocytes suggests that  $\text{Ca}^{2+}$  plays a role in activation of the NLRP3 inflammasome here too. Keratinocytes secreted IL-1 $\beta$  in a NLRP1- and NLRP3-dependent



manner in response to UVB irradiation. UVB irradiation caused an increase in intracellular  $\text{Ca}^{2+}$  concentration that when blocked with BAPTA-AM suppressed IL-1 $\beta$  secretion. An extracellular  $\text{Ca}^{2+}$  chelator was not effective in blocking IL-1 $\beta$  secretion, adding to evidence suggesting an intracellular source of  $\text{Ca}^{2+}$  flux [125].

The emerging mechanism describing how increases in intracellular  $\text{Ca}^{2+}$  activate the NLRP3 inflammasome is complex and may differ depending on the type of stimulus used. A few studies have begun to detail this complexity.

Multiple calcium release inhibitors including the two SERCA inhibitors thapsigargin and XeC, the Phospholipase C (PLC) inhibitor U73122, and the Inositol 1,4,5-trisphosphate receptor (IP3R) inhibitor 2-aminoethoxydiphenyl borate (2-APB) blocked ATP-, nigericin-, MSU- and LLMe-induced calcium mobilization and IL-1 $\beta$  secretion [126,127]. Knockdown of IP3R had the same effect [127].

Loss of the murine calcium sensing receptor (CASR) also suppressed ATP-induced Caspase 1 activation and IL-1 $\beta$  secretion and demonstrated the potential of extracellular  $\text{Ca}^{2+}$  to activate the NLRP3 inflammasome [127]. CASR interacted with both PLC and adenylate cyclase (ADCY), the protein responsible for generation of cAMP, and both pathways remained intact and responsive to direct activation in a CASR-deficient background. The PLC agonist m-3M3FBS, the ADCY inhibitor KH7, and knockdown of ADCY3, ADCY6, ADCY7 or ADCY9 led to spontaneous IL-1 $\beta$  secretion, suggesting that both arms of this calcium-sensitive pathway are critical for controlling NLRP3 inflammasome activity [127].

Increasing cAMP concentrations by activating ADCY with NKH477 or forskolin or decreasing cAMP hydrolysis by inhibiting phosphodiesterase (PDE) 4 with Ro-20-1724, (r)-(-)-

rolipram, or zardaverine suppressed IL-1 $\beta$  secretion and ASC oligomerization induced by ATP. cAMP bound directly to NLRP3 and binding was decreased with CAPS-associated NLRP3 mutations suggesting that the spontaneous activation of these mutations may be due to decreased cAMP binding. Furthermore, activating ADCY or inhibiting PDE4 to increase cAMP concentrations suppressed IL-1 $\beta$  secretion from PBMCs isolated from CAPS patients with NLRP3 mutations. Treatment with 2-APB or BAPTA also decreased IL-1 $\beta$  secretion from these cells [127].

A separate study confirmed that increased concentrations of extracellular Ca<sup>2+</sup> can activate the NLRP3 inflammasome, but came up with a different mechanism of action. Inhibition of CASR with Calhex231 and GPRC6A with NPS2143 separately blocked IL-1 $\beta$  secretion in response to extracellular Ca<sup>2+</sup> but had no effect on ATP-induced NLRP3 inflammasome activation [128]. This is in contrast to the previously discussed study on CASR that showed a role for CASR in ATP-induced NLRP3 inflammasome activation. That study made use of a CASR knockout though [127], raising the possibility that Calhex231 is not acting specifically on CASR in the presently discussed study. The requirement for CASR in extracellular Ca<sup>2+</sup>-mediated inflammasome activation in the current study is further supported by siRNA-mediated depletion of CASR that caused disruption of IL-1 $\beta$  secretion, not just the inhibitor [128].

Unlike in the previous study of ATP-induced Ca<sup>2+</sup> mobilization, cAMP had no role in extracellular Ca<sup>2+</sup> mediated-IL-1 $\beta$  secretion as treatment with forskolin, the ADCY activator, and SQ22536, an ADCY inhibitor, had no effect. Additionally, apyrase treatment to consume extracellular ATP had no effect on IL-1 $\beta$  secretion. Exposure of monocytes to necrotic cells (induced by 7-BIO treatment) caused IL-1 $\beta$  secretion that was suppressed by Calhex231 and GPRC6A knockout, suggesting that necrotic cells can act as a physiologic source of increased

extracellular calcium. *In vivo*, injection of aluminum ions, another agonist of GPRC6A, caused footpad swelling and IL-1 $\beta$  secretion that was not seen in GPRC6A deficient or Caspase 1 deficient mice [128].

The discrepancy in whether cAMP production through ADCY activation with forskolin regulates the NLRP3 inflammasome in the two studies of CASR might be explained by the dose of forskolin used. In the study that found an effect, the dose of forskolin to achieve suppression of IL-1 $\beta$  secretion was between 50 and 500 $\mu$ M [127] whereas the study that purported no effect only used concentrations as high as 20 $\mu$ M [128]. Strangely though, direct measurement of cAMP in both studies suggests that the dose used caused an increase in cAMP concentration of comparable magnitude. It is also possible that there is a difference in sensitivity of murine BMDMs and human monocytes to cAMP-mediated NLRP3 inflammasome regulation.

C/EPB homologous protein (CHOP) regulates ER Ca<sup>2+</sup> release through IP3R and was required for NLRP3 inflammasome activation in response to ATP stimulation. XeC did not further reduce IL-1 $\beta$  secretion in a CHOP deficient background suggesting that CHOP and IP3R are part of the same pathway. Calcium flux also led to production of mitochondrial ROS that was blocked by each of the aforementioned inhibitors used in this study. Lastly, potassium efflux was required for ATP-mediated Ca<sup>2+</sup> flux [126].

Sublytic concentrations of the complement-mediated membrane attack complex (MAC) can activate the NLRP3 inflammasome. Pretreatment with BAPTA-AM, XeC or 2-APB suppressed MAC-induced IL-1 $\beta$  secretion, as seen before with other stimuli. Additionally, the FDA-approved Ryanodine receptor inhibitor dantrolene also reduced MAC-triggered increases in cytosolic Ca<sup>2+</sup> and IL-1 $\beta$  secretion. Released Ca<sup>2+</sup> was found to accumulate in the

mitochondria using the Rhod2-AM probe and knockdown of the mitochondrial calcium uniporter (MCU) reduced both mitochondrial calcium uptake and IL-1 $\beta$  secretion, possibly providing a link between Ca<sup>2+</sup> mobilization and mitochondrial ROS production [129].

Another recent study has linked Ca<sup>2+</sup> mobilization and mitochondrial dysfunction through the MCU. *Pseudomonas aeruginosa* flagellin activated the NLRP3 inflammasome in IB3-1 cells, bronchial epithelial cells derived from a patient with cystic fibrosis. Treatment with purified flagellin induced MCU transport of Ca<sup>2+</sup> into the mitochondria leading to ROS production. Depletion of MCU in IB3-1 and CFTR $\Delta$ 508-expressing human primary cells blocked Ca<sup>2+</sup> accumulation in the mitochondria and decreased IL-1 $\beta$  secretion. Mutation of CFTR in cystic fibrosis has been shown to enhance cytosolic and mitochondria Ca<sup>2+</sup> mobilization, setting these cells up to be particularly sensitive to this mode of NLRP3 activation [130]. This study, in addition to the previously discussed study implicating MCU, raise the possibility that MCU may be a common tie between Ca<sup>2+</sup> and mitochondria dysfunction in NLRP3 inflammasome signaling.

Reliance on phagocytosis-mediated NLRP3 activation can turn the relationship between Ca<sup>2+</sup> mobilization and mitochondrial ROS production around. In this study, charged liposomes were used to activate the NLRP3 inflammasome and induce IL-1 $\beta$  secretion. Liposome-mediated NLRP3 activation required phagocytosis and could be blocked by cytochalasin D, much like alum-induced activation, but not like ATP-induced activation. These liposomes caused an increase in intracellular Ca<sup>2+</sup> that was not seen when Ca<sup>2+</sup> was removed from the media. It was discovered that liposome-, silica- and alum-mediated Ca<sup>2+</sup> flux required TRPM2 as Ca<sup>2+</sup> flux was blocked by TRPM2 knockout. TRPM2 is an ADP-ribose and Ca<sup>2+</sup> gated channel and as such was blocked by inhibition of PARP with DPQ to prevent ADP-ribose accumulation.

Additionally, treatment with the antioxidant DPI to suppress ROS generation also blocked  $\text{Ca}^{2+}$  flux. Loss of TRPM2 suppressed Caspase 1 activation and IL-1  $\beta$  secretion in response to liposomes, alum, silica and MSU and in MSU-induced peritonitis, but did not affect ROS generation, suggesting that ROS is upstream of TRPM2 [96].

Activation of the NLRP3 inflammasome by lysosome rupture triggered by LLME required  $\text{Ca}^{2+}$ -dependent TAK1-JNK signaling. While lysosome rupture was discussed in section 1.7.3, and kinase signaling will be discussed in more detail in section 1.7.7, the requirement for  $\text{Ca}^{2+}$  signaling will be discussed here. The intracellular calcium chelator BAPTA-AM, but not extracellular calcium chelators, suppressed TAK1-JNK signaling suggesting that lysosome rupture releases calcium ions that activate TAK1-JNK. The authors claim that the  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) inhibitor KN-93 suppressed LLME-induced TAK1-JNK signaling, however the effect is rather hard to appreciate. Additionally, KN-93 and its inactive derivative KN-92 both suppressed Caspase 1 activation and IL-1 $\beta$  secretion in response to LLME, although KN-92 to a lesser extent than KN-93 [112]. This observation is not consistent with CaMKII regulating NLRP3 inflammasome activation, a fact that is overlooked by the authors. KN-93 and KN-92 also exert CaMKII-independent effects through inhibition of L-type and N-type calcium channels at doses substantially lower than those used to show an effect on the NLRP3 inflammasome. The structurally unrelated CaMKII inhibitor AIP does not exert these effects and would be an easy means to test for this off-target effect [131]. Another kinase, death associated protein kinase (DAPK), contains a calcium/calmodulin-regulatory (CaM) domain and has been implicated in regulating the NLRP3 inflammasome. However, in the one study investigating DAPK, calcium studies were not conducted [132].

Ca<sup>2+</sup> release from the ER causes opening of store-operated Ca<sup>2+</sup> release-activated Ca<sup>2+</sup> (CRAC) channels in the plasma membrane via the interaction of stromal interaction molecule (STIM) 1 and STIM2. This allows ORAI1, the pore-forming subunit of the CRAC channel, to open and results in store-operated Ca<sup>2+</sup> entry (SOCE). To study the role of SOCE in innate immunity, STIM1 and STIM2 conditional knockout mice were generated. BMDMs from STIM1-deficient and STIM2-deficient mice secreted equivalent levels of IL-1 $\beta$  in response to ATP and MSU as wildtype BMDMs despite slight decreases in Ca<sup>2+</sup> mobilization. Although SOCE was not required, Ca<sup>2+</sup> inhibitors used previously including BAPTA and 2-APB were confirmed to block ATP-induced IL-1 $\beta$  secretion [133].

Despite this evidence for Ca<sup>2+</sup> signaling playing an important role in regulating the NLRP3 inflammasome, multiple studies contradict these findings.

The calcium ionophore ionomycin increased cytoplasmic calcium concentrations by triggering influx of extracellular calcium and release of ER calcium stores but did not trigger IL-1 $\beta$  secretion in BMDCs [134] or BMDMs [126], suggesting that calcium flux is not sufficient for NLRP3 inflammasome activation.

Nigericin-induced increases in cytosolic Ca<sup>2+</sup> concentration, but these Ca<sup>2+</sup> currents actually appeared to occur downstream of the NLRP3 inflammasome as loss of NLRP3 and Caspase 1 in BMDCs suppressed Ca<sup>2+</sup> mobilization. Incubation of BMDCs in calcium-deficient media prevented both ATP- and nigericin-induced increases in the cytosolic Ca<sup>2+</sup> concentration but has no effect on their ability to stimulate IL-1 $\beta$  secretion. LLME-induced IL-1 $\beta$  secretion was actually enhanced by culture in calcium-deficient media and LLME-induced calcium currents were observed to be NLRP3 inflammasome dependent. Thapsigargin treatment of

BMDCs caused an increase in intracellular  $\text{Ca}^{2+}$  that required calcium-containing media. The authors of this study reason that, while thapsigargin blocks SERCA activity, it also induced oligomerization of STIM family sensors in the ER that opened Orai family store-operated  $\text{Ca}^{2+}$  influx channels within juxtaposed domains of the plasma membrane. These channels facilitated  $\text{Ca}^{2+}$  influx to offset the loss of intracellular  $\text{Ca}^{2+}$  and replenish ER  $\text{Ca}^{2+}$ . Thapsigargin treatment in calcium-depleted media, in order to isolate ER  $\text{Ca}^{2+}$  disruption, also had no effect on nigericin- or ATP-induced IL-1 $\beta$  secretion. Unfortunately, only a small number of confirmatory experiments were conducted in BMDMs. Treatment of BMDMs with ATP or nigericin in calcium-depleted media or thapsigargin pretreatment did not decrease IL-1 $\beta$  secretion [134].

Interestingly, BAPTA-AM treatment suppressed nigericin-induced IL-1 $\beta$  secretion in BMDCs treated in calcium-deficient media. The authors reasons that since there was no observable increase in intracellular  $\text{Ca}^{2+}$  in nigericin-treated cells incubated in calcium-deficient media, the suppressive activity of BAPTA must have been the result of some other change. Similarly, treatment of BMDCs with 2-APB, the IP3R inhibitor, caused complete suppression of nigericin-induced IL-1 $\beta$  secretion. Rather than suppressing  $\text{Ca}^{2+}$  mobilization, as would be expected with an IP3R inhibitor, the intracellular  $\text{Ca}^{2+}$  concentration was increased dramatically, in both 2-APB and 2-APB plus nigericin treated cells and this was not observed with nigericin treatment alone [134]. While this study was very thorough and convincing, it was conducted almost entirely using BMDCs and not BMDMs as is more commonly used. This difference makes it difficult to compare directly between this and previously discussed studies.

Overall, the majority of conflicting findings in these studies pertain to responses seen with pharmacologic manipulation. Numerous knockouts and knockdowns have been made to substantiate findings and provide additional strength to experiments involving pharmacologic

manipulations. More widespread use of genetic approaches will continue to provide confidence in the role of calcium in regulating NLRP3 inflammasome activity. Surprisingly, the finding that loss of MCU protects mitochondria from MAC-mediated dysregulation has been largely overlooked by subsequent studies, but I feel it is an area worth investigating further.

### **1.7.6 Ubiquitination acts as a platform for regulator binding**

Classically, ubiquitination plays a role in regulating protein turnover by targeting proteins for proteasomal degradation [135,136]. However, ubiquitination, mainly by the LUBAC, is also now being appreciated as a non-proteasomal regulator of the NLRP3 inflammasome.

The LUBAC is required for NLRP3 inflammasome activation in response to a wide variety of triggers. BMDM deficient in HOIL-1L, a component of the LUBAC, have decreased IL-1 $\beta$  secretion in response to nigericin, ATP, silica, MSU, and dsDNA, an activator of the AIM2 inflammasome. Upon treatment of BMDMs with nigericin, linear ubiquitin-specific antibody staining co-localized with ASC foci in an HOIL-1L-dependent manner. This linear ubiquitin chain is bound directly to ASC. *In vivo*, HOIL-1L is required for both NLRP3-dependent MDP-induced peritonitis and LPS-induced septicemia demonstrating that linear ubiquitination of NLRP3 is an important regulator of NLRP3 activity [137]. Sharpin, another component of the LUBAC, is also required for NLRP3 inflammasome activation as loss of Sharpin expression almost completely eliminated ATP-induced IL-1 $\beta$  and IL-18 secretion and LDH release [75]. Strangely, while loss of Sharpin expression reduced cytokine secretion in response to nigericin, alum, flagellin and dsDNA, it had no effect on LDH release [138]. The



difference is unexpected and may suggest that ATP-induced cell death has a unique requirement for Sharpin.

Deubiquitination is also necessary to release baseline inhibition of NLRP3 to promote NLRP3 inflammasome activation. The small molecule DUB enzyme inhibitor G5 increased ubiquitination of the NACHT and LLR domains and blocked ATP-, nigericin-, silica and LLMe-induced IL-1 $\beta$  secretion. BRCC3 was identified through a series of co-immunoprecipitation, overexpression, and knockdown experiments to screen cellular DUBs as a critical DUB in NLRP3 inflammasome activation [139].

Steady-state ubiquitination may serve as a platform for negative regulators of the NLRP3 inflammasome. HDAC6, a negative regulator of the NLRP3 inflammasome, bound directly to NLRP3 through its Binder of Ubiquitin Zinc finger (Buz) domain. Loss of HDAC6 caused increased ASC oligomerization, Caspase 1 activation and IL-1 $\beta$  secretion in response to ATP and nigericin. Treatment with the DUB enzyme inhibitor PR619 caused increased NLRP3 ubiquitination, as expected, and also suppressed ATP-induced IL-1 $\beta$  secretion in an HDAC6-dependent manner [140]. At this point, it remains unclear how HDAC6 binding to NLRP3 suppressed NLRP3 inflammasome activity.

Ubiquitination also regulated the NLRP3 inflammasome indirectly. Tripartite motif 33 (TRIM33), like most proteins in the TRIM family, is a E3 ubiquitin ligase that interacts with E2 ubiquitin-conjugating enzymes via their RING domain and transfers ubiquitin from E1 ubiquitin activating enzymes [141,142]. TRIM33 interacted with DHX33, the cytosolic dsRNA sensor for the NLRP3 inflammasome [143], and induced K63-linked ubiquitination of DHX33 K218 upon

dsRNA stimulation. This ubiquitination promoted interaction between DHX33 and NLRP3. As a result, TRIM33 was essential for dsRNA-induced NLRP3 inflammasome activation [144].

A20, a ubiquitin-modifying enzyme that inhibits NF- $\kappa$ B, also regulated the NLRP3 inflammasome. A20 deficient macrophages treated with LPS alone spontaneously secreted IL-1 $\beta$  and IL-18, and dramatically increased IL-1 $\beta$  secretion when additionally stimulated with ATP. This effect was dependent on RIPK3 and RIPK1. A20 associated with pro-IL-1 $\beta$  in a complex including Caspase 1, Caspase 8, RIPK1 and RIPK3. Pro-IL-1 $\beta$  was found to be ubiquitinated with K63-linked ubiquitin chains and poly-ubiquitin at pro-IL-1 $\beta$  K133 before activation of the NLRP3 inflammasome as blocking potassium efflux had no effect on the ubiquitination status of pro-IL-1 $\beta$ . Overexpression of A20 suppressed K63-linked ubiquitination of pro-IL-1 $\beta$  and decreased Caspase 1 activation. Similarly, a pro-IL-1 $\beta$  K133R mutation resulted in decreased IL-1 $\beta$  secretion [145].

Ubiquitination is also hijacked by pathogens to trigger NLRP3 inflammasome activation. *Shigella flexneri*, the cause of the food borne illness Shigellosis, injects invasion plasmid antigen H (IpaH) 7.8, an E3 ubiquitin ligase, through its type 3 secretion system into the host cytosol. The presence of IpaH7.8 during infection of macrophages caused NLRP3- and NLRP4-dependent Caspase 1 activation, IL-1 $\beta$  and IL-18 secretion and LDH release. Treatment of cells with the ubiquitin-proteasome inhibitor MG132 blocked IpaH7.8-mediated cell death, implicating the E3 ligase activity of IpaH7.8 in this process. IpaH7.8 interacted with GLMN, a Cullin ring ligase inhibitor [146,147], hinting at a role for GLMN in NLRP3 inflammasome activation. Upon stimulation with LPS and ATP, GLMN localized in specks, similar to the NLRP3 inflammasome, and sometimes but not always co-localized with Caspase 1. IpaH7.8 forced this localization. Heterozygosity of GLMN (deletion was embryonic lethal) in murine

BMDMs led to increased IL-1 $\beta$  secretion and cell death in response to *Shigella* infection that required IpaH7.8. A similar role of GLMN in suppressing NLRP3 inflammasome activation was also seen for LPS and ATP treatment [148]. Exactly how GLMN regulates the NLRP3 inflammasome is unknown, but the authors of this paper suggest that it may be through inhibiting an E3 ubiquitin ligase that activates the NLRP3 inflammasome. Numerous other bacteria, such as enteropathogenic *Escherichia coli* and *Legionella pneumophila*, produce virulence factors that hijack the human ubiquitination system too [149]. Further research may uncover other pathogenic ubiquitin-manipulating NLRP3 inflammasome-activating factors

Ubiquitin targeting NLRP3 for degradation is also an important regulator of NLRP3 inflammasome activity. Dopamine binding to the DRD1 receptor triggered production of cAMP that binds to NLRP3 and promotes K48-linked polyubiquitination of NLRP3 by the E3 ligase MARCH7. As such, dopamine stimulation of BMDMs decreased IL-1 $\beta$  and IL-18 secretion induced by MSU, nigericin, ATP and alum. Loss of MARCH7 blocked dopamine-mediated NLRP3 inflammasome repression. Interestingly, the autophagy inhibitor 3-methyladenine (3-MA) blocked dopamine-induced NLRP3 degradation but the proteasome inhibitor MG132 did not. Dopamine-DRD1 signaling also suppressed inflammation in a variety of models including nigericin-induced neuroinflammation, LPS-induced systemic inflammation and MSU-induced peritoneal inflammation [150].

Each of these regulatory mechanisms is probably relied upon to a different extent in different tissues. For example, colonic macrophages suppressed NLRP3 and pro-IL-1 $\beta$  through ubiquitination and proteasomal degradation, as inhibition with MG-132 dramatically increased NLRP3 and pro-IL-1 $\beta$  protein levels in these macrophages, but not in BMDMs [151]. Further

regulatory differences will be discovered as more cell types and tissue types continue to be investigated.

### **1.7.7 Phosphorylation controls inflammasome oligomerization**

Numerous kinases have been identified that regulate the NLRP3 inflammasome in response to a wide range of stimuli. However, the exact mechanism of action for most of these kinases remains unknown. NLRP3 inflammasome-regulating kinases are an important area of study because the druggability of kinases [152] makes them viable targets for modulating NLRP3 inflammasome activity.

#### **1.7.7.1 Syk kinase couples C-type lectin domain family members to NLRP3 inflammasome signaling**

The first identified and most well studied NLRP3 inflammasome-regulating kinase is Spleen tyrosine kinase (Syk). Syk coupled the detection of fungal molecular patterns by Dectin1 (also known as CLEC7A) to NLRP3 inflammasome activation [90,153]. Specifically, Dectin-1 bound to beta-glucans, polysaccharides in fungal cell walls, including curdlan, glucan from baker's yeast, paramylon and zymosan [153]. Pharmacological inhibition or genetic depletion of Syk completely blocked NLRP3-dependent Caspase 1 activation and IL-1 $\beta$  and IL-18 secretion in response to *Candida albicans* [90] and beta-glucans [153]. Additionally, activation of NLRP3 by beta-glucans was blocked by cytochalasin D, an inhibitor of phagocytosis, CA-074-Me, an inhibitor of CTSB, butylated hydroxyanisole (BHA), an antioxidant, and extracellular potassium

[153]. Loss of NLRP3 increased susceptibility of mice to *C. albicans* infection, suggesting that the Dectin1-Syk-NLRP3 pathway is critical for generating a productive immune response [90]. Syk activation has also been shown to be required for NLRP3 inflammasome activation in response to *Aspergillus fumigatus* [154], acapsular *Cryptococcus neoformans* [155], and *Malassezia* yeasts [156], a cause of pityriasis versicolor.

Beyond detection of fungal pathogens, the role of Syk in NLRP3 inflammasome activation is mixed. Soluble schistosomal egg antigens (SEA) from *Schistosoma mansoni* bound to Dectin-2 (also known as CLEC6A) and activated Syk to induce NLRP3 inflammasome activation. Unlike Dectin-1/Syk recognition of fungal molecules, this response was not sensitive to inhibition by cytochalasin D but did require ROS and potassium efflux. Mice lacking ASC or NLRP3 infected with *S. mansoni* showed decreased immunopathology including smaller granulomas with no significant difference in parasite burden [157] suggesting that NLRP3 inflammasome activation is a detrimental immune response in this setting.

Evidence also suggests that Syk may play a role in NLRP3 inflammasome activation in response to dengue virus. NLRP3-dependent Caspase 1 activation, IL-1 $\beta$  and IL-18 secretion and cell death were observed in dengue virus infected macrophages [158]. Treatment with an antibody to block CLEC5A, a C-type lectin critical for dengue hemorrhagic fever [159] and Japanese encephalitis virus infection [160], inhibited NLRP3 inflammasome activation [158]. Activation of the NLRP3 inflammasome by dengue virus could be blocked by preventing potassium efflux, CTSB activation and Syk activation [158].

Dectin-1, Dectin-2 and CLEC5A are all C-type lectin domain family members, remarked for their similarity to the calcium-dependent carbohydrate binding domain from Mannose-

binding lectin [161]. A variety of other C-type lectin domain family members coupled to Syk and bound to molecules that can stimulate an inflammasome-like response [162] but a direct connection to NLRP3 has yet to be investigated. Ligands for CLEC9A are exposed upon cell death and CLEC9A is required for cross-presentation of dead cell-associated antigens by dendritic cells [163]. CLEC9A binding to dead cell-associated antigens is also required for development of IFN- $\gamma$  positive cells [163], a process that can be initiated and enhanced by IL-18 and IL-1 $\beta$  [164,165]. Macrophage-inducible C-type lectin (Mincle) bound SAP130, a component of small nuclear ribonucleoprotein released by dying cells [166], and also detected *Malassezia* [167], a Syk-dependent NLRP3 inflammasome-activating fungi [156]. Syk activation is also required to activate the NLRP3 inflammasome in response to DAP12-dependent (a C-type lectin-associated protein that also binds CLEC5A [159,160]), and DAP12-independent haptens that cause allergic contact dermatitis [168]. It has been postulated that these haptens are causing cell death leading to CLEC9A or Mincle-mediated Syk activation of the NLRP3 inflammasome, however direct evidence is lacking.

Additionally, Syk may play a larger role in NLRP3 inflammasome activation than is suggested by the previously discussed data on C-type lectin domain family members. Inhibition of Syk diminished Alum- [169], MSU-, ATP- and nigericin-induced Caspase 1 activation and IL-1 $\beta$  secretion [170]. Interestingly, early evidence suggests that NLRP3 inflammasome regulation by Syk is cell type specific. For example, in murine BMDCs, Syk was not required for nigericin-induced NLRP3 inflammasome activation [90] but was critical in murine BMDM and the U937 human macrophage cell line [171].

The Src family kinase Lyn appeared responsible for Syk activation in response to hemozoin (Hz), an inorganic crystal formed by *Plasmodium* during the heme detoxification

process. Lyn was activated by Hz and pharmacologic inhibition of Lyn or genetic disruption blocked Hz-induced Syk phosphorylation and NLRP3-dependent IL-1 $\beta$  secretion. However, this effect was not seen with MSU, suggesting that Lyn was specifically involved downstream of a Hz receptor. Hz-induced NLRP3 inflammasome activation was also dependent on phagocytosis, ROS production, potassium efflux and CTSB activation [84].

Syk appeared to regulate the NLRP3 inflammasome through direct phosphorylation of ASC [170]. ASC was phosphorylated during NLRP3 inflammasome activation in a Syk-dependent manner and inhibition of Syk prevented ASC speck formation [170,171]. ASC tyrosine residues at position 146 and 187 were phosphorylated by Syk and were required for Syk-dependent ASC oligomerization and IL-1 $\beta$  secretion [170].

#### **1.7.7.2 RIPK1/RIPK3- and NLRP3-dependent necrosis overlap**

RIPK1 and RIPK3 are additional well characterized kinases implicated in regulating the NLRP3 inflammasome. RIPK1 and RIPK3 form a necrosis-induced signaling node originally characterized in TNF- $\alpha$ -induced necrosis [172-174]. The RIPK1/RIPK3 complex bound MLKL and RIPK3 phosphorylated MLKL at threonine 357 and serine 358. Blocking this phosphorylation step by knocking down MLKL or through necrosulfonamide (NSA) treatment prevented necrosis [175,176]. RIPK3 also phosphorylated Phosphoglycerate mutase family member 5 (PGAM5), a mitochondrial protein phosphatase [177]. PGAM5 activated DRP1, a GTPase involved in mitochondrial fission, by dephosphorylating the serine 637 residue causing mitochondrial dysregulation and ultimately NLRP3 dependent cell death [177]. However, a variety of studies with conflicting results have produced confusion as to whether certain

pathogens and molecules activate RIPK1- and RIPK3-dependent NLRP3 inflammasome signaling.

In one report, double-stranded RNA (dsRNA) viruses including vesicular stomatitis virus (VSV), Sendai virus and influenza virus all induced Caspase 1 activation and IL-1 $\beta$  and IL-18 secretion in murine BMDMs in a RIPK3- and NLRP3-dependent fashion. RIPK3 activation in response to these RNA viruses led to RIPK1 activation, mitochondrial damage, activation and translocation of DRP1 to mitochondria, and ultimately NLRP3 inflammasome activation. dsRNA was sufficient to induce each of these changes and activated the NLRP3 inflammasome in a RIPK1-, RIPK3-, DRP1-dependent manner. Interestingly, dsRNA viruses induced RIPK1-, RIPK3-, MLKL- and PGAM5-dependent, and NLRP3-independent cell death, suggesting that RIPK1/RIPK3 signaling diverges to promote inflammatory signaling and necrosis separately [178]. This signaling was also specific to dsRNA viruses as DNA viruses including adenovirus or herpes simplex virus (HSV), as well as classic NLRP3 agonists including ATP, MSU, alum and nigericin, were RIPK3-independent [178].

However, in two sequential reports, RIPK3 was not required for VSV-induced NLRP3 inflammasome activation [71,179]. PGAM5 knockout mice were generated and both macrophages and dendritic cells demonstrated defective IL-1 $\beta$  secretion in response to NLRP3 agonists including VSV, nigericin, ATP, and silica. RIPK3 deficiency did not mirror the IL-1 $\beta$  secretion defect seen in PGAM5 knockout mice, suggesting PGAM5 is activated through a separate pathway under these conditions [179].

Caspase 8 is also thought to play a significant role in determining the fate of RIPK1 and RIPK3 signaling. Caspase 8 can cleave RIPK1 [180], so Caspase 8 deficient cells essentially



have RIPK1/RIPK3 upregulated signaling. In Caspase 8 deficient dendritic cells, NLRP3 inflammasome-priming agents alone were sufficient to induce secretion of IL-1 $\beta$  and IL-18 in a RIPK1-, RIPK3-, MLKL- and PGAM5-dependent manner. Deletion of NLRP3 or ASC in the Caspase 8 deficient background blocked this hypersensitivity to NLRP3 inflammasome priming [181]. Similarly, inhibitor of apoptosis proteins (IAPs) promoted ubiquitination of RIPK1 and RIPK3 to prevent NLRP3 inflammasome hyperactivation in response to priming agents. In the absence of IAPs, the NLRP3 inflammasome is activated in a Caspase 8-dependent and MLKL-independent or MLKL-dependent manner, depending on the presence or absence of Caspase 8 [182].

The role of RIPK1 and RIPK3 signaling in the context of bacterial infection is much more clear. Human neutrophils infected with USA300 strain community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) underwent necrosis in a Necrostatin-1 (Nec-1)-sensitive manner, implicating RIPK1 signaling in this process [183]. *S. aureus* also triggered RIPK3- and MLKL-dependent cell death in THP1 cells and mouse alveolar macrophages, and triggered Nec-1- and NSA-sensitive cell death in primary human macrophages. *S. aureus* cytolytic PFTs, including Hla, LukAB and PVL, were required to induced this cell death. *S. aureus*-mediated RIPK3 activation led to IL-1 $\beta$  secretion as RIPK3 deficient mice had less cytokine production and improved bacterial clearance when challenged with *S. aureus* pneumonia [184].

*Serratia marcescens*, *Streptococcus pneumoniae*, *Listeria monocytogenes*, and uropathogenic *Escherichia coli* (UPEC) all induced RIPK1-, RIPK3- and MLKL-dependent cell death through production of pore-forming toxins. Inhibition of RIPK1 with necrostatin-5 improved survival in a mouse model of *S. marcescens* hemorrhagic pneumonia [185].

As more data is collected on RIPK1/RIPK3-dependent cell death, the division with NLRP3-dependent cell death begins to blur. For example, both *S. aureus* [43] and UPEC [186] have been shown to induce NLRP3-dependent cell death. Carefully tailoring the exact conditions initiating necrotic cell death signaling is extremely important for understanding the contribution of these various pathways. For example, *S. aureus* LukAB induced NLRP3-dependent cell death when binding the plasma membrane of human monocytes, but when *S. aureus* was phagocytosed and LukAB binds to the phagosomal membrane, the NLRP3 inflammasome was still activated but necrotic cell death occurred independent of NLRP3 (Chapter 3, Figure 9) [45].

### **1.7.7.3 Kinases have a wide ranging role in regulating the NLRP3 inflammasome**

About a dozen other kinases have been implicated in regulating the NLRP3 inflammasome but have only preliminary evidence supporting their involvement – that evidence will be reviewed here.

RIPK2 acted outside of the RIPK1/RIPK3 signaling complex to influence NLRP3 inflammasome activation. RIPK2 deficiency caused defective mitochondrial autophagy leading to accumulation of damaged mitochondria and ROS and ultimately NLRP3 inflammasome activation. RIPK2 promoted autophagy by phosphorylating ULK1, and ULK1 deficiency similarly caused increased mitochondrial ROS and Caspase 1 activation. RIPK2 deficient mice were unusually susceptible to influenza A viral infection and subsequent disruption of IL-18, by deletion or antibody-mediated blockade, led to improved infection clearance and survival [97]. In contrast, deficiency of RIPK2 had no impact on ATP-induced IL-1 $\beta$  and IL-18 secretion [2], suggesting RIPK2 is not a universal requirement for NLRP3 inflammasome activation.

DAPK appeared necessary for Caspase 1 activation and IL-1 $\beta$  production, but its kinase activity may be dispensable. DAPK deficient murine BMDMs and THP1 cells showed decreased IL-1 $\beta$  production and Caspase 1 activation in response to MSU, nigericin, ATP and alum. Overexpression of DAPK and the kinase-dead mutant, DAPK K42A, led to increased Caspase 1 activation and IL-1 $\beta$  production. When expressed in 293T cells, DAPK and NLRP3 interacted through DAPK ankyrin repeats and death domain and NLRP3 leucine-rich repeat (LRR) motif [132]. While the authors suggest that DAPK plays a scaffolding role for the NLRP3 inflammasome, overexpression experiments with kinase-dead mutants were conducted in THP1 cells that expressed wildtype DAPK and it remains possible that wildtype DAPK function was not blocked by co-expression of mutant DAPK allowing it to promote NLRP3 inflammasome activation, necessitating only a minimal requirement for DAPK kinase activity. Expressing kinase-dead mutants in DAPK deficient cells or using DAPK kinase activity inhibitors would provide greater strength to the authors' argument.

Activation of the NLRP3 inflammasome required NEK7, a cell cycle regulating kinase of the “never in mitosis gene a” (NIMA)-related family, forcing cell division and inflammasome activation to be non-overlapping processes temporally. Loss of NEK7 resulted in decreased IL-1 $\beta$  and IL-18 secretion and LDH release in response to nigericin [187], ATP, and alum, and decreased IL-1 $\beta$  and IL-18 secretion alone in response to *E. coli* and *C. rodentium* [188]. NEK7 bound directly to the LRR domain of NLRP3 to promote NLRP3 inflammasome formation. Kinase-dead NEK7 K64M successfully rescued the secretion of IL-1 $\beta$  in response to nigericin or ATP in NEK7 deficient peritoneal macrophages, suggesting that kinase activity was not important. Interestingly, the association between NEK7 and NLRP3 was diminished during mitosis and cells arrested in mitosis by RO-3306 treatment had decreased IL-1 $\beta$  secretion in

response to nigericin that was restored upon RO-3306 release [188]. This was the first demonstration of a cell cycle effect on NLRP3 inflammasome activation and may suggest a role for other cell cycle regulating kinases in NLRP3 inflammasome activation.

RNA-dependent protein kinase (PKR, also known as EIF2AK2) autophosphorylation was induced by NLRP3 inflammasome agonists including dsRNA, ATP, MSU, and alum, as well as NLRP1, NLRC4 and AIM2 agonists. PKR interacted with NLRP3 and this interaction was enhanced upon stimulation of the NLRP3 inflammasome. PKR also interacted with NLRP1, NLRC4 and AIM2. Loss of PKR blocked NLRP3-dependent Caspase 1 activation, IL-1 $\beta$  secretion and HMGB1 release, a marker of necrotic cell death. Loss of PKR kinase activity (PKR K296R) also blocked Caspase 1 activation [189]. A second study has also confirmed that PKR inhibition blocked alum-induced Caspase 1 activation and IL-1 $\beta$  secretion [169]. Interestingly, activation of PKR in response to ATP required potassium efflux, a feature not typically seen in the activation of AIM2 inflammasomes [189]. How PKR is activated in response to diverse stimuli and regulates multiple inflammasomes remains an open question.

Bruton's tyrosine kinase (BTK) also physically interacted with NLRP3 and ASC to regulate the NLRP3 inflammasome. The BTK TK domain bound the ASC pyrin domain and both BTK SH2/3 and TK domains bound to the NLRP3 NACHT and LRR domains. Inhibitors of BTK, including Ibrutinib, an FDA approved BTK inhibitor for the treatment of B-cell malignancies, blocked alum-, MSU-, nigericin-, ATP and LLMe-induced Caspase 1 activation and IL-1 $\beta$  secretion but not dsDNA-induced AIM2 inflammasome activation [169].

IL-1 receptor-associated kinases (IRAKs) played a role in NLRP3 inflammasome activation by signaling through MyD88. When a TLR agonist such as LPS and an NLRP3

agonist such as ATP were added together, Caspase 1 was activated in an IRAK-4 and IRAK-1-dependent manner that required kinase activity of both enzymes. However, when LPS and ATP were added sequentially, with ample time for LPS to prime the NLRP3 inflammasome, IRAK-4 and IRAK-1 were not required. Loss of both IRAK-1 and IRAK-2 together compromised ATP-induced Caspase 1 activation in both immediate activation and primed cells. IRAK-1 associated with ASC upon stimulation of the NLRP3 inflammasome. This immediate response triggered through simultaneous exposure was important for IL-18 processing and secretion and necrotic cell death. This type of exposure is likely to mimic early exposure to pathogens, as most pathogens express both TLR-binding ligands that act as priming molecules and NLRP3 inflammasome activating molecules, and indeed, infection of BMDMs with *L. monocytogenes* activated Caspase 1, HMGB1 release and necrotic cell death in an IRAK-1 and IRAK-4-dependent manner [65,66].

Inhibitor of nuclear factor  $\kappa$ -B kinase subunit  $\alpha$  (IKK- $\alpha$ ) was a negative regulator of ASC-dependent inflammasomes including NLRP3. In resting macrophages, IKK- $\alpha$  bound ASC to sequester it in the nucleus. IKK-i facilitated translocation of IKK- $\alpha$  and ASC from the nucleus to the perinuclear area during NLRP3 inflammasome activation by ATP. Mutation of ASC serine 16 or serine 193 to alanine disrupted IKK- $\alpha$ -mediated regulation of ASC and mutation of ASC serine 58 to alanine disrupted IKK-i-mediated regulation of ASC. Loss of IKK-i, IKK- $\alpha$  or IKK- $\alpha$  kinase activity (K44A) caused NLRP3 inflammasome hyperactivation in response to both priming agents alone and classical NLRP3 activators. Protein phosphatase 2A (PP2A) interacted with IKK- $\alpha$  following treatment with LPS and ATP. Inhibition of PP2A by okadaic acid blocked this interaction and blocked dissociation of ASC and IKK- $\alpha$  suggesting that phosphatase activity was responsible for opposing the suppressive activity of IKK- $\alpha$  in regulating the NLRP3

inflammasome. Furthermore, knockdown and inhibition of PP2A disrupted Caspase 1 activation [190].

JNK, a stress-responsive mitogen-activated protein kinase (MAPK), was required for NLRP3 inflammasome activation in response to nigericin and dsRNA. Loss of JNK blocked Caspase 1 activation and IL-18 secretion by blocking ASC aggregation. JNK was required for ASC phosphorylation but a JNK-dependent ASC phosphorylation site was not identified, leaving it open as to whether JNK phosphorylated ASC directly [171]. JNK also was critical for lysosome rupture-induced NLRP3 inflammasome activation. Upon treatment with LLME or MSU, activation of JNK led to Caspase 1 activation and IL-1 $\beta$  secretion. Treatment with the TAK1 (also known as MAP3K7) inhibitor 5-Z-Oxozeaenol [113] or TAK1 siRNA blocked JNK activation in this setting. TAK1-JNK signaling helped promote ASC oligomerization and was dependent on intracellular Ca<sup>2+</sup> signaling, as discussed above [112].

Mitochondrial ROS triggered signaling through PI3K-AKT-ERK1/2 during ATP-induced NLRP3 inflammasome activation. Treatment of macrophages with NAC or the PI3K inhibitors blocked phosphorylation of AKT and ERK1/2. The ERK1/2 inhibitor PD98056 blocked ATP-induced Caspase 1 activation as well. However, this study was unusual in that its ATP treatments lasted for 6-12 hours and, as such, raised the possibility that what we are seeing is engagement of a long-term feedback mechanism and not a direct requirement of NLRP3 inflammasome activation [88]. However, two additional studies have shown that AKT inhibitors can block NLRP3 inflammasome signaling. In one, treatment of macrophages with the AKT inhibitor AKTx blocked ATP- and nigericin-induced IL-1 $\beta$  secretion [48]. In the second, treatment with AKT inhibitor IV blocked Caspase 1 activation during a 30 minute ATP treatment in LPS-primed THP1 cells [113].

Numerous phosphorylation sites on ASC have been identified and implicated in NLRP3 inflammasome activity. These include the previously mentioned S16 and S193 that were required for interaction with IKK- $\alpha$  [190], S58 that was required for interaction with IKK-i [190], Y146 and Y187 that were phosphorylated by Syk [170], as well as ASC Y144, S58, T125, T151, T152 and S153 [171], as disruption of any of these caused aberrant NLRP3 inflammasome activation.

Thus far, little direct evidence of NLRP3 or Caspase 1 phosphorylation has been produced, though early evidence suggests that NLRP3 and Caspase 1 are also regulated by phosphorylation. *H. pylori* induced NLRP3 inflammasome activation [191] and *H. pylori* LPS activated Caspase 1 and IL-1 $\beta$  secretion [192]. Interestingly, *H. pylori* LPS induced direct interaction between p21-activated kinase (PAK) 1 and Caspase 1 that required Caspase 1 S376. PAK1 was able to phosphorylate recombinant Caspase 1 but not the S376A mutant and LPS-induced Caspase 1 activation was blocked in cells transfected with the S376A mutant [192]. Additionally, NLRC4 was phosphorylated by PKC $\delta$  at S533 and depletion of PKC $\delta$  attenuated Caspase 1 activation and IL-1 $\beta$  secretion in response to *S. typhimurium*. NLRC4 S533A was unable to recruit pro-Caspase 1 and did not form visible specks [193]. NLRC4 contains a CARD allowing it to interact directly with pro-Caspase 1 without requiring the adapter ASC raising the possibility that phosphorylation of NLRC4 is more akin to ASC phosphorylation than NLRP3 phosphorylation, however, amino acid 533 lies between the NACHT and LRR domains raising the possibility that a corresponding residue in NLRP3 is similarly phosphorylated.

ATK	BTK	DAPK	ERK1/2	IKK- $\alpha$
IKK-i	IRAK1	IRAK2	IRAK4	JNK
Lyn	NEK7	PAK1	PI3K	PKR
RIPK1	RIPK2	RIPK3	Syk	TAK1

### **1.8 Canonical inflammasome activators: nigericin, ATP and monosodium urate**

After carefully reviewing the most well studied mechanisms of NLRP3 inflammasome activation, the lack of cohesion between studies resulting from the diversity of activating stimuli is now probably apparent to the reader. In this section, I will look specifically at the three most well studied NLRP3-activating molecules: nigericin, extracellular ATP and MSU. Each classical activator is thought to provoke a unique series of changes that leads to NLRP3 inflammasome activation: nigericin is a mitochondria-disrupting ionophore [194], extracellular ATP triggers P2RX7 causing potassium efflux [195], and MSU causes lysosome dysfunction [196]. Many critical regulatory elements in NLRP3 inflammasome signaling have been characterized using one of these activating molecules. Although the initial insults differ, the resultant signaling pathways have some overlap. In the subsections of 1.8, I will review the requirements for NLRP3 inflammasome activation in response to each of these three well-studied activators.



### 1.8.1 Nigericin is a potassium ionophore that activates the NLRP3 Inflammasome

Incubation of murine macrophages and human peripheral blood monocytes with the bacterial toxin nigericin, a potassium/proton ionophore produced by *Streptomyces hygroscopicus* [197,198], led to proteolytic processing of the 35-kDa pro-IL-1 $\beta$  and release of the 17-kDa active form [199]. Cells treated with nigericin experienced rapid and complete loss of the preloaded potassium analog  $^{86}\text{Rb}^+$ , whereas cells treated with valinomycin, a potassium ionophore that does not stimulate IL-1 $\beta$  processing, caused only a partial depletion of the radiolabeled cation. Nigericin-stimulated IL-1 $\beta$  processing was inhibited in cells grown in medium containing KCl rather than NaCl [200]. Caspase 1 (originally called interferon-converting enzyme – ICE) was responsible for nigericin-induced IL-1 $\beta$  processing and activation [201]. In THP1 cells, nigericin induced Caspase 1 activation and IL-18 secretion without LPS priming [202].

With this information in hand, and the discovery of NLRP3 as an activator of Caspase 1, nigericin was shortly thereafter demonstrated to activate the NLRP3 inflammasome. Nigericin activated Caspase 1 through the NLRP3 inflammasome followed by processing and secretion of mature IL-1 $\beta$  and IL-18, as genetic deletion of NLRP3 or ASC in macrophages disrupted this response [203]. Complementation of Caspase 1 deficient cells with a non-cleavable or catalytically inactive mutant of Caspase 1 also disrupted IL-1 $\beta$  secretion in response to nigericin [204]. Nigericin-induced Caspase 1 cleavage can be prevented by blocking potassium efflux [80]. Caspase 11 was not required for nigericin-mediated NLRP3 inflammasome signaling [51]. Nigericin-induced NLRP3-dependent IL-1 $\beta$  and IL-18 secretion also extended beyond the periphery and was triggered in microglia [205].

In addition to IL-1 $\beta$  and IL-18 secretion, nigericin induced Caspase 1-independent and CTSB-dependent cell death. Treatment of cells with nigericin caused rapid lysosomal leakage and translocation of CTSB to the cytoplasm. Both pharmacologic intervention, including the CTSB inhibitor CA-074-Me, and genetic disruption, including transformation with an endogenous cathepsin inhibitor cystatin A or antisense CTSB cDNA, blocked nigericin-induced cell death, and interestingly, IL-18 secretion [202]. Nigericin-induced cell death has also been shown to be NLRP3-dependent [206]. Nigericin-induced NLRP3-dependent cell death has been observed in non-immune cells including primary renal tubular epithelial cells [207].

In the absence of Caspase 1 and Caspase 11, prolonged treatment of BMDCs with nigericin triggered NLRP3-inflammasome dependent Caspase 8 activation and Caspase 8-dependent IL-1 $\beta$  secretion. Caspase 8 also was required for nigericin-induced cell death in this setting [208]. The precise role of Caspase 8 in responding to nigericin in wildtype cells, though, is unclear.

Further characterization of nigericin-induced NLRP3 inflammasome activation has proceeded in a piecemeal fashion largely as a comparator for other NLRP3-activating stimuli or to test for endogenous regulators of NLRP3 inflammasome activity.

Inhibition of Syk with R406, Syk inhibitor I, or BAY 61-3606, or knockdown of Syk expression blocked nigericin-induced IL-1 $\beta$  and IL-18 secretion from peritoneal macrophages. Similarly, inhibition of JNK with SP600125 or TAT-TI-JIP<sub>153-163</sub>, or knockout of MAPK8 or MAPK9, the genes encoding for JNK1 and JNK2, also blocked nigericin-induced IL-1 $\beta$  and IL-18 secretion. Similar results were also obtained in mouse BMDMs and the U937 human macrophages cell line. Decreased cytokine secretion resulted from decreased ASC speck

formation and Caspase 1 activation. Treatment of macrophages with nigericin induced phosphorylation of Syk and JNK but Syk inhibitors did not block phosphorylation of JNK, placing these two kinases into separate pathways. Overexpression of Syk or JNK resulted in phosphorylation of ASC that was blocked by a Y144F substitution, suggesting that this is the site targeted by these kinases. However, *in vitro* kinase assays using purified Syk or JNK and amino acids 139-150 of ASC did not consume ATP and could not confirm phosphorylation at Y144 [171]. Interestingly, two reports on BMDCs found Syk is not required in nigericin-induced NLRP3 inflammasome activation, pointing to cell type specific signaling [90,171].

Other kinases, such as NIMA-related kinase 7 (NEK7), which plays a role in nucleating microtubules at centrosomes during mitosis, have been implicated in NLRP3 inflammasome activation. Disruption of NEK7 by CRISPER/Cas9 protected macrophages from nigericin-induced cell death. Murine macrophages deficient in NEK7 had a largely blunted NLRP3 inflammasome response suggesting that NEK7 acted upstream of NLRP3 [187], however the precise mechanism has not been elucidated. BTK interacted with NLRP3 and ASC during nigericin treatment and was required for nigericin-induced ASC oligomerization [169]. The TAK1 inhibitor 5-Z-oxozeaenol blocked nigericin-induced Caspase 1 activation [113]. The AKT inhibitor AKTx also blocked nigericin-induced IL-1 $\beta$  secretion [48]. Lastly, DAPK was required for nigericin-induced IL-1 $\beta$  secretion. However, little is known about how the regulating modality of these kinases overlaps during nigericin-induced NLRP3 inflammasome activation [132].

The mitochondrial protein phosphatase PGAM5 promoted nigericin-induced NLRP3 inflammasome activation. Deletion of PGAM5 diminished IL-1 $\beta$  secretion, but not cell death, in

response to nigericin in BMDMs but not BMDCs. Nigericin-induced ASC oligomerization was blocked by PGAM5 deficiency [179].

The production of ROS, and more specifically mitochondrial ROS that perturb mitochondrial function, have been proposed as a major requirement for NLRP3 inflammasome activation. This finding is typically established through two observations: (1) that mitochondrial oxygen consumption or mitochondrial membrane potential becomes disrupted and (2) that antioxidant treatment prevents NLRP3 inflammasome activation.

Macrophages treated with nigericin showed a series of mitochondrial changes that can be separated into two groups, NLRP3-independent changes that are not suppressed by blocking potassium efflux and NLRP3-dependent changes that are suppressed by blocking potassium efflux.

Nigericin reduced the mitochondrial oxygen consumption rate and decreased cellular ATP, observations that were unchanged during incubation with high extracellular potassium. However, NLRP3 inflammasome activation by nigericin led to loss of mitochondrial membrane integrity, as measured using MitoTracker Red, loss of mitochondrial DNA, as measured by PicoGreen staining, and loss of lysosome staining, as measured using the LysoTracker Green dye, suggesting either loss of lysosomal acidity or loss of lysosomal membrane integrity. Exposure of phosphatidyl-serine on the plasma membrane also occurred in an NLRP3 inflammasome-dependent fashion. Neither nigericin-induced lysosome and mitochondria disruptions nor LDH release, a common measure of cell death, were blocked by the Caspase 1 inhibitor Ac-YVAD-CMK [206]. Direct disruption of mitochondrial function using rotenone, an inhibitor of the

mitochondrial electron transport chain, was not sufficient to induce NLRP3 inflammasome activation and actually suppressed nigericin-induced Caspase 1 activation [209].

In one report, high doses of NAC, a potent antioxidant, or the NOX2 inhibitor DPI did not block nigericin-induced Caspase 1 cleavage [80]. Mito-Tempo, a mitochondria-specific ROS scavenger, delayed lysosomal membrane permeabilization and reduced mitochondrial membrane permeabilization. The authors also claimed that IL-1 $\beta$  secretion and LDH release after nigericin exposure was dampened by Mito-Tempo, but the change was unconvincing and the difference may have been called based on inappropriate statistical tests [206]. XOR has been implicated in contributing to crystalline-mediated ROS production and NLRP3 inflammasome activation and febuxostat, a XOR inhibitor, also decreased IL-1 $\beta$  secretion in response to nigericin, possibly implicating XOR as a source of ROS [48]. Although mitochondrial ROS are detected in nigericin treated macrophages, inhibitors of Syk or JNK do not impact their production [171].

TRIM30 negatively regulated nigericin-induced NLRP3 activation through modulating ROS. Knockdown of TRIM30 increased nigericin-induced Caspase 1 activation and IL-1 $\beta$  production. Production of ROS, measured using CM-H2DCFDA labeling, was also increased in TRIM30 knockdown cells, and its increase was required for enhanced NLRP3 inflammasome activation as treatment with NAC blocked excess IL-1 $\beta$  production. Conversely, overexpression of TRIM30 attenuated ROS production and NLRP3 inflammasome activation [210].

Pinning down ROS-mediated effects can be extremely difficult as redox states of cells can differ even within the same cell types. TRIM30's effect on IL-1 $\beta$  secretion was demonstrated in murine BMDMs, but the ROS studies and suppression by NAC in knockdown TRIM30 cells were performed using murine J774 macrophages, a cell line derived from a reticulum cell

sarcoma. Future studies using murine TRIM30 knockout cells would provide more convincing evidence for TRIM30 in suppressing ROS-induced NLRP3 inflammasome activation.

Nigericin-induced NLRP3 inflammasome activation appeared to make use of the mitochondria as a scaffold for inflammasome assembly. Treatment of J774 cells with colchicine to disrupt microtubule dynamics blocked nigericin-induced IL-1 $\beta$  secretion. Nigericin induced microtubule-driven and dynein-dependent rearrangement of mitochondria that was critical for NLRP3 inflammasome activation. This resulted from decreased concentration of cellular NAD<sup>+</sup> leading to increased acetylated  $\alpha$ -tubulin, as discussed above [102]. SHP, which also binds NLRP3 at the mitochondria, inhibited nigericin-induced NLRP3 inflammasome activation [101].

Disruption of mitochondrial fission by knockdown of DRP1 increased nigericin-induced Caspase 1 activation and IL-1 $\beta$  secretion. CCCP, which induces mitochondria fission, attenuated nigericin-induced NLRP3 inflammasome activation in wild type treated cells. Mitochondrial fission appeared to block ASC oligomerization [99].

Another possible role for mitochondrial disruption in NLRP3 activation is through reduction in intracellular ATP. Artificial reduction of intracellular ATP by 2-deoxyglucose, a glycolysis inhibitor, caused mitochondrial membrane depolarization and IL-1 $\beta$  secretion via NLRP3 and Caspase 1 activation. Nigericin also lowered intracellular ATP through potassium and calcium-mediated mitochondrial dysfunction possibly leading to a positive feedback loop for enhanced NLRP3 inflammasome activation [211]. However, decreased cellular ATP in response to nigericin was unaffected by incubation with high extracellular potassium [206], putting these studies in conflict.

To summarize, current evidence suggests that nigericin damages mitochondria directly and NLRP3 inflammasome activation causes further damage, but the association between ROS production and NLRP3 inflammasome activation is weak. The suggestion made in Nomura et al. that ATP leakage might be involved in NLRP3 inflammasome activation is interesting in light of evidence that Pannexin 1, a membrane protein can act as a pore and associates with the ATP receptor P2RX7, is also required for nigericin-induced Caspase 1 processing and IL-1 $\beta$  secretion. Additionally, inhibition of Pannexin 1 did not block nigericin-induced potassium efflux, suggesting that Pannexin 1 is required downstream of this critical step [212]. Studies pointing to the mitochondria as a scaffold for inflammasome assembly during nigericin treatment are also compelling.

NLRP3 is also regulated by ubiquitination. The small molecule deubiquitinase inhibitor G5 blocked nigericin-induced IL-1 $\beta$  secretion by inhibiting Caspase 1 activation. G5 treatment induced appearance of a high molecular weight NLRP3 that reacted with anti-K48 and anti-K63 ubiquitin antibodies. The deubiquitinase BRCC3 co-immunoprecipitated with NLRP3 and was required for activation of the NLRP3 inflammasome [139]. In a separate study, histone deacetylase 6 (HDAC6) was found to negatively regulate nigericin-induced NLRP3 inflammasome activation through binding to ubiquitinated NLRP3. Knockdown of HDAC6 by shRNA led to increase nigericin-induced Caspase 1 processing and IL-1 $\beta$  secretion without affecting mRNA levels through NF- $\kappa$ B activation. In 293T cells, HDAC6 co-immunoprecipitated with NLRP3 and required the BUZ domain, a binder of ubiquitin zinc finger domain, for this association. Treatment of cells with PR619, a deubiquitinase inhibitor, enhanced association between HDAC6 and NLRP3 [140].

The LUBAC used ubiquitin to promote nigericin-induced NLRP3 inflammasome signaling. Disruption of LUBAC by deletion of HOIL-1L decreased nigericin-induced ASC oligomerization and IL-1 $\beta$  secretion [137]. Similarly, deletion of Sharpin, another LUBAC component, eliminated nigericin-induced IL-1 $\beta$  and IL-18 secretion but had no effect on LDH release, raising the possibility that LUBAC does not regulate NLRP3-dependent cell death [138].

Ubiquitin also promoted NLRP3 degradation to limit NLRP3 inflammasome signaling. Treating cells with dopamine signaled through DRD1 to activate the E3 ligase MARCH7. MARCH7 bound directly to the NLRP3 inflammasome and ubiquitinated it, targeting it for degradation. Treatment of cells with dopamine and nigericin decreased IL-1 $\beta$  secretion as compared to nigericin alone, and knockdown of MARCH7 by siRNA eliminated the dopamine-mediated reduction in IL-1 $\beta$  secretion [150].

Calcium is a common second messenger in many signaling processes. Nigericin-induced IL-1 $\beta$  secretion but not LDH release was sensitive to inhibition by BAPTA-AM and thapsigargin [120]. The SERCA inhibitor XeC, the PLC inhibitor U73122, and the IP3R inhibitor 2-APB all blocked nigericin-induced Caspase 1 activation and IL-1 $\beta$  secretion [126]. Depletion of CASR by siRNA suppressed nigericin-induced IL-1 $\beta$  secretion [127].

However, the mechanism of action of BAPTA and 2-APB appeared to be dissociated from their canonical or expected effects on calcium homeostasis. Calcium perturbations resulting from nigericin treatment were actually NLRP3 inflammasome-dependent, rather than signaling events responsible for initiating NLRP3 inflammasome activation [80,134].

Disruption of ribosome function has also been proposed as a common initiator signal for NLRP3 inflammasome activation. Ribosomal function inhibitors including ricin, cycloheximide,



puromycin, pactamycin, and anisomycin caused NLRP3 inflammasome-dependent IL-1 $\beta$  secretion. Nigericin treatment also disrupted protein synthesis in a manner dependent on potassium efflux [213]. However, convincing evidence is currently lacking to place ribosomal dysfunction upstream of NLRP3 inflammasome activation and no clear mechanism has been proposed to link the two events.

Loss of guanylate binding protein 5 (GBP5) dramatically decreased nigericin-induced ASC oligomerization, and IL-1 $\beta$  and IL-18 secretion. Tetrameric GBP5 associated with NLRP3 by binding the NLRP3 pyrin domain but little is known about the mechanism of regulation [214].  $\beta$ -arrestin 1, also promoted nigericin-induced NLRP3 activation, as loss of  $\beta$ -arrestin 1 decreased nigericin-induced Caspase 1 activation, IL-1 $\beta$  secretion and LDH release.  $\beta$ -arrestin 1 promoted NLRP3 self-oligomerization, an important step for NLRP3 inflammasome formation [215]. Again, few details have been ascertained about this interaction and regulation.

A variety of NLRP3 inflammasome inhibiting and dampening agents have also been characterized using nigericin. Ethanol had a dose-dependent effect in suppressing nigericin-induced IL-1 $\beta$  secretion without affecting potassium efflux. Ethanol blocked ASC speck formation [216]. Arsenic trioxide and sodium arsenite inhibited activation of multiple inflammasomes including NLRP1, NLRC4, and nigericin-induced NLRP3 through an unknown mechanism to suppress Caspase 1 cleavage and IL-1 $\beta$  secretion [217]. Carbon monoxide, a gaseous molecule physiologically produced in cells and tissues during heme catabolism, inhibited IL-18 secretion in response to nigericin treatment [218]. Unsaturated fatty acids oleate and linoleate suppressed IL-1 $\beta$  secretion triggered by nigericin [219].

Additional endogenous factors implicated in NLRP3 inflammasome activation and regulation by other stimuli have no demonstrated role in nigericin-induced NLRP3 inflammasome activation. Loss of RIPK3 in BMDMs did not impact nigericin-induced IL-1 $\beta$  signaling [178]. The proteasome inhibitor MG132 did not block Caspase 1 activation and cell death triggered by lipopolysaccharide and nigericin [220]. Mitochondrial antiviral signaling (MAVS), a common adaptor that induces NLRP3 activation in response to poly I:C, was not required for nigericin-mediated NLRP3 activation [221]. Promyelocytic leukemia protein (PML) was shown to increase and decrease NLRP3 inflammasome activation in separate conflicting studies [222,223], but loss of PML appeared to have no effect on nigericin-induced IL-1 $\beta$  secretion [217].

Much less is known about the mechanistic details of events downstream of nigericin-induced NLRP3 inflammasome activation. Recently, Gasdermin D (GSDMD) was shown to be cleaved by Caspase 1 upon activation by nigericin. GSDMD cleavage was required for cell death and IL-1 $\beta$  secretion but not proteolytic maturation of IL-1 $\beta$ . Expression of the N-terminal proteolytic fragment of GSDMD triggered cell death and modifications such as tagging with Flag sequence disrupted this function. Pro-Caspase 1 was capable of processing GSDMD and ASC was not essential for GSDMD to function, so the means by which GSDMD is regulated to limit cell death is unclear [224].

Lastly, on the organism level, elderly mice (8-9 month old) had decreased inflammasome activation in response to a mouse-adapted strain of influenza (HKx31 an H3N2 strain) but retained normal inflammasome activation in response to nigericin [225]. These data suggest that core NLRP3 inflammasome signaling remained intact during aging. Also, NLRP3 inflammasome activation by nigericin was observed in miniature and domestic pigs - important

pre-clinical models for toxicology studies. Porcine PBMCs secreted IL-1 $\beta$  at lower doses of nigericin than mouse BMDMs, though no comparison was made to human cells [226].

There are many gaps in knowledge in nigericin-induced NLRP3 inflammasome signaling. For example, of the kinases that have been implicated in regulating the NLRP3 inflammasome, only a small fraction have been evaluated in the context of nigericin. Very few studies have examined both cytokine secretion and cell death resulting from nigericin-induced NLRP3 activation. In the studies that have, though, we observed a role for Caspase 1 and Sharpin in cytokine secretion, but not cell death. Additionally, BAPTA-AM blocked IL-1 $\beta$  secretion but not nigericin-induced cell death. As is probably now clear, when examining the NLRP3 inflammasome signaling pathway from the context of a single activator, the landscape is littered with holes. We will continue our review of ATP and MSU now.

### **1.8.2 Extracellular ATP triggers NLRP3 activation by binding P2RX7**

Extracellular ATP is another commonly used NLRP3 inflammasome activator. Even before the discovery of the NLRP3 inflammasome, it was realized that treatment of mouse peritoneal macrophages with extracellular ATP caused processing and secretion of IL-1 $\beta$  that was dependent on intracellular potassium depletion with longer incubations also causing cell death. ADP was less effective at inducing cytokine secretion and AMP, GTP and UTP were completely inactive in that regard [200]. In cells lacking P2RX7, ATP did not activate Caspase 1 or cause IL-1 $\beta$  processing and secretion. This finding translated *in vivo* to mouse models including a LPS-septicemia model. ATP injection into LPS-primed wildtype mice caused IL-1 $\beta$

secretion that was not seen in P2RX7 deficient mice [227]. ATP-binding to P2RX7 activated the Pannexin 1 channel leading to large pore formation and IL-1 $\beta$  secretion [228].

The discovery of the NLRP3 inflammasome tied together ATP binding P2RX7 and Pannexin 1-mediated potassium efflux with Caspase 1 activation and IL-1 $\beta$  secretion. ATP bound to its plasma membrane receptor, P2RX7, decreased intracellular K<sup>+</sup> levels leading to NLRP3 inflammasome activation that resulted in Caspase 1 activation and IL-1 $\beta$  processing and secretion. Incubating cells in high extracellular potassium prior to ATP treatment blocked Caspase 1 activation and IL-1 $\beta$  secretion [80,203]. Deletion of Caspase 11 did not change ATP-mediated IL-1 $\beta$  secretion [51]. ATP also caused NLRP3- and Caspase 1/Caspase 11-dependent necrotic cell death [80].

The steps between potassium efflux and inflammasome activation are less well understood. When macrophages that phagocytosed rhodamine-labeled MDP were treated with ATP, rapid redistribution of the labeled MDP from phagolysosomes to the cytoplasm, consistent with lysosome disruption, was observed. This lysosomal disruption also required Pannexin 1, suggesting it may be in line with the NLRP3 inflammasome signaling pathway [229]. Consistent with this lysosomal disruption, ATP binding to P2RX7 triggers cathepsin release in macrophages that was blocked by pre-incubation with a P2RX7 inhibitor [230]. However, treatment with the CTSB inhibitor CA-074-Me or bafilomycin to block lysosome acidification did not block ATP-induced IL-1 $\beta$  secretion [108,231]. TAK1-JNK signaling has been implicated in responding to lysosome rupture in cells treated with LLME. Pretreatment of THP1 cells with the TAK1 inhibitor 5-Z-oxozeaenol or the JNK inhibitor SP600125 blocked ATP-induced Caspase 1 activation, however, this study did not look directly at whether ATP induced lysosome rupture. Additionally, LLMe-induced JNK phosphorylation was sensitivity to inhibition by CA-074-Me,

but this was not investigated for ATP [112]. A second study has confirmed that 5-Z-oxozeaenol inhibition of TAK1 blocked ATP-induced Caspase 1 activation [113]. These data show that ATP caused lysosome rupture but CTSB was not a critical component of signaling that rupture. It appears that TAK1 was necessary for ATP-induced NLRP3 inflammasome activation, but the partial responsiveness of JNK phosphorylation to CTSB inhibition would not be consistent with ATP requiring TAK1 but not CTSB. A focused study addressing these questions would help provide greater certainty than these piecemeal reports.

Treatment of macrophages with LPS and ATP caused mitochondria to swell and disrupted mitochondrial cristae. This was worsened by loss of LC3B and Beclin 1, proteins important in autophagy. These autophagy-defective macrophages produced more mitochondrial ROS and had enhanced NLRP3 inflammasome activation. Quenching mitochondrial ROS with Mito-TEMPO, a scavenger specific for mitochondrial ROS, inhibited secretion of IL-1 $\beta$  in response to LPS and ATP in both wildtype and autophagy-defective macrophages [104]. Rotenone, a mitochondrial complex I inhibitor, induced mitochondrial ROS production [232] and enhanced Caspase 1 activation and IL-1 $\beta$  secretion when added to cells with LPS and ATP [104,209].

Culturing cells in the presence of low levels of ethidium bromide inhibits replication of mtDNA and can produce cells lacking mitochondrial DNA altogether. Upon treatment with ATP, DNA can be found in the cytoplasm of wildtype but not mitochondrial DNA deficient J77A.1 macrophages, suggesting that the DNA in the cytosol originated in the mitochondria. Treatment of cells with cyclosporine A, an inhibitor of the mitochondrial membrane permeability transition pore, blocked ATP-induced mtDNA translocation to the cytoplasm. Cyclosporine A also inhibited NLRP3 inflammasome activation in both wildtype and autophagy-defective

macrophages. Cytosolic mtDNA appeared to activate the NLRP3 inflammasome as treatment with DNase I, but not heat-inactivated DNase I, blocked ATP-induced inflammasome activation. Additionally, transfection of AIM2 deficient cells (a cytosolic double stranded DNA sensor) with mtDNA caused Caspase 1 activation [104]. Interestingly though, NLRP3 deficiency suppressed release of mtDNA into the cytosol, but not mitochondrial ROS production in response to treatment with LPS and ATP suggesting that mitochondrial ROS may be upstream of inflammasome activation, and mtDNA translocation may be downstream and play an amplifying role [104,209].

Treatment of macrophages with ATP resulted in rapid ROS-dependent PI3K activation, AKT phosphorylation and ERK1/2 phosphorylation. Pretreatment with NAC or the PI3K inhibitors LY294002 and wortmannin blocked AKT and ERK1/2 phosphorylation. The PI3K inhibitors and the ERK1/2 inhibitor PD98056 also completely blocked ATP-induced Caspase 1 activation and IL-1 $\beta$  secretion, comparable to a direct Caspase 1 inhibitor. While this evidence suggests that ROS-PI3K-AKT-ERK1/2 signaling is required for ATP-mediated NLRP3 activation, this study used unusually long ATP treatment times, 6 and 12 hours, when assaying Caspase 1 activation and IL-1 $\beta$  secretion. The inflammasome is typically activated on a time scale on the order of minutes, not hours, raising the possibility that other pathways are being recruited for Caspase 1 activation and cytokine secretion in this setting [88]. However, two additional studies have shown that inhibition of AKT, using AKTx or AKT inhibitor IV, blocked ATP-induced Caspase 1 activation and IL-1 $\beta$  secretion during shorter ATP exposures [48,113].

Overexpression of Bcl2, an anti-apoptotic mitochondrial protein, suppressed ATP-induced Caspase 1 activation and IL-1 $\beta$  secretion while helping to preserve mitochondrial polarization. Similarly, knockdown of Bcl2 enhanced ATP-induced IL-1 $\beta$  secretion and cell

death. In this very important study, mtDNA co-localized and immunoprecipitated specifically with NLRP3. NLRP3 bound directly to oxidized DNA to promote Caspase 1 activation and subsequent cytokine processing [103]. This was the first demonstration of a direct ligand for NLRP3. However, it remains unclear how it fits in with previous studies that showed mtDNA translocation to the cytosol required NLRP3, rather than activated it.

XOR has been implicated in contributing to crystalline-mediated ROS production and NLRP3 inflammasome activation and Febuxostat, a XOR inhibitor, also decreased IL-1B secretion in response to ATP [48]. XOR produces ROS as a byproduct of its enzymatic activity and may be a source of ROS production during ATP treatment.

The  $\gamma$ -aminobutyric acid A receptor-associated protein (Gabarap), which functions in  $\gamma$ -aminobutyric acid A receptor trafficking and postsynaptic localization in neurons, has also been implicated in regulating mitochondrial-based NLRP3 inflammasome activation. Gabarap deficient macrophages treated with LPS and ATP had defective clearance of damaged mitochondria resulting in more mitochondrial ROS and mtDNA release into the cytosol. Additionally, ATP-induced Caspase 1 activation and IL-1 $\beta$  and IL-18 secretion were increased in Gabarap deficient macrophages [98].

Knockdown of TRIM30 enhanced ATP-induced Caspase 1 activation and production of IL-1 $\beta$  in BMDMs by increasing cellular ROS production. Treatment of TRIM30-depleted cells with NAC or DPI before ATP stimulation opposed the increased NLRP3 inflammasome activity by suppressing ROS. Overexpression of TRIM30 also attenuated ROS production and opposed ATP-induced NLRP3 inflammasome activation [210].

While the evidence supporting a role for ROS in ATP-induced NLRP3 inflammasome activation is generally strong, there have been occasional conflicting findings. For example, in one study, pretreatment of LPS-primed ATP-stimulated BMDMs with NAC or DPI was unable to specifically suppress Caspase 1 activation [80].

The evidence for calcium mobilization in the activation of the NLRP3 inflammasome in response to ATP is also somewhat conflicted. Incubation in calcium free media blocked extracellular calcium entry and attenuated ATP-induced Caspase 1 activation and IL-1 $\beta$  secretion in one study [126] but had no effect in another [134]. ATP-induced IL-1 $\beta$  secretion was also suppressed by BAPTA-AM, an intracellular calcium chelator [120,127,233].

Treatment of LPS-primed BMDMs with thapsigargin, an inhibitor of SERCA pumps and agonist of plasma membrane calcium channels that increases cytoplasmic calcium concentrations, has produced conflicting results. Thapsigargin treatment attenuated ATP-induced Caspase 1 activation and IL-1 $\beta$  secretion in two studies [120,126]. In cells treated with thapsigargin alone, without additional NLRP3 stimulus, thapsigargin activated Caspase 1 and IL-1 $\beta$  secretion in one study [127] and had no effect in another [120]. And to further complicate things, in a study using thapsigargin-treated LPS-primed BMDCs and macrophages, increased cytoplasmic calcium concentration was only observed when cells were in calcium-containing media and not calcium-free media suggesting that thapsigargin was causing calcium influx from the extracellular environment and not depletion of ER calcium stores. In comparison to the two previous studies, thapsigargin treatment in calcium-free media did not induce IL-1 $\beta$  secretion on its own and did not change ATP-induced IL-1 $\beta$  secretion. However, when the TLR2 ligand Pam3CSK4 or TNF- $\alpha$  were used to prime the dendritic cells, thapsigargin-treatment in calcium free media caused a modest but significant decrease in ATP-induced IL-1 $\beta$  secretion [134].



Interpretation of these studies together is difficult, especially given the use of dendritic cells and not macrophages in one. This series of studies, however, nicely highlights how more care needs to be taken in the field to replicate previous work to confirm or refute existing data and design new experiments to be more easily comparable.

Macrophages deficient in the murine CASR did not activate Caspase 1 or secrete IL-1 $\beta$  in response to ATP [127]. Interestingly, the synthetic CASR agonist R568 produced sustained calcium influx and led to barely detectable ASC oligomerization but failed to induce Caspase 1 activation or IL-1 $\beta$  secretion [134], suggesting that CASR is not sufficient for NLRP3 inflammasome activation. It is also possible that a critical threshold of calcium signaling was not reached by R568-CASR binding as peak cytosolic calcium concentration reached with R568 was nearly 5-fold less than with ATP [134].

Calcium mobilization or direct calcium binding usually leads to interaction of CASR with PLC at the plasma membrane [234]. Activated PLC hydrolyses phosphatidylinositol-4,5-bisphosphate into diacyl glycerol (DAG) and IP<sub>3</sub>. DAG activates protein kinase C (PKC) and IP<sub>3</sub> binds to IP<sub>3</sub>R in the ER to increase cytosolic calcium. The PLC inhibitors U73122 and the SERCA inhibitor XeC attenuated ATP-mediated Ca<sup>2+</sup> mobilization. Additionally, 2-APB, an inhibitor of store-operated calcium entry, a system for triggering extracellular calcium influx upon ER calcium depletion, also blocked calcium mobilization [126]. Edelfosine, another PLC inhibitor, U73122, XeC and 2-APB all also blocked ATP-induced Caspase 1 activation and IL-1 $\beta$  processing [126,127]. Knockdown of IP<sub>3</sub>R also had the same suppressive effect [127]. m-3M3FBS, a direct activator of PLC, caused IL-1 $\beta$  secretion in the absence of any other exogenous stimuli [127].

Interestingly, high extracellular potassium reduced ATP-mediated  $\text{Ca}^{2+}$  mobilization and U73122, XeC and 2-APB all reduced production of mitochondrial ROS, providing a link between  $\text{Ca}^{2+}$  mobilization and both of these NLRP3 inflammasome activation hypotheses [126].

CASR also interacted with and inhibited ADCY to reduce cellular cAMP levels. Increased cAMP levels, either through direct activation of ADCY and cAMP synthesis with NKH477 or forskolin or decreasing cAMP hydrolysis with PDE4 inhibitors Ro-20-1724, (R)-(-)-rolipram, or zardaverine, suppressed ATP-induced IL-1 $\beta$  secretion. The ADCY inhibitor KH7 induced dose-dependent secretion of IL-1 $\beta$  on its own in an NLRP3, ASC and Caspase 1-dependent manner. Spontaneous inflammasome activation was also observed in BMDM upon knockdown of several ADCYs found in macrophages including ADCY3, ADCY6, ADCY7 and ADCY9 [127].

cAMP does not influence inflammasome activation through protein kinase A (PKA), its usual pairing, as PKA inhibitors and knockdown of PRKACA caused no change in ATP-induced NLRP3 activation. Instead, cAMP interacted with endogenous NLRP3 in pull down experiments. This binding occurred in the nucleotide-binding domain of NLRP3 but did not interfere with ATP binding to NLRP3 [127].

Deletion of CHOP, a transcription factor that can regulate ER calcium release through the IP3R, reduced ATP-induced calcium mobilization and IL-1 $\beta$  secretion. Treatment with the SERCA inhibitor XeC that suppresses IP3R signaling did not reduce IL-1 $\beta$  secretion any further in CHOP deficient cells suggesting that these two changes are acting on the same pathway [126].

Activation of the NLRP3 inflammasome also triggered necrotic cell death, though the effects of calcium signaling on this process are far less well studied. While treatment of cells

with BAPTA-AM decreased ATP-induced IL-1 $\beta$  secretion, it had no effect on ATP-induced LDH release. Incubation in calcium-free media actually increased ATP-induced LDH release [120] and propidium iodide staining in BMDCs but not macrophages [134]. Treatment with thapsigargin had no effect on ATP-induced LDH release [120] but reversed increases in propidium iodide staining seen during stimulation in calcium-free media [134].

Nitric oxide (NO) is an important short-lived signaling molecules in the immune system. It also inhibited NLRP3 inflammasome activation. SNAP, an NO donor, markedly inhibited NLRP3 inflammasome activation in response to ATP without a significant effect on mitochondrial reactive oxygen species. NO may act directly on NLRP3 as S-nitrosylation of NLRP3 was detected in macrophages treated with SNAP [60]. Endogenous NO derived from iNOS also negatively regulated NLRP3 inflammasome activity. Depletion of iNOS caused increased accumulation of dysfunctional mitochondria in response to ATP. iNOS deficiency or pharmacological inhibition of NO production enhanced NLRP3-dependent cytokine production *in vivo* which was prevented by NLRP3 deficiency [235].

Treatment of macrophages with ATP induced PKR autophosphorylation and PKR disruption blocked IL-1 $\beta$  and IL-18 secretion as well as HMGB1 release, a marker of cell death. PKR interacted with numerous inflammasomes including NLRP3, NLRP1, NLRC4 and AIM2, suggesting a broad role in regulating inflammasome activity [189].

LPS and ATP stimulation induced deubiquitination of NLRP3. LPS-induced deubiquitination of NLRP3 was sensitive to treatment with NAC and Mito-TEMPO while ATP-induced deubiquitination was not [74].

A variety of other proteins contributed to the regulation of ATP-mediated NLRP3 inflammasome activation. mTORC1, a regulator of hexokinase 1 (HK1)-dependent glycolysis, controlled ATP-mediated activation of the NLRP3 inflammasome. Knockdown of mTORC1 or HK1 suppressed Caspase 1 activation and IL-1 $\beta$  secretion in response to ATP [236]. Acetylcholine, choline and phosphocholine signaling through the  $\alpha$ 7,  $\alpha$ 9, and  $\alpha$ 10 nicotinic acetylcholine receptors inhibited ATP-induced Caspase 1 activation and IL-1 $\beta$  with evidence suggesting they may have worked by preserving mitochondrial integrity [237,238]. Cellular FLICE-inhibitory protein (c-FLIP), an inhibitor of Caspase 8, also promoted Caspase 1 activation and IL-1 $\beta$  secretion in response to ATP. c-FLIP associated with the NLRP3 inflammasome and promoted NLRP3 mitochondrial localization [239].

Other proteins implicated in NLRP3 inflammasome regulation in response to diverse stimuli do not play a role in ATP-mediated NLRP3 regulation. For example, MAVS, the common mitochondrial adaptor for antiviral signaling, induced NLRP3 activation in response to poly(I:C) but did not play a role in response to ATP [221].

The physiologic significance of extracellular ATP-mediated NLRP3 inflammasome signaling is apparent when one considers that necrotic cells release ATP. Pressure-disrupted B16 mouse melanoma cells or complement-lysed 6F10 activated the NLRP3 inflammasome. When dying cells were fractionated, only isolated mitochondria were able to activate the NLRP3 inflammasome. P2RX7-deficient macrophages had significantly disrupted NLRP3 inflammasome activation in response to pressure-disrupted B16 cells, complement-lysed 6F10 cells and isolated mitochondria. However, this response was not disrupted entirely suggesting that other ligand-receptor pairs contributed [240]. Dying tumor cells released ATP that acted on P2RX7 in dendritic cells to activate the NLRP3 inflammasome. Interestingly, this has

consequences for chemotherapy as well. The tumoricidal activity of oxaliplatin was dramatically decreased in tumors established in P2RX7 deficient, NLRP3 deficient or Caspase 1/Caspase 11 deficient mice as compared to the parental mouse strain. Breast cancer patient data suggests these findings extend to humans as well. Anthracycline-treated breast cancer patients carrying a loss-of-function allele of P2RX7 developed metastatic disease more rapidly than individuals with normal alleles [241].

Extracellular ATP may also amplify the NLRP3 inflammasome response to crystalline molecules such as aluminum salt, silica, and uric acid. Crystalline-induced NLRP3 inflammasome activation is diminished in cells treated with purinergic receptor and connexin/pannexin channel inhibitors including A740003, carbenoxolone and flufenamic acid. Addition of less than 1mM ATP, ADP and UTP to BMDMs did not promote IL-1 $\beta$  secretion independently, but enhanced IL-1 $\beta$  secretion in response to silica and alum [242]. Calcium oxalate (CaOx) crystals, a common cause of kidney stones and nephropathy, induced renal tubular damage, cytokine secretion, neutrophil recruitment and renal failure in an NLRP3 inflammasome-dependent manner. CaOx-induced kidney damage was blocked by depletion of ATP with apyrase treatment, suggesting that ATP is important in amplifying the response to CaOx crystals [243]. Serum amyloid A (SAA) also formed particulate structures that activate the NLRP3 inflammasome. Similarly, inhibition of P2RX7 blocked SAA-induced IL-1 $\beta$  secretion [244].

Extracellular ATP has been implicated in triggering and amplifying inflammasome activation in response to a variety of pathogens. *Entamoeba histolytica* binding to  $\alpha 5\beta 1$  integrin caused NLRP3 to relocate into the intercellular junction. Activation of  $\alpha 5\beta 1$  integrin induced ATP release through Pannexin 1 into the extracellular space. There, ATP triggered P2RX7 leading

to NLRP3 inflammasome activation [245]. *Streptococcus pneumoniae* pore-forming toxin pneumolysin caused ATP-dependent IL-1 $\beta$  secretion. Inhibition of P2RX7 blocked pneumolysin-induced Caspase 1 activation and IL-1 $\beta$  secretion [246].

Complement C3a binding to C3aR caused IL-1 $\beta$  secretion from LPS-stimulated human macrophages and dendritic cells. C3a controlled release of intracellular ATP into the extracellular space in an ERK1/2-dependent fashion, that subsequently triggered NLRP3 inflammasome activation [247].

ATP is not always required and does not always amplify NLRP3 inflammasome activation. Heme activated the NLRP3 inflammasome in LPS-primed macrophages causing IL-1 $\beta$  secretion. This response required mitochondrial ROS and potassium efflux but was independent of ATP release and P2RX7 signaling [248].

Extracellular ATP has also been implicated in pulmonary fibrosis. Fibrotic patients have elevated ATP content in bronchoalveolar lavage (BAL) fluid as compared with non-fibrotic individuals. In mice, airway administration of bleomycin (BLM), a model of idiopathic pulmonary fibrosis, caused an early increase in ATP in BAL fluid. Apyrase treatment to degrade ATP reduced BLM-induced inflammatory cell recruitment and lung IL-1 $\beta$  while treatment with ATP $\gamma$ S, a non-hydrolyzable form of ATP, enhanced inflammation. BLM-induced inflammation depended on P2RX7 and Pannexin 1 [249].

ATP played a critical role in contact hypersensitivity (CHS), a type of allergic reaction. Mice lacking P2RX7 were resistant to CHS and failed to induce sensitization to contact allergens. Similarly, treatment of wildtype mice with P2RX7 antagonists, apyrase or the IL-1R

antagonist prevented CHS and exogenous IL-1 $\beta$  administration restored CHS in P2RX7 deficient mice [250].

Overall, the use of ATP as a model agonist for NLRP3 inflammasome activation and its direct relevance to numerous diseases has provided a large base of data to analyze ATP-induced NLRP3 inflammasome activation. Even with all we have to examine, though, there are still many gaps in our understanding of how ATP activates the NLRP3 inflammasome. The role for mitochondria is probably most well supported in the context of ATP stimulation than for any other agonist but the link between P2RX7 binding and mitochondrial damage is not clear. Mitochondria exchange potassium in the matrix with protons from the cytosol to concentrate a pool of protons for use in ATP synthesis [251]. Decreased extracellular potassium resulting from ATP stimulation may compromise mitochondrial respiration by limiting the exchange of potassium ions for protons and might be a connection worth investigating.

### **1.8.3 Monosodium urate disrupts lysosomes to trigger the NLRP3 inflammasome**

MSU is the last of the three classic NLRP3 agonists. MSU is a crystalline particulate NLRP3 agonist [196]; a class of agonists that also includes silica [83,108], asbestos [83], aluminum hydroxide [108,252,253], basic calcium phosphate crystals (octacalcium phosphate, carbonate-substituted apatite, and hydroxyapatite) [254], fibrillar amyloid-beta [231] and others. This section will primarily focus on MSU but will also touch on these other crystalline particulate molecules.

It has long been known that patients with gout have elevated serum uric acid. As such, the treatment for gout has largely focused on inhibiting the breakdown of purines to uric acid

with xanthine oxidase inhibitors, such as allopurinol, or increasing the excretion of uric acid with uricosurics, such as probenecid. The recent discovery that MSU triggers the NLRP3 inflammasome and promotes gouty inflammation through IL-1R signaling [196,255] has given patients with uncontrolled gout new options to reduce their inflammation and pain [256].

The importance of MSU-induced NLRP3 inflammasome activation likely extends beyond gout. Release of uric acid from dying mammalian cells is sensed broadly by the immune system leading to NLRP3 inflammasome activation, dendritic cell maturation, and CD8<sup>+</sup> T cell activation [257]. However, there is some debate on whether or not uric acid is a major contributor to this process [240,258].

MSU caused canonical Caspase 1 activation, IL-1 $\beta$  and IL-18 secretion and necrotic cell death and did not require Caspase 11 [51]. Particulate NLRP3 agonists are thought to act primarily through phagolysosome destabilization [108]. Inhibition of phagocytosis by blocking actin polymerization with cytochalasin D or latrunculin A caused a complete block of IL-1 $\beta$  secretion in particulate-stimulated phagocytes [83,108,254]. Blocking the H<sup>+</sup>-ATPase system, which is required for acidification of lysosomal compartments, with bafilomycin A blocked both formation of acidic lysosomes and prevented silica- and LLMe-induced IL-1 $\beta$  secretion [108,112]. Silica crystal treatment also activated CTSB, which was partly required for NLRP3 inflammasome activation. Interestingly, disruption of the lysosome using a rapid hypertonic to hypotonic media switch or the lysomotropic reagent LLMe was sufficient for causing NLRP3 inflammasome activation and IL-1 $\beta$  secretion [108]. Disruption of the lysosome by LLMe also activated TAK1-JNK signaling that was important for NLRP3 inflammasome activation, though this was discussed in more detail in a previous section on kinases [112].



It has been suggested that exposure of MSU to the acidic environment of the lysosome causes dissociation of sodium that places osmotic pressure on lysosomes. Inhibition of lysosomal acidification with CQ and NH<sub>4</sub>Cl, or inhibition of aquaporins (AQP) with HgCl<sub>2</sub> or phloretin, blocked MSU-induced IL-1 $\beta$  secretion. Similarly, knockdown of AQP0, AQP1, and AQP9 in THP1 cells resulted in decreased IL-1 $\beta$  secretion [259]. However, while sodium is a component of MSU, it is not typically associated with other particulate NLRP3 agonists. It would be important to test AQPs in the context of other particulate activators to better substantiate, or disprove, the osmotic pressure hypothesis.

Other studies have focused on the role of ROS in MSU-mediated NLRP3 inflammasome activation. THP1 cells treated with the antioxidants NAC or (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate (APDC) blocked MSU-induced IL-1 $\beta$  secretion [84,108,254], suggesting that ROS production was necessary for NLRP3 inflammasome activation. Loss of the NADPH oxidase subunit p22phox also led to decreased IL-1 $\beta$  secretion and loss of the ROS detoxifying protein TRX caused an increase [83]. This is in contrast to claims made by authors of another study that MSU treatment did not trigger respiratory oxidative burst as loss of gp91phox, a subunit of the phagosomal NADPH-oxidase cytochrome b, did not affect MSU-induced Caspase 1 or IL-1 $\beta$  secretion [108]. However, this data is presented in a manner that frustrates comparisons between WT and gp91Phox knockout cells. Attempts to compare IL-1 $\beta$  secretion between mouse strains (and between graphs) but within the same treatment group appears to suggest that loss of gp91phox actually increased IL-1 $\beta$  secretion. Gp91phox and p22phox are the two components of the NOX2 complex so different outcomes of knocking down these two proteins would be unexpected. Analysis of gp91phox was performed in BMDM from knockout mice and analysis

of p22phox was performed using shRNA in human THP1 cells, so it is certainly possible that the depletion strategy, cell type or species influenced the different outcomes.

A second study has further supported the role of the TRX system in NLRP3 inflammasome activation. TXNIP is triggered by ROS to dissociate from TRX and bind to NLRP3 in THP1 cells treated with MSU. Treatment with APDC to suppress ROS generation prevented dissociation of TRX and TXNIP and blocked association of TXNIP and NLRP3. Knockout of TXNIP in BMDMs or siRNA-depletion of TXNIP in THP1 cells suppressed MSU-induced Caspase 1 activation and IL-1 $\beta$  secretion. Similar results were observed in a model of MSU-induced peritonitis [94]. The exact mechanism leading from TXNIP and NLRP3 binding to NLRP3 inflammasome activation remains unclear.

A variety of kinases have also been shown to regulate MSU-induced NLRP3 inflammasome activation, though unfortunately without much integration of these findings with one another. Loss of DAPK in BMDM or THP1 cells led to a decrease in MSU-induced Caspase 1 activation and IL-1 $\beta$  secretion. DAPK deficiency also reduced neutrophil infiltrations and IL-1 $\beta$  secretion in a murine MSU-triggered peritonitis [132]. PKR is phosphorylated during exposure of BMDM to MSU. Knockout of PKR in BMDM eliminated MSU cytotoxicity and HMGB1 secretion, a marker of necrotic cell death [189]. NEK7, a kinase involved in cell cycle regulation, was also important in murine MSU-triggered peritonitis. Loss of NEK7 resulted in decreased peritoneal exudate cells, neutrophils and monocytes/macrophages in the peritoneum. The same result was seen in irradiated wildtype mice reconstituted with NEK7 deficient bone marrow treated with MSU. This decrease in infiltrating cells was accompanied by less IL-1 $\beta$  secretion and an improved clinical phenotype, reflecting less inflammation [188]. MSU treatment also induced phosphorylation of Syk and MSU-induced IL-1 $\beta$  secretion was blocked

by inhibition or genetic disruption of Syk [170]. However, MSU-induced phosphorylation of Syk in PMA-differentiated THP1 cells was not observed, suggesting a possible cell type or species difference [84]. The BTK inhibitor LFM-A13 and loss of BTK also prevented MSU-induced IL-1 $\beta$  secretion [169].

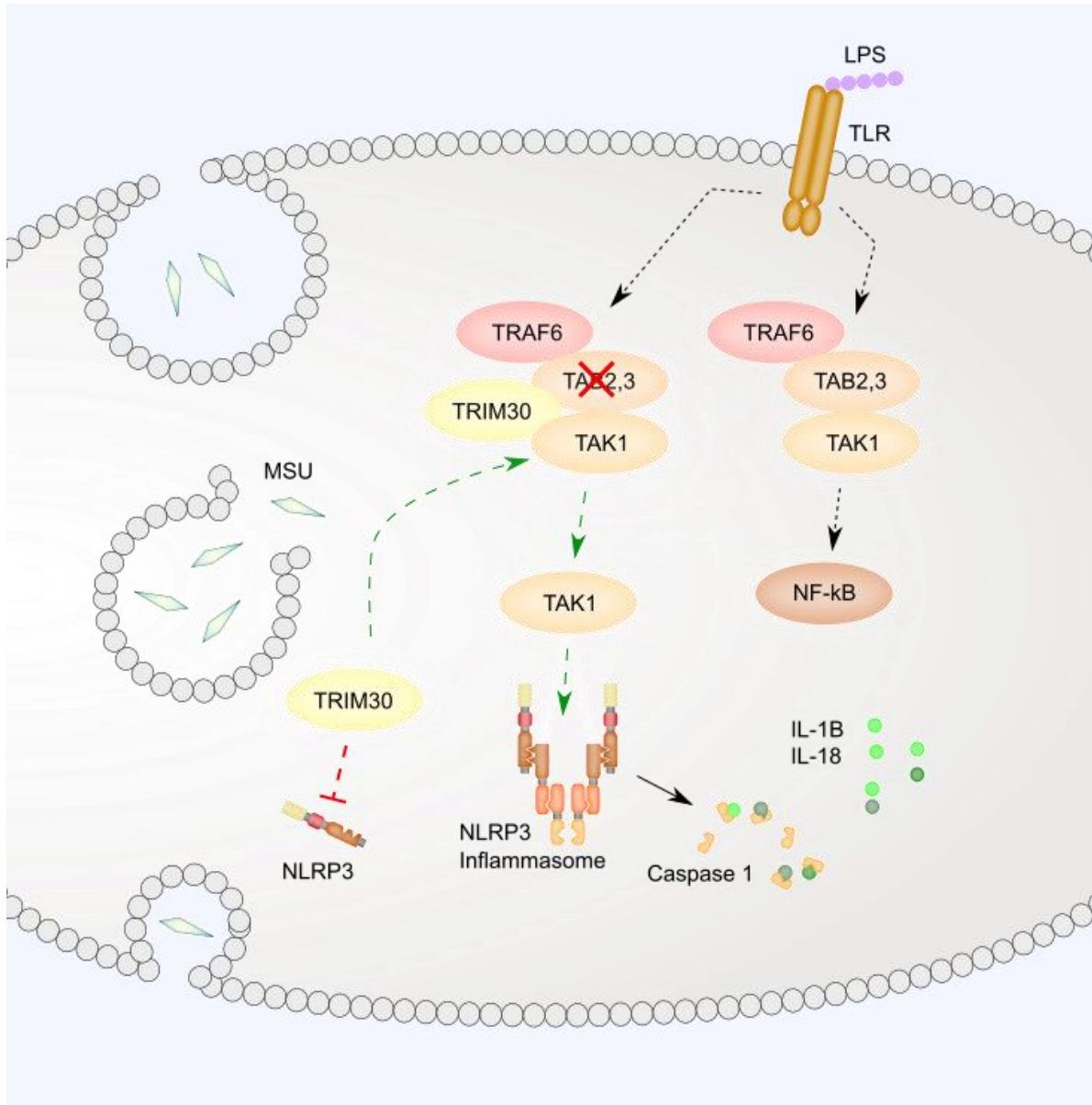
Some kinases that have been implicated in regulating the NLRP3 inflammasome in response to other stimuli, such as Lyn [84] and RIPK3 [178], have not been required for MSU-induced NLRP3 inflammasome activation.

One study utilized the human phospho-kinase array from R&D Systems to characterize MSU treatment of osteoblasts, a non-professional phagocyte. This screen is a semi-high throughput antibody based method of testing for kinase activation among a panel of 46 kinases. In this screen MSU treatment caused a significant increase in ERK1/2 T202/Y204, T185/Y187 phosphorylation and a significant decrease in TOR phosphorylation. A few other kinases were trending towards significance but unfortunately further investigation of these changes was focused on vacuole formation and not NLRP3 inflammasome activation [260]. At the very least, this study demonstrates that a high throughput approach can detect differences in kinase activity resulting from treatment with an NLRP3 agonist.

As with other NLRP3 agonists, a variety of other regulators have been identified in a piecemeal fashion. While there are clear opportunities to try to unify separate lines of evidence, the necessary experiments have not been carried out.

TRIM30 is one of the few negative regulators of MSU-induced NLRP3 inflammasome activation that has been identified. Knockdown of TRIM30 by siRNA caused increased IL-1 $\beta$  secretion in response to MSU challenge in J774 cells. Similarly, overexpression of TRIM30

(under control of the enhancer of the cytomegalovirus intermediate-early gene and the promoter of chicken beta-actin [261]) in murine MSU-induced peritonitis caused decrease neutrophil infiltration and IL-1 $\beta$  secretion [210]. A previous study showed that, in response to TLR agonists, TRIM30 interacted with TAK-1 binding protein 2 (TAB2), TAB3, and TAK1 and promoted degradation of TAB2 and TAB3. This was not blocked by inhibitors of the ubiquitin-proteasome pathway and TRIM30 could not undergo autoubiquitination, suggesting it lacks E3 ligase activity. Downregulation of TAB2 was blocked by NH<sub>4</sub>Cl and chloroquine, both inhibitors of lysosomal protein degradation [261]. TAB2 and TAB3 are required for activation of TAK1 by TRAF6 in TLR signaling [262,263]. Interestingly, this suggests that priming with TLR ligands is opposed by the pathway leading to NLRP3 inflammasome activation because TRIM30 promotes degradation of TAB2 and TAB3. However, TAK1 is activated in NLRP3 inflammasome signaling [112], so this shift may allow TAK1 to come under control of a second pathway responsible for NLRP3 activation.



**Figure 1.8. TLR signaling may shift TAK1 between priming and NLRP3 inflammasome roles.** TRIM30 is a negative regulator of the NLRP3 inflammasome. However, treatment of cells with LPS recruits TRIM30 to interact with TAB2, TAB3 and TAK1 leading to degradation of TAB2 and TAB3. TAB2 and TAB3 are required for TRAF6 to activate TAK1 in canonical TLR-NF-κB signaling. TRIM30-induced degradation of TAB2 and TAB3 may release TAK1 to activate the NLRP3 inflammasome, rather than NF-κB signaling.

PML is required for MSU-induced Caspase 1 activation and IL-1 $\beta$  secretion. Loss of PML also led to reduced CTSB activation in response to MSU [223]. BMDM from  $\beta$ -arrestin1, the G protein-coupled receptor signaling regulator, knockout mice showed decreased Caspase 1 activation, IL-1 $\beta$  secretion and LDH release in response to MSU. It was similarly important in MSU-induced peritonitis in these same mice, with loss of  $\beta$ -arrestin1 leading to decreased neutrophil infiltration and IL-1 $\beta$  secretion.  $\beta$ -arrestin1 interacted with NLRP3 when exogenously expressed in HEK293T cells and promoted NLRP3 self-oligomerization [215].

Interestingly, xanthine oxidase activity is rapidly increased in response to MSU and other crystalline activators leading to production of both uric acid and ROS. Uricase treatment had no effect on IL-1 $\beta$  secretion in this setting, suggesting that uric acid production was not responsible for downstream NLRP3 inflammasome activation. Instead, XOR activity increased PI3K-AKT-mTOR signaling and treatment with the XOR inhibitor febuxostat blocked phosphorylation of AKT and ROS production. Additionally, inhibitors of PI3K and AKT blocked MSU-induced IL-1 $\beta$  secretion [48].

The intracellular concentration of ATP has been implicated in NLRP3 inflammasome signaling. MSU treatment of murine BMDMs caused a significant decrease in intracellular ATP concentration that coincided in time with IL-1 $\beta$  secretion [211]. This loss of ATP might lead to mitochondrial dysfunction as mitochondrial respiration is increased to replenish the diminished ATP stores.

As briefly mentioned above, MSU has been a popular agonist to study because of its easy application to *in vivo* study in the murine MSU-induced peritonitis model and its direct role in gout. Below, we will examine a few studies focusing on *in vivo* effects of MSU.

Ventilator-induced lung injury (VILI) is thought to result from overly aggressive ventilation that causes mechanical stretch-induced inflammation and injury. In a recent study that modeled this cyclic stretching in isolated alveolar macrophages and in mice, the NLRP3 inflammasome was identified as the source of IL-1 $\beta$  production. While ROS contributed to NLRP3 inflammasome activation, gp91phox was not required as was seen above with MSU stimulation. Uric acid concentrations were vastly increased in the media after the cyclic stretch protocol. Treatment of alveolar macrophages with allopurinol diminished uric acid production, ROS generation and NLRP3 inflammasome activation. In a mouse model of VILI, high tidal volumes induced NLRP3 activation in a time dependent manner. This led to an increase in trans-alveolar protein permeability and lung edema. These consequences were reduced in mice pretreated with an IL-1 $\beta$  blocking antibody, supporting the NLRP3 etiology of these effects [264]. To better support the role for uric acid release in NLRP3 inflammasome activation *in vivo*, VILI should be assessed in mice treated with allopurinol, as was done for isolated macrophages, and uricase. Allopurinol inhibits XOR and might also reduce ROS independent of uric acid release, so uricase treatment would provide important supporting evidence by directly depleting uric acid levels.

Uric acid is liberated from hepatocytes during alcohol intoxication. This can be observed both in tissue culture and in human volunteers who when given alcohol experienced an increase in serum uric acid concentrations. This spike in uric acid concentration leads to increased IL-1 $\beta$  secretion in human PBMCs stimulated with hepatocyte conditioned media. The increase in IL-1 $\beta$  secretion in this setting was sensitive to uricase treatment, suggesting a causal role for uric acid. Most interestingly, NLRP3 knockout mice fed an alcohol containing diet to induce liver toxicity did not experience increased serum or liver IL-1 $\beta$  levels and were protected against liver damage

[265]. The authors try to connect the *in vitro* observation of ethanol-induced uric acid changes to the *in vivo* changes seen in ethanol fed mice. However, further experiments in mice to substantiate the role of uric acid are important to rule out a uric acid-independent effect of ethanol on the NLRP3 inflammasome. Even in this very same study, extracellular ATP was found to contribute to IL-1 $\beta$  secretion observed *in vitro* [265].

NLRP3 may not be the only inflammasome-like pathway activated by uric acid exposure. In a mouse model of radiation damage, radiation exposure led to cell damage and a local inflammatory response. Whole body radiation caused an increase in serum uric acid levels and activation of Caspase 1. In mice treated with allopurinol or Caspase 1 knockout mice, radiation induced immune cell loss was blunted, however, the effect, while statistically significant, was not dramatic. Unexpectedly, NLRP3 knockout mice subjected to whole body radiation experienced the same extent of Caspase 1 activation and immune cell loss as the parental strain mice. However, serum IL-1 $\beta$  was reduced in both Caspase 1 and NLRP3 knockout mice [266]. This study raises two important points. First, this study suggests that uric acid might be activating Caspase 1 in an NLRP3-independent manner. Since the discovery that MSU activates the NLRP3 inflammasome, most studies have relied on Caspase 1 activation and IL-1 $\beta$  secretion as their assay readouts, without confirming that the effect they are seeing was the result of NLRP3 inflammasome activation specifically. Second, the use of allopurinol to reduce uric acid might not be free from off-target effects. While this study tried to tie together radiation damage, increased serum uric acid, and allopurinol inhibition of Caspase 1 activation, the potential for a uric acid-induced NLRP3-independent inflammasome effect must further be addressed using additional methods to reduce uric acid.



## 1.9 Consequences of NLRP3 inflammasome signaling in pulmonary diseases

There is an increasing amount of research linking NLRP3 inflammasome signaling to outcomes for both infectious and non-infectious diseases in mouse models. In chapter 2, the discussion will focus on the interaction between *S. aureus* and host inflammasomes. In particular, the role of T<sub>H</sub>17 cells will be highlighted as a critical component of the immune response against *S. aureus* that depends on NLRP3-dependent IL-1 $\beta$  signaling. Additionally, a portion of the publication included in Appendix A will highlight the importance of the NLRP3 inflammasome to heart failure and how it might be targeted by novel drugs to improve heart failure outcomes.

Before concluding this introduction, I will briefly discuss the role of the NLRP3 inflammasome in lung diseases – a particular interest of mine – in order to highlight the wide ranging role of the NLRP3 in both infectious and non-infectious diseases. I will not comprehensively detail the pathophysiology of every disease discussed, I will only highlight recent reports on the role of the NLRP3 inflammasome. The reader is cautioned against reading too much into these results given the incomplete discussion of disease pathophysiology.

Additionally, since *Staphylococcus aureus* will be the focus of the body of this dissertation, it will be skipped over here and instead be included in Chapters 2, 3 and 4.

## **1.9.1 The NLRP3 inflammasome is a sentinel in pulmonary infections**

Bacteria, viruses and fungi can all infect upper and lower respiratory airways. While the NLRP3 inflammasome is activated in response to nearly every lung pathogen investigated, the consequence of that activation differs substantially.

### **1.9.1.1 NLRP3 inflammasome activation helps clear respiratory viruses**

In influenza infection, mice lacking NLRP3 and Caspase 1 were more susceptible than the parental strain, with approximately 60% of knockout mice from both groups dying after two weeks compared to only 30% from the parental strain. Knockout of NLRP3 or Caspase 1 had wide ranging depressive effects on cytokine production during influenza infection. Decreases were seen in IL-1 $\beta$ , IL-18, TNF- $\alpha$ , IFN- $\alpha$  IL-6, KC and MIP-2, demonstrating a cascade of immune signaling defects emanating from the defective NLRP3 inflammasome response. Additionally, lymphocyte chemotaxis was defective, as BAL fluid from infected knockout mice had significantly fewer neutrophils and monocytic dendritic cells than the infected parental strain mice. Surprisingly, these mice had no observed general defect in IgM, IgA or IgG production or CD8<sup>+</sup> T cell recruitment. They also had equivalent viral titers during the first week of infection. The increased mortality of the NLRP3 and Caspase 1 deficient mice appeared to result from uncontrolled pulmonary necrosis later in infection. Concentric layers of collagen were common in the distal airways and lung interstitium of both NLRP3 and Caspase 1 knockout mice and alveoli were occluded. This severely exacerbated lung pathology correlated with decreased blood oxygen partial pressure [267].

Elderly mice (15 months) infected with influenza experience a more severe disease course characterized by increased duration, higher viral burden, and higher mortality than in young (2-4 months) and middle-aged (8-9 months) mice. Elderly mice produce less IL-1 $\beta$  and IL-18 during the course of infection, suggesting that the NLRP3 inflammasome was compromised by aging in this setting. BMDCs demonstrated the same defect when infected *ex vivo*, and adoptive transfer of young dendritic cells to aged hosts partially rescued IL-1 $\beta$  secretion and improved their clinical progress, suggesting it is a cell intrinsic problem. Elderly influenza infected mice treated with nigericin have increased IL-1 $\beta$  secretion, decreased disease severity and dramatically improved survival [225]. Altogether, this data suggests that the ability of phagocytes sense influenza infections and transduce that signal into NLRP3 inflammasome activation is compromised, but NLRP3 inflammasome activation itself retains much of its youthful potential.

Respiratory syncytial virus (RSV) is a common cause of severe respiratory illness in children. NLRP3 inflammasome activation in response to RSV has been studied in tissue culture. NLRP3, ASC and Caspase 1 were all required for IL-1 $\beta$  secretion in response to RSV infection in BMDMs [268]. The RSV protein SH, a small hydrophobic protein that is classified as a viroporin and important for infectivity *in vivo* [269,270], is required for IL-1 $\beta$  secretion in infected lung epithelial cells as loss of SH completely eliminated the IL-1 $\beta$  response with no effect on IL-6 secretion [271]. However, the role of SH in activating the NLRP3 inflammasome has not been tested directly, nor has the role of NLRP3 been evaluated in the context of an actual respiratory RSV infection.

### **1.9.1.2 The NLRP3 inflammasome is differentially required for clearing extracellular and intracellular bacterial infections**

*Streptococcus pneumoniae* is one of the leading causes of pneumonia. Infection of mice with *S. pneumoniae* serotype 3 results in pneumonia typically without spreading to bacteremia [272]. NLRP3 deficient mice appeared to have a slight increase in infection severity with mortality generally occurring two days before the parental strain mice. The bacterial burden in the whole lung was slightly increased in NLRP3 deficient mice at 48 hours post infection, suggesting compromised control of bacteria. Surprisingly, no significant differences were recorded in BAL leukocytes or in IL-1 $\beta$ , IL-18, IL-10, IL-6, IFN-g or KC in BAL fluid. In this report, comparison of the concentration of serum albumin in BAL fluid to serum albumin in the serum was used as an indicator of damage to the lung vascular barrier. Mice deficient in NLRP3 had increased serum albumin in BAL fluid at 24 hours compared to the parental strain mice but both strains were equivalent at 48 hours. Additionally, dynamic lung compliance was compromised in NLRP3 deficient mice reflecting increased lung edema [273]. Overall, the contribution of NLRP3 to infection course was not outstanding. A subsequent study on serotypes 1 and 8 found that expression of non-hemolytic pneumolysin resulted in these serotypes being poor activators of IL-1 $\beta$  secretion [274]. This finding suggests that NLRP3 would be even less engaged in infections with these two serotypes, an important suggestion given that serotype 1 is the major cause of invasive disease worldwide.

*Klebsiella pneumoniae* most often occurs in patients with weakened immune systems, typically as a result of aspiration. It is characterized by production of “red-currant jelly” sputum and is extremely aggressive. Mice infected intratracheally with *Klebsiella pneumoniae* develop a severe pulmonary infection that reflects human infection. *Klebsiella* infected mice lacking

NLRP3 or ASC had an exacerbated disease course and worse outcomes than the parental strain infected mice. In contrast to what was seen with influenza, mice lacking NLRP3 had severely attenuated airway inflammation as seen by H&E staining. However, the bacterial burden measured in BAL fluid and lung homogenates was equivalent to the parental strain infected mice. As is expected, NLRP3 deficient mice produced less IL-1 $\beta$  and had a lower burden of necrosis *in vivo* but had increased levels of IL-6 [275].

*Mycobacterium tuberculosis* (*Mtb*) evades killing by alveolar macrophages to established infection of these cells. In tuberculosis, ESAT-6, a substrate of the ESX-1 secretion system implicated in membrane damage, was both necessary and sufficient for NLRP3-dependent Caspase 1 activation and IL-1 $\beta$  secretion [276]. Mice deficient in ASC, but not NLRP3 or Caspase 1, that were infected with aerosolized *Mtb* demonstrated a survival defect as compared to the parental strain mice but with no difference in bacterial burden or lung IL-1 $\beta$  detected. ASC deficient mice formed fewer granulomas and, despite a similar bacterial burden, were unable to contain bacteria within granulomas during chronic *Mtb* infection [277]. In a separate model for chronic tuberculosis, IFN- $\gamma$  activation of macrophages suppressed IL-1 $\beta$  production by specifically inhibiting the NLRP3 inflammasome. Loss of IFN- $\gamma$  led to increased IL-1 $\beta$  secretion, compromised infection control, increased neutrophil recruitment and tissue damage. IFN- $\gamma$  induced iNOS and stimulated NO production leading to S-nitrosylation of the NLRP3 [61]. Overall, for *Mtb*, this early research suggests that the adaptive immune response must suppress the NLRP3 inflammasome to progress to the chronic phase of infection. In the chronic phase, ASC is required for granuloma formation independent of its role in activating Caspase 1.

*Chlamydomphila pneumoniae* is an intracellular bacterium that causes atypical pneumonia. *C. pneumoniae*-infected macrophages secreted IL-1 $\beta$  and IL-18 that was almost completely

absent upon deletion of NLRP3 or ASC. Infection of mice with *C. pneumoniae* intranasally resulted in pneumonia. Infected mice deficient in IL-1R had increased TNF- $\alpha$ , IL-6, and IL-12 at day 3 of infection but these cytokines were equivalent to the parental strain by day 5. At day 3 and day 5, the infectious burden of both IL-1R deficient mice and the parental strain were equivalent, and nearly all mice cleared the infection by day 12. By histologic examination, IL-1R deficient mice had extensive proliferation of fibroblasts and decreased infiltration of neutrophils out to day 12. The IL-1R deficient mice had more significant fibrosis as observed by trichrome staining [278]. It would be additionally interesting to examine NLRP3 and IL-18 (or IL-18R) deficient mice in *Chlamydomphila pneumoniae*. Controlling this infection is known to depend heavily on IFN- $\gamma$  signaling [279] and IL-18 is an important IFN- $\gamma$ -inducing cytokine [280]. Early studies of *Chlamydomphila* suggested that IL-18 is critical for induction of IFN- $\gamma$  in response to infection too [281].

*Burkholderia pseudomallei* (previously known as *Pseudomonas pseudomallei*) is an intracellular bacterium that causes a lung infection called melioidosis endemic to southeast Asia and Australia. Mice deficient in ASC, Caspase 1, NLRC4 or NLRP3 were more susceptible to intranasal *B. pseudomallei* infection than the parental strain mice. Interestingly, NLRP3 deficient mice produced lower levels of IL-1 $\beta$  and IL-18, but NLRC4 deficient mice produced higher levels of IL-1 $\beta$  and IL-18. Cells from NLRC4 deficient mice did not undergo cell death and these mice had higher bacterial burdens whereas NLRP3 deficient mice were equivalent to the parental strain in bacterial burden. To determine how both enhanced and defective cytokine secretion could be detrimental, IL-1R and IL-18 knockout mice were examined. IL-1R knockout mice had improved survival during *B. pseudomallei* infection as compared to the parental strain whereas IL-18 knockout mice died extremely rapidly. Mice deficient in both IL-1R and IL-18 died at

similar rates to the IL-18 deficient mice, suggesting that IL-18 was a more significant determinant in infection outcomes. Daily injection of exogenous IFN- $\gamma$  in IL-18 deficient mice provided complete protection, demonstrating that IL-18 was important for induction of IFN- $\gamma$ . Surprisingly, daily injection of exogenous IL-1 receptor antagonist into wildtype mice protected mice from infection with a lethal dose of *B. pseudomallei*. The detrimental effect of IL-1 $\beta$  was the result of enhanced recruitment of neutrophils to the site of infection. Neutrophils did not undergo cell death in this setting and as such failed to limit replication of *B. pseudomallei*. Inhibition of neutrophil chemotaxis by injection of antileukinate, a CXCR2 antagonist, provided perfect protection in NLRC4 deficient mice [282]. The detailed work in this report nicely highlights how different inflammasomes can be activated in a single infection, and how the outcomes of inflammasome signaling – IL-1 $\beta$ , IL-18 and cell death – can each contribute to infection pathology or clearance in different ways.

### **1.9.1.3 The NLRP3 inflammasome responds to acute and chronic fungal infections**

Although numerous fungi have been shown to activate the NLRP3 inflammasome, little research has been conducted to investigate the importance of the NLRP3 inflammasome in fungal pulmonary infections. Fungal pneumonia is almost entirely found in immunocompromised patients and includes a wide variety of infections like histoplasmosis, coccidioidomycosis, pneumocystis pneumonia, cryptococcosis, aspergillosis, and more.

*Paracoccidioides brasiliensis*, the cause of paracoccidioidomycosis (PCM), activated the NLRP3 inflammasome leading to IL-1 $\beta$  and IL-18 secretion. NLRP3, ASC and Caspase 1 knockout mice were more susceptible to infection than the parental strain mice. Loss of

inflammasome function compromised control of fungal growth and led to higher fungal burdens in the course of infection. Loss of NLRP3 inflammasome components did not affect the appearance of granulomas but must have affected their function given the compromised fungal control. To study the downstream effects of NLRP3 inflammasome activation, IL-1R knockout mice and IL-18 knockout mice were studied. Loss of IL-18 mimicked the survival defect seen in NLRP3 inflammasome deficient mice whereas IL-1R knockout mice had similar mortality to the parental strain. Loss of IL-18 resulted in aggressive dissemination of the fungus as fungal counts were dramatically increased in the lung, liver and spleen. Loss of IL-18 resulted in decreased IFN- $\gamma$  production and injection of recombinant IL-18 improved infection control as seen by fungal burdens decreased to the level seen in the parental strain mice [283].

To model aspergillosis, a fungal pneumonia caused by *Aspergillus fumigatus*, mice were administered *A. fumigatus* conidia intranasally. These mice experienced a pulmonary infection characterized by pulmonary neutrophilia, histologic lung damage and upregulation of chemotactic cytokines CXCL1 and CXCL2. Administration of IL-37 intraperitoneally prior to infection resulted in decreased neutrophil influx and lung damage in a dose-dependent fashion. IL-37 decreased Caspase 1 activation and IL-1 $\beta$  production during aspergillosis, and also decreased mRNA levels of IFN-g, IL-17A, TNF- $\alpha$ , and IL-6. IL-10 was increased with IL-37 administration indicating that IL-37 tips the cytokine balance more towards an anti-inflammatory state. However, this anti-inflammatory state did not depend on IL-10 as the protective effect of IL-37 persisted in IL-10 deficient mice. To evaluate the importance of IL-37-dampened IL-1 $\beta$  signaling in aspergillosis, NLRP3 deficient mice were infected with *A. fumigatus*. NLRP3 deficient mice had decreased IL-1 $\beta$  production, as expected, and showed decreased signs of neutrophil infiltration. By histology, lungs of NLRP3 deficient mice had more well preserved



architecture and less inflammatory cell infiltrates than the parental strain infected mice. IL-37 had no additional benefit in NLRP3 deficient mice, suggesting that IL-37 downregulating NLRP3 activity is its primary mode of action. The protective effect of IL-37 required TIR-8/SIGIRR [284], a negative regulator of TLR signaling [285]. However, it is not clear from this study alone whether TIR-8 is responsible for reduced NLRP3 inflammasome signaling or simply acts in a complementary manner to suppress NLRP3 inflammasome signaling.

### **1.9.2 Chronic obstructive pulmonary disease is influenced by long term NLRP3 inflammasome stimulation**

Chronic obstructive pulmonary disease (COPD) is the most common chronic lung disease in the world. The primary cause of COPD is chronic exposure to tobacco smoke and pollution.

In order to study the role of acute inflammation in response to COPD-causing pollutants, mice were exposed to cigarette smoke for 3 days or 4 weeks and evaluated for pulmonary inflammation. Cigarette smoke caused a significant increase in IL-1 $\alpha$  and IL-1 $\beta$  production in the lungs of parental strain mice. The numbers of immune cells recovered in BAL fluid and in total lung homogenates, including neutrophils, macrophages, dendritic cells, and both CD4+ and CD8+ T cells, were all decreased in IL-1R knockout mice exposed to cigarette smoke as compared to the parental strain. Treatment of parental strain mice with neutralizing IL-1 $\alpha$  or IL-1 $\beta$  antibodies significantly reduced the recruitment of neutrophils to the lungs. Loss of NLRP3 or Caspase 1 had no effect on cigarette smoke-induced leukocyte infiltration of the lung or IL-1 $\alpha$  production, but completely eliminated IL-1 $\beta$  secretion [286]. The difference observed between an IL-1 $\beta$  neutralizing antibody, which decreased neutrophil infiltration, and depletion of NLRP3-

mediated production of IL-1 $\beta$ , which had no effect on neutrophil infiltration is a somewhat perplexing finding. It is possible that the level of IL-1 $\beta$  required for neutrophil infiltration is below the limit of detection of the assay used. It is also possible that IL-1 $\alpha$  production is increased to the extent necessary to compensate for the loss of IL-1 $\beta$  signaling at the IL-1R. To test this, the IL-1 $\alpha$  neutralizing antibody should be tested in NLRP3 knockout mice to see if it blocks neutrophil infiltration into the lung.

A functional mouse model of chronic cigarette smoke exposure, in which mice were exposed to cigarette smoke for up to 12 months, was also used to study the role of NLRP3 in COPD. Pulmonary function tests included small animal plethysmography, pleural pressure measurements, transpulmonary pressure calculations, and pressure-volume curve assessments. Parental strain mice exposed to cigarette smoke (COPD mice) had significantly increased respiratory system resistance after 10 and 12 months of cigarette smoke exposure, whereas the NLRP3 deficient mice had the same resistance as unexposed mice. Similarly, respiratory system compliance was significantly decreased in COPD mice, whereas the NLRP3 deficient mice mimicked the unexposed mice again. Immune cell infiltrates in the BAL fluid and IL-18 and IL-1 $\beta$  measured in the serum were nearly equivalent in unexposed and NLRP3 deficient mice and were significantly greater in COPD mice. Lung sections from COPD mice stained with H&E showed significant immune cell infiltration and destruction of normal alveolar architecture. Lung histology for the NLRP3 deficient mice was indistinguishable from control mice [287]. Unlike the rather acute exposure model seen above, this year long exposure showed a very significant role for the NLRP3 inflammasome in COPD. Although it is hard to say with complete certainty, this model probably more accurately reflects the physiologic experience of humans as COPD normally develops through decades of cigarette smoking or pollutant exposure.

BAL fluid was collected from never-smokers, asymptomatic smokers, and patients with COPD of different severity for measurement of extracellular ATP. ATP levels were increased in all smokers over never-smoker subjects and ATP levels in COPD patients were negatively correlated with lung function as measured by the amount of air forcefully expired in 1 second (FEV<sub>1</sub>). Some patients with severe COPD had an extracellular ATP concentration recovered in BAL fluid as high as 600 nmol/mL BAL fluid, whereas normal subjects were closer to 5 nmol/mL BAL fluid. Neutrophils and macrophages from patients with COPD were hyperresponsive to ATP stimulation, with neutrophils releasing more elastase and macrophages releasing more IL-1 $\beta$  and MMP-9 than healthy controls [288]. These data are very suggestive of NLRP3 inflammasome activation. If that is the case, the high extracellular ATP observed in COPD patients might be both the result of NLRP3 inflammasome activation and the cause, resulting in a positive feedback cycle that is exacerbated by smoke inhalation.

Although it would seem tempting to evaluate IL-1 $\beta$  disrupting therapies in patients with COPD, their increased risk for infection, even without cytokine antagonism, would be a major risk.

### **1.9.3 NLRP3 may contribute to airway hyperreactivity in asthma, allergy, obesity**

Airway hyperreactivity is a characteristic of asthma and other similar disorders in which airways are overly sensitive to a constrictor trigger. This can be tested for directly in a clinical or laboratory setting using methacholine challenge [289]. Although mild asthma is most commonly associated with eosinophil and mast cell infiltrates and histamine production, patients

with severe asthma have a predominantly neutrophil infiltrate that causes tissue damage [290]. Research studies have tried to model both conditions.

Segmental allergen challenge of patients with asthma caused a significant increase in extracellular ATP recovered in BAL fluid from the allergen challenged segment versus a saline challenge segment after 24 hours. The same increase was observed in mice sensitized with ovalbumin (OVA) and alum and challenged 10 days later with OVA. Twenty-four hours after the challenge, the concentration of ATP was increased in the BAL fluid of OVA- but not PBS-challenged mice. Administration of apyrase to the lung 30 minutes before OVA challenge greatly lowered the concentration of ATP in BAL fluid. This decrease was also accompanied by decreased inflammatory cell infiltrates and peribronchial inflammation. Inhibition of P2Rs with suramin prior to OVA challenge also decreased inflammatory cell infiltrates and peribronchial inflammation. To better appreciate the effect of extracellular ATP in this setting, ATP $\gamma$ S was administered to mice before OVA challenge. In this case, ATP $\gamma$ S dramatically increased inflammatory cell infiltrates and led to increased T<sub>H</sub>2 cytokine production, including IL-4, IL-5 and IL-13 [291]. Although this study did not measure NLRP3 inflammasome activation, it is important in that it established the abundance of an NLRP3 agonist in the OVA plus alum model of airway hypersensitivity.

Allergic lung inflammation can also be induced by OVA sensitization without an adjuvant for one week followed by intranasal challenge with OVA. This model leads to a predominantly eosinophilic infiltrate and production of T<sub>H</sub>2 cytokines. In NLRP3 deficient mice challenged with OVA, cell infiltrates recovered in BAL fluid were almost entirely absent as compared to parental strain mice challenged with OVA. NLRP3 deficient mice had a significant reduction in lung IL-1 $\beta$ , IL-6, IL-33, CCL5, CCL11, and CCL17, and BAL recovered IL-5.

CCL5 and CCL11 are known to play a role in eosinophil influx into the lung, and CCL17, IL5, and IL-33 are all T<sub>H</sub>2 cytokines. Cellular infiltrates in the lungs were evaluated by histology and confirmed that NLRP3 deficient mice had less cellular infiltration and less mucus production. OVA challenge in IL-1R knockout, IL-1 $\beta$  knockout and IL-1 $\alpha$  knockout mice showed the same results, that loss of IL-1 signaling decreased cellular infiltrates and T<sub>H</sub>2 cytokine production in this model. Increased levels of ATP were found in BAL fluid of OVA challenged mice, as was seen in the previous study that used OVA and alum sensitization, and may be the trigger for NLRP3 inflammasome activation [292].

However, another study of allergic airway disease in mice using alum-dependent and alum-independent OVA sensitization and acute and chronic house dust mite antigen exposure found no role for the NLRP3 inflammasome. Loss of NLRP3 did not impact inflammatory cell infiltration in BAL fluid or lung histology in any of the four sensitization modalities. It also did not impact airway resistance or tissue damping in the airways in response to methacholine [293]. This study points to the need for further investigation of NLRP3 in allergic airway diseases. Perhaps modifying factors exist, like the microbiome, as the authors of this study propose, that can explain the requirement for NLRP3 in previous studies and the lack in this one.

One possible explanation for how NLRP3 is able to so widely influence cytokine secretion comes from a study that has found NLRP3 acts separately from the inflammasome to activate transcription of a T<sub>H</sub>2 program. Differentiation of CD4<sup>+</sup> cells to a T<sub>H</sub>2 phenotype is normally accompanied by production of IL-4, IL-5, and IL-13. In NLRP3 deficient T cells, production of these three T<sub>H</sub>2 cytokines and more were dramatically decreased. T cell IL-2 induced STAT5-mediated NLRP3 mRNA transcription, as blocking IL-2 with an antibody or siRNA-mediated depletion of STAT5 prevented upregulation of NLRP3 transcripts. Loss of

ASC and Caspase 1 did not reproduce the defective cytokine secretion seen in T<sub>H</sub>2 cells, suggesting that NLRP3 was acting outside of its role in the inflammasome. Interestingly, in chromatin immunoprecipitation experiments, NLRP3 precipitated with hundreds of regions of DNA associated with transcription of T<sub>H</sub>2 phenotype genes including IL-4, IL-5 and IL-13. NLRP3 bound to a consensus motif of nGRRGGnRGAG, where 'n' is any nucleotide and 'R' is any purine. NLRP3 interacted with IRF4 to promote binding to T<sub>H</sub>2 promoters including IL-4 [294].

From these data, it is apparent that NLRP3 directly influenced T<sub>H</sub>2 cell fate. Turning back to airway hyperreactivity, loss of NLRP3 in a model of OVA sensitization without alum followed by OVA challenge, as above, led to decreased leukocyte infiltration in the lungs and decreased mucus production. These effects were not observed in ASC and Caspase 1 deficient mice, suggesting that NLRP3 was acting outside of its role in the inflammasome. NLRP3 deficient mice challenged with OVA produced less IL-4 and IL-5 than the parental strain mice. Transfer of NLRP3-containing T cells into NLRP3 deficient mice restored the OVA-induced airway inflammation and IL-4 and IL-5 production to the same level seen in parental strain mice [294]. This study is really quite stunning. Even separate from the compelling analysis of allergic airway disease, this study further complicates the interpretation of NLRP3 deletion studies in whole animal models by demonstrating a diminished differentiation of T<sub>H</sub>2 cells in NLRP3 deficient animals. This study points to a critical need for development of lineage specific knockouts of NLRP3 and a more thorough investigation of NLRP3 beyond its role in the NLRP3 inflammasome.

Obesity is often associated with the development of asthma. As such, mice fed a high-fat diet for 12 weeks became obese and developed airway hyperreactivity. Knockout of Rag1, a

protein required for differentiation of B and T cells, had no effect on the development of obesity-associated airway hyperreactivity. Instead, loss of IL-17 prevented the mice from developing airway hyperreactivity even in the context of obesity. Similarly, deletion of NLRP3 actually worsened obesity but protected mice from developing airway hyperreactivity. Parental strain mice with airway hyperreactivity had increased IL-1 $\beta$ , IL-6 and IL-23a mRNA in both adipose tissue and the lung. In these mice, IL-1 $\beta$  promoted development of innate lymphoid cells (ILC3 cells) that produced IL-17A which was critical for airway hyperreactivity. Treatment of obese parental strain mice with the IL-1R antagonist prevented development of ILC3 cells and prevented airway hyperreactivity. Administration of IL-1 $\beta$  into Rag2 deficient mice had the opposite effect, leading to robust airway hyperreactivity [295].

Serum amyloid A (SAA) is increased in subjects with severe allergic asthma. Saa3 is expressed in the lungs of mice exposed to several mixed T<sub>H</sub>2/T<sub>H</sub>17-polarizing allergic sensitization regimens. SAA drove production of multiple pro-inflammatory cytokines and promoted NLRP3-dependent secretion of IL-1 $\beta$  by dendritic cells and macrophages. This IL-1 $\beta$  triggered naive CD4<sup>+</sup> T cells to produce IL-17. On its own, SAA acted as an adjuvant to sensitize mice to inhaled OVA, but in IL-1R or NLRP3 deficient cells, it did not induce production of IL-17 or recruitment of eosinophils to the lung [296].

Overall, the variety of models used in these studies of allergic airway disease make understanding the relevance of their discoveries to human health more complicated. Each proposes to more accurately mirror the physiology seen in the human condition, but without any direct interventional studies in humans to corroborate these findings, it is hard to differentiate cause from effect. The NLRP3 inflammasome seems to play an interesting role in IL-17-mediated allergic airway disease but its role in T<sub>H</sub>2 oriented diseases is still an open question.

The possibility of NLRP3 acting outside of the context of the inflammasome to influence the T<sub>H</sub>2 program is also an important discovery that needs additional follow up and may have widespread implications in other T<sub>H</sub>2-driven diseases such as atopy.

#### **1.9.4 The NLRP3 inflammasome is critically activated in acute lung injury**

Acute lung injury (ALI) can result from a wide variety of different causes including hyperoxia, mechanical ventilation/stretch and more. ALI is characterized by extravasation of protein into alveoli resulting in high protein BAL fluid, pulmonary edema, and immune cell infiltration.

NLRP3 deficient and parental strain mice were exposed to 100% O<sub>2</sub> for 48-72 hours to induce hyperoxic acute lung injury (HALI). Mice lacking NLRP3 had improved pathological scores indicating less damage to the lung resulting from hyperoxia. Recruitment of inflammatory cells, including macrophages and neutrophils, and secretion of IL-1 $\beta$ , TNF- $\alpha$  and MIP-2, but not IL-6, were all decreased in NLRP3 deficient mice as compared to the parental strain [297]. This hyperoxic damage was thought to cause ROS production leading to NLRP3 inflammasome activation, however, at this point, that is not well established.

P2RX7 knockout mice and the parental strain were exposed to 100% oxygen for 72 hours. Knockout of P2RX7 increased survival during this trial. Hyperoxia caused an increase in BAL protein and infiltration of macrophages and neutrophils. Knockout of P2RX7 protected mice from hyperoxia-induced macrophage and neutrophil infiltration. Interestingly, IL-1 $\beta$  levels were substantially decreased in the P2RX7 mice as compared to the parental strain after hyperoxia. In cells exposed to hyperoxia *ex vivo*, P2RX7 exhibited substantially decreased



Caspase 1 activation, suggesting that hyperoxia caused release of ATP that triggered the NLRP3 inflammasome [298]. Unfortunately, as we have seen in many other studies that suggest a role for extracellular ATP in activating the NLRP3 inflammasome, apyrase should have been used to definitively diminish extracellular ATP levels in the parental strain mice.

While NLRP3 mediates HALI, PTEN-induced putative kinase 1 (PINK1), an initiator of mitophagy, mediates protection from HALI. First, NLRP3, ASC and Caspase 1 deficient mice were confirmed to have improved survival after HALI as compared to the parental strain mice. Loss of inflammasome components resulted in decreased BAL fluid protein, immune cell and IL-1 $\beta$  recovery. NLRP3 deficient mice had increased PINK1 expression, both at baseline and following HALI as compared to the parental strain mice. Deletion of PINK1 sensitized mice to HALI, including worsening BAL parameters such as protein recovery, and even reversed the improved survival seen in NLRP3 deficient mice suggesting that PINK1 is critical for protection against HALI. Specific knockdown of PINK1 in lung endothelial cells had the same effect as global knockout [299]. As has been seen in many *in vitro* studies, functional autophagy and mitochondrial repair/recycling was critical for avoiding NLRP3 inflammasome activation. When PINK1 was deleted producing a defect in mitophagy, HALI likely over activated the NLRP3 inflammasome, leading to greater cell death than see in the parental strain mice.

In a separate model of ALI induced by administration of LPS intratracheally, the NLRP3 inflammasome was required for both albumin and IL-1 $\beta$  recovery in BAL fluid. Additionally, loss of NLRP3 or Caspase 1 diminished infiltration of neutrophils into the lungs. Depletion of macrophages by clodronate liposomes or neutrophils by treatment with a neutrophil depleting antibody limited IL-1 $\beta$  production in this model of ALI. Although neutrophils did not produce IL-1 $\beta$  when stimulated *ex vivo*, neutrophils were a source of extracellular histones that stimulated

NLRP3-dependent IL-1 $\beta$  secretion from macrophages. Histone release during ALI required both NLRP3 and Caspase 1, suggesting that it resulted from pyroptotic cell death [233].

Inhibition of P2RX7 with A438079 also blocked LPS-induced ALI in mice. A438079 decreased neutrophil and protein recovery in BAL fluid of LPS treated mice. LPS-induced ALI resulted in IL-1 $\beta$ , IFN- $\gamma$  and IL-17A production in lung tissue. Blocking P2RX7 with A438079 decreased each of these cytokines, but had no effect on IL-10 secretion [300]. As before, while this picture of P2RX7-mediated production of IL-1 $\beta$  resembled activation of the NLRP3 inflammasome, without direct experimental evidence we cannot be certain.

Vimentin, an intermediate filament and component of the cytoskeleton is required for LPS-induced ALI in mice. Deletion of Vimentin improved survival, preserved alveolar architecture by lung histology, decreased lung edema, decreased protein content in BAL fluid and interestingly decreased Caspase 1 and IL-1 $\beta$  production. Alveolar macrophages from vimentin deficient mice exhibited a significant defect in NLRP3 inflammasome activation in response to LPS plus ATP, asbestos or MSU challenge *ex vivo*. Vimentin co-precipitated with both NLRP3 and Caspase 1 from human macrophage cell lysates and this association was enhanced following stimulation of cells with ATP [301]. These findings suggest that the NLRP3 inflammasome is regulated by the cytoskeletal intermediate filament vimentin that potentially acts as a scaffold for NLRP3 inflammasome assembly.

Mice receiving high tidal volume (HV) ventilation experience ventilator-induced lung injury, just as humans do. Treatment with HV ventilation liberated more uric acid and IL-1 $\beta$  than treatment with low tidal volume (LV) ventilation. NLRP3 knockout mice exposed to HV ventilation experience less lung edema, protein in the BAL fluid, neutrophil infiltration, IL-1 $\beta$

and IL-6 secretion than the parental strain, suggesting a role for NLRP3 in mediating HV ventilation lung damage. ASC knockout mice had less edema and neutrophil infiltration, but equivalent protein recovery and IL-1 $\beta$  secretion, an unexpected finding that might suggest another ASC-independent inflammasome compensates for the loss of ASC [302].

Depletion of alveolar macrophages by administration of clodronate liposomes largely attenuated HV ventilation lung damage as measured by neutrophils and protein recovered in BAL fluid and by histology. Injection of BMDMs prior to the start of HV ventilation restored the pre-depletion phenotype. Interestingly, treatment of BMDMs with siRNA targeting ATG5 to block autophagy prior to reconstituting mice resulted in decreased BAL fluid neutrophil and protein recovery following HV ventilation. Immunoprecipitation of NLRP3 and co-precipitation of ASC and Caspase 1 was used to assess inflammasome activation following HV ventilation. Mice depleted of macrophages, or reconstituted with siATG5 treated macrophages had significantly less ASC and Caspase 1 associated with NLRP3 than mice that were not treated with clodronate liposomes or reconstituted with BMDMs receiving a scrambled siRNA [303]. Overall, this study suggests that the NLRP3 inflammasome is being activated in mice during HV ventilation and correlates with the severity of lung damage. This study is unusual in that it suggested blocking autophagy decreased, rather than increased NLRP3 inflammasome activation.

Mouse alveolar macrophages that undergo a cyclic stretching (CS) protocol *ex vivo* experienced significant cell death and IL-1 $\beta$  secretion. CS macrophage IL-1 $\beta$  production was Caspase 1 dependent, as it was blocked by the Caspase 1 inhibitor ac-YVAD-fmk and siRNA against Caspase 1. Depletion of NLRP3 by siRNA also greatly diminished CS-induced IL-1 $\beta$  production. NLRP3 activation appeared to be the result of mitochondrial ROS production as

treatment with rotenone exacerbated IL-1 $\beta$  production and treatment with SS-31, a ROS scavenger, significantly reduced IL-1 $\beta$  production. Similar results were seen in whole mouse lungs. Pretreatment with SS-31 or depletion of macrophages by liposomes diminished IL-1 $\beta$  production resulting from HV ventilation [264].

Overall, the NLRP3 inflammasome appears sensitive to both mechanical-induced ventilator lung damage and hyperoxia-induced lung damage. However, these data would be more convincing if other inflammasomes were used as controls. In some instances, discrepancy between NLRP3 and ASC knockout mice in the severity of lung damage raises important questions about the involvement of other, non-ASC-containing inflammasome.

### **1.9.5 The NLRP3 inflammasome drives fibrotic changes in pulmonary fibrosis**

Pulmonary fibrosis occurs through widespread scarring in the lungs that stiffens lung tissue and increases the work of breathing. In most instances, the exact cause of pulmonary fibrosis is difficult to determine, resulting in classification as idiopathic pulmonary fibrosis. Without a clear cause, accurately recapitulating the mechanism of action in a mouse model is challenging, but many studies have focused in on bleomycin (BLM)-induced pulmonary fibrosis. BLM induces DNA strand breaks. Patients who receive BLM are at higher risk for pulmonary fibrosis and are cautioned against breathing high concentrations of oxygen, such as during some anesthesiology regimens, as it is believed that BLM treatment induces sensitivity to oxygen toxicity.

Patients with pulmonary fibrosis had increased ATP in BAL fluid as compared to healthy controls. Isolated lung epithelial cells from both human and mice released significant amounts of

ATP when challenged with BLM. In mice, administration of BLM led to an early increase in extracellular ATP, IL-1 $\beta$  production and neutrophil infiltration. Treatment of mice with apyrase opposed the changes seen with BLM, leading to less neutrophil infiltration and less IL-1 $\beta$  production. ATP $\gamma$ S treatment further exacerbated damage caused by BLM. The P2R family inhibitor suramin further implicated ATP in that blocking P2Rs similarly decreased neutrophil infiltration and IL-1 $\beta$  production in BLM-treated mice. Knockout of P2RX7 caused a significant reduction in these inflammatory markers as well, that is not further exacerbated by ATP $\gamma$ S administration. P2RX7 deficient mice had an improved clinical course and did not lose as much weight as the parental strain mice when treated with BLM. By histology, loss of P2RX7 reduced inflammatory cell infiltration and production of collagen in the lung [249]. Again, this pattern is highly suggestive of NLRP3 inflammasome-mediated IL-1 $\beta$  production, but without testing it directly, we are left with too many confounding possibilities to know for certain.

A subsequent study from this same group identified IL-1 $\beta$ , IL-23p19 and IL-17A as critical inflammatory mediators in BLM-induced pulmonary fibrosis. Administration of IL-1 $\beta$  without BLM caused a significant increase in IL-23p19 and IL-17A production and loss of the IL-1R diminished IL-17A production during BLM administration. Mice lacking IL-23p19 or IL-17A receptor experienced significantly less neutrophil infiltration into the lung during BLM exposure and treatment with an anti-IL-17A neutralizing antibody, but not an anti-IL-17F neutralizing antibody, had the same effect.  $\gamma\delta$ -T cells were determined by flow cytometry to be the primary source of IL-17A production in this setting. Loss of IL-23p19, IL-17A or treatment with a IL-17A neutralizing antibody all dramatically improved lung architecture by histology [304]. Combined IL-17A and IL-23p19 knockouts would have been interesting to examine in this setting. IL-23p19 is thought to upregulate matrix metalloprotease MMP9 and IL-17A is a

proinflammatory cytokine involved in neutrophil recruitment, so loss of both cytokines together may have had a complementary effect.

As was seen above with LPS-induced ALI, Vimentin was also required for BLM-induced pulmonary fibrosis in mice. Deletion of Vimentin preserved alveolar architecture by lung histology, decreased lung edema, decreased protein content in BAL fluid and, as seen in ALI, decreased Caspase 1 and IL-1 $\beta$  production. In the absence of vimentin, BLM-induced collagen deposition was significantly decreased as seen by Masson's trichrome and Picro-Sirius red stains for collagen. Mice also had significantly improved lung elasticity as measured by quasi-static compliance and elastic modulus testing by atomic force microscopy [301]. These findings suggest that the NLRP3 inflammasome is regulated by the cytoskeletal intermediate filament Vimentin and that disruption of this interaction can dramatically improve clinical measures of BLM-induced fibrosis.

Epidemiologic evidence suggests that statin use could increase risk for development of interstitial lung disease, a type of pulmonary fibrosis. In mice treated with BLM, co-administration of a statin increased markers of lung inflammation and fibrotic change, including IL-1 $\beta$  and IL-18 production and destruction of alveolar architecture as determined by histology. In isolated macrophages, treatment with pravastatin enhanced NLRP3 inflammasome activation in response to LPS plus ATP challenge as measured by Caspase 1 activation, IL-1 $\beta$  and IL-18 secretion. Deletion of NLRP3 or Caspase 1 completely eliminated secretion of these cytokines. Statin pretreatment was thought to increase mitochondrial ROS production, leading to exacerbation of NLRP3 inflammasome activity [305]. In light of the difficulty in identifying a cause of pulmonary fibrosis in patients, this study is particularly interesting. It suggests that even commonly used medications might contribute to pulmonary fibrosis in the right setting. Further

investigation of modifying factors, particularly with a focus on ROS production, might prove fruitful in identifying individuals at risk for development of pulmonary fibrosis or might provide a point of intervention to limit disease progression.

### **1.9.6 NLRP3 inflammasome signaling may predict risk of bronchopulmonary dysplasia in preterm infants**

Lastly, the NLRP3 inflammasome played a major role in the development of bronchopulmonary dysplasia (BPD). BPD is a chronic lung disease that affects newborns and is thought to result from damage to the lungs from mechanical ventilation and long-term oxygen supplementation. Hyperoxia-exposed neonatal mice (BPD mice) developed lung damage characterized by larger alveolar spaces and simplification of distal lung architecture. In mice deficient of NLRP3, alveoli were well formed and more closely approximated normoxia exposed mice. BPD mice had significantly increased pulmonary IL-1 $\beta$  production that was completely absent with loss of NLRP3, suggesting a critical role for the NLRP3 inflammasome in BPD pathogenesis. Treatment with IL-1R antagonist every other day during hyperoxic exposure dramatically improved mouse lung histology and more closely mirrored the lung architecture of NLRP3 deficient mice and normoxia mice indicating that NLRP3-dependent IL-1 $\beta$  signaling is critical. Interestingly, in preterm infants intubated for respiratory failure, tracheal aspirate IL-1 $\beta$  content was negatively correlated with gestational age, with the most preterm infants having the highest levels of IL-1 $\beta$ . Decreasing gestational age is an important risk factor for BPD. IL-1 $\beta$  content was also a predictor of death or BPD development in preterm infants and improved predictions made on the basis of gestational age alone [306].

Together, these data are very suggestive of a role for the NLRP3 inflammasome in the development and severity of BPD. Being able to predict which infants are at greatest risk of BPD is an important step for ultimately targeting preventive measures to the right patients. One could imagine that these patients would benefit from more tightly controlled respiratory settings or IL-1 $\beta$ -targeted therapy. It would be interesting to see if therapeutic intervention in mice that develop a BPD-like phenotype could reverse disease course and improve survival or long-term lung function. While treating preterm infants, who are already at increased risk for infections, with anti-cytokine therapy would be risky, a small bump in the right direction might provide a sufficient benefit to diminish the effects of BPD.

## **1.10 Concluding introduction**

Throughout this introduction we examined the role of NLRP3 in heritable fever disorders, the complex system of regulation of NLRP3 inflammasome activation both by regulatory modality and agonist modality, and have seen the widespread significance of NLRP3 in the pathogenesis of pulmonary diseases. Now, our focus will turn to the interaction between *Staphylococcus aureus* and inflammasomes generally. Following that review, we will turn to my dissertation research focused on elucidating the mechanism of *S. aureus* LukAB cytotoxicity and identifying critical kinases in mediating NLRP3 inflammasome activation in response to LukAB.



## REFERENCES

1. Ting JP, Lovering RC, Alnemri ES, Bertin J, Boss JM, et al. (2008) The NLR gene family: a standard nomenclature. *Immunity* 28: 285-287.
2. Mariathasan S, Newton K, Monack DM, Vucic D, French DM, et al. (2004) Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 430: 213-218.
3. Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD (2001) Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. *Nat Genet* 29: 301-305.
4. Feldmann J, Prieur AM, Quartier P, Berquin P, Certain S, et al. (2002) Chronic infantile neurological cutaneous and articular syndrome is caused by mutations in CIAS1, a gene highly expressed in polymorphonuclear cells and chondrocytes. *Am J Hum Genet* 71: 198-203.
5. Dode C, Le Du N, Cuisset L, Letourneur F, Berthelot JM, et al. (2002) New mutations of CIAS1 that are responsible for Muckle-Wells syndrome and familial cold urticaria: a novel mutation underlies both syndromes. *Am J Hum Genet* 70: 1498-1506.
6. Witherspoon FG, White CB, et al. (1948) Familial urticaria due to cold. *Arch Derm Syphilol* 58: 52-55.
7. Doeglas HM (1973) Familial cold urticaria. *Arch Dermatol* 107: 136-137.
8. Derbes VJ, Coleman WP (1972) Familial cold urticaria. *Ann Allergy* 30: 335-341.
9. Muckle TJ, Wellsm (1962) Urticaria, deafness, and amyloidosis: a new heredo-familial syndrome. *Q J Med* 31: 235-248.
10. Black JT (1969) Amyloidosis, deafness, urticaria, and limb pains: a hereditary syndrome. *Ann Intern Med* 70: 989-994.
11. Prieur AM, Griscelli C (1981) Arthropathy with rash, chronic meningitis, eye lesions, and mental retardation. *J Pediatr* 99: 79-83.
12. Hassink SG, Goldsmith DP (1983) Neonatal onset multisystem inflammatory disease. *Arthritis Rheum* 26: 668-673.
13. Torbiak RP, Dent PB, Cockshott WP (1989) NOMID--a neonatal syndrome of multisystem inflammation. *Skeletal Radiol* 18: 359-364.
14. Manji GA, Wang L, Geddes BJ, Brown M, Merriam S, et al. (2002) PYPAF1, a PYRIN-containing Apaf1-like protein that assembles with ASC and regulates activation of NF-kappa B. *J Biol Chem* 277: 11570-11575.

15. Wang L, Manji GA, Grenier JM, Al-Garawi A, Merriam S, et al. (2002) PYPAF7, a novel PYRIN-containing Apaf1-like protein that regulates activation of NF-kappa B and caspase-1-dependent cytokine processing. *J Biol Chem* 277: 29874-29880.
16. Agostini L, Martinon F, Burns K, McDermott MF, Hawkins PN, et al. (2004) NALP3 forms an IL-1 $\beta$ -processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* 20: 319-325.
17. Dowds TA, Masumoto J, Zhu L, Inohara N, Nunez G (2004) Cryopyrin-induced interleukin 1 $\beta$  secretion in monocytic cells: enhanced activity of disease-associated mutants and requirement for ASC. *J Biol Chem* 279: 21924-21928.
18. Rosengren S, Mueller JL, Anderson JP, Niehaus BL, Misaghi A, et al. (2007) Monocytes from familial cold autoinflammatory syndrome patients are activated by mild hypothermia. *J Allergy Clin Immunol* 119: 991-996.
19. Bonar SL, Brydges SD, Mueller JL, McGeough MD, Pena C, et al. (2012) Constitutively activated NLRP3 inflammasome causes inflammation and abnormal skeletal development in mice. *PLoS One* 7: e35979.
20. Hawkins PN, Lachmann HJ, McDermott MF (2003) Interleukin-1-receptor antagonist in the Muckle-Wells syndrome. *N Engl J Med* 348: 2583-2584.
21. Ter Haar N, Lachmann H, Ozen S, Woo P, Uziel Y, et al. (2013) Treatment of autoinflammatory diseases: results from the Eurofever Registry and a literature review. *Ann Rheum Dis* 72: 678-685.
22. Hoffman HM, Rosengren S, Boyle DL, Cho JY, Nayar J, et al. (2004) Prevention of cold-associated acute inflammation in familial cold autoinflammatory syndrome by interleukin-1 receptor antagonist. *Lancet* 364: 1779-1785.
23. Goldbach-Mansky R, Dailey NJ, Canna SW, Gelabert A, Jones J, et al. (2006) Neonatal-onset multisystem inflammatory disease responsive to interleukin-1 $\beta$  inhibition. *N Engl J Med* 355: 581-592.
24. ter Haar NM, Oswald M, Jeyaratnam J, Anton J, Barron KS, et al. (2015) Recommendations for the management of autoinflammatory diseases. *Ann Rheum Dis* 74: 1636-1644.
25. Hoffman HM, Throne ML, Amar NJ, Sebai M, Kivitz AJ, et al. (2008) Efficacy and safety of riloncept (interleukin-1 Trap) in patients with cryopyrin-associated periodic syndromes: results from two sequential placebo-controlled studies. *Arthritis Rheum* 58: 2443-2452.
26. Goldbach-Mansky R, Shroff SD, Wilson M, Snyder C, Plehn S, et al. (2008) A pilot study to evaluate the safety and efficacy of the long-acting interleukin-1 inhibitor riloncept (interleukin-1 Trap) in patients with familial cold autoinflammatory syndrome. *Arthritis Rheum* 58: 2432-2442.
27. Lachmann HJ, Kone-Paut I, Kuemmerle-Deschner JB, Leslie KS, Hachulla E, et al. (2009) Use of canakinumab in the cryopyrin-associated periodic syndrome. *N Engl J Med* 360: 2416-2425.

28. Leung VC, Lee KE (1985) Infantile cortical hyperostosis with intramedullary lesions. *J Pediatr Orthop* 5: 354-357.
29. Ivker RA, Grin-Jorgensen CM, Vega VK, Hoss DM, Grant-Kels JM (1993) Infantile generalized pustular psoriasis associated with lytic lesions of the bone. *Pediatr Dermatol* 10: 277-282.
30. Aksentijevich I, Masters SL, Ferguson PJ, Dancey P, Frenkel J, et al. (2009) An autoinflammatory disease with deficiency of the interleukin-1-receptor antagonist. *N Engl J Med* 360: 2426-2437.
31. (1997) Ancient missense mutations in a new member of the RoRet gene family are likely to cause familial Mediterranean fever. The International FMF Consortium. *Cell* 90: 797-807.
32. Dowds TA, Masumoto J, Chen FF, Ogura Y, Inohara N, et al. (2003) Regulation of cryopyrin/Pypaf1 signaling by pyrin, the familial Mediterranean fever gene product. *Biochem Biophys Res Commun* 302: 575-580.
33. Houten SM, Kuis W, Duran M, de Koning TJ, van Royen-Kerkhof A, et al. (1999) Mutations in MVK, encoding mevalonate kinase, cause hyperimmunoglobulinaemia D and periodic fever syndrome. *Nat Genet* 22: 175-177.
34. Drenth JP, Cuisset L, Grateau G, Vasseur C, van de Velde-Visser SD, et al. (1999) Mutations in the gene encoding mevalonate kinase cause hyper-IgD and periodic fever syndrome. International Hyper-IgD Study Group. *Nat Genet* 22: 178-181.
35. van der Burgh R, Nijhuis L, Pervolaraki K, Compeer EB, Jongeneel LH, et al. (2014) Defects in mitochondrial clearance predispose human monocytes to interleukin-1beta hypersecretion. *J Biol Chem* 289: 5000-5012.
36. Lindor NM, Arsenault TM, Solomon H, Seidman CE, McEvoy MT (1997) A new autosomal dominant disorder of pyogenic sterile arthritis, pyoderma gangrenosum, and acne: PAPA syndrome. *Mayo Clin Proc* 72: 611-615.
37. Wise CA, Gillum JD, Seidman CE, Lindor NM, Veile R, et al. (2002) Mutations in CD2BP1 disrupt binding to PTP PEST and are responsible for PAPA syndrome, an autoinflammatory disorder. *Hum Mol Genet* 11: 961-969.
38. Shoham NG, Centola M, Mansfield E, Hull KM, Wood G, et al. (2003) Pyrin binds the PSTPIP1/CD2BP1 protein, defining familial Mediterranean fever and PAPA syndrome as disorders in the same pathway. *Proc Natl Acad Sci U S A* 100: 13501-13506.
39. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, et al. (2005) IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 23: 479-490.
40. Shao W, Yeretssian G, Doiron K, Hussain SN, Saleh M (2007) The caspase-1 digestome identifies the glycolysis pathway as a target during infection and septic shock. *J Biol Chem* 282: 36321-36329.

41. Schmidt RL, Lenz LL (2012) Distinct licensing of IL-18 and IL-1 $\beta$  secretion in response to NLRP3 inflammasome activation. *PLoS One* 7: e45186.
42. Denes A, Lopez-Castejon G, Brough D (2012) Caspase-1: is IL-1 just the tip of the ICEberg? *Cell Death Dis* 3: e338.
43. Craven RR, Gao X, Allen IC, Gris D, Bubeck Wardenburg J, et al. (2009) *Staphylococcus aureus* alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells. *PLoS One* 4: e7446.
44. Holzinger D, Geldon L, Mysore V, Nippe N, Taxman DJ, et al. (2012) *Staphylococcus aureus* Panton-Valentine leukocidin induces an inflammatory response in human phagocytes via the NLRP3 inflammasome. *J Leukoc Biol* 92: 1069-1081.
45. Melehani JH, James DB, DuMont AL, Torres VJ, Duncan JA (2015) *Staphylococcus aureus* Leukocidin A/B (LukAB) Kills Human Monocytes via Host NLRP3 and ASC when Extracellular, but Not Intracellular. *PLoS Pathog* 11: e1004970.
46. Brydges SD, Mueller JL, McGeough MD, Pena CA, Misaghi A, et al. (2009) Inflammasome-mediated disease animal models reveal roles for innate but not adaptive immunity. *Immunity* 30: 875-887.
47. Brydges SD, Broderick L, McGeough MD, Pena CA, Mueller JL, et al. (2013) Divergence of IL-1, IL-18, and cell death in NLRP3 inflammasomopathies. *J Clin Invest* 123: 4695-4705.
48. Ives A, Nomura J, Martinon F, Roger T, LeRoy D, et al. (2015) Xanthine oxidoreductase regulates macrophage IL1 $\beta$  secretion upon NLRP3 inflammasome activation. *Nat Commun* 6: 6555.
49. Maier NK, Leppla SH, Moayeri M (2015) The cyclopentenone prostaglandin 15d-PGJ2 inhibits the NLRP1 and NLRP3 inflammasomes. *J Immunol* 194: 2776-2785.
50. Li P, Allen H, Banerjee S, Franklin S, Herzog L, et al. (1995) Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. *Cell* 80: 401-411.
51. Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, et al. (2011) Non-canonical inflammasome activation targets caspase-11. *Nature* 479: 117-121.
52. Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, et al. (2009) Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol* 183: 787-791.
53. Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, et al. (1997) Novel inhibitors of cytokine-induced IkappaB $\alpha$  phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. *J Biol Chem* 272: 21096-21103.

54. Eigenbrod T, Franchi L, Munoz-Planillo R, Kirschning CJ, Freudenberg MA, et al. (2012) Bacterial RNA mediates activation of caspase-1 and IL-1 $\beta$  release independently of TLRs 3, 7, 9 and TRIF but is dependent on UNC93B. *J Immunol* 189: 328-336.
55. Franchi L, Eigenbrod T, Nunez G (2009) Cutting edge: TNF-alpha mediates sensitization to ATP and silica via the NLRP3 inflammasome in the absence of microbial stimulation. *J Immunol* 183: 792-796.
56. Bauernfeind F, Bartok E, Rieger A, Franchi L, Nunez G, et al. (2011) Cutting edge: reactive oxygen species inhibitors block priming, but not activation, of the NLRP3 inflammasome. *J Immunol* 187: 613-617.
57. Schroder K, Sagulenko V, Zamoshnikova A, Richards AA, Cridland JA, et al. (2012) Acute lipopolysaccharide priming boosts inflammasome activation independently of inflammasome sensor induction. *Immunobiology* 217: 1325-1329.
58. Liao PC, Chao LK, Chou JC, Dong WC, Lin CN, et al. (2013) Lipopolysaccharide/adenosine triphosphate-mediated signal transduction in the regulation of NLRP3 protein expression and caspase-1-mediated interleukin-1 $\beta$  secretion. *Inflamm Res* 62: 89-96.
59. O'Neill LA, Golenbock D, Bowie AG (2013) The history of Toll-like receptors - redefining innate immunity. *Nat Rev Immunol* 13: 453-460.
60. Hernandez-Cuellar E, Tsuchiya K, Hara H, Fang R, Sakai S, et al. (2012) Cutting edge: nitric oxide inhibits the NLRP3 inflammasome. *J Immunol* 189: 5113-5117.
61. Mishra BB, Rathinam VA, Martens GW, Martinot AJ, Kornfeld H, et al. (2013) Nitric oxide controls the immunopathology of tuberculosis by inhibiting NLRP3 inflammasome-dependent processing of IL-1 $\beta$ . *Nat Immunol* 14: 52-60.
62. Patel MN, Bernard WG, Milev NB, Cawthorn WP, Figg N, et al. (2015) Hematopoietic IKBKE limits the chronicity of inflammasome priming and metaflammation. *Proc Natl Acad Sci U S A* 112: 506-511.
63. Xu P, Wen Z, Shi X, Li Y, Fan L, et al. (2013) Hemorrhagic shock augments Nlrp3 inflammasome activation in the lung through impaired pyrin induction. *J Immunol* 190: 5247-5255.
64. Gurung P, Li B, Subbarao Malireddi RK, Lamkanfi M, Geiger TL, et al. (2015) Chronic TLR Stimulation Controls NLRP3 Inflammasome Activation through IL-10 Mediated Regulation of NLRP3 Expression and Caspase-8 Activation. *Sci Rep* 5: 14488.
65. Fernandes-Alnemri T, Kang S, Anderson C, Sagara J, Fitzgerald KA, et al. (2013) Cutting edge: TLR signaling licenses IRAK1 for rapid activation of the NLRP3 inflammasome. *J Immunol* 191: 3995-3999.
66. Lin KM, Hu W, Troutman TD, Jennings M, Brewer T, et al. (2014) IRAK-1 bypasses priming and directly links TLRs to rapid NLRP3 inflammasome activation. *Proc Natl Acad Sci U S A* 111: 775-780.

67. Ghonime MG, Shamaa OR, Das S, Eldomany RA, Fernandes-Alnemri T, et al. (2014) Inflammasome priming by lipopolysaccharide is dependent upon ERK signaling and proteasome function. *J Immunol* 192: 3881-3888.
68. Rosenberg S, Zhang H, Zhang J (2011) FADD deficiency impairs early hematopoiesis in the bone marrow. *J Immunol* 186: 203-213.
69. Gurung P, Anand PK, Malireddi RK, Vande Walle L, Van Opdenbosch N, et al. (2014) FADD and caspase-8 mediate priming and activation of the canonical and noncanonical Nlrp3 inflammasomes. *J Immunol* 192: 1835-1846.
70. Allam R, Lawlor KE, Yu EC, Mildenhall AL, Moujalled DM, et al. (2014) Mitochondrial apoptosis is dispensable for NLRP3 inflammasome activation but non-apoptotic caspase-8 is required for inflammasome priming. *EMBO Rep* 15: 982-990.
71. Kang S, Fernandes-Alnemri T, Rogers C, Mayes L, Wang Y, et al. (2015) Caspase-8 scaffolding function and MLKL regulate NLRP3 inflammasome activation downstream of TLR3. *Nat Commun* 6: 7515.
72. Han S, Lear TB, Jerome JA, Rajbhandari S, Snaveley CA, et al. (2015) Lipopolysaccharide Primes the NALP3 Inflammasome by Inhibiting Its Ubiquitination and Degradation Mediated by the SCFFBXL2 E3 Ligase. *J Biol Chem* 290: 18124-18133.
73. Chen BB, Coon TA, Glasser JR, McVerry BJ, Zhao J, et al. (2013) A combinatorial F box protein directed pathway controls TRAF adaptor stability to regulate inflammation. *Nat Immunol* 14: 470-479.
74. Juliana C, Fernandes-Alnemri T, Kang S, Farias A, Qin F, et al. (2012) Non-transcriptional priming and deubiquitination regulate NLRP3 inflammasome activation. *J Biol Chem* 287: 36617-36622.
75. Gurung P, Lamkanfi M, Kanneganti TD (2015) Cutting edge: SHARPIN is required for optimal NLRP3 inflammasome activation. *J Immunol* 194: 2064-2067.
76. Bauernfeind F, Rieger A, Schildberg FA, Knolle PA, Schmid-Burgk JL, et al. (2012) NLRP3 inflammasome activity is negatively controlled by miR-223. *J Immunol* 189: 4175-4181.
77. Haneklaus M, Gerlic M, Kurowska-Stolarska M, Rainey AA, Pich D, et al. (2012) Cutting edge: miR-223 and EBV miR-BART15 regulate the NLRP3 inflammasome and IL-1 $\beta$  production. *J Immunol* 189: 3795-3799.
78. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, et al. (1980) Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int J Cancer* 26: 171-176.
79. Heng TS, Painter MW, Immunological Genome Project C (2008) The Immunological Genome Project: networks of gene expression in immune cells. *Nat Immunol* 9: 1091-1094.

80. Munoz-Planillo R, Kuffa P, Martinez-Colon G, Smith BL, Rajendiran TM, et al. (2013) K(+) efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity* 38: 1142-1153.
81. Petrilli V, Papin S, Dostert C, Mayor A, Martinon F, et al. (2007) Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ* 14: 1583-1589.
82. Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA (2008) Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* 453: 1122-1126.
83. Dostert C, Petrilli V, Van Bruggen R, Steele C, Mossman BT, et al. (2008) Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 320: 674-677.
84. Shio MT, Eisenbarth SC, Savaria M, Vinet AF, Bellemare MJ, et al. (2009) Malarial hemozoin activates the NLRP3 inflammasome through Lyn and Syk kinases. *PLoS Pathog* 5: e1000559.
85. Martinon F, Burns K, Tschopp J (2002) The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 10: 417-426.
86. Fernandes-Alnemri T, Wu J, Yu JW, Datta P, Miller B, et al. (2007) The pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation. *Cell Death Differ* 14: 1590-1604.
87. Arlehamn CS, Petrilli V, Gross O, Tschopp J, Evans TJ (2010) The role of potassium in inflammasome activation by bacteria. *J Biol Chem* 285: 10508-10518.
88. Cruz CM, Rinna A, Forman HJ, Ventura AL, Persechini PM, et al. (2007) ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. *J Biol Chem* 282: 2871-2879.
89. Hewinson J, Moore SF, Glover C, Watts AG, MacKenzie AB (2008) A key role for redox signaling in rapid P2X7 receptor-induced IL-1 beta processing in human monocytes. *J Immunol* 180: 8410-8420.
90. Gross O, Poeck H, Bscheider M, Dostert C, Hanneschlager N, et al. (2009) Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature* 459: 433-436.
91. Allen IC, Scull MA, Moore CB, Holl EK, McElvania-TeKippe E, et al. (2009) The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. *Immunity* 30: 556-565.
92. Cassel SL, Eisenbarth SC, Iyer SS, Sadler JJ, Colegio OR, et al. (2008) The Nalp3 inflammasome is essential for the development of silicosis. *Proc Natl Acad Sci U S A* 105: 9035-9040.

93. Iyer SS, He Q, Janczy JR, Elliott EI, Zhong Z, et al. (2013) Mitochondrial cardiolipin is required for Nlrp3 inflammasome activation. *Immunity* 39: 311-323.
94. Zhou R, Tardivel A, Thorens B, Choi I, Tschopp J (2010) Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat Immunol* 11: 136-140.
95. Fonfria E, Marshall IC, Benham CD, Boyfield I, Brown JD, et al. (2004) TRPM2 channel opening in response to oxidative stress is dependent on activation of poly(ADP-ribose) polymerase. *Br J Pharmacol* 143: 186-192.
96. Zhong Z, Zhai Y, Liang S, Mori Y, Han R, et al. (2013) TRPM2 links oxidative stress to NLRP3 inflammasome activation. *Nat Commun* 4: 1611.
97. Lupfer C, Thomas PG, Anand PK, Vogel P, Milasta S, et al. (2013) Receptor interacting protein kinase 2-mediated mitophagy regulates inflammasome activation during virus infection. *Nat Immunol* 14: 480-488.
98. Zhang Z, Xu X, Ma J, Wu J, Wang Y, et al. (2013) Gene deletion of Gabarap enhances Nlrp3 inflammasome-dependent inflammatory responses. *J Immunol* 190: 3517-3524.
99. Park S, Won JH, Hwang I, Hong S, Lee HK, et al. (2015) Defective mitochondrial fission augments NLRP3 inflammasome activation. *Sci Rep* 5: 15489.
100. Ichinohe T, Yamazaki T, Koshiba T, Yanagi Y (2013) Mitochondrial protein mitofusin 2 is required for NLRP3 inflammasome activation after RNA virus infection. *Proc Natl Acad Sci U S A* 110: 17963-17968.
101. Yang CS, Kim JJ, Kim TS, Lee PY, Kim SY, et al. (2015) Small heterodimer partner interacts with NLRP3 and negatively regulates activation of the NLRP3 inflammasome. *Nat Commun* 6: 6115.
102. Misawa T, Takahama M, Kozaki T, Lee H, Zou J, et al. (2013) Microtubule-driven spatial arrangement of mitochondria promotes activation of the NLRP3 inflammasome. *Nat Immunol* 14: 454-460.
103. Shimada K, Crother TR, Karlin J, Dagvadorj J, Chiba N, et al. (2012) Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. *Immunity* 36: 401-414.
104. Nakahira K, Haspel JA, Rathinam VA, Lee SJ, Dolinay T, et al. (2011) Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nat Immunol* 12: 222-230.
105. Fujisawa A, Kambe N, Saito M, Nishikomori R, Tanizaki H, et al. (2007) Disease-associated mutations in CIAS1 induce cathepsin B-dependent rapid cell death of human THP-1 monocytic cells. *Blood* 109: 2903-2911.
106. Yu J, Nagasu H, Murakami T, Hoang H, Broderick L, et al. (2014) Inflammasome activation leads to Caspase-1-dependent mitochondrial damage and block of mitophagy. *Proc Natl Acad Sci U S A* 111: 15514-15519.



107. Willingham SB, Bergstralh DT, O'Connor W, Morrison AC, Taxman DJ, et al. (2007) Microbial pathogen-induced necrotic cell death mediated by the inflammasome components CIAS1/cryopyrin/NLRP3 and ASC. *Cell Host Microbe* 2: 147-159.
108. Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, et al. (2008) Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* 9: 847-856.
109. Duncan JA, Gao X, Huang MT, O'Connor BP, Thomas CE, et al. (2009) *Neisseria gonorrhoeae* activates the proteinase cathepsin B to mediate the signaling activities of the NLRP3 and ASC-containing inflammasome. *J Immunol* 182: 6460-6469.
110. Orłowski GM, Colbert JD, Sharma S, Bogyo M, Robertson SA, et al. (2015) Multiple Cathepsins Promote Pro-IL-1 $\beta$  Synthesis and NLRP3-Mediated IL-1 $\beta$  Activation. *J Immunol* 195: 1685-1697.
111. Chen YT, Brinen LS, Kerr ID, Hansell E, Doyle PS, et al. (2010) In vitro and in vivo studies of the trypanocidal properties of WRR-483 against *Trypanosoma cruzi*. *PLoS Negl Trop Dis* 4.
112. Okada M, Matsuzawa A, Yoshimura A, Ichijo H (2014) The lysosome rupture-activated TAK1-JNK pathway regulates NLRP3 inflammasome activation. *J Biol Chem* 289: 32926-32936.
113. Gong YN, Wang X, Wang J, Yang Z, Li S, et al. (2010) Chemical probing reveals insights into the signaling mechanism of inflammasome activation. *Cell Res* 20: 1289-1305.
114. Menu P, Mayor A, Zhou R, Tardivel A, Ichijo H, et al. (2012) ER stress activates the NLRP3 inflammasome via an UPR-independent pathway. *Cell Death Dis* 3: e261.
115. Simard JC, Vallieres F, de Liz R, Lavastre V, Girard D (2015) Silver nanoparticles induce degradation of the endoplasmic reticulum stress sensor activating transcription factor-6 leading to activation of the NLRP-3 inflammasome. *J Biol Chem* 290: 5926-5939.
116. Osłowski CM, Hara T, O'Sullivan-Murphy B, Kanekura K, Lu S, et al. (2012) Thioredoxin-interacting protein mediates ER stress-induced beta cell death through initiation of the inflammasome. *Cell Metab* 16: 265-273.
117. Lerner AG, Upton JP, Praveen PV, Ghosh R, Nakagawa Y, et al. (2012) IRE1 $\alpha$  induces thioredoxin-interacting protein to activate the NLRP3 inflammasome and promote programmed cell death under irremediable ER stress. *Cell Metab* 16: 250-264.
118. Bronner DN, Abuaita BH, Chen X, Fitzgerald KA, Nunez G, et al. (2015) Endoplasmic Reticulum Stress Activates the Inflammasome via NLRP3- and Caspase-2-Driven Mitochondrial Damage. *Immunity* 43: 451-462.
119. Rubartelli A, Cozzolino F, Talio M, Sitia R (1990) A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. *EMBO J* 9: 1503-1510.

120. Brough D, Le Feuvre RA, Wheeler RD, Solovyova N, Hilfiker S, et al. (2003) Ca<sup>2+</sup> stores and Ca<sup>2+</sup> entry differentially contribute to the release of IL-1 beta and IL-1 alpha from murine macrophages. *J Immunol* 170: 3029-3036.
121. Chu J, Thomas LM, Watkins SC, Franchi L, Nunez G, et al. (2009) Cholesterol-dependent cytolysins induce rapid release of mature IL-1 $\beta$  from murine macrophages in a NLRP3 inflammasome and cathepsin B-dependent manner. *J Leukoc Biol* 86: 1227-1238.
122. Triantafilou K, Kar S, van Kuppeveld FJ, Triantafilou M (2013) Rhinovirus-induced calcium flux triggers NLRP3 and NLRC5 activation in bronchial cells. *Am J Respir Cell Mol Biol* 49: 923-934.
123. Ito M, Yanagi Y, Ichinohe T (2012) Encephalomyocarditis virus viroporin 2B activates NLRP3 inflammasome. *PLoS Pathog* 8: e1002857.
124. de Jong AS, de Mattia F, Van Dommelen MM, Lanke K, Melchers WJ, et al. (2008) Functional analysis of picornavirus 2B proteins: effects on calcium homeostasis and intracellular protein trafficking. *J Virol* 82: 3782-3790.
125. Feldmeyer L, Keller M, Niklaus G, Hohl D, Werner S, et al. (2007) The inflammasome mediates UVB-induced activation and secretion of interleukin-1beta by keratinocytes. *Curr Biol* 17: 1140-1145.
126. Murakami T, Ockinger J, Yu J, Byles V, McColl A, et al. (2012) Critical role for calcium mobilization in activation of the NLRP3 inflammasome. *Proc Natl Acad Sci U S A* 109: 11282-11287.
127. Lee GS, Subramanian N, Kim AI, Aksentijevich I, Goldbach-Mansky R, et al. (2012) The calcium-sensing receptor regulates the NLRP3 inflammasome through Ca<sup>2+</sup> and cAMP. *Nature* 492: 123-127.
128. Rossol M, Pierer M, Raulien N, Quandt D, Meusch U, et al. (2012) Extracellular Ca<sup>2+</sup> is a danger signal activating the NLRP3 inflammasome through G protein-coupled calcium sensing receptors. *Nat Commun* 3: 1329.
129. Triantafilou K, Hughes TR, Triantafilou M, Morgan BP (2013) The complement membrane attack complex triggers intracellular Ca<sup>2+</sup> fluxes leading to NLRP3 inflammasome activation. *J Cell Sci* 126: 2903-2913.
130. Rimessi A, Bezzerri V, Patergnani S, Marchi S, Cabrini G, et al. (2015) Mitochondrial Ca<sup>2+</sup>-dependent NLRP3 activation exacerbates the *Pseudomonas aeruginosa*-driven inflammatory response in cystic fibrosis. *Nat Commun* 6: 6201.
131. Gao L, Blair LA, Marshall J (2006) CaMKII-independent effects of KN93 and its inactive analog KN92: reversible inhibition of L-type calcium channels. *Biochem Biophys Res Commun* 345: 1606-1610.
132. Chuang YT, Lin YC, Lin KH, Chou TF, Kuo WC, et al. (2011) Tumor suppressor death-associated protein kinase is required for full IL-1 $\beta$  production. *Blood* 117: 960-970.

133. Vaeth M, Zee I, Concepcion AR, Maus M, Shaw P, et al. (2015) Ca<sup>2+</sup> Signaling but Not Store-Operated Ca<sup>2+</sup> Entry Is Required for the Function of Macrophages and Dendritic Cells. *J Immunol* 195: 1202-1217.
134. Katsnelson MA, Rucker LG, Russo HM, Dubyak GR (2015) K<sup>+</sup> efflux agonists induce NLRP3 inflammasome activation independently of Ca<sup>2+</sup> signaling. *J Immunol* 194: 3937-3952.
135. Wilkinson KD, Urban MK, Haas AL (1980) Ubiquitin is the ATP-dependent proteolysis factor I of rabbit reticulocytes. *J Biol Chem* 255: 7529-7532.
136. Wilkinson KD, Audhya TK (1981) Stimulation of ATP-dependent proteolysis requires ubiquitin with the COOH-terminal sequence Arg-Gly-Gly. *J Biol Chem* 256: 9235-9241.
137. Rodgers MA, Bowman JW, Fujita H, Orazio N, Shi M, et al. (2014) The linear ubiquitin assembly complex (LUBAC) is essential for NLRP3 inflammasome activation. *J Exp Med* 211: 1333-1347.
138. Douglas T, Champagne C, Morizot A, Lapointe JM, Saleh M (2015) The Inflammatory Caspases-1 and -11 Mediate the Pathogenesis of Dermatitis in Sharpin-Deficient Mice. *J Immunol* 195: 2365-2373.
139. Py BF, Kim MS, Vakifahmetoglu-Norberg H, Yuan J (2013) Deubiquitination of NLRP3 by BRCC3 critically regulates inflammasome activity. *Mol Cell* 49: 331-338.
140. Hwang I, Lee E, Jeon SA, Yu JW (2015) Histone deacetylase 6 negatively regulates NLRP3 inflammasome activation. *Biochem Biophys Res Commun* 467: 973-978.
141. Joazeiro CA, Weissman AM (2000) RING finger proteins: mediators of ubiquitin ligase activity. *Cell* 102: 549-552.
142. Rajsbaum R, Garcia-Sastre A, Versteeg GA (2014) TRIMmunity: the roles of the TRIM E3-ubiquitin ligase family in innate antiviral immunity. *J Mol Biol* 426: 1265-1284.
143. Mitoma H, Hanabuchi S, Kim T, Bao M, Zhang Z, et al. (2013) The DHX33 RNA helicase senses cytosolic RNA and activates the NLRP3 inflammasome. *Immunity* 39: 123-135.
144. Weng L, Mitoma H, Trichot C, Bao M, Liu Y, et al. (2014) The E3 ubiquitin ligase tripartite motif 33 is essential for cytosolic RNA-induced NLRP3 inflammasome activation. *J Immunol* 193: 3676-3682.
145. Duong BH, Onizawa M, Oses-Prieto JA, Advincula R, Burlingame A, et al. (2015) A20 restricts ubiquitination of pro-interleukin-1beta protein complexes and suppresses NLRP3 inflammasome activity. *Immunity* 42: 55-67.
146. Duda DM, Olszewski JL, Tron AE, Hammel M, Lambert LJ, et al. (2012) Structure of a glomulin-RBX1-CUL1 complex: inhibition of a RING E3 ligase through masking of its E2-binding surface. *Mol Cell* 47: 371-382.

147. Tron AE, Arai T, Duda DM, Kuwabara H, Olszewski JL, et al. (2012) The glomuvenous malformation protein Glomulin binds Rbx1 and regulates cullin RING ligase-mediated turnover of Fbw7. *Mol Cell* 46: 67-78.
148. Suzuki S, Mimuro H, Kim M, Ogawa M, Ashida H, et al. (2014) Shigella IpaH7.8 E3 ubiquitin ligase targets glomulin and activates inflammasomes to demolish macrophages. *Proc Natl Acad Sci U S A* 111: E4254-4263.
149. Ashida H, Kim M, Sasakawa C (2014) Exploitation of the host ubiquitin system by human bacterial pathogens. *Nat Rev Microbiol* 12: 399-413.
150. Yan Y, Jiang W, Liu L, Wang X, Ding C, et al. (2015) Dopamine controls systemic inflammation through inhibition of NLRP3 inflammasome. *Cell* 160: 62-73.
151. Filardy AA, He J, Bennink J, Yewdell J, Kelsall BL (2015) Posttranscriptional control of NLRP3 inflammasome activation in colonic macrophages. *Mucosal Immunol*.
152. Hopkins AL, Groom CR (2002) The druggable genome. *Nat Rev Drug Discov* 1: 727-730.
153. Kankkunen P, Teirila L, Rintahaka J, Alenius H, Wolff H, et al. (2010) (1,3)-beta-glucans activate both dectin-1 and NLRP3 inflammasome in human macrophages. *J Immunol* 184: 6335-6342.
154. Said-Sadier N, Padilla E, Langsley G, Ojcius DM (2010) *Aspergillus fumigatus* stimulates the NLRP3 inflammasome through a pathway requiring ROS production and the Syk tyrosine kinase. *PLoS One* 5: e10008.
155. Guo C, Chen M, Fa Z, Lu A, Fang W, et al. (2014) Acapsular *Cryptococcus neoformans* activates the NLRP3 inflammasome. *Microbes Infect* 16: 845-854.
156. Kistowska M, Fenini G, Jankovic D, Feldmeyer L, Kerl K, et al. (2014) *Malassezia* yeasts activate the NLRP3 inflammasome in antigen-presenting cells via Syk-kinase signalling. *Exp Dermatol* 23: 884-889.
157. Ritter M, Gross O, Kays S, Ruland J, Nimmerjahn F, et al. (2010) *Schistosoma mansoni* triggers Dectin-2, which activates the Nlrp3 inflammasome and alters adaptive immune responses. *Proc Natl Acad Sci U S A* 107: 20459-20464.
158. Wu MF, Chen ST, Yang AH, Lin WW, Lin YL, et al. (2013) CLEC5A is critical for dengue virus-induced inflammasome activation in human macrophages. *Blood* 121: 95-106.
159. Chen ST, Lin YL, Huang MT, Wu MF, Cheng SC, et al. (2008) CLEC5A is critical for dengue-virus-induced lethal disease. *Nature* 453: 672-676.
160. Chen ST, Liu RS, Wu MF, Lin YL, Chen SY, et al. (2012) CLEC5A regulates Japanese encephalitis virus-induced neuroinflammation and lethality. *PLoS Pathog* 8: e1002655.
161. Zelensky AN, Gready JE (2005) The C-type lectin-like domain superfamily. *FEBS J* 272: 6179-6217.

162. Kerrigan AM, Brown GD (2010) Syk-coupled C-type lectin receptors that mediate cellular activation via single tyrosine based activation motifs. *Immunol Rev* 234: 335-352.
163. Sancho D, Joffre OP, Keller AM, Rogers NC, Martinez D, et al. (2009) Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature* 458: 899-903.
164. Okamura H, Tsutsi H, Komatsu T, Yutsudo M, Hakura A, et al. (1995) Cloning of a new cytokine that induces IFN-gamma production by T cells. *Nature* 378: 88-91.
165. Tominaga K, Yoshimoto T, Torigoe K, Kurimoto M, Matsui K, et al. (2000) IL-12 synergizes with IL-18 or IL-1 $\beta$  for IFN-gamma production from human T cells. *Int Immunol* 12: 151-160.
166. Yamasaki S, Ishikawa E, Sakuma M, Hara H, Ogata K, et al. (2008) Mincle is an ITAM-coupled activating receptor that senses damaged cells. *Nat Immunol* 9: 1179-1188.
167. Yamasaki S, Matsumoto M, Takeuchi O, Matsuzawa T, Ishikawa E, et al. (2009) C-type lectin Mincle is an activating receptor for pathogenic fungus, *Malassezia*. *Proc Natl Acad Sci U S A* 106: 1897-1902.
168. Yasukawa S, Miyazaki Y, Yoshii C, Nakaya M, Ozaki N, et al. (2014) An ITAM-Syk-CARD9 signalling axis triggers contact hypersensitivity by stimulating IL-1 production in dendritic cells. *Nat Commun* 5: 3755.
169. Ito M, Shichita T, Okada M, Komine R, Noguchi Y, et al. (2015) Bruton's tyrosine kinase is essential for NLRP3 inflammasome activation and contributes to ischaemic brain injury. *Nat Commun* 6: 7360.
170. Lin YC, Huang DY, Wang JS, Lin YL, Hsieh SL, et al. (2015) Syk is involved in NLRP3 inflammasome-mediated caspase-1 activation through adaptor ASC phosphorylation and enhanced oligomerization. *J Leukoc Biol*.
171. Hara H, Tsuchiya K, Kawamura I, Fang R, Hernandez-Cuellar E, et al. (2013) Phosphorylation of the adaptor ASC acts as a molecular switch that controls the formation of speck-like aggregates and inflammasome activity. *Nat Immunol* 14: 1247-1255.
172. Cho YS, Challa S, Moquin D, Genga R, Ray TD, et al. (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell* 137: 1112-1123.
173. He S, Wang L, Miao L, Wang T, Du F, et al. (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. *Cell* 137: 1100-1111.
174. Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, et al. (2009) RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. *Science* 325: 332-336.
175. Sun L, Wang H, Wang Z, He S, Chen S, et al. (2012) Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell* 148: 213-227.

176. Zhao J, Jitkaew S, Cai Z, Choksi S, Li Q, et al. (2012) Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. *Proc Natl Acad Sci U S A* 109: 5322-5327.
177. Wang Z, Jiang H, Chen S, Du F, Wang X (2012) The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. *Cell* 148: 228-243.
178. Wang X, Jiang W, Yan Y, Gong T, Han J, et al. (2014) RNA viruses promote activation of the NLRP3 inflammasome through a RIP1-RIP3-DRP1 signaling pathway. *Nat Immunol* 15: 1126-1133.
179. Moriwaki K, Farias Luz N, Balaji S, De Rosa MJ, O'Donnell CL, et al. (2016) The Mitochondrial Phosphatase PGAM5 Is Dispensable for Necroptosis but Promotes Inflammasome Activation in Macrophages. *J Immunol* 196: 407-415.
180. Rajput A, Kovalenko A, Bogdanov K, Yang SH, Kang TB, et al. (2011) RIG-I RNA helicase activation of IRF3 transcription factor is negatively regulated by caspase-8-mediated cleavage of the RIP1 protein. *Immunity* 34: 340-351.
181. Kang TB, Yang SH, Toth B, Kovalenko A, Wallach D (2013) Caspase-8 blocks kinase RIPK3-mediated activation of the NLRP3 inflammasome. *Immunity* 38: 27-40.
182. Lawlor KE, Khan N, Mildenhall A, Gerlic M, Croker BA, et al. (2015) RIPK3 promotes cell death and NLRP3 inflammasome activation in the absence of MLKL. *Nat Commun* 6: 6282.
183. Greenlee-Wacker MC, Rigby KM, Kobayashi SD, Porter AR, DeLeo FR, et al. (2014) Phagocytosis of *Staphylococcus aureus* by human neutrophils prevents macrophage efferocytosis and induces programmed necrosis. *J Immunol* 192: 4709-4717.
184. Kitur K, Parker D, Nieto P, Ahn DS, Cohen TS, et al. (2015) Toxin-induced necroptosis is a major mechanism of *Staphylococcus aureus* lung damage. *PLoS Pathog* 11: e1004820.
185. Gonzalez-Juarbe N, Gilley RP, Hinojosa CA, Bradley KM, Kamei A, et al. (2015) Pore-Forming Toxins Induce Macrophage Necroptosis during Acute Bacterial Pneumonia. *PLoS Pathog* 11: e1005337.
186. Nagamatsu K, Hannan TJ, Guest RL, Kostakioti M, Hadjifrangiskou M, et al. (2015) Dysregulation of *Escherichia coli* alpha-hemolysin expression alters the course of acute and persistent urinary tract infection. *Proc Natl Acad Sci U S A* 112: E871-880.
187. Schmid-Burgk JL, Chauhan D, Schmidt T, Ebert TS, Reinhardt J, et al. (2016) A Genome-wide CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) Screen Identifies NEK7 as an Essential Component of NLRP3 Inflammasome Activation. *J Biol Chem* 291: 103-109.
188. Shi H, Wang Y, Li X, Zhan X, Tang M, et al. (2015) NLRP3 activation and mitosis are mutually exclusive events coordinated by NEK7, a new inflammasome component. *Nat Immunol*.

189. Lu B, Nakamura T, Inouye K, Li J, Tang Y, et al. (2012) Novel role of PKR in inflammasome activation and HMGB1 release. *Nature* 488: 670-674.
190. Martin BN, Wang C, Willette-Brown J, Herjan T, Gulen MF, et al. (2014) IKK $\alpha$  negatively regulates ASC-dependent inflammasome activation. *Nat Commun* 5: 4977.
191. Li X, Liu S, Luo J, Liu A, Tang S, et al. (2015) *Helicobacter pylori* induces IL-1 $\beta$  and IL-18 production in human monocytic cell line through activation of NLRP3 inflammasome via ROS signaling pathway. *Pathog Dis* 73.
192. Basak C, Pathak SK, Bhattacharyya A, Mandal D, Pathak S, et al. (2005) NF- $\kappa$ B- and C/EBP $\beta$ -driven interleukin-1 $\beta$  gene expression and PAK1-mediated caspase-1 activation play essential roles in interleukin-1 $\beta$  release from *Helicobacter pylori* lipopolysaccharide-stimulated macrophages. *J Biol Chem* 280: 4279-4288.
193. Qu Y, Misaghi S, Izrael-Tomasevic A, Newton K, Gilmour LL, et al. (2012) Phosphorylation of NLRC4 is critical for inflammasome activation. *Nature* 490: 539-542.
194. Kovac L, Bohmerova E, Butko P (1982) Ionophores and intact cells. I. Valinomycin and nigericin act preferentially on mitochondria and not on the plasma membrane of *Saccharomyces cerevisiae*. *Biochim Biophys Acta* 721: 341-348.
195. Surprenant A, Rassendren F, Kawashima E, North RA, Buell G (1996) The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). *Science* 272: 735-738.
196. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J (2006) Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440: 237-241.
197. Harned RL, Hidy PH, Corum CJ, Jones KL (1951) Nigericin a new crystalline antibiotic from an unidentified *Streptomyces*. *Antibiot Chemother (Northfield)* 1: 594-596.
198. Harold FM, Baarda JR (1968) Effects of nigericin and monactin on cation permeability of *Streptococcus faecalis* and metabolic capacities of potassium-depleted cells. *J Bacteriol* 95: 816-823.
199. Perregaux D, Barberia J, Lanzetti AJ, Geoghegan KF, Carty TJ, et al. (1992) IL-1 $\beta$  maturation: evidence that mature cytokine formation can be induced specifically by nigericin. *J Immunol* 149: 1294-1303.
200. Perregaux D, Gabel CA (1994) Interleukin-1 $\beta$  maturation and release in response to ATP and nigericin. Evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity. *J Biol Chem* 269: 15195-15203.
201. Cheneval D, Ramage P, Kastelic T, Szelestenyi T, Niggli H, et al. (1998) Increased mature interleukin-1 $\beta$  secretion from THP-1 cells induced by nigericin is a result of activation of p45 IL-1 $\beta$ -converting enzyme processing. *J Biol Chem* 273: 17846-17851.

202. Hentze H, Lin XY, Choi MS, Porter AG (2003) Critical role for cathepsin B in mediating caspase-1-dependent interleukin-18 maturation and caspase-1-independent necrosis triggered by the microbial toxin nigericin. *Cell Death Differ* 10: 956-968.
203. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, et al. (2006) Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440: 228-232.
204. Guey B, Bodnar M, Manie SN, Tardivel A, Petrilli V (2014) Caspase-1 autoproteolysis is differentially required for NLRP1b and NLRP3 inflammasome function. *Proc Natl Acad Sci U S A* 111: 17254-17259.
205. Gustin A, Kirchmeyer M, Koncina E, Felten P, Losciuto S, et al. (2015) NLRP3 Inflammasome Is Expressed and Functional in Mouse Brain Microglia but Not in Astrocytes. *PLoS One* 10: e0130624.
206. Heid ME, Keyel PA, Kamga C, Shiva S, Watkins SC, et al. (2013) Mitochondrial reactive oxygen species induces NLRP3-dependent lysosomal damage and inflammasome activation. *J Immunol* 191: 5230-5238.
207. Kasimsetty SG, DeWolf SE, Shigeoka AA, McKay DB (2014) Regulation of TLR2 and NLRP3 in primary murine renal tubular epithelial cells. *Nephron Clin Pract* 127: 119-123.
208. Antonopoulos C, Russo HM, El Sanadi C, Martin BN, Li X, et al. (2015) Caspase-8 as an Effector and Regulator of NLRP3 Inflammasome Signaling. *J Biol Chem* 290: 20167-20184.
209. Won JH, Park S, Hong S, Son S, Yu JW (2015) Rotenone-induced Impairment of Mitochondrial Electron Transport Chain Confers a Selective Priming Signal for NLRP3 Inflammasome Activation. *J Biol Chem* 290: 27425-27437.
210. Hu Y, Mao K, Zeng Y, Chen S, Tao Z, et al. (2010) Tripartite-motif protein 30 negatively regulates NLRP3 inflammasome activation by modulating reactive oxygen species production. *J Immunol* 185: 7699-7705.
211. Nomura J, So A, Tamura M, Busso N (2015) Intracellular ATP Decrease Mediates NLRP3 Inflammasome Activation upon Nigericin and Crystal Stimulation. *J Immunol* 195: 5718-5724.
212. Pelegrin P, Surprenant A (2007) Pannexin-1 couples to maitotoxin- and nigericin-induced interleukin-1beta release through a dye uptake-independent pathway. *J Biol Chem* 282: 2386-2394.
213. Vyleta ML, Wong J, Magun BE (2012) Suppression of ribosomal function triggers innate immune signaling through activation of the NLRP3 inflammasome. *PLoS One* 7: e36044.
214. Shenoy AR, Wellington DA, Kumar P, Kassa H, Booth CJ, et al. (2012) GBP5 promotes NLRP3 inflammasome assembly and immunity in mammals. *Science* 336: 481-485.



215. Mao K, Chen S, Wang Y, Zeng Y, Ma Y, et al. (2015) beta-arrestin1 is critical for the full activation of NLRP3 and NLRC4 inflammasomes. *J Immunol* 194: 1867-1873.
216. Nurmi K, Virkanen J, Rajamaki K, Niemi K, Kovanen PT, et al. (2013) Ethanol inhibits activation of NLRP3 and AIM2 inflammasomes in human macrophages--a novel anti-inflammatory action of alcohol. *PLoS One* 8: e78537.
217. Maier NK, Crown D, Liu J, Leppla SH, Moayeri M (2014) Arsenic trioxide and other arsenical compounds inhibit the NLRP1, NLRP3, and NAIP5/NLRC4 inflammasomes. *J Immunol* 192: 763-770.
218. Jung SS, Moon JS, Xu JF, Ifedigbo E, Ryter SW, et al. (2015) Carbon monoxide negatively regulates NLRP3 inflammasome activation in macrophages. *Am J Physiol Lung Cell Mol Physiol* 308: L1058-1067.
219. L'Homme L, Esser N, Riva L, Scheen A, Paquot N, et al. (2013) Unsaturated fatty acids prevent activation of NLRP3 inflammasome in human monocytes/macrophages. *J Lipid Res* 54: 2998-3008.
220. Squires RC, Muehlbauer SM, Brojatsch J (2007) Proteasomes control caspase-1 activation in anthrax lethal toxin-mediated cell killing. *J Biol Chem* 282: 34260-34267.
221. Franchi L, Eigenbrod T, Munoz-Planillo R, Ozkurede U, Kim YG, et al. (2014) Cytosolic double-stranded RNA activates the NLRP3 inflammasome via MAVS-induced membrane permeabilization and K<sup>+</sup> efflux. *J Immunol* 193: 4214-4222.
222. Dowling JK, Becker CE, Bourke NM, Corr SC, Connolly DJ, et al. (2014) Promyelocytic leukemia protein interacts with the apoptosis-associated speck-like protein to limit inflammasome activation. *J Biol Chem* 289: 6429-6437.
223. Lo YH, Huang YW, Wu YH, Tsai CS, Lin YC, et al. (2013) Selective inhibition of the NLRP3 inflammasome by targeting to promyelocytic leukemia protein in mouse and human. *Blood* 121: 3185-3194.
224. He WT, Wan H, Hu L, Chen P, Wang X, et al. (2015) Gasdermin D is an executor of pyroptosis and required for interleukin-1beta secretion. *Cell Res* 25: 1285-1298.
225. Stout-Delgado HW, Vaughan SE, Shirali AC, Jaramillo RJ, Harrod KS (2012) Impaired NLRP3 inflammasome function in elderly mice during influenza infection is rescued by treatment with nigericin. *J Immunol* 188: 2815-2824.
226. Kim J, Ahn H, Woo HM, Lee E, Lee GS (2014) Characterization of porcine NLRP3 inflammasome activation and its upstream mechanism. *Vet Res Commun* 38: 193-200.
227. Solle M, Labasi J, Perregaux DG, Stam E, Petrushova N, et al. (2001) Altered cytokine production in mice lacking P2X(7) receptors. *J Biol Chem* 276: 125-132.
228. Pelegrin P, Surprenant A (2006) Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. *EMBO J* 25: 5071-5082.

229. Marina-Garcia N, Franchi L, Kim YG, Miller D, McDonald C, et al. (2008) Pannexin-1-mediated intracellular delivery of muramyl dipeptide induces caspase-1 activation via cryopyrin/NLRP3 independently of Nod2. *J Immunol* 180: 4050-4057.
230. Lopez-Castejon G, Theaker J, Pelegrin P, Clifton AD, Braddock M, et al. (2010) P2X(7) receptor-mediated release of cathepsins from macrophages is a cytokine-independent mechanism potentially involved in joint diseases. *J Immunol* 185: 2611-2619.
231. Halle A, Hornung V, Petzold GC, Stewart CR, Monks BG, et al. (2008) The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. *Nat Immunol* 9: 857-865.
232. Li N, Ragheb K, Lawler G, Sturgis J, Rajwa B, et al. (2003) Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. *J Biol Chem* 278: 8516-8525.
233. Grailer JJ, Canning BA, Kalbitz M, Haggadone MD, Dhond RM, et al. (2014) Critical role for the NLRP3 inflammasome during acute lung injury. *J Immunol* 192: 5974-5983.
234. Hofer AM, Brown EM (2003) Extracellular calcium sensing and signalling. *Nat Rev Mol Cell Biol* 4: 530-538.
235. Mao K, Chen S, Chen M, Ma Y, Wang Y, et al. (2013) Nitric oxide suppresses NLRP3 inflammasome activation and protects against LPS-induced septic shock. *Cell Res* 23: 201-212.
236. Moon JS, Hisata S, Park MA, DeNicola GM, Ryter SW, et al. (2015) mTORC1-Induced HK1-Dependent Glycolysis Regulates NLRP3 Inflammasome Activation. *Cell Rep* 12: 102-115.
237. Lu B, Kwan K, Levine YA, Olofsson PS, Yang H, et al. (2014) alpha7 nicotinic acetylcholine receptor signaling inhibits inflammasome activation by preventing mitochondrial DNA release. *Mol Med* 20: 350-358.
238. Hecker A, Kullmar M, Wilker S, Richter K, Zakrzewicz A, et al. (2015) Phosphocholine-Modified Macromolecules and Canonical Nicotinic Agonists Inhibit ATP-Induced IL-1beta Release. *J Immunol* 195: 2325-2334.
239. Wu YH, Kuo WC, Wu YJ, Yang KT, Chen ST, et al. (2014) Participation of c-FLIP in NLRP3 and AIM2 inflammasome activation. *Cell Death Differ* 21: 451-461.
240. Iyer SS, Pulsikens WP, Sadler JJ, Butter LM, Teske GJ, et al. (2009) Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. *Proc Natl Acad Sci U S A* 106: 20388-20393.
241. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, et al. (2009) Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. *Nat Med* 15: 1170-1178.

242. Riteau N, Baron L, Villeret B, Guillou N, Savigny F, et al. (2012) ATP release and purinergic signaling: a common pathway for particle-mediated inflammasome activation. *Cell Death Dis* 3: e403.
243. Mulay SR, Kulkarni OP, Rupanagudi KV, Migliorini A, Darisipudi MN, et al. (2013) Calcium oxalate crystals induce renal inflammation by NLRP3-mediated IL-1 $\beta$  secretion. *J Clin Invest* 123: 236-246.
244. Niemi K, Teirila L, Lappalainen J, Rajamaki K, Baumann MH, et al. (2011) Serum amyloid A activates the NLRP3 inflammasome via P2X7 receptor and a cathepsin B-sensitive pathway. *J Immunol* 186: 6119-6128.
245. Mortimer L, Moreau F, Cornick S, Chadee K (2015) The NLRP3 Inflammasome Is a Pathogen Sensor for Invasive *Entamoeba histolytica* via Activation of  $\alpha$ 5 $\beta$ 1 Integrin at the Macrophage-Amebae Intercellular Junction. *PLoS Pathog* 11: e1004887.
246. Hoegen T, Tremel N, Klein M, Angele B, Wagner H, et al. (2011) The NLRP3 inflammasome contributes to brain injury in pneumococcal meningitis and is activated through ATP-dependent lysosomal cathepsin B release. *J Immunol* 187: 5440-5451.
247. Asgari E, Le Friec G, Yamamoto H, Perucha E, Sacks SS, et al. (2013) C3a modulates IL-1 $\beta$  secretion in human monocytes by regulating ATP efflux and subsequent NLRP3 inflammasome activation. *Blood* 122: 3473-3481.
248. Dutra FF, Alves LS, Rodrigues D, Fernandez PL, de Oliveira RB, et al. (2014) Hemolysis-induced lethality involves inflammasome activation by heme. *Proc Natl Acad Sci U S A* 111: E4110-4118.
249. Riteau N, Gasse P, Fauconnier L, Gombault A, Couegnat M, et al. (2010) Extracellular ATP is a danger signal activating P2X7 receptor in lung inflammation and fibrosis. *Am J Respir Crit Care Med* 182: 774-783.
250. Weber FC, Esser PR, Muller T, Ganesan J, Pellegatti P, et al. (2010) Lack of the purinergic receptor P2X(7) results in resistance to contact hypersensitivity. *J Exp Med* 207: 2609-2619.
251. Garlid KD, Paucek P (2003) Mitochondrial potassium transport: the K(+) cycle. *Biochim Biophys Acta* 1606: 23-41.
252. Franchi L, Nunez G (2008) The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1 $\beta$  secretion but dispensable for adjuvant activity. *Eur J Immunol* 38: 2085-2089.
253. Li H, Willingham SB, Ting JP, Re F (2008) Cutting edge: inflammasome activation by alum and alum's adjuvant effect are mediated by NLRP3. *J Immunol* 181: 17-21.
254. Pazar B, Ea HK, Narayan S, Kolly L, Bagnoud N, et al. (2011) Basic calcium phosphate crystals induce monocyte/macrophage IL-1 $\beta$  secretion through the NLRP3 inflammasome in vitro. *J Immunol* 186: 2495-2502.

255. Chen CJ, Shi Y, Hearn A, Fitzgerald K, Golenbock D, et al. (2006) MyD88-dependent IL-1 receptor signaling is essential for gouty inflammation stimulated by monosodium urate crystals. *J Clin Invest* 116: 2262-2271.
256. So A, De Smedt T, Revaz S, Tschopp J (2007) A pilot study of IL-1 inhibition by anakinra in acute gout. *Arthritis Res Ther* 9: R28.
257. Shi Y, Evans JE, Rock KL (2003) Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* 425: 516-521.
258. Li H, Ambade A, Re F (2009) Cutting edge: Necrosis activates the NLRP3 inflammasome. *J Immunol* 183: 1528-1532.
259. Schorn C, Frey B, Lauber K, Janko C, Stryio M, et al. (2011) Sodium overload and water influx activate the NALP3 inflammasome. *J Biol Chem* 286: 35-41.
260. Allaey I, Marceau F, Poubelle PE (2013) NLRP3 promotes autophagy of urate crystals phagocytized by human osteoblasts. *Arthritis Res Ther* 15: R176.
261. Shi M, Deng W, Bi E, Mao K, Ji Y, et al. (2008) TRIM30 alpha negatively regulates TLR-mediated NF-kappa B activation by targeting TAB2 and TAB3 for degradation. *Nat Immunol* 9: 369-377.
262. Kanayama A, Seth RB, Sun L, Ea CK, Hong M, et al. (2004) TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. *Mol Cell* 15: 535-548.
263. Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, et al. (2001) TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* 412: 346-351.
264. Wu J, Yan Z, Schwartz DE, Yu J, Malik AB, et al. (2013) Activation of NLRP3 inflammasome in alveolar macrophages contributes to mechanical stretch-induced lung inflammation and injury. *J Immunol* 190: 3590-3599.
265. Petrasek J, Iracheta-Vellve A, Saha B, Satishchandran A, Kodys K, et al. (2015) Metabolic danger signals, uric acid and ATP, mediate inflammatory cross-talk between hepatocytes and immune cells in alcoholic liver disease. *J Leukoc Biol* 98: 249-256.
266. Stoecklein VM, Osuka A, Ishikawa S, Lederer MR, Wanke-Jellinek L, et al. (2015) Radiation exposure induces inflammasome pathway activation in immune cells. *J Immunol* 194: 1178-1189.
267. Thomas PG, Dash P, Aldridge JR, Jr., Ellebedy AH, Reynolds C, et al. (2009) The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1. *Immunity* 30: 566-575.
268. Segovia J, Sabbah A, Mgbemena V, Tsai SY, Chang TH, et al. (2012) TLR2/MyD88/NF-kappaB pathway, reactive oxygen species, potassium efflux activates NLRP3/ASC inflammasome during respiratory syncytial virus infection. *PLoS One* 7: e29695.

269. Bukreyev A, Whitehead SS, Murphy BR, Collins PL (1997) Recombinant respiratory syncytial virus from which the entire SH gene has been deleted grows efficiently in cell culture and exhibits site-specific attenuation in the respiratory tract of the mouse. *J Virol* 71: 8973-8982.
270. Whitehead SS, Bukreyev A, Teng MN, Firestone CY, St Claire M, et al. (1999) Recombinant respiratory syncytial virus bearing a deletion of either the NS2 or SH gene is attenuated in chimpanzees. *J Virol* 73: 3438-3442.
271. Triantafilou K, Kar S, Vakakis E, Kotecha S, Triantafilou M (2013) Human respiratory syncytial virus viroporin SH: a viral recognition pathway used by the host to signal inflammasome activation. *Thorax* 68: 66-75.
272. Orihuela CJ, Gao G, McGee M, Yu J, Francis KP, et al. (2003) Organ-specific models of *Streptococcus pneumoniae* disease. *Scand J Infect Dis* 35: 647-652.
273. Witzenth M, Pache F, Lorenz D, Koppe U, Gutbier B, et al. (2011) The NLRP3 inflammasome is differentially activated by pneumolysin variants and contributes to host defense in pneumococcal pneumonia. *J Immunol* 187: 434-440.
274. Fatykhova D, Rabes A, Machnik C, Guruprasad K, Pache F, et al. (2015) Serotype 1 and 8 *Pneumococci* Evade Sensing by Inflammasomes in Human Lung Tissue. *PLoS One* 10: e0137108.
275. Willingham SB, Allen IC, Bergstralh DT, Brickey WJ, Huang MT, et al. (2009) NLRP3 (NALP3, Cryopyrin) facilitates in vivo caspase-1 activation, necrosis, and HMGB1 release via inflammasome-dependent and -independent pathways. *J Immunol* 183: 2008-2015.
276. Mishra BB, Moura-Alves P, Sonawane A, Hacohe N, Griffiths G, et al. (2010) *Mycobacterium tuberculosis* protein ESAT-6 is a potent activator of the NLRP3/ASC inflammasome. *Cell Microbiol* 12: 1046-1063.
277. McElvania Tekippe E, Allen IC, Hulseberg PD, Sullivan JT, McCann JR, et al. (2010) Granuloma formation and host defense in chronic *Mycobacterium tuberculosis* infection requires PYCARD/ASC but not NLRP3 or caspase-1. *PLoS One* 5: e12320.
278. He X, Mekasha S, Mavrogiorgos N, Fitzgerald KA, Lien E, et al. (2010) Inflammation and fibrosis during *Chlamydia pneumoniae* infection is regulated by IL-1 and the NLRP3/ASC inflammasome. *J Immunol* 184: 5743-5754.
279. Rottenberg ME, Gigliotti Rothfuchs AC, Gigliotti D, Svanholm C, Bandholtz L, et al. (1999) Role of innate and adaptive immunity in the outcome of primary infection with *Chlamydia pneumoniae*, as analyzed in genetically modified mice. *J Immunol* 162: 2829-2836.
280. Ghayur T, Banerjee S, Hugunin M, Butler D, Herzog L, et al. (1997) Caspase-1 processes IFN-gamma-inducing factor and regulates LPS-induced IFN-gamma production. *Nature* 386: 619-623.

281. Netea MG, Kullberg BJ, Jacobs LE, Verver-Jansen TJ, van der Ven-Jongekrijg J, et al. (2004) Chlamydia pneumoniae stimulates IFN-gamma synthesis through MyD88-dependent, TLR2- and TLR4-independent induction of IL-18 release. *J Immunol* 173: 1477-1482.
282. Ceballos-Olvera I, Sahoo M, Miller MA, Del Barrio L, Re F (2011) Inflammasome-dependent pyroptosis and IL-18 protect against Burkholderia pseudomallei lung infection while IL-1 $\beta$  is deleterious. *PLoS Pathog* 7: e1002452.
283. Ketelut-Carneiro N, Silva GK, Rocha FA, Milanezi CM, Cavalcanti-Neto FF, et al. (2015) IL-18 triggered by the Nlrp3 inflammasome induces host innate resistance in a pulmonary model of fungal infection. *J Immunol* 194: 4507-4517.
284. Moretti S, Bozza S, Oikonomou V, Renga G, Casagrande A, et al. (2014) IL-37 inhibits inflammasome activation and disease severity in murine aspergillosis. *PLoS Pathog* 10: e1004462.
285. Drexler SK, Kong P, Inglis J, Williams RO, Garlanda C, et al. (2010) SIGIRR/TIR-8 is an inhibitor of Toll-like receptor signaling in primary human cells and regulates inflammation in models of rheumatoid arthritis. *Arthritis Rheum* 62: 2249-2261.
286. Pauwels NS, Bracke KR, Dupont LL, Van Pottelberge GR, Provoost S, et al. (2011) Role of IL-1 $\alpha$  and the Nlrp3/caspase-1/IL-1 $\beta$  axis in cigarette smoke-induced pulmonary inflammation and COPD. *Eur Respir J* 38: 1019-1028.
287. Yang W, Ni H, Wang H, Gu H (2015) NLRP3 inflammasome is essential for the development of chronic obstructive pulmonary disease. *Int J Clin Exp Pathol* 8: 13209-13216.
288. Lommatzsch M, Cicko S, Muller T, Lucattelli M, Bratke K, et al. (2010) Extracellular adenosine triphosphate and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 181: 928-934.
289. O'Byrne PM, Inman MD (2003) Airway hyperresponsiveness. *Chest* 123: 411S-416S.
290. Lamblin C, Gosset P, Tillie-Leblond I, Saulnier F, Marquette CH, et al. (1998) Bronchial neutrophilia in patients with noninfectious status asthmaticus. *Am J Respir Crit Care Med* 157: 394-402.
291. Idzko M, Hammad H, van Nimwegen M, Kool M, Willart MA, et al. (2007) Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. *Nat Med* 13: 913-919.
292. Besnard AG, Guillou N, Tschopp J, Erard F, Couillin I, et al. (2011) NLRP3 inflammasome is required in murine asthma in the absence of aluminum adjuvant. *Allergy* 66: 1047-1057.
293. Allen IC, Jania CM, Wilson JE, Tekeppe EM, Hua X, et al. (2012) Analysis of NLRP3 in the development of allergic airway disease in mice. *J Immunol* 188: 2884-2893.

294. Bruchard M, Rebe C, Derangere V, Togbe D, Ryffel B, et al. (2015) The receptor NLRP3 is a transcriptional regulator of TH2 differentiation. *Nat Immunol* 16: 859-870.
295. Kim HY, Lee HJ, Chang YJ, Pichavant M, Shore SA, et al. (2014) Interleukin-17-producing innate lymphoid cells and the NLRP3 inflammasome facilitate obesity-associated airway hyperreactivity. *Nat Med* 20: 54-61.
296. Ather JL, Ckless K, Martin R, Foley KL, Suratt BT, et al. (2011) Serum amyloid A activates the NLRP3 inflammasome and promotes Th17 allergic asthma in mice. *J Immunol* 187: 64-73.
297. Fukumoto J, Fukumoto I, Parthasarathy PT, Cox R, Huynh B, et al. (2013) NLRP3 deletion protects from hyperoxia-induced acute lung injury. *Am J Physiol Cell Physiol* 305: C182-189.
298. Galam L, Rajan A, Failla A, Soundararajan R, Lockey RF, et al. (2016) Deletion of P2X7 attenuates hyperoxia-induced acute lung injury via inflammasome suppression. *Am J Physiol Lung Cell Mol Physiol*: ajplung 00417 02015.
299. Zhang Y, Sauler M, Shinn AS, Gong H, Haslip M, et al. (2014) Endothelial PINK1 mediates the protective effects of NLRP3 deficiency during lethal oxidant injury. *J Immunol* 192: 5296-5304.
300. Wang S, Zhao J, Wang H, Liang Y, Yang N, et al. (2015) Blockage of P2X7 attenuates acute lung injury in mice by inhibiting NLRP3 inflammasome. *Int Immunopharmacol* 27: 38-45.
301. dos Santos G, Rogel MR, Baker MA, Troken JR, Urich D, et al. (2015) Vimentin regulates activation of the NLRP3 inflammasome. *Nat Commun* 6: 6574.
302. Kuipers MT, Aslami H, Janczy JR, van der Sluijs KF, Vlaar AP, et al. (2012) Ventilator-induced lung injury is mediated by the NLRP3 inflammasome. *Anesthesiology* 116: 1104-1115.
303. Zhang Y, Liu G, Dull RO, Schwartz DE, Hu G (2014) Autophagy in pulmonary macrophages mediates lung inflammatory injury via NLRP3 inflammasome activation during mechanical ventilation. *Am J Physiol Lung Cell Mol Physiol* 307: L173-185.
304. Gasse P, Riteau N, Vacher R, Michel ML, Fautrel A, et al. (2011) IL-1 and IL-23 mediate early IL-17A production in pulmonary inflammation leading to late fibrosis. *PLoS One* 6: e23185.
305. Xu JF, Washko GR, Nakahira K, Hatabu H, Patel AS, et al. (2012) Statins and pulmonary fibrosis: the potential role of NLRP3 inflammasome activation. *Am J Respir Crit Care Med* 185: 547-556.
306. Liao J, Kapadia VS, Brown LS, Cheong N, Longoria C, et al. (2015) The NLRP3 inflammasome is critically involved in the development of bronchopulmonary dysplasia. *Nat Commun* 6: 8977.

## **Chapter 2. Inflammasome activation mediates tissue-specific pathogenesis in *Staphylococcus aureus* infection<sup>1</sup>**

### **2.1 Introduction**

*Staphylococcus aureus* is a Gram-positive coccus that interacts with human hosts on a spectrum from quiet commensal to deadly pathogen. *S. aureus* is capable of infecting nearly every tissue in the body resulting in cellulitis, pneumonia, osteomyelitis, endocarditis, brain abscesses, bacteremia and more. *S. aureus* has a wide range factors that promote infection and each site of infection triggers a different response in the human host. In particular, the different patterns of inflammasome activation mediate tissue-specific pathogenesis in *S. aureus* infection. Although still a nascent field, understanding the unique host-pathogen interactions in each infection and the role of inflammasomes in mediating pathogenesis may lead to novel strategies for treating *S. aureus* infections. Reviews addressing *S. aureus* virulence and pathogenesis [1], as well as epidemiology and pathophysiology [2], have recently been published. This review will focus on *S. aureus* factors that activate inflammasomes and their impact on innate immune signaling and bacterial survival.

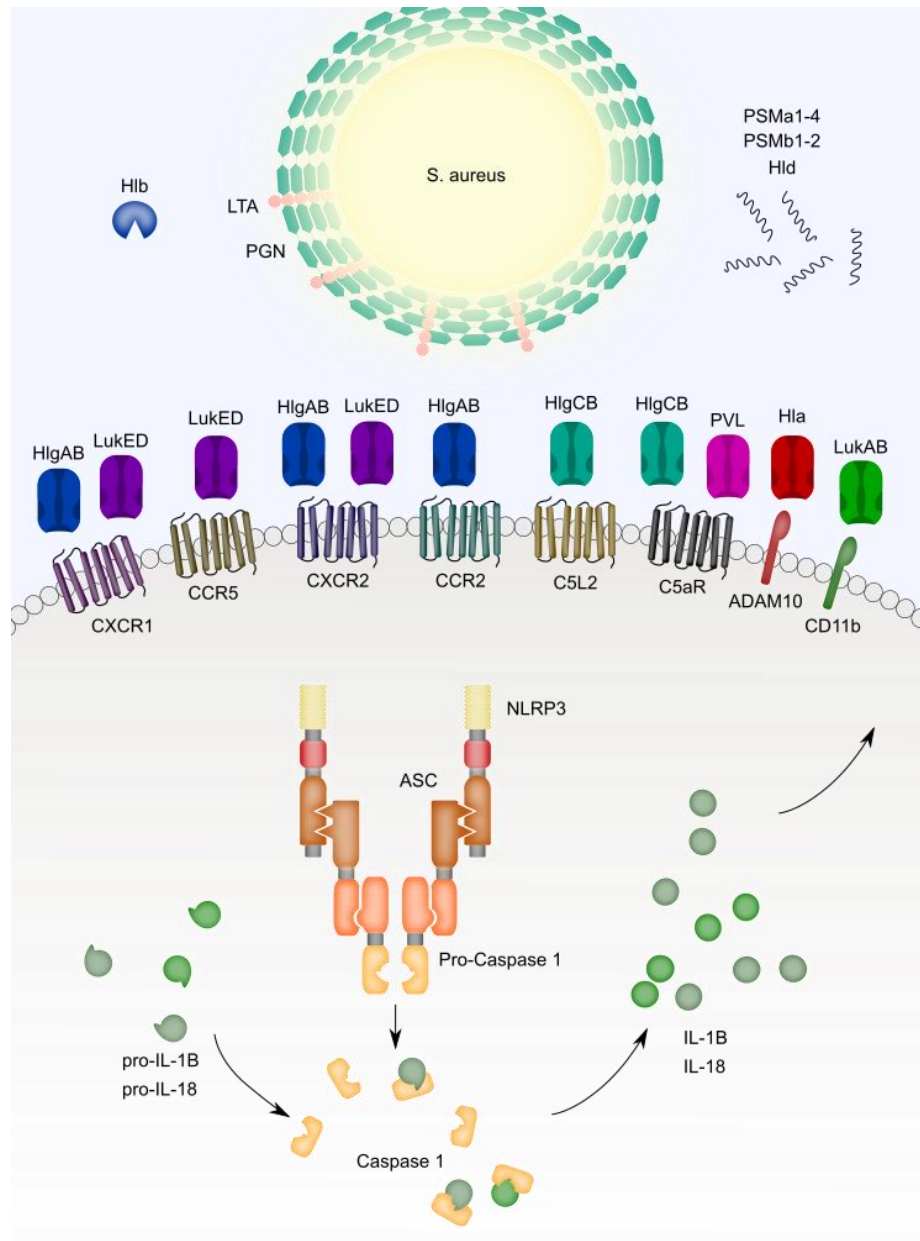
---

<sup>1</sup> This chapter was submitted as an invited review for publication to the journal Current Topics in Microbiology and Immunology. The citation was not available at time of submission.



## **2.2 *S. aureus* factors that activate inflammasomes**

*S. aureus* has numerous mechanisms to evade and subvert the immune system allowing it to produce infection broadly in immune-competent hosts. Many virulence factors, such as pore-forming toxins (PFTs) and phenol-soluble modulins (PSMs), and common bacterial constituents, such as bacterial RNA, lipoproteins and lipoteichoic acid – a constituent of the Gram-positive cell wall, activate inflammasomes and will be discussed here (Figure 1).



**Figure 2.1 *S. aureus* pathogen-associated molecular patterns and virulence factors activate inflammasome priming and signaling.** Activation of inflammasome signaling typically proceeds in two steps. First, Toll-like receptors (TLR) engage pathogen-associated molecular patterns (PAMPs) to prime inflammasome signaling. *S. aureus* lipoteichoic acid (LTA), a major constituent of the cell wall of Gram-positive bacteria binds TLR2 (not pictured). TLR signaling

activates NF- $\kappa$ B-mediated transcription of pro-interleukin-1 $\beta$  (IL-1 $\beta$ ) and also triggers post-translational modifications of the inflammasome signaling pathway. Second, a wide variety of *S. aureus* factors, including pore-forming toxins (PFTs), peptidoglycan (PGN), phenol-soluble modulins (PSMs and Hld), activate inflammasome signaling. Each PFT requires a receptor, as seen in the center of the figure, to promote binding and pore formation. The NLRP3 inflammasome is depicted as the primary example of inflammasome signaling in this setting. When activated, NLRP3 binds ASC through PYD-PYD interactions and ASC binds pro-Caspase 1 through CARD-CARD interactions. Formation of the NLRP3 inflammasome complex causes activation of Caspase 1 that can process pro-IL-1 $\beta$  and pro-IL-18 for activation and secretion.

*S. aureus* PFTs (i.e., alpha-hemolysin [Hla], gamma-hemolysin [HlgAB, HlgCB], leukocidin AB [LukAB, also known as LukGH], leukocidin ED [LukED], and Panton-Valentine leukocidin [PVL, also known as LukFS]) are, in most cases, two-component toxins that initiate attack when one component recognizes and binds to a cell surface receptor inserting itself into the host cell membrane. This is followed by recruitment of the second component and additional pairs to complete a hexameric beta-barrel pore [3-6]. The notable exception to this pattern is Hla which forms a pore by binding to its receptor then forming a heptameric beta-barrel pore made up of only a single component [7,8]. Each of these toxins is cytotoxic to a range of cells based on expression of their cognate receptor: Hla and a disintegrin and metalloprotease 10 (ADAM10) [9]; HlgAB and CXCR1, CXCR2 and CCR2; HlgCB and C5aR and C5L2 [10]; LukAB and CD11b [11], LukED and CCR5 [12], CXCR1 and CXCR2 [13]; and PVL and C5aR [14]. Many of these toxin-receptor pairs lend a level of species specificity as well – murine neutrophils are partially resistant to HlgAB and HlgCB because CCR2 is the only compatible Hlg murine receptor [10], LukAB binds to human CD11b at a 1000-fold higher affinity than murine [11], and both rabbit and human neutrophils are susceptible to PVL while murine and java monkey neutrophils are resistant [15].

Beta-hemolysin (Hlb) and delta-hemolysin (Hld) are similarly named but functionally distinct toxins that do not form a beta-barrel pore like the aforementioned toxins. Hlb is a hemolytic and cytotoxic sphingomyelinase [16,17]. Delta-hemolysin is a 26-amino acid membrane-damaging peptide that is classified as a phenol-soluble modulins [18].

Phenol-soluble modulins (PSMs – reviewed [19]) are cytotoxic and pro-inflammatory peptides produced by *S. aureus* [20]. *S. aureus* produces four PSM $\alpha$  peptides (PSM $\alpha$ 1-PSM $\alpha$ 4), two PSM $\beta$  peptides (PSM $\beta$ 1 and PSM $\beta$ 2), and delta-hemolysin (Hld). PSMs are sensed by

formyl peptide receptor 2 in human neutrophils [21]. PSMs have demonstrated roles in cytolysis, biofilm development, and immunomodulation and are thought to be partially responsible for the increased virulence of community-acquired *S. aureus* over hospital-acquired strains [22].

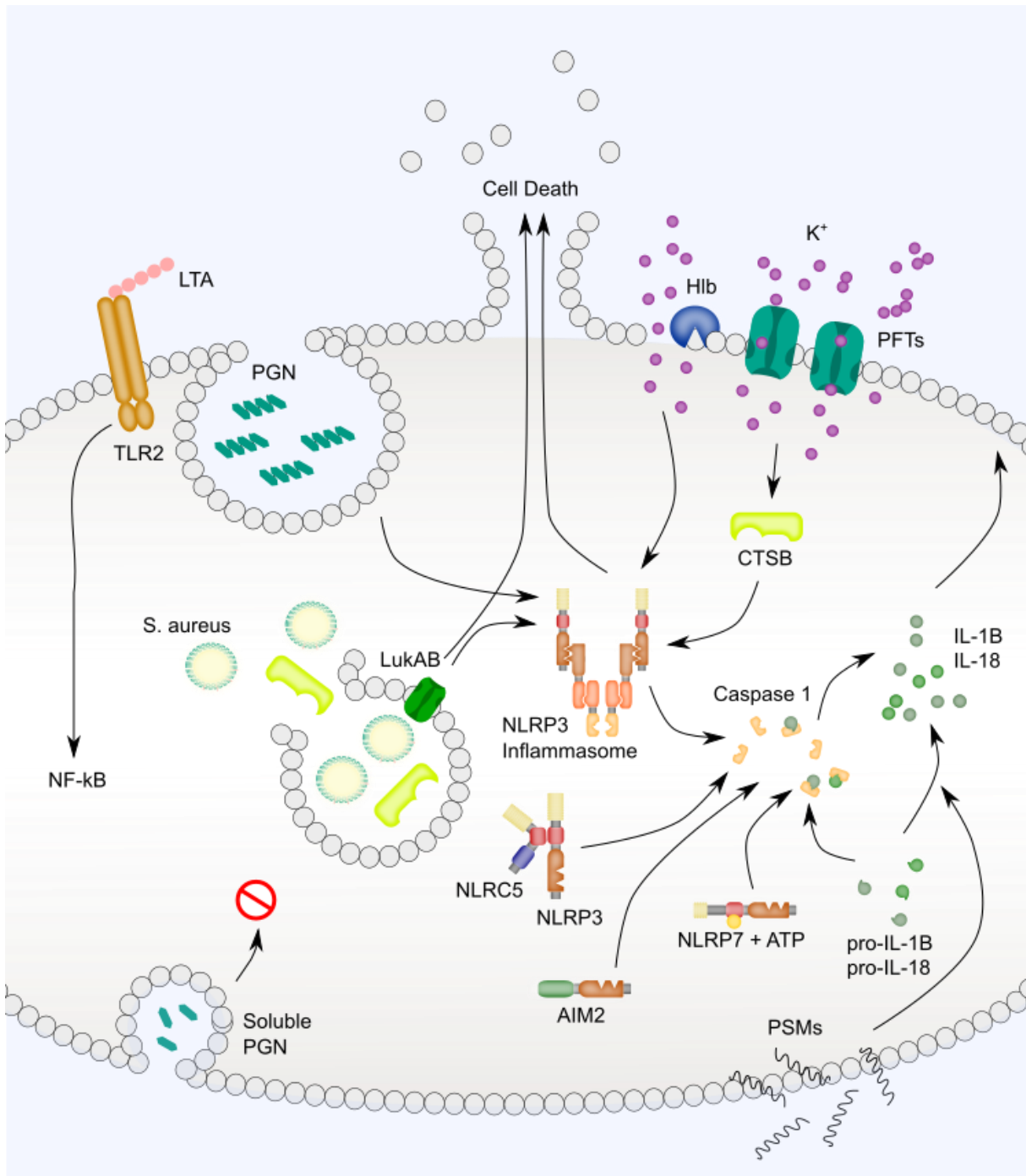
Lipoteichoic acid (LTA) is a component of the bacterial wall in Gram-positive bacteria that is essential for bacterial growth, cell division and survival [23]. Likely because of this integral requirement for LTA, the innate immune system has many robust strategies for detecting and responding to LTA, including Toll-like receptor 2, Scavenger Receptor A [24,25], CD36 [26], CD14 and soluble CD14 [27,28], Surfactant Protein D [29], Paired Ig-like Receptor B [30], and L-ficolin [31] (reviewed [32]). Having multiple methods for sensing LTA is probably important to fine tune the immune response to infections. For example, LTA binding to TLR2 upregulates cytokine production through NF- $\kappa$ B signaling, while LTA binding to Paired Ig-like receptor B suppresses IL-6 and IL-1 $\beta$  secretion [30]. The same LTA found in *S. aureus* is also found in other members of the *Firmicutes* phylum, such as *Bacillus subtilis* and *Listeria monocytogenes* [33], that share a last common ancestor approximately 1.5 billion years ago [34,35]. As such, LTA likely evolved well before the establishment of multicellular life suggesting that multiple strategies of LTA sensing in humans is the result of selective pressures favoring LTA detection rather than evolution of LTA to manipulate immune signaling. Additionally, other integral bacterial components such as bacterial RNA [36,37], lipoproteins [38], and peptidoglycan [39,40] are important molecular patterns detected by the immune system through inflammasomes.

### 2.3 Inflammasomes that are activated by *S. aureus*

A complex interplay of different inflammasomes activate during *S. aureus* infections (Figure 2). The first demonstration of inflammasome activation by *S. aureus* was the NLRP3 inflammasome [41]. Activation of the NLRP3 inflammasome involves two steps: (1) a priming step that involves NF- $\kappa$ B-mediated upregulation of pro-IL-1 $\beta$  transcription and post-translational modifications of inflammasome components and (2) NLRP3 inflammasome oligomerization leading to Caspase 1 activation and secretion of mature IL-1 $\beta$  and IL-18 and pyroptosis. In the case of an *S. aureus* infection, TLR2 detects LTA to provide an initial priming signal [42,43]. In experiments, in addition to purified LTA [44], priming is typically triggered by heat-killed *S. aureus* (HKSA) [45] or a xenobiotic TLR-ligand such as LPS.

Numerous studies have since delineated the contribution of various *S. aureus* virulence factors to NLRP3 inflammasome activation. All six PFTs from *S. aureus* activate the NLRP3 inflammasome in THP1 cells and primary human monocytes, leading to canonical cytokine processing and pyroptosis [38,44-46].

Caspase 1 is critical for processing IL-1 $\beta$  and IL-18 for secretion and is classically required for pyroptosis. However, Caspase 1 is not required for Hla-, PVL- or LukAB-triggered pyroptosis as shown with all three toxins using a Caspase 1 inhibitor in human cells treated with the toxins and by Caspase 1/Caspase 11 knockout mouse macrophages treated with Hla [44-46].



**Figure 2.2 NLRP3, NLRC5, NLRP7, AIM2 and other inflammasome-like signaling is activated in response to *S. aureus*.** Priming of inflammasome signaling in response to *S. aureus* typically occurs when lipoteichoic acid (LTA) binds Toll-like receptor 2 (TLR2) leading to upregulation of NF-κB-mediated transcription. The most well studied inflammasome activated

by *S. aureus* is the NLRP3 inflammasome. Activation of the NLRP3 inflammasome occurs through pore-mediated and phagocytosis-mediated actions. PFTs and Hlb damage the plasma membrane and cause potassium efflux necessary for NLRP3 inflammasome activation. PVL signals through CTSB to activate inflammasome signaling. PVL, Hla and LukAB also promote Caspase 1-independent cell death. PFTs may also destabilize lysophagosomes. In particular, LukAB has been shown to bind CD11b on the phagosomal membrane to promote *S. aureus* escape. This triggers NLRP3-dependent cytokine secretion and NLRP3-independent cell death. Phagocytosis of particulate peptidoglycan (PGN), but not soluble PGN, also leads to NLRP3 inflammasome activation. Lysophagosomal rupture is thought to lead to CTSB leakage into the cytoplasm, though this has not been shown directly with *S. aureus*.

A variety of other inflammasomes have also been implicated in sensing *S. aureus*. NLRC5 binds to NLRP3 and enhances cytokine secretion. NLRP7 binds to ATP and promotes cytokine secretion as well. AIM2 plays an important role in detecting *S. aureus* in central nervous system infections. PSMs also trigger secretion of IL-1 $\beta$  and IL-18, but through a Caspase 1-independent mechanism.



*S. aureus* activation of the NLRP3 inflammasome with PFTs, Hla [45], LukAB [44], and PVL [46], requires potassium efflux. PVL also causes activation of Cathepsin B (CTSB) and inhibition of CTSB protease activity by CA-074, a small molecule inhibitor, blocks cell death [46]. CTSB is a lysosomal cysteine protease required for NLRP3 activation by disruption of lysophagosomes after ingestion of inflammation inducing particulate matter like silica [47]. CTSB is thought to spill into the cytoplasm after lysophagosomal disruption, but the mechanism by which CTSB activates NLRP3 is unclear. It is also not known whether the requirement for CTSB in PVL-induced NLRP3 activation is toxin specific or common to all *S. aureus* PFTs. One possible explanation for how CTSB is liberated from lysophagosomes is that *S. aureus* PFTs can bind to their cognate receptor on phagosomal membranes. This binding promotes *S. aureus* escape from phagocytosis to enable survival [48]. Interestingly, the route of exposure (i.e., extracellular versus within a phagocytic vacuole) also seems to influence signaling in response to LukAB [44]. When LukAB interacts with CD11b at the plasma membrane, NLRP3 activation drives IL-1 $\beta$  and IL-18 secretion and causes cell death through pyroptosis. Alternatively, when LukAB expressed from phagocytosed *S. aureus* interact with CD11b on the phagocytic vacuole membrane, cell death becomes independent of NLRP3 activation, while NLRP3 is still required for IL-1 $\beta$  and IL-18 secretion. It is not clear whether this separate cell death mechanism engaged by phagosome-localized LukAB is activated in addition to NLRP3-dependent pathways or if the NLRP3-dependent pathways activated by extracellular toxin are not activated by toxin in this setting [44].

*S. aureus* can also trigger necroptosis, cell death dependent on RIPK1, RIPK3, and MLKL that can be blocked by necrostatin-1 and necrosulfonamide. Necrosulfonamide

diminished *S. aureus*- and purified Hla-induced IL-1 $\beta$  secretion suggesting this agent or the necroptotic pathway may tie in with inflammasome activation [49].

While the cytotoxicity of PFTs has been demonstrated widely in primary human monocytes, macrophages, neutrophils and dendritic cells, most studies of PFTs and inflammasome activation have been carried out in THP-1 cells, mouse macrophages or human peripheral blood monocytes. While it is easy to assume that the cytotoxicity across all these primary human cell types is NLRP3-mediated, this has not been demonstrated directly [46,50,51].

In primary human monocytes, the rank order potency of the PFTs is PVL and LukAB (tied), Hla and HlgCB (tied), HlgAB, then LukED [44]. The LukAB operon promoter is preferentially activated when USA300 strain *S. aureus* is exposed to human polymorphonuclear leukocytes (PMNs) [48]. Despite these hints at a prominent role for LukAB in *S. aureus* pathogenesis, the role of LukAB in infections has largely been overlooked because of its lack of effect in mice.

New infection models are sorely needed to better appreciate the role of human-restricted PFTs in pathogenesis. As an example, a recently developed humanized NSG mouse model of *S. aureus* skin infections required one to two log lower inoculum to induce consistent skin lesions as compared with non-humanized mice. This model aided in studying the role of PVL in dermonecrosis. Blocking the PVL receptor, human C5aR, with PMX53 or an anti-C5aR antibody eliminated the enhanced cytotoxicity of PVL-positive *S. aureus* but also reduced recruitment of neutrophils and exacerbated the infection [52].

Also important to note, the PFT rank order potency is almost certainly different in other cells based on the abundance of cell surface receptors for each toxin which may vary with

receptor gene polymorphisms. Further, binding affinities between toxins and receptors differ between species. For example, mouse bone marrow derived macrophages are resistant to purified Hla [45] and highly resistant PVL [46].

Few other mechanistic details between PFT exposure and NLRP3 inflammasome activation have been determined. Many other host proteins have been implicated along the NLRP3 inflammasome signaling cascade with other triggers, but have yet to be confirmed for *S. aureus*.

Particulate peptidoglycan (PGN), a component of the bacterial cell wall, can activate the NLRP3 inflammasome when internalized via phagocytosis [39,40]. The *S. aureus* enzyme PGN O-acetyltransferase A modifies PGN making the cell wall resistant to lysozyme digestion [53]. *S. aureus* lacking O-acetyltransferase A lead to enhanced inflammasome activation and inflammation *in vitro* and *in vivo* when compared to *S. aureus* with the enzyme. Loss of O-acetyltransferase A in *S. aureus* decreased bacterial survival in co-culture with BMDMs and in murine skin infections. However, skin lesion size was significantly increased in infections with O-acetyltransferase A-deficient *S. aureus* and treatment of mice with anti-IL-1 $\beta$  and IL-18 antibodies eliminated this increase [40].

Induction of *S. aureus* penicillin-binding protein 2A (encoded by the *mecA* gene) by beta-lactam treatment leads to a reduction in PGN cross-linking and enhanced PGN degradation. Phagocytosis and digestion of PGN from beta-lactam treated *S. aureus* caused NLRP3 inflammasome activation and robust IL-1 $\beta$  secretion in BMDMs and when injected into mice. These effects are lost upon peptidoglycan solubilization by pretreatment with lysostaphin. Mice infected subcutaneously with *S. aureus* then treated with nafcillin, a beta-lactam, had larger skin

lesions and more neutrophils in the lesion than PBS-treated mice. Bacterial burden was lower in nafcillin-treated mice, suggesting that a hyperactive immune response caused the lesion [39]. The exact nature of PGN crosslinking that induces NLRP3 inflammasome activation has not been determined. Mutanolysin, like lysozyme, cleaves the N-acetylmuramyl- $\beta$ (1-4)-N-acetylglucosamine linkages of PGN. While pretreatment of purified PGN with mutanolysin or lysostaphin prevents NLRP3 activation, most methods for purifying PGN generate lysozyme/mutanolysin-sensitive PGN [40]. Therefore, the ability of lysozyme-sensitive PGN in O-acetyltransferase A-deficient *S. aureus*, but not mutanolysin predigested purified PGN, to induce NLRP3 inflammasome activation must depend upon the timing and location of lysozyme digestion. The NLRP3 inflammasome is most likely activated by partially cross-linked PGN undergoing digestion in the phagosome. This strategy could help phagocytes differentiate PGN digested by secreted lysozyme from bacteria-associated PGN to better assess the proximity and severity of a bacteria threat. Consistent with that hypothesis, Muller et al. found that lysostaphin digestion of PGN could be titrated down to a point that promoted IL-1 $\beta$  secretion [39].

*S. aureus* Hlb-deficient mutants had no observable defect in inducing IL-1 $\beta$  and IL-18 secretion in mouse macrophages. *S. aureus* lacking the combination of Hla, Hlb, and Hlg caused very little IL-1 $\beta$  and IL-18 secretion when compared to *S. aureus* lacking any pair of these hemolysins [38]. This finding suggests that Hla, Hlb, and Hlg may have a synergistic effect on mouse macrophages, though the mechanism of which is unclear. In a study that preceded the discovery of the NLRP3 inflammasome, purified Hlb triggered human monocytes to secrete IL-1 $\beta$  and extracellular potassium suppressed this activity [16]. Suppression by extracellular potassium strongly suggests a role for the NLRP3 inflammasome in Hlb-induced IL-1 $\beta$  secretion, though this has not been directly demonstrated. These studies suggest that *S. aureus*

may have redundant mechanisms for activating the NLRP3 inflammasome. It is also possible that hemolysin production is context dependent and each hemolysin may be required in infection of separate tissues that has not been recapitulated in *in vitro* cell culture of immune cells. Hlb also limits production of IL-8, a potent neutrophil chemoattractant, by human endothelial cells [54] and as such, may play a dual role in establishing and promoting infection.

Deletion of lipoprotein diacylglycerol transferase ( $\Delta$ lgt), the protein responsible for catalyzing the first step of lipoprotein biosynthesis in *S. aureus*, was also used to explore secondary factors responsible for NLRP3 inflammasome activation. Macrophages exposed to purified Hla and supernatant from  $\Delta$ lgt *S. aureus* exhibited decreased Caspase 1 processing and IL-1 $\beta$  secretion as compared to macrophages exposed to supernatant from the isogenic parental *S. aureus* strain [38].

Other inflammasomes may interact with NLRP3 to enhance inflammatory signaling. In one instance, NLRC5 was shown to co-immunoprecipitate with NLRP3 and knockdown of NLRC5 in THP1 cells by shRNA led to dramatic reduction in IL-1 $\beta$  secretion in response to live *S. aureus* [55].

Beyond NLRP3, NLRP7, a sensor of acylated lipopeptides, appears to play a role in response to *S. aureus*. Knockdown of NLRP7 in THP1 cells resulted in decreased *S. aureus*-induced IL-1 $\beta$  secretion and increased intracellular bacteria following infection. However, loss of NLRP7 did not impact *S. aureus*-induced pyroptosis [56]. NLRP7 function requires ATP binding and hydrolysis as expression of a mutant NLRP7 defective in this process in THP1 cells led to decreased IL-1 $\beta$  secretion and pyroptosis, as measured by PI uptake, in response to *S. aureus* as compared to WT THP1 cells [57]. The role of NLRP7 in pyroptosis in these two

studies differed, both having used THP1 cells and live *S. aureus*, and the discrepancy remains unexplained.

AIM2 may also detect *S. aureus* during central nervous system infections leading to IL-1 $\beta$  production. *S. aureus* forms abscesses when injected intracranially into mice. Ultimately, the abscess is lethal to ASC knockout mice, but not NLRP3 knockout mice or the parental strain. To better understand why ASC, but not NLRP3, knockout mice succumb to infection, abscess homogenates were collected and analyzed with a multi-analyte microbead cytokine array. Loss of ASC dramatically decreased IL-1 $\beta$ , IL-6, CXCL1 and CXCL10 production, suggesting a defect in sensing and responding to *S. aureus*. Further investigation identified AIM2 as integral to the immune response against *S. aureus* in this brain abscess model as loss of AIM2 mirrored the cytokine secretion defect and mortality seen in ASC knockout mice [58].

Inflammasome-like or non-canonical inflammasome responses may also initiate an immune response against *S. aureus*. When purified, each of the seven *S. aureus* PSMs are sufficient to induce IL-18 secretion from human keratinocytes. *S. aureus* with all seven PSMs deleted induced lower levels of IL-18 and IL-1 $\beta$  secretion from keratinocytes. Unexpectedly, secretion of IL-18 or IL-1 $\beta$  from keratinocytes in response to live *S. aureus* could not be blocked by a panel of Caspase inhibitors [59].

While many studies of inflammasome activation by *S. aureus* have been conducted in *in vitro* cell culture using primary human monocytes as well as other cell types (THP1 cells, mouse macrophages, etc.), in natural infections, *S. aureus* and its secreted toxins interact with multiple cell types each with a unique capacity to sense and respond to the bacteria. This leads to the possibility that a variety of inflammasomes may be activated in different cell types over the course of an infection with variable effects on infection outcomes. To understand the role of

inflammasome activation in the natural course of infection, we turn our review to studies of *S. aureus* infection in animal models, primarily in mice. In most cases, mouse studies were carried out using only the C57BL/6 strain in which inflammasome components have been genetically deleted. Mice as a model for *S. aureus* infections have serious limitations as mice are relatively naturally resistant to *S. aureus* and non-responsive to many virulence factors that act specifically on human cells. These studies would benefit from incorporating more genetic diversity.

## **2.4 The role of inflammasomes in *S. aureus* infections**

### **2.4.1 The NLRP3 inflammasome responds to hemolysins to control *S. aureus* dermal infections**

*S. aureus* is the number one cause of skin infections. These infections range in severity from pimples and boils to abscesses and cellulitis. Neutrophils are an important component of controlling these infections [60] as neutrophil functional defects, such as chronic granulomatous disease, result in susceptibility to invasive *S. aureus* cutaneous infections [61]. A mouse model for *S. aureus* skin infections has been developed [62] and adapted to study the role of inflammasomes in this setting [63-65].

Tissue biopsies were collected from mice injected subcutaneously with a variety of isogenic hemolysin knockout *S. aureus* strains. When Hla, Hlb, and Hlg were deleted together, secreted IL-1 $\beta$  and IL-18 were significantly decreased as compared to any single or double hemolysin knockout and the parental *S. aureus* strain. Subcutaneous injection of mice with individual deletions of NLRP3, ASC, or Caspase 1, did not elicit IL-18 secretion, demonstrating a role for the NLRP3 inflammasome in this setting [38].

Mice deficient in IL-1 $\beta$  develop larger lesions with higher bacterial burdens and reduced neutrophil recruitment following subcutaneous injection of *S. aureus* as compared to parental strain mice [63]. Adoptive transfer of IL-1 $\beta$  deficient bone marrow into irradiated parental strain mice mirrored the defect seen in wholly IL-1 $\beta$  deficient mice, suggesting that immune cells are a critical source of IL-1 $\beta$  in this model skin infection. In IL-1 $\beta$  deficient mice, recombinant active IL-1 $\beta$  helped to control infections and promote bacterial clearance [63].

Using the same subcutaneous injection model, multispectral noninvasive imaging during infection revealed a strong functional and temporal association between neutrophil recruitment and IL-1 $\beta$  signaling. Adoptive transfer of IL-1 $\beta$ -expressing neutrophils into an IL-1 $\beta$  deficient host was sufficient to restore the impaired neutrophil abscess formation, suggesting neutrophils are the critical source of immune cell-derived IL-1 $\beta$  in this model. *S. aureus* induced IL-1 $\beta$  production during subcutaneous injection in mice in a Hla-dependent manner that required TLR2, NOD2, FPR1 and the NLRP3 inflammasome [64].

Although these studies in mice have been done with direct inoculation of bacteria through the epidermal barrier, *S. aureus* can cause infection by invading through a human keratinocyte barrier. In an organotypic culture of human keratinocytes grown at an air-liquid interface on a dermal substitute matrix, *S. aureus* can be seen by microscopy to disrupt the apical keratinocyte layer. Hla triggers Caspase 1 and Calpain (Ca<sup>2+</sup>-dependent intracellular protease) activation leading to IL-1 $\beta$  secretion and pyroptosis. If Caspase 1 or Calpain are inhibited during this process, invasion efficiency is partially decreased [66].

Patients with atopic dermatitis or psoriasis are commonly colonized with *S. aureus*; however, only those with atopic dermatitis suffer from increased risk of bacterial superinfections. Expression of NLRP3 and Caspase 1 by immunohistochemistry was reduced in skin in a cohort



of patients with lesional atopic dermatitis as compared to patients with psoriatic or healthy skin. Primary human keratinocytes from healthy skin downregulated NLRP3 and ASC expression in response to IL-4, IL-5 and IL-13 (cytokines abundant in atopic dermatitis) and upregulated NLRP3 in response to interferon- $\gamma$ . Similar results were seen in isolated monocytes. As a result, Hla-mediated Caspase 1-dependent IL-1 $\beta$  secretion was impaired in monocytes from patients with atopic dermatitis compared to patients with psoriasis and healthy controls [67]. The significance of a monocyte defect in *S. aureus* dermatitis, and whether neutrophils exhibit the same defect, however, is unclear. If it holds true that a Th2 dominant immune response opposes NLRP3 inflammasome activation, this might explain epidemiologic data suggesting patients with asthma and other atopic diseases are at increased risk of infection [68].

PSMs also play a role in pathogenesis of *S. aureus*-induced skin inflammation. Primary human keratinocytes exposed to *S. aureus* with all seven PSMs knocked out by allelic replacement or start codon disruption secreted significantly less IL-1 $\beta$ , IL-18, and LDH, a marker of cell death, than keratinocytes exposed to wild type *S. aureus*. When purified, all PSMs triggered IL-18 release but only the four PSMs and delta-toxin were cytotoxic. Interestingly, caspase inhibitors and extracellular potassium were unable to block cytokine secretion or cytotoxicity, suggesting that this combination of cell death and cytokine secretion that is normally associated with inflammasome activation of Caspase 1 is triggered by a Caspase 1-independent process in keratinocytes that remains unidentified. In mice challenged epicutaneously with *S. aureus* and an isogenic mutant lacking PSM, the parental *S. aureus* strain triggered a predominantly neutrophil infiltrate and the PSM-deficient *S. aureus* did not. This difference in host neutrophil recruitment to infection site persisted in mice lacking IL-18, suggesting that PSM-mediated killing of keratinocytes led to release of chemoattractive factors

or that PSM themselves are chemoattractive [59]. Previous research has shown that blocking formyl peptide receptor 2, the receptor for *S. aureus* PSMs, blocked PSM-mediated leukocyte infiltration in a mouse air pouch and mouse peritoneal infection model, suggesting that PSMs themselves are chemoattractive [21].

Currently though, the translatability of these models to the human condition remains an open question. Mouse skin differs significantly from human skin. Mouse epidermis is made up of only three cell layers and is less than 25 micrometers thick whereas human epidermis is usually 6-10 cell layers and is greater than 50 micrometers thick. The epithelial turnover in mouse skin is faster and mouse skin regenerates without significant scarring. These differences and more are reviewed in Gudjonsson et al [69]. Considerable interest exists in humanized mouse models of skin infections and these have been deployed to study the human specific pathogen *Neisseria meningitides* [70]. Caution must be taken in interpreting results, however, given changes observed in skin grafts over time [71]. Additionally, mice with human skin grafts would still carry myeloid-derived cells from mice which may be a significant cause of differences seen in *S. aureus* susceptibility between human and mice.

With those caveats in mind, current evidence suggests that *S. aureus* activates inflammasomes in keratinocytes to promote invasion and establish infection. PSMs that induce NLRP3-independent cell death in keratinocytes may provide a redundant mechanism to support invasion. Once infection is established, neutrophils are recruited and secrete IL-1 $\beta$ , probably through NLRP3 inflammasome activation, to promote clearance of *S. aureus* infections. If this holds true, therapeutic intervention to suppress inflammasome signaling may have a desirable effect in preventing invasion through keratinocytes but may unduly hinder clearance of already invaded *S. aureus*.

#### **2.4.2 *S. aureus* hijacks the NLRP3 inflammasome to exacerbate lung infection pathology**

*S. aureus* pneumonia is a difficult disease to effectively treat. Even with proper antimicrobial therapy, *S. aureus* triggers a massive immune response that causes significant tissue destruction with substantial lethality.

Hla is an essential virulence factor in mouse models of severe pneumonia. As in mouse skin infection models, *S. aureus* lacking Hla causes less lung pathology and death when delivered intranasally or intratracheally to the mouse lung when compared to *S. aureus* with an intact Hla gene. Blocking Hla with an antibody also promotes bacterial clearance from the lung, indicating that expression of Hla is important in the ability of the bacteria to cause lung infection [72,73]. Highly purified recombinant Hla, alone or in conjunction with HKSA, delivered intratracheally causes a clinical syndrome resembling *S. aureus* pulmonary infection in mice. Pathologic changes in the lung including destruction of normal alveolar architecture, tissue necrosis, hemorrhage, and inflammation are consistent with pathologic findings in human lungs with severe *S. aureus* pneumonia. This syndrome is largely abrogated when Hla is administered to mice lacking NLRP3. There was also no difference in bacterial burden or lung inflammation between wild type and NLRP3 null mice that were infected with *S. aureus* lacking Hla, suggesting Hla-induced activation of NLRP3 is a major cause of pneumonia pathology during *S. aureus* infection. Using an IL-1 receptor deficient mouse, it was shown that the pathogenesis of this severe inflammatory response and tissue destruction did not depend on IL-1 $\beta$  signaling [74]. These findings suggest that cell death and/or other Caspase 1-dependent cytokines downstream of NLRP3 activation were sufficient to cause severe lung pathology in the setting of *S. aureus* infection.

There is significant debate as to whether PVL is a critical virulence factor in pneumonia. Mice infected intranasally with PVL-expressing *S. aureus* experienced substantial neutrophil recruitment, inflammation in the lung parenchyma, bronchial epithelial damage, tissue necrosis and hemorrhage. These same effects were seen upon administration of both components of the purified PVL toxin but were absent in isogenic PVL-negative *S. aureus* or upon administration of a single component of the PVL toxin [75]. A second report replicated these findings, demonstrating that deletion of PVL from the LAC *S. aureus* strain improves Balb/c mouse survival following intranasal inoculation. Additionally, immunization against PVL showed a trend towards protection in subsequent *S. aureus* challenge by intranasal infection [76]. These results are in contrast to separate findings that deletion of PVL from the LAC *S. aureus* strain actually enhances virulence and decreases survival of Balb/c mice infected using the same protocol [77].

In primary human monocyte-derived macrophages, HKSA followed by administration of recombinant PVL induced secretion of high levels of IL-1 $\beta$ . IL-1 $\beta$  secretion was enhanced by co-administration PVL and the PSMs Hld and PSMalpha3, the PFTs Hlg and LukED, and Hlb, greater than the sum of their parts [78]. PVL also triggered IL-1 $\beta$  secretion in human alveolar macrophages that goes on to stimulate the secretion of neutrophil attracting chemokines IL-8 and MCP-1 by A549 human lung epithelial cells in a mixed culture model. Blocking the IL-1 receptor with IL-1R antagonist abolishes PVL-induced secretion of IL-8 and MCP-1 [78]. These data suggest that blockade of IL-1 $\beta$  signaling may diminish pathology in *S. aureus* pulmonary infection. However, a subsequent study in rabbits demonstrated that Anakinra, an IL-1 receptor antagonist, could block pathology induced by treatment with recombinant PVL and HKSA but not in live *S. aureus* infection. Furthermore, treatment of infected rabbits with Anakinra led to

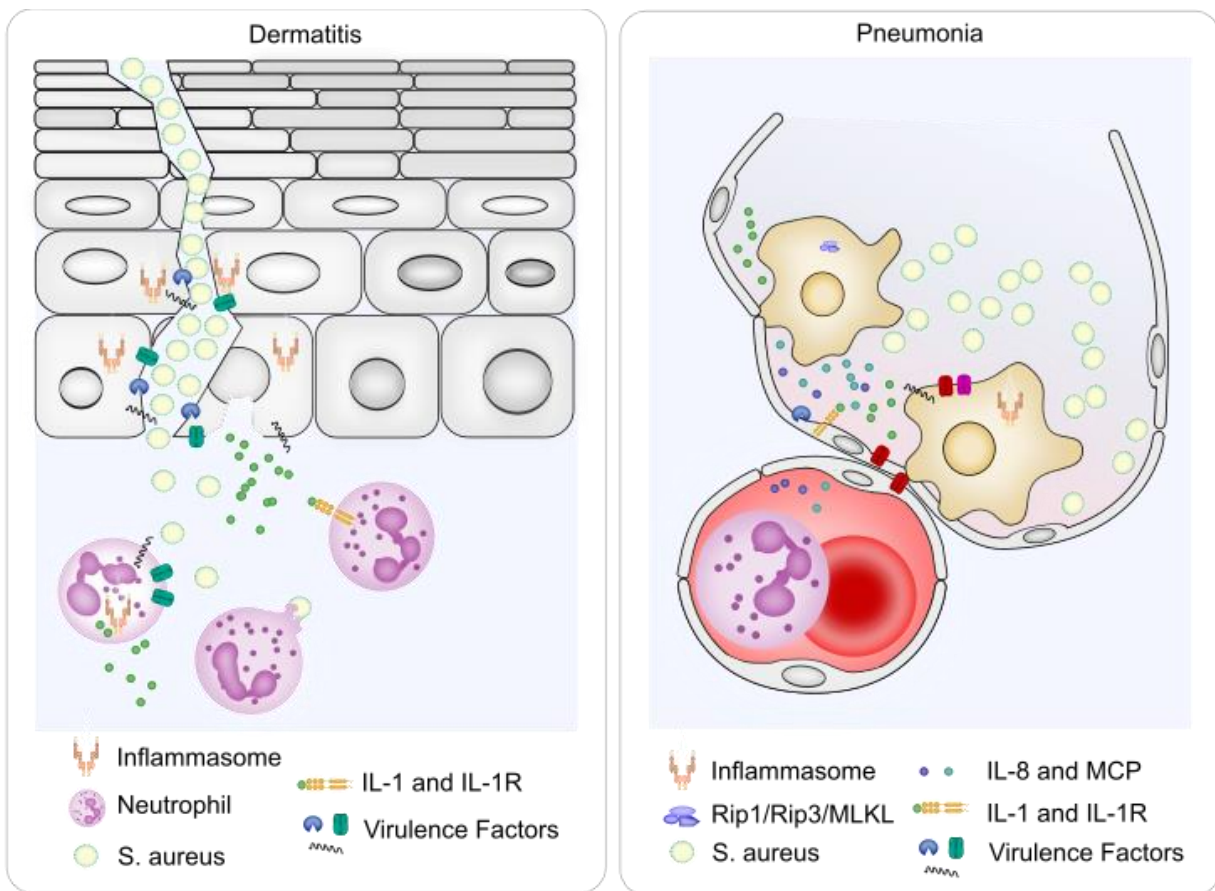
increased bacterial burden in the lungs [79]. These data demonstrate that IL-1 $\beta$  secretion triggered by PVL plays a protective role in *S. aureus* pneumonia in rabbits, where the toxin is active. While these findings seem at odds with the apparent pathologic role of NLRP3 inflammasome activation in mice with *S. aureus* pneumonia, it is worth noting that NLRP3-dependent IL-1 $\beta$  production was not the primary driver of pathology in mice.

Recently, *S. aureus* toxins were shown to induce necroptosis in the lung through RIPK1/RIPK3/MLKL signaling. In primary human macrophage co-culture with *S. aureus*, inhibitors of necroptosis, including necrostatin-1 and necrosulfonamide (NSA), or siRNA-mediated knockdown of RIPK3 or MLKL blocked *S. aureus*-induced cell death. NSA also decreased purified Hla-induced THP1 cell death. In murine *S. aureus* pneumonia, blocking necroptosis with NSA or knockout of RIPK3 improved bacterial clearance. RIPK3 deficient mice had improved lung architecture and less disruption of the pulmonary barrier, resulting in less protein in bronchoalveolar lavage fluid. Interestingly, NSA pretreatment of THP1 cells exposed to *S. aureus* supernatants in culture or RIPK3 knockout in *S. aureus* pneumonia led to decreased IL-1 $\beta$  secretion, suggesting a possible link between necroptosis and inflammasome activation [49].

Currently, there is strong evidence supporting the use of systemic corticosteroids in hospitalized patients with severe community acquired pneumonia [80]. Interestingly, a glucocorticosteroid-responsive negative regulatory element has been identified just upstream of the IL-1 $\beta$  transcription start site [81] and, as such, suppression of IL-1 $\beta$  production might be contributing to improved outcomes in these patients. However, conflicting results as to whether IL-1 signaling enhances or diminishes lung pathology will be important to clarify before targeted therapeutic immunosuppression is used clinically in the setting of severe *S. aureus* pneumonia.

Clinical trials of anakinra suggest patients with asthma or other pulmonary comorbidities might be at increased risk of infectious complications, though results were not statistically significant [82,83]. Suppression of necroptosis through RIPK3 inhibition may also be a viable strategy but understanding the contributions of necroptosis and NLRP3 inflammasome-mediated cell death to pathology and bacterial clearance is still in its infancy.

A summary comparison of *S. aureus* skin and lung infections is depicted (Figure 3).



**Figure 2.3 NLRP3 inflammasome signaling plays unique rolls in skin and lung infections**

Skin and lung infections are modeled here as an example of the unique effects of inflammasome signaling on *S. aureus* infections.

When injected subcutaneously, *S. aureus* Hla, Hlb, and Hlg activate the NLRP3 inflammasome leading to IL-1 $\beta$  and IL-18 secretion. NLRP3 activation in keratinocytes requires Caspase 1 and Calpain for IL-1 $\beta$  secretion and pyroptosis and promotes *S. aureus* invasion. PSMs also cause IL-1 $\beta$  and IL-18 secretion and cell death, though these are not blocked by potassium or Caspase inhibitors. IL-1 $\beta$ , primarily from bone marrow derived cells, is required for neutrophil recruitment and bacterial clearance. Loss of IL-18 does not impact that process.

In the lung Hla causes hyperactivation of the NLRP3 inflammasome leading to tissue destruction, as loss of NLRP3 seems to improve pathology with no change in bacterial burden. Loss of the IL-1 receptor does not impact the infection suggesting that IL-1 signaling is not required. In contrast, a separate study has shown that PVL triggers IL-1 $\beta$  that leads to IL-8 and MCP-1 secretion from lung epithelial cells and loss of the IL-1 receptor decreases Th17 activation. Hla, LukAB and PSMs also induce necroptosis, a RIPK1-, RIPK3-, MLKL-dependent cell death process. MLKL also plays a role in IL-1 $\beta$  secretion as loss of MLKL blocks Caspase 1 activation. RIPK3 knockout in pneumonia improves *S. aureus* clearance.

### **2.4.3 Microglia activate NLRP3 *in vitro* but depend on AIM2 for survival in *S. aureus* brain abscesses**

*S. aureus* can also cause brain abscesses usually as a complication of surgery, trauma or bacteremia. Microglia are immunologically competent cells in the brain activated early in the process of *S. aureus* abscess formation [84]. Exposure of primary microglia isolated from C57BL/6 mice to live *S. aureus* strain USA300 induced both IL-1 $\beta$  and IL-18 secretion. Microglia from mice lacking NLRP3 or ASC were deficient in IL-1 $\beta$  secretion but responded to live *S. aureus* with similar levels of IL-18 as wild type mice. Deletion of Hla or Hlg, but not

LukAB or LukED reduced Newman strain *S. aureus*-induced IL-1 $\beta$  secretion from microglia [85]. However, this is not surprising given the general resistance of mouse to LukAB [11] and the low level of expression of LukED [48] under the conditions the bacteria were cultured. Caspase 1 and CTSB inhibitors blocked *S. aureus*-induced microglial IL-1 $\beta$  secretion in these *in vitro* cell culture experiments. Consistent with a second pathway controlling IL-18 secretion in this setting, the CTSB inhibitor had no effect on IL-18 secretion [85].

In a model of brain abscess, ASC and Caspase 1/11 knockout mice had earlier mortality as compared to the parental strain. Unexpectedly, the survival of NLRP3 knockout mice was identical to the parental strain. Upon further investigation, loss of AIM2 mimicked the sensitivity to *S. aureus* infection seen in ASC deficient mice, suggesting that AIM2 is the primary inflammasome responsible for IL-1 $\beta$  production in an actual brain abscess. Besides IL-1 $\beta$ , other key inflammatory mediators, including IL-6, CXCL1, CXCL10, and CCL2 were significantly reduced in the CNS of AIM2 and ASC knockout mice [58]. Strangely, immune cell infiltrates, including neutrophils and macrophages, and other cytokines, such as IL-10, TNF- $\alpha$  and IFN- $\gamma$ , were not changed between ASC knockout and the parental strain mice. Also, the bacterial burden of AIM2 knockout mice and the parental strain were equivalent in the first 18 hours of infection. Most AIM2 knockout mice that died were recorded as having died at approximately 20 hours post infection, leaving us without a satisfying explanation for why these mice suddenly succumbed to disease given the similarities in bacterial burden and immune cell infiltrates [58]. Previous studies of *S. aureus* brain abscesses have demonstrated damage to the blood-brain barrier [86] and impaired capillary perfusion and parenchymal cell death has been noted in soft tissue infections [87], both explanation that might be worth further investigation in this setting. The discrepancy of which inflammasome is activated between *in vitro* and *in vivo* results points



to the need to push mechanistic studies through the translational pipeline to differentiate possible effects from actual effects. Further investigation of AIM2 knockout microglia *in vitro* would be beneficial for characterizing the response seen *in vivo*. Also, study of AIM2 knockout mice in the context of other *S. aureus* infections would help determine if the role of AIM2 is brain/microglia-specific or is a general feature of the immune response to *S. aureus*.

#### **2.4.4 IL-1 $\beta$ signaling is critical in soft tissue infections**

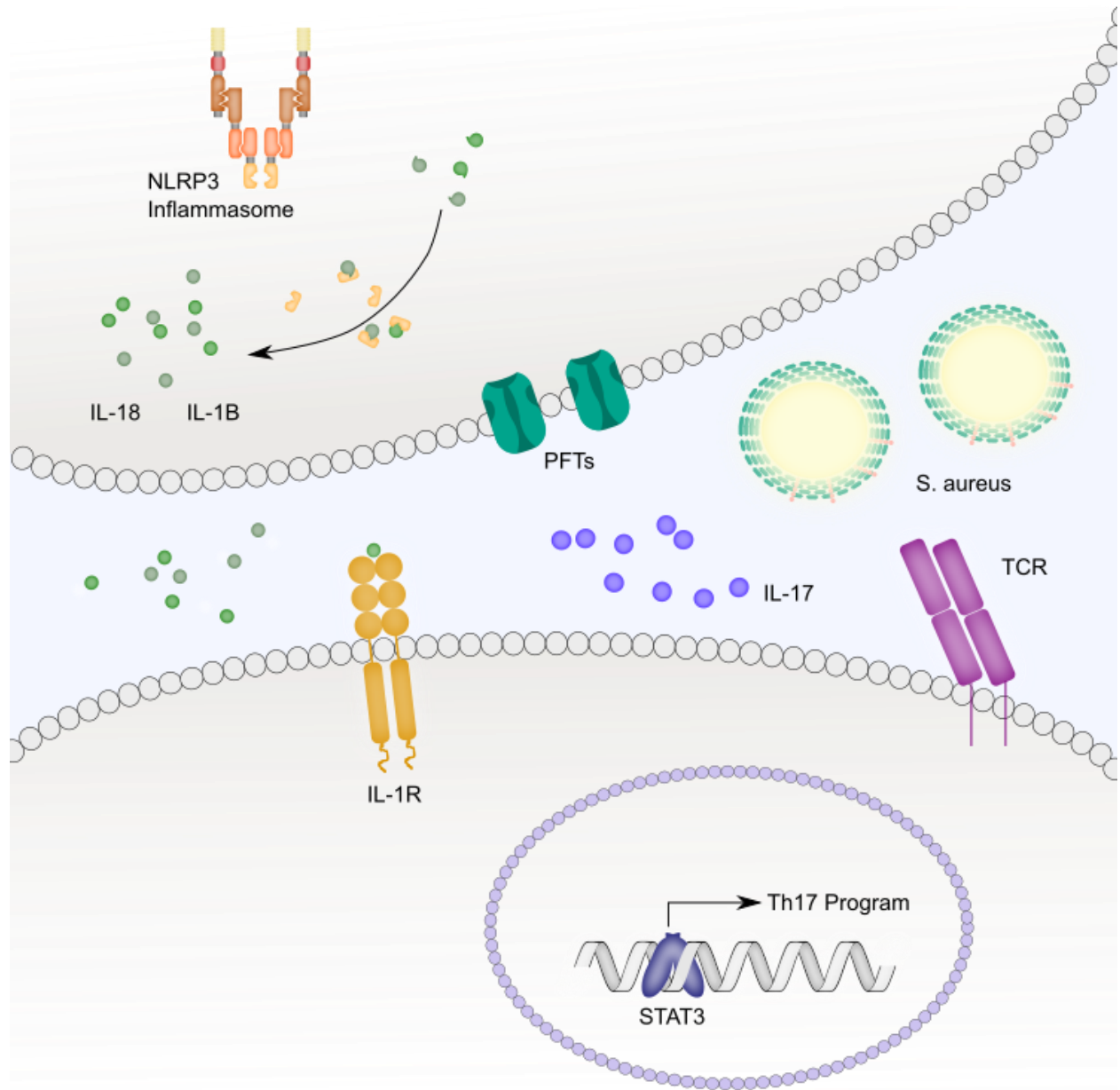
*Staphylococcus aureus* is a common culprit in soft tissue and medical device infections. Patients with ulcers, commonly resulting from advanced complications of diabetes, deep injuries, recent surgery, or indwelling medical devices are particularly at risk. Recent work has demonstrated a differential effect of *S. aureus* strains in inducing inflammasome activation and IL-1 $\beta$  secretion in surgical site infections, with strain PS80 leading to substantially more IL-1 $\beta$  than SH1000. In this study, loss of NLRP3 did not completely diminish IL-1 $\beta$  secretion, suggesting a coordinated role with other inflammasomes. However, deletion of NLRP3 or IL-1R compromised control of bacterial burden in infected surgical wounds [88]. Little else has been done to characterize the role of inflammasome signaling in soft tissue infections, though it is tempting to assume these would be similar to dermal infections once *S. aureus* crosses the keratinocyte barrier.

#### **2.5 How host inflammasomes can affect other immune processes**

The Th17 response has emerged as a major focus of studies looking downstream of inflammasome activation (Figure 4). The most direct hint that IL-17-driven responses are critical for defense against *S. aureus* is seen in patients with hyper IgE syndrome (HIES). In HIES,

mutation in STAT3 causes impaired Th17 cell function and recurrent and severe *S. aureus* infections [89]. Similar impairments have been recapitulated in mouse models of *S. aureus* infection.

NLRP3-mediated secretion of IL-1 $\beta$  by bone marrow dendritic cells promotes secretion of IL-17 by  $\gamma\delta$ -T cells, as loss of the IL-1 receptor limits this response. Importantly, the ability to control surgical site infections is enhanced by IL-1 $\beta$  stimulated  $\gamma\delta$ -T cells as bacterial burden is increased in  $\delta$ TCR deficient and IL-17R deficient mice [88]. In *S. aureus* cutaneous infections, loss of  $\gamma\delta$ -T cells led to larger skin lesions with higher bacterial counts as a result of impaired neutrophil recruitment. IL-17R-deficient mice had a similar phenotype. Treatment of  $\gamma\delta$ -T cell-deficient mice with a single dose of recombinant IL-17 rescued the impaired immune response to *S. aureus* [90]. A population of CD44<sup>+</sup> CD27<sup>-</sup> memory  $\gamma\delta$ -T cells that is expanded upon peritoneal infection of C57BL/6 mice with *S. aureus* produce high levels of IL-17 and promote bacterial clearance during reinfections. IL-1 signaling was not required for activation or expansion of memory  $\gamma\delta$ -T cells during reinfection [91].  $\gamma\delta$ -T cells also play a prominent role in defense against *S. aureus* pneumonia.  $\gamma\delta$ -T cell-deficient mice had impaired neutrophil recruitment and an increased bacterial burden in the lung. However, the absence of  $\gamma\delta$ -T cells decreases lung pathology and improves survival [92], similar to studies with loss of NLRP3 [74].



**Figure 2.4 Inflammasome signaling upregulates  $\gamma\delta$ -T cells and a Th17 response**

NLRP3-mediated secretion of IL-1 $\beta$  in response to *S. aureus* signals through the IL-1R to promote development of  $\gamma\delta$ -T cells that secrete IL-17.  $\gamma\delta$ -T cells and IL-17 contribute to defense against *S. aureus* in multiple mouse models including surgical site infections, cutaneous infections, peritoneal infections, and pneumonia. Loss of  $\gamma\delta$ -T cells or the IL-17 receptor leads to impaired neutrophil recruitment and higher bacterial counts; treatment of  $\gamma\delta$ -T cell deficient mice

with IL-17 rescues the impaired immune response and treatment of IL-17R deficient mice with IL-1 $\beta$  does not. In humans, patients with hyper IgE syndrome have impaired Th17 cell function and contract recurrent and severe *S. aureus* infections.

## **2.6 Integrating inflammasome studies to improve patient care**

Early evidence suggests that vaccines against PFTs may be beneficial in reducing virulence and severity of infections. In hospitalized patients with *S. aureus* infections, the risk of sepsis was significantly lower in those patients with higher levels of IgG against Hla, Hld, PVL, staphylococcal enterotoxin C-1 and PSM $\alpha$ 3 [93]. Some children with culture-proven *S. aureus* infection developed anti-LukAB antibodies that potently neutralize cytotoxicity *in vitro*, suggesting that the toxin is produced *in vivo* and that it elicits a humoral response [94].

Immunization of mice with a mutant form of Hla that cannot form pores, Hla H35L, generates antigen-specific immunoglobulin G responses and affords protection against staphylococcal pneumonia. Additionally, transfer of Hla-specific antibodies or anti-Hla monoclonal antibodies also protects naïve animals against *S. aureus* challenge and prevents the injury of human lung epithelial cells during infection [72,73]. This is presumably the result of decreased NLRP3 inflammasome activation in this setting, however, that has not been measured *in vivo* directly. Therapies targeting specific virulence factors may provide a useful adjuvant to antibiotic therapy. As recently declared in the Annals of Internal Medicine, many believe it is “time to change clinical practice” and recommend systemic administration of corticosteroids in patients with severe community acquired pneumonia. Theoretically, tailoring therapy more narrowly to reduce virulence and hyperactive immune responses without compromising bacterial clearance would provide further benefit over this broadly immunosuppressive therapy.

For understanding tissue-specific pathogenesis, we will need improved infection models that better recapitulate human tissues and immune cells to overcome the natural resistance of mice to *S. aureus*. Tissue-specific knockouts and bone marrow chimeras will also help differentiate tissue-driven immune responses from those of bone marrow origin. Ultimately, investigations into inflammasome-mediated responses to *S. aureus* infections may provide us with the therapeutic tools we need to push back against this growing threat.

## REFERENCES

1. Thammavongsa V, Kim HK, Missiakas D, Schneewind O (2015) Staphylococcal manipulation of host immune responses. *Nat Rev Microbiol* 13: 529-543.
2. Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler VG, Jr. (2015) Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin Microbiol Rev* 28: 603-661.
3. Badarau A, Rouha H, Malafa S, Logan DT, Hakansson M, et al. (2015) Structure-function analysis of heterodimer formation, oligomerization, and receptor binding of the Staphylococcus aureus bi-component toxin LukGH. *J Biol Chem* 290: 142-156.
4. Yamashita K, Kawai Y, Tanaka Y, Hirano N, Kaneko J, et al. (2011) Crystal structure of the octameric pore of staphylococcal gamma-hemolysin reveals the beta-barrel pore formation mechanism by two components. *Proc Natl Acad Sci U S A* 108: 17314-17319.
5. Pedelacq JD, Maveyraud L, Prevost G, Baba-Moussa L, Gonzalez A, et al. (1999) The structure of a Staphylococcus aureus leucocidin component (LukF-PV) reveals the fold of the water-soluble species of a family of transmembrane pore-forming toxins. *Structure* 7: 277-287.
6. Guillet V, Roblin P, Werner S, Coraiola M, Menestrina G, et al. (2004) Crystal structure of leucotoxin S component: new insight into the Staphylococcal beta-barrel pore-forming toxins. *J Biol Chem* 279: 41028-41037.
7. Sugawara T, Yamashita D, Kato K, Peng Z, Ueda J, et al. (2015) Structural basis for pore-forming mechanism of staphylococcal alpha-hemolysin. *Toxicon* 108: 226-231.
8. Gouaux E, Hobaugh M, Song L (1997) alpha-Hemolysin, gamma-hemolysin, and leukocidin from Staphylococcus aureus: distant in sequence but similar in structure. *Protein Sci* 6: 2631-2635.
9. Wilke GA, Bubeck Wardenburg J (2010) Role of a disintegrin and metalloprotease 10 in Staphylococcus aureus alpha-hemolysin-mediated cellular injury. *Proc Natl Acad Sci U S A* 107: 13473-13478.
10. Spaan AN, Vrieling M, Wallet P, Badiou C, Reyes-Robles T, et al. (2014) The staphylococcal toxins gamma-haemolysin AB and CB differentially target phagocytes by employing specific chemokine receptors. *Nat Commun* 5: 5438.
11. DuMont AL, Yoong P, Day CJ, Alonzo F, 3rd, McDonald WH, et al. (2013) Staphylococcus aureus LukAB cytotoxin kills human neutrophils by targeting the CD11b subunit of the integrin Mac-1. *Proc Natl Acad Sci U S A* 110: 10794-10799.
12. Alonzo F, 3rd, Kozhaya L, Rawlings SA, Reyes-Robles T, DuMont AL, et al. (2013) CCR5 is a receptor for Staphylococcus aureus leukotoxin ED. *Nature* 493: 51-55.

13. Reyes-Robles T, Alonzo F, 3rd, Kozhaya L, Lacy DB, Unutmaz D, et al. (2013) Staphylococcus aureus leukotoxin ED targets the chemokine receptors CXCR1 and CXCR2 to kill leukocytes and promote infection. *Cell Host Microbe* 14: 453-459.
14. Spaan AN, Henry T, van Rooijen WJ, Perret M, Badiou C, et al. (2013) The staphylococcal toxin Panton-Valentine Leukocidin targets human C5a receptors. *Cell Host Microbe* 13: 584-594.
15. Loffler B, Hussain M, Grundmeier M, Bruck M, Holzinger D, et al. (2010) Staphylococcus aureus panton-valentine leukocidin is a very potent cytotoxic factor for human neutrophils. *PLoS Pathog* 6: e1000715.
16. Walev I, Weller U, Strauch S, Foster T, Bhakdi S (1996) Selective killing of human monocytes and cytokine release provoked by sphingomyelinase (beta-toxin) of Staphylococcus aureus. *Infect Immun* 64: 2974-2979.
17. Huseby M, Shi K, Brown CK, Digre J, Mengistu F, et al. (2007) Structure and biological activities of beta toxin from Staphylococcus aureus. *J Bacteriol* 189: 8719-8726.
18. Fitton JE, Dell A, Shaw WV (1980) The amino acid sequence of the delta haemolysin of Staphylococcus aureus. *FEBS Lett* 115: 209-212.
19. Peschel A, Otto M (2013) Phenol-soluble modulins and staphylococcal infection. *Nat Rev Microbiol* 11: 667-673.
20. Wang R, Braughton KR, Kretschmer D, Bach TH, Queck SY, et al. (2007) Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat Med* 13: 1510-1514.
21. Kretschmer D, Gleske AK, Rautenberg M, Wang R, Koberle M, et al. (2010) Human formyl peptide receptor 2 senses highly pathogenic Staphylococcus aureus. *Cell Host Microbe* 7: 463-473.
22. Otto M (2010) Basis of virulence in community-associated methicillin-resistant Staphylococcus aureus. *Annu Rev Microbiol* 64: 143-162.
23. Grundling A, Schneewind O (2007) Synthesis of glycerol phosphate lipoteichoic acid in Staphylococcus aureus. *Proc Natl Acad Sci U S A* 104: 8478-8483.
24. Dunne DW, Resnick D, Greenberg J, Krieger M, Joiner KA (1994) The type I macrophage scavenger receptor binds to gram-positive bacteria and recognizes lipoteichoic acid. *Proc Natl Acad Sci U S A* 91: 1863-1867.
25. Greenberg JW, Fischer W, Joiner KA (1996) Influence of lipoteichoic acid structure on recognition by the macrophage scavenger receptor. *Infect Immun* 64: 3318-3325.
26. Hoebe K, Georgel P, Rutschmann S, Du X, Mudd S, et al. (2005) CD36 is a sensor of diacylglycerides. *Nature* 433: 523-527.

27. Hermann C, Spreitzer I, Schroder NW, Morath S, Lehner MD, et al. (2002) Cytokine induction by purified lipoteichoic acids from various bacterial species--role of LBP, sCD14, CD14 and failure to induce IL-12 and subsequent IFN-gamma release. *Eur J Immunol* 32: 541-551.
28. Han SH, Kim JH, Martin M, Michalek SM, Nahm MH (2003) Pneumococcal lipoteichoic acid (LTA) is not as potent as staphylococcal LTA in stimulating Toll-like receptor 2. *Infect Immun* 71: 5541-5548.
29. van de Wetering JK, van Eijk M, van Golde LM, Hartung T, van Strijp JA, et al. (2001) Characteristics of surfactant protein A and D binding to lipoteichoic acid and peptidoglycan, 2 major cell wall components of gram-positive bacteria. *J Infect Dis* 184: 1143-1151.
30. Nakayama M, Kurokawa K, Nakamura K, Lee BL, Sekimizu K, et al. (2012) Inhibitory receptor paired Ig-like receptor B is exploited by *Staphylococcus aureus* for virulence. *J Immunol* 189: 5903-5911.
31. Lynch NJ, Roscher S, Hartung T, Morath S, Matsushita M, et al. (2004) L-ficolin specifically binds to lipoteichoic acid, a cell wall constituent of Gram-positive bacteria, and activates the lectin pathway of complement. *J Immunol* 172: 1198-1202.
32. Weidenmaier C, Peschel A (2008) Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. *Nat Rev Microbiol* 6: 276-287.
33. Percy MG, Grundling A (2014) Lipoteichoic acid synthesis and function in gram-positive bacteria. *Annu Rev Microbiol* 68: 81-100.
34. Hedges SB, Dudley J, Kumar S (2006) TimeTree: a public knowledge-base of divergence times among organisms. *Bioinformatics* 22: 2971-2972.
35. Kumar S, Hedges SB (2011) TimeTree2: species divergence times on the iPhone. *Bioinformatics* 27: 2023-2024.
36. Sha W, Mitoma H, Hanabuchi S, Bao M, Weng L, et al. (2014) Human NLRP3 inflammasome senses multiple types of bacterial RNAs. *Proc Natl Acad Sci U S A* 111: 16059-16064.
37. Eigenbrod T, Franchi L, Munoz-Planillo R, Kirschning CJ, Freudenberg MA, et al. (2012) Bacterial RNA mediates activation of caspase-1 and IL-1beta release independently of TLRs 3, 7, 9 and TRIF but is dependent on UNC93B. *J Immunol* 189: 328-336.
38. Munoz-Planillo R, Franchi L, Miller LS, Nunez G (2009) A critical role for hemolysins and bacterial lipoproteins in *Staphylococcus aureus*-induced activation of the Nlrp3 inflammasome. *J Immunol* 183: 3942-3948.
39. Muller S, Wolf AJ, Iliev ID, Berg BL, Underhill DM, et al. (2015) Poorly Cross-Linked Peptidoglycan in MRSA Due to *mecA* Induction Activates the Inflammasome and Exacerbates Immunopathology. *Cell Host Microbe* 18: 604-612.



40. Shimada T, Park BG, Wolf AJ, Brikos C, Goodridge HS, et al. (2010) *Staphylococcus aureus* evades lysozyme-based peptidoglycan digestion that links phagocytosis, inflammasome activation, and IL-1 $\beta$  secretion. *Cell Host Microbe* 7: 38-49.
41. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, et al. (2006) Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440: 228-232.
42. Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ (1999) Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *J Biol Chem* 274: 17406-17409.
43. Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, et al. (1999) Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J Immunol* 163: 1-5.
44. Melehan JH, James DB, DuMont AL, Torres VJ, Duncan JA (2015) *Staphylococcus aureus* Leukocidin A/B (LukAB) Kills Human Monocytes via Host NLRP3 and ASC when Extracellular, but Not Intracellular. *PLoS Pathog* 11: e1004970.
45. Craven RR, Gao X, Allen IC, Gris D, Bubeck Wardenburg J, et al. (2009) *Staphylococcus aureus* alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells. *PLoS One* 4: e7446.
46. Holzinger D, Geldon L, Mysore V, Nippe N, Taxman DJ, et al. (2012) *Staphylococcus aureus* Pantone-Valentine leukocidin induces an inflammatory response in human phagocytes via the NLRP3 inflammasome. *J Leukoc Biol* 92: 1069-1081.
47. Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, et al. (2008) Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* 9: 847-856.
48. DuMont AL, Yoong P, Surewaard BG, Benson MA, Nijland R, et al. (2013) *Staphylococcus aureus* elaborates leukocidin AB to mediate escape from within human neutrophils. *Infect Immun* 81: 1830-1841.
49. Kitur K, Parker D, Nieto P, Ahn DS, Cohen TS, et al. (2015) Toxin-induced necroptosis is a major mechanism of *Staphylococcus aureus* lung damage. *PLoS Pathog* 11: e1004820.
50. Dumont AL, Nygaard TK, Watkins RL, Smith A, Kozhaya L, et al. (2011) Characterization of a new cytotoxin that contributes to *Staphylococcus aureus* pathogenesis. *Mol Microbiol* 79: 814-825.
51. Ventura CL, Malachowa N, Hammer CH, Nardone GA, Robinson MA, et al. (2010) Identification of a novel *Staphylococcus aureus* two-component leukotoxin using cell surface proteomics. *PLoS One* 5: e11634.
52. Tseng CW, Biancotti JC, Berg BL, Gate D, Kolar SL, et al. (2015) Increased Susceptibility of Humanized NSG Mice to Pantone-Valentine Leukocidin and *Staphylococcus aureus* Skin Infection. *PLoS Pathog* 11: e1005292.

53. Bera A, Herbert S, Jakob A, Vollmer W, Gotz F (2005) Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*. *Mol Microbiol* 55: 778-787.
54. Tajima A, Iwase T, Shinji H, Seki K, Mizunoe Y (2009) Inhibition of endothelial interleukin-8 production and neutrophil transmigration by *Staphylococcus aureus* beta-hemolysin. *Infect Immun* 77: 327-334.
55. Davis BK, Roberts RA, Huang MT, Willingham SB, Conti BJ, et al. (2011) Cutting edge: NLR5-dependent activation of the inflammasome. *J Immunol* 186: 1333-1337.
56. Khare S, Dorfleutner A, Bryan NB, Yun C, Radian AD, et al. (2012) An NLRP7-containing inflammasome mediates recognition of microbial lipopeptides in human macrophages. *Immunity* 36: 464-476.
57. Radian AD, Khare S, Chu LH, Dorfleutner A, Stehlik C (2015) ATP binding by NLRP7 is required for inflammasome activation in response to bacterial lipopeptides. *Mol Immunol* 67: 294-302.
58. Hanamsagar R, Aldrich A, Kielian T (2014) Critical role for the AIM2 inflammasome during acute CNS bacterial infection. *J Neurochem* 129: 704-711.
59. Syed AK, Reed TJ, Clark KL, Boles BR, Kahlenberg JM (2015) *Staphylococcus aureus* phenol-soluble modulins stimulate the release of proinflammatory cytokines from keratinocytes and are required for induction of skin inflammation. *Infect Immun* 83: 3428-3437.
60. Borregaard N (2010) Neutrophils, from marrow to microbes. *Immunity* 33: 657-670.
61. Miller LS, Cho JS (2011) Immunity against *Staphylococcus aureus* cutaneous infections. *Nat Rev Immunol* 11: 505-518.
62. Miller LS, O'Connell RM, Gutierrez MA, Pietras EM, Shahangian A, et al. (2006) MyD88 mediates neutrophil recruitment initiated by IL-1R but not TLR2 activation in immunity against *Staphylococcus aureus*. *Immunity* 24: 79-91.
63. Miller LS, Pietras EM, Uricchio LH, Hirano K, Rao S, et al. (2007) Inflammasome-mediated production of IL-1beta is required for neutrophil recruitment against *Staphylococcus aureus* in vivo. *J Immunol* 179: 6933-6942.
64. Cho JS, Guo Y, Ramos RI, Hebroni F, Plaisier SB, et al. (2012) Neutrophil-derived IL-1beta is sufficient for abscess formation in immunity against *Staphylococcus aureus* in mice. *PLoS Pathog* 8: e1003047.
65. Soong G, Paulino F, Wachtel S, Parker D, Wickersham M, et al. (2015) Methicillin-resistant *Staphylococcus aureus* adaptation to human keratinocytes. *MBio* 6.

66. Soong G, Chun J, Parker D, Prince A (2012) Staphylococcus aureus activation of caspase 1/calpain signaling mediates invasion through human keratinocytes. *J Infect Dis* 205: 1571-1579.
67. Niebuhr M, Baumert K, Heratizadeh A, Satzger I, Werfel T (2014) Impaired NLRP3 inflammasome expression and function in atopic dermatitis due to Th2 milieu. *Allergy* 69: 1058-1067.
68. Juhn YJ (2014) Risks for infection in patients with asthma (or other atopic conditions): is asthma more than a chronic airway disease? *J Allergy Clin Immunol* 134: 247-257; quiz 258-249.
69. Gudjonsson JE, Johnston A, Dyson M, Valdimarsson H, Elder JT (2007) Mouse models of psoriasis. *J Invest Dermatol* 127: 1292-1308.
70. Melican K, Michea Veloso P, Martin T, Bruneval P, Dumenil G (2013) Adhesion of *Neisseria meningitidis* to dermal vessels leads to local vascular damage and purpura in a humanized mouse model. *PLoS Pathog* 9: e1003139.
71. Kappes U, Schliemann-Willers S, Bankova L, Heinemann C, Fischer TW, et al. (2004) The quality of human skin xenografts on SCID mice: a noninvasive bioengineering approach. *Br J Dermatol* 151: 971-976.
72. Bubeck Wardenburg J, Schneewind O (2008) Vaccine protection against *Staphylococcus aureus* pneumonia. *J Exp Med* 205: 287-294.
73. Ragle BE, Bubeck Wardenburg J (2009) Anti-alpha-hemolysin monoclonal antibodies mediate protection against *Staphylococcus aureus* pneumonia. *Infect Immun* 77: 2712-2718.
74. Kebaier C, Chamberland RR, Allen IC, Gao X, Broglie PM, et al. (2012) *Staphylococcus aureus* alpha-hemolysin mediates virulence in a murine model of severe pneumonia through activation of the NLRP3 inflammasome. *J Infect Dis* 205: 807-817.
75. Labandeira-Rey M, Couzon F, Boisset S, Brown EL, Bes M, et al. (2007) *Staphylococcus aureus* Panton-Valentine leukocidin causes necrotizing pneumonia. *Science* 315: 1130-1133.
76. Brown EL, Dumitrescu O, Thomas D, Badiou C, Koers EM, et al. (2009) The Pantone-Valentine leukocidin vaccine protects mice against lung and skin infections caused by *Staphylococcus aureus* USA300. *Clin Microbiol Infect* 15: 156-164.
77. Bubeck Wardenburg J, Palazzolo-Ballance AM, Otto M, Schneewind O, DeLeo FR (2008) Pantone-Valentine leukocidin is not a virulence determinant in murine models of community-associated methicillin-resistant *Staphylococcus aureus* disease. *J Infect Dis* 198: 1166-1170.
78. Perret M, Badiou C, Lina G, Burbaud S, Benito Y, et al. (2012) Cross-talk between *Staphylococcus aureus* leukocidins-intoxicated macrophages and lung epithelial cells

- triggers chemokine secretion in an inflammasome-dependent manner. *Cell Microbiol* 14: 1019-1036.
79. Labrousse D, Perret M, Hayez D, Da Silva S, Badiou C, et al. (2014) Kineret(R)/IL-1ra blocks the IL-1/IL-8 inflammatory cascade during recombinant Panton Valentine Leukocidin-triggered pneumonia but not during *S. aureus* infection. *PLoS One* 9: e97546.
  80. Siemieniuk RA, Meade MO, Alonso-Coello P, Briel M, Evaniew N, et al. (2015) Corticosteroid Therapy for Patients Hospitalized With Community-Acquired Pneumonia: A Systematic Review and Meta-analysis. *Ann Intern Med* 163: 519-528.
  81. Zhang G, Zhang L, Duff GW (1997) A negative regulatory region containing a glucocorticosteroid response element (nGRE) in the human interleukin-1beta gene. *DNA Cell Biol* 16: 145-152.
  82. Schiff MH, DiVittorio G, Tesser J, Fleischmann R, Schechtman J, et al. (2004) The safety of anakinra in high-risk patients with active rheumatoid arthritis: six-month observations of patients with comorbid conditions. *Arthritis Rheum* 50: 1752-1760.
  83. Fleischmann RM, Schechtman J, Bennett R, Handel ML, Burmester GR, et al. (2003) Anakinra, a recombinant human interleukin-1 receptor antagonist (r-metHuIL-1ra), in patients with rheumatoid arthritis: A large, international, multicenter, placebo-controlled trial. *Arthritis Rheum* 48: 927-934.
  84. Kielian T (2004) Immunopathogenesis of brain abscess. *J Neuroinflammation* 1: 16.
  85. Hanamsagar R, Torres V, Kielian T (2011) Inflammasome activation and IL-1beta/IL-18 processing are influenced by distinct pathways in microglia. *J Neurochem* 119: 736-748.
  86. Kielian T, Barry B, Hickey WF (2001) CXC chemokine receptor-2 ligands are required for neutrophil-mediated host defense in experimental brain abscesses. *J Immunol* 166: 4634-4643.
  87. Harding MG, Zhang K, Conly J, Kubes P (2014) Neutrophil crawling in capillaries; a novel immune response to *Staphylococcus aureus*. *PLoS Pathog* 10: e1004379.
  88. Maher BM, Mulcahy ME, Murphy AG, Wilk M, O'Keeffe KM, et al. (2013) Nlrp-3-driven interleukin 17 production by gammadeltaT cells controls infection outcomes during *Staphylococcus aureus* surgical site infection. *Infect Immun* 81: 4478-4489.
  89. Milner JD, Brenchley JM, Laurence A, Freeman AF, Hill BJ, et al. (2008) Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature* 452: 773-776.
  90. Cho JS, Pietras EM, Garcia NC, Ramos RI, Farzam DM, et al. (2010) IL-17 is essential for host defense against cutaneous *Staphylococcus aureus* infection in mice. *J Clin Invest* 120: 1762-1773.

91. Murphy AG, O'Keefe KM, Lalor SJ, Maher BM, Mills KH, et al. (2014) Staphylococcus aureus infection of mice expands a population of memory gammadelta T cells that are protective against subsequent infection. *J Immunol* 192: 3697-3708.
92. Cheng P, Liu T, Zhou WY, Zhuang Y, Peng LS, et al. (2012) Role of gamma-delta T cells in host response against Staphylococcus aureus-induced pneumonia. *BMC Immunol* 13: 38.
93. Adhikari RP, Ajao AO, Aman MJ, Karauzum H, Sarwar J, et al. (2012) Lower antibody levels to Staphylococcus aureus exotoxins are associated with sepsis in hospitalized adults with invasive S. aureus infections. *J Infect Dis* 206: 915-923.
94. Thomsen IP, Dumont AL, James DB, Yoong P, Saville BR, et al. (2014) Children with invasive Staphylococcus aureus disease exhibit a potently neutralizing antibody response to the cytotoxin LukAB. *Infect Immun* 82: 1234-1242.

## **Chapter 3. *Staphylococcus aureus* Leukocidin A/B (LukAB) kills human monocytes by co-opting host NLRP3 and ASC when extracellular, but not intracellular<sup>2</sup>**

### **3.1 Introduction**

*S. aureus* is one of the most commonly identified causes of infection, and is responsible for a significant health and economic burden including approximately 100,000 life-threatening infections per year in the United States [1]. *S. aureus* can cause a variety of diseases that range from recurrent epidermal abscesses to life-threatening necrotizing pneumonias. To promote these infections, *S. aureus* produces many different virulence factors including several cytotoxic beta-barrel pore-forming toxins such as:  $\alpha$ -toxin (Hla), Leukocidin AB (LukAB), Leukocidin ED (LukED), Panton-Valentine leukocidin (PVL), and gamma hemolysins (HlgAB and HlgCB) [2,3]. Among these toxins, Hla and PVL are the most studied *in vivo*. In mouse studies, Hla has been implicated in enhancing virulence in numerous infectious models, including keratitis, mastitis, pneumonia, skin abscess, and lethal intraperitoneal infection [4-9]. In contrast, mouse models of keratitis, pneumonia, bone and muscle infections have demonstrated variable contributions to virulence by PVL [5,10-13]. Rabbit models of infection, however, highlight a clearer role of PVL in *S. aureus* virulence [14-17]. Rabbit neutrophils are significantly more susceptible to PVL than

---

<sup>2</sup> This chapter previously appeared as an article in the journal Public Library of Science Pathogens. The original citation is as follows: Melehani J.H., James D.B.A., DuMont A.L., Torres V.J., Duncan J.A. *Staphylococcus aureus* Leukocidin A/B (LukAB) kills human monocytes via host NLRP3 and ASC when extracellular, but not intracellular. PLOS Pathog. 2015;11:e1004970. doi: 10.1371/journal.ppat.1004970.

mouse neutrophils [18], but remain relatively resistant to the toxin when compared to human neutrophils, which is due to the species selectivity of PVL towards its cellular receptor, C5aR [19].

The most recently identified *S. aureus* leukotoxin is LukAB (also known as LukGH) [20,21]. LukAB kills primary human neutrophils, monocytes, macrophages, and dendritic cells [20]. As with PVL, LukAB also exhibits species specificity towards human leukocytes [22,23] and most of its characterization has been performed on primary human neutrophils. LukAB binds to CD11b, a component of the CD11b/CD18 integrin (also known as  $\alpha$ M/ $\beta$ 2, CR3, or Mac-1), to target and kill human neutrophils [22]. A glutamic acid at position 323 within the unique C-terminal region of the LukA subunit binds directly to the I-domain of human CD11b to promote cell binding and subsequent pore-mediated cell lysis [24]. Interestingly, sufficient differences exist between the mouse and human CD11b I-domain to render mouse leukocytes resistant to LukAB. Additionally, *S. aureus* escape from phagocytic killing by human neutrophils requires LukAB production, suggesting this toxin may play a unique and important role in bacterial survival and persistence [25].

Host innate immune response to combat *S. aureus* involves a diverse set of pattern recognition/danger responsive receptors including the intracellular NOD-like Receptor (NLR) protein 3 (NLRP3) [26]. NLRP3, together with proteins ASC and Caspase 1, form a cytoplasmic oligomeric complex known as the NLRP3 inflammasome, which plays a critical role in initiating innate immune responses [27]. *S. aureus* and its secreted toxins Hla, HlgACB, and PVL have been all found to activate the NLRP3 inflammasome leading to activation of Caspase 1, secretion of Caspase 1-processed pro-inflammatory cytokines IL-1 $\beta$  and IL-18, and induction of necrotic cell death [26,28-31]. In a mouse skin infection model, neutrophil NLRP3 inflammasome activation

and IL-1 $\beta$  secretion promotes inflammation and abscess formation that accompany bacterial clearance [32]. This is in contrast to murine *S. aureus* pneumonia where the NLRP3-driven response is not required for bacterial clearance but instead exacerbates the severity of disease pathology [26,33].

Herein, we sought to investigate how *S. aureus* directly kills human monocytes and whether the NLRP3 inflammasome contributes to this process. We utilized THP1 human monocytic cells as a model and to determine the molecular mechanism of cell death. This cell line expresses high levels of NLRP3, ASC, pro-Caspase 1 and is also extensively used to study inflammasome activation [34]. We show that *S. aureus* employs LukAB as a predominant toxin to promote necrotic cell death in THP1 monocytes. We demonstrate that purified LukAB is sufficient to activate Caspase 1, induce secretion of IL-1 $\beta$  and IL-18, and cause necrotic cell death. Importantly, these findings were replicated in primary CD14<sup>+</sup> human monocytes. Using shRNA knockdowns in THP1 monocytes, we confirmed that these responses were dependent on LukAB binding to its receptor CD11b, which leads to activation of the NLRP3 inflammasome. In contrast to LukAB binding to the host plasma membrane, LukAB secreted from within the phagosome of THP1 cells requires CD11b, but not ASC or NLRP3, to trigger cell death. Together these results provide a greater understanding of an important, yet previously underestimated, *S. aureus* virulence factor and the means by which this toxin targets and kills host monocytes.



## **3.2 Materials and Methods**

### **3.2.1 Ethics statement**

All protocols were conducted in accordance with National Institutes of Health guidelines for the care and use of human subjects. De-identified human blood packs were purchased from Gulf Coast Regional Blood Center or New York Blood Center. The use of the de-identified samples was reviewed by the UNC Office of Human Research Ethics, which determined that the proposed studies (Study #12-0024) do not constitute human subjects research as defined under federal regulations [45 CFR 46.102 (d or f) and 21 CFR 56.102(c)(e)(I)] and does not require further IRB approval. The New York City Blood Center obtained written informed consent from all participants involved in the study. This research was approved by the New York University School of Medicine institutional human subjects board.

### **3.2.2 Mammalian cell lines**

THP1 cells (ATCC TIB-202) were maintained in Roswell Park Memorial Institute medium 1640 (RPMI) medium (Cellgro) at 37°C with 5% CO<sub>2</sub>, where culture medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (0.1 mg/ml). Transduced THP1 cells were maintained in 1.3 µg/ml puromycin. All experiments utilized cells that 2 days prior reached a density of approximately 0.8 x 10<sup>6</sup> cells/ml before being split 1:2 with fresh media. Prior to infections or intoxications, the desired volume of cells was removed, centrifuged and suspended in fresh RPMI media and equilibrated for 1 hr at 37°C with 5% CO<sub>2</sub>. Unless specified, experiments were carried out in either 96-well or 48-well flat-bottom tissue culture treated plates. All experiments were conducted within approximately 1 month of thawing frozen cell stocks.

### 3.2.3 Purifying primary CD14+ human monocytes

Human blood from leukopacks was diluted 1:2 with 1x phosphate buffered saline (PBS) supplemented with 0.1% bovine serum albumin (BSA) and 2mM EDTA or Hank's Balanced Salt Solution (HBSS). Diluted blood was layered over Ficoll-Paque (GE Healthcare Life Sciences) and centrifuged for isolation of buffy coats. Purified buffy coats were washed, counted, concentrated by centrifugation and labeled with CD14+ magnetic beads (Miltenyi Biotec). Cells were then washed to remove excess beads and separated per manufacturer's instructions on a magnetic column. Purified primary CD14+ human cells were suspended in RPMI media with 10% (FBS) for 1 hour prior to intoxication experiments.

### 3.2.4 Bacterial strains, culture conditions and generating mutants

*S. aureus* isolate Newman [35] was used in all experiments as the “wild-type” (WT) strain (unless stated). *S. aureus* was grown on tryptic soy broth (TSB) solidified with 1.5% agar at 37°C. *S. aureus* cultures were grown in TSB or in RPMI (Invitrogen) supplemented with 1% Casamino Acids (RPMI+CAS), with shaking at 180 rpm. When appropriate, RPMI+CAS was supplemented with chloramphenicol (Cm) at a final concentration of 10 µg/ml for purification of toxins from *S. aureus*.

Bacterial strains are listed in Table 3.1. Generation of the *S. aureus* Newman  $\Delta\Delta\Delta$  ( $\Delta lukAB$ ,  $\Delta lukED$ ,  $\Delta hlg$ ,  $\Delta hla$ ) [VJT 31.57], and precursor strain *S. aureus* Newman  $\Delta lukAB/\Delta lukED/\Delta hlg$  [VJT 25.03] have been previously described [53]. To generate *S. aureus* Newman  $\Delta lukAB/\Delta lukED$  [VJT 25.50], a previous *lukAB* mutant [VJT 8.91] [20] was transduced with a phage encoding *lukED::kan*. To generate green fluorescent protein (GFP) *S. aureus* strains Newman and USA 300-BK18807, and respective isogenic *lukAB* mutant, were transformed with

*pOS1-P<sub>sarA</sub>-sod RBS-sgfp*, a plasmid that constitutively and robustly produces superfolded GFP [54].

### 3.2.5 Purifying toxins from *S. aureus*

A construct to co-purify recombinant LukAB from *S. aureus* (*pOS-1-P<sub>lukAB</sub>-sslukA-6His-lukA-lukB*) was generated as previously described [24] and transformed into Newman  $\Delta\Delta\Delta\Delta$  [53] to facilitate purification. This construct was also used to individually express toxin subunits from LukED, PVL, HlgABC [24]. Toxins were purified from *S. aureus* as previously described [24]. Briefly, strains were grown in TSB with 10  $\mu\text{g/ml}$  chloramphenicol for 5 h at 37°C, 180 rpm, to an optical density at 600 nm ( $\text{OD}_{600}$ ) of approximately 1.5 (which represents  $1 \times 10^9$  CFU/ml). The bacteria were then pelleted, and the supernatant was collected and filtered. Nickel-nitrilotriacetic acid (NTA) resin (Qiagen) was incubated with culture supernatant, washed, and eluted with 500 mM imidazole. The protein was dialyzed in  $1\times$  Tris-buffered saline (TBS) plus 10% glycerol at 4°C overnight and then stored at  $-80^\circ\text{C}$ .

### 3.2.6 Collecting culture filtrates

Culture filtrates were collected essentially as described previously [25]. Briefly, three-milliliter overnight cultures in RPMI+Cas were grown in 15-ml conical tubes held at a 45° angle and incubated at 37°C with shaking at 180 rpm. The following day, bacteria were subcultured at a 1:100 dilution and grown as described above for 5 h. Bacteria were then pelleted by centrifugation at 4,000 rpm [ $3220 \times g$ ] and 4°C for 10 min. Supernatants containing exoproteins were collected, filtered using a 0.2- $\mu\text{m}$  filter, and stored at  $-80^\circ\text{C}$ .

### 3.2.7 Transmission electron microscopy

THP1 cells were intoxicated with culture filtrates (10 % v/v) from WT *S. aureus* Newman, an isogenic *lukAB*-deficient mutant or culture media for 1 h at 37°C with 5% CO<sub>2</sub>. Cells were then fixed in 0.1 M sodium cacodylate buffer (pH 7.2), containing 2.5% glutaraldehyde and 2% paraformaldehyde for 2 h and post-fix stained with 1% osmium tetroxide for 1.5 h at room temperature, and en bloc stained with 1% uranyl acetate. The cells were dehydrated in ethanol then embedded in EMBED 812 (Electron Microscopy Sciences, Hatfield, PA). Semi-thin sections were cut at 1 µm and stained with 1% toluidine blue to evaluate the quality of preservation. Ultrathin sections (50 nm) were post stained with uranyl acetate and lead citrate and examined using Philips CM-12 electron microscope (FEI; Eindhoven, The Netherlands) and photographed with a Gatan (4 k × 2.7 k) digital camera (Gatan, Pleasanton, CA, USA).

### 3.2.8 Evaluating cell death by lactate dehydrogenase (LDH) release

For infection assays, *S. aureus* was cultured as described above for culture filtrate production then the bacterial pellet was washed twice with 5 ml of PBS. Bacteria were then normalized to an OD<sub>600</sub> 1.0, which represents approximately 1.0 X 10<sup>9</sup> CFU/ml using a Genesys 20 spectrophotometer (Thermo Scientific). Normalized *S. aureus* cultures were used to infect THP1 cells, seeded at 1 X 10<sup>5</sup> cells/well, at a multiplicity of infection (MOI) of 50 in a final volume of 100 µl for 2 h at 37°C and 5% CO<sub>2</sub>. Controls for 100% viability were composed of THP1 cells without *S. aureus*, while controls for 100% THP1 lysis included the addition of Triton X-100 (0.2%). Following infection, cells were pelleted by centrifugation at 1,500 rpm [450 x g] at 4°C for 5 min and lactate dehydrogenase (LDH) release was assayed as a measure of THP1 viability using the CytoTox-ONE homogeneous membrane integrity assay (Promega) per manufacturer

specifications. Briefly, 50  $\mu$ l of culture supernatant was removed and added to wells containing 50  $\mu$ l of LDH reagent and incubated for an additional 10 min at RT. Fluorescence was measured using a PerkinElmer Envision 2103 multilabel reader (excitation, 555 nm; emission, 590 nm), and data were normalized to 100% THP1 lysis.

THP1 cells were intoxicated with titrations of *S. aureus* culture filtrates (vol/vol) for 4 h at 37°C and 5% CO<sub>2</sub>. Controls for 100% viability were composed of cells with *S. aureus* growth medium (RPMI-CAS), while controls for 100% THP1 lysis included the addition of Triton X-100 (0.2 %) in RPMI-CAS. THP1 viability was assayed by measuring LDH release as described above.

### **3.2.9 Evaluating cell death by propidium iodide (PI) staining**

THP1 cells, seeded at  $1 \times 10^6$  cells/mL in 300  $\mu$ L/well, were intoxicated with culture filtrates or LukAB in the presence of propidium iodide (2.5  $\mu$ g/mL) for 60 minutes. Cells were fixed with a combination formaldehyde and methanol solution supplied by ImmunoChemistry Technologies. Fluorescence was measured by flow cytometry using an Accuri C6 flow cytometer (BD Biosciences).

### **3.2.10 Measuring cytokine secretion by AlphaLISA**

Culture supernatants from THP1 cells incubated with culture filtrates or LukAB were analyzed by alphaLISA for IL-1 $\beta$ , IL-18 and TNF- $\alpha$  according to the manufacturer's protocol for short incubation (Perkin Elmer) with reduced volumes. Briefly, 1 $\mu$ L of each sample or standard was added to a separate well in a 384-well plate with 4 $\mu$ L of acceptor beads and cytokine antibody. After a 1-hour incubation at room temperature shielded from light, 5 $\mu$ L of streptavidin-conjugated

donor beads was added to each well for 30 minutes. Luminescence was measured on an EnSpire Multimode Plate Reader (Perkin Elmer).

### **3.2.11 Measuring Caspase 1 activation with FLICA**

THP1 cells, seeded at  $1 \times 10^6$  cells/mL in 300uL/well, were intoxicated with culture filtrates or LukAB in the presence of the Caspase 1 inhibiting peptide FLICA-FMK bound to Alexa Fluor 660 (FLICA-1) (1:100 dilution) for 60 minutes. Cells were washed once with 1x PBS and resuspended in 1x PBS plus 8% fixative solution supplied by ImmunoChemistry Technologies. Fluorescence was measured by flow cytometry using an Accuri C6 flow cytometer (BD Biosciences).

### **3.2.12 Immunoblot Analysis**

THP1 cells were washed with 1x PBS and lysed with RIPA buffer (50mM Tris, pH 7.4, 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, protease inhibitor cocktail) for a concentration of  $1 \times 10^7$  cell equivalents/mL. Lysate was spun at full speed in a mini centrifuge for 10 minutes at 4°C. Lysate supernatant was mixed with Laemmli sample buffer and heated to 95°C for 5 minutes. Samples were stored at -80°C until analyzed.

Samples were loaded at  $1 \times 10^5$  cell equivalents per well in a pre-cast 4-12% Bis-Tris SDS-PAGE gel (Bio-Rad Laboratories, Inc.). Electrophoresis was run at 120 volts for 100 minutes. Transfer was conducted using Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Inc.). Membranes were blocked with 5% milk solids or 5% BSA in 1x TBS-T. Primary and HRP-conjugated secondary antibody incubation were performed overnight and for 1 hour, respectively, in blocking solution. Membranes were washed for 15 minutes three times after each antibody

incubation. Membranes were developed using Pierce ECL Western Blotting Substrate or SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) and imaged using a FluorChem E system (Protein Simple). All blots shown in the same figure are from the same experiment.

Antibodies used include anti-NLRP3, mAb (Cryo-2) at 1 to 1000 dilution (AdipoGen), anti-ASC, pAb antibody at 1 to 1000 dilution (Enzo Biosciences), anti-HMGB1 antibody (HAP46.5, ab12029) at 1:2000 dilution (Abcam), anti-Caspase 1 antibody (14F468) at a 1 to 1000 dilution (Novus Biologicals), anti-Actin antibody (SC-1615) at 1:5000 dilution (Santa Cruz Biotechnologies), Goat-anti Mouse antibody (SC-2005) at 1:5000 dilution (Santa Cruz Biotechnologies) and Goat-anti Rabbit antibody (SC-2004) at 1:5000 dilution (Santa Cruz Biotechnologies).

### **3.2.13 Measuring THP1 surface CD11b levels**

THP1 cells, seeded at  $1 \times 10^5$  cells/well, were stained with 1 ng/ $\mu$ l of APC-conjugated anti-CD11b (or isotype control) (Biolegend) in a final volume of 50  $\mu$ l for 30 min on ice. Cells were washed once with 1x PBS + 2% FBS + 0.05% sodium azide (FACS buffer), suspended in 50  $\mu$ l of FACS buffer, then analyzed using an LSR-II flow cytometer (Becton, Dickinson, BD).

### **3.2.14 Evaluating infection assays by flow cytometry**

THP1 infection assays under non-phagocytosing and phagocytosing conditions were modified from a previous study [25]. Briefly, GFP-expressing *S. aureus* Newman and the isogenic *lukAB*-deficient mutant were cultured and normalized to  $1.0 \times 10^9$  CFU/ml as described above. After normalization, bacteria were pelleted and suspended in phenol red-free RPMI with 10 mM

HEPES Buffer (RPMI+HEPES) for non-phagocytosing conditions or opsonized with RPMI+HEPES supplemented with 20% normal human serum (NHS) for phagocytosing conditions. To promote opsonization, bacteria were incubated at 37°C with rotation for 30 min, centrifuged and pellet suspended in equivalent volume of RPMI+HEPES. Ninety-six well plates used for phagocytosing conditions were first coated with 20% NHS in RPMI+HEPES for 30 min at 37°C and subsequently washed with RPMI+HEPES.

Prior to infection, THP1 cells were primed with 500 ng/ml of purified *S. aureus* lipoteichoic acid (LTA) for 3 hrs, centrifuged at 1,500 rpm [450 x g] and 4°C then suspended in equivalent volume of RPMI+HEPES. THP1 cells, plated at  $1 \times 10^5$  cells/well, were infected with GFP *S. aureus* Newman or the isogenic *lukAB*-deficient mutant at an MOI of 10. For phagocytosing conditions, THP1 cells and bacteria were centrifuged at 1,500 rpm [450 x g] and 4°C for 7 min to promote synchronization of phagocytosis. Post-synchronization, cells were treated with 2.5 µg/ml of polyclonal anti-LukA antibody affinity purified from rabbit sera along and lysostaphin (40 µg/ml; Ambi Products LLC) to reduce effects of extracellular *S. aureus* and LukAB, then incubated at 37°C and 5% CO<sub>2</sub> for 45 min. For non-phagocytosing conditions, bacteria were incubated at 37°C and 5% CO<sub>2</sub> for 120 min.

Post-infection, cells were washed 2 times in 200 µl of PBS then stained with a 1:5,000 dilution of a Fixable Viability Dye (eFluor 450; Affymetrix eBioscience) and a 1:150 dilution of FLICA-1 in a final volume of 20 µl for 20 min on ice. Cells were washed 2 times in 200 µL of FACS buffer before being suspended in 40 µl of fixing buffer (1 X PBS + 2% paraformaldehyde + 2% FBS + 0.05% sodium azide) and analyzed using an LSR-II flow cytometer to measure GFP and eFluor 450 fluorescence.



**Table 3.1 *Staphylococcus aureus* strains**

<b>Strain</b>	<b>Background</b>	<b>Description</b>	<b>Designation</b>	<b>Reference</b>
VJT 1.07	Newman	WT/pOS1	Newman ( <i>p</i> )	[20]
VJT 2.59	USA 200	WT	USA 200	[55]
VJT 2.84	USA 100	WT	USA 100	NARSA <sup>3</sup>
VJT 2.97	Newman	WT	WT or Newman	[35]
VJT 4.79	USA 400	MW2	USA 400	[56]
VJT 7.09	Newman	$\Delta hlgACB::tet$	$\Delta hlg$	[57]
VJT 8.16	Newman	$\Delta lukED$	$\Delta lukED$	[20]
VJT 8.91	Newman	$\Delta lukAB$	$\Delta lukAB$	[20]
VJT 9.72	Newman	$\Delta lukAB/pOS1$	Newman $\Delta lukAB$ ( <i>p</i> )	[20]
VJT 9.76	Newman	$\Delta lukAB/pOS1-lukAB$	Newman $\Delta lukAB$ ( <i>plukAB</i> )	[20]
VJT 10.21	USA 500	USA 500-2395	USA 500	[58]
VJT 12.61	USA 300	LAC	USA 300	[59]
VJT 15.15	USA 300	pOS1	LAC ( <i>p</i> )	[20]
VJT 15.17	USA 300	$\Delta lukAB/pOS1$	LAC $\Delta lukAB$ ( <i>p</i> )	[20]
VJT 15.18	USA 300	$\Delta lukAB/pOS1-lukAB$	LAC $\Delta lukAB$ ( <i>plukAB</i> )	[20]
VJT 15.19	USA 400	pOS1	MW2 ( <i>p</i> )	[20]

VJT 15.21	USA 400	$\Delta lukAB/pOS1$	MW2 $\Delta lukAB (p)$	[20]
VJT 15.22	USA 400	$\Delta lukAB/pOS1-lukAB$	MW2 $\Delta lukAB (plukAB)$	[20]
VJT 21.05	USA 700	WT	USA 700	NARSA <sup>3</sup>
VJT 21.06	USA 800	WT	USA 800	NARSA <sup>3</sup>
VJT 21.08	USA 1100	WT	USA 1100	NARSA <sup>3</sup>
VJT 38.71	Newman	$pOS1sGFP-P_{sarA-sod} RBS$	Newman GFP	This study
VJT 38.72	Newman	$\Delta lukAB pOS1sGFP-P_{sarA-sod} RBS$	Newman $\Delta lukAB$ GFP	This study
VJT 38.77	USA 300-BK	$pOS1sGFP-P_{sarA-sod} RBS$	USA 300 GFP	This study
VJT 38.78	USA 300	$\Delta lukAB pOS1sGFP-P_{sarA-sod} RBS$	USA 300 $\Delta lukAB$ GFP	This study

<sup>3</sup> NARSA, Network of Antimicrobial Resistance in *Staphylococcus aureus*.

### 3.3 Results

#### 3.3.1 *S. aureus* kills human monocytes in a LukAB dependent manner.

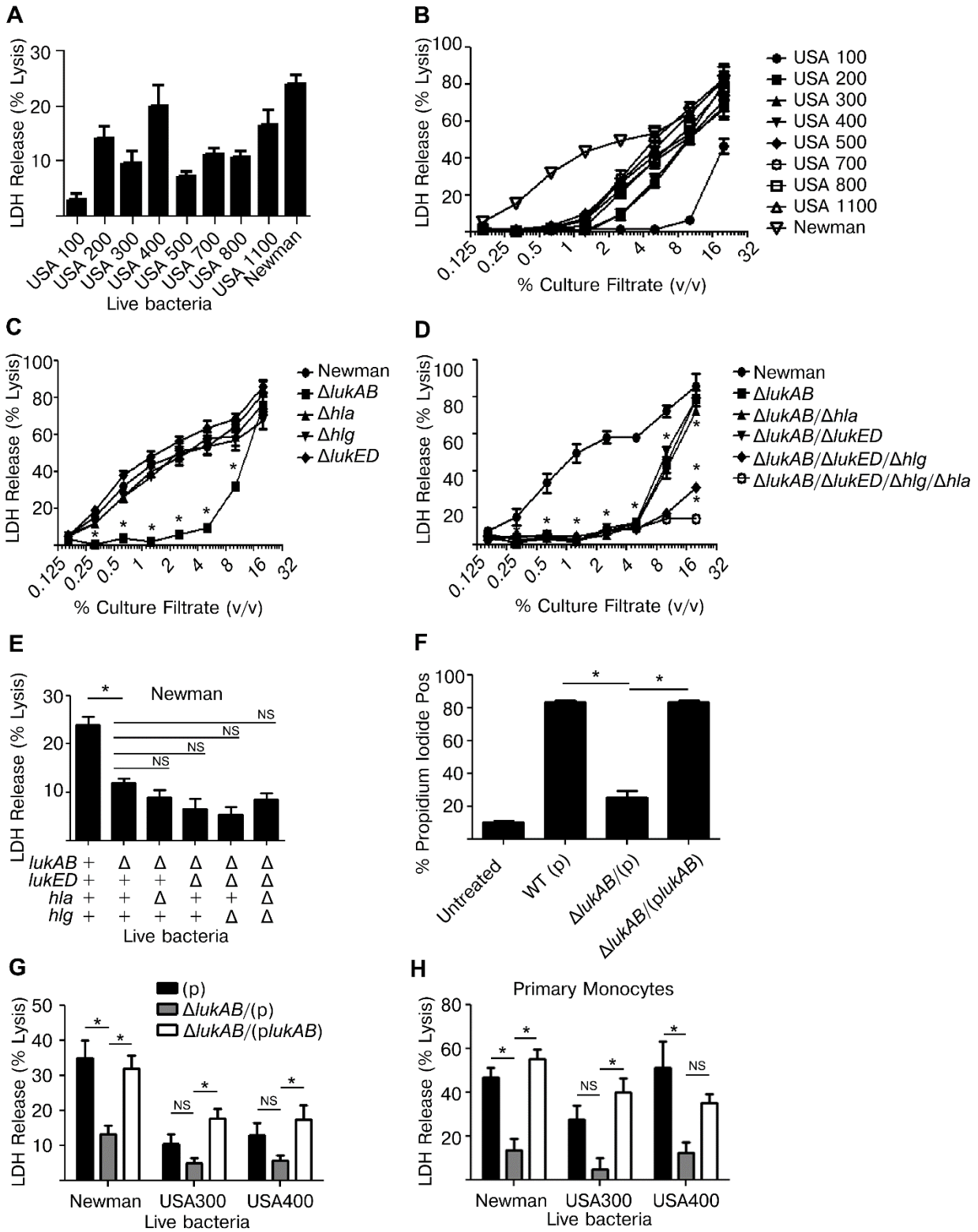
To investigate the ability of *S. aureus* to kill human monocytes, a variety of live *S. aureus* clinical isolates, representing different clonal lineages, were co-cultured with THP1 cells. Each *S. aureus* strain killed THP1 cells, albeit to varying degrees, as assessed by the amount of cytoplasmic lactate dehydrogenase (LDH) released into the culture supernatant (Fig. 3.1A). To evaluate the contribution of *S. aureus* secreted proteins to cell death, culture filtrates were collected from log-phase grown *S. aureus* and used to intoxicate THP1 cells. These culture filtrates were all capable of inducing cell death in a dose dependent manner (Fig. 3.1B).

To further dissect the role of specific secreted toxins, we selected *S. aureus* Newman, a well-characterized methicillin-sensitive clinical isolate [35] that exhibited potent cellular killing [20]. Here, THP1 cells were intoxicated with a titration of culture filtrates from *S. aureus* Newman or individual isogenic mutants deficient of *lukAB*, *hla*, *hlgACB*, or *lukED* and LDH release was evaluated (Fig. 3.1C). Only loss of LukAB had a significant effect on culture filtrate cytotoxicity, particularly at concentrations of  $\leq 5\%$  v/v. Culture filtrates lacking LukAB had residual cytotoxicity at higher concentrations, suggesting that additional factors can contribute to THP1 cell death (Fig. 3.1C). Using *S. aureus* strains lacking multiple toxins and in various combinations, we demonstrated that HlgACB and Hla in culture filtrates also contribute to THP1 killing at these higher concentrations (Fig. 3.1D), consistent with previous reports using murine cells [30,31,36]. We also utilized these strains to determine the relative effects of each toxin in live *S. aureus*-mediated killing of THP1 cells (Fig. 3.1E). Similar to our observations with culture filtrates, loss of LukAB significantly reduced *S. aureus* killing of THP1 cells, while the loss of the other toxins had little if any additional effect. To further validate the LDH data, we used propidium iodide (PI), a membrane-impermeable DNA-intercalating dye to monitor

membrane integrity of human monocytes using flow cytometry. In agreement with the LDH release data, LukAB was responsible for the propidium iodide staining observed with culture filtrates of *S. aureus* strain Newman (Fig. 3.1F).

*S. aureus* Newman naturally does not encode *pvl*, thus we next sought to determine whether LukAB also contributes to THP1 cell death in methicillin-resistant *S. aureus* (MRSA) strains that produce PVL. We assessed the cytotoxicity of wildtype and LukAB-deficient strains LAC (USA 300) and MW2 (USA 400), two representative MRSA clones, towards THP1 cells in models of live bacterial infection (Fig. 3.1G). As with Newman, strains LAC and MW2 lacking *lukAB* exhibited reduced cytotoxicity to THP1 cells when compared to the isogenic parental strain, a phenotype complemented by expressing *lukAB* from a plasmid episomally (Fig. 3.1G).

The impact of LukAB in killing human monocytes was also tested using live bacterial infection and culture filtrate intoxication of purified CD14+ primary human monocytes. The diminished cytotoxicity of *lukAB* deficient *S. aureus* strains seen in THP1 experiments was phenocopied in experiments with primary monocytes, as was the complementation of the phenotype by *lukAB*-expressing plasmids (Fig. 3.1H and 3.1I). Thus, these data demonstrate that LukAB is the predominant toxin secreted by *S. aureus* to kill human monocytes.



**Figure 3.1** *Staphylococcus aureus* LukAB induces cell death in human monocytic cells. (A)

THP1 cells were infected with the indicated *S. aureus* strains at a multiplicity of infection (MOI)

of 50 for 2 hours and culture supernatants were analyzed for LDH release as a measurement of cell lysis. (B) Culture filtrates, acquired from log-phase growth of *S. aureus* strains listed, were used to intoxicate THP1 cells at the indicated concentrations for 4 hours and LDH release was analyzed. (C and D) THP1 cells were intoxicated with culture filtrates from *S. aureus* Newman or the indicated isogenic mutants for 4 hours and LDH release was analyzed. (E) THP1 cells were infected with *S. aureus* Newman or the indicated isogenic mutants at an MOI of 50 and LDH release was analyzed after 2 hours. THP1 cells (F) and primary CD14+ human monocytes (G) were infected with *S. aureus* Newman, USA300 (LAC) or USA400 (MW2) or the respective *lukAB*-deficient mutants and complemented strains at an MOI of 50 (F) or 25 (G) and LDH release was analyzed after 2 hours. (H) Culture filtrates (1% v/v) from the indicated *S. aureus* strain and isogenic mutants were used to intoxicate primary CD14+ human monocytes for 1 hour and LDH release was analyzed. (I) THP1 cells were incubated with propidium iodide and intoxicated with culture filtrates from *S. aureus* Newman, *lukAB* mutant, or complemented strain at 1% (v/v) for 4 hours then analyzed by flow cytometry. Error bars represent the mean  $\pm$  standard error of the mean for at least two independent experiments, each performed in triplicate. Experiments with primary cell experiments include at least three independent donors. Asterisks indicate significance at a *p*-value of  $\leq 0.05$  by Tukey's multiple comparisons post-test for 1-way or 2-way ANOVA, as appropriate.

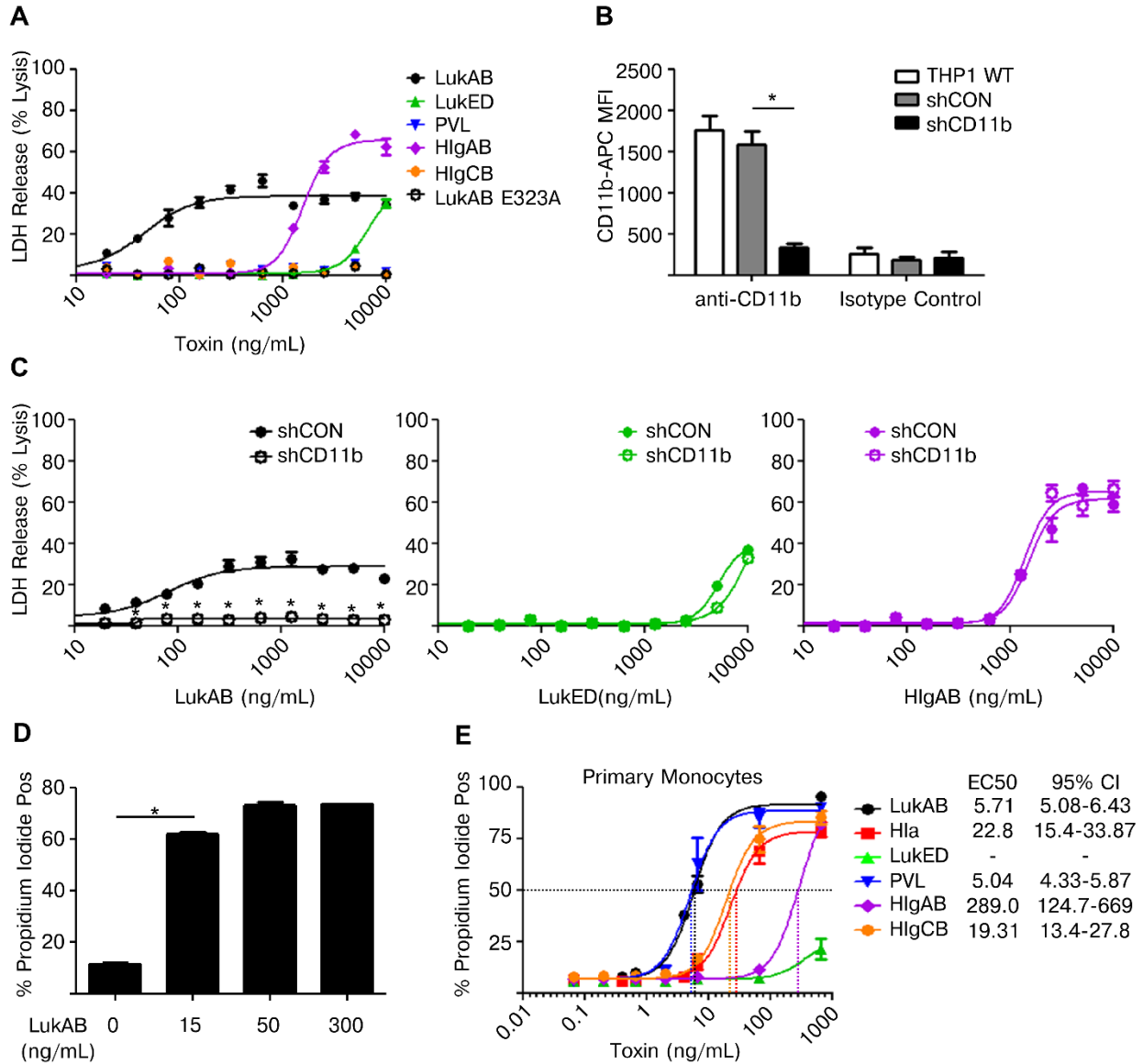
### **3.3.2 LukAB kills human monocytes by engaging its cellular receptor.**

To determine whether LukAB was sufficient to induce THP1 cell death, and to compare its relative cytotoxicity to that of the other bi-component pore forming toxins, THP1 cells were intoxicated with purified toxins. LukAB, LukED and HlgAB were all able to induce cytotoxicity in THP1 cells, as measured by release of LDH into the culture medium (Fig. 3.2A). In contrast, HlgCB and PVL were unable to lyse THP1 cells [29]. Among the toxins tested, LukAB was the most potent, capable of lysing THP1 cells at concentrations approximately 8- and 12-fold lower than HlgAB and LukED, respectively (Fig. 3.2A).

LukAB targets CD11b on human neutrophils to promote cell death [22], so we next sought to determine if LukAB-CD11b recognition was also required for LukAB activity towards human monocytes. We first utilized a previously characterized LukAB mutant that does not bind CD11b, LukAB E323A [24]. This mutant toxin failed to elicit LDH release from THP1 cells, indicating that CD11b interaction is required for LukAB-mediated death in this monocytic cell line (Fig. 3.2A). To further validate this finding, THP1 cells were transduced to stably express short hairpin RNA (shRNA) against CD11b or a non-targeting control (Fig. 3.2B). Knockdown of CD11b was confirmed through immunostaining and analysis by flow cytometry (Fig. 3.2B). These transduced cells were then intoxicated with purified LukAB, LukED or HlgAB (Fig. 3.2C). The CD11b shRNA knockdown significantly reduced LukAB-mediated cytotoxicity, but not cytotoxicity resulting from HlgAB or LukED intoxication, indicating that LukAB recognizes CD11b on human monocytes to induce cell death (Fig. 3.2C). The potency of LukAB in mediating THP1 cell permeability to PI paralleled the potency observed for release of LDH from target cells. (Fig. 3.2D).

We next sought to determine the potency to LukAB, relative to other pore-forming toxins in killing CD14+ primary human monocytes. As assessed by PI staining, dose-titrations of each toxin demonstrated that LukAB and PVL were the most potent in their ability to kill primary monocytes (Fig. 3.2E). Interestingly, primary monocytes exhibited a ten-fold increase in susceptibility to LukAB when compared to the THP1 cell line. This difference in toxin susceptibility was accentuated in PVL where primary monocytes were susceptible to the toxin while THP1 cells were essentially resistant.





**Figure 3.2 LukAB recognizes CD11b on human monocytes to potently induce cell death.**

(A) THP1 cells were intoxicated with titrations of the indicated purified toxins for 1 hour and LDH release was analyzed. (B) THP1 cells were transduced with either non-targeting shRNA or shRNA against CD11b and surface CD11b levels were evaluated by flow cytometry. (C) THP1 cells described in B were intoxicated with titrations of the indicated toxins for 1 hour and LDH release was analyzed. (D) THP1 cells were intoxicated with the indicated concentration of LukAB for 1 hour and analyzed by flow cytometry for permeability to propidium iodide. (E)

Primary CD14<sup>+</sup> human monocytes were intoxicated with titrations of the indicated toxins for 1 hour and analyzed by flow cytometry for permeability to propidium iodide. EC<sub>50</sub> values also shown. Error bars represent the mean  $\pm$  standard error of the mean for at least two independent experiments, each performed in triplicate. Primary cell experiments include three independent donors. Asterisks indicate significance at a *p*-value of  $\leq 0.05$  by Tukey's multiple comparisons post-test for 1-way or 2-way ANOVA, as appropriate.

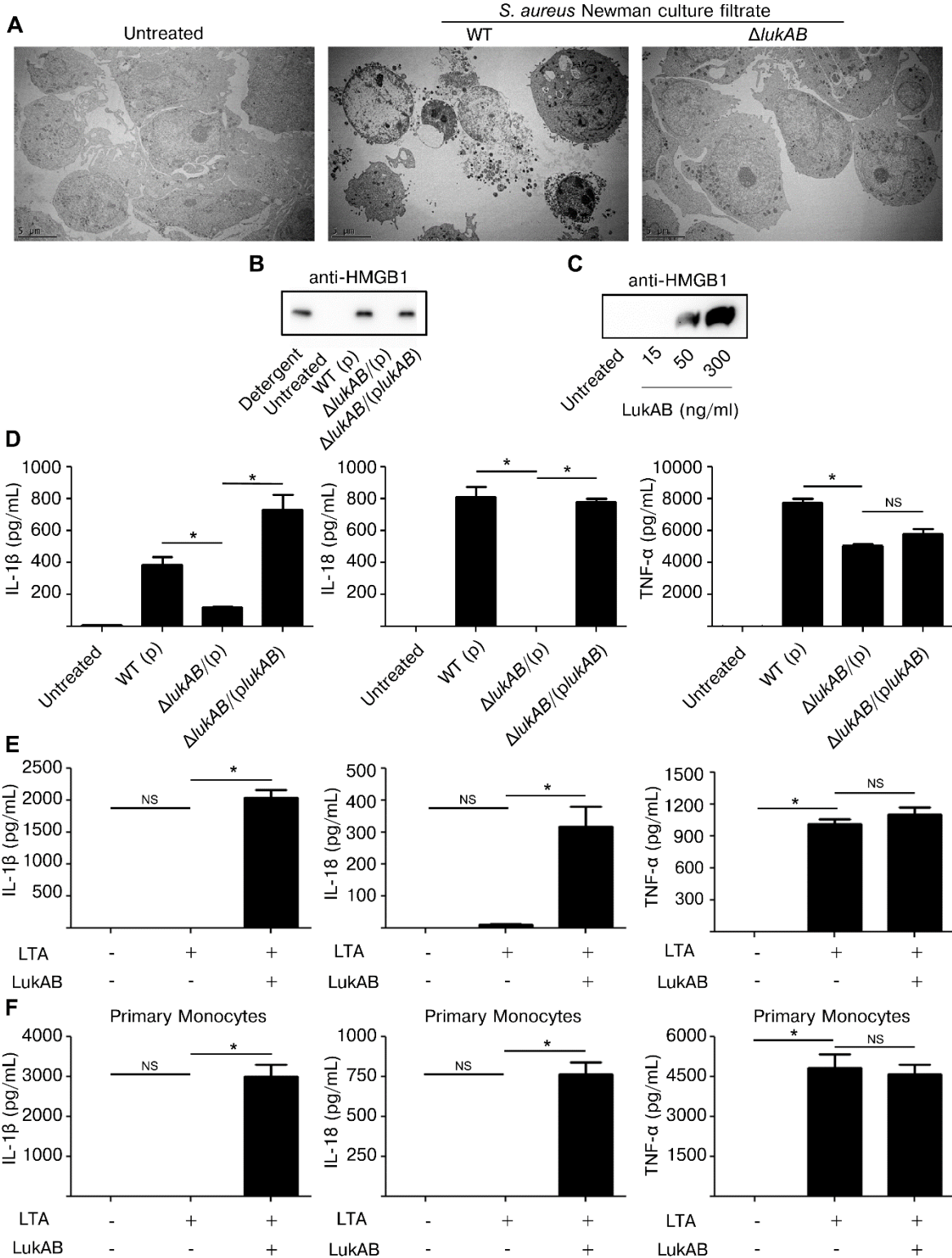
### 3.3.3 LukAB-induced cell death displays necrotic features.

Traditionally, programmed cell death can be morphologically categorized into necrotic or apoptotic phenotypes [37,38]. During necrotic cell death membrane integrity is rapidly lost, releasing cytosolic and nuclear contents into the extracellular milieu. Death with necrotic features leads to inflammation, as cytosolic contents act as endogenous danger signals triggering activation of innate immune signaling. In contrast, apoptosis is relatively immunologically silent as cytosolic and nuclear contents are broken down into small membrane bound bodies [39]. To further visualize the effect of LukAB on the membrane of THP1 cells, culture filtrates from *S. aureus* Newman or the isogenic *lukAB* deficient-mutant were used to intoxicate THP1 cells which were then examined by transmission electron microscopy (Fig. 3.3A). THP1 cells intoxicated with LukAB-containing culture filtrates displayed vacuolation of the cytoplasm, substantial plasma membrane compromise, and gross changes in nuclear morphology, all suggestive of necrotic cell death. This is in contrast to THP1 cells intoxicated with culture filtrates from an isogenic *lukAB* deficient-mutant or with the media control, where membranes were mostly intact and cytoplasmic contents preserved (Fig. 3.3A).

Another marker of necrosis is the release of High Mobility Group Box 1 protein (HMGB1), a nuclear protein that, when released, acts as a pro-inflammatory danger signal [40]. To test whether LukAB induced HMGB1 release, THP1 cells were intoxicated with *S. aureus* culture filtrates or purified LukAB and the culture supernatants were evaluated by immunoblot with antibodies specific to HMGB1. We observed that LukAB was necessary and sufficient to induce HMGB1 release (Fig. 3.3B and 3.3C).

Necrotic cell death paired with secretion of inflammatory cytokines IL-1 $\beta$  and IL-18 is termed pyroptosis [41-43]. To determine if LukAB causes THP1 cells to secrete IL-1 $\beta$  and IL-

18, we analyzed culture supernatants from THP1 cells intoxicated with Newman strain culture filtrates (Fig. 3.3D) and purified LukAB (Fig. 3.3E). In this setting, THP1 cells secreted IL-1 $\beta$ , IL-18 and TNF- $\alpha$  in response to Newman strain culture filtrates, though deletion of *lukAB* eliminated secretion of IL-1 $\beta$  and IL-18 with no evident effect on TNF- $\alpha$  secretion. THP1 cells do not express pro-IL-1 $\beta$  at baseline and thus require priming with a Toll-like receptor ligand such as lipoteichoic acid (LTA) prior to intoxication with purified LukAB. LTA induced TNF- $\alpha$  secretion, but LukAB was required for secretion of IL-1 $\beta$  and IL-18 (Fig. 3.3E). As with THP1, primary CD14<sup>+</sup> human monocytes also secreted IL-1 $\beta$  and IL-18 in response to LukAB (Fig. 3.3F).



**Figure 3.3** LukAB induces necrotic cell death and secretion of pro-inflammatory cytokines

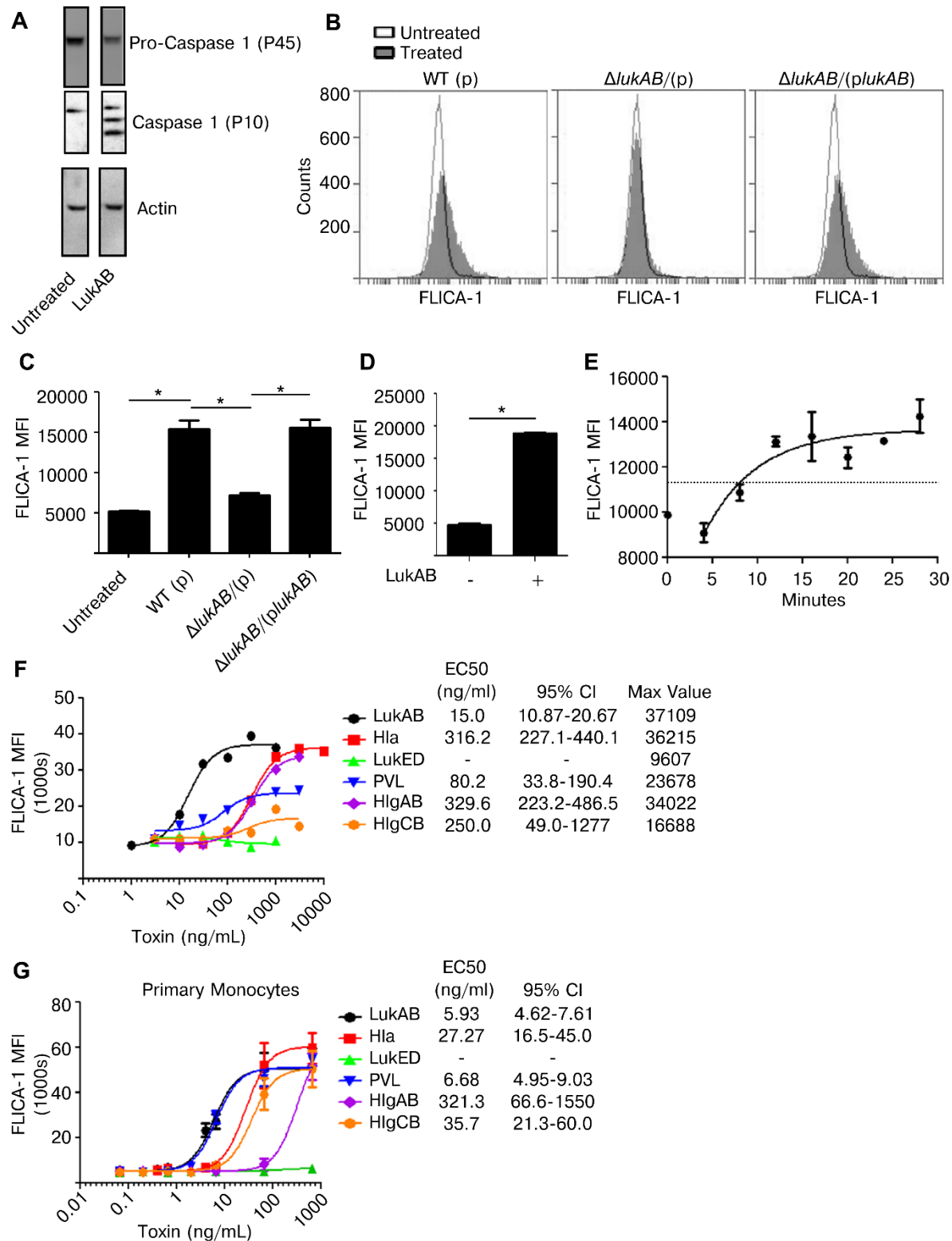
**IL-1 $\beta$  and IL-18.** (A) THP1 cells were intoxicated with culture filtrate from *S. aureus* Newman

(10% v/v), isogenic *lukAB* mutant or culture media for 1 hour. Cells were collected, prepared and imaged by transmission electron microscopy (see methods). (B) THP1 cells were intoxicated with 1% (v/v) culture filtrate from *S. aureus* Newman (1% v/v) and the indicated isogenic mutants and after 4 hours supernatants were collected and analyzed by immunoblot for HMGB1 release. (C) THP1 cells were intoxicated with purified LukAB at the indicated concentrations for 1 hour and supernatants were analyzed by immunoblot for HMGB1 release. (D) Primary CD14+ human monocytes were intoxicated with purified LukAB at the indicated concentrations for 1 hour and supernatants were analyzed by immunoblot for HMGB1 release. (E) THP1 cells were intoxicated with culture filtrates (1% v/v) from the indicated strains and supernatants were collected and analyzed for secretion of the indicated cytokines. THP1 cells (F) and primary CD14+ human monocytes (G) were primed for production of pro-IL-1 $\beta$  with 500 ng/mL LTA for 3 hours followed by intoxication with LukAB (THP1 with 50 ng/mL and CD14+ monocytes with 30 ng/mL) for 1 hour then supernatants were analyzed for secretion of the indicated cytokines. Error bars represent the mean  $\pm$  standard error of the mean for at least two independent experiments, each performed in triplicate. Primary cell experiments include three independent donors. Asterisks indicate significance at a *p*-value of  $\leq 0.05$  by Tukey's multiple comparisons post-test for 1-way or 2-way ANOVA, as appropriate.

### 3.3.4 LukAB induces activation of Caspase 1 in human monocytes.

Pyroptosis depends on the activation of Caspase 1 [39]. To determine if LukAB induced Caspase 1 activation, THP1 cells were incubated with purified LukAB and activation of Caspase 1 assessed using immunoblot analyses. The auto-proteolysis-derived P10 subunit, an indication of Caspase 1 activation, was observed in cells treated with purified LukAB (Fig. 3.4A). To quantitatively assess Caspase 1 activation, we used a flow cytometry based fluorescent-labeled peptide inhibitor of Caspase 1 (660-YVAD-FMK) assay (hereafter FLICA-1) [41]. THP1 cells treated with *S. aureus* culture filtrates demonstrated a marked increase in FLICA-1 fluorescence when compared to the isogenic *lukAB*-deficient mutant or untreated cells (Fig. 3.4B and 3.4C). The phenotype of the *lukAB*-deficient mutant could be complemented to that of the WT strain by expressing a plasmid encoding *lukAB*. As observed by immunoblot (Fig. 3.4A), LukAB treatment alone was sufficient to cause enhanced FLICA-1 activation in THP1 cells (Fig. 3.4D). Measurable change in FLICA-1 activation in THP1 cells lagged the introduction of toxin by approximately five minutes. After the lag period, FLICA-1 activation rapidly increased reaching a plateau over approximately 10 minutes after the FLICA-1 signal began to increase.

It has previously been reported that Hla and PVL activate Caspase 1 [28,29,36]. We next sought to determine the relative potency of each *S. aureus* leukotoxin in inducing Caspase 1 activation as measured by FLICA-1 in both THP1 cells (Fig. 3.4F) and primary CD14+ human monocytes (Fig. 3.4G). In THP1 cells, LukAB was the most potent FLICA-1 activator (Fig. 3.4F). Although PVL did not induce cell lysis in THP1 cells (Fig. 3.2A), it did induce FLICA-1 activation (Fig. 3.4F). In primary human monocytes, LukAB and PVL demonstrated equivalent potency in inducing FLICA-1 activation (Fig. 3.4G).



**Figure 3.4** LukAB is a potent activator of Caspase 1. (A) THP1 cells were intoxicated with 50 ng/mL LukAB for 1 hour and cell lysates were analyzed by immunoblot for Caspase 1 cleavage,



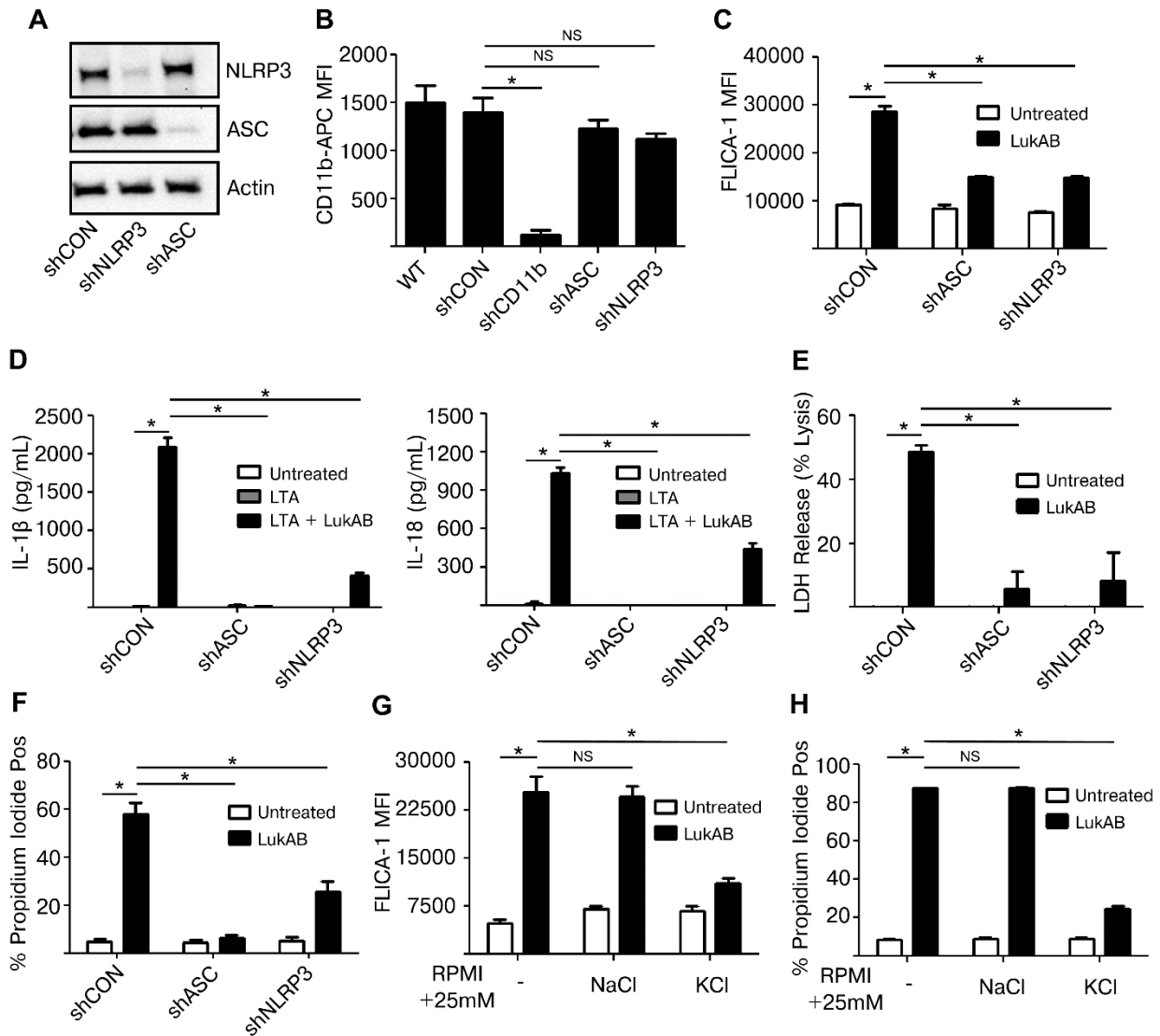
which indicates activation. (B and C) THP1 cells were incubated with FLICA-1 (660-YVAD-FMK) then intoxicated with culture filtrates (1% v/v) from the indicated *S. aureus* stains. After 1 hour, cells were washed, fixed and analyzed by flow cytometry. Panel B shows a representative flow plot from one experiment, and panel C shows the corresponding mean fluorescence intensities (MFI). (D) THP1 cells were incubated with FLICA-1, intoxicated with purified LukAB for 1 hour (50 ng/mL), then washed, fixed and analyzed by flow cytometry. (E) FLICA-1 was added to THP1 cells then LukAB was added (50 ng/mL) for time-course samples in descending order. All samples were washed and fixed at the same time, corresponding to different LukAB incubation times, then analyzed by flow cytometry. THP1 cells (F) and primary CD14+ human monocytes (G) were incubated with FLICA-1 and intoxicated with a dose titration of the indicated purified *S. aureus* leukotoxin for 1 hour. Cells were washed, fixed and analyzed by flow cytometry. Bars represent the standard error of the mean of triplicate samples. Each graph is representative of three experiments. Primary cell experiments include three independent donors. Asterisks indicate significance at a  $p$ -value of  $\leq 0.05$  by Tukey's multiple comparisons post-test for 1-way or 2-way ANOVA, as appropriate.

### **3.3.5 NLRP3 and ASC are necessary for LukAB-induced cytokine secretion and necrotic cell death.**

The activation of Caspase 1 by *S. aureus* Hla and PVL depends on host NLRP3 and ASC [26,29]. We next sought to determine whether LukAB-mediated Caspase 1 activation, secretion of IL-1 $\beta$  and IL-18 and cell death were also dependent on NLRP3 and ASC. To this end, THP1 cells were transduced to stably express shRNA constructs targeting inflammasome components ASC and NLRP3, or a non-targeting shRNA control. Knockdown of ASC and NLRP3 were confirmed by immunoblot analyses (Fig. 3.5A). Importantly, knock down of ASC or NLRP3 did not reduce levels of the LukAB receptor CD11b (Fig. 3.5B). Knock down of ASC or NLRP3 resulted in a significant reduction in LukAB-induced Caspase 1 activation (Fig. 3.5C) and secretion of the Caspase 1 dependent cytokines IL-1 $\beta$  and IL-18 (Fig. 3.5D). Thus, FLICA-1 is a reliable measure of NLRP3 inflammasome activation by LukAB. Moreover, LukAB-induced necrotic cell death as measured by LDH release (Fig. 3.5E) and membrane permeability to PI (Fig. 3.5F) was essentially eliminated when ASC or NLRP3 were depleted. Knock down of ASC or NLRP3 also had a similar effect on IL-1 $\beta$  and IL-18 secretion and cell death in THP1 cells intoxicated with Newman culture filtrates (Fig. 3.6). Residual cell death and cytokine release was consistently observed in the NLRP3 knock down, a result likely due to incomplete knockdown (Fig. 3.5C-F).

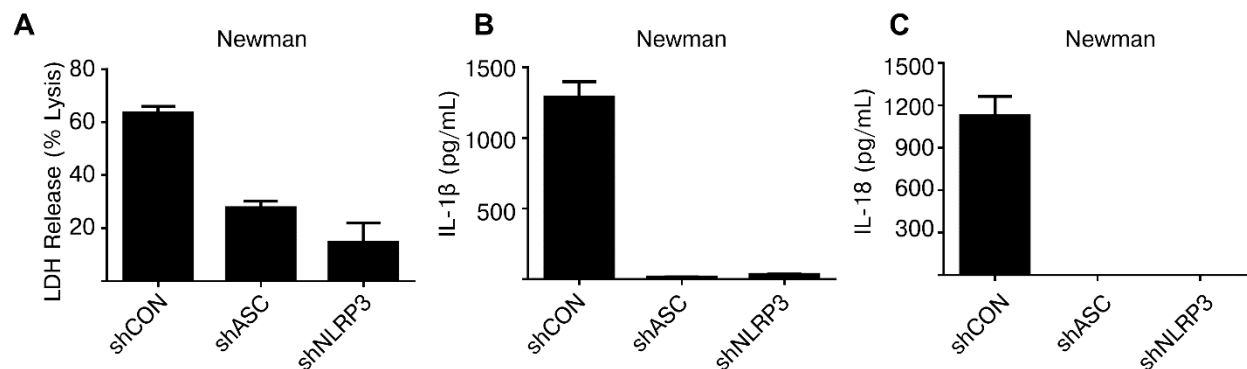
Activation of the NLRP3 inflammasome involves potassium efflux [42,43], thus we evaluated the role of potassium in LukAB-mediated inflammasome activation. THP1 cultures supplemented with potassium chloride, but not sodium chloride or choline chloride (Fig. 3.7), inhibited LukAB-induced NLRP3-inflammasome activation as assessed by FLICA-1 activation (Fig. 3.5G) and cell membrane PI permeability (Fig. 3.5H). Altogether, these results indicate that

LukAB activates the NLRP3 inflammasome to initiate Caspase 1-dependent cytokine release and necrotic cell death.

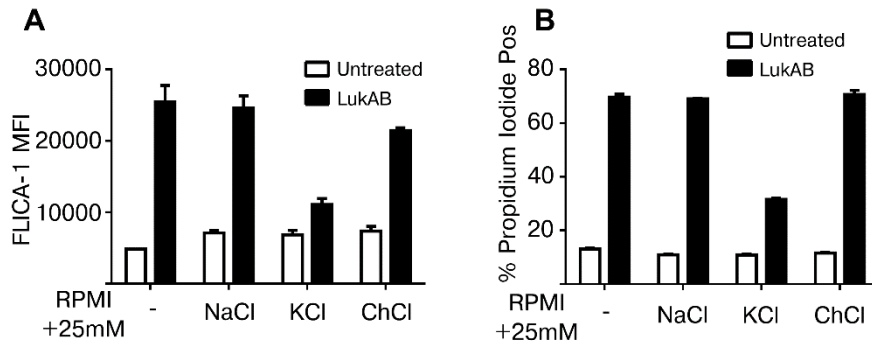


**Figure 3.5** LukAB activates the NLRP3 inflammasome leading to cell death and cytokine secretion. (A) THP1 cells were transduced with shRNA against NLRP3, ASC or a non-targeting control. Cells lysates were analyzed by immunoblot to confirm knockdown of NLRP3 and ASC. (B) Surface CD11b levels were evaluated by flow cytometry. (C) THP1 shRNA cells were incubated in the presence of FLICA-1 then intoxicated with LukAB (50 ng/mL) for 1 hour then

analyzed by flow cytometry. (D-E) THP1 shRNA cells were primed with 500 ng/mL LTA for 3 hours followed by intoxication with LukAB (50ng/mL) for one hour. Culture supernatants were collected and analyzed for secretion of the indicated cytokines. (F) THP1 shRNA cells were intoxicated with LukAB (50 ng/mL) for 1 hour and culture supernatants were collected for analysis of LDH release. (G) THP1 shRNA cells were incubated with propidium iodide then intoxicated with LukAB (50 ng/mL) for 1 hour then analyzed by flow cytometry. (H and I) THP1 cells were incubated in media supplemented with an additional 25 mM NaCl or KCl. Cells were incubated with FLICA-1 (H) or propidium iodide (I) then intoxicated with LukAB (50 ng/mL) for 1 hour and analyzed by flow cytometry. Bars represent the mean  $\pm$  standard error of the mean for at least two independent experiments, each performed in triplicate. Asterisks indicate significance at a  $p$ -value of  $\leq 0.05$  by Tukey's multiple comparisons post-test for 1-way or 2-way ANOVA, as appropriate.



**Figure 3.6 NLRP3 and ASC contribute to *S. aureus* culture filtrate-mediated cytokine secretion and THP1 cell death.** The indicated THP1 shRNA cells were intoxicated with culture filtrates (1% v/v) from *S. aureus* Newman. Culture supernatants were collected and analyzed for secretion of LDH release (A) and IL-1 $\beta$  (B) and IL-18 (C) secretion. Bars represent the mean  $\pm$  standard error of the mean for at least two independent experiments, each performed in triplicate.

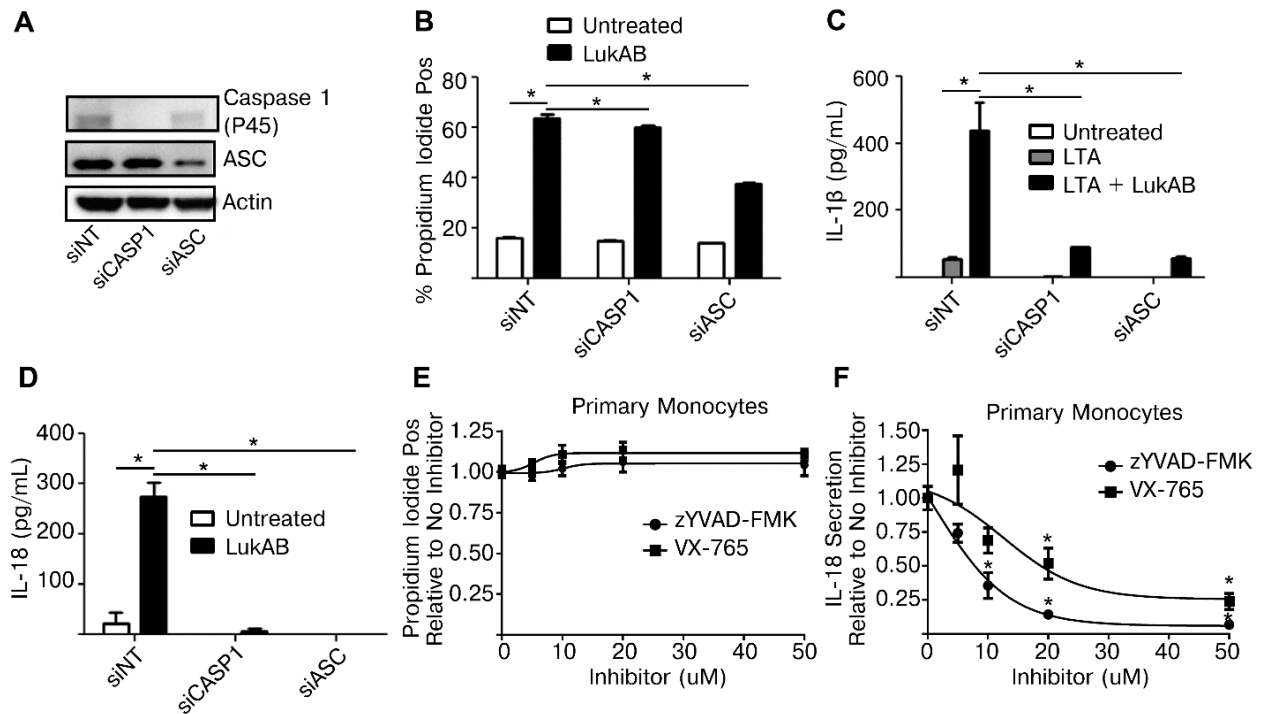


**Figure 3.7 Media supplementation with KCl inhibits LukAB-mediated FLICA-1 activation and cell death.** THP1 cells were incubated in media supplemented with an additional 25 mM NaCl, KCL or ChCl. Cells were incubated with FLICA-1 (A) or propidium iodide (B) then intoxicated with LukAB (50 ng/mL) for 1 hour and analyzed by flow cytometry. Bars represent the mean  $\pm$  standard error of the mean for at least two independent experiments, each performed in triplicate.

### 3.3.6 Caspase 1 is required for LukAB-induced cytokine secretion.

The dose-dependent cytotoxic activity exhibited by the different *S. aureus* pore-forming toxins towards primary CD14+ human monocytes (Fig. 3.2E) closely matched the results obtained by measuring FLICA-1 activation in these cells (Fig. 3.4G). These data raised the question as to whether Caspase 1 was required for cell death, a feature of the pyroptotic inflammatory cell death pathway. To test this, we transfected THP1 cells with small interfering RNA (siRNA) targeting Caspase 1 or ASC, as a positive control. After 72 hours, Caspase 1 levels were noticeably reduced as determined by immunoblot analyses (Fig. 3.8A). Loss of Caspase 1 had only a slight measurable impact on LukAB-induced PI staining (Fig. 3.8B) but nearly eliminated secretion of IL-1 $\alpha$  and IL-18 (Fig. 3.8C and 3.8D). Additionally, primary

CD14+ human monocytes were also treated with two pharmacologic inhibitors of Caspase 1: VX-765, a potent and selective small molecule inhibitor of Caspase 1 [44], and zYVAD-FMK, a peptide-based inhibitor of Caspase 1 [45]. Following pretreatment with a dose titration of both inhibitors, primary monocytes intoxicated with LukAB showed no difference in cell death (Fig. 3.8E), but both VX-765 and zYVAD-FMK suppressed IL-18 secretion (Fig. 3.8F).



**Figure 3.8 Genetic or pharmacologic disruption of Caspase 1 blocks LukAB-induced cytokine secretion but not cell death.** (A) THP1 cells were transfected with siRNA against Caspase 1, ASC or a non-targeting sequence. Cell lysates were collected and analyzed by immunoblot to confirm knockdown of pro-Caspase 1 and ASC. (B) THP1 siRNA cells were incubated with propidium iodide and intoxicated with 50 ng/mL LukAB for 1 hour then analyzed by flow cytometry. (C and D) THP1 siRNA cells were primed with LTA (500ng/mL) (C) or not (D) and intoxicated with LukAB (50ng/mL) and culture supernatants were analyzed for cytokine

release. (E and F) Primary CD14<sup>+</sup> human monocytes were incubated with the indicated concentration of z-YVAD-FMK or VX-765 for 30 minutes. Primary monocytes were incubated in the presence of propidium iodide (E) then intoxicated with LukAB (50ng/mL) and analyzed by flow cytometry. (F) THP1 cells were intoxicated with LukAB (50ng/mL) and culture supernatants were collected for cytokine analysis. Propidium iodide staining and IL-18 secretion are reported as a fraction of measurement in primary cells not treated with inhibitor. Bars represent the mean  $\pm$  standard error of the mean for at least two independent experiments, each performed in triplicate. Asterisks indicate significance at a *p*-value of  $\leq 0.05$  by Tukey's multiple comparisons post-test for 1-way or 2-way ANOVA, as appropriate.

### **3.3.7 LukAB promotes *S. aureus* escape from within human monocytes independent of NLRP3 or ASC, but dependent on CD11b.**

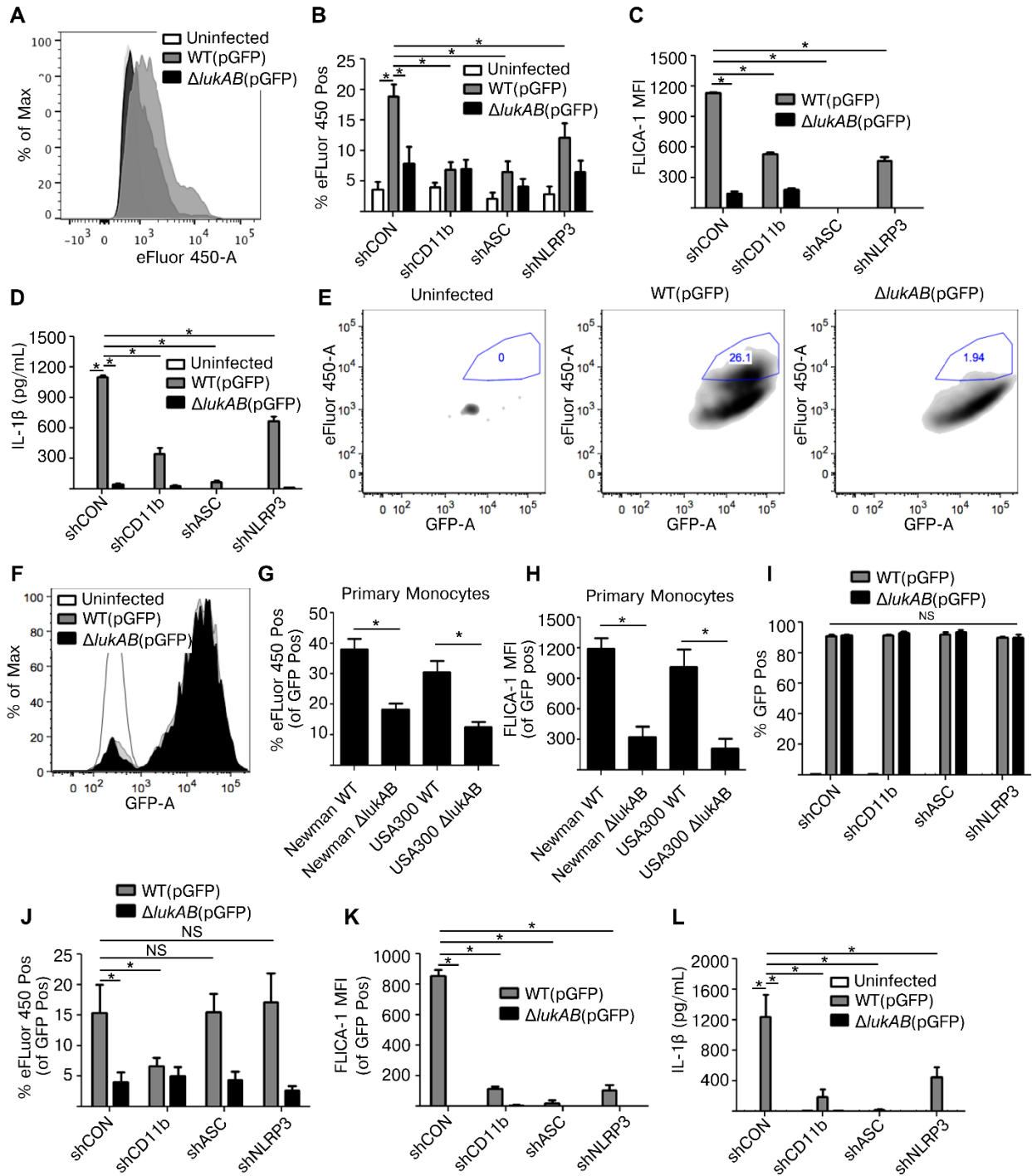
We next sought to evaluate the contribution of ASC and NLRP3 in infection models with live *S. aureus*. THP1 cells were first infected with *S. aureus* Newman and the isogenic *lukAB*-deficient mutant, both constitutively expressing GFP, in the absence of opsonization (i.e. extracellular infection). Following infection cells were stained with a fixable viability dye eFluor 450, a membrane damage and cell death marker, then analyzed by flow cytometry to determine the extent of THP1 cell death. In agreement with previous experiments using LDH and PI, THP1 cells infected with *S. aureus* lacking *lukAB* showed reduced eFluor 450 staining in comparison to THP1 cells infected with the wildtype strain (Fig. 3.9A). In shRNA-transduced cell lines, we observed LukAB-dependent cell death (Fig. 3.9B), FLICA-1 staining (Fig. 3.9C), and IL1- $\beta$  release (Fig. 3.9D) in the control shRNA cell line. Each of these LukAB-mediated effects was significantly reduced in the CD11b, ASC, and NLRP3 knockdown cell lines (Fig. 3.9B-D). The phenotypes observed in this extracellular infection model are consistent with our observations using purified toxins and culture filtrates.

LukAB targets CD11b to promote *S. aureus* escape from within human neutrophils [22,25]. We next sought to determine whether LukAB mediates similar escape from within human monocytes, and if NLRP3 or ASC contribute to this process. To evaluate cell death post-phagocytosis we modified our previously described method [25] to utilize GFP expressing strains and flow cytometric analyses. *S. aureus* Newman and the isogenic *lukAB*-deficient mutant were opsonized with human-serum and centrifuged in co-culture with THP1 cells to promote phagocytosis (Fig. 3.9E-9L). Following phagocytosis, lysostaphin, a potent enzyme that kills *S. aureus* by degrading the cell wall [46], was added in combination with anti-LukA neutralizing



antibodies to remove any remaining extracellular *S. aureus* and to block any residual extracellular LukAB [25]. We next quantified the proportion of cells that were both GFP positive and had maximal incorporation of eFluor® 450, as an indication of THP1 cells terminally injured by intracellular *S. aureus*. We observed that post-phagocytosis, LukAB contributed to *S. aureus*-mediated membrane damage and cell death (Fig. 3.9E). The distribution of GFP fluorescence in THP1 cells infected with WT *S. aureus* or the *lukAB*-deficient mutant were overlapping, indicating equal bacterial burden (Fig. 3.9F). Importantly, *ex vivo* infections of primary CD14<sup>+</sup> human monocytes with *S. aureus* Newman and a PVL<sup>+</sup> USA 300 strain revealed that LukAB is indeed responsible for cell death (Fig. 3.9G) and FLICA-1 activation (Fig. 3.9H) post-phagocytosis in human monocytes.

Using this model, we next evaluated the contributions of CD11b, ASC and NLRP3 to *S. aureus*-mediated THP1 cell death. Post-infection, approximately 90% of THP1 cells were GFP-positive across all cell lines and between *S. aureus* strains (Fig. 3.9I), indicating equivalent phagocytosis. We next quantified the proportion of THP1 cells killed by intracellular *S. aureus*. Remarkably, we observed that *S. aureus* induced THP1 cell death in a LukAB- and CD11b-dependent manner, but an ASC- and NLRP3-independent manner (Fig. 3.9J). Furthermore, we observed that while death was ASC- and NLRP3-independent, LukAB-mediated FLICA-1 activation (Fig. 3.9K) and IL-1 $\beta$  release (Fig. 3.9L) occurred through CD11b, ASC, and NLRP3. Thus, taken together these results demonstrate that LukAB, when secreted by *S. aureus* in the extracellular milieu, activates the host NLRP3 inflammasome to promote killing. However, when secreted from within monocytes, LukAB activates the NLRP3 inflammasome and induces cell death independently of NLRP3 or ASC.



**Figure 3.9** LukAB promotes the kill human monocytes by extracellular or phagocytized *S.*

*aureus*. (A) THP1 cells were infected with *S. aureus* Newman strains at an MOI of 10 for 120 min under non-phagocytosing conditions (extracellular infection; see methods), stained with the fixable viability dye eFluor 450, a membrane damage and cell death marker, and analyzed by

flow cytometry. Representative histogram is shown. (B-D) THP1 shRNA cells were infected *S. aureus* Newman, MOI 10 for 120 min, followed by flow cytometry analysis for maximal eFluor 450 staining (B) and FLICA-1 activation (C). (D) Culture supernatants were collected from intracellular infections and analyzed for IL-1 $\beta$  secretion. (E and F) THP1 cells were infected with GFP-expressing *S. aureus* Newman strains at an MOI of 10 for 45 min under phagocytosing conditions (intracellular infection; see methods). (E) THP1 cells were stained with the fixable viability dye eFluor 450. After infection, THP1 cells were analyzed by flow cytometry and GFP-positive THP1 cells were selected, indicative of *S. aureus* phagocytosis. Maximal eFluor 450 incorporation was gated among GFP-positive cells, indicative of THP1 death by intracellular *S. aureus*. Very few cells appear in the first plot corresponding to the background autofluorescent uninfected cells. Representative plots are shown. (F) Representative histogram of GFP fluorescence in *S. aureus* infected and uninfected THP1 cells is shown. (G-H) Purified primary CD14<sup>+</sup> human monocytes were infected with *S. aureus* Newman and USA300-BK18807 strains, along with respective isogenic *lukAB* mutants, at an MOI of 5 for 45 min then stained with eFluor 450 (G) or FLICA-1 (H) and analyzed by flow cytometry. Graphs reflect the fraction of cells that were GFP positive. (I-L) THP1 shRNA cells were infected with *S. aureus* Newman, MOI 10 for 45 min, and analyzed by flow cytometry for (I) GFP fluorescence indicating phagocytosis; (J) maximal eFluor 450 staining indicating cell death; (G) and FLICA-1 activation. (L) Culture supernatants were collected and analyzed for IL-1 $\beta$  secretion. Bars represent the mean  $\pm$  standard error of the mean for at least two independent experiments, each performed in triplicate. Experiments with primary cell experiments include at least three independent donors. Asterisks indicate significance at a *p*-value of  $\leq 0.05$  by Tukey's multiple comparisons post-test for 1-way or 2-way ANOVA, as appropriate.

### 3.4 Discussion

*Staphylococcus aureus* is a leading global cause of life threatening bacterial infections by virtue of its remarkable ability to invade practically all tissues in the human body, evade immune clearance, and subsequently proliferate. Its wide arsenal of virulence factors likely enables this broad tissue tropism and persistence. With regard to pore-forming toxins, many studies have focused on  $\alpha$ -toxin (Hla) and Panton-Valentine leukocidin (PVL). Herein, we demonstrate that LukAB, the most recently identified *S. aureus* toxin [20,21], is a predominant cytolytic leukotoxin responsible for inducing programmed inflammatory cell death in human monocytes. Both purified LukAB and PVL have exquisite potency in activating the NLRP3 inflammasome and inducing inflammatory cell death in primary CD14<sup>+</sup> human monocytes; a result consistent with high selectivity of these toxins toward human cells [3]. The dependency of *S. aureus* on any one toxin for survival and pathogenesis is likely tissue-, cell- and strain-specific. Unlike PVL, which is only encoded in about 15% of clinical isolates, LukAB is a part of the core genome of *S. aureus* and found in the vast majority of isolates (data publically available in NCBI sequenced *S. aureus* genomes). Our data points to an important role for LukAB in mediating virulence through interacting with CD11b on human monocytes. In fact, during *ex vivo* infection of primary CD14<sup>+</sup> human monocytes, LukAB seems to be the main factor produced by *S. aureus* responsible for targeting and killing monocytes. By activating the inflammasome in monocytes, LukAB is likely to induce uncontrolled production of pro-inflammatory cytokines such as IL-1 $\beta$  and IL-18, thus in theory worsening the outcome of *S. aureus* infections [23,47]. The presence of multiple toxins that activate a common host signaling pathway suggests there is selective advantage for *S. aureus* to activate the NLRP3-inflammasome, at least under some circumstances. Alternatively, if activation of the NLRP3-inflammasome is detrimental to *S. aureus* in its host, each of these NLRP3-inflammasome activating toxins must also provide

advantages to bacterial survival that are independent and outweigh the negative selective pressure inflammasome activation would exert on the organism.

By using both genetic and pharmacologic approaches, we demonstrate that LukAB-mediated IL-1 $\beta$  and IL-18 secretion depends on Caspase 1. Consistent with previous reports on Caspase 1 activity in programmed necrotic cell death [48], we have found that genetic depletion of Caspase 1 by siRNA in THP1 cells and pharmacologic inhibition with zYVAD-FMK and VX-765 in primary CD14<sup>+</sup> human monocytes does not alter LukAB-induced cell death. With these data we cannot rule out a role for Caspase 1 in programmed necrotic cell death, either as a longer half-life executioner that persists through the time course of siRNA experiments as suggested previously [48], or as a highly sensitive executioner resistant to total inhibition. These results suggest that LukAB-mediated death shares common features with a pyroptotic mechanism but cannot be strictly classified as pyroptosis without further experimentation in human immune cells completely lacking Caspase 1, which are currently lacking.

In contrast to other *S. aureus* bi-component pore-forming toxins, which are found as water-soluble monomers in solution, LukAB is isolated as a dimer in solution [24,49]. The recently solved crystal structure of LukAB identified three salt bridges, unique to LukAB, that are involved in dimer formation [49]. While it is tempting to speculate that the “dimeric” nature of LukAB accelerates LukAB-mediated Caspase 1 activation and monocyte death, we observed similar potencies with PVL, which is not isolated as a dimer in solution [24] nor targets CD11b. These results may then suggest that the signals downstream of receptor binding regulate the potencies of LukAB and PVL. Indeed, *S. aureus* pore-forming toxins LukAB, PVL and Hla all converge on a single signal transduction pathway in human phagocytes: activation of the

NLRP3-inflammasome, which ultimately leads to the generation of mature IL-1 $\beta$  and IL-18 and necrotic cell death.

Another novel feature of LukAB is the activity of this toxin from within human neutrophil phagosomes, which facilitates bacterial escape and promotes cell killing [20,22,25]. We show here that this characteristic is also evident in primary CD14+ human monocytes. Using THP1 cells as a model, we determined that LukAB-mediated cell death from within monocyte phagosomes is CD11b-dependent, but NLRP3 or ASC-independent manner. In contrast, the extracellular model of infection revealed that *S. aureus* LukAB killed monocytic cells in a CD11b-, ASC- and NLRP3-dependent manner. Previously, LLO from *Listeria monocytogenes* has been reported to activate S10H3 dephosphorylation in addition to activating the NLRP3-ASC inflammasome [50], and escape of *L. monocytogenes* into the cytoplasm has been shown to cause infrequent bacterial lysis leading to AIM2-ASC inflammasome activation [51]. This study, however, represents the first of its kind showing an inflammasome-dependent cell death initiated by extracellular toxin, but an ASC-containing-inflammasome-independent cell death initiated specifically by intracellular toxin. A possible explanation for these observations is that additional signaling cascades that lead to cell death are engaged by LukAB-CD11b recognition within the phagosome as compared to LukAB-CD11b recognition on the cell membrane. On the contrary, a recent report suggested that phagocytosis of *S. aureus* by murine phagocytes triggers early activation of the NLRP3 inflammasome and Caspase 1; an event that is required for bacterial clearance [52]. The apparent discrepancy between our results can be attributed to the inability of LukAB to lyse murine cells [22,23]. Similarly, we have previously shown that mice lacking NLRP3 are protected from *S. aureus* pneumonia [26]. The magnitude of the detrimental nature of NLRP3 activation in human *S. aureus* infection is likely to be underestimated due to reliance on

murine models which are resistant to LukAB-mediated lysis. LukAB binds to human CD11b with nearly 1000-fold higher affinity than to mouse CD11b [22], a feature that has led to dramatically underestimating the impact of this toxin in murine models [20]. Of note, a recent study revealed that children with invasive *S. aureus* disease exhibit a potent IgG response to LukAB [47], highlighting that LukAB is produced *in vivo* during human infection. These findings further support the notion that LukAB influences *S. aureus* pathophysiology in human infections. Ultimately, this study advances our understanding of how LukAB manipulates leukocytes, information critical to fully uncover how this virulence factor contributes to *S. aureus* infection.

## REFERENCES

1. Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, et al. (2007) Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA* 298: 1763-1771.
2. Alonzo F, 3rd, Torres VJ (2014) The bicomponent pore-forming leucocidins of *Staphylococcus aureus*. *Microbiol Mol Biol Rev* 78: 199-230.
3. DuMont AL, Torres VJ (2014) Cell targeting by the *Staphylococcus aureus* pore-forming toxins: it's not just about lipids. *Trends Microbiol* 22: 21-27.
4. Bramley AJ, Patel AH, O'Reilly M, Foster R, Foster TJ (1989) Roles of alpha-toxin and beta-toxin in virulence of *Staphylococcus aureus* for the mouse mammary gland. *Infect Immun* 57: 2489-2494.
5. Bubeck Wardenburg J, Bae T, Otto M, Deleo FR, Schneewind O (2007) Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in *Staphylococcus aureus* pneumonia. *Nat Med* 13: 1405-1406.
6. Bubeck Wardenburg J, Patel RJ, Schneewind O (2007) Surface proteins and exotoxins are required for the pathogenesis of *Staphylococcus aureus* pneumonia. *Infect Immun* 75: 1040-1044.
7. Girgis DO, Sloop GD, Reed JM, O'Callaghan RJ (2005) Effects of toxin production in a murine model of *Staphylococcus aureus* keratitis. *Invest Ophthalmol Vis Sci* 46: 2064-2070.
8. Kennedy AD, Bubeck Wardenburg J, Gardner DJ, Long D, Whitney AR, et al. (2010) Targeting of alpha-hemolysin by active or passive immunization decreases severity of USA300 skin infection in a mouse model. *J Infect Dis* 202: 1050-1058.
9. Rauch S, DeDent AC, Kim HK, Bubeck Wardenburg J, Missiakas DM, et al. (2012) Abscess formation and alpha-hemolysin induced toxicity in a mouse model of *Staphylococcus aureus* peritoneal infection. *Infect Immun* 80: 3721-3732.
10. Bubeck Wardenburg J, Palazzolo-Ballance AM, Otto M, Schneewind O, DeLeo FR (2008) Panton-Valentine leukocidin is not a virulence determinant in murine models of community-associated methicillin-resistant *Staphylococcus aureus* disease. *J Infect Dis* 198: 1166-1170.
11. Labandeira-Rey M, Couzon F, Boisset S, Brown EL, Bes M, et al. (2007) *Staphylococcus aureus* Panton-Valentine leukocidin causes necrotizing pneumonia. *Science* 315: 1130-1133.
12. Tseng CW, Kyme P, Low J, Rocha MA, Alsabeh R, et al. (2009) *Staphylococcus aureus* Panton-Valentine leukocidin contributes to inflammation and muscle tissue injury. *PLoS One* 4: e6387.



13. Zaidi T, Zaidi T, Yoong P, Pier GB (2013) Staphylococcus aureus corneal infections: effect of the Panton-Valentine leukocidin (PVL) and antibody to PVL on virulence and pathology. *Invest Ophthalmol Vis Sci* 54: 4430-4438.
14. Cremieux AC, Dumitrescu O, Lina G, Vallee C, Cote JF, et al. (2009) Panton-valentine leukocidin enhances the severity of community-associated methicillin-resistant Staphylococcus aureus rabbit osteomyelitis. *PLoS One* 4: e7204.
15. Lipinska U, Hermans K, Meulemans L, Dumitrescu O, Badiou C, et al. (2011) Panton-Valentine leukocidin does play a role in the early stage of Staphylococcus aureus skin infections: a rabbit model. *PLoS One* 6: e22864.
16. Diep BA, Palazzolo-Ballance AM, Tattevin P, Basuino L, Braughton KR, et al. (2008) Contribution of Panton-Valentine leukocidin in community-associated methicillin-resistant Staphylococcus aureus pathogenesis. *PLoS One* 3: e3198.
17. Diep BA, Chan L, Tattevin P, Kajikawa O, Martin TR, et al. (2010) Polymorphonuclear leukocytes mediate Staphylococcus aureus Panton-Valentine leukocidin-induced lung inflammation and injury. *Proc Natl Acad Sci U S A* 107: 5587-5592.
18. Loffler B, Hussain M, Grundmeier M, Bruck M, Holzinger D, et al. (2010) Staphylococcus aureus panton-valentine leukocidin is a very potent cytotoxic factor for human neutrophils. *PLoS Pathog* 6: e1000715.
19. Spaan AN, Henry T, van Rooijen WJ, Perret M, Badiou C, et al. (2013) The staphylococcal toxin Panton-Valentine Leukocidin targets human C5a receptors. *Cell Host Microbe* 13: 584-594.
20. Dumont AL, Nygaard TK, Watkins RL, Smith A, Kozhaya L, et al. (2011) Characterization of a new cytotoxin that contributes to Staphylococcus aureus pathogenesis. *Mol Microbiol* 79: 814-825.
21. Ventura CL, Malachowa N, Hammer CH, Nardone GA, Robinson MA, et al. (2010) Identification of a novel Staphylococcus aureus two-component leukotoxin using cell surface proteomics. *PLoS One* 5: e11634.
22. DuMont AL, Yoong P, Day CJ, Alonzo F, 3rd, McDonald WH, et al. (2013) Staphylococcus aureus LukAB cytotoxin kills human neutrophils by targeting the CD11b subunit of the integrin Mac-1. *Proc Natl Acad Sci U S A* 110: 10794-10799.
23. Malachowa N, Kobayashi SD, Braughton KR, Whitney AR, Parnell MJ, et al. (2012) Staphylococcus aureus leukotoxin GH promotes inflammation. *J Infect Dis* 206: 1185-1193.
24. DuMont AL, Yoong P, Liu X, Day CJ, Chumbler NM, et al. (2014) Identification of a crucial residue required for Staphylococcus aureus LukAB cytotoxicity and receptor recognition. *Infect Immun* 82: 1268-1276.

25. DuMont AL, Yoong P, Surewaard BG, Benson MA, Nijland R, et al. (2013) *Staphylococcus aureus* elaborates leukocidin AB to mediate escape from within human neutrophils. *Infect Immun* 81: 1830-1841.
26. Kebaier C, Chamberland RR, Allen IC, Gao X, Broglie PM, et al. (2012) *Staphylococcus aureus* alpha-hemolysin mediates virulence in a murine model of severe pneumonia through activation of the NLRP3 inflammasome. *J Infect Dis* 205: 807-817.
27. Davis B, Wen H, Ting J (2011) The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annual review of immunology* 29: 707-735.
28. Craven R, Gao X, Allen I, Gris D, Bubeck Wardenburg J, et al. (2009) *Staphylococcus aureus* alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells. *PLoS one* 4.
29. Holzinger D, Geldon L, Mysore V, Nippe N, Taxman D, et al. (2012) *Staphylococcus aureus* Panton-Valentine leukocidin induces an inflammatory response in human phagocytes via the NLRP3 inflammasome. *Journal of leukocyte biology* 92: 1069-1081.
30. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, et al. (2006) Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440: 228-232.
31. Hanamsagar R, Torres V, Kielian T (2011) Inflammasome activation and IL-1beta/IL-18 processing are influenced by distinct pathways in microglia. *J Neurochem* 119: 736-748.
32. Cho JS, Guo Y, Ramos RI, Hebroni F, Plaisier SB, et al. (2012) Neutrophil-derived IL-1beta is sufficient for abscess formation in immunity against *Staphylococcus aureus* in mice. *PLoS Pathog* 8: e1003047.
33. Confalonieri M, Annane D, Antonaglia C, Santagiuliana M, Borriello EM, et al. (2013) Is Prolonged Low-Dose Glucocorticoid Treatment Beneficial in Community-Acquired Pneumonia? *Curr Infect Dis Rep*.
34. Willingham SB, Bergstralh DT, O'Connor W, Morrison AC, Taxman DJ, et al. (2007) Microbial pathogen-induced necrotic cell death mediated by the inflammasome components CIAS1/cryopyrin/NLRP3 and ASC. *Cell Host Microbe* 2: 147-159.
35. Duthie ES, Lorenz LL (1952) Staphylococcal coagulase; mode of action and antigenicity. *J Gen Microbiol* 6: 95-107.
36. Munoz-Planillo R, Franchi L, Miller LS, Nunez G (2009) A critical role for hemolysins and bacterial lipoproteins in *Staphylococcus aureus*-induced activation of the Nlrp3 inflammasome. *J Immunol* 183: 3942-3948.
37. Duprez L, Wirawan E, Vanden Berghe T, Vandenabeele P (2009) Major cell death pathways at a glance. *Microbes Infect* 11: 1050-1062.
38. Edinger AL, Thompson CB (2004) Death by design: apoptosis, necrosis and autophagy. *Curr Opin Cell Biol* 16: 663-669.

39. Bergsbaken T, Fink SL, Cookson BT (2009) Pyroptosis: host cell death and inflammation. *Nat Rev Microbiol* 7: 99-109.
40. Scaffidi P, Misteli T, Bianchi ME (2002) Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 418: 191-195.
41. Bedner E, Smolewski P, Amstad P, Darzynkiewicz Z (2000) Activation of caspases measured in situ by binding of fluorochrome-labeled inhibitors of caspases (FLICA): correlation with DNA fragmentation. *Exp Cell Res* 259: 308-313.
42. Munoz-Planillo R, Kuffa P, Martinez-Colon G, Smith BL, Rajendiran TM, et al. (2013) K(+) efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity* 38: 1142-1153.
43. Warny M, Kelly CP (1999) Monocytic cell necrosis is mediated by potassium depletion and caspase-like proteases. *Am J Physiol* 276: C717-724.
44. Wannamaker W, Davies R, Namchuk M, Pollard J, Ford P, et al. (2007) (S)-1-((S)-2-[[1-(4-amino-3-chloro-phenyl)-methanoyl]-amino]-3,3-dimethyl-butanoyl)-pyrrolidine-2-carboxylic acid ((2R,3S)-2-ethoxy-5-oxo-tetrahydro-furan-3-yl)-amide (VX-765), an orally available selective interleukin (IL)-converting enzyme/caspase-1 inhibitor, exhibits potent anti-inflammatory activities by inhibiting the release of IL-1beta and IL-18. *J Pharmacol Exp Ther* 321: 509-516.
45. Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, et al. (1992) A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature* 356: 768-774.
46. Schindler CA, Schuhardt VT (1964) Lysostaphin: A New Bacteriolytic Agent for the Staphylococcus. *Proc Natl Acad Sci U S A* 51: 414-421.
47. Thomsen IP, Dumont AL, James DB, Yoong P, Saville BR, et al. (2014) Children with invasive Staphylococcus aureus disease exhibit a potentially neutralizing antibody response to the cytotoxin LukAB. *Infect Immun* 82: 1234-1242.
48. Motani K, Kushiyaama H, Imamura R, Kinoshita T, Nishiuchi T, et al. (2011) Caspase-1 protein induces apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC)-mediated necrosis independently of its catalytic activity. *J Biol Chem* 286: 33963-33972.
49. Badarau A, Rouha H, Malafa S, Logan DT, Hakansson M, et al. (2015) Structure-function analysis of heterodimer formation, oligomerization, and receptor binding of the Staphylococcus aureus bi-component toxin LukGH. *J Biol Chem* 290: 142-156.
50. Hamon MA, Cossart P (2011) K<sup>+</sup> efflux is required for histone H3 dephosphorylation by Listeria monocytogenes listeriolysin O and other pore-forming toxins. *Infect Immun* 79: 2839-2846.

51. Sauer JD, Witte CE, Zemansky J, Hanson B, Lauer P, et al. (2010) *Listeria monocytogenes* triggers AIM2-mediated pyroptosis upon infrequent bacteriolysis in the macrophage cytosol. *Cell Host Microbe* 7: 412-419.
52. Sokolovska A, Becker CE, Ip WK, Rathinam VA, Brudner M, et al. (2013) Activation of caspase-1 by the NLRP3 inflammasome regulates the NADPH oxidase NOX2 to control phagosome function. *Nat Immunol* 14: 543-553.
53. Reyes-Robles T, Alonzo F, 3rd, Kozhaya L, Lacy DB, Unutmaz D, et al. (2013) *Staphylococcus aureus* leukotoxin ED targets the chemokine receptors CXCR1 and CXCR2 to kill leukocytes and promote infection. *Cell Host Microbe* 14: 453-459.
54. Benson MA, Lilo S, Nygaard T, Voyich JM, Torres VJ (2012) Rot and SaeRS cooperate to activate expression of the staphylococcal superantigen-like exoproteins. *J Bacteriol* 194: 4355-4365.
55. Gillaspay AF, Hickmon SG, Skinner RA, Thomas JR, Nelson CL, et al. (1995) Role of the accessory gene regulator (*agr*) in pathogenesis of staphylococcal osteomyelitis. *Infect Immun* 63: 3373-3380.
56. Centers for Disease C, Prevention (1999) Four pediatric deaths from community-acquired methicillin-resistant *Staphylococcus aureus* - Minnesota and North Dakota, 1997-1999. *MMWR Morb Mortal Wkly Rep* 48: 707-710.
57. Supersac G, Piemont Y, Kubina M, Prevost G, Foster TJ (1998) Assessment of the role of gamma-toxin in experimental endophthalmitis using a *hlg*-deficient mutant of *Staphylococcus aureus*. *Microb Pathog* 24: 241-251.
58. Benson MA, Ohneck EA, Ryan C, Alonzo F, 3rd, Smith H, et al. (2014) Evolution of hypervirulence by a MRSA clone through acquisition of a transposable element. *Mol Microbiol* 93: 664-681.
59. Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, et al. (2006) Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. *Lancet* 367: 731-739.

## Chapter 4. Novel approaches to studying kinase-dependent regulation of the NLRP3 inflammasome

### 4.1 Introduction

The NLRP3 inflammasome is a critical immune signaling pathway that promotes secretion of interleukin-1B (IL-1 $\beta$ ) and interleukin-18 (IL-18), and causes necrotic cell death. Dysregulation and hyperactivation of the NLRP3 inflammasome can result in hyperinflammatory conditions such as Cryopyrin-associated periodic syndromes or exacerbate infection pathology, as in *S. aureus* pneumonia [1].

Dozens of pathogen- and danger-associated molecules activate the NLRP3 inflammasome. However, a comprehensive model for inflammation activation and regulation has yet to emerge. Post-translational modifications, including phosphorylation, are thought to play a major role in regulating the NLRP3 inflammasome.

Numerous different kinases are required for NLRP3 inflammasome activation under a variety of inciting conditions. But little is known about how these kinases control NLRP3 inflammasome activity. As discussed previously, kinases that regulate the NLRP3 inflammasome include Syk [2-8], Jnk/MAPK8/MAPK9 [7,9], Lyn [8], NEK7 [10,11], PKR/EIF2AK2 [12], BTK [13], RIPK1/RIPK3/MLKL [14,15], RIPK2 [16], TAK1/MAP3K7 [9], IKKi/CHUK and IKKa/IKBKE [17], IRAK1 [18,19], IRAK4 [19] and DAPK [20]. There is also evidence that NLRP3 inflammasome regulation is cell type specific. For example, loss of Syk has no effect on

nigericin-induced NLRP3 activation in murine dendritic cells [2] but diminishes nigericin-induced NLRP3 activation in murine bone marrow derived macrophages [7].

Multiple putative phosphorylation sites on NLRP3, ASC and pro-Caspase 1 exist. Phosphorylation of ASC has been demonstrated in three separate manuscripts. Both Syk and Jnk are required for ASC phosphorylation [7] and Syk phosphorylates ASC at Y146 and Y187 [6]. IKKa/IKBKE interacts directly with ASC in a manner that requires ASC S16 and S193 for phosphorylation, though it isn't clear if IKKa also phosphorylates these sites. IKKi interacts with ASC as well and phosphorylates ASC at S58 [17]. ASC phosphorylation appears to be critical for ASC oligomerization, as mutation of phosphorylation sites to alanine diminishes its ability to form oligomers in a cell-free system and deletion of Syk blocks Caspase 1 activity in cells [6,7,17].

The mechanisms by which the other identified kinases regulate NLRP3 inflammasome activity are less clear. By in large, their role has been established by showing that genetic disruption or pharmacologic inhibition increases or decreases IL-1 $\beta$  secretion or cell death in response to NLRP3-dependent stimuli.

Although direct phosphorylation of NLRP3 has been observed at T233, S387, S436 and S728 in phospho-proteome studies, the role of these phosphorylated residues in NLRP3 in unstimulated cells is not yet known. Phosphorylation of NLRC4 (a NLR-family protein that bypasses ASC to directly activate Caspase 1) by PKC $\delta$  at S533 is required for activation of the NLRC4 inflammasome in response to *Salmonella enterica* serovar Typhimurium [21]. A second study has found that PKC $\delta$  was dispensable for NLRC4-mediated Caspase 1 activation in response to *Shigella* infection [22], suggesting that it may not be a universal requirement.

LukAB is a recently identified PFT produced by *S. aureus* that kills human phagocytic cells [23,24] by binding to CD11b [25]. LukAB is one of the most potent toxins of the six *S. aureus* PFTs in activating the NLRP3 inflammasome and killing primary human monocytes [26]. When *S. aureus* is exposed to human polymorphonuclear cells (PMNs), transcription at the LukAB promoter increases dramatically, far more than any other toxin promoter. LukAB can also be used by *S. aureus* to escape phagocytosis by PMNs [27].

To determine whether kinase activity and NLRP3 phosphorylation are involved in NLRP3 activation in response to LukAB and possibly other *S. aureus* PFTs, we initiated a multi-pronged study to characterize changes in kinases and phosphorylation in response to *S. aureus* LukAB. First, borrowing from cancer kinome research [28], we used multiplexed kinase inhibitor bead chromatography and mass spectrometry (MIB/MS) to quantitate changes in activity in the THP1 cell kinome. Using this method, activated kinases are affinity purified from cell lysates over a column of beads conjugated to promiscuous kinase inhibitors that specifically bind activated kinases. After washing several times, purified kinases are eluted, trypsin digested and labeled with isobaric tags for mass spectrometry quantitation.

Guided by a carefully performed time course of LukAB-induced NLRP3 inflammasome activation, we compared THP1 cells at different stages of NLRP3 signaling to untreated cells in attempt to capture the full picture of kinase activity during NLRP3 inflammasome activation. We combined three experimental replicates through inverse variance weighting and found ten significant changes across the three time points involving a total of eight kinases.

Second, we investigated the role of DAPK activity in LukAB-induced NLRP3 inflammasome activation. Previous research has demonstrated a requirement for DAPK in NLRP3 inflammasome activation in response to monosodium urate, nigericin, alum and ATP.

When DAPK is overexpressed in THP1 cells, NLRP3 inflammasome activation leads to increased IL-1 $\beta$  secretion and knockdown of DAPK by siRNA leads to decreased IL-1 $\beta$  secretion [20]. Biochemical assays with recombinant NLRP3 and DAPK in our lab have shown that NLRP3 and DAPK interact and DAPK can phosphorylate NLRP3 on S198 and S201. In this second part, we used pharmacologic inhibition to show that DAPK is required for LukAB-induced IL-1 $\beta$  and IL-18 secretion and cell death.

Third, we investigated the importance of S198 and S201 phosphorylation in NLRP3 inflammasome activation by using a novel transfection system to overexpress NLRP3 mutants in THP1 cells. We show that NLRP3 S201D, a phosphomimetic mutation, leads to spontaneous IL-18 secretion whereas NLRP3 S198A and S198D did not.

Overall, this series of studies demonstrates the utility of these novel approaches to studying NLRP3 inflammasome regulation and provides a strong foundation for further research on kinase-dependent regulation in this system.

## **4.2 Materials and Methods**

### **4.2.1 Mammalian cell lines**

THP1 cells (ATCC TIB-202) were maintained in Roswell Park Memorial Institute medium 1640 (RPMI) medium (Cellgro) at 37°C with 5% CO<sub>2</sub>, where culture medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (0.1 mg/ml). Transduced THP1 cells were maintained in 1.3  $\mu$ g/ml puromycin. All experiments utilized cells that 2 days prior reached a density of approximately 0.8 x 10<sup>6</sup> cells/ml before being split 1:2 with fresh media. Prior to infections or intoxications, the desired volume of cells was removed, centrifuged and suspended in fresh RPMI media and equilibrated for 1 hr at



37°C with 5% CO<sub>2</sub>. Unless specified, experiments were carried out in either 96-well or 48-well flat-bottom tissue culture treated plates. All experiments were conducted within approximately 1 month of thawing frozen cell stocks.

#### **4.2.2 Purifying LukAB from *S. aureus***

A construct to co-purify recombinant LukAB from *S. aureus* (*pOS-1-P<sub>lukAB</sub>-sslukA-6His-lukA-lukB*) was generated as previously described [29] and transformed into Newman  $\Delta\Delta\Delta\Delta$  [30] to facilitate purification. LukAB was purified from *S. aureus* as previously described [29]. Briefly, strains were grown in TSB with 10  $\mu\text{g/ml}$  chloramphenicol for 5 h at 37°C, 180 rpm, to an optical density at 600 nm (OD<sub>600</sub>) of approximately 1.5 (which represents  $1 \times 10^9$  CFU/ml). The bacteria were then pelleted, and the supernatant was collected and filtered. Nickel-nitrilotriacetic acid (NTA) resin (Qiagen) was incubated with culture supernatant, washed, and eluted with 500 mM imidazole. The protein was dialyzed in  $1\times$  Tris-buffered saline (TBS) plus 10% glycerol at 4°C overnight and then stored at  $-80^\circ\text{C}$ .

#### **4.2.3 Evaluating cell death by propidium iodide (PI) staining**

THP1 cells, seeded at  $1 \times 10^6$  cells/mL in 300  $\mu\text{L}$ /well in a 48-well tissue culture plate, were intoxicated with LukAB in the presence of propidium iodide (2.5  $\mu\text{g/mL}$ ) for 60 minutes. Cells were fixed with a combination formaldehyde and methanol solution supplied by ImmunoChemistry Technologies. Fluorescence was measured by flow cytometry using an Accuri C6 flow cytometer (BD Biosciences).

#### **4.2.4 Measuring cytokine secretion by AlphaLISA**

THP1 cells, seeded at  $1 \times 10^6$  cells/mL in 300uL/well in a 48-well tissue culture plate, were intoxicated with LukAB. Culture supernatants were collected and analyzed by AlphaLISA for IL-1 $\beta$ , IL-18 and TNF- $\alpha$  according to the manufacturer's protocol for short incubation (Perkin Elmer) with reduced volumes. Briefly, 1uL of each sample or standard was added to a separate well in a 384-well plate with 4uL of acceptor beads and cytokine antibody cocktail. After a 1-hour incubation at room temperature shielded from light, 5uL of streptavidin-conjugated donor beads was added to each well for 30 minutes. Luminescence was measured on an EnSpire Multimode Plate Reader (Perkin Elmer).

#### **4.2.5 Measuring Caspase 1 Activation with FLICA**

THP1 cells, seeded at  $1 \times 10^6$  cells/mL in 300uL/well in a 48-well tissue culture plate, were intoxicated with LukAB in the presence of the Caspase 1 inhibiting peptide FLICA-FMK bound to Alexa Fluor 660 (FLICA-1) (1:100 dilution) for 60 minutes. Cells were washed once with 1x PBS and resuspended in 1x PBS plus 8% fixative solution supplied by ImmunoChemistry Technologies. Fluorescence was measured by flow cytometry using an Accuri C6 flow cytometer (BD Biosciences).

#### **4.2.6 Multiplex Inhibitor Bead Chromatography**

THP1 cells were collected and concentrated to  $1 \times 10^7$  cells/mL in approximately 60mL of media per sample. Each sample was treated with 200ng/mL LukAB for 10, 20 or 40 minutes, or left untreated. After experimental treatments, cells are harvested by lysing in detergent-containing phosphorylation-preserving buffer. Total protein is quantified using a Bradford assay.

Roughly 10mg of protein lysates per sample are equilibrated to 1M NaCl and passed through columns of broad spectrum inhibitor-conjugated beads, including Bisinodylmaleimide-X, SB203580, Lapatinib, Dasatinib, Purvalanol B, VI16832 and PP58, to enrich for protein kinases. These inhibitors preferentially bind to the active conformation of kinases which allows us to correlated captured kinase abundance with kinase activity. Beads are then washed with high- and low-salt buffers and 0.1% SDS-containing solution before elution by boiling in 0.5% SDS-containing solution.

#### **4.2.7 iTRAQ labeling**

Proteins eluted from the MIB column are then purified using chloroform/methanol extraction and resuspended in 50mM HEPES (pH 8) for iTRAQ labeling. Samples are trypsin digested overnight at 37°C with sequencing grade modified trypsin. iTRAQ labeling of digested peptides is carried out using iTRAQ 4-plex reagent for 2 hours at room temperature in the dark. Peptides are fractionated with Mini SCX Spin Columns and cleaned with PepClean C18 Spin Columns before separation by a Tempo LC MALDI Spotting System.

#### **4.2.8 Mass spectrometry**

MS data is acquired with a Q Exactive MS/MS and analyzed by ProteinPilot Software version 3.0 with a UniProtKB/Swiss-Prot database. Proteins are accepted when  $\geq 1$  unique peptide is identified at  $\geq 99\%$  confidence. Peptide quantitation by ProteinPilot is performed using the Pro Group Algorithm with appropriate normalization to correct for various technical biases including batch effect, background correction and unequal mixing of the different labeled samples. Standard output for the software includes the protein ratio of test to control samples, the

number of peptides used in computing the ratio, and the two-sided p-value, which help assess whether the change in protein detection is statistically significant.

#### **4.2.9 Exploratory Statistical Analysis of MIB/MS Results**

As an initial exploratory approach, we used a weighting scheme based on the inverse variance of calculated ratios across replicates. Statistically significant kinase identifications are declared when the 95% confidence interval of inverse variance weighted ratios does not cross the point of equivalence, 1. In order to improve robustness, significant hits were only declared if no replicates were weighted less than 20%. Correction for multiple hypotheses was not yet performed.

#### **4.2.10 DAPK inhibition experiments**

THP1 cells were cultured as described above for propidium iodide and AlphaLISA assays. Cells were treated with 500ng/mL LTA for 2.5 hours then the DAPK inhibitor (Calbiochem, CAS 315694-89-4) was added at the appropriate concentration per experiment for 30 minutes. Then, LukAB was added at 50ng/mL for 1 hour. Cells or culture supernatants were collected and analyzed as described above.

#### **4.2.11 Site directed mutagenesis of NLRP3**

NLRP3 mutants were generated from the wildtype sequencing using the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer's protocol. Briefly, primers containing the mutated sequence were used in a PCR reaction to mutate and amplify the NLRP3 containing plasmid. DpnI, a restriction enzyme that requires

DNA methylation, was used to digest the template plasmid. Beta-mercaptoethanol was added to XL10-Gold Ultracompetent *E. coli* cells that were then transformed with 1.5uL of DpnI-treated DNA from the QuikChange reaction. *E. coli* was then plated overnight. Colonies were isolated the subsequent day and analyzed by restriction digest to identify positive transformants. Sanger sequencing was used to verify mutagenesis.

#### **4.2.12 Cloning of NLRP3 mutants into T7 expression vector**

Primers were made to introduce new restriction digest sites at the ends of the mutated NLRP3 genes. PCR was performed, followed by restriction digest of the NLRP3 PCR product and the T7 promoter-containing vector pUNCH938. The vector was phosphatase treated with calf intestine phosphatase and the mutant gene was ligated with the vector. Max Efficiency DH5 Competent Cells were transformed with the ligation reaction and plated on selecting media overnight. Colonies were isolated the subsequent day and analyzed by restriction digest to identify clones containing NLRP3. Sanger sequencing was used to verify the sequence of the T7 promoter and NLRP3.

#### **4.2.13 *In vitro* transcription of capped and poly-adenylated mRNA**

The mMESSAGING mMACHINE T7 Ultra Kit (Life Technologies) was used for *in vitro* transcription of capped and poly-adenylated mRNA according to the manufacturer's protocol. Briefly, NLRP3-containing plasmids were linearized at the 3' end of the NLRP3 gene, column purified and quantified with UV spectroscopy. Transcription reactions were assembled at room temperature and incubated for 2 hours at 37°C. After transcription, reactions were spiked with 1uL TURBO DNase for 15 minutes at 37°C. Poly-adenylation was then carried out for 30 minutes at

37°C and poly-adenylated mRNA was purified using lithium chloride precipitation. mRNA was quantified using UV spectroscopy.

#### **4.2.14 Transfection of mRNA into THP1 cells**

Transfection of mRNA into THP1 cells was carried out using TransIT-mRNA Transfection Kit (Mirus). THP1 cells were resuspended in fresh media at a density of  $5 \times 10^5$  cells/mL and 500uL of cells were added to a 24-well tissue culture plate. In a separate tube, 50uL OptiMEM, an appropriate amount of mRNA, and 1uL of both the boost and transfection reagent per sample were combined. After a 3-minute incubation, 50uL was added over the top of the THP1 cells with effort to disperse the mixture as much as possible. Transfection of GFP mRNA was used as a control and to track protein expression over time.

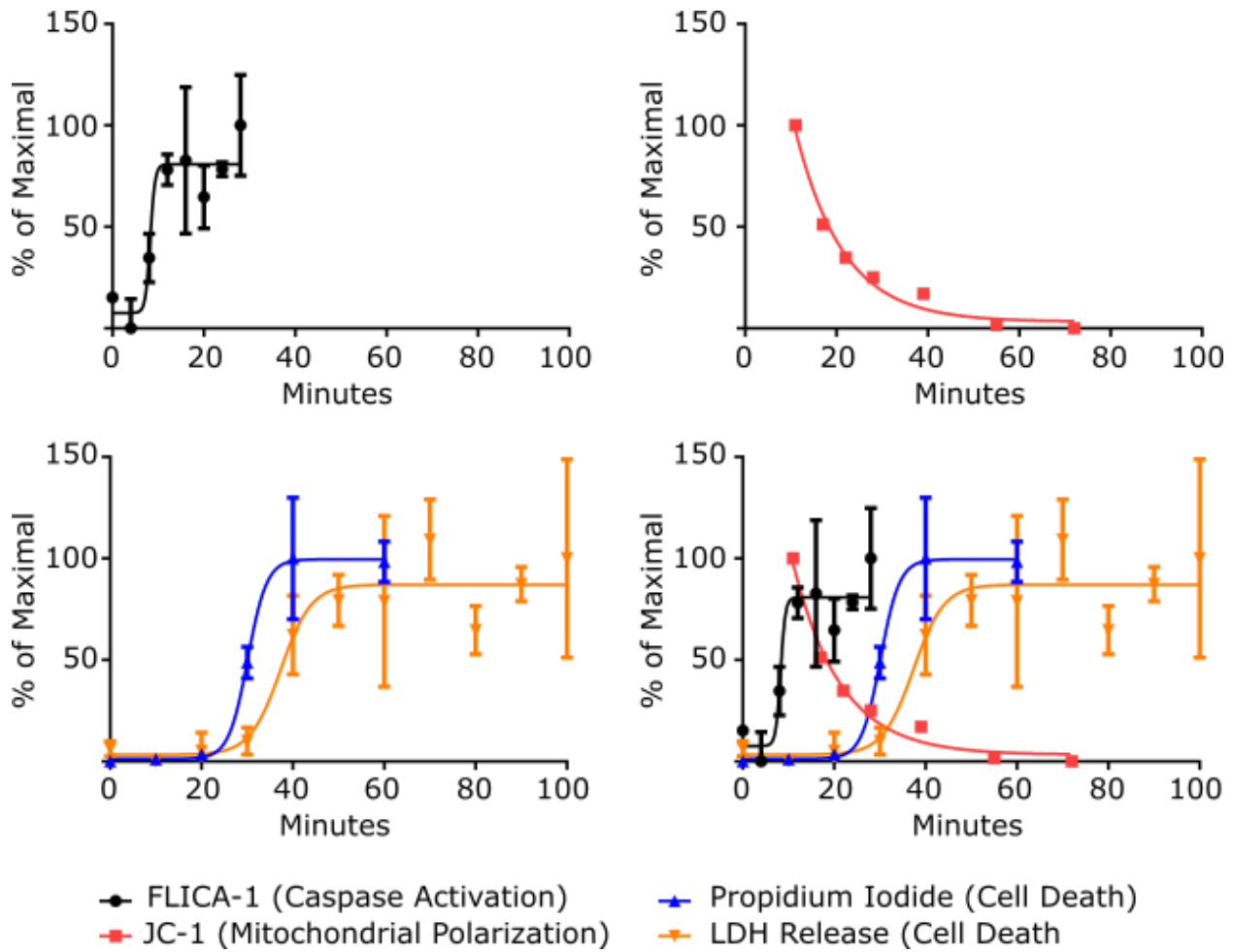
### **4.3 Results**

#### **4.3.1 LukAB causes rapid NLRP3 inflammasome activation and cell death**

In order to better understand the kinetics of LukAB-mediated NLRP3 inflammasome activation and cell death, we performed a careful time course analysis. THP1 cells were incubated separately with fluorescent probes for Caspase 1 activation (FLICA-1), mitochondrial polarization (JC-1) and plasma membrane integrity (PI). Then 50ng/mL LukAB was added and separate samples were analyzed by flow cytometry at time points ranging from 3 minutes to 100 minutes depending on the experiment. Additionally, culture supernatants were collected to analyze secretion of lactate dehydrogenase (LDH), a second marker of cell death. It is important to note that no priming agent was added to these cells.

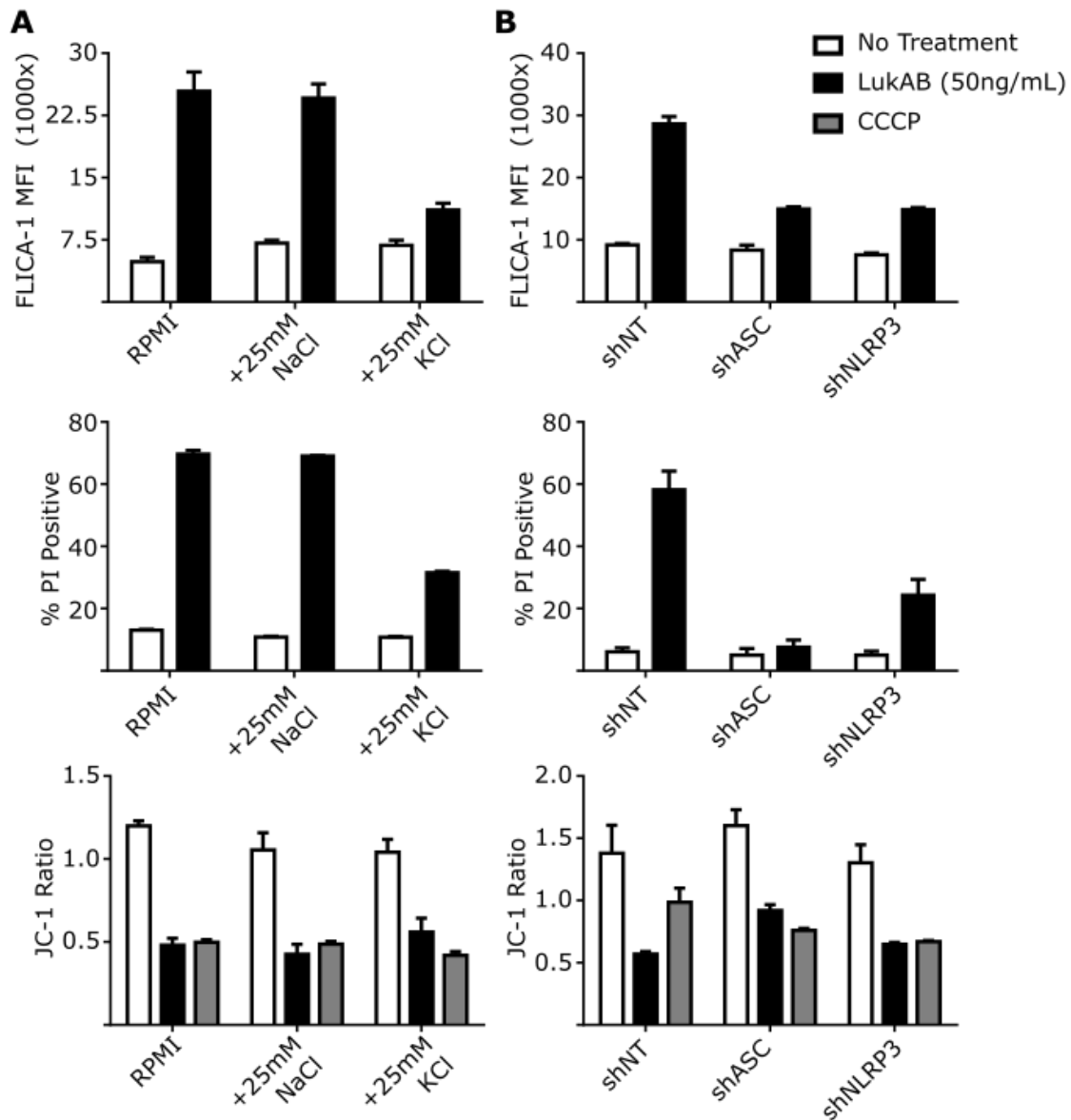
LukAB intoxication caused rapid activation of Caspase 1 starting at 4 minutes that was maximal by 12 minutes. Mitochondrial polarization was equivalent to untreated cells at 11 minutes, but dropped precipitously at 17 minutes and beyond, indicating disruption of mitochondrial integrity. Both markers of cell death increased following roughly 30 minutes of LukAB treatment, though the increasing PI signal was detectable before measurable levels of LDH activity were present in the culture supernatant (Figure 4.1).

While mitochondrial depolarization has been implicated in NLRP3 inflammasome activation in response to other triggers [31], it has not previously been assayed in response to LukAB or other *S. aureus* PFTs. In order to determine the relationship of mitochondrial depolarization to NLRP3 inflammasome activation, we measured mitochondrial polarization after treating cells with LukAB while using potassium inhibition and shRNA knockdown of NLRP3 and ASC to block upstream and downstream processes. We used CCCP as a control that should depolarize the mitochondria regardless of the activation of the NLRP3 inflammasome. Addition of 25mM KCl, but not 25mM NaCl, blocked LukAB-induced Caspase 1 activation and cell death, but had no effect on mitochondrial depolarization. Knockdown of NLRP3 or ASC with shRNA also blocked Caspase 1 activation and cell death, but had no effect on mitochondrial depolarization (Figure 4.2)



**Figure 4.1 LukAB intoxication leads to rapid NLRP3 inflammasome activation, mitochondrial depolarization and cell death.** THP1 cells were incubated separately with fluorescent probes for Caspase 1 activation (FLICA-1), mitochondrial polarization (JC-1) and cell death (PI). Cells were then treated with 50ng/mL LukAB and analyzed at various time points. Collected data was transformed to a 0 to 100 scale in order to compare points of maximal signal across assays. The lower right graph is a composite of the other three.





**Figure 4.2 Mitochondrial depolarization is not blocked by extracellular potassium or shRNA-mediated depletion of NLRP3 or ASC.** (A) THP1 cells were incubated in regular RPMI, or RPMI supplemented with 25mM NaCl or 25mM KCl for 30 minutes. Fluorescent probes to assess Caspase 1 activation (top), cell membrane permeabilization (middle) and mitochondrial polarization (bottom) were then added followed by 50ng/mL LukAB for 1 hour. Cells were analyzed by flow cytometry. (B) THP1 cells containing shRNA constructs targeted

at ASC, NLRP3 or a scrambled control, were incubated with fluorescent probes as in (A) then treated with 50ng/mL LukAB for 1 hour. CCCP was added as a positive control to depolarize mitochondria 15 minutes before analyzing cells. Cells were analyzed by flow cytometry

#### **4.3.2 MIB/MS identifies 344 kinases expressed in THP1 cells**

Given that LukAB treatment of THP1 cells causes Caspase 1 activation in approximately 10 minutes, and cell death at approximately 30 minutes, we decided to assay the THP1 kinome at 10, 20 and 40 minutes. These time points should correspond to kinases involved in activating the NLRP3 inflammasome at 10 minutes, kinases immediately changed in response to NLRP3 inflammasome activation at 20 minutes and kinases involved in triggering cell death at 40 minutes. Borrowing from well-established protocols in cancer kinome research [28], we compared the kinome of LukAB-treated THP1 cells at these three time points to untreated cells by using MIB/MS.

In total, between three biological replicates, 344 individual kinases were identified by mass spectrometry through identification of at least one unique peptide with >99% confidence in all four samples in a replicate. Of these kinases, 180 were identified in all three replicates, 50 were identified in two replicates, and 114 were identified in only one replicate. Kinases from all eleven families [32] were identified. Of the 16 previously mentioned kinases implicated in NLRP3 inflammasome regulation, 14 were identified in our survey.

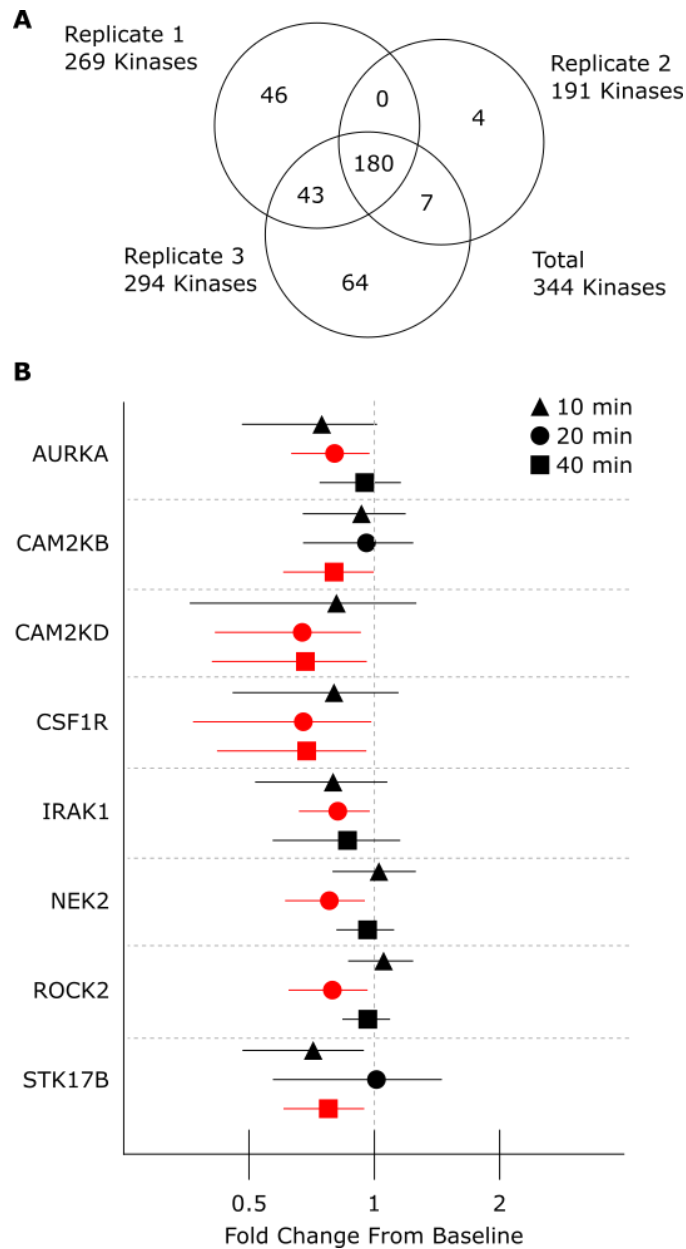
#### **4.3.3 Eight kinases are modulated in response to LukAB intoxication**

For each kinase identified by MIB/MS, a fold change ratio was calculated based on the unique peptides and mass spectrometry probe identified for that kinase and sample. To combine

replicates and prioritize candidate kinases based on their likelihood of truly being modulated in response to LukAB, the inverse variance weighted mean ratio and a new 95% confidence interval were calculated for each kinase. Inverse variance weighting has a propensity to allow one replicate with a small sample size and variance to sway the data. To counter this, we calculated the variance of the weights themselves for each replicate and used 0.1 as a cutoff that appeared to represent well balanced weighting between replicates.

A total of eight kinases had a 95% confidence interval that did not overlap one, indicating that they had changed relative to untreated cells, and met our robustness criteria of relatively balanced weights. These kinases included AURKA, CAM2KB, CAM2KD, CSF1R, IRAK1, NEK2, ROCK2 and STK17B. Figure 4.3 depicts the fold change ratio at each time point for these eight kinases. The data points colored in red are both significant and robust.

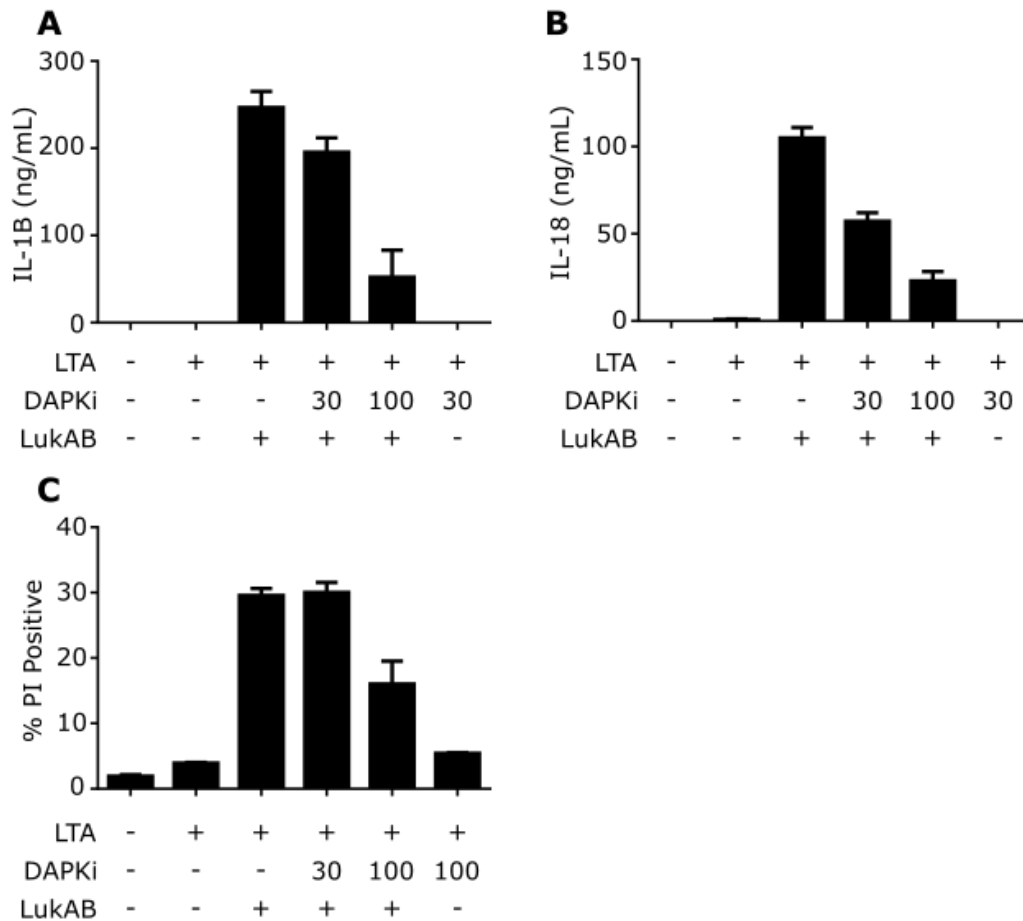
No kinase was changed at the earliest time point, though AURKA and STK17B trended close. Also, no significant increases in kinase activity were captured. Only CAM2KD and CSF1R were changed at more than one time-point; 20 minutes and 40 minutes. Notably, IRAK1 has previously been implicated in NLRP3 inflammasome regulation in response to LPS priming.



**Figure 4.3 Kinome analysis by MIB/MS identifies 344 kinases, 8 of which are significantly changed at 10 separate time points.** (A) A Venn diagram of the number of kinases identified in each biological replicate. The replicates and the total number of kinases identified are listed outside the diagram. (B) A graph depicting the fold change from baseline and 95% confidence interval for each of the eight significant kinases at all three time points. Those colored in red meet both significance and robustness criteria.

#### 4.3.4 Inhibiting DAPK suppresses LukAB cytotoxicity and IL-1 $\beta$ and IL-18 secretion

Building on other data from our lab that showed recombinant DAPK can bind and phosphorylate recombinant NLRP3 *in vitro* (data not shown), we undertook to analyze the functional role of DAPK in LukAB-induced NLRP3 inflammasome signaling. Using a commercially available DAPK inhibitor, we found that blocking DAPK reduces LukAB-induced IL-1 $\beta$  and IL-18 secretion and also blocks cell death (Figure 4.4).



**Figure 4.4 DAPK inhibition blocks LukAB-mediated IL-1 $\beta$  and IL-18 secretion and cell death in THP1 cells.** THP1 cells were primed with 500ng/mL lipoteichoic acid for 2.5 hours as indicated. Then DAPK inhibitor was added for 30 minutes at the indicated concentration ( $\mu$ M).

Finally, 50ng/mL LukAB was added for 1 hour. (A) IL-1 $\beta$  and (B) IL-18 secretion were analyzed by AlphaLISA and (C) propidium iodide staining was analyzed by flow cytometry.

#### **4.3.5 mRNA transfection of NLRP3 S198D/S201D into THP1 cells causes spontaneous IL-18 secretion not seen in S198D single mutant**

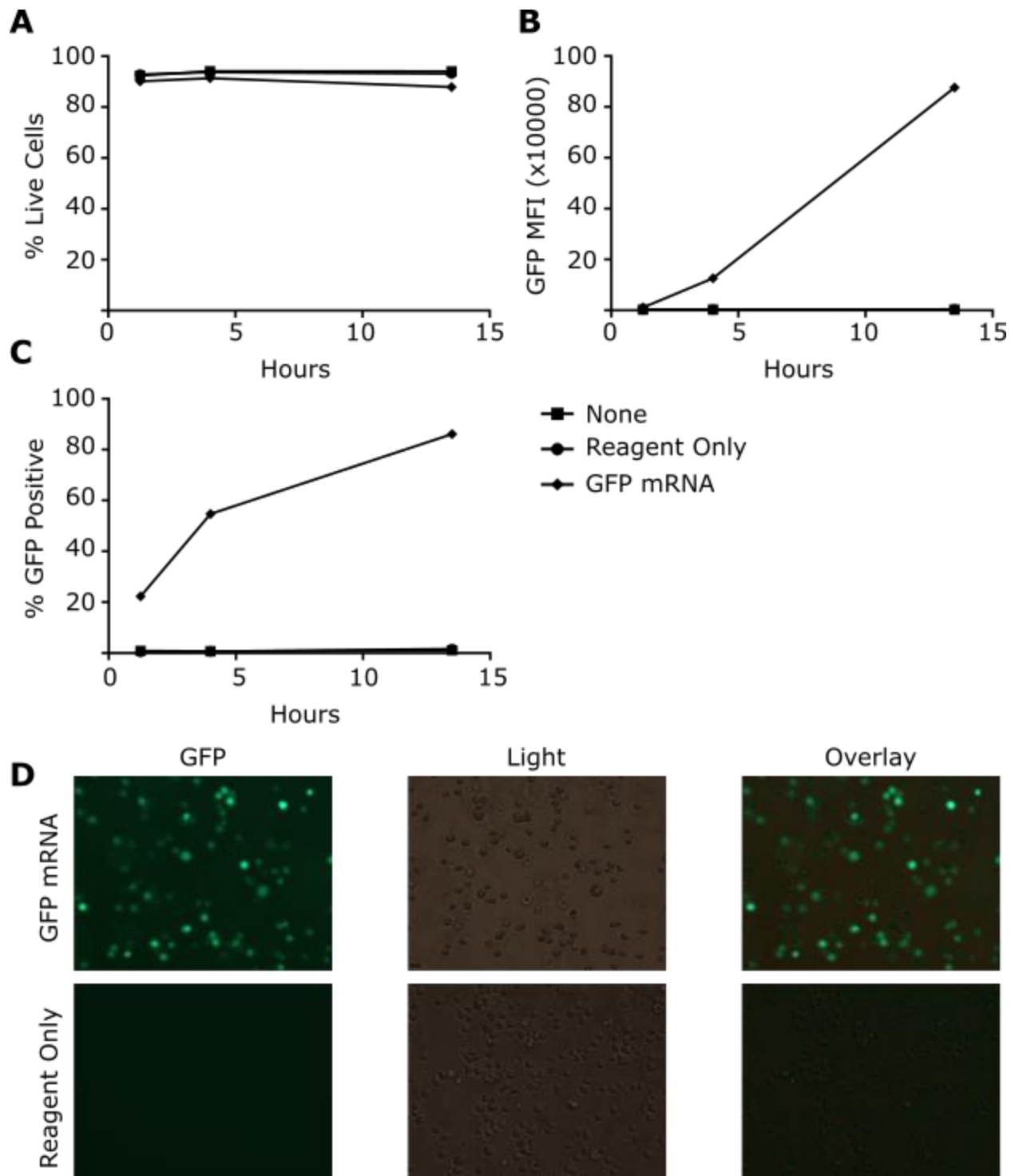
In order to determine if phosphorylation at S198 and S201, the sites identified in the *in vitro* recombinant DAPK-NLRP3 phosphorylation assay (data not shown), were necessary or sufficient for NLRP3 inflammasome activation, we constructed a series of mutants to both block and mimic phosphorylation. Having seen that DAPK inhibition blocks NLRP3 activation, we reasoned that if phosphorylation by DAPK was required for NLRP3 inflammasome activity then a phosphomimetic mutation, serine to aspartic acid, might be constitutively active when overexpressed in THP1 cells leading to Caspase 1 activation and cytokine secretion.

Expression of exogenous genes in THP1 cells is challenging because the cells readily initiate a cell death process when transfected. Viral transduction has been successful but is slow and labor intensive. In order to rapidly test functional consequences of phosphorylation site mutations, we utilized *in vitro* transcription to produce capped and poly-adenylated mRNA followed by transfection using the TransIT-mRNA Transfection Kit. We used GFP mRNA in initial trials of mRNA transfections in THP1 cells to be able to rapidly determine the transfection efficacy.

Transfection of GFP mRNA was well tolerated with almost no cell death observed (Figure 4.5A). Transfection of GFP mRNA resulted in detectable GFP fluorescence as early as 1-hour post transfection with approximately 20% of cells being GFP positive by flow cytometry. The fraction of GFP positive cells increased across 14 hours reaching a high of approximately

85% (Figure 4.5B-C). Beyond 14 hours, the fraction of GFP positive cells remained stable but the mean fluorescence intensity (MFI) continued to increase through approximately 48 hours post transfection (data not shown). Cells exposed only to the transfection reagents with no GFP mRNA failed to produce fluorescence by flow cytometry and microscopy (Figure 4.5D)

Upon testing transfection of *in vitro* transcribed NLRP3, we observed significant toxicity within the first 12 hours with the number of viable cells decreasing from 85% to 50%. This change was not concomitant with secretion of IL-18 and thus was not thought to be the result of NLRP3 inflammasome activation. However, transfection of the NLRP3 double mutant S198D/S201D, but not NLRP3 single mutant S198D or S198A led to a substantial increase in IL-18 secretion and an enhancement of cell death, consistent with S198D/S201D leading to spontaneous NLRP3 activation (Figure 4.6A-B).

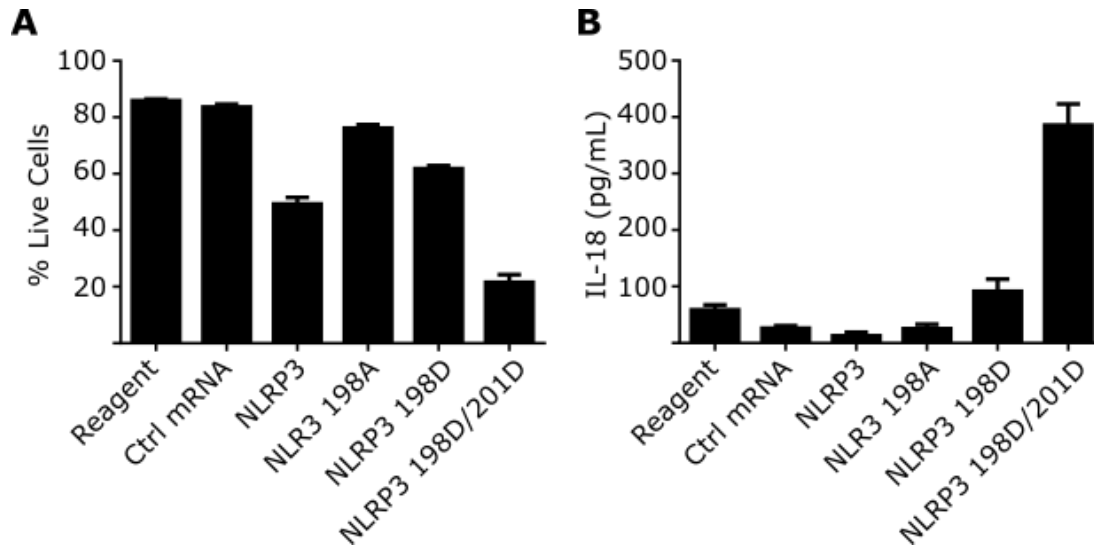


**Figure 4.5 GFP mRNA transfection efficiency is high and well tolerated in THP1 cells.**

THP1 cells were transfected with GFP mRNA using the TransIT-mRNA kit and were monitored by flow cytometry for 14 hours. (A) The fraction of viable cells, as determined by forward and



side scatter, is depicted. (B) The GFP mean fluorescence intensity of viable cells is depicted. (C) The fraction of GFP positive cells is depicted. Note, no change was observed with transfection reagent alone and as such this line overlaps with untreated cells. (D) Transfected cells were imaged by fluorescence microscopy at 14 hours post transfection.



**Figure 4.6 Phosphomimetic mutation of NLRP3 S198 and S201 leads to spontaneous NLRP3 inflammasome activity.** THP1 cells were transfected with a control mRNA (elongation factor a from *Xenopus*), wildtype sequence NLRP3 or NLRP3 containing the indicated amino acid changes. (A) Cell viability was determined at 13 hours post transfection by flow cytometry. (B) Culture supernatants were collected and analyzed by AlphaLISA for IL-18 secretion.

#### 4.4 Discussion

*S. aureus* PFTs all activate the NLRP3 inflammasome and LukAB is one of the most potent in killing human cells. Little is known, thus far, about how LukAB activates the NLRP3 inflammasome except that it requires potassium efflux [26]. In this study, we utilized a variety of

complementary strategies to characterize changes in the THP1 cell kinome in response to LukAB treatment.

Initially, we performed a careful time course analysis of NLRP3 inflammasome activation, mitochondrial depolarization and cell death. We observed extremely rapid activation of Caspase 1, the most proximal step resulting from NLRP3 inflammasome activation, by about 10 minutes after LukAB exposure. This rapid pace was not dependent on the cells being primed. We observed mitochondrial depolarization following NLRP3 inflammasome activation. At around 30 minutes, both PI and LDH indicated cells were undergoing necrosis. Both PI and LDH transit through holes in the plasma membrane and PI is considerably smaller than LDH, so the observation that cells became PI positive slightly before leaking LDH was not surprising.

While mitochondrial depolarization has been implicated in NLRP3 inflammasome signaling from other inciting agents [31], it has not previously been connected to PFT-mediated NLRP3 inflammasome signaling. We found that LukAB treatment consistently led to loss of mitochondrial polarization. However, LukAB-induced mitochondrial depolarization was not inhibited by extracellular potassium or by shRNA-mediated knockdown of NLRP3 or ASC. These data suggest that mitochondrial depolarization resulting from LukAB intoxication is either initiated upstream of potassium efflux or is a consequence of LukAB intoxication that is independent of NLRP3 inflammasome signaling. Although potassium efflux is thought to be a direct result of LukAB inserting into the cell membrane, LukAB may also activate secondary signaling events unrelated to the permeabilization of host cell membranes. One study has shown that LukA or LukB on their own can initiate NF- $\kappa$ B signaling leading to IL-8 production [33]. However, in the active kinome analysis, we observed a decrease in activity in members of the NF- $\kappa$ B signaling pathway including IRAK1.

Our kinome analysis represents the first application of this high throughput screening technology to the study of kinases in immune cells. Using a proteomics-based approach, we identified 344 unique kinases as being expressed in THP1 cells. While this may not be the full complement of kinases in THP1 cells, it is the largest catalog of kinase expression evidence to date. Each kinase was identified by at least one peptide that was found across four samples: the untreated, and 10, 20 and 40 minute LukAB treatment samples. As a result, we are extremely confident in declaring these kinases as expressed in THP1 cells.

We are also surprised to find many kinases missing from our analysis, most notably, DAPK and MLKL. These two kinases have been implicated in inflammasome activation under different inciting conditions. However, one limitation of this approach, as with all mass spectrometry approaches, is that low abundance kinases can easily be missed. Additionally, because peptides had to be identified across all four samples for the peptide to be included, it is possible that major changes in kinase activity would be missed because of extremely low activity in one sample that fails to register.

Surprisingly, in the kinome analysis, we observed a decrease in kinase activity far more frequently than an increase. The kinases that are decreased in activity may represent basal state inhibitors of the NLRP3 inflammasome signaling pathway. A large number of the modulated kinases are annotated as being involved in cell cycle regulation and cell division processes. This may be a bias in that cell cycle regulation research was one of the earliest areas to focus in on kinase signaling, but may also be a true indicator of NLRP3 inflammasome related processes. One recent report found that NEK7, a cell cycle regulating kinase, restricted NLRP3 inflammasome activation to interphase of the cell cycle. NEK7 bound directly to NLRP3 in a kinase-independent manner to promote inflammasome formation. Loss of NEK7 decreased IL-

1 $\beta$  secretion in response to canonical (nigericin, ATP, alum) and non-canonical NLRP3 stimuli (*E. coli* and *C. rodentium*) [11].

It is also possible that changes in kinase activity relate to other signaling events unrelated to the NLRP3 inflammasome. For example, a kinase could be involved in mitochondrial depolarization in response to LukAB, a phenotype that was not blocked by extracellular potassium or knockdown of the NLRP3 inflammasome. Further research will be needed to determine the roll of these kinases.

In the second part of our study, we demonstrated that DAPK is critical for LukAB-induced NLRP3 inflammasome activation. Also, using mRNA transfections to overexpress phosphomimetic mutants, we identified S201 as an important phosphorylation site on NLRP3. The S198D mutant did not stimulate IL-18 secretion alone, but the S198D/S201D mutant led to robust IL-18 secretion. It is possible that phosphorylation at both S198 and S201 is required for activation, rather than just phosphorylation at S201. Future experiments will have to address the S201D single mutant. Based on biochemical evidence not shown, recombinant DAPK appears to phosphorylate NLRP3 at S198 and S201, but further research is needed to determine if DAPK is responsible for the required activity at these sites in cells.

Transfection of GFP mRNA into THP1 cells was extremely well tolerated. However, transfection of similar amounts of NLRP3 mRNA led to cell death over the same time period. We currently believe that this is the result of NLRP3 activating a signaling pathway besides the inflammasome that leads to cell death without cytokine secretion. NLRP3 has been observed to trigger ASC- and Caspase-8-dependent apoptosis [34], but further investigation is required to determine if that is the cause of death in this setting.

Stepping back, the application of mRNA transfections to the study of protein signaling in THP1 cells is a significant advancement. Viral transduction, a slow and labor-intensive process, has been relied on in the past for overexpressing proteins in THP1 cells. Often times, this is so challenging that researches have opted to use standard plasmid transfections to reconstitute the NLRP3 inflammasome in non-immune cells. One major shortcoming of this approach is that regulators of the NLRP3 inflammasome that are present in immune cells may not be present in epithelial cells. As such, experiments in the reconstituted system may be misleading about the role for endogenous regulators.

With mRNA transfections, multiple proteins can be cloned, mutated and transfected into THP1 cells at a time. Titration of expression can occur through changing the dose of mRNA or changing the time at which subsequent evaluation is performed. The drawback of expressing mutant proteins over a background of wildtype proteins can be overcome with advanced planning to combine siRNA and mRNA transfections in which mutations have been introduced to evade siRNA-mediated destruction. Because siRNA-mediated depletion of proteins can take a few days and mRNA transfections lead to rapid protein production, we recommend transfecting siRNA well in advanced of transfecting mRNA. Ultimately, this technique has the potential to open a goldmine of new explorations in THP1 cells that have previously been impossible. For example, rapid alanine mutational scanning can now be used to identify critical residues in protein-protein interactions or protein signaling networks.

Overall, the data and techniques emerging from these early studies hold great promise in advancing our knowledge of LukAB intoxication and NLRP3 inflammasome signaling.

## REFERENCES

1. Kebaier C, Chamberland RR, Allen IC, Gao X, Broglie PM, et al. (2012) *Staphylococcus aureus* alpha-hemolysin mediates virulence in a murine model of severe pneumonia through activation of the NLRP3 inflammasome. *J Infect Dis* 205: 807-817.
2. Gross O, Poeck H, Bscheider M, Dostert C, Hanneschlager N, et al. (2009) Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature* 459: 433-436.
3. Kankkunen P, Teirila L, Rintahaka J, Alenius H, Wolff H, et al. (2010) (1,3)-beta-glucans activate both dectin-1 and NLRP3 inflammasome in human macrophages. *J Immunol* 184: 6335-6342.
4. Ritter M, Gross O, Kays S, Ruland J, Nimmerjahn F, et al. (2010) *Schistosoma mansoni* triggers Dectin-2, which activates the Nlrp3 inflammasome and alters adaptive immune responses. *Proc Natl Acad Sci U S A* 107: 20459-20464.
5. Callaway JB, Smith SA, McKinnon KP, de Silva AM, Crowe JE, Jr., et al. (2015) Spleen Tyrosine Kinase (Syk) Mediates IL-1beta Induction by Primary Human Monocytes during Antibody-enhanced Dengue Virus Infection. *J Biol Chem* 290: 17306-17320.
6. Lin YC, Huang DY, Wang JS, Lin YL, Hsieh SL, et al. (2015) Syk is involved in NLRP3 inflammasome-mediated caspase-1 activation through adaptor ASC phosphorylation and enhanced oligomerization. *J Leukoc Biol*.
7. Hara H, Tsuchiya K, Kawamura I, Fang R, Hernandez-Cuellar E, et al. (2013) Phosphorylation of the adaptor ASC acts as a molecular switch that controls the formation of speck-like aggregates and inflammasome activity. *Nat Immunol* 14: 1247-1255.
8. Shio MT, Eisenbarth SC, Savaria M, Vinet AF, Bellemare MJ, et al. (2009) Malarial hemozoin activates the NLRP3 inflammasome through Lyn and Syk kinases. *PLoS Pathog* 5: e1000559.
9. Okada M, Matsuzawa A, Yoshimura A, Ichijo H (2014) The lysosome rupture-activated TAK1-JNK pathway regulates NLRP3 inflammasome activation. *J Biol Chem* 289: 32926-32936.
10. Schmid-Burgk JL, Chauhan D, Schmidt T, Ebert TS, Reinhardt J, et al. (2016) A Genome-wide CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) Screen Identifies NEK7 as an Essential Component of NLRP3 Inflammasome Activation. *J Biol Chem* 291: 103-109.
11. Shi H, Wang Y, Li X, Zhan X, Tang M, et al. (2015) NLRP3 activation and mitosis are mutually exclusive events coordinated by NEK7, a new inflammasome component. *Nat Immunol*.

12. Lu B, Nakamura T, Inouye K, Li J, Tang Y, et al. (2012) Novel role of PKR in inflammasome activation and HMGB1 release. *Nature* 488: 670-674.
13. Ito M, Shichita T, Okada M, Komine R, Noguchi Y, et al. (2015) Bruton's tyrosine kinase is essential for NLRP3 inflammasome activation and contributes to ischaemic brain injury. *Nat Commun* 6: 7360.
14. Kang TB, Yang SH, Toth B, Kovalenko A, Wallach D (2013) Caspase-8 blocks kinase RIPK3-mediated activation of the NLRP3 inflammasome. *Immunity* 38: 27-40.
15. Wang X, Jiang W, Yan Y, Gong T, Han J, et al. (2014) RNA viruses promote activation of the NLRP3 inflammasome through a RIP1-RIP3-DRP1 signaling pathway. *Nat Immunol* 15: 1126-1133.
16. Lupfer C, Thomas PG, Anand PK, Vogel P, Milasta S, et al. (2013) Receptor interacting protein kinase 2-mediated mitophagy regulates inflammasome activation during virus infection. *Nat Immunol* 14: 480-488.
17. Martin BN, Wang C, Willette-Brown J, Herjan T, Gulen MF, et al. (2014) IKKalpha negatively regulates ASC-dependent inflammasome activation. *Nat Commun* 5: 4977.
18. Lin KM, Hu W, Troutman TD, Jennings M, Brewer T, et al. (2014) IRAK-1 bypasses priming and directly links TLRs to rapid NLRP3 inflammasome activation. *Proc Natl Acad Sci U S A* 111: 775-780.
19. Fernandes-Alnemri T, Kang S, Anderson C, Sagara J, Fitzgerald KA, et al. (2013) Cutting edge: TLR signaling licenses IRAK1 for rapid activation of the NLRP3 inflammasome. *J Immunol* 191: 3995-3999.
20. Chuang YT, Lin YC, Lin KH, Chou TF, Kuo WC, et al. (2011) Tumor suppressor death-associated protein kinase is required for full IL-1beta production. *Blood* 117: 960-970.
21. Qu Y, Misaghi S, Izrael-Tomasevic A, Newton K, Gilmour LL, et al. (2012) Phosphorylation of NLRC4 is critical for inflammasome activation. *Nature* 490: 539-542.
22. Suzuki S, Franchi L, He Y, Munoz-Planillo R, Mimuro H, et al. (2014) Shigella type III secretion protein MxiI is recognized by Naip2 to induce Nlrc4 inflammasome activation independently of Pkcdelta. *PLoS Pathog* 10: e1003926.
23. Dumont AL, Nygaard TK, Watkins RL, Smith A, Kozhaya L, et al. (2011) Characterization of a new cytotoxin that contributes to Staphylococcus aureus pathogenesis. *Mol Microbiol* 79: 814-825.
24. Ventura CL, Malachowa N, Hammer CH, Nardone GA, Robinson MA, et al. (2010) Identification of a novel Staphylococcus aureus two-component leukotoxin using cell surface proteomics. *PLoS One* 5: e11634.
25. DuMont AL, Yoong P, Day CJ, Alonzo F, 3rd, McDonald WH, et al. (2013) Staphylococcus aureus LukAB cytotoxin kills human neutrophils by targeting the CD11b subunit of the integrin Mac-1. *Proc Natl Acad Sci U S A* 110: 10794-10799.

26. Melehani JH, James DB, DuMont AL, Torres VJ, Duncan JA (2015) Staphylococcus aureus Leukocidin A/B (LukAB) Kills Human Monocytes via Host NLRP3 and ASC when Extracellular, but Not Intracellular. *PLoS Pathog* 11: e1004970.
27. DuMont AL, Yoong P, Surewaard BG, Benson MA, Nijland R, et al. (2013) Staphylococcus aureus elaborates leukocidin AB to mediate escape from within human neutrophils. *Infect Immun* 81: 1830-1841.
28. Duncan JS, Whittle MC, Nakamura K, Abell AN, Midland AA, et al. (2012) Dynamic reprogramming of the kinome in response to targeted MEK inhibition in triple-negative breast cancer. *Cell* 149: 307-321.
29. DuMont AL, Yoong P, Liu X, Day CJ, Chumbler NM, et al. (2014) Identification of a crucial residue required for Staphylococcus aureus LukAB cytotoxicity and receptor recognition. *Infect Immun* 82: 1268-1276.
30. Reyes-Robles T, Alonzo F, 3rd, Kozhaya L, Lacy DB, Unutmaz D, et al. (2013) Staphylococcus aureus leukotoxin ED targets the chemokine receptors CXCR1 and CXCR2 to kill leukocytes and promote infection. *Cell Host Microbe* 14: 453-459.
31. Gurung P, Lukens JR, Kanneganti TD (2015) Mitochondria: diversity in the regulation of the NLRP3 inflammasome. *Trends Mol Med* 21: 193-201.
32. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S (2002) The protein kinase complement of the human genome. *Science* 298: 1912-1934.
33. Yanai M, Rocha MA, Matolek AZ, Chintalacheruvu A, Taira Y, et al. (2014) Separately or combined, LukG/LukH is functionally unique compared to other staphylococcal bicomponent leukotoxins. *PLoS One* 9: e89308.
34. Sagulenko V, Thygesen SJ, Sester DP, Idris A, Cridland JA, et al. (2013) AIM2 and NLRP3 inflammasomes activate both apoptotic and pyroptotic death pathways via ASC. *Cell Death Differ* 20: 1149-1160.



## Chapter 5. Concluding thoughts and future directions

In recent years, our understanding of how *Staphylococcus aureus* interacts with the innate immune system has grown tremendously. Identification of novel PFTs and their receptors on the host cell membrane, characterization of their species specificity, and the discovery that the NLRP3 inflammasome is central in responding to all PFTs is an important starting foundation for understanding the contribution of the PFTs to disease pathogenesis. As the threat of drug resistant *S. aureus* continues to grow, novel strategies will be required for combating infections. Targeting *S. aureus* pore forming toxins may be one such strategy.

In the review contained in Chapter 2, I outlined the current scientific research pertaining to *S. aureus* and inflammasome interactions. Thus far, most studies have focused on the significance of Hla to disease pathogenesis. With the discovery of LukAB and its demonstrated potency in killing human cells, and its failure to kill mouse cells, it is now clear that new animal models are required to better capture the full effect of *S. aureus* PFTs in infection pathogenesis.

Introducing or replacing mouse PFT receptor genes with human sequences might help overcome this resistance, but the large number of toxins and receptor genes makes replacing all of them together more difficult. Additionally, the result of chimeric receptor expression on immune signaling more generally would be entirely unpredictable. For example, would replacing mouse CD11b with human CD11b affect its association with mouse CD18? Or would inclusion of both mouse and human CD11b sequences cause competition for CD11b-associated molecules that reduces signaling potential? Efforts are currently underway to develop these mice so the

opportunity to study LukAB *in vivo*, and the consequences of humanizing receptors in mice, will soon exist.

An alternative approach would be to develop humanized mouse models through bone marrow reconstitution. However, at best, this labor intensive process creates chimeric myeloid compartments with variable distribution of human cells to tissues such as the lung. There is also evidence that *S. aureus* virulence factors act on epithelial and endothelial cells which cannot be humanized. Mice are generally resistant to *S. aureus* and even humanized mice require direct inoculation with approximately 1 million *S. aureus* colony forming units to establish infection.

Although mice provide the best opportunity for genetic studies, a natural host may be more suitable for studies of *S. aureus* virulence. One such natural host is the domesticated chicken, *Gallus gallus domesticus*. *S. aureus* naturally causes a wide range of infections in chickens that mimic its infectivity in humans; this includes localized abscesses, synovitis, osteomyelitis, endocarditis and bacteremia. While most university animal facilities are not equipped for housing chickens, *S. aureus* can also infect the yolk sac, causing omphalitis. As such, *S. aureus* inoculation of chicken eggs may be a viable strategy for investigating *S. aureus* virulence in a natural host. However, many differences between chicken and human immune systems exist that would also raise questions about the generalizability and translatability of such a model.

Without new models that incorporate sensitivity to a wider range of *S. aureus* virulence factors, novel treatments may be misguided and ineffective.

In Chapter 3, I highlighted my work on investigating the mechanism of LukAB in killing human monocytes. This work demonstrated the significance of LukAB, as compared to the other *S. aureus* PFTs, in activating the NLRP3 inflammasome and killing human monocytes. We were

also surprised to find that when *S. aureus* was phagocytosed, LukAB-mediated bacterial escape did not require the NLRP3 inflammasome, even though the NLRP3 inflammasome was being activated. Loss of ASC did not diminish LukAB-induced cell death from within the phagosome, suggesting that this mode of cell death is not caused by an ASC-containing inflammasome. Future research will investigate the role of RIPK1/RIPK3 and Caspase 8 in this cell death process.

In Chapter 4, I discussed initial experiments characterizing important kinase and phosphorylation changes in the response to LukAB. In our data from the MIB/MS analysis, we were surprised to see no significant increases in kinase activity despite identifying approximately 180 kinases in all three replicates. It is possible that kinases that had very low activity in the basal state, but were upregulated in response to LukAB, were missed because of their low capture rate on the MIB/MS column in the untreated sample. We will have to go further analyze the raw mass spectrometry data to see if there are high abundance peptides associated with kinases that appear in LukAB-treated samples that were completely absent in untreated cells. We will also follow up on the eight kinases that decrease in activity in response to LukAB. By depleting each kinase with siRNA, we will test for increased sensitivity or spontaneous activity of the NLRP3 inflammasome. I suspect that these kinases, particularly those in the earlier time points, are involved in maintaining the off state of the NLRP3 inflammasome.

The development I am most excited for is the application of mRNA transfections to structure-function analyses in THP1 cells. My demonstration of high efficacy transfection with mRNA will allow researchers an opportunity to do broad-scale mutagenesis studies to identify critical residues in the proteins in innate immune cells, rather than having to create reconstituted systems in epithelial cells. The early product of this new strategy demonstrated that the

S198D/S201D NLRP3 mutant has spontaneous inflammasome activity. This is the first direct demonstration of a phosphomimetic mutation in NLRP3 with functional consequences.

Continued research can target all phosphorylation sites to better understand the consequences of kinases and phosphorylation in the regulation of the NLRP3 inflammasome.

Lastly, having spent considerable time with the literature in the NLRP3 inflammasome field, I believe a collaborative effort is needed to systematically address holes and generalizability of existing research. Dozens of different activators are used to study NLRP3 inflammasome activity leading to disparate findings. Cell type differences, species differences, unique activators and genetic versus pharmacologic interventions have resulted in a body of research that is hard to follow. A systematic and searchable cataloging of what experiments were done including the cells, activator, reagents, and results would help provide clarity. Additionally, the readouts of NLRP3 inflammasome activation, namely IL-1 $\beta$  and IL-18 secretion and cell death, are easily adapted to high throughput analysis. A research core set up to systematically compare inhibitors across different activators could also produce a wealth of data to help resolve conflicting findings.

Overall, the data described in this dissertation and the future research questions they highlight should continue to sustain a fruitful research program in the area of *S. aureus* host-pathogen interactions and NLRP3 inflammasome regulation.

## **Appendix A. Functional amyloid signaling via the inflammasome, necrosome and signalosome: new therapeutic targets in heart failure<sup>4</sup>**

### **A.1 Abstract**

As the most common cause of death and disability globally, heart disease remains an incompletely understood enigma. A growing number of cardiac diseases are being characterized by the presence of misfolded proteins underlying their pathophysiology, including cardiac amyloidosis and dilated cardiomyopathy (DCM). At least nine precursor proteins have been implicated in the development of cardiac amyloidosis, most commonly caused by multiple myeloma (MM) light chain disease and disease-causing mutant or wildtype transthyretin (TTR). Similarly aggregates with PSEN1 and COFILIN-2 have been identified in up to 1/3 of idiopathic DCM cases studied indicating the potential predominance of misfolded proteins in heart failure. In this review, we present recent evidence linking misfolded proteins mechanistically with heart failure and present multiple lines of new therapeutic approaches that target the prevention of misfolded proteins in cardiac TTR amyloid disease. These include multiple small molecule pharmacological chaperones now in clinical trials designed specifically to support TTR folding by rational design, such as tafamidis, and chaperones previously developed for other purposes, such as doxycycline and tauroursodeoxycholic acid. Lastly, we present newly discovered non-

---

<sup>4</sup> This article previously appeared as an article in the journal *Frontiers in Cardiovascular Medicine*. The original citation is as follows: Parry TL, Melehani JH, Ranek MJ, and Willis MS (2015) Functional amyloid signaling via the inflammasome, necrosome, and signalosome: new therapeutic targets in heart failure. *Front. Cardiovasc. Med.* 2:25. doi: 10.3389/fcvm.2015.00025

pathological “functional” amyloid structures, such as the inflammasome and necrosome signaling complexes, which can be activated directly by amyloid. These may represent future targets to successfully attenuate amyloid-induced proteotoxicity in heart failure as the inflammasome, for example, is being therapeutically inhibited experimentally in autoimmune disease. Together, these studies demonstrate multiple novel points in which new therapies may be used to primarily prevent misfolded proteins or to inhibit their downstream amyloid-mediated effectors, such as the inflammasome, to prevent proteotoxicity in heart failure.

## **A.2 Introduction**

As the most common cause of death and disability globally, heart disease remains an incompletely understood enigma. Despite vast improvements in our understanding of the neurohormonal basis of heart failure, current therapies targeting this system have reached a plateau for which new therapeutic paradigms might be considered. A great deal of insight has recently been achieved by our understanding of the role of protein homeostasis in the heart. The role of misfolded proteins, once reserved as the pivotal pathophysiological mechanism in neurodegenerative diseases, such as Alzheimer’s disease, Huntington’s disease, and Parkinson’s disease, has now been demonstrated to be present in many types of heart failure.

## **A.3 Misfolded proteins in cardiac disease**

A growing number of cardiac diseases are characterized by misfolded proteins underlying their pathophysiology, including cardiac amyloidosis and dilated cardiomyopathy. Cardiac amyloidosis represents a broad disease entity characterized by misfolded proteins forming insoluble aggregates that are deposited in the heart. While the physical morphology is shared by

all amyloid fibrils, the spectrum of affected organs and clinical presentation and prognosis varies greatly. The prognosis is often dependent upon the makeup of the amyloid precursor protein and its associated pathophysiology throughout the body. At least nine precursor proteins have been implicated in the development of cardiac amyloidosis [1]. The most common cause of cardiac amyloidosis is multiple myeloma (MM), specifically MM-secreted monoclonal Ig light chain disease [1], followed by disease-causing mutant or wildtype transthyretin (TTR) protein [2]. Functionally, infiltrative amyloid deposition causes a restrictive cardiomyopathy (diastolic dysfunction) and subsequent systolic heart failure.

The pathophysiology of AL (Ig light chain) cardiomyopathy was hypothesized to be due to the extracellular deposition of fibrils, resulting in increased passive stiffness and loss of cardiac parenchyma due to cell death [1]. The physiological impairment in diastolic filling in amyloid cardiomyopathy patients supported this idea [3,4]. However, evidence for precursor toxicity has gained traction with recent correlations of AL cardiomyopathy with ATTR cardiomyopathy function with similar degrees of fibril deposition [5]. Surprisingly, patients with equal fibril deposition due to AL cardiomyopathy are functionally worse than ATTR patients indicating that the infiltration itself is not completely responsible for the dysfunction [5]. Soluble factors found circulating in AL patients may be an additional contributor to the cardiac dysfunction. This has been shown in studies where experimental infusion of amyloidogenic light chain proteins from patients with severe cardiac involvement into healthy hearts. The infusion of amyloidogenic light chain proteins induced diastolic dysfunction in healthy unaffected isolated mouse hearts, while infusion of light chains from patients with myeloma and no amyloidosis do not [6-9]. The cardiac dysfunction in amyloid cardiomyopathy therefore appears to be related to

mechanisms induced by both infiltrative insoluble factors in the heart itself and circulating soluble factors from the systemic multiple myeloma.

Recognition of protein aggregation as a cause of heart failure is likely underestimated. Two recent studies illustrate the prevalence of protein aggregation as a cause of heart failure in patients that had heart failure clinically characterized by dilated cardiomyopathy. Two new sequence variants in the presenilin-1 (PSEN1) gene promoter were identified in nearly 1% (3/325) of patients of the idiopathic dilated cardiomyopathy patients investigated [10]. Interestingly, PSEN1 mutant patients have cardiac protein aggregates present, along with reduced *psen1* gene and PSEN1 protein expression [10]. Mechanistically, PSEN1 co-immunoprecipitates with SERCA2a illustrating one point in which PSEN1 may be affecting cardiac function [10]. With the PSEN1 oligomer interacting directly with the Ca<sup>2+</sup> channel, it's possible that changes in Ca<sup>2+</sup> and heart failure seen in these patients may be mechanistically linked by this interaction [10]. Similarly, tangles and plaque-like aggregates made of COFILIN-2 have been found in other dilated cardiomyopathy cases, estimated to involve nearly 1/3 of the cases [11]. Initial studies investigated the aggregate composition of aggregates extracted from human idiopathic DCM with Congo red positivity has been found to include COFILIN-2 in a high percentage of patients, which was confirmed in a larger cohort of samples (Subramanian, 2015). Aggregates had COFILIN-2 present, an actin depolymerizing protein known to participate in neurodegenerative diseases [12,13]. Understanding COFILIN-2's role in chronic degenerative diseases such as DCM offers a novel therapeutic target [11].

Mutations in heat shock proteins (HSPs), a critical component of the cellular “anti-folding” apparatus, also underlie human cardiac disease. HSP proteins assist protein folding in routine maintenance of the cardiomyocyte. However in the context of disease, their recruitment



to protein misfolding is critical with acquired conditions such as ischemia/reperfusion injury, or because of mutations which can modify protein structures [14]. In Long QT Syndrome 2, mutations in KCNH2 (aka human ether-a-go-go related gene/HERG) encoding the rapidly activating-delayed rectifier potassium channel Kv11.1 alpha-subunit alter cell repolarization of the ventricular action potential [15]. Characterized by prolonged QT interval and ventricular tachycardia, syncope, and sudden death, the largest number of HERG mutations (28/34) affect protein folding and trafficking [16]. Similarly, the desmin contractile apparatus linking nucleus, mitochondria, and sarcolemma is critical to cardiomyocyte function. Desmin deficiency or mutations in the chaperone proteins assisting desmin folding (e.g. HSPA/HSP70, HSPH (HSP110), DNAJ (HSP40), HSPB (small HSPs), SHPD, HSPE, CCT result in proteotoxicity mediated via aggregate formation [14].

#### **A.4 Protein folding, preamyloid oligomers, and aggregation**

In biological systems, multiple physical factors influence protein folding [17], including mutations, molecular chaperones, and protein quality control systems (such as the ubiquitin proteasome system) that prevent the formation of misfit conformations resulting from destabilized protein folding and/or aggregate formation [18,19]. Protein misfolding is driven by alterations in the protein sequence (i.e. mutations), malignant post-translational modifications, and oxidative stress among other environmental cues (Figure A.1A). These alterations initiate pathology through: 1) formation of a destabilized protein; 2) accumulation of intermediates with unstable folding, and 3) stabilization of misfolded conformations through the formation of aggregates (Figure A.1B-D). While native conformation stability is characterized as having achieved the lowest free energy state, this feature may also explain the stability of

aggregate/fibril formation in diseases, including heart failure in amyloidosis and non-amyloidosis-related states.

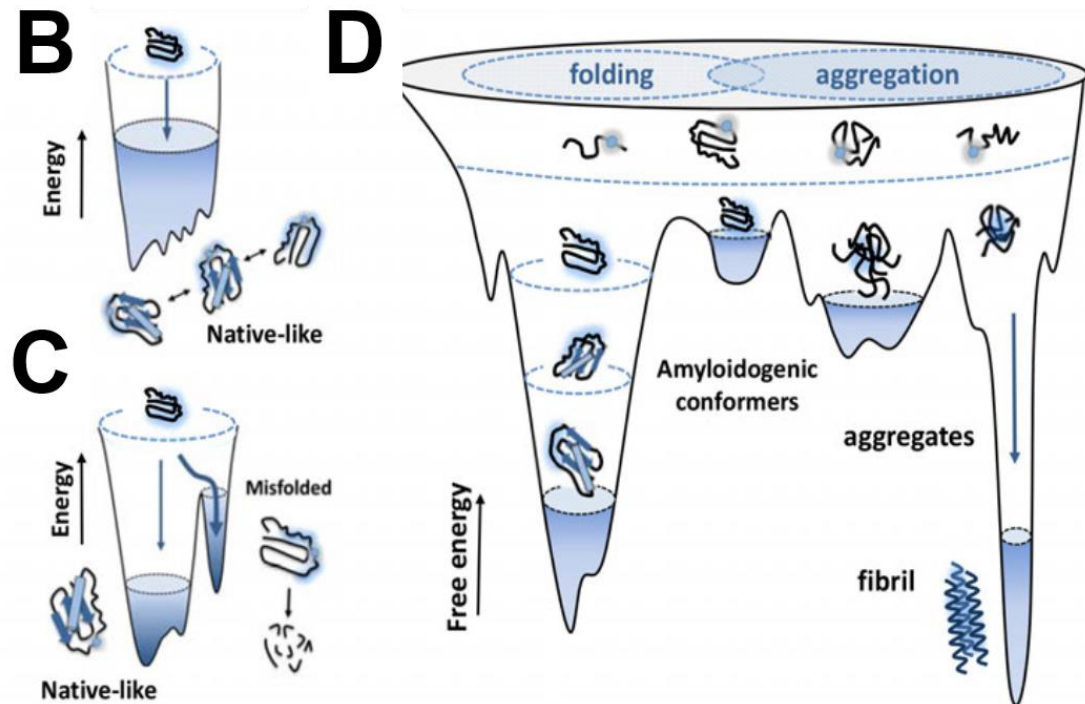
## A Protein Misfolding

### Causes:

- Post-translational Modifications
- Oxidative Stress
- Protein concentration
- Mutations
- Temperature
- Altered cell chemistry
- Crowding
- Membrane interactions

### Effects:

- Stabilizing interactions
- Folding pathway
- Hydrophobic core
- Catalytic sites
- Cofactor binding
- Quaternary structure
- Breathing dynamics
- PTM sites



**Figure A.1 Native, non-native, aggregates, and amyloid protein structures and the stressors that drive them.** (A) Proteins are prone misfolding by direct biological and indirect environmental stresses, including alterations in the protein sequence (mutations) and post-

translational modifications (e.g. those induced by oxidative stress), respectively, creating protein aggregates and amyloid. These toxic structures are dangerous to biological systems, driving amyloidosis in neurodegenerative and cardiac pathologies. (B) Unfolded protein reside at a high entropy state in an unstable “non-native” structure. As they become folded, they move towards a lower entropy and move towards stable and favorable “native” structure. (C) Biological and environmental stressors initiate alterations in protein sequence (i.e., mutations), forcing intermediates into unstable conformations. (D) Destabilized proteins accumulate resulting into misfolded protein aggregates and amyloid with more stable conformations. B-D: ©Bentham Science. Used with permission. Gomes, 2012 [21].

Protein folding is a process through which polypeptide chains wrap themselves to achieve optimal stability in their physiological environment (Figure A.1B) [20]. This process results from the lower energy “native-like” interactions that are more stable than “non-native-like” conformations (Figure A.1C) [21]. As favorable interactions are established, proteins transition from a high entropy state to one with a lower entropy structure. Proteins therefore have a range of free energies they can move through, inevitably proceeding through a set of conformations that narrow as the native state is achieved [22-24]. Typically for small globular proteins, this transition has been likened to that of a funnel, where inter- and intra-domain interactions cooperatively interact to establish a stable native confirmation [25]. Adverse conditions or mutations result in a degeneration of the energetic minimum, resulting in the lowering of barriers between native-like conformations (Figure A.1D). The resulting larger distribution results in higher energy amyloidogenic conformers in destabilized folding patterns, susceptible to degradation or consequent local unfolding that triggers aggregation. These conformers can then

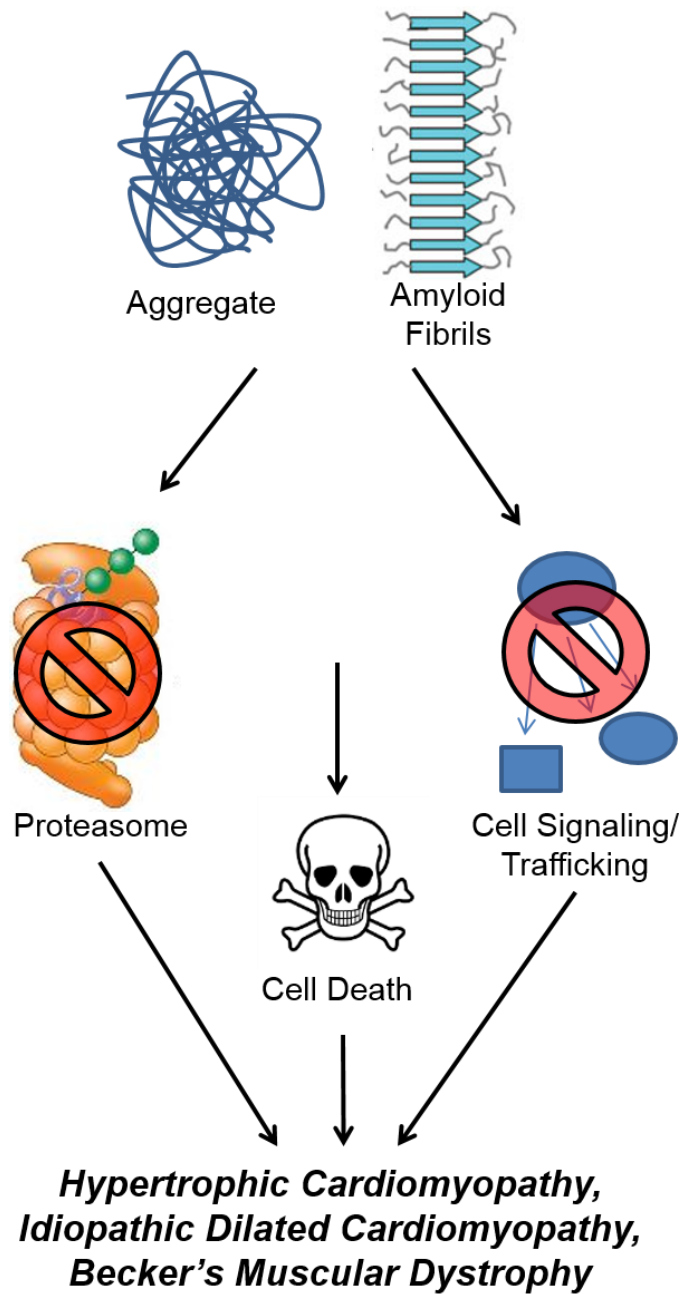
contribute to the assembly into amorphous organized aggregates into fibrils results from interactions between stabilized aggregates (Figure A.1D).

### **A.5 Protein aggregates, proteotoxicity, and heart failure**

Protein aggregates and their preceding intermediates induced cell death is a process described as proteotoxicity (Figure A.2). These designations refer to a protein's state, not its origin as many different types of proteins can form these misfolded proteins. However, most familiar examples of aggregates tend to be recognized for their specific sources, such as  $\beta$ -amyloid (AB) and  $\alpha$ -synuclein found in Alzheimer's and Parkinson's disease, respectively. Misfolded protein aggregation leading to heart failure has been found to be caused by misfolded transthyretin and excessive immunoglobulin resulting from aging or mutations and multiple myeloma, respectively. Soluble oligomers and aggregated proteins disrupt cellular function by interfering with proteasome function, disrupting cell signaling and protein trafficking, and directly support signals that induce cell death [26,27]. The accumulation of misfolded protein oligomers have been identified in patients with hypertrophic cardiomyopathy, idiopathic dilated cardiomyopathy, and Becker's muscular dystrophy, but not in healthy controls [28]. In dilated cardiomyopathy, cytosolic aggregates co-localize with ubiquitin and increased autophagy has been identified [29]. Autophagy, a mechanism for removing aggregated proteins, may be one way in which the heart clears the pathological buildup of misfolded proteins.

The link between misfolded proteins, proteotoxicity and heart failure has just recently been made. Early correlations between misfolded proteins and heart disease were made in mouse models of cardiac hypertrophy, where aggregation of misfolded proteins and aggresomes were identified [30]. Further, inhibiting proteasome activity resulted in the accumulation of

ubiquitinated protein aggregates and enhanced autophagy [30]. However, it was not until the toxic effects of misfolded proteins in the heart were tested directly that the causal link of misfolded proteins to proteotoxicity and heart failure was made. In these studies, mouse hearts expressing small oligomers prone to misfolding (Poly-Q83) were compared to hearts expressing a similar but non-oligomer forming peptide repeat (Poly-Q19) [31]. Mice with cardiac PQ83 developed heart failure by 5 months of age, whereas the non-aggregating control PQ19's heart function was completely normal [31]. These studies develop a new paradigm by which to consider how protein aggregation may contribute to heart failure and ischemic heart disease [32,33], as well as genetic diseases associated with protein misfolding and cardiac dysfunction.



**Figure A.2. Protein aggregate and amyloid stimulate cellular dysfunction.** Misfolded protein aggregates and amyloid inhibit normal proteasome function, interfere with cellular signaling and trafficking, and induced apoptotic cell signaling pathways. All of these perturbations have been shown to occur in pathologies, such as hypertrophic cardiomyopathy, idiopathic dilated cardiomyopathy, and Becker's muscular dystrophy.

## A.6 How endogenous systems prevent protein misfolding

Cells have adapted mechanisms to counteract the propensity of a protein to misfold, particularly in the face of common cellular challenges, such as oxidative stress, and the routine post-translational modifications that can accumulate on proteins. The ubiquitous heat shock proteins (HSPs) act as molecular chaperones to counteract the stress-induced denaturation of other proteins. In the heart, the cardio-protective effect of HSP90, HSP70, TRiC as well as a host of small HSPs ( $\alpha$ BCrystallin (aka HSPB5), HSP20 (aka HSPB6), HSP22 (aka HSPB8, H11 kinase, and  $\alpha$ CCrystallin), HSP27 (aka HSPB1 and HSPB2), and HSP60) has been described in the context of cardiac hypertrophy, heart failure, and ischemia reperfusion disease [34]. Their ability to protect the misfolding of specific targets, such as actin, tubulin, desmin, lipid membranes, and mediators of cell death (e.g. Bax/Bak) have been implicated to date, though a thorough description of their role is just beginning to be elucidated [34].

Not only do chaperones play a role in maintaining proper protein folding, they have overlapping functions that might be useful when thinking about new therapeutic interventions in targeting protein misfolding. HSPs have overlapping functions-the integrity of a single protein can be maintained by >1 HSP in most cases. Recent studies have provided a proof of concept of how this knowledge may be applied therapeutically. Briefly, cultured cardiac cells with disease causing mutations in CryAB mediate cell death and dysfunction; as the complementary HSPB1 is introduced into the cells, both the aggregate formation and associated toxicity is relieved [35]. As a consequence of this relationship, overexpressing HSPB1 facilitates the ubiquitination and proteasome degradation of protein aggregates induced by multiple CryAB misfolding mutations [35] illustrating that increasing HSP expression can help enhance both the solubility and degradation of misfolded proteins in cardiomyocytes. Similarly, treatment of the CryAB<sup>R120G</sup>

mutant mouse model of desmin-related cardiomyopathy with the drug geranylgeranyl-acetone (increasing HSPB8 and HSPB1) significantly reduces heart failure [36]. CryAB<sup>R120G</sup> transgenic mice treated with geranylgeranyl-acetone have reduced amyloid oligomer formation, decreased fibrosis, and recovery of heart function and overall survival [36].

### **A.7 Pharmacological chaperone therapies**

Establishing the role of misfolded proteins in heart failure gives context to the emerging development of small molecule pharmacological chaperones [37-39]. Pharmacological chaperones are defined as molecules which act specifically on misfolded or destabilized protein substrate(s) to specifically refold or stabilize these proteins, respectively [40]. Pharmacological stabilization of the protein transthyretin (TTR) and amyloid fibril disruptors are being developed for polyneuropathy. More recently, their applicability to amyloid disease in the heart has been demonstrated and they are now undergoing clinical trials for cardiac amyloidosis due to misfolded TTR. Mutations and aging cause misfolding to result in TTR amyloidosis and pharmacological chaperone therapy has successfully been used to treat both forms successfully.

Initial studies investigating pharmacological chaperone therapy focused on the neurological manifestations of TTR amyloidosis. Extracellular deposition of TTR is a hallmark of the fatal autosomal dominant neurodegenerative Familial Amyloidotic Polyneuropathy (FAP) [41], in which both mutant (familial forms) and wild type (senile/aging forms) aggregate. More than 80 different mutations destabilize TTR and prevent formation of the tetramer and promote aggregation of misfolded monomers [42]. Misfolded TTR, also known as pre-albumin, is a protein synthesized by the liver that forms a soluble homotetramer that acts to transport thyroid hormone (T4) and retinol. Extensive screening in aggregate-prone conditions led to the discovery



of multiple aromatic small molecules that stabilize mutant TTR [43]. These molecules, including specific NSAIDs and naturally-derived flavonoid and xanthenes, function by binding to thyroxine-binding sites in TTR to stabilize its structure [43]. This led to the development of a wide range of compounds from different structural families, including enoxazoles [44,45]. Tafamidis, a derivative enoxazole, was selected for clinical development based on its ability to stabilize mutant TTR *in vitro* and was later found to have a therapeutic effect in neurological disease models [46,47]. While occupying the thyroxine-binding sites, tafamidis's negative cooperativity acts to stabilize the TTR tetramer.

The first human study of tafamidis for the treatment of polyneuropathy associated with TTR mutations was reported in 2012. Patients with the V30M TTR mutation and FAP were treated with 20 mg daily for 18 months [46]. The primary objective was to monitor progression of neuropathy and evaluate drug safety, while a secondary objective was to determine if tafamidis exhibits a stabilizing effect on human V30M TTR. A total of 128 patients were randomized to tafamidis or placebo. Significant reduction in neuropathy impairment scores were identified as early as 6 months and continued through 18 months [46]. The changes in small nerve fiber function scores were attenuated by tafamidis, while allowing significant increases in BMI to occur (reflecting less muscle mass loss /atrophy resulting from the disease process) [46]. Oral tafamidis is now approved in the EU for the treatment of TTR amyloidosis in adult patients with early stage symptomatic polyneuropathy to delay neurologic impairment. In Argentina, Japan, and Mexico tafamidis is approved for use in delaying peripheral neurological impairment of TTR familial amyloid polyneuropathy [48]. In the US, the FDA has requested a second efficacy study before it will grant approval while noting that the data provided internal consistency and replication of effect.

In addition to neurodegeneration, patients with TTR-FAP develop cardiac disease. At least five clinical trials that investigate tafamidis in patients with TTR-related cardiomyopathy are active or have been completed (see Table A.1). The first one recently published was a randomized crossover study in healthy volunteers that determined tafamidis did not have any effect on QTc intervals or serious adverse events in 42 subjects [49]. Inhibition of TTR expression using siRNA therapy is yet another therapeutic approach being tested to treat TTR-related cardiomyopathy (see Table A.1, rows 6-7).

Pfizer's tafamidis and its potential to stabilize amyloid cardiomyopathy (TTR) is just the tip of the iceberg in the development of pharmacological chaperones for heart disease. Multiple other anti-amyloidogenic compounds have been discovered, including: 1) AG10; 2) doxycycline; 3) tauroursodeoxycholic acid (TUDCA); 4) (-)-epigallocatechin-3-gallate (EGCG); and 5) curcumin. High throughput screening for compounds that bind to the T4 pocket of TTR under physiological conditions identified AG10, which has higher affinity for TTR and greater pharmacokinetic stability while being more selective than tafamidis and diflunisal (a salicylic acid derivative) at physiological conditions [50]. In *in vitro* studies, AG10 was found to effectively inhibit the proteotoxicity of V122I-TTR in human cardiomyocytes [50]. AG10 inhibits aggregation of both wild-type TTR (associated with aging) and V122I-TTR significantly better than tafamidis and almost fully stabilizes TTR at 10  $\mu$ M plasma concentrations [42]. AG10 is a bivalent molecule and thus able to bind 2 TTR proteins per molecule, allowing it to simultaneously occupy adjacent T4 sites in the TTR tetramer [42].

**Table A.1. Clinical studies investigating tafamidis and siRNA TTR in transthyretin-associated (amyloid) cardiomyopathy.**  
TTR: Transthyretin. Created from data available at: ClinicalTrials.gov (Accessed 2 February 2015).

<p><b>1. TTR Cardiomyopathy</b></p> <p>ClinicalTrials.gov <a href="https://clinicaltrials.gov/ct2/show/study/NCT01775761">NCT01775761</a></p> <p>Estimated enrollment: 42</p> <p>Estimated completion: 2013 (For Primary Outcome Measure)</p>	<p><b>A Study To Determine Any Effect Of Tafamidis On Electrocardiographic Intervals, Specifically The Rate Corrected QT Interval (QTc)</b></p> <p><i>Endpoint: Safety</i></p> <p>Phase 1</p> <p><i>Randomized, Placebo, and Positive Controlled Cross-Over study</i></p>	<p><b>Completed/No study results posted.</b></p> <p><b>Primary outcome measures:</b> QTc interval using Fridericia's correction method (QTcF) of tafamidis and placebo (baseline-adjusted) at each post-dose time.</p> <p><b>Secondary outcome measures:</b> QTcF of moxifloxacin and placebo at historical moxifloxacin median T<sub>max</sub> of 3 hours.</p>
<p><b>2. TTR Cardiomyopathy</b></p> <p>ClinicalTrials.gov <a href="https://clinicaltrials.gov/ct2/show/study/NCT01655511">NCT01655511</a></p> <p>Enrollment: 9</p> <p>Estimated completion: September 2012 (For Primary Outcome Measure)</p>	<p><b>Safety And Pharmacokinetic Assessment Of Orally Administered Tafamidis In Healthy Volunteers</b></p> <p><i>Endpoint: Pharmacokinetics of Tafamidis &gt;120 mg as an oral solution</i></p> <p>Phase 1</p> <p><i>Randomized, Double-Blind, Cross-over, ascending Dose-Tolerance</i></p>	<p><b>Completed/No study results posted.</b></p> <p><b>Primary outcome measures:</b> Safety and tolerability of orally administered tafamidis in healthy volunteers</p> <p><b>Secondary outcome measures:</b> C<sub>max</sub>, T<sub>max</sub>, AUC 0-24, AUC last, AUC Inf, T<sub>1/2</sub> plasma decay, TTR blood concentration, TTR stabilization</p>

<b>Table A.1 Continued</b>		
<p><b>3. TTR-CM</b></p> <p>ClinicalTrials.gov <a href="https://clinicaltrials.gov/ct2/show/study/NCT00925002">NCT00925002</a></p> <p>Estimated enrollment: 110</p> <p>Estimated completion: 2021</p>	<p><b>Safety And Efficacy Evaluation Of Fx-1006a In Patients With V122i Or Wild-Type Transthyretin (TTR) Amyloid Cardiomyopathy</b></p> <p><i>Endpoint: Safety/Efficacy</i></p> <p>Phase 3</p> <p><i>Non-randomized, open-label evaluation of Tafamidis in patients with TTR amyloidosis</i></p>	<p><b>Active, currently recruiting.</b></p> <p><b>Primary outcome measures:</b> Long-term, open-label safety and efficacy in TTR amyloidosis patients (10 years)</p> <p><b>Secondary outcome measures:</b> Provide investigation product until market availability to ATTR who have completed protocol</p>
<p><b>4. Cardiomyopathy</b></p> <p>ClinicalTrials.gov <a href="https://clinicaltrials.gov/ct2/show/study/NCT00694161">NCT00694161</a></p> <p>Estimated enrollment: 35</p> <p>Estimated completion: 2010 (Final data collection for primary outcome measure)</p>	<p><b>The Effects Of Fx-1006A On Transthyretin Stabilization And Clinical Outcome Measures In Patients With V122I Or Wild-Type TTR Amyloid Cardiomyopathy</b></p> <p><i>Endpoint: Safety/Efficacy</i></p> <p>Phase 2</p> <p><i>Interventional, open label, of TTR Stabilization and Clinical outcomes in V122I or wildtype TTR amyloid cardiomyopathy</i></p>	<p><b>Completed (Has Results, below)</b></p> <p><b>Primary outcome measures:</b> Participants with TTR V122I) and wildtype TTR achieved TTR stabilization at Week 6 (97.1%, N=35).</p> <p><b>Secondary outcome measures:</b> Those achieving TTR stabilization continued through Month 12 (87.5% with stabilized TTR, N=34).</p>

<b>Table A.1 Continued</b>		
<p><b>5. Transthyretin (TTR) Amyloid Cardiomyopathy</b></p> <p>Tafamidis</p> <p>ClinicalTrials.gov <a href="https://clinicaltrials.gov/ct2/show/study/NCT01994889">NCT01994889</a></p> <p>Estimated enrollment: 400</p> <p>Estimated completion: August 2018</p>	<p><b>Tafamidis</b></p> <p><i>Endpoint: Safety /Efficacy</i></p> <p>Phase 3</p> <p><i>Multi-Center, International, Double-blind, placebo-controlled, randomized study to evaluate efficacy, safety, and tolerability of oral dosing of Tafamidis Meglumine in TTR-CM</i></p>	<p><b>Recruiting, no study results posted.</b></p> <p><b>Primary outcome measures:</b> All-cause mortality and frequency of CV-related hospitalization.</p> <p><b>Secondary outcome measures:</b> 6 min walk test, Kansas City Cardiomyopathy Questionnaire, CV-related mortality, Frequency of CV-related hospitalization, All-cause mortality, TTR stabilization at 1 month.</p>
<p><b>6. TTR-mediated Amyloidosis</b></p> <p>ALN-TTRSC</p> <p>ClinicalTrials.gov <a href="https://clinicaltrials.gov/ct2/show/study/NCT01814839">NCT01814839</a></p> <p>Estimated enrollment: 76</p> <p>Estimated completion: 2015</p>	<p><b>ALN-TTRSC (RNAi)</b></p> <p><i>Endpoint: Safety</i></p> <p>Phase 1</p> <p><i>Randomized, double-blind, placebo-controlled, single and multi-dose, dose escalating study</i></p>	<p><b>Completed, no study results posted.</b></p> <p><b>Primary outcome measures:</b> Adverse events, serious adverse events, study drug discontinuation</p> <p><b>Secondary outcome measures:</b> Pharmacokinetics of ALN-TTRSC, Effect of ALN-TTRSC on Vitamin A, Effect of AL-TTRSC on retinol binding protein</p>

<b>Table A.1 Continued</b>		
<p><b>7. TTR-mediated Amyloidosis</b></p> <p>ALN-TTRSC</p> <p>ClinicalTrials.gov <a href="https://clinicaltrials.gov/ct2/show/study/NCT01981837">NCT01981837</a></p> <p>Estimated enrollment: 25</p> <p>Estimated completion: January 2015</p>	<p>ALN-TTRSC (RNAi)</p> <p><i>Endpoint: Safety /Efficacy</i></p> <p>Phase 2</p> <p><i>Open-label trial to evaluate safety, pharmacokinetics, pharmacodynamics, and Exploratory Clinical Trial of ALLN-TTRSC activity in TTR Cardiac Amyloidosis Patients</i></p>	<p><b>Active, not recruiting, no study results posted.</b></p> <p><b>Primary outcome measures:</b> AEs, SAEs, Study Drug Discontinuation.</p> <p><b>Secondary outcome measures:</b> Pharmacokinetics of ALN-TTRSC, Effect of ALN-TTRSC on TTR</p>

The discovery that tetracycline antibiotics have anti-amyloidogenic activity comes from the observation that iododoxorubicin (an anthracycline) is able to reduce amyloid and improve end organ damage in amyloid light-chain (AL) amyloidosis [51,52]. Doxycycline was then shown to act as a fibril disruptor *in vitro*, without toxicity [53]. In mouse models of AL amyloidosis, doxycycline similarly disaggregated amyloid and improved tissue markers of TTR deposition, without effecting pre-fibril aggregates [54]. In combination with the water-soluble bile acid tauroursodeoxycholic acid (TUDCA), synergistic clearance of TTR deposits was observed [55]. Used in treating cholestasis in liver disease, TUDCA, like doxycycline, is an FDA approved drug for other indications. A phase II, open-label study evaluating the efficacy, tolerability, safety, and pharmacokinetics of doxycycline and TUDCA in TTR-related cardiomyopathy is currently underway (Table A.2).

Additionally, there are a number of naturally occurring polyphenols that have anti-amyloidogenic properties, including the polyphenols EGCG and curcumin. EGCG, found in green tea, appears to bind amyloidogenic proteins and exert its protective effects by redirecting the aggregation process. Specifically, alternate non-pathogenic oligomeric species are instead formed which then deplete the formation of pathogenic types [56,57]. In a phase II clinical trial, EGCG was effective in preventing the progression of cardiomyopathy over 12 months. Of the 14 patients completing the study, no significant changes in cardiac mass or wall thickness were found when treated with ~500-700 mg EGCG daily [58]. However, wide variation in bioavailability following oral EGCG intake is an issue complicating the clinical development of this compound [58,59], including suspected hepatic toxicity [60]. Still, at least three clinical trials evaluating EGCG for protein misfolding diseases are ongoing, two of which are in cardiac amyloidosis (Table A.3). The other polyphenol, curcumin, competes for T4 on TTR to stabilize

TTR's native structure [61], inhibiting amyloid fibril formation *in vitro*, and disrupting pre-formed TTR fibrils from generating small oligomers [62]. *In vivo* studies of transgenic TTR mice have demonstrated prevention of cytotoxicity and decreases in TTR deposition [63]. However, there are no ongoing clinical trials evaluating curcumin for this therapeutic indication.

Successful early studies of pharmacological chaperones in pre-clinical and now clinical trials of amyloid-based disease have supported the concept that reversing/stabilizing protein misfolding is possible and can attenuate and sometimes reverse the pathophysiology, even in the heart. The increasing number of clinical trials (both phase 1 and 2) with cardiac indication holds promise that we will better understand the efficacy and safety of these drugs in patients with heart failure soon. While specific pharmacological chaperones appear to be specific for their misfolded substrate (e.g. TTR), it will be interesting to see how applicable these chaperones are to other substrates and whether pharmacological chaperones can be created for other substrate(s) found in differing causes of heart failure. With limited options for patients with heart failure, this class of drug offers a new way to treat an old but common problem.

Are pharmacological chaperones the only way to think about inhibiting proteotoxicity? It is possible that multiple points exist to prevent the proteotoxicity from contributing to heart failure, understanding that the formation of pre-amyloid oligomers, aggregates, and amyloids itself are a continuum. Like the concept of proteotoxicity itself, some of the answers may come from emerging discoveries in neurodegenerative diseases caused by endogenous misfolded proteins (e.g. Tau protein in Alzheimer's disease) or exogenous proteins (e.g. caused by prions). Next, we introduce how the concept of altered proteostasis activates large signaling complexes, including the inflammasome and necrosome, as well as complexes to prevent misfolded protein-induced cell death (proteotoxicity), such as the signalosome.



**Table A.2. Clinical studies investigating doxycycline in transthyretin-associated (amyloid) cardiomyopathy. TTR:** Transthyretin; TUDCA: Tauroursodeoxycholic acid; UDCA: Ursodeoxycholic acid. Created from data available at: ClinicalTrials.gov (Accessed 2 February 2015).

<p><b>8. Transthyretin Amyloidosis</b></p> <p>Doxycycline + TUDCA</p> <p>ClinicalTrials.gov <a href="https://clinicaltrials.gov/ct2/show/study/NCT01171859">NCT01171859</a></p> <p>Estimated enrollment: 40</p> <p>Estimated completion: July 2015 (Final data collection date for primary outcome measure)</p>	<p><b>Doxycycline + TUDCA</b></p> <p><i>Endpoint: Safety /Efficacy</i></p> <p>Phase 2</p> <p><i>Single center, non-randomized, open-label, prospective study followed by 6 month withdrawal to evaluate efficacy, tolerability, safety, pharmacokinetics of TUDCA in TTR Amyloidosis</i></p>	<p><b>Active, not recruiting</b></p> <p><b>Primary outcome measures:</b> Response rate to TUDCA (Based in mBMI reduction &lt;10%, change in Neurologic Impairment Score-Lower Limbs (NIS-LL) &lt;2 and NT-proBNP &lt;30% (or &lt;300 pg/ml)</p> <p><b>Secondary outcome measures:</b> Treatment-emergent adverse events, Doxycycline PK, Response in autonomic dysfunction, Neuropathy and Visceral Organ Involvement, Incidence of patients discontinuing from study due to clinical or laboratory adverse events</p>
---	--	--

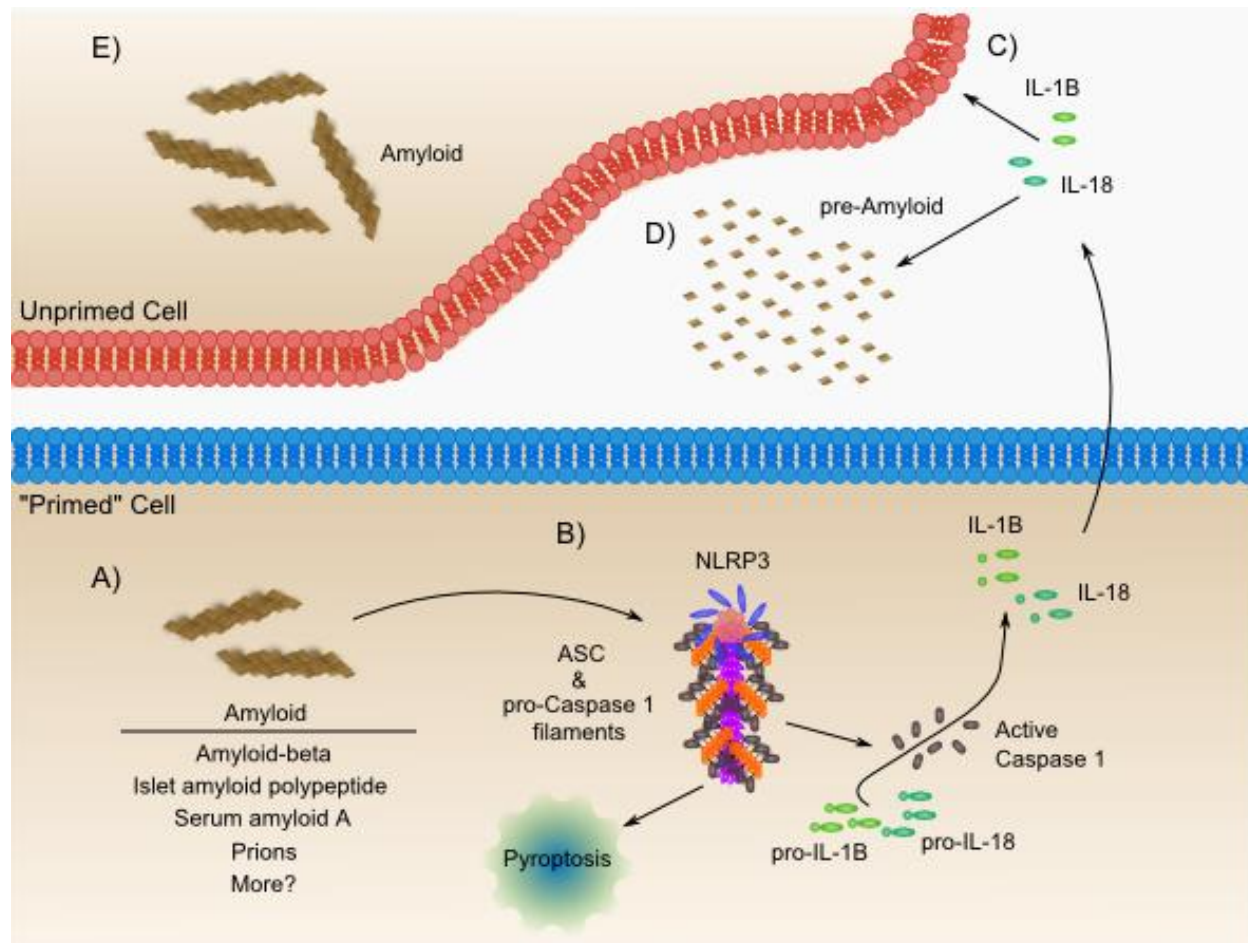
<b>Table A.2 Continued</b>		
<p><b>9. Amyloidosis; Heart (Manifestation); Senile Cardiac Amyloidosis</b></p> <p>Doxycycline + TUDCA</p> <p>ClinicalTrials.gov <a href="#">NCT01855360</a></p> <p>Estimated enrollment: 40</p> <p>Estimated completion: Sept 2015 (Final data collection date for primary outcome measure)</p>	<p><b>Doxycycline + TUDCA</b></p> <p><i>Endpoint: Safety /Efficacy</i></p> <p>Phase 1/2</p> <p><i>Open-label study of TUDCA in TTR-cardiomyopathy</i></p>	<p><b>Recruiting</b></p> <p><b>Primary outcome measures:</b> Rate of progression of TTR cardiac amyloidosis (Strain echocardiography)</p> <p><b>Secondary outcome measures:</b> Number of patients with adverse events to the medications over the period of therapy (18 months), Evaluate general and health related QoL in Senile and Familial TTR amyloidosis subjects.</p>
<p><b>10. Transthyretin Amyloidosis; Cardiomyopathy</b></p> <p>Doxycycline + UDCA</p> <p>ClinicalTrials.gov <a href="#">NCT02016365</a></p> <p>Estimated enrollment: 30</p> <p>Estimated completion: June 2015 (Final data collection date for primary outcome measure)</p>	<p><b>Doxycycline + UDCA</b></p> <p><i>Endpoint: Safety /Efficacy</i></p> <p>Phase 2</p> <p><i>Open-label study multi-center study of Safety and Efficacy of TUDCA in ATTR amyloidosis disease progression</i></p>	<p><b>Active, not recruiting</b></p> <p><b>Primary outcome measures:</b> efficacy on serum NT-proBNP (12 months).</p> <p><b>Secondary outcome measures:</b> Modified BMI, Increase in septum thickness, neurologic Kumamoto Scale, Number of patients with adverse events, blood work for potential drug-related adverse events.</p>

<b>Table A.3. Clinical studies investigating ECGG in primary cardiac amyloidosis and Alzheimer's disease. EGCG: Epigallocatechin-3-galleate. Created from data available at: ClinicalTrials.gov (Accessed 2 February 2015).</b>		
<p><b>11. Light Chain (AL) Amyloidosis, Cardiac Involvement</b></p> <p>ClinicalTrials.gov <a href="#">NCT02015312</a></p> <p>Estimated enrollment: 38</p> <p>Estimated completion: Sept 2017 (Final data collection date for primary outcome measure)</p>	<p><b>Green Tea Compound EGCG</b></p> <p><i>Safety/Efficacy</i></p> <p>Phase II</p> <p><i>Randomized, Double-Blind Study of Safety/Efficacy</i></p>	<p><b>Recruiting</b></p> <p><b>Primary outcome measures:</b> 12 month change in LV mass.</p> <p><b>Secondary outcome measures:</b> Change in QoL, Number of adverse events according to CTC criteria, change in cardiac biomarkers, hematological improvement, organ response (non-heart), overall survival.</p>
<p><b>12. Alzheimer's Disease</b></p> <p>ClinicalTrials.gov <a href="#">NCT00951834</a></p> <p>Estimated enrollment: 50</p> <p>Estimated completion: June 2015 (Final data collection date for primary outcome measure)</p>	<p><b>Green Tea Compound Epigallocatechin-3-galleate</b></p> <p><i>Efficacy Study</i></p> <p>Phase II/III</p> <p><i>Randomized, Double-Blind Study of Efficacy</i></p>	<p><b>Recruiting</b></p> <p><b>Primary outcome measures:</b> ADAS-COG (Score 0-70)</p> <p><b>Secondary outcome measures:</b> Safety and tolerability, MMSE Score after 18 months vs. baseline, Time to hospitalization and Time to death related to AD, brain atrophy via MRI, Baseline ADAS-COG and Baseline-MMSE as covariates, CIBIC+ and WHO QoL Bref, Trail Making Test and MVGT.</p>

<b>Table A.3 Continued</b>		
<p><b>13. Primary (Cardiac) Amyloidosis of Light Chain Type</b></p> <p>ClinicalTrials.gov <a href="https://clinicaltrials.gov/ct2/show/study/NCT01511263">NCT01511263</a></p> <p>Estimated enrollment: 86</p> <p>Estimated completion: December 2015 (Final data collection date for primary outcome measure)</p>	<p><b>Green Tea Compound Epigallocatechin-3-gallate</b></p> <p><i>Efficacy</i></p> <p>Phase II</p> <p><i>Open-label, Randomized Study of Dietary EGCG</i></p>	<p><b>Recruiting</b></p> <p><b>Primary outcome measures:</b> Cardiac response (Rate of cardiac response following chemotherapy in patients with AL amyloidosis). Endpoint at 6 months.</p> <p><b>Secondary outcome measures:</b> Rate of adverse events, cardiac progression, time to cardiac progression, rate of cardiac events, time to cardiac events, survival at 6 months.</p>

## **A.8 Amyloid proteins, heart failure, and activation of the inflammasome**

The inflammasome is a large oligomeric signaling structure that generates a robust pro-inflammatory response through coordinated necrotic cell death and secretion of IL-1 $\beta$  and IL-18. While the composition varies based on the activating signal, the inflammasome generally is made up of three proteins, including a cytosolic sensory receptor, an adaptor protein ASC, and a Caspase 1 enzyme [64]. The best characterized inflammasome, the NLRP3 inflammasome (**Figure A.3**), is activated by a diverse array of infectious and sterile stimuli, which initiate signaling through perturbation of cellular homeostasis – either through mitochondrial disruption, ER stress, calcium influx, potassium efflux, or some combination of these mechanisms [65,66]. When activated, these proteins come together to form an enormous fibrillar structure that acts as a platform for cytokine processing [67-69]. For example, NLRP3 mutations resulting in activation cause familial cold-induced inflammatory syndrome 1 (FCIS1), Muckle-Wells syndrome (MWS) and chronic neurologic cutaneous and articular syndrome (CINCA), all of which are characterized by hyperactive NLRP3 inflammasome signaling and chronic inflammation. Disruption of IL-1 signaling, with either the IL-1-receptor antagonist, Anakinra, or an anti-IL-1 $\beta$  monoclonal antibody, Canakinumab, provides rapid and dramatic resolution of symptoms [70,71].



**Figure A.3. The NLRP3 inflammasome is an amyloid-like fibrillar cytokine-processing platform, which senses amyloid and contributes to worsening heart failure.** (A) Amyloid fibrils from diverse sources including amyloid-beta, islet amyloid polypeptide, serum amyloid A, prions and possibly others, accumulate within cells and tissues. (B) The NLRP3 inflammasome can activate in response to these amyloid fibrils, leading to formation of a functional amyloid nucleated by NLRP3 and containing long polymeric repeats of ASC, the adaptor protein, and pro-Caspase 1. The NLRP3 inflammasome is capable of inducing a pro-inflammatory necrotic cell death, termed pyroptosis. Additionally, the proximity of pro-Caspase 1 proteins to one another leads to their proteolytic activation and release of active Caspase 1. Active Caspase 1 then processes pro-IL-1 $\beta$  and pro-IL-18, among other proteins, leading to the secretion of the

active form of these pro-inflammatory mediators. (C) Both IL-1 $\beta$  and IL-18 have been shown to directly cause cardiac dysfunction and may be targets for pharmacologic intervention as in the CANTOS trial. (D) IL-1 $\beta$  and IL-18 also can contribute to systemic inflammatory diseases characterized by massive production of acute phase reactants such as serum amyloid A. Serum amyloid A can then act as a seed for additional amyloid formation, which is sometimes seen in chronic inflammatory diseases such as Muckle-Wells syndrome (MWS) or Rheumatoid arthritis (RA). In many resting cells, which do not express components of the NLRP3 inflammasome, these components can be upregulated through Toll-like receptor-NF- $\kappa$ B mediated signaling. Upon upregulation of inflammasome components, these cells are “primed” for inflammasome activation. It still remains to be seen whether inflammasome activation originating in the heart or from phagocytes drives the pathogenesis of heart failure. Additionally, studies have yet to tease out the role of NLRP3 inflammasome-mediated pyroptosis in cardiac dysfunction. (E) The inflammatory IL-1 $\beta$  and IL-18 may contribute to the formation of amyloid in neighboring cells, through unclear mechanisms. IL-18 co-localizes with A $\beta$ -plaques and increases the hyperphosphorylation of tau-protein [105]. IL-18 enhanced cleavage of serum amyloid- $\beta$  precursor protein experimentally and may be one mechanism, of many remaining to be discovered.

Some patients with activating mutations of NLRP3 develop multi-organ serum amyloid A protein (AA)-type amyloidosis - likely the result of persistently elevated levels of this acute phase protein in response to chronic inflammation [72]. AA amyloidosis can involve deposits in the heart that lead to ventricular hypertrophy, atrial dilatation, and cardiac dysfunction [73-76]. Although typically thought of as rare, a recent survey of re-examined autopsy tissues from 369 rheumatoid arthritis patients found that 30% had evidence of AA amyloid deposits with cardiac

amyloid and renal amyloid appearing with equal frequency [77]. In two recent case reports of AA amyloidosis, treatment with anti-cytokine therapies reduced chronic inflammation and serum amyloid A levels resulting in stabilized or improved cardiac manifestations of the disease [78,79], providing further support for the hypothesis that limiting the availability of aggregation-prone proteins can limit amyloid formation.

While chronic inflammation can lead to amyloidosis, studies have also shown that the NLRP3 inflammasome can be activated in response to different types of amyloid, including amyloid-beta, islet amyloid polypeptide, serum amyloid A, prions, and curli fibers, a type of amyloid found in the biofilm of *E. coli* and *S. enteric* serovar Typhimurium [80 ,81-85]. This diverse collection of amyloid that trigger the NLRP3 inflammasome raise the possibility that NLRP3 is central in generating an inflammatory response to *all* amyloids.

We are just beginning to appreciate the role of the NLRP3 inflammasome in cardiac disease. In mice, cardiac-specific overexpression of a constitutively active form of the calcineurin A catalytic subunit leads to impaired cardiac function [86] and myocardial inflammation characterized by activation of Caspase 1 [87]. In this model, additional deletion of NLRP3 or pharmacologic inhibition of IL-1 $\beta$  signaling using the IL-1-receptor antagonist significantly reduced left ventricular dilatation and abrogated the progressive decrease in fractional shortening (FS) observed in wildtype and saline treated mice, thus implicating the NLRP3 inflammasome in this cardiac dysfunction [87].

In patients with idiopathic dilated cardiomyopathy, increased mRNA levels of NLRP3 inflammasome components were associated with worsening left ventricular ejection fraction and NLRP3 and IL-1 $\beta$  mRNA at the time of admission were independent predictors of 6-month re-hospitalization [88]. In support of a role for IL-1 $\beta$  signaling in cardiac dysfunction, human atrial



trabeculae tissue in an organ bath exhibited compromised systolic and diastolic function when exposed to TNF- $\alpha$  and IL-1 $\beta$  individually and had additive effects when combined at low doses [89]. Additionally, in patients with rheumatoid arthritis without concurrent cardiovascular disease, treatment with anakinra improved noninvasive measures of vascular and left ventricular function [90].

In 2011, a large multicenter trial called Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS), which planned to enroll 17,200 post myocardial infarction patients, was initiated to determine whether IL-1 $\beta$  inhibition can prevent recurrent myocardial infarction, stroke and cardiovascular death among stable patients with coronary artery disease who remain at high vascular risk [91]. This trial will provide the most comprehensive clinical evaluation to date of the inflammatory hypothesis in cardiac disease.

These studies highlight the emerging and pivotal role that the NLRP3 inflammasome and IL-1 $\beta$  signaling plays in the pathogenesis of heart failure. Continued interrogation of the NLRP3 inflammasome in heart failure is likely to aid in identifying new targets for therapeutic intervention, which may act synergistically with pharmacologic chaperones to break the cycle of amyloidosis and inflammation.

### **A.9 Amyloid proteins, heart failure, and activation of “functional amyloid”: Necrosome**

While amyloid has primarily been described as a key mediator of pathological processes, it now been recognized that when certain proteins form amyloid, it is part of their physiological role. That is, they act as a molecular switch to promote environmental adaptation and act as a regulator of intracellular signaling [92]. This includes the receptor-interacting protein kinases RIP1 and RIP3, which are found in the heart and activate cell death by forming the necrosome

[92]. Activation and assembly resulted from their ability to form a fibrillar complex with amyloid properties, which was observed during *in vitro* and *in vivo* experimental conditions [93]. The RIP homotypic interaction motifs (RHIMs) of RIP1 and RIP3 mediate the assembly of heterodimeric filamentous structures, which can trigger downstream signaling and cell death [93].

In the heart, myocardial ischemia induces RIP3 expression, which is sufficient to induce necroapoptosis [94]. Conversely, RIP3<sup>-/-</sup> mice challenged with MI had better function and less hypertrophy 30 days post- infarction, accompanied by decreased inflammatory response and decreased ROS production [94]. Similarly, inhibiting RIP1 with the small molecule Necrostatin-1 (Nec-1) prevents necrotic cell death in experimental models of cardiac ischemia [95]. This model also displayed less adverse remodeling, evidenced by less dilation, preserved systolic function, and reduced inflammation (TNF- $\alpha$  mRNA and ROS) [95]. Taken together, these studies illustrate both the importance of the RIP1/RIP3-mediated necrosis in ischemia, offering a new direction for therapy to disrupt physiologic amyloid-like proteins that contribute to pathology [96].

#### **A.10 The COP9 signalosome enhances protein degradation to reduce the misfolded protein burden**

Enhanced protein degradation clinically can be attempted through blockade of the bad signaling complexes (e.g. the inflammasome and necrosome), or by activation of the beneficial constitutive photomorphogenesis mutant 9 (COP9) signalosome (CSN). Analogous to ubiquitination, proteins can be neddylated via a series of enzymatic reactions to conjugate NEDD8 to substrate proteins, such as a Cullin-RING-Ligase (CRL) (detailed in **Figure A.4**),

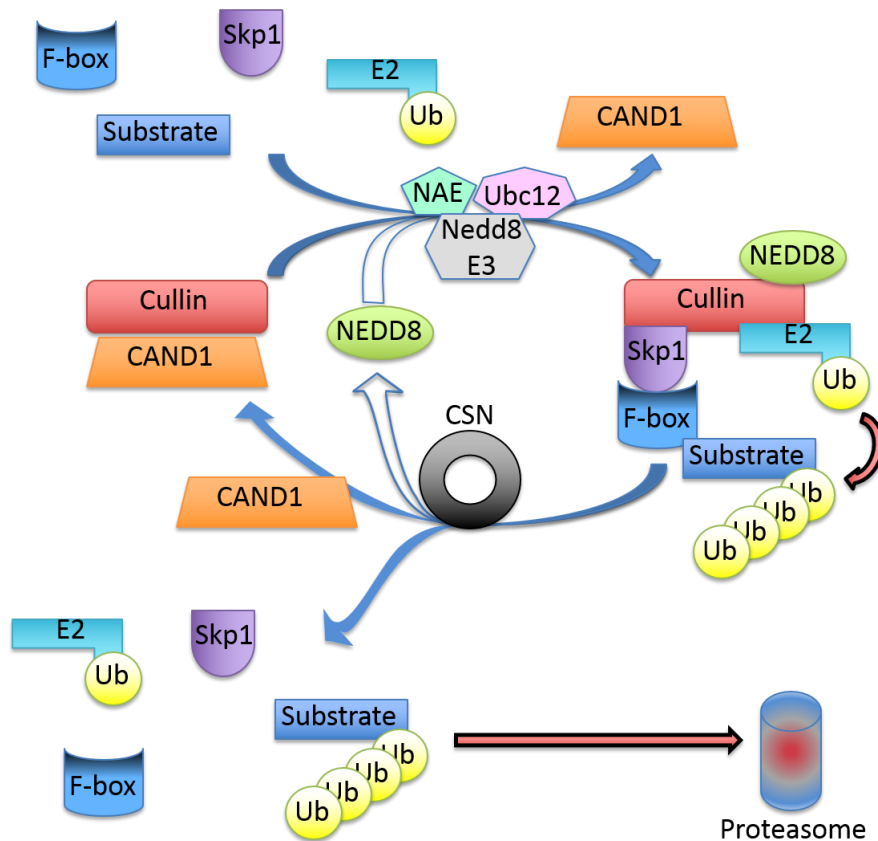
triggering the assembly of a functional CRL. CRLs are the largest family of ubiquitin ligases, including the ubiquitin ligases Parkin, Mdm2, Smurf1, and XIAP, in addition to transcription factors, such as E2F1, HIF1a, and p53 [97].

Only recently has the role of deneddylation been explored. The CSN is a highly conserved multiprotein complex composed of eight subunits (CSN1 to 8) to form a ~350 kDa complex that deneddylates proteins [98] to regulate the protein degradation by ubiquitin proteasome system [99]. CRL deneddylation is essential to disassemble the functional CRL and release the ubiquitinated substrate protein for subsequent degradation by the proteasome. The CSN crystal structure has two organization centers: 1) A horseshoe-shaped ring created by its six PCI domain proteins and a large bundle formed by the carboxy-terminal  $\alpha$ -helices of each subunit [100]. The CSN5-CSN6 dimer is found at the core of the helical bundle [100]. Neddylated CRL binding to CSN is sensed by CSN4, communicated to CSN5 (with CSN6), resulting in activation of the deneddylase and regulation of downstream ubiquitin ligases [100].

Recent studies have demonstrated the COP9 signalosome regulates autophagosome maturation *in vivo*. Mice with a cardiomyocyte-specific deletion of *csn8* resulted in striking increases in autophagic flux (evidenced by increased LC3-II/LC3I, autophagosomes by TEM) and increased p62 protein (a functional amyloid forming protein) [101,102]. CSN appears to regulate Rab7, which plays a critical role in autophagosomal formation and may explain its regulation of autophagy [102]. Furthermore, the CSN appears to influence proteasomal activity by directly interacting with the 26S proteasome and potentially competing with the 19S proteasome lid [103,104]. Therefore, it is hypothesized that the overt loss of protein quality control due to compromised autophagosome formation and removal paired with reduced

functionality of the UPS results in severe cardiomyocyte necrosis, leading to dilated cardiomyopathy, heart failure, and death.

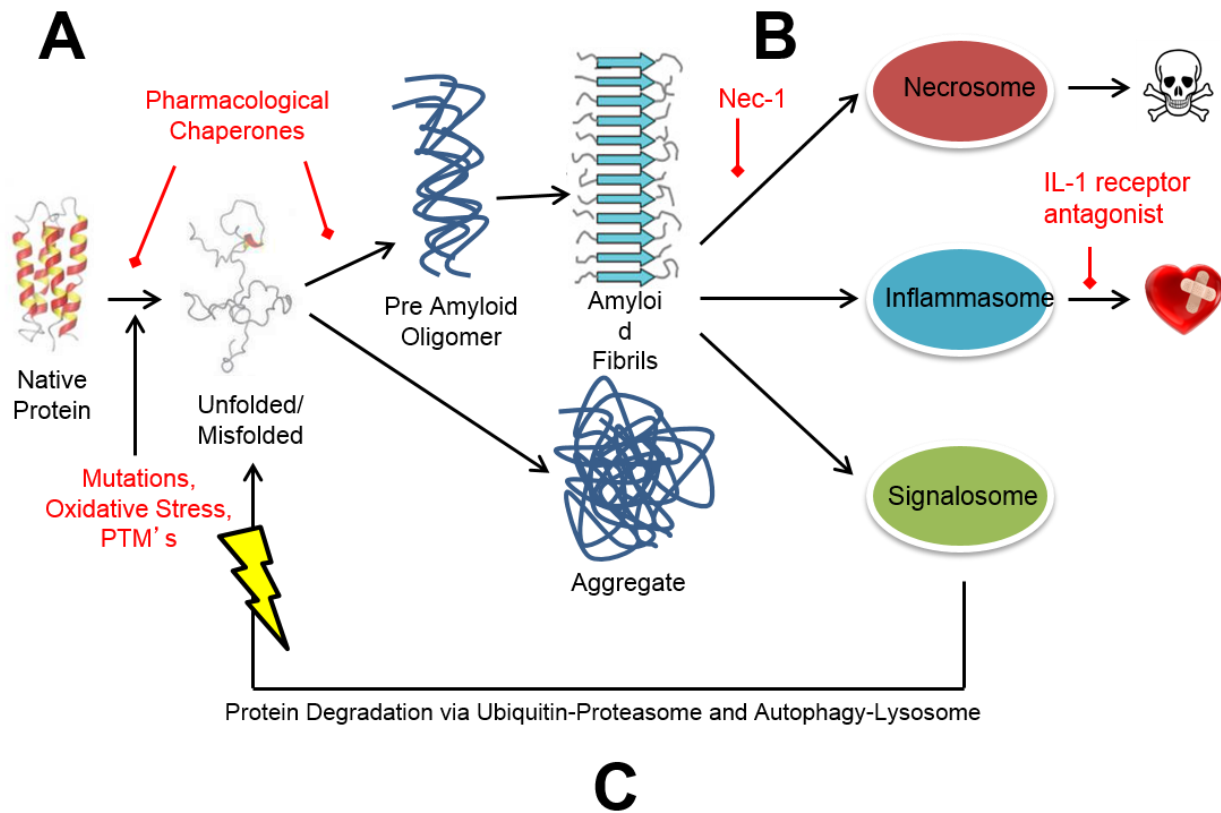
Inducible cardiac CSN8<sup>-/-</sup> mice demonstrated the role of CSN8 in regulating autophagic flux *in vivo*. The temporal cardiac Cre-LoxP ablation of CSN8 resulted in the accumulation of neddylated cullin (and non-cullin) proteins, increased ubiquitinated proteins, and a significant decrease in autophagic flux [101]. Autophagosomes were markedly increased, as were oxidized proteins and necrotic cardiomyocytes, resulting in a dilated cardiomyopathic phenotype [101]. How the COP9 signalosome is activated has not been clearly delineated. An intriguing possibility is that, similar to the proteasome, the COP9 signalosome can be regulated by post-translational modifications. Currently there are no known proteins that post-translationally regulate the CSN, however if found, this approach may have great therapeutic potential with small pharmacological modulators.



**Figure A.4. Cullin-RING ubiquitin ligase (CRL) activity is regulated via neddylation and deneddylation.** Cullin serves as a scaffold protein when neddylated (conjugated with NEDD8), a reaction catalyzed by NEDD8 activating enzyme (NAE), ubiquitin conjugating enzyme (Ubc12) and a NEDD8-specific ligase (NEDD E3). Cullin neddylation triggers displacement of the inhibitory CAND1 (cullin-associated NEDD8-dissociated protein 1), allowing cullin to interact with the adapter protein, Skp1, and a F-box protein forming a functional CRL. Next a ubiquitin charged E2 and a substrate are brought in close proximity to foster the transfer of ubiquitin to the substrate. After substrate ubiquitination, the COP9 Signalosome (CSN) will deneddylate (remove NEDD8) from cullin, which triggers the disassembly of the CRL and frees the substrate to be degraded by the proteasome. The freed cullin can be neddylated again to initiate another cycle or inhibited via CAND1 binding.

## A.11 Summary and Conclusion

The formation of amyloids has been observed as a pathological change implicated in neurodegenerative disease, including Alzheimer's disease, Parkinson's disease, and polyglutamine proteins (Huntington's disease). Paralleling these pathological findings and mechanisms, heart failure has similarly been found to involve these same biological processes [31]. This has led to the multiple clinical studies underway testing pharmacological interventions to stabilize the common TTR misfolding in cardiac amyloidosis (**Tables A.1-3**). However, the activation of amyloid formation is not only a pathological state; recent evidence suggests critical functional processes have similar aggregated states (**Table A.4**) [92]. Evidence that both the necrosome and inflammasome can be inhibited demonstrate the utility of targeting their activation when aggregates have already formed, complementing the pharmacological chaperones that largely prevent misfolded proteins from forming and possibly reversing/removing protein aggregation. Complementary to these three pathways, activation of the COP9 signalosome by a yet to be determined pathway, may prove to be another way in which misfolded cardiac proteins can be cleared to reduce future aggregate burdens. The multiple levels in which heart failure may be targeted illustrates many new opportunities for treating the heavy burden of heart failure where limited therapies exist, none of which currently target the underlying protein quality control issues outlined in this review (**Figure A.5**).



**Figure A.5. Targeting pathological and functional aggregates in heart failure.** Alterations drive protein unfolding and misfolding, resulting in the formation of misfolded, toxic protein aggregates and amyloid. Such misfolded protein structures activate pathological pro-inflammatory and necrotic signaling complexes such as the inflammasome and necrosome. However, protein aggregates can also activate the signalosome to assist in clearing of mis/unfolded proteins. The continuum of protein misfolding, to protein aggregation and amyloid formation, to activation of large signaling complexes provides multiple levels for potential pharmacological therapeutic targeting. (A) Pharmacological chaperones target misfolded and unfolded proteins to stabilize protein conformation. Such drugs include tafamidis, doxycycline and tauroursodeoxycholic acid. (B) Targeting and inhibiting large signaling structures like the inflammasome and necrosome offer new and possibly complementary methods of treating

amyloid based diseases. To date, these targets have been inhibited through treatment with an IL-1 antagonist (inflammasome) and Necrostatin-1 (necrosome). (C) Alternately, the COP9 signalosome may offer yet another therapeutic target to reduce the amyloidosis burden. Increasing activity of the signalosome could function alone or in tandem with other therapies to assist in clearance of misfolded protein aggregates and amyloid.



**Table A.4. Proteins forming amyloid as part of their functional role in cellular responses to the environment.** ©2013 Elsevier Publishing. Used with permission. From: Furukawa and Nukina, 2013 [92].

<i>Proteins</i>	<i>Species</i>	<i>Roles</i>		<i>Detection methods for aggregates<sup>5</sup></i>
		<i>Soluble state</i>	<i>Aggregated state</i>	
Sup35	Yeast	Translation terminator	Functional	ThT, CR, EM, X-ray, <i>etc.</i>
Mod5	Yeast	tRNA isopentenyltransferase	Functional	ThT, EM
CPEB	Marine snail	Transcriptional activator/repressor	Functional	ThT, EM
	Fruit fly			
Pmel17	Human	Melanin synthesis	Functional	ThT, CR, EM, X-ray
RIP1, RIP3	Human	Kinases	Functional	ThT, CR, EM, X-ray
p62	Human	Sequestosome formation	Functional	ThT, EM
GW182	Human	P-body formation	Functional	EM
RCK/p54	Human	P-body formation	Functional	EM
TIA-1	Human	Stress granule formation	Functional	ThT, CR, EM
TDP-43	Human	DNA/RNA binding protein	Pathological/functional?	ThT, CR, EM
FUS/TLS	Human	DNA/RNA binding protein	Pathological/functional?	EM

<sup>5</sup> ThT, Thioflavin T; CR, Congo red; EM, Electron microscopy; X-ray, X-ray diffraction.

## REFERENCES

1. Guan J, Mishra S, Falk RH, Liao R (2012) Current perspectives on cardiac amyloidosis. *Am J Physiol Heart Circ Physiol* 302: H544-552.
2. Kirshenblat Ia D, Serbeniuk VN (1966) [The effect of the nervous system on the sensitivity of rat ovaries to lactogenic hormone]. *Probl Endokrinol Gormonoter* 12: 100-105.
3. Falk RH, Comenzo RL, Skinner M (1997) The systemic amyloidoses. *N Engl J Med* 337: 898-909.
4. Klein AL, Hatle LK, Burstow DJ, Seward JB, Kyle RA, et al. (1989) Doppler characterization of left ventricular diastolic function in cardiac amyloidosis. *J Am Coll Cardiol* 13: 1017-1026.
5. Dubrey SW, Cha K, Skinner M, LaValley M, Falk RH (1997) Familial and primary (AL) cardiac amyloidosis: echocardiographically similar diseases with distinctly different clinical outcomes. *Heart* 78: 74-82.
6. Brenner DA, Jain M, Pimentel DR, Wang B, Connors LH, et al. (2004) Human amyloidogenic light chains directly impair cardiomyocyte function through an increase in cellular oxidant stress. *Circ Res* 94: 1008-1010.
7. Migrino RQ, Hari P, Gutterman DD, Bright M, Truran S, et al. (2010) Systemic and microvascular oxidative stress induced by light chain amyloidosis. *Int J Cardiol* 145: 67-68.
8. Sikkink LA, Ramirez-Alvarado M (2010) Cytotoxicity of amyloidogenic immunoglobulin light chains in cell culture. *Cell Death Dis* 1: e98.
9. Migrino RQ, Truran S, Gutterman DD, Franco DA, Bright M, et al. (2011) Human microvascular dysfunction and apoptotic injury induced by AL amyloidosis light chain proteins. *Am J Physiol Heart Circ Physiol* 301: H2305-2312.
10. Gianni D, Li A, Tesco G, McKay KM, Moore J, et al. (2010) Protein aggregates and novel presenilin gene variants in idiopathic dilated cardiomyopathy. *Circulation* 121: 1216-1226.
11. Subramanian K, Gianni D, Balla C, Assenza GE, Joshi M, et al. (2015) Cofilin-2 phosphorylation and sequestration in myocardial aggregates: novel pathogenetic mechanisms for idiopathic dilated cardiomyopathy. *J Am Coll Cardiol* 65: 1199-1214.
12. Munsie LN, Truant R (2012) The role of the cofilin-actin rod stress response in neurodegenerative diseases uncovers potential new drug targets. *Bioarchitecture* 2: 204-208.
13. Maloney MT, Bamberg JR (2007) Cofilin-mediated neurodegeneration in Alzheimer's disease and other amyloidopathies. *Mol Neurobiol* 35: 21-44.

14. Christians ES, Mustafi SB, Benjamin IJ (2014) Chaperones and cardiac misfolding protein diseases. *Curr Protein Pept Sci* 15: 189-204.
15. Mehta A, Sequiera GL, Ramachandra CJ, Sudibyo Y, Chung Y, et al. (2014) Re-trafficking of hERG reverses long QT syndrome 2 phenotype in human iPS-derived cardiomyocytes. *Cardiovasc Res* 102: 497-506.
16. Anderson CL, Delisle BP, Anson BD, Kilby JA, Will ML, et al. (2006) Most LQT2 mutations reduce Kv11.1 (hERG) current by a class 2 (trafficking-deficient) mechanism. *Circulation* 113: 365-373.
17. Braakman I, Bulleid NJ (2011) Protein folding and modification in the mammalian endoplasmic reticulum. *Annu Rev Biochem* 80: 71-99.
18. Chiti F, Dobson CM (2006) Protein misfolding, functional amyloid, and human disease. *Annu Rev Biochem* 75: 333-366.
19. Gregersen N, Bross P, Vang S, Christensen JH (2006) Protein misfolding and human disease. *Annu Rev Genomics Hum Genet* 7: 103-124.
20. Dobson CM (2006) The generic nature of protein folding and misfolding. *Protein Misfolding, Aggregation, and Conformational Diseases*: Springer. pp. 21-41.
21. Gomes CM (2012) Protein misfolding in disease and small molecule therapies. *Curr Top Med Chem* 12: 2460-2469.
22. Onuchic JN, Wolynes PG (2004) Theory of protein folding. *Curr Opin Struct Biol* 14: 70-75.
23. Thirumalai D, O'Brien EP, Morrison G, Hyeon C (2010) Theoretical perspectives on protein folding. *Annu Rev Biophys* 39: 159-183.
24. Dill KA, Chan HS (1997) From Levinthal to pathways to funnels. *Nat Struct Biol* 4: 10-19.
25. Han JH, Batey S, Nickson AA, Teichmann SA, Clarke J (2007) The folding and evolution of multidomain proteins. *Nat Rev Mol Cell Biol* 8: 319-330.
26. Ross CA, Poirier MA (2004) Protein aggregation and neurodegenerative disease. *Nat Med* 10 Suppl: S10-17.
27. Ross CA, Poirier MA (2005) Opinion: What is the role of protein aggregation in neurodegeneration? *Nat Rev Mol Cell Biol* 6: 891-898.
28. Sanbe A, Osinska H, Saffitz JE, Glabe CG, Kaye R, et al. (2004) Desmin-related cardiomyopathy in transgenic mice: A cardiac amyloidosis. *Proceedings of the National Academy of Sciences of the United States of America* 101: 10132-10136.
29. Kostin S, Pool L, Elsasser A, Hein S, Drexler HC, et al. (2003) Myocytes die by multiple mechanisms in failing human hearts. *Circ Res* 92: 715-724.

30. Tannous P, Zhu H, Nemchenko A, Berry JM, Johnstone JL, et al. (2008) Intracellular protein aggregation is a proximal trigger of cardiomyocyte autophagy. *Circulation* 117: 3070-3078.
31. Pattison JS, Sanbe A, Maloyan A, Osinska H, Klevitsky R, et al. (2008) Cardiomyocyte expression of a polyglutamine preamyloid oligomer causes heart failure. *Circulation* 117: 2743-2751.
32. Bulteau AL, Lundberg KC, Humphries KM, Sadek HA, Szweda PA, et al. (2001) Oxidative modification and inactivation of the proteasome during coronary occlusion/reperfusion. *J Biol Chem* 276: 30057-30063.
33. Tian Z, Zheng H, Li J, Li Y, Su H, et al. (2012) Genetically induced moderate inhibition of the proteasome in cardiomyocytes exacerbates myocardial ischemia-reperfusion injury in mice. *Circ Res* 111: 532-542.
34. Willis MS, Patterson C (2010) Hold me tight: Role of the heat shock protein family of chaperones in cardiac disease. *Circulation* 122: 1740-1751.
35. Zhang H, Rajasekaran NS, Orosz A, Xiao X, Rechsteiner M, et al. (2010) Selective degradation of aggregate-prone CryAB mutants by HSPB1 is mediated by ubiquitin-proteasome pathways. *J Mol Cell Cardiol* 49: 918-930.
36. Sanbe A, Daicho T, Mizutani R, Endo T, Miyauchi N, et al. (2009) Protective effect of geranylgeranylacetone via enhancement of HSPB8 induction in desmin-related cardiomyopathy. *PLoS ONE* 4: e5351.
37. Morello JP, Petaja-Repo UE, Bichet DG, Bouvier M (2000) Pharmacological chaperones: a new twist on receptor folding. *Trends Pharmacol Sci* 21: 466-469.
38. Morello JP, Salahpour A, Laperriere A, Bernier V, Arthus MF, et al. (2000) Pharmacological chaperones rescue cell-surface expression and function of misfolded V2 vasopressin receptor mutants. *J Clin Invest* 105: 887-895.
39. Serradeil-Le Gal C, Lacour C, Valette G, Garcia G, Foulon L, et al. (1996) Characterization of SR 121463A, a highly potent and selective, orally active vasopressin V2 receptor antagonist. *J Clin Invest* 98: 2729-2738.
40. Leandro P, Gomes CM (2008) Protein misfolding in conformational disorders: rescue of folding defects and chemical chaperoning. *Mini Rev Med Chem* 8: 901-911.
41. Saraiva MJ, Magalhaes J, Ferreira N, Almeida MR (2012) Transthyretin deposition in familial amyloidotic polyneuropathy. *Curr Med Chem* 19: 2304-2311.
42. Obici L, Merlini G (2014) An overview of drugs currently under investigation for the treatment of transthyretin-related hereditary amyloidosis. *Expert Opin Investig Drugs* 23: 1239-1251.

43. Johnson SM, Connelly S, Fearn C, Powers ET, Kelly JW (2012) The transthyretin amyloidoses: from delineating the molecular mechanism of aggregation linked to pathology to a regulatory-agency-approved drug. *J Mol Biol* 421: 185-203.
44. Razavi H, Palaninathan SK, Powers ET, Wiseman RL, Purkey HE, et al. (2003) Benzoxazoles as transthyretin amyloid fibril inhibitors: synthesis, evaluation, and mechanism of action. *Angew Chem Int Ed Engl* 42: 2758-2761.
45. Johnson SM, Connelly S, Wilson IA, Kelly JW (2008) Biochemical and structural evaluation of highly selective 2-arylbenzoxazole-based transthyretin amyloidogenesis inhibitors. *J Med Chem* 51: 260-270.
46. Coelho T, Maia LF, Martins da Silva A, Waddington Cruz M, Plante-Bordeneuve V, et al. (2012) Tafamidis for transthyretin familial amyloid polyneuropathy: a randomized, controlled trial. *Neurology* 79: 785-792.
47. Coelho T, Maia LF, da Silva AM, Cruz MW, Plante-Bordeneuve V, et al. (2013) Long-term effects of tafamidis for the treatment of transthyretin familial amyloid polyneuropathy. *J Neurol* 260: 2802-2814.
48. Scott LJ (2014) Tafamidis: a review of its use in familial amyloid polyneuropathy. *Drugs* 74: 1371-1378.
49. Klamerus KJ, Watsky E, Moller R, Wang R, Riley S (2014) The effect of tafamidis on the QT interval in healthy subjects. *Br J Clin Pharmacol*.
50. Alhamadsheh MM, Connelly S, Cho A, Reixach N, Powers ET, et al. (2011) Potent kinetic stabilizers that prevent transthyretin-mediated cardiomyocyte proteotoxicity. *Sci Transl Med* 3: 97ra81.
51. Merlini G, Ascari E, Amboldi N, Bellotti V, Arbustini E, et al. (1995) Interaction of the anthracycline 4'-iodo-4'-deoxydoxorubicin with amyloid fibrils: inhibition of amyloidogenesis. *Proc Natl Acad Sci U S A* 92: 2959-2963.
52. Gianni L, Bellotti V, Gianni AM, Merlini G (1995) New drug therapy of amyloidoses: resorption of AL-type deposits with 4'-iodo-4'-deoxydoxorubicin. *Blood* 86: 855-861.
53. Cardoso I, Merlini G, Saraiva MJ (2003) 4'-iodo-4'-deoxydoxorubicin and tetracyclines disrupt transthyretin amyloid fibrils in vitro producing noncytotoxic species: screening for TTR fibril disrupters. *FASEB J* 17: 803-809.
54. Cardoso I, Saraiva MJ (2006) Doxycycline disrupts transthyretin amyloid: evidence from studies in a FAP transgenic mice model. *FASEB J* 20: 234-239.
55. Macedo B, Batista AR, Ferreira N, Almeida MR, Saraiva MJ (2008) Anti-apoptotic treatment reduces transthyretin deposition in a transgenic mouse model of Familial Amyloidotic Polyneuropathy. *Biochim Biophys Acta* 1782: 517-522.
56. Hyung SJ, DeToma AS, Brender JR, Lee S, Vivekanandan S, et al. (2013) Insights into anti-amyloidogenic properties of the green tea extract (-)-epigallocatechin-3-gallate

- toward metal-associated amyloid-beta species. *Proc Natl Acad Sci U S A* 110: 3743-3748.
57. Lopez del Amo JM, Fink U, Dasari M, Grelle G, Wanker EE, et al. (2012) Structural properties of EGCG-induced, nontoxic Alzheimer's disease A $\beta$  oligomers. *J Mol Biol* 421: 517-524.
  58. Kristen AV, Lehrke S, Buss S, Mereles D, Steen H, et al. (2012) Green tea halts progression of cardiac transthyretin amyloidosis: an observational report. *Clin Res Cardiol* 101: 805-813.
  59. Dube A, Nicolazzo JA, Larson I (2010) Chitosan nanoparticles enhance the intestinal absorption of the green tea catechins (+)-catechin and (-)-epigallocatechin gallate. *Eur J Pharm Sci* 41: 219-225.
  60. Mazzanti G, Menniti-Ippolito F, Moro PA, Cassetti F, Raschetti R, et al. (2009) Hepatotoxicity from green tea: a review of the literature and two unpublished cases. *Eur J Clin Pharmacol* 65: 331-341.
  61. Pullakhandam R, Srinivas PN, Nair MK, Reddy GB (2009) Binding and stabilization of transthyretin by curcumin. *Arch Biochem Biophys* 485: 115-119.
  62. Ferreira N, Saraiva MJ, Almeida MR (2011) Natural polyphenols inhibit different steps of the process of transthyretin (TTR) amyloid fibril formation. *FEBS Lett* 585: 2424-2430.
  63. Ferreira N, Santos SA, Domingues MR, Saraiva MJ, Almeida MR (2013) Dietary curcumin counteracts extracellular transthyretin deposition: insights on the mechanism of amyloid inhibition. *Biochim Biophys Acta* 1832: 39-45.
  64. Walsh JG, Muruve DA, Power C (2014) Inflammasomes in the CNS. *Nat Rev Neurosci* 15: 84-97.
  65. Latz E, Xiao TS, Stutz A (2013) Activation and regulation of the inflammasomes. *Nat Rev Immunol* 13: 397-411.
  66. Wen H, Miao EA, Ting JP (2013) Mechanisms of NOD-like receptor-associated inflammasome activation. *Immunity* 39: 432-441.
  67. Lu A, Magupalli VG, Ruan J, Yin Q, Atianand MK, et al. (2014) Unified polymerization mechanism for the assembly of ASC-dependent inflammasomes. *Cell* 156: 1193-1206.
  68. Cai X, Chen J, Xu H, Liu S, Jiang QX, et al. (2014) Prion-like polymerization underlies signal transduction in antiviral immune defense and inflammasome activation. *Cell* 156: 1207-1222.
  69. Franklin BS, Bossaller L, De Nardo D, Ratter JM, Stutz A, et al. (2014) The adaptor ASC has extracellular and 'prionoid' activities that propagate inflammation. *Nat Immunol* 15: 727-737.

70. Goldbach-Mansky R, Dailey NJ, Canna SW, Gelabert A, Jones J, et al. (2006) Neonatal-onset multisystem inflammatory disease responsive to interleukin-1beta inhibition. *N Engl J Med* 355: 581-592.
71. Lachmann HJ, Kone-Paut I, Kuemmerle-Deschner JB, Leslie KS, Hachulla E, et al. (2009) Use of canakinumab in the cryopyrin-associated periodic syndrome. *N Engl J Med* 360: 2416-2425.
72. van der Hilst JC, Simon A, Drenth JP (2005) Hereditary periodic fever and reactive amyloidosis. *Clin Exp Med* 5: 87-98.
73. Matsui M, Okayama S, Tsushima H, Samejima K, Kanki T, et al. (2014) Therapeutic Benefits of Tocilizumab Vary in Different Organs of a Patient with AA Amyloidosis. *Case Rep Nephrol* 2014: 823093.
74. Simons JP, Al-Shawi R, Ellmerich S, Speck I, Aslam S, et al. (2013) Pathogenetic mechanisms of amyloid A amyloidosis. *Proc Natl Acad Sci U S A* 110: 16115-16120.
75. Ha SJ, Kim WS, Hwang SJ, Woo JS, Shon IS, et al. (2009) A case of systemic amyloidosis following ankylosing spondylitis associated with congestive heart failure. *J Am Soc Echocardiogr* 22: 542 e545-547.
76. Isabel C, Georgin-Lavialle S, Aouba A, Delarue R, Nochy D, et al. (2013) [Cardiac amyloidosis: a case series of 14 patients, description and prognosis]. *Rev Med Interne* 34: 671-678.
77. Koivuniemi R, Paimela L, Suomalainen R, Tornroth T, Leirisalo-Repo M (2008) Amyloidosis is frequently undetected in patients with rheumatoid arthritis. *Amyloid* 15: 262-268.
78. Hattori Y, Ubara Y, Sumida K, Hiramatsu R, Hasegawa E, et al. (2012) Tocilizumab improves cardiac disease in a hemodialysis patient with AA amyloidosis secondary to rheumatoid arthritis. *Amyloid* 19: 37-40.
79. Wada Y, Kobayashi D, Murakami S, Oda M, Hanawa H, et al. (2011) Cardiac AA amyloidosis in a patient with rheumatoid arthritis and systemic sclerosis: the therapeutic potential of biological reagents. *Scand J Rheumatol* 40: 402-404.
80. Masters SL, Dunne A, Subramanian SL, Hull RL, Tannahill GM, et al. (2010) Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1beta in type 2 diabetes. *Nat Immunol* 11: 897-904.
81. Halle A, Hornung V, Petzold GC, Stewart CR, Monks BG, et al. (2008) The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. *Nat Immunol* 9: 857-865.
82. Niemi K, Teirila L, Lappalainen J, Rajamaki K, Baumann MH, et al. (2011) Serum amyloid A activates the NLRP3 inflammasome via P2X7 receptor and a cathepsin B-sensitive pathway. *J Immunol* 186: 6119-6128.

83. Ather JL, Ckless K, Martin R, Foley KL, Suratt BT, et al. (2011) Serum amyloid A activates the NLRP3 inflammasome and promotes Th17 allergic asthma in mice. *J Immunol* 187: 64-73.
84. Hafner-Bratkovic I, Bencina M, Fitzgerald KA, Golenbock D, Jerala R (2012) NLRP3 inflammasome activation in macrophage cell lines by prion protein fibrils as the source of IL-1beta and neuronal toxicity. *Cell Mol Life Sci* 69: 4215-4228.
85. Rapsinski GJ, Wynosky-Dolfi MA, Oppong GO, Tursi SA, Wilson RP, et al. (2015) Toll-like receptor 2 and NLRP3 cooperate to recognize a functional bacterial amyloid, curli. *Infect Immun* 83: 693-701.
86. Molkenin JD, Lu JR, Antos CL, Markham B, Richardson J, et al. (1998) A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* 93: 215-228.
87. Bracey NA, Beck PL, Muruve DA, Hirota SA, Guo J, et al. (2013) The Nlrp3 inflammasome promotes myocardial dysfunction in structural cardiomyopathy through interleukin-1beta. *Exp Physiol* 98: 462-472.
88. Luo B, Wang F, Li B, Dong Z, Liu X, et al. (2013) Association of nucleotide-binding oligomerization domain-like receptor 3 inflammasome and adverse clinical outcomes in patients with idiopathic dilated cardiomyopathy. *Clin Chem Lab Med* 51: 1521-1528.
89. Cain BS, Meldrum DR, Dinarello CA, Meng X, Joo KS, et al. (1999) Tumor necrosis factor-alpha and interleukin-1beta synergistically depress human myocardial function. *Crit Care Med* 27: 1309-1318.
90. Ikonomidis I, Lekakis JP, Nikolaou M, Paraskevaidis I, Andreadou I, et al. (2008) Inhibition of interleukin-1 by anakinra improves vascular and left ventricular function in patients with rheumatoid arthritis. *Circulation* 117: 2662-2669.
91. Ridker PM, Thuren T, Zalewski A, Libby P (2011) Interleukin-1beta inhibition and the prevention of recurrent cardiovascular events: rationale and design of the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS). *Am Heart J* 162: 597-605.
92. Furukawa Y, Nukina N (2013) Functional diversity of protein fibrillar aggregates from physiology to RNA granules to neurodegenerative diseases. *Biochim Biophys Acta* 1832: 1271-1278.
93. Li J, McQuade T, Siemer AB, Napetschnig J, Moriwaki K, et al. (2012) The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. *Cell* 150: 339-350.
94. Luedde M, Lutz M, Carter N, Sosna J, Jacoby C, et al. (2014) RIP3, a kinase promoting necroptotic cell death, mediates adverse remodelling after myocardial infarction. *Cardiovasc Res* 103: 206-216.
95. Oerlemans MI, Liu J, Arslan F, den Ouden K, van Middelaar BJ, et al. (2012) Inhibition of RIP1-dependent necrosis prevents adverse cardiac remodeling after myocardial ischemia-reperfusion in vivo. *Basic Res Cardiol* 107: 270.



96. Moriwaki K, Chan FK (2014) Necrosis-dependent and independent signaling of the RIP kinases in inflammation. *Cytokine Growth Factor Rev* 25: 167-174.
97. Kandala S, Kim IM, Su H (2014) Neddylation and deneddylation in cardiac biology. *Am J Cardiovasc Dis* 4: 140-158.
98. Wei N, Chamovitz DA, Deng XW (1994) Arabidopsis COP9 is a component of a novel signaling complex mediating light control of development. *Cell* 78: 117-124.
99. Wei N, Deng XW (2003) The COP9 signalosome. *Annual review of cell and developmental biology* 19: 261-286.
100. Lingaraju GM, Bunker RD, Cavadini S, Hess D, Hassiepen U, et al. (2014) Crystal structure of the human COP9 signalosome. *Nature* 512: 161-165.
101. Su H, Li J, Osinska H, Li F, Robbins J, et al. (2013) The COP9 signalosome is required for autophagy, proteasome-mediated proteolysis, and cardiomyocyte survival in adult mice. *Circulation: Heart Failure* 6: 1049-1057.
102. Su H, Li F, Ranek MJ, Wei N, Wang X (2011) COP9 signalosome regulates autophagosome maturation. *Circulation* 124: 2117-2128.
103. Huang X, Hetfeld BK, Seifert U, Kähne T, Kloetzel PM, et al. (2005) Consequences of COP9 signalosome and 26S proteasome interaction. *FEBS Journal* 272: 3909-3917.
104. Enchev RI, Schreiber A, Beuron F, Morris EP (2010) Structural Insights into the COP9 Signalosome and Its Common Architecture with the 26S Proteasome Lid and eIF3. *Structure* 18: 518-527.
105. Sutinen EM, Pirttila T, Anderson G, Salminen A, Ojala JO (2012) Pro-inflammatory interleukin-18 increases Alzheimer's disease-associated amyloid-beta production in human neuron-like cells. *J Neuroinflammation* 9: 199.